Immune Regulation of Intracellular Bacterial Infection and Allergy: Implications of Dendritic Cells & Natural Killer T Cells

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A Thesis submitted to the Faculty of Graduate Studies of

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Abstract

As the burden of infectious diseases becomes reduced in many countries, a remarkable increase in the incidence of allergies has occurred. The basis for the rise in atopic disorders as a correlate of the decline in infectious diseases has not been defined. In the present study, we tested experimentally whether prior *Chlamydia muridarum* (*Chlamydia muridarum*) infection or *Mycobacterium bovis* BCG had any effect on ragweed (RW) + $Al(OH)_3$ (alum)-induced allergic responses and the potentially underlying mechanisms. The data showed that RW-induced eosinophilia and goblet cell development were significantly inhibited by prior infection with *Chlamydia muridarum*. The data also demonstrate that BCG infection delivered during the course of a RW-induced allergic response could inhibit the existing allergic phenotype. The abrogation of RW-induced mucus production and pulmonary eosinophilia in *Chlamydia muridarum*- or BCG-infected mice correlated with significantly decreased Th2 cytokine (IL-4, IL-5, IL-9 and IL-13) production and increased IFN-γ and IL-12 production. Additionally, the production of eosinophil-chemoattractant chemokine, eotaxin, followed by RW exposure were significantly reduced in the lungs of *Chlamydia muridarum* infected mice. However, *Chlamydia muridarum* infection did not reduce the levels of RW-specific IgE and IgG1 production in the sera, nor did it diminish the level of total serum IgE. These data provide direct evidence that intracellular bacterial infection (i.e. Chlamydia or BCG) can inhibit airway inflammation induced an environmental allergen.

Since polarized T cell responses are downstream from the events that initiate the immune response, we examined whether innate immune cells, dendritic cells (DCs) and natural killer T (NKT) cells, can be activated by *Chlamydia muridarum* infection, and the potential roles played by these cells in infection-mediated inhibition of the allergic response.

We first examined the role played by dendritic cells (DCs) in chlamydial infection-mediated modulation of allergic airway inflammation. The results showed that DC freshly isolated from Chlamydia-infected mice, unlike those from naive control mice, exhibited higher surface expression of CD8α and

Toll-like receptor 9 (TLR9) as well as higher IL-10 and IL-12 production than DC isolated from naïve mice. Interestingly, the blockade of IL-10 or IL-12 showed a partial reversal in the inhibiting effect on allergen-driven Th2 cytokine production. Furthermore, adoptive transfer of DC from Chlamydia- or BCG-infected mice, but not DC from naïve mice, could inhibit systemic and local eosinophilia induced by allergen exposure. The inhibitory effect of transfer of DC from infected mice could be partially reversed by administration of anti-IL-10 antibody to block DC's IL-10 production. As well, we observed a partial reversal of the reduction of the RW-driven allergic response by delivery of neutralizing IL-10 or IL-12 antibody following DC transfer from bacteria-infected mice. In an additional adoptive transfer system, we also saw that the CD8α+-expressing DC isolated from *Chlamydia muridarum*-infected mice, but not CD8α- DC, could inhibit the RW-driven allergic phenotype and that these cells also produced significantly higher levels of both IL-10 and IL-12. The data demonstrate a critical role played by IL-10-producing DCs from Chlamydia-infected mice in the infection-mediated inhibition of allergic airway inflammation.

To further investigate the role of other innate cells in allergy and infection, we examined the role of NKT cells in the individual allergy and infection models. The data demonstrate that, first, in the allergy model, mice deficient in NKT cells exhibit less RW-driven IL-4 production and this is associated with lower levels of eotaxin production, lung eosinophilia and mucus oversecretion by goblet cells compared to wild-type mice treated with RW. Secondly, in the *Chlamydia muridarum* infection model, NKT cell-deficient mice also demonstrate lower levels of *Chlamydia muridarum*-induced IL-4 production and less *Chlamydia muridarum*-induced pathology as well as *in vivo* chlamydial growth. Overall, the date suggest that the innate immunity activated by chlamydial infection is very important in determining the outcomes of T cell responses upon allergen exposure and that particular cell types of the innate immune response, specifically DC and NKT cells, have an effect on both the allergen-induced and infection processes.

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Abbreviations

α-GalCer	alpha-Galactosylceramide
Ab	Antibody
mAb	Monoclonal antibody
PAS	Periodic Acid Schiff
AHR	Airway hyperresponsiveness
Alum	Aluminum hydroxide adjuvant
APC	Antigen presenting cell
BAL	Broncho-alveolar lavage
BALF	Broncho-alveolar lavage fluid
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CFA	Complete Freund's adjuvant
ConA	Concanavalin A
CPM	Counts per minute
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
ELISA	Enzyme Linked ImmunoSorbent Assay
FACS	Flow assisted cell sorting
FBS	Fetal bovine serum
HMI	Histological mucus index
HRP	Horse radish peroxidase
H&E	Hematoxylin and eosin
Ig	Immunoglobulin
IFU	Inclusion-forming units
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneal
IFN	Interferon
KO	Gene knockout
Chlamydia muridarum	*C. muridarum* mouse pneumonitis
NKT cell	Natural Killer T Cell
OVA	Chicken Egg Ovalbumin

PBS	Phosphate buffered saline
PMA	Phorbol myristic acid
RW	Ragweed
SDS	Sodium dodecyl sulfate
SPG	Sucrose-phosphate-glutamic acid buffer
TBS	Tris (tris(hydroxymethyl)aminomethane) Buffered Saline
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll like receptor

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Part I. General Introduction

Medical conditions such as allergic asthma, inflammatory bowel disease and diabetes have risen sharply in recent years stimulating the attention of the medical world. Asthma, in particular, has been the focus of much research with a greater understanding of the immunological processes involved in airway pathologies. Many thought-provoking explanations have been proposed for the rising prevalence of asthma, with the hygiene hypothesis gaining much favour. This hypothesis is breaking new ground in allergic and other dysregulated inflammatory disorders and proposes that some aspects of infectious agents can be beneficial, maintaining immunological homeostasis. The recently observed inverse association between infections and allergic diseases have helped to shape the hygiene hypothesis from a simple Th1-Th2 dichotomy into a multilateral regulatory model, and may explain rising trends of a wide range of inflammatory disorders. The following introduction highlights key events in this exciting area, summarising the complex disease state of asthma with a cellular-based model of airway allergy. The influence of genetic pre-dispositions and environmental risk factors are discussed, with a detailed analysis of the hygiene hypothesis and the influence of infectious pathogens on allergic disease outcome. Attention is focused on intracellular bacterial infections and their exceptional ability to modify responses to allergens. This introduction concludes with proposed mechanisms of intracellular bacterial infection-associated protection from allergies and highlights the need and justification for this thesis.

Escalating Allergic and Inflammatory diseases

Asthma exerts a global burden of 300 million sufferers (Masoli, Fabian et al. 2004) affecting 1 in 5 individuals in developed countries (Umetsu, McIntire et al. 2002), accounting for almost 1 in every 250 deaths worldwide. It is predicted to rise by a further 100 million cases by 2025 (Masoli, Fabian et al. 2004) posing one of the most common chronic diseases in the world. Since the 1950's, allergic symptoms in general have progressively increased (Isolauri, Huurre et al. 2004). Childhood asthma symptoms in the USA are now the major cause of school absenteeism, are the most common cause of

hospitalisation, and have resulted in a doubling of asthma-related deaths from 1980 to 1992 (O'Connell 2004). Within North America, an approximate threefold increase in allergy-related visits to hospitals has been observed over the past 20 years, burdening the health service by $900 million per annum. These numbers are expected to rise not only within North America but also other westernized countries such as the UK (Johnson, Ownby et al. 2002). Furthermore, multi-system-allergic diseases are more common, with patients presenting multiple sensitivities culminating in airway, food borne and skin allergies within the same individual .

Several reports have assessed the global burden of asthma and other atopic diseases. Namely, the 'The International Study of Asthma and Allergies in Childhood (ISAAC), which exposed a peak prevalence of asthma symptoms in North America, United Kingdom, New Zealand, Australia and Republic of Ireland, compared to lower prevalence in non-industrialised countries, Indonesia (2%), Albania, Romania, and Georgia (ISAACSubject 1998). Similarly, the European Community Respiratory Health Survey (ECRHS) identified a higher prevalence of allergic diseases in developed countries than in lesser developed eastern Europe, North Africa and India . Combining these and other reports, the Global Initiative for Asthma (GINA) made an effort to raise awareness among public health bodies, government officials, health care workers and the general public that asthma is on the increase (Masoli, Fabian et al. 2004). These epidemiological data sets paint a convincing picture of elevating asthma symptoms; however thankfully, several reports also describe a plateau and even decline in asthmatic episodes (Anderson, Ruggles et al. 2004; Sunderland and Fleming 2004), Australia (Toelle, Ng et al. 2004) and Italy (Ronchetti, Villa et al. 2001). Improved disease management and allergen avoidance may contribute to these findings; however explanations for these reductions are as elusive as explanations for the initial increase. Interestingly, a considerable disparity in the prevalence of allergic symptoms between developed and developing countries of the world has been unearthed. The many differences between these regions may highlight factors protecting individuals from allergic diseases in developing countries.

The disparity in asthma symptoms is also found between rural and urban areas within both industrial and non-industrial countries (Yemaneberhan, Bekele et al. 1997; Beasley 1998; Beasley, Crane et al. 2000; Leynaert, Neukirch et al. 2001; Dagoye, Bekele et al. 2003; Masoli, Fabian et al. 2004). In particular, within African countries allergies are more common in the upper socioeconomic bracket of urban residents (Weinberg 2000), suggesting that urbanisation and the adoption of a 'western lifestyle' correlate with allergic symptoms. Other immune-mediated diseases are also rising in similar geographical regions. Autoimmunity, such as type 1 diabetes (IDDM) (2000; Simpson, Anderson et al. 2002) multiple sclerosis (MS) (Rosati, Aiello et al. 1988) and coeliac disease (CD) (Farrokhyar, Swarbrick et al. 2001) are increasing in a manner similar to allergic disorders (Stene and Nafstad 2001; Bach 2002) (Fig.1.1). Most significantly, allergy and autoimmunity can coincide in the same individuals, indeed children with Th1 associated CD or Rheumatoid Arthriris (RA) have a higher incidence of Th2 associated allergies (Kero, Gissler et al. 2001; Simpson, Anderson et al. 2002).

Many potential factors underlying the rise in immune-reactive disorders have been suggested. Asthma genetics has progressed significantly with genome wide screens to identify previously unrecognised genes, while candidate gene approaches have revealed several asthma susceptibility genes (Wills-Karp and Ewart 2004) as highlighted below.

Environmental risk factors, such as changes in lifestyle and behavioural patterns, the influence of viral infections during childhood and changes in allergen exposure are all uncovering potential risk factors associated with the increased allergy epidemic, and will be discussed further below.

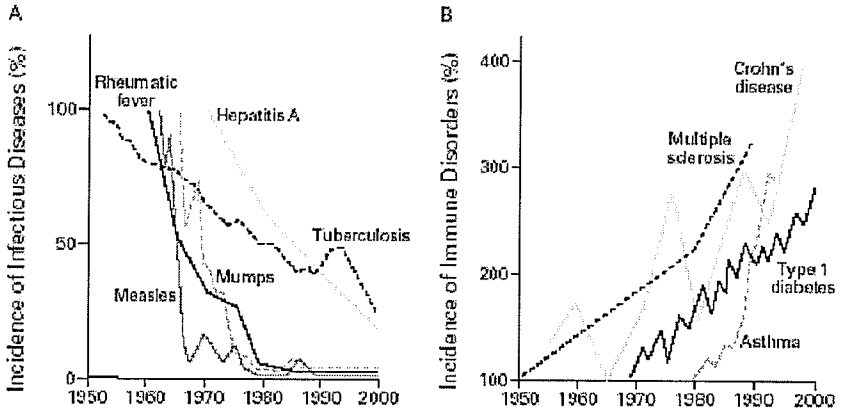

Fig 1.1 Inverse Relation between the Incidence of Infectious Diseases (A) and the Incidence of Immune Disorders (B) from 1950 to 2000 (Bach 2002)

Asthma - Polygenic inheritance combined with Environmental allergen exposure

Allergic diseases are thought to result from multigenic predispositions combined with specific environmental interactions. The most characteristic feature of allergic responses is the generation of allergen-specific-IgE. The production of IgE appears to be protective against pathological parasitic infections (Jankovic, Kullberg et al. 1997). The production of IgE may have provided an evolutionary advantage, limiting helminth infections for example. However, an aberration from our helminth burdened past and relative freedom from parasitic infections may have resulted in the development of IgE associated allergic diseases.

A 'genotype of susceptibility', identifying those at a higher risk of developing allergies, has yet to be resolved. The task is obstructed because asthma, for example, includes several different phenotypes, likely reflecting different pathogenic mechanisms with aetiological heterogeneity. The association with environmental factors further complicates the identification of a 'genotype of susceptibility' as environmental factors, such as diet and housing, are themselves clustered within families. Nevertheless, efforts are being made to identify genes, alleles and chromosomal regions, which confer susceptibility to asthma. Familial aggregation, parent-of-origin (most predominantly maternal

linkage, suggesting immune interactions through placenta and breast milk) and genomic imprinting are the strongest risk factors for asthma susceptibility (Geha 2003). Th2 cytokines within the 5q31 region (Cookson and Moffatt 2000), cluster of differentiation 14 (CD14), and several other chromosomal regions (Ober, Cox et al. 1998; Howard, Wiesch et al. 1999) associate with a number of intermediate phenotypes of asthma. Of interest, ADAM33 (Van Eerdewegh, Little et al. 2002), Tim1 (McIntire, Umetsu et al. 2001) and complement factor 5 (C5) (Karp, Grupe et al. 2000) are some of the most recent and exciting genes identified in humans and mice. These will be discussed later. An inspiring review of this topic, beyond the requirements of this thesis can be seen in (Wills-Karp and Ewart 2004).

Approximately 40% of allergic diseases can be attributed to genetically variable factors (Holgate 1998) such as atopy and IgE (Pearce, Pekkanen et al. 1999), however, for disease to manifest there is an absolute requirement for environmental interactions with the insulting allergen. The most important environmental allergens derive from invertebrates, such as house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*) and cockroaches, domestic animals, fungi, pollen and grasses. *In utero* sensitisation to aeroallergens, such as Der p1, via maternal inhalation might represent the earliest environmental exposure (Piccinni, Mecacci et al. 1993), with further sensitisation and allergen-specific IgE responses developing and progressing throughout life. The relatively rapid rise in allergic diseases over a short period of time suggests environmental factors, not changes in genotype, are causes of the allergy epidemic. How might environmental factors, beyond allergens themselves, influence the tendency to become sensitised or responsive to innocuous antigens? To address this, we must first appreciate how allergic responses unfold and then examine possible environmental risk factors.

Immunology of Asthma

Allergic asthma represents an immune-mediated disorder, characterised by a state of immune hypersensitivity with elevated levels of allergen-specific IgE. Subsequent allergen encounters and cross linking of two or more mast cell

bound IgE molecules provoke mast cell dregranulation and an accumulation of mast cells and eosinophils into air spaces and tissues of the lung, instigated by Th2 cells (Fig.1.2). Mechanisms associated with allergic asthma are described below in terms of cellular induction and soluble mediators, which play a direct role in allergic inflammation.

Immunological surveillance in the airways depends on dendritic cells (DC's) in the sub-epithelial layers and intercellular spaces between epithelial cells lining the alveolar walls of the lung (Gong, McCarthy et al. 1992; Moller, Overbeek et al. 1996; Lambrecht, Hoogsteden et al. 2001; Julia, Hessel et al. 2002).

Allergen encounter and capture by DC, possibly by disruption of epithelial tight junctions (Wan, Winton et al. 1999) or contact with the probing dendrites of airway DCs (Rescigno, Urbano et al. 2001), accompanied by appropriate pathogen associated molecular pattern (PAMP) stimulation (Eisenbarth, Piggott et al. 2002), activates DCs and triggers a migration to the draining lymph nodes (Vermaelen, Carro-Muino et al. 2001).

Once DCs reach the local draining lymph nodes (Holt, Oliver et al. 1993; Vermaelen, Carro-Muino et al. 2001), they mature and express high levels of peptide-loaded MHC and co-stimulatory molecules, (CD80, CD86, OX40L, and ICOSL), and migrate to the T-cell zones. MHC-TCR engagement (Mempel, Henrickson et al. 2004) with appropriate co-stimulation and Interleukin (IL)-4 signalling, generates allergen-reactive Th2 cells. Th2 cells then egress from lymph nodes back to the site of initial allergen encounter (Herrick and Bottomly 2003) following chemotactic gradients of chemokines such as RANTES (regulated on activation, normal T-cell expressed and secreted), TARC (Thymus-associated and regulated chemokine) MDC (macrophage derived chemokine) (Bonecchi, Sozzani et al. 1998; Soumelis, Reche et al. 2002) and most recently prostaglandin D_2 (PGD_2) (Hirai, Tanaka et al. 2001; Honda, Arima et al. 2003). Allergen re-encounter by DCs and activation of antigen-specific Th2 cells, results in a barrage of Th2 cell derived cytokine secretions.

The central role for Th2 cells in asthma is apparent with both specific IgE production and the Th2-cytokine milieu found in the allergic response (Tournoy, Kips et al. 2001). Animal models of allergic airway inflammation have shown that GATA-3 and STAT-6 disruption, which inhibits Th2 cell signalling, severely attenuates all key features of allergic airway inflammation (Tomkinson, Kanehiro et al. 1999; Zhang, Yang et al. 1999). Alternative studies have depleted $CD4^+$ Th2 cells or knocked out IL-4 and found similar inhibitions of allergen induced airway inflammation (Brusselle, Kips et al. 1994; Gavett, Chen et al. 1994). Furthermore, the allergic airway inflammatory response can be induced by the transfer of $CD4^+$ T-cell receptor transgenic Th2, but not Th1, cells (Cohn, Tepper et al. 1998). Thus, $CD4^+$ Th2 cells, recognising allergen peptides via their TCR and releasing interleukins orchestrating the allergic inflammatory response, are absolutely essential in the aetiology of allergic asthma.

Allergen-specific IgE responses define allergic patients. However, it was not until the Th1-Th2 dichotomy was classified (Mosmann and Coffman 1989) based on cytokine profiles, that the basis for IgE induction was understood. Th2-cell derived IL-4 induced transcription of Cε (Corry and Kheradmand 1999) and CD40-CD40L interactions between B- and T-cells (de Lafaille, Muriglan et al. 2001) induce B cell migration into lymphoid follicles, or bronchial associated lymphoid tissue (BALT) (Chvatchko, Kosco_Vilbois et al. 1996), culminating in allergen specific IgE production (Burrows, Martinez et al. 1989; Saini and MacGlashan 2002). However, allergen-specific-IgE only represents a very small fraction of the total serum immunoglobulins (Burrows, Martinez et al. 1989).

Due to the low concentrations of IgE, its function is related to the cell-bound high affinity receptor, FcεR1, found on mast cells, basophils and human eosinophils (Yamaguchi, Sayama et al. 1999). Allergen re-encounter and cross linking of two FcεR1 bound IgE molecules on tissue mast cells or blood basophils, in addition to activation of Th2 cells, results in phosphorylation and dephosphorylation of a series of proteins triggering activation and degranulation (Holgate 1998; Gould, Sutton et al. 2003). Mast cells abound

with inflammatory mediators including; histamine, proteolytic enzymes, complex carbohydrate cleaving enzymes, cytokines and chemokines, prostanoids and leukotrienes (Bradding, Roberts et al. 1994; Bradding and Holgate 1999; Miller, Wright et al. 1999; Kobayashi, Miura et al. 2000; Wedemeyer, Tsai et al. 2000; Hart 2001). Mast cells and their products are undoubtedly important components of the allergic inflammatory cascade (Kobayashi, Miura et al. 2000; Mayr, Zuberi et al. 2002; Taube, Dakhama et al. 2004) however, their importance in animal models of acute allergic airway inflammation is debatable. Comparable airway inflammation, airway hyperresponsiveness (AHR) or anaphylaxis (Oettgen, Martin et al. 1994) has been achieved in the absence of IgE (Mehlhop, van de Rijn et al. 1997; Hamelmann, Cieslewicz et al. 1999), B-cells (Hamelmann, Cieslewicz et al. 1999; MacLean, Sauty et al. 1999), CD23 (Haczku, Takeda et al. 1997) or mast cells (Takeda, Hamelmann et al. 1997) but not T-cells (Gavett, Chen et al. 1994).

Th2 cytokines; IL-4, IL-5, IL-9 and IL-13 can be attributed to almost all intermediate stages of airway inflammation and pathophysiological asthma (Romagnani 2001). The archetypal Th2 cytokine IL-4, plays essential roles in Th2 generation, however, interestingly once Th2 priming is initiated, IL-5 takes centre stage. Beyond Th2 polarisation, IL-4 is not necessary for airway inflammation (Coyle, Le Gros et al. 1995; Ying, Humbert et al. 1997; Hamelmann, Cieslewicz et al. 1999). Thus, very early sources of IL-4, possibly from NKT cells (Akbari, Stock et al. 2003) and basophils (Min, Prout et al. 2004) are fundamental for Th2 differentiation.

IL-13, although sharing many properties, and even receptor complexes, with IL-4, has greater functional properties at the effector cell level. Mucus hypersecretion due to mucus cell metaplasia and goblet cell hyperplasia (Zhu, Homer et al. 1999) have been closely associated with IL-13 (Zhu, Lee et al. 2001) and IL-9 (Longphre, Li et al. 1999) secretions. IL-13 also promotes cellular recruitment by upregulating chemokine expression (Hart 2001) and is critical for the development of AHR (Walter, McIntire et al. 2001). Furthermore, initial IgE class switching (Finkelman, Katona et al. 1988),

airway eosinophilia (Cohn, Herrick et al. 2001) and pro-fibrotic developments in chronic asthma (Postlethwaite, Holness et al. 1992) are promoted by IL-4 and / or IL-13. The recently discovered cytokine IL-25, is also secreted by Th2 cells and accentuates IL-4, IL-5 and IL-13 gene expression, amplifying the Th2 environment (Fort, Cheung et al. 2001).

In allergic asthma, IL-5 works in close collaboration with the predominant eosinophil chemoattractant, eotaxin (Jose, Adcock et al. 1994). Th2 cell derived IL-4 and IL-13, along with TNF-α released from macrophages, stimulate eotaxin release from lung fibroblasts, epithelial cells, and smooth muscle cells (Rankin, Conroy et al. 2000). Additional to the chemotactic properties of eotaxin, local eotaxin production enhances; intracellular calcium levels- increasing respiratory burst activity (Tenscher, Metzner et al. 1996), eosinophil adhesion to endothelial cells, via VCAM-1 on endothelial cells (Burke-Gaffney and Hellewell 1996), corroborating eosinophil extravasation and degranulation in local tissues (Rankin, Conroy et al. 2000). In the periphery, eotaxin and IL-5 enter the blood stream and act synergistically to mobilise the large reserve of mature eosinophils within bone marrow (Sklar, Tsuji et al. 2004). IL-5, in addition to its chemotactic attributes is a priming agent and growth factor for eosinophils, increases eosinophil responsiveness and differentiation (Hamblin 1993) and promotes T-cell dependent airway mucus production (Justice, Crosby et al. 2002), AHR and tissue damage. IL-5 is a potential therapeutic target, but although depleting >70% of circulating and sputum eosinophils with anti-IL-5 treatment, clinical asthma such as AHR was found not to be altered (Leckie, ten Brinke et al. 2000). The recruitment of eosinophils into the allergic lung and draining lymph nodes (van Rijt, Vos et al. 2003) is a hallmark of allergic airway inflammation. Allergen induced degranulation of eosinophils, releasing major basic protein (MBP) (Lefort, Nahori et al. 1996), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) is most likely responsible for lung tissue damage and AHR (Rankin, Conroy et al. 2000). Mucus hypersecretion and goblet cell hyperplasia, following allergen provocation, is another pathophysiological feature of asthma (Rogers 2003). Cytokines IL-4, IL-9 and most importantly IL-13 (Zhu, Lee et al. 2001) as well as oxidants from Th2 cells upregulate

mucin production and epidermal growth factor (EGF). These pathophysiological changes can be easily observed in biopsies or paraffin embedded murine lung sections stained with Alcian blue and periodic-acid Schiff.

Collectively, a considerable cellular influx into the airways following allergen provocation, accompanied by tissue damage and excessive secretion of mediators, cytokines, chemokines and mucus are thought to ultimately lead to airway remodelling, and irreversible changes in lung function independent of inflammatory episodes.

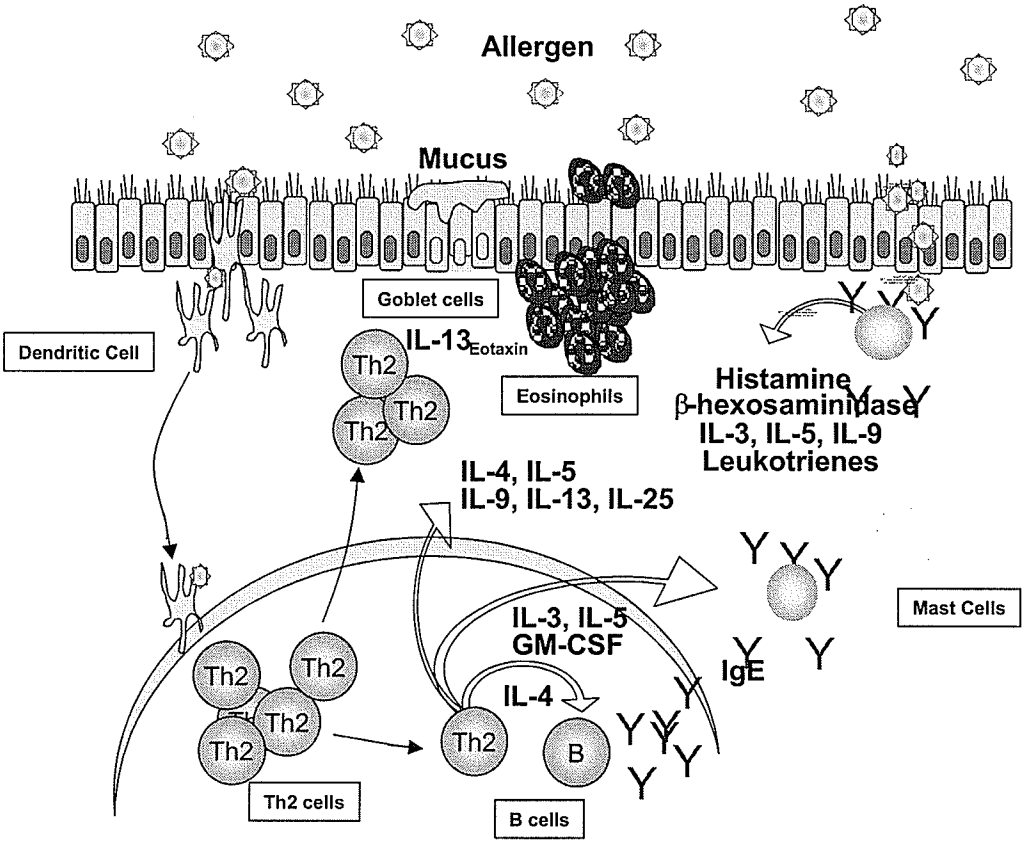

Fig.1.2. Key events in the Allergic Cascade. Airway dendritic cells capture antigen and migrate to local lymph nodes, where Th2 polarisation and differentiation occurs. Th2 cells egress to the site of allergen contact secreting IL-4, IL-5, IL-9 and IL-13, recruiting mast cells and eosinophils. Th2 cells promote B cell maturation and IgE class switching, with soluble IgE binding to its Fc receptor on mast cells. Cross linking of two IgE molecules triggers mast cell degranulation with a plethora of inflammatory mediators.

Airway remodelling refers to the non-inflammatory alterations in airway cells and tissues following chronic inflammation and is a major component of clinical asthma. Briefly, thickening of the airway wall smooth muscle, edema, inflammatory cell infiltrates, glandular hypertrophy and connective tissue deposition result in airway narrowing, leading to reduced baseline airway calibre and hyper-responsiveness (Elias 2000; Chiappara, Gagliardo et al. 2001; Zhu, Lee et al. 2001).

The prevalence and involvement of various cell types during the course of allergic inflammation have become evident. Natural killer T (NKT) cells have also been demonstrated to play a pertinent role in the intiation of allergic inflammation. NKT cells are a T cell subset that was initially defined by the co-expression of both T cell and NK cell markers, such as the T cell antigen receptor (TCR) αβ, and C-type lectin NK1.1, respectively Although the cells conforming to this broad definition are heterogeneous and can be divided into at least three groups, many of them are characterized by their dependence on recognition of the MHC Class I-like CD1d molecule for positive thymic selection and subsequent activation in the periphery (Bendelac, Lantz et al. 1995). The major subset of CD1d-restricted T cells recognizes CD1d through the expression of antigen receptors comprised of an invariant TCR α chain encoded in mice by Vα14 rearranged to Jα18, and in humans by a strikingly homologous Vα24-Jα18 rearrangement. These invariant TCR α chains pair with TCR β chains with limited heterogeneity due to marked skewing of Vβ chain usage (most prominently Vβ8.2 in mice, and a closely homologous Vβ11 in humans). This population of cells is highly conserved in many aspects of their phenotype, distribution and functions in most mammals that have been studied to date (Matsuda, Gapin et al. 2002; Godfrey, MacDonald et al. 2004; Godfrey, McCluskey et al. 2005).

It is now clear from many studies that NKT cells are potent regulatory T cells that have the capacity to either initiate or shut down a wide variety of immune responses. For example, it has been found that activation of NKT cells occurs early in a number of microbial infection models in mice, and that such activation can lead to an "adjuvant cascade" that reinforces innate immunity and promotes subsequent adaptive immunity. The range of pathogens that

can trigger this antimicrobial activity of NKT cells is astonishingly broad, and is known to include a variety of bacteria, viruses and parasites. In addition, NKT cells have been implicated in a variety of anti-tumour responses in mice, and it appears that they may be activated by certain types of neoplasia to mediate immune surveillance. However, in the absence of a microbial or neoplastic trigger, the dominant effect of NKT cells may be to regulate the adaptive immune system in a way that prevents harmful autoimmunity. The evidence for this is particularly strong from mouse models of autoimmune disease, including experimental allergic encephalomyelitis and type 1 diabetes mellitus (Singh, Wilson et al. 2001; Rocha-Campos, Melki et al. 2006).

Interestingly, NKT cells are reported to be involved in the promotion of both Th1-like reactions in response to intracellular pathogens as well as the production of Th2 cytokines. A specific marine sponge glycolipid, α-galactosylceramide (α-GalCer), binds to CD1d and is recognized by and activates mouse Vα14 and human Vα24 T cells, however the specific glycolipid antigens that are normally recognized by NKT cells *in vivo* are not known. More recently, it has been demonstrated that NKT cells are capable of recognizing bacterial glycosphingolipids (Kinjo, Wu et al. 2005). When activated, however, NKT cells rapidly produce large quantities of IL-4 and interferon (IFN-γ), which seem to influence subsequent adaptive immune responses and the polarization of conventional αβ-TCR T cells. NKT cells therefore seem to have important regulatory functions for both Th1 and Th2 arms of the immune response, although a clear role for NKT cells in the respiratory tract has not been shown. Many reports concur on the essential role for NKT cells following allergen exposure. These cells appear to affect the subsequent development of allergen-induced airway hyperreactivity as well as the initiation of T cell recruitment for elicitation of contact sensitivity (Akbari, Stock et al. 2003; Campos, Szczepanik et al. 2003; Lisbonne, Diem et al. 2003; Araujo, Lefort et al. 2004; Kim, Kim et al. 2004; Lisbonne, Hachem et al. 2004). Additionally, several studies have examined the frequency of NKT cells in human peripheral blood samples, comparing IL-4 transcripts in atopic and non-atopic individuals. These studies have revealed that NKT cells become activated and contribute to the initial pool of IL-4 and promote the

development of allergies at early time points following allergen exposure (Prell, Konstantopoulos et al. 2003). In addition, previous studies investigating the role of T cells in the development of airway hyperresponsiveness, and have revealed a significant contribution by NKT cells, with greater reductions in AHR following CD4+ and NKT cell depletion than with CD4+ T cell depletion alone. (Joetham, Takeda et al. 2005). NKT cells are capable of rapidly secreting key influential cytokines such as IL-4 and IFN-γ, with the ability to modulate allergic diseases. However, comprehensive studies regarding the role of NKT cells and CD1 molecules in allergic responses especially in asthmatic reactions are lacking. In particular, the limited studies on NKT cells in allergic responses generated very controversial data on the potential role of NKT cells in the development of the allergic reaction, especially IgE responses (Yoshimoto, Bendelac et al. 1995; Dougherty, Brown et al. 1996; Zhang, Rogers et al. 1996; Matsuda, Gapin et al. 2003; Morishima, Ishii et al. 2005). Some studies suggest that NKT cells are not essential for the allergic reaction and their activation can even inhibit allergen-specific IgE responses (Dougherty, Brown et al. 1996; Zhang, Rogers et al. 1996). However, Akbari *et al* reported that NKT cells play an essential role in the development of ovalbumin-induced airway hyperreactivity (Akbari, Stock et al. 2003).

Overall, the immunology and pathogenesis of allergic responses associated with allergic responses is complicated and encompasses many immune cell types from both innate and adaptive immunity. As outlined above, there is a greater understanding of the immunological events that occur from the very early stages, including dendritic cells and NKT cells in addition to those events involving adaptive mechanisms further downstream responsible for sustaining the allergen-induced airway inflammation. It is only through this initial understanding of these cellular and humoral events that we can begin to discuss the "manipulation" of said immunological events and how these may be altered following exposure to various agents which could potentially prevent and/or change the course of allergic disease.

The Hygiene Hypothesis - Risk factors and the pliancy of allergic diseases to environmental factors.

Numerous risk factors in industrialised and urban living have been suggested to be potential origins of the allergy epidemic. One credible theory for the recent rise in allergic diseases that takes into account the changes in pathogen exposure, accounts for our evolutionary past and genetic predispositions, which dissects the pathogenesis of allergic diseases and proposes tangible mechanisms, is the hygiene hypothesis.

Genetic pre-dispositions

Asthma manifests from complex gene-by-environment interactions (Barnes 1999). Therefore, the allergy epidemic may possibly reflect an increase in susceptibility to allergic diseases, as well as changes in environmental patterns. The hygiene hypothesis integrates epidemiology with genetics, recognising that gene-environment interactions can also influence disease. Although there may not have been enough time for genetic factors to change sufficiently to cause the current rise in allergies, epigenetic changes, as a result of environmental cues (Jaenisch and Bird 2003) may certainly contribute to the present epidemic.

Umetsu and co-workers unravelled an enticing scenario in which *Tim1*, a recently identified asthma susceptibility gene in mice, may provide evidence for a role of hepatitis A virus (HAV) and protection from allergic manifestations in humans. The gene product of Tim1 has been shown to regulate Th2 cell development and AHR in mice (McIntire, Umetsu et al. 2001) with polymorphisms in *Tim1* associated with varying development of AHR. The human homologue of Tim1 encodes for the receptor HAV. Thus, interaction of HAV with its receptor (hu*Tim1*) may directly modify the Th2 cell pool, inhibiting excessive Th2 cellular development and proliferation and prevent the development of allergic asthma (Umetsu, McIntire et al. 2002).

A single nucleotide polymorphism (SNP) in the CD14 promoter region, C-159T, associated with increased soluble CD14 (sCD14) and decreased total serum IgE (Baldini, Lohman et al. 1999; Hubacek, Rothe et al. 1999), was

initially proposed as an LPS-related mechanism regulating IgE levels and subsequent allergen sensitisation. However, elevated sCD14 has also been observed in the BAL fluid of asthmatic patients (Vercelli, Baldini et al. 2001) possibly enhancing allergic inflammation (Kusunoki, Nakahata et al. 2002). Thus, the complex interactions between CD14, LPS and IgE, although unresolved, highlight another gene-environment interaction, which may regulate allergic reactivity.

Finally, children born and raised on a farm have been reported to develop fewer allergies than non-farm dwelling children (described in more detail below). The protective effect of farm lifestyles, however, is only observed in children with a T allele for TLR2/-16934, with those children homozygous for the A allele displaying similar levels of atopy and allergic responsiveness as non-farm dwelling children (Waser, Schierl et al. 2004). Thus, exposure to a farm environment may modify an individual's genotype enough to alter their susceptibility to allergies. These studies help to identify candidate genes for allergy and the roles they play in the immunological development of allergic inflammation.

Environmental Risk Factors

Environmental exposures or lifestyle changes, such as diet and nutrition, have changed dramatically over the past several decades, with more processed and sterile packaged foods available. Consequences of diet changes are vast. The 'Barker hypothesis'- which associates foetal programming, particularly undernourished development, with chronic diseases (Barker 1998) may dissect the allergy epidemic. Indeed, children with lower birth weights and smoking parents are at a greater risk of developing asthma (Magnus, Berg et al. 1985). However, enhanced maternal nutrition has also been described as a risk factor (Beasley, Crane et al. 2000). Thus nutritional status may only bear relevance in combination with other risk factors such as sterile food use and poor diets (Sears 1997).

Atmospheric changes have also been profound. Assaulting the airways with tobacco smoke during pregnancy or exposing infants to smoke increases wheezing and bronchial hyperreactivity during childhood (Chilmonczyk, Salmun et al. 1993; Stein, Holberg et al. 1999). Experimental systems addressing smoke exposure have ascribed adjuvant-like properties to tobacco smoke (Seymour, Pinkerton et al. 1997; Rumold, Jyrala et al. 2001) and diesel exhaust particles (Diaz-Sanchez, Tsien et al. 1997), enhancing specific-IgE production. Reports of lower rates of asthma amongst smokers (Beasley 1998) are most likely the "healthy smoker syndrome"- the tendency of those with asthma not to smoke, rather than any actual protection from allergy. Therefore, smoking and carbon-based air pollutants may certainly exacerbate existing airway allergy but there is no evidence that smoking or pollution creates new cases. Increased allergen release from pollen grains and longer-lasting pollen counts by some, but not all (Behrendt and Becker 2001), air pollutants may represent changes in the composition of allergens encountered, resulting in increased exposure. However, air pollution can not solely account for the rise in prevalence, as regions such as China and Eastern Europe, where some of the highest levels of particulate matter and SO_2 are found (Jones 1998), have lower allergy rates than less polluted regions such as North America, New Zealand and the UK (Beasley 1998; ISAACSubject 1998).

We are exposed to more indoor allergens, such as HDM and mould spores, than in past decades, following the desire to insulate homes with wall-to-wall carpeting and installing central heating systems. Increased allergen exposure during the pollen season or after thunderstorms (Jarvis and Burney 1998) results in bouts of patients presenting allergy-related symptoms. However, the annual pattern of allergen release, at least in Japan, has not changed significantly (Sato, Nakazawa et al. 1997). More contentiously, pet ownership has also increased (Custovic, Simpson et al. 2003), contributing to increased allergen levels (Wahn, Lau et al. 1997). Interestingly, pet ownership may pose as a protective factor rather than a risk factor for some individuals. This will be discussed below.

Therefore, increased allergen exposure is unquestionably the biggest risk factor faced for allergic patients, however the effects of increased exposure on new allergy cases remains to be elucidated.

The paradoxical effect of dog and cat ownership (a major source of allergens themselves) protecting from allergic diseases has been highlighted by Thomas Platts-Mills and others (Platts-Mills 2002). Reports of exposure to domestic cats and dogs during infancy (Hesselmar, Aberg et al. 1999; Roost, Kunzli et al. 1999) and preteen years (Perzanowski, Ronmark et al. 2002) providing protection from allergic diseases and allergen sensitisations (Ownby, Johnson et al. 2002) added another dimension to the hygiene hypothesis. Individuals exposed to animals, whether farm animals or domestic cats and dogs, are exposed to significantly more environmental endotoxins (Platts-Mills 2002), suggesting that similar immunological mechanisms, such as Th1 immune deviation, govern animal-exposed-protection from allergy. However, the fact that cats and dogs are major sources of allergens, Fel d1, and Can f1, respectively, suggests multifaceted mechanisms. Indeed, children who did not respond to Fel d1, with skin prick tests had significant levels of Fel d1-specifc-IgG4 (Platts-Mills, Vaughan et al. 2001) and IL-10 secretions from peripheral T-cells (Reefer, Carneiro et al. 2004). As IgG4 production requires IL-4, similar to IgE, the occurrence of IgG4 and IL-10 without IgE has been described as a 'Modified Th2' response (Platts-Mills, Vaughan et al. 2001). Furthermore, exposure to cats can also prevent sensitisations to other allergens such as dog allergens (Custovic, Simpson et al. 2003). Recent studies have found further evidence of a modified Th2 response, with higher IL-10 and IL-13 cytokine secretion profiles after 1 year, and reduced allergic sensitization and atopic dermatitis in children exposed to dogs during the first year of life (Gern, Reardon et al. 2004). Polymorphisms in genes encoding CD14 were also observed in this study, however, which may have confounding or additive protection from allergy when combined with dog exposure. The 'Modified Th2 response' can conceivably provide protection from allergic reactivity by competing for allergen binding, IgG4 vs. IgE of similar specificities, and also by suppressing T-cell proliferation via IL-10, however the exact mechanism is still unresolved.

Intracellular bacterial infections and polarized Th1 immunity: Chlamydia

Some of the largest challenges encountered for the treatment and/or vaccination against allergic disease is identifying those agents which may induce an immunological response which could prevent &/or inhibit ongoing allergic responses. One area of study which has been focused upon over the last decade is the deviation of the allergic response away from a Th2 bias. Apart from attempting to exploit different regulatory mechanisms as described above, one major focus has been an attempt to enhance the Th1 arm of the immune response in the hopes of deviating away from a Th2 allergic response. Prior exposure to various agents which elicit a Th1 immune response have been studied, particularly in the context of exposure to various bacterial agents which a Th1-type response. In our previous and ongoing studies, one agent which has been a focus is that of Chlamydia infection. The development of an effective vaccine has been a long-term goal for controlling diseases cause by Chlamydial infection including trachoma (blindness caused by *Chlamydia trachomatis* infection) as well as sexually transmitted chlamydial infection and for preventing the sequelae of these diseases that also include infertility and ectopic pregnancy. As a result of more intense studies aimed towards new vaccine strategies against this intracellular bacterial infection, much knowledge has emerged regarding this organism and how the immune response against it could serve as a useful tool in re-directing a Th2-biased immune response to allergen. Many vaccine-based studies have been tested in order to understand this response, including, but not limited to, immunization with whole organisms, membrane proteins, and synthetic peptides. In the following paragraphs, a more detailed description of chlamydial immunobiology as well as the overall response, which makes it a good tool to study the effect of previous bacterial infection on the impact of the allergic response.

Recent immunoepidemiological studies of trachoma demonstrated the extreme importance of cell-mediated immunity in human defence against chlamydial infection. In particular, Mabey and colleagues (Mabey and Bailey 1996, Bailey, 1995 #204, Holland, 1993 #205) found that patients with

conjunctival scarring caused by trachoma had markedly depressed lymphocyte proliferation to chlamydial antigens compared with control individuals without scarring. Individuals with trachoma more frequently had chlamydial heat shock protein 60 (hsp60) antigen-specific IL-4 secreting lymphocytes and less frequently had chlamydial major outer membrane protein (MOMP) antigen-specific IFN-γ-producing lymphocytes. Similarly, Bobo *et al* (Bobo, Novak et al. 1996) reported that individuals with scarring trachoma tended to have IFN-γ and IL-12 gene transcripts less often than non-scarred individuals. Moreover, they also reported that the ocular secretions of scarring trachoma individuals exhibited significant increases in TGF-β, a cytokine known to shift the type of antigen-presenting cells and results in T-helper 2 (Th2) polarized CD4 T cell responses (King, Davies et al. 1998). More recent animal experimental studies, especially those using gene knockout mice, have focused on the (T helper type 1) Th1-Th2 paradigm for chlamydia immunity versus immunopathology. These studies confirm the role of Th1-like CD4 T cells in protection against chlamydial infection.

Gene knockout mice represent the most popular and effective experimental approach in studies of chlamydial immunobiology. The advantage of this approach is that it allows direct examination of the role of a particular cell population or molecule in a complex biological or physiological process. Studies using knockout mice demonstrated the predominant role of $CD4^{+}\alpha\beta$ T cells for host defence against chlamydial infection. Yang *et al* (Yang, Hayglass et al. 1998) reported that T-cell-deficient mice, similar to T and B cell (recombinase activating gene; $RAG^{-/-}$)-deficient mice, had higher mortality and chlamydial *in vivo* growth rates compared with wild-type control mice after lung infection with *Chlamydia trachomatis* mouse pneumonitis (*Chlamydia muridarum*). They also demonstrated impaired delayed-type hypersensitivity (DTH) and IFN-γ production in αβ T cell knockout mice. In contrast, γδ T cell knockout mice had intact DTH responses and their mortality and chlamydial growth rates were comparable to those of wild-type mice. This study confirmed the finding by Perry *et al* (Perry, Feilzer et al. 1997) that showed similar microbiological kinetics during genital infection in αβ T cell knockout and wild-type mice although the results differed slightly from those of Williams

et al (Williams, Grubbs et al. 1996), who showed transient higher chlamydial growth in γδ T cell knockout mice in the early stages of infection. In studies of T cell subsets, Johansson *et al* (Johansson, Schon et al. 1997) reported that CD4 for but CD8 knockout mice exhibited significantly higher chlamydial shedding from the genital tract compared with wild-type control mice after intravaginal infection with a human *C. trachomatis* strain (serovar D). The results suggested that CD4, but not CD8, T cells are critical for protection against chlamydial genital tract infection. Moreover, Williams *et al* (Williams, Grubbs et al. 1997) found that the role of $CD4^+$ T cells in host defence against chlamydial infection is one that can not be replaced by alternative compensatory mechanisms. Studies of B cell knockout mice have yielded inconsistent results when the lung and genital infection models were independently tested. Studies examining genital tract *Chlamydia muridarum* infection using B cell knockout mice carried out by Su *et al* (Su, Feilzer et al. 1997) and Johansson *et al* (Johansson, Ward et al. 1997) failed to show any protective role played by B cells. In contrast, Yang and Brunham (Yang and Brunham 1998) reported significantly higher mortality and *in vivo* chlamydial growth rates in B cell KO mice compared with wild-type mice after *Chlamydia muridarum* lung infection. Moreover, the study by Yang and Brunham suggested that B cells play a role in initiating T cell responses to chlamydial infection.

Cytokine KO mice are also widely used in chlamydial research, and the results have emphasized the importance of Th1-related cytokines in host defence against chlamydial infection. Johansson *et al* (Johansson, Schon et al. 1997) demonstrated that IFN-γ KO, but not IL-4 KO, mice failed to resolve *C. trachomatis* serovar D genital infection. They also found that IL-12 KO and IFN-γ receptor (IFN-γR) KO mice showed impaired clearance of genital infection and that IFN-γR KO mice failed to resist re-infection after antibiotic treatment-induced resolution of primary infection. Cotter *et al* (Cotter, Ramsey et al. 1997) found that IFN-γ KO mice vaginally infected with *Chlamydia muridarum* suffered disseminated chlamydial infection.

IL-6 is a cytokine that has drawn a great deal of attention in the field of host defence, especially after Ramsay *et al* (Ramsay, Husband et al. 1994)

reported that IL-6 KO mice failed to produce secretory IgA after mucosal immunization with recombinant virus. Studies carried out by Perry *et al* (Perry, Feilzer et al. 1998), however, showed that IL-6 knockout mice had the kinetics of chlamydial shedding and a course of infection similar to that of wild-type mice. Serum and mucosal antibody production including mucosal IgA in IL-6 KO mice was nearly identical to that of wild-type mice. Williams *et al* (Williams, Grubbs et al. 1998) demonstrated slightly different findings using the lung infection model. They found significantly increased mortality and increased chlamydial *in vivo* growth in IL-6 KO mice compared with wild-type control mice. IL-6 KO mice, however, eventually showed levels of cell-mediated and antibody responses similar to those of wild-type mice.

Overall, it is obvious that infection with Chlamydia, both in mice and in humans, yields a Th1-biased immune response. As mentioned above, the mechanisms through which the immune response attempts to clear the infection oppose those mechanisms exhibited by the Th2 bias of allergic airway inflammatory reactions to allergens. As the Hygiene Hypothesis suggests, previous exposure to bacteria/bacterial products which elicit a Th1 type Immune response may reduce and therefore influence the outcome to allergens. Using the outlined knowledge of the mechanisms initiated by an intracellular bacterial infection such as Chlamydia in both human and animal models, this may serve as a useful tool in order to deliver a Th1-inducing infectious agent and a means to test the Hygiene Hypothesis at the experimental level. Another prominent intracellular bacterial infection with much epidemiological impact on the Hygiene Hypothesis, namely *Mycobacterium bovis* BCG, is also considered below.

Intracellular bacterial infections and polarized Th1 immunity: Mycobacteria

As mentioned above, immunoglobulin-E (IgE)-mediated allergic disorders, in particular allergic asthma, are mediated primarily by Th2 lymphocytes. Th1 and Th2 cells are reciprocally regulate each other through the cytokines they secrete. For example, IFN-γ and IL-12 inhibit Th2 cells and IL-4 and IL-10 down-regulate the activity of Th1 cells (Romagnani 1994, Mocci, 1995).

Association of allergic disorders with the Th2 cytokine patterns raises the possibility to design prophylactic and therapeutic modalities in a way that would promote a Th1 immune response and thereby down-regulate the Th2 immune response (Mocci and Coffman 1995, Holt, 1994). Humans are initially exposed to environmental allergens during infancy or early childhood and there is a growing consensus that high-level allergen exposure during the first few months of life predisposes to allergic sensitization, which manifests in later childhood as Th2 reactivity. Early childhood may be considered also as an opportunity to regulate the development of normal anti-allergen immunity in unsensitized children.

Mycobacterium species, particularly bacilli Calmette-Guerin (BCG) used as a vaccine to prevent human tuberculosis, are known to be potent inducers of Th1 responses (Mosmann and Sad 1996, Del Prete, 1991). *M. tuberculosis,* in particular, is a facultative intracellular pathogen that survives and grows primarily within macrophages in the host. In both mice and humans, the immune response has difficulty eliminating a *M. tuberculosis* infection, resulting in the establishment of a latent infection in humans and a chronic disease state in mice. Similar to other intracellular pathogens, immunity to *M. tuberculosis* is cell-mediated. Murine studies using *in vivo* antibody depletion, gene-disrupted mice, or adoptive transfer indicate that $CD4^+$ T cells are key to the control of infection. Mice with disruptions in the genes for β_2-microglobulin, TAP, or CD8 are more susceptible to *M. tuberculosis* than wild-type mice, also implicating a role for $CD8^+$ T cells and the MHC Class I pathway. Both $CD4^+$ and $CD8^+$ T cells have been shown to produce IFN-γ during an *M. tuberculosis* infection. In experimental models, IL-12 has a central role in regulating Th1 T cells production of IFN-γ, and IFN-γ is clearly key in the activation of macrophages. These findings, along with the extreme susceptibility of people and mice with disruptions in the IFN-γ or p40 gene to *M. tuberculosis* infection, has led to proposed use of IFN-γ as a correlate of protection for new vaccines against tuberculosis.

With regards to the use of this knowledge of the immune response against Mycobacteria, recent studies in mice have demonstrated that mycobacterial infections (Herz, Gerhold et al. 1998, Tukenmez, 1999, Yang, 1999) prior to

allergen sensitization inhibit Th2 immune responses to the allergen. These studies concluded that this is predominantly due to the skewing of the immune response away from a Th2 allergic response to a more Th1 bias, and hence, a less allergic reaction. Wang and Rook (Wang and Rook 1998) have also reported that a single injection of killed *Mycobacterium vaccae* into allergen-pre-sensitized mice suppressed serum IgE and allergen-specific IL-5 synthesis.

In light of the outlined immune response induce by Mycobacteria infection as well as the studies which have pioneered an insight into the effects of previous inoculation with these bacteria on the outcome of the allergic response in mouse models, further mechanistic studies are required in order to examine how this inhibition of allergy as a result of infection occurs. Specifically, in addition to Chlamydia infection, much knowledge has been acquired regarding the immune response to a prominent intracellular bacterial infection such as Mycobacteria for the purpose of vaccine studies. This same information is useful in examining the effect of previous intracellular bacterial infection on the outcome of a model of allergic airway inflammation. Both Chlamydia and Mycobacteria could be used to investigate this question as well as further deduction of particular cell types involved in the infection-induced inhibition of allergic disease.

