The Innate Immune Mechanisms
Underlying the Interplay Between
Respiratory Infections and Asthma

By

Gerard Emil Kaiko BBiomedical Sci (Hons)

Research Centre for Asthma and Respiratory Diseases,
Discipline of Immunology and Microbiology,
Faculty of Health,
The University of Newcastle
NSW, Australia

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THESIS STATEMENTS

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

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I hereby certify that this thesis is submitted in the form of a series of publications of which I am first author. I have included as part of the thesis a written statement from each co-author; and endorsed by the Deputy Head of Faculty (Research), attesting to my contribution to the joint publications.

For all three publications my work includes conceptualising the studies, designing the studies, performing the studies, and writing the manuscripts. For all three publications Professor Paul Foster, Dr. Simon Phipps, and Professor Kenneth Beagley were involved in conceptualising and designing the studies, and editing the drafts of the manuscripts. All other co-authors provided either a critical reagent, or mouse strain.

This thesis consists of a comprehensive introduction to the topic encapsulating the three papers and the rationale for the work. Followed by the three individual manuscripts with specific introduction, methods, results and discussion sections, the first published in 2008, the second published in 2010, and the third in submission. These publications have been re-formatted for consistent styling, as their original format was journal-specific. Please note: In the case of the third publication the introduction, methods, results, and discussion sections have also been lengthened to provide further detail beyond the word restrictions of the journal. The thesis is concluded with an overall discussion of the three publications to place them in the context of an established body of knowledge.

Gerard Kaiko
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Many thanks also to the CRC for Asthma and Airways (Australia) for their support during my PhD studies.
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PUBLICATION #1


PUBLICATION #2


PUBLICATION #3


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LIST OF ABBREVIATIONS

RSV; Respiratory Syncytial Virus
PVM; Pneumonia Virus of Mice
Cpn; Chlamydophila pneumoniae
Cmu; Chlamydia muridarum
LRT; lower respiratory tract
URT; upper respiratory tract
MHC; Major Histocompatibility
TCR; T cell receptor
PFU; plaque forming units
IFU; infection forming units
JAK; Janus-activated kinase
STAT; Signal transducer and activator of transcription
HDM; house dust mite
TNF; tumour necrosis factor
IL; interleukin
IFN; interferon
CXCL; chemokine ligand
CXCR; chemokine receptor
dC; dendritic cell
APC; antigen presenting cell
GATA-3; GATA binding protein 3
T-box; T-box expressed in T cells
ROR-γt; retinoic acid receptor-related orphan receptor γ-t
TLR; Toll-like receptor
PRR; pathogen recognition receptor
PAMP; pathogen associated molecular pattern
DAMP; damage associated molecular pattern
OVA; ovalbumin
LPS; lipopolysaccharide
i.n.; intranasal
i.v.; intravenous
i.p.; intraperitoneal
MyD88; myeloid differentiation factor 88
Th; CD4 T helper cell
Th1; CD4 T helper cell type 1
Th2; CD4 T helper cell type 2
Th17; CD4 T helper cell type 17
Treg; Regulatory T cell
TGF; transforming growth factor
NHC; natural helper cell
MPPT2; multipotent progenitor cell type 2
NK; natural killer
BAL; bronchoalveolar lavage
AHR; airways hyper-reactivity
MSC; mucous secreting cell
PAS; periodic-acid Schiff
BM; basement membrane
BMDC; bone marrow-derived dendritic cell
NOD; Nucleotide-binding oligomerisation domain-containing protein
DC-SIGN; Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
NS protein; non-structural protein
MIP; macrophage inflammatory protein
MCP; monocyte chemotactic protein
RANTES; regulated upon activation, normal T cell expressed and secreted
ICAM; inter-cellular adhesion molecule
VCAM; vascular cell adhesion molecule
VEGF; vascular endothelial growth factor
GM-CSF; granulocyte-macrophage colony stimulating factor
Df; Dermatophagoides farinae
RIG-I; retinoic acid-inducible gene-I
LT; leukotriene
Ag; antigen
GWAS; genome wide association study
SNP; single nucleotide polymorphism
DEP; diesel exhaust particles
ETS; environmental tobacco smoke
EB; elementary body
RB; reticulate body
PB; persistent body
ECP; eosinophil cationic protein
FI; formalin-inactivated
TSLP; thymic stromal lymphopoietin
HPC; hematopoietic progenitor cell
PAR; protease activated receptor
pDC; plasmacytoid dendritic cell
mDC; myeloid dendritic cell
TARC; thymus and activation-regulated chemokine
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Appendix 1:

ABSTRACT

Asthma is a complex heterogeneous disease, which may involve a dynamic interplay between multiple gene and environmental factors. There is a growing need to develop better therapies for the treatment and prevention of asthma. In order for this to occur an improved understanding of disease origins and pathogenesis is required. This thesis utilises experimental animal modelling and in vitro culture systems in order to dissect the pathways and mechanisms underlying the interaction between respiratory infections, the innate immune system, and asthma. This thesis consists of three publications. The first publication examines the role of immune evasion of *Chlamydophila pneumoniae* in its association with acute exacerbations of asthma, and how immune deviation of dendritic cells may be central to both outcomes. The second publication investigates the association between Respiratory Syncytial Virus (RSV) and asthma. This work defines a mechanism by which the innate immune system may induce a viral-specific Th2 response. The third publication attempts to delineate a possible 2-hit hypothesis of asthma pathogenesis. This involves mouse modelling of a specific genetic susceptibility (identified in humans) and an early-life viral infection acting as an environmental insult to the respiratory tract. This work evaluates whether an interplay between these two factors and the innate immune system could potentially predispose to the hallmark features of asthma in later-life.
CHAPTER I: INTRODUCTION
I.1 Overview of Asthmatic Disease

Asthma is the most common chronic lung disease worldwide and is currently estimated to affect upwards of 200 million people (Eder et al., 2006, Adcock et al., 2008). Rather than existing as a single defined disease presenting with clear clinical and pathological symptoms asthma is a complex heterogeneous syndrome with many clinical phenotypes and is instead considered in terms of its hallmarks (Busse and Lemanske, 2001). Asthma is a chronic inflammatory disease of the airways (trachea and bronchi) that involves an intricate interplay between inflammatory cells, immune mediators and respiratory mesenchymal cells (Busse and Lemanske, 2001). It is characterized by mucous hypersecretion, inflammation, reversible airway obstruction, and airway hyper-reactivity (AHR) or bronchial constriction to a variety of non-specific stimuli (Shepherd et al., 2002, Taube et al., 2004). The prevalence of this disease in the developed world has experienced a rapid increase (75%) over the last quarter of a century to the stage where around 10% of the adult population (and even higher in children) are now directly affected, especially in North America, Western Europe, and Australia (Eder et al., 2006). Despite decades of research asthma remains a disease without cure or prevention, with significant progress in the development of new treatments having not been made since the introduction of inhaled glucocorticosteroids over 20 years ago (Adcock et al., 2008, Eder et al., 2006). Although mortality rates have dropped over the past 20 years the ongoing treatments now impose a sizeable financial burden on the health care system, with the cumulative cost of treating asthma worldwide now exceeding $6 billion annually (Barnes, 2004, Shepherd et al., 2002). This will become an even greater concern over the next 10-20 years as the prevalence of asthma has already begun to rise in semi-developed parts of the world such as Latin America, Asia, and Eastern Europe (Adcock et al., 2008, Eder et al., 2006).
The clinically manifested symptoms of asthma include narrowing of the airways leading to difficulty breathing and recurrent wheezing, dyspnea, chest tightness and cough (Tillie et al., 2005). The majority of asthmatics (approximately 75-80%) are also atopic, which is the genetic predisposition for the development of hypersensitivity reactions to common allergens leading to an allergic phenotype (Cookson, 1999, Kay, 2001). Such a state is characterised by immunoglobulin (Ig) E-sensitisation to allergens. However as mentioned above it is important to point out that clinically there is great heterogeneity in asthma, and as such it cannot be classified as having just one disease pathology. Indeed, asthma can be classified or endotyped into multiple different forms of the disease based on the predominant type of inflammatory cell found in the bronchoalveolar lavage (BAL) and sputum (Anderson, 2008, Baines et al., 2011). Although these subtypes may be helpful in classifying severity of disease, the degree to which this endotyping indicates differences in pathogenesis as a pose to differences in exposure to environmental triggers, bacterial colonization, and treatment resistance remains unclear at this stage. Notably, in a percentage of asthmatic cases (~10%) the disease is refractory to the mainstay of conventional treatment with glucocorticosteroids (Barnes and Adcock, 2009, Holgate and Polosa, 2006). This leads to the severest form of disease, referred to as steroid-resistant asthma (Holgate and Polosa, 2006).

I.2 Pathology and Immunology of Asthma

In this literature review the emphasis has been placed on allergic or atopic asthma, as this represents the majority of asthmatics and the focus of the research in this particular thesis. However, asthma may also occur through non-allergic mechanisms, involving non-specific stimuli, which is generally manifested later in life (Maddox and Schwartz, 2002). Allergic asthma is typically considered to be a disease predominately driven by CD4 T helper (Th) 2 lymphocytes. Importantly, there can also be involvement from other Th cell subsets that result in increases in the
severity of this disease and convey its heterogeneity. The immunological mechanisms underlying the prominent inflammation and pathological hallmarks of asthma requires an understanding of the pathways leading to Th cell differentiation. Briefly, antigen presenting cells (APCs), in particular dendritic cells (DCs), detect pathogens through pathogen associated molecular patterns (PAMPs), which activate pathogen recognition receptors (PRRs) (Janeway and Medzhitov, 2002, Medzhitov and Janeway, 2000). In this manner APCs can directly detect the class of pathogen. Alternatively, PAMPs can be detected by other innate immune cells leading to the release of a variety of endogenous tissue factors or damage associated molecular patterns (DAMPs), which then activate APCs as an indirect means of pathogen detection (Janeway and Medzhitov, 2002, Medzhitov and Janeway, 2000, Kaiko et al., 2008). These APCs migrate to secondary lymphoid tissues where they regulate the differentiation of Th cell subsets. The diagrams below summarise the classical pathways of Th cell differentiation leading to the polarization of the different subsets including: regulatory T cells (Treg), Th1, Th2, and Th17 cells (Figure 1 and 2) (Kaiko et al., 2008). Th1 cells (producing IFN-γ) primarily mediate clearance of intracellular bacterial and viral infections. Th2 cells (producing IL-3, IL-4, IL-5, IL-9, and IL-13) mediate protection against large extracellular parasites, such as Helminths. Th17 cells (producing IL-17, IL-22, TNF-α, and IL-6) mediate protection against extracellular bacteria and fungi. Treg cells (producing IL-10 and TGF-β) mediate suppression of Th1, Th2, and Th17 responses and maintain homeostatic balance to protect against overt immunopathology (Kaiko et al., 2008). Th1 and Th2 cells were initially discovered over 20 years ago (Mosmann et al., 1986), however Th17 cells have only risen to prominence over the last 5 years for their role in autoimmune diseases (Harrington et al., 2005, Park et al., 2005a, Aggarwal et al., 2003). For simplicity the broad pathways of the original Th subsets are depicted in Figure 1, and Th17 is added in Figure 2 with the inclusion of greater signaling detail. For a much more detailed explanation of the immunological pathways involved in T cell subset differentiation and the responses produced by each subset please see Appendix 1 review article. The innate
immune pathways involved in this process will also be covered in detail in section I.9 of this Introduction.

Figure 1: Pathways Involved in the Creation of T helper cells. Depicts the events, stages, and molecular factors involved in the interaction between antigen presenting cells (APCs) and naïve T helper (Th0) cells that culminate in the decision to become either a T helper type 1 (Th1), T helper type 2 (Th2) or regulatory T (Treg) cell. PAMP, pathogen associated molecular patterns; PRR, pathogen recognition receptor; TCR, T-cell receptor; Ag, antigen peptide derived from pathogen; MHCII, major histocompatibility complex class II. Reproduced from (Kaiko et al.,
Figure 2: Factors Involved in T cell polarisation to Th1, Th2, and Th17. IFN-γ and IL-12 induce Janus-activated kinase (JAK)1/2 and signal transducer and activator of transcription (STAT)1/3/4 to stimulate T-bet and further IFN-γ production, resulting in a T helper 1 (Th1) response, whereas IL-4 triggers JAK1/3 and STAT6 to activate GATA-3 and a T helper 2 (Th2) response.
response. Features of Th1 cells: Th1 cytokines (IFN-γ, tumour necrosis factor (TNF)-β), and decreased protein kinase C and GATA-3 (IFN-γ and IL-12 mediated). Features of Th2 cells: Th2 cytokines (IL-3, -4, -5, -9, 13), and increased protein kinase C. IL-6 acts to induce IL-21 secretion from T cells, which then acts in concert with transforming growth factor-β (TGF-β) to induce the development of T helper 17 (Th17) cells from naive T cells. Th17 cells are activated by binding of IL-23 to the IL-23 receptor (IL-23R), which is up-regulated by TGF-β and IL-21 stimulation. TGF-β and IL-21 activate STAT3, which induces the transcription factor retinoic acid receptor-related orphan receptor γ-t (RORγt), responsible for Th17 differentiation. Features of Th17 cells: release of IL-17A, IL-17F, IL-6, IL-22, and TNF-α, which activate a variety of innate immune and structural cells. These cells release inflammatory mediators and chemokines that recruit neutrophils and induce tissue inflammation. Reproduced from (Kaiko et al., 2008). © 2008 John Wiley & Sons, permission to copy and communicate this work has been granted by John Wiley & Sons.
In general, because inhalation of innocuous antigen into the respiratory tract is particularly common the body has developed mechanisms to avoid a detrimental inflammatory response. This involves inhaled antigen through the action of DCs rendering CD4 T cells anergic or inducing these naïve T cells to become Treg cells allowing the development of quiescent tolerance to the antigen (McMenamin and Holt, 1993, Chang et al., 2002, Martin et al., 2003). In certain susceptible individuals an innocuous antigen in the respiratory tract may fail to induce tolerance, and in the absence of an overt inflammatory signal then a Th2 response to this antigen is commonly the result. Whether this occurs because of allergic predisposition, a “default” Th2 response of the lung microenvironment, specific Th2-inducing mechanisms of certain allergens, or a combination of these factors remains to be determined (Stumbles et al., 1998, Saenz et al., 2008). Understanding the latter along with how genes and environment interact to create these predisposed individuals forms the basis of much of this thesis.

An allergic Th2 inflammatory response to an innocuous inhaled allergen can lead to the beginnings of an asthmatic phenotype. Th2 cells (as well as basophils and mast cells) secrete a range of cytokines including IL-4 and IL-13, which propagate B cells and create antibody class switching to secrete allergen-specific IgE (Punnonen et al., 1993, Gauchat et al., 1993). Evidence of the critical nature of these Th2 cells in mediating the pathogenesis of asthma was provided in the 1990s by numerous studies demonstrating that lung tissue from asthmatics is infiltrated with activated Th2 cells, and IL-4, IL-5, IL-9 and IL-13 are up-regulated in asthmatic patients (Huang et al., 1995, Robinson et al., 1992, Shimbara et al., 2000). IgE is released into the blood where it binds high affinity IgE receptors (FccRI) on the surface of mast cells and basophils (Busse and Lemanske, 2001, Gauchat et al., 1993). Post-sensitisation exposure to allergen initiates cross-linking of the allergen-specific IgE and mast cell/basophil degranulation (Mossmann et al., 1974, Patterson et al., 1976, Rivera et al., 2008). The constituents of the preformed granules include mainly histamine,
tryptase, chymase, eicosanoids, free radicals, prostaglandin D₂ and E₂, leukotriene C₄, bradykinin, and cytokines (IL-3, -4, -5, -13 and TNF-α) (Clark et al., 1995, Lorentz et al., 2003, Conroy et al., 1990, Rivera et al., 2008). These molecules together induce contraction of airway smooth muscle, mucus secretion, degradation of the epithelium, inhibition of ciliary activity, and vascular leakage (Schroeder et al., 1997, Clark et al., 1995, Ahmed et al., 1981). This sequence of events dominated by mast cells constitutes the early phase reaction of asthma and onsets after minutes lasting a couple of hours (Alam et al., 1996, MacGlashan et al., 1994, Busse and Lemanske, 2001). The release of leukotrienes and inflammatory cytokines elicits the recruitment and activation of eosinophils, Th2 cells, macrophages, and in severe cases neutrophils, which lead to the prolonged late phase inflammatory reaction presenting 4-6 hours later (Durham et al., 1984, Busse and Lemanske, 2001). In addition to allergen, non-specific stimuli including exercise and chemicals (such as diesel exhaust and toluene di-isocyanate) may also precipitate degranulation and AHR (O’Brien et al., 1979, Wardlaw, 1993, Lee et al., 1984).

The late phase reaction is dominated by eosinophilic infiltration and Th2 cells, which become the primary mediators of chronic inflammation in asthma (Durham et al., 1984, Busse and Lemanske, 2001). IL-5 is responsible for selective differentiation of the eosinophil from bone marrow precursors along with its exodus into the circulation and survival (Adachi et al., 1995, Mould et al., 1997, Sehmi et al., 1992, van Oosterhout et al., 1995). A complex network of chemokines released by mast cells, T cells, macrophages, and epithelial cells are attributed to the recruitment of eosinophils into the lung. These chemokines, most of which operate through the chemokine receptor 3 (CCR3), include macrophage inflammatory protein 1α, monocyte chemoattractant protein 3 and 4, eotaxin 1 and 2, and leukotriene C₄ and B₄ (Fonteh et al., 2001, Underwood et al., 1996, Ying et al., 1997, Ying et al., 1999). Eosinophil numbers in the airway wall correlate with disease severity (Walker et al., 1991). Eosinophils once activated are capable of releasing a
cornucopia of inflammatory mediators that ultimately elicit epithelial damage, edema, and perpetuate eosinophil and Th2 cell recruitment to initiate chronic inflammation, AHR and airway remodeling (Egan et al., 1995, Flood-Page et al., 2003, Mauser et al., 1995, Trifilieff et al., 2001, Foster et al., 1996).

Th2 cells are attracted to the lungs via multiple chemokines acting through CCR3 or CCR4 (Bochner et al., 2003). In murine models of asthma, besides playing an integral role in the initiation of allergic disease Th2 cells are also prominent at the effector stage through the secretion of various cytokines IL-4, IL-5, IL-9 and IL-13. IL-4 is important in the initial sensitization to allergen and development of Th2 polarised cells, however it is not critical for the effector phase of the response involving inflammation and AHR (Coyle et al., 1995, Lukacs et al., 1994). In contrast, inhibition of IL-13 during the effector phase abrogates the development of AHR, goblet cell (mucous secreting cell) hyperplasia, and airway remodeling (Wills-Karp et al., 1998, Grunig et al., 1998, Naseer et al., 1997, Zhu et al., 1999). The sources of IL-13 aside from Th2 cells include basophils, eosinophils, mast cells, smooth muscle cells, and CD8+ cytotoxic lymphocytes (Miyahara et al., 2004, Grunstein et al., 2002, Burd et al., 1995, Stassen et al., 2001, Masuda et al., 2002, Hoshino et al., 1999b, Wills-Karp, 2001). IL-13 also upregulates the expression of multiple gene families (including adhesion molecules on endothelium) leading to recruitment and extravasation of further inflammatory cells into the airway spaces (Zimmermann et al., 2003, Faffe et al., 2003, Zhu et al., 1999). It is noteworthy that the prototypical Th1 cytokine IFN-γ and neutrophils can both be elevated in the airways of allergic asthmatics, correlating with severity of disease, and IFN-γ is also capable of activating both neutrophils and eosinophils (Hartnell et al., 1993, Randolph et al., 1999, Hollams et al., 2009). This paradigm will be explored further in other chapters of this thesis.
The chronic aspect of asthmatic inflammation induces a process of ineffective tissue repair and remodeling in the lungs eventuating in structural narrowing of the airways. Briefly, the process involves airway wall thickening as a consequence of increased collagen deposition in the subepithelial basement membrane, epithelial fibrosis, goblet cell hyperplasia, proliferation of myofibroblasts and myocytes, and increased smooth muscle mass (Tschumperlin, 2011, Kuwano et al., 1993). These changes are mediated primarily by elevated levels of leukotrienes, matrix metalloproteases, and transforming growth factor (TGF)-β (Hoshino et al., 1999a, Vignola et al., 1998, Kurosawa et al., 1994, Lee et al., 2001). Airflow obstruction ensues due to a combination of mucous plug formation, edema, and airway narrowing due to remodeling and AHR.
I.3 Etiology of Asthma: Environmental Factors Influencing the Origin of Asthma

Genetics clearly plays a key role in the etiology of asthmatic disease as evidenced by greater concordance in familial and monozygotic twin studies. However, asthma is a complex disease and is not fully heritable and therefore environmental factors play a key role in the origins of asthma (Skadhauge et al., 1999). This is clearly conveyed by the rapid increase of the disease in the developed world over the last 40-50 years far outpacing the rate of genetic drift. Changes in western lifestyle are prime candidates for explaining not just this increase in prevalence but also the etiology of asthma. Genome wide association studies (GWAS) and analysis of single nucleotide polymorphisms (SNPs) have revealed several promising candidate genes, however so far these studies have failed to yield consistency across multiple populations. This is likely a result of the heterogeneity of the disease, differing environmental factors, and diversity among different racial and ethnic groups (Miller and Ho, 2008). Potentially important environmental factors have included maternal in utero conditioning, changes in western diet, increased exposure to indoor allergens, smoking and air pollution, the ‘hygiene hypothesis’, and early-life respiratory infections (Miller and Ho, 2008). Precisely how these environmental factors may contribute to the pathogenesis of asthma remains an area of ongoing investigation. One might hypothesise that these factors may influence programming of the innate immune system, or alternatively act as environmental insults to the respiratory epithelium allowing increased access of antigen/allergen to the underlying immune compartments.

I.3.1 Diet

Many obvious changes have occurred in western diets over the last 50 years that have coincided with increasing rates of allergic diseases, for instance the fat content of western diets has markedly increased. During fetal development there is evidence to suggest that low maternal intake of foods containing vitamin E and zinc may increase the risk of childhood asthma (Devereux et al., 2006).
In contrast, higher maternal intake during pregnancy of antioxidant-rich foods, fruits, vegetables, and certain fish may decrease the risk (Martindale et al., 2005, Miyake et al., 2010). Likewise, diets high in fat are associated with a worsening severity of pre-existing asthma and the opposite appears to be the case for diets high in antioxidants (Wood et al., 2011, Wood and Gibson, 2009). This area of research is only in its infancy and many more prospective studies utilising direct dietary supplementation are required.

### I.3.2 Increased Exposure to Indoor Allergens

An increasingly urbanised lifestyle during the latter half of the 20th century may have led to exposure to higher levels of common inhaled indoor allergens, such as house dust mite (HDM) and molds. Indeed, sensitisation to HDM allergen during infancy is one of the single most important predictive risk factors for the development of childhood asthma (Holt et al., 2010, Sporik et al., 1990) (serum levels of HDM-specific IgE are positively correlated with asthma risk). Moreover, studies observed that exposure to escalating amounts of HDM early in life increased the risk of asthma (Cole Johnson et al., 2004, Sporik et al., 1990). This work originally suggested that exposure to higher doses of HDM was itself a precipitating factor in the inception of asthma. However, this remains somewhat controversial as more recent studies have failed to replicate these dose-related effects instead suggesting that higher levels of HDM exposure lead to increased risk of IgE-sensitization, but are not correlated with the incidence of asthma or wheezing (Carlsten et al., 2010, Carter et al., 2003). Furthermore, the allergen dose effect has also not been supported by studies concerning other allergens, such as animal dander (Platts-Mills et al., 2001). This seemingly suggests that the relative risk for asthma does not differ between exposure to different doses of allergen in an unselected population. The latter findings support the concept that sensitization to indoor allergen plays a critical role in disease pathogenesis in predisposed individuals, however allergen alone is insufficient to initiate asthma.
I.3.3 Smoking and Air Pollution
It is well acknowledged that air pollution in the form of diesel exhaust particles (DEP), sulphur and nitrogen dioxides, and environmental tobacco smoke (ETS) all lead to a worsening severity of pre-existing asthma (McCreanor et al., 2007, Mackay et al., 2010, Baena-Cagnani et al., 2009, Chauhan et al., 2003). More recently however, these pollutants have been linked to a greater risk of an asthma diagnosis. Exposure to these chemicals either in infancy or prenatally, in the maternal in utero environment, is associated with a higher incidence of asthma (Alati et al., 2006, Fedulov et al., 2008, Magnusson et al., 2005, Ponsonby et al., 2001). Interestingly, in urban regions of China, which have one of the world’s highest rates of smoking and 16 of the world’s worst 20 cities for air pollution, the prevalence of asthma is estimated to have risen by 40% between 2000-2005 in parallel with worsening air quality (Watts, 2006)

I.3.4 The ‘Hygiene Hypothesis’
The hallmark publication by Strachan et al., in 1989, generated the original concept that the decreasing incidence of infection and environmental microbial exposure in developed countries underlies the origin of the increasing incidence of allergic diseases (Strachan, 1989). This became known as the ‘hygiene hypothesis’. This original epidemiological evidence pointed to an inverse correlation between hay fever and the number of older siblings inferring that shared infections between siblings in early-life protected against allergic diseases. Similar evidence has been provided for day care attendance in the first 6 months of life and reduced risk of asthma (Ball et al., 2000). Throughout the 20th century public health measures in developed countries led to decreasing incidences of infection due to decontamination of the water supply, sterilization of milk and other foods, vaccination, use of antibiotics, and smaller family sizes (Okada et al., 2010). In fact the geographical distribution of allergic diseases is a mirror image of the geographical distribution of declining infectious diseases such as hepatitis A, gastrointestinal diarrhea infections, and parasitic infections (Bach, 2002). Furthermore, Schistosoma, Toxoplasma gondii, hepatitis A, and herpes
simplex virus-1 infections have all been directly associated with a protective effect against asthma (Matricardi et al., 2002, van den Biggelaar et al., 2000). These infections are particularly common in Africa and parts of Asia where health standards are lagging, although the same protective effect can be seen on an individual basis within the USA. Perhaps the most impressive of all epidemiological findings in support of the ‘hygiene hypothesis’ are those derived from the study of children of European farmers. Children who grow up in farming environments show lower levels of allergy and asthma, especially if the mother is exposed during pregnancy, compared with children of the same age living in the same villages, but not in farming environments (Riedler et al., 2001). Further investigation suggested that this difference is due to early-life contact with cowsheds, farm animals, gastrointestinal exposure to unpasteurised milk, and greater inhaled endotoxin exposure in dust (Gereda et al., 2000, Riedler et al., 2001).

A causal relationship between these observations can only be provided by animal models and interventional human studies. In this regard, evidence is still in its infancy, however, neonatal BCG vaccination reduces asthma risk in humans and OVA-induced airway inflammation in mice (Hopfenspirger et al., 2001, Linehan et al., 2007). Furthermore, administration of a bacterial species commonly identified in farm barn dust, Acinetobacter Iwofi, postnatally or prenatally to pregnant dams has been demonstrated to impart a strong resistance against allergic reactions in mice (Conrad et al., 2009, Debarry et al., 2007). This suggests that controlled microbial stimulation at the mucosal surface of the lung during pregnancy, eliciting sub-clinical inflammation, is sufficient to dampen immune activity and allergic disease in the offspring. This effect was dependent on maternal TLR signaling. Interestingly, the intestinal flora of allergic children has also been suggested to differ to that of non-allergic children, with more aerobic bacteria, such as Staphylococcus aureus and Clostridia, and a decreased proportion of Lactobacilli, or anaerobes such as Bifidobacterium and Bacteroides (Bjorksten et al., 1999, Kalliomaki et al., 2001a).
Following on from this work, several randomized placebo-controlled trials of *Lactobacillus* supplementation in pregnant mothers have been conducted. However, after showing initial promise in protection against allergy and asthma, more recent trials have been quite disappointing (Kalliomaki et al., 2001b, Kalliomaki et al., 2007, Kopp et al., 2008, Kuitunen et al., 2009, Kukkonen et al., 2011). The possible mechanisms underlying the ‘hygiene hypothesis’ are a matter of ongoing debate. One possibility is insufficient immune deviation from Th2 to Th1, due to a lack of shifting of the allergen-specific response away from a neonatal Th2 phenotype due to reduced microbial burden. Alternatively, allergic inflammation may occur as the result of defective immune suppression created by a lack of microbial-induced Treg activity.

**I.3.5 Early-life Respiratory Infections**
This environmental factor is the subject matter of this thesis and will be reviewed in greater detail in section I.5.2 of this Introduction. Early-life viral bronchiolitis dramatically increases the risk for the development of childhood asthma (Sigurs et al., 2000, Holt et al., 2010). It is impossible to determine whether the rates of early-life respiratory infections due to Respiratory Syncytial Virus (RSV), Rhinovirus (RV), and others have changed over the last century as many of these viruses were only fully identified less than half a century ago. However, even if the rates have remained constant along side the increasing prevalence of asthma this by no means indicates that these infections are not critical. Rather the increasing rates of asthmatic disease may involve an interplay between these respiratory infections and another environmental factor that has seen more recent evolution, such as a lower microbial burden. Respiratory infections are also a major cause of acute exacerbation of pre-existing asthma.
1.4 *Chlamydomphila pneumoniae*

There are three known species of *Chlamydia* that can act as obligate intracellular human pathogens. *Chlamydia trachomatis* is the causative agent of genital tract and ocular infections (Beagley and Timms, 2000). *Chlamydomphila pneumoniae* (Cpn) infects the respiratory tract and is responsible for 10-20% of community acquired pneumonia (Kuo et al., 1995, Gibbs et al., 1998, Hahn, 1999). *C. psittaci* (Chlamydomphila psittaci) is primarily a pathogen of psittacine birds but infection may lead to atypical pneumonia or pyrexia in man (Eugster, 1980). Cpn is an atypical gram-negative intracellular bacteria that can elicit chronic persistent infection in various diseases such as pneumoniae, bronchitis and sinusitis (5% worldwide cases) (Kuo et al., 1995). Interestingly, recent evidence has also established an association between Cpn and both atherosclerosis and asthma (Gibbs et al., 1998, Hahn, 1999). It is estimated that approximately 50-80% of adults have Cpn-specific antibodies to the bacterium, hence most individuals will experience a Cpn infection at some point in their lifetime (Kuo et al., 1995). Chlamydia has an intracellular development cycle (Figure 3), which involves the ability to form non-replicating bodies under adverse environmental conditions (e.g. unfavourable immune response, antibiotic treatment) that retain their viability in a dormant state and can be reactivated to permit a latent infection (Beatty et al., 1995). Cpn requires a strong cell-mediated Th1 immune response, dominated by IFN-γ and activated macrophages, for protective clearance (Ito and Lyons, 1999, Rottenberg et al., 1999). An inability to mount an adequate *Chlamydia*-specific Th1 response can often lead to persistence of the bacteria and associated immunopathology in the host. Notably, DC-derived IL-12 is essential for the generation of this Th1 response (Farrar et al., 2002, Lu et al., 2000). Cpn is primarily a pathogen of epithelial cells, however, it can infect and proliferate in a range of cell types of the respiratory tract including vascular endothelial, and smooth muscle cells as well as immune cells such as alveolar macrophages,
neutrophils, and DCs (Godzik et al., 1995, Gaydos et al., 1996, Matyszak et al., 2002, Hook et al., 2005). Infection of the mesenchymal cells exhibits the release of a variety of pro-inflammatory mediators such as TNF-α, IL-1, IL-8, and IL-12 (Ingalls et al., 1995). Cpn also has the ability to inhibit ciliary function in bronchial epithelial cells, and create deleterious airway inflammation (Holland et al., 1993, Shemer-Avni and Lieberman, 1995).

