

# **The Mechanistic Study on The Role of Dendritic Cell in Modulating Immune Response in Infections and Allergy**

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**DOCTOR OF PHILOSOPHY**

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## *Abstract*

The study mainly focuses on the role of dendritic cells (DCs), especially DC subsets, in modulating intracellular bacterial infections and the modulating effect of these infections on allergic responses. Specifically, it investigated the molecular mechanisms of 1) mycobacterial infection, 2) chlamydia infection and 3) the effect of these infections on allergic reactions.

The results demonstrated that *Bacillus Calmette-Guérin* (BCG) vaccination promoted the expansion of CD8<sup>+</sup>DCs, which showed a more mature phenotype and produced more IL-12 compared to CD8<sup>-</sup>DCs. *In vivo* BCG-primed CD8<sup>+</sup>DC, but not CD8<sup>-</sup>DC, was the dominant DC subset in inducing protective immunity against BCG infection. Further, the results showed that adoptive transfer of different DC subsets from BCG-infected mice inhibited OVA-induced allergic reactions through immune deviation and immune regulation mechanisms. In addition, the results showed that CD8<sup>+</sup>DCs primed by Chlamydia, another intracellular bacterial pathogen, inhibited the allergic responses through IL-10 dependent pathway. Further, the data demonstrated that IL-10 inhibited the expansion of protective Th17 cells following Chlamydia infection. DCs from IL-10 KO mice showed significantly higher ICOS ligand (ICOS-L) expression compared to WT mice. Th17 from Chlamydia infected mice, but not Th1 cells, expressed high levels of ICOS. Blockade of ICOS/ICOSL signaling virtually abolished the Th17 promoting effect of DCs from IL-10 KO mice but had no significant effect on Th1 cells.

This study provided new knowledge on the immune regulation of DC, especially

its subsets, which may be helpful for the rational development of new preventive or therapeutic methods to tuberculosis / Chlamydia infections and allergic diseases.

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## Abbreviations

|         |  |
|---------|--|
| Ab      | Antibody   |
| Ag      | Antigen  |
| AHR     | Airway hyperreactivity                               |
| Alum    | Aluminum hydroxide adjuvant                          |
| APC     | Allophycocyanin conjugated                           |
| APCs    | Antigen presenting cells                             |
| BAL     | Bronchoalveolar lavage                               |
| BCG     | <i>Mycobacterium bovis</i> -bacillus Calmette-Guerin |
| BSA     | Bovine serum albumin                                 |
| CCR3    | CC chemokine receptor                                |
| CD      | Cluster of Differentiation                           |
| CMI     | Cell-mediated immunity                               |
| CMV     | Cytomegalovirus                                      |
| C. m.   | Chlamydia muridarum                                  |
| CTLA-4  | Cytotoxic T lymphocyte antigen                       |
| DC      | Dendritic cells                                      |
| DLN     | Draining lymph nodes                                 |
| DTH     | Delayed type hypersensitivity                        |
| EB      | Elementary body                                      |
| ECP     | Eosinophil cationic protein                          |
| EDN/EPX | Eosinophil-derived neutrophil/eosinophil protein-X   |

|        |                                    |
|--------|------------------------------------|
| ELISA  | Enzyme linked immunesorbent assay  |
| FACs   | fluorescent activated cell sorting |
| FBS    | Fetal bovine serum                 |
| FcR    | Immunoglobulin Fc receptors        |
| FITC   | Fluorescein isothiocyanate         |
| HMI    | Histological mucus index           |
| H&E    | Hematoxylin and eosin              |
| HBSS   | Hank's balanced salt solution      |
| HLA    | human leukocyte antigen            |
| HSP    | heat shock protein                 |
| ICAM-1 | intercellular adhesion molecule 1  |
| ICOS   | inducible co-stimulator            |
| ICOSL  | ICOS ligand                        |
| IDO    | indoleamine dioxygenase            |
| IFN    | Interferon                         |
| IFU    | Inclusion-forming units            |
| Ig     | Immunoglobulin                     |
| IL     | Interleukin                        |
| i.n.   | Intranasal                         |
| i.p.   | Intraperitoneal                    |
| MCP    | Monocyte chemoattractant protein   |
| MHC    | Major histocompatibility complex   |

|         |   |
|---------|---|
| MoPn    | <i>Chlamydia muridarum</i>  |
| mRNA    | messenger RNA   |
| NK      | Natural killer  |
| OVA     | Chicken Egg Ovalbumin   |
| PAMP    | Pathogen-associated molecular pattern   |
| PBMC    | Peripheral blood mononuclear cells  |
| PBS     | Phosphate buffered saline   |
| PE      | Phycoerythrin   |
| pDC     | Plasmacytoid DC   |
| PMA     | Phorbol myristate acetate   |
| PRR     | Pattern recognition receptors   |
| RB      | Reticulate body   |
| RT-PCR  | Reverse transcriptase polymerase chain reaction   |
| Sham-DC | Dendritic cells from adult mice neonatally exposed to<br>Sucrose-phosphate-glutamic acid buffer |
| SPG     | Sucrose-phosphate-glutamic acid buffer  |
| TCR     | T cell receptor   |
| Th      | T helper cell   |
| TLR     | Toll-like receptor  |
| TNF     | Tumor necrosis factor   |
| Treg    | Regulatory T cell   |
| UV      | Ultraviolet   |

|            |   |
|------------|---|
| UV-MoPn-DC | Dendritic cells from adult mice neonatally exposed to<br>UV-killed <i>C.muridarum</i> |
| VCAM-1     | Vascular cell adhesion molecule I   |
| VLA-4      | Very late activation antigen 4  |
| WT         | Wild type   |

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## **PART I: GENERAL INTRODUCTION**

### **I: Allergic Diseases**

#### **1.1 Allergy and Asthma—Definition and Clinical Manifestations**

Allergy is an undesirable clinical manifestation in which the immune system reacts to normally harmless environmental materials, called allergens. Common allergens include pollen, dust, mites, molds and food components. Allergy is also referred to as hypersensitivity. Allergic reactions are manifested in a variety of forms, such as eczema, hives, asthma and food allergies. The reaction to the stinging insects, like wasps and bees, and medicines, like aspirin and antibiotics, also fall within the category of allergic diseases. Interestingly, allergic diseases are extremely common in developed countries, like USA and Canada. It is estimated that over 50 million North Americans suffer from allergic diseases(1).

Asthma is a serious global health concern. Although asthma affects people in all age groups, it is the most common allergic disease in children. It is a complex, heterogeneous and inflammatory disease of lung characterized by chronic airway obstruction and bronchial hyper-responsiveness. Based on the symptoms, airway inflammation and abnormalities of airway functions, allergy has been defined in a variety of ways. The Global Initiative for Asthma (GINA) defines asthma as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. This chronic inflammation leads to recurrent episodes of wheezing, breathlessness, chest tightness, and accompanying coughing, particularly at night or in the early morning. These episodes are usually associated with airflow obstruction

within lung that is often reversible either spontaneously or with treatment(2). Although this definition is widely accepted, it does not fully define the phenotypic heterogeneity of asthma in terms of its presentation, etiology and pathophysiology. The genetic background, age of patients and environmental triggers are related to the severity and variability of this disease. Asthmatic patients suffer from various clinical symptoms. They usually have sleep disturbances, impairment of lung function and requirement for rescue medications. In addition, asthmatic symptoms may be intermittent and non-specific, thus making its diagnosis quite difficult for physicians. The accurate diagnosis of asthma and the knowledge of other key pathologic changes are essential for the appropriate treatment of asthma(3). It is clear that eosinophilic inflammation in asthma is well controlled with small dose of corticosteroid, while non-eosinophilic exacerbations, commonly neutrophilic infiltration in the airway, are unresponsive to steroid treatment(4). Measurement of lung function, airway responsiveness and allergic status may help establish a diagnosis for asthma.

## **1.2 Epidemiology of Asthma**

Asthma is a high priority disease in many governmental health strategies. Poor control of asthma causes sufficient burden on health care. It also leads to loss of productivity in family life, including absence from school or work and premature death. The World Health Organization (WHO) stated that asthma currently affects more than 300 million people globally(5). The worldwide mortality rate of asthma is around 180,000 annually, which accounts for 1 in every 250 death, even with the

improved understanding of the pathogenesis of asthma, availability of medical care and the ability to effectively control it(6). The substantial prevalence and high mortality of asthma have intrigued numerous studies to characterize this disorder. The Global Initiative for Asthma (GINA) program initiates an effort to raise awareness among public health and government officers. They summarized the prevalence of asthma in different countries and stated that 45–84% of asthma patients suffered from frequent symptoms and 9–31% hospitalized for further treatment (5). The International Study of Asthma and Allergies in Childhood (ISAAC) was designed to compare the prevalence of these disorders between different countries and conducted at the interval of 5-10 years in 56 countries in children aged 13–14 years and in 37 countries in children aged 6–7 years (7). They confirmed that asthma is increasing globally. In some Asian countries, the prevalence of asthma has increased by three to four times over the past 20 years (8). European Community Respiratory Health Survey (ECRHS) found the 7% prevalence rate of asthma in France and Germany, 11% in the United States of America (U.S.A) and 15–18% in the United Kingdom. In Canada, the Canadian Health Infant Longitudinal Development (CHILD) studies reflect that the prevalence of asthma has increased sharply in the past 20 years. 6.1% of adults over 15 years of age had asthma in 1994, while only 2.3% in 1979 (9). Currently, around 8.4% of adults and 12% of children suffer from asthma in Canada. This is why further efforts are highly desirable to develop prophylactic and/or therapeutic strategies to control asthma.

### **1.3 Etiology and risk factors for asthma**

Despite dedicating many years and much effort, the etiology of asthma remains poorly understood. Multiple factors contribute to the pathogenesis of asthma. Based on these factors, asthma can be classified into several types, such as viral induced, allergic, non-allergic, seasonal, exercise induced, occupational and persistent. Allergic asthma is normally induced by allergens via mediation of Th2 immune responses. Non-allergic asthma can be initiated by air pollution or infections, whereas genes can be involved in development of spontaneous asthma.

Genetics is important in the development of asthma(10). More than 100 major and minor susceptibility genes have been found involved in this process(11, 12). However, these genes likely interact with environmental triggers to determine susceptibility to asthma(13, 14). Studies on immigrant population suggest that the environmental triggers determine difference in prevalence of asthma between different countries. The interaction between gene and environment factors probably explains much of geographic variation in both prevalence rates and the magnitude of the increase((15, 16, 17). Prenatal risk factors for asthma include maternal smoking, diet and nutrition, stress, use of antibiotics, etc. Causative factors of childhood asthma can include allergic sensitization, maternal tobacco exposure, animal contact, environmental pollution and gender. Adult-onset asthma can occur as a relapse of childhood asthma. Also, occupational exposure constitutes a common risk factor for adult asthma(9). In addition, maturation of immune system and chances of infectious exposure during infancy are important factors modifying the risk of asthma. A better

understanding of these important risk factors, and how these factors interact with genetic determinants for asthma may lead to more opportunities to develop primary preventive strategies against the development of asthma and related allergic disorders.

#### **1.4 Pathogenesis of Asthma**

As stated above, asthma is an inflammatory disorder in the airway that multiple immune cells and multiple mediators are involved in, causing characteristic pathophysiological changes(18, 19). Many studies have concluded that the pattern of inflammation found in asthma patients is consistent, and characterized by the infiltration of activated mast cells, eosinophils, NKT and T helper 2 lymphocytes (Th2) into the airway. In allergic individuals, initial allergen exposure results in the production of allergen-specific IgE, namely allergic sensitization. The early-phase hypersensitivity reaction is initiated through cross-linking of FcεRI on the surface of mast cells/basophils, resulting in mast cell degranulation and the release of histamine, heparin, tryptase and a range of cytokines. Activated mast cell also releases inflammatory mediators, like prostaglandins, leukotriens and platelet-activating factors. These pro-inflammatory mediators initiate cascade events, resulting in the late phase reaction (LPR) and persistent tissue inflammation. In most cases, the late phase reactions are responsible for clinical symptoms, such as further wheezing, sustained blockage of the airway and eczemas. The activation of adhesion molecules, influx of inflammatory cells and release of cytokines and mediators are involved in LPR (20). Bronchoalveolar lavage (BAL) examination and biopsy indicate that the activities of

mast cells, eosinophils, basophils and T cells are increased in the respiratory system of asthmatic patients. However, in some persistent cases of asthma, neutrophils are the dominant inflammatory cell in the airway (21). Airway narrowing is common for patients suffering from severe asthma, which is consequence of the combined effort of several factors, such as airway smooth muscle contraction in response to some bronchoconstrictors, airway thickening due to airway remodeling, and mucus hypersecretion. All of these observations suggest that allergic inflammation is the foundation of the pathogenesis of asthma. A thorough understanding of the molecular and cellular mechanisms underlying allergic inflammation may provide insights into the pathogenesis of allergic disorders.

## **1.5 Inflammatory component in asthma**

Although several types of asthma have been identified, majority of studies have focused on allergic asthma, the most common type of asthma. Specific type 2 immune response, high levels of serum IgE, activated mast cells, and accumulation of eosinophils are hallmarks of allergic asthma.

### **1.5.1 Th2 immune response**

Substantial studies support that initiation and severity of allergic asthma depend on the allergen-specific type 2 T helper cells (Th2). Th2 cells are dominant in the BAL or airway of asthmatic patients and cause the development of eosinophilic inflammation and AHR (22, 23). Th2 cells are necessary and sufficient for the allergic responses. The depletion of T cells with anti-CD3 antibody inhibits the initiation of

allergic response in allergen sensitized mice (24). Similarly, mice depleted of CD4 T cells (25) or deficient in Th2-related cytokines (26) do not develop AHR in their airway. In contrast, mice deficient in the Th1-related transcription factor T-bet have spontaneous eosinophil inflammation and global cell hyperplasia in their lung (27). The inhibition of GATA-3, a typical transcription factor for Th2 development, dramatically reduces the eosinophil inflammation in the allergic mice (28, 29). Adoptive transfer of Th2 cells induces airway mucus production and allergic inflammation in recipient mice (30).

Th2 cells produce large amount of cytokines, which undoubtedly contribute to the ongoing inflammatory response characterized by the recruitment of inflammatory cells (macrophages, basophils and eosinophils), mucus over-production and airway obstruction (31). IL-4, a prototypical Th2 cytokine, polarizes Th2 phenotype development, enhances mast cell activation, promotes IgE class switch, increases VCAM-1 expression on endothelial cells and induces eotaxin production (32). IL-4 blockage attenuates airway allergic inflammation and serum IgE production (33). But IL-4 antibody fails to block allergic response when delivered during challenge phase (34). IL-5 is important in regulating lung eosinophil recruitment. IL-5 KO mice or mice treated with anti-IL-5 antibody showed much less eosinophil in serum and lung tissue than the WT (35, 36). IL-13 is another cytokine produced by Th2 cells, in addition to NK cell, mast cells and eosinophils. IL-13 is particularly critical for AHR and mucus hypersecretion (37, 38). IL-13 blockade by the exogenous administration of IL-13 neutralizing antibody prevented the goblet cell hyperplasia in mice (39, 40).

Also, IL-9 is able to stimulate mast cell growth and differentiation, goblet cell hyperplasia and IgE production (41, 42). IL-6 promotes immunoglobulin production by B cells, and enhances Th2 formation with cooperation with IL-4 (43).

### **1.5.2 IgE**

IgE is critical for asthmatic pathogenesis. IgE is used as a diagnostic indicator for asthma, since dramatically increased serum IgE level has been found in asthma patients (44). Elevated IgE is also regarded as a risk factor even in non-allergic individuals because IgE is associated with an increased likelihood of development of asthma in atopic families (45). In animal models, the transfer of IgE can incur acute or late-phase response. Coyle et al. showed that anti-IgE treatment blocked both eosinophil inflammation and AHR in allergic mice (46). Although IgE contributes to some aspects of asthmatic pathogenesis, T cells are required for the full expression of allergic responses (47, 48). Asthmatic mice without thymus do not recruit eosinophil into airway after allergen exposure. IgE also serves as a positive regulator for its receptors, FcεR I. In human, IgE antibody treatment decreased FcεR expression on basophils (49). Taken together, IgE is able to facilitate antigen uptake and processing, modulate IgE receptor expression, and trigger mast cell-mediated airflow obstruction. Therefore, IgE is a good candidate for therapeutics.

### **1.5.3 Eosinophils**

A very prominent cell in airway of asthmatic patients is eosinophil, which is

present not only in the airway wall, but also in large numbers in the sputum and BAL (50, 51). IL-3, GM-CSF and eotaxins 1-3 are responsible for the early development of eosinophils from its bone marrow precursor cells, while IL-5 contributes to their maturation and recruitment into lung (52). After being stimulated, eosinophils play an important inflammatory role by producing Th2 related cytokines (IL-4, IL-5, IL-10, IL-13) and TNF $\alpha$ . In some but not all experimental mice models, eosinophils are required for AHR (53, 54). Eosinophils contribute to airway remodeling through promoting TGF- $\beta$  1 generation, fibroblast proliferation, collagen synthesis and myofibroblast maturation (55). The observation that the dramatic reduction in eosinophil number is closely related to the efficiency of treatment and improvement of lung function further supports that eosinophils are the principal inflammatory cells in asthma (56).

#### **1.5.4 Mast cells**

Mast cells have long been recognized as associated with asthmatic reaction. Mast cells are present in the airway epithelium, submucosa and mucosa. They are fundamental for some of the inflammation in chronic asthma(57). As mentioned before, the early phase of allergic reaction following inhaled allergen is provoked by mast cell activation and degranulation. Activated mast cells release inflammatory mediators, including histamine, tryptase, proteases, heparin and cytokines (TNF- $\alpha$ , IL-4, IL-5)(57). These mediators are potent agents for smooth muscle contraction and increasing microvascular permeability(57). Mast cells also contribute to chronic

asthma, since the interaction of mast cells with airway smooth muscle cells lead to fibrogenesis, a part of 'remodeling' responses(58, 59).

## **1.6 Animal Models of Asthma**

Animal models are widely used to study the pathogenesis or immune responses of asthma, and to evaluate the efficacy of new asthma drugs. Different animal species have been developed to establish allergic animal models. Among them, rodents (mice and rats) and guinea pigs are most commonly used, because they are easily sensitized to antigens and their allergic responses exhibit homology to the responses in humans. The advantages of murine allergic models include: **1.** immune responses and genetic background of murine are well-known compared to any other species. **2.** commercial availability of a vast array of molecular and immunological probes facilitates the investigation. **3.** the development of gene knock-out and transgenic technologies makes it easy to study specific molecular functions.

Allergic mice models of asthma are established by the first intraperitoneal sensitization to a foreign allergen, most commonly ovalbumin (OVA), along with an adjuvant, typically aluminium. Some studies also utilized ragweed (60) or house dust mite(HDM) (61), which are more representative of antigens that occurred naturally. OVA is widely used as an allergen in murine models. It takes several weeks for the host immune system to mount a reaction against the antigen. Then the mice receive a further allergen exposure directly to the lung, by the postnasal drip of soluble OVA or aerosolized OVA (or ragweed instead) inhalation. This elicits an allergic response developed in the sensitized mice, characterized by an eosinophil influx, Th2 cytokine

production, airway hyperresponsiveness, IgE production and goblet cell hyperplasia. The features of asthma reach their peak in about 5-8 days, and both early and late phase reactions can be detected in allergic mice following OVA challenge(62). Investigations in this model have elucidated the crucial role for a range of cellular and soluble mediators in the development of allergic responses and in coordination of airway chronic inflammation. Obviously, this experimental mouse model does not reflect all forms of asthma or intend to replace clinical study, but it does facilitate our understanding of the role of Th2 and adaptive immunity in human asthma. It is common to use ozone repeat exposure to develop another type of asthma, which is independent of Th2 cell and causes neutrophil-mediated airway inflammation. Intrinsic AHR can be observed in a particular strain of mice, A/J strain. These mice develop asthma spontaneously without manipulation.

However, there are limitations with present murine models of asthma. Firstly, most rodent strains like BN rat or A/J mouse have no symptoms of allergic disease as observed in humans, despite demonstrating the eosinophilic inflammation and Th2 response to antigens. Secondly, the immunology and anatomy of mice are different from human beings in many aspects. For instance, eosinophils in murine model of asthma rarely degranulate. In contrast, the mediators released by eosinophilic degranulation are an important pathogenic factor in human asthma. Thirdly, mice do not develop chronic pulmonary inflammation. Unlike allergic humans, who maintain sensitivity to allergens for years without allergen exposure, rodents used for asthma models generally lose their 'allergic' situation when no further exposed to the

allergens. An intense, chronic and pulmonary inflammatory response observed in human patients is not found in animal models. The failure to develop chronic immune responses in rodent models limits scientific study in acute pulmonary responses rather than chronic response to allergens. Therefore, these drawbacks have to be considered carefully when we evaluate the significance of conclusions acquired from animal models and predict or understand its pathogenesis in human.

## **II. The Hygiene Hypothesis**

### **2.1 Overview and Epidemiological Evidence**

The 'hygiene hypothesis', first proposed in 1989 (63), offers an explanation that the increased prevalence of allergic diseases in past decades has been ascribed to the reduction of overt and unapparent infections with viruses and bacteria(64).

Many epidemiological observations are in support of this provocative hypothesis(11, 65-68). In particular, through well-performed studies, it is clearly demonstrated that there is higher prevalence of asthma in developed nations than in developing ones. Asthma is higher in affluent people compared to poor, higher in urban than rural areas. Younger children with older siblings are at high risk of infections but show less allergic diseases, including asthma in later life (69-72). These large scale, relatively well controlled, serial epidemiological studies have provided strong evidence for the imprinting effect of infection on immune system and on the prevention of allergic diseases. A hallmark epidemiological study in 1997 suggested a strong inverse relationship between cutaneous BCG vaccination and the several

features of atopy, including both antigen-specific IgE and symptoms in Japanese children(65). Recently, a systematic thorough review on the meta-analysis of all twenty-three published papers (up to June 2008) on the relationship between BCG exposure and asthma has been published (236). Based on the history of BCG vaccination, tuberculin response and scar diameter, and asthma including 10 cohort, 5 case-control and 8 cross-sectional studies, the review suggests a clear inhibitory role for BCG vaccination in development of asthma (236). All these epidemiological studies point out that reduced exposure to microbes in early life (e.g. improved sanitation, wide-used antibiotics and vaccination) influences the development of host's immune system vis-à-vis the prevention of allergic diseases.

The original epidemiological observations have subsequently been confirmed by other disciplines, such as immunology, that infections or unhygienic contact might confer protection against the development of allergic illnesses (73). Alm et al. took an experimental approach and determined or suggested that BCG vaccination in infancy was sufficient to divert the immune system of infants from the development of later childhood atopy, but no effect on the high risk population (children with atopic heredity)(74). Our previous work shows that *C. muridarum* and BCG infections have a significant imprinting effect on the newborn or young adult C57BL/6 and Balb/c mice for the inhibition of allergy/asthma induced by natural or model allergens, ragweed or ovalbumin (OVA), in later life (60, 75-80). In particular, *Clamydia* infection showed a strong imprinting effect on the inhibition of allergy even when the infection had long been cleared. Moreover, recent studies have also demonstrated an

inhibitory effect of parasitic infections on allergy/asthma(58, 81-83). Over the course of numerous discussions, researchers have agreed that infections or noninvasive microbial component exposures may influence a subject's innate and adaptive immune response.

On the other hand, this hypothesis currently faces numerous challenges(84-86). Some epidemiological studies, performed on populations with different genetic background, culture, economic status, health care system and geographical location, showed either no inverse relationship between infection and allergy, especially asthma or even increased allergic asthma in association with infection(85-89). In experimental models, some infections showed exacerbating effects on already established allergy/asthma by acute infection or enhanced allergic reactions when allergen and infectious agents were co-administered (84). In general, an imprinting effect of prior bacterial infections on the inhibition of the development or pathogenesis of allergy and asthma is more commonly observed than the recent or concurrent infections.

## **2.2 Mechanisms**

On the basis of these observations, several researchers have focused on examining the underlying mechanisms of the impact of infected component exposure on allergy (90, 91). The underlying mechanism of the infection-mediated inhibition of allergy is associated with the interaction between the two forms of immune responses: innate and adaptive. In particular, it is generally believed that microbial infections

may modulate Th2-like allergic responses by promoting immune deviation towards Th1 (balance of Th1/Th2), primarily an augmented IFN- $\gamma$  secretion(92). The other mechanism of enhancement of immune regulation, such as the induction of regulatory T cells and IL-10–dependent mechanisms, might be responsible for the suppressive effects as well (93).

### **2.2.1 Immune deviation**

The identification of Th1/Th2 paradigm has provided a valuable framework for understanding some unresolved immunobiological phenomena. Th1 cells secrete IFN- $\gamma$ , which is involved in monocyte/ macrophage activation and plays an important role in the host defense against intracellular bacterial and viral infections (94, 95). Th2 cells are critical in initiating allergic response by secreting IL-4 and IL-5, which promote mast cell or eosinophil proliferation and function (reviewed in (96, 97)). It is shown that Th1 and Th2 cells secrete specific cytokines, which serve as their autocrine factors and exert mutually antagonistic effects on each other's development and activity. For example, IFN- $\gamma$  produced by Th1 cells amplify its own development and inhibit Th2 cell proliferation(98, 99), while Th2 cytokines promote Th2 cell differentiation and suppress Th1 development by inhibiting IFN- $\gamma$  production(100).

In addition, it has been demonstrated that Th1 and Th2 can cross-regulate their transcription factors. T-bet is a Th1 specific transcription factor that can dampen Th2 genetic program and represses the development of Th2 programs (101), while GATA-3 regulates Th2 cytokines expression and shuts down Th1 development(102).

The finding that Th1/Th2 counter-regulates each other implies that Th1 environment induced by the infection might attenuate the development of Th2 response when exposed to the allergens(73) .

Indeed, cytokine presence in the early T cell activation directs the subsequent T cell response. APC-derived IL-12 has been shown to promote the naïve T cell differentiation towards Th1 response, producing large amount of IFN- $\gamma$ , while IL-4 favors the development of Th2 phenotype. As a highly heterogeneous population, DCs have many subpopulations as has been described in mice(103) and humans(104). In mice spleen, CD8<sup>+</sup> and CD8<sup>-</sup> DCs are functionally distinct subtypes. Both CD8<sup>+</sup> and CD8<sup>-</sup>DCs can sensitize naïve T lymphocytes and direct distinct T cell development. CD8<sup>+</sup>DCs induce high IFN- $\gamma$  and IL-2 production, whereas CD8<sup>-</sup>DCs induce IL-4 and IL-10 production in some conditions(105). Adoptive transfer of DC subsets directs the development of distinct Th cells. Adoptive transfer of CD8<sup>+</sup> DCs leads to the development of polarized Th1 type immune response, which is dependent on IL-12(106). In contrast, antigen-pulsed CD8<sup>-</sup>DCs induce a Th2-type response(106, 107). In addition, CD8<sup>-</sup>DCs might favor Th2 development through IL-10 production that indirectly inhibits Th1. However, later studies challenged this “one cell type-one type of response” concept by showing that DCs with distinct functional properties could emerge from the same precursors(108, 109), and CD8<sup>+</sup>DCs could also play a regulatory role.

### **2.2.2 Immune Regulation**

Currently, it is recognized that the Th1/Th2 paradigm is too simple to explain the relationship between increased allergic diseases and declined infections. There are several immunobiological phenomena, which cannot be supported by the simple switch between Th1 and Th2. First, the prevalence of autoimmune diseases, which is dominant in Th1 immunity, has increased concomitantly with allergic diseases in western countries in last few decades. Also, some respiratory viral infections, such as influenza A, induce high IFN- $\gamma$  production in host, but it exacerbates the established asthmatic reactions(87). In addition, Th2 dominant helminth infection can protect the host against the development of allergic diseases(110). Also, in experimental mice model, the transfer of IFN- $\gamma$  producing T cells fails to counterbalance allergen-induced airway hyperreactivity, and leads to severe inflammation (111).

The identification of regulatory T cells has provided a new concert to explore the mechanisms behind the hygiene hypothesis. The lack of exposure to the infectious components in industrialized society may dampen the development of regulatory network, which results in the increased trend in both autoimmune diseases and allergic disorders (112). The ability of Treg to inhibit the differentiation of Th2 clones has been documented in mice. Adoptive transfer of nTreg into mice did not decrease IgE level once IgE response was established, but it prevented further Th2 differentiation(113).

DCs isolated from infected individuals have tolerogenic DC function. Recently, a study showed that adoptive transfer of DCs from *Schistosoma japonicum* infected mice dramatically decreased airway allergic inflammation in the recipients, which was

associated with significant decrease in IL-4/IL-5 production but increase in IL-10 production(114). The modulating effect by DCs was associated with the development of Treg. Not only the parasitic infections, but also bacterial infections have also been shown to modulate DC functions to inhibit allergic responses. Bacterial products such as filamentous hemagglutinin expressed by *Bordetella pertussis* were found to be able to enhance IL-10 production by DCs, which promoted Tr1 cells(115). We found that DC from *C. muridarum* infected mice produced significantly higher levels of IL-10 and IL-12 than those from naïve mice. Further, these 'infected' DCs expressed higher levels of ICOSL and that the ICOSL expression potentiated the modulating effect by working together with IL-10 produced by the DCs(77). In conclusion, the DCs from bacteria-infected mice may contain tolerogenic DCs with a phenotype of higher IL-10 production and ICOSL expression, possibly expressed by the heterogeneous subsets.

Therefore, microbial infections may modulate Th2-like allergic responses by promoting immune deviation (towards Th1 phenotype) and/or enhancing immune regulation, depending on multiple factors, including, but not limited to, type of infection/microbial substance, time and duration of exposure, and genetic factors. In particular, different infectious organisms and even various components of the same infectious agent may have different influences on allergy. The relationship between infection and asthma is more complicated because not all types of asthma are related to allergy. Therefore, although the hygiene hypothesis is stimulating, it is unable to provide a sole explanation for the observed increase of allergic diseases including asthma in past decades. Therefore, it is not surprising that inconsistent findings are

often reported in this research area. This emphasizes the critical importance of mechanistic studies on the relationship between infection and allergy/asthma using well established experimental models, especially those with well controlled parameters on the type of infection, condition of infection and host genetic background. The recent findings on the critical role of DCs in infection-mediated inhibition of allergy and asthma represent a significant advancement in elucidating the mechanism underlying the hygiene hypothesis. Further investigation on the role and mechanisms by which DCs are “educated” by infection or exposure to microbial substances at different stages of host development will be helpful for understanding immune regulation in disease settings and the rational design of preventive/therapeutic approaches to allergy/asthma and infections.

### **III Chlamydia Infection**

#### **3.1 Epidemiology and Life cycle**

Chlamydiae are a unique family of Gram-negative obligate intracellular pathogens. The family comprises a group of pathogens, including *Chlamydia trachomatis* and *Chlamydia pneumoniae*. *C. trachomatis* infects the genital and ocular mucosa of humans, causing pelvic inflammatory disease, infertility, and blinding trachoma. It is a major agent of sexually transmitted diseases (STD) in the western world and a major reason for blindness (trachoma) in developing countries. Since a large proportion of Chlamydia infection are asymptomatic, infected women are at high risk to develop complications, like pelvic inflammatory disease (PID), which

may induce tubal infertility and ectopic pregnancy(116). *C. pneumoniae* is another important human pathogen, causing respiratory infections and is responsible for 12-15% of community- acquired pneumonia(117). Both chlamydial species are restricted to the epithelia of the genital, ocular, and respiratory mucosa, although *C. pneumoniae* is implicated in the atherosclerotic cardiovascular diseases and some neurodegenerative diseases(118, 119). *C. trachomatis* consists of 18 major serovars and additional serovariants(120). Among them, the closely related murine strain was reclassified as *C. muridarum*(121) in 1999, originally designated as MoPn (for mouse pneumonitis). *C. muridarum* is a natural murine pathogen and Chlamydia mouse model has been especially helpful in our understanding the features of this disease.

Epidemiologic studies highlight that Chlamydia infection is currently a major burden globally and is a significant public health concern. It is estimated that over 90 million STD's are caused by Chlamydia each year worldwide. Over two-third of cases occur in the developing countries, especially in Sub-Saharan Africa, southern and Southeast Asia. 15 million new cases in Africa and 45 million in southern Asia have been reported annually (122). The prevalence in China is even higher. A study concludes that 2.5% of people at the age of 20-64 are infected with Chlamydia (123). Some programs have been practiced to control this infection, but most developing countries cannot afford it. So the development of vaccine is essential for infection control. However, a human vaccine is not available yet because of the insufficient understanding of the mechanism of pathogenesis and host protective immune

responses.

The unique biphasic developmental life cycle of Chlamydia is the key to understand the pathophysiology of the Chlamydia disease. Chlamydia infection starts from its attachment to a susceptible host cell in the form of metabolically inert elementary body (EB), and enters the cells within membrane-bound vesicles, referred to as inclusions. Then the EB rapidly differentiates into a reticulate body (RB). RB is bigger than EB and actively metabolic and non-infectious. RB is efficiently able to replicate through binary fission within the confines of the inclusion. After several rounds of replication, RBs are converted to EBs, which are released by cytolysis to initiate new infections.

### ***3.2 host Immune Response***

The elucidation of immunity to infection is essential for the vaccine development. Mice models of both genital and lung infection have been widely used to investigate the immune mechanisms of Chlamydia infection diseases. Body weight loss in infected mice is a good indicator of disease morbidity, and usually wide type (WT) mice would gain its body weight after 2-3 weeks of infection (124). *C. muridarum* has evolved different strategies to escape from host immune defense, such as increasing survival ability inside host cells and avoid host immune response (122). However, host employs both innate and adaptive immune responses to control the growth and spread of this organism during infection, and finally succeeds in clearance of the organism (in several months in humans and several weeks in mice). Numerous

studies demonstrated that cell-mediated immunity (CMI) and Th1 responses (introduced in part 5) are the major protective factors against Chlamydia infection, in contrast, Th2 type response is related to the dissemination of infection (125, 126). The Th1 cytokine IFN- $\gamma$  is widely accepted as a protective factor in Chlamydial infection. Multiple mechanisms are involved in the IFN- $\gamma$  mediated protection, such as enhancing macrophage phagocytic ability, increasing oxidative and non-oxidative mechanisms, inhibiting chlamydial growth and depleting intracellular tryptophan.

#### **IV. Mycobacterial Tuberculosis Infection**

##### **4.1 Epidemiology**

Tuberculosis is a type of mycobacterial infection that causes serious public health problem worldwide. The World Health Organization (WHO) estimates that about 2 billion people, meaning one third of the world's population, are infected with *Mycobacterium tuberculosis* (*Mtb*) (127). More importantly, the incidence of tuberculosis continues to rise due to the spread of HIV infection and the emergence of multi-drug-resistant *Mtb* strains (128, 129). *Mtb* infection is the leading cause of death in AIDS patients. Every year, 2 million patients die of tuberculosis, and 8 million new cases are reported, mostly in developing countries (127). BCG (Bacille Calmette -Guérin) is the only registered human vaccine for prevention of *Mtb* infection. Although its protective effect has been well established, its efficacy is quite variable and far from the ideal (130). A better understanding of the mechanism by which BCG immunization protects the host from challenge infection may be useful for the

possible improvement of the efficacy of this particular vaccine, and may have implications in the rational development of new vaccines for tuberculosis.

#### **4.2 Immune Response**

Typically, tuberculosis infects host through the respiratory system, although it does not exclude other ways. After inhalation, the organism is phagocytosed by macrophages. Most organisms are killed at this stage, but some may evade the host surveillance and multiply in the macrophage. Both DC and macrophage are recruited to the infected site to phagocytose the organisms and initiate specific T cell responses. T cell mediated immunity promotes granuloma formation and provides a “dormant” site for tuberculosis, known as latent tuberculosis infection. Disease reactivation easily happens in latent infected individual under temporary or continuous immune compromised conditions, such as HIV infection, physical stress, aging, malnutrition and chemotherapy.

Although the complete mechanism remains unclear, the host protection against Mtb infection has been found to be largely dependent on T cell mediated immunity, especially type-1 T cell responses. The influence of type-2 T cells and humoral immune responses to the host defense is limited, and even may be detrimental in certain circumstances. Human studies have shown that Th1 response is essential for the host protection from mycobacterial infections (131). The human subjects deficient in receptors for IFN- $\gamma$  and IL-12 are profoundly susceptible to mycobacterial infections (132, 133). Experimental studies using animal models also confirm the critical importance of T cell responses in protective immunity and control of

pathological reactions (80,81).

## **V. Dendritic cells**

### **5.1 Immunobiology of Dendritic cells**

The activation of Th cell starts from the interaction of its TCR (T cell receptor) with antigenic peptides presented by antigen-presenting cells (APC) via MHCII molecules. Dendritic cells (DCs) are the most potent APCs, and central to the initiation of immune responses. They determine the fate and intensity of an immune response to pathogenic microbes and harmless allergens. DCs are a trace population, but notably forms a network in most tissues, such as skin, trachea and intestine. In the peripheral tissues, DCs serve as 'sentinels' monitoring the antigen invasion. Furthermore, DCs are considered immature when they are poor in stimulating T lymphocyte responses but efficient in antigen uptake. After the uptake of antigen, DCs migrate to the lymphoid organs. This process is associated with functional and phenotypic maturation of DCs that up-regulate the expression of MHCII, CD80, CD86 and CD40 surface molecules involved in the stimulation of T lymphocytes. DCs are not only excellent stimulators, but also potent immune regulators for immune response. The breakdown of DC's regulatory functions can lead to the loss of tolerance and result in immune disorders. DCs play an important role in allergic response, since they express high affinity IgE receptor and are efficient in capture and presentation of allergens to T cells (134).

DCs originate from bone marrow and circulate in blood in an immature form.

The origin of DCs are controversial but are thought to be from either myeloid progenitors or from lymphoid progenitors. The multiple differentiation pathways, which lead to the origin of DCs, have been proposed (135). DCs demonstrate considerable heterogeneity in both phenotype (i.e. cell surface marker expression) and function. Based on the phenotype and function, they can be classified into distinct subsets. Both the number of distinct DC subsets and the distribution of the DC subsets can differ dramatically depending on the site or tissue of origin (136-138). DC subsets also differ in function (139).

## **5.2 Mechanism of Dendritic Cell Polarization**

DCs, as the most efficient professional APC, not only provide processed peptides to the naive T cell, but also secrete different cytokines with Th cell polarizing capacities. The differentiation of Th0 towards Th1, Th2, Th17 or Treg mainly depends on the particular DC subsets that intimately contact with T cells. DC can phagocytose bacteria and modify its function and phenotype following bacterial uptake(140). Then that DCs become functional and mature with various potentialities to bias the Th1, Th2, Th17 or Treg cells development. The nature and level of antigen they encounter, the time and duration of exposure, the type and levels of costimulatory molecules they express and the type and concentration of cytokines they produce, all determine DC functions and phenotypes that subsequently direct the different type of T cell responses. In addition, DCs are extremely plastic and complex that they are able to adapt their phenotype and function according to the characteristics of the cytokine

milieu or stimulators. The panel of co-stimulatory molecules expressed by DCs elicits different types of T cell responses. For example, CD86 and OX40L contribute to the development of pathogenic Th2 cells(141, 142), while ICOS-L and PD-1 promote the regulatory T cells development(143, 144). The cytokine producing DCs are directly responsible for inducing the development of T cell responses. Well documented examples include IL-12 and IFN- $\gamma$ , which are typical Th1-polarizing factors(145). MCP1(monocytic chemotactic protein 1) and OX40 ligand are Th2 polarizing factors(146). IL-10 and TGF- $\beta$  (transforming-growth factor  $\beta$ ) polarize regulatory T cells(147).