The Influence of Infection on Allergic Disease

In search of an environmental aetiology of the allergy epidemic, the decline in common infectious diseases, highlighted by the inverse relationship between the prevalence of infectious pathogens and that of allergic disease (Fig 1.1) (Shirakawa, Enomoto et al. 1997; Matricardi and Bonini 2000; Matricardi and Bonini 2000; Bach 2002) is one of the most attractive. Conceivably, if changes in our lifestyle with the introduction of air pollutants, smoking, changes in diet and increased allergen exposure may affect the incidence of asthma, then the inverse is also plausible, namely the loss of other protective environmental exposures.

It is generally thought that diseases reduce, rather than increase, our reproductive fitness. However, the hygiene hypothesis approaches this

concept from the reverse, assessing our immunologically burdened past and appraising the benefit rather than the burden of infection.

Hygiene Hypothesis

Increased allergic diseases accompanied with reduced childhood infections through vaccination programmes, antibiotic development and general improved hygiene, contrast with a lower prevalence of allergic diseases in areas where childhood infections are more common (Strachan 1989; Wills-Karp, Santeliz et al. 2001; Yazdanbakhsh, Kremsner et al. 2002; Romagnani 2004). These observations helped shape the early hygiene hypothesis.

Original observations almost 30 years ago, indicating that infections might protect from allergy, set the stage for later studies (Gerrard, Geddes et al. 1976). Despite higher levels of serum IgE (275.4 u/ml compared to 81.3 u/ml) with evidence of helminth, viral and bacterial infections, Métis residents of Saskatchewan, Canada, had relatively fewer cases of asthma, eczema and urticaria, compared to their relatively uninfected, causasian countrymen (Gerrard, Geddes et al. 1976). Helminth infected populations in the Gambia and Zimbabwe (formerly Rhodesia) with fewer allergies were reported during the mid 1970's (Godfrey 1975), with self experimentation using hookworm alleviating hay fever symptoms (Turton 1976). Thirteen years later, the hygiene hypothesis was coined from observations made by Strachan (Strachan 1989), relating large household sizes with reduced hay fever. Strachan proposed that increased transmission of viral infections between siblings could protect from the development of allergic rhinitis. Immunological interpretation of smaller family sizes and reduced bacterial and viral infection, as impaired Th1 responses and thus impaired counter regulation of Th2- pro-allergic responses, was the founding of the Th1-Th2 hygiene hypothesis (Rook and Stanford 1998; Matricardi, Rosmini et al. 2000).

Although stimulating much new thinking on allergy, this position did not account for the parallel increase in Th1-mediated autoimmune diseases during a similar time frame (Stene and Nafstad 2001; Bach 2002) and even

within the same individuals (Kero, Gissler et al. 2001; Simpson, Anderson et al. 2002). Moreover, in developed countries, Th2-inducing helminth infections (such as *Ascaris* and the once ubiquitous pinworm) diminished in prevalence alongside *Mycobacterium tuberculosis* and other Th1-driving pathogens (Mao, Sun et al. 2000; Gale 2002). Interestingly, the paradox emerged that allergies are less prevalent in helminth-infected populations, who show strong Th2 responses.

Newer studies, described below, imply that ongoing and chronic infections may be as important as early-life immunological imprinting in determining the mode of an immune response to allergenic substances. Furthermore, the spotlight is moving firmly into infection *per se*, rather than the Th1- or Th2-stimulating nature of different microbes, and the questions now centre more on the generic response to infection rather than the consequence of any one pathogen. In these ways, the hygiene hypothesis has evolved and is often discussed in the context of disordered immunoregulation, rather than a dysfunctional Th1/Th2 balance (Wills-Karp, Santeliz et al. 2001; Yazdanbakhsh, van den Biggelaar et al. 2001; Gale 2002; Umetsu, McIntire et al. 2002; Yazdanbakhsh, Kremsner et al. 2002)

Whether the hygiene hypothesis is posed in its original (imprinting) or later (regulatory) context, it has provoked a barrage of fascinating epidemiological studies. Traditional lifestyles associated with farming (Von Ehrenstein, Von Mutius et al. 2000; Leynaert, Neukirch et al. 2001; Riedler, Braun-Fahrlander et al. 2001) or anthroposophic communities, where a diet rich in lactobacilli and a limited use of antibiotics and vaccinations is common (Matricardi and Bonini 2000) appear to be protected from allergies. More akin with the developed world lifestyle, those attending childcare in early months of life (Kramer, Heinrich et al. 1999; Ball, Castro-Rodriguez et al. 2000) or from larger families (Matricardi, Franzinelli et al. 1998; Cooper, Chico et al. 2004; Stabell Benn, Wohlfahrt et al. 2004) also appear to be protected from allergies. Presumably these lifestyles and environments provide an arena where infections are transmitted more readily. Infectious burden in the developing world, due to problems of sanitation, access to clean drinking

water, inadequate nutrition and crowding, where allergies are less common, far out weigh that of the developed world, further suggest that infections may be protective. Indeed, migrants from Albania with relatively high levels of present or previous infection (covering *Toxoplasma gondii*, herpes simplex virus, hepatitis A virus (HAV) and *Helicobacter pylori*) and low allergic symptoms, manifest with time an increasing prevalence of sensitisation to local allergens (Ventura, Munno et al. 2004).

Thus, rather ironically, the medical endeavour to eradicate may of these infections and the lifestyle changes associated with more affluent living may have left us at greater risk of developing allergic diseases. However, 'unhygienic' living is not a simple resolution to alleviate allergic diseases, indeed, poor and unhygienic inner city dwellings have seen an increase in severe asthma cases (Platts-Mills 1997). Therefore, an understanding of specific bacterial, viral or helminth pathogens is required to piece together how particular pathogens and their products can protect us from allergies.

Viral Infections and Allergic disease

Immune responses to viral infections characteristically involve NK cells, $CD8^+$ T-cells and $CD4^+$ T-cells secreting IFN-γ and IL-12 resulting in a polarised Th1 profile. As described above the counter-regulatory capacity of Th1 responses can provide a possible mechanism of bacterial-induced protection and thus presumably viral protection from allergic disease. However, viral infections in relation to allergic disease, work both ways. Early childhood viral infections increase wheezing and airway irritation with positive associations with the development of allergic diseases, whereas later viral infections and positive serology from prospective studies indicate a negative association with allergic disease.

For example, children with evidence of respiratory infections, such respiratory syncytial virus (RSV) infections, bronchitis, or pneumonia, during infancy were found to have a higher risk of developing bronchial obstruction during the first 2 years of life and of having asthma at 4 years of age (Nafstad, Magnus et al. 2000; Sigurs, Bjarnason et al. 2000). Prospective studies conducted in

Australia (Ponsonby, Couper et al. 1999) and Germany (Illi, von Mutius et al. 2001) have also confirmed that respiratory tract infections during the first 6 weeks of life (Illi, von Mutius et al. 2001), or within the first year of life (Ponsonby, Couper et al. 1999), increase the risk of asthma. The most common form of virally-exacerbated asthma is caused by rhinovirus (RV). Although these viruses can induce asthma like responses themselves, most virally-related asthma symptoms are lost by the age of 13 (Herz, Lacy et al. 2000), suggesting that the viral-associated-asthmatic phenotype observed in childhood, may not actually lead to asthma in the long run.

Experimental models, however, support an exacerbation of airway allergy following viral infection; including increased allergen-specific IgE production and AHR (Suzuki, Suzuki et al. 1998) and even anaphylactic shock (O'Donnell and Openshaw 1998) with influenza A virus or RSV infection (Schwarze, Hamelmann et al. 1997; Barends, Boelen et al. 2002).

Culley and colleagues (2002) have elegantly shown that the timing of RSV infection in a juvenile mouse study can have significant effects on airway allergy, with later RSV infections inducing less viral-exacerbated-disease and heightened IFN-γ responses (Culley, Pollott et al. 2002).

The inflammatory response and tissue damage incurred during early respiratory viral infections may enhance allergen capture and sensitisation, contributing to allergy exacerbations (Holt and Sly 2002). In addition, allergic airway inflammation and viral respiratory infections both induce AHR (Folkerts, Walzl et al. 2000) and may synergise to enhance AHR and associated airway conditions (Makela, Kanehiro et al. 2002).

An allergy-like phenotype following viral infection can also be replicated in animal models. RSV and parainfluenza-3 (PI3) virus infections can recruit eosinophils and enhance IL-5 secretions (Schwarze, Hamelmann et al. 1997; Harrison, Bonville et al. 1999; Scheerens, Folkerts et al. 1999; Schwarze, Cieslewicz et al. 1999) and potentiate RANTES secretions from eosinophils (Lacy, Mahmudi-Azer et al. 1999). Influenza A viral infection, with increased IFN-γ production, also enhanced airway inflammation by promoting both

allergen-specific Th1 and Th2 responses, orchestrated, in part, by altered DC function (Dahl, Dabbagh et al. 2004). Thus, it is clear that respiratory viral infections can exacerbate airway allergy, through mechanical disruption, exacerbated Th2 cytokine secretions, DC manipulation and induction of AHR.

Evidence of gastrointestinal viral infections has, however, shown a stronger negative association with allergic diseases. For example, fewer allergic symptoms have been observed in Hepatitis A virus (HAV) seropositive Italian military students, compared to seronegative colleagues (Matricardi, Rosmini et al. 1997). As described above, a role of HAV and protection from allergic manifestations in humans is emerging with interaction of HAV with its receptor (hu*Tim1*) potentially modifying the Th2 cell pool and preventing the development of asthma (Umetsu, McIntire et al. 2002). Recent studies of German and Spanish students found an inverse association between Hepatitis B virus (HBV) seropositivity and the development of allergic rhinitis, however in this study no association was seen with Hepatitis A, *H. pylori* or herpes simplex virus-1 (Uter, Stock et al. 2003).

HIV-1 infection and the effects on allergic disease outcome are varied. It has recently been proposed that HIV-1 shed gp120 virions are allergenic (Becker 2004) and can induce IL-4, Th2 cell differentiation and IgE production. Furthermore, a deviated Th2 response can significantly limit the induction of Th1 cells and cytotoxic T cell (CTL) responses following anti-viral treatment. However, hypersensitivities to anti-viral drugs are more commonly discussed in HIV infected patients (Carr, Cooper et al. 1991; Dikeacou, Katsambas et al. 1993; Marshall 1999; Montero Mora, Suarez Nunez et al. 2004), with multiple drug sensitivities reported.

In conclusion, the association between viral infections and the development of asthma and allergic diseases is highly variable. Genotypic differences, viral species variation, timing of infection and the final infection niche are just some of the parameters to consider. Tacking a course through the sea of epidemiological and experimental literature has uncovered contradictions and diverging mechanistic explanations. However, despite the differences, there

still appears to be evidence for protective viral pathogens or particles in relation to allergic diseases.

The exact pathogens and mechanisms remain unresolved, with some of the strongest possibilities to date discussed above. Future studies require prospective, long-term birth cohort studies, taking into account immunological profiling combined with diagnostic methods capable of detecting all common viral infections (de Kluijver, Evertse et al. 2003; Zambrano, Carper et al. 2003; Murray, Simpson et al. 2004).

Helminth Infections and Allergic disease: modified Th2, IL-10 & IgG4

While the suppressive influence of mycobacteria, for example, has been well established in animal models of Th2-type allergic lung inflammation (Erb, Holloway et al. 1998; Wang and Rook 1998; Yang, Wang et al. 1999; Hopfenspirger, Parr et al. 2001; Zuany-Amorim, Manlius et al. 2002; Zuany-Amorim, Sawicka et al. 2002) studies on helminth parasites at this level have only recently been initiated. Nevertheless, recent data now provide experimental evidence for mitigation of allergic outcomes by helminth infections.

Among the first reports, Wang *et al* (Wang, Nolan et al. 2001) infected mice with two doses of the human parasite *Strongyloides stercoralis,* which produces a short-lived tissue infection. Mice sensitized to ovalbumin (OVA) shortly after the second infection showed greatly reduced allergen-specific IgE production. At the effector level, asthma-like responses can be measured in terms of cytokine secretion and leukocyte exodus into the bronchoalveolar lavage fluid (BALF) following intratracheal challenge with specific allergen. *S. stercoralis* infected mice showed reduced eotaxin secretion, but no overall change in pulmonary eosinophilia or the local recruitment of other lymphocytes in the BAL fluid.

Because *S. stercoralis* introduces an abortive and highly immunogenic infective episode, its effects may be less far-reaching than a long-lived chronic

infection; nevertheless these data show significant inhibition on components of both the sensitisation (IgE) and effector (eotaxin) phases.

Bashir *et al* (Bashir, Andersen et al. 2002) showed that allergic responses to a food allergen (from peanut) is down-modulated by infection with the gastrointestinal nematode *Heligmosomoides polygyrus*; both allergen-specific IgE and IL-13 were inhibited, but the whole effect was reversed in animals given anti-IL-10. This implicates a suppressive mechanism involving an immunoregulatory pathway. These results indicate ablation of both sensitisation and effector mechanisms. As discussed above, IL-13 is a crucial initiator of allergic responses in mice including airway mucus production, and IL-10 is pivotal in its inhibitory effects in experimental allergy.

Most recently, Wohlleben and colleagues (Wohlleben, Trujillo et al. 2004) showed that airway allergy, including airway eosinophilia, eotaxin levels and OVA-specific IgE and IgG1 in BALF, was suppressed with chronic (4 week and 8 week pre-challenge) but not acute (1 week and 2 week pre-challenge) *Nippostrongylus brasiliensis* infections, pre OVA challenge. This effect was attributed to IL-10, with increased $CD4^+$ $IL\text{-}10^+$ T-cells in BAL fluid of chronically infected OVA-challenged mice. IL-10 deficient animals showed no signs of protection with infection. This is the first experimental study to support a regulatory mechanism generated during helminth infection, with the capacity to down regulate airway allergy.

One study has gone so far as to demonstrate that helminth products can modulate allergic disease, without the presence of live infection. Inhibition of lung inflammation and hyper-responsiveness was observed following *Ascaris suum* extract implants (Lima, Perini et al. 2002). Suppressed IL-4, IL-5 and Eotaxin in the BALF may help to explain such an inhibitory effect, however IgE and IgG levels were also significantly decreased in both serum and BALF. Such experiments promise to quickly elucidate candidate modulators with potential to dampen allergies *in vivo*.

Importantly, not all experimental helminth infections modulate allergic disease. Under conditions of existing elevated IL-4 and IL-5 and consequent IgE and

localised eosinophilia, an incoming wave of helminth larvae migrating through the respiratory system may exacerbate airway hyperresponsiveness (Silveira, Nunes et al. 2002; Negrao-Correa, Silveira et al. 2003). As with the human situation, a key distinction may lie between acute and chronic helminth infections, and the extent to which the allergic state pre-exists at the time of parasite invasion.

The 'modified Th2' response is associated with elevated IL-10 and IgG4 responses and could be regarded as a form of tolerance (Platts-Mills, Vaughan et al. 2001). Chronic parasite diseases such as Bancroftian (Hussain, Poindexter et al. 1992) and Brugian (Kurniawan, Yazdanbakhsh et al. 1993) filariasis, display a similar modified Th2 response. In these infections, extremely high levels of IgG4 predominate, a curious feature as this isotype of antibody is poorly cytophilic and is not associated with any effector responses. Moreover, filarial antigen-specific IgG4 has been shown to block IgE reactivity *in vitro* (Hussain, Poindexter et al. 1992).

The IL-10-regulated dichotomy between IgE and IgG4 production (Jeannin, Lecoanet et al. 1998) would, if replicated in a helminth infection, provide a link between the cellular and humoral mechanisms involved in chronic infection and allergy. Evidence that this may indeed be the case comes from recent studies in Ecuador in which children seropositive for IgG4 to *Ascaris* had a lower frequency of atopy to common allergens than those who were IgG4-negative (Cooper, Chico et al. 2003). Interestingly, in this study high total IgE was independently associated with protection from allergy, emphasising that multiple mechanisms of mitigating atopy may be at play.

One of the most direct models to explain helminth down-regulation of allergic responses is that infection results in both parasite-antigen-specific IgE, and very high levels of polyclonal IgE. The resulting elevation in total serum IgE would either, or both, dilute out allergen-specific IgE and saturate the FcεRI receptors on mast cells and basophils (Lynch, Goldblatt et al. 1999). For example, non-allergic children in Venezuela with high rates of helminth infection showed the highest levels of polyclonal IgE and relatively low levels of either parasite- or environmental allergen-specific IgE (Hagel, Lynch et al.

1993; Lynch, Hagel et al. 1993). Many other studies, however, do not reproduce these findings; for example, in the Gabonese study described earlier, there were no differences in polyclonal IgE levels between atopic and non-atopic *S. haematobium*-infected individuals (van den Biggelaar, van Ree et al. 2000). An important caveat is that the regulation of FcεRI receptor expression, and the absolute number of mast cells, are both influenced by IgE concentration (Kawakami and Galli 2002), calling into question whether mast cell FcεRI receptors on a systemic level can reach saturation even in individuals producing high levels of IgE. Furthermore, allergic airway hyperreactivity is intact in mice carrying a null mutation of Cε and are thus unable to produce IgE (Mehlhop, van de Rijn et al. 1997). A more general reservation is that regulation via saturation of FcεRI would operate only in allergy and could not explain how, for example, autoimmune reactivities are also down-regulated in parasite infection.

In a recent review of parasite-allergy interactions, Yazdanbakhsh and colleagues temptingly speculate, that a cross sensitisation may occur during chronic helminth infections, whereby sensitisation to parasites may induce clinically irrelevant cross-reaction to house dust mites, in a similar manner to cross-sensitisation between pollen and foods (Yazdanbakhsh, Kremsner et al. 2002). However this requires much work to identify potential cross-reactive epitopes within parasite antigens and allergens.

T Regulatory cells and alteration of allergic diseases

A model has been proposed that the increasing level of overt allergy is linked to under-development of general down-regulatory controls of the immune system, such as the regulatory T cell population (Wills-Karp, Santeliz et al. 2001; Yazdanbakhsh, van den Biggelaar et al. 2001; Herrick and Bottomly 2003). This revised hygiene hypothesis postulates that the protective effect observed with infections may be attributed to appropriate development or education of the immune system, arguably under the conditions for which it has optimally evolved. This hypothesis also takes into account the parallel

increase in autoimmune diseases, as a common consequence of an ill-balanced immune system and deficient regulatory population.

Several phenotypes of Treg cells have been described, based on their mode of action, generation, phenotype, function, location, surface receptor expression and antigen specificity (Battaglia, Blazar et al. 2002). Beyond Th1 vs. Th2 antagonism, a unifying feature of Treg phenotypes; ($CD4^+CD25^+$, $CD45RB^{lo}$ $CD4^+$ ($CD25^{+/-}$), T regulatory 1 (Tr1), Th3, $CD8^+$ T-reg, TCR $\gamma\delta^+$T cells, NKT cells) is their ability to regulate the magnitude of immune responses. In one form 'natural' Treg ($CD4^+CD25^+$) are induced before leaving the thymus and mature as essential components of the peripheral immune response, without which various spontaneous organ-specific autoimmune diseases ensue (Sakaguchi, Sakaguchi et al. 2001). In other guises, they mediate tolerance to extraneous specificities derived from commensal gut flora as well as food antigens.

Whether natural Treg cells are expanded or stimulated during infection or whether DCs or microbial products convert peripheral Th0 cells into Treg functioning cells is unknown. Coherence is emerging, with three commonly studied and described Treg phenotypes; Natural- $CD4^+CD25^+$ regulating self reactive T-cells in the periphery; Th3 cells, activated in the mucosal surface of the gut and secreting IL-10 and TGF-β and finally; IL-10 secreting Tr1 cells.

Evidence is accumulating to support the peripheral development of Treg cells similar to those of natural, thymic emigrant Treg cells (Apostolou, Sarukhan et al. 2002; Belkaid, Piccirillo et al. 2002; McGuirk, McCann et al. 2002). Treg function is associated with IL-10, TGF-β and CTLA-4. Whether these molecules are required for the generation of T-reg cells or simply involved in their function is not yet fully understood. Additional markers such as the surface receptors CD25 (IL-2Rα) and GITR, as well as the transcription factor Foxp3 (Fontenot, Gavin et al. 2003; Khattri, Cox et al. 2003) are now available to chart the development and activity of this cell population. Foxp3 may be a critical component of Treg cell differentiation, as its mutation or genetic dysfunction results in several autoimmune disorders in humans (IpEX/XLAAD syndrome) and mice (Scurfy)(Kasprowicz, Smallwood et al. 2003).

Furthermore, over expression of Foxp3 induces a Treg phenotype from cells that did not express a Treg phenotype capable of inhibiting autoimmune disease (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003; Khattri, Cox et al. 2003).

There is growing support for regulatory models of allergy, in which some form of T_{reg} population is at the centre of an anti-inflammatory network (Wills-Karp, Santeliz et al. 2001; Akbari, Freeman et al. 2002; Bach 2002; Herrick and Bottomly 2003; Umetsu, Akbari et al. 2003), with particularly strong evidence that T cells transfected with IL-10 or TGF-β, to confer regulatory function (Hansen, McIntire et al. 2000; Oh, Seroogy et al. 2002), can prevent allergic airway inflammation or autoimmune disease models (Kohm, Carpentier et al. 2002). Treg cells can suppress both Th1 (Kohm, Carpentier et al. 2002) and Th2 (Cottrez, Hurst et al. 2000) effector responses. However, cells with Treg markers from allergic patients show a deficiency in the ability to suppress effector responses (Bellinghausen, Klostermann et al. 2003; Cavani, Nasorri et al. 2003; Akdis, Verhagen et al. 2004; Grindebacke, Wing et al. 2004; Ling, Smith et al. 2004). This deficiency in Treg function and lack of control with respect to pro-allergic-Th2 responses, may be an important finding in relation to increasing allergies.

Strong evidence of Treg cells in allergy, derive from allergen-specific immunotherapy (SIT). Successful SIT for bee (Muller, Akdis et al. 1998) and wasp venom allergy (Pierkes, Bellinghausen et al. 1999) often results in T-cell hyporesponsiveness to allergen stimulation, accompanied with enhanced IL-10 secretions. IL-10 and TGF-β production appear to be essential components of successful treatment in humans (Akdis and Blaser 1999; Bellinghausen, Knop et al. 2001) and mice (Hall, Houghton et al. 2003; Vissers, van Esch et al. 2004). High dose Der p1 immunotherapy induced significant reductions of Der p1 specific IL-5, IL-13 and IFN-γ from T-cells with a paralleled increase in $CD4^+CD25^+$ derived IL-10 and TGF-β. Furthermore, Treg cells isolated following SIT have shown classical suppression of allergen-responsive effector T-cells in vitro (Jutel, Akdis et al. 2003). SIT is also associated with an increase in IgG4 with a decrease in IgE production

(Pierkes, Bellinghausen et al. 1999; van Neerven, Wikborg et al. 1999) comparable to a 'modified Th2' response described with natural exposure to high levels of cat or dog allergen. IL-10 treatment of B-cells from individuals hyperimmune to phospholipase A, increases phospholipase A-specific IgG4 responses and decreases IgE levels (Muller, Akdis et al. 1998), supporting the work of Jeannin (Jeannin, Lecoanet et al. 1998), with IL-10 promoting IgG4 and suppressing IgE (Punnonen, de Waal Malefyt et al. 1993).

Animal studies have identified Treg cells in allergic airway inflammation (Hadeiba and Locksley 2003; Jaffar, Sivakuru et al. 2004) and allowed the dissection of possible roles Treg cells may play. Using monoclonal populations of HA-specific-B cells and OVA-specific T-cells, de Lafaille (de Lafaille, Muriglan et al. 2001) showed that a single immunisation with a cross-linked OVA-HA antigen induced a hyper IgE state, which could be prevented with the infusion of Treg cells.

CTLA-4 is actively involved in down-regulating T cell activation and maintaining lymphocyte homeostasis and has been associated with Treg function (Vasu, Prabhakar et al. 2004). Blocking CTLA-4 *in vivo*, with several injections of anti-CTLA-4 mAb, Hellings and colleagues (Hellings, Vandenberghe et al. 2002), were able to show an exacerbated allergy phenotype, suggesting that Tregs use CTLA-4 to regulate airway allergy.

OVA-specific Tr1 cells can suppress Th2 cell proliferation and cytokine secretion (Cottrez, Hurst et al. 2000). Akbari and colleagues (Akbari, Freeman et al. 2002) demonstrated that mature pulmonary DC's exposed to allergen can induce IL-10 secreting Tr1 cells, which can significantly suppress allergen-induced airway reactivity. Recent studies from this group have further identified that Tr cells, derived from naïve $CD4^+CD25^-$ T-cells, which express $Foxp3^+$ and $IL\text{-}10^+$ T-cells, can inhibit AHR.

Interestingly, these Treg cells cells also expressed Tbet and IFN-γ, indicating that they are either related to, or contain contaminant, Th1 cells (Stock, Akbari et al. 2004). The dependence of ICOS ligation in Treg cell activity has also been demonstrated in a murine model of diabetes, with blockade of ICOS

progressing insulitis to diabetes (Herman, Freeman et al. 2004). Disease progression was associated with a shift in the balance between Treg and T-effector cells.

In summary, a deficiency of Treg cells, or Treg cell function and development, is a feature of human allergy and has been confirmed in experimental settings. Moreover, allergy can be suppressed, through restoration of the Treg compartment, through successful immunotherapy in humans or adoptive transfer experiments in animal model systems.

Bacterial infections and Allergic disease

Bacteria and bacterial products have received much attention for their allergy-preventing effects, presumably due to the wealth of stimuli for the developing immune system and our close association with commensal gut flora. A further extension of the hygiene hypothesis indicated that antibiotic 'over-use' has resulted in a general reduction of bacterial colonisation and bacterial turnover rate, conditions observed in developed countries, and may have removed the beneficial effects of these 'old bacterial friends' (Rook and Stanford 1998; Bjorksten, Sepp et al. 2001). Indeed, a positive correlation with increased antibiotic use and allergic reactivity can be observed, at the epidemiological level (Farooqi and Hopkin 1998; Wickens, Pearce et al. 1999).

Approximately a third of the developing world carry *Mycobacterium tuberculosis*, however 90-95% do not develop disease, presumably due to efficient anti-bacterial responses of a Th1 nature (Rook and Stanford 1998). Japanese school children have indeed shown an inverse association between reactivity to tuberculin and allergic responsiveness, with greater Th1:Th2 cytokine ratios (Shirakawa, Enomoto et al. 1997). Vaccinations with Bacilli Calmette-Guérin (BCG), resulting in tuberculin conversion, reduces allergic symptoms in atopic individuals (Shirakawa, Enomoto et al. 1997) and significantly reduces the development of allergic diseases (Romagnani 2004). However, several studies contest the allergy-protecting nature of BCG

vaccination, indicating that vaccination per se does not prevent the development of allergy and asthma (Alm, Lilja et al. 1998).

Bjorksten proposed that bacteria colonising the gastrointestinal tract provide persistent pressure on the developing immune system, in a manner protective to the host (Bjorksten, Sepp et al. 2001). A model involving the mucosal associated lymphoid tissue (MALT) network linking the gastric MALT (GALT) and bronchial MALT (BALT) allowing T cells to migrate between the two MALT sites, has been proposed.

Prospective studies found atopic patients had lower Bifidobacterium colonisation relative to allergy-free patients (Watanabe, Narisawa et al. 2003). Other colonising bacteria, however, measured by seropositivity were associated with a higher prevalence of allergic disease (Linneberg, Ostergaard et al. 2003), suggesting that only specific bacterial exposures harbour anti-allergy properties with the balance of protective:pathogenic explaining the variable epidemiological data. Attempts to restore or modulate bacteria colonisation with probiotics have uncovered some encouraging results with reduced allergic symptoms following lyophilised Bifidobacteria treatment (Hattori, Yamamoto et al. 2003) and Lactobacillus ingestion (Kalliomaki, Salminen et al. 2001), presumably shifting the balance towards the protective species.

Endotoxins, such as lipopolysacharides (LPS) comprised of an antigenic O-specific side chain, a core oligosacharide and a toxic lipid component, are derived from the outer-membrane of Gram-negative bacteria and are a major source of bacterial stimulus. Lower levels of endotoxin exposure, resulting in reduced IFN-γ secretions from peripheral T-cells, have been associated with increased skin reactivity to house dust mite (Gereda, Leung et al. 2000) and Alternaria, a common fungal allergen in Arizona (Martinez and Holt 1999). Endotoxin levels in unpasteurised milk and exposure patterns of pregnant mothers and newborns may be responsible for the observed protection from allergy seen in children raised on farms (Leynaert, Neukirch et al. 2001; Waser, Schierl et al. 2004).

Endotoxins can trigger significant IL-12 secretions from DC's and Th2 cells, upon ligation with its receptor, CD14 (Triantafilou and Triantafilou 2002). As described earlier, the potential gene-environment interactions with LPS and CD14, extend the protective effect of LPS exposure. Furthermore, it was also reported that BCG vaccination early in infancy might prevent the development of atopy in African children (Aaby, Shaheen et al. 2000). A multicentre study including 23 countries in Europe as well as the USA, Canada, Australia, and New Zealand demonstrated that the increase in the tuberculosis notification rates was significantly inversely associated with the prevalence of wheezing, asthma or allergic rhinoconjunctivitis (von Mutius, Pearce et al. 2000). A lower prevalence of asthma was also found in Finnish women who had tuberculosis before the age of 20 (von Hertzen, Klaukka et al. 1999). Taken together, these findings suggest that the exposure to live mycobacteria helps in reducing the risk of developing allergic disorders. However, susceptibility to mycobacterial disease or atopy may be mutually exclusive. The finding that asthmatic BCG-vaccinated children developed smaller BCG scars or weaker tuberculin reactions compared to nonasthmatics (Sarinho, Schor et al. 2000) supports this view. Furthermore, other studies have found no protective effect of BCG vaccination on the development of allergic disorders (Alm, Lilja et al. 1997), (Yilmaz, Bingol et al. 2000), (Omenaas, Jentoft et al. 2000), (Wong, Hui et al. 2001), (Anderson, Poloniecki et al. 2001), (Jang and Son 2002), (Jentoft, Omenaas et al. 2002). Differences in the BCG strain and doses used for vaccination may account for the conflicting results as they exhibit variable immunogenic properties.

Model Systems of Bacterial Infection and Allergic Airway Inflammation

Model systems offer great opportunities to dissect the interplay between pathogens and allergic disease. These data focus on experimental models addressing the question if infections with bacteria or the exposure to bacterial products can inhibit the development of atopic disorders and the possible mechanisms mediating this effect.

Many experimental models have investigated the effect of mycobacterial infection and the subsequent development of allergic disorders. One of the hallmarks of infections with mycobacteria (in particular *Mycobacterium*

tuberculosis and *Mycobacterium bovis*) is the induction of profound Th1 responses, with high levels of IFN-γ secreted predominantly by $CD4^+$ T cells. As IFN-γ has been shown to have inhibitory effects on Th2 responses, many authors have speculated that infections with mycobacteria may protect humans from developing allergies.

In contrast to epidemiological studies, animal experiments and various models have shown more conclusive results and further support for the inverse relationship between the exposure to infection and subsequent reduction in the allergic phenotype, especially with respect to the influence of mycobacterial infection on the development of atopy. Additionally, Nahori et al and Hunt el al demonstrated the protective effects of *Mycobacterium bovis* BCG or *Mycobacterium vaccae*, respectively, on the development of allergic inflammation in addition to bronchial hyperrresponsiveness (BHR). Mice vaccinated as newborns did not develpop BHR or bronchial hypereosinophilia (Nahori, Lagranderie et al. 2001, Hunt, 2005). As well, increased levels of IFN-γ after *in vitro* stimulation of tracheo-bronchial lymph node cells accompanied this blockade of BHR. *Mycobacterium vaccae*, a rapid-growing soil organism, was shown by Wang and Rook (Wang and Rook 1998) to inhibit not only the production of allergen-specific IgE but also OVA-induced IL-5 production by spleen cells (Wang and Rook 1998) Numerous studies have also demonstrated that infection with live BCG prevents airway eosinophilia, the development of airway hyper-reactivity (AHR) and induces a partial reduction of allergen-specific IgE/IgG1 serum antibodies in the models of both ovalbumin (OVA)-induced allergic disease in mice, rats and guinea pigs (Yang, Fan et al. 2002), (Erb, Holloway et al. 1998), (Koh, Choi et al. 2001), (Su, Peng et al. 2001), (Hopfenspirger, Parr et al. 2001). A reduction in the levels of Th2 cytokines detected in draining lymph node and spleen cell cultures or bronchoalveolar lavages (BAL) is frequently associated with these findings. Interestingly, it appears that bacteria do not necessarily have to be live to prevent allergic responses, as killed BCG also showed a protective effect (Major, Wohlleben et al. 2002). Nahori et al reported that even in newborn Th2-susceptible hyper-IgE BP2 mice, vaccination with live BCG 16 weeks prior to OVA challenge inhibited the bronchial hypereosinophilia and

AHR and reduced IgE levels (Nahori, Lagranderie et al. 2001). Other studies have also shown decreased levels of total serum IgE in Th2-predisposed brown Norway rats treated with live BCG simultaneously with OVA sensitization, although there was no difference in the pulmonary eosinophilia in comparison to non-treated OVA-immunized rats (Hylkema, Timens et al. 2002). The results obtained using OVA as the model allergen have been more recently confirmed using the mite allergen *Dermatophagoides pteronyssinus* group 2 (Der p2) in mice. A subcutaneous injection of live BCG down-regulated IgG1 levels in the serum and decreased airway inflammation in animals sensitized with Der p2 (Tsai, Liu et al. 2002).

These observations raise the question: do bacteria and their products protect from allergy by simply deviating responses towards a Th1 nature?

Bacterial products harbour some of the most potent Th1-inducing immunogens, acting via TLRs on DC's (Matricardi and Bonini 2000; Akdis, Kussebi et al. 2003). Such bacterial encounter during allergen sensitisation and T-cell priming may be enough to deviate responses away from a pro-allergic Th2 response (Matricardi and Bonini 2000). Active bacterial infections with Mycobacterium (Yang, Wang et al. 1999; Walzl, Humphreys et al. 2003), *Chlamydia muridarum* (Bilenki, Wang et al. 2002) or M*ycoplasm pneumoniae* (Chu, Honour et al. 2003), inducing a systemic Th1 response prior to allergen sensitisation, reduce subsequent allergic airway inflammation in animal models, suggesting that an 'educated' or polarised immune response protects from allergy. Supporting this notion, crude endotoxin or LPS (Tulic, Knight et al. 2001; Gerhold, Blumchen et al. 2002), bacterial DNA containing many CpG motifs (Kim, Kwon et al. 2004) or a bacterial-derived lipopeptide exposure, prevented subsequent sensitisations (Tulic, Wale et al. 2000), IgE responses and airway eosinophilia (Erb, Holloway et al. 1998; Hopfenspirger and Agrawal 2002). Similarly, the inhibition of airway allergy has been observed following treatment with killed *Listeria monocytogenes*, *M. vaccae* or *Lactobacillus plantarum*, implying that live bacteria and active infections are not necessary, but rather signature molecules or potent Th1 inducing agents are sufficient (Wang and Rook 1998; Herz, Lacy et al. 2000; Zuany-Amorim, Manlius et al. 2002). Animal studies have conclusively shown that in wild type,

but not IFN-γ receptor deficient mice, Th1 clones can suppress Th2 clones when co-transferred, inhibiting airway eosinophilia upon airway challenge (Cohn, Homer et al. 1999). Presumably, bacterial infections may protect from allergy in a similar manner, by deviating immune responses towards a Th1 profile, fitting with the original hygiene hypothesis.

Bacterial infections, however, invoke more than an anti-bacterial Th1 response. An anti-inflammatory, regulatory response is also induced, with the capacity to regulate allergic reactivity. Treatment of mice with SRP299, a killed *Mycobacterium vaccae*-suspension, induced allergen-specific $CD4^+CD45RB^{low}$ regulatory T cells (Treg), capable of protecting recipient mice from allergen provoked airway inflammation, in an IL-10 and TGF-β dependent manner (Zuany-Amorim, Sawicka et al. 2002).

In addition to animal models that test the effects of previous mycobacterial infections on allergen reactivity, other bacterial species have also been shown to suppress the development of allergic responses. For example, infection with *Listeria monocytogenes* can suppress OVA-induced Th2 responses (Mizuki, Miura et al. 2001). This Gram-positive, facultative intracellular bacterium has the capacity to escape from the phagosome/endosome and replicate in the cytoplasm. This leads to the processing of listerial antigens through the MHC Class I pathway and the stimulation of specific $CD8^+$ T cells. In addition, *Listeria* also induces IL-12 production by macrophages. Interestingly, heat-killed *Listeria monocytogenes* (HKL) as an adjuvant can also inhibit the development of Th2 immune responses (Yeung, Gieni et al. 1998). This effect is dependent on IL-12. Hansen et al also showed that one subcutaneous application of HKL within the OVA-immunization period prevented the development of AHR in BALB/c mice and significantly reduced the airway hypereosinophilia and the mucus production (Hansen, Yeung et al. 2000). These findings were associated with a significant decrease in OVA-specific IgE and enhanced levels of OVA-specific IgG2a in serum. Lung lymph node cells taken from OVA-immunized mice injected with HKL also showed a reduced OVA-specific IL-4 production and greatly enhanced levels of OVA-specific IFN-γ after *in vitro* stimulation (Hansen, Yeung et al. 2000).

Neutralization of IL-12 or depletion of $CD8^+$ T cells with specific monoclonal antibodies restored OVA-induced AHR in the HKL-treated mice, suggesting that HKL induced a protective Th1 response. This is supported by increased IL-18 mRNA expression in popliteal LN cells. Taken together, these results suggest that live or HKL inhibit the development of allergic responses in mice by inducing the production of IL-12/IL-18 leading to the generation of $CD8^+$ T cells secreting IFN-γ.

In addition to Listeria, other prominent intracellular bacterial infection models have been demonstrated an inhibitory effect on allergen-induced allergic responses. Of note, to further examine the hygiene hypothesis, our group has previously shown that *Chlamydia trachomatis* mouse pneumonitis (*Chlamydia muridarum*), which normally causes murine respiratory tract infection and predominantly induces Th1-like responses IFN-γ/IL-12 production in C57BL/6 mice (Yang, HayGlass et al. 1996, Yang, 1998, Yang, 1993, Bilenki, 2002), can reduce airway inflammation. The results in our study showed that, in comparison with those mice treated with ragweed (RW) alone, a common human allergen which afflicts many seasonally allergic individuals, *Chlamydia muridarum*-infected, RW-treated mice showed significantly lower levels of RW-driven IL-4, IL-5, IL-10, IL-13, and eotaxin production and higher levels of IFN-γ/IL-12 production. Both bronchial mucus production and pulmonary eosinophilic infiltration, which were evident in mice with solely RW sensitization/challenge, were dramatically decreased in mice with previous *Chlamydia muridarum* infection. Surprisingly, the levels of serum total IgE were increased following RW sensitization/challenge in the mice with previous *Chlamydia muridarum* infection. Together, our previous data also provide evidence for the ability of another prominent intracellular bacterium, *C. muridarum* to abrogate the allergic airway inflammation induced by a natural common allergen, ragweed.

Not only intracellular bacteria, such as mycobacteria, *Listeria* and Chlamydia, mediate suppressive effects on allergic Th2 responses, probiotic bacteria have also been associated with the improvement of atopic diseases. Probiotics are cultures of bacteria derived from the commensal gut microflora. They have been shown to have several effects on the immune system, which

could potentially promote anti-allergic responses. Some of these bacteria are potent inductors of IL-12, promoting the generation of Th1 responses, which in turn down-regulate Th2 cell development (Murosaki, Yamamoto et al. 1998). In addition, they induce the production of TGF-β, an anti-inflammatory cytokine which plays a role in the effector functions of Tr cells and is also important for the induction of oral tolerance (Rautava, Kalliomaki et al. 2002). Furthermore, the application of probiotic bacteria has been shown to prevent the increase in intestinal permeability induced by food allergens. This could limit the access of these antigens to other organs thereby inhibiting the development of hypersentivity reactions. In a double-blind, randomized placebo-controlled trial, *Lactobacillus rhamnosus* given prenatally to mothers effectively decreased the incidence of atopic eczema in breast-fed children at high risk (Kalliomaki, Salminen et al. 2001). This treatment was also shown to ameliorate the intestinal inflammation in infants with cow milk allergy (Majamaa and Isolauri 1997). However, oral administration of *Lactobacillus sp.* could not alleviate the symptoms in adults with already established asthma or food allergy (Helin, Haahtela et al. 2002, {Wheeler, 2001). The experimental and epidemiological data reviewed above clearly indicate that bacterial infections can modulate the development of allergen-specific Th2 responses.

Innate Immunity and bacterial infections: Cells, receptors and signalling pathways.

Janeway and colleagues (Janeway 1989) coined the pioneering concept that innate immunity-associated cells recognize pathogens through pattern-recognition receptors (PRRs)- a set of evolutionary conserved proteins that mediate cell activation. With time, this definition was broadened and PRRs are now considered to include molecules that mediate opsonization, endocytosis, activation of complement and coagulation cascades, activation of inflammatory signalling pathways and/or induction of apoptosis (Janeway and Medzhitov 2002). PRRs directly recognize conserved microbial molecules, known as pathogen-associated molecular patterns (PAMPs), many of which are shared by different classes of pathogen.

Innate immunity is activated very quickly after infection or exposure to foreign antigen in the organism and precedes the development of acquired immunity. Innate immunity is the first of all an ancient system, present in most organisms. The receptors of the innate immune system recognize conserved structures present in a large group of pathogens, for example lipopolysaccharide (LPS) in all Gram-negative bacteria, while the receptors of the acquired system recognize single epitopes expressed on a single pathogen. The innate system includes killers (NKs), dendritic cells (DCs), macrophages, γδT lymphocytes, neutrophils, and B-1 cells.

Recently, considerable progress has been observed in the knowledge of receptors of the innate immune system. Several types of innate immunity receptors have been identified and the list is constantly expanding. The most important receptors belong to the family known as the Toll-like receptors (TLRs). The *toll* gene was first discovered in the early 1980s by the German researchers Nusslein-Volhard and Weischaus in the genome of *Drosophilia melanogaster*. This gene was responsible for dorsal-ventral development of the fly (Anderson 1998). Almost 10 years later, this gene was found to be responsible for antifungal resistance of the *Drosophila* (Lemaitre, Nicolas et al. 1996). Similar genes and gene products were recongized in other invertebrates; i.e. *Coenorhabdis elegant* and *Anopheles gambiae* (Blandin and Levashina 2004, Coutinho, 2003, Pujol, 2001), and in plants (Holt, Hubert et al. 2003, Jones, 2004) and mammals. The products of genes similar to *toll* were names Toll-like receptors. Similar structures were found in mice and humans. The intracellular domain of the IL-1 receptor was found to be identical to that of TLRs. TLRs are now considered the main receptors recognizing pathogen-associated molecular patterns (PAMPs). All the receptors have domain structures with leucine repetitions and all have the same intracellular domains and similar pathways of intracellular signalling (Kopp and Medzhitov 2003). The signalling molecules include: adaptor molecule, which is myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK), TNF receptor-associated factor (TRAF)6, mitogen-activated protein (MAP) kinases, and nuclear factor (NF)-κB. Further study,

however, revealed that the signalling pathway may not sometimes include all the same elements.

Ten human and nine mouse TLR-family members have been identified so far, which recognize various microbial molecules. TLRs might be expressed as homo- or heterodimers. The importance of these receptors in innate immunity is highlighted by their wide distribution of expression by innate immune cells and various resident tissue cells, including fibroblasts, epithelial cells and endothelial cells. TLR-expression profiles show a broad variation. So far, the significance of these variable TLR-expression profiles is unclear, but they point to specialized roles in host defence.

Each TLR binds to a different ligand, and some pathogen structures are recognized by a homodimer of one receptor, some of a heterodimer consisting of two different TLRs. TLRs were at first considered receptors recognizing only bacterial; later it was found that TLRs could recognize viruses and also play a role in anti-tumour immunity (Heil, Ahmad-Nejad et al. 2003, Hertzog, 2003, Kopp, 2003, Pitha, 2004, Underhill, 2002, Vaidya, 2003). Four TLRs are engaged in anti-viral immunity: 3, 4, 7, and 9, whereas TLR2, 4, 9, 5 as well as the combination of heterodimers recognize bacterial products such as peptidoglycan and the LPS of Gram-negative bacteria. Their binding and subsequent activation of the innate cells which possess these receptors upon first encounter with pathogen-associated components results in various cytokine and chemokine production aimed at host protection and pathogen clearance. This is an essential component in the subsequent activation of the adaptive arm of the response specific for each pathogen.

Dendritic cells: Infection, allergy, DC subsets, and their influence on T-cell polarization

DCs can modify and adapt the T-cell response to the type of invading pathogen or occurring allergen, by developing into stable functional DC phenotypes that initiate either Th1-, Th2- or regulatory T-cell responses.

DCs are generated in the bone marrow and migrate as precursor cells to sites of potential entry of pathogens and/or allergens. Classical myeloid $CD11c^{hi}$

DCs reside as immature cells in the epithelia of skin and mucosal tissues for a period of time (Banchereau and Steinman 1998), depending on antigenic pressure. Simplistically, immature DCs are primed by pathogens to provide pathogen-specific T cells with signals that they require to become protective effector T cells. However, DC ontogeny is heterogeneous, comprising $CD11c^{hi}$ myeloid and $CD11c^{low}$ plasmacytoid DC subsets.

Both mouse and human DC subsets vary in their ability to recognize various antigens, and further differ in their T-cell polarizing abilities. However, they essentially are flexible and, at least *in vitro*, can adopt mature Th1-, Th2- or regulatory T-cell-inducing phenotypes, instructed by the priming signals from microbial and tissue-derived factors.

Distinct subtypes were initially more evident among mouse DCs than among human DCs because of the ready availability of different murine lymphoid tissues and the expression on mouse DCs of markers not present on humans DCs. Mouse DCs that are classed as "mature" express CD11c (the integrin-α_x chain) and the co-stimulator molecules CD80, CD86 and CD40, and have moderate to high surface levels of MHC Class II, although the levels of all of these can be further elevated upon activation. These features always correlate with a striking ability to induce proliferation of allogeneic T cells. Such mature DCs are heterogeneous in normal, uninfected laboratory mice. Surprisingly, the T cells markers CD4 and CD8 are expressed on mouse DCs and are useful for segregating subtypes. CD8 on DCs is in the form of a αα-homodimer rather than a αβ-heterodimer that is typical of T cells. Although CD4 is also present on human DCs, CD8α is not. So far, there is no evidence that either marker is functionally significant on mouse DCs. Other markers that are useful for segregating mouse DC subtypes include CD11b (the integrin α_M chain of Mac-1) and the interdigitating DC marker CD205 (the multilectin domain molecules DEC205, originally known as NLDC-145). Using these surface markers, five DC subtypes are consistently found in the lymphoid tissues of uninfected laboratory mice.

Spleen contains three DC subsets: $CD4^-CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ DCs (Vremec, Pooley et al. 2000). $CD8^+$ DCs are concentrated in the T-cell areas and $CD8^-$ in the marginal zones of most laboratory mice, but $CD8^-$ DCs

migrate into the T-cell zones on stimulation with microbial products (Iwasaki and Kelsall 2000, Reis e Sousa, 1997, De Smedt, 1996). $CD4^-CD8^+$ DC subtypes, which are $CD205^+CD11b^-$, is also found at moderate levels in LNs, but is the dominant subtype among thymic DCs. LNs contain two extra DC subsets that are not normally found in spleen, which have apparently arrived in LNs through the lymphatic system (Iwasaki and Kelsall 2000, Anjuere, 1999, Henri, 2001). Found in all LNs, $CD4^-CD8^-CD11b^+$ DCs express moderate levels of CD205, in contrast to spleen $CD8^-$ DCs. This LN DC subtype is believed to be the mature form of tissue interstitial DCs. Another distinctive DC subtype, found only in skin-draining LNs, expresses high levels of langerin, a characteristic marker of epidermal Langerhnas cells, and is believed to be the mature form of this Langerhnas cell (Henri, Vremec et al. 2001). It also expresses a range of myeloid markers, including CD11b, stains at low levels for CD8α and has high surface levels of CD205, as high as on the $CD8^{hi}$ DC subtype.

The different subtypes of mouse DC share a common capacity to present antigens to T cells and to promote cell-cycle progression. However, they differ in the other aspects of DC-T-cell signalling that determine the subsequent fate of the T cells that they activate. The most striking biological difference so far discovered is the ability of the $CD8^+$ DCs to induce a Th1-biased cytokine response in reactive $CD4^+$ T cells, whereas $CD8^-$ DCs tend to induce a Th2-biased response (Moser and Murphy 2000, Maldonado-Lopez, 1999, Pulendran, 1999). The main factor in this difference is the much higher level of IL-12p70 produced by $CD8^+$ DCs under certain experimental conditions.

Natural Killer T (NKT) cells: In Infection, Allergy and Immune Polarization

Natural killer T cells (NKT cells) constitute a T cell subset that was initially defined by the co-expression of both T cell and NK cell markers, including the T cell antigen receptor (TCR) αβ and the C-type lectin NK1.1. Although the cells conforming to this broad definition are heterogeneous and can be

divided into at least three groups (Godfrey, MacDonald et al. 2004), many of them are characterized by their dependence on recognition of the MHC Class-like CD1d molecules for positive thymic selection and subsequent activation in the periphery. The major subset of CD1d-restricted T cells recognizes CD1d through the expression of antigen receptors comprised of an invariant TCR α chain encoded in mice by Vα14 rearranged to Jα18, and in humans by a strikingly homologous Vα24-Jα18 rearrangement. These invariant TCR α chains pair with TCR β chains with limited heterogeneity due to marked skewing of Vβ gene usage (most prominently Vβ8.2 in mice, and a closely homologous Vβ11 in humans). This population of cells, referred to here as "invariant NKT cells" (iNKT cells), is highly conserved in many aspects of its phenotype, distribution and functions in most mammals that have been studies to date (Tomura, Yu et al. 1999; Matsuda, Gapin et al. 2002; Prell, Konstantopoulos et al. 2003).

It is now clear from many studies that iNKT cells are influential cells that have the capacity to manipulate a wide variety of immune responses. iNKT cells occurs early in a number of microbial infection models in mice, and that such activation can lead to an "adjuvant cascade" that reinforce innate immunity and promotes subsequent adaptive immunity (Lisbonne, Diem et al. 2003). The range of pathogens that can trigger this antimicrobial activity of iNKT cells is astonishingly broad, and is known to include a variety of bacteria, viruses and parasites (Kakimi, Guidotti et al. 2000; Kawakami, Kinjo et al. 2002; Lisbonne, Diem et al. 2003; Matsuda, Suda et al. 2005). In addition, iNKT cells have been implicated in a variety of anti-tumour and allergic responses in mice. They may be activated by certain types of neoplasia to mediate immune surveillance. However, in the absence of a microbial or neoplastic trigger, the dominant effect of iNKT cells may be to regulate the adaptive immune system in a way that prevents harmful autoimmunity. The evidence for this is particularly strong from mouse models of autoimmune disease, including experimental allergic encephalomyelitis and type 1 diabetes mellitus. Thus, NOD mice, which spontaneously develop autoimmune diabetes, have fewer iNKT cells than normal mice and also show a pronounced defect in the capacity of these cells to produce anti-inflammatory cytokines such as IL-4

(Gombert, Herbelin et al. 1996, Hammond, 2001). NOD mice that carry a homozygous CD1d knockout allele, which entirely lack iNKT cells, show acceleration of diabetes of diabetes development compared to NOD mice that express CD1d (Wang, Geng et al. 2001). Remarkably, similar iNKT cell defects have been found in some studies of humans with type 1 diabetes, and deficiencies of iNKT cells have been documented in human subjects and other autoimmune diseases. Below is reviewed the specific properties of specific glycolipid ligand recognition by these T cells, and how a more detailed understanding of this process might be exploited to harness their immunomodulatory properties.

The discovery of synthetic glycolipids in the α-galactosylceramide (α-GalCer) family that act as activators of iNKT cells has created many opportunities to study the effects of *in vivo* activation of these cells. Recent studies indicate that structurally modified forms of α-GalCer exist that can evoke substantially altered responses from CD1d-restricted iNKT cells and thus lead to markedly different effects on the overall immune response. By identifying those compounds which give particular types of iNKT cell responses in terms of cytokine production and other parameters of iNKT cell function, further immunomodulatory agents can be developed which will be useful for the treatment of the above range of diseases.