**Figure 3: Schematic Diagram of the Development Cycle of Chlamydia.** 1) Elementary bodies (EBs) attach to the membrane of the host cell. 2) EBs are endocytosed and become encapsulated in a phagosome that does not fuse with lysosomes. 3) EBs transform into reticular bodies (RBs), which begin to replicate. 4) RBs begin to transform back into EBs. 5) Chlamydial bodies are released by cytolysis. EBs are then able to reinitiate the cycle in other host cells. 6) Under adverse conditions, such as antibiotic therapy, RBs are able to form dormant persistent bodies (PBs), which are able to re-enter the development cycle as RBs when conditions become more favourable.
I.4.1 Association of Chlamydophila pneumoniae with Acute Exacerbations of Pre-existing Asthma

Epidemiologically there are significant links between Cpn infection and asthma exacerbation, especially in adults, as Cpn infection is more prevalent in asthmatics than healthy controls (Ronchetti et al., 2005). Interestingly, the increasing Cpn seroprevalence in the population also correlates with an increasing prevalence of asthma in the community over the last quarter of a century (Ferrari et al., 2002, Park et al., 2005b, Puolakkainen et al., 1989). Additionally, many adults with diagnosed asthma and many children with high frequencies of asthmatic exacerbations have evidence of a chronic infection with Cpn (Cunningham et al., 1998). One study in particular carried out by Black et al. demonstrated a correlation between increases in asthma severity and increases in patient antibody titres to Cpn (Black et al., 2000). Furthermore, acute Cpn infection has been suggested to precipitate exacerbation of asthma, as Wark et al. observed that as many as 38% of asthmatic subjects with acute exacerbations demonstrated clear serological evidence of an acute Cpn infection (Pizzichini et al., 1997, Wark et al., 2002). Based on this evidence Cpn has been associated with the exacerbation of asthma and increases in severity of the disease. This is particularly poignant as current therapies are ineffective against both acute exacerbations and severe asthma. However, it remains possible that this association could be a reflection of opportunistic behaviour of Cpn, infecting asthmatic individuals with their ineffectual Th2 dominance failing to quickly eliminate the bacteria. To answer these questions improved clinical studies must be designed with larger study populations and interventional antibiotic therapy against Cpn. More direct experiments using murine models have also begun to be employed by using the mouse homolog to Cpn known as Chlamydia muridarum (Cmu) (Fan et al., 1999). Increased expression of IL-10 in response to Cmu has been shown to suppress Th1 responses and delay resolution of infection (Yang et al., 1996, Yang et al., 1999). In a murine model of ovalbumin (OVA)-driven allergic airways inflammation, superimposing a Cmu
infection led to enhancement of inflammation but a mixed Th1/Th2 cell response (Horvat et al., 2007). In contrast, other data suggests that Cmu inhibits airway inflammation and Th2 responses to ragweed (Bilenki et al., 2002). The discrepancy in these studies likely lies with the genetic background of the mice, as the latter study used C57BL/6 mice, which are very strong Th1 responders to Chlamydia.
I.5 Respiratory Syncytial Virus (RSV)

RSV is a member of the family Paramyxoviridae genus *Pneumovirus*. It is a pleomorphic enveloped virus with a helical nucleocapsid within which lies a negative sense single stranded RNA genome containing 10 genes that encode 10 proteins (Collins and Graham, 2008, Graham et al., 2002, Melero et al., 1997). These proteins include the envelope G glycoprotein (see Figure 4), which mediates attachment of the virus to respiratory epithelial cells (Collins and Graham, 2008, Graham et al., 2002, Melero et al., 1997). The F glycoprotein facilitates fusion of the viral envelope with the plasma membrane of the host cell, permits insertion of the nucleocapsid and RNA into the cellular cytoplasm, and promotes cell-to-cell fusion creating multinucleated giant cells or syncytia (Collins and Graham, 2008, Graham et al., 2002, Melero et al., 1997). The G and F proteins are the major antigenic determinants of RSV and induce production of neutralising antibodies. The G protein is variable and forms the basis for subgroup classification into RSV type A and B (Melero et al., 1997). Other RSV proteins include those that are envelope associated, the small hydrophobic (SH) protein, the matrix (M) protein, and the M2 protein, nucleocapsid proteins like the nucleoprotein (N), the phosphoprotein (P), and the large nucleoprotein (L) (Collins and Graham, 2008, Graham et al., 2002, Melero et al., 1997). Non-structural proteins 1 and 2 (NS1 and NS2) are found only in infected cells but not in virions (Simoes, 1999, Ruuskanen and Ogra, 1993, Openshaw et al., 2003). Like other members of the Paramyxoviridae family, RSV is transmitted in respiratory droplets and initiates infection in the upper respiratory tract before spreading to the lower airways (Wennergren and Kristjansson, 2001). Importantly, RSV is a human virus typically restricted to the respiratory tract but is capable of replicating to a certain extent in other animal species (Ruuskanen and Ogra, 1993).
I.5.1 RSV and Disease
RSV is the primary cause of hospitalisation in the first year of life for children worldwide, and is responsible for the majority of lower respiratory tract (LRT) infections in infants (Nair et al., 2010, Simoes, 1999). By 2 years of age more than 95% of infants have been infected with RSV (Graham et al., 2002, Simoes, 1999, Sigurs, 2002). RSV infection can range in disease from upper respiratory tract (URT) infection, and LRT infection, to acute respiratory distress syndrome and respiratory failure. The LRT infection, which is often the symptomatic expression of a primary RSV infection in the first 2 years of life, typically manifests clinically as bronchiolitis (Nair et al., 2010, Simoes, 1999). This is characterised by coryza, low-grade fever, cough, tachypnoea, hyperinflation of the chest, cyanosis, apnoea, and wheezing, and in some cases can lead to pneumonia (Smyth and Openshaw, 2006). The majority of infants initially exposed to RSV develop LRT infection, however only a proportion of these infants develop severe bronchiolitis disease. Approximately, 2-3% of all infected children (virtually the entire infant population) are hospitalised for severe bronchiolitis annually (Nair et al., 2010, Simoes, 1999). In the USA alone this equates to 100,000-200,000 hospitalisations per year at a cost in excess of US$300 million (Sigurs, 2002, Openshaw, 2005, Henrickson et al., 2004). Worldwide there is a seasonal pattern of annual epidemics that occur during the winter months. The risk factors for severe RSV bronchiolitis include age less than 6 months, siblings, day care exposure, male gender, and birth during the initial part of the RSV season (Simoes, 2003). RSV URT infection is more prevalent in a healthy adult, the symptoms of which include those of a common cold. The groups with increased mortality to RSV infection include premature neonates, the elderly, patients with cardiac abnormalities, and immunosuppressed individuals (Nair et al., 2010, Simoes, 1999, Ogra, 2004).
The pathophysiology of RSV infection involves a viral incubation period of 3-5 days, after which infection of the bronchial epithelial cells leads to mucosal inflammation, oedema, inflammatory cell influx, tissue necrosis, mucus plugs and altered surfactant composition, all of which eventuates in airway obstruction (Wennergren and Kristjansson, 2001). It is becoming increasingly apparent that the physiological signs of the disease are created less by the direct viral cytopathic effect and more by the host’s aberrant immune response to the infection (Openshaw, 2005, Varga and Braciale, 2002). One of the most unique characteristics pertaining to this acute infection is that it fails to induce long term protective immunity, and therefore the host is conducive to re-infection throughout life with the same serotype of RSV (Bueno et al., 2008, Chang and Braciale, 2002, Graham et al., 2002). The reason for the paucity of immune memory remains largely unknown. The latter is of even greater significance considering that there is still no truly effective treatment or vaccine available for RSV. Although passive immunisation of the monoclonal antibody Palivizumab has shown efficacy in preventing severe infection in preterm infants this antibody can only be administered prophylactically and only to known high-risk patients, which greatly limits its use (Hu and Robinson, 2010). Treatment for severe RSV infection remains as supportive care, including pulmonary/nasal toilet, supplemental oxygen, and in the most severe cases mechanical ventilation (Lusebrink et al., 2009).

I.5.2 RSV and Asthma
It has long been recognised that infants suffering from a severe RSV infection, leading to infantile bronchiolitis, are at significantly greater risk for the development of asthma in later childhood (Sigurs et al., 2000, Sigurs et al., 2005, Simoes, 1999, Henderson et al., 2005, Kusel et al., 2007, Stein et al., 1999, Pereira et al., 2007). This relationship was first intimated as early as 1978 when a retrospective study, from Sims et al., assessed 8-year old children who had been previously hospitalised with RSV bronchiolitis in infancy. They discovered that 51% of these index children showed the presence of recurrent wheezing/asthma versus only 3% in the control
group (Sims et al., 1978). Although this association was consistently exhibited clinically it was not until 2000 that large scale prospective studies could confirm its validity. Sigurs et al. reported on the first prospective study where a cohort of infants hospitalised for RSV bronchiolitis in early-life were then examined at ages 1, 3, and 7 years for the presence of asthma. At 7 years asthma was 10-times more prevalent in the RSV group than the controls (Sigurs et al., 2000). Overall, between 25-50% of children admitted to hospital for RSV bronchiolitis are observed in later years to develop asthma, with the variation depending on the study population (see Table 1) (Sigurs, 2002, Sims et al., 1978, Gurwitz et al., 1981, Sigurs et al., 2000). Importantly, recent studies now show that this association between RSV bronchiolitis and later asthma can even be traced out to 18 years of age (Sigurs et al., 2010)
Table 1: Summary of the clinical studies examining the association between RSV bronchiolitis and recurrent wheezing

<table>
<thead>
<tr>
<th>Year of publication</th>
<th>Number of subjects (index/control)</th>
<th>Hospitalised in infancy</th>
<th>Age at follow up (years)</th>
<th>Presence of recurrent wheezing/asthma (index vs control)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1978</td>
<td>35/35</td>
<td>Yes</td>
<td>8</td>
<td>51% vs 3%*</td>
<td>(Sims et al., 1978)</td>
</tr>
<tr>
<td>1982</td>
<td>130/111</td>
<td>Yes</td>
<td>10</td>
<td>42% vs 19%*</td>
<td>(Pullan and Hey, 1982)</td>
</tr>
<tr>
<td>1984</td>
<td>59/177</td>
<td>No</td>
<td>8</td>
<td>44% vs 14%^</td>
<td>(McConnachie and Roghmann, 1984)</td>
</tr>
<tr>
<td>1987</td>
<td>51/24</td>
<td>Yes</td>
<td>2</td>
<td>60% vs 4%^</td>
<td>(Carlsen et al., 1987)</td>
</tr>
<tr>
<td>1992</td>
<td>73/73</td>
<td>Yes</td>
<td>6</td>
<td>43% vs 15%^</td>
<td>(Murray et al., 1992)</td>
</tr>
<tr>
<td>1993</td>
<td>70/70</td>
<td>Yes</td>
<td>2</td>
<td>44% vs 13%^</td>
<td>(Osundwa et al., 1993)</td>
</tr>
<tr>
<td>1995</td>
<td>47/93</td>
<td>Yes</td>
<td>3</td>
<td>23% vs 1%^</td>
<td>(Sigurs et al., 1995)</td>
</tr>
<tr>
<td>1997</td>
<td>61/47</td>
<td>Yes</td>
<td>9</td>
<td>34% vs 13%^</td>
<td>(Noble et al., 1997)</td>
</tr>
<tr>
<td>2000</td>
<td>47/93</td>
<td>Yes</td>
<td>7</td>
<td>30% vs 3%*</td>
<td>(Sigurs et al., 2000)</td>
</tr>
<tr>
<td>2010</td>
<td>46/92</td>
<td>Yes</td>
<td>18</td>
<td>39% vs 9%*</td>
<td>(Sigurs et al., 2010)</td>
</tr>
</tbody>
</table>

* Cumulative rates of wheezing or asthma, ^ rates at the specific age of follow up.

Whether RSV bronchiolitis is associated with increased risk of subsequent allergic sensitisation or atopy remains controversial. For instance, Sigurs et al. also reported that children admitted to hospital with RSV bronchiolitis as infants had significantly greater risk of allergic sensitisation and allergic rhinoconjunctivitis at both 7 and 18 years of age (Sigurs et al., 2000, Sigurs et al.,
2010). However, a more recent birth cohort study suggested that there was no difference in the rates of atopic disease compared to controls by 7 years of age (Henderson et al., 2005). The disparity in these results could potentially be a consequence of the inability to control for different genetic backgrounds and environmental allergen exposures when selecting these cohorts.

Aside from a predisposition to atopy, the most significant risk factor for the development of asthma is one or more early-life LRT viral infections, typically with RSV or Rhinovirus (RV) (Simoes, 1999, Simoes, 2001, Sigurs et al., 2000). Furthermore, the moderate predictive risk of asthma that is associated with IgE sensitisation to house dust mite by 2 years of age is increased dramatically (up to 7-fold) with increasing numbers of severe early-life LRT infections (RSV or RV) (Holt et al., 2010). Similarly, for children with at least one atopic parent the predictive risk of asthma is dramatically increased (5-fold) following RSV bronchiolitis in the first year of life (Sigurs et al., 2010).

A long-term prospective study from Simoes et al., attempted to further delineate whether this was a causal association between bronchiolitis and asthma by examining respiratory outcomes following passive immunisation against RSV (using Palivizumab) (Simoes et al., 2007). By preventing hospitalization due to severe RSV infection the risk of recurrent wheezing in later childhood was reduced by 50%. The relative risk was reduced by as much as 80% up to 5 years later, in children with no past family history of atopy (Simoes et al., 2010). Treatment with polyclonal immune globulins against RSV has also been demonstrated to reduce the incidence of childhood asthma 7 years later (Wenzel et al., 2002). Collectively, these studies have highlighted that severe RSV infection is not simply a marker of asthma predisposition but instead they provide support for a causal relationship between early-life viral exposure and childhood asthma.
Severe RSV infection in infancy alone, is however insufficient to initiate asthmatic disease as many of the affected infants do not go on to develop asthma. A genetic predisposition to asthma is most likely also required (Skadhauge et al., 1999). How the genes, environment, and respiratory infection interact in a multi-hit hypothesis for the inception of asthma remains to be discovered. One hypothesis might be that viral bronchiolitis at a critical time in the development of both the respiratory and immune systems, when occurring in individuals who are genetically/epigenetically susceptible to cytokine dysregulation, might lead to the outgrowth of persistent wheezing and then asthma in childhood (Figure 5).

**Figure 5: Two-hit hypothesis of asthma.** Genetic and environmental factors interact at a critical point in the development of the respiratory or immune systems to induce an asthmatic phenotype.
Aside from a role in the pathogenesis of asthma and wheezing, re-infections with RSV in later-life are responsible for acute exacerbations of pre-existing asthma. Viral respiratory infections can be detected in 85% of childhood asthma exacerbations (Johnston et al., 1995), and over 50% of adult exacerbations (Nicholson et al., 1993). RV appears to be responsible for the vast majority of these exacerbations, however RSV is still an important factor behind the seasonal peaks of asthmatic hospital admissions, especially in younger children (Johnston et al., 1995, Schaller et al., 2006, Message et al., 2008).
I.6 RSV and CD4 T Helper (Th) Responses

I.6.1 The CD4 T Helper Cell Response to RSV in Humans

Over the past 3 decades there have been numerous clinical studies attempting to delineate the precise nature of the T cell response induced by RSV in humans. This has been an important avenue of investigation not only to understand the acute immunopathology induced by the virus but also the longer term persistent wheeze and relationship of RSV with childhood asthma. As virtually all individuals are exposed to RSV within the first 2 years of life, the majority of infections having a mild-course, the acute Th cell response to RSV must be studied in an infant as adult re-exposure to the virus involves the interference of immunological memory. Studies attempting to understand the Th response have employed tissue collection ranging from serum and upper and lower airway secretions to T cell specific analysis in PBMCs. An imbalance between Th1 and Th2 responses to RSV, which requires Th1 and CD8 T cell-mediated immunity for clearance, would clearly represent a deleterious response. In response to infantile RSV infection some studies suggest the emergence of a predominately Th1 response, whereas others suggest the induction of a mixed Th cell response or “cytokine storm” with a Th2 component (see detailed summary of studies in Table 2). Importantly, when patients are stratified for increasing clinical severity such that RSV LRT bronchiolitis is compared to RSV URT infection and healthy controls, the Th2 cytokines appear to have greater presence within bronchiolitis patients. Furthermore, with increasing clinical severity of bronchiolitis, measured by persistent wheezing and degree of hypoxia (saturation of oxygen; SaO₂), there is also a propensity for elevated Th2 cytokines compared to more mild forms of LRT infection. Similarly, levels of IFN-γ from PBMCs appear to be reduced in infants with the severest forms of RSV bronchiolitis and persist at low levels long-term in those who subsequently develop asthma (Renzi et al., 1999). However, other studies utilising nasopharyngeal upper airway secretions rather than lymphocytes
have been unable to replicate these findings for IFN-γ (Pino et al., 2009). Interestingly, plasma cortisol levels are also inversely correlated in infantile bronchiolitis with IFN-γ (Pinto et al., 2006). As Th1 and Th2 are mutually antagonistic responses it is important in studies of this nature to not simply examine these cytokines in exclusivity but to look at the ratio of Th2 cytokines with IFN-γ. Other studies have suggested that there might be a global increase in both Th1, Th2, and Th17 cytokines (“cytokine storm”) in the acute phase of infection persisting up to one year post-bronchiolitis (Pino et al., 2009). The elevated levels of Th2 cytokines are not simply restricted to CD4 T cells either, as CD8 T cells from infantile RSV bronchiolitis have been shown to produce increased IL-4 by flow cytometry (Bendelja et al., 2000). Interestingly, when infants with RSV-bronchiolitis were subdivided into those with or without eosinophil infiltrates in the bronchoalveolar lavage (BAL), the levels of Th2 cytokines were discovered to be substantially higher in the eosinophil-positive group compared to eosinophil-negative or healthy infants. Furthermore, the levels of IL-5 and IFN-γ from infants with eosinophilic RSV-bronchiolitis closely resembled the high levels typically found in older children suffering from asthma (Kim et al., 2003). Overall, the bulk of evidence does appear to suggest that a mild RSV infection induces a robust Th1 response, however in the case of RSV bronchiolitis more of a mixed Th1/Th2 response seems more prominent. Due to the diversity of studies and contradictory nature of some results between different populations, it is problematic to make any strong conclusions in this regard. Perhaps what can be concluded from these studies is that rather than all cases of infantile bronchiolitis necessarily producing Th2 cytokines, a subset of these infants which are likely genetically predisposed (eosinophil-positive) develop a strong and lasting inappropriate Th2 response as a result of early-life viral infection. This would reflect a 2-hit hypothesis for the development of recurrent wheezing and asthma.

One novel approach to examining the nature of RSV-induced T cell responses, involved using overlapping peptides spanning the RSV G protein in order to stimulate recall responses of
PBMCs (Hancock et al., 2001). This study revealed that PBMCs from asthmatics more frequently produced IL-13 and IFN-γ in response to the G protein, compared to healthy donors whose PBMC response was dominated by IL-10.

The question as to whether a mixed Th1/Th2 response in cases of RSV bronchiolitis are viral-specific or determined instead by the age of initial exposure to any LRT infection has been the subject of much conjecture. One hypothesis is that any severe viral infection descending into the lower airways at a critical time of early-life immune and respiratory development is likely to lead the outgrowth of Th2 responses and airway dysfunction. Kristjansson et al., attempted to address this point finding that although RSV infection increased IL-4 and eosinophil cationic protein (ECP) in nasal lavage compared to healthy infants, there was no difference in the levels of these mediators between infants less than 3 months old with RSV, influenza A or parainfluenza (Kristjansson et al., 2005). There are, however, major caveats in this study, firstly the actual relative ratio of IL-4:IFN-γ was higher with RSV compared to the other viruses. Secondly and most importantly, these infants were not clinically selected for RSV bronchiolitis and so mild RSV infection is included. Furthermore, although an age of less than 6 months at initial infection is a risk factor for increased severity of RSV bronchiolitis, the development of this disease before and after 6 months of age have both been shown to give rise to the outgrowth of Th2 cytokines and chemokines (Bendelja et al., 2000, Barens et al., 2003, Becker et al., 1997). An earlier study examining a similar dynamic demonstrated that elevated IL-4 and IL-5 in the serum of RSV bronchiolitis infants was indeed viral-specific as age matched influenza-infected infants produced significantly lower levels of these cytokines (Sung et al., 2001). Comparisons specifically with rhinovirus to address the above question are in most cases inherently misleading due to the fact that rhinovirus infections typically manifest in older infants with a much higher rate of atopy. Hence, comparisons that have been made concerning Th2 cytokines and eosinophils in rhinovirus-induced wheezing episodes compared to RSV-induced wheezing are
difficult to interpret (Jartti et al., 2009). One critical question that all these studies do not address is whether a skewed Th1/Th2 response exists prior to infection (at birth) or whether the innate anti-viral response intrinsic to these individuals is different (during infection) leading to viral spread and to an aberrant Th1/Th2 balance. In other words does the skewed cytokine pattern exist prior to RSV infection (defects in cytokine genes) or is it induced by the course of severe infection.

Table 2: Summary of human studies examining Th1 vs Th2 cytokine production in RSV infection

<table>
<thead>
<tr>
<th>Group comparison</th>
<th>Scale of study *</th>
<th>Th1 vs Th2</th>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventilated RSV/bronch compared to non-ventilated RSV/bronch</td>
<td>Medium</td>
<td>Lower proliferation, IL-4, and IFN-γ, and higher IL-8</td>
<td>PBMCs stimulated with PHA</td>
<td>(Bont et al., 1999)</td>
</tr>
<tr>
<td>RSV/bronch compared to healthy infants</td>
<td>Medium</td>
<td>Higher ratio IL-4:IFN-γ</td>
<td>PBMCs stimulated with PHA</td>
<td>(Roman et al., 1997)</td>
</tr>
<tr>
<td>RSV/bronch compared to healthy infants</td>
<td>Medium</td>
<td>Higher IL-4, lower IFN-γ</td>
<td>PBMCs stimulated with PMA/iono</td>
<td>(Bendelja et al., 2000)</td>
</tr>
<tr>
<td>Eosinophil-positive RSV/bronch compared to eosinophil-negative or healthy infants</td>
<td>Medium</td>
<td>Higher IL-5 no difference in IFN-γ</td>
<td>Bronchoalveolar lavage fluid</td>
<td>(Kim et al., 2003)</td>
</tr>
<tr>
<td>Eosinophil-positive RSV/bronch compared to acute asthma</td>
<td>Medium</td>
<td>No difference in IL-5 or IFN-γ</td>
<td>Bronchoalveolar lavage fluid</td>
<td>(Kim et al., 2003)</td>
</tr>
<tr>
<td>RSV/bronch compared to healthy infants</td>
<td>Medium</td>
<td>Short-term: higher IL-4, IL-5, IL-8, IFN-γ, 1 year follow-up: higher IL-13 and IFN-γ</td>
<td>Nasopharyngeal aspirate</td>
<td>(Pino et al., 2009)</td>
</tr>
<tr>
<td>RSV/bronch compared to RSV URTI (with at least one atopic parent with asthma)</td>
<td>Medium</td>
<td>Higher ratio of IL-4: IFN-γ</td>
<td>Nasopharyngeal aspirate and PBMCs stimulated with PHA</td>
<td>(Legg et al., 2003)</td>
</tr>
<tr>
<td>RSV-infected infants compared to infants with other respiratory viral infection (influenza and parainfluenza)</td>
<td>Large</td>
<td>No difference in IL-4 but lower IFN-γ and IL-5</td>
<td>Nasopharyngeal aspirate</td>
<td>(Kristjansson et al., 2005)</td>
</tr>
<tr>
<td>RSV-infected infants compared to healthy infants</td>
<td>Large</td>
<td>Higher IL-4 and no difference in IL-5</td>
<td>Nasopharyngeal aspirate</td>
<td>(Kristjansson et al., 2005)</td>
</tr>
<tr>
<td>Rhinovirus infected wheezing infants compared to RSV-infected wheezing infants and healthy infants</td>
<td>Large</td>
<td>Higher IL-5, IL-13, and IFN-γ. No difference in IL-4</td>
<td>Serum</td>
<td>(Jartti et al., 2009)</td>
</tr>
<tr>
<td>RSV/bronch (hypoxic)</td>
<td>Large</td>
<td>Hypoxic: lower IL-13</td>
<td>Nasopharyngeal</td>
<td>(Garofalo et al., 2005)</td>
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and non-hypoxic) compared to RSV URTI

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Study Scale</th>
<th>Observations</th>
<th>Sample Type</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>RSV-infected infants compared to influenza infected infants</td>
<td>Medium</td>
<td>Higher IL-4 and IL-5</td>
<td>Serum</td>
<td>(Sung et al., 2001)</td>
</tr>
<tr>
<td>RSV/bronch compared to URTI</td>
<td>Medium</td>
<td>Lower ratio of IL-4: IFN-$\gamma$</td>
<td>Nasopharyngeal aspirate</td>
<td>(van Schaik et al., 1999)</td>
</tr>
<tr>
<td>Tonsillar lymphocytes from children with no respiratory complications bound to RSV-infected epithelial cells compared to unbound lymphocytes</td>
<td>Small</td>
<td>Higher IL-4 and IL-5 but no change in IFN-$\gamma$</td>
<td>Tonsillar lymphocytes stimulated with PHA</td>
<td>(Matsuzaki et al., 1996)</td>
</tr>
<tr>
<td>Severe RSV/bronch compared to mild RSV infection</td>
<td>Small</td>
<td>Lower IFN-$\gamma$</td>
<td>PBMCs stimulated with RSV</td>
<td>(Brandenburg et al., 2000)</td>
</tr>
<tr>
<td>RSV/bronch compared to healthy infants</td>
<td>Large</td>
<td>Higher IFN-$\gamma$ and IL-8</td>
<td>Nasopharyngeal aspirate</td>
<td>(Choi et al., 2010)</td>
</tr>
<tr>
<td>Severe RSV/bronch compared to mild RSV/bronch</td>
<td>Large</td>
<td>Lower IFN-$\gamma$ no difference in IL-4 and IL-5</td>
<td>PBMCs</td>
<td>(Pinto et al., 2006)</td>
</tr>
<tr>
<td>RSV/bronch compared to non-RSV/bronch, and severe RSV/bronch compared to mild RSV/bronch</td>
<td>Large</td>
<td>Higher ratio of IL-4: IFN-$\gamma$</td>
<td>Serum</td>
<td>(Hassan et al., 2008)</td>
</tr>
</tbody>
</table>

*Study scale described as small (<20 patients), medium (20-50 patients), large (>50 patients).

RSV/bronch, RSV bronchiolitis; URTI, upper respiratory tract infection; PHA, phytohaemagglutinin; PMA/iono, phorbol myristate acetate and ionomycin

### I.6.2 RSV and the IgE Response in Humans

Despite being notoriously difficult to detect a strong indicator of both allergy and the outgrowth of a Th2 response to a particular antigen is the appearance of antigen-specific IgE. RSV-specific IgE was initially linked to RSV bronchiolitis in a study some 30 years ago, when it was detected within nasopharyngeal secretions of the majority of cases of RSV-induced wheezing but in only 5% of cases of mild RSV in the acute phase of infection (Welliver et al., 1981). Furthermore, the IgE titre was positively correlated with both the degree of hypoxia and levels of the eosinophil and mast cell inflammatory mediator leukotriene C4 (LTC4) (Volovitz et al., 1988, Welliver et
al., 1981). Other groups have demonstrated RSV-specific IgE in acute phase serum also correlates with disease severity (Volovitz et al., 1988, Bui et al., 1987). Furthermore, B cells from RSV-infected patients express elevated levels of CD23, the low-affinity IgE receptor (Rabatic et al., 1997). Despite this, the existence of RSV-specific IgE remains somewhat controversial as more recent studies have failed to re-produce these results in similar patient cohorts (De Alarcon et al., 2001, Toms et al., 1996). Intriguingly the converse of this relationship appears to exist in allergic asthma, where higher titres of IgG antibodies to the RSV G protein have been observed in asthmatic patients compared to healthy controls (Hancock et al., 2001). Indeed the levels of these antibodies were positively correlated with the clinical severity of asthma. This data reflects the epidemiological evidence suggesting that RSV bronchiolitis is a major risk factor for the sensitisation to inhaled allergens in later childhood (Sigurs et al., 1995).

I.6.3 RSV, Eosinophils and Mast Cells in Humans

A key observation linking the Th2 effector cell, the eosinophil, to RSV-induced wheezing is that persistence of eosinophilia in the peripheral blood during bronchiolitis predicts which particular infants will go on to develop long-term recurrent wheezing through school age (Ehlenfield et al., 2000). These observations concur with eosinophil-positive RSV-bronchiolitis patients having a substantially higher IL-5:IFN-γ ratio compared to eosinophil-negative bronchiolitis (Kim et al., 2003). Infants with RSV bronchiolitis appear to have higher numbers of peripheral blood eosinophils than RSV URT infections, however this trend is relatively weak perhaps because sampling of peripheral blood conceals the possibility of heightened eosinophil extravasation into the inflamed lung tissue (Garofalo et al., 1994). In support of this possibility there is strong evidence that the level of degranulated ECP in nasopharyngeal aspirates positively correlates with the severity of RSV infection (Garofalo et al., 1994, Garofalo et al., 1992, Oymar et al., 1996). ECP concentrations were also observed to be substantially higher in infants with RSV
bronchiolitis compared to RSV URT infection, non-RSV URT infection, other non-RSV bronchiolitis, pneumonia, and healthy controls. Although this likely indicates an important role for these cells in disease, it is important to note that the predominant inflammatory cell in the airways during RSV bronchiolitis is the neutrophil, which accounts for approximately 90% of cells from nasopharyngeal aspirates (Everard et al., 1994, Marguet et al., 2008). The levels of the neutrophil chemoattractant IL-8 are also greatly elevated in the severest forms of RSV bronchiolitis as are the numbers of infiltrating neutrophils themselves (Pino et al., 2009, Bont et al., 1999).

Macrophage inflammatory protein-1α (MIP-1α; CCL3) and regulated upon activation normal T cell expressed and presumably secreted (RANTES; CCL5) are potent chemotactic factors for eosinophils, basophils, and monocytes but not neutrophils, induce enhanced B cell production of IgE, and are commonly found in the airways of asthmatics (Alam et al., 1996). Interestingly, the levels of MIP-1α in the nasopharyngeal secretions of infants with RSV infection are positively correlated with severity of infection and inversely correlated with SaO2 (Garofalo et al., 2001, Harrison et al., 1999). Similarly, RANTES is elevated in the nasal secretions of RSV-infected children compared to healthy controls or infants with a non-RSV respiratory viral infection (Kristjansson et al., 2005, Sung et al., 2001, Becker et al., 1997, Harrison et al., 1999). Eosinophil counts and RANTES concentrations tend to be higher in the recovery phase of RSV bronchiolitis than in the acute phase of hospital admission (Kawasaki et al., 2006). This perhaps suggest a mechanism for long-term wheezing.

Mast cells and their mediators including histamine, tryptases, and the cysteinyl leukotrienes (LTC4, LTD4, LTE4) play pivotal roles in the wheezing and inflammatory processes responsible for acute exacerbations of asthma. Mast cell histamine and cysteinyl leukotrienes are also
detected in higher concentrations during RSV bronchiolitis than any other form of RSV illness (Welliver et al., 1981, Kim et al., 2006, Volovitz et al., 1988). Similarly, patients intubated for severe RSV bronchiolitis have elevated mast cell tryptase in their BAL compared to infants intubated for non-infectious reasons (Everard et al., 1995). Based on these findings the cysteinyi leukotriene antagonist Montelukast has been trialled in the clinic for post-RSV bronchiolitis wheezing. Although initially producing promising results, longer-term larger scale studies showed very limited efficacy (Bisgaard et al., 2003, Bisgaard et al., 2008).

I.6.4 Mouse Models of RSV Vaccine-Enhanced Disease

In the 1960s the failure of a formalin-inactivated (FI) vaccine against RSV, administered parenterally to young infants, was associated with deleterious responses upon natural infection with RSV later in infancy (Belshe et al., 1982, Kapikian et al., 1969). In many cases seronegative infants experienced worse disease and increased mortality compared to non-vaccinated infants upon subsequent exposure to RSV (Belshe et al., 1982, Kapikian et al., 1969). This set the field back decades with future vaccine candidates meeting with significant reservation. Although initially the immunological mechanisms for this were not clear several studies of vaccine recipient samples and rodent models suggested that Th2 responses and lung eosinophilia were associated with the atypical disease (Connors et al., 1994, Kim et al., 1976). In order to understand how the adaptive immune system is manipulated by protein components of these inactivated vaccines, and how they can be made more effective numerous groups have investigated the response to the major RSV proteins, the F (fusion) and G (attachment) glycoproteins. Priming with the G protein of RSV induces G-specific T cells that are almost exclusively Th2 CD4 T cells, whereas F-specific cells are a mixture of cytolytic CD8 T cells and Th1 CD4 T cells, and M2-specific cells are almost exclusively cytolytic CD8 T cells (Alwan et al., 1994). Mice primed with the G protein combined with an adjuvant and later challenged with
RSV developed significant pulmonary pathology characterised by enhanced Th2 cytokines, low cytolytic activity, enhanced weight loss, and eosinophilic infiltrates that markedly resembled FI-RSV vaccination (Hancock et al., 1996). In contrast, mice primed with F protein developed a strong Th1 IFN-γ response, mononuclear cell infiltrate, greater cytolytic activity, increased weight loss compared to priming with live RSV, but no eosinophilia after RSV challenge. These phenotypes are dependent on the genetic background of the mouse as eosinophilia induced by G protein priming is drastically reduced in C57BL/6 mice compared to the BALB/c strain (Hussell et al., 1998). CD8 T cells are crucial to the dichotomy of lung disease produced by these two proteins as priming with the F protein in β2-microglobulin-deficient mice (CD8 T cell-deficient) produced an eosinophilic response similar to that of the G protein upon RSV challenge (Srikitakhachorn and Braciale, 1997). Similarly, CD8 T cell-deficient C57BL/6 mice primed with the G protein produce a more comparable response to that of wild-type BALB/c mice (Culley et al., 2006). The key Th2-related molecules IL-33 receptor, monocyte chemotactic protein-1 (MCP-1; CCL2), eotaxin-1 (CCL11), and IL-9 are all up-regulated as a consequence of G protein priming followed by RSV challenge, and antibody-mediated blockade of these molecules has the capacity to reverse atypical lung disease (Dodd et al., 2009, Matthews et al., 2005, Walzl et al., 2001, Culley et al., 2006). In contrast, mice primed with the F protein tend to exhibit higher levels of MIP-1α (Culley et al., 2006). On the gene expression front the divergent phenotypes that emerge after FI-RSV vaccination versus natural RSV infection appear to be caused by prolonged activation of innate immune genes upon secondary RSV infection, which eventually leads to Th2 genes being switched on (Schuurhof et al., 2010).

Although these studies reveal how priming with a major glycoprotein of RSV can lead to Th2-dominated responses this work does not reflect the clinical situation of natural RSV infection or infantile bronchiolitis. These studies more closely resemble FI-RSV vaccination. The RSV G
protein is only one immunogenic component of the live virus and while it may induce a Th2-biased response, the F protein component induces an antagonistic Th1-biased response. Evidence of this is provided by studies where as little as two of the viral proteins are combined into one vaccination wherein the Th2 response is suppressed and pulmonary pathology does not occur (Hancock et al., 1996). Moreover, natural RSV infection would not only combine these two proteins but also additional Th1 immunogens including the M2 protein, which stimulates a robust CD8 T cell IFN-γ response, and ssRNA-dependent Type I IFN stimulation.