Bidirectional interaction between DC and antigen-specific T cells initiate either an immunogenic or a tolerogenic pathway. Two general hypotheses have been put forward to explain how DCs induce tolerance as well as immunity(137). The first is that a specific DC subset is involved in immunity or regulation. The second is that all DCs have a capacity for initiating immunity and tolerance, but the specific immune response depends on the maturation of DCs, immature DCs induce tolerance, whereas mature DCs confer immunity. The factors which mainly influence the efficiency of DCs in T cell polarization can be of four types, (a) subsets of DCs, (b) nature of the stimuli that activate DCs, (c) kinetics of DC activation, and (d) costimulatory molecules.

### **5.2.1 DC subsets**

T cell differentiation into Th1, Th2, Th17 cells or Tregs may depend on the

type of DC subpopulation that interacts with the T cell. The CD8 molecule is found on the DC surface, and can be used for classifying DC subtypes. CD8 on DC is usually found in the form of  $\alpha\alpha$ -homodimer rather than the  $\alpha\beta$ -heterodimer, which is typical for T cells. Numerous reports have studied the phenotypic and functional characteristics of CD8<sup>+</sup> and CD8<sup>-</sup>DC, and concluded that they are functionally distinct subpopulations (137). The origin of CD8<sup>+</sup> and CD8<sup>-</sup>DC are debatable. Not only lymphoid progenitors, but also myeloid progenitors could differentiate into CD8<sup>+</sup> or CD8<sup>-</sup> DC (148). CD8<sup>-</sup>DCs are located mainly in marginal zone and subepithelial dome, whereas CD8<sup>+</sup>DCs are found mostly in the T-cell areas of the lymphoid organs (149). Although CD8<sup>-</sup>DC seems to have a higher endocytic and phagocytic capacity than CD8<sup>+</sup>DC (149, 150), Lyoda et.al. have suggested that CD8<sup>+</sup>, but not CD8<sup>-</sup> DC, can internalize apoptotic cells (151). Referring to the direct T cell differentiation, CD8<sup>-</sup>DC mainly induce Th2 responses, whereas CD8<sup>+</sup>DC elicit strong Th1-responses through production of IL-12 (105, 152).

DCs, being highly heterogeneous, can be grouped into two distinct DC subsets, namely myeloid DC (mDC) and plasmacytoid DC (pDC), in terms of their origin, morphology, phenotype and function in human and mice. In mice, mDC shows a unique morphology with more abundant cytoplasm, dendritic and irregular nuclei, and expresses high level of CD11c. Murine pDCs have a plasmacytoid morphology, which is characterized by a round shape, smooth surface and eccentric nucleus, with a phenotype, CD11b<sup>-</sup>Ly6C<sup>+</sup>B220<sup>+</sup>CD11c<sup>int</sup> (136). In

addition, they play divergent roles in induction and regulation of the immune responses(105, 153). The mDCs mainly serve as sentinels in the peripheral tissues. Although pDC may not be essential for initiating the immune responses, they are very important for the maintenance of immune homeostasis. mDCs exhibit an immunogenic ability, whereas pDCs have been found to play regulatory roles in RSV infection (154). pDC is well known as natural interferon-alpha producing cell (NIPC) and secretes large amounts of type I IFN in response to many microorganisms (136, 155). Type I IFNs exert broad effect on both innate and adaptive immune response by signaling through IFN- $\alpha$  receptor1 (IFNAR1) and IFNAR2(156). These effects include maturation of efficient antigen-presenting monocyte-derived DCs, stimulation of B lymphocytes (157, 158), activation of B cells class switching(159), and enhancement of mature lymphocyte survival (160, 161). pDCs originate from both lymphoid and myeloid precursors (162), and have been suggested to be involved in the maintenance of T-cell tolerance by inducing differentiation of regulatory T cells through IL-10 dependent mechanisms (163, 164).

### **5.2.2 Nature of stimuli**

Some studies have shown that Th1 or Th2-inducing function of human monocyte-derived DCs is not an intrinsic characteristic but depends on environmental instruction. Cytokines present in peripheral tissues during the DCs maturation can modulate DCs functions in directing naïve T cell development. DCs that mature in the presence of IFN- $\gamma$  induce Th1 response, whereas DCs that mature in the presence

prostaglandin E2 induce Th2 responses. The presence of IL-10 during maturation has been shown to lead to the development of regulatory T cells (Treg)(165). This provides the evidence for the adaptation of DC function to the conditions that they encounter in the pathogen-invaded tissue.

The antigen in itself can regulate DC function. Studies indicate that myeloid DCs produce IL-12 in response to some bacteria, or viruses, and parasites (reviewed in (166)), but fail to do so when activated by other stimuli (167). IL-12 producing DCs can prime Th1 response through positive feedback of IL-12 on the IFN- $\gamma$  production. DCs prime Th2 responses in the presence of IL-4 (168). In conclusion, such a flexible DC system might determine the balance of immunity and tolerance in host immune response against a pathogen.

### **5.2.3 Kinetics of DC activation**

The kinetics of DC activation may influence its capacity to induce different types of T cell response. DCs are sequentially exposed to various stimuli, first to invasive pathogens and inflammatory cytokines in peripheral tissues and then to T cells once they are mature and have reached the lymph nodes. DCs produce IL-12 during a narrow time window and afterwards become refractory to further stimulation(169). Thus, only freshly activated DCs can efficiently promote Th1 proliferation, whereas 'exhausted' DCs preferentially prime Th2 or nonpolarized T cell responses. The temporal restriction suggests a dynamic regulation of DC function in balancing Th1, Th2, Th17 and Tregs during the immune responses.

## **5.2.4 Costimulatory molecules**

Engagement of costimulatory molecules in the process of interaction between DC and T cells is critical for the efficient initiation of effector or regulatory T cell response (reviewed in (170)). DCs provide an antigen-specific signal 1 and a co-stimulatory signal 2 to naive T cells. Both signals contribute to the initial commitment of naïve T helper cells into Th1, Th2, Treg or Th17 subsets. Costimulation can increase the antigen-specific signals delivered by the MHC-TCR interaction. The majority of costimulatory molecules can be divided into 2 separate families, immunoglobulin superfamily including B7-1/B7-2 and ICOS, and tumor necrosis factor receptor superfamily, including CD40 and OX40 (170).

### **5.2.4.1 CD80/CD86/CD40**

The B7 pathway consists of two B7 family members, B7-1 (CD80) and B7-2 (CD86) that bind to the same receptor, CD28 or cytotoxic T lymphocyte antigen (CTLA)-4. The B7 pathways not only provide positive second signal to promote and sustain T cell responses, but also create a negative signal to limit, terminate or attenuate T cell responses (171, 172). This is partly due to its dual ability to bind to stimulatory receptor CD28 and the inhibitory receptor CTLA-4 (CD152) (173, 174). The distinct kinetics of the expression and affinities for B7 explain the bidirectional signals observed. Firstly, the kinetics of the expression of CD28/CTLA-4 may influence the regulatory role of B7 family on immune response. CD28 is constitutively expressed during early stage of immune response. In contrast, CTLA-4

is expressed later and it mediates inhibitory signals at the later stage of immune responses (175). The avidity of the interactions between B7 and its ligands could also account for the function of B7(176). When a pathogen invades, the low level of B7 can interact with high affinity costimulatory receptor, resulting in augmented T cell proliferation and cytokine production. When the pathogen is eradicated, the high level of B7 would then interact with the low affinity receptor resulting in suppression of the immune response(176). In addition, the interaction of B7-1 or B7-2 with CD28 has different effects on some disease pathogenesis. Treatment with anti B7-1 mAb had protective effects, while anti-B7-2 led to exacerbating disease in relapsing model of experimental autoimmune encephalomyelitis (EAE) (177). The relative significance of B7 family in the development of allergen-induced inflammation is not clear (178, 179). Mathur et al. showed that either of CD80 or CD86 activation is sufficient to induce allergic airway inflammation in mice(134, 180). CD80 expression was upregulated on lung cells within 24 h, and continued to be significantly expressed at 72 h after antigen challenge, while expression of CD86 elevated within 24 h, but returned to baseline levels by 72 h after antigen challenge(180). Cheng et al. indicated that only CD80, but not CD86, were upregulated on spleen DC from OVA-sensitized and challenged mice. He showed that spleen-derived DCs have undergone long time of migration after they captured the inhaled antigen in lung(179). B7 family is also important for development of Treg, and subsequent suppression of allergic inflammation(181, 182). However, the clinical studies of autoimmunity and anti-tumor responses indicate that B7/CD28 interactions are more potent in promoting

the effector T cell responses than regulatory T cell responses(183).

CD40 is expressed on DCs and B cells, and its ligand, CD154, on activated T cells. The interaction of CD40/CD154 provides an early signal for T cell activation. The signals induced by CD40/CD154 engagement are critical for the development of both Th1 and Th2(184, 185).

#### **5.2.4.2 Inducible costimulator (ICOS) ligand**

ICOS ligand exclusively binds to ICOS whose expression is up-regulated on activated T cells. Like CD28 and CTLA-4, ICOS is a glycosylated disulfide-linked homodimer. Both Th1 and Th2 cells express ICOS. ICOS, however, persists at higher levels on Th2 cells than does on Th1 cells. The blocking of ICOS/ICOS ligand pathway has different effects on T cell mediated immune response. In the OVA-induced allergic mice, the expression of ICOS-L is up-regulated on the surface of DCs in draining lymph nodes (186). ICOS appears to regulate ongoing Th2 response. In ICOS KO mice, defects of antigen-specific IgE and Th2-related cytokine production were observed (187). In addition, ICOS-ICOS-L interaction is also critical for the development of tolerance in allergic mice. The development of IL-10 producing Treg depends on ICOS signals (143). Recently, studies also suggest an important role for ICOS in immunobiology of infectious diseases. The protective Th1 response to the *Listeria monocytogenes* was impaired following ICOS blockade (188).

### **5.3 The role of DC in *Chlamydia muridarum* infection**

During Chlamydia infection, local resident DCs are activated through pattern recognition receptors (PRR) dependent or independent recognition of Chlamydia pathogen-associated molecular pattern (PAMP). Once immature DC phagocytoses Chlamydia, it upregulates CCR7, chemokine receptors and costimulatory molecules, then migrates to local draining lymph nodes, where it presents processed antigens to T cells through MHC-antigen-TCR interaction, resulting into the activation of antigen specific T cells. These T cells proliferate and eventually migrate back to the site of infection to function there.

After internalization, Chlamydia fuse with lysosome and undergo degradation, then it is presented to both CD4 and CD8 T cells to initiate the adaptive immune response (189, 190). At the same time, the mature DCs express a complex profile of co-stimulatory molecules and cytokines like MHC-II, CD80, CD86, CCR7, IL-12 and IP-10 (191, 192).

Cytokine production plays an important role in Chlamydia-mediated DC maturation. Our previous work showed that IL-10 deficient mice demonstrated stronger host defense and enhanced Th1 responses(125, 193). Mice acquired anti-chlamydia immunity when pretreated with chlamydia-pulsed IL-10 deficient APC(194). Our laboratory also contributes to DC biology in chlamydia infection. We found different DC subsets showing different abilities to initiate protective Th1-like immune responses. After adoptive transfer, CD8<sup>+</sup>DCs educated by Chlamydia infection were more effective in protecting the host against *C.m* infection than CD8<sup>-</sup>DCs (191) .

#### 5.4 The role of DC in *Mycobacterium tuberculosis* infection

DCs take part in both innate and adaptive immunity to *Mtb* and bridge them. Upon phagocytosis of organisms, DCs upregulate costimulatory molecule and adhesion molecule expression on their surface, and undergo a process of maturation. Activated DCs migrate to secondary lymphoid tissues, and present *Mtb* antigens to naïve T cells through MHC-I, MHC-II and CD1d. Activated CD4 T cells, CD8 T cells and gamma-delta T cells are recruited to the infection site to initiate the adaptive immune response. It has been shown that *Mtb*-infected DCs, but not macrophages, can polarize naïve CD4<sup>+</sup> T cells towards Th1 pathway (195). The lung DCs play a central role in initiating the immune response to tuberculosis (196). DCs can influence the polarization of naïve T cells to different types of effector T cells (197, 198). Giacomini et al. showed that IL-12 produced by DCs was critical for Th1 induction(199). Flynn's lab has demonstrated that IL-12 supplemental therapy can enhance Balb/c mice survival, but the survival advantage is lost when IFN- $\gamma$  gene has been disrupted. It emphasizes that IL-12 function is required for the downstream signaling from IFN- $\gamma$ .

Since DCs are a heterogeneous population, different DC subtypes may contribute differently on activation and polarization of mycobacterial antigen-specific T cells. Indeed, phenotypic and functional alterations in DCs have been reported in individuals with mycobacterial infection(200). In human, CD16<sup>-</sup> mDC and CD16<sup>+</sup> mDC subsets have been found to be different in preferential activation of

memory T cells. It has shown that CD16<sup>+</sup>mDC elicits stronger IFN $\gamma$  response than CD16<sup>-</sup> mDC (201). In mice, splenic CD8<sup>+</sup> and CD8<sup>-</sup>DC subsets have been extensively studied and some functional differences between the two subsets have been reported in several model systems. However, some studies have shown the overlapping functions of the two subsets (103, 105, 106, 202, 203). Little is known about the functional involvement of DC subsets in BCG induced protective immunity against mycobacterial infection.

## **VI. T cell mediated immune response**

CD4 T cells play a central role in adaptive immunity to a variety of pathogens. They help B cells to produce antibodies, enhance CD8 T cell function, regulate APC function, coordinate the immune response, modulate immune response to adjust its magnitude and persistence of responses, and maintain immunologic memory. They are involved in autoimmunity, asthma, tumor response and infectious diseases. CD4 effector T cells have been divided into functional subsets, based on the pattern of cytokine production and transcription factor expression. The cytokines play a major role in inducing distinctive transcription factor expression that determines naïve CD4 T cell differentiation(204). This differentiation from naïve T cells can result in different lineages of Th cells, including Th1, Th2, Th17 and Tregs. Cell-based pathogens, such as viruses and intracellular bacteria, trigger type I immune response, whereas type II immune response is elicited by extracellular pathogens, such as parasitic helminths and allergens.

## 6.1 Th1

Th1 immunity is characterized by production of interferon- $\gamma$  (IFN- $\gamma$ ). IL-12 plays a major role in development of IFN- $\gamma$ -dependent host immune responses. IL-12 production appears to be dependent on two signals: a microbial priming signal and a T cell-derived signal. It suggests that CD40 ligation induces a significant increase in IL-12p35 and IL-12p70 heterodimer production. An important determinant of Th1 development from CD4<sup>+</sup>T cells is STAT4 activation by IL-12, although a STAT-4 independent pathway has been also reported (205). The transcription factor T-bet is selectively expressed in Th1 cell, and plays a crucial role in Th1 differentiation. Upregulated T-bet polarizes naïve T cells to Th1 (206). The inhibition of GATA-3 expression by STAT-4 is one of the mechanisms by which IL-12 mediates the inhibition of Th2 (206).

IL-12 is secreted by APCs such as dendritic cells and macrophages that phagocytose intracytoplasmic microorganisms (including *M. tuberculosis*), and induce differentiation of Th0 into Th1 cells(207). The naïve T cells are not responsive to IL-12 because of the lack of IL-12 receptor  $\beta$ 2 (IL-12R  $\beta$ 2) expression, which forms IL-12 receptor together with IL-12R  $\beta$ 1 (208). In humans, the expression of IL-12R  $\beta$ 2 is induced by TCR signaling and STAT4 (signal transducer and activator of transcription 4). In mouse, the IFN- $\gamma$  signals through STAT1 and T bet (T-box expressed in T cells) are important in IL-12R  $\beta$ 2 expression(209, 210). Mycobacteria are such a strong IL-12 inducer that mycobacterial infection can skew the response of

a secondary antigen towards Th1 phenotype(211).

## **6.2 Th2**

Type II CD4 T cell differentiation occurs in the presence of IL-4 and IL-2. Matured Th2 can produce large amount of IL-4, IL-5, IL-9 and IL-13(212). Allergic asthma is associated with Th2 type immune response and Th2-related cytokines, which cause many features of the disease (213, 214). IL-5 is responsible for linking of innate and adaptive immune response by promoting eosinophil proliferation in bone marrow and subsequent migration to lung. IL-4 and IL-13 are implicated in dominance of IgE in allergic asthma(215).

## **6.3 Th17**

Th1/Th2 paradigm has dominated the field of infection and immunity for decades. Recently, T helper cell family has included a Th17 cell subset. This broadens our knowledge on adaptive immune response, thereby helping us further understand the mechanism of protective immunity, which is not fully explained by Th1/Th2 dichotomy. Th17 is characterized by the production of IL-17A(originally named as IL-17), IL-17F and IL-22 as signature cytokines, which are not produced by Th1 and Th2(216). The biological activity of IL-17A or IL-17F depends on heterodimeric complex, IL-17 receptor (IL-17R), which is comprised of IL-17RA and IL-17RC subunits. The bonding of IL-17 with its receptor produces proinflammatory cytokines and chemokines(217). IL-17RA recognizes IL-17A. IL-17RB serves as a receptor for

both IL-17B and IL-17E, but with higher binding avidity to IL-17E. Most of the IL-17 receptor family members exhibit broad tissue expression. This diverse expression pattern implies that IL-17 family may possess a unique immunologically modulated function and is critical in maintenance of immune homeostasis. Recently, IL-17 is recognized as an inflammatory cytokine capable of inducing chemokine gradients and initiating inflammation, particularly in the lung(218-220). IL-22 is another cytokine produced by Th17. The elucidation of Th17 promoting factors and their transcriptional profiles establish Th17 cells as the third effector T cell subset (221). The master transcription factors of Th17 differentiation are ROR  $\gamma$  t and ROR $\alpha$ .

The differentiation of Th17 takes place when naïve CD4 T cells are exposed to polarized cytokines. The requirement of cytokine milieu for Th17 cell differentiation in humans and mice is controversial. The transforming growth factor (TGF)- $\beta$  is demonstrated to be essential for ROR  $\gamma$  t production in both humans and mice(222, 223). IL-6, together with IL-21 in mice and IL-1 or IL-21 in humans, triggers IL-17 production in the presence of TGF- $\beta$  (224-227). TGF- $\beta$  contributes to Th17 differentiation through suppression of the development of Th1/Th2 in the presence of IL-6, while Th17 is stabilized and/or maintained by IL-23 signaling (228-230).

Th17 was initially identified as an important cell subset that secreted dominant pro-inflammatory cytokines driving autoimmune diseases, like multiple sclerosis, collagen- induced arthritis and experimental colitis (231, 232). In general, Th17 is believed to mount host defense against extracellular bacteria and fungi through secretion of IL-17 and recruitment of neutrophils (see review (233)). IL-17-dependent

induction of macrophage inflammatory protein-2 (MIP-2) and G-CSF are required for recruitment of neutrophils and pathogen clearance in *K. pneumoniae* infection (234). Another study has also suggested an important role for IL-17 in host defense against another extracellular pathogen, *Streptococcus pneumoniae* (235).

In contrast to the well described role of Th17 in host defense against extracellular pathogens, the effects of Th17 extend to intracellular bacteria infection in recent years (236, 237). In *Mtb* infection, the balance of Th1 and Th17 response during disease progress is critical for the disease pathogenesis. It appears that Th17 cells can balance Th1 mediated inflammation, Th1 cells are required to balance Th17 mediated inflammation although (238). The relative levels of IL-12 and IL-23 in mycobacterial infection are important for the balance between Th1 and Th17 cells. In primary *Mtb* infection, Th17 and Th1 cells are induced with the same kinetics, but Th1 cells expand 5-10 folds compared to Th17 cells (239, 240). Th17 is rapidly suppressed by the Th1 response (240).

However, the role of Th17 in allergic immune response remains unclear. In asthmatic patients, IL-17A expression was found in the lung and serum(241). Indeed, IL-17 can orchestrate local inflammation via promoting release of proinflammatory factors, such as TNF- $\alpha$ , IL-1  $\beta$ , IL-6, CXCL1, CXCL8, etc.(242). It seems that IL-17 is needed for the initiation of airway inflammation in mice as adoptive transfer of Th17 promotes neutrophil recruitment (219). Also, the interference of IL-17 with epithelial cells promotes eotaxin production (243). Thus, IL-17 may be critical for neutrophil- eosinophil balance in the lung.

## 6.4 Regulatory T cells

Although the immune system detects the potential dangerous invader, excessive inflammatory response is harmful to the host. A dysfunctional Th2 response leads to the development of allergy and asthma, while dysregulated Th1 and Th17 responses result in autoimmune inflammation. Therefore, the strict control of the actions of these effectors is necessary for the immune homeostasis.

The existence of a minor population of T cells with suppressive function, which could prevent autoimmunity and terminate conventional immune responses was described 40 years ago(244). During last few decades the identification of CD4<sup>+</sup> T cell population capable of preventing the development of autoimmunity(245) further broadened our understanding of immunity.

Two broad subsets of regulatory T cells, natural Treg (nTreg) and induced Treg (iTreg), have been described. nTreg constitutes 5-10% of CD4<sup>+</sup> T cells in naïve mice and healthy human (246). nTreg produced in the thymus prevents spontaneous inflammatory diseases(247). They are characterized by high expression of CD25 and Forkhead box P3 (FoxP3)(245, 248). FoxP3 has been identified as the lineage-specific transcriptional factor for the regulatory T cells(245). Foxp3 can be detected in both CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells, but it is much more abundant in CD4<sup>+</sup>CD25<sup>+</sup>. In lymphoid organs (dLN and spleen), most CD4<sup>+</sup>CD25<sup>+</sup> T cells express Foxp3, however, some Foxp3<sup>+</sup>CD4<sup>+</sup> cells do not express CD25. In lung, most Foxp3<sup>+</sup>CD4<sup>+</sup> T cells do not express CD25 (249). Furthermore, iTreg can be driven by the T cell receptor

(TCR) stimulation. They are subdivided into the Tr1 (secrete anti-inflammatory cytokines, IL-10) and Th3 (secrete TGF- $\beta$ ) (250, 251).

Studies have clarified that FoxP3 can interact directly with transcriptional factors, that are involved in both Th17 (ROR $\gamma$ t) and Th2 (interferon regulatory factor-4, Irf-4) lineage commitment (252, 253). FoxP3 can suppress inflammation directly through preventing the activation of pro-inflammatory elements. However, some studies have indicated that Treg can also play a role in host pathology. Treg expressing an attenuated level of FoxP3 can differentiate into Th2 and show Th2-driven pathology (254). This may explain that high levels of FoxP3 expression are necessary to prevent Th2 differentiation. In contrast, a reduced level of FoxP3 expression is sufficient to suppress the occurrence of Th1 response. The maintenance of FoxP3-dependent suppression of Th1 requires continued expression of FoxP3 (255). Therefore, it appears that Th1 responses are acutely sensitive to Treg-mediated inhibition, while Th2 responses appear to be very resistant. The basis for differential sensitivity of Th1/Th2 to Treg remains unclear.

Multiple mechanisms are involved in Treg mediated suppression of immune responses, and the relative importance of these mechanisms in different diseases is variable (256). The regulatory mechanisms likely involve 1) induction of various types of suppressive cytokines; 2) cell contact-dependent mechanism; and 3) CTLA-4 dependent cell unresponsiveness.

**(a) The role of suppressive cytokines:** TGF- $\beta$  and IL-10 are essential for maintaining tolerance in different diseases, e.g. IBD in mice model (257, 258). Their

immunosuppressive properties are most likely dependent upon inhibition of APC functions and direct suppression of the effector T cell proliferation (258-260). IL-10 can be produced by a variety of lymphoid cells, like Tr1 cell, CD5<sup>+</sup> B cells, mast cells and macrophages (261). Regulatory T cells can be primed to produce IL-10 by immature DCs(262, 263). IL-10 was originally recognized as Th2-derived factor, which could efficiently inhibit the synthesis of cytokines, particularly IFN- $\gamma$ , by Th1 cells. Currently, it is widely accepted that IL-10 efficiently suppresses both Th1 and Th2. IL-10 acts as a double-edged sword. On one hand, to evade the host's immune system the microbes utilize IL-10, but on the other hand, IL-10 production helps the host control over-exuberant inflammatory response, which may be detrimental (264, 265). IL-10 KO mice show enhanced Th1 response and confer resistance to *Cm* infection(193).

Most inhibitory effects of IL-10 are exhibited through modulation of macrophage and DCs(261). The immuno-suppressive properties of IL-10 on DCs are caused by a decline in expression of MHC-II, co-stimulatory and adhesion molecules, as well as by the inhibition of the production of inflammatory cytokines (IL-1, IL-6, TNF-a, IL-12). Of note, IL-10 modulates the function of immature DCs, but has little effect on mature DCs(266). The expansion of Th1 is particularly sensitive to IL-10, while some Th2 responses are less stringently affected by IL-10 than Th1(261).

**(b) The cell contact-dependent mechanism:** Treg may express surface molecules that bind to its receptors on target T cells and induce cell cycle arrest(267). Whether the cell contact-dependent inhibition is mediated via APCs remains

controversial. Certain surface molecules on APCs, such as ICOS-L, CD58, OX-2 and PD-1 ligand, can promote the development of Treg (268-270), while a direct  $T_{reg}$ - $T_{eff}$  interaction, which is independent of APCs, has been reported too(267). Levings et al. proposed a model of two-step differentiation of Treg(271): As a first step, naive T cells encounter antigen presented by DC in the presence of IL-10 and possibly  $TGF\beta$ , and become hyporesponsive to the antigen through a cell-cell contact dependent process. As a second step, following repeated antigen exposure, these T cells gain their ability to produce IL-10 and  $TGF\beta$ , mediating suppression through a cytokine-dependent, cell-cell contact independent mechanism. The fully differentiated Treg is more potent in its suppressive activity than anergic T cells.

**(c)The role of CTLA-4:** CTLA-4 expression is primarily restricted to  $CD4^+CD25^+$  T cells, and the interaction of ligation of CTLA-4 with its ligand on APCs (CD80 and CD86) strongly inhibits T cell activation (272). However, the precise mechanism of CTLA-4 mediated regulatory functions is unknown. One possibility is that it preferentially binds to B7 molecules due to its high affinity, thereby preventing CD28-mediated signals that might otherwise abrogate the suppressive function of Treg. Alternatively,  $TGF\beta$  production is enhanced when the cross-linking of CTLA-4 on CD4 T cells occurs (273).

In conclusion, antigenic stimulation can drive the polarization of naïve T cells to become Th1, Th2, Th17 or Treg cells. The balance and timing of the appearance of these different populations depend on the nature of the antigen and the cytokine milieu. It is clear that Treg is a principal factor for peripheral tolerance(274). However,

it is uncertain how DCs direct the Th0 differentiation and the relationship of Th1/Th17, Treg/Th17.

**Summary:**

Taken together, the balance between stimulatory and inhibitory signals and different types of T cell responses are required for effective immune responses to pathogens and allergens. DCs are the major regulators of this critical balance in the organization of immune response. It is not clear that whether a specific DC subpopulations are responsible for the regulation, activation, inhibition or fine running of T cell responses. Further study and exploration of the biological role of DC/DC subsets individually and their interplay in the course of allergic responses and infectious diseases should provide more insights into mechanisms of pathogenesis, and make it possible for a new and powerful therapeutic and prophylactic intervention. This research would inform clinically applicable therapeutic strategies in the future.

## **PART II : Research Questions and Hypotheses:**

The scope of this PhD thesis was to explore the mechanisms of modulation of DC subsets in intracellular bacterial infections and allergic reactions, as well as the mechanisms underlying the infection-mediated inhibition of allergy.

**Question 1. The role of CD8<sup>+</sup>DCs and CD8<sup>-</sup>DCs from BCG infected mice in host defense against mycobacterial infection**

**Hypothesis: CD8<sup>+</sup>DCs and CD8<sup>-</sup>DCs contribute differently in initiation of protective T cells immune responses in mycobacterial infection.**

It has been shown that the type of antigen DCs become exposed to can determine the downstream T-cell response. DCs present relevant antigen-derived peptides, in combination with co-stimulatory molecules/cytokines, to antigen-specific Th cells. The expression/production of co-stimulatory molecules/cytokines largely determines the function of DCs in directing Th cell responses. There are many subtypes of DCs, e.g. DCreg inducing tolerence, DC1 favoring Th1 and DC2 favoring Th2 responses. It has been shown that *Mtb*-infected DCs, can drive naïve CD4<sup>+</sup> T cells to Th1 pathway(195). Indeed, alterations in DC phenotype and function have been reported in individuals with mycobacterial infection(200). Our hypothesis is that *Mtb* infection may modulate the DCs subtypes differently and they would have different effects on the activation and polarization of mycobacterial antigen-specific T cells. To characterize DC subsets modulated by BCG, in *part III, chapter 1*, we first investigated the phenotype of CD8<sup>+</sup> and CD8<sup>-</sup>DCs and then adoptively transferred

them to recipient mice to explore their *in vivo* functions in generating type-1 T cell responses and protection against challenge infection

**Question 2. The role of CD8<sup>+</sup>DCs and CD8<sup>-</sup>DCs from BCG infected mice in infection-mediated inhibition of allergy**

**Hypothesis: CD8<sup>+</sup>DCs and CD8<sup>-</sup>DCs inhibit allergic reactions but through different mechanisms, including immune deviation and immune regulation.**

DCs play a critical role in BCG mediated inhibition of allergy. The adoptive transfer of DCs from BCG infected mice significantly inhibited *de novo* or established allergic inflammation and Th2 responses. Since different DC subsets have been found to be different in modulating T cell responses, in *part III chapter 2*, we extended our efforts to examine the role of DC subsets isolated from BCG-infected mice on allergic reaction. We hypothesized that different DC subsets might modulate allergic reactions differently. To achieve this, we adoptively transferred CD8<sup>+</sup>DC or CD8<sup>-</sup>DCs from BCG-infected mice to recipient mice following the OVA sensitization and challenge. We checked mucus production, pulmonary eosinophilia inflammation and patterns of T cell response in allergic mice.

**Question 3. The role of IL-10 in the DC subsets mediated inhibition of allergic responses.**

**Hypothesis: DC subsets inhibit the allergic responses through IL-10 dependent mechanisms**

We have shown that, in *Chlamydia muridarum* infected mice, spleen CD8<sup>+</sup> and

CD8<sup>-</sup> DCs are different in cytokine production and costimulatory molecule expression. Both DC subsets, especially CD8<sup>+</sup>DCs which produced high levels of IL-12 and IL-10, can inhibit allergic reactions. Here our hypothesis is that the inhibition of CD8<sup>+</sup>DCs from *Chlamydia* infected mice on allergic reactions may depend on IL-10 and regulatory T cells may be involved in this process. We checked the role of IL-10 in the CD8<sup>+</sup>DCs mediated inhibition of ragweed induced allergic reaction in ***part III chapter 3***.

**Question 4. The role of IL-10 in the host defence against *C.m* infection**

**Hypothesis: ICOS signaling is important in the host defence against *Chlamydia* infection through Th17, which is regulated by IL-10.**

Next, in ***part III chapter 4***, we extended our study to investigate the role and molecular mechanisms of IL-10 in host defence against *C.m* infection. IL-10 KO mice are more resistant to *C.m* infection with less pathological inflammation than WT mice. We reported that ICOSL positive DCs dramatically expanded in *C.m* infection and ICOSL KO mice showed dramatically reduced Th17 response following *C.m* infection. Since IL-17/Th17 also contributes to host immunity against *C.m* infection and some studies have suggested a suppressive role of IL-10 on Th17 responses, we hypothesize that Th17 responses may be detrimented by the IL-10, and ICOSL may be involved in the Th17 development. To address this, we checked the Th17 response in IL-10 deficient mice and used ICOSL blocking antibody *in vitro* to demonstrate the essential molecular mechanism of Th17 development in *C.m* infection.

### **Part III. Materials and Methods**

#### ***Mice and culture medium***

Female C57BL/6 mice were bred at the University of Manitoba breeding facility and hosted under specific-pathogen-free conditions. Animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care. Female IL-10 KO mice (IL-10<sup>-/-</sup>; C57BL/6-Il-10<sup>tm1Cgn</sup>) were purchased from the Jackson Laboratories (Bar Harbor, ME). Age and sex-matched wild-type C57BL/6 mice were purchased from Charles River Canada (St. Constant, Canada). Six- to eight-week-old mice were used in the study. The animal experimental protocol was approved by the ethical committee of The University of Manitoba.

Complete RPMI 1640 medium supplemented with 10% heat –inactivated fetal bovine serum, 1%L-glutamine, 25µg/ml gentamycin and 5×10<sup>-5</sup>M 2-mercaptoethanol was used for cell culture.

#### ***Organisms***

The original BCG vaccine was produced by Aventid Pasteur Limited (Toronto, Ontario, Canada). For expansion of the vaccine, BCG was grown in the Middlebrook's 7H9 broth (Difco Laboratories Inc., Detroit, MI) containing 0.2%(v/v) glycerol and 0.05%(v/v) Tween-80 and supplied with 10%(v/v) Middlebrook ADC enrichment (Difco) for 21days. The number of BCG bacilli, counted as colony-forming units (CFU), was measured by plating diluted culture on plates of Middlebrook 7H11 agar (Difco) containing 0.5%(v/v)glycerol and supplied

with Middlebrook OADC enrichment(Difco). The BCG stock was stored at -80°C until use. For inactivation of BCG, live BCG was put at 65 °C for 1 hour, which lead to complete killing of BCG confirmed by viability testing.

*C. muridarum* (*Cm*) was grown in HeLa 229 cells and purified by discontinuous density gradient centrifugation as described previously(77, 275). The partially purified organisms were resuspended in sucrose-phosphate-glutamic acid (SPG) buffer, and frozen at -80°C until used. The original infectivity of the stock *Cm*, as measured by inclusion-forming units (IFUs), was determined by infection of HeLa 229 cells and enumeration of inclusions that were incubated with a *Chlamydia* genus-specific murine mAb and stained with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) and developed with substrate (4-chloro-1-naphthol; Sigma-Aldrich). The same seed stock of *Cm* was used throughout the study.

### ***Mice immunization with BCG***

Mice were injected intravenously with  $5 \times 10^5$  CFU BCG in 200µL sterile protein-free PBS and sacrificed at day 21 after infection. Spleens were aseptically isolated and treated as following: Briefly, the spleens were digested in 1.5mg/ml collagenase D at 37 °C for 30 min and EDTA was applied at the last 5 min to disrupt DC-T complexes. The cell suspensions were then pipetted up and down several times and filtered.

### ***Mice treatment and quantitation of chlamydial growth in vivo***

*C. muridarum* (Cm) was grown in HeLa 229 cells and purified by discontinuous density gradient centrifugation as described previously(193). Infectivity of the purified Cm elementary bodies (EBs) was titrated in HeLa cell culture and demonstrated as inclusion-forming units (IFUs) as described (124). The same batch of Cm preparation was used throughout the study. IL-10 KO and WT mice were inoculated intranasally (i. n.) with 1000 IFU of Cm in 40ul sterile, protein-free sucrose-phosphate-glutamic acid (SPG) buffer as described(124, 193). In the designated experiments, IL-17 function in IL-10 KO mice was neutralized using mAb as described(276). Briefly, 10 µg of anti-mouse IL-17 mAb (R&D, Minneapolis, MN) in 40ul of PBS were administered i.n. to IL-10 KO mice 2 hours after inoculation of *Cm* and was repeatedly administered every 48h until mice were sacrificed at day 7 p.i.. The mice were monitored daily for body weight changes. The growth of Cm in the lung was determined as described(124, 193).

### ***OVA sensitization and challenge***

$5 \times 10^5$  DC subset cells in 200µl sterile protein-free PBS were injected intravenously to recipient mice. Control mice were treated with 200µl PBS. 2 h after DC subsets transfer, recipients mice were sensitized intraperitoneally (i.p.) with 2 µg OVA in alum followed by intranasally (i.n.) challenge with 50µg OVA (40µl) at 14 days later. Mice were sacrificed 7 days later for analysis of airway inflammation and immune responses as described(77, 80).

### ***Ragweed sensitization and challenge***

$5 \times 10^5$  DC subset cells (in 200 $\mu$ l sterile protein-free PBS) isolated from Chlamydia infected mice were injected intravenously to recipient mice. Control mice were treated with 200 $\mu$ l PBS. 2 h after DC subsets transfer. The mice were sensitized with the 100  $\mu$ g Ragweed i.p (Hollister-Stier Canada Co., Toronto, Ontario, Canada) in 2 mg of Al(OH)<sub>3</sub> adjuvant (alum). On day 14 post-RW sensitization, the recipient mice were challenged with 150 $\mu$ g ragweed intranasally. The mice were sacrificed 7 days later for further analysis.

### ***DC and its subsets isolation***

For total DCs (CD11c<sup>+</sup>) isolation, the splenocytes after RBC lyses with ACK lyses buffer (150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA) were incubated with the MACS (Miltenyi Biotec, Auburn, CA) CD11c beads for 15 min at 4 °C. The cells were washed and passed through the LS columns for selection of CD11c<sup>+</sup> cells. For isolation of DC subsets (CD11c<sup>+</sup>CD8<sup>+</sup>DCs and CD11c<sup>+</sup>CD8<sup>-</sup>DCs), MACS CD8<sup>+</sup>DC positive selection kit was used according to the manufacturer's instructions. Briefly, spleen cells were incubated with cocktail of biotin-conjugated antibodies against antibodies (CD90, CD45R, CD49b), followed by incubation with anti-biotin microbeads to deplete of T, B and NK cells. The CD8<sup>+</sup>DC subsets were isolated from the T, B and NK cell depleted preparation by incubating it with CD8<sup>+</sup> cell selecting beads for 30 min on ice and passing through LD columns. The CD8<sup>-</sup>DC subsets were further isolated from this preparation using CD11c column. The purity of the total DCs and DC subset was > 95% based on flow cytometry analyses.

### ***Adoptive transfer of DC subsets and BCG challenge infection***

Two models were used for BCG challenge. For model 1, an intravenous route was used. Purified CD8<sup>+</sup> DCs and CD8<sup>-</sup> DCs from BCG infected mice were injected intravenously (i.v.) into syngeneic recipient mice at the amount of  $5 \times 10^5$  cells in 200  $\mu$ l sterile protein-free PBS. The mice only received the PBS (sham-treatment) or DC subsets from naive mice were used as control groups. At day 7 after adoptive transfer, the mice were challenged with  $5 \times 10^5$  CFU BCG i.v. and were sacrificed at day 21 after challenge. The lungs and livers were homogenized in 10 ml PBS and plated in serial dilutions onto the Middlebrook 7H11 agar with Middlebrook ADC enrichment. The culture was allowed to proceed for 21 days at 37°C in an atmosphere of 9% CO<sub>2</sub> and the number of CFUs was counted. For model 2, the DC subsets were adoptively transferred to recipient mice by intranasal (i.n.) route at the amount of  $2 \times 10^5$  cells in 40  $\mu$ l sterile protein-free PBS. Two hours later, the recipient mice were challenged with  $2 \times 10^5$  CFU BCG i.n. and killed at day 21 after challenge infection. The BCG CFU in the lung homogenates were measured as above.