Early studies of iNKT cells and T-T hybridomas derived from them showed that these are often autoreactive to CD1d-bearing APCs, indicating the possibility that an endogenous ligand that can be recognized by NKT cells. Several candidate natural lipid ligands have been investigated. Glycosylphosphotidylinositols have been demonstrated to bind CD1d, but there is little if any evidence to suggest that these are strong stimulators of iNKT cells (Joyce, Woods et al. 1998). One iNKT hybridoma has been isolated that recognizes cellular phospholipids such as phosphoethanolamine and phosphotidylinositol, though this is not a general characteristic of other iNKT hybridomas that have been studied (Gumperz, Roy et al. 2000). Most recently, a strong candidate for a physiologically relevant natural ligand of iNKT cells has been identified. This is a lysosomal glycolipid, isoglobotrihexosylceramide (iGb3), which in either natural or synthetic forms

has the ability to activate most human or mouse iNKT cells *in vitro*. Most notably, the absence of this glycolipid in mice lacking the enzyme β-hexoaminidase B results in a dramatic loss of iNKT cell numbers. This suggests that iGb3 may be a major selecting ligand for iNKT cells *in vivo* (Zhou, Mattner et al. 2004). There has also been some success in identification for foreign microbial glycolipid ligands of CD1d that can activate iNKT cells. Most notably, certain glycosylceramides derived from lipopolysaccharide-negative *Sphingomonas* bacteria also activate iNKT cells (Kinjo, Wu et al. 2005, Mattner, 2005); this suggests a role for iNKT cells in innate response against pathogens which do not activate classical pattern-recognition receptors such as TLR4. In addition, one study has described a glycolipid identified as phophatidylinositol tetramannoside (PIM_4) produced by *Mycobacterium bovis* BCG that is also bound by CD1d, and appears to activate a subpopulation of iNKT cells (Fischer, Scotet et al. 2004).

Based on the above and also the studies of Brigl et al (Brigl, Bry et al. 2003), it is now believed that at least two mechanisms exist to account for the activation of iNKT cells by a wide variety of different microbial pathogens. In both mechanisms, a CD1d complex that engages the TCR of the iNKT cell is required. In one case, the ligand recognized by the TCR is most likely a self-lipid or glycolipid, which is responsible for the low level of CD1d-restricted autoreactivity that is characteristic of iNKT cells. This signal, when delivered alone, is not sufficient to activate pro-inflammatory "adjuvant cascade" that contributes to innate immunity and promoted development of Th1-type adaptive immunity. However, when this weak signal is combined with a sufficient amount of IL-12, the pro-inflammatory iNKT cell response is triggered as a result of a situation that is referred to as "enhanced autoreactivity". As many pathogens trigger strong IL-12 production as a result of signals delivered through toll-like receptors or other pattern-recognition receptors, this mechanism may account for innate activity of iNKT cells against a very broad array of different pathogens, many of which may not even harbour specific lipid antigens that are presented by CD1d. However, there do appear to be at least some cases in which microbial pathogens harbour significant levels of CD1d-predented lipids that can directly trigger

iNKT cells through TCR recognition. This situation is exemplified by the responses to *Sphingomonas* species (Sriram, Du et al. 2005), and also is possibly involved in responses to mycobacteria that produce PIM_4 (Fischer, Scotet et al. 2004). In this case, the strength of the signal that is delivered through the TCR is postulated to be much greater than the resulting from recognition of self-antigens presented by CD1d. This could potentially result in strong iNKT cell activation and initiation of the adjuvant cascade even in the absence of production of significant amounts of IL-12.

Progress in understanding iNKT cell biology has been greatly facilitated by the discovery that KRN7000, a synthetic analogue of α-linked galactosylceramides found in the marine sponge *Agelas mauritianus*, is a glycolipid that is potently recognized by iNKT cells (Kawano, Cui et al. 1997). Previous reports had shown that KRN7000 is a very strong inducer of the immune response against B16 melanoma in C57BL/6 mice (Kobayashi, Motoki et al. 1995). KRN7000 is an unusual ligand in that its sugar moiety is linked in the α-anomeric position, while mammalian glycolipids typically exist in the β-anomeric form. Nonetheless, this glycolipid in the context of CD1d binds the iNKT TCR with high affinity, and this allows construction of fluorescently-labelled KRN7000/CD1d tetramers that can be used to stain human and mouse iNKT cells in models of autoimmune disease, tumour rejection, and responses against infectious agents. In particular, in some models with a strong iNKT cell involvement, such as the type 1 diabetes-prone NOD mouse, activation of iNKT cells with KRN7000 causes a dramatic delay in disease induction (Hong, Wilson et al. 2001, Sharif, 2001). Even in other diseases in which it has been difficult to demonstrate an effect of deletion of CD1d or iNKT cells, such as infection with *Mycobacterium tuberculosis*, iNKT cell activation with KRN7000 can alter the overall immune response and have a beneficial impact on disease progression. On the other hand, in disease models where iNKT cell plays a pathogenic role such as the NZB x NZW F1 model of lupus nephritis, the apolipoprotein E-knockout mouse model of atherosclerosis or a mouse model of *Chlamydia muridarum*,

iNKT cell activation with KRN7000 can aggravate disease (Tupin, Nicoletti et al. 2004, Nakai, 2004, Zeng, 2003, Bilenki, 2005).

Much is known about the sequence of events that follow KRN7000 administration. In wild-type C57BL/6 mice, Kupffer cells in the liver and $CD11c^{hi}$ DCs in the spleen play dominant roles in antigen presentation to and activation of the iNKT cell populations in their respective organs (Schmieg, Yang et al. 2005). Once iNKT cells recognize the glycolipid in the context of CD1d, they produce both IL-4 and IFN-γ, as seen by measurements in both intracellular FACS staining and in serum levels. This earliest response is very rapid – within 1-2 hours post-administration – and this initial Th0 cytokine profile cannot be altered towards either Th1 or Th2 by changing the dose or mode of administration of KRN7000. The presence of pre-formed mRNA for IL-4 and IFN-γ in resting iNKT cells is the most likely explanation of this phenotype (Matsuda, Gapin et al. 2003). In tandem with this cytokine production, iNKT cells seem to disappear, as observed with flow cytometry. While this has originally been interpreted as induction of apoptosis in iNKT cells, it is now clear that down-modulation of the TCR and NK1.1 markers used to identify them is the main explanation (Crowe, Uldrich et al. 2003, Wilson, 2003 #194).

One of the most dramatic downstream events after iNKT cell activation is the *trans*-activation of bystander cells, including NK cells that produce IFN-γ 2-24 hours post-activation (Carnaud, Lee et al. 1999). In fact, this population is responsible for the bulk of the "late-phase" IFN-γ observed in serum following *in vivo* administration of KRN7000, as well as the efficacy of this glycolipid as an anti-tumour agent. IFN-γ, probably derived from the iNKT cell itself, as well as CD40/CD40L interactions plays some indirect role in this late NK cell activation. The immediate agonist for NK cells, however, is probably IL-12, though there is disagreement in the field whether macrophages or $CD8\alpha^{+}$ DCs are the key cells that link iNKT cell activation to the downstream NK cell response.

DCs are also *trans*-activated by KRN7000. Several maturation markers – MHC class II, CD8 and CD86 – are up-regulated as early as 24 hours post-administration (Fujii, Shimizu et al. 2003). NKT cell-derived IL-4 also induced

up-regulation of the activation marker CD69 in the vast majority of peripheral B cells. An increase in serum levels of Th2-associated antibody isotypes, most notably IgE, occurs within a week of administering KRN7000. An increase in total serum IgM from 7 to 14 days post-administration has also been observed. The source or significance of this transient rise in IgM is currently unclear.

Several recent studies have implicated mouse NKT cells in allergic in and inflammatory responses. In each case, the degree to which NKT cells serve as regulators, as opposed to effectors of immune-mediated damage, remains to be determined. The very small numbers of NKT cells that can be isolated from target organs, such as the lungs, suggest these cells are acting primarily in a regulatory capacity. The best-studied system is airway hypersensitivity, which can be induced in mice by sensitization with antigen followed by intranasal antigen challenge. The first study of the role of NKT cells in this process, using OVA as a model sensitizing antigen, compared NKT cell-deficient $J\alpha18^{-/-}$ mice with WT mice and failed to reveal a role for NKT cells in OVA-induced airway hypersensitivity (Korsgren, Persson et al. 1999). However, 2 more recent studies indicated that NKT ells are required for the induction of airway sensitivity (Lisbonne, Diem et al. 2003, Akbari, 2003). Transfer of NKT cells to $J\alpha18^{-/-}$ mice restored susceptibility and enabled the demonstration that either IL-$4^{-/-}$ or IL-$13^{-/-}$ NKT cells were competent to do this, while NKT cells deficient for both cytokine genes were not (Akbari, Stock et al. 2003). These findings are not confined to OVA immunization, as $CD1d^{-/-}$ mice used in our studies also showed a decrease response to the ragweed allergen when airway eosinophilia, mucus production, and anti-ragweed IgE were measured (Bilenki, Yang et al. 2004). Despite this, in humans the frequency of Vα24-expressing $CD161^{+}$ perihpheral blood lymphocytes was equivalent in atopic and non-atopic individuals (Prell, Konstantopoulos et al. 2003), which indicates that caution is required when extrapolating from animal models to humans. While at face value, this might suggest that NKT cells are not involved in human atopy, this may again reflect the problem that NKT cell levels in peripheral blood do not necessarily mirror those in other tissues. A more meaningful assessment of NKT cells in human airway

hyperresponsiveness might come from studying these cells in bronchoalveolar lavage samples. Considering the Th2 cytokine production by NKT cells in humans, and abundant data in support for their role in allergy in murine models, we strongly feel that further investigation into NKT cells in human allergy in warranted.

Mouse NKT cells have been implicated in several other contexts where stimulation of the immune system leads to inflammation or pathology. Contact hypersensitivity in mice has been shown to require IL-4 production by NKT cells (Campos, Szczepanik et al. 2003). This IL-4 acts rapidly in a cascade that involves activation of B-1 cells and mast cells, which leads to vascular permeability and the recruitment of effector T cells to the site where antigen is located in the skin (Campos, Szczepanik et al. 2003). In this case, therefore, it has been established that NKT cell IL-4 is playing a regulatory role in terms of modulating the activity of subsets of B lymphocytes as well as mast cells. Immunity and inflammation mediated by the innate and adaptive immune systems have been implicated in the pathogenesis of atherosclerosis. Interestingly, in atherosclerosis-prone *apo*E$^{-/-}$ mice crossed with CD1d$^{-/-}$ mice, lesion sizes in the arteries were decreased compared with control *apo*E$^{-/-}$ mice (Tupin, Nicoletti et al. 2004). Furthermore, administration of α-GalCer increased lesion size in *apo*E$^{-/-}$ mice that were CD1d positive. These results suggest that NKT cells exacerbate atherosclerosis, although no mechanism was defined. Overall, the study of NKT cells in the context of both allergic as well as infectious disease, needs to be extended.

Mechanisms of Bacterial-associated Protection from Allergies

A well-characterized PAMP is lipopolysaccharide (LPS). LPS (also referred to as endotoxin) is a conserved bacterial component and one of the most potent activators of the innate immune system (through CD14/TLR4-mediated signaling). This molecule is an integral constituent of the outer membrane of Gram-negative bacteria. Its well-characterized structure consists of lipid A, a conserved polysaccharide core and the highly variable O-polysaccharide. Lipid A is the hydrophobic and endotoxic part of the molecule. When peripheral immature DCs, monocytes and macrophages are exposed to LPS,

the maturation of the APCs is induced, increasing the expression of MHC II and co-stimulatory molecules and stimulating the production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. In addition, LPS induces the production of IL-12 in these cells stimulating the generation of Th1 responses. These immunomodulatory properties of LPS have been suggested to influence the development and maintenance of atopic disease. Results of epidemiological studies indicate that indoor endotoxin exposure early in life may protect against allergic sensitization (Von Ehrenstein, Von Mutius et al. 2000, Riedler, 2000). More recently, a study showed a correlation between concentrations of house-dust endotoxin and the frequency of IFN-γ-secreting T cells in the blood (Gereda, Leung et al. 2000). Furthermore, Lauener et al reported in 2002 that blood cells from farmers' children express significantly higher amounts of CD14 and TLR2 (Lauener, Birchler et al. 2002). These findings indicate that exposure to LPS may lead to the generation of appropriate Th1 immunity to environmental allergens. However, Braun-Fahrlander et al reported in 2002 that the production of pro-inflammatory cytokines by leukocytes from children showing no or little symptoms of atopy but high amounts of endotoxin in their bedding was significantly lower than the amounts of cytokines produced by leukocytes from atopic children (Braun-Fahrlander, Riedler et al. 2002). The authors suggest that endotoxin exposure may therefore lead to the establishment of immunological tolerance towards ubiquitous allergens. There are also some animal experiments suggesting that the exposure to LPS reduces the development of allergic inflammation. Vannier et al reported that intravenous application of LPS reduces OVA-induced bronchoconstriction in guinea pigs by reducing histamine release from lung mast cells (Vannier, Lefort et al. 1991). Furthermore, Gerhold et al also showed that systemic LPS administration before OVA sensitization reduces OVA-specific IgE levels, Th2 cytokine production by splenic mononuclear cells and the airway eosinophilia in mice (Gerhold, Blumchen et al. 2002). These effects were IL-12-dependent. However, the bronchial hyperreactivity was not affected by LPS exposure.

Although the above discussed results indicate that the exposure to LPS protects against the development of allergic disorders, LPS appears to have a dual role in the induction of allergic inflammation. It was shown that inhalation of endotoxin later in life, in contrast to exposure in early life, causes chronic bronchitis and is associated with airway hyperresponsiveness exacerbating pre-existing asthma (Reed and Milton 2001). Furthermore, it was more recently demonstrated that LPS induced Th2 cell-associated cytokine production (IL-5, IL-10 and IL-13) from mast cells *in vitro* and also synergistically enhanced production of these cytokines induced by IgE crosslinking (Masuda, Yoshikai et al. 2002). In addition, Tulic et al showed that exposure of rats to LPS aerosol 24 hours before 1, 2 or 4 days after OVA sensitization decreased hypereosinophilia in the BAL and OVA-specific IgE levels in the serum (Tulic, Wale et al. 2000; Tulic, Knight et al. 2001). Conversely, LPS given 6, 8 or 10 days after exposure increased the allergic responses.

Based on the observations discussed above, it may be also possible to use LPS in order to inhibit the development of allergic Th2 responses in humans. However, serious side effects due to the strong activation of the innate immune system need to be ruled out. The potential side effects of LPS, in particular toxic shock, may be ruled out by using a detoxified derivative of LPS, for example monophosphoryl lipid A (MPL). Supporting this view is the finding that immunization of mice with OVA or ragweed pollen extract in combination with MPL resulted in allergen-specific Th1 responses and reduction of Th2 responses (Wheeler, Marshall et al. 2001). The application of MPL in combination with allergen is currently being tested as an anti-allergy vaccine.

Bacterial infections, however, invoke more than an anti-bacterial Th1 response. An anti-inflammatory, regulatory response is also induced, with the capacity to regulate allergic reactivity. Treatment of mice with SRP299, a killed *Mycobacterium vaccae*-suspension, induced allergen-specific $CD4^{+}CD45RB^{low}$ regulatory T cells (Treg), capable of protecting recipient mice from allergen provoked airway inflammation, in an IL-10 and TGF-β dependent manner (Zuany-Amorim, Sawicka et al. 2002).

Furthermore, work form the same group has now shown that $CD11c^+$ DCs isolated from mice treated with *M.vaccae* express elevated TGF-β and IFN-α mRNA, suggesting that upstream mechanisms involving DCs may be responsible for protection from airway inflammation (Adams, Hunt et al. 2004). Similarly, Lactobacillus treatment of atopic children increased serum IL-10 and mitogen-stimulated PBMC IL-10 secretion in association with reduced allergic reactivity (Pessi, Sutas et al. 2000). Taken together, these studies suggest that in addition to immune deviation during bacterial infection / treatment, a regulatory compartment is expanded, providing an additional mechanism of protection from allergic reactivity. One mechanism by which LPS may trigger Th1-type immunity is via biding to the LPS receptor, CD14, which is expressed by APCs, particularly monocytes. Binding of LPS to CD14 results in marked stimulation of IL-12 production, which seems to be one of the most important inducers of Th1 responses. It is of interest that a polymorphism in the 5'-flanking region of the CD14 gene has been associated with the increased intensity of atopy (Baldini, Lohman et al. 1999). More recently, a study showed a correlation between concentrations of house-dust endotoxin and the frequency of IFN-γ-secreting T cells in the blood (Gereda, Leung et al. 2000).

These findings indicate that exposure to LPS may be of great biological relevance in generating appropriate Th1 immunity to environmental allergens. Furthermore, Stumbles, *et al* (Stumbles, Thomas et al. 1998) reported that the resident DC population of the respiratory tract of rats preferentially induces the development of Th2 cells. In the presence of GM-CSF or TNF-α, the DCs induced as response that was skewed towards the development of a Th1 response. Some LPS leads to the secretion of TNF-α by macrophages, it may be possible that LPS exposure leads to increased Th1 responses against inhaled allergens by altering the stimulatory quality of the mucosal DCs from Th2-type towards Th1-type. However, exposure to LPS was shown to have bi-directional effects in animal models: LPS given after airway allergen sensitization increased IgE production and airway inflammation; in contrast, exposure to LPS after allergen challenge abolished airway inflammation and bronchoconstriction.

Taken together, there is circumstantial evidence suggesting a role for LPS in the development of allergic disorders. The source and route of exposure to LPS may also be of importance, since LPS is produced by several bacterial pathogens (e.g. *Haemophilus* and *Salmonella*) and commensals colonizing the gut (e.g. *Escherichia coli*). Interestingly, Bjorksen *et al* (Bjorksten, Naaber et al. 1999) found that the colonization of the gastrointestinal tract from newborn babies who had lactobacillus and eubacteria (compared with whose who had *Clostridium difficile*) correlated with a decrease in atopic disorders later on in life. This suggests that commensal gut bacteria may play an important, as yet undefined role in predisposing children towards the atopic phenotype.

PAMPs are widely conserved among different bacterial species and play an important role in the activation of both innate and adaptive immune responses. Most (but not all) PAMPs bind to Toll-like receptors (TLRs) expressed by a wide variety of immune cells leading to cell activation (Kaisho and Akira 2002). A prominent example of PAMPs are CpG-oligonucleotides (ODN). CpG-ODN are non-coding non-methylated DNA sequences with central C-G dinucleotides commonly found in bacterial DNA and bind to TLR9. Viruses and eukaryotic organisms also contain CpG motifs, however, they are methylated and do not activate eukaryotic cells. Bacterial CpG-ODN exert multiple stimulatory effects on many cell types including DC, macrophages, B cells, NK cells, and T cells (Krieg 2002). CpG-ODN enhances the antigen-presenting capacity of DCs and macrophages through the up-regulation of co-stimulatory molecules and the induction of pro-inflammatory cytokines such as IL-12 and IFN-γ, promoting the development of Th1 responses. For this reason, CpG-ODN have been used as adjuvants in vaccines designed to protect against the development of allergic diseases. Several animal studies have shown that CpG-ODN could down-regulate allergen-induced Th2 responses (Kline, Waldschmidt et al. 1998, Shirota, 2000, Serebrisky, 2000). As predicted, inhibition of allergic responses seems to be an immune deviation from the allergen-specific Th2 to a Th1 response (Wohlleben and Erb 2001). Kline, et al reported that IFN-γ and IL-12 are not needed for CpG-

ODN-mediated inhibition of allergic responses (Kline, Krieg et al. 1999). However, in the absence of either IL-12 or IFN-γ, mice required 10 times as much CpG-ODN to be protected against the induction of airway eosinophilia.

Taken together these studies indicate that the application of CpG-ODN efficiently inhibits the development of allergic Th2 responses. Shirota et al have shown that conjugation of specific allergens to CpG-ODN is 100-fold more efficient in inhibiting airway eosinophilia and inducing Th1 differentiation in an IL-12-dependent manner *in vitro* than the unconjugated mixture of allergen and CpG-ODN (Shirota, Sano et al. 2000, Shirota, 2000). This strategy could allow the use of low amounts of CpG-ODN, by-passing potential side-effects observed with CpG ODNs while increasing anti-allergy effects.

Although it seems clear that infections that induce Th1 responses have the capacity to inhibit Th2 responses, there may be situations where this does not occur. For example, infections with *M. bovis*-BCG strongly inhibited the development of a local Th2 response in the lung, but not the systemic allergic response (Erb, Holloway et al. 1998, Herz, 1998). This suggests that the infection needs to occur at the same site as the allergic response to have the maximal suppressive effect. If this is not the case, it is plausible that Th1 and Th2 responses can co-exist simultaneously at different sites in the body. Furthermore, the time point of the infection in relation to the age of the individual may also be crucial for the suppression of the atopic phenotype. The neonatal immune response of non-atopics is biased towards Th2 responses, which then shifts towards Th1 responses during the first years of life. By contrast, children who become atopic do not lose the Th2 response. A recent report attributed the lack of Th2 response suppression in atopic children to the inability of these children to produce sufficient amounts of IFN-γ as neonates (Prescott, Sly et al. 1998). This suggests that infectious diseases that induce the production of IFN-γ should have the greatest impact on the inhibition of atopic disorders during the immediate post-natal period or early childhood, with infections later in life having no suppressive effect. The observation that non-atopic children develop allergen-specific Th1 patterns and non atopy-inducing Th2 patterns before the age of five years supports

this view (Yabuhara, Macaubas et al. 1997). Additionally, other theories that are highly plausible involve the infection with infectious agents, particularly parasites and helminths, which induce a high level of IL-10, and therefore have immunoregulatory properties rather a Th1 or Th2 bias (Wilson and Maizels 2004, Kamradt, 2005).

Overall, the mechanisms which govern the inhibition of allergen-induced airway inflammation are numerous. In addition to what has been overviewed above, there are many infection-induced mechanisms that are being considered within the scope of the Hygiene Hypothesis and why there is increased allergy as the rate of infectious diseases decrease. A few cytokines in particular, namely IL-12 and IL-10, have been postulated to be more highly involved in this inhibitory process of allergy. Evidence of these studies is outlined below and lead to the hypotheses and aims of the present study.

Induction of immunoregulatory factors: IL-12 versus IL-10

In general, the beneficial or harmful effect of a particular cytokine can be investigated through a) exogenous administration of the cytokine or its blocking antibody, or b) through the study of mice in which the gene has been selectively disrupted (knock-out models) or over-expressed (transgenic models). These methods have been used on many levels in order to discern which cells, molecules, cytokines or chemokines are key in inducing and/or sustaining allergic responses. In the context of investigating the various immunoregulatory models within the scope of the Hygiene Hypothesis, a few cytokines have been studied in greater detail. Specifically, IL-12 has been regarded as a crucial cytokine that regulates allergy as its expression is induced in many bacterial infections, which are said to prevent the development of allergy. The mechanism through which IL-12 may regulate the Th2 arm of the immune response has been outlined in the previous section. As well, IL-12 has been shown to be expressed by many innate cells of the immune system, especially macrophages and dendritic cells. Another cytokine which has received more attention over the last few years is IL-10.

The global immunosuppressive effects of IL-10 have been demonstrated by administration of IL-10 to mice. In one study of footpad inoculation with alloantigens and sheep erythrocytes, IL-10 administration inhibited a number of immunologic effects, including DTH, alterations, in vascular permeability and increases in footpad cytokine production (Li, Elliott et al. 1994). Conversely, IL-10 transgenic mice were unable to limit growth of immunogenic tumour cells. Administration of anti-IL-10 antibodies restored the anti-tumour responses, indicating that cell-mediated responses were globally suppressed in mice producing high levels of IL-10. IL-10 KO mice demonstrate a state of chronic inflammation characterized by an over-expression of Th1 cytokines (Yang, Gartner et al. 1999). IL-10 KO mice are growth-retarded and anemic and feature a form of enterocolitis which is similar to human inflammatory bowel disease and which can be partially ameliorated by exogenous administration of IL-10. This finding has resulted in a great deal of experimental activity relating to cytokine expression and Th1/Th2 balance in both gut and airway inflammatory diseases. The role of IL-10 in the immunopathogenesis of other inflammatory and autoimmune diseases has also been studied using the IL-10 KO mouse. These mice demonstrate more severe disease in experimental allergic encephalomyelitis, in virus-induced encephalomyelitis, in hypersensitivity pneumonitis, and in silica-induced intrapulmonary inflammation, suggesting that IL-10 plays a beneficial role in controlling the harmful inflammatory response in the natural histories of these conditions.

There have been numerous studies indicating IL-10 plays an immunoregulatory role in controlling allergen-induced airway inflammation. There is much experimental evidence to support the assumption that Th1 cytokines counterbalance Th2 cells; however, this has been developed primarily in infectious disease models. For example, in models of *Leishmania* infection, Th1 cells, which are responsible for protective immunity, inhibit the development of detrimental Th2 responses that cause dissemination of the infection. In addition, epidemiologic data demonstrate that individuals predisposed toward the production of Th1 cytokines (e.g. those infected with *Mycobacterium tuberculosis* or patients with multiple sclerosis) have a

reduced likelihood of developing allergic disease and asthma. Furthermore, studies concerning allergen immunotherapy in humans, administration of IL-12 or IL-12-fusion proteins, or plasmids containing complementary DNA (cDNA) for allergens together suggest that replacing allergen-specific Th2 cells with Th1 cells, which counterbalance the pathologic effects of Th2 cells, protects against asthma.

If Th1 cells, however, really counterbalance Th2 cells in the airway, then one would predict that Th1 cells would be prominent in the lungs of normal, non-asthmatic patients. This does not appear to be the case. The lungs of healthy people have very few lymphocytes, suggesting that other mechanisms are important in down-regulating and preventing asthmatic symptoms in non-asthmatic persons. Moreover, the presence of large numbers of Th1-effector cells in lung is associated with a tissue-damaging inflammatory response. For example, activated Th1 cells in the lung induce acute, neutrophilic airway inflammation and fail to inhibit Th2-cell-induced airway hyperreactivity. In addition, studies with Th1 and Th2 cells in autoimmune encephalomyelitis and diabetes mellitus indicate that Th1 and Th2 cells may not always antagonize each other, and that polarized Th2 cells, which are generally assumed to be protective in autoimmunity, may in some cases be unexpectedly harmful. Together, these studies indicate that the regulation of asthma and allergic disease may not be reflected in a simple, dichotomous balance of polarized cells, as suggested by the Th1/YTh2 paradigm, and that Th1 cells may not be as beneficial in asthma as initially thought.

The studies of Stampfli and co-workers suggest that IL-10 may provide the mechanism by which allergic inflammatory processes in the lungs are down-regulated. IL-10 can inhibit airway inflammation by reducing inflammatory cytokine and chemokine production and by inhibiting antigen presentation to T cells by limiting class II CD80 (B7.1)- and CD86 (B7.2)-expression on APCs (Borish 1998). Indeed, the absence of IL-10 results in severe allergen-induced airway inflammation and exaggerated production of IL-4, IL-5, and IFN-γ compared with that of wild-type mice. Consistent with this data is the observation that significantly less IL-10 is found in the lungs of patients with asthma (John, Lim et al. 1998, Tomita, 2002 #248). The same phenomenon

occurred when monocytes were acquired from children with persistent cow's milk allergy (Tiemessen, Van Ieperen-Van Dijk et al. 2004). Additionally, oral administration of specific antigens to allergy-prone infant dogs induced the expression of both IL-10 and TGF-β and prevents allergy in their adult life (Zemann, Schwaerzler et al. 2003). As well as these studies, it has been shown that airway gene transfer of IL-10 altered the immune response to OVA in a way that resulted in inhibition of airway eosinophilia (Stampfli, Cwiartka et al. 1999). Pulmonary DCs producing IL-10 mediate tolerance induced by respiratory exposure to antigen (Akbari, DeKruyff et al. 2001). As well as DCs, $CD4^+$ T helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation in a mouse model of airway inflammation (Oh, Seroogy et al. 2002) and that ideas of human asthma treatment have been extended from these findings by the observation that glucocorticoids up-regulate constitutive IL-10 production in human monocytes (Mozo, Suarez et al. 2004).

In the context of the Hygiene Hypothesis, IL-10 has been a more recent focus, with many studies further supporting its expression within the scope of infection models. For example, there is increasing evidence that helminth infections can protect the host against Th2-mediated allergic pathologies, even though helminths themselves are strong inducers of Th2 immunity. In more recent murine model systems, alleviation of allergy is not achieved through complete deviation to Th1, but is linked to IL-10-producing cells, particularly DCs as well as regulatory T cells. For example, schistosomiasis-induced IL-10 suppresses allergy prevalence as reported by Taylor-Robinson, *et al* (Taylor-Robinson 2001). In addition to this allergy model involving infection-induced IL-10 production and reduced allergy, both human and animal model data indicate that Treg cells activated in helminth infection can actively suppress airway allergic inflammation and it has been proposed that a major component of the immunological interaction between infection and allergy is mediated by Treg cells (Maizels 2005, Wilson, 2004, Wilson, 2005). This concept has also been acknowledged and reviewed in detail by Maizels in the past year (Maizels 2005).

The exact mechanism of how this is achieved is still not understood. Thus far, it appears that BCG and *Listeria* suppress the development of allergic disorders by inducing Th1 responses leading to the production of IL-12 and/or IFN-γ. In contrast, the anti-allergy effects of *M. vaccae* seem to be associated with the induction of Tr cells and the production of IL-10 and/or TGF-β. In humans, it is still unclear if, and which bacterial infections could lead to decreased atopic responses. However, the finding of Wickens et al, demonstrating that the use of antibiotics during infancy correlates with an increased risk of developing asthma, suggests that bacterial infections early in life may help to inhibit the development of asthma (Wickens, Pearce et al. 1999). Furthermore, it also appears that infections with *M. tuberculosis* may confer some protection against the development of atopic disorders. Taken together, it remains to be clearly shown that bacterial infections of a particular type or the exposure to bacterial products helps to suppress the development of allergic disease, particularly in established models. Therefore, the usefulness of the data generated in the aforementioned animal models and additional future models provide better insight than that previously known.

Overall, in the interest of the Hygiene Hypothesis, there is much support for the inhibition/intervention of the allergic response by infection, using intracellular pathogens as well as helminth and parasitic infections. Additionally, the role of IL-10 and its induced expression following these infections appears to have a more crucial role in controlling allergic disease than what was previously known.

Hypotheses & Thesis Aims

The regulation and manipulation of immune responses during intracellular bacterial infection is becoming increasingly appreciated. From many of the above-mentioned studies, mechanisms of infection-related-protection from allergy remain unresolved. In particular, how intracellular bacteria infections such as Chlamydia and BCG protect from allergic and other inflammatory diseases, is not fully understood. Detailed research has uncovered many ways of regulating allergic airway inflammation, particularly the pliancy of

allergic responses to cellular regulatory events involving immunoregulatory cytokines, such IL-12 and IL-10.

We propose that intracellular bacterial infections, namely Chlamydia and *Mycobacterium bovis* BCG, induce separately a Th1-biased immune response, resulting in an overall suppression of allergic and indeed other inflammatory responses. Additionally, these individual infections not only result in a reduction of allergen-induced allergic airway inflammation, but also the expression of DCs of a particular phenotype. An immunoregulatory mechanism rather than a complete reversal from Th1 to Th2 would support the recent findings of DC subtypes and production of IL-10-producing cells found in various infections and the deficiency or dysfunction of DCs found in allergic patients, and could explain the inverse association between intracellular bacterial infections and allergy.

We will use a murine model of RW-induced allergic inflammation and two independent intracellular bacterial infection models, namely *Chlamydia muridarum* (formerly known as *Chlamydia trachomatis* mouse pneumonitis) and *Mycobacterium bovis* BCG, we aim to address the following key objectives:

1. **To determine if an intracellular bacteria infection suppress allergic airway inflammation, and, if so**

2. **Determine the basis for the cellular modulation of the allergic response?**

As well, there is a greater appreciation and speculation of the involvement of another regulatory cell type, namely natural killer T (NKT) cells in both allergic disease development as well as in infectious disease manifestation. In addition to the Hygiene Hypothesis at the experimental level, we investigate the role (if any) of NKT cells in the development of both ragweed allergy and the resolution of Chlamydia or BCG infection.

In an attempt to understand the mechanisms which govern the development of the allergic response, the overall goal of this thesis work is to study the causal effect of intracellular bacterial infection on allergy development as well as an investigation of a couple of key innate cell populations which may display crucial roles in one or both of these processes.

Part II. Materials and Methods

Animals

Female C57BL/6 mice (7-10 weeks old) were bred at the University of Manitoba breeding facility (Winnipeg, Manitoba, Canada). Female BALB/c mice (7-10 weeks old) were purchased from Charles River Canada (Montreal, Canada). Breeding pairs of CD1 KO mice, originally developed in 129 background and had been backcrossed to BALB/c 11 times (129-*Cd1*^{tm1Gru}, 7-10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). The CD1 KO mice used in these studies were bred at the University of Manitoba breeding facility (Winnipeg, Canada). Control 129 mice were purchased from the Jackson Laboratory. All animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care.

Intracellular bacterial organisms

The culture and preparation of *C. trachomatis* mouse pneumonitis (*C. muridarum*) was performed in the laboratory at the University of Manitoba. The mouse pneumonitis biovar of *C. muridarum* (*Chlamydia muridarum*) was grown in HeLa 229 cells in Eagle's MEM containing 10% FBS and 2nM L-glutamine for 48 h. For inoculum preparation, infected cells were harvested with sterile glass beads and partially purified by successive 15-min 500 x *g* and 30-min 30,000 x *g* centrifugations. The partially purified organisms were resuspended in sucrose-phosphate-glutamic acid (SPG) buffer, and frozen at -80°C until used. The same seed stock of *C. muridarum* was used throughout the studies. Chlamydial antigens for the anti-*Chlamydia* antibody ELISA was prepared from *C. muridarum* elementary body preparations and purified by step gradient centrifugation using 35% Renografin (Squibb).

Allergen (Ragweed) Preparations

Infected or naïve mice were immunized intraperitoneally (i.p.) with 100 µg of ragweed purchased from Hollister-Stier Canada, Co., Toronto, Ontario, Canada. Ragweed extract was diluted in HBSS and mixed with 2mg of $Al(OH)_3$ adjuvant alum. For intranasal challenge, the ragweed extract stock was diluted to a concentration of 150µg/40µl in HBSS medium.

Models

C. muridarum infection

For the induction of infection, *C. muridarum* was cultured in HeLa 229 cells in Eagle's MEM containing 10% fetal bovine serum and 2 mM L-glutamine for 48 h. For inoculum preparation, infected cells were harvested with sterile glass beads, ultrasonically disrupted. For inoculum preparation, the cell culture organisms were partially purified by successive 15-min 500 x *g* and 30-min 30,000 x *g* centrifugations. They were resuspended in sucrose-phosphate-glutamic acid (SPG), and frozen at -80°C until used. Our previous studies showed that intranasal infection of C57BL/6 mice with *Chlamydia muridarum* can readily cause pneumonia, demonstrated by pulmonary inflammatory cellular infiltration and recovery of chlamydial organisms from the lungs (Yang, Hayglass et al. 1998). The mice were intranasally inoculated with *C. muridarum Chlamydia muridarum* in 40 µl SPG at a concentration of 2.5×10^3 inclusion forming units (IFUs).

Mycobacterium bovis BCG infection

For induction of infection, intravenous BCG was given to mice via the tail vein. Lyophilised BCG vaccine (freeze-dried, Pasteur Merieux Connaught Group, Connaught Laboratories Ltd.) was dissolved in 1.5 ml diluent. Each vial contained 1 mg/l of freeze-dried BCG vaccine and was diluted to a concentration of 1×10^6 CFU.

Allergen-induced Airway Inflammation

Mice infected with *C. muridarum*, BCG or naïve mice were immunized intraperitoneally (i.p.) with 100 µg of ragweed (RW) (Hollister-Stier Canada Co., Toronto, Ontario, Canada) in 2 mg of $Al(OH)_3$ adjuvant (alum) at 30-45 days following the infection for *Chlamydia muridarum* or 21 days post-BCG infection. On day 14 post-RW sensitization, mice were challenged intranasally (i.n.) with 150 µg of RW (40 µl) and were killed 6-8 days later for further analysis.

C. muridarum and Allergy (1 host)

Mice were intranasally (i.n.) infected with 2.5×10^3 inclusion-forming units (IFUs) of *C. muridarum* in a volume of 40 µl. Dilution of the seed stock of *Chlamydia muridarum* was done using SPG medium as described above. An acute *Chlamydia muridarum* infection (confirmed by chlamydial inclusion recovery in the lungs) was achieved by day 7. Mice were immunized by i.p. inoculation with 100 µg RW adsorbed to alum on day 30 or 45 days post-infection, and boosted again with 150 µg RW i.n. 14 days later on day 45. On day 6 post-challenge, mice were sacrificed by cervical dislocation or CO_2 fixation. The duration of infection (30 or 45 days) prior to the first allergen sensitization was established and determined in the course of this thesis and allowed us to address questions pertaining to an infection and allergic reactivity. Days 30 or 45 represent a time point at which mice have cleared the Chlamydial infection.

Mycobacterium bovis BCG and Allergy (1 host)

Mice were infected intravenously (i.v.) with *Mycobacterium bovis* BCG at a concentration of 1.0×10^6 colony-forming units (CFUs). Forty-five days following infection, mice were sensitized with 100 µg ragweed

intraperitoneally (i.p.) in conjunction with alum adjuvant. Two weeks after sensitization, mice were given an airway challenge intranasally (i.n.) with 150 µg ragweed and sacrificed 6-8 days post-challenge.

Established allergy and *Mycobacterium bovis* BCG infection (1 host)

Mice were initially sensitized intraperitoneally (i.p.) with 100 µg ragweed along with 2 mg alum adjuvant. Two weeks following sensitization, mice were challenged with 150µg ragweed i.n., and subsequently infected intravenously with *Mycobacterium bovis* BCG [1.0 x 10^6 colony-forming units (CFU)] immediately after challenge and then challenged intranasally (i.n.) with 150 µg ragweed (40 µl) 21 days post-BCG infection. Mice were killed and analyzed for allergic and immune responses 6-8 days following final allergen challenge.

Infection of mice with *C. muridarum* and transfer of Dendritic cells

Mice were inoculated intranasally (i.n.) with 2.5 x 10^3 inclusion-forming units (IFUs) of *C. muridarum* (*Chlamydia muridarum*) to generate respiratory tract infection as previously described. Seven days post-infection, spleens were aseptically removed. DC were isolated using a MACS (Miltenyi Biotech, Auburn, CA) CD11c column according to the manufacturer's instructions. Spleens were digested with Collagenase D (Boehringer Manheim Biochemicals, Germany). Single-cells suspensions were prepared in PBS with 0.5% bovine serum albumin. MACS CD11c microbeads were added to cell suspensions and were isolated using positive selection columns specific for $CD11c^+$ cells. Purified DCs were then analyzed for purity and expression of cell surface markers characteristic for splenic dendritic cells, namely FITC or PE anti-mouse CD11c (Pharmingen, clone HL3, Armenian Hamster IgG1, λ) and either FITC anti-mouse CD8α (clone 53-6.7, Rat (LOU/Ws1/M IgG2a, κ), PE anti-mouse CD80 (clone 16-10A1, Armenian Hamster IgG2, κ), PE anti-mouse CD86 (clone GL1, Rat (Louvain) IgG2a, κ) and FITC anti-mouse $I\text{-}A^b$ (clone 26-9-17, Mouse (C3H) IgG2a, κ) using a FACSCaliber II and CellQuest software (BD Bioscience, CA, USA). Fluorescence-conjugated

monoclonal antibodies with appropriate isotype controls were purchased from Pharmingen. Purity of the isolated $CD11c^+$ DC was 96-98% based on flow cytometry. For adoptive transfer, isolated $CD11c^+$ cells were first washed in protein-free PBS, and 5×10^5 DCs were injected intravenously to syngeneic recipient mice. Recipient mice were naïve C57BL/6 mice and were subsequently sensitized immediately following DC transfer and challenged with ragweed allergen 14 days later as described above.

Mycobacterium bovis BCG infection and Allergy: Dendritic cell adoptive transfer

Infection of mice, DC isolation and subsequent adoptive transfer of DCs from either naïve or *M. bovis* BCG-infected mice to either naïve or RW-sensitized mice were similar as those procedures in the previous section. Briefly, mice were inoculated with *Mycobacterium bovis* BCG [1.0×10^6 colony-forming units (CFU)]. Twenty-one days post BCG-infection, spleens were aseptically removed. DCs were isolated using a MACS (Miltenyi Biotech, Auburn, CA) CD11c column according to the manufacturer's instructions. Spleens were digested with Collagenase D (Boehringer Manheim Biochemicals, Germany). Single-cells suspensions were prepared in PBS with 0.5% bovine serum albumin. MACS CD11c microbeads were added to cell suspensions and were isolated using positive selection columns specific for $CD11c^+$ cells. Purified DCs were then analyzed for purity and expression of cell surface markers characteristic for splenic dendritic cells, namely FITC or PE anti-mouse CD11c (Pharmingen, clone HL3, Armenian Hamster IgG1, λ) and either FITC anti-mouse CD8α (clone 53-6.7, Rat (LOU/Ws1/M IgG2a, κ), PE anti-mouse CD80 (clone 16-10A1, Armenian Hamster IgG2, κ), PE anti-mouse CD86 (clone GL1, Rat (Louvain) IgG2a, κ) and FITC anti-mouse $I\text{-}A^b$ (clone 26-9-17, Mouse (C3H) IgG2a, κ) using a FACSCaliber II and CellQuest software (BD Bioscience, CA, USA). Fluorescence-conjugated monoclonal antibodies with appropriate isotype controls were purchased from Pharmingen. Purity of the isolated $CD11c^+$ DC was 96-98% based on flow cytometry. For adoptive transfer, isolated $CD11c^+$ cells were first washed in

protein-free PBS, and 5 x 10^5 DCs were injected intravenously to syngeneic recipient mice. These recipient mice were naïve C57BL/6 mice and were subsequently sensitized and challenged with ragweed allergen as described above.

C. muridarum infection and Allergy: Dendritic cell subset adoptive transfer

Mice were inoculated intranasally (i.n.) with 2.5 x 10^3 inclusion-forming units (IFUs) of *C. muridarum* to generate respiratory tract infection. Seven days post-infection, spleens were aseptically removed. DC were isolated using a MACS (Miltenyi Biotech, Auburn, CA) CD11c column according to the manufacturer's instructions. Spleens were digested with Collagenase D (Boehringer Manheim Biochemicals, Germany). Single-cells suspensions were prepared in PBS with 0.5% bovine serum albumin. MACS CD11c microbeads were added to cell suspensions and were isolated using positive selection columns specific for $CD11c^+$ cells. Purified DCs were then analyzed for purity and expression of cell surface markers characteristic for splenic dendritic cells, namely FITC or PE anti-mouse CD11c (Pahrmingen, clone HL3, Armenian Hamster IgG1, λ) and either FITC anti-mouse CD8α (clone 53-6.7, Rat (LOU/Ws1/M IgG2a, κ), PE anti-mouse CD80 (clone 16-10A1, Armenian Hamster IgG2, κ), PE anti-mouse CD86 (clone GL1, Rat (Louvain) IgG2a, κ) and FITC anti-mouse $I\text{-}A^b$ (clone 26-9-17, Mouse (C3H) IgG2a, κ) using a FACSCaliber II and CellQuest software (BD Bioscience, CA, USA). Fluorescence-conjugated monoclonal antibodies with appropriate isotype controls were purchased from Pharmingen. Purity of the isolated $CD11c^+$ DC was 96-98% based on flow cytometry. Freshly-isolated DCs from naïve or *Chlamydia muridarum*-infected mice were also co-stained with PE anti-mouse CD11c (clone HL3, Armenian Hamster IgG1, λ) and FITC anti-mouse CD8α (clone 53-6.7, Rat (LOU/Ws1/M) IgG2a, κ) and sorted based on CD11c positive, CD8α positive cell or CD11c positive, CD8α negative cells using FACSCaliber II (BD Biosciences, CA, USA). Sorted DC subsets isolated from naïve or infected donors were washed once with PBS. 5.0 x 10^5 sorted DC subsets from either phenotype were injected intravenously (i.v.) to naïve, syngeneic recipient C57BL/6 mice. Following DC subset adoptive transfer,

recipients were sensitized with 100 µg RW and alum adjuvant i.p., followed by a challenge with 150 µg RW i.n., as described above.

C. muridarum infection and Protective Immunity: DC subset Adoptive transfer

Mice were inoculated intranasally (i.n.) with 2.5 x 10^3 inclusion-forming units (IFUs) of *C. muridarum* to generate respiratory tract infection. Seven days post-infection, spleens were aseptically removed. DCs were isolated using a MACS (Miltenyi Biotech, Auburn, CA) CD11c column according to the manufacturer's instructions. Spleens were digested with Collagenase D (Boehringer Manheim Biochemicals, Germany). Single-cells suspensions were prepared in PBS with 0.5% bovine serum albumin. MACS CD11c microbeads were added to cell suspensions and were isolated using positive selection columns specific for $CD11c^+$ cells. Purified DCs were then analyzed for purity and expression of cell surface markers characteristic for splenic dendritic cells, namely FITC or PE anti-mouse CD11c (Pharmingen, clone HL3, Armenian Hamster IgG1, λ) and either FITC anti-mouse CD8α (clone 53-6.7, Rat (LOU/Ws1/M IgG2a, κ), PE anti-mouse CD80 (clone 16-10A1, Armenian Hamster IgG2, κ), PE anti-mouse CD86 (clone GL1, Rat (Louvain) IgG2a, κ) and FITC anti-mouse $I\text{-}A^b$ (clone 26-9-17, Mouse (C3H) IgG2a, κ) using a FACSCaliber II and CellQuest software (BD Bioscience, CA, USA). Fluorescence-conjugated monoclonal antibodies with appropriate isotype controls were purchased from Pharmingen. Purity of the isolated $CD11c^+$ DC was 96-98% based on flow cytometry. Freshly-isolated DCs from naïve or *Chlamydia muridarum*-infected mice were also co-stained with PE anti-mouse CD11c (clone HL3, Armenian Hamster IgG1, λ) and FITC anti-mouse CD8α (clone 53-6.7, Rat (LOU/Ws1/M) IgG2a, κ) and sorted based on CD11c positive, CD8α positive cell or CD11c positive, CD8α negative cells using FACSCaliber II (BD Biosciences, CA, USA). Sorted DC subsets isolated from naïve or infected donors were washed once with PBS. Adoptive transfer of sorted DC subsets was executed on naive recipients. 5.0 x 10^5 sorted DC subsets from either phenotype were injected intravenously (i.v.) to naïve,

syngeneic recipient mice. Following DC subset adoptive transfer, recipients were given a challenge infection with 1.0 x 10^3 IFU *C. muridarum* intranasally and were sacrificed 12 days post-infection.

Mycobacterium bovis BCG infection and Allergy: DC adoptive transfer and antibody neutralization with anti-IL-10 and anti-IL-12 antibody (1 host)

Mice were treated using two different protocols. Mice were initially sensitized intraperitoneally (i.p.) with 100 µg of ragweed extract (RW) (Hollister-Stier Canada Co., Toronto, Ontario, Canada) in 2 mg alum adjuvant. On day 14 post-sensitization, mice were challenged intranasally (i.n.) with 150 µg RW (40 µl). Naïve mice were simultaneously infected intravenously (i.v.) with *Mycobacterium bovis* BCG [2.5 x 10^5 colony-forming units (CFUs)]. BCG-infected mice were killed on day 21 post-infection and spleens were aseptically removed for dendritic cell isolation as described above. RW-sensitized/challenged mice were injected intravenously (i.v.) with dendritic cells from BCG-infected or naïve mice, with or without neutralizing IL-10 (1 mg/mouse, i.p.) or neutralizing IL-12 antibody (kindly provided by Dr. David Gray, University of Edinburgh, UK) (1 mg/mouse, i.p.) and re-challenged with RW i.n. (150 µg in 40 µl). Mice were killed 6 days later and analyzed for allergic and immune responses to RW.

α-Galactosylceramide treatment (KRN7000) and anti-IL-4 monoclonal antibody treatment: Allergy and Infection models

α-GalCer, which is 2S, 3S, 4R, 1-O-(α-galactopyranosyl)-2-(N-hexasosanoylamino)-1, 3,4 –octadecanetriol, was synthesized by Pharmaceutical Research Laboratories, Kirin Brewery (Gunma, Japan). The 200 µg/ml stock solution was dissolved to a final concentration of 4 µg with PBS. BALB/c, CD1 KO or 129 mice received a single intravenous injection of 2-4 µg α-GalCer diluted in PBS. Control mice of the same strains were injected with an identical volume of vehicle solution alone (0.025%

polysorbate-20). Mice were then either sensitized with RW, 2 h following α-GalCer or polysorbate injection or infected with 1.0 x 10^3 inclusion-forming units of *C. muridarum Chlamydia muridarum* infection 2 h following administration of α-GalCer.

Determination of Allergic Phenotype

Bronchoalveolar lavage (BAL)

Six to eight days after the final airway challenge, mice were sacrificed by means of cervical dislocation. The mouse trachea was cannulated and the lungs were washed twice with 1 ml of sterile PBS.

BAL Differential Cell Counts

The bronchoalveolar lavage (BAL) fluids were centrifuged immediately following the washing of the mouse lungs. The supernatants were collected for measurement of cytokines and eotaxin. The cell pellets were resuspended to prepare bronchoalveolar lavage smears. The slide was air-dried, fixed with ethanol, and then stained with the Fisher Leukostat Stain Kit (Fisher Scientific, Nepean, Ontario, Canada) to stain the leukocytes. The numbers of neutrophils, monocytes, lymphocytes, and eosinophils per 200 cells were counted based on their morphology and staining characteristics.

Peripheral blood differential cell counts

Blood smears were prepared from naïve, *Chlamydia muridarum*- or BCG-infected and RW-treated mice, treated with RW alone or *Chlamydia muridarum* infection alone. Several drops of blood were collected from each mouse and air-dried on glass slides. Blood smears were then stained using a Hema 3 Stain Set (Fisher Scientific) containing a cell fixative solution and eosin Y stain. After samples were fixed, stained, and air-dried, the number of leukocytes, including neutrophils, monocytes, lymphocytes, and eosinophils

were counted. This count was determined based on cellular morphology and staining characteristics. In the allergy and allergy/infection models, special attention was aimed at the number of eosinophils in each sample.

Mucus production: Histological Mucus Index

Lungs were collected aseptically and fixed in 10% buffered formalin. The fixed lungs were then embedded in paraffin, sectioned at a width of 10 μm, stained and examined for pathological changes. Bronchial mucus and mucus-containing goblet cells were stained by thionin using the method of Mallory and were analyzed with a histological mucus index (HMI), a quantitative way of measuring mucus secretion. The HMI was determined by examining slides at a final magnification of 400X with a rectangular 10-mm^2 reticule grid inserted into one eyepiece. Intersections of airway epithelium with reticule grid were counted, distinguishing mucus-containing or normal epithelium. The HMI represents the ratio of the total number of mucus-positive intersections and the total number of all intersections.

Histological analysis: Hematoxylin and Eosin staining

Lungs were collected aseptically and fixed in 10% buffered formalin. The fixed lungs were then embedded in paraffin, sectioned at a width of 10 μm, stained and examined for inflammation and histopathological changes as a result of exposure to allergen or infection under light microscopy. Fixed lung sections were stained with a hematoxylin and eosin (H & E) stain and examined for inflammatory cell infiltrates based on cell morphology and staining characteristics. H & E staining of lung sections also revealed any epithelia cell hyperplasia.

Immunohistochemical staining: Eotaxin

Lungs were aseptically removed from naïve or treated mice and were snap-frozen in liquid nitrogen and kept at -80°C until sectioning. Sections were mounted onto slides and fixed with 100% acetone. Frozen lung sections were prepared in this manner for the purpose of immunohistochemical staining of eotaxin. Primary goat anti-mouse eotaxin antibody was purchased from R & D Systems (Minneapolis, MN, USA). Secondary rabbit anti-goat antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA), and the developing AEC substrate was purchased from Dako (Mississauga, Ontario, Canada).