I.6.5 RSV and Mouse Models of Allergy

To examine whether RSV could impact upon models of airway disease many authors have utilised the ovalbumin (OVA)-driven allergic airway inflammation model and superimposed viral infection. Acute RSV infection in an adult mouse induces Th1 cytokines and neutrophils, however RSV infection followed by sustained exposure to aerosolised OVA has been demonstrated to bring about an increase in AHR, airway eosinophils, neutrophils, IL-4, and a decrease in IFN-γ compared to OVA alone (Schwarze et al., 1997). Sensitisation and challenge with OVA prior to viral infection also led to similar increases in AHR, eosinophils, neutrophils, and mucous secreting cell (MSC) numbers, compared to either RSV or OVA alone (Makela et al., 2003). RSV exposure during OVA challenges has also been observed to increase lung histopathology and lung remodelling through collagen deposition (Becnel et al., 2005). The exacerbation of the OVA-induced AHR and allergic airway inflammation that occurs upon RSV infection can be further enhanced in IFN-γR-deficient mice (Barends et al., 2003). Exacerbation of inflammation by RSV is mediated, at least partly, via IL-13 and RANTES (John et al., 2003, Park et al., 2003, Lukacs et al., 2001).
Depending on the genetic strain of mice RSV may have a differential impact on augmenting allergic airway inflammation. In C57BL/6 mice inoculation with RSV has been demonstrated to suppress sensitisation to cockroach allergen (CRA) (Smit et al., 2007). This differential effect appears to be dependent on IFN-γ and CD8 T cells. Utilising a protease allergen model (such as CRA or dust mite) provides superior replication of the mechanism by which sensitisation and the innate immune system are activated by allergens in asthmatic patients. Matsuse et al., repeatedly exposed allergen-sensitised mice to live RSV with concomitant allergen (Matsuse et al., 2000). By exposing mice to RSV during challenge with the allergen Dermatophagoides farinae (Df), AHR was exacerbated compared to Df alone. This observation could be replicated by sequential re-infections with RSV as the mice aged. This data suggests that RSV can exacerbate pre-existing allergic inflammation.

I.6.6 Mouse Models of Natural RSV Infection

The immune responses elicited by RSV can only truly be studied by employing a natural RSV infection without vaccine priming or concomitant antigen. In experiments performed using primary RSV infection in adult mice Schwarze et al., originally observed that RSV induced an increase in AHR, eosinophils, neutrophils, and IFN-γ (Schwarze et al., 1999a, Schwarze et al., 1997). The AHR was suggested to be dependent on IL-5. However, one could not convincingly conclude that the levels of IL-5 were actually increased by RSV, which seems somewhat incongruous. Furthermore, these findings remain controversial as although other groups have confirmed the presence of mild changes in AHR post-RSV exposure, most studies indicate that the response to primary infection with the A2 strain of RSV is dominated by neutrophils, mononuclear cells, and IFN-γ, and does not induce significant Th2 responses or eosinophilia (Chavez-Bueno et al., 2005, Jafri et al., 2004, Mejias et al., 2008). The changes in AHR after primary infection may even be independent of the immune system, and instead depend on over-
expression of reactive oxygen species, activation of sensory C-fibres, and release of airway neuropeptide substance P leading to broncho-constriction (Larsen and Colasurdo, 1999, Castro et al., 2006, Dakhama et al., 2005a). This mechanism suggests the possibility that a latent RSV infection in the respiratory tract might create continual stimulation of the neurogenic system and explain persistent AHR/wheezing. IFN-γ, however, does play an important negative regulatory role in RSV infection as mice deficient in this cytokine (or its receptor) have enhanced airway inflammation following RSV inoculation. Interestingly, CD8 T cells, somewhat surprisingly given their propensity to secrete IFN-γ, were observed to be essential for the presence of the eosinophilia and AHR (Boelen et al., 2002, Schwarze et al., 1999a, Schwarze et al., 1999b).

As virtually 100% of humans are infected with RSV in the first 2 years of life it was necessary to determine whether the age of the developing lung and immune system had an impact on the severity of lung pathology and nature of immune responses induced by RSV. Moreover, RSV typically re-infects individuals throughout life and these subsequent exposures tend to be linked to exacerbations of asthma (Sigurs, 2002). Therefore, neonatal infection with RSV followed by later repeated infection may lead to the development of asthmatic-like disease. Based on this rationale the seminal study by Culley et al., revealed disparate responses between initial RSV infection in neonatal (1 day old) versus adult mice (8 weeks old) upon subsequent re-challenge with the virus in later-life (12 weeks of age) (Culley et al., 2002). The age of primary RSV infection was critical as those mice first exposed as neonates and later re-infected with RSV developed more severe weight loss, greater eosinophilic and neutrophilic airway infiltrates, greater IL-4-secreting Th2 cells, and reduced IFN-γ-secreting Th1 cells, compared to adult mice. Priming of neonatal mice also led to a greater predominance of cytotoxic CD8 T cells relative to CD4 T cells in the lungs upon secondary challenge. Interestingly, the influx of these CD8 T cells was somewhat delayed and associated with elevated levels of the inflammatory chemokines
MCP-1, RANTES, MIP-1α, and TNF-α compared to adult primed mice (Tasker et al., 2008). However, effective anti-viral memory developed regardless of age of initial infection (Culley et al., 2002, Tasker et al., 2008). Perhaps a clue as to why delaying initial infection leads to a less severe pathology without eosinophilia may lie with the observation that neonatal mice have a repressed IFN-γ response to primary infection, potentially generating long-lived RSV-specific memory Th2 cells. This would parallel the association of severe RSV bronchiolitis with deficient IFN-γ production in humans (Renzi et al., 1999). Bocking experiments revealed that these responses were critically dependent on IFN-γ during primary infection, and IL-13 during secondary infection (Dakhama et al., 2005b, Lee et al., 2008, Harker et al., 2010, Dakhama et al., 2009, Ripple et al., 2010).

The role of mast cells and basophils as critical effector cells in the asthmatic response is well understood, however their role in viral-induced airway dysfunction has only recently begun to be explored. Similar to a limited number of studies of human RSV bronchiolitis, which have detected RSV-specific IgE, neonatal mice infected with RSV also produce viral-specific IgE (Welliver et al., 1981, Dakhama et al., 2009). Dakhama et al., observed that the AHR and Th2 responses that develop following neonatal infection and adult re-infection were suppressed in FcεRI-deficient mice and restored by adoptive transfer of wild-type mast cells or infusion with anti-IgE (Dakhama et al., 2009). Likewise cysteiny1 leukotrienes are increased in the BAL of mice post-RSV infection and treatment with the receptor antagonist, Montelkust, also inhibited the long-term atypical Th2 disease patterns (Han et al., 2010). In all these models suppression of Th2 responses was associated with increased IFN-γ. These data suggest a critical role for mast cells and basophils in the long-term airway sequelae, however as described above Montelkust has proved ineffective as a therapy in humans.
Although these previous studies present pre-eminent findings they do not necessarily reflect the complete clinical paradigm of RSV infection. Epidemiologically only a small percentage of even the very young infants (who are <6 months) that are infected with RSV actually develop severe disease (~4-14%) (Mohapatra and Boyapalle, 2008, Nair et al., 2010). An even lower proportion of these infants go on to develop later Th2 responses and asthma (Mohapatra and Boyapalle, 2008, Nair et al., 2010). Therefore, age of infection alone does not necessarily lead to Th2 responses nor susceptibility to viral-induced asthma. Instead in humans there are likely predisposing factor/s, possibly genetic, that select which infants develop severe infection, and later asthma. An earlier age of infection would conceivably lead to increased risk of severe disease because of a couple of reasons. Firstly, the physical size of the airways is smaller and hence more easily obstructed by inflammation, oedema, mucous, and epithelial sloughing. Second, early developmental factors in the neuromuscular apparatus may influence airway function. However, the majority of young infants do not develop severe LRT infection suggesting that other factors, not necessarily related to development of the lung, are probably also involved. This is an important concept for future investigation as it is not taken into account in these studies. Earlier age of RSV infection is important but only in a subset of the population that are genetically/epigenetically susceptible.

One other issue with these neonatal studies is that they suggest that the disease sequelae and pathology all occur after the secondary infection with RSV in adult mice. This however is not the case in humans where the illness (bronchiolitis) occurs during the primary infection in infants. The secondary RSV infection, usually in later childhood, induces an exacerbation of wheeze and/or asthma but not viral illness, or weight loss. This may be a consequence of the nature of RSV replication in the mouse.
I.7 Pneumonia Virus of Mice (PVM)

No animal model can replicate all facets of a human disease and this is particularly relevant when investigating infectious pathogens because of the specific host tropisms. Although human RSV in mice has provided great insights into certain aspects of immunity there has been a lack of a good animal model for the severe forms of RSV infection in humans. Human RSV is a host-restricted pathogen, and does not replicate efficiently or productively in rodents, and hence very large numbers of inoculated virions are required to induce inflammation. However, this does not detract from the significant advantages of utilising RSV in rodent models to answer many experimental questions. Pneumonia virus of mice (PVM) is a member of the same family (Paramyxoviridae) and genus (Pneumovirus) as for human RSV (Rosenberg et al., 2005). PVM is likewise an enveloped, single-stranded, non-segmented, negative-sense RNA virus (Rosenberg et al., 2005). In contrast to RSV, PVM is a natural rodent pathogen with an evolutionarily relevant host-pathogen relationship. PVM has the advantage of undergoing exponential replication in situ following intranasal inoculation of a minimal virus inoculum and inducing symptomatic disease (Rosenberg et al., 2005, Rosenberg and Domachowske, 2008). This is accompanied by an acute influx of granulocytes in the lungs and airways analogous to what has been observed for severe RSV infection in human infants (Harrison et al., 1999). PVM infection is localised specifically to the bronchiolar epithelium (Bonville et al., 2006). Molecular cloning has revealed that the 3’ to 5’ gene order remains constant between RSV and PVM with a high degree of similarity between many genes. The main difference is the overlap between the M2 and L genes present in RSV, but absent in PVM. Restricted cross-reactivity between the nucleocapsid protein (N) and the phosphoprotein (P) of PVM and RSV has been described. However, the external proteins do not show cross-reactivity allowing for distinction in neutralisation assays (Chambers et al., 1990). A schematic of the genomes of RSV and PVM
highlighting their close similarities and differences is shown in Figure 6. One of the possible areas in which PVM may produce a more relevant model of the inflammation associated with RSV infection in humans is when examining PRR/TLR activation and the initiation of the Type I IFN pathways. This is because these pathways are sensitive to replicating virus.

Figure 6: Viral genomic structure. Diagrams of the Genomic Structure of (A) RSV and (B) PVM in 3’ to 5’ gene order. Both viruses have negative-sense RNA genomes of ~15kb. The sizes of the coding and non-coding sequences are displayed. Source: (Rosenberg et al., 2005). © 2005 Elsevier. Permission to copy and communicate this work has been granted by Elsevier.
I.7.1 PVM as a Model of Severe RSV Infection

PVM replication in mice is insufficient in isolation to promote virulence as the pathogenic (mouse-passage) strain J3666 mirrors the viral kinetics of the non-pathogenic (tissue-culture passage) strain 15 (Domachowske et al., 2002). This indicates that both replication and virulence factors are required to promote complete antiviral inflammation and immunopathology. PVM strain J3666 induces exponentially greater weight loss, clinical scores, and granulocyte infiltration than strain 15. The most striking differences between these two strains lie in regions of the G protein, which may contribute to the differing virulence (Krempl and Collins, 2004). From herein unless otherwise stated references to PVM will refer to the pathogenic strain J3666.

The clinical pattern of PVM disease mirrors that of RSV LRT infection in humans. The symptoms produced as a result of PVM infection arise the day before viral load peaks, remain high until the virus is eliminated in between which time weight loss peaks, and thereafter subside over the course of the following 7 days (Bonville et al., 2006). The specific time-points vary with mouse strain and inoculum used. PVM induces the expression of an array of pro-inflammatory mediators that closely resemble that produced by RSV in humans including MCP-1, MIP-1α, RANTES, macrophage inflammatory protein-2 (MIP-2; mouse ortholog of IL-8), eotaxin-1, TARC, CXCL1, CXCL9, CXCL10, thymus and activated-regulated chemokine (TARC; CCL17), IFN-γ, and other IFN-related genes (Bonville et al., 2010, Bonville et al., 2006, Domachowske et al., 2002). Similar to RSV in humans the expression of the chemokines MCP-1, MIP-1α, and MIP-2 appears to be positively correlated with disease severity and respiratory dysfunction (Bonville et al., 2006, Welliver et al., 2002). Furthermore, the expression profile of MCP-1, MIP-1α, and MIP-2 mirrors that of the clinical symptoms by rising with viral load, then being sustained at elevated levels for a few days before slowly subsiding. Indeed the critical nature of MIP-1α was observed when mice deleted of this chemokine (or its receptor) displayed
a vast $10^5$-fold suppression of inflammatory cell influx along with reduced viral clearance and survival following PVM infection (Domachowske et al., 2000).

PVM viral clearance is dependent on CD4 and CD8 T cells (Frey et al., 2008). The non-structural (NS) 2 protein of PVM, as with RSV, provides a potent inhibitory effect on Type I IFNs, thus permitting viral replication in IFN-competent cells and mice (Buchholz et al., 2009). Deletion of the NS2 protein of PVM leads to an earlier Type I IFN and CXCL10 dominated response, associated with reduced viral load, clinical illness, and mortality, compared to PVM in wild-type mice. Furthermore, IFN-α/β-deficient mice infected with PVM display reductions in CXCL10 and increases in viral load (Garvey et al., 2005). Interestingly, these mice have enormous reductions in the numbers of CD8 T cells in the BAL and consequent large increases in eosinophils, eosinophil chemokines, and ribonucleases compared to wild-type controls. Perhaps due to the balancing act of an increase in viral cytopathic effect but decrease in CD8 T cell-mediated immunopathology there is little to no change in the clinical illness of these mice. Interestingly, similar to observations from Culley et al., concerning primary RSV infection, PVM infection also induces an attenuated IFN-γ response in young mice compared to older mice (Bonville et al., 2010, Culley et al., 2002).
1.8 Innate Immune Responses to RSV

RSV is detected by a wide variety of PRRs including TLR2, TLR3, TLR4, and TLR7, along with the retinoic acid-inducible gene-I (RIG-I) pathway (Klein Klouwenberg et al., 2009, Liu et al., 2007a). The redundancy that exists in RSV-stimulated pathways may be due to the need to counteract the inhibition of Type I IFN production by the NS1 and 2 viral proteins. These viral proteins exert their effects as a form of immune evasion through inhibition of interferon regulatory factor (IRF) 3 and STAT2 (Spann et al., 2004, Zhang et al., 2005, Lo et al., 2005). Myeloid differentiation factor-88 (MyD88)-deficient mice infected with RSV demonstrate a reduced infiltration and activation of myeloid DCs (mDCs), which in turn suppresses the capacity to prime anti-viral CD8 and CD4 T cells to produce IFN-γ, and induce neutrophil influx (Rudd et al., 2007). Instead MyD88-deficiency leads to a pronounced eosinophilia and Th2 response. MyD88-mediated up-regulation of both IL-12 and delta like ligand 4 (dll4) was necessary in order to instruct for Th1, and antagonise Th2 responses and the infiltration of perforin-producing CD8 T cells (Schaller et al., 2007). MyD88 signalling exerted its effect at the level of the DC and not epithelium, as wild-type DCs transferred into MyD88-deficient mice were able to reverse the phenotype.

Following RSV infection numbers of plasmacytoid DCs (pDCs) and mDCs are increased within the lung compartment and persist at elevated levels throughout the resolution phase of infection (Smit et al., 2006, Wang et al., 2006). pDCs express TLR7 and constitutively high levels of its downstream effector interferon regulatory factor 7 (IRF7) (Hengel et al., 2005). pDCs are permissive to RSV infection and exposure to RSV induces the production of large amounts of IFN-α, as well as IL-6 and IL-10, however their capacity to stimulate T cell proliferation remains relatively unchanged (Boogaard et al., 2007, Smit et al., 2008). In contrast, exposure of mDCs to RSV induces production of IL-12, IL-6, IL-10, and CXCL10, and increased induction of T cell
proliferation and Th1/Th2 cytokine production (Boogaard et al., 2007, Smit et al., 2008). Some authors have suggested that the potent induction of IFN-α does not extend to all strains of RSV, and that the virus actually inhibits IFN-α production by pDCs before and after administration of synthetic TLR7 or TLR9 agonists (Schlender et al., 2005). Seminal work published by Smit et al., revealed that depletion of pDCs during the course of RSV infection led to increases in viral load, histopathology scores, along with heightened AHR and Th2 responses (Smit et al., 2006). Supplementing these mice with recombinant IFN-α reversed only viral clearance, and therefore pDC play a protective role against RSV-induced inflammation through an IFN-α- and viral clearance-independent mechanism. This work has since been confirmed and expanded upon to show that pDCs also boost the RSV-specific CD8 T cell response (Smit et al., 2008, Wang et al., 2006). Interestingly, in humans lower numbers of circulating pDCs in the first year of life are inversely associated with risk of RSV bronchiolitis and a later diagnosis of asthma (Matsuda et al., 2002, Hagendorens et al., 2003, Upham et al., 2009).

The respiratory epithelium is the site of RSV infection and replication but it is also the first barrier to the spread of the virus. The role played by the epithelium in the innate immune response to RSV remains an active area of investigation. Numerous studies have revealed the diverse production of inflammatory chemokines and cytokines in vitro by RSV-infected human epithelial cells (see Table 3) (Domachowske et al., 2001). However, the contribution of the epithelium to the innate antiviral process in vivo is relatively defined. Knockout studies have demonstrated that MIP-1α is a key chemokine in the early and late phase response to RSV. MIP-1α regulates inflammatory cell recruitment and an over-exuberant CD8 T cell response that leads to immunopathology and weight loss (Tregoning et al., 2010).
Critically, the mechanism by which RSV interacts with the innate immune system to induce a Th2 response remains unknown.

Table 3: Major alterations in gene expression of human epithelial cells in response to RSV infection in vitro

<table>
<thead>
<tr>
<th>Gene Grouping</th>
<th>Main Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-surface molecules</td>
<td>ICAM-1, CD18, VCAM, MHC class I and II, CD14, CD15, Fas, Muscarinic receptors, β2 adrenergic receptors, Lewis antigen,</td>
</tr>
<tr>
<td>Chemokines and cytokines</td>
<td>IL-8, IL-11, IL-6, soluble TNF receptor, RANTES, MCP-1, MIP-1α, GM-CSF, IL-1β, IFN-β, IL-1α</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>NF-κB, NF-IL-6</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Inducible nitric oxide synthase, VEGF, Endothelin-1, 5-lipoxygenase, Leukotrienes, cytokeratin-17</td>
</tr>
</tbody>
</table>
I.9 Broad Overview of the Innate Immune System
The innate immune system comprises the immediate phase of defense against invading pathogens. It consists of both soluble (complement and cytokines) and cellular factors (epithelial cells, macrophages, granulocytes, and NK cells) (Biron, 2010, Kawai and Akira, 2010). Innate immunity involves a bi-directional response network whereby activation elicits responses aimed at early pathogen clearance but also initiation of a pathogen-specific response from the longer-lasting slower onset adaptive arm of the immune system. The adaptive system feeds back onto the innate system in order to facilitate more directed activation of innate immune factors. Detection of various classes of pathogens from extracellular fungi to intracellular viruses is achieved through pathogen recognition receptors (PRRs) (Biron, 2010, Kawai and Akira, 2010). These receptors recognize either PAMPs or tissue-derived factors of inflammation referred to as DAMPs (Biron, 2010, Kawai and Akira, 2010). Briefly, the main activities performed by the innate immune system include the following: at mucosal surfaces epithelial cells release anti-microbial peptide defensins; an anti-viral state is established by the induction of Type I IFNs; the alternative complement pathway is activated; macrophages and neutrophils phagocytose pathogens; pro-inflammatory cytokines, chemokines and granule mediators induce edema, recruit inflammatory cells, and stimulate bone marrow hematopoiesis; and finally APCs endocytose antigen and become primed by the milieu of pro-inflammatory signals before migrating to secondary lymphoid tissue where they initiate and control the adaptive response (Janeway and Medzhitov, 2002, Medzhitov and Janeway, 2000). Activation of the immune system can be harmful to the host when the antigens concerned are either self antigens or innocuous environmental antigens, the resulting immune responses could lead to autoimmune or allergic disease. How the immune system determines the origin of the antigen, and whether or not to induce an immune response depends largely on the innate immune system.
I.9.1 The Innate Immune System and Th2 Immunity
Until recently, the cell and molecular mechanisms responsible for the induction of a Th2 response was a proverbial ‘black hole’ in the understanding of Th immunity. This compares in stark contrast with those involved in the generation of Th1, Th17, and Treg responses, which are known to be reliant on TLR (and other PRR) ligand stimulation of DCs. The various innate immune pathways responsible for the generation of a pro-allergic Th2 response are still incompletely understood and remain an area of active investigation. However, over the last couple of years there have been several breakthroughs unraveling some of the key mechanisms involved in the generation of Th2 immunity.

I.9.2 Th2-inducing Cytokines
The innate immune cytokines interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) were discovered for their potent Th2-inducing capacity. Protease allergens or parasitic antigens can stimulate the epithelium to induce the release of these Th2-priming cytokines. The fundamental role of these cytokines in priming the immune system has generated great interest in their role in the pathogenesis of asthma and allergic inflammatory disorders. These cytokines have the ability to induce Th2 differentiation either directly, or indirectly, through effects on DCs and granulocytes. Furthermore, as initiators of inflammation, IL-25, IL-33, and TSLP may hold therapeutic potential as targeted interventions at earlier stages in the progression towards chronic allergic disease.

I.9.2.1 IL-25
IL-25 was originally discovered as a cytokine that when administered to naïve mice induced the whole gamete of Th2 responses including IL-4, IL-5, and IL-13 expression, increased serum IgE, eosinophilia, mucous production, and epithelial cell hyperplasia (Fort et al., 2001). Although first described as being produced by polarised Th2 cells, this has not been confirmed by subsequent studies, which instead suggest that IL-25 is produced by epithelial cells, basophils, and eosinophils.
IL-25 is expressed during protease allergen and OVA antigen-induced models of allergic airway inflammation and promotes the development of Th2 cells (Sharkhuu et al., 2006, Wang et al., 2007, Angkasekwinai et al., 2007). Allergic airways inflammation can also be suppressed by treatment with a monoclonal antibody raised against IL-25, while IL-25 transgenic over-expression or intratracheal administration of recombinant protein enhances allergic inflammation through a STAT6-dependent mechanism (Sharkhuu et al., 2006, Tamachi et al., 2006, Fort et al., 2001). Importantly, human biopsy specimens of asthmatic airways and atopic dermatitis lesions demonstrate evidence for up-regulation of both IL-25 and its receptor (Wang et al., 2007). The IL-25 receptor (IL-17RB) is expressed on eosinophils, a population of alveolar macrophages, DC-like cells, NK T cells, and Th2 memory cells (Cheung et al., 2006, Claudio et al., 2009, Gratchev et al., 2004). Interestingly, the expression of IL-17RB on airway smooth muscle cells is down-regulated by IFN-γ and up-regulated by TNF-α signalling (Lajoie-Kadoch et al., 2006). From a cellular mechanistic perspective at least part of the action of IL-25 during the induction of AHR in mouse models of asthma, is due to its effect on NK T cells, which express the cognate receptor IL-17RB (Terashima et al., 2008, Stock et al., 2009). Other groups have shown that IL-25 also induces Th2 differentiation by direct action on naïve CD4 T cells (Angkasekwinai et al., 2007). Furthermore, in conjunction with TSLP-treated DCs, IL-25 in humans activates and expands receptor bearing memory Th2 cells in an IL-4-independent manner (Wang et al., 2007). Although IL-25 is a member of the IL-17 family of cytokines it has opposing roles to that of IL-17. IL-25 inhibits the development of experimental autoimmune encephalomyelitis through suppression of Th17 driven pro-inflammatory cytokine release (IL-1β, IL-6, and IL-23) from DCs (Kleinschek et al., 2007).

**I.9.2.3 IL-33**
IL-33 was initially discovered as a member of the IL-1 family of cytokines that acted through the IL-1 family orphan receptor ST2, a negative regulator of Toll-like receptor-IL-1 signalling (Cayrol
and Girard, 2009, Luthi et al., 2009, Ohno et al., 2009). Unlike the other members of the IL-1 family, full-length IL-33 does not require caspase-1 processing to achieve activation. In contrast, IL-33 bioactivity is attenuated by caspase-mediated proteolysis and is more susceptible to the apoptotic caspases-3 and -7 (Cayrol and Girard, 2009, Luthi et al., 2009, Ohno et al., 2009). IL-33 binds to its receptor complex T1/ST2, expressed on Th2 cells, to enhance the secretion of the Th2 cytokines IL-5 and IL-13 and initiate chemotaxis of differentiated Th2 cells (Schmitz et al., 2005, Komai-Koma et al., 2007). IL-33 induces allergic airway inflammation (AHR, mucous hypersecretion, eosinophilia, IgE, and Th2 cytokines) in both the presence and absence of the adaptive immune system (Zhiguang et al., 2010). IL-33-induced responses are dependent on MyD88 adaptor signalling but independent of IL-4 (Kondo et al., 2008, Kurowska-Stolarska et al., 2008). One might postulate that this effect may be achieved through several mechanisms as IL-33 enhances the eosinophil-mediated differentiation of alternatively activated macrophages in the airways (Stolarski et al., 2010). In addition, IL-33 activates both IgE-dependent and -independent pathways in basophils and mast cells (Kondo et al., 2008, Kurowska-Stolarska et al., 2008, Stolarski et al., 2010). Conversely, the absence or blockade of T1/ST2 has been demonstrated to attenuate allergic airways inflammation (Kearley et al., 2009, Kurowska-Stolarska et al., 2008).

The IL-33 receptor possesses a soluble form, which similar to IL-33 has been shown to be highly up-regulated early on during OVA-induced airway inflammation (Hayakawa et al., 2007). The soluble form of ST2 appears to play an antagonistic role towards IL-33 in vitro, however further study will be required to more precisely outline the role of this receptor in vivo. Original studies on the source of IL-33 found that in the mouse and the human the mesenchymal cells were the primary producers including epithelial cells, smooth muscle cells, fibroblasts, and keratinocytes of various tissues (Schmitz et al., 2005). More recently, studies have revealed that LPS-stimulated human monocytes and mouse macrophages are also capable of producing substantial amounts of
IL-33 (Nile et al., 2010, Ohno et al., 2009). T1/ST2 receptor bearing IL-33-responsive cells include DCs, which when incubated with IL-33 increase MHC class II and IL-6 expression leading to the induction of a Th2 phenotype in naïve CD4 T cells (Rank et al., 2009). IL-33 also acts both alone and in concert with TSLP to accelerate the in vitro pro-inflammatory cytokine release from human mast cells, which express high levels of ST2 (Allakhverdi et al., 2007). Similarly, acting in synergy with IL-3, IL-33 is able to promote IL-4, IL-5, IL-8, and IL-13 production from human basophils and eosinophils (Pecaric-Petkovic et al., 2009). IL-33 however, does not enhance the release of granule mediators. Importantly, the IL-33 pathway may have potential therapeutic value in asthma as bronchial biopsies from asthmatics have increased protein levels of IL-33 (found in both airway smooth muscle and epithelial cells) compared to healthy controls, which were positively correlated with severity (Prefontaine et al., 2009, Prefontaine et al., 2010).

Despite the bulk of evidence pointing to a role for IL-33 in Th2 inflammation this cytokine can also activate natural killer (NK) cells and NK T cells to secrete large amounts of IL-12-dependent IFN-γ (Bourgeois et al., 2009, Smithgall et al., 2008). This suggests that IL-33 can have pleiotropic function in both Th1 and Th2 inflammation allowing it perhaps to play a role in multiple different subtypes of asthma. Indeed, many mesenchymal cells store large amounts of IL-33, which are released upon necrosis, allowing IL-33 to perhaps act as an alarmin-type inflammatory response to airway injury.

1.9.2.4 TSLP

The original clues that TSLP may play an integral role in Th2 immunity came from seminal work by Soumelis et al., who demonstrated that DCs were sensitive to epithelial-derived TSLP and were potently stimulated to produce CCL17 (a Th2 chemoattractant), and induce Th2 differentiation of naïve CD4 T cells (Soumelis et al., 2002). The transgenic over-expression of TSLP in epithelial
cells of the lung results in the development of severe Th2 cytokine-mediated inflammation (Zhou et al., 2005). In contrast, TSLP-deficient mice are less susceptible to OVA-induced models of allergic asthma (Al-Shami et al., 2005). More in depth analysis has revealed that TSLP inhibits the production of IL-12 in DCs, while promoting the expression of OX40-ligand, which leads to Th2 differentiation (Ito et al., 2005). Further studies on the cellular targets of TSLP have revealed that this cytokine can also act directly on CD4 T cells following TCR stimulation, resulting in IL-4 expression, and in the presence of a pro-inflammatory environment (IL-1 or TNF-α) can induce mast cell production of IL-5 and IL-13 (Allakhverdi et al., 2007, Rochman et al., 2007). This suggests that innate cells other than the epithelium may also play a direct role in TSLP-induced Th2 inflammation. As mentioned previously for IL-25 and IL-33, the potential for monoclonal antibody therapy was investigated by blockade of the TSLP receptor. This led to a reduction in allergic airways inflammation via suppressing the migration of airway DCs and a diminution in T cell priming (Shi et al., 2008). More recently, Kouzaki et al., nicely demonstrated that TSLP production by epithelial cells can be initiated at the level of the protease receptor (Kouzaki et al., 2009). Common airborne fungal and protease allergens acted through the protease-activated receptor-2 (PAR-2) G-protein coupled receptor, whose signalling could be enhanced by IL-4 and diminished by IFN-γ. This suggests TSLP might have a function in initial activation of the innate host defence response at the epithelium.

Interestingly, the transgenic overproduction of TSLP specifically in mouse skin keratinocytes, which acts as a trigger for atopic dermatitis in the skin, can also simultaneously lead to a worsening of pre-existing airways inflammation (Zhang et al., 2009b). This suggests a possible link between TSLP and the “atopic march”. Two key human studies have provided strong evidence that TSLP could be involved in the pathogenesis of asthma. Firstly, elevated levels of TSLP have been detected in the airways of asthmatic patients and these levels were positively correlated with
disease severity (Ying et al., 2005). The TSLP in this BAL was specifically identified in vitro to inhibit Treg function. Secondly, single nucleotide polymorphisms enhancing expression of the TSLP gene have been associated with an increased susceptibility to asthma (Harada et al., 2010).
I.9.3 Cells Involved in the Induction of Th2 Responses
Summarised in Figure 7 below.

I.9.3.1 Dendritic Cells Induce Th2 Differentiation

Much recent focus has been placed on the types of innate immune cells that may initiate Th2 differentiation in the lymph nodes. Although DCs have a well-defined role in secreting specific cytokines and expressing co-stimulatory molecules in order to induce either a Th1, Th17, or Treg response, it remains incompletely understood how these cells may promote a Th2 response. For many years it was considered that because DCs were reportedly unable to secrete IL-4 that they played a lesser role in Th2 differentiation other than provision of the necessary antigen. Furthermore, there is no strong evidence that DCs can produce IL-25, IL-33, or TSLP, neither is there evidence to suggest that they can respond to these cytokines with the production of the prototypical Th2 cytokines. As a result of this many studies focused on IL-4-independent induction of Th2 responses involving other DC-derived signals. TSLP can directly activate DCs to up-regulate OX40-ligand and suppress IL-12 and thereby induce Th2 responses in airway inflammation (Ito et al., 2005, Soumelis et al., 2002). Furthermore, DCs expressing high levels of Jagged1 and low levels of dll4 are capable of inducing Th2 cells in a STAT6/IL-4-independent fashion (Amsen et al., 2004). Schistosoma masoni eggs contain a glycoprotein, omega-1, that is responsible for driving DCs to induce Th2 polarisation through conditioning for dampened activation (Everts et al., 2009, Steinfeld et al., 2009). Interestingly, this mechanism occurs independently of IL-4, however these eggs do induce IL-4-secreting basophils in vivo, suggesting possible immunological redundancy. The Th2-inducing capacity of DCs has also been demonstrated for fungi, cholera toxin, histamine, prostaglandin E2, and allergens (Caron et al., 2001, d'Ostiani et al., 2000, Lamhamedi-Cherradi et al., 2008, Mazzoni et al., 2001, Braun et al., 1999, Ghaemmaghami et al., 2002, Kobayashi et al., 2009, Shreffler et al., 2006, Traidl-Hoffmann et al., 2005, Kalinski et al., 1997). However, the mechanisms by which this is achieved are largely
undefined in these studies, which instead suggest some kind of default response following inhibition of IL-12.

Different DC subsets also appear to have differing effects on T cell differentiation. Adoptive transfer of antigen-pulsed CD8α⁺ versus CD8α⁻ mouse splenic DCs differentially induces Th1 versus Th2 responses, respectively (Maldonado-Lopez et al., 1999, Pulendran et al., 1999). Resting respiratory tract myeloid DCs preferentially stimulate Th2 differentiation to inhaled antigen leading to eosinophilic airway inflammation (Lambrecht et al., 2000, Stumbles et al., 1998). There is a great deal of evidence demonstrating that TLR2 ligands can directly activate DCs to induce Th2 responses (Agrawal et al., 2003, Liu et al., 2007b, van Riet et al., 2009, Yang et al., 2008, Redecke et al., 2004). DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) expressed on DCs can differentially activate these cells for Th2 induction dependent on the pathogen. The Lewis antigen of Helicobacter pylori induces Th2-biased responses by binding to DC-SIGN (Bergman et al., 2004). In contrast, mycobacteria utilise DC-SIGN for immune evasion through suppression of DC function. The main glycoprotein allergen from peanuts, Ara h1, which is a potent Th2 adjuvant is also a ligand of DC-SIGN (Shreffler et al., 2006). Furthermore, FcγRIII ligation on DCs by antibody-complexes suppresses IL-12 induction and enhances IL-10 production, skewing DCs to promote Th2 responses and airway inflammation (Bandukwala et al., 2007). It will be important for future studies to determine whether DCs themselves can directly detect proteases from fungi, Helminths, and allergens.