### ***Lung mononuclear cell preparation***

Lung mononuclear cells were prepared by collagenase and DNAase digestion of the lung tissue and Percoll gradient isolation(277). Briefly, the lung tissues were minced into small pieces and incubated in digestive buffer (containing 2 mg/ml collagenase type XI (Sigma-Aldrich)) for 60 min at 37°C. The mononuclear cells

(MNC) population was purified by centrifugation through a Percoll gradient. Cell suspension was gently mixed with 35% Percoll and centrifuged for 20min at 750g. The pellet was collected and resuspended in FACS buffer. The erythrocytes were lysed with ACK lysis buffer (150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA) followed by two washes in RPMI 1640 with 5% FCS and resuspended in complete RPMI 1640 medium.

### *Flow Cytometric Analysis*

For analysing total DCs, purified CD11c<sup>+</sup> DCs (2x10<sup>6</sup> the cells) were pre-incubated with anti-mouse CD16/32 mAb for 15 min to block FcR binding before staining with specific antibodies. Cells were then incubated with specific mAbs at 4 °C for 30 min in dark. After washing with a staining buffer [Dulbecco's PBS (Sigma-Aldrich) without Ca<sup>2+</sup> and Mg<sup>2+</sup> containing 2% heat-inactivated FCS and 0.05% NaN<sub>3</sub>], the cells were fixed and analysed using FACS Calibur flow cytometer (BD Biosciences) and the data were analyzed using Cell Quest<sup>TM</sup> software (Becton Dickinson). To analyzed surface marker expression on DC subsets, three color-staining was performed. CD11c- allophycocyanin (Hamster IgG) and anti CD8-PE-Cy7 (Rat IgG2a κ) were used for gating DC subsets and FITC conjugated anti-CD80 (Hamster IgG), CD86 (Rat IgG2b κ), CD40 (Rat IgG2a κ) and MHC-II (Rat IgG2b) were used for the specific markers. The florescence-conjugated appropriate isotype controls antibodies were used as control.

Intracellular cytokines were analyzed as we previously described(278). Briefly,

freshly isolated draining lymph node cells ( $2 \times 10^6$  cells) were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., USA) and 1  $\mu$ g/ml ionomycin (Sigma) for 6 hours. 20  $\mu$ g/ml brefeldin A (Sigma) was added in at the last 3 hours in order to accumulate cytokines intracellularly. After washing with a staining buffer, cells were incubated with anti-mouse CD16/32 (Fc block, e-Bioscience) for 30 min on ice to block the FcR non-specific binding and subsequently stained for surface markers with PE-anti-CD4, FITC-anti-CD3 $\epsilon$ , PerCy-anti-CD8 or isotype controls for 30 min on ice. The cells were fixed and permeabilized with Cytofix/Cytoperm™ buffer (BD BioScience) for 20 minutes at 4 °C and incubated with allophycocyanin anti-IFN- $\gamma$  or corresponding isotype controls (eBioscience) for 20 minutes on ice. The raw sample data were collected using a FACS Calibur flow cytometer (BD Biosciences) and the data were analyzed using FlowJo (BD Biosciences).

For Foxp3 expression measurement, the cells from lung mononuclear cells and the cells collected from DC:T cell co-culture system, draining LNs were collected and stained for cell surface markers (CD3 $\epsilon$ , CD4, CD25) without stimulation as described by the manufactory instruction. After surface marker staining, cells were permeabilized and stained intracellularly with a specific Allophycocyanin-conjugated antibody for Foxp3 (eBiosciences) or corresponding isotype control Abs in permeabilization buffer (BD PharMingen).

### ***Histopathological analysis***

Lung and liver tissues of the mice with challenge infection were routinely fixed in 10% buffered formalin, embedded in paraffin, sectioned by a microtome, stained by hematoxylin and eosin (H&E) and examined under a light microscope. Infiltrating inflammatory cells were identified based on cellular morphology and characteristics.

Bronchial mucus and mucus-containing goblet cells within airway bronchial epithelium were stained by a periodic-acid Schiff (PAS) staining kit (Sigma) as described. The mucus secretion was quantified by histological mucus index (HMI), which represents the percentage of the area of mucus-positive epithelium(Goblet cells) in the total area of airway epithelium, using Image-Pro Plus software (Media Cybernetics)(92).

#### ***Cytokine response by DC subsets analysis***

To test the spontaneous cytokines production by the freshly isolated DC subsets, the DC subsets cells were cultured with complete medium using 96-well culture plates at  $5 \times 10^5$  cells /well for 72 hours. The levels of IL-12p70 and IL-10 in the culture supernatants were measured by ELISA.

#### ***In vitro restimulation assays and cytokine measurements***

Mice treated with different approaches were killed and the spleen, draining (mediastinum) lymph nodes and lungs were aseptically removed. To analyze cytokine production, single-cell suspensions were prepared from spleens, draining lymph nodes and lungs as described previously(277, 279). The cells were cultured at a

concentration of  $7.5 \times 10^6$  cells /ml (splenocytes) or  $5.0 \times 10^6$  cells /ml (LN, lung cells) respectively in complete culture medium with or without stimulation of UV- killed *chlamydia* ( $10^5$  IFU/ml), heat-killed (HK)-BCG ( $7.5 \times 10^5$  CFU) or OVA or Ragweed.. Cultured supernatants were harvested at 72h and cytokine concentrations in the supernatant were measured by ELISA using Abs purchased from eBioscience (specific for IFN- $\gamma$ , IL-17 and IL-23) or BD PharMingen (specific for IL-6, and TGF- $\beta$ ).

#### ***DC subsets –T cell co-culture***

Model 1, to directly assess the T cell modulating abilities of DC subsets, purified DC subsets were cultured with CD4 T cells isolated from BCG-infected mice as we previously described(278). CD4 T cells were isolated from the spleen using the CD4 T cell isolation column from MACS. The purity of CD4 T cells were >95%. DC subsets ( $1 \times 10^5$  cells/well) and CD4 T cells ( $1 \times 10^6$  cells/well) were co-cultured in 96-well plates in the presence of HK-BCG ( $5 \times 10^4$  CFU/well) in 200 $\mu$ L complete RPMI medium for 72 hours and the concentration of IFN- $\gamma$  and IL-4 in the supernatants were measured by ELISA.

Model 2, naive CD4<sup>+</sup> T cells were isolated from the spleens of DO11.10 OVA peptide-specific TCR- $\alpha\beta$  transgenic mice (Balb/c background) using a MACS LS CD4 positive selection column (Miltenyi Biotec). The purified CD4<sup>+</sup> T cells ( $5 \times 10^6$  cells/ well) were co-cultured with DC subsets isolated from BCG-infected Balb/c mice ( $5 \times 10^5$  cells/ well) with OVA (0.1 mg/ml) stimulation as described(77, 79).

### ***Quantitation of gene expression by real-time RT-PCR***

To analyze the BCG gene expression in the isolated DC subsets from BCG infected mice, real-time RT-PCR were performed. cDNA synthesis is performed using total mRNA primed with random primer. The thermal profile for RT was incubated at 25°C for 5 min, 50°C for 1 hour. Inactivation of the reaction was done by heating at 70°C for 15 min. The real-time RT-PCR was carried out on the MiniOpticon™ System using an iG™ SYBR Green Supermix (BioRad Laboratories, Hercules, California, USA). The PCR was performed in a 48-well plate in a reaction volume of 25 µl containing 12.5 µl 2x iG™ SYBR Green Supermix Mix, 200 nM each of forward and reverse primer, 0.5 µl template cDNA, added nuclease-free water to a final volume of 25 µl. The thermal profile for PCR was 95 °C for 10 min , followed by 40 cycles of 95 °C for 30 s and 58 °C for 30 s, and 72 °C for 60 s. Fluorescent signals were read and the data were collected at each annealing temperature. For each sample, the amplification plot and the corresponding dissociation curve were examined. The melting curve analysis, determining the specificity of the reaction, was carried out immediately after the final PCR cycle by measuring the changes in fluorescence during slowly heating the amplicon/probe heteroduplex. The threshold cycle (*Ct*) used in the real-time PCR quantification was defined as the PCR cycle number that a noticeable increase in reporter fluorescence above a baseline signal. The efficiency of target gene and GAPDH is similar and a comparative *Ct* method was used for calculations(280). GAPDH was used as an endogenous control gene for normalization

of the amount of RNA added to the reactions.  $\Delta Ct = Ct(\text{BCG}) - Ct(\text{GAPDH})$ , which means the difference between the threshold cycle of BCG and the threshold cycle of the corresponding GAPDH in the same sample. For each experiment, a negative control of nuclease-free water and a positive control (with known  $Ct$  value) were run in triplicate. The specific primers used in quantitative real-time PCR were: BCG IS6110 (123bp): 5'CCTGCGAGCGTAGGCGTCGG; 3'CTCGTCCAGCGCCGCTTCGG. GAPDH (191bp): 5'AACGACCCCTTCATTGAC, 3'CACGACTCATACAGCACCT

To analyze the expression of retinoic acid-related orphan receptor gamma (ROR- $\gamma$ t) transcripts, the mRNA was prepared from lung tissues using Trizol (Invitrogen, Burlington, Ontario, Canada) reagent protocol(275).  $\beta$ -actin was used as a loading control. Gene Amp PCR System 2400(Perkin Elmer) was used at 36 cycles for ROR- $\gamma$ t : Forward: 5' ACA CCG AGG GCT TAA CAA GAC ACT-3', Reverse: 5'-TGT GTG GTT GTT GGC ATT GTA GGC-3'  $\beta$ -actin, Forward: 5'-GTG GGC CGC CCT AGG CAC CA-3', Reverse: 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3'. Real-time PCR was conducted on the MJ mini Thermal Cycler (BIO-RAD) using iQ<sup>TM</sup> SYBR Green Supermix.

### ***Statistic analysis***

One-way ANOVA (One-way analysis of variance) and further Newman-Keulse test were used to determined statistic significance among groups. IFU of  $Cm$  was converted to logarithmic values and analyzed using the ANOVA test. The value of

$p < 0.05$  was considered significant.

## Part IV: Results

Chapter 1 (Published at the PloS One (Feb, 2010))

### **CD8<sup>+</sup> DCs, but not CD8<sup>-</sup>DCs, isolated from BCG-infected mice reduce pathological reactions induced by mycobacterial challenge infection**

#### Introduction

Tuberculosis is a type of mycobacterial infection that causes serious public health problems worldwide. BCG (Bacille Calmette -Guérin) is the only registered human vaccine for prevention of *Mtb* infection and diseases, but its efficacy is quite variable and far from ideal (130). A better understanding of the mechanism by which BCG immunization protects host from challenge infection may be not only useful for the possible improvement of the efficacy of this particular vaccine but also have implications in the rational development of new vaccines for tuberculosis.

Although the mechanism remains unclear, the host protection against *Mtb* infection has been found to be largely dependent on T cell mediated immunity especially type-1 T cell responses. Dendritic cells (DCs) are a type of the front line cells that encounter *Mycobacteria* in the infection sites. DCs are the key APCs in the activation of primary CD4 T cells and in the polarization of Th1/Th2 subsets. It has been shown that *Mtb*-infected DCs, but not macrophages, can drive naïve CD4<sup>+</sup> T cells polarize to Th1 pathway (195). The DCs in the lung play a central role in initiating the immune response to tuberculosis (196). DCs can influence the polarization of naive T cell to different types of effector T cells (197, 198). Since DCs

are a heterogeneous population, different DC subtypes may contribute differently on the activation and polarization of mycobacterial antigen-specific T cells. In mice, splenic CD8 $\alpha^+$  and CD8 $\alpha^-$  DC subsets have been extensively studied and some functional differences between the two subsets have been reported in several model systems including infections, although some studies showed overlapping functions of the two subsets (103, 105, 106, 202, 203). In particular, we have reported that CD8 $^+$ DCs isolated from *Chlamydia muridarum* infected mice are more potent in inducing protection against challenge infection with the same Chlamydia species than CD8 $^-$ DCs(191). Little is known about the functional involvement of DC subsets in BCG induced protective immunity against mycobacterial infection.

In the present study, we examined the relative effectiveness of splenic CD8 $^+$  and CD8 $^-$ DC subsets from BCG-infected mice in generating type-1 T cell responses and protection against challenge infection with an intention to know more on the mechanism by which a host defends against *Mtb* infection. The data showed that the distinct DC subsets primed by in vivo BCG infected mice are significantly different in their potency to induce protective immunity against challenge mycobacterial infection. Specifically, we found that CD8 $^+$ DCs are much more potent than CD8 $^-$ DCs for inducing type-1 T cell responses in both in vitro and in vivo conditions. This difference is unlikely due to the potential difference of the subsets in uptaking mycobacterial organisms because quantitative RT-PCR test showed similar levels of bacterial messages in the isolated DC subsets. Rather, it is more likely due to the functional difference of the DC subsets in modulating T cell responses.

## **Results:**

*BCG immunization induces preferential expansion of CD8<sup>+</sup> DC subset which exhibits differential expression of surface markers and the production of cytokines compared to CD8<sup>-</sup> DC subset.*

To analyze the effect of BCG immunization on DC subset, we measured CD8 molecule expression on the total DCs (CD11c<sup>+</sup> cells) isolated from the spleens of BCG infected and naïve C57BL/6 mice. As shown in Figure 1A, DCs isolated from BCG infected mice showed a higher percentage of CD8<sup>+</sup> subpopulation than the DCs from naïve mice (37% vs 21%), suggesting a preferential expansion of CD8<sup>+</sup>DCz following BCG immunization. Since the function of DCs in modulating immune responses is largely dependent on their expression of co-stimulatory molecules and the production of cytokines, we further analyzed the surface markers on the DC subsets by three-color staining (CD11c, CD8 and a particular surface marker). As shown in Figure 1B, in comparison with the CD8<sup>-</sup> DCs isolated from BCG infected mice iCD8<sup>-</sup>DCs, the CD8<sup>+</sup> DCs isolated from the mice with the same infection iCD8<sup>+</sup> DCs expressed higher CD80 (65.66% vs 17.43%), CD86 (57.43% vs 30%) and CD40 (44% vs 35%) molecules. Similar differences were observed in comparison of the density (mean fluorescence intensity, MFI) of these molecules expressed on the surface of these cells. Although the iCD8<sup>+</sup> DC and iCD8<sup>-</sup> DC subsets showed similar MHC-II in percentage (97% vs 95%), the MFI of MHC-II was much higher in iCD8<sup>+</sup> DC. Similar pattern of differences in expression levels of surface markers were found in naïve DC subsets, but the absolute levels in the naïve mice were lower than those

of infected mice, suggesting a significant impact of immunization on both DC subsets.

To further analyse the cytokine production by the DC subsets, we purified iCD8<sup>+</sup> DC and iCD8<sup>-</sup> DC using MACS columns. As shown in Figure 2A, the sorted DC subsets were in high purity. To assay the cytokines production pattern of the DC subsets, the purified iCD8<sup>+</sup> DC and iCD8<sup>-</sup> DC were cultured and the cytokine levels in the 72 h culture supernatants were measured by ELISA. As shown in Figure 2B, iCD8<sup>+</sup>DC produced significantly higher level of IL-12p70 than iCD8<sup>-</sup>DC, which produced more IL-10. Taken together, the results indicate that BCG immunization leads to a preferential expansion of CD8<sup>+</sup> DC which shows a different profile of co-stimulatory surface molecules and cytokines from CD8<sup>-</sup> DC.

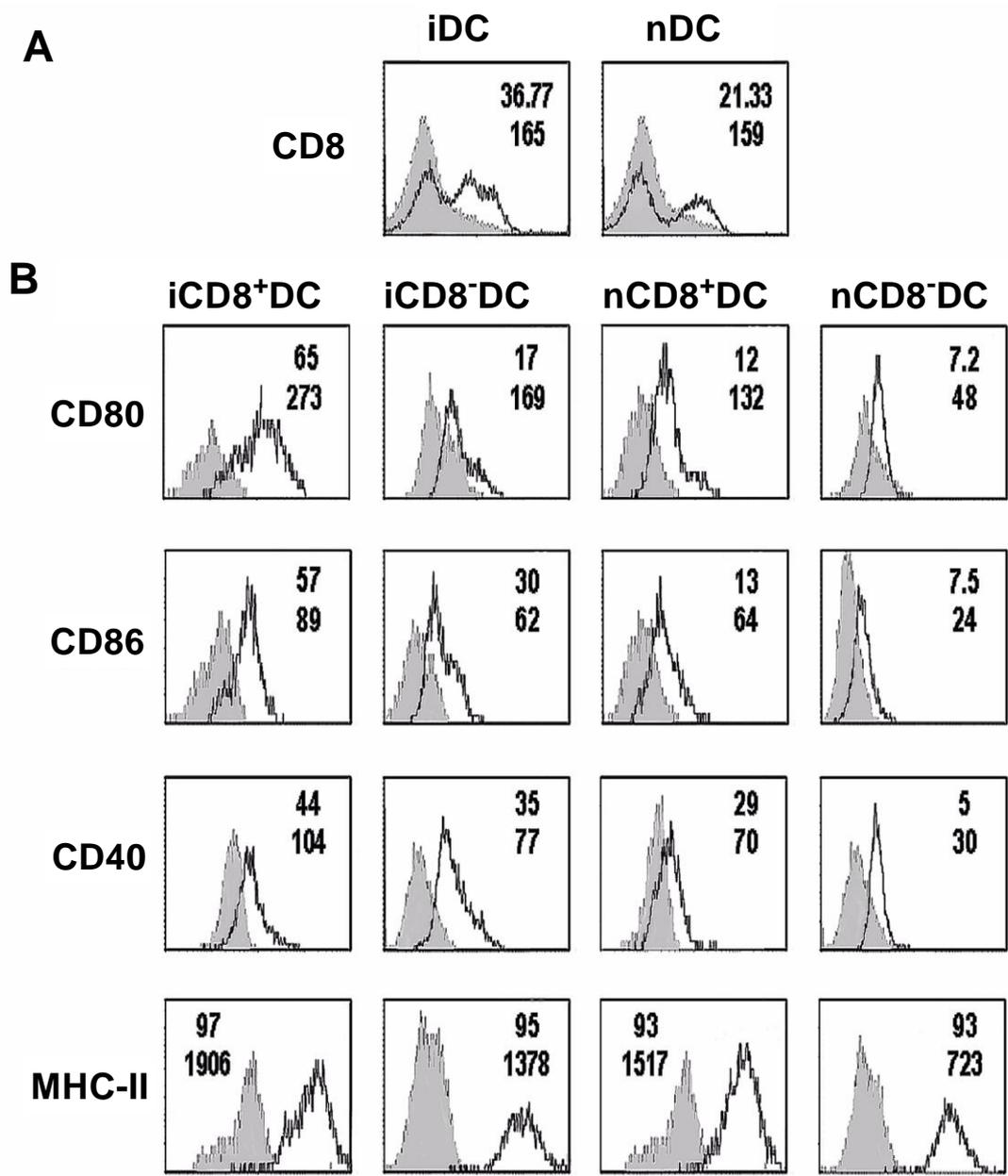
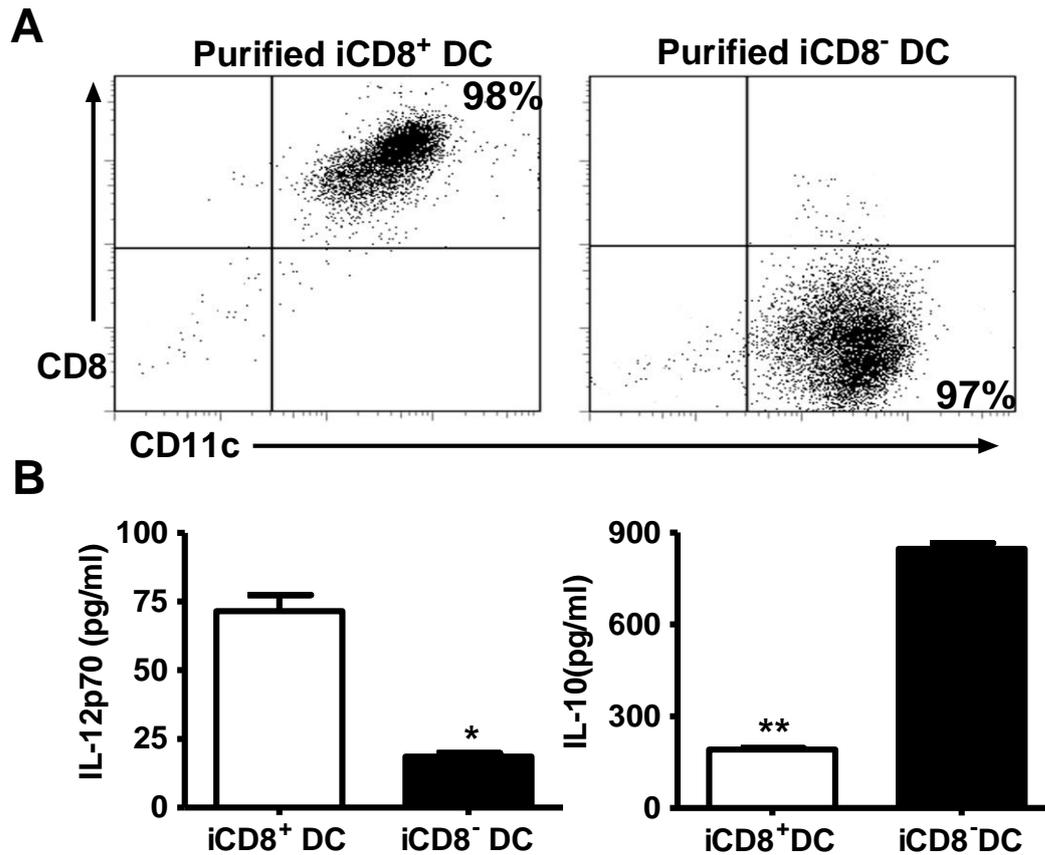


figure 1

**Figure 1.** BCG immunization induced the expansion of CD8<sup>+</sup> DC which expressed higher levels of co-stimulatory molecules compared with CD8<sup>-</sup> DC. Mice (C57BL/6, n=4/ group) were infected i.v. with 5x10<sup>5</sup> CFU of BCG and sacrificed at 21 days after immunization. Total DCs from infected and naïve mice were purified using the MACS CD11c<sup>+</sup> isolation column. Purified DCs were co-stained with APC-conjugated anti-CD11c, PE-Cy7-conjugated anti-CD8 and FITC-conjugated Ab specific for one of the surface markers (CD80, CD86, CD40 or MHCII). The surface marker expression (solid lines) or matched Ab isotype control (shaded histogram) are shown respectively. All histogram were based on 10,000 cells satisfying a gate set of forward vs side scatter light histogram. A, purified DCs were gated on CD11c positive cells showing CD8<sup>+</sup> DC population in infected (iDC) and naïve (nDC) mice. B, purified DCs were gated on either CD11c<sup>+</sup> CD8<sup>+</sup> DC (CD8<sup>+</sup>DC) or CD11c<sup>+</sup>CD8<sup>-</sup> DC (CD8<sup>-</sup>DC) and the surface molecules on the DC subsets were shown. The percentages of positive cells and mean fluorescence intensity (MFI) for the molecules were shown at the top and bottom lines respectively at the right upper corner of each histogram.



**figure 2**

**Figure 2: Different levels of cytokine production by DC subsets.** DC subsets were isolated from spleens of BCG infected (i.v.) mice at day 21 post-immunization as described in the Materials and Methods. A, purified DC subsets were analyzed by flow cytometry for purity. The  $5 \times 10^5$  sorted cells were co-stained with APC-conjugated anti-CD11c and PE-conjugated anti-CD8. The purities of the sorted iCD8<sup>+</sup> DC (left) and iCD8<sup>-</sup> DC (right) are indicated at the right upper corner and right lower corner respectively. B, freshly isolated iCD8<sup>+</sup> DC and iCD8<sup>-</sup> DC subsets were cultured. The culture supernatants were harvested at 72h and tested for IL-10 and IL-12p70 by ELISA. Data are shown as mean $\pm$ SD of each group. One representative

experiment of three independent experiments with similar results is shown.\* $p < 0.05$ ,  
\*\* $p < 0.01$ .

***iCD8<sup>+</sup>DC are more efficient in promoting Th1 (IFN $\gamma$ ) cytokine production while  
iCD8<sup>-</sup> DC are more efficient in promoting Th2 cytokine (IL-4) production by T cells  
primed with BCG in vivo.***

Since the analyses shown above on co-stimulatory molecule expression and cytokine production suggest a functional difference of the DC subsets isolated from BCG immunized mice, we performed experiments to address this by co-culturing iCD8<sup>+</sup> DC and iCD8<sup>-</sup> DC subsets separately with CD4 T cells isolated from BCG primed mice. As shown in Fig 3, iCD8<sup>+</sup> DC promoted dominant Th1-cytokine (IFN $\gamma$ ) production, while the iCD8<sup>-</sup>DC induced a dominant Th2 (IL-4) cytokine response by antigen-specific CD4 T cells. The experiments using an in vitro approach demonstrated that the DC subsets were functionally different in directing BCG antigen-specific T cells.

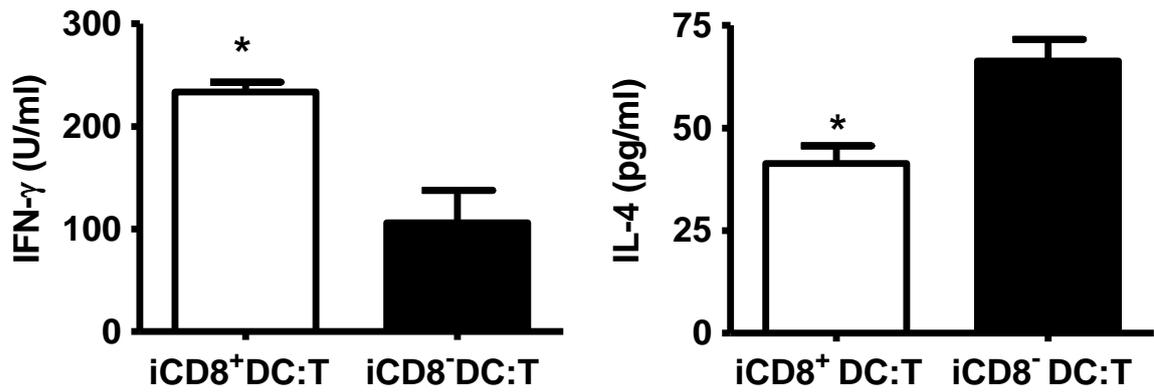


figure 3

**Figure 3. iCD8<sup>+</sup> DC and iCD8<sup>-</sup> DC subsets generate different patterns of BCG-driven T cell cytokine production.** BCG-specific CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) cell from BCG-infected mice were co-cultured with iCD8<sup>+</sup> DC or iCD8<sup>-</sup> DC ( $5 \times 10^4$  cells/well) in the presence of HK-BCG. Culture supernatants were collected at 72h. IFN-γ and IL-4 were measured by ELISA. The experiment was repeated twice showing similar results.\*p<0.05.

***Adoptive transfer of CD8<sup>+</sup> DC, but not CD8<sup>-</sup> DC, enhanced bacterial clearance and reduced pathological reactions in the infected tissues following challenge infection***

To further examine the functional difference of the DC subsets, we adoptively transferred the iCD8<sup>+</sup> DC and iCD8<sup>-</sup> DC subsets and tested protection in the recipients of the different DC subsets to challenge infection. DC subsets were purified from BCG-infected mice at day 21 post immunization and were adoptively transferred i.v. to naïve C57BL/6 mice. At 7 days after cell transfer, the recipient mice were challenged i.v. with BCG. Mice that received PBS (sham treatment) or DC subsets from naïve mice with the same challenge infection were used as controls. Twenty-one days after challenge infection, mice were sacrificed and the bacterial loads in the lung and liver were measured. As shown in Fig 4, a significant reduction in tissue bacterial loads were observed in the mouse group receiving iCD8<sup>+</sup> DC compared with the mice without DC transfer (sham treated control mice). In contrast, although the recipients of iCD8<sup>-</sup> DC also appeared to have a trend of lower CFU in the lung and liver compared to PBS controls, the differences were not statistically significant. As controls, the recipients of either DC subsets from naïve mice (nCD8<sup>+</sup> DC or nCD8<sup>-</sup> DC) showed comparable levels of bacterial loads with the PBS treated control mice, therefore not generating protection.

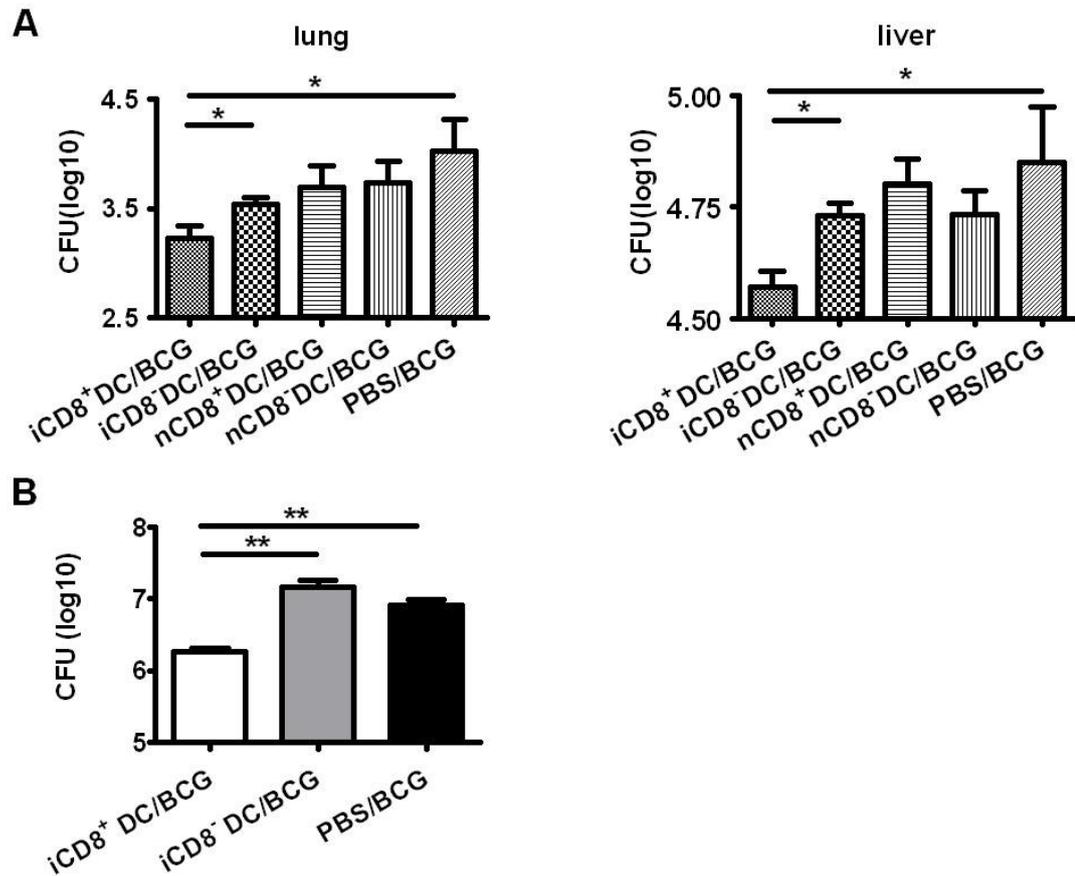
More remarkably, further histopathological analysis (Figure 5) showed that the recipient of iCD8<sup>+</sup> DC only had mild pathological changes in the lung and liver. In contrast, the sham-treated mice and the recipients of iCD8<sup>-</sup> DC showed much more severe pathological inflammatory reactions. Interestingly, although both sham-treated

mice and iCD8<sup>-</sup> DC recipients showed severe pathological reactions, their pattern of changes were different. Sham-treated mice showed multiple granulomas in both lung and liver tissues, characterized by dominant epithelioid cells (indicated by green arrows in Fig 5) at centre with surrounding lymphocytes, neutrophil and monocytes in peribronchial and perivascular areas (lung) and peri-central vein areas of the hepatic lobules (liver). Multinucleate giant cells were also observed in the sham-treated mice. In contrast, the inflammation in iCD8<sup>-</sup> DC recipients was diffused without notable granulomas and lack of typical epithelioid cells. The infiltrating cells in these mice were mainly neutrophils and mononuclear cell.

Since tuberculosis is mainly a pulmonary disease, we further analysed the protective function of the DC subsets using a model of intranasal challenge infection. DC subsets were adoptive transferred to recipient mice followed by i.n. challenge infection with live BCG. Similar to what observed in the i.v. infection model, the recipients of iCD8<sup>+</sup> DC showed lest organism growth (Figure 4B) and mildest pathological changes (not shown) in the lung among the groups, exhibiting very few inflammatory cells following intranasal challenge. In contrast, evident pathological changes were found in the sham treated control group (multiple granuloma and heavy inflammation) and iCD8<sup>-</sup> DC recipients (disseminated heavy inflammation). Taken together, the results indicate that only the CD8<sup>+</sup> DC subset from infected mice can generate significant protective immunity in vivo. Moreover, since granuloma formation is a way for host to control infection (although not very efficient), the failure of iCD8<sup>-</sup> DC recipients to have granuloma formation instead showing heavily

diffused inflammation suggest it may promote an inflammatory reaction which is not protective.

**figure 4**



**Figure 4. Adoptive transfer of iCD8<sup>+</sup>DC, but not iCD8<sup>-</sup> DC, reduced bacterial growths in recipients following either i.v. or i.n. challenge infection.** DC subsets were sorted from BCG-infected (i.v.) mice or naïve mice and adoptively transferred to syngeneic recipients (C57BL/6, n=4/group) by i.v. (A&B, 5x10<sup>5</sup> DC/mouse) or i.n. routes (C, 2x10<sup>5</sup> DC/mouse). Mice were challenged with BCG through i.v. (A) or i.n. (B) routes, respectively, as described in Materials and Methods. All mice were sacrificed at day 21 post challenge infection. Homogenized lung or liver tissues were measured for BCG CFU. The CFUs of BCG were converted to logarithmic values and

presented as mean±SD of each group. One representative experiment of three independent experiments is shown.\* p<0.05;\*\* , p<0.01.

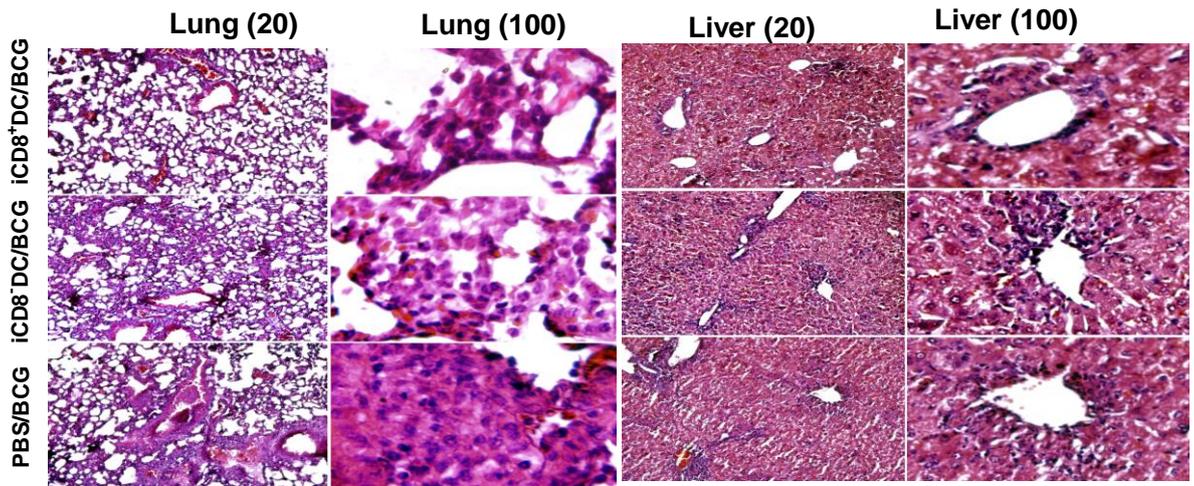


figure 5

**Figure 5. Significantly milder pathological changes in recipients of iCD8<sup>+</sup>DC following intravenous challenge infection.** Mice were treated /challenged as described in the legend to Figure 4 and analyzed for histopathological changes in lung and liver at day 21 post challenge infection by H&E staining. Low magnification(x20) and high magnification (x100) were shown respectively.

Since proinflammatory cytokine and chemokine responses contribute to the inflammation of local tissues and correlate with the degree of pathological reactions in some circumstances, we further measured the MIP-1 $\alpha$  (CCL3), IL-6 and TNF- $\alpha$  concentration in the lungs and liver. Consistent with the lowest degree of inflammation in the iCD8<sup>+</sup>DC recipients (Figure 5), this group of mice showed the lowest levels of IL-6, MIP-1 $\alpha$  and TNF- $\alpha$  response in the local tissues (Figure 6). In contrast, the recipient of iCD8<sup>-</sup>DC showed highest levels of these cytokines, even higher than the sham-treated, infected mice. Potentially, in the infected tissues many different types of inflammatory cells can produce these cytokines/chemokine including, but not limited to macrophages, DCs, and lymphocytes. The higher levels of these molecules in the lung of the iCD8<sup>-</sup>DC recipients suggest a non-protective or less protective severe inflammation in the lung, which is not efficient for clearing the infection but contributes to pathological changes. This data are consistent with the severe and diffused inflammation in the iCD8<sup>-</sup>DC recipients (Figure 5). The data suggest that the transfer of iCD8<sup>+</sup>DC, but not iCD8<sup>-</sup>DC, can reduce the pathological inflammatory responses in the local tissues.