Immunohistochemical staining: Vascular Cell Adhesion Molecule-1 (VCAM-1)

Lung tissues were aseptically removed and snap-frozen in liquid N_2 and kept at -80°C until sectioning. Sections were mounted on glass slides and fixed by 100% acetone. Purified rat anti-mouse VCAM-1 antibody and Isotype-matched control antibodies (Pharmingen, San Diego, CA, USA) were left on slides for 1 h at room temperature and successively washed using wash buffer. The sections were then incubated with secondary antibody (rabbit anti-rat antibody) conjugated with horseradish peroxidase (HRP) and developed with 3-amino-9-ethylcarbazol chromogen. The intensity of VCAM-1 expression on the pulmonary vascular endothelium was classified according to the criteria defined by Brisco. A score of 0 is faint staining of several vessels, 2+ is moderate intensity staining of most vessels, and 3+ is intense staining of most vessels (Yang, Wang et al. 1999). The sections were examined by two observers in a blind manner, and the average of the two determinations for each section was used for calculation of VCAM-1 expression.

Total serum IgE detection by ELISA

The level of total IgE was measured by capture ELISA. Monoclonal anti IgE (BD PharMingen) was diluted to 2μg/ml in 0.06M carbonate buffer and coated onto plates at 50μl/well and incubated overnight at 4°C. Wells were blocked with 5% Foetal calf serum (FCS) in carbonate buffer at 37°C. Doubling dilutions in TBS/0.05% Tween (Sigma-Aldrich, Canada) of serum samples were added to the plate and incubated overnight at 4°C. Biotinylated anti-IgE (BD PharMingen) was added at a concentration of 2μg/ml in TBST/FCS, 50ul/well. Avidin–alkaline phosphate (Sigma-Aldrich, Canada) was added to the plate at 4μg/ml for 1 hour at 37°C. Finally, *p*-nitrophenyl substrate was added and the reaction was allowed to develop before monitoring at 405nm. A standard curve was generated using dilutions of 500ng/ml to 0.122 ng/ml of mIgE (BD PharMingen). Between each layer the plates were washed five times with TBS/Tween.

C. muridarum Infection model parameters

Body weight measurements

To examine the susceptibility of mice to chlamydial infection, one of the parameters measured immediately after organism inoculation until the day of sacrifice was body weight loss of each mouse. Original mouse body weights prior to infection were noted and similar body weight was used at the beginning of each study. Body weight loss was recorded throughout the course of the studies and is a good indicator of morbidity (in conjunction with other infection model parameters).

Quantification of Chlamydial *In vivo* Growth

Mice were inoculated intranasally with various doses of *Chlamydia muridarum* in a volume of 40 μl. Mice were monitored daily for body weight changes as described above, and sacrificed by means of cervical dislocation on selected

days after infection. To determine *in vivo* growth of the chlamydial organism, the lungs, liver, heart, and kidneys of each infected mouse were aseptically removed and placed in sucrose phosphate glutamine (SPG) buffer. Each organ was placed in an autoclaved homogenizer and homogenized in 4 ml of SPG buffer. Tissue homogenates were then centrifuged at 500 x *g* for 10 min at 4°C, and the supernatant were collected and divided into aliquots of 1 ml/vial and kept at -70°C until tested. All samples from the same experiment were tested in the same titration assay. For *Chlamydia muridarum* quantification, Hela n299 cells were grown to confluence in 96-well flat-bottom microtiter plates and washed in 100 µl of HBSS media. The monolayer was then inoculated in triplicate with 100 µl of lung, liver, heart or kidney tissue supernatants from mice infected with *Chlamydia muridarum* or naïve mice. After 2 h incubation at 37°C on a rocker platform, plates were washed, and 200 µl of MEM containing 1.5 µg/ml cyclohexamide, gentamycin, and vancomycin was added. The plates were incubated for 48 h at 37°C in 5% CO_2. Culture medium was then discarded and the cell monolayer was fixed with absolute methanol. To identify inclusion-containing cells, plates were incubated with a *Chlamydia* genus-specific murine monoclonal antibody and stained with goat anti-mouse IgG conjugated to horseradish peroxidase. The number of inclusions were counted under a microscope at 200X magnification. At least five fields through the midline of each well were counted. The chlamydial infectivity in each organ supernatant was then calculated based on dilution titers.

Chlamydial challenge and Delayed-Type Hypersensitivity Measurements

Delayed-type hypersensitivity (DTH) was determined by measuring the increase in footpad thickness following local challenge with heat-inactivated (56°C for 10 min) *Chlamydia muridarum.* A 27-gauge needle was used to inject mice in the hind footpads with 0.025 ml *Chlamydia muridarum* (5×10^4 IFU) in the left foot and the same volume of Hela cell material in the right foot. The thickness of the footpads were measured 24, 48 and 72 h post-injection using a dial-gauge calliper (Walter Stern 601A, Fisher Scientific, Ottawa, ON,

Canada). The difference between the thickness of the two footpads was used as a measure of the DTH response. No measurable changes were observed following Hela cell material injection alone in naïve or *Chlamydia muridarum*-infected mice nor following heat-inactivated *Chlamydia muridarum* injection in naïve mice.

Chlamydial Inclusion Fluorescent Staining

Lungs from *C. muridarum*-infected or naive BALB/c, 129 and CD1 KO mice were collected aseptically as outlined above. One lung from each mouse was snap-frozen in liquid nitrogen for detecting chlamydial inclusions using immunofluorescent staining. For this analysis, 10 µm lung sections were cut from frozen tissue, treated with blocking buffer and incubated with mouse anti-*Chlamydia* major outer membrane protein antibody (Chemicon). After incubation, the sections were treated with FITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich). To stain epithelial cells in the same lung samples, tissue sections were treated with mouse anti-pan-cytokeratin antibody (Calbiochem-Novabiochem). The sections were washed, incubated with biotinylated rabbit anti-mouse antibody (Dako) and then developed with Texas Red Avidin D (Vector Laboratories).

Proliferation Assay

All *in vitro* cultures were performed in RPMI 1640 medium supplemented with penicillin (100U/ml) Streptomycin (100 µg/ml) (Sigma, Canada) and glutamine (Sigma-Aldrich, Canada), plus 10% fetal calf serum (FCS) (Sigma-Aldrich, Canada), unless otherwise stated. Single cell suspensions from lymph nodes or spleen were incubated in quadruplicate at $3x10^6$ cells/ well of a round-bottomed 96 well plate in a total of 200µl at 37°C. For cell proliferation measurements, 3.0×10^6 cells were either incubated alone or with 4 µg/ml Concanavalin A (ConA) (Sigma, Canada) in 96-well cell culture plates for 48 h. After incubation, 1 µCi of [^{3}H]thymidine (Sigma-Aldrich, Canada) was

added to each well of culture and incubated at 37˚C for 6h. [^{3}H]Thymidine incorporation was analyzed using a TopCount NXT microplate scintillation and luminescence counter (Canberra Biosciences).

Cytokine and Chemokine Detection by Enzyme-Linked Immunosorbent Assay (ELISA)

Culture supernatants collected after 54 hours of incubation, or BAL fluid, was assayed for cytokine secretions. Cytokines were measured by ELISA according to suppliers' guidelines. Between each layer the plates were washed five times with TBS/Tween. ELISA capture monoclonal antibodies were prepared in 0.06% carbonate buffer (0.1 mM $NaHCO_3$, pH 8.2) and incubated overnight at 4°C at the following concentrations (Table 2.1), with 50μl added per well of an ELISA plate. Following incubation, plates were blocked with 100 μl/well of 1% bovine serum albumin (BSA) in TBS, incubated for 2 hours at 37°C. 50μl/well of BAL fluid or supernatant was then added with a standard curve performed, with doubling dilutions of the recombinant cytokine. Following incubation and washing, 50 μl/well of matched biotinylated detection antibody was added and incubated at 37°C for 2 hours or overnight at 4°C. Streptavidin-alkaline phosphatase was added, 50 μl/well at 1μg/ml and incubated at 37°C for a further hour. Finally, plates were washed and 100μl of *p*-nitrophenyl phosphate substrate (Sigma-Aldrich, Canada) was added. Once colour had developed, optical densities, at 405 nm, were read, with cytokine concentration determined by extrapolation from the standard curve. Standard curves were obtained using recombinant murine IFN-γ, IL-4, IL-9, IL-10, IL-12 (BD Pharmingen), and IL-5, Eotaxin, and IL-13 (R & D Systems, Minneapolis, MN, USA) and are expressed in picograms per milliliter +/- standard error of the mean (SEM), unless otherwise stated. The buffers, substrates and concentrations of reagents used were adopted from previous protocols from this laboratory or from in house testing and optimization.

Monoclonal Capture Antibodies (Concentration used to coat plate)	Supplier	Biotinylated Monoclonal Detection Antibodies	Supplier
IL-4 (4μg/ml),	Pharmingen	0.5 μg/ml	Pharmingen
IL-5 (2μg/ml),	Pharmingen	1μg/ml	Pharmingen
IL- 9 (4μg/ml)	Pharmingen	1μg/ml	Pharmingen
IL-10 (4μg/ml)	Pharmingen	2μg/ml	Pharmingen
IL-13 (2μg/ml)	R&D	0.1μg/ml	R&D
IL-12 (2.5μg/ml)	Pharmingen	1μg/ml	Pharmingen
IFN-γ (3 μg/ml)	Pharmingen	1μg/ml	Pharmingen
Eotaxin (0.4μg/ml)	R&D	0.4μg/ml	R&D

Table 2.1. ELISA Antibodies

Allergen- and Chlamydia-specific antibody isotype detection by ELISA

Allergen-specific IgE, IgG1 and IgG2a antibodies were determined by ELISA. Unless otherwise stated, serum samples collected 6-8 days after final airway challenge were diluted in TBS/Tween and wells washed between each incubation step with TBS/Tween. Flat-bottomed 96 well plates were coated with 20 μg/ml of RW allergen diluted in HBSS. After washing, non-specific binding was blocked by incubating wells with 5% BSA (Fraction V, Gibco) in carbonate buffer, for 2 hours at 37°C. Doubling dilutions of serum from 1:10 to 1:1280 was then added and plates were incubated overnight at 4°C. For measurement of allergen-specific-IgE, IgG was first depleted as allergen-specific-IgG antibodies, which may be in more than 100-fold excess to IgE antibodies, interfere with the detection of allergen-specific IgE, possibly by competitive binding to allergenic epitopes. Therefore, serum samples were pre-treated by incubating twice with 50% slurry of protein G-Sepharose

(Pharmacia Biotech, Uppsala, Sweden) to remove the competing IgG antibodies. IgG-depleted serum was recovered by centrifugation, diluted in TBS/Tween and added to allergen-coated plates. Following extensive washing, biotinylated anti-IgE antibody was added. The plates were developed using avidin-conjugated alkaline phosphatase and measured by a microplate reader (Versamax; Molecular Devices, Sunnyvale, CA, USA).

For IgG1, HRP conjugated anti-mouse IgG1 (BD PharMingen) was added at 1:6000 dilution, HRP conjugated anti-mouse IgG2a (BD PharMingen) was used at 1:4000 dilution. Following incubation, 50µl/well of ABTS peroxidase substrate system was added, left to develop and monitored at 405nm.

To determine *C. muridarum*-specific serum antibodies, ELISA plates were coated overnight with *C. muridarum* elementary bodies in bicarbonate buffer (0.05M, pH 9.6). After blocking for 90 min with 2% BSA, 0.05% Tween 20 solution and extensive washing, serially diluted sera were incubated for 2 h at 37°C. The plates were washed, and biotinylated goat anti-mouse antibody was added and incubated overnight at 4°C. Alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Bio/Can Scientific) was added, and the plates were kept for incubation at room temperature for 45 min. After extensive washing of the plates, *p*-nitrophenyl phosphate (in 0.5 mM $MgCl_2$, 10% diethanolamine, pH 9.8) was added, and the reaction was allowed to proceed for 60 min. The plates were read with the same above microplate reader at 405 nm.

Spleen & Lymph Node Cell Cultures and Single-Cell suspensions

In allergy, allergy/infection, and infection models, mice were sacrificed by means of CO_2 fixation. Spleens and draining (mediastinum) lymph nodes were aseptically removed. Spleen and lymph nodes were homogenized manually and rinsed once with RPMI Complete medium RPMI 1640 containing 10% heat-inactivated FBS, 25 µg/ml gentamicin, 2 mM L-glutamine and 5×10^{-5} 2-ME (Kodak). Cell suspensions were then centrifuged and resuspended in RPMI Complete medium. Spleen and lymph node cells were

then cultured at a concentration of 7.5 and 5.0 x 10^6 cells/ml, respectively, alone or with RW (100 µg/ml) or with heat-inactivated *C. muridarum* (1 x 10 IFU/ml) at 37˚C in Complete culture medium. Duplicate cultures were established from the spleen and lymph node cells of individual mice in each group. Culture supernatants were harvested at 72 h for the measurement of cytokines by ELISA using purified (capture) and biotinylated (detection) antibodies as described below.

Isolation of total RNA

To determine the levels of total RNA, tissues were homogenized in 1 ml of TRIzol reagent per 50-100 mg of tissue and stored at -80°C until used. Samples were thawed and 0.2 ml of chloroform added to each sample. Tubes were shaken vigorously by hand for 30 seconds and incubated at 15-30°C for 2-3 minutes. Samples were centrifuged at 2-8°C for 15 minutes at 13,000 x g. Following centrifugation, the aqueous phase was removed and transferred to fresh tubes. The RNA was precipitated from each sample's aqueous phase by mixing with isopropyl alcohol. 1 ml of isopropyl alcohol was used per 1 ml of TRIzol reagent. Samples were incubated at 15-30°C for 10 minutes and centrifuged at 15,000 x g for 15 minutes at 2-8°C. Supernatants were removed by aspiration and the RNA pellet was washed once with 1 ml 75% ethanol. Samples were mixed by vortexing and centrifuged at 7,500 x g for 5 minutes at 2-8°C. RNA was dissolved in 10 to 50 µl Rnase-free water and vortexed. Samples were incubated for 10 minutes at 55-60°C. RNA was diluted by 100-fold and OD values were determined for each sample (using UV light) at 260nm and 280 nm. To determine individual RNA concentrations = 40 x A_{260} x dilution factor.

PCR Analysis

For analysis of TLR gene expression in the isolated DC, total cellular RNA was extracted from isolated DC using the phenol-guanidinium method (TRIzol) reagents, Invitrogen), followed by thanol precipitation. The first-

strand nDNA was generated from 1.2μg totla RNA in a final volume of 15μl using M-MLV reveres transcriptase and oligo (dT) primer. One microliter of cDNA was used for each PCR reaction. The presence pf RNA specific for TLR2, TLR4, and TLR9 was determined by semi-quantitative PCR. The PCR primer sets used were as follows: TLR2, 5'-GCTCCAGGTCTTTCACCTCTATTC-3' (sense) and 5'-TCCAGCAGGAAAGCAGACTCGCTTA (anti-sense); TLR4, 5'-GGAAGCTTGAATCCCTGCATAGAG-3' (sense) and 5'-TCCACATGTACTAGGTTCGTCAG-3' (anti-sense); TLR9, 5-AACCTGCGGCAGCTGAACCTCAA-3' (sense) and 5'-GAGTTCAGTGTATGGAGAGAGCTG-3' (anti-sense). β-actin was used as internal control: 5'-GTGGGCCGCCCTAGGCACCA-3' (sense) and 5'-CTCTTTGATGTCACGCACGAATTTC-3' (anti-sense). The reaction conditions for PCR were as follows: 1 cycle at 95°C for 5 min; 31 cycles (for TLR2) or 34 cycles (for TLR4 and TLR9) at 95°C for 1 min, at 55°C for 1 min, and 72°C for 1 min. β-actin was used as a loading control. PCR products were run on a 1% agarose gel containing 0.1 mg/ml ethidium bromide. Image analysis was performed using GelDoc 2000 gel documentation system (BioRad, Hercules, CA) and quantified using Scion Image Software (Scion Corporation, Frederick MD).

Statistical Analyses

Student's t test was used for statistical comparisons between groups with equal distributions. *P* values <0.05 were considered significant and were calculated using GraphPad Prism.

Part III: Results

The results section is divided into six chapters based on the various themes of study. Each chapter begins with an introduction to that section of study, followed by the experimental results and a small discussion.

3.1 Chapter 1: The effect of intracellular bacterial infection on the allergic response induced by ragweed allergen.

In this section, we intend to examine the "Hygiene Hypothesis" using a common environmental allergen and a natural respiratory tract infection model. The allergen used is ragweed extract (RW) which afflicts many seasonally allergic individuals, and the infectious agent is *Chlamydia trachomatis* mouse pneumonitis (*Chlamydia muridarum*) which normally causes murine respiratory tract infection. The murine *Chlamydia muridarum* infection model is well characterized and our previous studies have shown that *Chlamydia muridarum* infection predominantly induces Th1-like responses and IFN-γ/IL-12 production in C57BL/6 mice.

3.1.1 Development of allergic inflammation phenotype following ragweed (RW) exposure in mice.

To appreciate the immunological kinetics of a ragweed extract exposure in mice, C57BL/6 mice were administered 100 μg ragweed extract in alum intraperitoneally (i.p.) and subsequently challenged intranasally (i.n.) with 150 μg ragweed extract. On day 7 post-challenge, mice were killed and spleens and peribronchial lymph nodes were removed for *in vitro* T cell recall responses; sera were also taken for antibody detection.

As this was a new allergy model to the laboratory, some basic *in vitro* parameters were initially established with lymph node cells and splenocytes recovered from mice 6 days post challenge. Optimal antigen concentration for

in vitro re-stimulation was found to be at 100 μg/ml with optimal antigen specific stimulation established with 5 x 10^6 lymph node cells and 7.5 x 10^6 splenocytes per well of a 48-well plate. Spleen and lymph node cells recovered at day 6 post-challenge and re-stimulated *in vitro* with either ragweed extract antigen or with the T-cell stimulant anti-CD3e produced large amounts of IL-4, IL-5, IL-10 and IL-13 (**Figure 1**). The Th1 markers, IFN-γ and IL-12, were minimally expressed (or undetectable) in both lymph node and splenocyte cultures (**Figure 2**), indicative of a governing Th2 response.

Isotype responses, measured in the sera of infected mice, revealed a Th2-dominated phenotype with significantly elevated serum RW-specific IgE and IgG1 with significantly elevated total serum IgE (**Figure 2**). The data shown are from C57BL/6 mice. BALB/c mice were found to respond in a similar way.

In agreement with the aforementioned RW-induced allergic phenotype, the administration of RW allergen sensitization/challenge was also shown to induce cellular infiltration into the lung. Eosinophils made up greater than 50% of the total number of infiltrating cells into the BAL (**Figure 3A**) in RW-treated mice in comparison to PBS-treated controls. Additionally, the level of mucus production demonstrated by the histological mucus index (HMI) in the mice sensitized/challenged with RW was significantly higher than that in mice with PBS alone (**Figure 3B**).

Together, these results validate the use of RW allergen extract as a suitable allergen to induce allergic airway inflammation akin to human asthma, with eosinophilia and mucus production, which significantly contributes to the pathology in asthma.

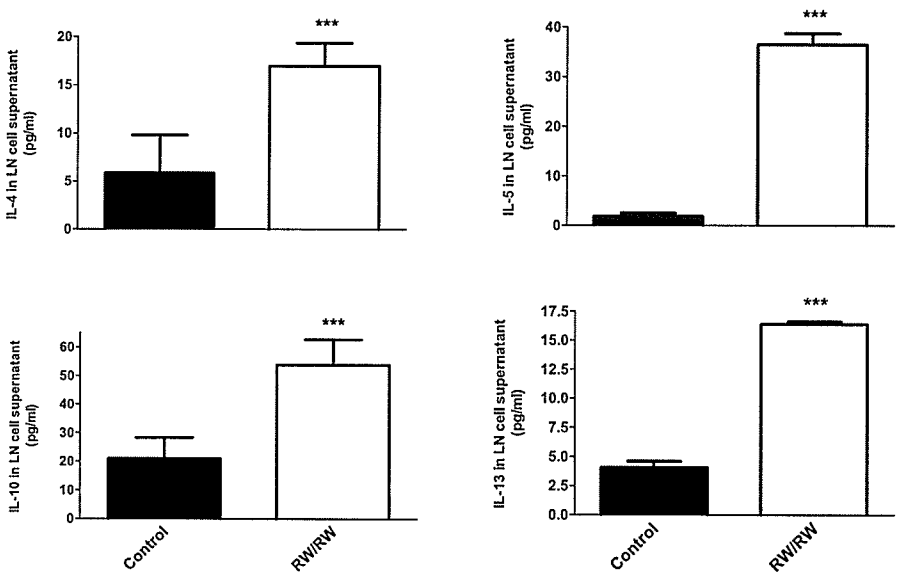

Figure 1. Local lymph node cells (peribronchial) from naïve C57BL/6 mice were cultured in 48-well plates in the presence or absence of 100μg/ml ragweed extract Ag or 1μg/ml of anti-CD3e antibody. Supernatants were collected 72 hours after stimulation with cytokine secretions measured by ELISA. Data presented are mean ± SEM of duplicate wells from 3 mice per group. *, $p<0.05$, **, $p<0.01$

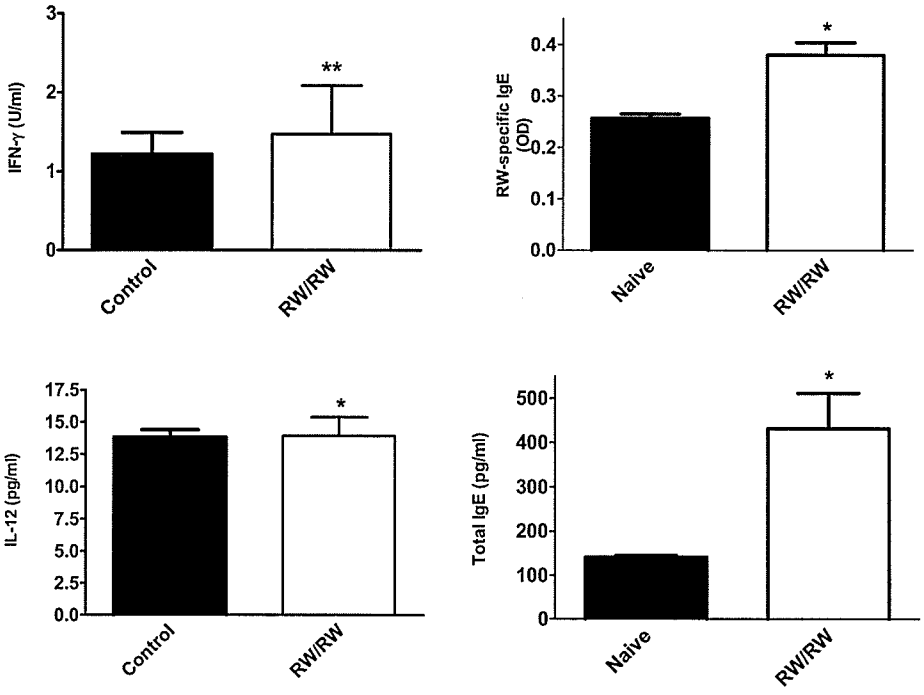

Figure 2. Local lymph node cells (peribronchial) from naïve C57BL/6 mice were cultured in 48-well plates in the presence or absence of 100μg/ml ragweed extract Ag or 1μg/ml of anti-CD3e antibody. Supernatants were collected 72 hours after stimulation with cytokine secretions measured by ELISA. Antibody isotype profiling from sera, was carried out on infected and naïve BALB/c blood samples. Antibody isotypes were measured by ELISA. Data presented are mean ± SEM of duplicate wells from 3 mice per group. *, $p<0.05$, **, $p<0.01$.

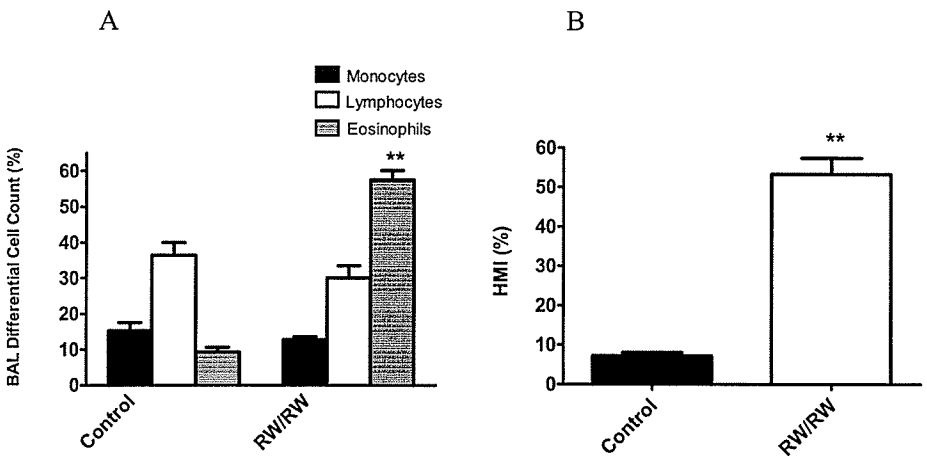

Figure 3. (A) Naïve or mice sensitized/challenged with RW show a significant reduction in BAL of eosinophils following RW sensitization and challenge compared to mice treated with RW only. Mice were sensitized i.p. with RW (100μg) in alum and were challenged with RW (150μg) on day 14 following sensitization. Mice were sacrificed day 6 post-challenge and mice tracheas were cannulated and BAL fluids acquired by washing lungs twice with sterile PBS. BAL cellular components were analyzed by differential cell counts using Fisher Leukostat Stain Kit. (B) Mice were treated as described in Figure 3. Lung tissues were fixed and sections were stained with thionin for mucus and mucus-containing goblet cells. Histological mucus index (HMI) was calculated based on the percentage of the mucus-positive area over the total area of the airway epithelium. Each group was composed of three mice. Data represent means +/- SD.

3.1.2 Local *Chlamydia muridarum* Infection Inhibits Pulmonary Eosinophilia and Bronchial Mucus Production

Our previous studies using *Chlamydia muridarum* in murine respiratory infection models have demonstrated that the *Chlamydia muridarum* lung infection in C57BL/6 mice are cleared within 20-25 days following inoculation (Wang, Fan et al. 1999). We intended in the present study to examine the effect of prior infection (when the infection has been cleared and the mice have recovered from the disease) on RW induced asthma-like reaction, thus choosing 30–45 days post infection as the starting point for RW sensitization. As both eosinophil infiltration into the lung as well as mucus over-secretion are the hallmarks of the asthma-like reaction, a direct comparison was made between the differential cell count from the BAL of uninfected, RW-treated mice and the *Chlamydia muridarum*-infected, RW-treated mice. The results

showed that the mice with previous *Chlamydia muridarum* infection had significantly less cellular infiltration into the lung following RW challenge compared to those mice treated with RW only. Significantly eosinophilic infiltration was dramatically reduced (2×10^5 eosinophils in mice with RW treatment only compared to 3×10^4 eosinophils in the *Chlamydia muridarum*-infected, RW-treated mice). Eosinophils made up greater than 50% of the total cell number of infiltrating cells into the BAL of the mice treated with RW only, whereas eosinophils in the *Chlamydia muridarum*-infected, RW-treated mice represented <25% of the total BAL cells ($p<0.05$) (**Figure 4**). Lymphocyte numbers within the BALs of both groups was unchanged. In addition, histological analysis also showed remarkably reduced levels of infiltrating eosinophils into the bronchial and pulmonary tissues of the RW-treated mice with previous *Chlamydia muridarum* infection, regardless of whether the mice were sensitized with RW at 30 or 45 days post-infection (**Figure 5**). Mice that had been treated with RW alone displayed significant infiltration of eosinophils in the bronchial submucosa, alveolar, and perivascular sheaths, accompanied by a small amount of infiltrating monocytes and lymphocytes. In contrast, mice with prior *Chlamydia muridarum* infection displayed milder cellular infiltrations that consisted mostly of macrophages and lymphocytes, accompanied by a minute number of infiltrating eosinophils (**Figure 5**). In addition, there was increased goblet cell staining as well as epithelial hyperplasia in the mice with RW treatment only, which was abrogated significantly in mice infected with *Chlamydia muridarum* infection prior to RW exposure (**Figure 6 & 7**). Collectively, these results indicate that a local *Chlamydia muridarum* infection which has been cleared prior to RW immunization is capable of inhibiting the local allergic inflammatory response, especially eosinophilia, and reducing mucus production which greatly contributes to the pathology in the asthma-like reaction.

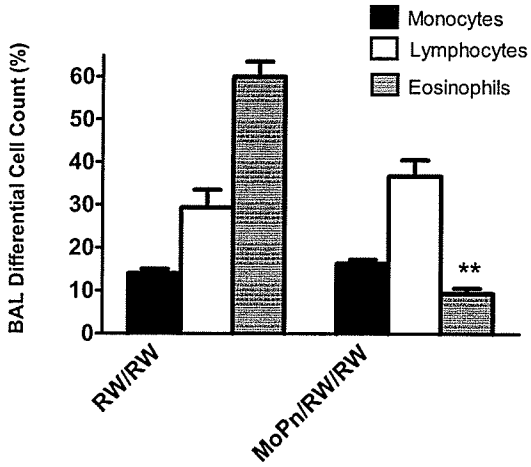

Figure 4. *Chlamydia muridarum*-, RW-treated mice or mice treated with RW alone show a significant reduction in BAL eosinophils following RW sensitization and challenge compared to mice treated with RW only. One group of mice were infected i.n. with 5 x 10^3 IFUs *Chlamydia muridarum*. Thirty days post-infection, all mice were sensitized i.p. with RW (100μg) in alum and were challenged with RW (150μg) on day 14 following sensitization. Mice were sacrificed day 6 post-challenge and mice tracheas were cannulated and BAL fluids acquired by washing lungs twice with sterile PBS. BAL cellular components were analyzed by differential cell counts using Fisher Leukostat Stain Kit.

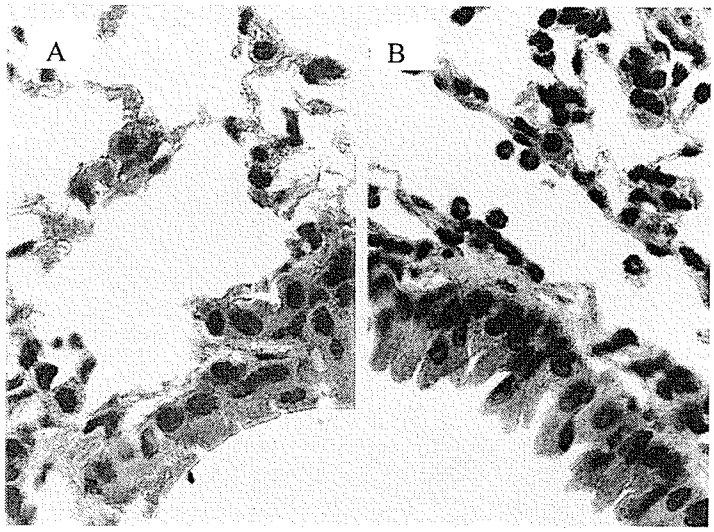

Figure 5. Mice infected with *Chlamydia muridarum* prior to RW sensitization show a significant reduction in peribronchial eosinophilic inflammation following RW challenge compared to mice treated with RW only. One group of mice was infected with 5 x 10^3 IFU Chlamydia trachomatis (*Chlamydia muridarum*) and were allowed to clear the infection for 30 days. On day 30, all mice were sensitized i.p. with RW (100μg) in alum and were challenged with RW (150μg) on day 14 following sensitization. Mice were sacrificed on day 8 post-challenge and the lungs were removed and fixed in formalin. Lung were sectioned and stained with H & E and examined in a double blind manner. Sections were photographed at low (x 100) magnification. Panel A: *Chlamydia muridarum*-infected, RW-treated mice; Panel B, RW-treated mice without previous *Chlamydia muridarum* infection.

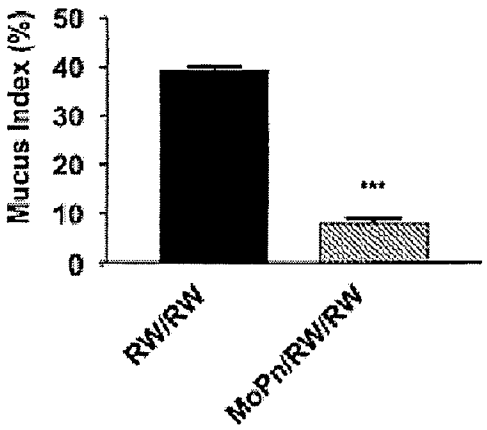

Figure 6. Dramatic reduction in mucus secretion in mice infected with *Chlamydia muridarum* 30 days before RW sensitization/challenge. Mice were killed on day 6 post-RW challenge. Lung tissues were fixed and stained for mucus and goblet cells. Histological mucus index (HMI) was calculated based on the percentage of the mucus-positive area over the total area of the airway epithelium. Each group was composed of three mice. Data represent

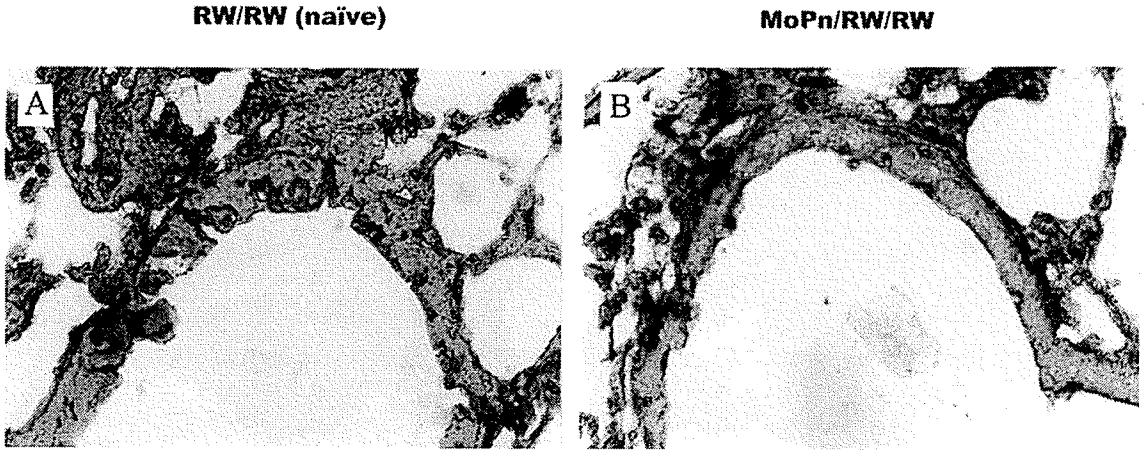

Figure 7. Dramatic reduction in mucus secretion in mice infected with *Chlamydia muridarum* 30 days before RW sensitization/challenge. Mice were killed on day 6 post-RW challenge. Lung tissues were fixed and stained for mucus and goblet cells. Mucus is stained as brownish red by thionin staining and cytoplasm is stained as orange by counterstaining with

3.1.3 Local *Chlamydia muridarum* Infection Reduces Blood Eosinophil Levels Induced by RW Allergen

Another pertinent hallmark of allergic disease which occurs frequently within atopic patients is the elevation of blood eosinophilia compared to non-atopic individuals upon allergen exposure. To address whether previous *Chlamydia muridarum* infection was also capable of reducing blood eosinophilia levels in response to allergen, the number of blood eosinophils in *Chlamydia muridarum*-infected, RW-treated mice were compared to those mice administered with RW only. As seen in **Table 1**, RW exposure induced high levels of blood eosinophilia in mice without previous *Chlamydia muridarum* infection. In contrast, those mice with previous *Chlamydia muridarum* infection displayed a significant decrease in their levels of blood eosinophilia in response to the RW treatment. The results demonstrate a clear ability of the prior *Chlamydia muridarum* infection to not only reduce the level of eosinophilia at the site of infection and allergen exposure, but also its ability to reduce the level of circulating eosinophils within the blood in response to a local allergen challenge.

TABLE 1

Circulating Eosinophils In Peripheral Blood of RW-Treated Mice versus MoPn-Infected, RW-Treated Mice

	Naive	RW/RW	MoPn/RW/RW
Lymphocytes	77.4 ± 4.1	59.5 ± 1.38	60.2 ± 3.18
Neutrophils	11.1 ± 1.1	27.5 ± 1.53	31.4 ± 3.54
Monocytes	9.3 ± 4.6	12.7 ± 1.32	8.23 ± 0.45
Eosinophils	0.13 ± 0.2	0.33 ± 0.067	0.19 ± 0.072*

Note. There is a significant reduction in the circulating eosinophils measured in the peripheral blood of MoPn-infected, RW-treated mice compared to mice treated with RW only. Mice (four mice/group) were treated as described in the legend to Fig. 1. Eight days post-RW challenge, all mice were bled, and the blood smears were stained using a Hema 3 Stain Set (Fisher Scientific). Differential cells were counted based on cell characteristics and cellular morphology. *$P < 0.05$, comparison between the MoPn-infected, RW-treated (MoPn/RW/RW) group and the uninfected, RW-treated (RW/RW) group. The cellular profile of naive mice without any treatment is also shown for comparison. One representative experiment from three independent experiments is shown.

3.1.4 Local *Chlamydia muridarum* infection Alters Cytokine and Chemokine Patterns Induced by Allergen

To directly examine the effect of *Chlamydia muridarum* infection on the subsequent response to RW allergen, a direct comparison was made between Th1-like cytokines, Th2-like cytokines, and a C-C chemokine, eotaxin, produced by RW-treated mice with or without prior *Chlamydia muridarum* infection. The latter has been documented to be crucial in eosinophilic chemoattraction to the site of the allergic inflammation. According to the cytokine profile patterns of spleen and draining lymph node cells from *Chlamydia muridarum*-infected, RW-treated mice re-stimulated with RW allergen in vitro, the levels of Th2-like cytokines, namely IL-4, IL-5, IL-10, and IL-13, were dramatically decreased compared to mice with RW treatment only (**Figure 8**). BAL fluid samples also demonstrated a significant decrease in the Th2-like cytokines in mice with previous *Chlamydia muridarum* infection (**Figure 8**). Furthermore, the levels of eotaxin, within BAL fluids were significantly reduced in those samples obtained from *Chlamydia muridarum*-infected, RW-treated mice, in comparison with those without prior *Chlamydia muridarum* infection (**Figure 9**). In contrast, either RW or polyclonal T-cell (anti-CD3) stimulation (data not shown), the levels of IFN-γ produced by spleen or lymph node cells from *Chlamydia muridarum*-infected, RW-treated mice was significantly higher compared to RW-treated mice without prior *Chlamydia muridarum* infection (**Figure 10**). Similar increases in IFN-γ production in the RW-treated mice with previous *Chlamydia muridarum* infection was observed in BAL samples. Moreover, IL-12 in the lymphocyte culture supernatant of *Chlamydia muridarum*-infected mice was also significantly higher than in mice without infection (**Figure 10**). Based upon these data, there was an obvious increase in the Th1/Th2 ratio in the *Chlamydia muridarum*-infected, RW-treated mice due to both a decrease in Th2 cytokine production (IL-4, IL-5, IL-10, & IL-13), and an increase in Th1 cytokine production (IFN-γ & IL-12) by these mice. These results clearly demonstrate an alteration in the profile of cytokines, both local and systemic, produced in mice that were infected previously with *Chlamydia muridarum* compared to those who were not infected before exposure to RW.

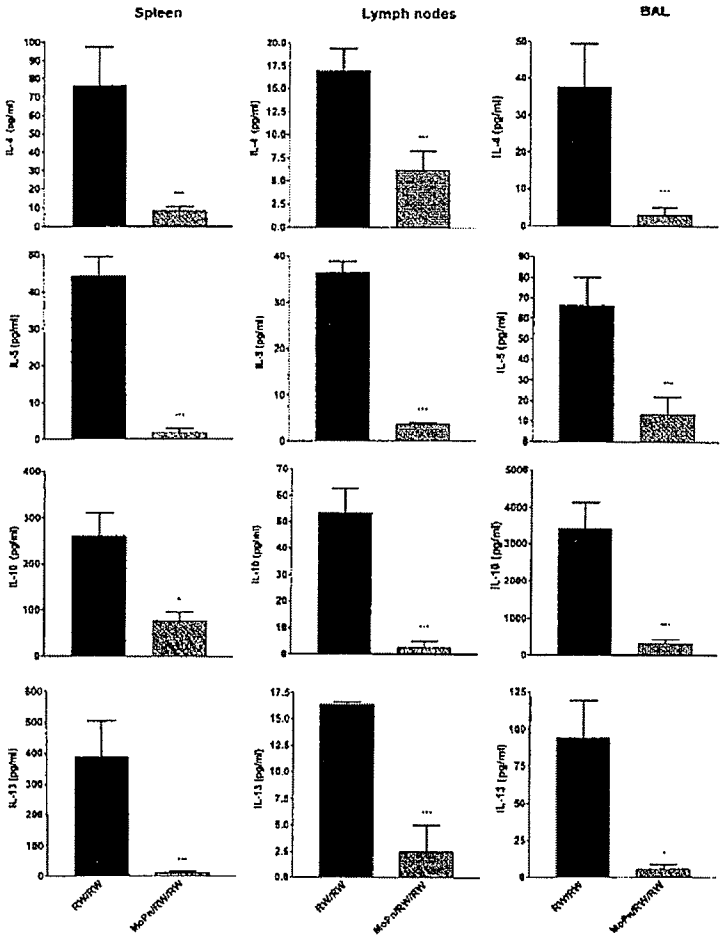

Figure 8. Drastic reduction in Th2 cytokines in spleen and lymph node cell cultures and BAL of *Chlamydia muridarum*-infected, RW-treated mice compared to mice treated with RW only. Mice were infected with 5 x 10^3 or treated with PBS, sensitized with 100μg RW in alum and 14 days later, challenged with 150μg RW and sacrificed at 6 days post-challenge. Spleen and lymph node cells were cultured at 7.5 x 10^6 cells/ml and 5.0 x 10^6 cells/ml, respectively and BAL collected from aseptically acquired lungs. Culture supernatants were harvested at 72 hours and tested for various Th2 cytokines using ELISA. Data are presented as the mean +/- SD of each group. *, $p<0.05$, ***, $p<0.0001$, cytokine production in infected mice versus uninfected mice.

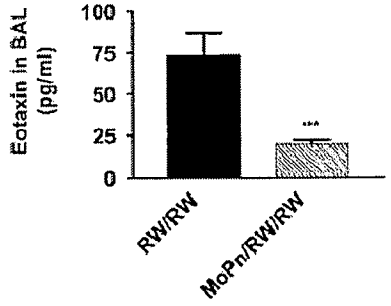

Figure 9. A significant reduction was seen in the eotaxin measured in the BALs of *Chlamydia muridarum*-infected, RW-treated mice compared to mice treated with RW only. Mice (three mice/group) were treated as mentioned in the legend to Figure 8. Six days post-RW challenge, mice were sacrificed and the lungs of mice were flushed with sterile PBS to obtain BALs. Eotaxin in the BALs was measured by ELISA. Data are presented as the means +/- SD of each group. ***, $p<0.0001$, comparison between infected and uninfected mice following RW sensitization/challenge.

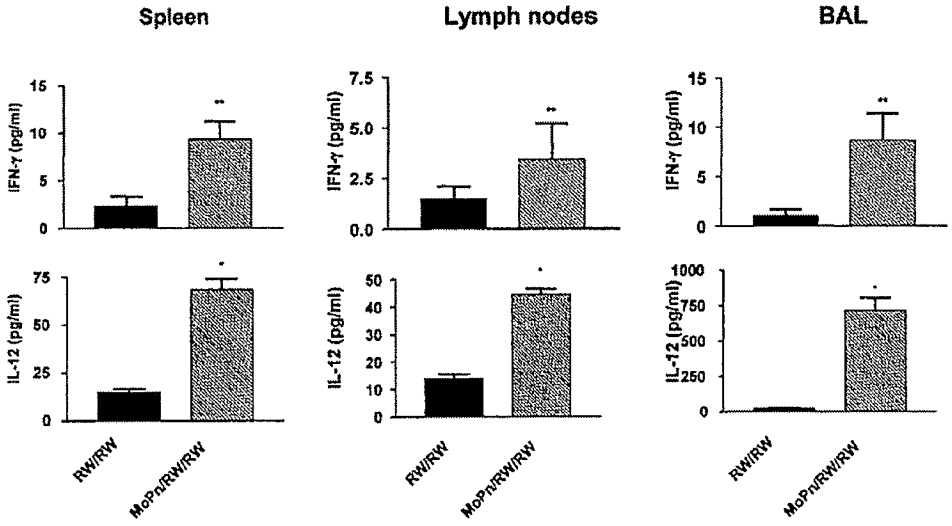

Figure 10. A marked increase was seen in IFN-γ and IL-12 production by both spleen and lymph node cells and in BALs from mice infected with *Chlamydia muridarum* prior to RW sensitization/challenge compared to mice without *Chlamydia muridarum* before RW treatment. Seventy-two-hour culture supernatants of spleen and lymph node cells and BALs as described in the legend to Figure 8 were determined for IL-12 and IFN-γ by ELISA. Data are presented as the means +/- SD of each group. *, $P<0.05$, **, $P<0.001$, cytokine production in mice with previous *Chlamydia muridarum* infection versus mice treated with RW only.

3.1.5 Local *Chlamydia muridarum* infection Alters Cytokine and Chemokine Patterns Induced by Allergen

Serum antibodies specific for the RW allergen were measured in both groups of mice; i.e. *Chlamydia muridarum*-infected, RW-treated mice and mice treated with RW only. When mice from both groups were sacrificed on day 6 post-RW challenge, which is the peak of IgE responses, the level of RW-specific IgE in RW-treated mice was significantly increased ($p<0.05$) compared to the levels in *Chlamydia muridarum*-infected, RW-treated mice. Unexpectedly, the infected and uninfected mice showed comparable levels of RW-specific IgE, IgG1 and IgG2a responses. Interestingly, those mice who had prior *Chlamydia muridarum* infection before RW sensitization/challenge displayed exacerbated levels of total IgE compared to mice treated with RW only (**Figure 11**). The data suggests that previous *Chlamydia muridarum* infection is ineffective at reducing the levels of both total and RW-specific IgE antibodies in the serum.

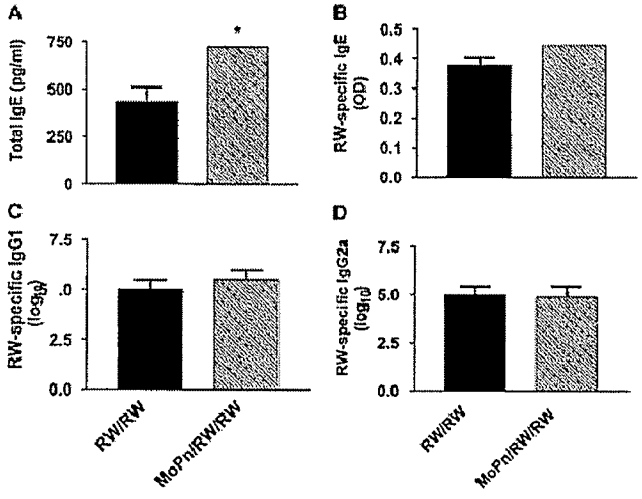

Figure 11. A significant increase was seen in total IgE levels in mice infected with *Chlamydia muridarum* prior to RW sensitization/challenge. Mice (three mice/group) were treated as described in the legend to Figure 8 and were bled on day 6 post-RW challenge. The serum RW-specific IgE, IgG1, IgG2a, and total IgE were determined using ELISA. ELISA titers for RW-specific antibodies were converted to log 10 and presented as the means +/- SEM. *, $P<0.05$, infected versus uninfected mice.

3.1.6 BCG infection Alters Cytokine and Chemokine Patterns, Lung Eosinophilia, and Bronchial Mucus production Induced by Ragweed Allergen and can re-direct the established allergic response

As demonstrated above, the delivery of a Th1-inducing infection such as Chlamydia is capable of inhibiting the development of the asthma-like reaction in a murine model of allergic airway inflammation.

Another model of intracellular bacterial infection was also established in our laboratory, namely *Mycobacterium bovis* BCG. We therefore tested the ability of the *Mycobacterium bovis* BCG to re-direct an already-established allergic phenotype induced by exposure to ragweed allergen.

Mice were sensitized with 100µg RW in conjunction with alum adjuvant and challenged 14 days later with 150µg RW. Following RW challenge, mice were infected with *Mycobacterium bovis* BCG [1.0×10^6 colony-forming units (CFU)] and then further challenged intranasally (i.n.) with 150µg ragweed (40µl) 21 days post-BCG infection. Mice were killed and analyzed for allergic and immune responses 6 days following final allergen challenge.

To directly examine the effect of *Mycobacterium bovis* BCG infection on the established allergic response to RW allergen, a direct comparison was made between Th2-like cytokines and Th1-like cytokines produced by RW-treated mice with or without BCG infection. Spleen and draining lymph node cells from BCG-infected, RW-treated mice re-stimulated with RW allergen *in vitro*, had dramatically decreased Th2-like cytokines, namely IL-4, IL-5, IL-10, and IL-13, were compared to mice with RW treatment only (**Figure 12**).

In contrast, either RW-specific or polyclonal T-cell (anti-CD3) stimulation, the levels of IFN-γ produced by spleen and lymph node cells from BCG-infected, RW-treated mice was significantly higher compared to those RW-treated mice

without BCG infection (**Figure 13**). Moreover, IL-12 in the lymphocyte culture supernatant of *Chlamydia muridarum*-infected mice was also significantly higher than in mice without infection (**Figure 13**).

Serum antibodies specific for the RW allergen were measured in both groups of mice; i.e. BCG-infected, RW-treated mice and mice treated with RW only. Mice from both groups were sacrificed on day 6 post-RW challenge, which is the peak of IgE responses, RW-specific IgE in RW-treated mice was significantly increased ($p<0.05$) compared to the levels of BCG-infected, RW-treated mice. Infected and uninfected mice showed comparable levels of IgG2a responses. Interestingly, mice who had received BCG infection after RW sensitization/challenge displayed exacerbated levels of total RW-specific IgE, RW-specific IgG2a and total IgE compared to those mice treated with RW only (**Figure 14**). The data indicate that BCG infection is ineffective at reducing the levels of both total and RW-specific IgE antibodies in the serum, but rather increases these levels above RW-treated only mice.

These results demonstrate that mice with BCG infection after allergen challenge significantly reduce cellular infiltration into the lung following RW challenge compared to mice treated with RW only, especially in eosinophilic infiltration (**Figure 15**). Eosinophils made up greater than 50% of the total cell number of infiltrating cells into the BAL of the mice treated with RW only, whereas eosinophils in the BCG-infected, RW-treated mice represented <25% of the total BAL cells ($p<0.05$) (**Figure 15**). The level of lymphocytes within the BALs of both groups of mice were comparable.

In addition, there was significantly more goblet cell staining in those mice with RW treatment only, which was abrogated significantly in those mice infected with BCG infection in the midst of established allergic airway inflammation induced by exposure to RW allergen (**Figure 16**).

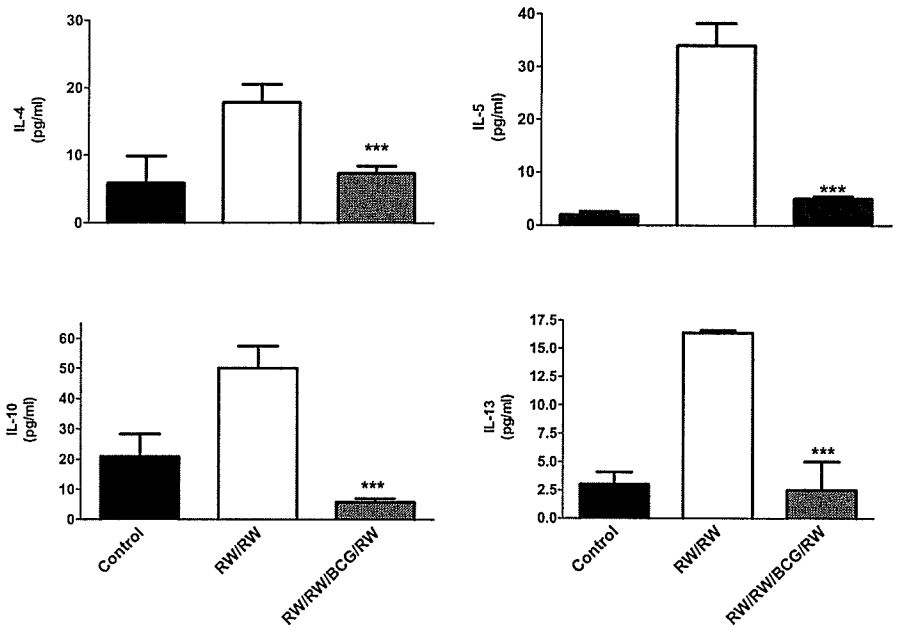

Figure 12. Drastic reduction in Th2 cytokines in lymph node cell cultures of BCG-infected, RW-treated mice compared to mice treated with RW only. Mice were sensitized with 100μg RW in alum and 14 days later, challenged with 150μg RW. Mice were then infected or not with infected with 1 x 10^6 CFUs BCG or treated with PBS, challenged with 150μg RW and sacrificed 6 days post-challenge. Lymph node cells were cultured at 5.0 x 10^6 cells/ml.. Culture supernatants were harvested at 72 hours and tested for various Th2 cytokines using ELISA. Data are presented as the mean +/- SD of each group. *, $p<0.05$, ***, $p<0.0001$, cytokine production in infected mice versus uninfected mice.