Recently, DCs have again moved into the spotlight with the identification of a new subset by morphological, phenotypic, and genetic analysis. These cells designated late-activator APCs (LAPCs) were initially identified in the lymph nodes of naïve and influenza-infected mice (Yoo et al., 2010). These LAPCs enter the lymph nodes with delayed kinetics compared to conventional
DCs and express lower levels of most co-stimulatory molecules and MHC class II. Despite these differences influenza-activated LAPCs were capable of stimulating the same degree of T cell activation as for conventional DCs with a strong bias towards Th2 cell differentiation. However, the mechanism remains unknown. Although an elegant study the conclusions on LAPCs need to be tempered somewhat by the reliance on a very weak Th2-inducing model - the influenza infection. It remains to be seen how relevant LAPCs prove to be in models involving a robust response to typical Th2 stimuli (allergens and parasites). Furthermore, because of the lack of depletion, or knockout experiments the author’s do not actually provide any direct evidence that these LAPCs are responsible for a Th2 phenotype \textit{in vivo}.

According to the classical model of DC activation, microbial stimuli directly program DCs to induce distinct Th responses, in reality however many accessory cell types also influence DC function. In addition to direct DC activation by microbes, DCs also orchestrate the combined action of a network of cell types.

\textbf{I.9.3.2 Basophils Induce Th2 Differentiation}

The lack of a soluble Th2-inducing factor produced by DCs led to the search for an alternate hematopoietic non-CD4 T cell source of IL-4 to initiate STAT6-dependent Th2 differentiation. As basophils have the ability to rapidly release IL-4, IL-9, IL-25, and TSLP in response to FcεRI cross-linking, protease allergen or cytokine stimulation they were a prime candidate. These circulating granulocytes, despite their relative obscurity in peripheral blood (1% leukocytes), have long been appreciated as key Th2 effector cells (Sokol and Medzhitov, 2010). A seminal study by Sokol \textit{et al.}, transformed this paradigm by demonstrating that subcutaneous immunisation with the protease allergen papain induced basophils to migrate to lymph nodes and promote Th2 differentiation. This was dependent on basophil secretion of IL-4 and TSLP, and was diminished
by the depletion of these cells through targeting of FcεRI (Sokol et al., 2008). This observation has since been replicated by other groups to show that basophils are the enigmatic source of “early” non-CD4 T cell-derived IL-4 in various Th2 models. More recently the role of basophils has been attributed even greater significance due to the demonstration of the potent antigen presenting capacity of these cells as Th2-inducing APC. (Perrigoue et al., 2009, Sokol et al., 2009, Yoshimoto et al., 2009). These more recent studies neatly demonstrated that induction of a Th2 response to papain is also dependent on basophil expression of MHC class II and antigen presentation by these cells and not solely their cytokine secretion. The authors of these studies also suggested the irrelevance of DCs in this context as they were neither necessary for Th2 differentiation by basophils, nor were these cells capable of instituting such a differentiation program themselves. Although these findings were conveyed in various models of Th2 responses including protease allergen, soluble antigen ovalbumin, complexes of antigen/IgE, and helminthic parasite infection (Trichuris muris and Schistosoma mansoni) they remain controversial for several reasons. Firstly, they contradict a history of studies that directly indicated DCs have at least an APC role to play in Th2 responses (as previously detailed) (Bozza et al., 2009, Caron et al., 2001, Kool et al., 2008, Lamhamedi-Cherradi et al., 2008, MacDonald et al., 2001, Whelan et al., 2000). Secondly, in these basophil studies when parasite antigen presentation was restricted to DCs either by use of transgenic MHC class II expression or in vitro culture with DCs alone (neutral conditions) a Th2 response would not develop, however, when DCs were exposed to a biased exogenous cytokine milieu (IL-4) they did push naïve CD4 T cells to Th2 as has been previously demonstrated (Yoshimoto et al., 2009). Thirdly, during Helminth parasite Nippostrongylus brasiliensis infestation migrating basophils (removed by either IL-3/-/- mice or direct depletion) are dispensable for the generation of Th2 immunity (Kim et al., 2010). Fourthly, the antibody employed for direct depletion of basophils in the aforementioned studies is in fact not specific for basophils, but instead also expressed on mast cells and a subset of dendritic cells. A very elegant study recently published
by Tang et al., appears to provide some reconciliation between the two sides of this debate by suggesting an alternative to mutual exclusivity with instead a cooperative relationship existing between IL-4-secreting basophils and DCs (Tang et al., 2010). The authors demonstrated that protease papain allergen activates reactive oxygen species (ROS) in DCs and the epithelium, which induced oxidised lipids that signalled through TLR4 in the epithelium to initiate the release of TSLP. ROS induced DC-derived CCL7, which mediated the recruitment of TSLP activated IL-4-secreting basophils. Papain induction of ROS in DCs programmed them to suppress Th1 and promote Th2 responses. Critically, both DCs and basophils were necessary to complete Th2 differentiation in the lymph nodes. The relative role of the basophil and DC in Th2 induction may also be specific for particular stimuli, in particular whether or not the allergenic preparation contains solely protease activity or also DC-activating PRR ligands, is likely to be important.

I.9.4 Newly Discovered Innate Immune Cells as Key Effectors of Th2 immunity

A trilogy of papers recently published in Nature have independently identified new types of immune cells derived from the mucosal gut-associated lymphoid tissue of both mice and humans. These cells uniquely respond to the innate Th2-type-inducing cytokines IL-33 and/or IL-25 (boosted in the presence of IL-2) by releasing a burst of IL-4, or IL-5 and IL-13. The discovery of these cells is a veritable breakthrough in the study of Th2 immunity. The three different cell types coined ‘nuocytes’ (Lineage−c-kit+CD45+ICOS+IL-7Rα+IL-17RB+T1/ST2+) (Neill et al., 2010), ‘natural helper cells’ (Lineage−c-kit+CD45+Sca-1+CD27+IL-7Rα+IL-17RB+T1/ST2+) (Moro et al., 2010), and ‘multi-potent progenitor (MPP)type2 cells’ (Lineage−c-kit+Sca-1+IL-7Rα+IL-17RB+T1/ST2) (Saenz et al., 2010) were shown in mice to be critical for the in vivo protection against Helminth parasites and the mucosal inflammation induced by the delivery of the recombinant cytokines IL-25 and/or IL-33. It is plausible that at least two of these cell types ‘nuocytes’ and ‘natural helper cells’ identified by independent groups as different cells may
actually be the same cell population as both seemingly bare a marked phenotypic resemblance. Both nuocytes and natural helper cells were observed to be the predominant source of early IL-5 and IL-13 in response to *N. brasiliensis*. These cells were dependent on IL-33 and/or IL-25 for expansion *in vivo* and were essential for mucous production, eosinophilia, and worm expulsion. Interestingly, natural helper cells were also capable of inducing B1 cell proliferation and IgA secretion in the gut, linking innate Th2 immunity to humoral responses at mucosal surfaces. The other cell type, MPP\textsuperscript{type2} cells identified as the IL-25-responsive undefined non-B non-T cell (discovered by Andrew McKenzie’s group several years earlier (Fallon et al., 2006)), are quite distinct. These cells are expanded and activated solely in response to recombinant IL-25 or IL-25-derived from *N. brasiliensis* and *Trichuris muris* infestation. These cells secrete IL-4 and induce differentiation of Th2 cells in both OVA and parasitic Th2 models. Moreover, these cells are heterogeneous for IL-4 production and this heterogeneity dictates the multipotent progenitor capacity of the cell wherein the IL-4-producers inherently differentiate into mast cells and the non-IL-4-producers differentiate into a range of cells *in vitro* including mast cells, basophils, macrophages, and a population of CD11b\textsuperscript{+}MHC-II\textsuperscript{+} APCs. One potential caveat of these findings is that they fail to convincingly exclude the possibility that these cells are simply circulating hematopoietic progenitor cells, which have been previously identified as innate Th2-like cells in asthma (see below). Lineage tracing experiments *in vivo* would be necessary to account for the effect of different microenvironments. It remains to be seen whether these innate Th2-cytokine producing cell types are also important in the Th2 inflammation underlying allergy and IgE-sensitisation.
I.9.5 Hematopoietic Progenitor Cells as Effector Cells of Th2 Immunity

Circulating hematopoietic progenitors extruded from the bone marrow into the blood play an important role in generating many of the immune system’s effector cells in peripheral tissues. Hematopoietic progenitors have long been recognised to play a crucial role in asthma through their capacity to migrate to allergic sites of inflammation in order to differentiate into mast cells, basophils, and eosinophils (Dorman et al., 2004, Massberg et al., 2007, Mastrandrea et al., 1999, Otsuka et al., 1987). A recent publication from Allakherdi et al., has extended this role, by utilising human cells ex vivo, the authors show that these hematopoietic progenitors also function as direct effector cells during the asthmatic response (Allakhverdi et al., 2009). The hematopoietic progenitors, derived from umbilical cord blood or adult peripheral blood, when stimulated with IL-33 or TSLP were able to produce an array of Th2 (but not Th1 or Th17) cytokines. These responses were enhanced by exposure to other cytokines commonly found at the site of allergic inflammation including IL-4, TNF-α, IFN-γ, prostaglandins and leukotrienes, but inhibited by the direct effects of TGF-β. When these progenitor cells were cultured in the presence of the supernatants from stimulated airway epithelial cells or nasal explants from rhinosinusitis patients they secreted IL-5 in a TSLP-dependent, IL-33-independent manner. Finally, the authors went on to show that these IL-5 and IL-13-producing hematopoietic progenitors exist in the sputum of patients with allergic asthma but not healthy controls, and that their numbers were rapidly increased after allergen challenge.

Despite these advances in our understanding of the initiation of Th2 immunity, the mechanism underlying viral-induction of Th2 responses remains unclear.
Figure 7: The innate immune pathways involved in the induction of Th2 cells

Protease allergens and Helminth parasites induce Th2 immune responses via interaction with the mucosal epithelium, leading to secretion of the cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). TSLP and IL-33 can activate hematopoietic progenitor cells (HPC) to secrete IL-5, IL-6, IL-13 and GM-CSF, which can be inhibited by the action of TGF-β. TSLP can induce DCs to up-regulate OX40 ligand and present antigen via MHC class II. Basophils can secrete IL-4, TSLP and present antigen via MHC class II in order to initiate Th2 differentiation. Nuocytes and natural helper cells in the gut are activated in response to IL-25 and IL-33 to release large amounts of IL-5 and IL-13, however their capacity to initiate Th2 differentiation as a pose to effector function has not been demonstrated. MPPtype2 cells are stimulated by IL-25 to
produce IL-4, and have the capacity to differentiate into multiple types of innate cells, some of which can present antigen via MHC class II to induce Th2 cells. DCs can also be activated directly to induce Th2 responses, rather than indirectly through the epithelium. This occurs via stimulation of the surface receptors: Toll-like receptor 2 (TLR2), DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), and FcγRIII by pathogens or antibody-antigen complexes, respectively. Source: Kaiko G.E., Foster P.S. 2011. New insights into the generation of Th2 immunity and potential therapeutic targets for the treatment of asthma. Current Opinion in Allergy and Clinical Immunology. 11(1):39-45. © 2011 Wolters Kluwer Health. Permission to copy and communicate this work has been granted by Wolters Kluwer Health.
I.9.6 Dendritic Cells and Re-activation of Th2 cells in Asthma

DCs form a network in the airway epithelium that continuously sample the tissue environment for antigen and migrate to the lymph nodes under inflammatory as well as non-inflammatory conditions (Vermaelen et al., 2001). The pivotal role of DCs in the initial phase of allergic asthma relate to their role in the induction of Th2 responses (as described previously). DCs encounter and endocytose antigen, become activated and help to elicit a Th2 cell-inducing response that leads to the initial sensitization to antigen in allergic diseases. However, the importance of the DC does not cease at the induction stage, rather DCs are crucial for maintaining the cardinal features of asthma. Following challenge of sensitized mice or humans with allergen there is a dramatic recruitment of DCs into the airway lumen and lymph nodes (Huh et al., 2003, Upham et al., 2002, Vermaelen and Pauwels, 2003). These DCs have been found to form clusters with primed T cells at these sites (Huh et al., 2003). In a seminal paper from van Rijt et al. it was eloquently demonstrated that selective depletion of CD11c⁺ DCs from the lungs of mice, immediately prior to allergen re-challenge, in mice that were already sensitized to allergen, abolished all features of airway inflammation (Lambrecht et al., 1998, van Rijt et al., 2005). Upon adoptive transfer of CD11c⁺ DCs, but not CD11c⁺ macrophages, back into these mice, eosinophilic infiltration and Th2 cytokine secretion were restored. To further impress that this was a secondary function of DCs in the airways and not a consequence of a lack of initial priming of Th2 cells, ex vivo activated Th2 cells were transferred into the mice but failed to induce Th2 cytokine secretion in the absence of CD11c⁺ DCs. This and other studies with similar depletion strategies clearly convey that lung DCs are essential for the maintenance of airway inflammation through local reactivation of allergen-specific Th2 cells at the sites of inflammation. In addition, DCs also maintain airway inflammation through the recruitment of memory Th2 cells to the lungs via secretion of the Th2 cell selective chemokine ligands 17 and 22 (CCL17 and CCL22) (Hammad et al., 2003).
I.10 Role of Toll-like Receptors (TLRs) in Asthma

Although the role of PRRs and TLRs are well understood in infection and immunity less is known about their role in overzealous inflammatory responses such as those involved in allergy and asthma. To date studies investigating the role of TLRs in asthma and allergy have largely focused around mechanisms of the Hygiene Hypothesis (reviewed in Chapter I.3). Several groups have measured the effects of the D299G TLR4 polymorphism on asthma. The rationale for these studies is based off two separate and somewhat contradictory observations of TLR4 function in asthma. Exposure to LPS can either suppress or exacerbate asthma depending on the timing of early-life versus later-life exposure, respectively (Braun-Fahrlander et al., 2002, Michel et al., 1996). Perhaps not surprisingly then, genetic association studies have produced a mixture of conclusions on the effect of this common TLR4 variant on asthma. Some studies suggested no effect of D299G TLR4 polymorphism on the incidence of asthma (Raby et al., 2002). However, other investigators have attributed both increased risk of allergen sensitisation, and decreased severity of asthma to this SNP (Werner et al., 2003, Yang et al., 2004). As TLR4 activation can increase both Th1 cytokine production and IL-10, individuals with the D299G TLR4 polymorphism may have decreased production of these cytokines (Higgins et al., 2003). Polymorphisms in CD14 (involved in LPS signalling) in concert with a farming lifestyle are protective against asthma (Smit et al., 2009). Further genetic analysis of TLRs amongst asthmatics revealed an association between SNPs in the TLR2 heterodimers (TLR2/TLR1 and TLR2/TLR6), which increased expression of the respective TLR proteins, and a protective effect against atopic asthma (Kormann et al., 2008). Furthermore, these SNPs were associated with increases in Th1 and decreases in Th2 cytokine production after stimulation of PBMCs. Similarly, other investigators have found that expression of TLR4 and TLR5 was lower in asthmatics, and that stimulation of asthmatic PBMCs by the respective ligands produced less pro-inflammatory cytokines compared to
healthy controls (Lun et al., 2009). In experimental models dsRNA stimulation of TLR3 has been implicated in exacerbating established OVA-induced airway inflammation, this process was enhanced by addition of Th2 cytokines (Torres et al., 2010). Although the mechanism underlying this was not delineated there was an association with increased release of chemokines from the airway epithelium. In stark contrast other studies demonstrate that TLR3 ligands actually suppress both exacerbation and sensitisation of OVA-induced Th2 responses (Sel et al., 2007). Moreover, in humans altered TLR3 function does not appear to be associated with asthma (Roponen et al., 2009, Lun et al., 2009). Similarly, human epidemiological data for TLR2 and TLR4 polymorphisms would suggest that stimulation of these receptors may protect against the onset of asthma. However, experimental studies with ligands to these receptors in mice suggest that they may in fact contribute to the initiation of airway inflammation (Hammad et al., 2009, Redecke et al., 2004). Overall, there is clearly a divergence between mouse and human studies in this area creating a need for better experimental models to mimic the critical time periods (prenatal and postnatal) for exposure to TLR ligands in the development of asthma.
I.11 SUMMARY AND RATIONALE

Asthma is a complex disease believed to involve polygenic gene-environment interactions, the nature of which remains largely unknown. In order to identify targets for improved therapies, a better understanding of the disease pathogenesis is required. One of the primary environmental factors associated with the development of asthma is early-life viral bronchiolitis. Furthermore, mounting evidence from human interventional studies suggests that this relationship may be more causal in nature rather than simply marking predisposition to asthma. However, the precise nature of a possible pathogenic relationship between RSV-induced bronchiolitis and the development of asthma remains unclear. It is apparent that RSV-induced bronchiolitis is by itself insufficient to predispose to asthma as some afflicted infants develop normal respiratory function. However, a subset of infants that suffer a bronchiolitis episode develop an imbalance between Th1/Th2 responses, persisting eosinophilia, and a deficiency in IFN-γ production. These immunological characteristics post-bronchiolitis appear to be predictive of a later diagnosis of asthma. We hypothesise that a severe RSV infection must first interact with a specific genetic susceptibility in these infants, during a critical time in postnatal development, in order to increase the risk of childhood asthma. Previous experimental models have not accounted for the complexity of this gene-environment relationship. The innate immune system is critical for viral detection, through PRRs, and regulation of the adaptive immune response. What role the innate immune system may have in an interplay between RSV and genetic susceptibility to asthma remains unknown.

In recent years there has been great progress in defining the innate immune pathways involved in the induction of Th2 responses following exposure to allergens and parasites. Emerging evidence suggests that a dynamic interplay exists between the epithelium, innate
immune cells, and the innate cytokines IL-25, IL-33, and TSLP. However, the cell and molecular mechanisms by which a viral infection might induce a non-protective Th2 response remain relatively unknown.

Severe asthma and acute exacerbations of disease in adults, which are both poorly treated and frequently the cause of hospital admission, have been epidemiologically associated with chronic *Chlamydia pneumoniae* infection. A possible mechanism underlying this association remains unexplored, however a role for immune evasion and persistence of the bacteria in these individuals may be implicated.

Elucidating the pathways involved in the pathogenesis of viral-induced asthma and infection-driven exacerbations may provide potential targets for future therapies, or even biomarkers for early identification of susceptible individuals.
I.12 SPECIFIC AIMS OF THESIS

1. To determine whether the atypical bacteria *Chlamydia* can infect dendritic cells and utilise this as a means of immune evasion.

2. To determine a mechanism by which the bacteria *Chlamydia* may contribute to acute exacerbation of pre-existing asthma, using a mouse model of allergic airways inflammation.

3. To determine the cell and molecular pathway underlying viral-induced Th2 responses, with specific focus on the interaction between RSV, the innate immune system, and the Th2-inducing cytokines.

4. To dissect the relationship underlying early-life RSV infection, bronchiolitis, and later development of asthma using mouse models of disease.

5. To determine whether TLR7 and/or natural killer cells play a critical role in the relationship between early-life RSV bronchiolitis and the pathogenesis of the hallmark features of asthma.
CHAPTER II: PUBLICATION #1

_Chlamydia muridarum_ Infection Subverts Dendritic Cell Function to Promote Th2 Immunity and Airways Hyper-reactivity


**Gerard E. Kaiko**<sup>1</sup>, Simon Phipps<sup>1</sup>, Danica K. Hickey<sup>1</sup>, Chuan En Lam<sup>1</sup>, Philip M. Hansbro<sup>1</sup>, Paul S. Foster<sup>1,2</sup>, and Kenneth W. Beagley<sup>1,3</sup>

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1. School of Biomedical Sciences and The Asthma and Respiratory Diseases Priority Research Centre, The University of Newcastle and Hunter Medical Research Institute, Newcastle, NSW 2300, Australia
2. John Curtin School for Medical Research, Australian National University, Canberra, 0200, ACT, Australia
3. Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, 4059, QLD
II.1 ABSTRACT

There is strong epidemiological evidence that *Chlamydia* infection can lead to exacerbation of asthma. However, the mechanism(s) whereby chlamydial infection, which normally elicits a strong Th1 immune response, can exacerbate asthma, a disease characterized by dominant Th2 immune responses, remains unclear. In the present study we show that *C. muridarum* infection of murine bone marrow-derived dendritic cells (BMDC) modulates the phenotype, cytokine secretion profile and antigen-presenting capability of these BMDC. *Chlamydia*-infected BMDC express lower levels of CD80 and increased CD86 compared to non-infected BMDC. When infected with *Chlamydia* BMDC secrete increased TNF-α, IL-6, IL-10, IL-12, and IL-13. Ovalbumin (OVA) peptide-pulsed infected BMDC induced significant proliferation of transgenic CD4⁺ DO11.10 (D10) T cells, strongly inhibited interferon gamma (IFN-γ) secretion by D10 cells and promoted a Th2 phenotype. Intra-tracheal transfer of infected, but not control non-infected, OVA peptide-pulsed BMDC to naïve BALB/c mice, that had been intravenously infused with naive D10 T cells, resulted in increased levels of IL-10 and IL-13 in bronchoalveolar lavage (BAL) fluid. Recipients of these infected BMDC showed significant increases in airways resistance and decreased airways compliance compared to mice that had received non-infected BMDC, indicative of the development of airways hyper-reactivity (AHR). Collectively these data suggest that *Chlamydia* infection of DCs allows the pathogen to deviate the induced immune response from a protective Th1 to a non-protective Th2 response that could permit ongoing chronic infection. In the setting of allergic airways inflammation this infection may then contribute to exacerbation of the asthmatic phenotype.
**II.2 INTRODUCTION**

*Chlamydia* are obligate intracellular Gram-negative bacteria and three species are known human pathogens. *Chlamydia trachomatis* is the causative agent of genital tract and ocular infections (Beagley and Timms, 2000). *C. pneumoniae (Chlamydiaphila pneumoniae)* infects the respiratory tract and is responsible for 10-20% of community acquired pneumonia (Kuo et al., 1995, Gibbs et al., 1998, Hahn, 1999). *C. psittaci (Chlamydiaphila psittaci)* is primarily a pathogen of psittacine birds but infection may lead to atypical pneumonia or pyrexia in man (Eugster, 1980). *Chlamydia* are primarily pathogens of epithelial cells, however they can infect a range of cell types including smooth muscle cells, vascular endothelial cells, and immune cells, such as macrophages and dendritic cells (DCs) (Godzik et al., 1995, Gaydos et al., 1996, Matyszak et al., 2002, Hook et al., 2005). Resolution of infection in the mouse requires a cell-mediated immune response driven by interferon gamma (IFN-γ)-secreting CD4+ T helper type 1 (Th1) cells. An inability to mount an adequate *Chlamydia*-specific Th1 response can often lead to persistence of the bacteria and associated immunopathology in the host. The central role of IFN-γ in clearance is evidenced by prolonged infections that occur in IFN-γ, and IFN-γ receptor, -deficient mice (Ito and Lyons, 1999, Rottenberg et al., 1999). Notably, DC-derived IL-12 is essential for the generation of this Th1 response (Farrar et al., 2002, Lu et al., 2000).

Allergic asthma is a chronic inflammatory disease of the airways that results in reversible airflow obstruction, airways hyper-reactivity (AHR), mucous overproduction, antigen specific IgE production and the development of airway specific lesions (collectively termed remodeling of the airways). The inflammatory response is complex but CD4+ T helper type 2 (Th2) cells are a predominant feature of cellular infiltrates in the airways (Shepherd et al., 2002). Furthermore, Th2 cytokines, which include IL-4, IL-5, IL-10, and IL-13, induce many of the hallmark features of asthma. In particular, IL-13 is the central regulator of the effector phase of
the asthmatic response due to its capacity to induce AHR, mucous hypersecretion, inflammation, and many of the lesions associated with airway wall remodelling (Grunig et al., 1998, Zhu et al., 1999, Wills-Karp et al., 1998). It is well recognized that DCs play a central role in the initial priming of Th2 responses during allergic sensitization. Recent investigations have demonstrated that DCs are also essential for the maintenance of many of the cardinal features of asthma through local reactivation of allergen-specific Th2 cells in the airways (van Rijt et al., 2005, Lambrecht et al., 1998).

Although allergens are common triggers of asthma the role of respiratory tract infections in predisposition and exacerbations is becoming increasingly recognized and is a common cause of the difficulty in managing steroid-insensitive asthma. Emerging epidemiological and clinical evidence has strongly associated C. pneumoniae lung infection as a trigger for the development of asthma and as a common cause of exacerbations of this disorder (Cunningham et al., 1998, Black et al., 2000, Wark et al., 2002, Webley et al., 2005). The mechanism(s) whereby infection with C. pneumoniae, a bacterium associated with a Th1 response, can exacerbate or trigger asthma, a disease that is often characterized by a Th2 phenotype, remains unclear. The observation that Chlamydia can infect many different cell types, including immune cells, suggests that this mechanism may also be linked to the processes that trigger infection-induced asthma. As DCs are pivotal in regulating both Th1 and Th2 immune responses we hypothesized that a Chlamydia infection of these antigen-presenting cells may subvert their ability to elicit protective Th1 immunity and instead promote Th2 responses that may amplify pre-existing allergic inflammation. This mechanism could operate to induce exacerbations of asthma and also perpetuate Chlamydia survival and persistent infection.
II.3 MATERIALS AND METHODS

Reagents

Biotinylated mouse CD4⁺ T lymphocyte enrichment cocktail, IMag Streptavidin Particles Plus-DM (direct magnet), BD IMagnet, anti-CD45R/B220 DM beads, anti-CD8α DM beads were all obtained from Becton Dickinson Biosciences (BD Biosciences, New Jersey). The ovalbumin (OVA) peptide (molecular weight = 1773.94), consisting of amino acids 323-339 (ISQAVHAAHAEINEAGR) of the OVA protein was synthesised at the Biomolecular Resource Facility, John Curtin School of Medical Research, Canberra. Recombinant murine (rm) GM-CSF was produced in yeast using an expression construct provided by Dr Tracey Wilson, Walter and Eliza Hall Institute, Melbourne. Recombinant mouse IL-4 was obtained from Peprotech (rmIL-4, Peprotech, California). All antibodies were purchased from BD Pharmingen.

Mice

Male BALB/c mice (6-8 weeks old) were obtained from the central animal house, the University of Newcastle. DO11.10 mice on a Rag2 -/- background expressing a transgenic TCR that recognizes only the OVA323-339 peptide in the context of I-A<sup>d</sup>, and IL-13 -/- mice (IL-13 KO, gift from Dr A Mackenzie, Cambridge University) on a BALB/c background were obtained from the John Curtin School of Medical Research. All studies were approved by and carried out in accordance with guidelines set out by the University of Newcastle Animal Care and Ethics Committee.

Dendritic cell culture and purification

DC were generated based on a method previously described (Inaba et al., 1992) with several modifications. Briefly, femurs and tibias of naive adult male wild-type BALB/c or IL-13
knockout mice were collected and bone marrow flushed out with growth medium (RPMI 1640, 5 x 10⁻⁵ M 2-mercaptoethanol, 10% heat-inactivated FCS, 2 mM L-glutamine, 20 mM hepes, 100 μg/ml penicillin and 100 μg/ml streptomycin). Red blood cells (RBCs) were lysed and cells were washed through a 70 mm nylon cell strainer with RPMI media. Magnetic nanoparticle-labelled anti-CD8a and anti-CD45R/B220 monoclonal antibodies were used to deplete cells according to the manufacturer’s instructions. The negative cell fraction was re-suspended at 1x10⁶ cells/ml and supplemented with 10 ng/ml of both rmGM-CSF and rmIL-4. On day 3 and 6 of culture, 75% of the medium was removed and replaced with fresh growth medium re-supplemented with cytokines. On day 8 the non-adherent and semi-adherent cells were harvested. Dendritic cell purity was increased by CD11c positive selection using incubation with a biotin-conjugated anti-mouse CD11c antibody and streptavidin-conjugated DM magnetic nanoparticles. The DCs were plated out in Chlamydia media (RPMI, 10% heat-inactivated FCS, 2 mM L-glutamine, 20 mM hepes, 5 μg/ml gentamycin and 100 μg/ml streptomycin sulphate) at 4x10⁵ cells/ml.

**Infection assay**

*C. muridarum* (ATCC VR-123, formerly the Mouse Pneumonitis strain, Cmu) was obtained from the American Type Culture Collection. Stock cultures were prepared as previously described (Berry et al., 2004) and stored at −80°C. Cmu was added to DCs at a 2:1 multiplicity of infection (MOI). The cultures were then incubated for 36 hours at 37°C in 5% CO₂. After 24 hours, *Escherichia coli* lipopolysaccharide (LPS) (Sigma, UK) at 1 mg/ml was added where appropriate for 12 hours. DCs were harvested and analysed by flow cytometry and supernatants collected for cytokine analysis. To examine DC infection by fluorescence microscopy, 10 mm diameter glass coverslips were placed into the wells of culture plates prior to cell seeding. Similarly, to assess the viability of infection after the 36-hour incubation with
Cmu, the DCs were harvested and washed to remove any extracellular Cmu. The cells were then lysed by sonication followed by vortexing to release any intracellular Cmu. The cell lysate was centrifuged to remove cell debris from the supernatant, which was then added to McCoy cells, plated on 10 mm diameter glass coverslips, and incubated for 3 hours at 37°C in 5% CO₂. Media was removed and replaced with fresh media containing 1 mg/ml cyclohexamide and the cells were incubated for a further 48 hours or until active infection could be visualised.

Fluorescence microscopy

After infection with Cmu, coverslips were washed to remove any cell debris, and cells fixed with methanol for 10 minutes. The cells were stained with FITC-conjugated anti-Chlamydia-specific LPS (Celllabs, UK) for 30 minutes at 37°C followed by washing. Cells were photographed using an Olympus fluorescence microscope (model BX51), digital camera (Olympus DP70) and DP software (Olympus, California). The percentage of infected cells was quantitated by counting a total of 200 cells per slide. Infected cells were defined as those containing large FITC-positive granular inclusions.

Flow cytometry

Cells were washed three times in PBS + 2% FCS and incubated with anti-mouse CD16/32 Fc receptor block at 1 mg/1x10⁶ cells for 10 minutes on ice. Cells (1x10⁶) were then incubated with the relevant antibody on ice for 20 minutes (CD80-biotin, CD86-biotin, I-A^d^-PE, CD11c-PE) and washed twice. For biotinylated antibodies a streptavidin conjugated fluorochrome (streptavidin-APC) was added at 4°C for 30 minutes. Cells were fixed in 2% formaldehyde (Polysciences, California) in PBS and analysed on a FACSCanto (BD Biosciences).
Antigen presentation and T cell proliferation assay

DCs were exposed to 5 mg/ml OVA peptide for 6 hours to allow uptake. DCs were then washed twice to remove non-acquired OVA peptide. CD4+ T cells were purified from spleens of naïve adult male D011.10 mice on a Rag2-/- background. Spleens were excised and tissue matrix digested with collagenase (1 mg/ml). The splenic material was then homogenized with RBC lysis and cells re-suspended in media. Mixed splenocytes were plated out in 6 well plates at 5-10x10^6 cells/ml and incubated for 2 hours at 37°C 5% CO₂, to deplete adherent leukocytes. Non-adherent cells were collected, washed and CD4+ T cells enriched by negative selection using the biotinylated mouse CD4 T lymphocyte enrichment cocktail and magnetic nanoparticles, according to manufacturer’s instructions. CD4+ T cell purity was readily assessed to be >95%. Purified T cells were stained with CFSE (Molecular Probes, UK) as previously described (Lyons and Parish, 1994). Cells were then washed twice, re-suspended in Chlamydia media and added to DCs at a DC:T cell ratio of 1:4. For cytokine analysis T cells were not labeled with CFSE. Cells were then centrifuged (1500 rpm for 2 minutes at room temperature) to initiate immediate DC and T cell contact. Culture was continued for 4 days at 37°C in 5% CO₂. Supernatants were collected and non-adherent cells were harvested for detection of proliferation by CFSE fluorescence.

In vivo adoptive transfer experiments

On day 0, DO11.10 Rag2-/- CD4+ T cells were purified as above and re-suspended in PBS before 2 x10^6 cells/200 µl were transferred intravenously (i.v.) via the tail vein into adult male BALB/c mice. DCs were cultured and infected as described. After 36 hours of infection, DCs were pulsed with 5 µg/ml OVA peptide overnight. On day 1, infected or non-infected DCs were washed twice to remove free peptide, re-suspended in PBS and transferred intratracheally (i.t.) at 3 x10^5 cells/50 µl to each mouse. On day 4, mice were sacrificed and airway function
was assessed. Bronchoalveolar lavage fluid (BALF) was also collected by administering 1ml Hanks Buffered Salt Solution to the trachea and extracting fluid and cells.

**Assessment of airway function**

Airway hyper-reactivity (AHR) was measured as previously described (Schwarze et al., 1997). Briefly, assessment was performed on anesthetised, mechanically ventilated mice with a plethysmograph (Buxco, USA) by measuring changes in lung resistance and compliance in response to increasing doses of inhaled methacholine.

**Cytokine Analysis**

The concentration of tumour necrosis factor-α (TNF-α) in supernatants was determined by ELISA according to manufacturer's instructions (mouse Duoset TNFα kit, R&D Systems, Minneapolis). A custom designed 7 cytokine (IL-4, -5, -6, -10, -12(p70), -13, and IFN-γ) multiplex analysis kit was purchased from Bio-Rad (Hercules, California) and the assay performed according to the manufacturer’s instructions. Plates were analysed for fluorescence using a Bio-Rad bioplex reader.