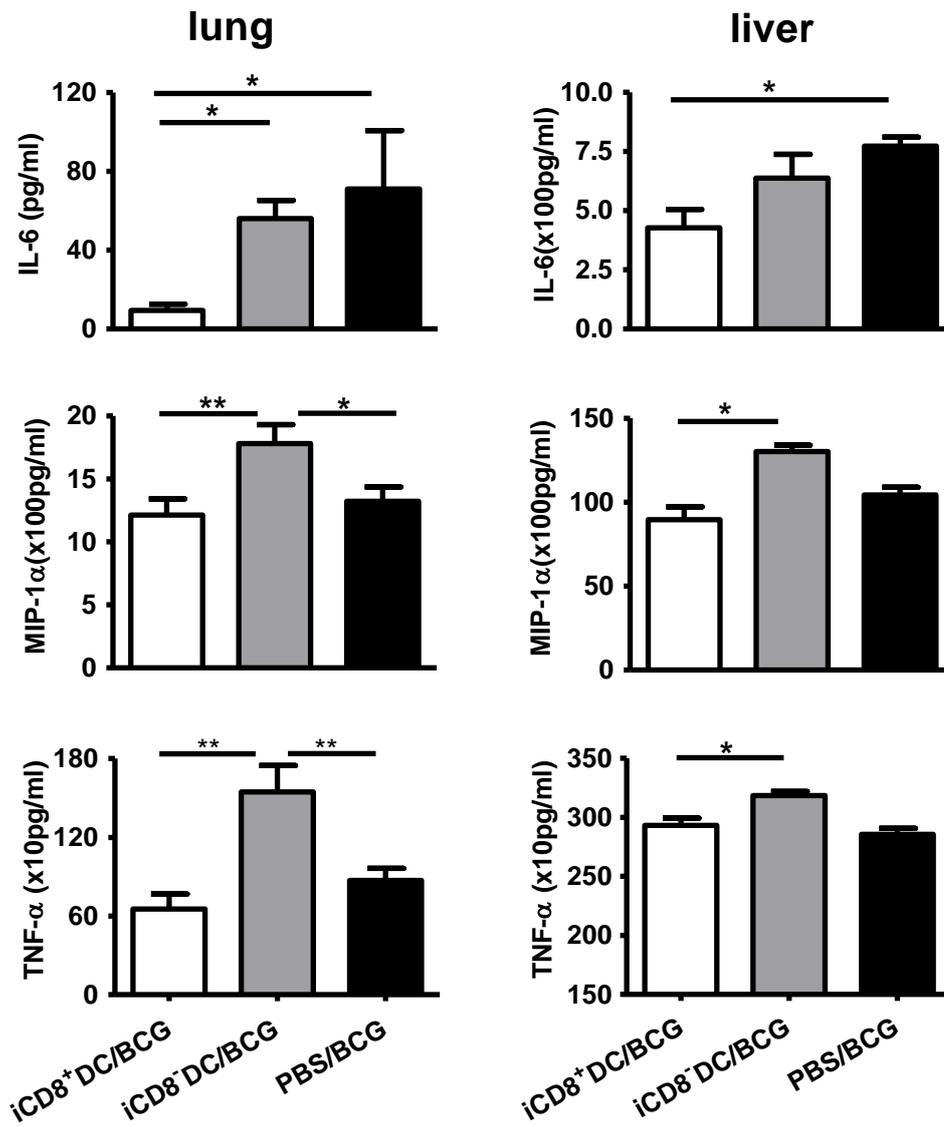
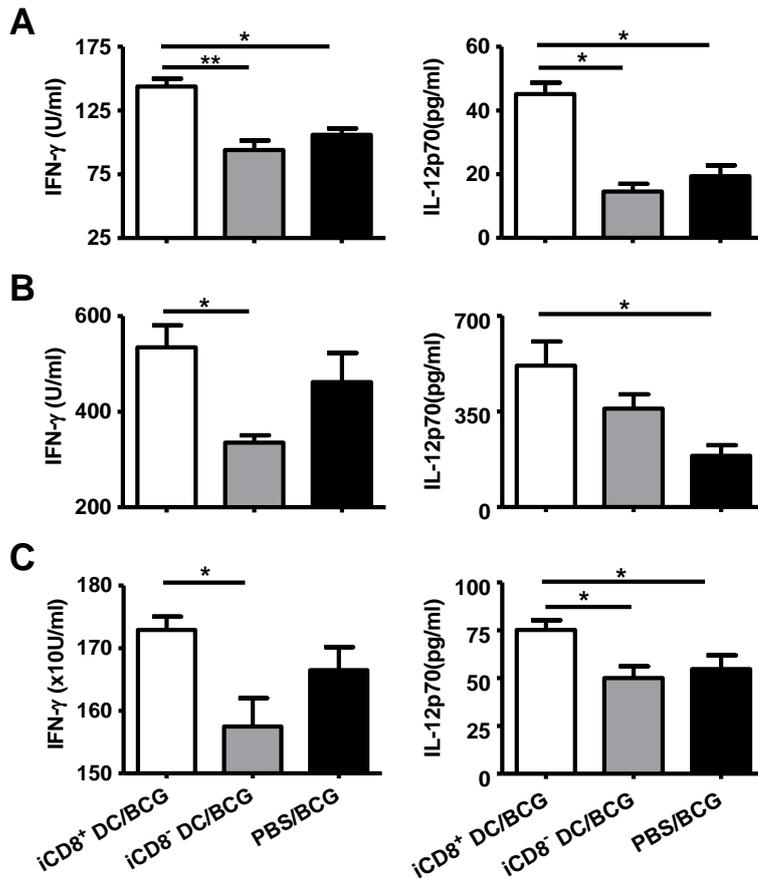


figure 6

Figure 6. Pro-inflammatory cytokine/chemokine levels in local tissues in mice treated with different DC subsets following challenge infection. The mice were treated as described in the legend to Fig 4 and the levels of IL-6, MIP-1 $\alpha$  and TNF- $\alpha$  proteins in the homogenates of lung and liver tissues were measure by ELISA.

***The adoptive transfer of iCD8<sup>+</sup> DC, but not iCD8<sup>-</sup> DC, enhances type 1 immune responses following challenge infection***

Since the type of immune responses plays a critical role in protection against mycobacterial infection, we further examined the T cell cytokine patterns and antibody responses in the recipients of different DC subsets in order to elucidate the mechanism underlying the difference in protection. As shown in Figure 7A, significant difference in the production of BCG driven type-1 related cytokines (IFN- $\gamma$  and IL-12) by bulk cultured spleen cells was observed between the different groups following challenge infection. Specifically, the recipient of iCD8<sup>+</sup>DC showed significantly higher IFN- $\gamma$  production than the control mice without cell transfer. Analysis of cytokine patterns in the local tissues also showed higher IL-12 and IFN- $\gamma$  levels in the lung (Figure 7B) and liver (Figure 7C) of the iCD8<sup>+</sup> recipient mice compared to sham control groups and iCD8<sup>-</sup> DC recipient mice. Serum antibody analysis showed low titers of BCG specific IgG2a and IgG1 antibodies in all the groups and no significant difference was observed among the groups (data not shown).

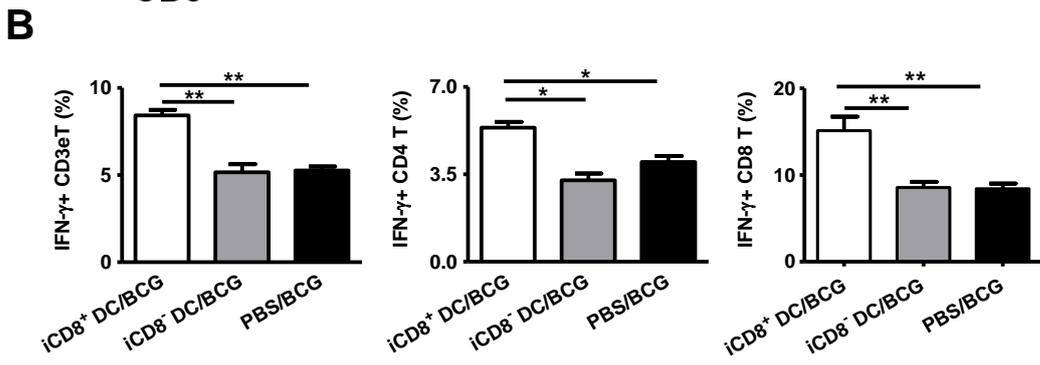
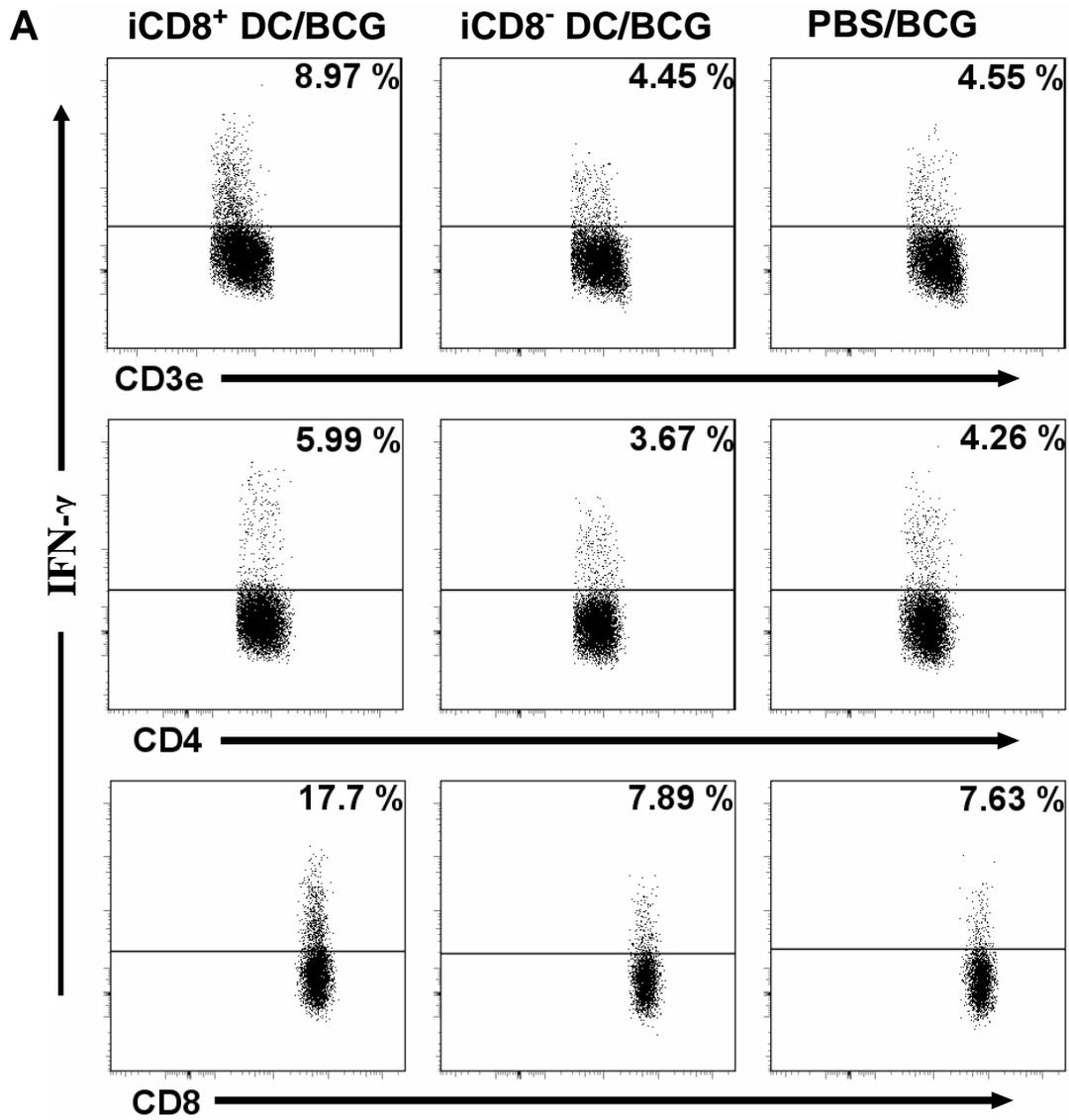


**figure 7**

**Figure 7. Effects of different DC subsets adoptive transfer on Th1-related cytokine production by recipient mice following challenge infection. A:** The recipient mice (C57BL/6, n=4/group) of i.v. adoptive transferred iCD8<sup>+</sup> DC or iCD8<sup>-</sup> DC subsets and PBS treated control mice were challenged i.v. with BCG and sacrificed at day 21 post challenge as described in Materials and Methods. A, splenocytes were cultured at the concentration of  $7.5 \times 10^6$  cells/well using HK-BCG as stimulator. Culture supernatants were harvested at 72h and the cytokines were measured by ELISA. B&C, lungs and livers were homogenized in 10ml cold protein-free D-PBS and centrifuged. The cytokines levels in the lung (B) and liver (C) were measured by ELISA. Data are presented as mean $\pm$ SD of each group. One representative experiment of three independent experiments is shown.\*p<0.05; \*\*,

p<0.01.

To further examine the local T cell responses, we performed intracellular cytokine staining of T cells from the draining lymph nodes following intranasal challenge infection. As shown in Figure 8, the adoptive transfer of iCD8<sup>+</sup> DC induced more IFN- $\gamma$  producing T cells than iCD8<sup>-</sup> DC. In particular, recipients of iCD8<sup>+</sup> DC mounted more than two fold higher IFN- $\gamma$  producing CD8 T cells than the sham-treated mice. Similarly, more IFN $\gamma$ -producing CD4 T cells were also found in the recipients of iCD8<sup>+</sup> DC. In contrast, adoptive transfer of iCD8<sup>-</sup> DC failed to enhance IFN $\gamma$  response by either CD4 or CD8 T cell. Taken together, the results suggest that iCD8<sup>+</sup> DCs have a stronger ability to promote type-1 T cell responses to mycobacterial challenge infection, which may be the basis for their strong capacity to generate protection against both systemic and local challenge infections.

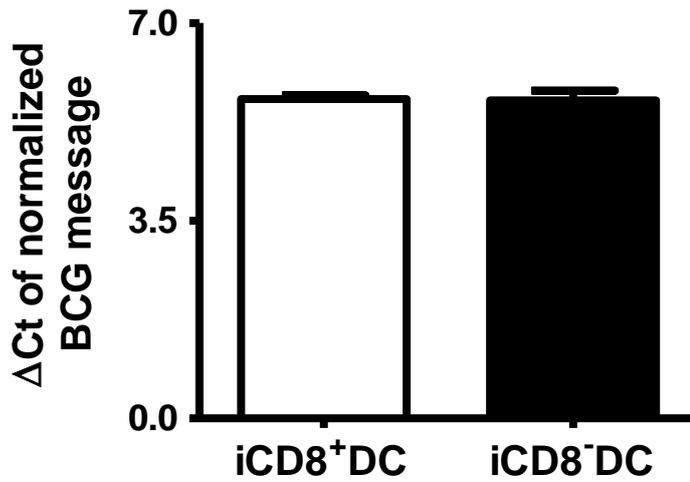


**figure 8**

**Figure 8. Intranasal adoptive transfer of iCD8<sup>+</sup>DC, but not iCD8<sup>-</sup>DC, enhanced IFN- $\gamma$  production by T cell.** Mice were adoptively transferred i.n. with iCD8<sup>+</sup>DC or iCD8<sup>-</sup>DC subsets followed by the challenge with BCG through i.n route as described in Materials and Methods. Draining lymph nodes was isolated aseptically and single cell suspension was prepared in cold staining buffer. Cells were co-stained with FITC-anti CD3 $\epsilon$ , PE-anti CD4, PerCP-anti CD8 Abs and stained for intracellular IFN- $\gamma$  using allophycocyanin -conjugated anti-IFN $\gamma$  Ab as described in Materials and Methods. Cells were gated on CD3 $\epsilon$ <sup>+</sup>T cells, CD3 $\epsilon$ <sup>+</sup>CD4<sup>+</sup>T cell and CD3 $\epsilon$ <sup>+</sup>CD8<sup>+</sup>T cell respectively and the percentage of positive cells for IFN- $\gamma$  is indicated in upper right corner (A). Pooled data in each group are shown as mean $\pm$ SD (B). One representative experiment of two independent experiments is shown.\*p<0.05; \*\*, p<0.01.

***DC subsets isolated from BCG infected mice show similar levels of BCG messages***

A question which needed to be addressed was if the differences observed above for the DC subsets isolated from infected mice in generating protective immunity was due to the potential difference of the subsets in BCG loads, thus the amount of antigens carried. To answer this question, we further analyzed the bacterial loads of the CD8<sup>+</sup>DC and CD8<sup>-</sup>DC subsets from BCG infected mice using quantitative RT-PCR. As shown in Figure 9, the BCG mRNA levels in the two DC subsets demonstrated in real-time PCR analysis were comparable. The *in vitro* culture of either DC subsets showed negative results for viable BCG. The data suggest that the DC subsets are not significantly different in carrying BCG and possibly its antigens.



**figure 9**

**Figure 9. DC subsets from BCG-infected mice show similar BCG load.** A real-time RT-PCR using the green fluorescent dye SYBR Green I was applied to determine the relative concentration of BCG mRNA in  $iCD8^+DC$  and  $iCD8^-DC$ . The BCG mRNA in different DC subsets was amplified as described in Materials and Methods. The BCG mRNA level was normalized to GAPDH mRNA in different DC subsets demonstrated as  $\Delta Ct$ .  $\Delta Ct = Ct (BCG) - Ct (GAPDH)$ , the threshold cycle of a BCG and the threshold cycle of the corresponding GAPDH in the same sample.

## Discussion

This chapter assessed the capacity of DC subsets in BCG infected mice in generating protective immunity against mycobacterial challenge infection. The data revealed that CD8<sup>+</sup> DCs, but not CD8<sup>-</sup>DCs, from the infected mice are able to induce protection and reduce pathological reactions in the local tissues of the recipient mice. This conclusion is supported by the facts that the recipients of iCD8<sup>+</sup> DCs exhibited significantly less bacterial growth, much milder histopathological changes and significantly stronger type-1 immune responses compared to the mice without cell transfer or received iCD8<sup>-</sup>DCs. To our knowledge, this is the first report showing the different capacity of DC subsets primed by *in vivo* immunization by BCG in reducing bacterial loads and pathological reactions following systemic and local mycobacterial challenge infection.

Several published studies have used DCs which are infected by BCG *in vitro* or are loaded with mycobacterial antigens to investigate the role of DC in inducing T cell immune responses and protection using cell adoptive transfer approaches (281, 282). Demangel et al found that bone marrow-derived DCs that were infected with BCG showed increased expression/production of MHC class II antigens, co-stimulatory molecules and immune regulatory cytokines. Intratracheally adoptive transfer of these infected DCs induced a potent protection against aerosol Mtb challenge, similar to those induced by *in vivo* BCG immunization (281). Gonzalez-Juarrero et al reported that lung DCs pulsed with Ag85 (LDC-Ag85) *in vitro* were able to prime naive CD4<sup>+</sup> T cells *in vivo* after adoptively transfer (282).

However, the LDC-Ag85 recipients were not more resistant to Mtb challenge than those receiving DCs pulsed with an irrelevant protein, instead, showing more serious consolidation in the lung. Comparing to these reported studies, our study using DCs primed/infected with BCG *in vivo* addressed the function of DCs in a more physiological and relevant way. Most importantly, our data not only showed the critical role of DCs in BCG-induced protection against mycobacterial challenge infection, but the different capacity of DC subsets in this matter.

In theory, a potential possibility for the difference of the DC subsets in generating protective immunity is because the DC subsets are different in uptaking and/or hosting BCG thus leading to differential immune responses. This concern is reasonable because some early studies have shown a poor phagocytic capacity of CD8<sup>+</sup>DC in certain infections (149, 283). However, our analysis on BCG gene expression in the two DC subsets showed similar levels of BCG mRNA in the isolated DC subsets (Figure 9). This is consistent with a reported study showing the similar efficiency of CD8<sup>+</sup> DC and CD8<sup>-</sup> DC in acquiring mycobacteria-derived antigens (284). This is also consistent with other reports which demonstrate a similar efficacy of the CD8<sup>+</sup> DC and CD8<sup>-</sup> DC subsets in phagocytosis (285, 286). Therefore, the difference in immune responses generated by the adoptive transfer of the DC subsets observed in our study is more likely reflecting a functional difference of the subsets in activating/modulating T cell responses rather than their difference in handling the microbes/antigens, especially considering the dramatic difference of the DC subsets in the expression of co-stimulatory molecules and the production of immunoregulatory

cytokines.

The expression of co-stimulatory cell surface molecules and the production of cytokines have been shown to be the most important bases by which DCs modulate the function/polarization of T cell responses. Our data showing the significant differences of the DC subsets in the expression and production of these molecules and cytokines fit well with the functional difference of the DC subsets in inducing the different types of immune response and protection. In our study, a higher proportion of iCD8<sup>+</sup> DC expressed costimulatory surface markers CD80 (65.66% vs. 17.43%), CD86 (57.43% vs. 30%) and CD40 (44% vs. 35%) compared with iCD8<sup>-</sup> DC. So were the densities (MFI) of the expressed molecules. More importantly, iCD8<sup>+</sup> DC predominantly produced Th1 promoting cytokine, IL-12p70 and IL-12p40 (not shown) while iCD8<sup>-</sup> DC produced higher IL-10. This is inline with a reported study showing CD8<sup>-</sup> DCs induced a Th2-type immune response, while the CD8<sup>+</sup> DCs led to the Th1 differentiation (287). IL-12 producing DC has been found to be powerful in generating protection against tuberculosis infection (288, 289). IFN- $\gamma$  is closely related to protective immunity to tuberculosis infection and is the principal macrophage-activating cytokine(290), which can stimulate the synthesis of reactive oxygen intermediates and nitric oxide by inducing production of phagocyte oxidase and nitric oxide synthase within lysosomes. IFN- $\gamma$  induced production of reactive nitrogen intermediates is one of the most important mechanisms for controlling mycobacterial infections (291). Therefore, the higher production of IL-12 by iCD8<sup>+</sup>DC and the subsequently enhanced IFN- $\gamma$  production induced by the adoptive

transfer of this DC subset is likely the major reason for the better protection of the iCD8<sup>+</sup>DC recipients observed in the present study. In addition to modulate CD4 T cell response, we also found a significantly higher IFN- $\gamma$  production by CD8 T cell in the recipients of iCD8<sup>+</sup>DC. In fact, the difference in CD8 T cells for IFN- $\gamma$  production by the different experimental groups was even more apparent than in CD4 T cells. This finding is inline with recent reports showing an exceptional ability of CD8<sup>+</sup>DC to present exogenous antigens through a process of cross-presentation (292, 293) and suggests the importance for enhancing CD8<sup>+</sup> T cell responses in promoting host defense against mycobacterial infections.

A notable characteristic of iCD8<sup>-</sup> DC observed in the present study is its dramatically higher production of IL-10 compared to that of iCD8<sup>+</sup> DC. It has been reported that, although IL-10 knockout mice showed little difference in resistance to Mtb infection than wild-type mice(294), gene transgenic mice that overly expressed IL-10 exhibited increased reactivation of chronic infection(295). Our data showed that adoptive transfer iCD8<sup>-</sup> DC had no significant effect on BCG loads in the lung and liver following challenge infection. However, histological analysis showed that the inflammation in the lung and liver tissues of iCD8<sup>-</sup> DC recipients was not only much heavier than the iCD8<sup>+</sup> DC recipients but also more severe than the mice without previous DC transfer. Typical multinucleate giant cells were found around the granulomas in mice without DC transfer. However, the iCD8<sup>-</sup> DC recipients showed more diffused inflammation. Granuloma formation is an important way for the host to control mycobacterial infection, although its efficiency is not absolute. The most

successful control of infection would lead to resolution of inflammation without granuloma formation. However, the lack of granuloma formation with diffused inflammation observed in the iCD8<sup>-</sup> DC recipients implies a lack of efficient control for the infection and the development of more severe pathological inflammation. The highest level of inflammatory cytokines/chemokine (IL-6, MIP-1 $\alpha$ , TNF- $\alpha$ ) in the iCD8<sup>-</sup> DC recipients confirms the severe inflammation in these mice. Since granuloma in mycobacterial infection is a type-1 granuloma that is promoted by IFN $\gamma$  and inhibited by IL-10, the higher IL-10 production by iCD8<sup>-</sup> DC may be the basis for the significantly reduced granuloma formation in its recipients.

It should be noted, however, that the challenge infection used in the study is BCG instead of virulent *Mtb* strains which are the real cause of tuberculosis. It is possible that immunity against challenge by virulent *Mtb* requires additional parameters. Therefore cautions are needed in interpreting the data from this study. Further study using virulent *Mtb* as challenge infection would be important. On the other hand, since many similarities in immune responses to BCG and *Mtb* have been reported and BCG has been used for modeling host defense against *Mtb* in many studies, the data from current study are very useful for guiding future studies and for understanding host defense mechanisms against *Mtb* infection.

In conclusion, this chapter has demonstrated that different DC subsets from BCG-infected mice are different in capacity to generate protective immunity. The functional difference may be related to their expression of co-stimulatory molecules and cytokine production patterns. The data suggest that proper targeting and/or

modulating “right” DC subset may have the potential to improve the efficiency of vaccination to generate protective immunity. This knowledge may have implications in the development and improvement of the approaches for prevention and therapy of tuberculosis infection and subsequent sequelae.

## **Part IV Chapter 2**

### **CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> dendritic cell (DC) subsets from BCG-infected mice inhibit established allergic Th2 responses through enhancing Th1 and Treg activity respectively**

#### **Introduction**

The hygiene hypothesis has been raised for more than two decades(63). Although conflicting results have been reported, it is generally accepted that some infections especially intracellular bacterial and parasitic infections may have negative impact on the development of allergic and autoimmune diseases(64, 73, 296, 297). In particular, a protective role of BCG infection/vaccination on the development of allergic diseases has been demonstrated by numerous epidemiological and experimental studies(65).

The mechanism of hygiene hypothesis has been believed to be related to immune deviation and/or immune regulation(67, 297-300). Indeed, some bacterial infections including BCG infection have been found to alter allergen-driven Th2 response to Th1 dominated response(65, 73, 75, 80, 90-92). More recently, the promoting effect of infections especially helminth parasitic infections on regulatory T cells was shown to be even more relevant to the inhibitory role of infection in allergic diseases(67, 301-303). DCs are critical in bridging infection mediated inhibition of allergy(76, 77, 79, 93, 304, 305). The data have demonstrated that DC play a critical role in infection mediated inhibition of allergy. In these studies, the adoptive transfer of DC from infected mice significantly inhibited de novo or established allergic

inflammation and Th2 responses. The reduction of allergic reactions and Th2 responses was either associated with enhanced Th1 response(76) or enhanced regulatory function(93, 304, 305) or both(77, 80, 305). In particular, using an adoptive transfer approach, we showed that DC play a pivotal role in BCG-mediated modulation of the allergic responses to ragweed, a common environmental allergen (80). More importantly, we found that the mechanism by which transferred DC from BCG-infected mice modulate allergic reactions involves both IL-12 and IL-10 mediated mechanism(80). The recipients of DC from BCG infected mice showed Th1-like allergen-driven cytokine production instead of Th2-like responses, suggesting a mechanism of immune deviation. This was consistent with the higher production of IL-12 by these DC. However, the DC from BCG infected mice also produce higher IL-10 than the DC from naïve mice. More importantly, the blockage of either IL-12 or IL-10 reversed the inhibitory effect of adoptively transferred DC from infected mice in inhibiting allergic reactions and Th2 responses(80). Since IL-10 produced by DC is a major cytokine for the induction of regulatory T cells, the data raised the possibility that DC from BCG infected mice might be able to induce regulatory T cells in addition to induce Th1 cells, thus inhibiting allergic reactions through both immune deviation and regulation mechanism. Since different DC subsets have been found to be different for modulating T cell responses (106, 283, 287, 306-308) and since we recently found CD8 $\alpha^+$  and CD8 $\alpha^-$  DC subsets from BCG infected mice showed higher IL-12 and IL-10 production respectively(309), we hypothesized that the two different DC subsets may modulate allergic reactions

through different mechanisms. The data generated from this study supported this hypothesis by showing that adoptive transfer of either CD8 $\alpha^+$  or CD8 $\alpha^-$  DC from BCG-infected mice inhibited OVA-induced mucus over-production and pulmonary eosinophilia inflammation, but the patterns of T cell response are different. Specifically, the transfer of CD8 $\alpha^+$ DC which predominantly produced IL-12 enhanced Th1 responses, while the transfer of CD8 $\alpha^-$  DC which predominantly produced IL-10 significantly increased the frequency of regulatory T cells.

## **Results**

### **CD8 $\alpha^+$ DC and CD8 $\alpha^-$ DC isolated from BCG-infected mice showed different patterns of surface markers and cytokine profile**

DC function mainly relies on surface costimulatory molecule expression and cytokine production. We recently reported that BCG infection induced significant expansion of CD8 $\alpha^+$ DC, which showed different patterns of surface markers and cytokine profile in C<sub>57</sub>BL/6 mice(309). In this study we further analyze the effect of BCG infection on the phenotype of CD8 $\alpha^+$  DC and CD8 $\alpha^-$ DC in BALB/c mice because this mouse strain was used for the following experiments. As shown in Figure 10, CD8 $\alpha^+$  DC and CD8 $\alpha^-$ DC from BCG infected mice showed significantly higher CD80 and CD40 expression than those from naïve DC. MHC-II expression was higher on CD8 $\alpha^+$ DC than CD8 $\alpha^-$ DC but not relevant to infection. Interestingly, BCG infection enhanced CD86 expression on CD8 $\alpha^-$ DC but not on CD8 $\alpha^+$ DC. More interestingly, the CD86 expression on CD8 $\alpha^-$ DC was significantly higher than

CD8 $\alpha$ <sup>+</sup>DC in BCG infected mice. The results suggest that BCG infection has differential influences on surface marker expression on CD8 $\alpha$ <sup>+</sup>DC than CD8 $\alpha$ <sup>-</sup>DC subset in BALB/c mice, especially on CD86 expression.

We further examined cytokine production by the DC subsets. Real-time PCR were used to analyze the mRNA messages of IL-12 and IL-10 in freshly purified CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC (Fig 10C). CD8 $\alpha$ <sup>+</sup>DC showed higher IL-12p40 mRNA message while CD8 $\alpha$ <sup>-</sup>DC has higher IL-10 mRNA expression. To further confirm the difference in IL-12 and IL-10 production by DC subsets, the purified DC subsets were cultured in complete medium for 72h and the culture supernatants were tested by ELISA. In line with the difference in message expression, the cultured DC showed similar pattern in cytokine protein production (Figure 10D). Therefore, BCG infection indeed had influence on DC subsets which were different in the expression of surface co-stimulatory markers and cytokines production.

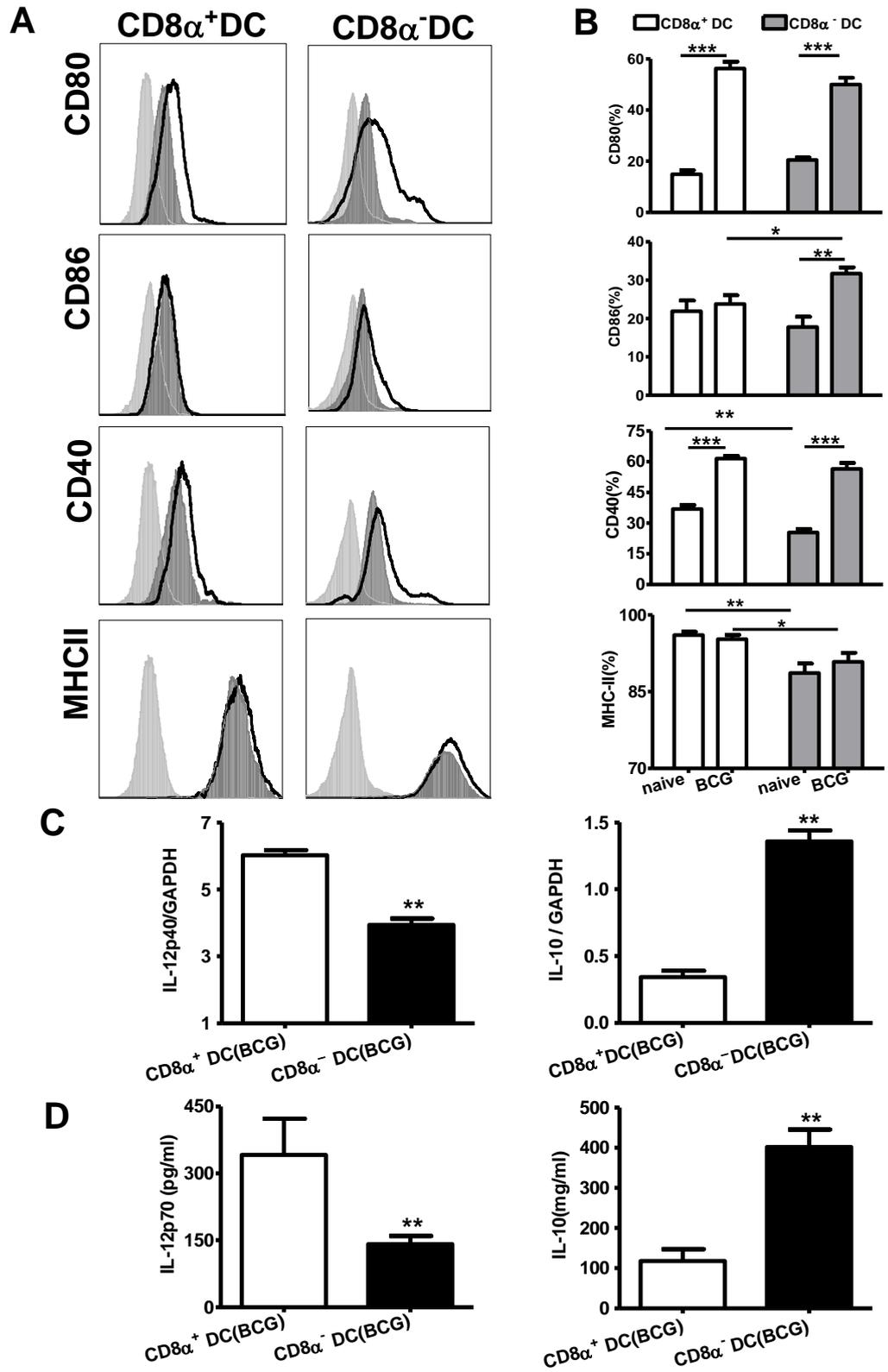


figure 10

**Figure 10. Differential surface co-stimulatory marker expression and cytokine profiles of DC subsets isolated from BCG infected mice.** BALB/c mice were killed at 21 days after intravenously infection with BCG ( $5 \times 10^5$  CFUs). DC were isolated from the spleen of BCG infected mice or naïve mice using CD11c microbeads and MACS LD columns as described in *Materials and Methods*. A, cells were stained with FITC-anti-CD11c, PE-anti-CD8 $\alpha$  and Allophycocyanin-conjugated antibodies for surface molecules (CD80, CD86, CD40, MHC-II) and were analyzed using flow cytometry. Light shaded area, isotype control; dark shaded area, naïve DC subsets; solid line, DC subsets isolated from BCG infected mice. B, summary data on surface marker expression by DC subsets from naïve and BCG infected mice. C. DC in the spleen of BCG-infected mice were sorted by a flow cytometer based on the CD11c and CD8 $\alpha$  markers to CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup> subsets as described in *Materials and Methods*. The expression of mRNA of IL-12 and IL-10 in the CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC was measured by real-time PCR. The ratio of copies of IL-12 or IL-10 to GAPDH is shown as mean  $\pm$  SEM for pooled data from three independent experiments. D, The sorted DC subsets were cultured in complete medium for 72 h. The proteins of IL-12p70 and IL-10 in the supernatant of DC subset cultures were measure by ELISA. The data are shown as mean  $\pm$  SD. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

## **Adoptive transfer of either CD8 $\alpha$ <sup>+</sup> DC or CD8 $\alpha$ <sup>-</sup> DC isolated from BCG-infected mice inhibited the allergic response but CD8 $\alpha$ <sup>+</sup> DC were more effective**

Our previous works have demonstrated that mycobacterial infection can inhibit allergic responses induced by ovalbumin (OVA)(75), and adoptive transferring of DC isolated from BCG-infected mice are capable of modulating allergic responses(80). In the present study, we intended to further examine the functional relevance of DC subsets in the inhibition of allergic reactions by BCG infection. We adoptively transferred CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC subsets isolated from BCG infected mice to recipient mice two hour before the mice were sensitized with OVA. The control mice received PBS only with the same OVA treatment. After further intranasal challenge with OVA, the mice were killed and examined for airway inflammation, mucus production and IgE responses. Analysis of inflammatory cells in the bronchoalveolar lavage fluids (BALFs) showed that the recipients of either CD8 $\alpha$ <sup>+</sup>DC or CD8 $\alpha$ <sup>-</sup>DC subsets mounted significantly reduced airway inflammation than the control mice (PBS) measured by total infiltrating cells (Figure 11A) and eosinophils (Figure 11B), while the reduction was more dramatic in the recipients of CD8 $\alpha$ <sup>+</sup>DC. Similar pattern of difference was observed in histopathological analysis (H&E staining) of the lung tissues (Figure 11C). Control mice exhibited massive and diffuse eosinophilic infiltration in alveolar, peribronchial and perivascular areas, which was remarkably lower in the recipients of CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC subsets especially those received CD8 $\alpha$ <sup>+</sup>DC. Moreover, the goblet cell and mucus staining by periodic acid-schiff

method showed less mucus production in the DC recipients than control mice (Figure 11C). HMI, a quantitative method for measuring the goblet cell development and mucus secretion, showed that the percentage of mucinous airway epithelium was 30% in control mice, while it was 8% and 15%, respectively, in CD8 $\alpha$ <sup>+</sup> DC and CD8 $\alpha$ <sup>-</sup> DC recipients (Figure 11D). Consistent with the changes in local allergic inflammation, the recipients of CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC subsets showed significantly lower serum OVA-specific IgE responses than control mice (Figure 11E). The results suggest that both CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC subsets from BCG infected mice can inhibit airway allergic reactions and that CD8 $\alpha$ <sup>+</sup>DC subset is more efficient in mediating this inhibition.