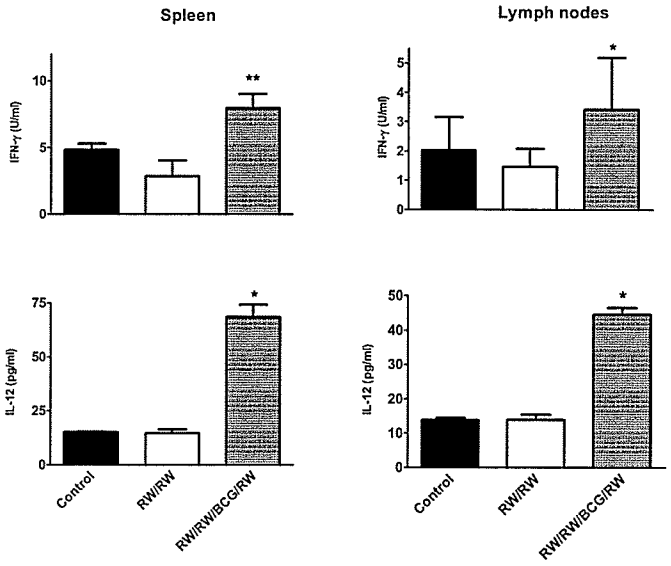

Figure 13. A marked increase was seen in IFN-γ and IL-12 production by both spleen and lymph node cells from mice infected with BCG following to RW sensitization/challenge compared to mice without BCG during established RW allergic inflammation. Seventy-two-hour culture supernatants of spleen and lymph node cells as described in the legend to Figure 8 were determined for IL-12 and IFN-γ by ELISA. Data are presented as the means +/- SD of each group. *, $P<0.05$, **, $P<0.001$, cytokine production in mice with previous BCG infection versus mice treated with RW only.

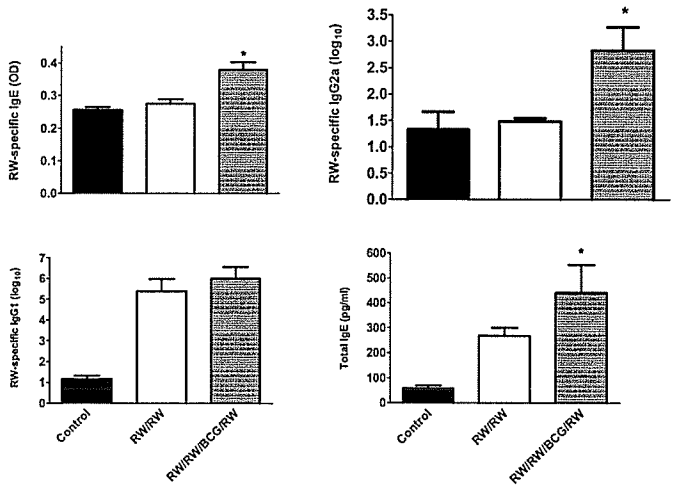

Figure 14. A significant increase was seen in total serum IgE and serum RW-specific IgE levels in mice infected with BCG infection and treated with RW. Mice (three mice/group) were treated as described in the legend to Figure 8 and were bled on day 6 post-RW challenge. The serum RW-specific IgE, IgG1, IgG2a, and total IgE were determined using ELISA. *, $P<0.05$, infected versus uninfected mice.

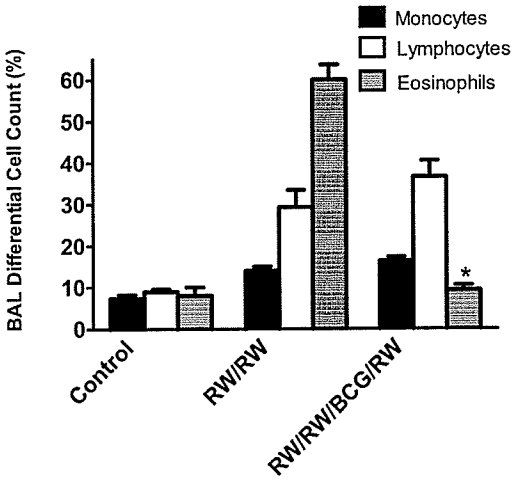

Figure 15. BCG-, RW-treated mice or mice treated with RW alone show a significant reduction in BAL eosinophils following RW sensitization and challenge compared to mice treated with RW only. Mice were sensitized i.p. with 100μg RW in alum and challenged 14 days later with 150μg RW i.n. One group of mice were then infected i.v. with 1.0 x 10^6 CFUs *Mycobacterium bovis* BCG. Mice were challenged with RW (150μg) and sacrificed 6 days post RW re-challenge. Tracheas were cannulated and BAL fluids acquired by washing lungs twice with sterile PBS. BAL cellular components were analyzed by differential cell counts using Fisher Leukostat Stain Kit. *, $P<0.05$, infected versus uninfected mice.

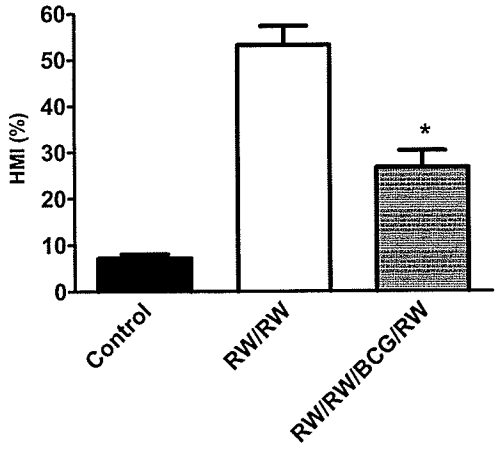

Figure 16. Dramatic reduction in mucus secretion in mice infected with BCG following RW sensitization/challenge. Mice were killed on day 6 post-RW challenge. Lung tissues were fixed and stained for mucus and goblet cells. Histological mucus index (HMI) was calculated based on the percentage of the mucus-positive area over the total area of the airway epithelium. Each group was composed of three mice. Data represent means +/- SD. *, $P<0.05$, comparison between RW/RW-treated mice and RW/RW/BCG/RW-treated mice.

3.1.7 Discussion

Previous studies, in addition to our present study, demonstrate that infection of mice with mycobacteria is capable of suppressing allergen-induced airway eosinophilia and local inflammatory Th2 responses in the lung (Alm, Lilja et al. 1997; Alm, Lilja et al. 1998; Aaby, Shaheen et al. 2000). In this study, we extended and confirmed these observations by demonstrating the alteration of the allergen-induced asthma-like response in mice with previous respiratory tract *Chlamydia trachomatis* (*Chlamydia muridarum*) infection. More importantly, the choice of allergen in this study, namely ragweed, has not cenn previously tested in animal models examining the relationship between allergy and infection. Most studies to date have used mouse models of asthma induced by ovalbumin (OVA) exposure. Compared to OVA, ragweed is a natural allergen existing in the environment and is a major cause of clinical asthma in humans. This portion of this Hygiene Hypothesis-based study was clear in demonstrating both the ability of the intracellular bacterial infection (*Chlamydia muridarum* or BCG) to inhibit the subsequent development of the allergic response induced by the ragweed antigens, especially eosinophilic infiltration and mucus production by goblet cells. Eosinophils have been the focus of many investigative studies of asthma, both in mice and in humans. Airway eosinophils induce inflammatory reactions, with increased surface expression of adhesion molecules and superoxide anion (O_2^-) release. In addition, the mucus overproduction by epithelial goblet cells is another hallmark of the asthmatic reaction, which, as a result, is a major factor in human deaths due to asthma. The reduction in these parameters in infected mice indicates significantly inhibited asthma-like reaction by the following prior infection with Chlamydia or post-allergen challenge infection with BCG. Moreover, Bettiol *et al* reported that not only atopic asthma, but also intrinsic asthma patients displayed increased levels of blood eosinophilia (Bettiol, Bartsch et al. 2000). This is likely due to impaired release of IFN-γ from peripheral blood leukocytes and an increased production of IL-4 compared to healthy, non-atopic subjects (Bettiol, Bartsch et al. 2000). Our murine model is consistent with an elevated level of blood eosinophils in RW treated mice compared to naïve mice, with a significant

reduction in blood eosinophilia numbers with prior *Chlamydia muridarum* infection or the with the delivery of BCG infection during established RW allergy. The inhibitory effect of prior *Chlamydia muridarum* infection or BCG infection on the asthma-like reaction and peripheral eosinophilia induced by RW is evidently well beyond the clearance of infection, as the *Chlamydia muridarum* infection within this strain of mouse (C57BL/6) is cleared within 20-25 days after inoculation in the case of *Chlamydia muridarum*, or beyond 21 days in the case of BCG infection. An interesting finding that has not yet been reported is the ability of either intracellular bacterial infection to reduce the levels of eotaxin in the lungs of allergen-treated mice. This is key as eotaxin is the most important chemokine in eosinophil recruitment. In addition, eotaxin can induce eosinophil degranulation, via CCR3 expressed on the eosinophil cell surface, which may also contribute to eosinophilic inflammation in the airway (Fujisawa, Kato et al. 2000). More recently, it was reported that eotaxin elicits rapid vesicular transport-mediated release of preformed IL-4 from human eosinophils (Bandeira-Melo, Sugiyama et al. 2001). The decrease in eotaxin levels within the lungs of *Chlamydia muridarum*-infected, RW-treated mice or RW-treated, BCG-infected mice may explain the reason for the reduction in eosinophilia within the lungs of these mice. This phenomenon was not observed in other studies using OVA with previous *Chlamydia muridarum* infection (Han, Fan et al. 2004). However, similar reduced airway eosinophilic inflammation was observed. The difference in eotaxin levels suggest that the mechanism by which infection alters airway inflammation may be different depending on the nature of the allergen, thus emphasizing the importance to examine the responses caused by natural common environmental allergens.

Another interesting finding in the present study involves the obvious elevation of both total serum IgE in mice infected with *Chlamydia muridarum* prior to RW sensitization/challenge or with the delivery of BCG during established RW allergy. Numerous studies have concluded that the levels of serum IgE measured in diseases such as asthma is not necessarily a concrete parameter for determining and/or predicting human airway reactivity and hyperresponsiveness (van der Pouw Kraan, Aalberse et al. 1994).

Numerous other studies demonstrated that the level of IgE is not always consistent with asthmatic symptoms and eosinophilic reaction (Wilder, Collie et al. 1999). Some cytokines are capable of exacerbating the level of serum IgE while still significantly decreasing other parameters which are associated with allergic diseases. IL-18, also known as interferon-gamma-inducing factor, is involved in enhancing IFN-γ production and promoting a Th1 response, however Yoshimoto *et al* have clearly shown that IL-18 alone has the potential to stimulate basophils (Yoshimoto, Tsutsui et al. 1999). These cells, upon degranulation, release a variety of chemical mediators such as serotonin and histamine and a set of cytokines including IL-4 and IL-13, which can contribute to class switching of B cells to IgE production. This suggests that IL-18, in conjunction with other pro-inflammatory cytokines could contribute also to the differentiation process of precursor CD4 T cells, working in synergy with IL-4, into the Th2 phenotype. Our previous data have shown that *Chlamydia muridarum* infection induces an IL-18 response (Lu, Yang et al. 2000). Therefore, the previous infection may contribute to the generation of cytokines that might exacerbate IgE production, with reduction of airway eosinophilia. Another potential cellular mechanism which may contribute to both eosinophil reduction and IgE increase is NKT cells. NKT cells appear to be an extremely versatile group of cells that are involved in the promotion of a Th1 reaction induced by intracellular bacterial infection, and the production of Th2 cytokines such as IL-4. It is possible that NKT cells may play a dual role in inhibiting airway eosinophilia by producing IFN-γ and in enhancing IgE production as a result of IL-4 production. Data that supports this unusual finding is discussed and data shown in a later.

The findings in this chapter also suggest that it is possible to re-direct an immune response to allergens that may normally be detrimental to the responding host with an intracellular bacterial infection. The altering effect of infection, suppressing the development of the asthma-like reaction, extends well beyond the time frame of active infection. The establishment of the role of cytokines in the regulation of immune responses provides an explanation for the possible link between increase in allergy and decreases in infection.Some studies have demonstrated an inverse correlation between

childhood infection and the development of asthma in later life (Alm, Lilja et al. 1997; Ball, Castro-Rodriguez et al. 2000; Anderson, Ruggles et al. 2004). It is also reported that the occurrence of allergy within an individual family inversely correlates with the birth order of each sibling and that the youngest child is the least likely to develop an allergic disease (Ball, Castro-Rodriguez et al. 2000; Beasley, Crane et al. 2000). The present study provided experimental evidence that microbial infection may be potent modulators of the allergic process.

3.2 Chapter 2: NKT cells and Allergic Disease.

In this section, we intended to perform a comprehensive investigation into the role of NKT cells in the development of the asthma-like reaction using a common environmental allergen, ragweed (RW). CD1 is constitutively expressed by cortical double-positive $CD4^+8^+$ thymocytes, which positively select for developing NKT cells. CD1 KO mice are thus deficient in NKT cells (Bendelac, Lantz et al. 1995; Bendelac, Rivera et al. 1997; Coles and Raulet 2000). Using a well-characterized asthma-like reaction model (Zhu, Greenstein et al. 1995), we found that, in comparison with BALB/c control mice, the NKT cell-deficient CD1 KO mice (backcrossed to BALB/c for 11 times) showed significantly lower pulmonary eosinophilia and bronchial mucus production following ragweed sensitization and local challenge. In addition, the levels of both RW-specific and total serum IgE antibody responses in the CD1 KO mice were significantly lower than control BALB/c mice following RW sensitization and challenge. The reduction of allergic reaction in CD1 KO mice was associated with an impaired allergen-driven IL-4 production by spleen and draining lymph node cells and reduced eotaxin production in the lung. Moreover, stimulation of NKT activity with α-galactosylceramide (α-GalCer) enhanced airway eosinophilia, IL-4, and eotaxin production in wild-type BALB/c mice, but not in CD1 KO mice. These data provide evidence for the involvement of NKT cells in allergic reaction and in the development of Th2-type immune responses induced by environmental allergens such as ragweed and dysregulated airway inflammation, characteristic of airway allergy.

3.2.1 CD1 KO mice display reduced pulmonary eosinophilia and bronchial mucus production.

Our previous studies using RW allergen have demonstrated that RW is capable of inducing an allergic response and local lung pathology characteristic of the asthma-like reaction (Bilenki, Wang et al. 2002). In order to examine the role of NKT cells in the allergic response induced by RW allergen, CD1 KO mice and BALB/c control mice were analyzed for airway

inflammatory reaction and mucus secretion following RW sensitization and challenge. These two parameters were analyzed because eosinophilic infiltration and mucus oversecretion by goblet cells in the airway epithelium are the classical hallmarks of an asthma-like reaction. CD1 KO mice demonstrated significantly less cellular infiltration into the lung following RW challenge, compared to control BALB/c mice with the same treatment (**Figure 17**). Specifically, the number of infiltrating eosinophils were significantly reduced (4.1×10^5 +/- 0.5 in CD1 KO mice versus 1.6×10^6 +/- 0.6 in BALB/c mice; $P<0.05$). In addition, histological analysis also showed remarkable difference in cellular infiltration to the lung of CD1 KO and control BALB/c mice following RW treatment (**Figure 18**). BALB/c control mice displayed significant infiltration of eosinophils in the bronchial submucosa, alveolar, and perivascular sheaths, accompanied by a small amount of infiltrating monocytes. CD1 KO mice had significantly fewer infiltrating cells, especially eosinophils. In addition, the levels of mucus production within the bronchial epithelium of RW-treated CD1 KO mice were significantly lower than those found in RW-treated BALB/c mice (**Figure 19**). These results demonstrate that CD1 KO mice mount less asthma-like reaction to a natural allergen, implicating a role for NKT cells in the development of allergic pathological reactions in the lung.

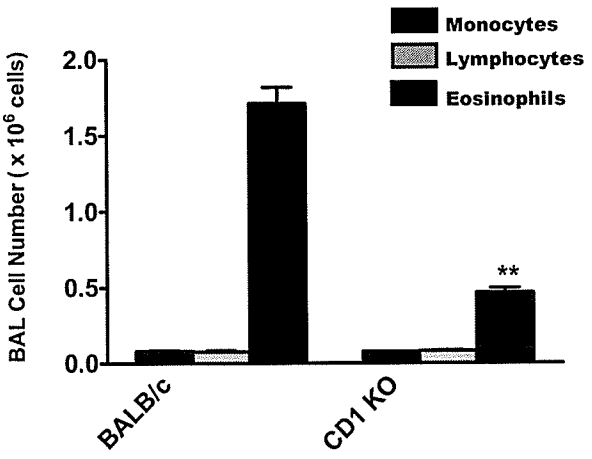

Figure 17. CD1 KO mice show decreased pulmonary eosinophilic infiltration induced by RW. Mice were sensitized intraperitoneally (i.p.) with 100μg RW in alum. The mice were intranasally challenged with 150μg RW at day 14 following RW sensitization and were sacrificed 6 days post-challenge. Pulmonary cellular infiltration was examined by differential cell counts of the bronchoalveolar lavage fluid cells. Data presented represent three independent experiments. **,represents $P<0.05$, CD1 KO versus BALB/c mice.

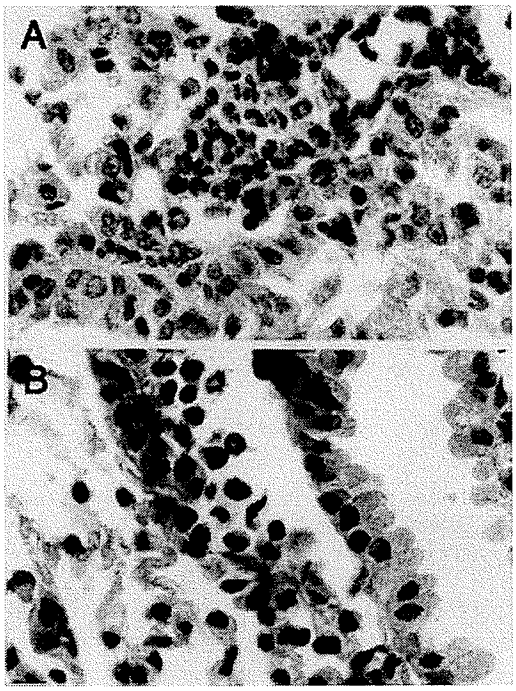

Figure 18. Ragweed-treated CD1 KO mice display a dramatic reduction in infiltrating eosinophils into the lung tissues compared to RW-treated BALB/c mice. Mice were sensitized intraperitoneally (i.p.) with 100μg RW in alum. The mice were intranasally challenged with 150μg RW at day 14 following RW sensitization and were sacrificed 6 days post-challenge. Lungs from BALB/c and CD1 KO mice were aseptically excised and fixed in 10% buffered formalin. Lungs were cut into 10μm thick sections and stained with hematoxylin and eosin (H & E) staining and examined at 400X magnification. Panel A, BALB/c mice; Panel B, CD1 KO mice.

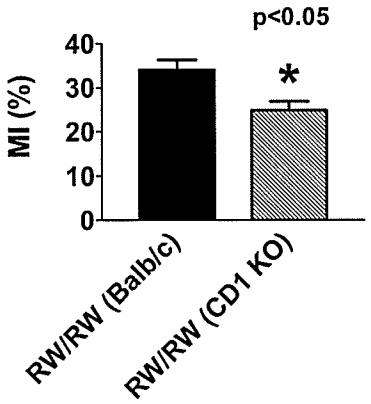

Figure 19. CD1 KO mice show reduced mucus production induced by RW exposure in comparison with BALB/c mice. Mice were treated as described in Figure 3. Mice were sensitized intraperitoneally (i.p.) with 100μg RW in alum. The mice were intranasally challenged with 150μg RW at day 14 following RW sensitization and were sacrificed 6 days post-challenge. Lung tissues were fixed and sections were stained with thionin for mucus and mucus-containing goblet cells. Histological mucus index (HMI) was calculated based on the percentage of the mucus-positive area over the total area of the airway epithelium. Each group was composed of four mice. Data represent means +/- SD. One representative experiment of three independent is shown. *$P< 0.05$, HMI in RW-treated CD1 KO mice versus BALB/c mice treated with RW.

3.2.2 CD1 KO mice exhibit reduced circulating eosinophils following RW exposure.

Airway eosinophilia in asthma is often associated with elevated peripheral blood eosinophil levels, resulting from increased eosinophil production and/or release from the bone marrow. We therefore further tested the impact of NKT cell deficiency on the development of systemic eosinophilia caused by RW exposure. As seen in **Table 2**, although RW exposure induced blood eosinophilia in both BALB/c and CD1 KO mice, the latter showed significantly less increase in blood eosinophils than the former. These results demonstrate that NKT cells are involved in the development of the allergic reaction at both local and systemic levels.

Table 2
Circulating Eosinophils in Peripheral Blood of RW-treated CD1 KO mice versus BALB/c mice treated with RW

	BALB/c	CD1 KO
Lymphocytes	60.0 ± 0.05	64.8 ± 0.37
Neutrophils	38.1 ± 0.69	30.4 ± 0.21
Monocytes	2.91 ± 0.37	4.33 ± 0.41
Eosinophils	**0.650 ± 0.183**	**0.333 ± 0.0318***

Table 2. There is a significant reduction in the circulating eosinophils measured in the peripheral blood of RW-treated CD1 KO mice compared to RW-treated BALB/c mice. Mice (three mice/group) were sensitized with 100μg RW with alum i.p. and were challenged fourteen days later with 150μg RW i.n. Six days post-challenge, all mice were bled, and the blood smears were stained using a Hema 3 Stain Set (Fisher Scientific). Differential cells were counted based on cell characteristics and cellular morphology. *, $P<0.05$, comparison between RW-treated CD1 KO and BALB/c mice. One representative experiment from three independent experiments is shown.

3.2.3 The Absence of NKT cells results in Altered IL-4 and Eotaxin production induced by RW allergen exposure.

In order to examine the mechanism by which NKT cells regulate allergic responses to the RW allergen, we studied the cytokine and chemokine response in RW-treated BALB/c and CD1 KO mice. The results, as shown in **Figure 20**, demonstrated that upon re-stimulation with the RW allergen, both spleen and draining lymph nodes from RW-treated CD1 KO mice produced significantly lower levels of IL-4, a stereotypic Th2-like cytokine, compared with those from RW-treated BALB/c mice. In addition, CD1 KO mice exhibited significantly lower levels of eotaxin, a C-C chemokine crucial for eosinophil recruitment, in their bronchoalveolar lavage fluids following RW challenge (**Figure 21**). Interestingly, CD1 KO mice exhibited comparable levels of allergen-driven Th1-like cytokine (IFN-γ and IL-12) production to BALB/c mice (**Figure 22**). Interestingly, IL-13 and IL-5, which have also been shown to play a role in the maintenance of the allergic response, were not significantly affected in the CD1 KO mice (**Figure 20**). Thus, NKT cells play a critical role in allergen-driven IL-4 and eotaxin production.

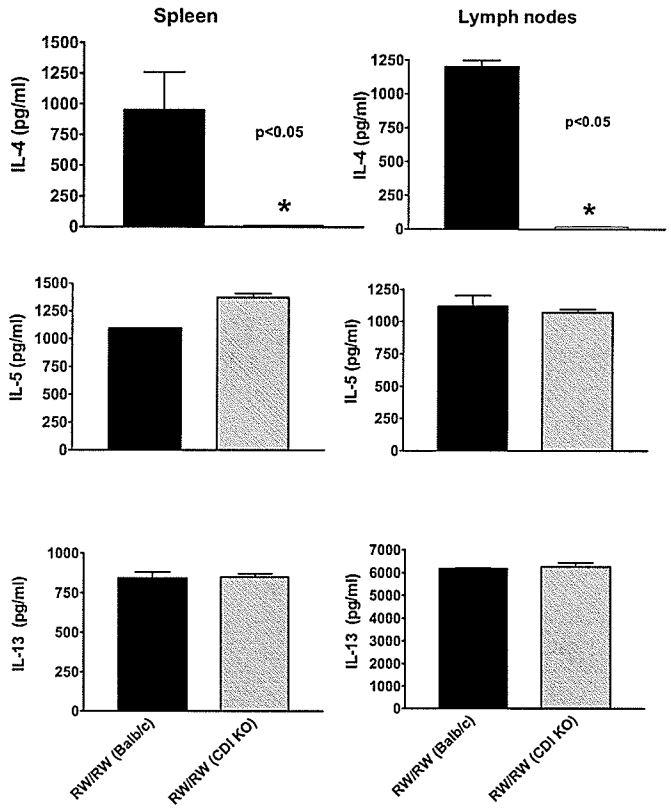

Figure 20. Mice (three mice/group) were sensitized with 100μg RW with alum i.p. and were challenged fourteen days later with 150μg RW i.n. Six days post-challenge, local lymph node cells (peribronchial) and spleen cells from RW-treated BALB/c and CD1 KO mice were cultured in 48-well plates in the presence or absence of 100μg/ml ragweed extract Ag or 1μg/ml of anti-CD3e antibody. Supernatants were collected 72 hours after stimulation with cytokine secretions measured by ELISA. Data presented are mean ± SEM of duplicate wells from 3 mice per group. *, $p<0.05$.

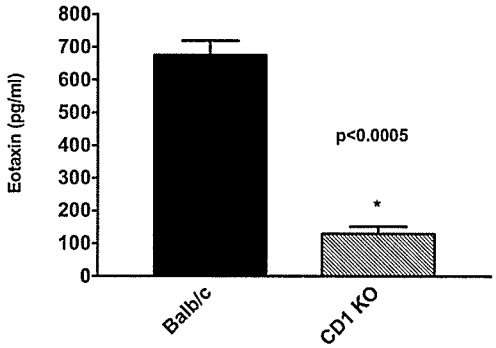

Figure 21. Mice (three mice/group) were sensitized with 100μg RW with alum i.p. and were challenged fourteen days later with 150μg RW i.n.. For the determination of eotaxin, lungs were washed twice with PBS. Bronchoalveolar lavage fluid was centrifuged and supernatants were collected. Eotaxin levels were measured by ELISA. *, $P<0.0005$, eotaxin production in RW-treated CD1 KO mice versus RW-treated BALB/c mice. One representative experiment of three independent experiments is shown. Data presented are mean ± SEM of duplicate wells from 3 mice per group.

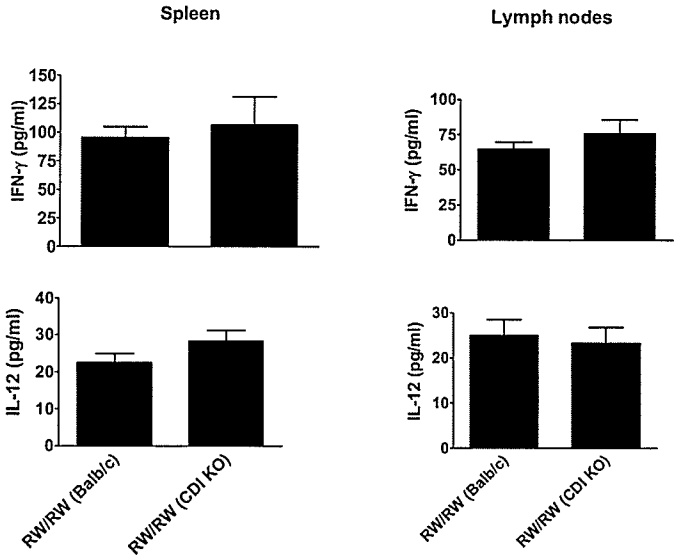

Figure 22. Mice (three mice/group) were sensitized with 100μg RW with alum i.p. and were challenged fourteen days later with 150μg RW i.n.. Local lymph node cells (peribronchial) and spleen cells from RW-treated BALB/c and RW-treated CD1 KO mice were cultured in 48- well plates in the presence or absence of 100μg/ml ragweed extract Ag or 1μg/ml of anti-CD3e antibody. Supernatants were collected 72 hours after stimulation with cytokine secretions measured by ELISA. One representative experiment of three independent experiments is shown. Data presented are mean ± SEM of duplicate wells from 3 mice per group.

3.2.4 CD1 KO and wild-type BALB/c mice display comparable levels of lymphocytes number and function.

To exclude the possibility that the difference observed between CD1 KO and BALB/c mice is caused by an intrinsic defect in other immune cells of the former mice rather than due to NKT deficiency, we analyzed the frequency of immune cells including conventional T lymphocytes, namely CD3-, CD4-, CD8-positive cells, and B lymphocytes; i.e. CD19-expressing cells in NKT cell-deficient CD1 KO mice. As shown in **Figure 23**, the percentage of CD3-, CD4-, CD8-, and CD19-positive cells found in the spleen of CD1 KO mice was comparable to those found in the BALB/c control mice. In addition, the levels of NK (DX5+) cells and γδ T cells were also comparable between these mice (**Figure 23**). We next examined whether the function of lymphocytes from CD1 KO mice are functionally intact. Spleen cells from both naïve BALB/c wild-type mice and CD1 KO mice were cultured alone or in the presence of a

polyclonal activator, ConA. Following ConA stimulation, spleen cells from CD1 KO and wild-type mice showed comparable levels of proliferative reaction (**Figure 24A**) and IL-4 production (**Figure 24B**). Therefore, although the CD1 KO mice are deficient in NKT cells, their key immune cells, namely CD3-, CD4-, CD8-, and CD19-expressing lymphocytes appear normal in frequency and function. The data suggest that the reduction of allergic reaction in CD1 KO mice is due to the deficiency of NKT cells rather than defect in conventional immune cells.

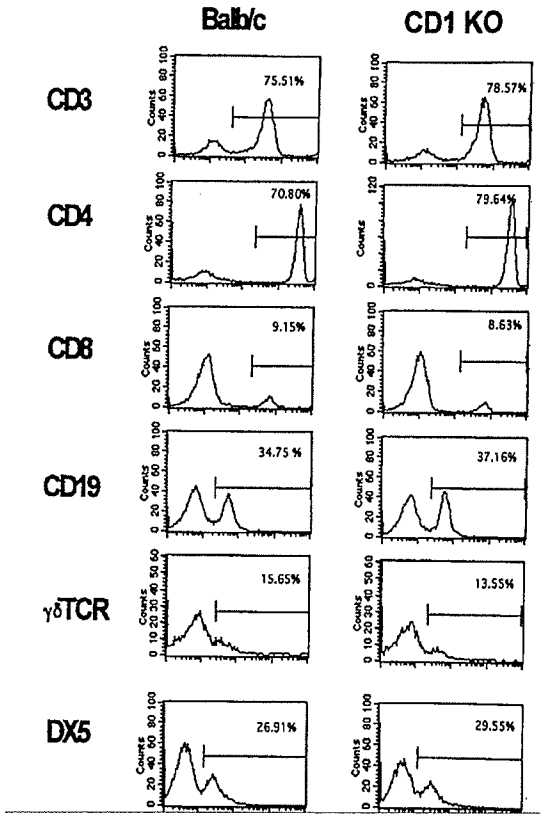

Figure 23. Naïve CD1 KO mice display comparable levels of CD3-, CD4-, CD8-, CD19- (B cells), γδ TcR-, and DX5- (NK cells)-positive cells compared to BALB/c control mice. Single spleen cell suspensions from naïve CD1 KO and BALB/c mice were stained for T, B, and NK cell markers using fluorescence-labeled monoclonal antibodies, followed by fixation with 4% paraformaldehyde. Cells were analyzed by flow cytometry. Data presented represent three independent experiments using a FACSCaliber II and CellQuest program. The figure shows the percentage of each cell type.

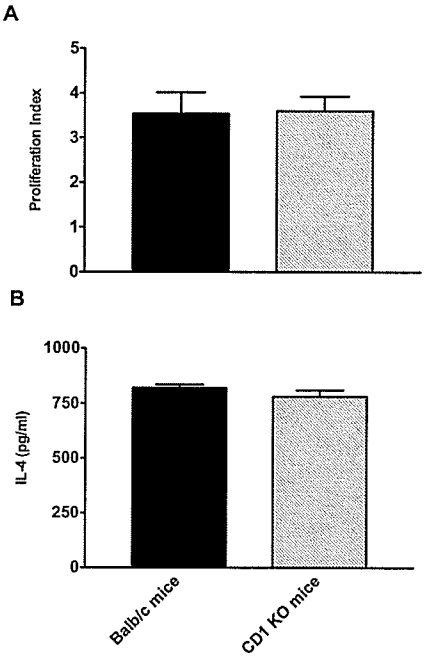

Figure 24. Lymphocytes from CD1 KO mice show normal levels of cellular proliferation and IL-4 production following polyclonal stimulation. Spleens from naïve CD1 KO and BALB/c mice were aseptically removed. Spleen cells were cultured in the presence or absence of ConA polyclonal stimulation. **(A)** ConA-stimulated cells were pulsed with [^{3}H]thymidine and [^{3}H]thymidine incorporation was assessed using a TopCount NXT microplate scintillation and luminescence counter. Simulation index (SI) was calculated based on the fold of counts un Con A-stimulated cells over the control wells. **(B)** IL-4 levels in the 72 h-culture supernatants were measured by ELISA.

3.2.5 Stimulation of NKT activity with α-GalCer (KRN7000) enhanced allergic reactions to RW exposure, which was correlated with enhanced IL-4 and eotaxin production.

To confirm the role of NKT cells in the development of allergic reaction, we further tested the effect of α-galactosylceramide (α-GalCer) treatment on the allergic reaction in wild-type BALB/c and CD1 KO mice. α-GalCer is a natural ligand of NKT cells, which can specifically stimulate NKT cell activity *in vivo* (Burdin, Brossay et al. 1999; Cui, Watanabe et al. 1999). As shown in **Figure 25**, BALB/c mice treated with α-GalCer (KRN7000) showed dramatically enhanced allergen-driven IL-4 production by lymphocytes, eotaxin expression in airway tissues, RW-specific IgE production and eosinophil infiltration in the

BAL. Similar responses to α-GalCer stimulation were observed in wild-type 129 mice (data not shown). In contrast, CD1 KO mice, which are deficient in NKT cells, failed to respond to α-GalCer treatment in the perspectives of IL-4, eotaxin and RW-specific IgE production and airway eosinophilic infiltration. Therefore, the enhanced effect on the allergic reaction and cytokine (IL-4)/chemokine (eotaxin) production seen in wild-type mice following α-GalCer treatment reflect the *in vivo* function of NKT cells. This experiment, by enhancing NKT activity, confirms the finding obtained from the experiments using NKT deficient mice as shown above, reinforcing a critical role played by NKT cells in the development of the allergic reaction. Moreover, the increase in IL-4 and eotaxin production and enhancement of the allergic reaction (eosinophilia and IgE production) by *in vivo* activation of NKT cells demonstrate that NKT cells play a role in the development of the allergic response, possibly by enhancing IL-4 and eotaxin production.

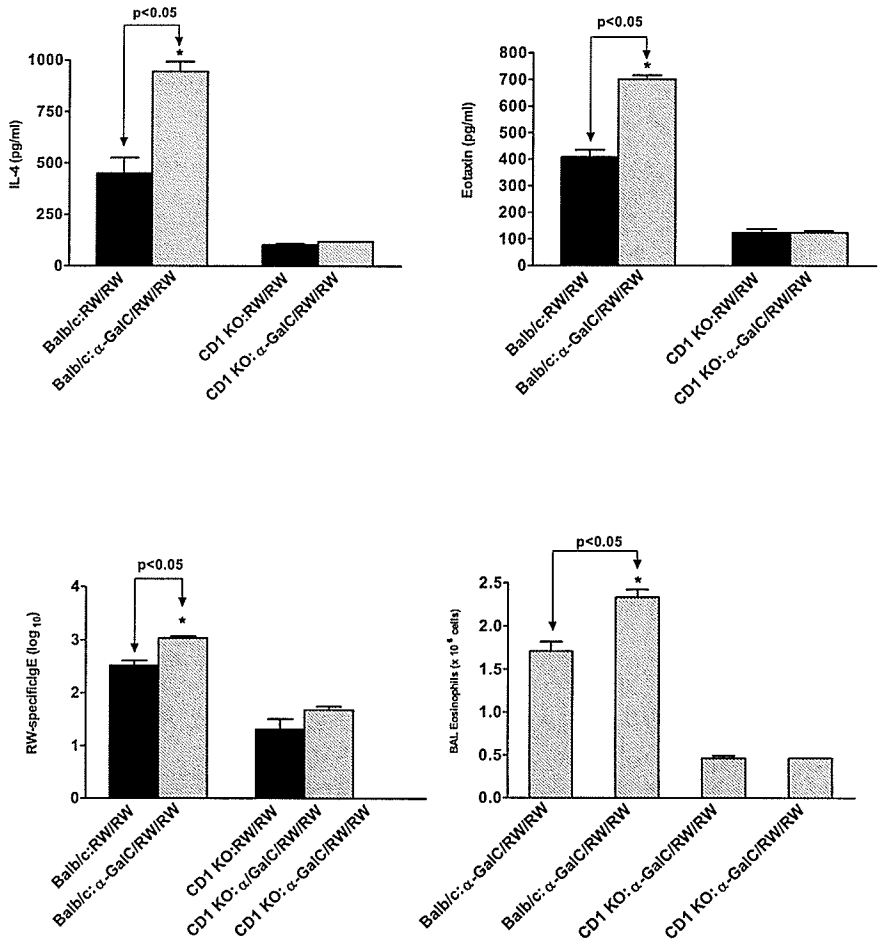

Figure 25. *In vivo* activation of NKT cells results in exacerbated allergen-driven IL-4 production, eotaxin expression, RW-specific IgE measurements, and eosinophilia in wild-type BALB/c mice compared to CD1 KO mice. Naïve CD1 KO and BALB/c mice were injected i.v. with 4μg α-GalCer in PBS or polysorbate vehicle control. Mice were sensitized 2 h following α-GalCer or vehicle injection with RW and challenged. Mice were sacrificed six days post-challenge and spleens were cultured in the presence of RW for allergen-driven IL-4 measurements. BAL fluid was collected and used to measure eotaxin and eosinophils levels, and sera were measured for RW-specific IgE levels. Data are presented as the means +/- SD of each group. One representative experiment of four independent experiments is shown.

3.2.6 Discussion.

This section, using a common environmental allergen, ragweed, provides evidence that NKT cells play an important role in the outcome of the allergic responses. Airway eosinophilic inflammation induced by RW was significantly reduced in NKT cell deficient CD1 KO mice, which is with impaired IL-4 and

eotaxin production. CD1 KO mice also displayed a significant decrease in serum allergen-specific and total IgE levels in their sera compared to BALB/c mice. The difference between CD1 KO and wild type BALB/c mice is unlikely due to potential intrinsic deficiencies of CD1 KO mice in the development of immune cells as the frequency of T and B cells in the two types of mice are comparable (**Figure 23**) and more importantly, lymphocytes from both strains of naïve mice showed similar levels of cell proliferation and cytokine production upon polyclonal stimulation (**Figures 24A & 24B**). The inability of CD1 KO mice to produce IL-4 in response to allergen-specific re-stimulation provides evidence that NKT cells are responsible for the development of IL-4 responses to natural allergen, capable of affecting downstream allergic disease parameters. In contrast, the Th1 arm of the immune response (IFN-γ and IL-12) appears unaffected by the lack of NKT cells in our model. The explanation for such a phenomenon may be two-fold. Firstly, the study model is an asthma-like reaction, which is dominated by Th2-like responses, therefore the impact of NKT deficiency may be more visible with regards to the parameters of Th2-type responses. Notably, the allergen-driven IFN-γ production even in wild-type mice is very low. Secondly, it is possible that NKT cells play a more dominant role in IL-4 responses, although they are capable of producing IFN-γ in certain circumstances (Apostolou, Takahama et al. 1999). Th2 cytokines other than IL-4, such as IL-5 and IL-13, remain largely unaffected by the absence of NKT cells in the allergen-treated CD1 KO mice (**Figure 20**), suggesting that it is likely that NKT cells are particularly critical for allergen-driven IL-4 responses.

Another novel finding is the dependence on NKT cells for eotaxin production. Several Th2 cytokines such as IL-4, IL-5, and IL-13 may be able to promote eotaxin production. The present study, however, showed that allergen-driven IL-5 and IL-13 production in CD1 KO mice were normal, while eotaxin production in these mice was significantly reduced, which paralleled with significantly decreased allergen-driven IL-4 production (**Figure 21**). The association between NKT cells and reduced allergen-driven IL-4 and eotaxin production was also confirmed in the study involving α-GalCer treatment, which showed that *in vivo* stimulation of NKT cells with α-GalCer significantly

increased both IL-4 and eotaxin responses (**Figure 25**). It has been reported that IL-4 can bind to its receptors expressed on the surface of airway epithelial cells and induce eotaxin production (Romagnani 2002). The results in the present study suggest that NKT cells play a particularly important role in eotaxin production through either a direct effect or indirect mechanisms such as enhancing IL-4 production.

A novel but not surprising finding in this section study is the association between NKT cells and mucus overproduction by epithelial goblet cells following allergen exposure. Mucus overproduction by goblet cells within the airway epithelial layer is a classical hallmark of the asthma-like reaction and is a major factor in human asthmatic deaths. There are no previous links established between mucus production by epithelial goblet cells and NKT cells in an allergic response, thus the present data represent a key piece of evidence that implicates the role played by NKT cells in the development of the multiple components of allergic disease pathology. This finding is particularly interesting because previous studies have shown that IL-13 plays a critical role in mucus production (Shim, Dabbagh et al. 2001; Kuperman, Huang et al. 2002). The present study, however, showed that, although IL-13 levels are comparable between CD1 KO and control mice, the former exhibited significantly lower mucus production. The data suggest that factors other than IL-13, which are directly or indirectly related to NKT cells, may independently enhance allergen-driven mucus overproduction.

The findings in the present study has provided *in vivo* evidence that NKT cells play an important role in the development of allergic responses. The demonstration of the involvement of NKT cell in allergic responses extends our understanding on the function of NKT cell in modulating immune responses in natural diseases. The use of the ragweed allergen in this model is particularly significant as many atopic individuals suffer seasonal allergies as a result of the abundance of RW in the environment and some studies on NKT cells have used less relevant allergens.

3.3 Chapter 3: Chlamydia Infection and NKT cells

Functional studies on NKT have suggested an important role of these cells in immunoregulation. NKT cells are reported to be involved in the promotion of both Th1-like reaction (Singh, Wilson et al. 2001), as well as the production of Th2 cytokines (Leite-De-Moraes, Hameg et al. 2001; Kukreja and Maclaren 2002). They appear to have essential roles in tumour rejection as well as the prevention of the development of autoimmune diseases (Smyth, Thia et al. 2000; Yang, Bao et al. 2001). Moreover, a role of NKT in host defense against infections has been documented in virus, parasite and bacterial infection models (Apostolou, Takahama et al. 1999; Gonzalez-Aseguinolaza, de Oliveira et al. 2000; Kakimi, Guidotti et al. 2000; Exley, Bigley et al. 2001; Grubor-Bauk, Simmons et al. 2003). For example, studies on NKT with α-GalCer inhibited hepatitis B viral replication in HBV transgenic mice (Kakimi, Guidotti et al. 2000) while CD1 KO mice showed substantial impairment in antiviral response to encephalomyocarditis virus infection (Exley, Bigley et al. 2001) and herpes simplex type 1 virus infections (Grubor-Bauk, Simmons et al. 2003). Moreover, α-GalCer treatment significantly enhanced protective anti-malaria immunity (Gonzalez-Aseguinolaza, de Oliveira et al. 2000) and reduced bacterial burden and tissue injury in a *Mycobacterium tuberculosis* mouse models (Apostolou, Takahama et al. 1999). Furthermore, CD1 KO mice are less resistant to *Borrelia burgdorferi* infection (Kumar, Belperron et al. 2000) and showed impaired eradication of *Pseudomonas aeruginosa* infection (Nieuwenhuis, Matsumoto et al. 2002). These studies strongly suggest a protective role of NKT in host defence against infectious diseases.

As an obligate intracellular bacterial pathogen, *Chlamydia trachomatis* is a major cause of sexually transmitted diseases, blindness, and infertility in humans (Brunham, Zhang et al. 2000). A clear understanding of the adaptive and innate immune responses to chlamydial infection is critical in the rational development of an effective vaccine to this infection. The studies on the role played by distinct CD4 T cell subsets and the cytokines have provided insight into the mechanisms of the adaptive immune response to chlamydial

infection. The difference in the cytokine patterns have been correlated with the severity of disease progression in both human subjects and animal models. In particular, Th1 responses have been found to be associated with protection while Th2 responses are associated with immunopathology {Holland, 1996 #587; Su, 1995 #588; Yang, 1999 #244; Wang, 1999 #589). So far, there is no report on the role of NKT cells in chlamydial infection and the effect of NKT on adaptive immune responses to chlamydial infection. In the present study, we examined the role of NKT in chlamydial lung infection using two approaches. Firstly, we used CD1 gene knockout (KO) mice, which lack NKT cells, and secondly, we treated mice with α-galactosylceramide (α-GalCer), a natural ligand of NKT cell, to stimulate these cells.

3.3.1 CD1 KO mice show reduced morbidity, lower chlamydial *in vivo* growth, and less pathological changes in the lung.

To examine the role of NKT cells in the susceptibility to chlamydial infection, CD1 KO and BALB/c wild-type mice were intranasally infected with a sublethal dose (1 x 10^3 IFU) of *C. muridarum* and monitored for body weight changes and *in vivo* growth of the organism. As shown in **Figure 26 A,** the body weight loss was significantly less in CD1 KO mice than wild-type mice. The overall physical condition of CD1 KO mice was also better (i.e. more activity, less fur ruffling and dehydration) than that of wild-type controls. The chlamydial levels in the lung in CD1 KO mice was close to 100-fold lower than control mice (**Figure 26 B & C**). The same is true for the chlamydial burdens in the liver. More importantly, although wild-type mice showed detectable level of *Chlamydia* in the heart, CD1 KO mice consistently exhibited negativity of *Chlamydia* in this organ. Immunofluorescence staining of the lung sections also showed less chlamydial inclusions in the lung tissues of CD1KO mice than BALB/c mice (**Figure 27 C, F).** Interestingly, the size of the inclusions in the lung were significantly smaller in CD1 KO mice than in wild-type mice **(Figure 27 C, F).** The results indicate higher resistance of CD1 KO mice to chlamydial infection, including the dissemination of the infection.

The histological analysis showed differential changes in the lung in the two types of mice (**Figure 27**). BALB/c mice showed severe tissue inflammation

with inflammatory exudates and diffused cellular infiltration in the lung, indicative of a significant pathological reaction. The cellular infiltrates were not only comprised of lymphocytes and macrophages, but also a significant portion of neutrophils (**Figure 27 B**). In contrast, in the CD1 KO mice, the inflammatory changes in the lungs were much less severe, characterized by more localized and less dense cellular infiltration with mainly macrophages and lymphocytes (**Figure 27 E**). Neutrophils were scarcely seen in the lung of infected CD1 KO mice. These observations demonstrate that CD1 KO mice suffer less severe tissue damage during the process of chlamydial infection.

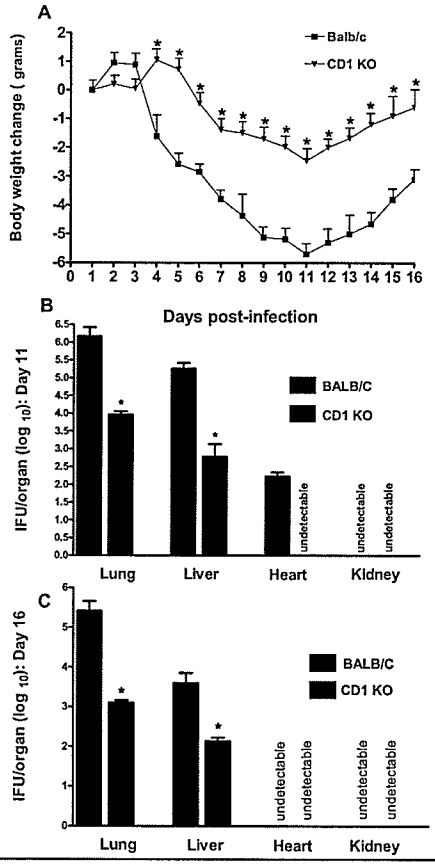

Figure 26. Less body weight loss (**A**) and chlamydial growth *in vivo* (**B & C**) after chlamydial infection in CD1 KO mice than in control infected BALB/c mice. Mice were intranasally infected with *C. muridarum* (1000 IFUs) and were monitored daily for body weight changes. Each point represents the mean +/- SD of three mice. The original body weights of the mice were similar between the groups. Mice were sacrificed on days 11 and 16 in panels B & C, respectively, post-infection, and the different organs (lung, liver, heart, and kidney) were analyzed for *in vivo* chlamydial as described in *Materials and Methods*. One representative experiment of three independent experiments with similar results is shown. *, $P<0.05$, KO versus BALB/c mice.

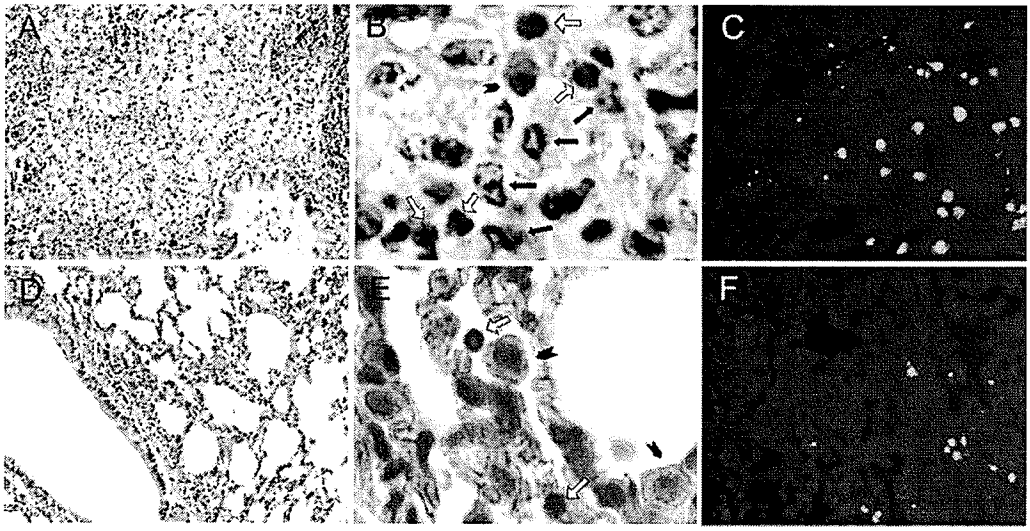

Figure 27. **CD1 KO mice exhibit less inflammatory responses and chlamydial growth in the lung than wild-type BALB/c mice.** The lungs from mice intranasally infected with *C. muridarum* (1000 IFU) at day 11 post-infection were analyzed for histological changes by H&E staining (Panels A, B, D & E) and chlamydial inclusions by immunofluorescence using *C. muridarum* major outer membrane protein-specific mAb (Panels C & F) as described in *Materials and Methods*. Top panel shows BALB/c mice. Bottom panel shows CD1 KO mice. Graphs A&D are at the magnification of 200x and the rest of the graphs are at the magnification of 400x. Graphs C & F, yellow color represents chlamydial inclusions in the lung. In graphs B&E, open arrows indicate lymphocytes; thin black arrows indicate neutrophils; and wide black arrows indicate macrophages.