**Statistical analysis**

Data presented are the means ±SEM. A non-parametric Mann-Whitney test (two-tailed) was used to establish statistical significance between any two separate groups with different means. For AHR data an ANOVA was used to analyse the difference between groups. A significant difference between any two groups was considered to exist when the p value was <0.05. Prism software version 4 was used for all calculations.
II.4 RESULTS

*C. muridarum* infects murine BMDC

To determine whether murine DCs could be infected with Cmu, BMDC were exposed to Cmu at a MOI of 2:1. After 36 hours BMDC were fixed and stained with FITC anti-*Chlamydia* LPS. Figure 1A shows that BMDC stained positive for chlamydial inclusions (60% of BMDC; data not shown), which were notably granular in appearance indicating replicating bacteria (infection with Cmu). To confirm that this was a viable infection rather than phagocytic uptake by the DC, BMDC were harvested from primary cultures, washed to remove any extracellular Cmu, then lysed to release intracellular Cmu. The cell lysate was then plated on the epithelial McCoy cell line. Cmu isolated from infected BMDC were able to infect and form sizeable inclusions within McCoy cells (Figure 1B). Infectivity of Cmu in McCoy cells was reduced following growth in BMDC ($1.5 \times 10^4 \pm 0.3 \times 10^4$ IFU per million DC), however it is possible this may be increased at later time points. At the time points studied, DC cell death post-infection with *Chlamydia* was negligible as determined by staining with trypan blue. This is a novel observation and indicates that *Chlamydia muridarum* can readily form viable infections within murine DCs.
Figure 1. *Chlamydia muridarum* forms a viable infection within murine dendritic cells. BMDC generated by culture in GM-CSF and IL-4 were exposed to Chlamydia muridarum (Cmu) for 36 hours. Cell monolayers were then stained with FITC-conjugated anti-Chlamydia LPS to detect the presence of inclusion bodies within BMDC. (A) A typical field is shown. To demonstrate that infection of BMDC resulted in viable Cmu progeny, infected BMDC were washed, lysed and cell lysates plated on McCoy cells. (B) Inclusions characteristic of a typical Cmu infection were observed and indicated with arrows. Scale bars represent 5μm (A) and 25μm (B). Green (FITC) represents Cmu inclusion bodies and red (rhodamine) represents a
general cellular stain. Fluorescent micrographs are from one experiment representative of four independent experiments.
C. muridarum infection induces unique phenotypic changes in murine BMDC

Following infection with Cmu, BMDC were stained with anti-MHC class II, anti-CD80 and anti-CD86 and analysed by flow cytometry. Because DC generated in vitro by culture with GM-CSF and IL-4 are not fully matured, BMDC were also activated in culture with LPS (E. coli) and included as positive controls. MHC class II surface expression was increased by exposure to LPS. In contrast expression by infected BMDC did not significantly differ from that of non-infected BMDC, indicating Cmu infection failed to up-regulate this DC maturation marker (Figure 2A). Interestingly, Cmu infection of BMDC reduced the number of cells positively expressing the co-stimulatory marker CD80 compared with non-infected BMDC, while as expected LPS increased the number of cells expressing this co-stimulatory molecule (Figure 2B). By contrast, CD86 expression was significantly increased (p<0.05) on both infected and LPS-stimulated BMDC as compared to non-infected controls (Figure 2C). These data reveal that in comparison to LPS stimulation (MHC class II⁺, CD80hi, CD86hi) Cmu induces a distinct DC phenotype characterized by MHC class II⁺⁻, CD80lo, and CD86hi levels of expression.
Figure 2. *Chlamydia* infection of BMDC prevents up-regulation of MHC class II and CD80 but increases CD86 expression. *Chlamydia muridarum* (Cmu)-infected BMDC (shaded), non-infected control BMDC (shaded), or non-infected BMDC cultured for 24 hours then stimulated with LPS for the final 12 hours (shaded) were each stained with (A) anti-MHC class II, (B) anti-CD80 and (C) anti-CD86 and analysed by flow cytometry. The dashed line (unshaded area) represents the isotype control. Values depicted reflect the percentage of cells expressing the relevant surface molecule. Results are representative of four independent experiments.
C. muridarum infection alters the cytokine secretion profile of murine BMDC

To determine whether Cmu infection altered the DC cytokine secretion profile, supernatants from control non-infected, Cmu-infected, and LPS-stimulated BMDC were collected after 36 hours and analysed by a multiplex bead assay. This experiment was also performed for Toll-like receptor (TLR)-2 agonist peptidoglycan (PGN)-stimulated BMDC. Infected BMDC secreted significantly more IL-12, TNF-α, IL-6, IL-10 and IL-13 (Figure 3A-E) than non-infected BMDC. With the exception of IL-10 and TNF-α, cytokine levels were approximately equivalent between infected and LPS-stimulated BMDC (Figure 3A-E), and likewise for PGN-stimulated BMDC (data not shown). TNF-α was produced by infected BMDC at less than 75% of the level induced by LPS-stimulation (Figure 3B), this was despite the large dose of Cmu administered. Similarly, TNF-α was lower in cultures of PGN-stimulated BMDC compared to LPS-stimulated BMDC (data not shown). Interestingly, IL-10 was ~3-fold higher in cultures of infected BMDC compared to both LPS-stimulated (Figure 3D) and PGN-stimulated BMDC (data not shown), suggesting an important role for this Th1-inhibiting cytokine. Furthermore, very high levels of IL-6, a regulatory T cell inhibitory cytokine and activator of Th2 immunity, were detected from Cmu-infected BMDC.
Figure 3. *Chlamydia* infection of BMDC increases the secretion of a diverse array of cytokines including Th2-inducers. *Chlamydia muridarum* (Cmu)-infected (black) and non-infected BMDC (unshaded) were cultured for 36 hours or non-infected BMDC were cultured for 24 hours then stimulated with LPS for the final 12 hours (grey). Cytokines in BMDC supernatants were determined by multiplex bead array system, (A) IL-12(p70), (B) TNF-α, (C) IL-6, (D) IL-10, (E) IL-13. All values are concentrations of pg/ml. * p<0.05 compared to non-infected BMDC, ^ p<0.05 compared to LPS-stimulated BMDC. Data represent mean ±SEM.
Chlamydia-infected BMDC induce T cell proliferation and a Th2 phenotype in D10 cells

To evaluate the potential of Cmu-infected BMDC to polarize T cells to a specific phenotype, infected or non-infected BMDC were pulsed with the dominant antigenic peptide (OVA_{323-339} peptide) of the archetypal allergen ovalbumin (OVA). The cells were washed and then cultured with CFSE-labeled naive CD4^{+} OVA peptide-specific DO11.10 T (D10) cells. After 4 days, 60-70% of D10 cells co-cultured with infected BMDC had undergone ≥ 3 cell divisions in contrast to only 10-12% of T cells activated by non-infected BMDC (Figure 4A). Conversely, Cmu infection of BMDC produced a significantly lower proportion of static or poorly proliferative (0-2 divisions) D10 cells compared to co-culture of non-infected BMDC with D10 cells. This data clearly demonstrates that Cmu infection of BMDC stimulates significantly greater allergen-specific T cell proliferation. BMDC exposed to LPS and OVA and then cultured with T cells did not produce any significant increase in proliferation compared to non-infected BMDC controls (data not shown). This strongly suggests that the enhanced proliferation was specific to Chlamydia infection and not simply a result of maturation of the BMDC. The T cell cytokine production was determined after 4 days of co-culture with infected or non-infected, OVA peptide-pulsed BMDC. Infected and non-infected BMDC cultured alone were included as controls. In addition, non-peptide-pulsed unstimulated BMDC cultured with D10 cells were also used as controls and produced very low levels of all cytokines (Figure 4B-D). Not only did Cmu-infected BMDC secrete significantly more TNF-α, IL-10 and IL-13 as compared to non-infected controls (as shown in Figure 3), but they also drove D10 cells to secrete additional levels of these cytokines (Figure 4B-D). D10 cells cultured with infected BMDC produced much higher levels of TNF-α, and the Th2 cytokines IL-10 and IL-13, compared to D10 cells cultured with non-infected BMDC. At baseline, activation of peptide-specific D10 cells by BMDC pulsed with OVA peptide resulted in IFN-γ secretion. Notably,
while D10 cells activated by non-infected BMDC produced substantial levels of IFN-γ, Cmu infection of BMDC significantly attenuated this response (Figure 4E). In fact infection of BMDCs elicited a near total reduction (>20-fold) in the secretion of the prototypical Th1 cytokine, IFN-γ, by D10 cells. Collectively these results suggest that chlamydial infection of DCs promotes the proliferation of allergen-specific T cells to a bystander antigen, and this is associated with commitment to a Th2 phenotype and suppression of Th1 responses.
Figure 4. Co-culture of antigen-specific T cells with *Chlamydia*-infected BMDC creates a highly proliferative Th2 cell. *Chlamydia muridarum* (Cmu)-infected and non-infected BMDC were pulsed with OVA\textsubscript{323-339} peptide then cultured for 4 days with DO11.10 T cells. In some experiments T cells were labeled with CFSE and T cell division was determined by a decrease of CFSE fluorescence. (A) The data represents the percentage of cells that have undergone between 0 and 2 divisions versus 3 or more cell divisions, for unstimulated T cells (grey), non-infected (unshaded) and Cmu-infected (black) BMDC pulsed with OVA\textsubscript{323-339} peptide and cultured with CFSE-labeled DO11.10 T cells. * p<0.05 compared to unstimulated T cells, # p<0.05 compared to BMDC + OVA + T cells. Co-culture supernatants were assayed by
multiplex bead array system to determine concentrations of the cytokines, (B) TNF-α, (C) IL-10, (D) IL-13, and (E) IFN-γ. All values are concentrations of pg/ml. Controls included unstimulated T cells (BMDC + T cells), and Cmu-infected and non-infected BMDC pulsed with OVA peptide. * p<0.05 compared to BMDC + OVA + T cells. Data represent mean ±SEM.
Adoptive transfer of *Chlamydia*-infected BMDC plus D10 cells causes airways hyper-reactivity in naïve BALB/c mice

Next we determined if chlamydial infection of BMDC could induce AHR, a critical clinical feature of infection-induced exacerbations of asthma. Naïve BALB/c mice were intravenously infused with 2 x 10^6 D10 cells one day prior to the intratracheal (i.t.) administration of 3 x 10^5 Cmu-infected or non-infected, OVA peptide-pulsed BMDC. Three days following transfer of BMDC, BAL fluid from recipient mice was collected. IL-10 and IL-13 concentrations in BAL fluid were significantly increased in recipients of infected BMDC and D10 cells as compared to recipients of non-infected BMDC and D10 cells (Figure 5A-B), or D10 cells alone (data not shown). To examine whether the increased IL-13 was DC or T cell-derived, BMDC from IL-13-deficient (IL-13/-) mice were utilized. The IL-13 levels in BAL fluid from recipients of infected versus non-infected IL-13/- BMDC was not significantly different (Figure 5B), demonstrating that the IL-13 was derived directly from Cmu-infected DCs themselves, consistent with our *in vitro* data (Figure 3E). Similar to BMDCs from wild-type mice, IL-10 levels were significantly greater in recipients of Cmu-infected versus non-infected IL-13/- BMDC (Figure 5A). We then determined if adoptive transfer of Cmu-infected BMDC and D10 cells affected the development of AHR in BALB/c mice, as IL-13 is a critical regulator of enhanced bronchoconstriction in mouse models of asthma. Mice were mechanically ventilated and challenged with two doses of methacholine prior to measurement of airways resistance and compliance. Airways resistance (Figure 5C) was significantly greater in recipients of infected BMDC as compared to recipients of non-infected BMDC. Similarly, airways compliance was significantly decreased in recipients of infected BMDC compared to recipients of non-infected BMDC (Figure 5D). A similar trend was also observed for mucous secreting cells with greater numbers detected in the airways of recipients of infected versus non-infected BMDC (data not shown). Overall, these data reveal that a *Chlamydia* infection of DCs significantly increases
AHR, which is associated with increased IL-13 production. Importantly, this pathway is also critical in inducing AHR in models of allergic asthma and thus identifies a common mechanism for infection-induced exacerbation of asthma.
Figure 5. Adoptive transfer of *Chlamydia*-infected BMDC leads to DC-derived IL-13 in the BALF and increased AHR. DO11.10 T cells (2 x 10^6) were adoptively transferred i.v. to naïve BALB/c mice on day 0. Mice received 3 x 10^5 OVA_{323-339} peptide-pulsed BMDC (*Chlamydia* (Cmu)-infected or non-infected) i.t. on day 1. On day 3, animals were sacrificed and the levels of (A) IL-10, and (B) IL-13 in bronchoalveolar lavage fluid were determined. Statistical significance and p values are depicted. Mice were mechanically ventilated and (C) airways resistance and (D) compliance were determined by methacholine exposure for mice receiving Cmu-infected BMDC (shaded boxes) or non-infected BMDC (unshaded boxes). * p<0.05, **p<0.01 compared to non-infected BMDC control. Data represent mean ±SEM. WT; wild-type bone marrow, IL-13/-/-; IL-13 knockout bone marrow.
II.5 DISCUSSION

There is a strong epidemiological link between infection with the intracellular bacteria *C. pneumoniae* and asthma exacerbation. A recent investigation also shows that early life infection with the mouse pneumonitis biovar *C. muridarum* exacerbates allergic asthma in a mouse model of the disease (Horvat et al., 2007). However, the cellular and molecular mechanisms that underpin this association are poorly understood. In this investigation we show that *C. muridarum* (Cmu) can form a viable infection within BMDC and that infection subverts DC function. *Chlamydia* infection of BMDC inhibited the expression of the constitutive co-stimulatory marker CD80 and enhanced CD86 expression, a phenotype initially reported to promote Th2 responses (Kuchroo et al., 1995). However, the actual effect on the polarization of T cells is perhaps more complex (Bell et al., 2003, Elloso and Scott, 1999). *Chlamydia* failed to up-regulate the antigen presenting molecule MHC class II. This result complements a study from Zhong et al., which showed that in cervical and airway epithelial cell lines *Chlamydia* infection inhibits IFNγ-inducible MHC class II expression by degrading the transcription factor upstream stimulator factor-1 (USF-1) (Zhong et al., 1999). This deviation of MHC class II expression combined with the lower co-stimulatory CD80 expression may assist the bacteria in evading recognition by the immune system. *Chlamydia*-infected BMDC spontaneously secreted high levels of IL-6, and IL-10, cytokines that promote Th2 responses, as well as the inflammatory cytokine TNF-α. PGN-stimulated BMDC produced a very similar cytokine secretion pattern to that of *Chlamydia* infection other than the respective results for IL-10. As PGN is a known agonist of both TLR2 and the NOD1 intracellular pathogen recognition receptor, our results complement previous reports that indicate *Chlamydia* activates both TLR2 and NOD1. Interestingly, IL-13 secretion, which to the author’s knowledge, has not previously been shown to be produced by DCs in response to a pathogen, was also induced by *Chlamydia* infection. Whilst infected BMDC also produced IL-12, which is normally
associated with the induction of Th1 responses, co-culture of infected, OVA peptide-pulsed BMDC with naïve D10 cells inhibited the development of a Th1 phenotype through suppression of IFN-γ. In contrast, *Chlamydia*-infected BMDC promoted the expression of the Th2 cytokines IL-10 and IL-13 from D10 cells. Furthermore, *Chlamydia*-infected BMDC induced substantially greater expansion of allergen-specific T cells. Collectively, the chlamydial infection of BMDC generates an APC that favors the induction of a highly proliferative Th2 response to a bystander antigen (OVA peptide). These results were substantiated *in vivo* by adoptive transfer studies. Recipients of OVA peptide-pulsed, *Chlamydia*-infected BMDC and D10 cells had higher levels of IL-10 and IL-13 in the BAL fluid as compared to recipients of non-infected BMDC. The increased production of Th2 cytokines was associated with increased airways resistance and decreased compliance (enhanced AHR) and a trend to greater numbers of mucous secreting cells. Therefore, this study provides a novel mechanism to explain the association between *Chlamydia* lung infection and infection-induced exacerbations of asthma.

Interestingly, *C. pneumoniae* has been detected in the cytoplasm of DCs *in vivo* from patients with atherosclerosis, and human DCs can also be infected with *C. pneumoniae in vitro* (Bobryshev et al., 2004, Gervassi et al., 2004, Wittkop et al., 2006). Moreover, a study by Wittkop et al., demonstrated that infectious progeny are present within DCs as much as 25 days after infection (Wittkop et al., 2006). Our studies suggest the possibility that *Chlamydia*-infected pulmonary DCs could take up allergen in the lung, migrate to lung-draining lymph nodes and stimulate a greater Th2 response that contributes to allergic disease. Alternatively, infected DCs may reside in the respiratory epithelium and induce greater re-stimulation of allergen-specific Th2 cells, possibly via the secretion of IL-6 (Doganci et al., 2005). Our studies uniquely demonstrate that *Chlamydia*-infected murine DCs can release the key
asthmatic effector cytokine IL-13, and the enhanced secretion of this cytokine due to infection associates with enhanced AHR and mucous secretion. This provides a mechanism by which Chlamydia-infected pulmonary DCs could directly exacerbate features of asthma, especially infection-associated bronchial hyper-reactivity. Thus, we have identified a common aetiopathological pathway where infections and allergens may converge to exacerbate asthma.

Although the mechanisms of clearance are less well-defined in humans, immune protection against murine chlamydial infection requires a Th1 response, with the mechanism(s) of clearance critically dependent on the production of IFN-γ (Morrison and Caldwell, 2002). DC-derived IL-12 plays an important role in the induction of this anti-chlamydial Th1 response. Administration of recombinant IL-12 has been shown to confer protection to Chlamydia-infected mice, whereas IL-12-deficient mice develop reduced clearance of Chlamydia that is associated with severe tissue lesions due to low IFN-γ production (Huang et al., 1999, Lu et al., 2000). We have demonstrated that Chlamydia-infection of BMDCs suppresses a Th1 response by dramatically inhibiting the IFN-γ secretion by D10 T cells. In combination with our finding that Chlamydia-infected BMDC secrete IL-6, IL-10 and IL-13, which collectively induce Th2 responses and inhibit macrophage-dependent innate immunity, this could potentially offer a survival advantage for Chlamydia within the host. Further study is required to determine whether this may contribute to the development of persistent infections (Hogan et al., 2004).

Although IFN-γ production was inhibited, IL-12 was still secreted by infected BMDCs, which implicates the involvement of other factors downstream of IL-12 in disrupting the induction of a Th1 response. IL-6 was produced by infected BMDC. Secretion of this cytokine by pulmonary DCs has been shown to suppress IL-12-mediated Th1 responses (Dodge et al., 2003). Furthermore, IL-6 enhances local Th2 responses by allergen-specific T cells in the lung
and promotes T cell proliferation (Doganci et al., 2005). Hence the induction of an adaptive immune response to *Chlamydia* infection, in the presence of IL-6, could favor a non-protective Th2 response. IL-10 was also secreted by *Chlamydia*-infected BMDC and from T cells stimulated by these DC. IL-10 is a potent inhibitor of IL-12-induced Th1 differentiation. Protective anti-chlamydial Th1 cells are elicited far more effectively in IL-10 knockout mice than in wild-type mice. Igie et al, demonstrated this to be a consequence of the predilection of the DCs from IL-10 knockout mice to being potent initiators of a Th1 response (Yang et al., 1999, Igietseme et al., 2000). Therefore, the significant IL-10 levels measured from our infected BMDCs are likely a major factor for the inhibition of the Th1 response in our T cell experiments. Studies of another inflammatory disease caused by *Chlamydia*, namely reactive arthritis, have also shown that the IL-10/IL-12 balance is critical for determining the outcome of the immune response (Yin et al., 1997). Maintenance of the Th2 cytokine pattern that drives both reactive arthritis and chlamydial persistence depended on IL-10 suppression of IL-12-mediated protective immunity. Thus excess IL-10 can result in Th2 immunity even in the presence of IL-12. Indeed, overproduction of IL-10 is a key feature in the impaired cell-mediated response to *C. trachomatis* infection in patients with trachoma (Ohman et al., 2006). Therefore, the production of IL-10 early in the innate immune response to intracellular pathogens such as *Chlamydia* may promote the establishment of persistent infections and the potential for Th2-mediated pathology.

In this study, IL-13 was produced by both *Chlamydia*-infected BMDC and from T cells stimulated by these DC. Currently, there is a paucity of data concerning the production of IL-13 by DCs, and to date only one such study exists in mice. This study demonstrated that murine DCs secrete low levels of IL-13 in response to Toll-like receptor (TLR) 2-stimulation by the synthetic ligand Pam3Cys (Redecke et al., 2004). This stimulation of DC IL-13
production was associated with the exacerbation of allergic airways disease, which further supports our observations. Bellinghausen et al., demonstrated that human DCs can produce IL-13 in response to allergen exposure, wherein this cytokine then acts on the T cell in a signal transducer and activator of transcription-6 (STAT6)-dependent manner to contribute to the induction of Th2 cells (Bellinghausen et al., 2003). In the context of our study IL-13 production by infected DCs may contribute to the development of non-protective anti-chlamydial immunity by suppressing macrophage function. IL-13 is known to inhibit macrophage production of NO (McKenzie et al., 1993, Doherty et al., 1993, Davidson et al., 2007), IFN-γ-induced tryptophan degradation (Chaves et al., 2001), and production of inflammatory cytokines, such as IL-1 (Muzio et al., 1994, Zurawski and de Vries, 1994). These are all important mechanisms for resolution of chlamydial infection. Unpublished data from our laboratory also shows that IL-13-deficient mice clear a respiratory Cmu infection more rapidly than wild-type mice (unpublished data), suggesting that IL-13 regulates the innate response to Cmu infection. By inducing the secretion of IL-13 through viable infection of the DC, *Chlamydia* may suppress the innate immune response to infection to enhance bacterial survival, while simultaneously having the deleterious effects on airway reactivity.

In summary, *Chlamydia* infection has been linked with the exacerbation of asthma. Here we provide a mechanism whereby infection of DCs subverts the function of these crucial immune cells to promote Th2 immune responses that are non-protective with regards to infection but have the potential to exacerbate preexisting allergic inflammation and induce AHR. Notably, allergen or infection both induce common effector pathways, converging on the IL-13 operated STAT6 signaling cascade that is critical for the development of enhanced bronchoconstriction (AHR). This data provides the first mechanistic explanation for the association between *Chlamydia* lung infection and exacerbation of asthma.
CHAPTER III: PUBLICATION #2

Natural Killer Cell Deficiency Predisposes to Viral-Induced Th2-type Allergic Inflammation via Epithelial-Derived IL-25


Gerard E. Kaiko¹², Simon Phipps¹, Pornpimon Angkasekwinai³, Chen Dong³, and Paul S. Foster¹²

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1. Centre for Asthma and Respiratory Diseases, School of Biomedical Sciences and Pharmacy, Faculty of Health, The University of Newcastle, Newcastle, NSW, 2300, Australia
2. Cooperative Research Centre (CRC) for Asthma and Airways, Australia
3. Department of Immunology, M. D. Anderson Cancer Center, Houston, TX, 77030, USA
III.1 ABSTRACT
Severe respiratory syncytial virus (RSV) infection has long been associated with an increased risk for the development of childhood asthma and exacerbations of this disorder. Despite much research into the induction of T helper 2 (Th2) responses by allergens and Helminths, the factors associated with viral infection that predispose to Th2-regulated asthma remain unknown. Recently, clinical studies have shown reduced numbers of natural killer (NK) cells in infants suffering from a severe RSV infection. Here we demonstrate that NK cell deficiency during primary RSV infection of BALB/c mice results in the suppression of IFN-γ production and the development of an RSV-specific Th2 response and subsequent allergic lung disease. The outgrowth of the Th2 responses was dependent on airway epithelial cell-derived IL-25, which induced the up-regulation of the notch ligand Jagged 1 on dendritic cells. This study identifies a novel pathway underlying viral-driven Th2 responses that may have functional relevance to viral-associated asthma.
III.2 INTRODUCTION

Respiratory Syncytial Virus (RSV) is a single-stranded negative sense RNA virus from the paramyxoviridae family (Simoes, 1999). RSV is the primary cause of hospitalisation in the first year of life for children worldwide (Simoes, 1999). It has long been recognised that infants suffering from a severe RSV infection, leading to bronchiolitis, are at significantly greater risk for the development of asthma (a CD4 T helper 2 lymphocyte (Th2)-dominated immune condition) and wheezing in later childhood (Sigurs et al., 2000, Sigurs et al., 2005, Simoes, 1999, Henderson et al., 2005, Kusel et al., 2007, Stein et al., 1999, Pereira et al., 2007). A long-term prospective study from Simoes et al., attempted to further delineate whether this was a causal association by examining respiratory outcomes following passive immunisation against RSV (using Palivizumab) (Simoes et al., 2007). This study revealed that by preventing hospitalization due to severe RSV infection the risk of recurrent wheezing in later childhood was reduced by 50%. Similarly, treatment with polyclonal immune globulins against RSV has also been demonstrated to reduce the incidence of childhood asthma 7-10 years later (Wenzel et al., 2002). These studies and others suggest that severe RSV infection is not simply a marker of asthma predisposition but instead they provide support for a causal relationship between early-life viral exposure and childhood asthma.

An emerging trend born from multiple clinical studies of severe RSV infected infants is a failure to generate a robust natural killer (NK) cell response (De Weerd et al., 1998, Welliver et al., 2007, Welliver et al., 2008, Larranaga et al., 2009). Furthermore, the magnitude of the deficiency increases with worsening severity of disease indicating an inverse relationship (De Weerd et al., 1998, Larranaga et al., 2009). For instance, although the numbers of NK cells were reduced in patients admitted to hospital with RSV bronchiolitis, a subpopulation of
infants who were ventilated exhibited an even greater (3-fold) reduction in NK cells, while fatal cases displayed a near absence of NK cells in lung autopsy specimens. Thus, deficiency in NK cell numbers is critically linked to severity of RSV-induced disease.

NK cells were originally thought to solely function as early innate inflammatory cells for host defence against pathogens and malignancy. It is now recognised that these cells play a crucial role in the priming of adaptive immune responses against a variety of viral infections (Andoniou et al., 2006). Indeed the recruitment and activation of IFN-γ producing NK cells to the site of inflammation plays a critical role in the subsequent development of an effector CD4 T helper 1 lymphocyte (Th1) and cytotoxic T lymphocyte response (Mailliard et al., 2003). This may occur indirectly through NK cell licensing of dendritic cells (DCs). During this bidirectional crosstalk IFN-γ released by NK cells activates DCs to produce IL-12, which in turn feeds back on the NK cell to further amplify IFN-γ secretion (Mailliard et al., 2003, Orange and Biron, 1996). Of note, defective NK cell function is strongly linked with the development of Th2-dominated immune responses in atopic eczema (Hall et al., 1985, Jensen et al., 1984, Sakai et al., 2003).

The absence of inflammatory signals in the lung micro-environment has been proposed to default naïve T cells towards a Th2 phenotype (Stumbles et al., 1998), however recent reports indicate that specific Th2-inducing mechanisms also exist. Thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 have all been identified as innate immune mediators that promote Th2 differentiation during sensitisation to allergens and/or helminthic parasites (Saenz et al., 2008). Although both macrophages and granulocytes secrete TSLP, IL-25, and IL-33, airway epithelial cells are also a rich source of these cytokines supporting the paradigm that the activation of stromal cells influences the development of Th2 immunity (Soumelis et al., 2002,
Angkasekwinai et al., 2007, Hammad et al., 2009). These cytokines have the ability to induce Th2 cell differentiation, by either acting directly on naïve T cells or indirectly through modulation of other cell types (Soumelis et al., 2002, Angkasekwinai et al., 2007). Although these factors contribute to the generation of Th2 immune responses during exposure to allergens it remains unknown how viral infections (such as RSV) may induce Th2 rather than protective Th1 responses.

Collectively, clinical observations suggest that impaired or deficient NK cell activity may play a role in the manifestation of severe RSV induced bronchiolitis, a disease often associated with the development of childhood asthma. Data is also emerging suggesting that epithelial cell-derived cytokines (TSLP, IL-25, and IL-33) may promote Th2 immunity at mucosal surfaces. In this study we examine the role of NK cells in the regulation of RSV infection and in particular how a viral infection may influence the Th2 immune compartment. By employing a mouse model of RSV infection and NK cell depletion we demonstrate a critical role for this cell in protection against the generation of viral- and bystander allergen-specific Th2 responses. Airway epithelial cell derived-IL-25 was significantly up-regulated due to the reduced levels of IFN-γ in the absence of NK cells in infected mice, demonstrating a unique inverse relationship between these factors. IL-25 was critical to the development of Th2 cells and inflammation in response to RSV, at least in part, by the up-regulation of the notch ligand Jagged1 on lymph node DCs. IL-25 activated a novel Th2-inducing pathway during respiratory viral infection.
III.3 MATERIALS AND METHODS

Mice and Infections

Male BALB/c mice (8-10 wk old) were used in all studies. Mice were administered virus via the intranasal route under light isoflurane induced anaesthesia. Respiratory Syncytial Virus (RSV) (long strain, type A) was obtained from the American Type Culture Collection (ATCC) and was propagated in monolayers of HEp-2 cells for 4 days. Supernatant and cells were collected with 50 mM HEPES and 100 mM MgSO4 and centrifuged to produce clarified supernatant. Resulting volume was layered onto a two-step sucrose gradient and ultracentrifuged at 100,000g for 1 hour at 4°C. RSV titer was determined by plaque assay. Mice were administered 5x10^5 PFU of RSV in 50 μl PBS on day 0. Influenza strain A/PR/8/34 was propagated and purified as previously described (Oh and Eichelberger, 1999). Mice were administered 1000 PFU of influenza in 50 μl PBS on day 0. All studies were approved by and conducted in accordance with guidelines set out by the University of Newcastle Animal Care and Ethics Committee.

Mouse Treatment Regimes

Mice were administered intraperitoneal (ip) injections of anti-ASIALO GM1 (Wako) on days -1, 1, and 4, in order to deplete NK cells, or purified antibody clone YTS 169.4 on days -3, -1, 2, and 5, in order to deplete CD8 T cells, or treated with isotype-matched controls. Treatments were confirmed by flow cytometry to deplete 95% of NK cells, and 98% of CD8 T cells, respectively. Mice that were depleted of NK cells were also treated in separate experiments with an IL-25 neutralising antibody clone 35B (kindly provided by Chen Dong of the MD Anderson Cancer Center, USA) or rat IgG isotype control (Sigma-Aldrich) (both 250 μg in 200μl PBS) by ip injection on days -1, 1, and 3, or recombinant mouse IFN-γ (BD Biosciences).
(1 μg in 30 μl PBS) by intranasal instillation on days 1, 2, and 3. To deplete basophils mice were treated with anti-FcεRI (clone MAR-1) or an isotype control as previous (Denzel et al., 2008). To determine the impact on an inhaled antigen model, mice were exposed to ovalbumin (OVA) protein via the intranasal route without adjuvant. Mice were given 100μg OVA on days 3, 5, and 7 and after 2 weeks challenged with 25 μg OVA on days 21, 22, 25, 26, and sacrificed 24 hours later.

**Lymph Node Stimulation Assays**

Lymph node cells were prepared and placed in culture at 2x10⁶ cells/well/200 μl growth medium (RPMI 1640, 5x10⁻⁵ M 2-ME, 10% heat-inactivated FCS, 2 mM L-glutamine, 20 mM HEPES, 100 μg/ml penicillin ,and 100 μg/ml streptomycin, 0.1mM sodium pyruvate). Cells were treated with either 50 μg/ml anti-Jagged1 (Sigma-Aldrich) or a goat IgG isotype control, or left untreated and cultured for 3 days. For the OVA model, lymph node cells were stimulated with 200 μg/ml OVA protein or cultured without stimulation for 4 days.

**Purified CD4 T Cell Stimulation Assay**

Naïve T cell-depleted splenocytes were inactivated by mitomycin C treatment according to manufacturer’s instructions (Sigma-Aldrich). Splenocyte feeder cells were plated out at 5x10⁵ cells/well/100 μl growth medium and exposed to UV-inactivated RSV (MOI of 0.5) or unstimulated. Lymph node cells were isolated from treated mice, and CD4 T cells were purified by negative selection using the CD4 T Lymphocyte Enrichment kit (BD Biosciences) and plated out at 2x10⁵ cells/well/100 μl growth medium together with the feeder cells for 3 days culture.
Histology

Lung tissue was fixed in 10% formalin and paraffin-embedded for sectioning. Stains included Chromotrope 2R and Hematoxylin to enumerate eosinophils, or Periodic Acid-Schiff to enumerate mucous secreting cells. Cells were counted around major airways for a minimum of 10 fields at 100 x magnification.

Real-Time Quantitative PCR

Lungs and lymph nodes were excised and frozen in RNAlater solution (Ambion) at -80°C. Total RNA was isolated using TriReagent solution (Ambion) according to manufacturer’s instructions. Reverse transcription and quantitative PCR were conducted with Superscript III and Express Sybr Green with ROX (Invitrogen) according to the manufacturer’s instructions. Intron spanning primer sequences were designed for the following (5’ to 3’): IL-4 (TTGAGAGAGATCATCGGCATTTTG and TCAAGCATGGAGTTTTCCCATGT), IL-12p35 (GCTGAAGACCACAGATGACATGGT and TCATAGATGCTACCAAGGCACAGG), IL-13 (AGCTGAGCAACATCACACAAGACC and TGGGCTACTTCGATTTTGGTATCG), IL-25 (CTGAAGTGAGCTCTGCATCTGTG and GTCCATGTGGGAGCCTGTCTGTAG), IFN-γ (TCTTGAAAGACAATCAGCCCATCA and GAATCAGCAGCGACTCCTTCC), Eotaxin-1 (CCCCACACACTACTGAAGAGCTACAA and TTTGCCCAACCTGGTGCTTG), Eotaxin-2 (TAGCCTGCGGTGTGGCATCTTCC and TAAACCTCGGTGTATTTGCCACGG), HPRT (AGGCCAGACTTTGTGGATTTGAA and CAACTTGCGCTCATCTTAGGCTTT), RSV A strain N gene (CATCCAGCAATACACCATCCA and TTCTGCACATCATAATTAGGAGTATCAA). RT-PCR was performed using the Realplex Mastercycler ep (Eppendorf) using the following cycling conditions: 1 x 50°C 2min, 1 x 95°C
2min, 40 x 95°C 15sec, 40 x 60°C 1 min, plus standard melting curve. Relative gene expression was determined using a HPRT housekeeping gene, whereas quantification of RSV viral titre was determined using plasmid copy number standards for RSV N gene and HPRT.