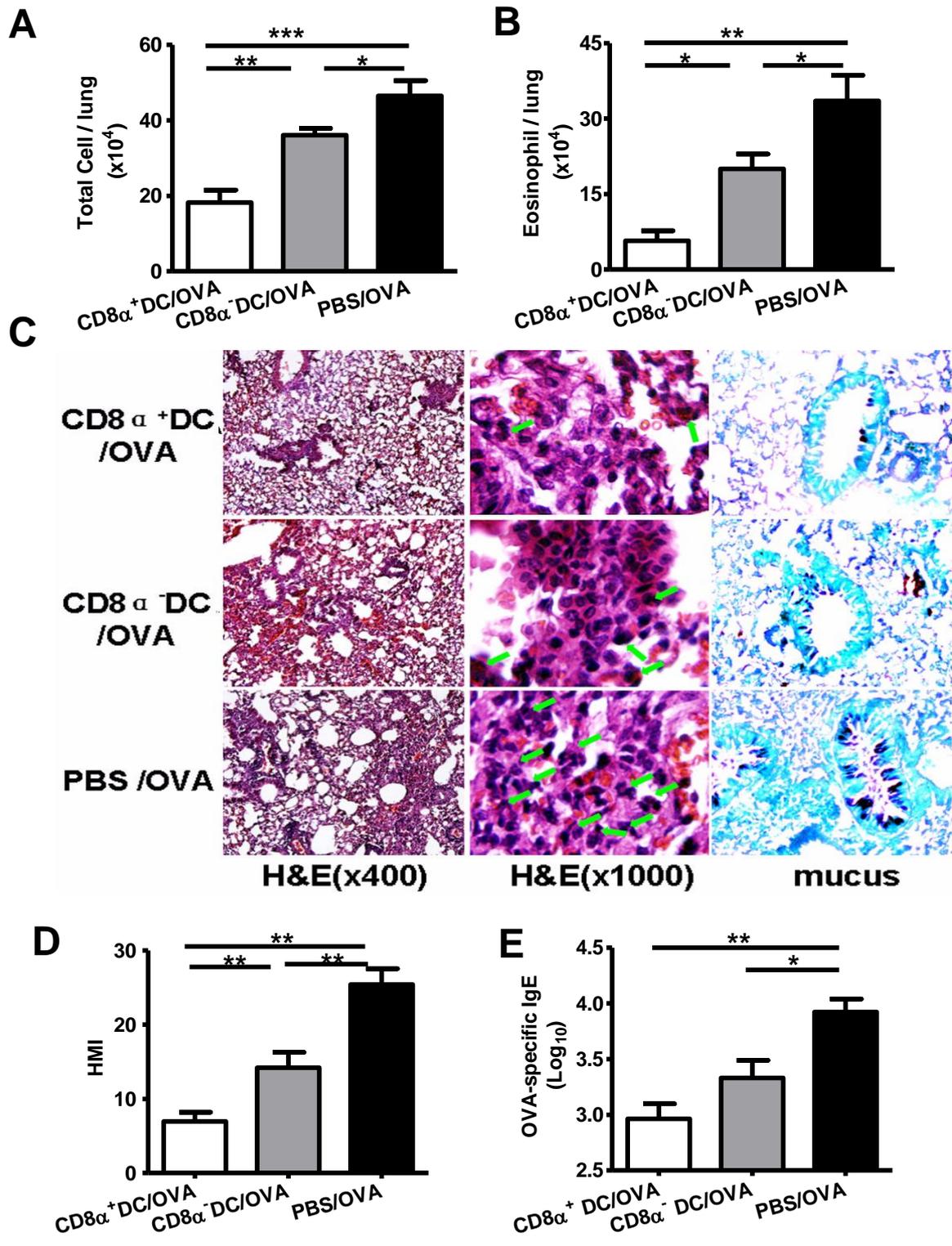


figure 11

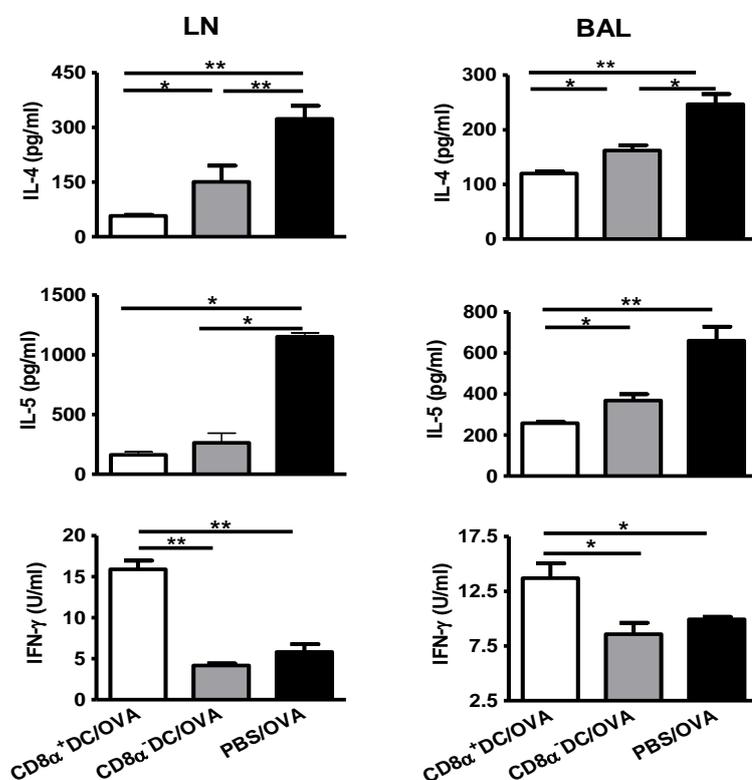
**Figure 11. The effect of adoptive transfer of DC subsets isolated from BCG-infected mice on airway inflammatory and mucus production in the lung.**

BALB/c mice (n=12) were infected with BCG intravenously. On day21 post infection, CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup> DC subsets in the spleen were sorted by a flow cytometer and injected intravenously (tail vein) to recipient mice (5x10<sup>5</sup> cells in 200 $\mu$ l PBS). Two hours after the cell transfer, the mice were sensitized i.p. with 4 $\mu$ gOVA (in alum) followed by intranasal challenged 50 $\mu$ g OVA (in 40 $\mu$ l PBS) at day 14 days after sensitization. Control mice received the same OVA sensitization and challenge but only pretreated with PBS instead of DC transfer. Mice were sacrificed at day 7 after intranasal OVA challenge and analyzed for lung inflammation, mucus production and serum IgE responses. A&B, cells in the bronchoalveolar lavage (BAL) fluids were counted and slides of cell pellets stained by Fisher Leukostat Stain kit. The absolute number of total infiltrating cells (A) and eosinophils (B) in the different groups are shown. C, Lung tissues were fixed with formalin, and the sections (5 $\mu$ m) were stained with H&E for inflammation (left and middle columns) or a periodic acid-schiff (PAS) staining kit for mucus production (right column). Green arrows point eosinophils in the lung tissue (middle column). D, Mucus producing epithelium was quantified as histological mucus index (HMI) as described in the *Materials and Methods*. E, OVA-specific serum IgE levels in different groups are shown. One representative experiment of four independent experiments is shown. Data are shown as the mean  $\pm$ SD of each group. \*  $p$ <0.05; \*\*  $p$ <0.01; \*\*\*  $p$ <0.001.

### **3. Adoptive transfer of either CD8 $\alpha$ <sup>+</sup> DC or CD8 $\alpha$ <sup>-</sup>DC isolated from BCG-infected mice reduced allergen-driven Th2 cytokine production but only the CD8 $\alpha$ <sup>+</sup> DC subset increased Th1 cytokine production**

To explore the molecular basis for the altered eosinophilic inflammation and mucus production in the airway, we further examined the allergen-driven cytokine production in the different groups of mice. As shown in Figure 12, the recipients of CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC subsets showed significantly lower OVA-driven Th2 cytokine (IL-4 and IL-5) production than PBS treated control mice in the culture of draining lymph node (LNs) cells and direct measurement of BALF cytokine (BAL). The reduction of Th2 cytokines was more significant in CD8 $\alpha$ <sup>+</sup>DC recipients. In sharp contrast, the recipients of CD8 $\alpha$ <sup>+</sup>DC showed dramatically higher allergen driven IFN $\gamma$  production by the cultured draining LNs and in the BALFs than the other groups of mice. Notably, IFN $\gamma$  production in the recipients of CD8 $\alpha$ <sup>-</sup>DC was similar to control mice, significantly lower than the recipients of CD8 $\alpha$ <sup>+</sup>DC. The data demonstrate that adoptive transfer of either DC subsets can reduce Th2 cytokine production induced by allergen treatment but only CD8 $\alpha$ <sup>+</sup>DC subset can enhance Th1 cytokine production. Since the Th1 and Th2 related cytokines can be produced by multiple cells, we further did CD4<sup>+</sup> T cell intracellular cytokine staining to confirm the changes in Th1 and Th2 cells. As shown in Figure 13, both CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC recipients showed lower percentage of IL-4 producing CD4<sup>+</sup> T cells, while the recipients of CD8 $\alpha$ <sup>+</sup>DC showed higher percentage of IFN $\gamma$  producing CD4<sup>+</sup> T cells than control mice. Therefore,

adoptive transfer of either CD8 $\alpha$ <sup>+</sup>DC or CD8 $\alpha$ <sup>-</sup>DC indeed inhibited Th2 cells but only CD8 $\alpha$ <sup>+</sup>DC enhanced Th1 cells, implying that the two DC subsets from BCG infected mice may inhibit allergen-driven Th2 cells by different mechanisms.



**figure 12**

**Figure 12. Effect of DC subsets transfer on allergen-driven cytokine production by draining LNs cells and in the BAL.** Recipients (BALB/c, n=4/group) of DC subsets isolated from mice with or without BCG-infection were sensitized and challenged with OVA as described in legend of Fig 11. At day 7 after OVA challenge, the draining lymph nodes were collected and the cells ( $5 \times 10^6$  cells/ml) were cultured for 72h with re-stimulation of OVA. Cytokines in the culture supernatants of draining LNs cells and in BAL were measured by ELISA. Data are presented as mean $\pm$ SD. One representative experiment of four independent experiments with similar results is

shown. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

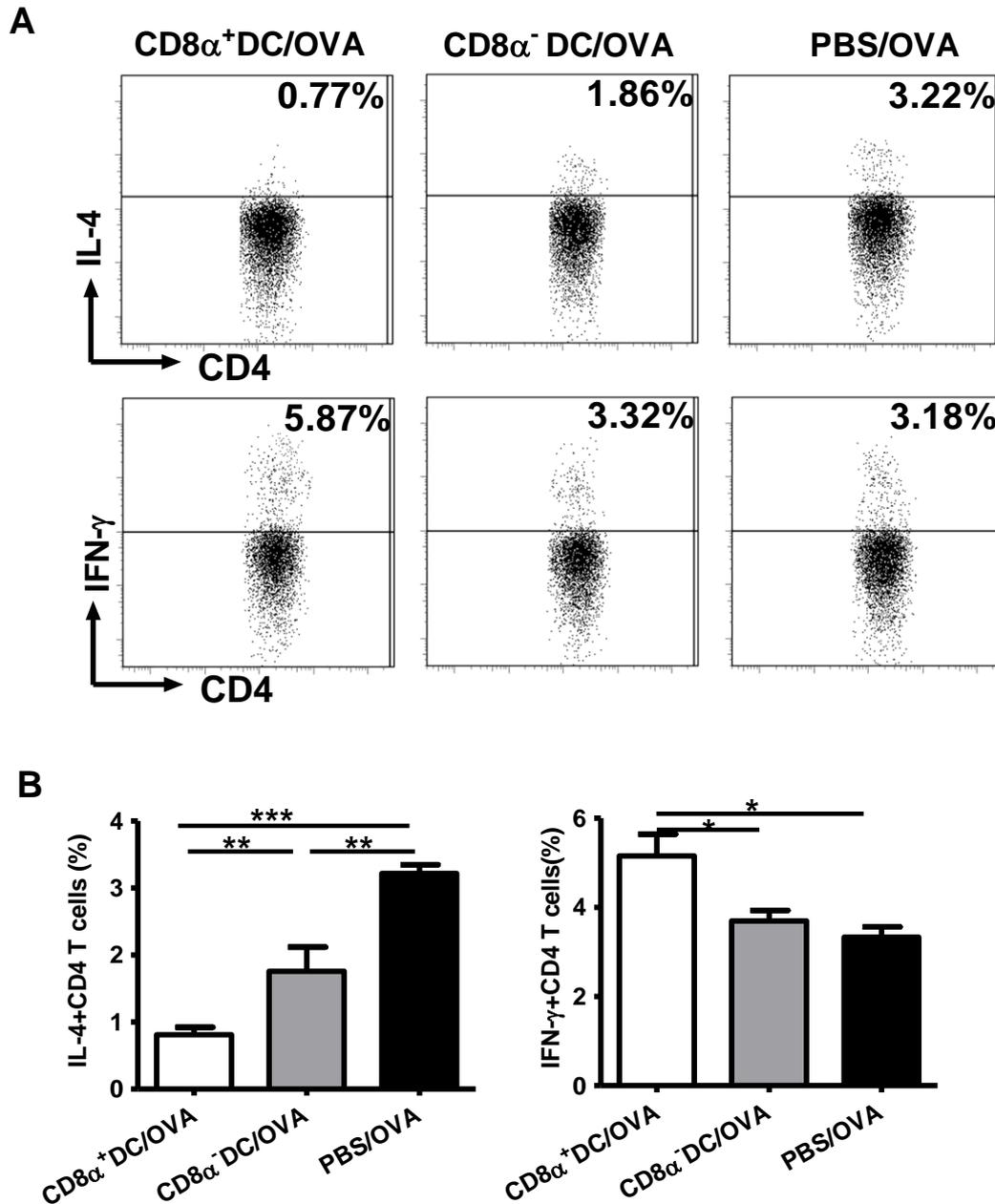


figure 13

Figure 13. Effect of DC subsets transfer on IL-4 and IFN $\gamma$  production by CD4<sup>+</sup> T cells in draining LNs following OVA sensitization and challenge. The recipient

mice of DC subsets isolated from BCG-infected mice were sensitized and challenged as described in legend to Fig11. The draining LNs were homogenized and  $2 \times 10^6$  single cells were stimulated with PMA/ionomycin for 6 hrs in complete RPMI-1640 medium at 37°C. Brefeldin A was added at the last 3 hrs incubation to accumulate cytokines intracellularly. Cytokines produced by  $CD3\epsilon^+CD4^+$ T cells were analyzed by intracellular cytokine staining. The draining LNs cells were co-stained with FITC-anti- $CD3\epsilon$  and PE-anti- $CD4$  in addition to Allophycocyanin conjugated antibodies specifically for  $IFN\gamma$  or IL-4. Cells were gated on  $CD3\epsilon^+CD4^+$ T cell. A, The representative images of the IL-4-producing and  $IFN\gamma$ -producing  $CD4^+$  T cells from three independent experiments are shown. B, summary of the frequencies of IL-4<sup>+</sup> producing and  $IFN-\gamma^+$  producing  $CD4$  T cell in draining LNs of each group. Data represent three independent experiments. \* $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

## **CD8 $\alpha$ <sup>+</sup> DC promote the differentiation of naïve allergen-specific CD4<sup>+</sup> T cells to Th1 direction**

To more directly determine the capacity of CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC subsets from BCG infected mice in directing allergen-specific T cell responses, we co-cultured the different DC subsets with naïve OVA-specific CD4<sup>+</sup> T cells isolated from DO11.10 mice for 48 h, in the presence of OVA stimulation. As showed in figure 14A&B, a significantly lower percentage of OVA-specific CD4<sup>+</sup> T cells developed to IL-4 producing cells when co-cultured with CD8 $\alpha$ <sup>+</sup>DC than those cultured with CD8 $\alpha$ <sup>-</sup>DC. In contrast, a significantly high percentage of CD4<sup>+</sup> T cells became IFN $\gamma$  producing cells when co-cultured with CD8 $\alpha$ <sup>+</sup>DC (Figure 14A&B). Measurement of cytokine proteins in the supernatants of the co-culture system showed similar pattern of Th2 and Th1 cytokines production (Figure 14C).

Since we had found different pattern of cytokine production by the DC subsets from BCG infected mice (Figure 10), we further tested the role of IL-12 and IL-10 which were predominantly produced by CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC, respectively, in influencing the T cell cytokine production. We used anti-IL-12 and anti-IL-10 mAbs to neutralize the function of corresponding cytokines in the DC subsets: CD4<sup>+</sup> T cell co-culture system. The data showed that in the CD8 $\alpha$ <sup>+</sup>DC:T cell co-culture system, blockade of IL-12 led to dramatic increase of IL-4 cytokine production (> 10 folds) in line with a significant reduction of IFN $\gamma$  production (Figure 14C). The blockade of IL-10 in this system had much milder effect (2.5 folds). In contrast, The blockade of IL-12 in the co-culture of CD8 $\alpha$ <sup>-</sup>DC:T cells had no significant effect on

IL-4 and IFN $\gamma$  production, while the blockade of IL-10 led to significant increase of IL-4 production. The results suggest that the higher IL-12 production by CD8 $\alpha^+$ DC is critical for the development of antigen-specific Th1 cells which may be the major mechanism for inhibiting allergic Th2 responses while the higher IL-10 production by CD8 $\alpha^-$ DC may be the major mechanism for inhibiting Th2 responses from the perspective of cytokine production by DC.

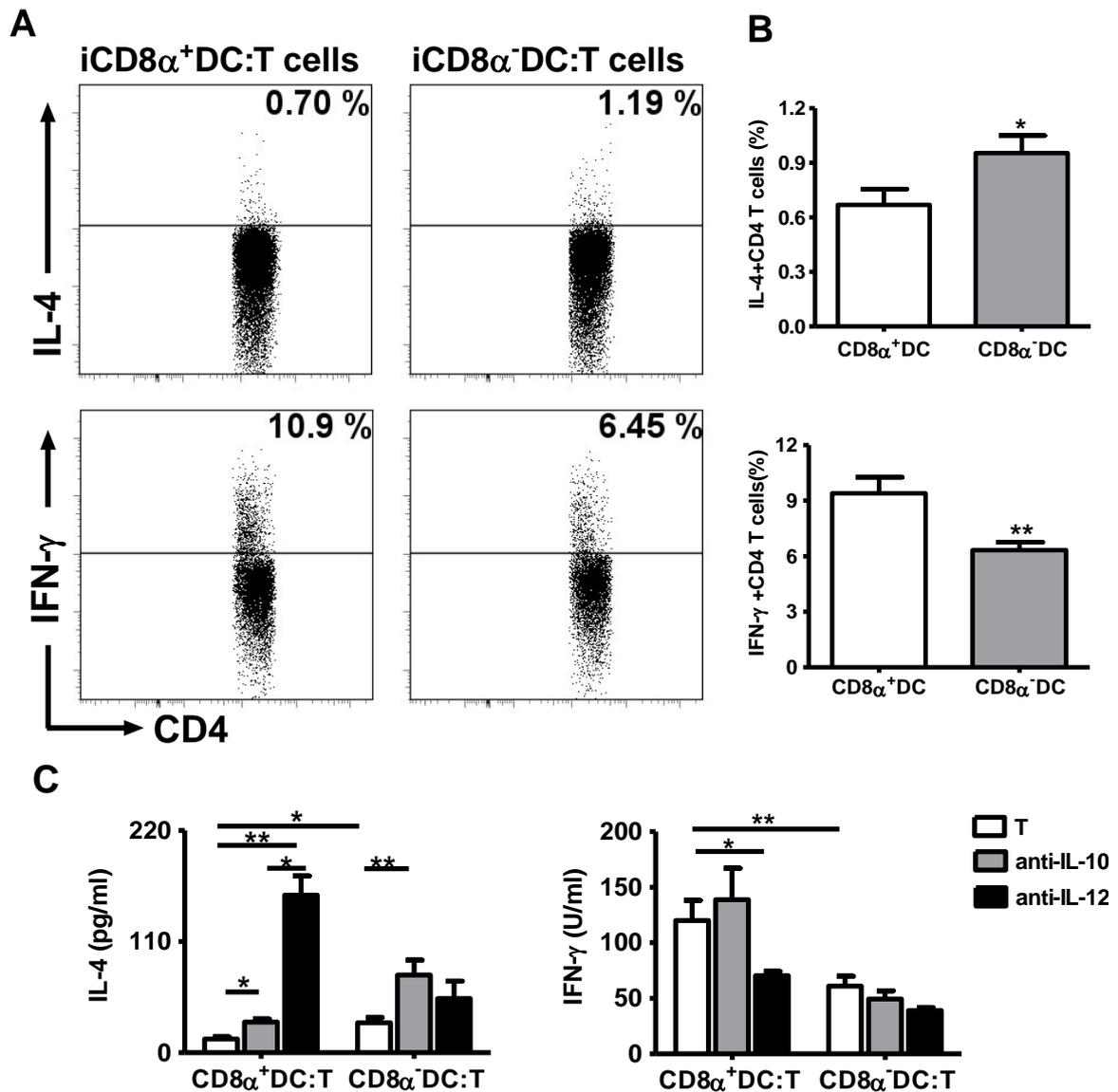


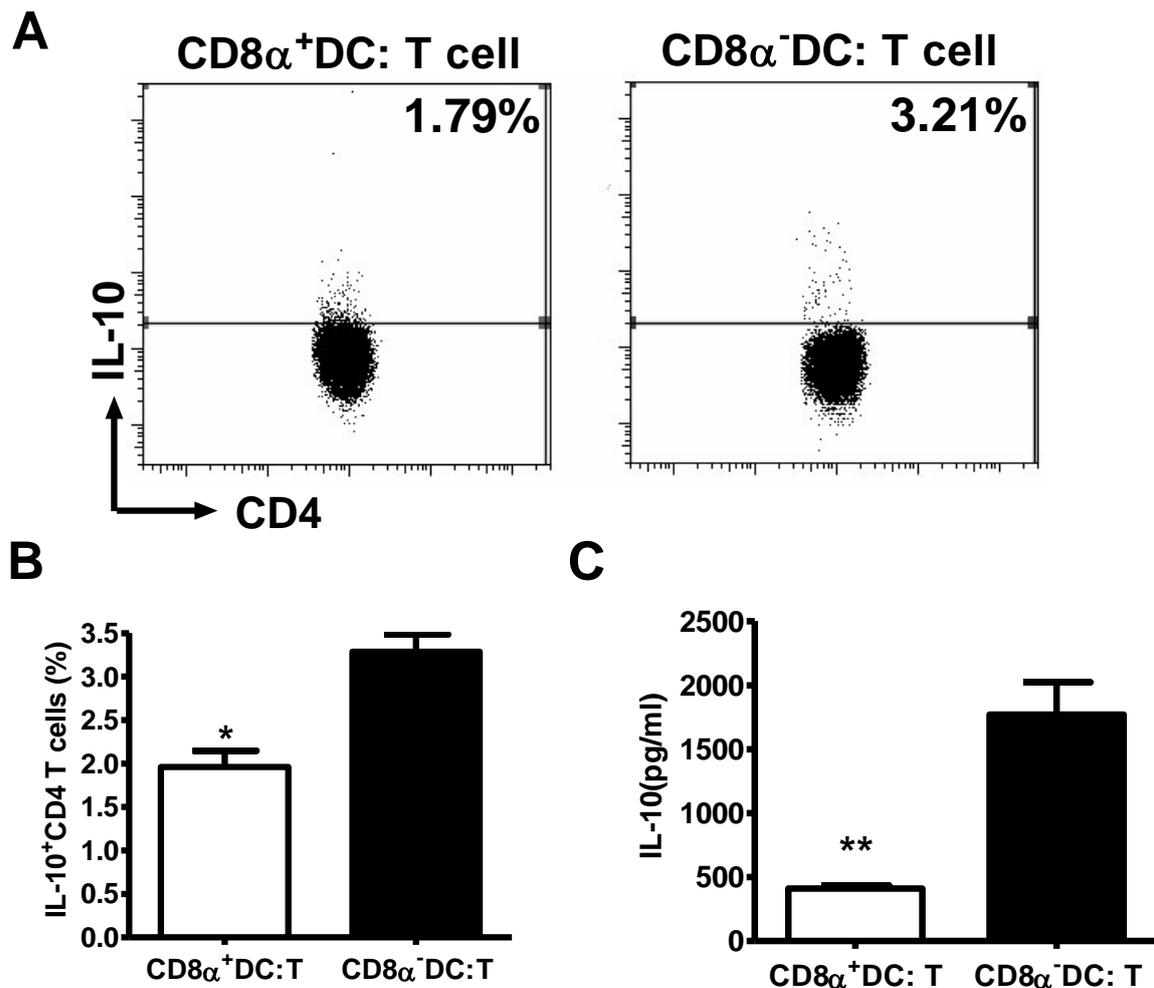
figure 14

**Figure 14. Differential ability of DC subsets isolated from BCG-infected mice to direct IL-4 and IFN $\gamma$  production by naïve OVA-specific CD4 T cells and the contribution of IL-10 and IL-12.** OVA peptide-specific CD4<sup>+</sup> T cells isolated from naïve DO11.10 transgenic mice were co-cultured with freshly isolated DC subsets from BCG-infected mice (DC:T ratio, 5x10<sup>5</sup>:5x10<sup>6</sup>) with OVA stimulation (0.1 mg/ml). A, the co-culture was allowed to proceed for 48 h. The CD4<sup>+</sup> T cells were

analyzed for intracellular cytokines by co-staining with FITC-anti-CD3 $\epsilon$ , PE-anti-CD4 and Allophycocyanin conjugated antibodies for IFN $\gamma$  or IL-4. The frequencies of IL-4<sup>+</sup> producing and IFN- $\gamma$ <sup>+</sup> producing CD4<sup>+</sup> T cells were measured by flow cytometry. B, summary of the frequencies of IL-4<sup>+</sup> producing and IFN- $\gamma$ <sup>+</sup> producing CD4 T cells (mean $\pm$  SD). C, The OVA-specific CD4 T cells were co-cultured with DC subsets from BCG-infected mice with OVA in the absence or presence of anti-IL-10 or anti-IL-12 mAb for 72 h as described in *Materials and Methods*. The levels of IFN $\gamma$  and IL-4 in culture supernatants were determined by ELISA. One representative experiment of three independent experiments with similar results is shown.\* $p$ <0.05, \*\* $p$ <0.01,

## **CD8 $\alpha$ <sup>-</sup>DC induced more IL-10 producing OVA-specific CD4<sup>+</sup> T cells than CD8 $\alpha$ <sup>+</sup> DC**

Having shown the major role of IL-10 production by CD8 $\alpha$ <sup>-</sup>DC on modulation of OVA-specific CD4<sup>+</sup> T cells, we further examined the capacity of this DC subset in inducing IL-10 production by OVA-specific T cells. We co-cultured CD4<sup>+</sup> T cells isolated from naïve DO11.10 mice with CD8 $\alpha$ <sup>+</sup>DC and CD8 $\alpha$ <sup>-</sup>DC, respectively, isolated from BCG infected mice for 48 h and examined the percentage of IL-10 producing CD4<sup>+</sup> T cells after the co-culture and level of IL-10 in the culture supernatants. As shown in Figure 15A&B, co-culture with CD8 $\alpha$ <sup>-</sup>DC, compared to CD8 $\alpha$ <sup>+</sup>DC, led to significantly higher percentage of IL-10 producing allergen-specific CD4<sup>+</sup> T cells. The results were confirmed by the significantly higher levels of IL-10 in the co-culture with CD8 $\alpha$ <sup>-</sup>DC than CD8 $\alpha$ <sup>+</sup>DC (Figure 15C). In combination with the data on Th1 cytokine analysis in vivo and in the co-culture system (Figures 13&14), the results suggest that CD8 $\alpha$ <sup>+</sup>DC from BCG infected mice may modulate allergic Th2 cells mainly by enhancing immune deviation (change Th1/Th2 balance) while CD8 $\alpha$ <sup>-</sup>DC from BCG infected mice may inhibit Th2 response mainly through induction of IL-10 producing Treg cells and/or direct inhibitory effect of Th2 cells.



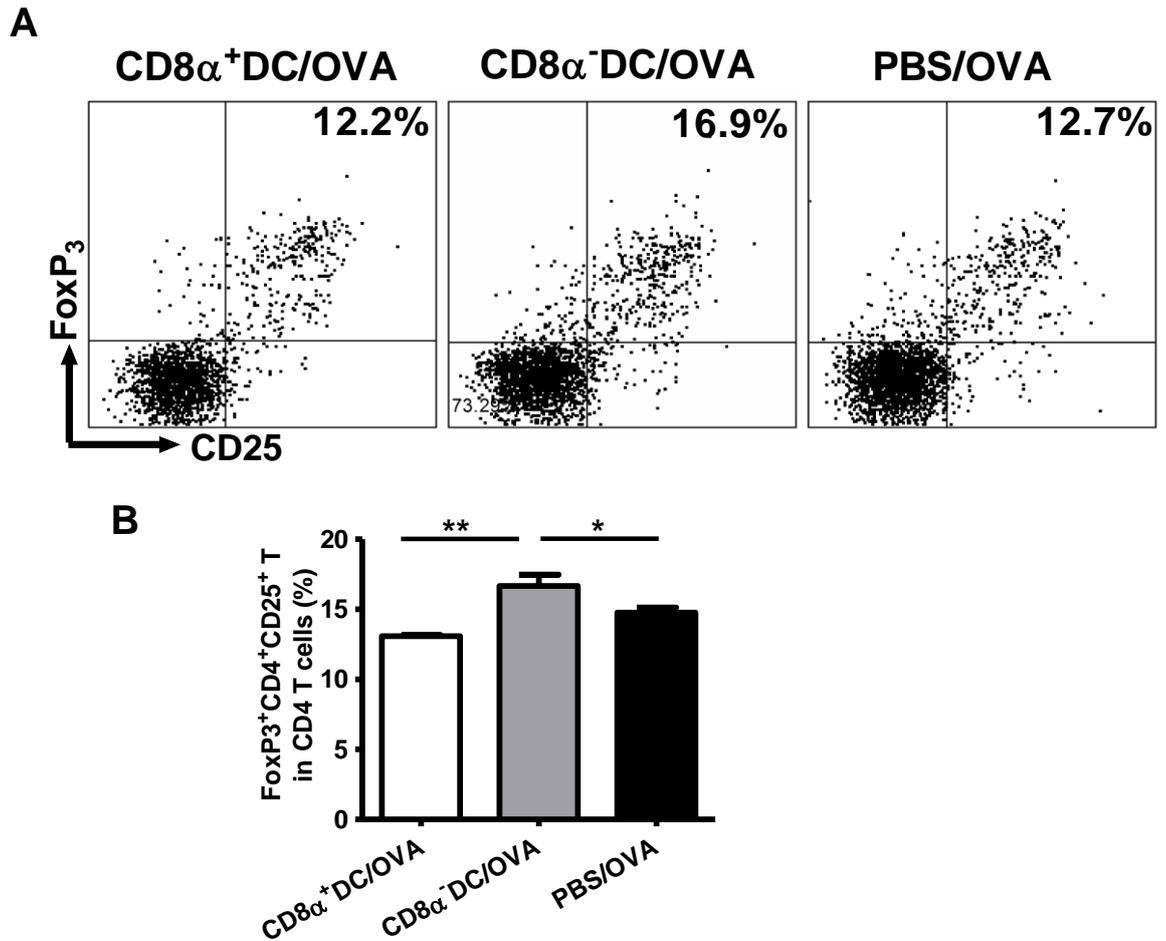
**figure 15**

**Figure 15. CD8 $\alpha^-$  DC are more efficient for promoting IL-10 producing CD4 T cells than CD8 $\alpha^+$  DC.** OVA peptide-specific CD4 $^+$  T cells isolated from naïve DO11.10 transgenic mice were co-cultured with freshly isolated CD11c $^+$ CD8 $\alpha^+$  and CD11c $^+$ CD8 $\alpha^-$  DC subsets from BCG-infected mice (DC:T ratio, 5x10 $^5$ :5x10 $^6$ ) with OVA stimulation (0.1 mg/ml). A, the co-culture was allowed to proceed for 48 h. The cells were collected and boomed with PMA/ionomycin for 8 h and BFA was used at last 4 hrs. The CD4 $^+$  T cells were analyzed for intracellular cytokines by co-staining

with FITC-anti-CD3 $\epsilon$ , PE-anti-CD4 and Allophycocyanin-anti-IL-10. The frequency of IL-10 producing CD4 T cells was measured by flow cytometry. A, representative dot plots for intracellular IL-10 staining are shown. Cells were gated for CD3 $\epsilon$ <sup>+</sup> cells. B, summary of the percentage of IL-10 producing CD4<sup>+</sup> T cells in co-culture with CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup> DC subsets. C, the 72 h co-culture supernatants of each group were tested for IL-10 protein levels by ELISA. The data are shown as mean $\pm$ SD. One representative experiment of two independent experiments with similar results is shown.\* $p$ <0.05, \*\* $p$ <0.01.

**Adoptive transfer of CD8 $\alpha$ <sup>-</sup>DC isolated from BCG-infected mice promoted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cell responses with higher surface expression of TGF $\beta$  and IL-10 production**

To directly examine the capacity of CD8 $\alpha$ <sup>-</sup>DC in inducing Treg in vivo, we adoptively transferred CD8 $\alpha$ <sup>-</sup>DC and CD8 $\alpha$ <sup>+</sup>DC isolated from BCG infected mice and examined IL-10 production by cells in draining LNs and in the BALFs and the development of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cell following OVA sensitization and challenged as described above. We found that similar to being observed in the co-culture experiments, the adoptive transfer of CD8 $\alpha$ <sup>-</sup>DC, but not CD8 $\alpha$ <sup>+</sup>DC, significantly increased IL-10 production in vivo (date not shown). In fact, the transfer of CD8 $\alpha$ <sup>+</sup>DC inhibited, instead of increased, IL-10 production. More specific analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg showed significantly enhanced CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg in the recipients of CD8 $\alpha$ <sup>-</sup>DC, but not CD8 $\alpha$ <sup>+</sup>DC by measure percentage (Figure 16A&B) of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells.



**figure 16**

**Figure 16. Adoptive transfer of CD8 $\alpha$ <sup>-</sup> DC, but not CD8 $\alpha$ <sup>+</sup> DC, enhanced CD4<sup>+</sup>CD25<sup>+</sup> FoxP<sub>3</sub><sup>+</sup> T cells following OVA sensitization and challenge.** Mice were treated with DC subset transfer and OVA as described in the legend to Figure 11. At day 7 following intranasal OVA challenge, 2x10<sup>6</sup> draining LNs cells were co-stained with FITC-anti-CD3 $\epsilon$ , PE-anti-CD4, PE-Cy7-anti-CD25 and Allophycocyanin-anti-FoxP<sub>3</sub> and analyzed by flow cytometry as described in *Materials and Methods*. A, representative dot plots of FoxP<sub>3</sub><sup>+</sup>CD25<sup>+</sup> cells in total CD4<sup>+</sup> T cells are shown. CD3 $\epsilon$ <sup>+</sup>CD4<sup>+</sup> cells were gated. B, summer of the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP<sub>3</sub><sup>+</sup> cells CD4 T cells in draining LNs. Data are shown as mean $\pm$ SD. One representative experiment of three independent experiments with similar results is shown.\**p*<0.05,

**\*\*** $p < 0.01$ .

Since surface expressed TGF $\beta$  and the production of IL-10 have been found to be related to the function of regulatory T cells (310, 311), we further examined CD4<sup>+</sup> T cells from the recipients of CD8 $\alpha$ <sup>-</sup>DC, the only DC subset inducing CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg, in the expression of TGF $\beta$  and production of IL-10. As shown in Figure 17, the transfer of CD8 $\alpha$ <sup>-</sup>DC significantly enhanced the percentage of surface-TGF $\beta$ -expressing CD4<sup>+</sup> T cells and IL-10 producing T cells in the draining LNs of the mice treated with OVA sensitization and challenge. Due to technique limitations, we were unable to directly examine TGF $\beta$  expression and IL-10 production in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg, but the consistency of these parameters in the separate analyses suggest the possible induction of TGF $\beta$  expressing and IL-10 producing CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg by CD8 $\alpha$ <sup>-</sup>DC from BCG infected mice although other types of TGF $\beta$  expressing and/or IL-10 producing regulatory T cells may also be induced.

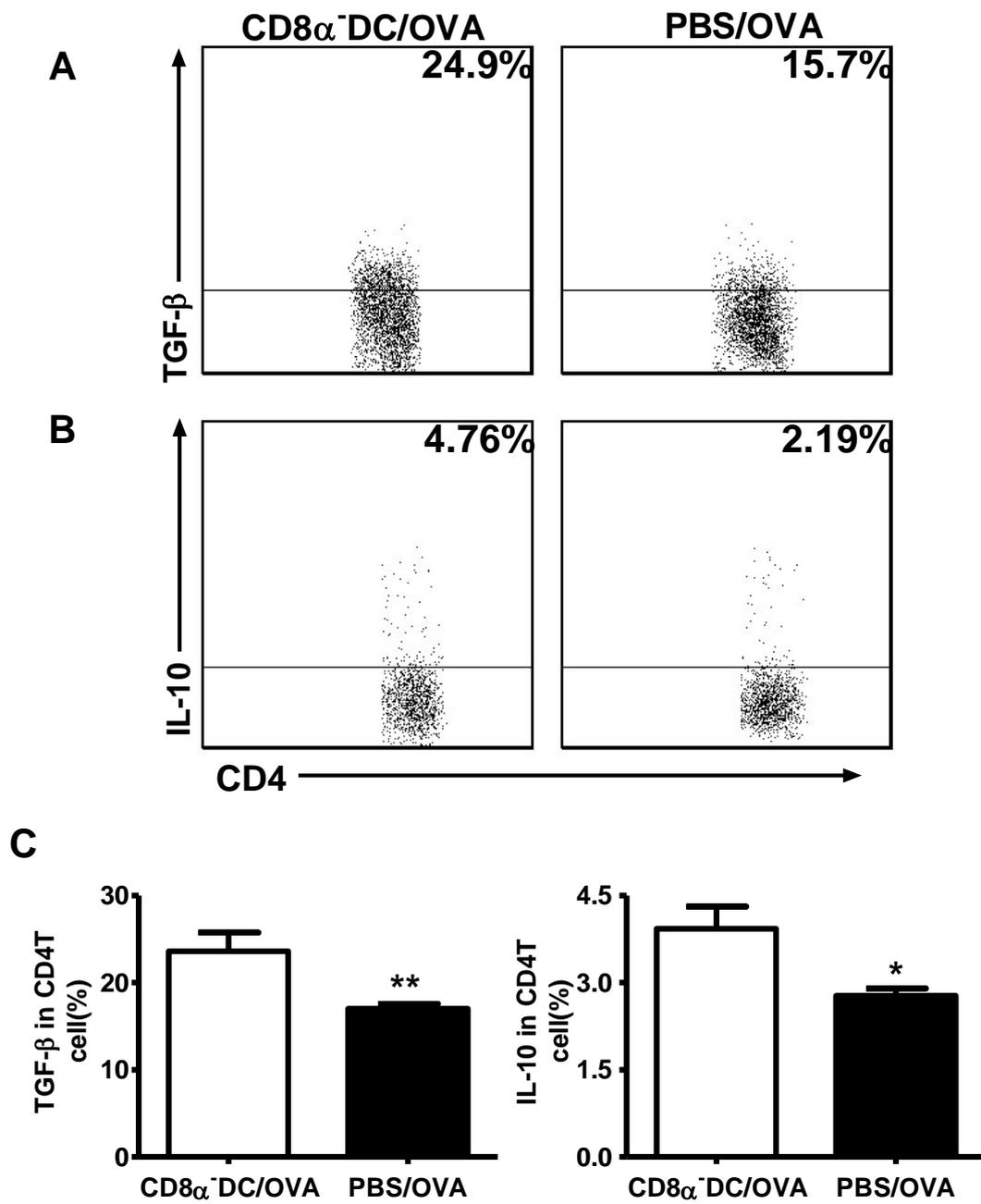


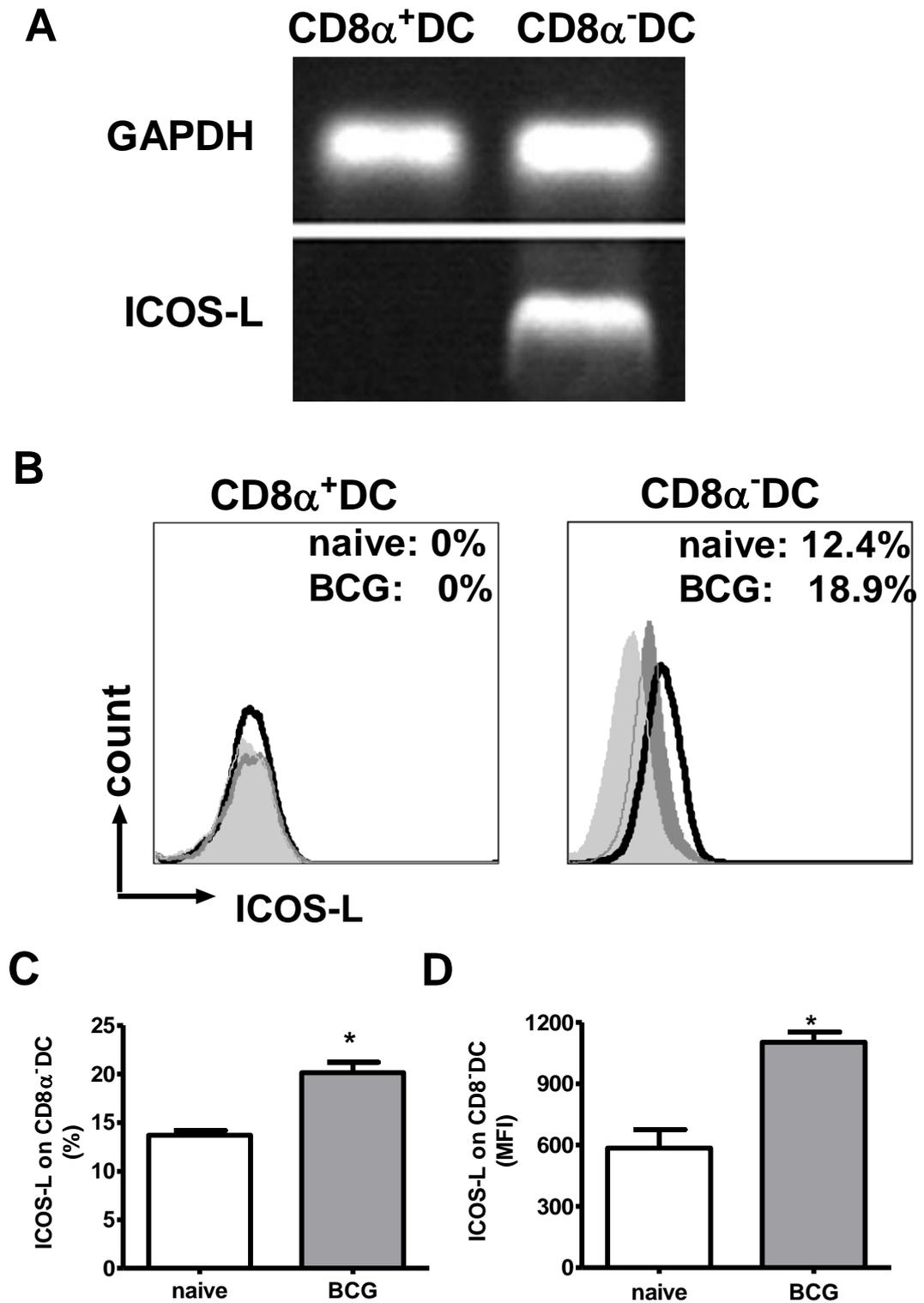
figure 17

Figure 17. Lung CD4<sup>+</sup>CD25<sup>+</sup> T cells in the recipients of CD8 $\alpha$ <sup>-</sup> DC expressed higher membrane-bound TGF $\beta$  and produced higher IL-10 than control mice

**following OVA exposure.** Mice were pre-treated with CD8 $\alpha$ DC from BCG infected mice or PBS followed by OVA sensitized and challenged as described in the legend to figure 11. The lung was collected at day 7 after challenge and digested by collagenase XI. Mononuclear cells were prepared by Percoll. A,  $2 \times 10^6$  cells were stained with FITC-anti CD3 $\epsilon$ , Allophycocyanin -anti CD4, and PE-anti TGF $\beta$  and analyzed by flow cytometry. CD3 $\epsilon^+$  CD4 $^+$  cells were gated. The percentage of member TGF- $\beta$  was shown on the left corner. B, intracellular IL-10 production by lung CD4 $^+$  T cells were done by stimulating lung mononuclear cells with PMA/ ionomycin and permeabilizing the cells with cytopermeabilization buffer as described in the *Materials and Methods*. The cells were gated on CD3 $\epsilon^+$  CD4 $^+$  cells. C&D, summary data on the frequencies of TGF $\beta$  expressing (C) and IL-10 producing (D) CD3 $\epsilon^+$ CD4 $^+$  T cells in the recipients of CD8 $\alpha$ DC and control group. One representative experiment of two independent experiments with similar results is shown.\* $p < 0.05$ , \*\* $p < 0.01$ .