3.3.2 CD1 KO mice exhibit reduced *C. muridarum*-specific Th2 cytokine production.

To examine the effect of NKT activation on T cell responses to chlamydial infection, we analyzed the *C. muridarum*-driven cytokine production by spleen and lymph node cells from infected CD1 KO and BALB/c mice. The results revealed that both spleen and lymph node cells of *C. muridarum*-infected CD1 KO mice produced significantly lower levels of IL-4 and IL-5 compared to that of the infected wild-type mice. The production of other Th2-like cytokines, such as IL-13 and IL-10, were also reduced in the *C. muridarum*-infected CD1 KO mice, although the differences were not statistically significant (**Figure 28**). Analysis of Th1-like cytokines showed similar levels of *C. muridarum*-driven IFN-γ production between CD1 KO and wild-type mice. Similarly, the two strains of mice showed similar levels of pro-Th1 (IL-12 and IL-18) and

pro-inflammatory cytokine (TNF-α) production **(Figure 29)**. Our results suggest that NKT cells promote a Th2-type cytokine response to chlamydial infection.

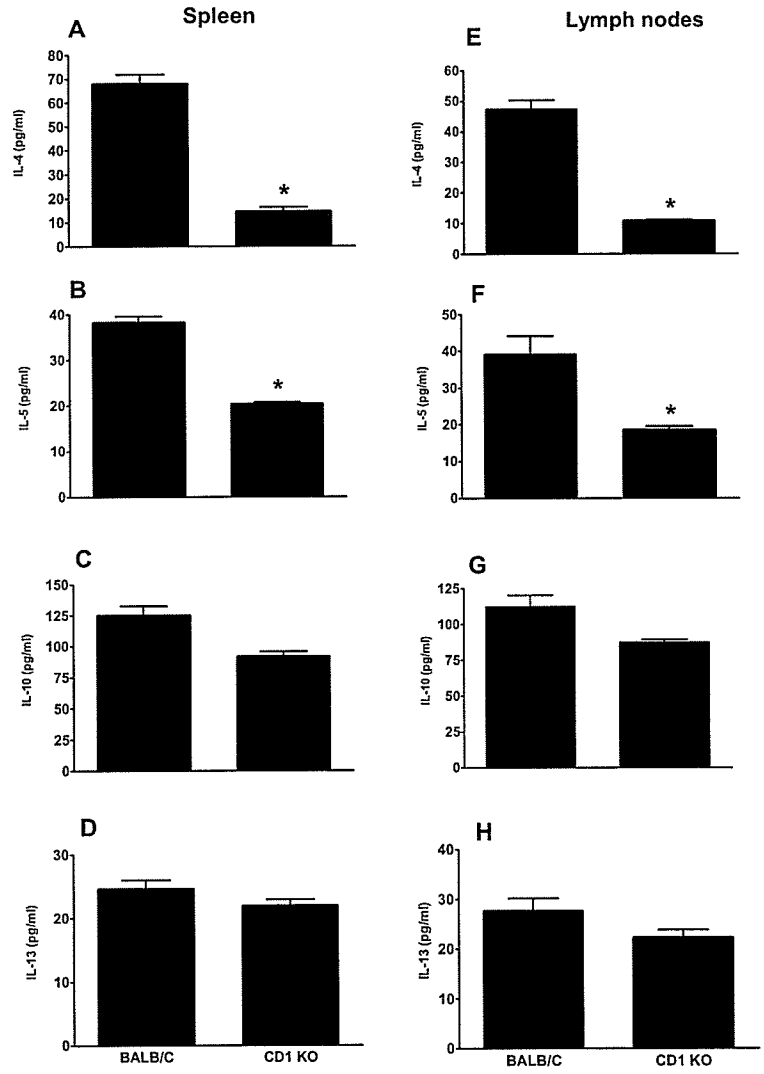

Figure 28. Lower C. muridarum-driven Th2 cytokine production in CD1 KO mice compared to BALB/c mice. Mice (three mice/group) were infected intranasally with *C. muridarum* as described in the legend to Fig.3, and were sacrificed 11 days post-infection. Spleen and lymph node cells were cultured with heat-inactivated *C. muridarum* as described in *Materials and Methods*. Th2 cytokines (IL-4, IL-5, IL-10 and IL-12) in 72h culture supernatants were determined by ELISA. Data are presented as the mean +/- SD of each group. *, $p<0.05$, cytokine production in *C. muridarum*-infected CD1 KO mice versus BALB/c mice. One representative experiment of three independent experiments is shown.

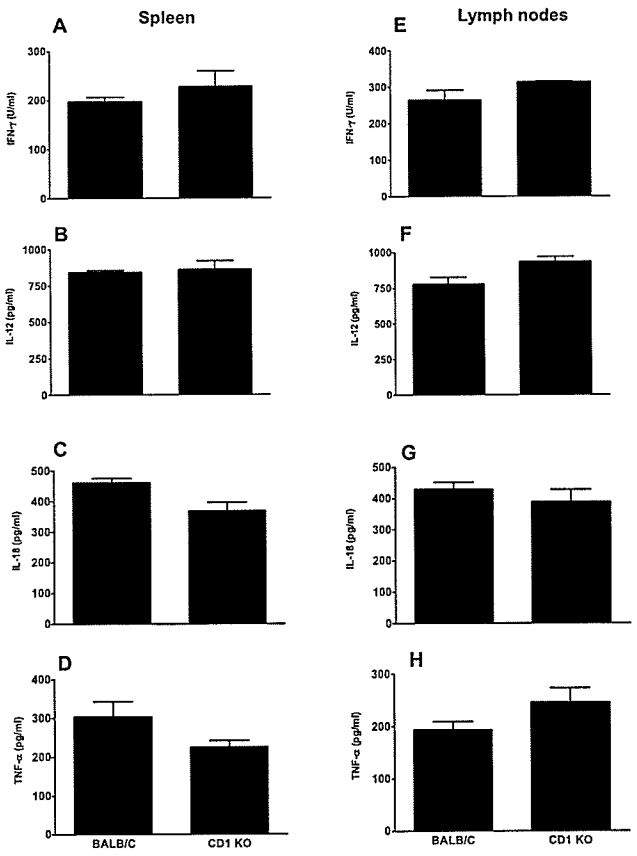

Figure 29. **Similar Th1 and pro-inflammatory cytokine production by spleen and lymph node cells from C. muridarum-infected CD1 KO mice and BALB/c control mice.** Cytokine determination for IFN-γ, IL-12, IL-18 and TNFα was performed with 72h culture supernatants from the experiments shown in Figure 28 by ELISA. Data are presented as the mean +/- SD of each group. One representative experiment of three independent experiments is shown.

3.3.3 CD1 KO mice show significantly lower serum *C. muridarum*-specific IgE and IgG1 antibody responses.

To see if changes in cytokine responses influenced humoral responses, we then measured the levels of total IgE as well as *C. muridarum*-specific IgE, *C. muridarum*-specific IgG1 and IgG2a in serum samples from CD1 KO and BALB/c control mice prior to and after chlamydial infection. Following infection, the levels of total IgE, as well as *C. muridarum*-specific IgE were increased in CD1 KO and BALB/c mice, but the levels of IgE antibodies were significantly lower ($P<0.05$) in CD1 KO mice than wild-type mice (**Figure 30 A & B**). While the *C. muridarum*-specific serum IgG2a levels were similar between the two groups of mice, the IgG1 levels were significantly lower in the CD1 KO mice than in the wild type mice (**Figure 30 C & D**). The results indicate that the production of antibody isotypes which are regulated by Th2 cytokines, especially IL-4 were significantly affected in NKT deficient CD1 KO mice.

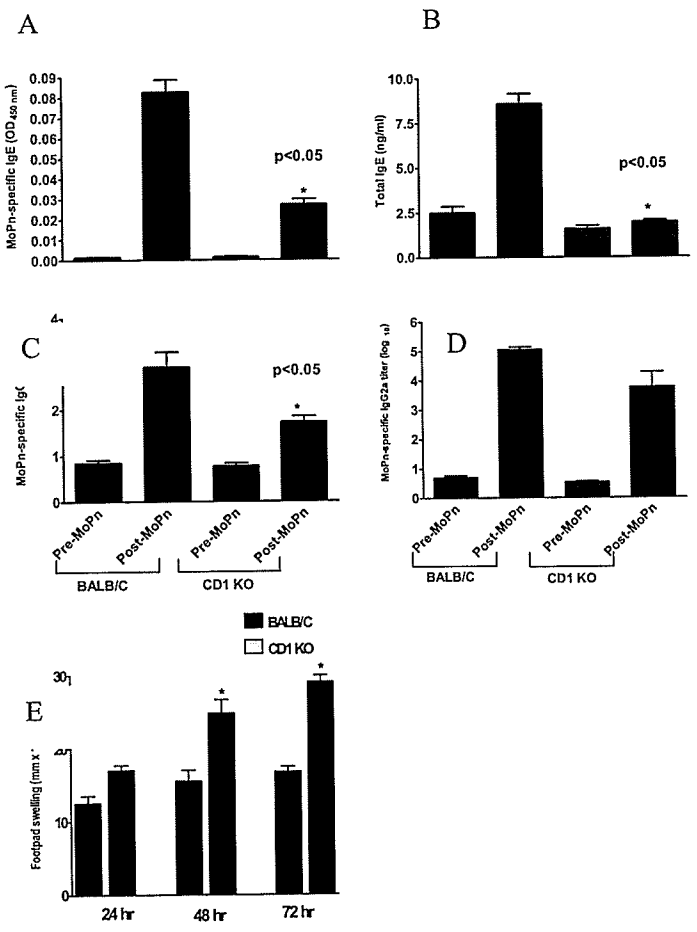

Figure 30. **Significantly lower levels of IgE and IgG1 production and stronger DTH responses in CD1 KO mice compared to control BALB/c mice.** Top two panels: the sera collected from mice (three mice/group) prior to and post (day 11) intransal *C. muridarum* infection (1000 IFUs) were determined for total IgE, *C. muridarum*-specific IgE, IgG1 and IgG2a using ELISA. For detection of *C. muridarum*-specific IgE, the sera were pre-treated twice with 50% slurry of protein G sepharose to remove IgG. Untreated samples were tested for *C. muridarum*-specific IgG2a and IgG1. ELISA readings (optical density, OD) at proper dilutions are presented as the mean +/- SEM. *, $p< 0.05$, *C. muridarum*-infected CD1 KO mice versus BALB/c control mice infected with *C. muridarum*. Pooled data for three experiments (3 mice in each group) are presented. For DTH response (bottom panel), mice (three mice/group) were intranasally infected with *C. muridarum* (1000 IFU) and challenged with heat-inactivated *C. muridarum* in the footpads at 11 days post-infection. Footpad swelling was measured at 24, 48, and 72 h following dead *C. muridarum* injection. The difference in the thickness of footpads with or without *C. muridarum* injection was used as a measure of DTH responses as described in *Material and Methods*. Data show the mean +/- SD. * represents $p<0.05$, comparison between CD1 KO and wild-type mice. One of three independent experiments with similar results is shown.

3.3.4 CD1 KO mice exhibit enhanced DTH response after *C. muridarum* infection.

To examine whether the increased resistance to chlamydial infection observed in CD1 KO mice was correlated with alterations in cell-mediated immune responses, we tested DTH responses in these mice following infection. While DTH response could be observed in both CD1 KO and wild-type mice, the levels of these responses were markedly stronger in the former than in the latter. Statistically significant enhancement in DTH responses in CD1 KO mice was determined at 48hrs and 72hrs following footpad challenge with heat-inactivated EBs (**Figure 30 E**). The data suggest that NKT cells play an immunoregulatory role in T cell-mediated immune responses *in vivo* during chlamydial infection.

3.3.5 Similar differences between CD1 KO mice and wild-type controls are observed in experiments using 129 mice.

As CD1 KO mice used in the study were originally developed in a 129 background, they may still carry some 129 background genes despite being backcrossed to BALB/c for 11 generations. Furthermore, previous studies have shown that different mouse strains are variable in susceptibility to chlamydial infection {Yang, 1996 #34; Darville, 2001 #590; Ramsey, 2001 #591}, this it is important to exclude the possibility that the differences observed between CD1 KO mice and BALB/c controls shown above are caused by the potential slight differences of the mice in genetic backgrounds. To this end, we also performed some experiments using 129 mice as controls. As shown in **Figure 31**, similar to the differences observed between CD1 KO and BALB/c mice, a greater body weight loss and *Chlamydia* growth was observed in 129 mice compared to CD1 KO. Again, the IL-4 production in CD1 KO mice was significantly lower than in wild-type (129) mice. The results suggest that the difference observed between CD1 KO mice and wild-type mice is due to the deficiency in NKT rather than any potential slight difference in genetic background between these mice.

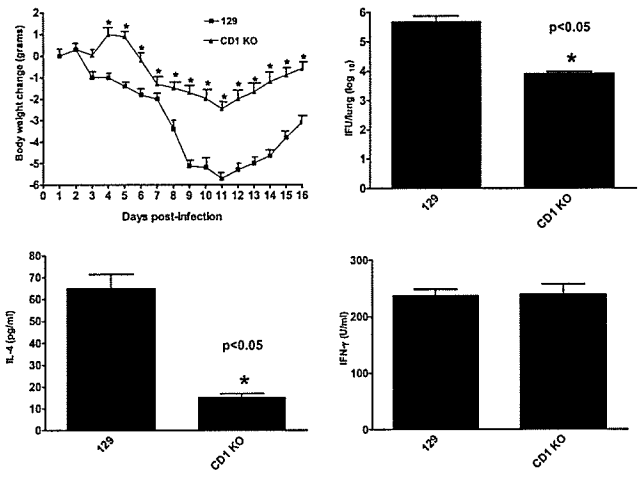

Figure 31. Similar differences between CD1 KO and wild-type mice were observed in experiments using 129 mice as controls. CD1 KO and 129 mice were intranasally infected with *C. muridarum* (1000 IFU) and monitored for body weight changes. At day 16 following infection, mice were killed and chlamydial *in vivo* growth in the lung and organism-driven cytokine production by spleen cells were analyzed using the methods described in Materials and Methods. * represents $p<0.05$, 129 versus CD1 KO mice.

3.3.6 CD1 KO and wild-type controls display similar levels of lymphocyte number and function.

To exclude the possibility that the altered susceptibility and immune responses observed in CD1 KO mice following *C. muridarum* infection might be due to the potential alteration of other immune cells in the genetically modified mice, we analyzed the frequency of T, B and NK cells in CD1 KO mice. As shown in **Table 3**, the percentages of T cells (CD3-, CD4-, CD8-positive cells), γδ T cells, NK cells (DX5 positive cells), B cells (CD19-positive cells) and dendritic cells (CD11c+ cells) in the spleen of CD1 KO mice were similar to those found in the two types of wild-type control mice. Similar results were obtained in analysis using peripheral blood cells (data not shown). Moreover, we found that the levels of neutrophils and monocytes in the peripheral blood were similar between BALB/c and CD1 KO mice (**Table 4**). We also examined the functional integrity of the T lymphocytes from CD1

KO mice. Spleen cells from BALB/c and CD1 KO mice were cultured alone or in the presence of ConA, a polyclonal T activator. Following ConA stimulation, spleen cells from CD1 KO mice and wild-type mice showed similar levels of proliferation and production of IL-4 (**Table 3**). These data suggest that, while the CD1 KO mice are deficient in NKT cells, their major immune cells are normal in amount and function. Therefore, the alteration in host susceptibility to chlamydial infection observed in CD1 KO mice is unlikely due to potential intrinsic changes in immune cells other than NKT cells.

Table 3
Comparison between intrinsic properties of BALB/C and CD1 KO mice

	% cells in Spleen	
	BALB/C	**CD1 KO**
T cells	73.7 ± 0.9	77.5 ± 0.7
CD4+	65.7 ± 1.9	70.8 ± 1.2
CD8+	10.1 ± 2.0	7.90 ± 1.3
B cells	21.5 ± 1.4	22.3 ± 0.9
γδT cells	12.3 ± 0.7	13.2 ± 1.2
NK cells	17.9 ± 1.4	19.2 ± 2.3
Dendritic cells	1.0 ± 0.1	1.1 ± 0.1
NKT cells	0.8 ± 0.1	0.2 ± 0.1*
*, $p<0.05$		
Proliferation index	4.1	4.6
IL-4 production (pg/ml)	880	916

Table 3. Naïve CD1 KO mice display similar levels of major immune cells and the T cell function. Spleen cells from naïve BALB/c and CD1 KO mice were prepared and stained for T, B (CD19), NK (DX5+), dendritic cell (CD11c+) and NKT (CD1d/PBS57 ligand) cell markers using fluorescence labeled mAbs/tetramer and analyzed by flow using a FACSCaliber II and CellQuest program. For cellular proliferation measurements, single spleen cell suspensions were cultured in the presence or absence of concanavalin A (ConA) stimulation. ConA-stimulated cells were pulsed with [^{3}H]thymidine and [^{3}H]thymidine incorporation was assessed using a TopCount NXT microplate scintillation and luminescence counter. Stimulation index (S.I.) was calculated based on the folds of counts in ConA-stimulated cells over the control wells; IL-4 levels following polyclonal stimulation in the 72h culture supernatants were measured by ELISA.

Table 4
Comparison between intrinsic properties of Balb/c, 129, and CD1 KO mice

% cells in peripheral blood	BALB/C	CD1 KO
Lymphocytes	70.6 ± 0.96	71.5 ± 0.70
Neutrophils	15.7 ± 0.41	12.1 ± 0.74
Monocytes	15.4 ± 0.23	11.0 ± 0.56
Eosinophils	0.14 ± 0.32	0.20 ± 0.67

Table 4. Blood smears from naïve BALB/c and CD1 KO mice were stained using a Hema 3 Stain Set (Fisher Scientific). Differential cells were counted based on cell characteristics and cellular morphology.

3.3.7 Stimulation of NKT activity with α-GalCer enhances chlamydial growth *in vivo*, which is correlated with enhanced Th2 cytokine/IgE production and reduced DTH responses.

α-GalCer is a natural ligand of type 1 NKT, which can specifically stimulate NKT activity in the context of CD1 *in vivo* (Burdin, Brossay et al. 1999; Brigl, Bry et al. 2003). To further confirm the regulatory role of NKT cells in chlamydial infection, we tested the effect of α-GalCer treatment in wild-type BALB/c and CD1 KO mice prior to infection with *C. muridarum*. BALB/c mice treated with α-GalCer showed dramatically enhanced chlamydial growth in the lung (**Figure 32**). In addition, the α-GalCer treated BALB/c mice displayed significantly increased *C. muridarum*-driven IL-4 and IL-5 production and *C. muridarum*-specific IgE production, compared to untreated mice. Moreover, the α-GalCer treated mice had less pronounced DTH responses following *C. muridarum* infection **(Figure 32)**. Interestingly, IFNγ levels were similar between the *C. muridarum*-infected mice with or without α-GalCer treatment. As expected, CD1 KO mice failed to respond to α-GalCer treatment, with unchanged chlamydial growth, IL-4, IgE, and DTH responses, confirming the specificity of α-GalCer on CD1/NKT. Moreover, neutralization of IL-4 activity in vivo in αGalCer treated BALB/c mice significantly reduced the promoting effect of α-GalCer treatment on chlamydial growth in vivo **(Figure 32)**. Taken together, these observations provide firm evidence that NKT cells play an

important role in modulating the immune responses to *Chlamydia* during a lung infection, thus significantly influencing the process of this infection. In addition, the production of IL-4 by NKT cells is a key mechanism for the immunomodulating and infection-promoting effect of these cells.

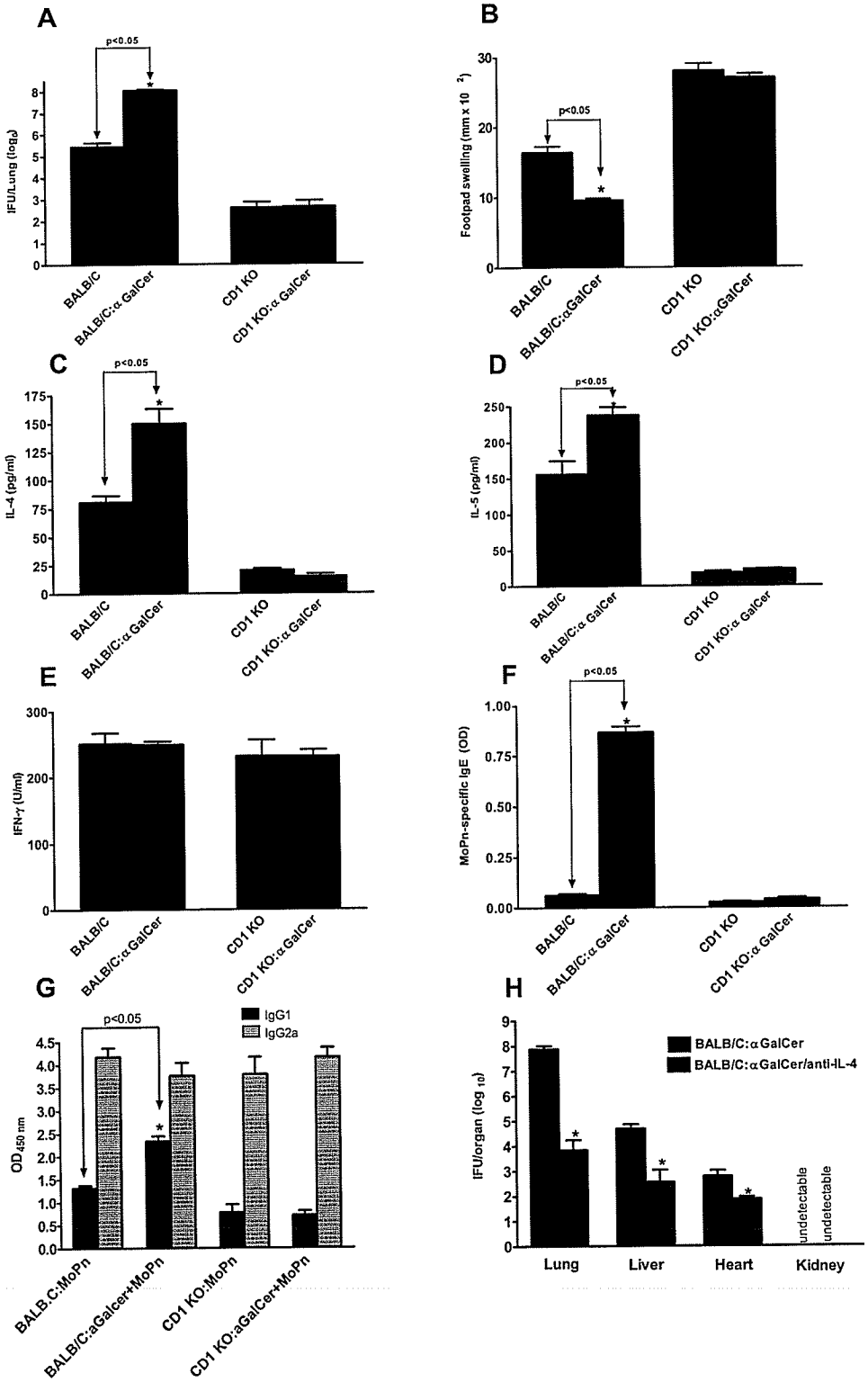
A
p<0.05
IFU/Lung (log10)
BALB/C
BALB/C:α GalCer
CD1 KO
CD1 KO:α GalCer
B
p<0.05
Footpad swelling (mm x 10 -2)
BALB/C
BALB/C:αGalCer
CD1 KO
CD1 KO:αGalCer
C
p<0.05
IL-4 (pg/ml)
BALB/C
BALB/C:α GalCer
CD1 KO
CD1 KO:α GalCer
D
p<0.05
IL-5 (pg/ml)
BALB/C
BALB/C:α GalCer
CD1 KO
CD1 KO:α GalCer
E
IFN-γ (U/ml)
BALB/C
BALB/C:α GalCer
CD1 KO
CD1 KO:α GalCer
F
p<0.05
MoPn-specific IgE (OD)
BALB/C
BALB/C:α GalCer
CD1 KO
CD1 KO:α GalCer
G
p<0.05
IgG1
IgG2a
OD450 nm
BALB.C:MoPn
BALB/C:aGalcer+MoPn
CD1 KO:MoPn
CD1 KO:aGalCer+MoPn
H
BALB/C:αGalCer
BALB/C:αGalCer/anti-IL-4
IFU/organ (log 10)
undetectable
undetectable
Lung
Liver
Heart
Kidney

Figure 32. **Effect of *in vivo* stimulation of NKT by α-GalCer treatment on host response to *Chlamydia* and the role of IL-4. Panels A-G**: Naïve CD1 KO and BALB/c mice (three mice/group) were injected i.v. with 4 μg α-GalCer in PBS or with polysorbate vehicle control. Mice were intranasally infected with *C. muridarum* (1000 IFU), 2 h following α-GalCer or vehicle injection. Mice were sacrificed on day 11 for analysis of serum IgE, *C. muridarum*-specific cytokine production by spleen cells and chlamydial growth in the lung using the methods described in *Materials and Methods*. For DTH responses, *C. muridarum* infected mice were challenged in footpads with dead *C. muridarum* as described in Figure 5 and the footpad swelling at 72 h post footpad challenge is shown. * represents $p<0.05$. One representative experiment of three independent experiments is shown. **Panel H:**, neutralization of IL-4 activity in vivo reduced the promoting effect of α-GalCer treatment on chlamydial in vivo growth. BALB/c mice (three mice /group) received a single i.v. injection of 4μg α-GalCer diluted in PBS. Immediately following α-GalCer injection, mice were treated or not intraperitoneally with 0.5 mg anti-IL-4 antibody (clone 11B11) in PBS. One hour later, both groups were infected with 1.0×10^3 IFU *C. muridarum* intranasally. Five days post-infection, mice initially treated with anti-IL-4 mAb were re-injected i.p. with this antibody at the same dose. Both groups were sacrificed Day 11 post-infection and the chlamydial burden in various organs was determined as described in Figure 1. * represents $p<0.001$.

3.3.8 Discussion.

In the present section, we investigated the role of NKT cells in modulating immune responses against *C. muridarum* infection. CD1 KO mice, lacking NKT cells, mounted more protective immune responses than the wild-type control (BALB/c and 129) mice following *C. muridarum* infection. *C. muridarum*-infected CD1 KO mice did not lose as much body weight, had lower chlamydial growth and milder pathological changes during infection. Moreover, stimulation of NKT cells using α-GalCer increased host susceptibility to chlamydial infection. The data indicate that CD1d-restricted NKT cells can modulate the immune response to Chlamydia infection and contribute to pathological outcome caused by the infection. As only classical type 1, but not type 2 CD1-dependent NKT are responsive to α-GalCer stimulation (Godfrey, MacDonald et al. 2004), the results suggest that the NKT cells, which play immunomodulatory role during chlamydial infection in the lung are type 1 NKT cells.

In this section, we further demonstrated that the reduced pathology and decreased bacterial burden in CD1 KO mice correlated with a reduced Th2 type immune response, especially lower levels of *C. muridarum*-driven IL-4 and IL-5 production. Initial IL-4 production by NKT cells may be one mechanism how these cells modulate adaptive immune response and promote host susceptibility to chlamydial infection. This idea is reinforced

from observations when neutralization of IL-4 immediately following α-GalCer treatment significantly reduced the promoting effect of this NKT ligand on chlamydial growth in vivo (**Figure 32**). The reduction in Th2 cytokines in CD1 KO mice was associated with a significant reduction in serum total IgE, *C. muridarum*-specific IgE and IgG1 responses. As far as we know, this is the first report regarding *Chlamydia*-specific IgE in mouse models and its correlation with susceptibility to chlamydial infection.

The present study found that CD1 KO mice mounted significantly stronger DTH response than wild-type mice following *C. muridarum* infection. Our previous study demonstrated that a DTH response is one of the major protective mechanisms against chlamydial infection, which is highly associated with enhanced Th1 response such as IFNγ, IL-12 and IL-18 production (Wang, Fan et al. 1999; Yang 2001). Interestingly, the results in the present study revealed that the Th1-related cytokines (IFN-γ, IL-12 and IL-18) were not significantly changed by the lack of NKT cells in CD1 KO mice although their Th2 cytokine production appeared to be significantly reduced. Therefore, it is possible that Th2 cytokines (IL-4 and IL-5) have a direct inhibitory effect on cell-mediated DTH responses during chlamydial infection. It is also possible and even more likely that the change in the balance of Th1/Th2 cytokines, rather than the absolute level of individual cytokines, caused by the lack of NKT cells accounts for the alteration in DTH response. Overall, the comparison of cytokine responses between CD1 KO and wild-type mice suggest that NKT cells play a crucial role in polarizing immune responses towards a Th2 phenotype, increasing susceptibility and downstream pathological outcomes caused by chlamydial infection.

Collectively, the findings suggest that NKT cells play a crucial role in the regulation of the immune responses to chlamydial infection. Our data reconfirm that Th2-like immune responses mediate host susceptibility to, and pathological consequences of, chlamydial infection. More importantly, the data suggest that NKT cells may not be protective, but rather NKT cells may enhance host susceptibility to certain infections. It would be important to further evaluate the role of NKT cells in various infections and the mechanisms underlying the variability in the role of NKT cells to derive new preventive and therapeutic strategies for infectious diseases.

3.4 Chapter 4: Re-direction of Ragweed-Induced Allergic Inflammation by Dendritic Cells from BCG-infected mice.

The previous sections have demonstrated that prior mycobacterial infection as well as *Chlamydia trachomatis* mouse pneumonitis (*Chlamydia muridarum*) can inhibit established allergic responses induced by either ovalbumin (OVA) or ragweed (RW) (Bilenki, Wang et al. 2002; Yang, Fan et al. 2002). The cellular and molecular basis of infection-mediated inhibition of de novo and established allergy remains unclear. As a key point in determining T helper cell differentiation is the interaction between DCs and T cells, we hypothesize that DCs play a central role in *Mycobacterium bovis* BCG infection mediated inhibition of allergic asthma-like reaction with ragweed. To test this hypothesis, the present study examines the ability of freshly isolated DCs, from BCG-infected mice, adoptively transferred to RW sensitized/challenged mice to re-direct and polarize the response to RW *in vivo*. Our results demonstrate that DCs isolated from mice infected with BCG are capable of re-directing the allergic response induced by RW, in contrast with DCs from naïve mice, which fail to alter the allergic phenotype. The modulating effect of DCs from BCG infected mice on established allergic response to ragweed was associated with a switch of allergen-driven cytokine production and reduced VCAM-1 and eotaxin expression. Comparative analysis of DCs from BCG infected and those from naive mice showed that the former had higher percentage of CD8α+ cells and expressed higher levels of TLR-9. Moreover, the data suggest a particularly important role played by DCs in infection-mediated inhibition of allergy and demonstrated the importance of IL-10 and IL-12 produced by DCs in the inhibition process. The data also indicate the effectiveness of adoptive transfer of DCs in modulating established allergic responses.

3.4.7 Adoptive transfer of DCs from BCG-infected mice to RW-sensitized/challenged mice inhibits eosinophilia and mucus overproduction.

Our previous studies using RW allergen have demonstrated that the RW allergen is capable of inducing an allergic response and lung pathology characteristic of the asthma-like reaction, including Th2-like cytokine production and allergen-specific IgE responses. In addition, our lab has demonstrated that mycobacterial infection can inhibit established allergic inflammatory responses induced by allergen (Yang, Fan et al. 2002). DCs are key antigen-presenting cells capable of substantially directing T helper cell differentiation, we therefore analyzed the effect of DCs isolated from BCG-infected mice on the asthma-like reactions induced by local challenge with RW in animals which had established allergy (IgE and Th2 responses). Mice were first sensitized with RW, subsequently challenged, administered DCs intravenously from BCG-infected mice, and further challenged with RW. Control mice had the same established allergy induced by RW, but received DCs from naïve mice. At 6 days post-challenge, mice were bled and sacrificed and analyzed for airway inflammatory responses and mucus secretion following RW challenge. These two parameters were analyzed because eosinophilic infiltration and mucus oversecretion by goblet cells in the airway epithelium are the classical hallmarks of an asthma-like reaction. Mice that received DCs from BCG-infected donors demonstrated significantly less cellular infiltration into the lung following RW challenge than the control mice that received DCs from naïve animals (**Figure 32A**). In particular, the number of infiltrating eosinophils were significantly reduced ($1.6 \pm 0.2 \times 10^6$ in recipients of DCs from naive mice versus $0.28 \pm 0.08 \times 10^6$ in the recipient of DCs from infected mice). This is also reflected in the percentage of infiltrating eosinophils into the lung tissue following RW re-challenge (**Figure 32B**). In addition, histological analysis also showed remarkable differences in cellular infiltration into the lung of the recipients of DCs from BCG-infected mice compared to those that received DCs from naive mice following RW challenge (**Figure 33A-D**). RW-treated recipients of DCs from naïve donors displayed massive infiltration of eosinophils in the bronchial submucosa, alveolar, and

perivascular sheaths (**Figures 33A & B** , whereas recipients of DCs from BCG-infected mice with the same RW treatment showed a remarkably lower level of infiltrating cells, especially eosinophils (**Figures 33C & D**). In addition, the levels of mucus production within the bronchial epithelium of mice that received DCs isolated from BCG-infected mice (**Figure 34B**) were significantly lower than those found in RW-treated mice given DCs from naïve donors (**Figure 34A**). The results clearly indicate that DCs taken from BCG-infected mice are able to inhibit established airway eosinophilic inflammation and mucus over-production elicited by re-challenge with the RW allergen.

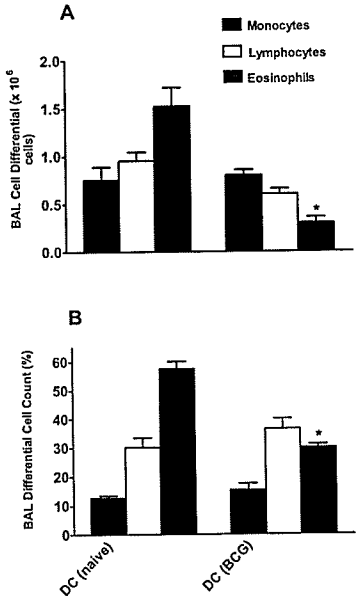

Figure 32. Mice treated with DCs isolated from BCG-infected mice during established RW allergy show decreased pulmonary eosinophilic infiltration induced by RW. Mice were sensitized intraperitoneally (i.p.) with 100μg RW in alum. The mice were intranasally challenged with 150μg RW at day 14 following RW sensitization and were administered DCs from naïve or BCG-infected donors. Mice were re-challenged with 150μg RW and sacrificed 6 days post-challenge. Pulmonary cellular infiltration was examined by differential cell counts of the bronchoalveolar lavage fluid cells, with particular attention given to eosinophil counts. Data presented represent three independent experiments. *, represents $P<0.05$, RW/RW/DC(BCG)/RW versus RW/RW BCG(Naïve)/RW-treated mice.

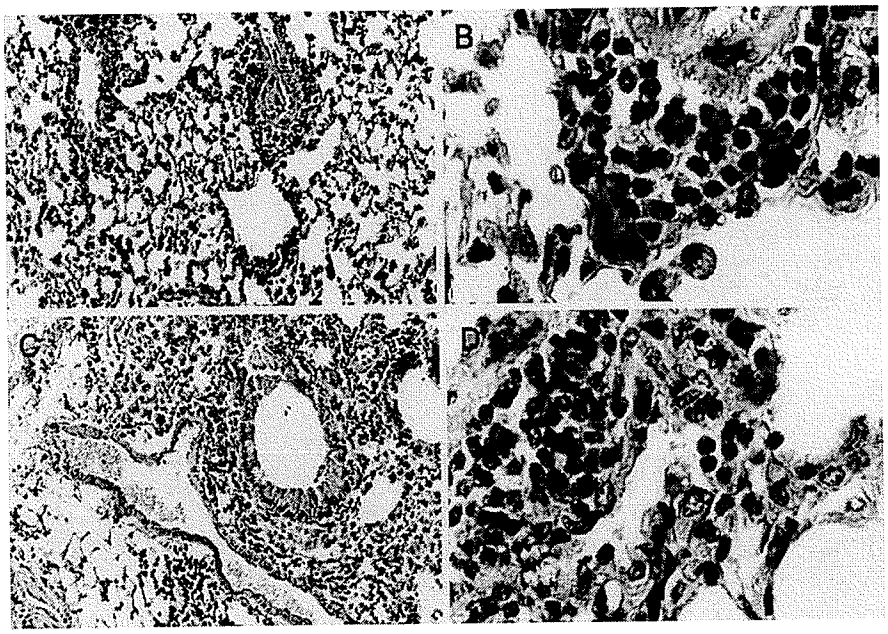

Figure 33. Adoptively transferred DCs from BCG-infected mice during established RW allergy dramatically reduced infiltrating eosinophils into the lung tissues compared to RW-allergic mice given DCs from naïve donors. Lungs from both groups of mice were aseptically excised and fixed in 10% buffered formalin. Lungs were cut into 10μm thick sections and stained with hematoxylin and eosin (H & E) staining and examined at 100X and 400X magnifications. Panels A & B, RW/RW/DC/RW/ (naïve) mice; Panels C & D, RW/RW/DC/RW (BCG) mice.

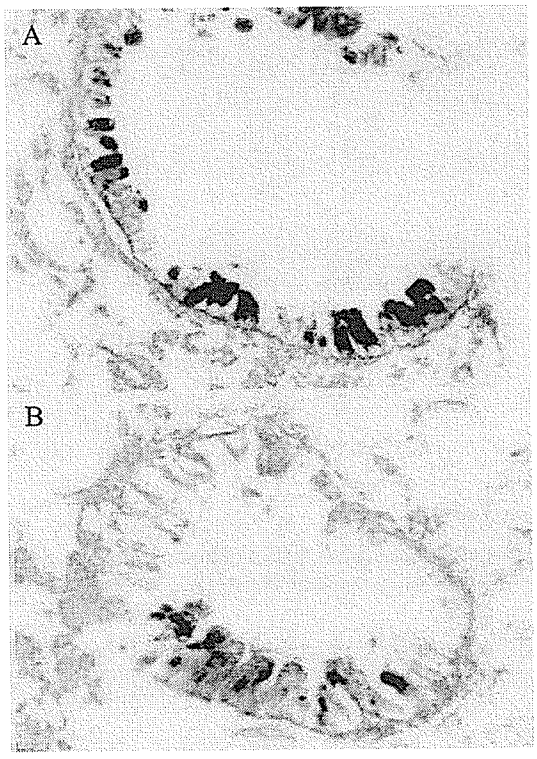

Figure 34. Dramatic reduction in mucus secretion in mice administered DC from BCG-infected mice following RW sensitization/challenge compared to mice given DCs from naïve mice following challenge. Mice were killed on day 6 post-RW challenge. Lung tissues were snap-frozen and stained for mucus and goblet cells by means of the Periodic Acid-Schiff (PAS) staining method. (A) RW/RW/DC/RW (Naïve); (B) RW/RW/DC/RW (BCG).

3.4.8 Adoptive transfer of DCs from BCG-infected mice results in reduced circulating eosinophils following RW re-challenge.

Airway eosinophilia in asthma is often associated with elevated peripheral blood eosinophil levels, resulting from increased eosinophil production and/or release from the bone marrow. Therefore, we further tested the impact of adoptively transferred DCs from BCG-infected mice to mice with established RW allergy on the development of systemic eosinophilia caused by RW exposure. As seen in **Table 5**, although RW exposure induced blood eosinophilia in both groups of mice, the mice that received DCs from BCG-infected donors had significantly fewer blood eosinophils compared to mice that received DCs from naïve mice. Taken together, these data demonstrate that DCs taken from BCG-infected mice and delivered to mice with

established RW allergy can inhibit allergic eosinophilia at both local and systemic levels.

Table 5

Differential Cell Count in DC (Naïve) versus DC (BCG)-treated mice during established allergy induced by ragweed allergen (x 10^3 cells/mm^3)

	DC (Naïve)	DC (BCG)
Monocytes	5.68 ± 0.342	3.44 ± 1.95
Lymphocytes	7.92 ± 1.65	2.72 ± 0.354
Neutrophils	6.24 ± 0.323	2.04 ± 0.149
Eosinophils	**2.31 ± 0.0213**	**0.900 ± 0.0432***

Table 5. There is a significant reduction in the circulating eosinophils measured in the peripheral blood of RW-treated mice given DCs from BCG-infected mice. Mice (three mice/group) were sensitized with 100μg RW with alum i.p. and were challenged fourteen days later with 150μg RW i.n. Mice were administered DCs from naïve or BCG-infected donors. Mice were re-challenged with 150μg RW and sacrificed 6 days post-challenge. All mice were bled and the blood smears were stained using a Hema 3 Stain Set (Fisher Scientific). Differential cells were counted based on cell characteristics and cellular morphology. *, $P<0.05$, comparison between RW-treated mice given DCs isolated from BCG-infected mice versus those given RW and DCs from naïve mice. One representative experiment from three independent experiments is shown.

3.4.9 Adoptively-transferred DCs from BCG-infected mice alter established, allergen-driven cytokine and chemokine production.

As we have reported previously, RW alum sensitization followed by intranasal challenge induces Th2-like cytokine responses, characterized by predominant IL-4 and IL-5 production with minimal IFN-γ and IL-12 production. To test whether DCs transferred from infected mice could affect allergen-driven cytokine and chemokine responses *in vivo*, we examined the cytokine and chemokine profiles of both spleen and draining lymph node cells from mice receiving DC from BCG-infected mice or from naïve mice. As shown in **Figures 35**, both spleen and draining lymph node cells from mice receiving DCs from BCG-infected donors produced significantly lower levels of Th2-related cytokines, namely IL-4, IL-5, IL-9, and IL-13, in comparison with

recipients of DCs from naïve. In addition, mice treated with DCs from BCG-infected mice exhibited significantly lower levels of eotaxin in their bronchoalveolar lavage fluids following RW re-challenge (**Figure 35**). The results also showed that mice receiving DC isolated from BCG-infected mice during established RW allergy displayed significantly higher levels of Th1-related cytokines, namely IL-12 and IFN-γ in comparison with those recipients of DCs isolated from naïve mice upon re-stimulation with RW allergen (**Figure 36**).

We further examined the effect of adoptively transferred DCs from BCG-infected mice, compared to those from naïve mice, on established IgE responses. Both RW-specific and total serum IgE were analyzed. Consistent with significantly lower Th2 cytokines, mice with DC transfer from BCG-infected mice following RW sensitization/challenge and prior to RW re-challenge showed significantly reduced levels of both RW-specific and total IgE production compared to those with naïve DC transfer (**Figure 37**). Additionally, the results demonstrated a significant increase in the levels of RW-specific IgG2a production in mice that received DC from BCG-infected donors compared to those that received DCs isolated from naïve donors. Taken together, the results demonstrate that adoptive transfer of DCs from infected mice can alter established allergen-driven Th2 cytokine production and IgE responses, which provide the basis for the reduction of local eosinophilic inflammation and bronchial mucus production.

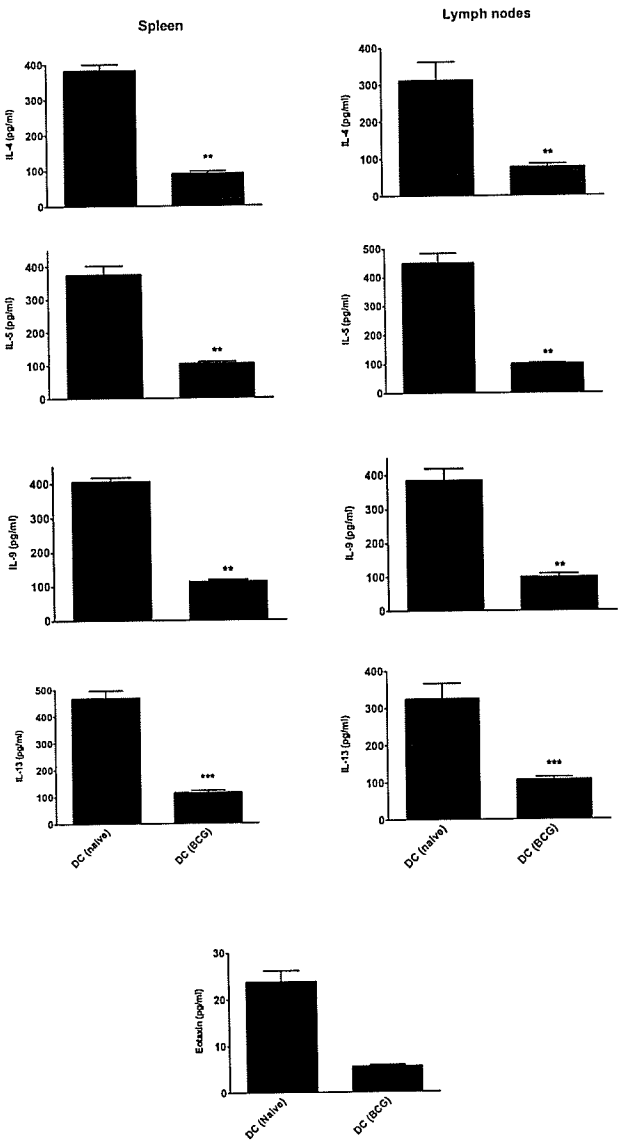

Figure 35. Dramatic reduction in allergy-driven Th2 cytokine production in mice treated with DCs from BCG-infected mice during established RW allergy compared to those treated with DCs from naïve mice. Mice (four mice/group) were sensitized intraperitoneally (i.p.) with 100μg RW in alum. Mice were intranasally challenged with 150μg RW at day 14 following RW sensitization. Mice were injected intravenously (i.v.) with DCs isolated from BCG-infected or naïve mouse spleens and re-challenged with 150 μg RW i.n. and sacrificed 6-8 days post-RW re-challenge. Spleen and lymph node cells were cultured at 7.5 x 10^6 cells/ml and 5.0 x 10^6 cells/ml, respectively, in the presence of RW (0.1 mg/ml), and the culture supernatants were harvested at 72h. Th2 cytokines in the culture supernatants were determined by ELISA. Data are presented as the means +/- SD of each group. *P<0.05, cytokine production in RW/RW/DC/RW (BCG) versus RW/RW/DC/RW (naïve) mice. One representative experiment of three independent experiments is shown. Bronchoalveolar lavage fluid was centrifuged and supernatants were collected. Eotaxin levels in the supernatants were determined by ELISA. Data are presented as mean±SD. One representative experiment of two independent experiments is shown.

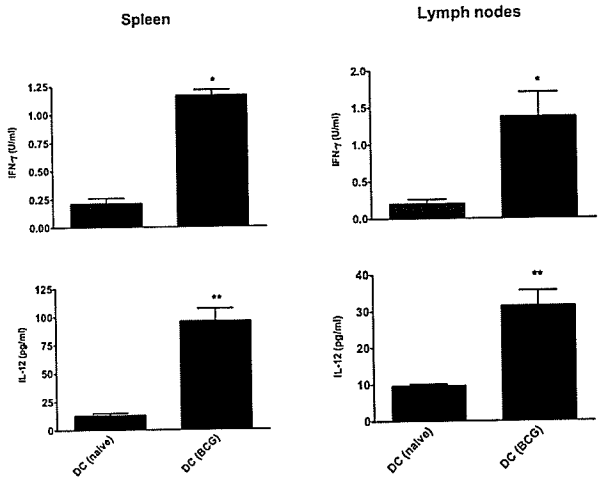

Figure 36. Dramatic reduction in allergy-driven Th1 cytokine production in mice treated with DCs from BCG-infected mice during established RW allergy compared to those treated with DCs from naïve mice. Mice (three mice/group) were sensitized intraperitoneally (i.p.) with 100μg RW in alum. Mice were intranasally challenged with 150μg RW at day 14 following RW sensitization. Mice were injected intravenously (i.v.) with DCs isolated from BCG-infected or naïve mouse spleens and re-challenged with 150 μg RW i.n. and sacrificed 6-8 days post-RW re-challenge. Spleen and lymph node cells were cultured at 7.5 x 10^6 cells/ml and 5.0 x 10^6 cells/ml, respectively, in the presence of RW (0.1 mg/ml), and the culture supernatants were harvested at 72h. Th1 cytokines in the culture supernatants were determined by ELISA. Data are presented as the means +/- SD of each group. *P<0.05, cytokine production in RW/RW/DC/RW (BCG) versus RW/RW/DC/RW (naïve) mice. One representative experiment of three independent experiments is shown.

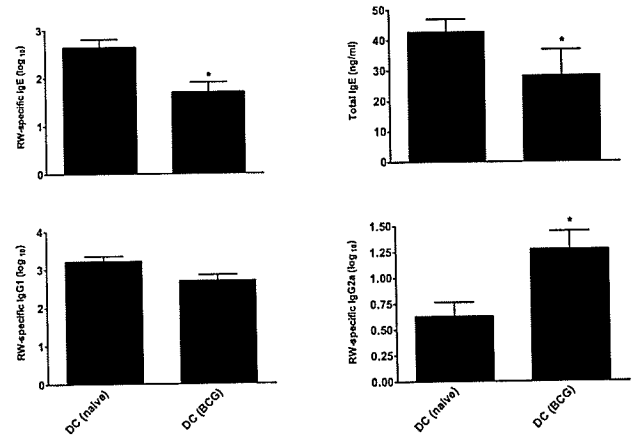

Figure 37. Mice administered DCs from BCG-infected mice during established RW allergy display significant decrease in RW-specific IgE, IgG1, and total serum IgE. Mice (three mice/group) were treated as described in the legend to Fig.1 and were bled 6-8 days post-RW re-challenge. The RW-specific IgE, IgG1, IgG2a, and total IgE in the sera collected post-RW treatment were determined as described in Materials and Methods. ELISA titers for RW-specific antibodies were converted to log 10 and presented as the means +/- SEM. *, p<0.05, RW/RW/DC/RW (BCG) versus RW/RW/DC/RW (naïve) mice.

3.4.10 DCs from BCG-infected mice display altered expression of TLR9 compared to those from naïve mice.

The results of the adoptive transfer experiments suggest that the DCs from BCG-infected and naive mice function differently in modulating allergic reaction. To investigate the basis for functional difference of these DC populations, we examined the relative expression of various TLR messages and cytokine production associated with DC phenotype. RT-PCR analysis of messenger RNA for TLRs showed that DCs from infected mice expressed higher TLR-9 message (**Figure 38**). As seen in **Figure 39**, a population of DCs isolated from BCG-infected mice expressed significantly higher levels of CD8α on their cell surface than those from naïve mice.

We also examined the spontaneous cytokine production profile of DCs isolated from either BCG-infected or naïve mice. As shown in **Figure 40**, DCs isolated from BCG-infected mice, when placed in culture, spontaneously produce significantly higher levels of both IL-10 and IL-12 in comparison with those isolated from naïve mice. Thus DCs isolated from BCG-infected mice differ with regards to the cellular marker expression and cytokines secretion compared to DCs from naïve mice.

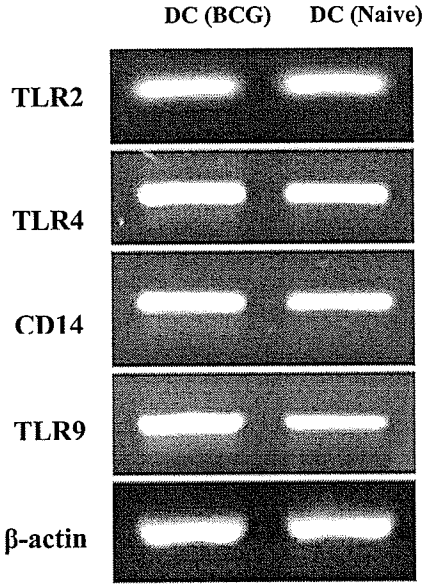

Figure 38. DCs from BCG-infected mice express higher levels of TLR9 mRNA than DC isolated from naïve mice. DCs were isolated using CD11c column (MACS) from the spleen from naïve (DC (Naive)) or BCG-infected mice (DC (BCG)). Total cellular RNA was extracted and RT-PCR was executed for measuring mRNA of TLR2, TLR4, CD14, TLR9, and β-actin. Electrophoretic visualization of the amplicons in one representative of three independent experiments with similar results is shown.