**Immunohistochemistry**

Paraffin-embedded sections were cut, rehydrated and exposed to antigen retrieval using heating in sodium citrate buffer. Sections were incubated in 0.3% Triton X-100/PBS for 10 minutes to permeabilize the cells before blocking non-specific binding with 5% rabbit immunoglobulins (Sigma-Aldrich) for 1 hour. Sections were washed in 0.3% Triton X-100/PBS and incubated with 60 μg/ml rat anti-mouse IL-25 (clone 35B) or a rat IgG isotype control (Sigma-Aldrich) overnight at 4°C. Sections were washed and incubated with a 1/100 dilution of rabbit anti-rat IgG-biotin (Dako) for 1 hour, and washed and incubated with a 1/100 dilution of streptavidin-alkaline phosphatase (Amersham Biosciences) for 30 minutes. Washes in TBS were followed by colour development using the FAST RED TR/Naphthol system (Sigma-Aldrich) with levamisole endogenous alkaline phosphatase inhibitor (Dako), and hematoxylin counterstain. Photographs were captured at room temperature at 40x magnification using an Olympus microscope (model BX51), digital camera (Olympus DP70) and DP software (Olympus, California).

**Flow cytometry/Intracellular Cytokine Staining**

Mediastinal lymph node cells or lung cells were isolated and incubated with anti-mouse CD16/32 Fc receptor block at 20 μg/ml/10^6 cells for 15 min on ice. Cells were incubated with the relevant antibody on ice for 20min in dark. Antibodies included anti-mouse MHC class II-APC, FcεRI-biotin, CD3-PECy7, and c-kit-PECy7 (all from eBioscience), DX5-PE, CD11c-PE, Gr-1-APC, CCR3-APC, and IL-4-FITC (all from BD Biosciences), delta-like ligand 4-
biotin (R & D Systems), and purified rabbit anti-mouse Jagged1 (Santa Cruz Biotechnology). All isotype control antibodies were obtained from BD Biosciences. For biotinylated antibodies, a streptavidin-conjugated PerCP (BD Biosciences) was added and for Jagged1 a goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Invitrogen) was added at 4°C for 30 min. Intracellular staining was performed by incubating lymph node cells for 4 hours unstimulated in growth media containing Brefeldin A (Sigma-Aldrich) at 5 μg/ml. Cells were first stained for DX5, FcεRI, c-kit, CD3, CCR3, Gr-1, fixed, permeabilized, and then stained for IL-4 according to standard intracellular staining procedures before being fixed. Cells were analyzed on a FACSCanto (BD Biosciences). DCs in the draining lymph nodes were gated based on their expression of CD11c^+MHIClassII^high^.

**BAL Analysis**

A cannula was placed into the trachea and bronchoalveolar lavage (BAL) conducted using two 0.7 ml injections of Hank’s Buffered Salt Solution, cells were spun down, and RBCs lysed. Cells were then spun onto slides using cytospin centrifugation and stained with the May-Grünwald and Giemsa stains. A minimum of 400 total cells were counted to determine differential WBC percentages.

**ELISA**

Serum concentrations of total IgE were determined using reagents from BD Biosciences. OVA-specific IgG1 and IgG2a concentrations in the serum and BAL fluid were determined by coating plates with 20 μg/ml OVA protein and using antibodies from Southern Biotech. Cell culture supernatants were analysed for the cytokines IL-4, -5, and -IFN-γ using antibodies from BD biosciences and IL-13 using antibodies from R & D Systems. All standards were obtained from BD Biosciences.
Statistical analyses

Statistical analyses were performed using GraphPad Prism version 4.0 software with the Mann-Whitney t test and one way ANOVA tests. A p value less than 0.05 was considered statistically significant.
III.4 RESULTS

NK cells protect against viral-mediated Th2 allergic airways inflammation

To investigate the role of NK cells in regulating RSV-infection we compared immune and morphological responses between wild type isotype control-treated (WT control) mice and NK cell-depleted mice. The influx of NK cells into the lungs peaked 4 days after inoculation with live RSV, and NK cell numbers were reduced by 95% in the lung (80% in the mediastinal lymph nodes) confirming NK cell antibody-mediated depletion (Supplementary Figure S1A and B). Inoculation of NK cell depleted mice with RSV resulted in significantly reduced levels (5-fold reduction) of IFN-γ mRNA expression in the lungs compared to both WT control and CD8 T cell-depleted mice at 4 days post-infection (dpi) (Figure 1A). As determined by intracellular cytokine staining, NK cells directly produce IFN-γ protein, and furthermore are the major source of this cytokine in the lungs at 4 days post RSV infection (Supplementary Figure S2A and B). Depletion of NK cells led to increased mRNA levels of the Th2-type cytokines (IL-4 and IL-13) and eosinophil-active chemokines (eotaxin-1 and -2) in the lungs at 9 dpi (Figure 1B). Furthermore, other classical markers of Th2-mediated allergic inflammation were significantly elevated in the absence of NK cells as NK-depleted mice had increased serum IgE, and increased numbers of tissue eosinophils and mucus secreting cells (Figure 1C-E). The increased mucus cell metaplasia in NK-depleted mice also coincided with the appearance of mucus plugging in the airways as is depicted visually in the micrographs of Supplementary Figure S3. This increased Th2 pathology was reflected in a delayed viral clearance in the later stages of infection (Supplementary Figure S4). A trend to greater numbers of mast cells was also observed (Supplementary Figure S5). To demonstrate the specificity of NK cell depletion for the induction of the Th2 immune phenotype, mice were also depleted of IFN-γ-producing CD8 T cells. Depletion of this T cell subset did not promote
Th2 responses, however a small increase in eosinophils was observed (Figure 1 B-E). Thus, the reduction in IFN-γ and the development of an RSV-driven Th2 phenotype was induced specifically through the absence of NK cells and independently of CD8 T cells. Importantly, in order to confirm that the cellular source of the Th2 cytokines was the differentiation of viral-specific Th2 lymphocytes, CD4 T cells were purified from the mediastinal lymph nodes of mice (9 dpi) and stimulated with feeder splenocytes pulsed with UV-irradiated RSV. This approach eliminated any interference from other potentially virus-activated cells within the lymph nodes. RSV-specific CD4 T cell-derived IL-4, -5, and -13 were all significantly elevated in the absence of NK cells compared to WT controls but not in the absence of CD8 T cells, while levels of RSV-specific IFN-γ production were reduced (Figure 1F). Thus, viral-specific Th2 cells develop in the absence of NK cells.
Figure 1. NK Cells Protect Against Viral-mediated Th2 Responses. Mice were treated with anti-ASIALO GM1 (NK-depleted), YTS169.4 (CD8-depleted), or isotope control (WT control) antibodies, as described in materials and methods, and mice were inoculated with RSV on day 0. (A) At day 4, relative mRNA expression of IFN-γ was measured in the lungs by quantitative
PCR and normalised to Hprt. Data are expressed as the fold change over naïve mice and represent the mean ± SEM. **p<0.01 compared to WT control mice. (B) Relative mRNA expression of IL-4, IL-13, and eotaxin-1, and -2 was measured in the lungs on day 9. Data represent the mean ± SEM. ***p<0.001, **p<0.01, and * p<0.05 compared to WT control mice. (C) Total IgE was determined in the serum of mice on day 9. Results represent the mean ± SEM. ** p<0.01 compared to WT control mice. (D) Eosinophils were enumerated in the airway parenchyma per 100μm basement membrane (BM) on day 9. Data represent the mean ± SEM. *p<0.05, and ***p<0.001 compared to WT control mice. (E) The percentage of epithelial cells in the airways staining positive for mucous was enumerated on day 9. Data represent the mean ± SEM. *** p<0.001 compared to WT control mice. (F) At 9 days feeder splenocytes were pulsed with UV-inactivated RSV and plated out with lymph node CD4 T cells from each mouse. Supernatants analysed for IL-4, -5, -13, and IFN-γ. Data represent the mean ± SEM. * p<0.05 compared to WT control mice. ND; not detected. Results represent 2 independent experiments with n ≥ 5 mice per group.
**Viral-specific Th2 responses persist into long-term memory**

Although NK cells were depleted during a primary infection they returned to normal levels by 16 dpi (data not shown). In the previously NK cell depleted mice a secondary inoculation in the presence of NK cells, 42 days after primary RSV exposure, induced CD4 T cells to secrete increased levels of the Th2 effector cytokines IL-5 and IL-13, by comparison to WT control mice (Figure 2A). Furthermore, both the numbers of mucus secreting cells and tissue eosinophils in the lung were significantly elevated above controls (Figure 2B and C). The morphological changes appear milder than at 9 dpi, which may reflect the presence of NK cells during the secondary infection, but may also reflect the impact of protective immunity to RSV elicited by a strong secondary neutralizing antibody response. Nevertheless, this data demonstrates that the Th2 cells generated by a primary infection in the absence of NK cells can persist long-term and can be reactivated by viral antigens, associated with airway changes characteristic of allergic inflammation.
Figure 2. Th2 responses persist long-term. Mice were inoculated with RSV on day 0 and left for 42 days before being re-inoculated with RSV. At 48 days, feeder splenocytes were pulsed with UV-inactivated RSV and plated out with lymph node CD4 T cells from each mouse, supernatants were analysed for (A) IL-5, and IL-13. Data represent the mean ± SEM. * p<0.05 compared to WT control mice. ND; not detected. (B) The percentage of epithelial cells in the airways staining positive for mucous was enumerated; and (C) the number of eosinophils in the airway parenchyma per 100 μm basement membrane (BM) was counted. Data represent the
mean ± SEM. * p<0.05 compared to WT control mice. Results represent 2 independent experiments with n ≥ 5 mice per group.

**NK cell deficiency during RSV infection induces allergic airways inflammation in response to an innocuous bystander antigen**

To examine concurrent exposure to both RSV and airborne allergens (to more closely replicate the situation in humans), we next evaluated the role of NK cells in the modulation of viral infection concomitantly with exposure of the airways to an innocuous antigen. Direct exposure of the airways to intranasal ovalbumin (OVA) without adjuvant is known to result in immunological tolerance to this protein (Holt et al., 1987). To determine if the Th2 response generated by RSV infection in the absence of NK cells impacted upon immunological tolerance to inhaled bystander antigen, the WT control or NK cell depleted mice were infected with RSV whilst being simultaneously exposed to inhaled OVA (see study design Figure 3A).

*In vitro* re-stimulation of lymph nodes cells from mice that had been exposed to virus and OVA in the absence of NK cells (NK-depleted RSV OVA) induced substantial increases in IL-4, -5, and -13 production, as compared to the WT control mice (WT RSV OVA) (Figure 3B). Both naïve and the OVA alone (OVA) mice had low levels of all cytokines measured. Hallmark features of allergic inflammation were also observed in NK-depleted RSV OVA treated mice. The numbers of epithelial cells secreting mucous and eosinophils in the bronchoalveolar lavage (BAL) fluid and lung tissue, were significantly increased compared to WT RSV OVA mice (Figure 3C-E). Furthermore, the humoral response generated in NK-depleted RSV OVA mice was strongly biased towards Th2, as evidenced by increased serum and BAL fluid concentrations of OVA-specific IgG1 in contrast to decreased levels of OVA-specific IgG2a (Figure 3F). Interestingly, mice that did not receive an RSV infection but were nevertheless depleted of NK cells (NK-depleted OVA) produced significantly lower OVA-specific IL-4 and IL-13, however these mice produced significantly higher levels of IL-5,
compared to mice infected with RSV. This higher IL-5 secretion was associated with elevated eosinophil numbers in the BAL fluid. This result also indicates a direct role for NK cells in suppressing pulmonary Th2 responses and maintaining tolerance to inhaled antigens. However, the strongest Th2 response (range of cytokines generated, development of a humoral response and degree of lung inflammation) occurred in the absence of NK cells during exposure to RSV. Thus, NK cells play an important role in inhibiting the development of Th2 responses to both viral and innocuous inhaled antigens.
Figure 3. NK Cell Depletion During RSV Infection Induces Th2 Responses to OVA. (A) Study design: all mice except naïve controls were exposed to Ovalbumin (OVA) via intranasal route without adjuvant. (B) Lymph node cells were cultured with or without OVA and supernatants analysed for IL-4, IL-5, and IL-13. Data represent the mean ± SEM. * p<0.05,
p<0.01 compared to WT RSV OVA mice. # p<0.05 compared to OVA mice. (C) Eosinophils in the airway lumen BAL were counted and expressed as the number of eosinophils per ml of BAL fluid. Data represent the mean ± SEM. * p<0.05 compared to WT RSV OVA mice. # p<0.05 compared to OVA mice. (D) Eosinophils were enumerated in the airway parenchyma per 100μm basement membrane (BM). Data represent the mean ± SEM. ** p<0.01 compared to WT RSV OVA mice. # p<0.05 compared to OVA mice. (E) The percentage of epithelial cells in the airways staining positive for mucous was enumerated. Data represent the mean ± SEM. * p<0.05 compared to WT RSV OVA mice. # p<0.05 compared to OVA mice. (F) OVA-specific IgG1 and IgG2a was measured in the serum and OVA-specific IgG1 was measured in the BAL. Data represent the mean ± SEM. ***p<0.001, ** p<0.01, *p<0.05 compared to WT RSV OVA mice. ND; not detected. Results represent 2 independent experiments with n ≥ 8 mice per group.
The RSV-induced Th2 immune response is initiated early in the lymph nodes and reversed by IFN-γ

Cytokine expression in the lymph nodes at 4 dpi was examined in order to determine whether alteration in T helper polarising signals occurred during the early phase of T cell differentiation. Indeed, in NK-depleted mice significant alterations in the mRNA expression of IL-4 (3-fold increased), IL-12 and IFN-γ (both 3-fold decreased) were observed in the lymph nodes compared to WT control mice (Figure 4A). Similarly, when these lymph nodes cells were cultured for 3 days there was also an increase in the protein level of IL-13, (likewise for IL-4 and IL-5; Supplementary Figure S6), coupled with a decrease in IFN-γ (Figure 4B). The skewing of these responses towards Th2 was not due to higher RSV load, as there was no detectable difference in viral titre between WT control and NK-depleted mice during T cell differentiation at 4 dpi (Supplementary Figure S7). To determine the impact of a reduced level of IFN-γ in the lungs due to the absence of NK cells, NK-depleted mice were treated with recombinant IFN-γ by repeated intranasal instillation. By utilising this treatment the cytokine pattern induced by NK cell depletion was significantly reversed to reflect that of WT controls and a Th1 response (Figure 4A and B). This data demonstrates a critical role for the IFN-γ signal from NK cells in the regulation of viral induced Th2 immune responses. As IL-4 was elevated in the lymph nodes at 4 dpi, and given recent reports of basophils as a major source of IL-4 contributing to the induction of Th2 responses to allergens in the lymph nodes, we determined the influence of this cell type on the development of RSV-driven Th2 pathology. FcRεI⁺CD49b⁺c-kitCCR3Gr-1’CD3⁺ basophils were observed within the lymph nodes of RSV-infected mice at similar percentages to that of influenza infected and naive control mice (Supplementary Figure S8). After intracellular staining approximately 50% of the basophils isolated from RSV-infected mice were found to secrete IL-4 (Figure 4C). By contrast, those
cells from naive or influenza-infected mice did not secrete IL-4 at all, suggesting this to be a unique effect of RSV. However, basophils were recruited to the lymph nodes independently of NK cells as there was no significant difference between WT control versus NK-depleted mice. Furthermore, depletion of these cells (using MAR-1 monoclonal antibody) failed to alter the generation of the Th2 phenotype (Figure 4D). Despite basophil depletion leading to an increase in RSV-specific CD4 T cell IFN-γ, there was no significant effect on the Th2 responses (no significant change in IL-4, IL-5 or IL-13 expression). Therefore, while these IL-4-secreting basophils may make some contribution to the generation of Th2 responses under some conditions they are not critical to the generation of the RSV-specific Th2 responses.
Figure 4. The RSV-induced Th2 Phenotype is Reversible by Intranasal IFN-γ. (A) At day 4, relative mRNA expression of IL-4, IL-12, and IFN-γ was measured in mediastinal lymph nodes by quantitative PCR and normalised to Hprt. Data are expressed as the fold change over naïve mice and represent the mean ± SEM. ** p<0.01, * p<0.05 compared to WT control mice. # p<0.05 compared to NK-depleted mice. (B) At day 4, lymph node cells were cultured
unstimulated for 3 days and supernatants were analysed for IL-13, and IFN-γ. Data represent the mean ± SEM. * p<0.05 compared to WT control mice. # p<0.05 compared to NK-depleted mice. (C) At day 4, lymph node cells were cultured unstimulated with brefeldin A followed by detection of IL-4 expression. Plots are gated on FcRε⁺CD49b⁺c-kit⁺CCR3⁻Gr-1⁻CD3⁻cells and the boxes represent IL-4⁺ basophils as a percentage of total basophils (noted in the top right corner). Each plot is from a single experiment representative of four independent experiments. Mice were infected with influenza as a control. (D) NK-depleted mice were treated with MAR-1 or an isotype control twice daily from -1 to 4 days post-RSV inoculation. At day 9 feeder splenocytes were pulsed with UV-inactivated RSV and plated out with lymph node CD4 T cells from each mouse. Supernatants analysed for IL-4, -5, -13, and IFN-γ. Data represent the mean ± SEM. * p<0.05 compared to Isotype control. Results represent 2 independent experiments with n ≥ 8 mice per group.
**IL-25 expression is induced in the respiratory epithelium by RSV and sustained in the absence of NK cells and IFN-γ**

We next determined the mechanism where by NK cell/IFN-γ deficiency resulted in the generation of Th2 responses to RSV, in order to answer the question of whether there was a Th2 default in the lung in the absence of these factors, or whether the NK cell/IFN-γ suppresses a Th2-inducing mechanism. Given the emerging role of the cytokines TSLP, IL-25 and IL-33 in the initiation of Th2 immunity, we measured the expression of these cytokines in the lung compartment at 2 and 4 dpi. Only the levels of the IL-17 family member IL-25 (IL-17E), were significantly increased (~5-fold) in the absence of NK cells, and this correlated with significantly reduced IFN-γ levels in the lung (~5-fold) at this time (Figure 5A). By contrast, the expression level of the other cytokines (TSLP, IL-33, and IL-4) involved in polarisation of Th2-cells were not altered in the lung (data not shown). When the airways of NK-depleted mice were repeatedly treated with recombinant IFN-γ, levels of IL-25 were significantly reduced both at 2 and 4 dpi (Figure 5B). The expression pattern of IL-25 and IFN-γ identified a unique inverse relationship, wherein IFN-γ levels remained at baseline while IL-25 expression increased at 2 dpi independently of NK cells. When NK cells infiltrate into the lungs at 4 dpi a disparate IFN-γ response emerges between the RSV infected WT control and NK cell depleted mice. As the expression of IFN-γ increases this suppresses the expression of IL-25. Conversely, in the absence of NK cell influx and IFN-γ production, IL-25 expression is not suppressed and continues to increase in lung tissue. By using immunohistochemistry, we identified that the IL-25 signal was localised to the respiratory epithelium, the same site known to be infected by RSV (Figure 5C). These immunohistochemistry results also demonstrated that at 4 dpi WT control mice displayed negligible levels of IL-25, which was comparable to that of the naïve mice and also NK-depleted RSV sections stained with the isotype control.
antibody, thus confirming the mRNA expression data. Thus, only in the absence of NK cells did RSV maintain a high level of IL-25 expression (4 dpi) in the respiratory epithelium.
Figure 5. RSV Induces IL-25 Expression in the Respiratory Epithelium. (A) At day 2 and 4 post-infection (dpi), relative mRNA expression of IFN-γ and IL-25 were measured in the lungs by quantitative PCR and normalised to Hprt. Data are expressed as the fold change over
naïve mice and represent the mean ± SEM. **p<0.01 compared to WT control mice. (B) Relative mRNA expression of IL-25 was measured in the lungs of NK-depleted mice treated with rmIFN-γ. Data represent the mean ± SEM. *p<0.05, **p<0.01 compared to NK-depleted mice. (C) At day 4, IL-25 protein expression was measured in lung sections by immunohistochemistry. The deep red colour is specific for IL-25 protein. Scale is as depicted on the micrograph. Results represent 2 independent experiments with n ≥ 5 mice per group.
RSV-induced Th2 responses are IL-25-dependent

To identify a role for IL-25 in the induction of the Th2 response in RSV-infected NK-depleted mice this cytokine was blocked in vivo. NK-depleted mice were treated with anti-IL-25 monoclonal antibody or an isotype control. As only temporary blockade was sort for a specific period gene-deficient mice were not considered a preferable option. Lymph node CD4 T cells isolated from anti-IL-25 treated mice at 9 dpi, and subsequently stimulated with UV-irradiated RSV, secreted significantly less RSV-specific IL-5 and IL-13 than mice treated with the isotype control (Figure 6A). A small decrease in IL-4 was also detectable but was not statistically significant (data not shown). This data indicates that IL-25 plays a critical role in the differentiation of viral-specific Th2 cells. The attenuation of the Th2 cell responses by anti-IL-25 treatment also resulted in suppression of inflammation and histopathological changes in the lungs at 9 dpi. Both the number of mucous secreting cells and eosinophils were significantly reduced (approximately 2-fold and 3-fold, respectively) compared to the isotype control treatment (Figure 6B and C). Furthermore, the expression in the lung of IL-4 and -13 and the chemokine eotaxin-2 (factors that induce allergic inflammation) were all significantly reduced after anti-IL-25 treatment compared to the isotype control (Figure 6D). By contrast, IFN-γ levels were unchanged, suggesting an exclusive effect of IL-25 on Th2- rather than Th1-mediated immune responses. This data identifies a crucial role for IL-25 in the induction of Th2 responses by RSV.
Figure 6. RSV-induced Th2 Responses are IL-25-dependent. Mice were treated with anti-IL-25 or an isotype control. (A) At day 9, feeder splenocytes were pulsed with UV-inactivated RSV and plated out with lymph node CD4 T cells from each mouse. Supernatants analysed for IL-5 and IL-13. Data represent the mean ± SEM. * p<0.05 compared to isotype control. (B) The percentage of epithelial cells in the airways staining positive for mucous was enumerated.
on day 9. Data represent the mean ± SEM. * p<0.05 compared to isotype control. (C) Eosinophils were enumerated in the airway parenchyma per 100μm basement membrane (BM) on day 9. Data represent the mean ± SEM. * p<0.05 compared to isotype control. (D) Relative mRNA expression of IL-4, IL-13, eotaxin-1, -2, and IFN-γ was measured in the lungs on day 9 by quantitative PCR and normalised to Hprt. Anti-IL-25 treatments (shaded bars) are expressed as the fold reduction over the isotype control (dotted line) and represent the mean ± SEM. **p<0.01, and * p<0.05 compared to isotype control. Results represent 2 independent experiments with n ≥ 5 mice per group.
IL-25-dependent Jagged1 expression on DCs is important for RSV-induced Th2 differentiation

To investigate how the IL-25 signal might drive the generation of the Th2 phenotype we examined IL-25 expression in the mediastinal lymph nodes. We were unable to detect mRNA or protein for this cytokine within these lymphoid tissues in any RSV-infected mice (data not shown). This suggested that IL-25 did not act directly to prime naïve T cells within the lymph node compartment. Next we examined if an indirect effect of IL-25 on Th2 differentiation occurred through a migrating intermediary cell, such as the dendritic cell (DC). DC phenotype was characterised in the lymph nodes by the expression of the notch ligands associated with Th1 (delta-like ligand 4; dll4) and Th2 (Jagged1) polarisation. The DCs expressed either ligand on the cell surface but not both on the same cell. Cells isolated from the lymph nodes at 4 dpi displayed a marked up-regulation in the number of DCs expressing Jagged1 compared to the number expressing dll4, in NK-depleted mice compared to WT control mice (Figure 7A). In particular, this was brought about by increased DC Jagged1 expression in the NK-depleted mice (Figure 7A). This was not an effect of DC migration as numbers of lymph node DCs remained unaltered between the groups (Supplementary Figure S9A). Although there was an increase in the numbers of lung DC in the NK-depleted mice there was no change in the numbers expressing Jagged1 (Supplementary Figure S9A and B). To determine a role for this elevated Jagged1 expression, this notch ligand pathway was inhibited using anti-Jagged1 monoclonal antibody. The lymph node cells from mice at 4 dpi were cultured with anti-Jagged1 or an isotype control for 3 days. Blockade reduced levels of IL-13 by ~3-fold, with negligible impact on IFN-γ (Figure 7B) indicating that Jagged1 may regulate the induction of the Th2 response in the lymph nodes. In order to confirm that the observations of Jagged1- and IL-25-mediated Th2-dependence were inter-related, mice were treated again with anti-IL-25 and the lymph nodes examined during initial T cell differentiation (4 dpi). DCs isolated from
the lymph nodes of the NK-depleted mice treated with anti-IL-25, displayed a significantly reduced expression of Jagged1 compared to isotype controls (Figure 7C). These data demonstrate that IL-25 exerts its Th2 polarising effect, at least in part, by increasing the expression of Jagged1 on DCs.
Figure 7. RSV-induced Th2 Differentiation is Dependent on DC Jagged1. (A) At day 4, mediastinal lymph node cells were analysed by flow cytometry for the ratio of Jagged-1 to dll4 expressing CD11c⁺ MHC class II⁺ DCs (left panel), or percentage of Jagged1 expressing CD11c⁺ MHC class II⁺ DCs (right panel). Data represent the mean ± SEM. * p<0.05 compared
to WT control mice. (B) At day 4, lymph node cells from NK-depleted RSV infected mice were treated with anti-Jagged-1 neutralizing Ab or an isotype control goat IgG and cultured for 3 days. Supernatants analysed for IL-13, and IFN-γ. Data represent the mean ± SEM. * p<0.05 compared to WT control mice, ns, not significant. (C) At day 4, mediastinal lymph node cells were isolated from mice treated with anti-IL-25 or the isotype control, and analysed by flow cytometry for the ratio of Jagged-1 to dll4 expressing CD11c⁺ MHC class II⁺ DCs (left panel), or percentage of Jagged1 expressing CD11c⁺ MHC class II⁺ DCs (right panel). Data represent the mean ± SEM.* p<0.05 compared to isotype control. Results represent 2 independent experiments with n ≥ 8 mice per group.
Supplementary Figure S1. NK cell numbers in the lungs and lymph nodes. Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV on day 0. At various time points post inoculation (A) lung cells, or (B) lymph node cells were isolated and stained for NK cell surface markers. The percentage of lung cells with the phenotype DX5⁺NKp46⁺CD3⁻ are graphed. Data represent mean ± SEM, ***p<0.001 compared to WT control, #p<0.05 compared to day 0.
Supplementary Figure S2. NK cells producing IFN-γ in the lungs. Mice were inoculated with RSV on day 0. At day 4, lung cells were isolated, cultured with Brefeldin A and stimulated with or without (A) IL-12 (20 ng/ml) for 5 hours and stained for NK cell markers DX5^+NKp46^+CD3^−, or stimulated with or without (B) PMA (0.1 μg/ml) and ionomycin (1
μg/ml) for 5 hours and stained for the T cell marker CD3⁺. IFN-γ was then detected by ICS and IFN-γ⁺ cells gated based on isotype controls. The percentage of NK cells (gated on DX5⁺NKp46⁺CD3⁻) or T cells (gated on CD3⁺) producing IFN-γ is depicted in the boxes for unstimulated and stimulated cells from both naïve and RSV-infected mice. Data are from one experiment representative of 3 or 4 independent experiments.
Supplementary Figure S3. Micrographs of airway mucus secreting cells and mucus plugging. Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV on day 0. Mucus secreting cells were visualised in lung sections using the PAS stain.
Supplementary Figure S4. RSV viral titre during late stages of infection. Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV on day 0. Whole lung tissue was isolated on day 9 and used to determine the viral titre by quantitative PCR. The number of copies of RSV N gene (viral genome) was compared to the housekeeping gene HPRT.
Supplementary Figure S5. Mast cells in lung tissue. Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV on day 0. The number of mast cells in the airway parenchyma per 100μm basement membrane (BM) were counted on day 9 using CAE stain.
Supplementary Figure S6. IL-4 and IL-5 protein produced by lymph node cells in culture. Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV on day 0. Some mice were treated with recombinant murine (rm) IFN-γ on days 1, 2, and 3. Lymph node cells were isolated on day 4 and cultured unstimulated for 3 days. Data represent mean ± SEM, *p<0.05 compared to WT control, ##p<0.01 compared to NK-depleted.
**Supplementary Figure S7. RSV viral titre early during T cell differentiation.** Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV on day 0. Whole lung tissue was isolated on day 4 and used to determine the viral titre by quantitative PCR. The number of copies of RSV N gene (viral genome) was compared to the housekeeping gene HPRT.
Supplementary Figure S8. Presence of basophils in the lymph nodes. Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV or influenza on day 0, and 4 days later lymph node cells were isolated and stained for basophil surface markers. The percentage of lymph node cells with the phenotype FeRεI⁺CD49b⁺c-kit⁻CCR3⁻Gr-1⁻CD3⁻ are graphed.
Supplementary Figure S9. Changes in lung DC numbers but not phenotype. Mice were treated with anti-ASIALO GM1 (NK-depleted), or isotype (WT control), and then inoculated with RSV on day 0. On day 4, lung cells, or lymph node cells were isolated and analysed by flow cytometry for DC surface markers CD11c, CD11b and MHC class II, as well as the notch ligand Jagged1. The numbers of cells expressing (A) CD11c⁺CD11b⁺MHCclassII⁺, and (B) CD11c⁺CD11b⁺MHCclassII⁺Jagged1⁺ are graphed. Data represent mean ± SEM, **p<0.01 compared to WT control.
III.6 DISCUSSION

Severe RSV infection has been linked to the development of childhood asthma and exacerbations of this disorder (Sigurs et al., 2000, Sigurs et al., 2005, Henderson et al., 2005, Johnston et al., 1995). Recent clinical studies indicate that impaired NK cell function can readily occur during severe RSV infection (De Weerd et al., 1998, Welliver et al., 2008, Larranaga et al., 2009), and as such these studies provide new evidence to suggest that this deficiency may underpin the association with asthma. Here we demonstrate the central importance of NK cells in maintaining appropriate protective immunity against viral infection and inhaled antigens. Furthermore, during RSV infection, we identify a critical role for NK cells and the production of IFN-γ for the prevention of deleterious viral-specific Th2 responses. This study is the first to demonstrate that NK cells negatively regulate the development of viral-specific Th2 responses, which has implications for how severe RSV infections may exert deleterious effects in promoting the pathogenesis of asthma.

The secretion of IFN-γ from NK cells is known to play a central role in the innate host defence response to viral infection (Orange et al., 1995). In this study we demonstrate the critical importance of these factors, particularly early in the immune response to RSV infection, for limiting the long-term programming of viral-specific Th2 immune responses. NK cells and IFN-γ deficiency predisposed to the development of viral specific Th2 effector cells, and the onset of immune and pathological features of allergic airways disease (recruitment of eosinophils into the airways, mucus hypersecretion and increased production of IgE). In an acute infection setting we were unable to reconstitute these mice with NK cells due to the use of the depleting antibody. Notably, these Th2 cells persisted long-term into memory and could be reactivated by secondary viral infection (42 days after primary infection) in the presence of normal NK cell numbers. Thus, factors that predispose to the impairment of NK cell and IFN-γ
function during the acute phases of RSV exposure may have profound effects on the subsequent phenotype of immune response elicited long after infection. In the context of asthma, induction and re-enforcement of Th2 responses by RSV infection would significantly contribute to a mechanism of viral-induced pathogenesis. In a previous study depletion of NK cells using a model of Bordetella pertussis infection has also been shown to enhance Th2 responses, however the downstream regulatory pathways were not investigated (Byrne et al., 2004).

Notably, during the early phase of RSV infection (2-4dpi) the Th2 polarising cytokine, IL-25, is produced from respiratory epithelial cells. Furthermore, the early production of IFN-γ from NK cells appears to play a critical role in suppression of IL-25 expression, and in directing a protective anti-viral immune response. The influx of NK cells 4 dpi results in a pronounced increase in the levels of IFN-γ in the lung, and the concomitant inhibition of IL-25 production from infected airway epithelial cells. However, in the absence of NK cells and IFN-γ the IL-25 signal is not inhibited, and viral specific Th2 cells are generated (between 4-9 dpi). Furthermore, the delivery of recombinant IFN-γ to the airways of NK cell depleted mice infected with RSV, which suppressed the production of IL-25, also inhibited Th2 differentiation. To the authors knowledge this paper is the first to establish an inverse relationship between IFN-γ and the regulation of IL-25 expression. This finding complements the observation that the expression of the IL-25 receptor (IL-17BR) on airway smooth muscle cells is down-regulated by IFN-γ and up-regulated by TNF-α signalling (Lajoie-Kadoch et al., 2006).