## **CD8 $\alpha$ <sup>-</sup>DC but not CD8 $\alpha$ <sup>+</sup> DC isolated from BCG-infected mice expressed ICOS-L**

The above results strongly suggest a tolerogenic nature of CD8 $\alpha$ <sup>-</sup>DC isolated from BCG infected mice. Since our and others recent study has shown an important role of ICOS-L expression on the tolerogenic function of DC(77, 312), we further examined ICOS-L expression by the CD8 $\alpha$ <sup>-</sup>DC and CD8 $\alpha$ <sup>+</sup>DC isolated from BCG infected mice. RT-PCR analysis of the DC subsets freshly isolated from BCG infected mice showed a clear ICOS-L message expression in the CD8 $\alpha$ <sup>-</sup>DC but not CD8 $\alpha$ <sup>+</sup>DC (Figure 18A). Moreover, flow cytometric analysis of these DC subsets showed significant surface expression of ICOS-L in the CD8 $\alpha$ <sup>-</sup>DC but not CD8 $\alpha$ <sup>+</sup>DC (Figure 18B). In addition, comparison of ICOS-L expression by CD8 $\alpha$ <sup>-</sup>DC from naïve and BCG infected mice showed a promoting effect of BCG infection on the percentage of ICOS-L positive CD8 $\alpha$ <sup>-</sup>DC (Figure 18C) and the density of ICOS-L expression on these DC (Figure 18D). The data showed significant expression of ICOS-L on CD8 $\alpha$ <sup>-</sup>DC which was enhanced by BCG infection, which was consistent with the above observed Treg promoting effect of this DC subset, thus likely a molecular basis for its tolerogenic function in addition to its higher IL-10 production.



**figure 18**

**Figure 18. BCG infection enhanced ICOS-L expression on CD8 $\alpha$ <sup>-</sup>DC but not**

**CD8 $\alpha$ <sup>+</sup>DC.** Mice were infected with BCG intravenously. At 21days after infection, mice were sacrificed and spleen DC were analyzed for ICOS-L expression by RT-PCR and flow cytometry. A, CD8 $\alpha$ <sup>-</sup>DC but not CD8 $\alpha$ <sup>+</sup>DC subsets were sorted by flow cytometry and semi-quantitative RT-PCR was performed to detect the mRNA levels of ICOS-L on it. B. MACS column isolated total DC (CD11c<sup>+</sup>) were triple-stained with FITC-anti-CD11c, Allophycocyanin-anti-CD8 $\alpha$  and PE-anti-ICOS-L mAbs. Cells were gated on double positive (CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup>) and single positive (CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup>DC) cells, respectively. Representative flow histograms are showed. Light shaded areas, isotype control; Dark shaded areas, naïve DC; solid lines, DC subsets isolated from BCG infected mice. C, summary of the percentages of ICOS-L positive CD8 $\alpha$ <sup>-</sup> DC on CD8 $\alpha$ <sup>-</sup> DC in naïve and BCG infected mice. D, the density (MFI) of ICOS-L on CD8 $\alpha$ <sup>-</sup> DC in naïve and BCG infected mice. Data are shown as mean $\pm$ SD. One representative experiments of two independent experiments with similar results is shown.\* $p$ <0.05.

## Discussion

In this chapter, we have demonstrated that both CD8 $\alpha^+$  and CD8 $\alpha^-$  DC isolated from BCG-infected mice can inhibit the development of allergic airway inflammation in BALB/c mice. More importantly we found the two DC subsets can inhibit allergic reactions through different mechanisms. Specifically, CD8 $\alpha^+$  DC inhibited OVA-induced airway eosinophilic inflammation mainly through switching Th2 dominant allergen-driving CD4 $^+$  T cell response to Th1 dominant response while CD8 $\alpha^-$  DC inhibited the allergic reactions mainly via increasing IL-10 production and generating regulatory T cells. Indeed, we found that more CD4 $^+$  T cells isolated from naïve OVA TCR transgenic mice (DO11.10) co-cultured with CD8 $\alpha^+$  DC became Th1 (IFN $\gamma$  producing) cells (Figure 14) while the same CD4 $^+$  T cells tended to developed Treg (IL-10 producing) cells when co-cultured with CD8 $\alpha^-$  DC (Figure 15). Moreover, adoptive transfer of either DC subset significantly inhibited the allergic airway eosinophilic inflammation and mucus over-production, serum OVA-specific IgE production and Th2 cytokine (IL-4 and IL-5) responses induced by OVA sensitization and challenge. Notably, the adoptive transfer of CD8 $\alpha^+$  DC led to significant increase of IFN $\gamma$  in the local tissues (lung) and its production by cells from draining LNs and the spleen following OVA-specific re-stimulation. In contrast, the adoptive transfer of CD8 $\alpha^-$  DC significantly enhanced the level of IL-10 in the local tissues and, more interestingly, CD4 $^+$ CD25 $^+$ Foxp3 $^+$  Treg responses. The strong capacity of the CD8 $\alpha^-$  DC in inducing regulatory T cells and tolerance was confirmed by the enhanced induction of IL-10 producing and membrane TGF $\beta$ -expressing CD4 $^+$  T cells (Figure

17). These results generated from both *in vitro* and *in vivo* studies confirmed our previous reports on the important role of DC in infection-mediated inhibition of allergic responses and further demonstrated the involvement of variable mechanisms used by different DC subsets in the inhibition of allergy.

The finding on the involvement of different mechanisms related to DC subsets has implication on understanding the mechanism of hygiene hypothesis. Notable debates are currently ongoing on the mechanisms of hygiene hypothesis especially for the modulating effect on allergen-driven Th2 responses by infections. The major controversy is whether the modulating effect is mediated by immune deviation (Th2 switching to Th1) or by immune regulation (tolerance and Treg development)(297, 298). Numerous reports have shown the involvement of either mechanism in various infections. In the present study, we showed that both mechanisms can operate in a single type of infection and this coordination can be done by DC subsets. Indeed, the CD8 $\alpha$ <sup>+</sup> DC mainly enhance allergen-driven Th1 response, thus modulating the Th2 response through immune deviation while the CD8 $\alpha$ <sup>-</sup> DC mainly induced Treg, thus reducing Th2 via immune regulation and tolerance. Therefore, both mechanisms are valid and are important for the inhibition of allergy by infections. On the other hand, it was found in this BCG infection model, that CD8 $\alpha$ <sup>+</sup> DC were more powerful than CD8 $\alpha$ <sup>-</sup> DC in inhibiting allergic Th2 responses and airway inflammation. Therefore, although BCG infection can inhibit allergy through both mechanisms, immune deviation is likely a more dominant mechanism than immune regulation in this intracellular bacterial infection. This is consistent with numerous previous reports

showing significantly enhanced allergen driven Th1 response in BCG infected/vaccinated mice and humans(65, 75, 80, 90-92). This is also consistent with the nature of mycobacterial infections which are found to mainly induce Th1 type responses(75, 92, 140). Notably, however, *Mycobacterium vaccae* infection has reported to induce Treg cells which are inhibitory for allergic responses(93). Our data demonstrated that even for an infection which mainly induces Th1 response, it is still able to modulate immune response through multiple mechanisms, for which different DC subsets likely play a critical role for modulating the respective mechanisms.

Our data identified several characteristics of CD8 $\alpha$ <sup>-</sup> DC which might be related to their tolerogenic function. First, the CD8 $\alpha$ <sup>-</sup> DC produce higher levels of IL-10 than CD8 $\alpha$ <sup>+</sup>DC. This was demonstrated by quantitative RT-PCR of freshly isolated DC and ex vivo culture of these cells. The importance of IL-10 production for the function of tolerogenic DC has been found in many studies (115, 313). Second, CD8 $\alpha$ <sup>-</sup>DC expressed ICOS-L on their surface, which was significantly increased following BCG infection. It has been reported that expression of ICOS-L on DC is important for the maintenance of immune homeostasis. ICOS/ICOS-L signaling is essential for IL-10 producing tolerogenic DC to induce T cell anergy(314). Without ICOS-L co-stimulation by DC, IL-10 failed to influence the differentiation and cytokine production by CD4<sup>+</sup> T cells(314). Moreover, several studies have shown the importance of ICOS/ICOS-L signaling in regulatory T cell responses(315-317). The co-expression/production of ICOS-L and higher levels of IL-10 by the CD8 $\alpha$ <sup>-</sup>DC from BCG infected mice shown in the present study provided a molecular basis for the

synergistic effect in inducing allergic Th2 cell anergy and Treg. On this respect, the CD8 $\alpha$ <sup>+</sup>DC showed a quite different pattern in phenotype, displaying higher IL-12 production and lower IL-10 production. The contribution of the differentially produced cytokines by the different DC subsets in inducing Th1 response and suppressing allergic Th2 response was confirmed in the co-culture experiments with neutralization of IL-10 and IL-12 activity, respectively (Figure 14). Moreover, it was found that CD8 $\alpha$ <sup>+</sup>DC from both naïve and BCG infected mice did not express ICOS-L, shown by RT-PCR and flow cytometric analyses, demonstrating a selective influence of the infection on ICOS-L expression on CD8 $\alpha$ <sup>-</sup>DC. This sharp contrast in cytokine production and ICOS-L expression provided a basis for the difference of these DC subsets in inducing different type of T cells, particularly Treg and Th1 cells. Another interesting finding on surface molecules is the difference of the DC subsets in surface CD86 expression. Unlike CD80 and CD40 molecules which were significantly enhanced in levels in both CD8 $\alpha$ <sup>-</sup> and CD8 $\alpha$ <sup>+</sup> DC in BCG infected mice, the expression of CD86 was only increased in CD8 $\alpha$ <sup>-</sup>DC (Figure 10). The preferential increase of CD86 by CD8 $\alpha$ <sup>-</sup>DC might also contribute the suppressive function of these DC on allergic Th2 responses. Notably, it has been reported that CD86 expression controls the suppressive function of DC in mycobacterial infection(318). Moreover, although having been found to be important for the induction of Th1 responses in numerous studies, CD40/CD40L signaling is also critical for inducing IL-10 production by tolerogenic DC(319). Therefore, some co-stimulatory molecules may be preferentially important for a subset of T cell response, such as ICOS/ICOS-L

signaling particularly for Treg, while others for multiple T cell subsets, such as CD80 and CD40 signaling for both Th1 and Treg, depending on the expression/production of other molecules by a particular DC. Further study on the relevance of individual molecules and, more importantly, the combination of these molecules in the induction of different T cell subsets, particularly the induction of regulatory T cells, would be very helpful for understanding the mechanisms by which different DC subsets from infected mice inhibit allergic Th2 reactions.

In summary, our data have demonstrated the co-existence of immune deviation and regulatory mechanisms for the modulating effect on Th2 allergic reactions by an intracellular bacterial infection. Moreover, we have shown the role of different DC subsets in infection-mediated inhibition of allergy in determining the initiation of the different inhibition mechanisms. This study has provided new insight into the mechanism of hygiene hypothesis. Further study on the relative contribution and interaction of the different mechanisms in modulation of allergic diseases mediated by different types of infections and the cellular and molecular basis for the induction and maintenance of the different mechanisms *in vivo* will be helpful for better understanding immune regulation and for developing new preventive and therapeutic strategies for allergic and autoimmune diseases.

## Part IV Chapter 3

### **IL-10 dependent inhibition of allergic responses by CD8 $\alpha$ <sup>+</sup> DC from Chlamydia muridarum-infected mice through induction of Treg**

#### Introduction

Allergic asthma has increased substantially over the past few decades in the developed countries, which is associated with increased morbidity and mortality rate(320). Th2 response plays a major role in their pathologic process(11, 321) and induce the recruitment and proliferation of eosinophils to local tissue(322). Eosinophils can promote airway inflammation through release of several inflammatory mediators(323). The response can also promote mucus overproduction and airway remodeling and airway hyperresponsiveness (AHR).

Almost two decades of studies in this area have suggest that microbial infections may modulate Th2-like allergic responses by promoting immune deviation (toward Th1), and thus shifting of Th2 to Th1 phenotype (325, 326), and/or enhancing regulatory mechanisms, such as generating regulatory T cells to inhibit Th2 responses and allergic reactions(298, 327). Regulatory T cells are a highly heterogenous family, which includes, but not limited to, type 3 Th (Th3) cells, T regulatory 1 (Tr1) cells, and CD4<sup>+</sup>CD25<sup>+</sup>T cells, so the molecular difference have been documented among these different types of Treg but not exclusively. TGF- $\beta$  dependent mechanism appears more contributing to regulatory function of Th3 cells (328), whereas Tr1 cells mainly produce IL-10 (329). In contrast, the regulatory function of CD4<sup>+</sup>CD25<sup>+</sup> T cells appears more related to the activity of both membrane cytotoxic T lymphocyte associated antigen-4 (CTLA)-4, membrane TGF- $\beta$  (330) and transcription factor

Foxp3 that is essential for CD4<sup>+</sup>CD25<sup>+</sup> Treg cells development and function(331). Mutation of Foxp3 results in the extensive inflammation or autoimmune disease(245). The involvement of Treg in bacterial infection-mediated modulation of allergy/asthma is much less studied but indeed reported(93, 115, 332).

Our previous data have shown that *Chlamydia trachomatis*, an obligate intracellular bacterial pathogen, can inhibit allergic response induced by ragweed and ovalbumin (OVA), and the inhibition was mediated by the modification of DC. CD8<sup>+</sup>DCs are more potent in inducing protection against allergic airway inflammation than CD8<sup>-</sup>DCs. One of the features of CD8<sup>+</sup>DCs isolated from *C.m* infected mice is higher production of IL-10. Our studies have shown that DCs educated by *C.m* infection can significantly inhibit established allergic airway inflammations, which was associated with IL-10 production by DCs. However, how IL-10 produced by DC inhibit allergic responses and its relationship with DC subsets remains largely unknown. We hypothesized that the higher IL-10 producing CD8<sup>+</sup>DCs from *C.m* infected mice may inhibit allergic reactions through inducing Treg cells.

In this chapter, we tested this hypothesis by comparing the inhibitory capacity of CD8<sup>+</sup> and CD8<sup>-</sup>DCs, the dependent of the inhibition on IL-10 produced by DCs and the dependent of IL-10 produced by DCs on the generation of Treg following allergen exposure. Our results confirmed that CD8<sup>+</sup>DC are more potent in inducing protection against ragweed induced allergic inflammation than CD8<sup>-</sup>DC. We further found the inhibition of CD8<sup>+</sup>DC on allergic reactions was dependent on IL-10 production while CD8<sup>-</sup>DC does not. Further, we found that CD8<sup>+</sup> DC from WT, but not IL-10 KO mice,

was capable of inducing more FoxP3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, which expressed surface TGF-β<sup>+</sup> and CTLA-4<sup>+</sup>.

## Results

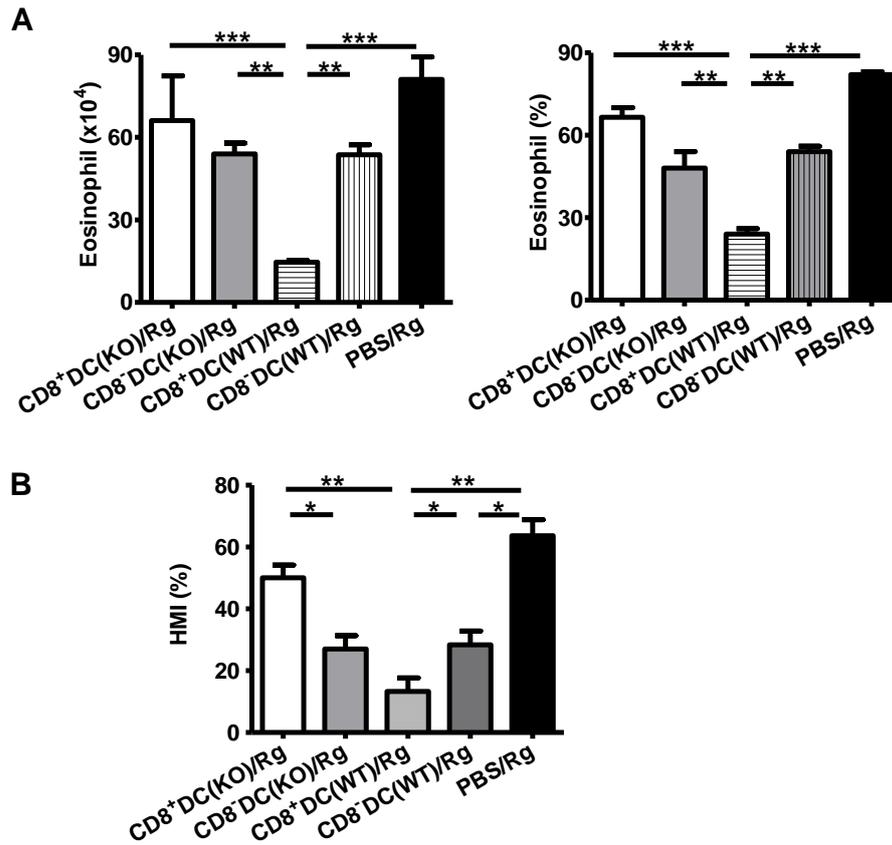
### 1. CD8<sup>+</sup>DC loss its ability to inhibit ragweed induced allergic responses in the absence of IL-10.

Our previous work have found that both CD8<sup>+</sup> and CD8<sup>-</sup>DC subsets from *C.m* infected mice are able to inhibit ragweed, a nature allergen, initiated allergic responses, whereas CD8<sup>+</sup>DC are more potent in this inhibition(Laure, et al. in review). We also found that CD8<sup>+</sup>DCs from *C.m* infected mice produced dramatically higher IL-10 than CD8<sup>-</sup>DCs(191). In order to directly test the role of IL-10 in DC subsets mediated protection, we sorted the CD8<sup>+</sup> and CD8<sup>-</sup>DC subsets from both IL-10 KO and WT mice whose were previously infected by the *C.m*. Then, the syngeneic mice were pretreated, by adoptive transfer, with one of the DC subsets isolated from IL-10KO mice or WT mice followed by immediate ragweed sensitization. Fourteen days after sensitization, all groups were challenged with ragweed i.n.. The control mice received ragweed sensitization and challenge only. The different groups of mice were sacrificed 7 days afer allergic challenge. We found that both the proportion and the absolute amounts of eosinophils (Fig 19A) in the BAL fluids were dramatically reduced in the CD8<sup>+</sup>DC(WT) pretreated mice. The one received CD8<sup>-</sup>DC(WT) also reduced eosinophils although in a lesser degree. In sharp contrast, the eosinophil inflammation of the mice received CD8<sup>+</sup> or CD8<sup>-</sup>DC(KO) subsets was comparable in

levels to control mice received Rg treatment only.

In addition, the levels of airway mucus production demonstrated by PAS staining in the mice which received CD8<sup>+</sup>DC(KO) was significantly higher compared to those with CD8<sup>+</sup>DC(WT) transfer prior to ragweed exposure(Fig 19B and Fig20). We qualified the eosinophils in the BAL since eosinophils infiltration into the lung is one of the hallmarks of the asthma-like reactions. We found more eosinophil in CD8<sup>+</sup>DC(KO) treated mice compared to CD8<sup>+</sup>DC(WT) treatment. Lungs were collected and lung sections were prepared for pathological evaluation. As showed in Fig20, CD8<sup>+</sup>DC(WT) pretreated mice had less cellular infiltration, especially eosinophil, in the lung compared to CD8<sup>+</sup>DC(WT) and PBS control groups. However, we found that mice treated with CD8<sup>+</sup>DC (KO) which were deficient in IL-10 production exhibited heavy eosinophilia in the airway. Our result demonstrated that CD8<sup>+</sup>DC loss its suppressive effect on the eosinophil inflammation in the allergic mice in the absence of IL-10.

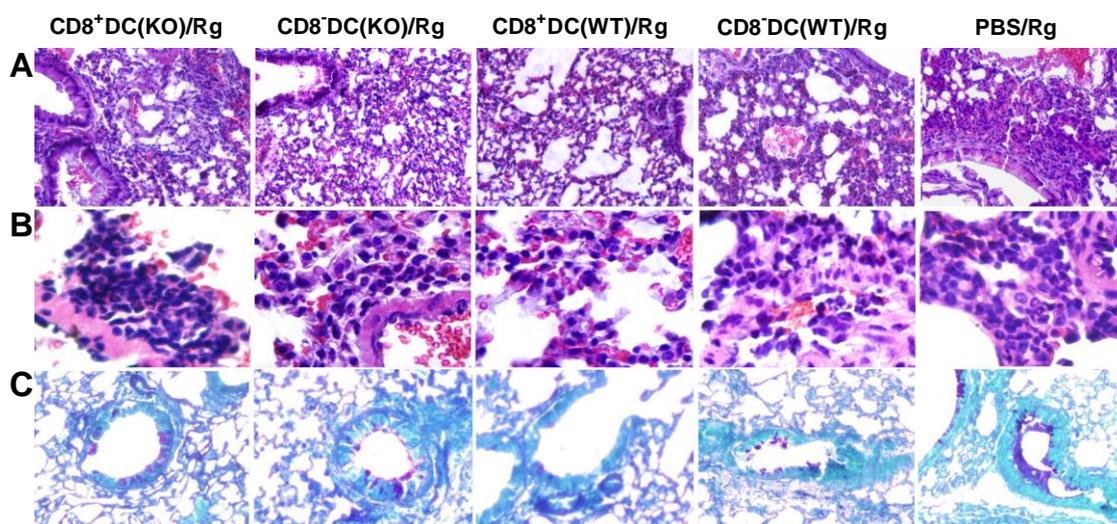
Taken together, the data demonstrated that the development of IL-10 production by DC subsets from infected mice is critical in the modulation of allergic reactions.



**Fig 19**

**Figure 19, IL-10 dependent-reduced airway eosinophilic infiltration and mucus production in iCD8<sup>+</sup> DC pretreated recipient mice.** DC subsets isolated from the spleens of *C.m.* infected WT or IL-10 KO mice (day 7 p.i.) were adoptively transferred (i.v.) to naive syngeneic WT mice followed by sensitization /challenge with RW. Mice that received PBS served as controls. The mice were killed at day 7 post challenge. The lungs were washed with 2 ml PBS and BALs were collected as described in *Material and Methods*. The cells in bronchoalveolar lavage (BAL) was stained using a Fisher Leukostat Stain kit. The absolute number of total and each type of infiltrating cells in the BAL were calculated. The amount and the proportion (A) of eosinophil in each group were selected to show. B, Mucus producing epithelium was quantified as histological mucus index (HMI). One representative experiment of three

independent experiments is shown. Values are shown as the mean value  $\pm$ SD of each group. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .



**Fig 20**

**Figure 20 Adoptive transfer of CD8<sup>+</sup>DC from WT, But not those from IL-10KO mice, inhibit allergic inflammation in vivo.** C<sub>57</sub> BL/6 mice and IL-10 KO mice were inoculated intranasally (i.n.) with *C. muridarum* (*Cm*) for seven days. Spleen DC subsets were isolated using a MACS CD8 positive column (Miltenyi Biotech, Auburn, CA) as mentioned in *Material and Methods*. Purified DC subsets isolated from *Cm* infected IL-10 KO or WT donors were adoptive transferred intravenously (i.v.) to naïve, syngeneic recipient mice. 2 hours after DC subset adoptive transfer, recipients were sensitized with 100  $\mu$ g RW and alum adjuvant i.p., followed by a challenge with 150  $\mu$ g RW intranasally in 14 days. Mice were sacrificed on day 7 following ragweed challenge. Lung pathological changes and mucus production were analyzed. The lung sections were stained by haematoxylin and eosin for inflammation (A, 40x; B, 100x)

and by PAS staining for mucus production (C). One representative experiment of three independent experiments is shown. Data are expressed as mean  $\pm$  SD. One representative of the two independent experiments is shown.

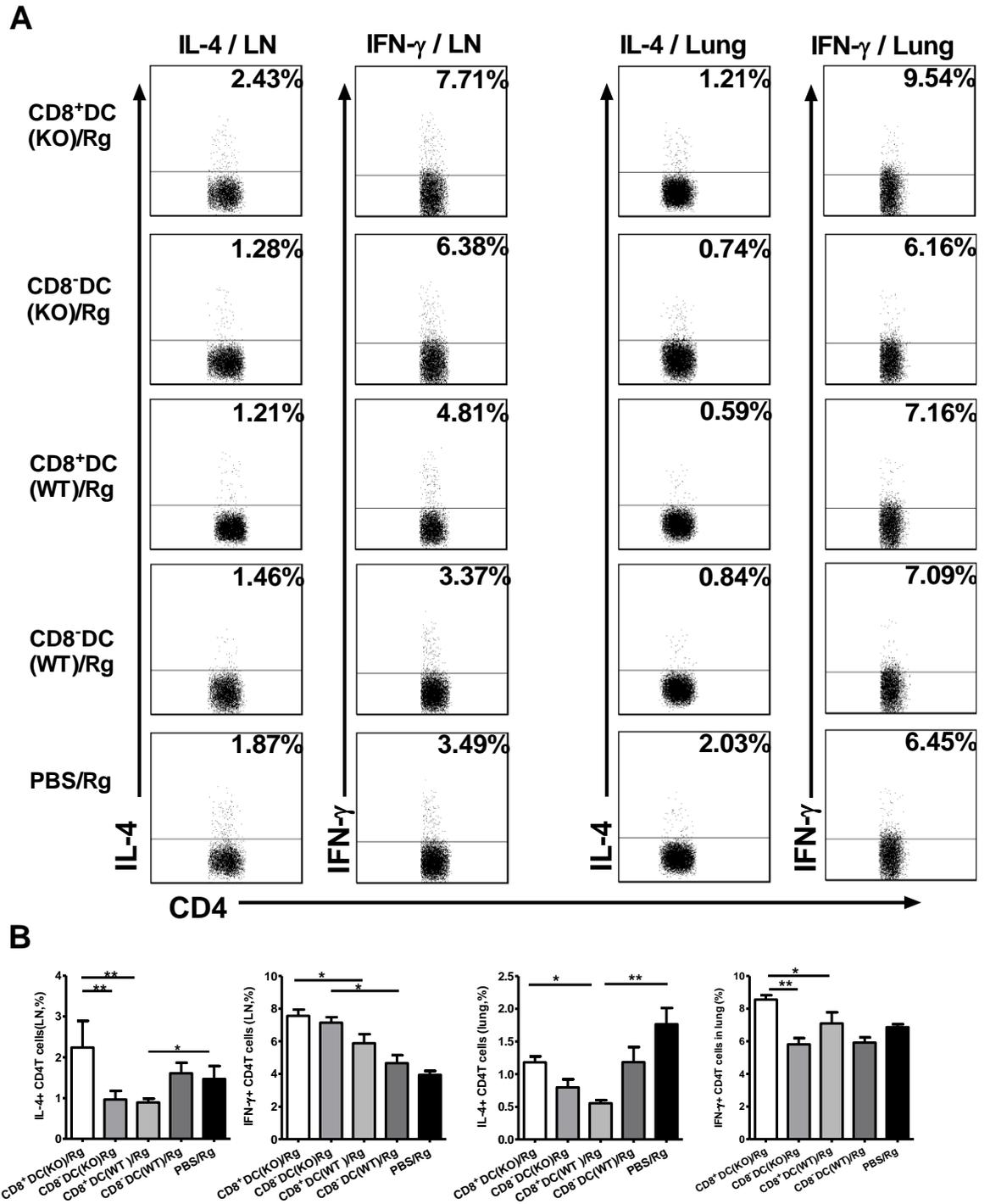
## **2, The adoptive transfer of DC subsets from *C.m* infected IL-10 KO mice failed to inhibit IL-4 but increase IFN- $\gamma$ production by CD4<sup>+</sup> T cells.**

To further explore the effect of the transfer of different DC subsets on Th2 responses, we used intracellular cytokines staining to measure the Th1 (IFN- $\gamma$ ) and Th2 (IL-4) polarization in the draining LN (dLN) and the lung. As shown in the Fig 21, ragweed exposure in control mice induced higher IL-4 producing CD4 T cells in both dLN and lung, which was dramatically inhibited by the transfer of CD8<sup>+</sup>DC(WT). However, the transfer of CD8<sup>+</sup>DC(KO) recipient mice failed to do so. The data suggested that IL-10 is critical for the CD8<sup>+</sup>DC(WT) to inhibit the development of allergic Th2 responses. Interestingly, we found that Th1 response had no significantly changes in recipients of both CD8<sup>+</sup> and CD8<sup>-</sup>DC(WT). The transfer of the DC subset from IL-10 KO mice even increased the percentage of IFN- $\gamma$  producing cells in dLN(Fig 21).

We further tested the effect of adoptive transfer of DC subsets isolated from IL-10 KO infected mice versus WT mice on the allergen-driven IL-4 and IFN- $\gamma$  production in population levels in dLNs, the lung and BAL fluid. The results showed that the recipients of CD8<sup>+</sup>DC(WT) produced significantly lower levels of IL-4 in comparison to those who were recipients of CD8<sup>-</sup>DC(WT) and control groups (Figure

22). In contrast, CD8<sup>+</sup>DC (KO) fail to downregulate the IL-4 production. The data were consistent with what we found in intracellular staining (Fig 21).

Since eotaxin has been documented to be critical in eosinophilic chemoattraction in allergic inflammations, we also directly compared the production of this chemokine among these groups. As showed in Fig23, the transfer of CD8<sup>+</sup>DC(WT) dramatically reduced the eotaxin production compared to control mice, while CD8<sup>-</sup>DC showed significantly less extent of effect. More importantly, both DC subsets of IL-10 KO mice failed in downregulation of eotaxin levels.



**Fig 21**

Figure 21 Th cell polarizations in recipient mice pretreated with DC subsets isolated from IL-10 KO and WT mice. Draining lymph nodes (dLN) and lung were collected at day 7 after ragweed challenge. Single cells were prepared and analyzed

for cytokine production by intracellular cytokine staining, as described in *Materials and Methods*. Cells were gated on CD3<sup>+</sup> CD4<sup>+</sup> T cells. A, Representative flow cytometry images with the percentages of the cytokine-producing CD4<sup>+</sup> T cells in draining LN and lung mononuclear cells are shown. B. Graphical summaries represent percentage of IL-4 produced CD4 T cells and IFN- $\gamma$  produced CD4 T cells, respectively, in dLN and lungs of each group. Two independent experiments with four mice in each group were performed, and one representative experiment is shown. The results were expressed as mean $\pm$ SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

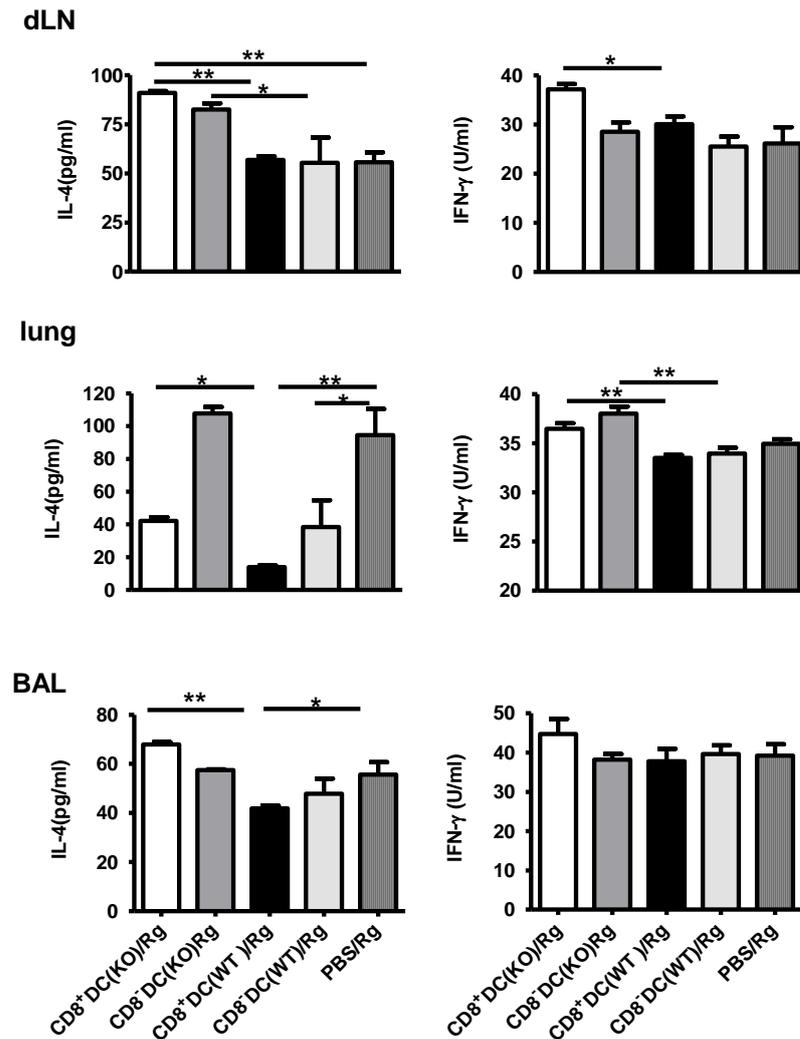
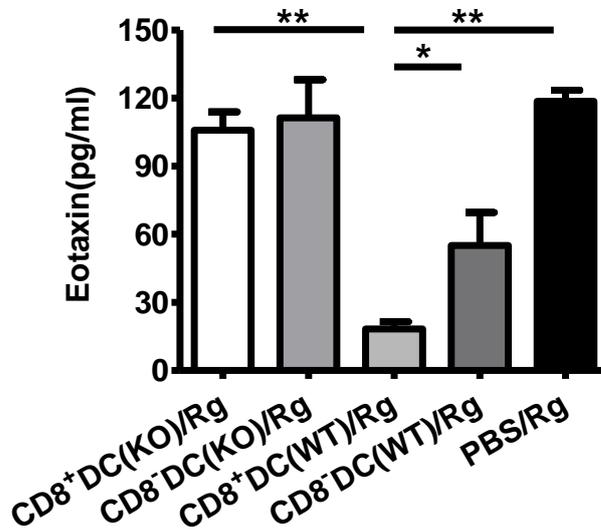


Fig 22

Figure 22. mice pretreated with CD8<sup>+</sup>DC from IL-10 deficient mice showed dominant Th2 cytokine production compared to recipients prior exposed to CD8<sup>+</sup>DC from WT mice. Mice were pretreated with DC subsets as described in the legend to Fig.19. At 7 days after i.n. ragweed challenge, mice were sacrificed and the draining LN cells and lung cells from individual mice were put into culture with ragweed-specific stimulation for 72h. The concentrations of IL-4, IL-5 and IFN-γ in culture supernatants were measured using ELISA. BALs were collected as described in *Material and Methods* and cytokine concentrations in BAL were also measure by

ELISA. One representative experiment of three independent experiments is shown. \*

$p < 0.05$ ; \*\*  $p < 0.01$ .



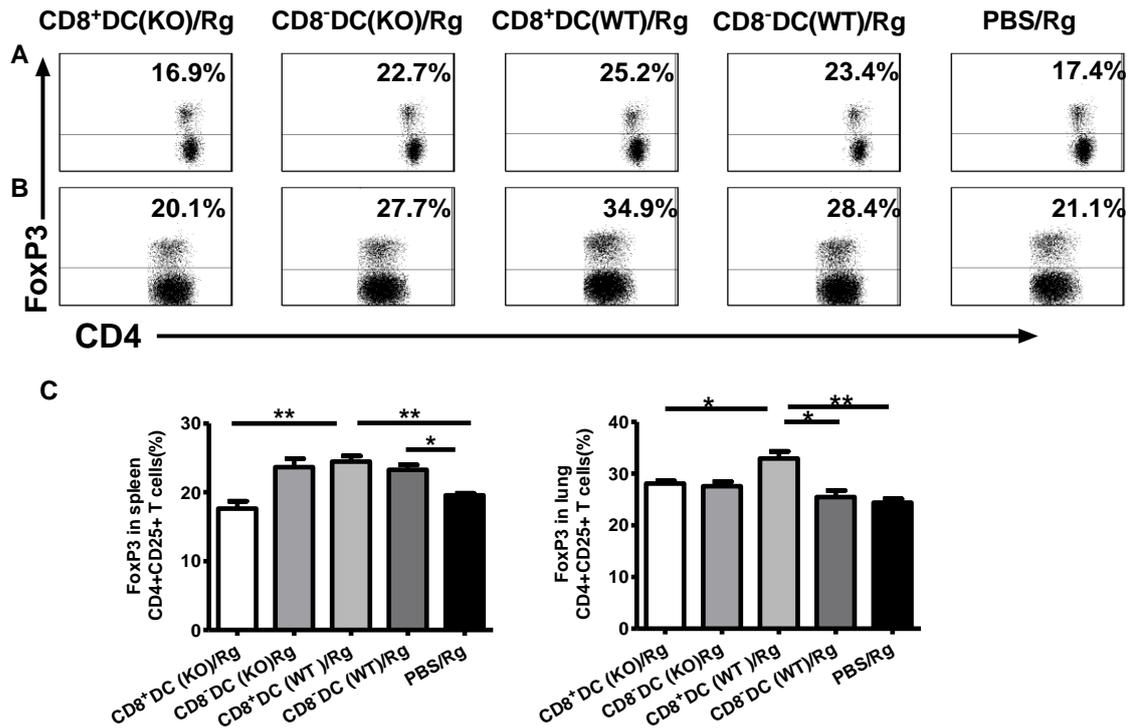
**Fig 23**

**Figure 23, IL-10 dependent-reduced eotaxin production in iCD8<sup>+</sup> DCs pretreated recipient mice.** DC subsets isolated from the spleens of *C.m.* infected WT or IL-10 KO mice (day 7 p.i.) were adoptively transferred (i.v.) to naive syngeneic WT mice followed by sensitization /challenge with RW. Mice that received PBS (PBS) served as controls. The mice were killed at day 7 post challenge. The lungs were washed with 2 ml PBS and eotaxin concentration in BAL was detected by ELISA. One representative experiment of three independent experiments is shown. Values are shown as the mean value  $\pm$ SD of each group. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

### **3, IL-10 production by CD8<sup>+</sup>DCs is critical for the generation CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg.**

To further exam the mechanism by which IL-10 produced by DC subsets from *C.m* infected mice in inhibiting allergic reactions and Th2 cytokines responses, we examined the development of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg in the different group of mice.