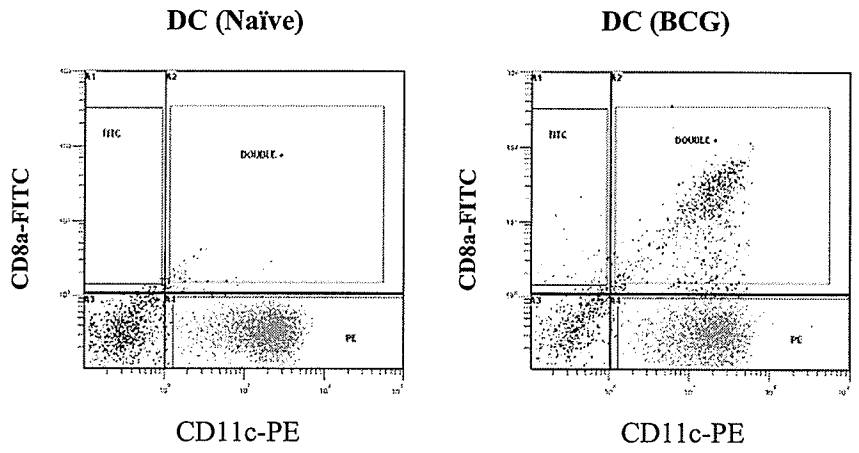

Figure 39. Characterization of DCs isolated from BCG-infected and naïve mice. Naïve mice or mice infected with BCG were incubated for 21 days. Following incubation, mice were sacrificed and spleens were aseptically excised. DCs were positively selected by means of CD11c-coated MACS beads. Single spleen cell suspensions from naïve or BCG-infected mice were prepared and stained for mature DC cell surface markers using fluorescence labeled mAbs, particularly CD11c-PE and CD8a-FITC followed by fixation with 4% paraformaldehyde. Cells were analyzed by flow cytometry. Data presented represent three independent experiments using a FACSCaliber II and CellQuest program.

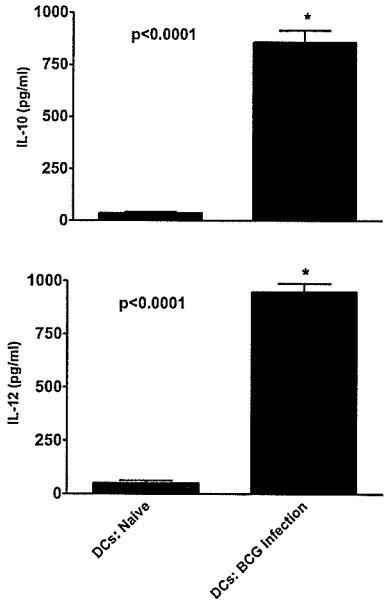

Figure 40. Characterization of DCs isolated from BCG-infected and naïve mice. Naïve mice or mice infected with BCG were incubated for 21 days. Following incubation, mice were sacrificed and spleens were aseptically excised. DCs were positively selected by means of CD11c-coated MACS beads. Single cell suspensions from naïve or BCG-infected C57BL/6 mice were prepared and 1.0×10^6 cells were placed into culture and incubated at 37°C for 48 hours. Following incubation, DC supernatants were collected and IL-10 and IL-12 cytokines were detected by means of ELISA. *, $p<0.001$, DCs (BCG) versus DCs (naïve).

3.4.11 *In vivo* neutralization of IL-10 or IL-12 results in partial reversal of the inhibitory effect of DC (BCG) delivery to RW-allergic mice.

To investigate the role of IL-12 and IL-10 produced by DCs in the inhibition of allergic reactions, we treated RW sensitized/challenged mice with subsequent administration of anti-IL-10 or anti-IL-12 neutralizing antibody. The results showed that mice receiving DCs from BCG-infected mice in conjunction with anti-IL-10 or anti-IL-12 antibody exhibited partial, but significant, reversal of the inhibitory effect on allergic reactions (**Figure 41**) and allergen-driven Th2 cytokine production **(Figure 42).** Of note, mice sensitized/challenged with RW and administered DCs from naïve mice in conjunction with anti-IL-10 or anti-IL-12 antibody did not increase Th2 cytokine production (**Figure 42**). The data indicate that DCs isolated from BCG-infected mice are able to re-direct the *in vivo* allergic response induced by RW and their ability to secrete IL-10 and IL-12 is at least partially responsible for this phenomenon with regards to key Th2 cytokine production patterns.

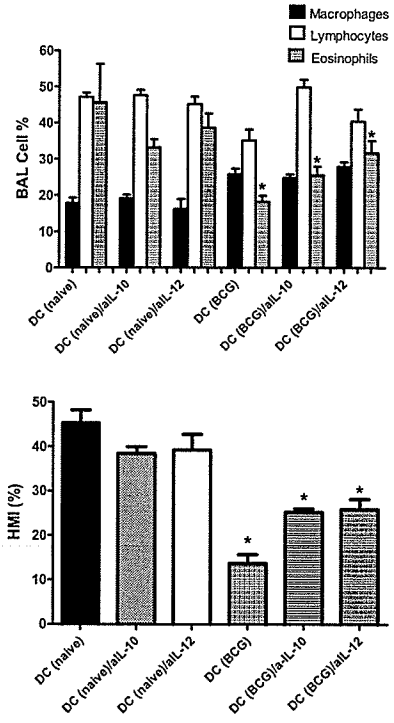

Figure 41. Mice delivered neutralizing IL-10 or IL-12 antibodies exhibit partial reversal of allergic airway inflammation. Lung tissues were fixed and sections were stained with thionin for mucus and mucus-containing goblet cells. Histological mucus index (HMI) was calculated based on the percentage of the mucus-positive area over the total area of the airway epithelium. *$P<0.05$, HMI in RW-treated mice administered DCs from BCG-infected mice compared to mice administered DCs from naïve mice. Pulmonary cellular infiltration was examined by differential cell counts of the bronchoalveolar lavage fluid cells. Data presented represent three independent experiments. *represents $P<0.05$, DC (BCG) versus DC (BCG)/a-IL-10 or versus DC (BCG)/a-IL-12. Data represents means +/- SD. One representative experiment of three independent experiments is shown.

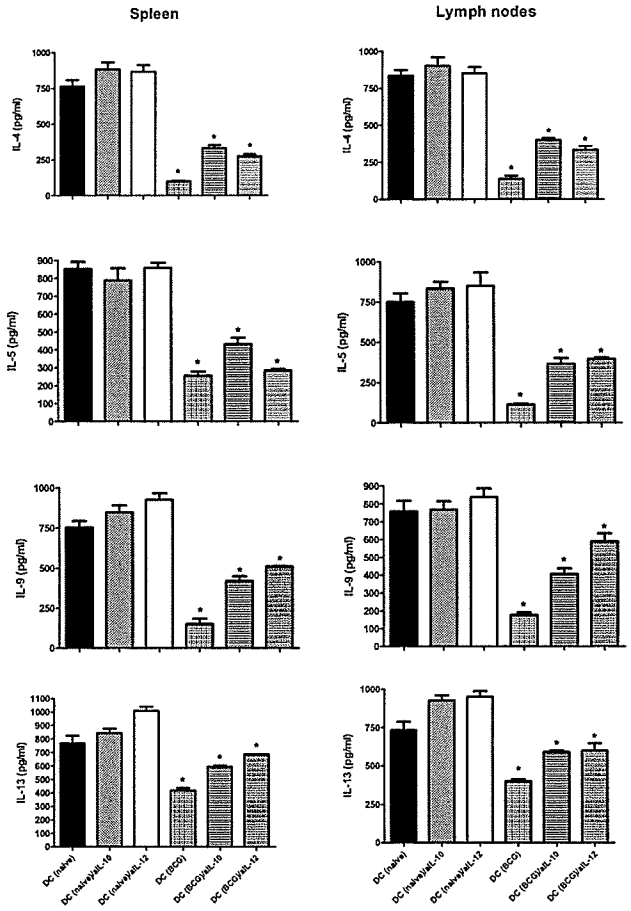

Figure 42. Neutralizing IL-10 or IL-12 antibodies partially reverse the inhibition of Th2 cytokines that is significantly higher than those mice delivered DCs from BCG-infected mice without antibodies. Mice (three mice/group) were sensitized intraperitoneally (i.p.) with 100μg RW in alum. Mice were intranasally challenged with 150μg RW at day 14 following RW sensitization. Mice were injected intravenously (i.v.) with DCs isolated from BCG-infected or naïve mouse spleens. Immediately following DC adoptive transfer from naïve or BCG-infected mice, mice were administered 1mg/ml of anti-IL-10 or anti-IL-12 antibody intraperitoneally and subsequently re-challenged with 150 μg RW intranasally. Six days post-re-challenge, mice were sacrificed and spleen and lymph node cells were cultured at 7.5 x 10^6 cells/ml and 5.0 x 10^6 cells/ml, respectively, in the presence of RW (0.1 mg/ml). Culture supernatants were harvested at 72h. Th2 cytokines in the culture supernatants were determined by ELISA. Data are presented as the means +/- SD of each group. *P<0.05, cytokine production in RW/RW/DC/a-IL-10/RW versus RW/RW/DC (BCG)/RW or RW/RW/DC/a-IL-12/RW versus RW/RW/DC(BCG)/RW.

3.4.12 Discussion.

In the present section, using an adoptive transfer approach, we showed that DCs play a pivotal role in the infection-mediated modulation of the allergic responses to ragweed. We found that adoptive transfer of DCs isolated from infected mice is capable of reducing the established Th2-like cytokine response and eosinophilic inflammation induced by RW. In addition, this DC transfer also reduced systemic eosinophilia in established RW allergy. These data provide clear evidence that DCs isolated from mice infected with *Mycobacterium bovis* BCG can efficiently modify and re-direct key T cell responses involved in the allergic asthma-like reaction. This is a novel finding as, although previously reported data have demonstrated a reduction in pulmonary eosinophilia (induced by OVA) as a result of BCG infection in de novo and established allergy models (Erb, Holloway et al. 1998; Major, Wohlleben et al. 2002), the present finding extends towards the cellular basis of the infection mediated inhibition of allergy.

The mechanism by which transferred DCs from BCG-infected mice mediate protection from allergic reactivity appears to influence allergen-specific CD4 T cell differentiation. It is clear that the recipients of DCs from infected mice showed Th1-like allergen-driven cytokine production instead of Th2-like responses. The transferred DCs may present allergen directly to RW-specific T cells and/or modulate the function of the existing DCs in the recipients. The cytokine microenvironment generated by the DCs from BCG-infected mice may lead to an overall decrease in the development of the established Th2 phenotype and an increase of Th1-like RW-specific T cells. Indeed, our data showed that the adoptive transfer of DCs isolated from BCG-infected mice switched allergen-specific T cell cytokine pattern from established Th2 responses to Th1 phenotype. Specifically, our results showed that spleen and lymph node cells cultured in the presence of RW antigen obtained from mice with adoptive transfer of DCs from infected mice demonstrate significantly lower levels of allergen-driven IL-4, IL-5, IL-9, and IL-13 compared to mice given DCs from naïve mice during established RW allergy.

In addition to the change in cytokine patterns, this data demonstrate significant reaction in chemokine production and adhesion molecule

expression related to eosinophil infiltration. Recipients of DCs from BCG-infected mice showed reduced levels of eotaxin in the lungs of allergen-treated mice. This is important because eotaxin is not only a key chemokine in eosinophil recruitment but a inducer of eosinophil degranulation via CCR3 expressed on the eosinophil cell surface, which may also contribute to eosinophilic inflammation in the airway (Bandeira-Melo, Sugiyama et al. 2001). Further elucidation of the mechanisms by which DCs from infected suppress eotaxin and expression may significantly enhance our understanding of the regulation of allergy by DCs.

In summary, these results have demonstrated a pivotal role played by DCs in infection-mediated modulation of established allergic reaction induced by RW allergen. The present data suggest that the function of DCs in re-directing the established allergic response is capable of highly influencing changes in the microenvironment initially established in a BCG-infected host. It is clear that, within the present model, the re-direction of the asthma-like reaction induced by RW proposes that T-cell re-polarization could be determined at several levels, including: the pathogen, pathogen recognition receptors on DCs, DC subsets, the microenvironment, and cytokines released by DCs and other cells in the vicinity. Moreover, the present study provides clear evidence for the ability of an intracellular bacterial infection such as *Mycobacterium bovis* BCG to induce a population of DCs capable of secreting key immunomodulatory cytokines such as IL-10 and IL-12 which contributes to the infection-mediated inhibition of allergy. Further studies investigating the relative contributions to this pool of either IL-10 or IL-12 by various DC subsets and their relationship with CD8α and TLR-9 marker expression could potentially elucidate a mechanism by which DCs play a crucial role in mediating the allergic response in human diseases.

3.5 Chapter 5: Protection against *Chlamydia muridarum* Infection *In Vivo* by Dendritic Cell Subsets.

DCs are the most efficient APCs in priming T cells. Based on functional preferences in directing Th1 or Th2 differentiation, APCs have been recently divided into type-1 or type-2 APCs (Kalinski, Hilkens et al. 1999; Kapsenberg 2003; Cerundolo, Hermans et al. 2004). Lineage and phenotypic differences have been identified between type 1 and type 2 DC. Similarly, human studies have found that monocyte-derived CD11c+ DC (DC1) cells polarize naive T cells towards Th1-like while CD11c- DC (DC2) directs T cells to Th2-like cells (Palucka, Taquet et al. 1998; Cella, Jarrossay et al. 1999). Mouse DC normally express CD11c and can be divided into CD8α+ and CD8α- subsets (Grabbe, Kampgen et al. 2000). Several studies found that CD8α+ lymphoid DC (DC1) primes naive CD4 T cells to Th1, whereas CD8α- myeloid DC (DC2) primes CD4 T cells toward Th2 differentiation (Maldonado-Lopez, De Smedt et al. 1999; Pulendran, Smith et al. 1999).

Su *et al* showed that vaccination with murine bone marrow-derived DC pulsed with *Chlamydia* induced protective immune response in a genital infection model (Su, Messer et al. 1998). Similar results were obtained in a study using cultured DC line (D3SC/1) (Ojcius, Bravo de Alba et al. 1998). Lu and Zhong reported that IL-12 production is required for chlamydial antigen-pulsed DC to induce protection against infection (Lu and Zhong 1999). We also reported that *Chlamydia muridarum* infection in the peritoneum induced the maturation of DC (Zhang, Yang et al. 1999) and DC from *Chlamydia* infected C57BL/6 mice express higher levels of IL-12 than those from naïve mice (Han, Fan et al. 2004). However, a critical analysis of the DC subsets relating to differential host responses to *Chlamydia* has yet to be performed.

In this section, we demonstrated an expansion of CD8α+ DC population in mice infected with *Chlamydia trachomatis* mouse pneumonitis (*Chlamydia muridarum*), more recently called *C. muridarum*. We compared the ability of sorted $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets in transferring protection against challenge infection and show that CD8α+ DC subset was more protective than CD8α- subset. Additionally, we have analyzed cytokine patterns of DC

subsets and the immune responses generated in the recipients of different DC subsets to elucidate potential mechanisms involved in protection against chlamydial infection by transferred DC subsets.

3.5.1 Adoptive transfer of CD11c+CD8α+ DCs from infected mice reduced body weight loss and chlamydial growth *in vivo*.

To compare the effectiveness of DC subsets in inducing protection against chlamydial infection, naïve C57BL/6 mice were adoptively transferred intravenously (i.v.) with DP DC (double-positive DC, CD11c+, CD8α+) or SPDC (single-positive DC, CD11c+ CD8α+) isolated from syngeneic *Chlamydia muridarum*-infected or naïve mice followed by intranasal challenge infection with *Chlamydia muridarum*. As shown in **Figure 43,** the body weight loss was significantly less in mice given DP DC from infected mice (iDPDC) before *Chlamydia muridarum* challenge infection, compared to mice without DC adoptive transfer. The overall physical condition of mice delivered iDPDC was also better (i.e. more activity, less fur ruffling and dehydration). The recipients of SP DC from infected mice (iSPDC) also showed reduced body weight loss compared to control mice. In contrast, the adoptive transfer of DC subsets from naïve mice (CD11c+CD8α+, nDPDC, and CD11c+CD8α-, nSPDC) had no significant effect on mouse conditions. The analysis of chlamydial levels in the lung showed that the recipients of iDPDC had significantly lower (1000 folds less) in vivo chlamydial growth than the control mice treated with PBS (**Figure 44**). Similar to the finding in body weight changes, the recipients of iSPDC also showed less chlamdydial growth in the lung than control mice, but their chlamydial levels were still 100 times higher than the iDPDC recipients (**Figure 44**). The adoptive transfer of DPDC and SPDC from naïve mice failed to show protective effects (**Figure 43 & 44**). The results indicate that the adoptive transfer of DC subsets from infected mice can induce significant protective immunity in recipient mice with but CD8α+ DC being most potent in inducing protection.

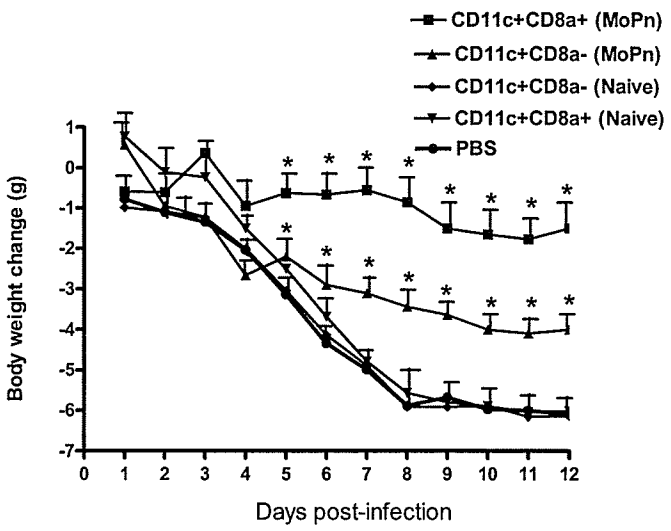

Figure 43. Less body weight loss following chlamydial infection in mice administered $CD8\alpha^+$ DC and exposed to *C. muridarum* infection. Naïve recipient mice (C57BL/6) were delivered $CD8\alpha^+$ or $CD8\alpha^-$ DC isolated from the spleen of *Chlamydia muridarum*-infected (intransal, 1000 IFU) mice or naïve C57BL/6 mice followed by intranasal challenge infection with same chlamydial strain, *Chlamydia muridarum* (3000 IFU). Mice were monitored daily for body weight changes. Each point represents the mean +/- SD of four mice. The original body weights of the mice were similar between the groups. *, $p<0.05$, compared to mice without cell transfer. One representative experiment of three independent experiments with similar results is shown.

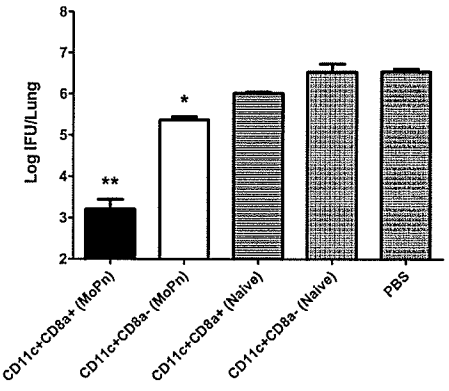

Figure 44. Less chlamydial growth *in vivo* following *C. muridarum* infection in mice treated with $CD8\alpha^+$ DC isolated from Chlamydia-infected mice. The different groups of mice shown in Figure 1 were sacrificed on day 12 post-infection and the different organs (lung, liver, and heart) were analyzed for *in vivo* chlamydial growth as described in *Materials and Methods*. One representative experiment of three independent experiments with similar results is shown. * represents $p<0.05$, compared to mice without cell transfer.

3.5.2 CD8α+ DC induce stronger DTH responses and *Chlamydia muridarum*-specific IgG2a antibody protection than CD8α- DC.

To examine the relationship between the degree of protection observed in mice delivered different DC subsets and the types of immune response, we tested DTH and antibody responses in these mice following challenge *Chlamydia muridarum* infection. As shown in **Figure 45**, while DTH responses were observed in all groups of mice, the levels in recipients of DPDC from infected mice were markedly stronger than other groups of mice. The data suggest that $CD8\alpha^+$ DC from infected mice are more powerful in inducing cell-mediated immune responses *in vivo* during chlamydial infection.

Antibody measurement showed that the levels of *Chlamydia muridarum*-specific IgG2a were significantly higher in mice treated with DPDC from infected mice compared to those treated with PBS (**Figure 46**). The transfer of SPDC from infected mice or DC subsets from naïve mice had no significant effect on IgG2a production. In contrast, both the levels of *Chlamydia muridarum*-specific IgG1 and IgE were significantly lower in the mice pre-treated with DPDC or SPDC from infected mice than the control mice. (**Figure 46**). Taken together, the results indicate that the immune responses including DTH, and the production of antibody isotypes, were significantly increased in mice pre-treated with DC isolated from *Chlamydia muridarum*-infected donors.

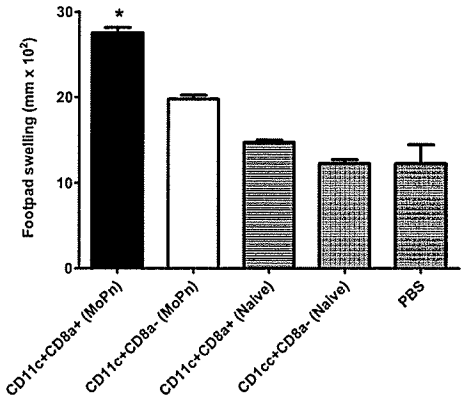

Figure 45. Significantly stronger DTH responses in mice treated with $CD8\alpha^+$ DC and infected with *C. muridarum*. For DTH response, mice (three mice/group) were treated and infected as described in Figure 1. At 11 days post intranasal challenge infection, the footpads of different groups of mice were challenged with heat-inactivated *Chlamydia muridarum* (left footpad) or SPG (right footpad) and the thickness of the footpads were measured at 24, 48, and 72 h following dead *Chlamydia muridarum* injection. The difference in the thickness of footpads with or without *C. muridarum* injection was used as a measure of DTH responses as described in *Material and Methods*. Data show the mean +/- SD. * represents $p<0.05$, compared to mice without DC adoptive transfer. One of three independent experiments with similar results is shown.

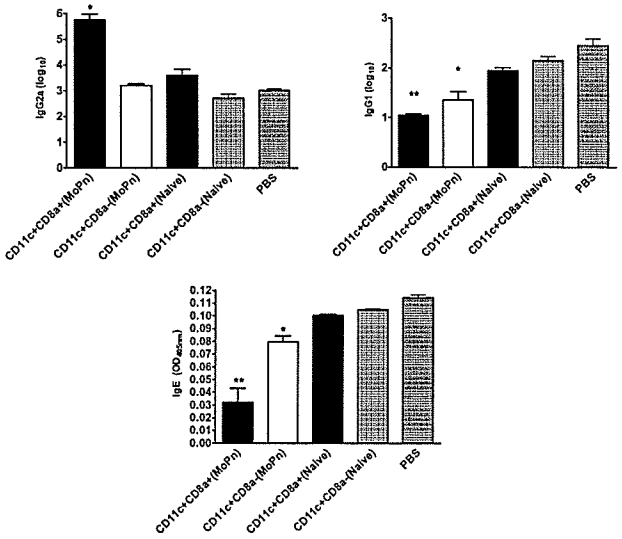

Figure 46. Recipients of DC from infected mice produce significantly lower levels of serum IgE and IgG1 following challenge infection. The sera collected from mice (three mice/group) post adoptive transfer of DC and post (day 12) intransal *Chlamydia muridarum* infection as described in Figure 1 were determined for *Chlamydia muridarum*-specific IgE, IgG1 and IgG2a using ELISA. For detection of *Chlamydia muridarum*-specific IgE, the sera were pre-treated twice with 50% slurry of protein G sepharose to remove IgG. Data are presented as mean ± SD. *, $p<0.05$, **, $p<0.001$; compared to mice without DC adoptive transfer. One representative experiment of three independent experiments is shown.

3.5.3 Mice pre-treated with CD8α+ DC isolated from *Chlamydia muridarum*-infected mice exhibit *Chlamydia muridarum*-driven Th1 and Th2 cytokine production upon chlamydial infection.

We examined the cellular immune responses in mice receiving DC subsets before challenge infection, by re-stimulating spleen and draining lymph node cells *in vitro*. Both spleen and lymph node cells of *Chlamydia muridarum*-infected mice pre-treated with iDPDC produced significantly higher levels of TNF-α, IFN-γ and IL-12 compared to mice without DC adoptive transfer (**Figure 47**). Notably, recipients of iSPDC also showed increased of Th1 cytokines however to a lesser degree but than the iDPDC recipients. Interestingly, the recipients of nDPDC also showed significant increase of TNFα and IFNγ although they were lower than the recipients of DC subsets from infected mice. In contrast, Th2 cytokines such as IL-4, IL-5 and IL-13 were significantly reduced in iDPDC treated mice (**Figure 48**). The transfer of iSPDC also appeared to decrease Th2 cytokine production, but in a lesser degree. Together, the data demonstrate a powerful role of $CD8\alpha^+$ DC in promoting protective Th1 type immune responses to chlamydial infection.

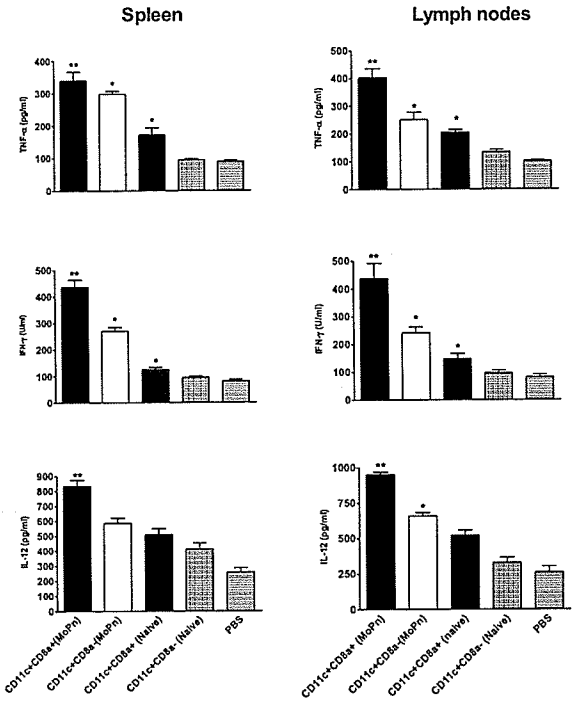

Figure 47. Recipients of DC from infected mice produce significantly higher *Chlamydia muridarum*-driven Th1 cytokines following challenge infection. Mice (three mice/group) were treated and infected as described in the legend to Fig.1, and were sacrificed at 12 days post-infection. Spleen and lymph node cells were cultured with UV-inactivated *Chlamydia muridarum* as described in *Materials and Methods*. TNF-α, IFN-γ and IL-12 in 72h culture supernatants were determined by ELISA. Data are presented as the mean +/- SD of each group. *, $p<0.05$, **, $p<0.001$; compared to mice without DC adoptive transfer. One representative experiment of three independent experiments is shown.

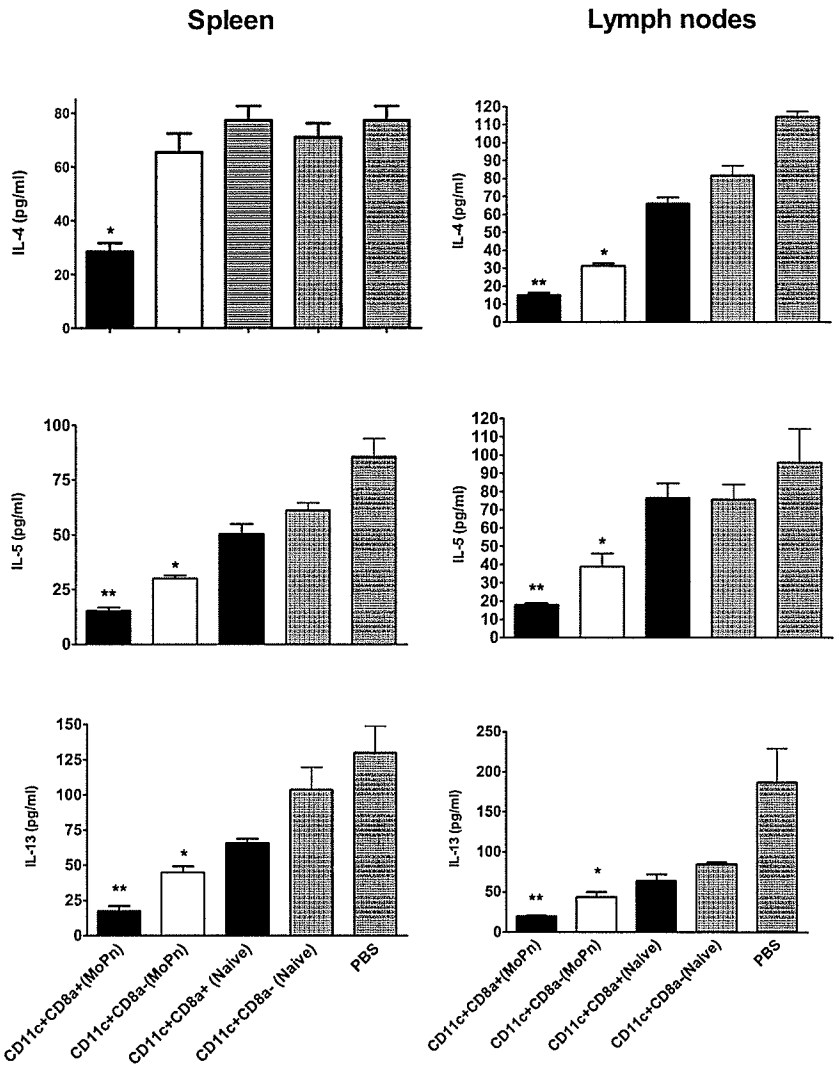

Figure 48. Recipients of DC from infected mice produce significantly lower *Chlamydia muridarum*-driven Th2 cytokines following challenge infection. Spleen and lymph node cell culture supernatants from experiments described in Figure 4 were determined for Th2 cytokines (IL-4, IL-5 and IL-13) by ELISA. Data are presented as the mean +/- SD of each group. *, $p<0.05$, **, $p<0.001$; compared to mice without DC adoptive transfer. One representative experiment of three independent experiments is shown.

3.5.4 *Chlamydia muridarum* infection induces expansion of CD8α+ DC population which show different cytokine production patterns.

To test the cytokine production pattern of the DC subsets, sorted DPDC and SPDC were placed in culture and the spontaneous cytokine production was measured by ELISA in recovered supernatants. As shown in **Figure 49**, DPDC produced significantly higher levels of IL-12 and IL-10 than SPDC. The results indicate that CD8+ DC from infected mice produce higher immune regulatory cytokines than CD8- DC.

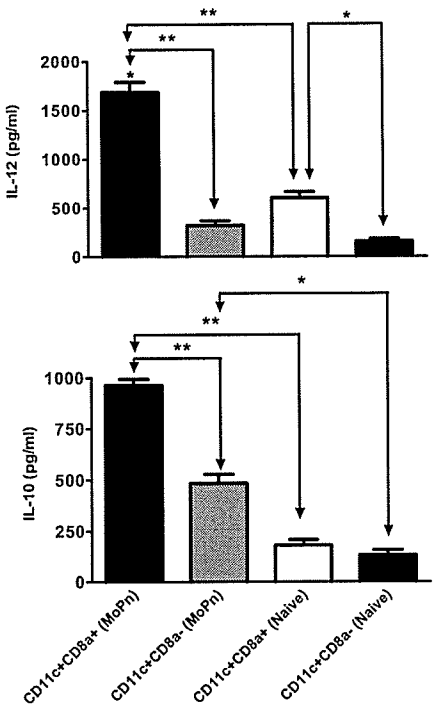

Figure 49. $CD8\alpha^+$ DC isolated from *Chlamydia muridarum*-infected mice produce significantly higher levels of IL-12 and IL-10 compared to $CD8\alpha^-$ DC isolated from infected mice or DC isolated from naïve mice. Mice were infected intranasally with *Chlamydia muridarum* (1000 IFU) and were sacrificed 7 days post-infection. Naïve mice were used as controls. Spleen cells were surface stained for CD11c and CD8α and subjected to cell sorting resulting in $CD11c^+CD8\alpha^+$ and $CD11c^+CD8\alpha^-$ subpopulations from infected and naïve mice. The DC subsets were placed in culture for 72 hrs and cytokines in the supernatants were measured by ELISA. *, $p<0.05$, **, $p<0.001$. One representative experiment of three independent experiments is shown.

3.5.5 Discussion.

In the present model we have shown that the transfer of CD8α+ DC, compared to CD8α- DC, isolated from *C. trachomatis Chlamydia muridarum*-infected mice are much more capable of transferring protective immunity to naïve mice to subsequent challenge infection. To our knowledge, this is the first report showing variable capacity of DC subsets in inducing protective immunity against chlamydial infection. Our results showed that the DC subsets were different in cytokine production, in particular, CD8α+ DCs secrete IL-12 and IL-10 at significantly higher levels compared to CD8α- DC isolated from infected mice or either subset taken from naïve mice. The ability of iDPDC to produce higher levels of these cytokines indicates a pertinent role for these cytokines secreted in the transfer of protective immunity against subsequent infection.

The approach used in this study is more physiological than most previously reported studies on the function of DC subsets. Most previous studies examined bone marrow derived, or peripheral monocyte derived DCs which were manipulated in *in vitro* culture conditions to generate different DC subsets.

Notably, although our data strongly suggest that CD8α+ DC are DC1-like cells which induce preferentially induce Th1-like responses, the CD8α- DC in our model are not typical CD2-like cells. In fact, the adoptive transfer of CD8α- DC from infected mice also enhanced Th1-related cytokine production (**Figure 47**) and inhibited Th2-related cytokine production (**Figure 48**). One possible reason for this is that the CD8α- DCs are still a heterogeneous population which includes both DC1- and DC2-like cells. Indeed, we found that within the CD8α- DC population, further separation based on CD4 expression could obtain distinct CD4+ and CD4- DC subsets and the former produced least IL-12 in *in vitro* culture (Han et al, unpublished data). Therefore, using CD8α as a sole discriminative marker for DC1- and DC2-like cells may be an over-simplification.

A rather surprising but also very interesting finding in the present study is the higher IL-10 (in addition to higher IL-12) production by CD8α+ DC from

infected mice which confer strong protective immunity against chlamydial challenge infection. Although IL-12 has been consistently found to be associated with protection (Lu and Zhong 1999; Lu, Yang et al. 2000), higher IL-10 production has been previously linked to susceptibility to *C. trachomatis* infection and pathology in mouse and human chlamydial diseases (Yang, HayGlass et al. 1996; Cohen, Plummer et al. 1999; Yang, Gartner et al. 1999). It remains unclear why DC subsets which produce higher levels of these two apparently functionally counter cytokines showed dominant protective phenotype. However, several possibilities may exist accounting for this phenomenon. First, the ratio and the degree of increase of these two cytokines may enhance IL-12 production by the CD8α+ DC from infected mice. Indeed, these cells produce 2.5 folds higher IL-10, 7.5 fold higher IL-12, than CD8α- DC. It is likely that the relative degree of IL-12/IL-10 production, rather than the absolute value of IL-10 or IL-12, is more important in determining the type of immune response and protection. Notably, the transfer of CD8α+ DC from infected mice not only induced significantly higher IFN-γ and TNF-α production but lower Th2 cytokine (IL-4, IL-5 and IL-13) production by *Chlamydia*-specific T cells, demonstrated by organism-specific re-stimulation studies (**Figures 47 & 48**). As the recipients of CD8α+ DC demonstrated higher cell-mediated immunity (DTH) and lower IgE and IgG1 antibody production, the results further confirm the importance of Th1 type immunity in host defense against Chlamydia infection. The finding that the approaches which induce higher IL-12 production elicit strong protection even with increased IL-10 is not without precedents. We have demonstrated in previous studies that DNA vaccination using chlamdydial MOMP gene induces both IL-12 and IL-10 production but provide significant protection (Zhang, Yang et al. 1997). Moreover, we have shown that live, compared to heat-inactivated, chlamydial vaccination induced higher IL-10 (and IL-12) and also provide strong protection. Therefore, in addition to increased Th1 responses by higher IL-12 production and higher IL-12/IL-10 ratio, CD8α+ DCs might play an inhibitory role on the development of Th2 cytokines by way of its higher IL-10 production. Recent studies have showed that IL-10 production by tolerogenic DC can induce T regulatory cells which can inhibit Th2 cells in allergy model (Zhang, Yang et al. 1999). It is likely that the

CD8α+ DC population is still heterogeneous, meaning that some CD8α+ DC produce higher IL-12 thus DC1-like while others produce high IL-10, like tolerogenic DC, which may lead to unresponsiveness of Th2 cells or the development of regulatory T cells. In general, we feel the results in the present study showing lower Th2 cytokine production in iCD8α+ DC recipients may be a combinational outcome of higher IL-12 production by these DC and subsequent IFN-γ production by T cells and the inhibitory effect of IL-10 on Th2 cell development via direct suppression of Th2 cells and/or the development of regulatory T cells. Further studies on the heterogeneity of CD8α+ DC and taking the approach of analyzing DC from IL-10 gene knockout mice or blocking its IL-10 production by RNA interference techniques may be helpful for addressing this question.

Collectively, the findings of the present study suggest that $CD8\alpha^+$ DC, in contrast to $CD8\alpha^-$ DC, play a crucial role in the mounting of protective immune responses to active chlamydial infection. The difference in cytokine profiles of the various DC subsets could present a possible mechanism by which the subsets from *C. trachomatis Chlamydia muridarum*-infected mice provide different levels of protective immunity to recipient mice. It would be important to further investigate the conditions and the chlamydial components which predominantly induce DC subset(s) which are beneficial for preventive and therapeutic strategies for chlamydial diseases.

3.6 Chapter 6: Infection-Mediated Inhibition of Allergic Airway Inflammation by Dendritic Cell Subsets.

In previous studies, we have shown that prior *Chlamydia muridarum* and mycobacterial infections can inhibit allergic asthmatic responses in murine models, which is correlated with enhanced Th1-type cytokine (IFN-γ) production and reduced Th2-type cytokine (IL-4, IL-5 and IL-13) production (Akira, Takeda et al. 2001; Lanzavecchia and Sallusto 2001; Liu, Kanzler et al. 2001). However, the mechanism for the alteration in T cell cytokine production and allergic inflammation mediated by these infections remains unclear. As DC play an important role in directing T cell differentiation and different types of immune responses, we were interested in examining the role played (if any) in the chlamydial infection-mediated inhibition of the allergic response. Additionally, as a result of previous studies executed by other groups, we wanted to probe further the involvement of particular DC subsets isolated from naïve or Chlamydia-infected mice. Many studies have already shown data to support the heterogeneity of DC sub-populations and their differential capacity to prime for various disease states such as allergy (Hammad, de Vries et al. 2004). Different subsets of DC, identified in mouse spleen by their differential expression of CD8α can induce different T helper cell responses after systemic administration. CD8α- DC have been shown to preferentially induce Th type 2 responses whereas CD8α+ DC induce Th1 responses (Hammad, de Vries et al. 2004). Other groups have also shown that the type of antigen DC become exposed to can determine the downstream T-cell response (Langenkamp, Messi et al. 2000). For example, Charbonnier *et al* have demonstrated that Der p 1-pulsed DC, a common dust mite allergen, isolated particularly from dust mite-sensitized allergic patients, can dysregulate the immune response towards Th2 (Charbonnier, Hammad et al. 2003). Also, as we hypothesize that CD8α+ DC isolated from Chlamydia-infected mice can inhibit the allergic response, others have demonstrated that other infections, such as *Mycobacterium vaccae*, can induce a population of pulmonary CD11c+ cells with the ability to regulate allergic disease (Adams, Hunt et al. 2004). Additionally, Manickasingham *et al* have recently shown

that the ability of murine DC subsets to direct T helper cell differentiation is dependent upon exposure to bacterial antigens and the exposure to various microbial signals (Manickasingham, Edwards et al. 2003).

The results in the following section shows that firstly, previous *Chlamydia muridarum* infection in mice, followed by sensitization and challenge with ragweed (RW) can inhibit the production of RW-specific, Th2-type cytokine responses. BAL cell numbers, particularly eosinophils, were also reduced in *Chlamydia muridarum*-infected, RW-treated mice compared to mice administered RW only. DC isolated from *Chlamydia muridarum*-infected mice and transferred to naïve mice and also had the same inhibitory effect on the RW-induced allergic response following RW exposure with regards to Th2-type cytokines, lung eosinophils numbers and mucus production in the airways. The inhibition of RW-induced allergic response did not occur if mice were administered DC from naïve mice. Additionally, to test which DC sub-population from *Chlamydia muridarum*-infected mice are the most potent inhibitors of the allergic response, mice were infected with *Chlamydia muridarum* and CD11c+CD8α+ (DP) DC as well as CD11c+CD8α- (SP) were. The data show that DP DC isolated from *Chlamydia muridarum*-infected mice were capable of inhibiting the allergic response induced by RW sensitization/challenge. This was evident in terms of RW-driven Th2 cytokines, BAL eosinophils numbers, eotaxin production, and mucus production by airway epithelia goblet cells. This effect was not seen in those mice delivered SP DC isolated from *Chlamydia muridarum*-infected mice, and even less so in mice given DP or SP DC isolated from naïve donors. Cell sorting of these two subsets from *Chlamydia muridarum*-infected mice and spontaneous cytokine measurements in culture showed a significantly higher level of both IL-10 and IL-12 production from those DP compared to SP. The data demonstrate a critical role played specifically by the CD11c+CD8α+ DC subset (DP) isolated from *Chlamydia muridarum*-infected mice in chlamydial infection-mediated inhibition of the allergic response.

3.6.1 Local *Chlamydia muridarum* Infection Alters Cytokine Patterns Induced by Allergen as well as Pulmonary Eosinophilia.

Our previous studies using *Chlamydia muridarum* in murine respiratory infection models have demonstrated that the *Chlamydia muridarum* lung infection in C57BL/6 mice is cleared within 20-25 days following inoculation. In the present study, we examined the effect of DC subset delivery from *Chlamydia muridarum*-infected mice on a subsequent RW-induced asthma-like reaction. To first demonstrate the effect of Chlamydial infection on the outcome of RW-specific Th2 cytokine production, we infected mice and then sensitized with RW when the infection had been cleared and the mice had recovered. Thirty days post-infection, mice were sensitized with RW and subsequently challenged. As both RW-specific Th2-type cytokine production and eosinophils infiltration into the lung are classic hallmarks of the asthma-like reaction, a direct comparison was made between Th2-like cytokines produced by RW-treated mice, with or without prior *Chlamydia muridarum* infection. According to the profile of the cytokine patterns of draining lymph node cells (peribronchial) from *Chlamydia muridarum*-infected, RW-treated mice (three mice/group) re-stimulated with RW allergen *in vitro*, their levels of Th2-like cytokines, namely IL-4, IL-5, IL-10 and IL-13, were dramatically decreased compared to mice with RW treatment only (**Figure 50**).

A direct comparison was also made between the differential cell counts from the BAL of uninfected, RW-treated mice and that of the *Chlamydia muridarum*-infected, RW-treated mice. The results showed that the group (three mice/group) of mice with previous *Chlamydia muridarum* infection demonstrated significantly less cellular infiltration into the lung following RW challenge than mice treated with RW only, especially in eosinophilic infiltration ($1.2 \times 10^6 \pm 0.4$ eosinophils in mice with RW treatment only compared to $2.0 \times 10^5 \pm 0.4$ eosinophils in the *Chlamydia muridarum*-infected, RW-treated mice; $P<0.05$). Eosinophils made up greater than 44% ± 0.3% of the total number of infiltrating cells into the BAL of the mice treated with RW only, whereas eosinophils in the *Chlamydia muridarum*-infected, RW-treated mice represented 21.3% ± 0.6% of the total BAL cells ($P<0.05$) (**Figure 51**). The experiments were repeated three times, and similar results were obtained.

Together, these results show that the local *Chlamydia muridarum* infection which has been cleared prior to RW immunization is capable of inhibiting the local allergic inflammatory response.

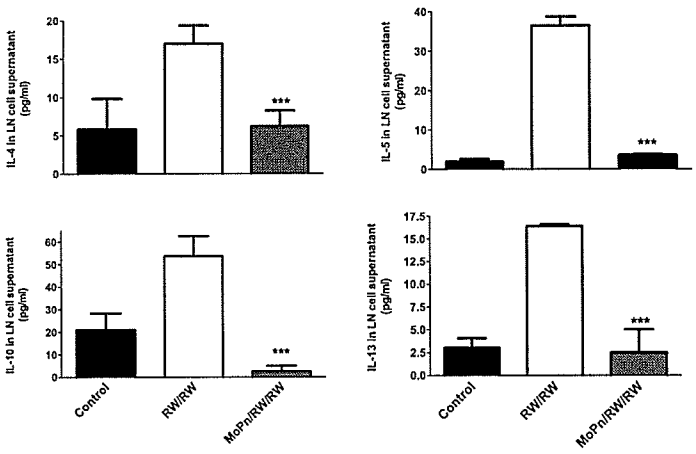

Figure 50. Drastic reduction in Th2 cytokines in draining lymph node cell cultures of *Chlamydia muridarum*-infected, RW-treated mice compared to mice treated with RW only. Mice were infected with 1 x 10^3 IFU 30 days before sensitization with RW. All mice were sensitized/challenged with RW and sacrificed 6 days post-challenge. Lymph node cells were cultured at a concentration of 5.0 x 10^6 cells/ml. Culture supernatants were harvested at 72 hours and tested for various Th2 cytokines using ELISA. Data are presented as the mean +/- SD of each group. ***, $p<0.0001$, cytokine production in infected mice compared to those levels measured in uninfected mice.

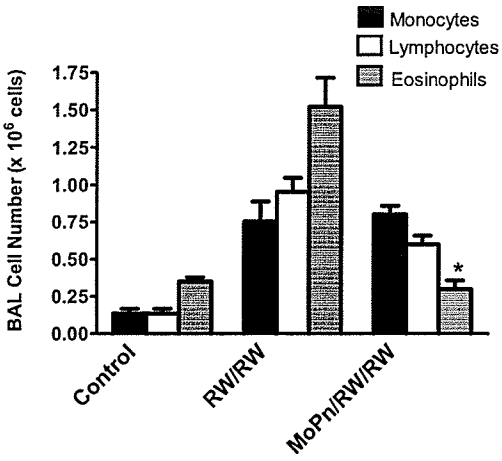

Figure 51. Mice infected with *Chlamydia muridarum* prior to RW sensitization show a significant reduction in BAL of eosinophils following RW sensitization and challenge compared to mice treated with RW only. One group of mice was infected with 1 x 10^4 IFU *Chlamydia trachomatis* (*Chlamydia muridarum*) and were allowed to clear the infection for 30 days. On day 30, all mice were sensitized i.p. with RW (100μg) in alum and were challenged with RW (150μg) on day 14 following sensitization. Mice were sacrificed day 6 post-challenge and mice tracheas were cannulated and BAL fluids acquired by washing lungs twice with sterile PBS. BAL cellular components were analyzed by differential cell counts using Fisher Leukostat Stain Kit. The data shows the proportion of each cell population in the BAL fluids of both groups of mice. *, $p<0.05$, eosinophils cell number in infected mice compared to those levels measured in uninfected mice.

3.6.2 Inhibition of the allergic response mediated by DC from *Chlamydia muridarum*-infected mice.

To test whether transferred DC isolated from *Chlamydia muridarum*-infected mice could interfere with allergen-driven cytokine profiles, we isolated draining lymph node cells from mice following DC transfer from *Chlamydia muridarum*-infected or naïve controls. Local lymph node cells from mice receiving DC from infected mice produced significantly lower levels of Th2-type cytokines (IL-4, IL-5, IL-9 and IL-13) in comparison to recipients of DC isolated from naïve mice ($P<0.05$) (**Figure 52**). Additionally, we examined the effect of the prior delivery of DC from *Chlamydia muridarum*-infected donors on the outcome of lung eosinophilia following RW sensitization/challenge. As shown in **Figure 53A**, mice that received DC isolated from infected mice had significantly less cellular infiltration into the lung following RW challenge than those mice treated with RW only, especially in the percentage of eosinophilic infiltration (36% ± 0.6% eosinophils in mice with RW treatment only compared

to 20% ± 0.4% eosinophils in the *Chlamydia muridarum*-infected, RW-treated mice; $P<0.05$). Finally, the level of mucus production, demonstrated by HMI, in mice that received DC from *Chlamydia muridarum*-infected donors was significantly reduced (**Figure 53B**). Taken together, the data demonstrate that adoptive transfer of DC isolated from *Chlamydia muridarum*-infected mice can modulate not only Th2 cytokine patterns to allergen exposure, but also classic hallmarks of allergic airway inflammation such as lung eosinophil infiltration, as well as mucus production by airway epithelial goblet cells.

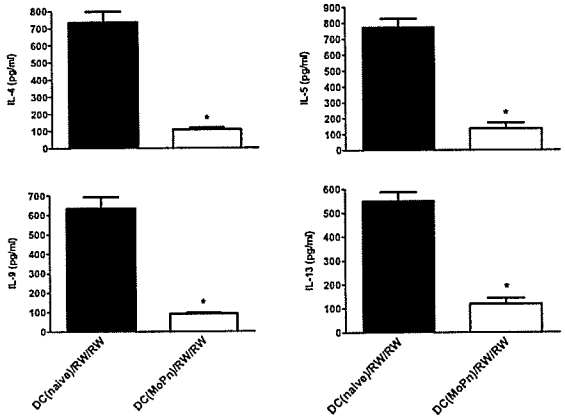

Figure 52. Effect of whole DC transfer on allergen-driven cytokine production by draining lymph node cells. Recipient mice (C57BL/6, n=3/group) of adoptively transferred DC from *Chlamydia muridarum*-infected or naïve mice from syngeneic mice were sensitized i.p. with RW (100 μg in alum). At fourteen days following sensitization, mice were challenged i.n. with 150 μg RW and sacrificed 6 days post-challenge. Draining lymph node cells from different groups of mice were cultured in the presence of RW (0.1 mg/ml). Cytokines in the supernatants were analyzed by ELISA. Data are presented as mean ± SD. One representative of three independent experiments with similar results is shown. *, $P<0.05$, cytokine production in mice delivered DC from *Chlamydia muridarum*-infected mice compared to levels detected in mice given DC from naïve mice prior to RW sensitization/challenge.

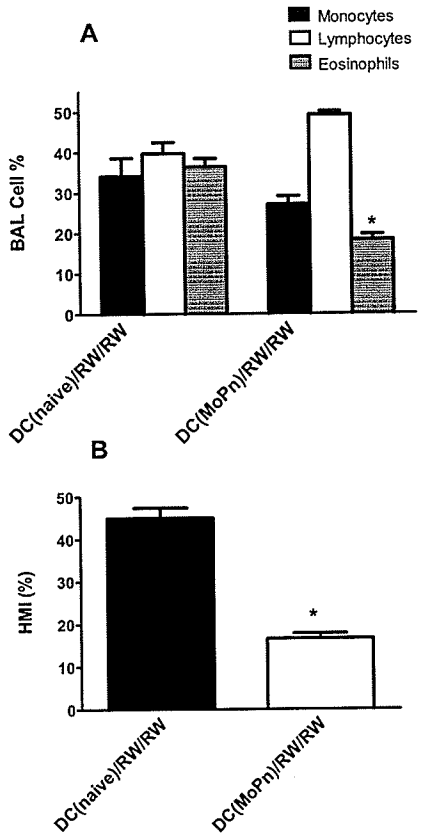

Figure 53. Dramatic reduction in the percentage of BAL eosinophils as well as mucus production in mice administered whole DC isolated *Chlamydia muridarum* infection prior to RW sensitization/challenge compared to mice which received RW treatment only. One group of mice was infected with 1 x 10^4 IFU *Chlamydia trachomatis* (*Chlamydia muridarum*). On day 7 post-infection, DC were isolated using CD11c beads using MACS system. 2 h following DC adoptive transfer from *Chlamydia muridarum*-infected or naïve mice, all mice were sensitized i.p. with RW (100μg) in alum and were challenged with RW (150μg) on day 14 following sensitization. **(A)** Mice were sacrificed day 6 post-challenge and mice tracheas were cannulated and BAL fluids acquired by washing lungs twice with sterile PBS. BAL cellular components were analyzed by differential cell counts using Fisher Leukostat Stain Kit. **(B)** Lung tissues were fixed and stained. Mucus was stained by means of the Periodic Acid-Schiff stain. (PAS) and the Histological Mucus Index (HMI) was calculated by based on the percentage of the mucus-positive area over the total area of the airway epithelium. Bronchoalveolar lavages were used to measure eotaxin levels by ELISA and were also used for a differential cell count. Data are presented as the mean +/- SD of each group. *, $P<0.05$.

3.6.3 CD11c+CD8α+ DC (DP) isolated from *Chlamydia muridarum*-infected mice and adoptively transferred to naïve mice can inhibit Th2 cytokines, lung eosinophilia and eotaxin production induced by RW exposure.