The ability of IL-25 to promote the development of Th2 cells in models of allergic asthma and Helminth worm infestation (Wang et al., 2007, Tamachi et al., 2006, Sharkhuu et al., 2006,
Fort et al., 2001, Fallon et al., 2006) is well established. However, a role for IL-25 in the development of viral induced Th2 responses has not been described. By depleting IL-25 in mice where NK cell and IFN-γ function was impaired, we demonstrate a critical role of this cytokine in generating RSV-specific Th2 responses. This viral Th2 response was generated, at least in part, by the IL-25-dependent up-regulation of the co-stimulatory molecule Jagged1 on lymph node DCs. The similar numbers of Jagged1 expressing DCs in the lungs of both treatment groups, suggests that up-regulation of Jagged1 in the absence of NK cells must occur either en route from the lungs to the lymph nodes or within the lymphoid tissue itself. When expressed on dendritic cells the notch ligands, Jagged1 and dll4, have been suggested to polarize naïve CD4 T cells toward a Th2 or Th1 phenotype, respectively (Amsen et al., 2004). Jagged1 expression on DCs can polarise both human (Liotta et al., 2008) and mouse (Okamoto et al., 2009) T cells to the Th2 phenotype independently of IL-4. Furthermore, treatment of mice with Jagged1-Fc fusion protein has been shown to enhance inflammation in a model of allergic airways disease (Okamoto et al., 2009). Our data reveals an important role for IL-25 in the regulation of Jagged1 expression on mediastinal lymph node DCs that significantly contributes to the generation of viral-induced Th2 responses. Interestingly, Schaller et al., demonstrated that during RSV infection blockade of dll4 signalling directs the immune response away from a robust protective Th1 phenotype, and enhances the generation of Th2 cytokines, mucous secreting cells, and eosinophils (Schaller et al., 2007). Thus the latter study combined with our own results indicates that the appropriate control of notch ligand expression during RSV infection is critical to the programming of the adaptive CD4 T cell phenotype, which has significant implications for protective immunity. Under conditions where host defence pathways are impaired, RSV infection may induce prolonged production of epithelial-derived IL-25, which can alter DC co-stimulatory molecule expression to promote a Th2 differentiation program.
The precise way in which IL-25 modulates Jagged1 expression on lymph node DCs is not clear. IL-25 has been shown to act through its cognate receptor to drive allergic inflammatory processes by activating a subset of NK T cells, and by inducing the differentiation of Th2 cells from naïve CD4 T cells (Angkasekwinai et al., 2007, Terashima et al., 2008). In our depletion model we were unable to detect IL-25 within the lymph nodes of RSV infected mice suggesting that these mechanisms did not operate. The IL-25 receptor is also expressed on eosinophils, a population of alveolar macrophages, and DC-like cells (Cheung et al., 2006, Gratchev et al., 2004, Claudio et al., 2009). IL-25 may act directly on DCs within the airway mucosa to up-regulate Jagged1 expression before they migrate to the regional lymph nodes. IL-25 may have additional effects on as yet unidentified lung cells, which may also assist in promoting a Th2 polarizing environment.

Recently, IL-4 producing basophils have been shown to play a critical role in the differentiation of naive T cells into Th2 effector cells in response to specific allergens (Sokol et al., 2008, Charles et al., 2009, Yoshimoto et al., 2009). Thus, we investigated if this cell may also contribute along with IL-25 in the generation of viral-specific Th2 responses. We observed that IL-4 producing basophils were specifically recruited to pulmonary lymph nodes in response to RSV infection and this correlated with the development of Th2 cells. IL-4-secreting basophils have also been found in the parenchyma of STAT1-/- mice infected with RSV (Moore et al., 2009). However, depletion of basophils during exposure to RSV did not inhibit the development of viral-induced Th2 cells or features of allergic inflammation. Thus, a role for basophils may be limited to specific Th2 allergens, and their role in viral-induced Th2 differentiation may be redundant, or they simply secrete IL-4 as a bystander effect of Th2 differentiation.
The mechanism whereby individuals become sensitised to normally innocuous environmental antigens and develop asthma remains largely unknown. Here we define a causal relationship between RSV infection and impairment of NK cell/IFN-γ host defence mechanisms with sensitisation to a bystander antigen leading to the generation of OVA-specific Th2 cells. Although Th2 cells were generated in response to NK cell depletion alone, the effect on polarisation and inflammatory changes, both systemically and in the airways, was weak by comparison to the effect of concurrent infection with RSV. In this manner NK cells appear to act as a tolerance “brake” in the absence of which sensitisation to innocuous antigens can occur. The persistence of these viral-specific Th2 cells long-term (as we demonstrated) may mean that re-exposure to RSV could potentially re-activate an allergic airways disease phenotype “asthma like” to both the virus and any bystander allergen present at the time. One previous study suggested that NK cell depletion has limited impact on OVA induced allergic airway inflammation. However, in this model sensitisation to OVA was achieved by intraperitoneal injection and without an inflammatory stimulus (ie a virus), which obfuscates any direct activation of innate immune pathways, such as NK cells, in the airways (Wang et al., 1998). Although we did not specifically clarify the mechanism underlying sensitisation to OVA during RSV infection in the absence of NK cells, it is also likely to be linked to increased epithelial cell-derived IL-25 secretion arising from impaired NK cell IFN-γ production. Indeed, IL-25 has been shown to directly promote OVA induced allergic airways disease in mouse models of asthma (Tamachi et al., 2006). Alternatively, the underlying mechanism may involve “collateral priming” through adaptive immune signals (Eisenbarth et al., 2004). In this scenario RSV-specific Th2 cells would provide the polarising signals, through secretion of IL-4, for the generation of the OVA-specific Th2 cells from naïve bystander T cells.
The role of the innate immune system and epithelial cell-derived cytokines in the initiation of Th2 immunity and allergic responses is being increasingly recognized (Saenz et al., 2008, Barrett and Austen, 2009). Despite this recent trend our understanding of the mechanisms predisposing to viral-induced Th2 responses remains largely unclear. In this study we demonstrate the importance of NK cells and IFN-γ as negative regulators of Th2 immunity to viral infection and foreign allergens. Impairment of these host defence mechanisms generates enhanced production of epithelial-derived IL-25 and the induction of Jagged1 expression on DCs leading to the development of RSV-induced Th2 responses and hallmark features of allergic inflammation. This defines for the first time a dynamic pathway by which the host epithelium interacts with innate immune cells to induce a viral-specific Th2 response. These findings also provide a potential mechanism whereby severe RSV infection may predispose to and/or exacerbate asthma in susceptible individuals, however further human studies would be required in order to more thoroughly explore this relationship.
CHAPTER IV: PUBLICATION #3

Early-life viral infection and TLR7 susceptibility gene interaction predispose toward the development of asthma in later-life

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Gerard E. Kaiko$^{1,3}$, Amit Lalwani$^2$, Paul S. Foster$^{1,3}$, and Simon Phipps$^2$

1. Centre for Asthma and Respiratory Diseases, School of Biomedical Sciences and Pharmacy, Faculty of Health, The University of Newcastle, Newcastle, NSW, 2300, Australia
2. School of Biomedical Sciences, University of Queensland, St. Lucia, QLD, 4072, Australia
3. Cooperative Research Centre (CRC) for Asthma and Airways, Australia
IV.1 ABSTRACT
Respiratory viruses are a major risk factor for asthma inception in childhood and the most common cause of acute exacerbations in both children and adults. An aberrant anti-viral host defense response caused by a defect in toll-like receptor (TLR)7-mediated signals may underlie both associations. Here, using a neonatal mouse model of pneumovirus infection, we demonstrated that the absence of TLR7 led to a severe bronchiolitis characterised by increased viral load, epithelial sloughing, peribronchial inflammation, a mild eosinophilia, and T helper 2 (Th2) priming. This response was associated with attenuated Type I and II IFN production. Reinfection of TLR7-deficient but not TLR7-sufficient mice in later-life induced all of the cardinal pathophysiologic features of asthma, including Th2 cytokine and IgE production, eosinophilia, mast cell hyperplasia, smooth muscle airway remodelling, and airways hyper-reactivity in a memory CD4 T cell-dependent manner. Lastly, we show that TLR7 gene expression in sputum cells is significantly lower in human asthmatics compared with healthy-controls. Our findings highlight how a clinically relevant gene-environment interaction could increase susceptibility to the development of asthma.
IV.2 INTRODUCTION
Asthma is conventionally considered to be a CD4+ T helper 2 cell-dominated disease (Robinson et al., 1992). Although the inflammatory response may be heterogeneous, giving rise to the concept of distinct endophenotypes of asthma (Anderson, 2008), in both children and adults the great vast majority of asthma exacerbations are attributed to a viral infection (Weiss et al., 2000, Johnston et al., 1995). These acute episodes are the major cause of morbidity and hospital visits (Weiss et al., 2000, Johnston et al., 1995), yet mainstay treatment with broad spectrum anti-inflammatory agents including corticosteroids has shown limited benefit (Doull et al., 1997, Oommen et al., 2003).

Wheezy lower respiratory tract (LRT) viral infections are a major individual risk factor for the development of asthma and recurrent wheezing in later childhood (Sigurs et al., 2000, Sigurs et al., 2005, Simoes, 1999, Henderson et al., 2005, Kusel et al., 2007, Stein et al., 1999, Pereira et al., 2007, Sigurs et al., 2010). This is typically associated with respiratory syncytial virus (RSV) or rhinovirus (RV). Of note, IgE sensitisation to house dust mite by 2 years of age is associated with a moderate predictive risk of asthma, however this risk increases dramatically (up to 7-fold) with increasing numbers of severe early-life LRT infections (Holt et al., 2010). Similarly, for children with at least one atopic parent the predictive risk of asthma is dramatically increased (5-fold) following RSV bronchiolitis in the first year of life (Sigurs et al., 2010). A long-term prospective study from Simoes et al., attempted to further delineate whether this was a causal association by examining respiratory outcomes following passive immunisation against RSV (using Palivizumab) (Simoes et al., 2007). By preventing hospitalization due to severe RSV infection the risk of recurrent wheezing in later childhood was reduced by 50%. The relative risk was reduced by as much as 80% up to 5 years later, in children with no family history of atopy (Simoes et al., 2010). Collectively, these studies and
others suggest that severe RSV infection is not simply a marker of asthma predisposition but instead they provide support for a causal relationship between early-life viral exposure and childhood asthma. Severe RSV infection in infancy alone is, however, insufficient to initiate asthmatic disease as many infants do not go on to develop asthma. A genetic predisposition to asthma is also required, however exactly which genes are implicated and how many genetic hits are involved is yet to be fully elucidated (Skadhauge et al., 1999).

Importantly, severe RSV bronchiolitis in infants, or an experimental RV infection in asthmatics both lead to increased viral load, reduced lung function, reduced lymphocyte numbers, deficient IFN-γ responses, and enhanced Th2 cytokine production from bronchoalveolar lavage cells compared to mild RSV infection, or RV infection in non-asthmatics, respectively (Message et al., 2008, Buckingham et al., 2000, Renzi et al., 1999, Larranaga et al., 2009, Kim et al., 2003). These studies show that neither RSV nor RV naturally induce a predominant Th2 response, however under certain conditions, perhaps including genetic susceptibility to asthma, both viruses are able to do so (Bendelja et al., 2000, Bermejo-Martin et al., 2007). Recent studies have provided important insights into how anti-viral innate immunity may be linked to asthma. Asthmatic bronchial epithelial cells infected with rhinovirus produce less IFN-β and -λ relative to healthy donors (Contoli et al., 2006, Wark et al., 2005). Alveolar macrophages and circulating leukocytes from asthmatics also appear to have deficient type I and type III IFN production (Bufe et al., 2002, Contoli et al., 2006, Gehlhar et al., 2006), suggesting that an impaired anti-viral response in asthma involves both structural cells of the lung, and migratory immune cells.

A specific subtype of DC known as the plasmacytoid DC (pDC) plays a specialised role in anti-viral defense and in some circumstances immune tolerance. pDCs express large amounts
of antiviral Type I IFNs upon activation due to constitutively high levels of interferon regulatory factor 7 (IRF7) (Hengel et al., 2005). Studies of peripheral blood have suggested that asthmatics have reduced levels of circulating pDCs compared to healthy subjects (Matsuda et al., 2002, Hagendorens et al., 2003). Children who experience an episode of RSV bronchiolitis in infancy and go on to develop asthma have markedly reduced numbers of circulating pDCs at 6 years of age compared to those that do not develop asthma (Silver et al., 2009). Moreover, Upham et al., observed that the levels of circulating pDC (and not myeloid DCs) during the first year of life were inversely associated with the incidence of severe LRT infections in early-life and a diagnosis of asthma at 5 years of age (Upham et al., 2009). In both murine models of allergic asthma and RSV infection, depletion of pDCs exacerbates airway inflammation and Th2 responses, whereas adoptive transfer of pDC reverses this response (de Heer et al., 2004, Smit et al., 2006). Collectively, lower numbers of pDCs during infancy appear to be a risk factor for asthma and bronchiolitis while higher numbers appear to be protective.

The molecular basis for this apparent diminished anti-viral innate immunity in asthma and its causal relationship, if any, to the development of asthma remains unclear. A gene encoding a protein that is both responsible for induction of Type I IFNs and highly expressed on pDCs is Toll-like receptor (TLR) 7. Genome wide association studies (GWAS) have revealed strong linkage between asthma and the sex chromosome Xp22 (Brasch-Andersen et al., 2008, Haagerup et al., 2004). This susceptibility loci harbours the genes for TLR7 and TLR8. Importantly, recent studies have demonstrated that TLR7 and TLR8 SNPs have a very strong association with asthma across diverse populations (Moller-Larsen et al., 2008, Zhang et al., 2009a). Whether the TLR7 SNPs affect TLR7 expression and/or protein function remains unknown, although it is noteworthy that stimulating PBMCs with the TLR7 ligand imiquimod,
but not TLR3 ligands, induces significantly lower levels of innate anti-viral molecules in asthmatics compared to non-asthmatics (Roponen et al., 2009). Despite this it remains unknown how defective/deficient TLR7 may impact upon asthmatic disease and whether there is a pathogenic relationship between TLR7, IFNs, and asthma.

RSV is a single stranded RNA virus and thus should be detected by a pathogen recognition receptor (PRR) such as TLR7/8. Indeed, RSV has been shown to activate endosomal TLR7, expressed in pDCs, and induce Type I IFN production (Hornung et al., 2004). Human RSV is a host-restricted pathogen that does not replicate productively in rodents. Therefore, we employed pneumonia virus of mice (PVM), a member of the same family (Paramyxoviridae) and genus (Pneumovirus) as human RSV that replicates exponentially in situ and inducing symptomatic disease analogous to that observed for RSV infection in humans (Harrison et al., 1999). We have utilised PVM in these studies as it represents a more relevant model of RSV infection in humans when examining TLR activation and the initiation of the innate Type I IFN cascades, which are sensitive to replicating virus.

We investigated a 2-hit hypothesis for the inception of viral-associated asthma, to determine whether early-life viral stimulation of TLR7 and the Type I IFN pathway could underlie the relationship between viral infections and asthma. Here we show that an early-life respiratory viral infection in the presence of a genetic TLR7-deficiency leads to a severe bronchiolitis-like phenotype, deficient Type I and II IFN responses, and increased viral load, upon primary infection. Upon secondary exposure to virus in later-life these same mice develop enhanced Th2 responses, IgE, eosinophilia, mast cell influx, airway remodelling, and substantial airways hyper-reactivity (AHR), dependent on memory CD4 T cells. Furthermore, sputum cells from human asthmatics express lower levels of TLR7 than non-asthmatics. TLR7 may provide a
novel susceptibility gene for asthma and this study provides evidence of how a specific gene and viral infection may interact in the development of disease.
IV.3 MATERIALS AND METHODS

Mice, PVM, and Treatment Regimes

Wild-type and TLR7-deficient BALB/c mice (supplied by Dr. Shizuo Akira, University of Osaka, Japan) were used at 7 days old in all studies. Mice were administered virus via the intranasal route under light isofluorane induced anaesthesia. Neonatal mice were administered 5 PFU of mouse passaged PVM (J3666 strain) in 10 μl vehicle (DMEM + 10% fetal calf serum) by inoculation on day 0 (day 7 of life). Adult BALB/c mice were re-inoculated with 200 PFU of PVM in 40 μl vehicle at 42 days post primary-infection (dpi), and likewise at 84 dpi. Control mice received vehicle alone. Each mouse was weighed individually every day for 2 weeks following PVM infection. To deplete mice of CD4 T cells prior to secondary infection, neonatal PVM-infected mice at 3 weeks of age were injected intraperitoneally with 300 μg of purified GK1.5 antibody or rat IgG isotype control in 200 μl PBS. Injections were performed at 21, 23, and 25 dpi and mice were allowed to rest for 2.5 weeks to allow de novo CD4 T cells to regenerate before re-inoculation with PVM. All studies were approved by and conducted in accordance with guidelines set out by the University of Newcastle and University of Queensland Animal Care and Ethics Committees.

Real Time-Quantitative PCR

For each time point lungs were excised and frozen in RNAlater solution (Ambion). Total RNA was isolated using TriReagent solution (Ambion) according to manufacturer’s instructions. Primer sequences and reaction conditions used for reverse transcription and real-time quantitative PCR are available upon request. PVM viral titre was conducted using standards consisting of plasmids containing either the SH domain of the PVM genome, or the housekeeping gene hprt at a known copy number. For human sputum cDNA samples the housekeeping gene used was 18S ribosomal RNA and all gene expression, including TLR3, -7,
-8, and -9, was determined using real-time quantitative PCR with commercially available Taqman kits (ABI) according to the manufacturer’s instructions.

**Lymph node assays**

Single cell suspensions of lymph node cells were prepared and placed in culture at 5x10^5 cells/well/200 µl growth medium (RPMI 1640, 5x10^{-5} M 2-ME, 10% heat-inactivated FCS, 2 mM L-glutamine, 20 mM HEPES, 100 µg/ml penicillin, and 100 µg/ml streptomycin, 0.1mM sodium pyruvate). Supernatants were collected and frozen at -80°C for ELISA.

**Flow cytometry**

Isolated single cell suspensions of lung cells were washed in PBS plus 2% FCS and incubated with anti-mouse CD16/32 Fc receptor block at 20 µg/ml/10^6 cells for 15 min on ice. Cells were incubated with the relevant antibody on ice for 20min in dark. Antibodies included anti-mouse CD4 APC, Gr-1 APC, CD11c FITC, CD11b PerCP, MHC class II PE, CD3 FITC (all from BD Biosciences). All isotype control antibodies were obtained from BD Biosciences. Cells were washed three times as previous, fixed in 1% formaldehyde (Polysciences) in PBS, and analysed on the FACSCanto (BD Biosciences).

**ELISA**

Serum concentrations of IgE were determined using reagents from BD Biosciences. Cell culture supernatants were analysed for the cytokines IL-5, and -IFN-γ using antibodies and standards from BD biosciences, IL-17A using reagents from eBiosciences. PVM-specific IgG titres were detected in mouse sera using a SMART-spot ELISA kit according to manufacturer’s instructions (Biotech trading partners). Eotaxin-2 concentration was determined by
homogenising whole lungs in RIPA buffer (Sigma) and using ELISA reagents from R&D systems.

**Assessment of airway function**

Airway hyper-reactivity (AHR) was measured as previously described (Weckmann et al., 2007). Briefly, airway responsiveness was determined by direct measurements of airway resistance (RI) and compliance (Cdyn) in anaesthetized (0.2 mg/10g xylazine and ketamine 0.4mg/10g), and tracheotomised mice in response to nebulised methacholine (Sigma) at concentrations of 1.25-20 mg/ml for 1 min. Percentage increase over baseline (saline) in response to increasing doses of methacholine was calculated.

**Histology**

Lung tissue was fixed in 10% formalin and paraffin-embedded for sectioning. Stains included Chromotrope 2R and Hematoxylin to enumerate eosinophils, Toluidine blue to enumerate mast cells, Periodic acid-Schiff for mucous-secreting cells, and Hematoxylin and Eosin to analyse histopathology. Cells were counted around major airways for a minimum of 10 fields at 100 x magnification. For measurement of epithelial loss the total perimeter of airways was measured using Aperio Image Scope software (Aperio) followed by the length of the airway/basement membrane with detached epithelial cells. The percentage of the latter was then calculated and averaged for all medium and large airways on a given mouse’s lung section. Photographs were captured at room temperature at 40x or 100x magnification using an Olympus microscope (model BX51), digital camera (Olympus DP70) and DP software (Olympus).
**Immunohistochemistry**

Paraffin-embedded sections were cut, rehydrated and exposed to antigen retrieval using heating in sodium citrate buffer. Sections were incubated in PBS-Tween for 10 minutes. For immunofluorescent staining of smooth muscle actin, sections were blocked with 25% normal goat serum for 30 minutes and washed in PBS-Tween. Sections were then incubated with 2% anti-mouse smooth muscle actin (Dako) in PBS for 1 hour at 37°C, and washed 3 times in water followed by mounting with fluorescent mounting medium.

**Clinical Samples**

Adults with stable asthma (n=43) were recruited from the John Hunter Hospital, Newcastle, Australia. Asthma was diagnosed according to American Thoracic Society guidelines on the basis of current (past 12 months) episodic respiratory symptoms, doctor’s diagnosis, and demonstrated evidence of airway hyper-responsiveness to hypertonic saline. Healthy controls without asthma had no acute or chronic respiratory illness and normal lung function and were recruited by advertisement (n=14). Exclusion criteria included recent (past month) respiratory tract infection, recent asthma exacerbation, recent unstable asthma or change in maintenance therapy, and current smoking. All participants gave written informed consent before their inclusion in the study, and the Hunter New England Area Health Service and the University of Newcastle Research Ethics Committee approved this study. Sputum induction with hypertonic saline (4.5%) was performed. For inflammatory cell counts, selected sputum was dispersed by using dithiothreitol, and a total cell count viability was performed. Cytospins were prepared and stained (May-Grunwald Giemsa), and a differential cell count was obtained from 400 non-squamous cells. For gene expression microarray analysis, 100 μL selected sputum was added to Buffer RLT (Qiagen) and stored at –80°C prior to RNA extraction. RNA was extracted using
the RNeasy kit (Qiagen) and reverse-transcribed to cDNA by using the high capacity cDNA reverse transcription kit (Applied Biosystems).

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism version 5.0 software with the Mann-Whitney t test and one way ANOVA tests. Statistical analysis of human samples used the Mann-Whitney t-test between asthmatic and healthy groups with a non-Gaussian distribution. A p value less than 0.05 was considered statistically significant.
IV.4 RESULTS

TLR7 protects against severe infection and pathology during neonatal respiratory viral infection

To examine the effect of an inherent defect in TLR7 expression/function on respiratory virus infection, neonatal wild-type (WT), and TLR7-deficient (TLR7-/−) mice (7 days of life) were infected with the mouse-adapted Pneumovirus, Pneumoniae Virus of Mice (PVM, 5 PFU). Day 7 of life was chosen for technical reasons as this is the earliest age at which the neonatal mice were properly able to inhale the intranasal dose of PVM. We have previously shown that TLR7 is expressed by both the mouse epithelium and pDCs following viral infection (Davidson et al., 2011). In TLR7-/− neonatal mice, PVM replication was increased (>5-fold) and viral clearance was significantly delayed compared to WT mice (Figure 1A). This reduced clearance of PVM was associated with enhanced gross histopathology including peribronchiolar infiltration of inflammatory cells and parenchymal consolidation (Figure 1B). Similarly, reduced viral clearance was associated with increased airway epithelial shedding observed in TLR7-/− mice compared to WT mice at 7 days post-infection (dpi) (Figure 1C). In Figure 1C, this epithelial shedding was quantified as the length of airway basement membrane with detached epithelial cells as a percentage of the total airway perimeter. This measurement is an average of all airways and the difference between strains is even greater within large airways alone (data not shown). This loss of epithelium reaches approximately 25% of the airway circumference during the peak of the PVM infection, and reflects observations of necrosis of ciliated epithelial cells and sloughing of the bronchial epithelium in infants with severe RSV bronchiolitis (Aherne et al., 1970, Noah et al., 2002). TLR7-/− neonates also displayed increased mortality in the course of the 2-week follow-up after viral infection compared to WT mice (Figure 1D). This data indicates that in the absence of TLR7, respiratory viral infection with PVM leads to a more severe pathology, which more closely resembles the pathological
features of RSV-induced bronchiolitis in human infants, compared to the more mild disease induced in WT mice.

A

![Graph showing PVM copies/million HPRT over dpi (days post injection).]

B

WT  TLR7−/−

**Vehicle**

**PVM**
Figure 1. Reduced viral clearance and enhanced pathology occurs in the absence of TLR7. (A) Viral load in the lungs of mice was determined using quantitative PCR and HPRT as a housekeeping control. (B and C) Micrographs of lung sections stained with hematoxylin and eosin at 7dpi showing (B) gross histopathology and peribronchial infiltration, scale bar represents 200μm, and (C) respiratory epithelium, scale bar represents 50μm. Micrographs in (C) are quantified by a graph of % epithelial cell loss, which represents the length of the basement membrane with detached epithelial cells as a % of the total airway perimeter averaged for all airways. (D) Survival of mice infected with PVM was assessed between 1-14 dpi. dpi; days post infection. ***p<0.001 compared to PVM WT mice.
The absence of TLR7 leads to reduced antiviral responses and skewed DC subset recruitment

Neonatal PVM infection in the absence of TLR7 leads to a dramatic reduction in the expression of Type I IFN-α and -β production in the lung, whereas WT mice exhibit robust production of these innate cytokines (log scale) (Figure 2A). Interferon regulatory factor 7 (IRF7), the key downstream signalling molecule activated by TLR7, and the chemokine interferon-γ inducible protein-10 (IP-10; CXCL10), produced in response to Type I and II IFN stimulation, are both highly expressed in the lung following PVM infection at 4 dpi, but remain at baseline levels in TLR7/-/- mice (Figure 2B). These results very closely reflect observations of human asthmatic PBMCs stimulated with the TLR7 ligand imiquimod, which showed a diminished capacity to produce IP-10 compared to healthy controls (Roponen et al., 2009). Furthermore, antiviral IFN-γ defences are compromised in the absence of TLR7 as TLR7/-/- mice demonstrate a significant delay in the production of IFN-γ over the first 10 days of infection compared to WT mice (Figure 2C). Interestingly, a lack of TLR7 activation also had a significant effect on the recruitment of different DC subsets to the lungs. WT mice had minimal changes in myeloid DC (mDCs) numbers compared to TLR7/-/- mice, which showed a 3-fold increase in this cell type within the lung at 7 dpi (Figure 2D). Conversely, we have previously shown that TLR7/-/- mice lack the normal expansion of plasmacytoid DCs (pDCs) within the lung as observed for WT mice (Davidson et al., 2011). This data demonstrates that an absence of TLR7 during PVM infection leads to a switch from a predominance of pDCs to a predominance of mDCs in the lungs. Although numerically the predominant lung inflammatory infiltrate in both WT and TLR7/-/- mice remained the neutrophil the recruitment of these cells was also delayed in TLR7/-/- (Supplementary Figure S1). This data indicates that the absence of TLR7 during neonatal PVM infection leads to a deficient or delayed innate antiviral response creating an inverse effect on DC subset recruitment.
Figure 2. Diminished or delayed antiviral Type I and II IFN production occurs in the absence of TLR7. (A-C) Gene expression in the lungs for IFN-α, -β, -γ, were determined on the days as indicated and IRF7 and IP-10 expression at 4dpi using quantitative PCR and HPRT as a housekeeping gene, expression was normalised to vehicle controls. (D) Myeloid DCs (CD11c⁺CD11b⁺MHCclassII⁺) were enumerated in the lungs by flow cytometry and expressed as a percentage of total lung cells. dpi; days post infection. *p<0.05, **p<0.01, ***p<0.001 compared to PVM TLR7⁻/⁻ mice.
Mixed Th1/Th2 responses are promoted in the absence of TLR7

The expression of IL-13 revealed an inverse pattern to that of IFN-γ, as TLR7-/− mice demonstrated significantly higher expression of this Th2 effector cytokine during viral clearance from 7-10dpi compared to WT mice (Figure 3A). Likewise IL-4 and the Th2 cell chemoattractant CCL22 were also expressed at higher levels in the absence of TLR7. Although increased in both WT and TLR7-/− mice above baseline levels, there was no statistical difference in eotaxin-1 levels between the two strains (Figure 3B). However, the levels of the potent eosinophil chemoattractants CCL5 and eotaxin-2 were elevated above baseline in the PVM-infected TLR7-/− mice and not WT mice (Figure 3B). Eotaxin-2 protein levels were confirmed to be increased in lung homogenates at 7 dpi from these same mice. The increase in these eosinophil chemoattractants was mirrored by a similar increase in the number of tissue eosinophils in TLR7-/− mice as identified around the bronchiolar epithelium (Figure 3C). To account for any possible impact of viral load on the cytokine responses, at 10 dpi once the virus was cleared in both WT and TLR7-/− mice, the mediastinal lymph node cells from these mice were isolated and cultured in vitro. The cells derived from TLR7-/− mice produced greater IL-5 than WT mice and equivalent amounts of IFN-γ (Figure 3D). This reflected the results within the lung showing delayed IFN-γ, which eventually equalised between the strains by 10 dpi. As we have previously shown that in the absence of TLR7 PVM can activate the Pyrin domain containing Nod-like receptors (NLRP3), we determined whether this may contribute to the induction of the Th2 phenotype by examining the expression of the inflammasome-associated Th2-inducing cytokine IL-33. We found that IL-33 levels were significantly higher in the absence of TLR7 during viral infection (Figure 3E). The expression of IL-17A and IL-17F at the mRNA level was not altered at any timepoint during neonatal PVM infection compared to baseline levels (vehicle control) in either the WT or TLR7-/− mice (data not shown). In addition the production of IL-17A protein in the lymph nodes also showed no difference between PVM-
infected WT and TLR7−/− mice (Figure 3D). Overall, this data suggests that the absence of TLR7 during neonatal infection leads to a skewing of the immune response away from a protective Th1 response towards priming of Th2 cells, chemokines, and eosinophils. However, it should be noted that these were quantitatively relatively mild Th2 responses in the neonate.
Figure 3. Outgrowth of a mixed Th response after neonatal Pneumoviral infection in the absence of TLR7. (A, B, and E). Gene expression in the lungs was determined using quantitative PCR and HPRT as a housekeeping gene, expression was normalised to vehicle controls. (B) Eotaxin-2 protein was measured by ELISA in lung homogenates at 7 dpi. (C) Eosinophils were enumerated around the airways by counting the number of cells per 100μm epithelial basement membrane in lung sections stained with hematoxylin and chromotrope. (D) Mediastinal lymph nodes cells were isolated and cultured for 3 days and concentrations of IL-5, IFN-γ, and IL-17A were measured by ELISA in the supernatants. dpi; days post infection. *p<0.05, **p<0.01, ***p<0.001 compared to PVM WT mice.
**TLR7 does not impact upon long-term virus-specific antibody production or protection against re-infection**

PVM-infected neonatal mice were allowed to mature into adulthood before being re-infected with PVM at a proportionally higher dose (100 PFU) 6 weeks later to examine the long-term immunity to this virus (Figure 4A). All parameters were measured 6 days following PVM inoculation as this coincides with the peak of T cell infiltration (data not shown). Both WT and TLR7-/- mice were protected against re-infection with PVM as both strains rapidly cleared the virus back to the detection limit of the vehicle controls by 6 dpi (Figure 4B). This data suggests that the ability to more rapidly clear the virus through immunological memory was intact, regardless of TLR7 expression, as it was eliminated in both strains of mice (6 dpi) prior to the time at which it reached peak viral titre in the primary infection (7dpi). The levels of PVM-specific IgG in the serum were also unaffected by TLR7, indicating that the generation of robust B cell memory was independent of TLR7 (Figure 4C). Furthermore, unlike the primary infection when robust viral replication and Type I IFN induction was observed, during the secondary infection the virus induced very low levels of Type I IFN only slightly above baseline vehicle controls (Figure 4D).
Figure 4. Secondary protective memory responses are generated independently of TLR7.

(A) Timeline of infection protocol. (B) Viral load in the lungs of mice was determined at 6 days after secondary infection using quantitative PCR and HPRT as a housekeeping control. (C) Concentration of serum PVM-specific IgG was determined at 6 days after secondary infection using ELISA. (D) Gene expression in the lungs was determined 6 days after secondary infection using quantitative PCR and HPRT as a housekeeping gene, expression was normalised to vehicle controls. dpi; days post infection, PFU; plaque forming unit, wk; week.
Allergic airway inflammation develops upon re-exposure to Pneumovirus in later-life in the absence of TLR7

In humans, frequent re-infection with respiratory viruses can occur in both childhood and adulthood often leading to an acute asthmatic exacerbation in predisposed individuals (Johnston et al., 1995). To model this we determined the impact of a Pneumovirus infection and TLR7-deficiency, during the critical period of neonatal development of the immune and respiratory systems, on the outcome of re-exposure to virus in later-life. As previously stated (see Figure 4A) WT or TLR7-/- mice were re-infected with PVM 6 weeks after the initial primary exposure. TLR7-/- mice, which had experienced a more severe primary infection, displayed a dramatic outgrowth of Th2 cytokines, and airway inflammation upon secondary exposure to PVM as compared to WT mice. TLR7-/- mice expressed substantially higher levels of IL-4, IL-5 and IL-13, as well as the eosinophil chemoattractants eotaxin-1 and -2 (although the latter was not statistically significant) within the lung compared to WT mice (Figure 5A). The mice strains showed no difference in Muc-5a expression or numbers of mucous positive cells, as both WT and TLR7-/- mice expressed profuse mucous, which made any distinction between such levels difficult to detect (Figure 5A and Supplementary Figure S2). Interestingly, TLR7-/- mice also expressed slightly higher levels of IFN-γ in the lung, however this did not appear to be the case for the mediastinal lymph node cells cultured in vitro where there was no difference between WT and TLR7-/- mice in terms of IFN-γ protein production (Figure 5B). The levels of IL-5 protein produced by the lymph node cells were elevated in TLR7-/- compared to WT mice, indicating a higher IL-5:IFN-γ ratio in the absence of TLR7. This is often observed in clinical samples from asthmatic patients (Kim et al., 2003). IL-17A protein was detected in only very low amounts from lymph node cultures and no difference was discernible between WT and TLR7-/- mice (Figure 5B). Once again this reflected the expression of IL-17A in the lung as it was virtually undetectable in all mice (data not shown).
Upon secondary PVM exposure TLR7/-/- mice showed a significant influx of eosinophils around the respiratory epithelium amounting to more than 3-fold that of WT mice (WT not significantly different from baseline) (Figure 5C). Furthermore, the number of medium-to-large sized airways surrounded by a contiguous layer of smooth muscle increased by nearly 50% in TLR7/-/- mice following secondary PVM infection (Figure 5D). In contrast, WT mice maintained the same number of airways surrounded by a layer of smooth muscle independent of PVM infection indicating that TLR7 may protect against the initiation of certain remodelling processes following viral infection. Most importantly, after secondary exposure to PVM, TLR7/-/- mice showed a dramatic increase in both airways resistance and dynamic compliance, in response to increasing doses of aerosolised methacholine, when compared to either WT PVM-infected mice or WT and TLR7/-/- vehicle-treated mice (Figure 5E). This data suggests that re-exposure to PVM induces increased airways hyper-reactivity (AHR) in the absence of TLR7 signalling.