We multi-stained the spleen and lung cells with fluorochrome conjugated anti CD3, CD4, CD25 and intracellular Foxp3 antibodies. As showed in Fig 24, the levels of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in the recipients of CD8<sup>+</sup>DC(WT) were significantly higher compared to control group. However, this Treg enhancing effect lost in CD8<sup>+</sup>DC(KO) treated mice(Fig 24). The result suggested that DC subsets from infected mice, especially CD8<sup>+</sup>DCs can induce Treg following allergen exposure. Moreover, the Treg inducing capacity of the DC subsets is largely dependent on IL-10 production.



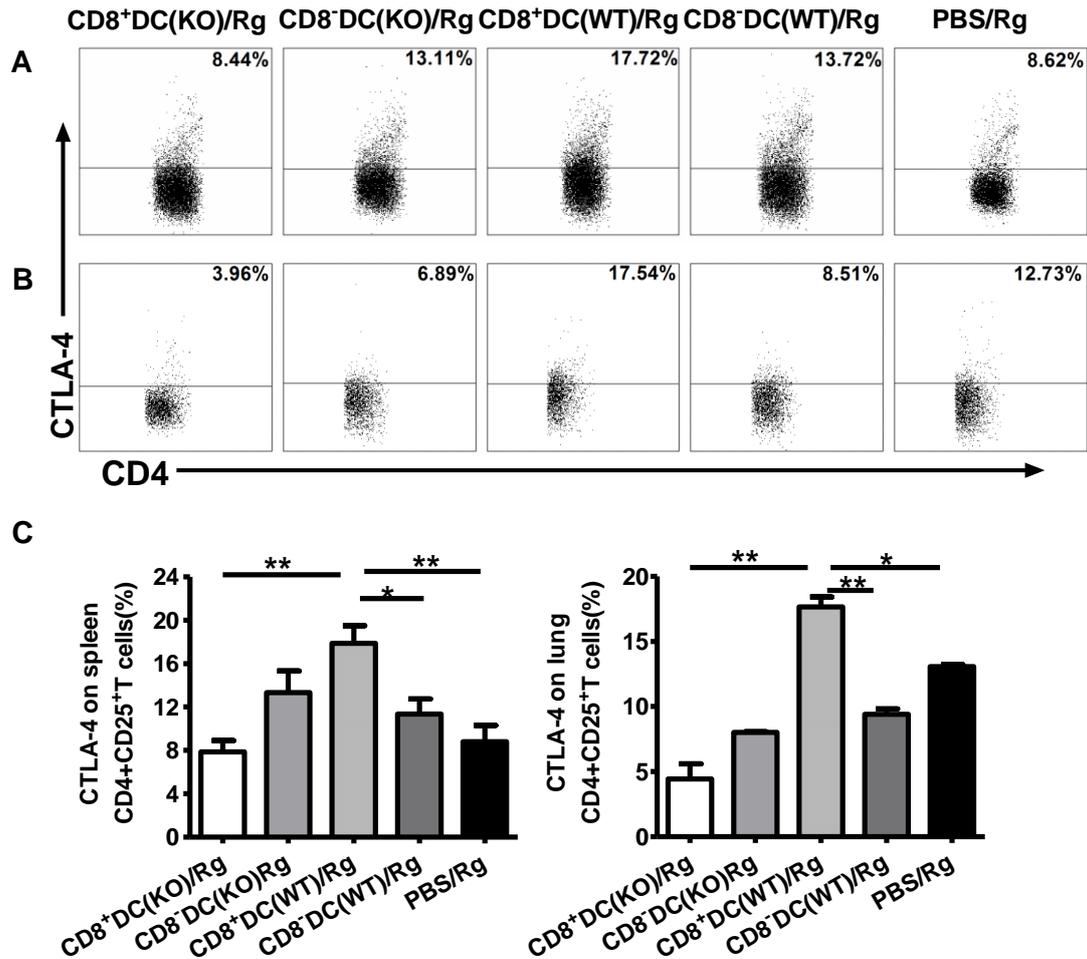
**Fig 24**

**Figure 24. Foxp3 expression is dependent on IL-10 in recipient of iCD8<sup>+</sup>DC(WT)**

Single cells from spleens or lungs were stained with FITC-anti CD3, APC-anti CD4, PE-Cy7-anti CD25 and intracellular PE-anti Foxp3 as described in *Material and Methods*. A, the percentage of Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup>T cells in spleen T cell(A) or lung cells (B) were measured using flow cytometry. C, summary of the percentage of Foxp3<sup>+</sup> cells in CD25<sup>+</sup>CD4<sup>+</sup> T cells in spleen (left) and lung MNC (right). Data are presented as mean  $\pm$  SD of each group. One representative experiment of two independent experiments is shown. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.

#### **4. The relationship of IL-10 with CTLA-4 positive CD4 T cells**

CTLA-4 expression on Treg is important for its function, particularly it constitutively expresses on the naturally occurred regulatory T cells and is essential for the Treg to maintain immune homeostasis(334). One study reported that crosslinking of CTLA-4 enhances TGF- $\beta$  production by CD4 T cells (273). We therefore further investigate the effect of adoptive transfer of DC subsets on CTLA-4 expression on CD4<sup>+</sup>CD25<sup>+</sup> T cells and the involvement of IL-10 production by the DC subsets in the CTLA-4 expression. Not surprisingly, CTLA-4 expressing Treg (CD4<sup>+</sup>CD25<sup>+</sup> T cells) have expanded in CD8<sup>+</sup>DC(WT) recipient mice, which depended on IL-10. CD8<sup>+</sup>DC(KO) fails to promote the development of CTLA-4<sup>+</sup> Treg in both spleen and lung of recipient mice(Fig 25).



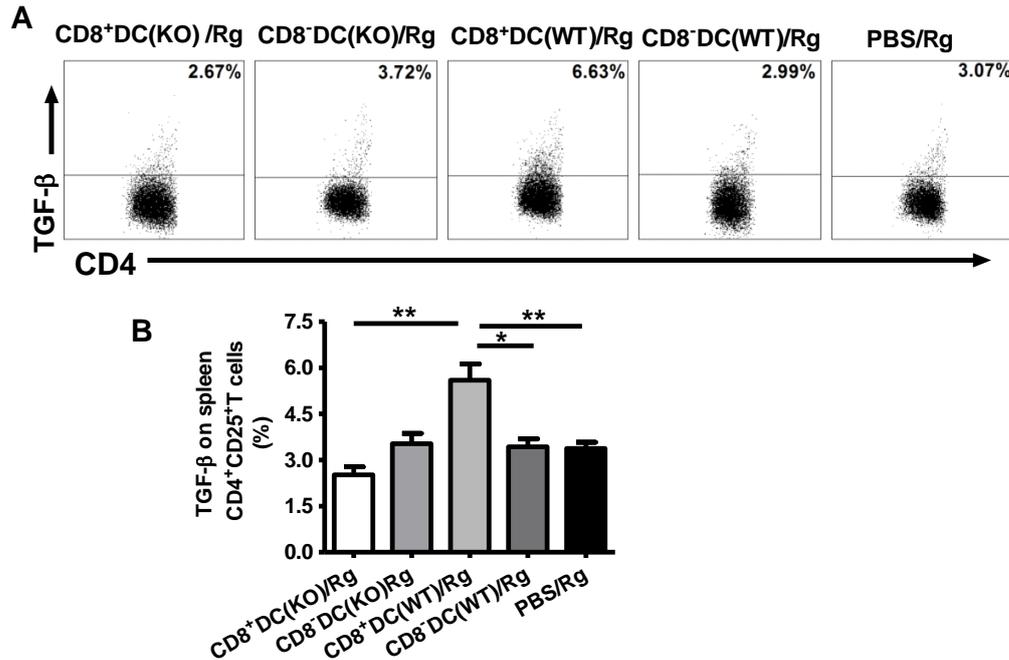
**Fig 25**

**Figure 25. IL-10 promoted the surface expression of CTLA-4 on CD4<sup>+</sup>CD25<sup>+</sup> T cells in both spleen and lung.** Single cells prepared from spleens or lungs were stained with FITC-anti CD3e, APC-anti CD4, PE-Cy7-anti CD25 and PE-anti CTLA-4. A, the percentage of CTLA-4 expression in CD4<sup>+</sup>CD25<sup>+</sup>T cells in spleen T cell(A) or lung cells (B) were measured using flow cytometry. C, summary of the percentage of CTLA-4expression on CD4<sup>+</sup>CD25<sup>+</sup>T cells of spleen (left) or lung cells (right) of recipient mice. Data are presented as mean ± SD of each group. One representative experiment of two independent experiments is shown. \* p<0.05; \*\*

p<0.01; \*\*\* p<0.001.

**5. Expression of membrane-bound TGF- $\beta$  in spleen was regulated by DC subsets through IL-10 dependend pathway.**

In addition to CTLA-4, we also exam TGF- $\beta$ , a cytokine known to be important for Treg developement and function(335, 336), especially the one expressed on CD25<sup>+</sup>CD4<sup>+</sup> T cells. We gated live cells on CD25<sup>+</sup>CD4<sup>+</sup> T cells to analyze the surface TGF- $\beta$  expression (Fig 26). Our result showed that TGF- $\beta$  expression on the CD4<sup>+</sup>CD25<sup>+</sup> T cells is upregulated in the mice received CD8<sup>+</sup>DC(WT) but not those received CD8<sup>-</sup>DC(WT) in comparison with the control group. The enhanced TGF- $\beta$  expression on Treg relied on the IL-10 because the recipients of CD8<sup>+</sup>DC(KO) showed much lower level of TGF- $\beta$  on the CD4<sup>+</sup>CD25<sup>+</sup> T cell surface compared to recipient mice treated with CD8<sup>+</sup>DC(WT). The results suggest that the CD8<sup>+</sup>DC transfer induced surface TGF $\beta$  expressing Treg, which was dependent on IL-10.



**Fig 26**

**Figure 26. The effect of IL-10 production on the expression of TGF- $\beta$  on CD4 T cells.** Mice were treated with DC subsets (KO) or DC subsets (WT) prior sensitization and challenge with ragweed. Single cells prepared from spleens were stained with FITC-anti CD3e, APC-anti CD4, PE-Cy7-anti CD25 as well as with PE-anti TGF- $\beta$  as described in *Material and Methods*. A, the percentage of TGF- $\beta$ <sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>+</sup>T cells in spleen T cell(A) or lung mononuclear cells (B) were measured using flow cytometry. B, summary of the percentage of membrane TGF- $\beta$  expression on CD4<sup>+</sup> T cells of spleen of recipient mice. Data are presented as mean  $\pm$  SD of each group. One representative experiment of two independent experiments is shown. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

## Discussion

In this chapter, we provided evidence that DC subsets from *C.m* infected mice, especially, CD8<sup>+</sup>DC, possess the ability to suppress ragweed mediated allergic responses through IL-10 dependent mechanisms. We found that the adoptive transfer of CD8<sup>+</sup>DC(WT) was capable of transferring protection against RW-induced allergy, including airway eosinophilia inflammation, mucus over-production and Th2 related cytokines production in the draining LN and lung. Moreover, we provided evidence that CD8<sup>+</sup>DC from *C.m* infected mice increased CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg through IL-10 dependent mechanism. We also found enhanced CTLA-4 and TGFβ on Treg in the recipient of CD8<sup>+</sup>DC (WT) from *C.m* infection and the dependence of their expression on IL-10.

The most important findings in the present study are the dependency of IL-10 production by DC subsets from infected mice on the inhibition to allergic reactions following subsequent allergen (ragweed) exposure and the association of the inhibiting activity with the induction of Treg. Although various types of regulatory T cells have been documented in the literature, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells are the most typical Treg. One possible mechanism of the immunosuppression of CD4<sup>+</sup>CD25<sup>+</sup> T cells is that these cells can secrete by themselves, or cause secretion of TGF-β. In addition, Treg can express CTLA-4, a potent negative regulator of T cell immune responses. Recent study confirmed that CTLA-4 is essential for Treg to maintain immune homeostasis(334). We have reported previously that *C.m* infection can inhibit the development of allergic reactions (60) and demonstrated the importance of

DCs in the *C.m*-mediated inhibition (77). We also reported that the inhibition of allergy by DC from *C.m* infected mice was associated with higher IL-10 production by DCs(77). In the present study, we extended our previous findings by directly confining the contribution by IL-10 production by DC on the mediation of allergy inhibition, and the relationship between IL-10 production by DCs and the induction of Treg. We showed evidence that CD8<sup>+</sup>DC(WT), but not CD8<sup>+</sup>DC(KO), can induce CD4<sup>+</sup>CD25<sup>+</sup>Treg which express higher CTLA-4 and TGF- $\beta$ . We also provide evidence that the Th2 response was reduced in the recipients of CD8<sup>+</sup>DC(WT), but not CD8<sup>+</sup>DC(KO) in both single cells (Fig 21) and population levels in the dLN, lung and BAL fluids(Fig 22), suggesting an inhibitory foundation of the Treg in vivo.

The present study provided evidence on the critical importance of immune regulation on the *C.m*-mediated inhibition of allergy. Notably, the modulating effect of infection on allergies has been found to be related to immune deviation (increased Th1 to inhibit Th2) and/or immune regulation (tolerogenic DC and Treg). *C.m* infection, as an intracellular bacterial infection, has been found to induce Th1 responses. However, in this allergen sensitization and airway challenge model, it was found that the decrease of Th2 cytokine (IL-4), rather than increased Th1 cytokines (IFN- $\gamma$ ), was associated with the inhibitory allergic reactions. In particular, the transfer of DC subsets from IL-10 KO mice fail to inhibit allergic reaction even though they induced significantly higher IFN- $\gamma$  production (Fig 21B). In contrast, the failure of DC subsets from IL-10 KO mice to inhibit allergic reaction showed close

association with significantly decreased CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg which express CTLA-4 and TGFβ (Fig 24-26). Therefore, even in this intracellular bacterial infection model, immune regulation rather than immune deviation appears to be the predominant mechanism by which the infection inhibits allergy.

In summary, the results presented here support a critical role of IL-10 producing DC in infection mediated inhibition of allergy and its relationship with the development of Treg. Even in intracellular bacterial infection, the DCs may modulate allergic reactions predominantly through immune regulation (tolerogenic DC and Treg) rather than immune deviation. Further work to characterize the nature of regulatory T cells and the contribution of the CTLA-4, TGF-β and Foxp3 in in vivo suppressive function of Treg in allergic inflammation is needed. Further understanding of the activity of these molecules may be helpful for designing more effective prevention and therapeutic methods for allergic diseases.

## Part IV Chapter 4

# Enhanced ICOSL expression on DC of IL-10 knockout mice is critical for Th17 but not Th1 response in chlamydial infection

## INTRODUCTION

*Chlamydia* cause large spectrum of human diseases involving eye, genital tract and the respiratory system (337, 338). No human vaccine is available to prevent chlamydial infections. Better understanding of the mechanisms related to protection and pathogenesis of chlamydial infections is essential for rational development of a safe and effective vaccine(339). Previous studies have demonstrated that T cells, especially type 1 CD4<sup>+</sup> T (Th1) cells, play a central role in host defense against chlamydial infection (124, 125, 193, 340-342). In contrast, higher Th2 responses and higher IL-10 production appear to be associated with increased susceptibility to chlamydial infection and pathological responses (124, 193).

The Th17 cell is a newly identified CD4<sup>+</sup> T cell subset characterized by its predominant production of IL-17. IL-17 is a group of cytokines belonged to IL-17 family including IL-17A and IL-17F, which are produced not only by Th17 cell but other cells such as some  $\gamma\delta$ T cells, CD8<sup>+</sup> T cells and NK T cells (216). Th17 is distinct from Th1 and Th2 cell in its developmental pathway. It has been demonstrated that [transforming growth factor beta](#) (TGF- $\beta$ ), IL-6 and IL-23 production is particularly important for Th17 responses (223, 343). TGF- $\beta$  and IL-6 are critical for initiation of Th17 cell differentiation, while IL-23 signaling is more important for the stabilization and/or maintenance of Th17 responses (228-230). Although Th17 was initially mainly found associated with overly enhanced inflammations in autoimmune diseases (344, 345), its protective role in host defense against infections especially extracellular

bacterial and fungi infections was well documented lately (reviewed in (233)). Very recently, the protective role of Th17/IL-17 in intracellular bacterial infections was also reported (236, 237, 276, 346). In particular, we and another group recently reported that IL-17 played an important role in host defense against chlamydial lung infection (276, 346).

Inducible co-stimulator (ICOS) is a surface molecule on activated T cells belonging to CD28 superfamily (347). ICOS is not expressed on naïve T cells but can be up-regulated quickly when the cells are activated (348, 349). ICOS can be expressed by both Th1 and Th2 cells, but more dominantly by Th2 cells(187, 350-356). The ligand for ICOS (ICOSL or B7RP-1) is expressed on DC, macrophages and some epithelial cells and ICOSL is the only ligand of ICOS (176). Notably, the limited studies on the role of ICOS/ICOSL interaction in Th17 responses have reported inconsistent results(357-360). For example, one study found that Th17, like Th2, expresses higher ICOS than Th1 cells(358) while another study showed that blockage of ICOS increased Th17 responses(359). We recently reported that chlamydia infection induced ICOS-L expression on DC(77) and that ICOS-L KO mice showed dramatically reduced Th17 response but enhanced Th1 response following Cm infection (361).

We and others have reported previously that IL-10 KO mice are more resistance to chlamydia infection with less pathological reactions than WT mice in both lung(193) and genital(194) models. The enhanced protection in IL-10 KO mice has been attributed to an increased Th1 response. Indeed, the IL-10 KO mice produced significantly higher IL-12 and IFN $\gamma$  following chlamydia infection(193). Moreover, adoptive transfer of DC from IL-10 KO mice induced significantly higher IFN $\gamma$  production by CD4<sup>+</sup> T cells, which was associated with better protection

compared to transfer of DC from WT mice(194). Since recent studies have shown that IL-17/Th17 also contributes to protection against chlamydial infection and since some recently studies have suggested a suppressive role of IL-10 on Th17 responses (362), the nature and mechanism of Th17 response in IL-10 deficient mice became an important question to be addressed. Answer to this question can provide important new knowledge on the immune regulation of chlamydial infection. Notably, some studies showed parallel Th1/Th17 responses while other showed mutually inhibitory effect of Th1 and Th17 responses(358). Indeed, IL-17 can suppress Th1 cell differentiation in the presence of exogenous IL-12(358). IL-12 or IFN $\gamma$  can negatively regulate Th17 cell differentiation in the presence of antigen-presenting cells(358). Considering the reported higher Th1 response in IL-10 KO mice (193, 194) and the discordance of Th1 and Th17 responses observed in ICOSL KO mice (363) following chlamydial infection, further study on Th17 responses in IL-10 KO mice is likely providing new knowledge on the relationship between Th1 and Th17 responses in a setting of natural intracellular bacterial infection.

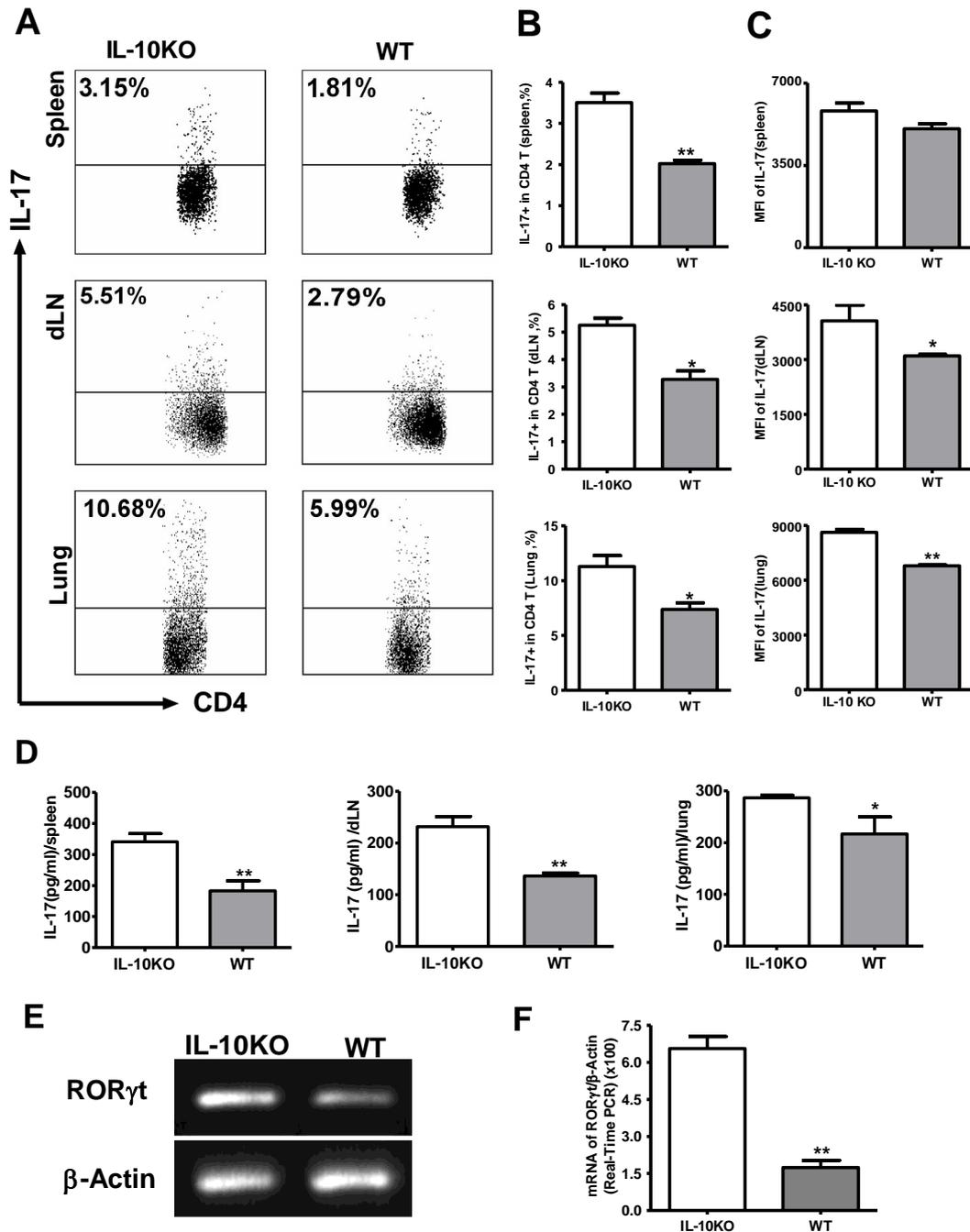
We, therefore, performed experiments in this chapter to examine the nature of Th17 response in IL-10 KO mice mainly focusing on the level of Th17 response, the mechanism especially its relationship with ICOS/ICOSL signaling, the relationship between Th17 and Th1 response and the contribution of Th17 response to protection against chlamydial infection.

## **RESULTS**

### ***IL-10 KO mice show enhanced Th17 responses following Cm lung infection***

We first compared the Th17 responses in IL-10 KO and WT mice to *Cm* lung challenge infection. The mice were infected i.n. with *Cm* and were euthanized on day

7 after infection. The cells isolated from the spleen, draining LNs and lung were analyzed for CD4 T cell markers (CD3 and CD4) and intracellular IL-17 by flow cytometry. As shown in Figure 27A and 27B, the percentage of IL-17 producing CD4<sup>+</sup> T cells was significantly higher in IL-10 KO mice in all the tested tissues in comparison with WT mice. Moreover, the mean channel fluorescence intensity (MFI) of IL-17 positive cells in IL-10 KO mice was also higher than that in WT mice (Figure 27C), suggesting a higher capacity of Th17 cells in IL-10 KO mice to produce IL-17. The bulk culture of splenocytes and the cells isolated from draining LNs and lung of IL-10 KO mice showed significantly higher IL-17 (IL-17A) levels in the culture supernatants, suggesting a higher Th17 response in a population level also (Figure 27D). Since retinoid-related orphan receptor (ROR)- $\gamma$ t is a central transcription factor specific for Th17 cells (364), we further analyzed the message of ROR $\gamma$ t in the lung tissues. In line with the increased Th17/IL-17 responses, both semi-quantitative (Figure 27E) and real-time (Figure 27F) RT-PCR showed significantly higher ROR $\gamma$ t transcripts in the IL-10 KO mice. Collectively, the results demonstrate that IL-10 deficiency led to enhanced Th17 responses in lymphoid organs and local tissues following *Chlamydia* lung infection.



**figure 27**

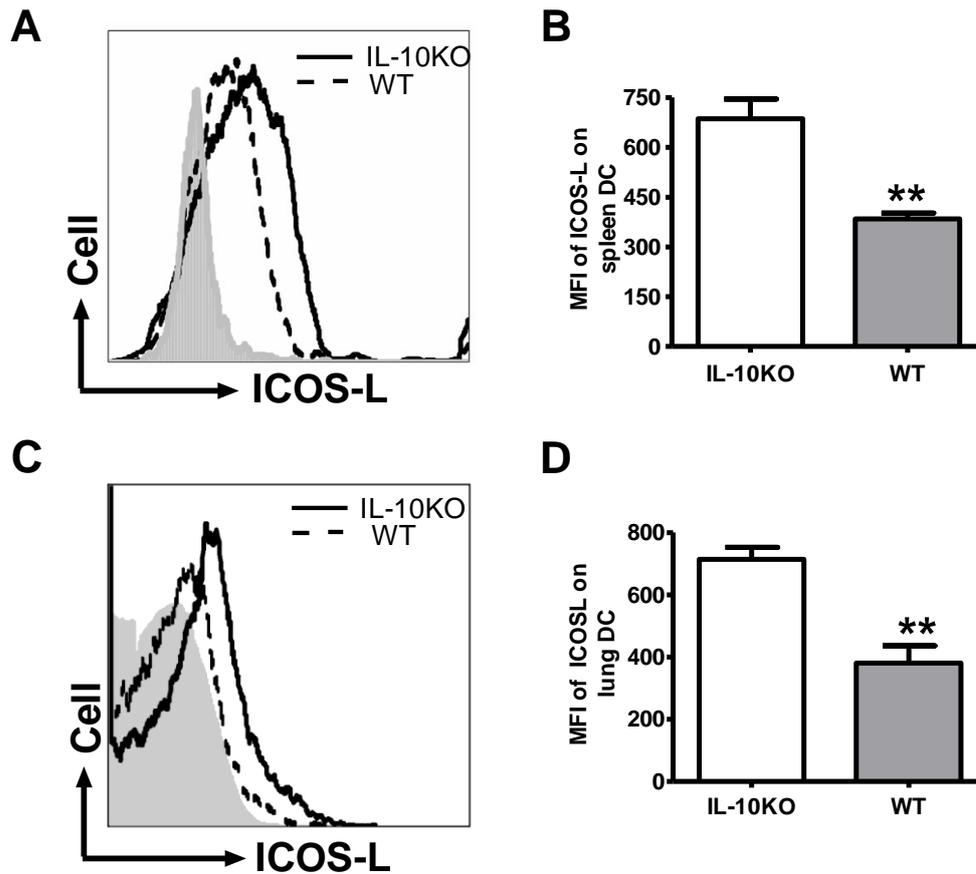
**Figure 27. Enhanced Th17 cell expansion and IL-17 production in the peripheral lymphoid organs and lung tissues of IL-10 KO mice after *Cm* infection.**

A-C, Single cell suspensions of spleen and draining lymph nodes (dLN) and lung mononuclear cells of IL-10 KO and WT mice were prepared at day 7 after i.n. *Cm* lung infection ( $1 \times 10^3$  IFUs) as described in *Materials and Methods*. Intracellular

cytokine staining was used to detect the percentage of IL-17-producing CD4<sup>+</sup> T cells using fluorescence conjugated antibodies including FITC-CD3ε, PE-CD4, and APC-IL-17A. A, Representative data of intracellular IL-17 staining. Cells were gated on CD3<sup>+</sup> CD4<sup>+</sup> cells. B, Summary of the percentage of Th17 cells in spleen, dLN, and lung. C, the mean fluorescence intensity (MFI) of IL-17 producing CD4<sup>+</sup> T cell. D, the single spleen cells ( $7.5 \times 10^5$ ), draining LN cells ( $5 \times 10^5$ ) and lung mononuclear cells ( $5 \times 10^5$ ) were cultured for 72 h and the culture supernatants were tested for IL-17 protein by ELISA. E&F, Total RNA extracted from lung tissues of IL-10 KO or WT mice were assayed for RORγt expression by semi-quantitative reversed transcription PCR (E) and real-time PCR (F), respectively. Three independent experiments with four mice in each group were performed. Data is shown as mean ±SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . Representative data of three independent experiments are shown.

**DC from IL-10 KO mice show significantly higher ICOS-L expression following *Cm* infection.**

Our previous study has shown a significantly reduced Th17 response in ICOS-L KO mice following *Cm* infection, suggesting an important role of ICOS/ICOS-L interaction in Th17 response(363). In order to explore the mechanism for the enhanced Th17 response in IL-10 KO mice, we further compared the ICOS-L expression on the DC of IL-10 KO mice with WT mice following *Cm* infection. The purified DC were double stained with anti-CD11c and anti-ICOS-L mAbs and analyzed by flow cytometry. As shown in Figure 28A, a significantly higher proportion of DC from IL-10 KO mice expressed ICOS-L than WT mice (27.0 % vs 7.3%). The density of ICOS-L expression on DC of IL-10 KO mice was also significantly higher than WT mice, suggested by MFI measurement (Fig. 20B). Same trend has been found on lung DC too.



## figure 28

**Figure 28. ICOS-L expression was significantly higher on DC from IL-10 KO than WT mice following Cm infection.** DC were isolated from spleens (A&B) or lung (C&D) using CD11c microbeads and LS MACs columns as described in *Materials and Methods*. The DC were stained with anti-CD11c-FITC, anti-ICOS-L-PE mAb for 20min on ice and analyzed by flow cytometry. A&C: One representative histogram of ICOS-L expression on DC is shown. Solid line: DC from IL-10 KO mice (KO), dash line: DC from WT mice (WT), shaded histogram: isotype control. B&D, Mean fluorescence intensity (MFI) of ICOS-L on spleen DC or lung DC is shown as mean  $\pm$  SD for each group (n=4). \*\*, p<0.01. Representative data of three independent experiments are shown.

### **IL-17 producing CD4<sup>+</sup> T cells (Th17) are mainly ICOS positive cells**

Having identified the higher ICOS-L expression on DC of IL-10 KO mice, we further examined the expression of ICOS, the corresponding molecule of ICOS-L, on Th17 cells in the Cm-infected mice. Spleen cells and lung mononuclear cells were collected from infected mice and stained for intracellular IL-17 and surface ICOS in conjunction with CD3 and CD4. It was found that most of the IL-17 producing CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD4<sup>+</sup>) were ICOS positive in spleen and lung of both IL-10 and WT mice (Figure 29). The measurement of IL-17 production by MFI showed that ICOS<sup>+</sup> Th17 cells produced significantly higher levels of IL-17 than ICOS<sup>-</sup> Th17 cells (Figure 29B). Therefore, the ICOS<sup>+</sup> Th17 cells were not only the major portion of Th17 population but also the higher producers of IL-17. The data, in combination with the observed higher expression of ICOS-L on DC of IL-10 KO mice, suggest the possible involvement of ICOS/ICOS-L interaction in the development of Th17 cells especially those with higher IL-17 producing capacity.

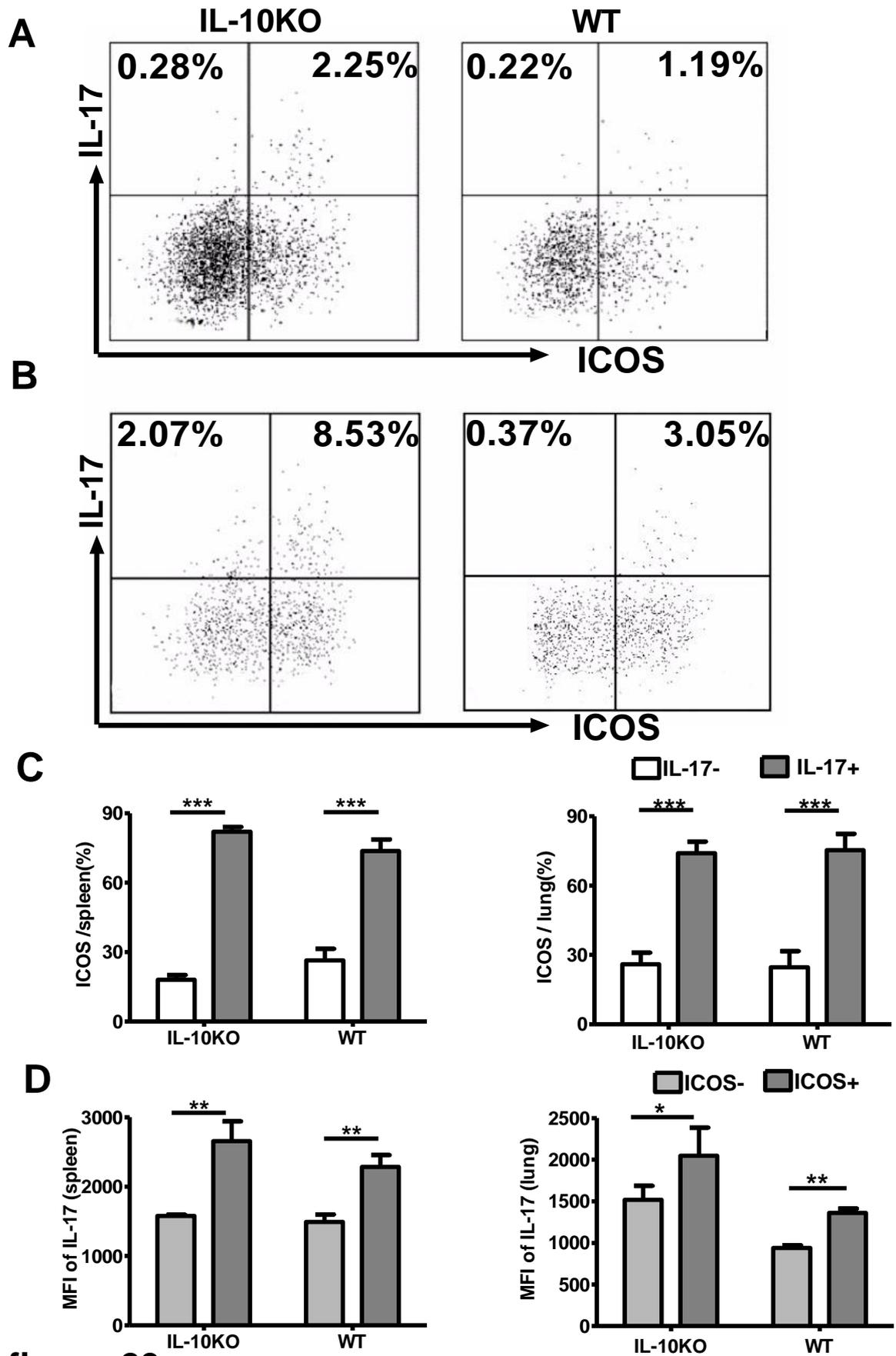


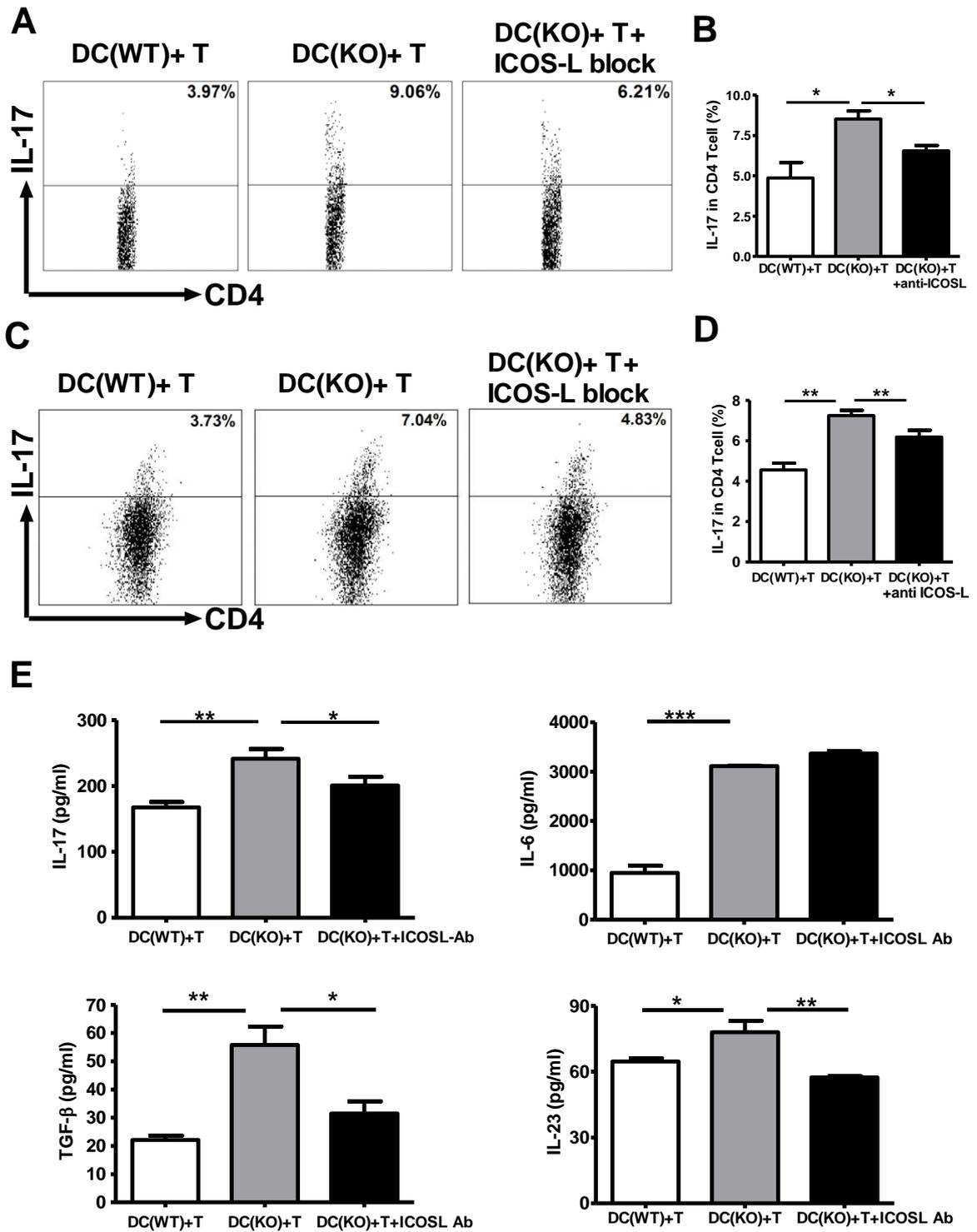
figure 29

**Figure 29. Most IL-17 producing CD4<sup>+</sup> T cells (Th17) are ICOS positive and ICOS<sup>+</sup> Th17 cells produce higher IL-17 than ICOS<sup>-</sup> Th17 cells.** Single spleen cells (A) or lung mononuclear cells (B) isolated from IL-10 KO (left panel) and WT mice (right panel) at day 7 after i.n. *Cm* lung infection (1x10<sup>3</sup> IFUs) were analyzed by four color staining on surface CD3 $\epsilon$ , CD4 and ICOS and intracellular IL-17A. Representative dot plots of ICOS expression on Th17 cells in spleen (A) and lung (B) are shown. Cells were gated on CD4<sup>+</sup>CD3<sup>+</sup> population. C, summary of the percentage of ICOS<sup>+</sup> within IL-17<sup>+</sup> and IL-17<sup>-</sup> CD4<sup>+</sup> T cells. D, the MFI of IL-17 in ICOS<sup>+</sup> and ICOS<sup>-</sup> Th17 cells (CD3 $\epsilon$ <sup>+</sup>CD4<sup>+</sup>IL-17<sup>+</sup>). Data are shown as the mean $\pm$  SD for each group (n=4). \*, p<0.05, \*\*, p<0.01, \*\*\*, p<0.001. Representative data of three independent experiments are shown.