To examine the potential sub-populations of DC capable of regulating the responses to RW allergen, we were interested in sorting whole DC isolated from either naïve or infected mice, adoptively transferring them to naïve mice, and sensitizing/challenging mice with RW. Donor mice were infected intranasally with *Chlamydia muridarum* and sacrificed seven days later. Naïve mice were used as uninfected controls. Seven days post-infection, infected or naïve mice were sacrificed and spleens were aseptically excised. Single cell suspensions were made and cells were isolated based on CD11c expression using MACS beads (see *Materials and Methods*). CD11c+ cells were then further subjected to cell sorting based on CD11c+CD8α+ (DP) or CD11c+CD8α- (SP) cell-surface expression. Sorted DC subsets, namely DP and SP DC, collected from *Chlamydia muridarum*-infected mice or DP and SP DC collected from naïve mice, were adoptively transferred to naïve, syngeneic recipients, and 2 h later, were sensitized with RW allergen i.p. Fourteen days later, all four groups were challenged with RW i.n. and sacrificed 6 days later to assess the severity of the allergic reaction. As shown in **Figure 54**, upon RW re-stimulation *in vitro*, local draining lymph node cells from DP DC (*Chlamydia muridarum*)-, RW-treated mice produced significantly lower levels of Th2-related cytokines, namely IL-4, IL-5, IL-9 and IL-13 in comparison to mice treated with either SP DC from *Chlamydia muridarum*-infected mice or either subset isolated from naïve donors. Furthermore, DP DC from *Chlamydia muridarum*-infected mice could significantly reduce cellular infiltration into the lung following RW challenge, compared to mice given CD8α- DC from infected mice or either subset sorted from naïve mice (**Figure 55A**). Eotaxin levels within BAL fluids were significantly reduced in samples obtained from mice treated with CD8α+ DC sorted from *Chlamydia muridarum*-infected mice, in comparison to those mice treated with RW following delivery of CD8α- DC from *Chlamydia muridarum*-infected mice or either subset isolated from naïve donor mice (**Figure 55B**). These results

clearly demonstrate an alteration in the profile of RW-induced Th2-related cytokines, lung eosinophilia as well as eotaxin production in mice pre-treated with CD8α+ (DP) DC isolated from Chlamydia-infected mice.

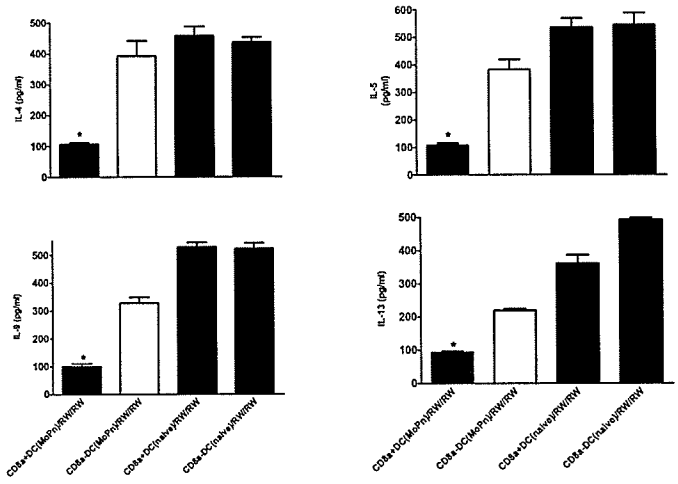

Figure 54. Recipients of CD8α+ DC from infected mice produce significantly lower RW-driven Th2 cytokines following challenge infection. Draining lymph node cell culture supernatants from experiments described in were determined for Th2 cytokines (IL-4, IL-5, IL-9 and IL-13) by ELISA. Data are presented as the mean +/- SD of each group. *, $p<0.05$, compared to mice with DC adoptive transfer from naïve mice. One representative experiment of three independent experiments is shown.

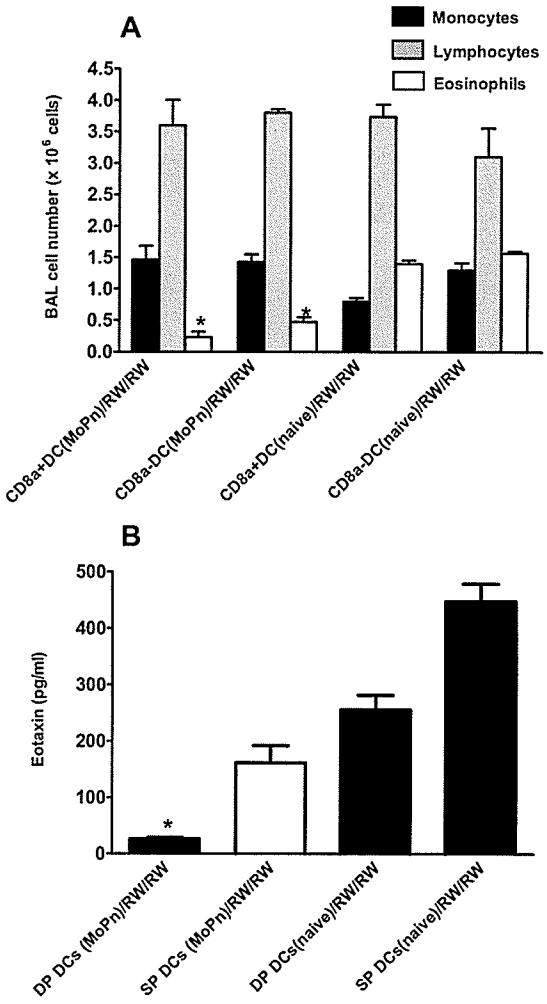

Figure 55. Dramatic reduction in the percentage of BAL eosinophils as well as mucus production in mice administered CD8α+ DC isolated *Chlamydia muridarum*-infected mice prior to RW sensitization/challenge. One group of mice was infected with 1 x 10^4 IFU *Chlamydia trachomatis* (*Chlamydia muridarum*). On day 7 post-infection, DC were isolated using CD11c beads using MACS system and sorted based on cell-surface expression of CD8α. 2 h following DC adoptive transfer from *Chlamydia muridarum*-infected or naïve mice, all mice were sensitized i.p. with RW (100μg) in alum and were challenged with RW (150μg) on day 14 following sensitization. Mice were sacrificed day 6 post-challenge and mice tracheas were cannulated and BAL fluids acquired by washing lungs twice with sterile PBS. **(A)** BAL cellular components were analyzed by differential cell counts using Fisher Leukostat Stain Kit. Lung tissues were fixed and stained. **(B)** Bronchoalveolar lavages were used to measure eotaxin levels by ELISA and were also used for a differential cell count. Data are presented as the mean +/- SD of each group. *, $P<0.05$.

3.6.4 Delivery of CD11c+CD8α+ (DP) DC from *Chlamydia muridarum*-infected mice inhibits pulmonary eosinophilia and bronchial mucus production induced by RW.

As both eosinophils infiltration into the lung and mucus oversecretion are the hallmarks of the asthma-like reaction, a direct comparison was made between the histological analysis of these groups of mice and the subset of dendritic cells received prior to exposure to RW allergen. Histological analysis also showed remarkably reduced levels of infiltrating eosinophils into the bronchial and pulmonary tissues of the RW-treated mice with previous CD8α+ DC isolated from *Chlamydia muridarum*-infected mice. Mice that had been treated with CD8α- DC taken from *Chlamydia muridarum*-infected mice, CD8α+ DC from naïve mice or CD8α- DC isolated from naïve mice displayed massive infiltration of eosinophils in the bronchial submucosa, alveolar, and perivascular sheaths, accompanied by a small amount of infiltrating monocytes and lymphocytes (**Figure 56, #1**). In addition, the level of mucus production demonstrated by Periodic Acid-Schiff (PAS) staining in mice given CD8α+ DC followed by RW exposure was significantly lower than that of those mice pre-treated with CD8α- DC from *Chlamydia muridarum*-infected mice or either subset isolated from naive mice (**Figure 56, #2**). Collectively, these results show that the adoptive transfer of CD8α+ DC sorted from *Chlamydia muridarum*-infected mice prior to RW sensitization/challenge is capable of inhibiting the local allergic inflammatory response, especially lung tissue eosinophilia (the site of allergen challenge), and of reducing the mucus production which greatly contributes to the pathology in the asthma-like reaction.

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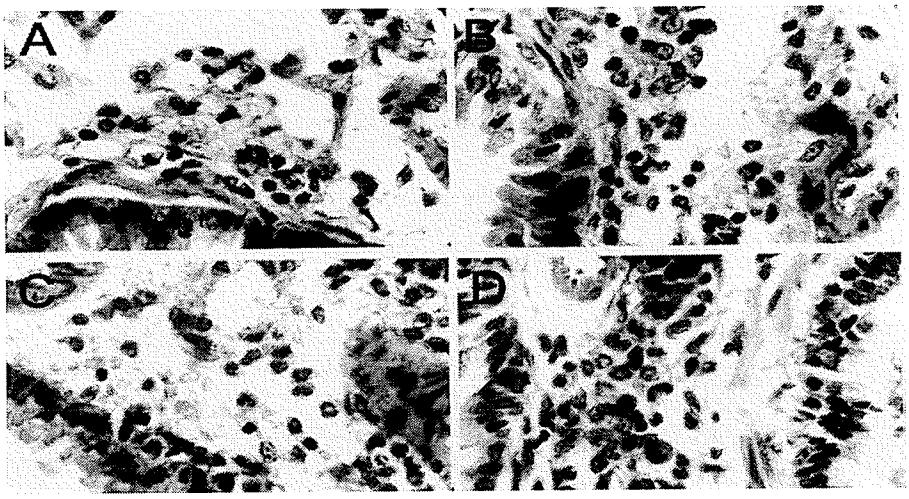

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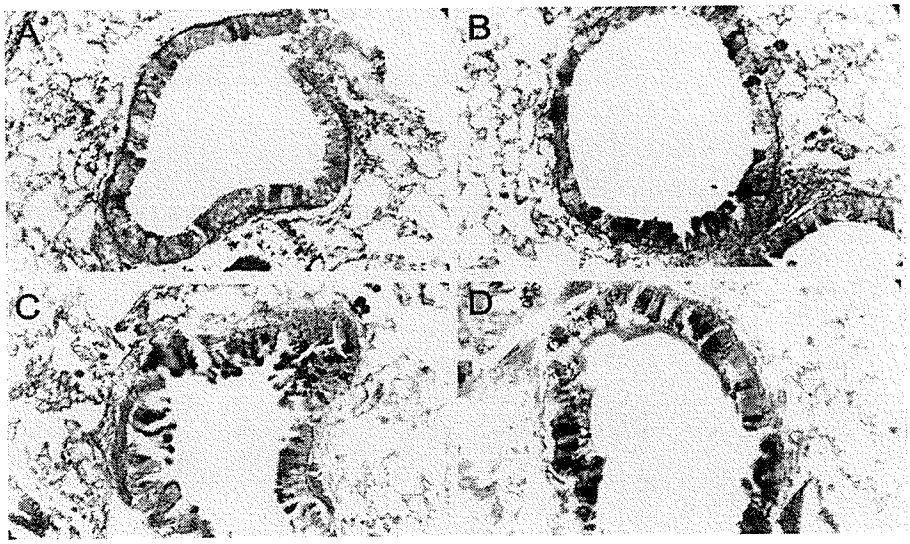

Figure 56. Significant reduction in peribronchial eosinophilic inflammation and bronchial mucus production in mice previously administered CD8α+ DC prior to RW sensitization/challenge. Lungs were treated with DC subsets (DP or SP from *Chlamydia muridarum*-infected or naïve mice) prior to RW sensitization were aseptically removed and fixed in formalin. Lungs were sectioned and stained with H & E. Sections were photographed at low (x 100) magnification; Mucus staining was performed using the PAS technique; mucus –positive cells stain as red/purple, and cytoplasm as blue. All sections were photographed at the same magnification (x 200). For both figures 1 & 2: Figure A: CD8α+ (*Chlamydia muridarum*)/RW/RW, B: CD8α- (*Chlamydia muridarum*)/RW/RW, C: CD8α+ (Naïve)/RW/RW, D: CD8α- (Naïve)/RW/RW).

3.6.5 *Chlamydia muridarum* Infection induces expansion of CD8α+ DC population which show difference cytokine production patterns.

We next examined the effect of *Chlamydia muridarum* infection on DC population in vivo. . To test the cytokine production pattern of the DC subsets, sorted DPDC and SPDC were placed in culture and the spontaneous cytokine production was measured. As shown in **Figure 57**, DPDC produced significantly higher levels of IL-12 and IL-10 that SPDC. The results indicate that CD8α+ DC from infected mice produce more immune regulatory cytokines than CD8α- DC.

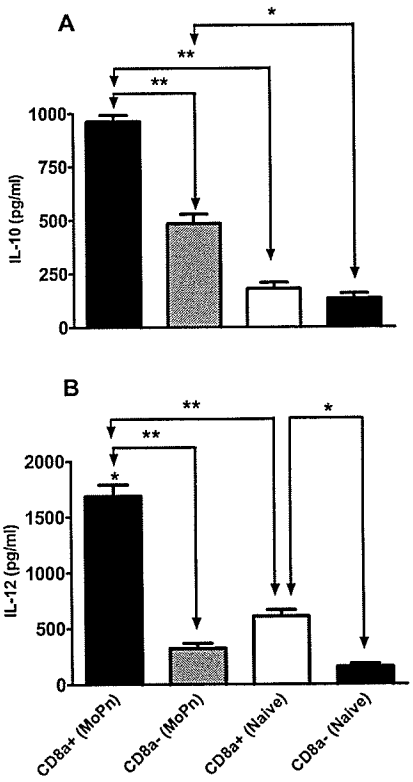

Figure 57. $CD8\alpha^+$ DC isolated from *Chlamydia muridarum*-infected mice produce significantly higher levels of IL-12 and IL-10 compared to $CD8\alpha^-$ DC isolated from infected mice or DC isolated from naïve mice. Mice were infected intranasally with *Chlamydia muridarum* (1000 IFU) and were sacrificed 7 days post-infection. Naïve mice were used as controls. Spleen cells were surface stained for CD11c and CD8α and subjected to cell sorting resulting in $CD11c^+CD8\alpha^+$ and $CD11c^+CD8\alpha^-$ subpopulations from infected and naïve mice. The DC subsets were placed in culture for 72 hrs and cytokines in the supernatants were measured by ELISA. *, $p<0.05$, **, $p<0.001$. One representative experiment of three independent experiments is shown.

3.6.6 Discussion.

In the present model we have shown that the transfer of CD8α+ DC, compared to CD8α- DC, isolated from *C. trachomatis Chlamydia muridarum*-infected mice are much more capable of transferring protection against RW-induced allergy to naïve mice. To our knowledge, this is the first report showing variable capacity of DC subsets in inducing protection against RW-induced allergy in a murine model of allergic airway inflammation. Our results showed that the DC subsets were different in cytokine production, in particular, CD8α+ DC secrete IL-12 and IL-10 at significantly higher levels compared to CD8α- DC isolated from infected mice or either subset taken from naïve mice. The ability of CD8α+ DC from *Chlamydia muridarum*-infected mice to produce higher levels of these cytokines indicate a pertinent role for these cytokines secreted in the transfer of protective immunity against subsequent allergy induced by RW delivery.

The finding that different DC subsets induce different levels of inhibition may be helpful in understanding the previous reports on the role of DC in allergic disease mechanisms. For example, Shaw et al have reported that adoptive transfer of whole *Chlamydia* organism-pulsed bone marrow derived DC induce strong Th1 responses and protection to challenge infection (Su, Messer et al. 1998) while major outer membrane protein (MOMP)-pulsed bone marrow derived DC induce Th2 type responses and failed to provide protection (Shaw, Grund et al. 2002). They also found that the DC pulsed with whole organism produce higher IL-12 than the DC pulsed with MOMP protein. It is therefore likely that the two system preferentially elicit different DC subsets, i.e., the former mainly induce DC1-like cells which are functionally similar to the DP DC in our model, thus inducing responses favouring an arm of immunity which is skewed away from the typical Th2-type response normally induced by RW allergen. O'Connell *et al* have also reported that DC isolated from mouse liver are also phenotypically distinct with regards to CD8α+ and CD8α- DC subsets (O'Connell, Son et al. 2003). These similarities between DC subsets in various tissues may not necessarily represent identical populations and also be tissue specific.

Collectively, the findings of the present study indicate that $CD8\alpha^+$ DC from infected donors, in contrast to $CD8\alpha^-$ DC, play a crucial role in the infection-mediated inhibition of allergic response induced by RW. The difference in cytokine profiles of the various DC subset isolated from *Chlamydia muridarum* or naïve mice could present a possible mechanism by which the subsets from *C. trachomatis Chlamydia muridarum*-infected mice provide different levels of protection against allergy to recipient mice. As DC are exquisitely sensitive to environmental signals, there is considerable interest in the development of therapeutic interventions targeted specifically at DC. Whether switching from a Th2 to a more Th1-type immune response, or inducing an IL-10-producing regulatory DC population remains controversial, and there is clearly a need to develop a greater understanding of how DC and their subsets can be induced and hence manipulated to induce an inhibitory response against allergen, for the prevention and/or treatment of allergic disease.

Part IV. General Discussion

Human epidemiological studies support an inverse association between exposure to infections and allergic responsiveness (reviewed by (Yazdanbakhsh, van den Biggelaar et al. 2001)). This thesis supports these observations with experimental data and provides evidence of bacterial-induced responses restraining allergic airway inflammation.

Using experimental murine models, this thesis aimed to dissect the relationship between infection with a prominent intracellular bacteria such as *C. muridarum* as well as *Mycobacterium bovis* BCG and allergic reactivity. We hypothesized that immunological events invoked during either one of these infections and allergic reactivity interact with the net effect of a suppressed allergic response to a common environmental allergen, namely ragweed. Additionally, we hypothesized that other key innate immune cells, such as natural killer T (NKT) cells have a key role to play in the response to either ragweed allergen or the Chlamydia intracellular bacterial infection. To address this aim and test our hypotheses, we used a murine model of allergic airway inflammation using ragweed allergenic extract, and two individual models of intracellular bacterial infection, *C. muridarum* (*Chlamydia muridarum*) as well as *Mycobacterium bovis* BCG. These models allowed us to observe modifications in cellular recruitment, cytokine and chemokine expression, and airway pathology in the presence of an intracellular bacterial infection with this allergen. As well, the use of mice deficient in NKT cells and the murine Chlamydia infection model as well as murine RW allergy model allowed us investigate the involvement of NKT cells in the immune response against RW or *Chlamydia muridarum.*

RW-induced allergic airway inflammation in a mouse model

Allergy, particularly allergic asthma, is a complex syndrome, characterized as a chronic inflammatory disorder of the airways associated with airway obstruction and airway hyperresponsiveness (AHR). Airway inflammation is thought to play a central role in mediating other pathophysiological features. Murine asthma models are useful tool to study the complicated mechanism of human pulmonary inflammation, since human and murine immune systems have similar patterns of cytokine and immunoglobulin production. However, despite the similarity, mice do not spontaneously develop asthma, but rather asthmatic reactions can be induced in mice by using different exposure protocols. In our model, allergic responses were achieved by systemic sensitization of mice to RW plus alum, followed by intranasal challenge with RW pollen extract. Using this protocol, mice developed robust eosinophilic inflammation, mucus overproduction, RW-specific antibody production as well as elevated total serum IgE levels and Th2-polarized T cell cytokine production patterns.

In contrast to this model, some studies have used systemic sensitization followed by several aerosolized allergen challenges to induce allergic responses, because one challenge with brief exposure to nebulized allergen is not sufficient to fully establish the allergic responses in the lung (Hamelmann, Tadeda et al. 1999). This might be due to the fact that the actual amount of allergen delivered to the lung by nebulization is difficult to control. In contrast, a single intranasal application of allergen in sensitized mice can induce significant allergic airway disease in our model, indicating adequate amount of allergen has been delivered to the lung. In some other studies, development of allergic asthma was induced by systemic administration of allergen alone without adjuvant followed by airway challenge, or, in the absence of systemic sensitization, by repeated administration of allergen directly into the lung either by inhalation, intranasal, or intratracheal instillation (Renz, Smith et al. 1992; Yu, Shieh et al. 1999; Williams and Galli 2000). Compared to our present model of RW allergy, all

of the aforementioned protocols, which avoid sensitization or adjuvant, elicit less prominent airway inflammation, develop weaker Th2 response, and take a longer period of time in order to establish the allergic response. More importantly, the choice of allergen in this study, ragweed, has not as yet been tested in models examining the relationship between allergy and infection. Most studies to date have used mouse models of asthma induced by ovalbumin (OVA) exposure. Compared to OVA, ragweed is a natural allergen existing in the environment and is a major cause of clinical asthma. To investigate the effect of infection on the allergic response, it was essential to establish a reproducible allergic airway inflammation model induced by ragweed exposure. The data showed that RW sensitization and challenge was able to successfully induce the classic Th2 allergic phenotype, including RW-driven Th2 cytokines, lung tissue as well as systemic eosinophilia, mucus overproduction and elevated RW-specific and total serum IgE compared to mice without RW exposure.

The effect of *Chlamydia muridarum* Infection or BCG infection on the Development of the RW-induced Allergic Response

The results presented in this part of the study clealyr demonstrated that prior *C. trachomatis* (*Chlamydia muridarum*) infection inhibits RW-induced allergic reactions, characterized by significant reductions of systemic and local eosinophilic inflammation and mucus production. The reduction of airway pathogenesis was associated with a switch of allergen-driven cytokine production patterns of local CD4 T cells towards a Th1 phenotype, and this was accompanied by a dramatic decrease of VCAM-1 expression and eotaxin production in the lung, suggesting intracellular bacterial infection, which induces Th1 dominant immune responses, may re-direct the immune response to the RW allergen. Additionally, the inhibitory effect of prior *Chlamydia muridarum* infection on the development of allergic responses lasted beyond the time frame of active infection. These results support the

hypothesis that a reduction of infectious diseases may contribute to the increased prevalence of atopic disorders in humans.

The data suggest that alteration in cytokine patterns is crucial for bacterial infection-mediated suppression of the allergic response. Immune responses to newly encountered antigens may be generally divided into two classes: Th1, which are characterized by the generation of IFN-γ and IL-12; and Th2, which are associated with a release of IL-4 and IL-5 (Piccinni, Mecacci et al. 1993; Holgate 1998). Bacterial infections are generally a strong trigger for Th1 responses. Asthma, on the other hand, results from the inappropriate generation of Th2 responses to environmental antigens, accompanied by pulmonary inflammation (Yabuhara, Macaubas et al. 1997). *Chlamydia muridarum*, a Gram-negative, intracellular bacterium, elicits a strong cell-mediated, Th1 dominant immune response in mouse model (Yang 2003). Our results showed that a previous *Chlamydia muridarum* infection in mice induced a Th1 rather than Th2 immune response to subsequent RW sensitization, characterized by increased IFN-γ and IL-12 production, as well as decreased IL-4, IL-5, IL-9 and IL-13 production.

How can prior intracellular bacterial infection modulate the host's cytokine-producing patterns and airway reactivity induced by allergen? The commitment to Th1 or Th2 cells is determined by a variety of factors, including the cytokine milieu in which the antigen is initially presented to specific lymphocytes (Seder, Paul et al. 1992), whose selective differentiation is affected by microenvironmental factors that regulate transcriptional activation of cassettes of specific cytokine genes (Constant and Bottomly 1997; Lohoff, Ferrick et al. 1997; Zheng and Flavell 1997). The early presence of IFN-γ and IL-12 favours Th1 polarization whereas IL-4 is the potent stimulus for Th2 polarization. IL-12 can activate transcription factors related to the Th1 phenotype, including Stat4 (Kaplan, Sun et al. 1996; Magram, Connaughton et al. 1996; Morinobu, Gadina et al. 2002). In addition, mice with germline disruption of the IL-4 gene fail to generate Th2-like cells and fail to produce IgE antibodies (Kuhn, Rajewsky et al. 1991; Kopf, Le Gros et al. 1993; Lederer, Perez et al. 1996). IL-4 induces the development of Th2-like cells, via signaling, through the activation of Stat6, a transcriptional factor for the

Th2 phenotype (Lederer, Perez et al. 1996). Intracellular bacteria often induce Th1-like responses because they can innately activate macrophages and dendritic cells, which produce IFN-γ and IL-12, and natural killer (NK) cells, which produce IFN-γ, thereby establishing a setting for Th1-like T-cell priming. In contrast, allergens may fail to establish a setting for Th1 priming when being exposed *in vivo*, resulting in the development of Th2-like cells. It is possible therefore that the Th1-like setting in the microenvironment of antigen-presenting cell–allergen peptide–T-cell interaction, established by previous intracellular bacterial infection, can direct the allergen-specific T cells to Th1-like cells when allergen is subsequently exposed.

One of the most significant differences of allergic responses between mice with or without prior infection is the suppression of systemic and local eosinophilia. Reduction of IL-5 production in the lung is one of the major factors responsible for the observed inhibition of airway eosinophilia, since accumulation of eosinophils into the airway is highly dependent upon IL-5 (Coffman, Seymour et al. 1989; Kopf, Le Gros et al. 1993). Treatment with anti-IL-5 antibodies can inhibit eosinophilic infiltration in allergen-sensitized and allergen-challenged mice (Nagai, Yamaguchi et al. 1993). Furthermore, using IL-5 deficient mice, airway eosinophilia could not be induced, and IL-5 transgenic mice displayed eosinophilic infiltration and increased AHR in the absence of allergen challenges (Kopf, Le Gros et al. 1993). Erb *et al.* reported infection of mice with BCG suppressed allergen-induced airway eosinophilia, and administration of IL-5 into the lung only partly restored airway eosinophilia (20-25% of the levels observed in control mice) (Erb, Holloway et al. 1998). Since IL-5 administration into the lung did not fully restore eosinophil accumulation into the airway, infection may induce some other factors, contributing to the suppression of airway eosinophilia. An interesting finding that has not yet been reported is the ability of intracellular bacterial infection to reduce the levels of eotaxin in the lungs of allergen-treated mice. Eotaxin is the most important chemokine in eosinophil recruitment. In addition, eotaxin can induce eosinophil degranulation via CCR3 expressed on the eosinophil cell surface, which may also contribute to eosinophilic inflammation in the airway (Fujisawa, Kato et al. 2000). More recently, it was reported that eotaxin elicits rapid vesicular transport-mediated

release of pre-formed IL-4 from human eosinophils (Bandeira-Melo, Sugiyama et al. 2001). The decrease in eotaxin levels within the lungs of *Chlamydia muridarum*-infected, RW-treated mice or RW-treated, BCG-infected mice could be a reason for the reduction in eosinophilia within the lungs of infected mice. Several animal models have demonstrated that eotaxin levels correlate with the number of eosinophils infiltrating into the lung tissue (Yang, Loy et al. 1998). In multiple allergen challenge experiments in mice, an antibody to eotaxin reduced eosinophil recruitment and abolished hyperresponsiveness of the lung (Campbell, Kunkel et al. 1998). Cytokines that are synthesized by Th2 lymphocytes, such as interleukin-4, interleukin-13 and interleukin-5, are potential intermediaries in eotaxin production (Rankin, Conroy et al. 2000; Conroy and Williams 2001). Our results showed that prior *Chlamydia muridarum* infection in mice produced less Th2 cytokine upon RW challenge, which provided a possible mechanism that linked Th2 cells, eotaxin production and eosinophil recruitment in infection-mediated inhibition of allergic responses.

Another interesting finding in the present study was the elevation of total serum IgE in mice infected with either *Chlamydia muridarum* prior to RW sensitization/challenge or with the delivery of BCG during established RW allergy. Numerous studies have concluded that the levels of serum IgE are not a concrete parameter for determining and/or predicting human airway reactivity, hyperresponsiveness (van der Pouw Kraan, Aalberse et al. 1994), asthmatic symptoms or eosinophilic reaction (Wilder, Collie et al. 1999). Some cytokines are capable of exacerbating the level of serum IgE while still significantly decreasing other parameters which are associated with allergic diseases. IL-18, also known as interferon-gamma-inducing factor, is involved in enhancing IFN-γ production and therefore a Th1 response, Yoshimoto *et al* have clearly shown that IL-18 alone has the potential to stimulate basophils (Yoshimoto, Tsutsui et al. 1999). These cells, upon degranulation, release a variety of chemical mediators such as serotonin and histamine and a set of cytokines such as IL-4 and IL-13, which can contribute to class switching of B cells to IgE production. This suggests that IL-18, in conjunction with other pro-inflammatory cytokines, could contribute to the differentiation process of

precursor CD4 T cells, working in synergy with IL-4, into the Th2 phenotype. Our previous data have shown that *Chlamydia muridarum* infection induces IL-18 responses (Lu, Yang et al. 2000). Therefore, a previous bacterial infection may contribute to the generation of cytokines that might exacerbate IgE production, while also reducing airway eosinophilia. Another potential cellular mechanism which may contribute to both eosinophil reductions and IgE increase is NKT cells. NKT cells are an extremely versatile group of cells that are involved in the promotion of a Th1 reaction induced by intracellular bacterial infection, and the production of Th2 cytokines such as IL-4. It is possible that NKT cells may play a dual role in inhibiting airway eosinophilia by producing IFN-γ and in enhancing IgE production as a result of IL-4 production. Data that supports this unusual finding is discussed.

The findings in the present study also suggest that it is possible to re-direct an immune response to allergens following BCG infection, which may normally be detrimental to the responding host. The otherwise protective effect of infection, in suppressing the development of the asthma-like reaction, extends well beyond the time frame of active infection. Other studies have demonstrated an inverse correlation between childhood infection and the development of asthma in later life (Alm, Lilja et al. 1997; Ball, Castro-Rodriguez et al. 2000; Anderson, Ruggles et al. 2004). It is also reported that the occurrence of allergy within an individual family inversely correlates with the birth order of each sibling and that the youngest child is the least likely to develop an allergic disease (Ball, Castro-Rodriguez et al. 2000; Beasley, Crane et al. 2000). The present study provided experimental evidence that microbial infections may be potent modulators of the allergic process, providing potential explanation of these observations.

Dendritic Cells from Infected mice in the Regulation of the Allergic Response

In the present study, using both in vitro and in vivo approaches, we demonstrated a crucial role played by DCs in the infection-mediated modulation of allergen-specific CD4+ T cell responses and allergic inflammation. We found that DC isolated from BCG infected mice expressed significantly higher levels of CD8α and TLR9, and produced higher levels of IL-10 and IL-12 than DC from naïve mice. The blockade of IL-10 or IL-12 abolished the protective effect of DC on Th2 cytokine production by allergen-specific CD4 T cells. Furthermore, we showed that adoptive transfer of DCs isolated from infected mice are capable of reducing the development of Th2-like cytokine responses and eosinophilic inflammation induced by allergen. The data further extend our knowledge on the mechanisms by which infection inhibit allergy, especially the role of DC in this process.

We have shown that, similar to that observed in natural infection, the adoptive transfer of iIDC is capable of suppressing allergic airway inflammation following OVA exposure (Han, Fan et al. 2004). The data provide clear evidences that DCs educated by chlamydial infection in vivo can efficiently modulate T cell responses to allergen. Although previous studies have shown the modulating effect of microbial infection and microbial products on DC function, direct evidence is lacking for the modulating effect of DCs educated by microbial infection on allergen-specific CD4 T cells. The results suggest that intracellular bacterial infection may prevent the development of Th2-like allergic responses by deviating the immune responses away from a Th2 profile towards a Th1 profile through the modulation of DC function. There are at least two mechanisms, which may account for the modulating effect of transferred iIDC on CD4 T cell response to allergen in vivo. One is that the iIDCs which are educated by microbial infection can directly present allergen to T cells in recipient mice, as shown in the in vitro experiments in this study, and the other is that transferred iIDCs can further influence the function of DCs in the recipient mice, leading to decreased Th2-like cell development.

A novel finding in the present study is the high TLR9 expression on DCs isolated from BCG-infected mice. TLRs provide important links between innate and adaptive immune responses (Akira, Takeda et al. 2001; Edwards, Diebold et al. 2003). Several studies comparing farm and non-farm children in the development of allergy and asthma have shown significantly fewer allergies in the children growing up in a farming environment who arguably have higher exposure to microbial products (Akira, Takeda et al. 2001). A recent study showed that the expression of TLRs on peripheral blood cells of farm and non-farm children are different, suggesting the contribution of the alteration in innate immunity caused by microbial exposure in the modulation of allergic reaction (Lauener, Birchler et al. 2002). Thus, higher TLR-9 expression in the present study is particularly interesting because TLR-9 is the receptor for bacterial CpG motifs. Numerous studies have shown that CpG can inhibit de novo and established allergic responses in animal and human models (Bohle 2002). Therefore, we can speculate that BCG infection, via modulation of TLR expression, can change the phenotype of DCs, leading to decreased allergen-specific Th2 cell and, to a lesser degree, enhanced Th1 cell development. By examining the effect of DCs with different TLR expression on allergic responses in vivo, our present study provides a closer link between the changes in innate immunity and the alteration of adaptive immune responses to allergen.

How do DCs from infected mice modulate allergen-specific T cell cytokine production? It has been previously reported that the ability of DCs to modulate T cell responses is largely dependent upon their cytokine patterns. DCs are the principal cell required for induction of primary peptide-specific Th1 activation and clonal expansion (Macatonia, Hsieh et al. 1993; Langenkamp, Messi et al. 2000; Moser and Murphy 2000; Vieira, de Jong et al. 2000; MacDonald and Pearce 2002). Moreover, down-regulatory molecules which are produced by DCs such as IL-10, transforming growth factor beta (TGF-β), and nitric oxide (NO) have been implicated in the suppression of various arms of the immune system (Levings, Bacchetta et al. 2002; Zemann, Schwaerzler et al. 2003). Interestingly, our data showed that DCs from infected mice produced higher levels of both IL-12 and IL-10 and the blockade of either could partially reverse the inhibition effect of DCs from infected mice on the

allergic responses. It is likely that the DCs from BCG infected mice are heterogeneous in terms of cytokine production. One population may be higher IL-12 producers, enhancing the development of Th1 cells which inhibit allergen-specific Th2 cells, while another population may produce higher IL-10 (tolerogenic DC or DCreg), leading to unresponsiveness of allergen-specific Th2 cells or the development of Treg. In line with this thought, DCs from BCG infected mice also express higher levels of TLR-9, a receptor for CPG which is strong inducer of Th1 responses, which, in combination with higher IL-12 production, suggests the development of DC1-like cells. On the other hand, amagaloid DC, a newly defined DC subset which produce IL-10, are often tolerogenic DCs, also express higher TLR-9, therefore supporting the existence of a tolerogenic subset in DCs from BCG infected mice. Moreover, we found that DCs from BCG-infected mice showed a significantly larger CD8α+ population than the DCs from naïve mice. It is likely that this larger population of $CD8\alpha^+$ DCs from BCG-infected mice play a prominent role in re-directing the established Th2 allergic phenotype towards a more Th1-like reaction following RW re-challenge. It has been previously reported that, although $CD8\alpha^+$ DCs are comparable to $CD8\alpha^-$ DCs with regards to other cell surface marker expression and can both capture soluble antigens, process them, and prime antigen-specific $CD4^+$ and $CD8^+$ T cells efficiently *in vivo,* the $CD8\alpha^+$ DC subset induce a predominant Th1-like response (Pulendran, Banchereau et al. 2001). The significantly higher percentage of Th1-inducing $CD8\alpha^+$ DCs present in BCG-infected mice compared to naïve mice could be a mechanism by which the adoptive transfer of DCs from infected mice alters the established allergic response to RW allergen. Further studies on the subsets of the DCs from BCG infected mice will lead to better understanding of the mechanisms by which DCs modulate allergic responses.

In the present model we have shown that the transfer of CD8α+ DC, compared to CD8α- DC, isolated from *C. trachomatis Chlamydia muridarum*-infected mice are much more capable of transferring protection against RW-induced allergy to naïve mice. To our knowledge, this is the first report showing variable capacity of DC subsets in inducing protection against RW-induced allergy in a murine model of allergic airway inflammation. Our results showed that the DC subsets were different in cytokine production, in

particular, CD8α+ DC secrete IL-12 and IL-10 at significantly higher levels compared to CD8α- DC isolated from infected mice or either subset taken from naïve mice. The ability of CD8α+ DC from *Chlamydia muridarum*-infected mice to produce higher levels of these cytokines indicate a pertinent role for these cytokines secreted in the transfer of protective immunity against subsequent allergy induced by RW delivery.

An interesting finding in this study was the elevated IL-10 production from whole DC isolated from BCG-infected mice, similar to the CD8α+ subset of DC from *Chlamydia muridarum*-infected mice. We therefore tested the involvement of this immunoregulatory cytokine in DC-mediated inhibition of allergen-driven Th2 responses, and found that partial inhibition of the infection-mediated protection from allergic inflammation could be achieved by *in vivo* delivery of anti-IL-10 neutralizing antibody. As well, in the adoptive transfer model of DC subsets and their induction of protective immunity against *C. muridarum* infection, there was a significantly higher production of both IL-10 and IL-12 from CD8α+ DC taken from *Chlamydia muridarum*-infected mice which correlated with a higher level of protection against a challenge infection with *Chlamydia muridarum.* Consistently, in both models of infection alone and infection-mediated inhibition of allergy by transfer of DCs from *Chlamydia muridarum*- or BCG-infected mice, respectively, IL-10 and IL-12 were both significantly elevated. As a result, mice were either protected from subsequent infection, had severe RW-induced pathology prior to RW sensitization or could inhibit an already-established RW allergy phenotype. It is becoming increasingly apparent that cytokines produced by DC play a central role in directing T cell polarization and in manipulating immune responses. IL-10 which is produced by DC has been shown to be related to the induction of immunological unresponsiveness and the suppression of immune reactions. The present study suggests that IL-10 may play an important role in DC-mediated inhibition of allergen-driven Th2 cytokine production and allergic responses. There are at least three potential mechanisms which might account for the inhibitory effect of IL-10 produced by DC from mice infected with either intracellular bacteria on the allergic. First, IL-10 may have directly regulatory function on allergen-specific T cell differentiation and allergic reaction (Moore, de Waal Malefyt et al. 2001). For

example, the presence of IL-10 may result in non-responsiveness of allergen specific Th2 cell progenitors during DC:CD4+ T cell interaction, thus reducing general Th2 cytokine production. As well, IL-10 can reduce eosinophil survival and eotaxin production, resulting in reduced eosinophil recruitment to the sites of allergic inflammation (Bellinghausen, Knop et al. 2001). Secondly, IL-10 producing DC may induce regulatory T cells such as Tr1 cells (Bilsborough, George et al. 2003). Akbari et al have found that IL-10 producing DC can stimulate the development of antigen-specific T regulatory cells that also produce high levels of IL-10, resulting in the inhibition of allergic responses (Akbari, DeKruyff et al. 2001). Interestingly, we found, in the present study, that DC from BCG-infected mice as well CD8α+ DC isolated from *Chlamydia muridarum*-infected mice produced higher levels of IL-10. More importantly, we found that blocking of IL-10 increased Th2 cytokine production by allergen-specific CD4+ T cells. Lastly, IL-10 which is produced by DC isolated from BCG- or *Chlamydia muridarum*-infected mice may influence the development and the function of endogenous DC in recipient mice, which subsequently inhibit the development of allergen-specific Th2 cells. Further studies are necessary to clarify whether some or all of the mechanisms play a role in the DC-mediated inhibition of allergy and allergen-specific Th2 cell development as well as their role in protective immunity against infection in vaccine-based studies.

NKT cells in Infection and Allergic Airway Inflammation

Our present study using a common environmental allergen provides evidence that NKT cells play an important role in allergic responses. The data show that airway eosinophilic inflammation induced by RW is significantly reduced in NKT deficient CD1 KO mice, which correlates with impaired IL-4 and eotaxin production. CD1 KO mice also displayed a significant decrease in serum allergen-specific and total IgE levels compared to BALB/c mice. The difference between CD1 KO and wild type BALB/c mice is unlikely due to other intrinsic immunological deficiencies of CD1 KO mice as the

development of immune cells,T and B cells, in the two types of mice are comparable (**Figure 23**) and more importantly, showed similar levels of T-cell proliferation and cytokine production upon polyclonal stimulation (**Figures 24A & 24B**). The inability of CD1 KO mice to produce IL-4 in response to allergen-specific re-stimulation provides evidence that NKT cells are responsible for the development of IL-4 responses to natural allergen, thus capable of affecting downstream allergic disease parameters. In contrast, the Th1 arm of the immune response (IFN-γ and IL-12) appears unaffected by the lack of NKT cells in our research model. The explanation for such a phenomenon may be two-fold. Firstly, the study model is an asthma-like reaction, which is dominated by Th2-like responses, therefore the impact of NKT deficiency may be more visible with regards to the parameters of Th2-type responses. Notably, the allergen-driven IFN-γ production even in wild-type mice is very low. Secondly, it is possible that NKT cells play more dominant role in IL-4 responses, although they are capable of producing IFN-γ in certain circumstances. Since Th2 cytokines other than IL-4 such as IL-5 and IL-13 remain largely unaffected by the absence of NKT cells in the allergen-treated CD1 KO mice (**Figure 20**), it is likely that NKT cells are particularly critical for allergen-driven IL-4 responses. Our data is in contrast with a previous report, which shows that NK cells, rather than NKT cells, play a significant role in allergic reaction in a mouse model (Korsgren, Persson et al. 1999). The reason for this discrepancy between the studies is unclear. However, our data collected from a comprehensive study analyzing airway eosinophilia, mucus secretion, antibody production and cytokine/chemokine responses focuses primarily on NKT cells, whereas the other study only partially assess the implication of NKT cells. More importantly, the study using α-GalCer treatment which showed enhanced allergic reaction in wild-type BALB/c, but not CD1 KO mice (**Figure 25**), provided convincing evidence that confirms the involvement of NKT cell in the allergic reaction. Moreover, the use of ragweed, a common environmental human allergen, in the present study further emphasizes the relevance of this finding to human asthma-like reaction. The results are also inconsistent with another study, which showed α-GalCer treatment inhibits IgE production (Cui, Watanabe et al. 1999).

Notably, the strain of mice (C57BL/6 vs. BALB/c) and allergen used (ovalbumin vs. ragweed) in these studies were different. It remains unclear whether these differences may account for the different observations. However, it should be pointed out that the present data are consistent with the original finding that NKT cells can promote IgE responses (Burdin, Brossay et al. 1999).

In addition to the role of NKT cells in our model of RW-induced allergic airway inflammation, we also showed another prominent role for NKT cells, namely their involvement in the development of *C. muridarum*-induced lung pathology. Overall, in this murine model of mouse pneumonitis (*Chlamydia muridarum*) infection, we found that CD1 KO mice, lacking NKT cells, mounted a stronger protective immune response than the wild-type control (BALB/c or 129) mice following *C. muridarum* infection. Furthermore, *C. muridarum*-infected CD1 KO mice showed significantly less body weight loss, lower chlamydial *in vivo* growth, and milder pathological changes during the infection. Moreover, it is supported by the finding that stimulation of NKT using α-GalCer increased host susceptibility to chlamydial infection. The data indicate that CD1d-restricted NKT cells can modulate the immune response to *Chlamydia* infection and contribute to pathological outcome caused by the infection. As only classical type 1, but not type 2 CD1-dependent NKT cells are responsive to α-GalCer stimulation (Godfrey, MacDonald et al. 2004), the results suggest that the NKT cells which play an immunomodulatory role during chlamydial infection in the lung are type 1 NKT cells. Cytokine analyses have shed light on the mechanism by which NKT influence host susceptibility to chlamydial infection. Previous animal and human studies in our lab (Yang, HayGlass et al. 1996; Wang, Fan et al. 1999) and by other researchers (Holland, Bailey et al. 1993; Holland, Bailey et al. 1996) have shown that Th2 cytokine responses are associated with susceptibility to chlamydial infection and accompanying pathology. In the present study, we demonstrated that the reduced pathology and decreased bacterial burden in CD1 KO mice were correlated with a reduced Th2 type immune response, especially lower levels of *C. muridarum*-driven IL-4 and IL-5 production. The initial IL-4 production by NKT may be the mechanism by which these cells

modulate adaptive immune response and promote host susceptibility to chlamydial infection, as neutralization of IL-4 immediately following α-GalCer treatment significantly reduced the promoting effect of this NKT ligand on chlamydial growth in vivo.

Innate Responses: Potential Exploitation of Key Innate Immune Cells to Control Allergic Asthma & Infection

Allergic asthma is a multifactorial respiratory disease involving both genetic and environmental factors which, in combination, lead to the development of a strongly polarized Th2 immune response towards innocuous antigens, characterized by IgE production and tissue inflammation including eosinophilia. In airways, inflammation is accompanied by tissue remodelling (fibrosis) and mucus hypersecretion leading to airway hyperreactivity. In the classical view, the immunological factors involved in the development of asthmatic reactions include, but are not limited to, IL-5 (which is associated with eosinophilia) plus IL-4 and IL-13 (which are individually or jointly associated with IgE production). At the cellular level, in addition to bronchial epithelial cells which contribute to the pathology, **DCs** (prototypical antigen-presenting cells) are considered to be important factors in the initiation, and according to some authors, the maintenance of the inflammatory phenotype. As has been shown in the presented data, it is increasingly clear that DC have a key role to play in the regulation of allergic airway inflammation. Through the exposure of mice to Th1-inducing agents, such as Chlamydia or BCG infection, a particular phenotype of DC, with regards to cell-surface molecule expression and cytokine production patterns, is generated. The ability of this IL-12- and IL-10-expressing DC to inhibit Th2-type reactions to RW sensitization and challenge (or even an already-established allergic phenotype) is clear by the use of the adoptive transfer studies from these infected mice and subsequent RW immunization and challenge and the showing reduced allergen-induced inflammatory response. This infection-mediated inhibition by DC isolated from infected mice appears to be partially dependent on the ability of these DC to produce IL-10 and IL-12 as

neutralization of either cytokine *in* vivo following transfer of these DC has a partial reversal of the inhibitory effect. The induction of the DC1-like phenotype, capable of inhibiting the allergic response induced by RW delivery, is also consistent with the phenotype of DC induced by Chlamydial infection and the subsequent protective immunity seen in mice pre-treated with DC isolated from Chlamydia-infected mice. Also, following the adoptive transfer of DC from *Chlamydia muridarum*-infected mice we see an enhanced protection against the pathology normally induced in infection with Chlamydia. This protection against Chlamydial infection as a result of this adoptive transfer of DC from infected mice also appears to be dependent upon the expression of CD8α+- and production of IL-10 and IL-12, as seen in the allergy model. These are key findings in both models, and particularly with regards to allergic disease. Lambrecht *et al* have recently suggested that DC have such a key role to play, that they follow the Koch's postulates in human asthma: 1) Allergic airway inflammation is accompanied by an increase in airway DC; 2) Administration of DC induces and exacerbates asthmatic features; and 3) Inhibition of DC function reduces asthmatic features (Lambrecht and Hammad 2003). As such, the therapeutic intervention with DC taken from an infection model may perhaps hold even more potential with such a strong influence of these particular cells on the outcome of allergic disease.

In addition to DC, it is evident that from the outlined results, that NKT cells might also exert agonistic affects on the outcome of asthma, at least in mouse experimental models. At this time, it is unrealistic to conclude that NKT cells are a solid, single target for allergy immunotherapy. This thesis' data strongly suggests a consistency of NKT cells in the ability to rapidly secrete cytokines, particularly IL-4, both in response to RW and Chlamydia, and influence the severity of allergic disease and Chlamydia pathology, respectively. In contrast, three papers have demonstrated that NKT cell activation by α-GalCer, whose administration suppresses tumor cell growth and autoimmune pathologies in various experimental models, inhibits allergic airway inflammation in an IFN-γ-dependent manner (Hachem, Lisbonne et al. 2005; Matsuda, Suda et al. 2005; Morishima, Ishii et al. 2005); however, the papers

differ regarding some of the parameters affected by the treatment, as well as the mechanisms involved. Hachem *et al* demonstrate that both intranasal and intravenous treatments with α-GalCer immediately prior to intranasal antigen challenge inhibit AHR, eosinophilia and IgE production, an effect abrogated by treatment with anti-IFN-γ but not with anti-IL-10 receptor (Hachem, Lisbonne et al. 2005). The effect of α-GalCer injection is mimicked by the transfer of sorted NKT cells or spleen cells from α-GalCer-treated wild-type but not α-GalCer-treated Jα18-/- or IFN-γ-/- animals. The total NKT cell frequency and cytokine balance within lung or spleen NKT cells was directly affected by the treatment, with decreased IL-4-producing and increased IFN-γ-producing NKT cell frequency. In a model using aerosolized antigen challenges, Matsuda *et al* demonstrate that, in addition to the parameters mentioned above (with the exception of IgE concentrations), IL-13 production and goblet cell hyperplasia are also decreased upon intraperitoneal α-GalCer injection, while IgG2a (a Th1 isotype in mouse) levels are increased (Matsuda, Suda et al. 2005). Most of these effects are abrogated in IFN-γ-/- animals, again suggesting a Th2 to Th1 shift in the immune response. Intriguingly, Morishima *et al* demonstrate the opposite effects of α-GalCer treatment with continuous α-GalCer administration increasing eosinophilia and IgE production, while a single subcutaneous delivery at the time of intranasal antigen challenge abrogates eosinophilia, fibrosis and AHR in wild-type but not in Jα281-/- mice (Morishima, Ishii et al. 2005). A stronger sensitization protocol and a more Th2-prone background might explain these differences. In addition to the altered Th1-Th2 balance observed following α-GalCer treatment, VCAM-1 expression, which promotes eosinophils recruitment, is inhibited by α-GalCer treatment. Interestingly, while the first two papers (Hachem, Lisbonne et al. 2005; Matsuda, Suda et al. 2005) indicate a direct effect of α-GalCer on cytokine production by NKT cells, the last one (Morishima, Ishii et al. 2005) shows that NK, rather than NKT, cells account for the increased IFN-γ production. It is likely that NKT cells are involved in the initial trigger of the process due to a very rapid, but transient, cytokine production, while NK cells have been associated with induction of asthmatic pathology (Korsgren, Persson et al. 1999).

Future Perspectives

Data in this thesis demonstrate an intricate role for both DC and NKT cells in infection and allergy models supports a more current view of NKT cell and DC interactions. The exposure to infectious products as well as allergens appear to differentially induce the expansion and cytokine secretion patterns of DC. Although the data provides evidence for the differential expression of cell-surface molecules (such as CD8α) and cytokine profiles (enhanced IL-10 and IL-12 production) in Chlamydia and BCG infections as well as RW allergy, there is also the potential for a change in expression of other key molecules on the surface of the DC. The expression of other cell-surface molecules may change in different microenvironments and affect the activation of other key innate cells capable of shaping the subsequent adaptive immune response. For example, Hayakawa *et al* have demonstrated that the regulation of NKT cells and their ability to become activated in a Th1 or Th2 manner is dependent upon their ligation by CD28 and CD40 co-stimulatory pathways (Hayakawa, Takeda et al. 2001). They showed that CD28-deficient mice showed impaired IFN-γ and IL-4 production in response to specific NKT cell activation with α-GalCer stimulation both *in vitro* and *in vivo*, whereas production of IFN-γ but not IL-4 was impaired in CD40-deficient mice. These may be one of many signals that NKT cells require, and could be differentially expressed by DC in certain microenvironments, in both infection and/or allergic scenarios.

The present rise in allergic and autoimmune diseases may be accounted for by a lack of immune regulation (Tsitoura, Blumenthal et al. 2000; Wills-Karp, Santeliz et al. 2001; Bach 2002; Umetsu, McIntire et al. 2002; Zuany-Amorim, Sawicka et al. 2002; Akbari, Stock et al. 2003; Herrick and Bottomly 2003; Jutel, Akdis et al. 2003; Karlsson, Rugtveit et al. 2004). Restoring regulatory networks with both a reduction in key Th2-associated cytokines such as IL-4 in combination with elevated IL-10-producing cells, such as DC, may reduce these diseases, as demonstrated in this thesis.

One way in which this knowledge could be applied is the design of new vaccines to prevent sensitization to inhaled allergen or even within the realm of DC vaccination strategies for Chlamydia infection. Furthermore, a greater appreciation of how DC react to both allergens and microbial patterns could be used in immunotherapy of asthma as well as infectious diseases. Additionally, the understanding of how other quick-responding innate cells such as NKT cells would be affected, either directly or in a bystander effect-type manner would also be an asset.

Part V. References

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