In order to examine the potential for long-term re-exacerbation of this allergic airways disease phenotype mice were rested for a further 6 weeks before a third exposure to PVM was investigated (Supplementary Figure S3A). Similar to the secondary infection the tertiary exposure to PVM elicited robust antiviral memory and clearance was rapidly achieved with no difference in viral titre between either WT or TLR7/-/- mice (data not shown). The expression of IL-4 and IL-5 was once again significantly elevated in the lungs of TLR7/-/- mice over WT mice, however increases in IL-13 and eotaxin-1 and -2 were equivalent between the strains (Supplementary Figure S3B). Once again there was an increase in the ratio of IL-5:IFN-γ protein levels produced by lymph node cells, and no difference in IL-17A levels (Supplementary Figure S3C). This suggests that neither was a Th17 response generated by PVM nor was it attenuated by the absence of TLR7. The changes in lung pathology reflected
that of the secondary infection with an increase in both the influx of eosinophils and elevated smooth muscle layering in TLR7−/− mice as compared to WT mice (Supplementary Figure S3D and E). Finally, there was also a significant exacerbation of AHR upon tertiary infection exclusively in the TLR7−/− mice (Supplementary Figure 3F).
Figure 5. Re-infection with virus in later-life in the absence of TLR7 leads to Th2 responses, airway remodelling, and AHR. All endpoints were examined 6 days after secondary infection. (A) Gene expression in the lungs was determined using quantitative PCR and HPRT as a housekeeping gene, expression for all genes was normalised to vehicle controls. (B) Mediastinal lymph nodes cells were isolated and cultured for 3 days and concentrations of
IL-5, IFN-γ, and IL-17A were measured by ELISA in the supernatants. (C) Eosinophils were enumerated around the airways by counting the number of cells per 100μm epithelial basement membrane in lung sections stained with hematoxylin and chromotrope. (D) The number of airways with a contiguous surrounding layer of smooth muscle were counted in lung sections fluorescently stained with anti-mouse α-smooth muscle actin. (E) Airways resistance (left) and airways compliance (right) in response to increasing doses of aerosolised methacholine (MCh) were determined and expressed as a percentage change over saline. *p<0.05, **p<0.01, ***p<0.001 compared to PVM WT mice.
TLR7 protects against the outgrowth of mast cells and IgE after repeated Pneumovirus infection

The early-phase of the asthmatic response in humans is dependent on mast cells and IgE (Kay, 2001). In the absence of TLR7, each sequential PVM infection led to a near linear outgrowth of both the level of total IgE in the serum (Figure 6A), and tissue mast cells residing within 100 μm of the airway epithelium and smooth muscle layer (Figure 6B). This increase was unique to TLR7-/- mice and did not occur in WT mice that were likewise repeatedly exposed to PVM over time. A clear distinction in these responses between these two strains did not begin to arise until 6 days post the secondary infection with PVM as both sets of mice demonstrated the same negligible levels of IgE and mast cells after the initial neonatal infection. This suggests that repeated exposure to Pneumovirus, in the absence of TLR7, critically induces a robust Th2 response and expansion of mast cells and IgE in later life.
Figure 6. Mast cell numbers and IgE increase with each subsequent viral infection. Measurements were conducted 7 days, 6 days, and 6 days following the 1st, 2nd, and 3rd PVM infections, respectively. (A) Serum IgE concentrations were assessed by ELISA (B) Mast cells were enumerated around the airways by counting the number of cells per 100μm epithelial basement membrane in lung sections stained with mast cell-specific toluidine blue. **p<0.01, ***p<0.001 compared to PVM WT mice.
Viral-induced airway inflammation is dependent upon memory CD4 T cells

The Th2 responses to PVM generated in the absence of TLR7 were enhanced by repeated exposure to the virus. Hence, we postulated that this phenomenon might be dependent on the development of aberrant memory CD4 T cells. In order to investigate the underlying mechanism we setup a depletion strategy such that newly generated memory CD4 T cells would be depleted after the PVM infection and inflammation had subsided (3 weeks post-infection so as not to impact upon the primary response). Then de novo CD4$^+$ cells including CD4 T cells were allowed to regenerate for 2.5 weeks before re-infection with PVM (Figure 7A and Supplementary Figure S4). Utilising this method, upon re-exposure to PVM the TLR7-/ mice could mount only a primary effector CD4 T cell response, without the effects of a CD4 T cell memory component. PVM was cleared by 6 dpi in TLR7-/ mice depleted of CD4 T cells (CD4- mice) and likewise in the TLR7-/ mice treated with an isotype control (Isotype) (data not shown). In CD4- TLR7-/ mice all Th2-associated cytokines and chemokines in the lung were diminished compared to Isotype TLR7-/ mice, including IL-4, -5, -13, eotaxin-1, and -2, however the expression of IFN-$\gamma$ remained unchanged (Figure 7B). Once again expression of the goblet cell associated gene muc-5a was unchanged between the treatment groups. Levels of both IL-5 and IFN-$\gamma$ protein made by lung-draining lymph node cells in culture were reduced in CD4- TLR7-/ as compared to Isotype controls (Figure 7C), whereas IL-17A expression within the lung or by lymph node cells in culture was unchanged (Figure 7C, and data not shown). By depleting memory CD4 T cells the AHR previously observed in TLR7-/ mice was significantly suppressed compared to that of the Isotype control groups (Figure 7D). Therefore, the robust Th2 responses and AHR generated by the Pneumoviral infection in the absence of TLR7 were dependent on memory CD4 T cells. It is noteworthy that the absence of memory CD4 T cells did not completely ablate AHR (to baseline vehicle control levels) suggesting that there may be a partial effect delivered by a non-CD4 T cell source.
Figure 7. Long term airway inflammation and reactivity generated in the absence of TLR7 is dependent on memory CD4 T cells. (A) Timeline of experimental protocol. All endpoints were examined 6 days after secondary infection. (B) Gene expression in the lungs was determined using quantitative PCR and HPRT as a housekeeping gene, expression for all genes was normalised to vehicle controls. (C) Mediastinal lymph nodes cells were isolated and cultured for 3 days and concentrations of IL-5, IFN-γ, and IL-17A were measured by ELISA in
the supernatants. (D) Airways resistance (left) and airways compliance in response to increasing doses of aerosolised methacholine (MCh) were determined and expressed as a percentage change over saline. dpi; days post infection, PFU; plaque forming unit, wk; week, ND; not detected. *p<0.05, **p<0.01 compared to PVM TLR7-/- Isotype mice.
Human asthmatics express lower levels of TLR7 than healthy controls

Although specific SNPs in the TLR7 gene have been strongly linked with an increased risk of asthma through gene association studies little is known about the actual level of TLR7 expression in asthmatics (Moller-Larsen et al., 2008). We collected sputum from stable adult asthmatics not currently undergoing any form of acute exacerbation and analysed for the mRNA expression of the nucleic acid receptors TLR3, TLR7, TLR8, and TLR9. We investigated expression levels in stable asthmatics to avoid transient changes in the inflammatory state due to a concurrent respiratory infection, unstable asthma, or changes in maintenance therapy. We have previously shown that the expression of TLR2 is increased in the sputum cells of stable asthmatics compared to healthy controls whereas TLR4 expression remains unchanged (Wood et al., 2010). In this current study we observed that the sputum cells collected from asthmatics expressed a significantly lower level of TLR7 as compared to healthy control patients (Figure 8A). This represented a sizeable reduction as the expression level was almost 2-fold lower in asthmatics. Importantly, this difference appeared to be exclusive to TLR7, as no such difference was detectable for TLR3, TLR8, or TLR9 (Figure 8B-D). As TLR9 has relatively homologous cell type expression to TLR7 in humans, this suggests that the observed difference in TLR7 expression is not simply due to an artefact of different sputum cellular content between asthmatics and healthy control patients. Instead this suggests a difference on a per cell basis. Interestingly, there was no difference in TLR7 mRNA expression between the bronchial epithelium of asthmatics and healthy controls (data not shown), suggesting that this disparity is limited to immune cells.
Figure 8. Sputum cells from human asthmatics express lower levels of TLR7.

Sputum cells from stable asthmatics and healthy controls were collected and RNA extracted (see materials and methods section for detailed description). Gene expression for (A) TLR7, (B) TLR3, (C) TLR8, and (D) TLR9 was determined using quantitative PCR with Taqman kits and 18S ribosomal RNA as the housekeeping gene, expression for all genes was normalised to healthy controls. In box whisker plots, the line inside the box represents the median, upper box border represents the 75th quartile, lower border represents 25th quartile, whisker are 5th and 95th percentiles. **p<0.01 compared to healthy controls.
IV.5 SUPPLEMENTARY DATA

Supplementary Figure S1. Neutrophil influx after PVM infection. During the primary infection neutrophils were enumerated in the lungs of PVM-infected mice by flow cytometry to determine the percentage of total lungs cells that expressed the neutrophil markers CD11b^Gr-1^+. ***p<0.001 compared to PVM TLR7^/-^ mice. dpi; days post infection.
Supplementary Figure S2. Hypersecretion of mucous following PVM infection. At 6 days after secondary infection lung sections of PVM-infected mice were stained with Periodic-acid Schiff to show mucous produced by airway epithelial cells. Micrographs are at 20x magnification and scale bar represents 100\(\mu\)m.
Supplementary Figure S3. Tertiary infection in the absence of TLR7 leads to exacerbation of Th2 responses and AHR. (A) All endpoints were examined 6 days after tertiary infection according to the timeline of infection protocol. (B) Gene expression in the lungs was determined using quantitative PCR and HPRT as a housekeeping gene, expression for all genes was normalised to vehicle controls. (C) Mediastinal lymph nodes cells were isolated and cultured for 3 days and concentrations of IL-5, IFN-γ, and IL-17A were measured by ELISA in the supernatants. (D) Eosinophils were enumerated around the airways by counting the number of cells per 100μm epithelial basement membrane in lung sections stained with hematoxylin and chromotrope. (E) The number of airways with a contiguous surrounding
layer of smooth muscle were counted in lung sections fluorescently stained with anti-mouse α-smooth muscle actin. (F) Airways resistance (left) and airways compliance (right) in response to increasing doses of aerosolised methacholine (MCh) were determined and expressed as a percentage change over saline. *p<0.05, **p<0.01 compared to PVM WT mice.
Supplementary Figure S4. Depletion CD4+ cells and re-generation of antigen-naïve CD4 T cell population. CD4 T cells were depleted as detailed in materials and methods section. Flow cytometry histograms showing CD4 T cells in the lungs and lymph nodes before depletion, during depletion treatment, and 2.5 weeks after depletion just prior to PVM re-infection. The numbers in the top right hand corner of plots denotes CD4 T cells as a percentage of totals cells in either mediastinal lymph nodes or lungs.
IV.6 DISCUSSION

Early-life viral infections are a major risk factor for the later development of childhood asthma (Simoes, 1999, Simoes, 2001). In particular, severe RSV infection resulting in LRT bronchiolitis in the first year of life has been demonstrated to substantially enhance the risk of long-term wheezing and later asthma diagnosis in childhood, which can be traced as far out as 18 years of age (Sigurs et al., 2000, Sigurs et al., 2005, Simoes, 1999, Henderson et al., 2005, Kusel et al., 2007, Stein et al., 1999, Pereira et al., 2007, Sigurs et al., 2010). Approximately 30-40% of the affected infants (depending on the study population) subsequently develop asthma (Sigurs et al., 2010, Camara et al., 2004). Furthermore, the relative risk increases with repeated infections in infancy (including both RSV and RV) or a family history of atopy (Holt et al., 2010). Despite these observations, there is very limited understanding of the complex nature of the association between infections and asthma, or the role that pre-existing genetic susceptibility may play.

Seminal work from Culley et al., proposed that the RSV infection itself in the mouse could be involved in the induction of Th2 responses (Culley et al., 2002). This work, and subsequently that of others, suggested that by inoculating neonatal mice with RSV and re-infecting them as adult mice the early age of infection led to outgrowth of Th2 responses and airways reactivity (Dakhama et al., 2009, Dakhama et al., 2005b). However, clinically only a small percentage of even the very young infants (< 6 months) that are infected with RSV (asymptomatic, mild or severe) actually develop severe sequelae requiring hospitalisation (~4-14%) and even less go on to develop later asthma (Mohapatra and Boyapalle, 2008, Nair et al., 2010). Therefore, these previous experimental models suggesting that age of infection itself leads to susceptibility to Th2 responses and viral-induced asthma do not necessarily reflect the complete clinical paradigm and the complexity of the gene-environment relationship. Instead in humans there are
likely predisposing factor/s, possibly genetic, that have influence over which infants develop severe infection, allergic responses, and later asthma.

We investigated whether genetic susceptibility in the gene encoding TLR7, containing SNPs with strong linkage to asthma and producing a receptor that operates within the innate antiviral Type I IFN pathway, might provide a mechanism linking viral infection to asthma. In the presence of an early-life viral infection with a productively replicating respiratory virus, PVM, mice deficient in TLR7 experience a bronchiolitis-like disease exhibiting marked epithelial sloughing, granulocytic and peribronchial infiltration, parenchymal consolidation, increased virus replication, and slightly increased mortality compared to WT mice. Intriguingly, there is epidemiological evidence to suggest that RSV bronchiolitis is more common in male than female infants and given that TLR7 is located on the X chromosome this could provide a plausible explanation for this trend (Pezzotti et al., 2009, Wainwright, 2010, Wickman et al., 1998). The increase in severity of infection in our model was associated with suppression of Type I IFN production, a switch in the predominance of myeloid DCs over plasmacytoid DCs in the lungs, and a delayed IFN-γ response. A reduction in Type I IFNs and pDCs mirrors what is seen both in asthmatics, and in the case of pDCs, children at risk of severe LRT bronchiolitis (Upham et al., 2009). Deficient IFN-γ responses have been observed in infants with RSV bronchiolitis, which was also predictive of the later development of asthma (Renzi et al., 1999).

In the absence of TLR7, the delayed antiviral response induced by PVM failed to completely suppress the emergence of several key Th2 molecules. We demonstrated mixed Th1/Th2 cell priming in TLR7-deficient mice, which was reflected in a mild increase in eosinophil numbers surrounding the lower airways. Overall, this bears a striking similarity to what has been observed for a subset of infants suffering RSV bronchiolitis. In humans, RSV infection itself does not induce a Th2 response as the majority of infected infants experience a relatively mild
course with a predominant Th1 response. However, in severe cases, possibly where a genetic susceptibility exists, the response induced can involve delayed lymphocyte recruitment, a mixed Th1/Th2 cell response, and increases in eosinophil numbers and eosinophil degranulation in the respiratory tract (Buckingham et al., 2000, Renzi et al., 1999, Larranaga et al., 2009, Kim et al., 2003, Ehlenfield et al., 2000, Garofalo et al., 1994, Harrison et al., 1999). Importantly, it is the infants that express this immunological pattern post-bronchiolitis that appear to be most at risk of subsequent asthma.

When mice deficient in TLR7 were re-infected in later life we observed a dramatic induction of Th2 responses, IgE, smooth muscle airway remodelling, and AHR. In human asthmatics, aside from the classical Th2 cytokines, IgE sensitisation, and atopic family history, the levels of IFN-γ and numbers of neutrophils in the blood are also significant risk factors for the severity of allergic asthma (Hollams et al., 2009). We also observed higher levels of IFN-γ in the lungs of these TLR7-deficient mice upon secondary infection. This is therefore supported by clinical reports suggesting IFN-γ acts as a co-factor in the presence of Th2 memory responses to induce greater inflammation. The altered secondary response to virus in our model was due to antigen-based re-activation of CD4 memory T cells. Although we used a depletion strategy to eliminate all CD4+ cells, we allowed these cells to regenerate prior to infection. This was done such that the only missing CD4+ factor upon secondary infection would be the memory component (i.e. CD4 memory T cells). However, because all T helper subsets would have been depleted by the antibody treatment and this resulted in both a reduction in Th1 and Th2 cytokines in the lymph nodes (although in the lungs only Th2 cytokines were diminished), it remains unknown whether the critical CD4 memory T cells were Th1, Th2 or both. Th17 cells appeared to play no role. This distinction may be somewhat superfluous given the heterogeneity of T cell responses even in allergic asthma, and the role of IFN-γ as mentioned above.
Our data also revealed that in the longer-term the absence of TLR7 during a subsequent third infection with PVM led to an exacerbation of disease and the outgrowth of mast cells and IgE. This data parallels clinical evidence that the risk of IgE sensitisation to allergens is positively correlated with the number of LRT infections in childhood (Schauer et al., 2002, Majkowska-Wojciechowska et al., 2007). Perhaps repeated viral exacerbations increase the risk of inhaled allergen sensitisation due to a greater mast cell presence within the lungs.

In humans, despite the prevalence of RSV very little is known about its engagement with the single stranded RNA receptors TLR7 and TLR8. Similarly, although genetic SNPs in TLR7 are strongly associated with asthma it is unknown how expression levels are affected in asthmatics (Moller-Larsen et al., 2008). We have shown for the first time that TLR7 expression is significantly reduced in the sputum cells of asthmatic patients compared to healthy control subjects. Importantly, these studies were carried out in stable asthmatics to avoid caveats of viral exacerbation or changes in maintenance therapy impacting upon inflammatory status. It remains to be determined whether this observed difference in TLR7 expression arises from defects at the level of receptor transcription, due to genetic SNPs in the promoter region, or a mutated form of the receptor.

There is mounting evidence indicating that synthetic TLR7/8 agonists are capable of suppressing Th2 responses during OVA-driven models of airway inflammation (Camateros et al., 2007, Moisan et al., 2006, Sel et al., 2007, Xirakia et al., 2010). The effect of these agonists can be achieved by either therapeutic or prophylactic administration. Our study undoubtedly provides evidence to support the potential future use of TLR7 agonists as a treatment of asthma and viral-associated exacerbations. Interestingly, clinical studies are currently investigating the
ability of low dose Type I IFNs to treat severe asthma and viral exacerbations, however at this stage it would be premature to draw any conclusions from these studies. Smaller scale studies in the past have shown promising efficacy, however significant side-effects were also apparent (Simon et al., 2003, Satoh et al., 1999, Kroegel et al., 2009).

Collectively this study suggests that a genetic predisposition, perhaps in the TLR7 gene or another as yet undefined mediator of the Type I IFN pathway, may create susceptibility to early-life LRT viral infections. These two factors could form the basis of a complex gene-environment interaction leading to Th2 cell priming. This appears to have a consequent long-term effect on the CD4 T cell memory immune compartment and one can also speculate (although not defined here) long-term changes in the airway epithelium. Re-exposure to virus in later-life, as is frequently seen in humans for RSV or RV, leads to re-activation of these memory cells and T cell-driven responses creating airway inflammation, AHR, remodelling and the expansion of mast cells and IgE. If this were to occur with concomitant allergen exposure it could potentially feed into allergen-specific IgE sensitisation and the perpetuation into chronic asthmatic disease. Improved understanding of the pathogenesis of virus-induced asthma should enable the design of more targeted therapies and the prediction of which children will go on to develop asthma due to a severe bronchiolitis episode in infancy. This could pave the way for passive immunisation to prevent or at least delay LRT infections in susceptible infants and perhaps reduce the burden of chronic asthmatic disease.
This thesis explores the gene-environment interactions along with other cell and molecular processes underlying the relationship between respiratory infections and asthma. Moreover, it attempts to delineate how respiratory infections might activate and manipulate the innate immune system, and how this may be involved in the pathogenesis of the hallmark features of asthma.

The first publication in this thesis demonstrated that *Chlamydia* could infect and survive in bone marrow-derived murine DCs and that by infecting these cells the bacteria could not only evade immune detection but also divert the immune response away from a protective Th1 IFN-\( \gamma \) response, to a less protective and more proliferative Th2 response. *Chlamydia* was able to survive inside these DCs but whether the individual bacterium took the form of active cycling elementary bodies or dormant persisting bodies remains to be determined. The implication of these observations is that *Chlamydia* infection of DCs enables persistence of the bacteria by usurping the immune response. However, as DCs are a highly migratory cell, transitioning between peripheral tissues and lymphoid organs, it is not without reason that *Chlamydia* could also utilise DCs as a transport vessel. These cells may in fact enable the spread of infection to other tissues in the host. This seems an intriguing possibility given that the main portal of entry and infection for the bacteria is the respiratory tract (*Chlamydophila pneumoniae* (Cpn)) or genital tract (*Chlamydia trachomatis*), however *Chlamydia* species have been identified in distal organs, including atherosclerotic plaques of coronary arteries (Bobryshev et al., 2004). This suggests a new paradigm of bacterial immune evasion wherein rather than simply interfering with the generation of an appropriate immune response, pathogens may actually utilise the migratory nature of immune cells in order to disseminate throughout the host. This underlies the importance of future investigations into a possible role of DCs as a transport vessel harbouring and disseminating *Chlamydia* species in systemic diseases, such as heart
disease. Although perhaps not as likely to undergo a productive infection away from the confines of the mucosal epithelium, the bacteria would be more than capable of inducing a low level of pathologic inflammation in certain tissues. In order to investigate this possibility it would be necessary to use bacterial tracing experiments in both normal and immune-compromised hosts.

By infecting DCs *Chlamydia* was able to increase the production of IL-13 from these cells, as well as from antigen-specific T cells responding to a bystander antigen. *Chlamydia*-infected DCs were also able to exacerbate OVA-induced AHR. Interestingly, *Chlamydia* species have been shown to also infect human DCs (Bobryshev et al., 2004, Matyszak et al., 2002). It remains to be tested how closely our experimental model mimics a possible bystander effect of Cpn on allergen-specific T cells in human asthmatics. Finally, it is conceivable that Cpn may also initiate acute exacerbations of asthma through alternative non-specific mechanisms. Cpn may chronically infect epithelial cells through its capacity to evade immune detection by down-regulating epithelial MHC molecules (Zhong et al., 2000). This may create underlying inflammation in asthmatics through mild epithelial sloughing, mucous production, and neutrophil influx into the airways. Such an effect could lead to reduced responsiveness to steroid treatment. The importance of immune evasion of Cpn in relation to acute exacerbation of asthma in humans will depend on two future lines of investigation. Firstly, whether the use of specific anti-Chlamydial antibiotics in interventional studies for pre-existing asthma improves AHR, and secondly, whether direct evidence (not based on Ig titres) can be demonstrated for persistent Cpn colonisation of asthmatic airways.

The second and third publications in this thesis are closely related. These chapters describe two separate but novel mechanisms that may underlie the enigmatic relationship between
respiratory viral infections and the development of asthma. The general implication of this work is that deficiencies in the innate immune system impart a critical effect on long-term regulation of the adaptive T cell response. This suggests the novel paradigm that dysregulation of innate cells (NK cells), cytokines (Type I and II IFNs, IL-25), or PRRs (TLR7), when triggered by an environmental insult such as an early-life LRT infection, may predispose to long-term asthmatic disease. A similar mechanism could feasibly exist in numerous chronic inflammatory disorders, including but not limited to, autoimmune disease. This is especially likely given the strong associations of various autoimmune diseases with innate immune mediators, both through GWAS and transcriptional studies, and their epidemiological association with infections and stress.

The conclusions of the second publication suggest the following novel cell and molecular mechanism underlying viral-induced Th2 responses (summarised in Figure 1 below). Under normal circumstances RSV infecting the respiratory epithelium is detected by NK cells, which produce large amounts of IFN-γ following activation. IFN-γ then inhibits IL-25, which is produced by the respiratory epithelial cells following RSV infection. IFN-γ also establishes a positive feedback loop with myeloid DCs and IL-12. This feedback up-regulates dll4 on the DCs, which along with the IL-12 production enables the DC to migrate to the lymph nodes and induce Th1 differentiation. Under circumstances of severe infection with RSV, involving reduced NK cell numbers, there is an attenuation of IFN-γ. Deficient IFN-γ removes the inhibitory effect on the epithelium allowing for enhanced production of IL-25. This IL-25 up-regulates the expression of Jagged1 on myeloid DCs instead of dll4. These DCs then migrate to the lymph nodes and initiate Th2 differentiation. An aspect of this mechanism that was not established in this publication was whether a secondary cell, also activated by epithelial-derived IL-25, could provide a soluble signal, such as IL-4, to enhance Th2 differentiation. The
identity of such a secondary cell is purely speculative, although interesting candidates might include one of the recently discovered gut-derived innate Th2-like cells, such as the ‘nuocyte’ or ‘MPPtype2’ cell. At this stage a role for these cells in the respiratory tract remains unclear. Future studies will need to investigate the presence or absence of these cells in the lungs during models of allergy and/or infection. Furthermore, it is also feasible that the mechanism underlying viral-induced Th2 responses in the respiratory tract was IL-4/STAT6-independent, and instead reliant on other soluble factors expressed by cells such as the ‘nuocyte’. Future experiments utilising monoclonal antibody blockade, or IL-4/STAT6-deficient mice will be necessary to answer these questions and determine whether there are indeed other unidentified soluble factors involved.
Figure 1: Proposed mechanism of Th2 induction by RSV. The effects of RSV in the presence of normal NK cell function are depicted in black text. The effects of severe RSV infection in the presence of reduced numbers of NK cells are depicted by red text and arrows. ?? indicates an unknown aspect of the pathway.Dll4; delta-like ligand 4, DC; dendritic cells, NK; natural killer, RSV; respiratory syncytial virus.
The following is a proposed mechanism based upon the findings from the third publication (summarised in Figure 2 below). During early-life exposure to pneumovirus activation of a TLR7-MyD88 signalling cascade following recognition of viral single stranded RNA induces the secretion of type I IFNs. These IFNs initiate an anti-viral immune response characterised by pDCs, NK cells, neutrophils, and IFN-γ-producing CD4 Th1 cells. This leads to robust viral clearance and acute mild/transient disease. In a state of TLR7-deficiency, these responses are markedely diminished and viral clearance is delayed leading to a disease pathologically quite similar to bronchiolitis. Furthermore, a Th2-type memory response is primed in early-life, which upon re-exposure to the same pneumovirus in later-life, promotes eosinophilia, elevated levels of IgE, mast cells, airway remodelling, and AHR. This deleterious response, which reflects the hallmark features of asthmatic pathology, is regulated by memory CD4 T cells. As asthmatic individuals express lower levels of TLR7 on airway inflammatory cells, a similar scenario to that described above may occur in humans. Those individuals carrying hypofunctional TLR7 SNPs may display a similarly IFN-deficient response to early-life LRT infection, and subsequent seasonal exposure to RSV or RV may trigger airway inflammation and asthma.

Future investigations should determine the relative role of IFN-α versus IFN-β in this model, and the potential for exogenous supplementation of these molecules to reverse the effect of TLR7-deficiency. This may be a potential intervention with a narrow therapeutic window in early-life to prevent severe bronchiolitis and the eventual onset of chronic asthmatic disease. The dependency of the Th2 response on MyD88, in the absence of TLR7, also remains to be determined. However, the lethality of this virus in MyD88-deficient mice poses an immense caveat to these studies. Likewise the precise cell and molecular source responsible for inducing the Th2 response in the absence of Type I and II IFN production is unclear at this stage.
Increases in IL-33 and myeloid DCs were both associated with the outgrowth of the Th2 phenotype, but would require depletion experiments to be directly implicated. It is possible that the pneumovirus may stimulate pro-inflammatory pathways in a state of IFN-deficiency through two distinct mechanisms. This includes altered dominance of specific viral proteins at the PRR level that would otherwise be suppressed by the TLR7-IFN pathway. One example is the RSV F protein interacting with TLR4 (Kurt-Jones et al., 2000) in a more immunodominant manner than usual. Alternatively, the virus may promote activation of DAMPs (such as heat shock proteins or high mobility group nucleosome binding protein-1), which then act as endogenous alarmin signals to promote the release of IL-33. Another interesting aspect of this work that will require future investigation is the possibility that the virus-induced Th2 response may lead to bystander allergen sensitisation. In the absence of TLR7 the linear increase in IgE and airway mast cells that was observed with each subsequent pneumoviral infection suggests the propensity for enhanced sensitisation to inhaled antigens, similar to what has been observed in human infants (Holt et al., 2010). To examine this possibility the PVM infection model should be superimposed with an allergen, such as OVA or house dust mite, to determine alterations in 1) specific IgE-sensitisation, and 2) exacerbation of airway disease. Finally, it would provide tremendous clinical applicability in terms of biomarker identification to conduct a genetic epidemiological investigation into whether infants that are more susceptible to viral bronchiolitis carry hypofunctional TLR7 SNPs, and lower levels of TLR7 expression, and whether such infants are more prone to transition from LRT infection to a diagnosis of asthma in later childhood.
Figure 2: Proposed mechanism underlying the development of a Th2 memory response in a state of TLR7-deficiency. In a TLR7-sufficient individual exposure to respiratory pneumovirus infection in early-life induces a robust Type I IFN response derived from pDCs signalling through MyD88. This initiates rapid viral clearance via activation of IFN-γ-producing Th1 cells, NK cells, and neutrophils. The result of this response is a mild disease due to the acute lower respiratory tract infection. In contrast, in a TLR7-deficient (or TLR7-hypofunctional) individual the Type I IFN pathway is not activated in response to virus leading to a delay in the induction of IFN-γ. This delay leads to slower viral clearance and pronounced...
pathology in the airways eventuating in a severe lower respiratory tract infection or bronchiolitis-like disease. In the absence of Type I IFNs alternative T helper cell induction signals emerge, which potentially includes IL-25 and/or IL-33 (signalling through the receptor TS1/ST2). These innate cytokines likely prime Th2 cells that eventually lead to increased AHR, airway smooth muscle remodelling, airway eosinophilia, mast cells, and IgE. pDC; plasmacytoid dendritic cell, NK; natural killer, AHR; airways hyper-reactivity, MyD88; myeloid differentiation factor 88, TLR7; toll-like receptor 7.

Increasingly, there are examples of susceptibility gene-environment interactions as the basis of many complex diseases. The classic example of course is tumour carcinogenesis. However, more recently specific virus-susceptibility gene interactions have been identified for the autoimmune inflammatory bowel disease. Infection with a specific gastrointestinal virus, in the presence of mutations in the Crohn’s disease susceptibility gene Atg16L1, when combined with an additional environmental insult to the gastrointestinal lining, precipitates intestinal pathologies in mice that closely resemble human Crohn’s disease (Cadwell et al., 2010). Although this is an area that has received a great deal of attention for several chronic diseases it is only just beginning to be properly investigated in respiratory disease, despite a mountain of epidemiological evidence alluding to the critical nature of gene-environment interactions. Previous work in the area of viral-associated asthma has largely attributed cause to either genetic/immune defects, or alternatively environmental changes (early-life infection, pollution or allergen exposure). Clearly the work presented in this thesis reveals that in order to properly dissect the complex nature of asthma these two factors cannot be considered in isolation. A multi-hit gene-environment hypothesis (such as the one presented in this thesis) may explain the relationship between early-life LRT infections and childhood asthma, however the precise mechanism may vary depending upon atopic status (Simoes et al., 2010). For instance, in
infants from a non-atopic background, exposure to a LRT viral infection, at a critical time of postnatal development, in the presence of genetic susceptibility within the innate immune system, may lead to severe pulmonary sequelae. Ultimately the inability to generate an efficient IFN-driven response to viral infection may prime an inappropriate long-lived T cell response. Such a memory response can be exacerbated by re-exposure to virus in later-life. The outgrowth of IgE and mast cells in the lungs with each subsequent viral exacerbation may increase the likelihood of inhaled allergen sensitisation leading to chronic asthmatic disease. An alternative mechanism may exist in infants from an atopic background. Genetic susceptibility may select for severe viral infection, and the ensuing denudation of the respiratory epithelial barrier, may simply target systemic allergic predisposition to the airways. This would occur by increasing the underlying immune compartments exposure to inhaled allergens.

Collectively, the work in this thesis may help to improve insight into the etiology of virus-associated asthma, the pathogenesis of acute exacerbations, and the concept of gene-environment interactions precipitating chronic inflammatory diseases. An overall greater understanding of the role that respiratory infections play in the pathogenesis of asthma will enable the early identification of susceptible infants in the clinic. This could pave the way to use vaccination to prevent, or at least delay, LRT infections in order to reduce the burden of chronic asthmatic disease. Alternatively, improved understanding of disease mechanisms could lead to the design of more targeted molecular therapies aimed at either suppressing inflammation or remediating defects in the innate immune system.
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