## **Enhanced ICOS-L expression on DC of IL-10 KO mice contributes to Th17 expansion which was associated with promotion of Th17-driving cytokines production**

To confirm the involvement of ICOS/ICOS-L interaction in Th17 response in *Cm* infection, we tested the effect of blocking ICOS/ICOS-L pathway on the development of *Cm*-specific CD4<sup>+</sup> Th17 cells using a DC:CD4<sup>+</sup> T cell co-culture system. The CD4<sup>+</sup> T cells were isolated from *Cm*-immunized mice and cultured together with DC isolated from IL-10 KO or WT mice for 48 hours. The development/expansion of Th17 cells in the co-culture was determined by double staining of surface CD4 and intracellular IL-17. As shown in Figure 30, both spleen DC or lung DC from IL-10 KO mice which expressed higher ICOS-L induced significantly higher percentage of Th17 cells than those from WT mice (9% versus 4% in spleen DC co-culture, 7% vs.3.7% in lung DC co-culture), suggesting a dramatically higher capacity of the DC from IL-10 KO mice in enhancing Th17 cell development. More importantly, the blockade of ICOS/ICOS-L interaction by anti-ICOS-L mAb in the co-culture largely reduced the capacity of the DC from IL-10 KO mice to increase Th17 response (6.2% versus 9.06% in spleen DC co-culture, 5% versus 7.04% in lung DC co-culture). Similar pattern of changes in Th17 response was found in population level by measuring IL-17 in the co-culture supernatants (Figure 30E). Interestingly, the higher ICOS-L expression on DC from IL-10 KO mice was associated with the production of higher levels of Th17-driving cytokines (IL-6, TGF $\beta$  and IL-23) in the co-culture and the blockade of ICOS/ICOS-L interaction by

anti-ICOS-L mAb dramatically reduced the production of TGF $\beta$  and IL-23 (Figure 30E). Therefore, ICOS/ICOS-L interaction indeed contributes to the enhanced Th17 response in IL-10 KO mice following chlamydial infection and this interaction is critical for the production of Th17-driving cytokines, especially TGF $\beta$  and IL-23.



**figure 30**

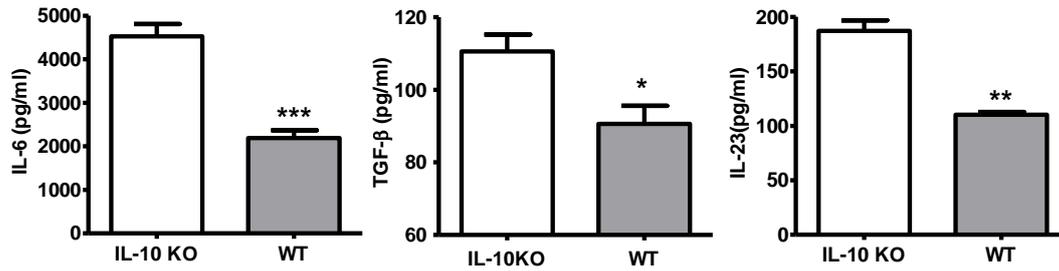
Figure 30. Higher ICOS-L expression is critical for DC from IL-10 KO mice to promote the development of Th17 cells and the production of Th17 driving

**cytokines (TGF $\beta$  and IL-23) in DC:CD4<sup>+</sup> T cell co-culture.** CD4 T cells isolated from *Cm* immunized mice were co-cultured with freshly purified lung DC (A) or spleen DC (C) from *Cm*-infected WT [DC(WT)] or IL-10 KO [DC(KO)] mice (DC :T cells, 5x10<sup>5</sup>:2x10<sup>6</sup>) in the presence of killed *Cm* stimulation with or without anti ICOS-L mAb. After 48 h cultures, cells were analyzed for surface CD3 $\epsilon$  and CD4 and intracellular IL-17 as described in *Materials and Methods*. A&C, flow graph showing intracellular IL-17 staining in different culture condition. Cells were gated on CD3 $\epsilon$ <sup>+</sup> CD4<sup>+</sup> cells. The percentages of IL-17<sup>+</sup>CD4<sup>+</sup> T cells in total CD4<sup>+</sup> T cells (CD3 $\epsilon$ <sup>+</sup>CD4<sup>+</sup>) are indicated at the right upper corner. B&D, summary of the percentages of IL-17<sup>+</sup> CD4<sup>+</sup> T cells in total CD4<sup>+</sup> T cells in lung DC+T coculture (B) or spleen DC+T co-culture (D). E, The IL-17A, IL-6, IL-23 and TGF $\beta$  protein levels in the spleen DC +T co-culture supernatants of each condition were detected by the ELISA. Data are shown as mean $\pm$  SD (n=4). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Representative data of three independent experiments are shown.

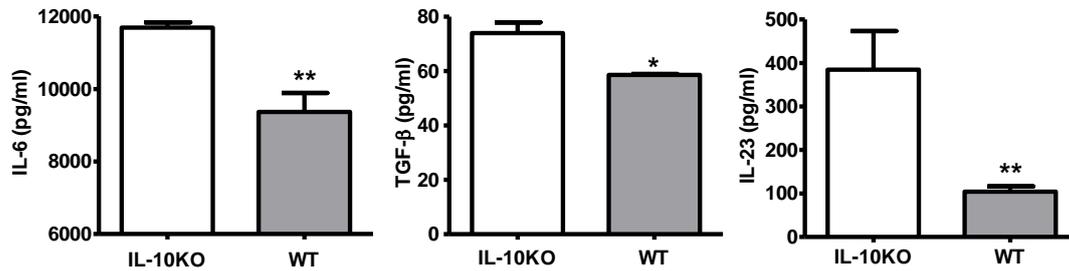
**IL-10 KO mice show dramatically increased production of Th17-driving cytokines.**

Inspired by the finding on Th17 driving cytokines in relationship with ICOS/ICOS-L expression in the co-culture system, we further examined the production of these cytokines in the Cm-infected IL-10 KO and WT mice with known difference in ICOS-L expression on DC. As shown in Figure 31, the levels of IL-6, TGF- $\beta$  and IL-23 production by the cells from spleen, draining LNs and the lung were significantly higher in IL-10 KO mice. The data provide in vivo evidence for the association of ICOS-L expression on DC with the level of Th17 driving cytokines following Cm infection.

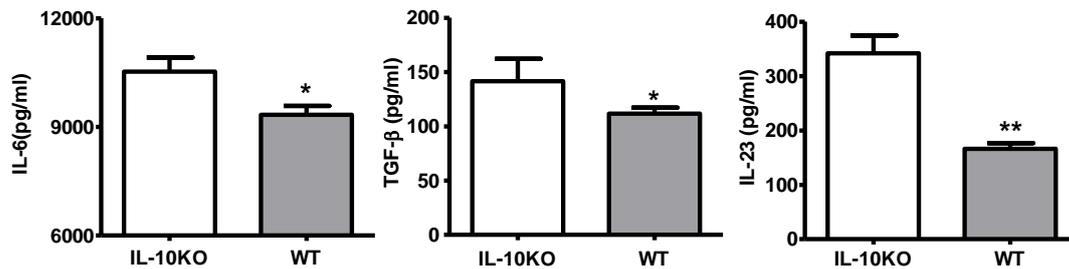
## A(spleen)



## B(LN)



## C(lung)



**figure 31**

**Figure 31. IL-10 KO mice showed higher Th17 driving cytokine production than WT following *Cm* infection.** Single spleen (A) and dLN (B) cells and lung mononuclear cells (C) isolated from IL-10 KO and WT mice at day 7 p.i. (four mice in each group) were cultured for 72h with killed *Cm*. The levels of IL-6, TGF $\beta$  and IL-23 in the culture supernatants were measured by ELISA. Data are shown as the mean  $\pm$  SD for each group (n=4). \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001. Representative data of three independent experiments are shown.

## **IL-10 KO mice show enhanced Th1 response which is independent of ICOS/ICOS-L interaction**

We and others have reported that IL-10 KO mice mount stronger Th1 response than WT following *Cm* infection(193, 194). We confirmed this finding in the present study by intracellular IFN $\gamma$  staining of CD4<sup>+</sup> T cells (data not shown). We then examined the expression of ICOS on the IFN $\gamma$  producing CD4<sup>+</sup> T cells (Th1). Interestingly, in contrast to Th17 cells which mostly were ICOS positive (Figure 29), only a small portion of Th1 cells were ICOS positive especially in IL-10 KO mice (Figure 32A&B). To more directly examine the influence of ICOS/ICOS-L interaction on Th1 cell responses, we co-cultured CD4<sup>+</sup> T cells isolated from *Cm*-immunized mice with DC from *Cm*-infected IL-10 KO mice in the presence or absence of ICOS-L blocking mAb. Similar to the observed enhancing effect on IL-17 producing cells (Figure 30A&B), the DC from IL-10 KO mice showed stronger promoting effect on the development of IFN $\gamma$  producing cells compared to those from WT mice (18.91% vs. 10.34%,  $p=0.009$ ; Figure 32C&D). However, the blockade of ICOS/ICOS-L interaction by neutralizing mAb had no significant effect on IFN $\gamma$  production by CD4<sup>+</sup> T cells, in sharp contrast to the virtual abolishment of the enhanced IL-17 responses by the blockade in the same co-culture system (Figure 30A&B). The failure to change IFN $\gamma$  production by blockade of ICOS/ICOS-L interaction was also confirmed by testing IFN $\gamma$  in the culture supernatants (Figure 32E). The results suggest that the higher Th1 response in IL-10 KO mice, unlike Th17 response, is independent of their higher ICOS-L expression on DC.

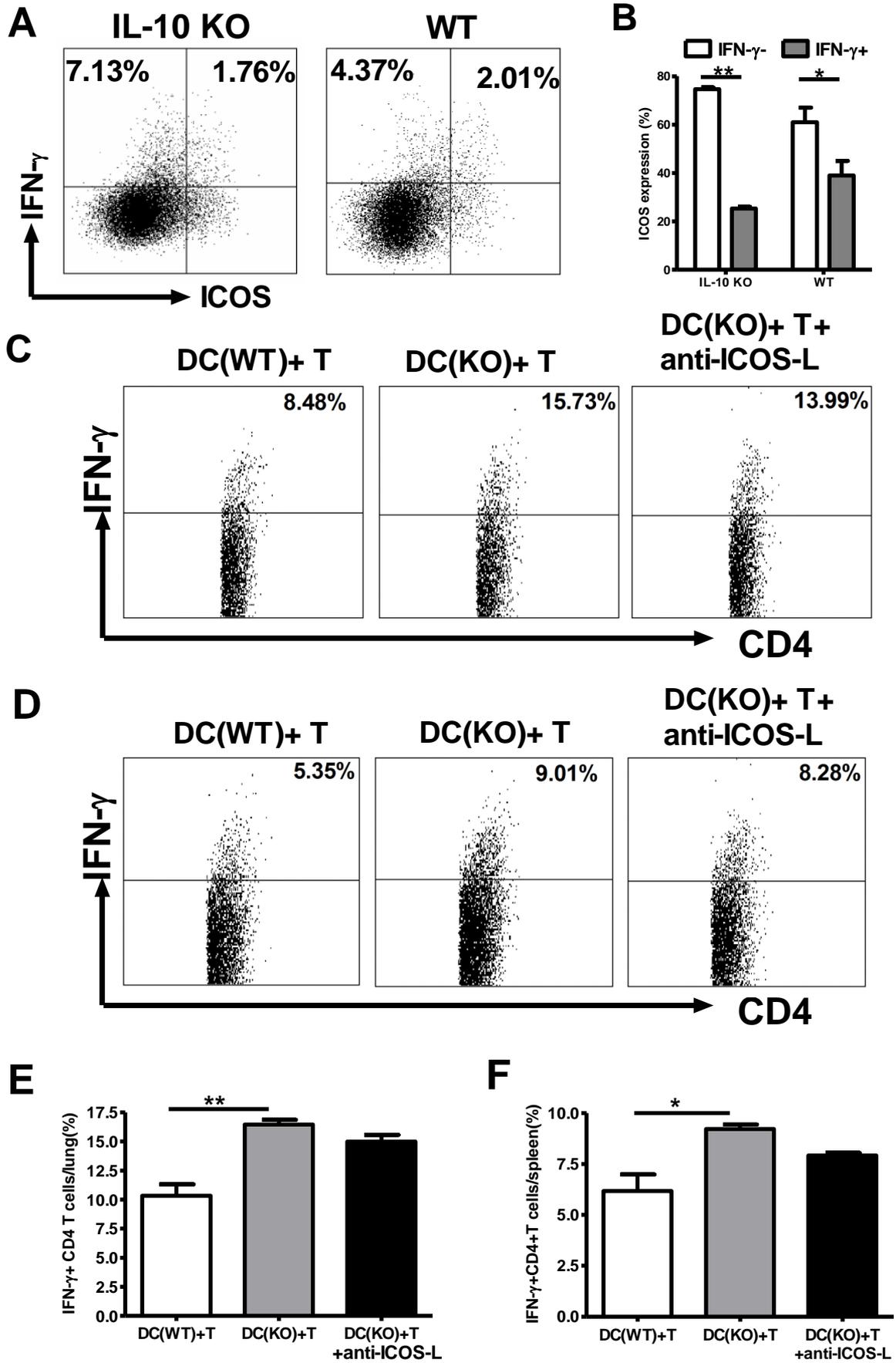


figure 32

**Figure 32. Most Th1 cells are ICOS negative and the development of Th1 response is independent of ICOS/ICOS-L signaling.** A&B, Single lung cells were prepared from IL-KO and WT mice at day 7 post i.n. Cm infection. The cells were analyzed by four colors staining on surface CD3 $\epsilon$ , CD4 and ICOS and intracellular IFN $\gamma$ . A, representative graph of flow cytometry. The cells were gated on CD3 $\epsilon$ <sup>+</sup>CD4<sup>+</sup> cells. B, summary of the percentage of ICOS<sup>+</sup> within IFN $\gamma$ <sup>+</sup> and IFN $\gamma$ <sup>-</sup> CD4<sup>+</sup> T cells. C&D, lung (C)/spleen (D) DC:T co-culture system was set up as described in legend to figure 30. The percentage of IFN $\gamma$ <sup>+</sup> producing CD4<sup>+</sup> T cells (CD3 $\epsilon$ <sup>+</sup>CD4<sup>+</sup>) was measured by the intracellular cytokine staining and representative dot plot is shown. Cells were gated on CD4<sup>+</sup> T cells. E&F, summary of the percentage of IFN $\gamma$  producing cells in the co-culture. E, lung DC<sup>+</sup> T cells .F: spleen DC<sup>+</sup> T cells. Data are shown as the mean $\pm$  SD for each group (n=4). \*, p<0.05, \*\*, p<0.01. Representative data of three independent experiments are shown.

## **Blockade of Th17 function reverses the better protection of IL-10 KO mice against chlamydial infection**

Having shown the significant enhancement of Th17 response in IL-10 KO mice, we further tested whether the enhanced Th17 response in these mice was relevant to the previously reported better protection of these mice against chlamydial infection(193, 194). WT and IL-10 KO mice were infected i.n. with Cm and one group of IL-10 KO mice were treated i.n. with 10 $\mu$ g of anti-mouse IL-17 mAb at 2 h p.i. and every 48 hours thereafter until the mice were sacrificed at day 7 p.i.. The severity of disease and chlamydial growth in different groups of mice were examined by monitoring body loss, lung chlamydial loads and histopathological analysis. As reported previously, the IL-10 KO mice showed less body loss (Figure 33A), lower chlamydial growth (Figure 33B) and less cellular infiltration in the lung (Figure 33C), confirming the better protection in the IL-10 KO mice. However, the neutralization of IL-17 virtually abolished the better protection observed in IL-10 KO mice from all the three aspects: body weight change, in vivo chlamydial load and pathology (Figure 33). The treatment with isotype control antibody has no effect on the disease process in WT mice(276). The data clearly indicate that the enhanced Th17 response in IL-10 KO mice contributes significantly to the better protection of these mice against Cm infection.

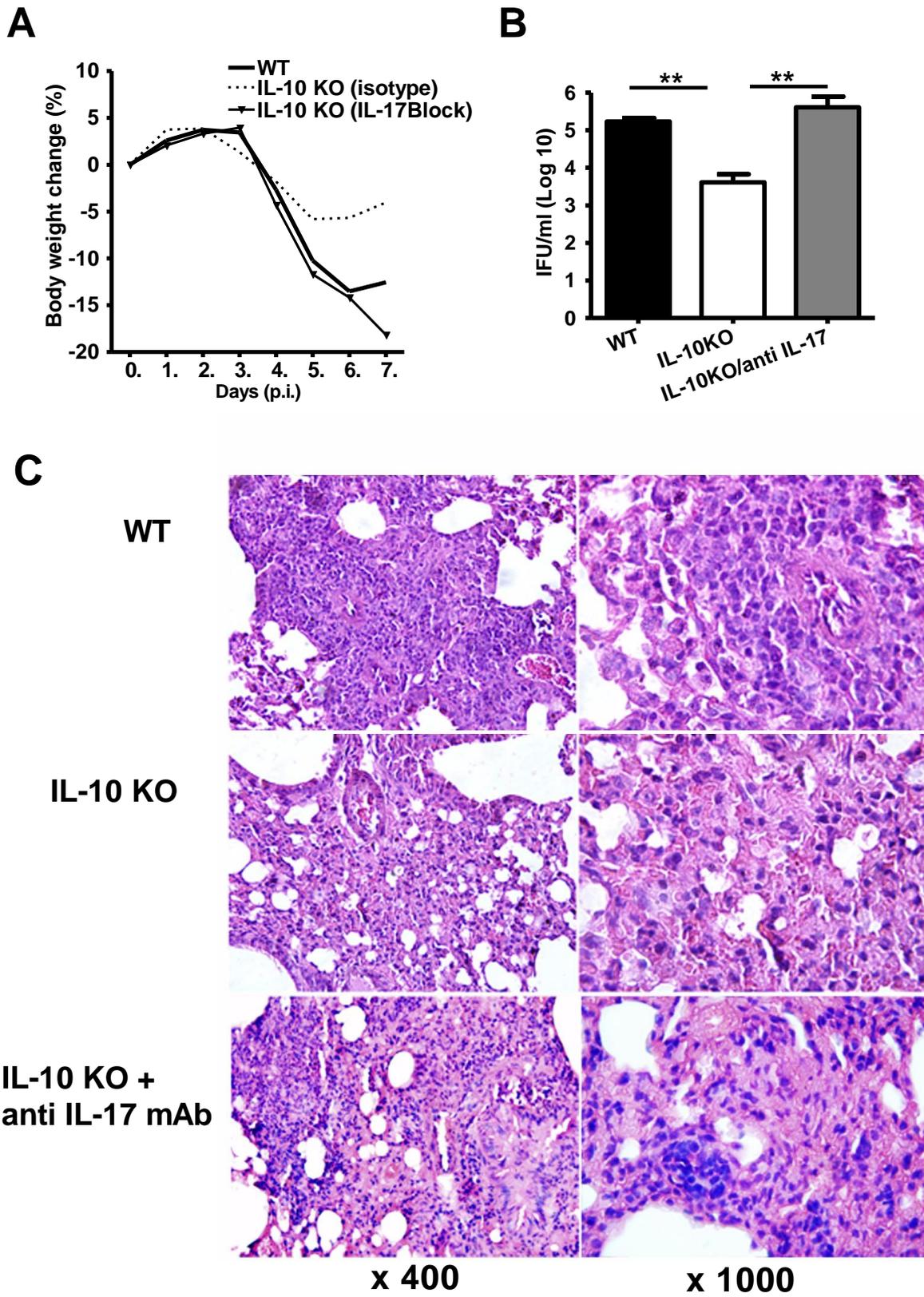


figure 33

Figure 33. IL-10 KO mice show better protection following Cm infection which is

**virtually abolished by in vivo neutralization of IL-17.** Aged matched WT and IL-10 KO mice were infected i.n. with Cm ( $1 \times 10^3$  IFUs). Two hours later, IL-10 KO mice were administered i.n. with 10  $\mu$ g of anti-mouse IL-17 mAb or isotype control (IgG2a) antibody. The same antibody treatment was given at days 2 and 4 p.i. A, Mice were monitored daily for body weight change. The original body weights of the different groups of mice were similar. B&C, Mice were sacrificed on day 7 p.i., the bacterial loads (B) and pathologic changes (C) in the lung were analysis as described in *Materials and Methods*. Data are shown as the mean  $\pm$  SD for each group (n=4). \*\*, p<0.01. Representative data of three independent experiments are shown.

## **Neutralization of IL-17 activity dramatically reduces type-1 T cell responses in IL-10 KO mice**

Since it has been shown that type-1 T cell responses (Th1 and Tc1) are critical for controlling chlamydial lung infection while IL-17 had no directly inhibitory effect on chlamydial growth(346), we further examined the effect of IL-17 neutralization on the type-1 T cell responses induced by Cm infection in IL-10 KO mice. We tested IFN $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cell and total T cells in Cm-infected WT and IL-10 KO mice which were treated with anti- IL-17 mAb or isotype control antibody. The results showed much higher percentage of IFN $\gamma$  producing CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in both lymphoid organs (spleen and draining LNs) and the local tissues (lung) in IL-10 KO mice compared to WT mice (Fig. 34). Pooled data of intracellular IFN $\gamma$  analysis showed statistically significant differences between the groups (Table 1). Similar difference between WT and IL-10 KO mice in IFN $\gamma$  production was observed in the bulk culture of cells isolated from the spleen, draining LNs and lung (Table 1), confirming our previous report(193). Remarkably, IL-17 neutralization in vivo completely reversed the enhanced IFN $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells observed in the IL-10 KO mice (Figures 34 and Tables 1). The results suggest that the enhanced Th17 response in IL-10 KO mice has significant promoting effect on type-1 T cell responses against chlamydial infection.

|        |                     | Intracellular IFN- $\gamma$ (%) |              |              | Bulk Culture (U/ml) |
|--------|---------------------|---------------------------------|--------------|--------------|---------------------|
|        |                     | CD3e                            | CD4          | CD8          |                     |
| Spleen | WT                  | 3.84±0.33                       | 5.06±0.76    | 7.66±0.44    | 304.2±5.60          |
|        | IL-10 KO            | 6.72±0.78 *                     | 9.28±0.69 *  | 11.3±0.88 *  | 358.5±13.2*         |
|        | IL-10KO/anti IL-17  | 3.63±0.68 #                     | 3.55±0.78 #  | 6.88±0.71 #  | 291.2±15.19#        |
| dLN    | WT                  | 5.67±0.34                       | 6.58±0.73    | 12.07±1.22   | 103.6±8.86          |
|        | IL-10 KO            | 7.98±0.55 *                     | 10.71±0.49 * | 18.33±1.03 * | 135.0±12.99*        |
|        | IL-10KO/ anti IL-17 | 4.08±0.89 #                     | 6.10±0.92 #  | 10.75±1.21 # | 68.78±16.25##       |
| Lung   | WT                  | 5.31±0.38                       | 5.19±0.77    | 15.00±1.29   | 170.5±6.15          |
|        | IL-10 KO            | 8.05±0.67 *                     | 7.51±0.67 *  | 21.77±1.98 * | 316.1±14.81**       |
|        | IL-10KO/ anti IL-17 | 4.53±0.87 #                     | 4.70±0.87 #  | 13.89±1.84 # | 173.4±15.59##       |

**Table 1. Summary of the proportion of IFN- $\gamma$  producing CD3e, CD4, CD8 T cells and IFN- $\gamma$  production in bulk culture of cells from WT mice and IL-10 KO mice with or without anti-IL-17 mAb treatment.** Single cells were collected from the spleen, dLNs and lung of WT mice and IL-10 KO mice with or without anti-IL-17 mAb treatment at day 7 p.i.. Cells were analyzed for intracellular IFN $\gamma$  production or put into culture for 72h with killed Cm.. The percentages of IFN- $\gamma$  producing cells in the total T cell and CD4+ and CD8+ T cell subsets in different groups (n=8) were detected by FACs and IFN- $\gamma$  levels in the culture supernatants were measured by ELISA. Data are summarized as mean  $\pm$  SD. \*,  $p < 0.05$  compared to WT mice; #,  $p < 0.05$  compared to IL-10 KO mice.

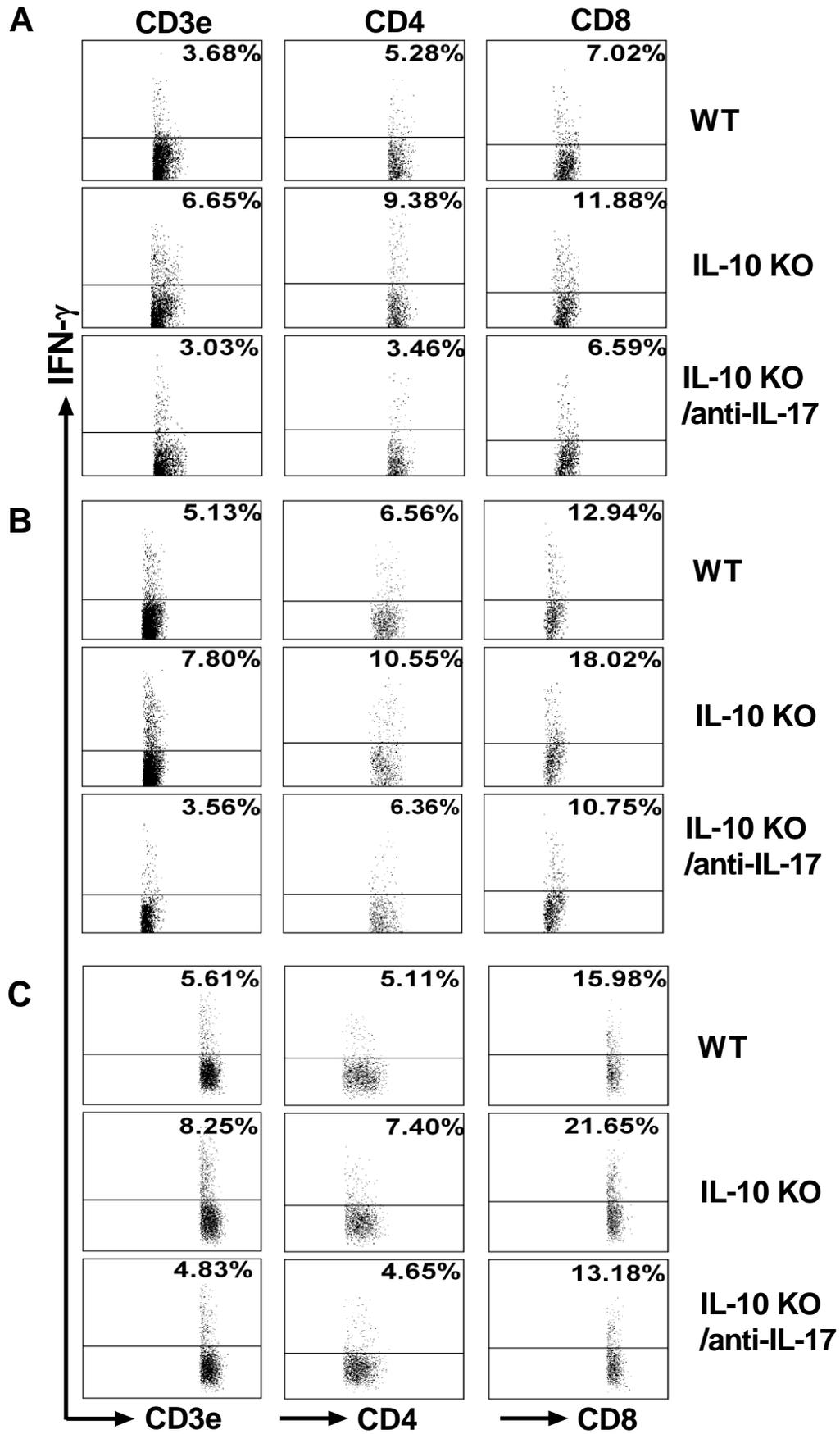


figure 34

**Figure 34. IL-10 KO mice show higher type-1 CD4+ (Th1) and CD8+ (Tc1) T cell responses than WT mice following *Cm* lung infection, which is abolished by in vivo neutralization of IL-17.** Spleen (A), draining LNs (B) and lung mononuclear cells (C) were collected from mice treated as described in the legends to Figure 33 at day 7 p.i.. The cells were analyzed by four color staining for surface CD3 $\epsilon$ , CD4 and CD8 and intracellular IFN $\gamma$ . The representative flow cytometry dot plots are shown. The percentages of IFN $\gamma$  producing are shown in the upper right corner. Representative data of three independent experiments are shown.

## **IL-17-neutralized IL-10 KO mice show altered co-stimulatory molecule expression on DC**

To further explore the molecular basis for the reduced type-1 T cell responses in anti-IL-17 mAb treated IL-10 KO mice, we further examined the expression of co-stimulatory molecules on DCs of Cm-infected IL-10 KO mice with or without in vivo neutralization of IL-17. Purified lung DC were stained with fluorescence conjugated anti-CD11c and mAbs specific for co-stimulatory molecules related to DC functions (CD80, CD86 and CD40). As shown in Table 2, lung DC from IL-10 KO mice showed statistically significantly higher expression, compared to WT DC, of all the tested molecules, including CD80 (46.22% vs 26.27%), CD86 (36.23% vs 25.45 %) and CD40(15.97% vs 7.45%) following Cm infection. Interestingly, although its influence on CD80 expression was minimal, the neutralization of IL-17 dramatically reduced CD86 and CD40 expression on DC of IL-10 KO mice. Therefore, modulating DC function through altering co-stimulatory molecule expression is likely a mechanism by which the enhanced Th17 response enhances type-1 T cell responses in IL-10 KO mice following Cm infection.

|      | WT           | IL-10 KO       | IL-10 KO<br>+anti-IL-17 |
|------|--------------|----------------|-------------------------|
| CD80 | 26.27 ± 2.70 | 46.22 ± 1.97*  | 42.93 ± 1.44            |
| CD86 | 25.45 ± 1.72 | 36.23 ± 1.904* | 20.53 ± 2.04#           |
| CD40 | 7.45 ± 0.21  | 15.97 ± 0.760* | 9.985 ± 0.24#           |

**Table 2. In vivo neutralization of IL-17 reduced co-stimulatory marker**

**expression on DC from IL-10 KO mice following *Cm* infection.** Lungs of WT mice and IL-10 KO mice with or without anti-IL-17 mAb treatment were collected at day 7 p.i. and lung DCs were isolated using CD11c microbeads and LS column as described in *Material and Methods*. Cells were staining for surface markers and analyzed by flow cytometry. The percentage of CD80, CD86, CD40 positive CD11c<sup>+</sup> cells were summarized as mean±SD. \*,  $p < 0.05$  compared to WT mice; #,  $p < 0.05$  compared to IL-10 KO mice.

## Discussion

This chapter has three novel findings on the mechanism and function of Th17 responses. First, the study provided clear evidence that Th17 response is enhanced in the conditions of IL-10 deficiency following chlamydial infection. The enhanced Th17 response in IL-10 KO mice is supported by increased ROR $\gamma$ t expression in the infected tissues (lung), higher proportion of IL-17 producing CD3<sup>+</sup>CD4<sup>+</sup> T cells in a single cell level (intracellular IL-17 staining) and higher IL-17 protein production in a population level in the bulk cultures of spleen, draining LN and lung mononuclear cells. Notably, this is the first report showing that IL-10 KO mice mount enhanced Th17 response in an infection model. Second, the study showed a critical role of ICOS/ICOSL interaction in the development of Th17, but not Th1 responses. The DC of IL-10 KO mice expressed significantly higher levels of ICOS-L than those of WT mice following chlamydial lung infection. Most Th17 cells are ICOS positive while most Th1 cells are ICOS negative. Blockade of ICOS/ICOSL interaction dramatically decreased Th17 response without significant effect on Th1 response. Third, the enhanced Th17 response in IL-10 KO mice contributes significantly to host defense against chlamydial infection largely through promoting Th1 response via modulating DC function. It has been reported that IL-10 KO mice are better protected than WT mice following Cm lung infection(193, 194). The present study confirmed this finding and, more importantly, showed that the neutralization of IL-17 in these mice completely abolished the better protection in the IL-10 KO mice (Figure 33). Analysis of cytokine patterns of T cells and DC surface markers showed significant reduction of IFN $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 34) and the expression of

CD86 and CD40 molecules on the lung DC (Table 2) of IL-10 KO mice following neutralization of in vivo IL-17 activity.

The most important finding in this study is the critical role of ICOS/ICOS-L signaling for Th17 response in IL-10 KO mice. Notably, although the functional distinction of different CD4<sup>+</sup> T cell subsets (Th1/Th2/Th17) have been well documented, the molecular basis determining the skewing of the different T cell subsets remains unclear. Our present data showed that ICOS/ICOS-L interaction was particularly important for Th17 response but not for Th1 response, although the IL-10 KO mice showed enhanced responses for both Th17 and Th1 cells. Indeed, blockade of ICOS-L in the co-culture of DC:CD4 T cells significantly reduced the expansion of Th17 cells but failed to change Th1 response (Figure 30 vs. Figure 32). The results were consistent with our recent finding that ICOS-L KO mice mounted significantly lower Th17 response than WT mice following chlamydial infection (363). Although more mechanisms are likely involved, our data suggest a critical role of ICOS/ICOS-L interaction in creating a microenvironment suitable for Th17 responses including local cytokines. Specifically, in the DC:CD4<sup>+</sup> T cell co-culture experiments, we found that the levels of Th17 driving cytokines (IL-6, TGF $\beta$  and IL-23) were significantly higher in the co-culture with DC from IL-10 KO mice than with DC from WT mice. More importantly, the blockade of ICOS-L in the co-culture led to dramatic reduction of TGF $\beta$  and IL-23 although IL-6 levels remained high (Fig 30). Therefore, the ICOS/ICOS-L interaction is important for TGF $\beta$  and IL-23 responses. It has been well demonstrated that IL-6 alone cannot promote Th17 response, rather the co-existence

of TGF $\beta$  and IL-23 is critical for Th17 response. It has been well documented that TGF $\beta$  and IL-6 are critically important for the induction of Th17 cell differentiation while IL-23 is required for terminal differentiation and stabilization of Th17 cells (216). The association of the reduction of TGF $\beta$  and IL-23 caused by blockade of ICOS-L with the decreased Th17 responses suggests that the enhanced ICOS-L expression by DC in IL-10 KO mice may promote Th17 response not only through ICOS/ICOS-L interaction per se but also through the consequent enhancement of TGF $\beta$  and IL-23 levels in the microenvironment of T:DC interaction. Therefore, promoting Th17 driving cytokine production is likely an important mechanism by which ICOS/ICOS-L interaction directing Th17 response. This point is further supported by the fact that TGF $\beta$  and IL-23 production is higher in IL-10 KO mice than WT mice in addition to higher IL-6 production in all the tested organs including spleen, draining LNs and lung.

The importance of ICOS/ICOS-L signaling for Th17 response is not only reflected by its effect to increase IL-17 producing cells, but by its influence on the capacity of individual Th17 cells to produce IL-17. We found that not all Th17 cells express ICOS although most cells do, suggesting that not all Th17 cells need ICOS signalling for development. This is line with previous reports showing that ICOS KO mice displayed certain degree of Th17 response in chronic colitis mice models(216) and ICOS-L KO mice still had Th17 response although the level was significantly lower than WT mice following *Cm* infection(363). Importantly, we found in the present study that the “quality” of the ICOS<sup>-</sup> and ICOS<sup>+</sup> Th17 cells was different

when measured by their capacity to produce IL-17. Indeed, the MFI of IL-17 in ICOS<sup>+</sup> Th17 cells was significantly higher than that of ICOS<sup>-</sup> Th17 cells in the Cm-infected IL-10 KO and WT mice (Figure 29D). It is likely that the combination of the higher proportion of DC expressing ICOS-L and the higher levels (MFI) of ICOS-L on individual DC in the IL-10 KO mice (Figure 28) equipped these DC to be more effective and powerful for promoting ICOS positive, thus higher IL-17 producing, Th17 cells following chlamydial infection.

The present study has clearly shown a close association of enhanced Th17 response with the better protection of IL-10 KO mice against chlamydial lung infection. However, the protection is unlikely directly mediated by IL-17/Th17 through inhibiting chlamydial growth because IL-17 alone failed to inhibit chlamydial growth ((346)and data not shown). Instead, the enhanced Th17 response in IL-10 KO mice is more likely playing its protective role through promoting Th1 responses. As it was previously reported, the IL-10 KO mice mounted strong Th1 responses following Cm lung infection(193). The present study confirmed and extended this by showing higher IFN $\gamma$  producing CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in the spleen, draining LNs and lung tissues (Figure 34 and Table 1). More importantly, the study showed a close correlation between the Th17 response and type-1 T cell responses in the IL-10 KO mice. When IL-17 activity in IL-10 KO mice was blocked in vivo, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced much less IFN $\gamma$  to a level similar to, even lower than, WT mice. Although a direct effect cannot be excluded, the promoting effect of Th17 on type-1 T cell responses (Th1/Tc1) is more likely through modulating DC function. It

has been reported that DC of IL-10 KO mice expressed significantly higher levels of surface co-stimulatory molecules following chlamydial infection(194), which was confirmed by the present study (Table 2). Interestingly, we found that the blockade of IL-17 virtually completely abolished the enhanced CD40 and CD86 expression in IL-10 KO DC. We have shown previously that the expression of CD40 and CD86 by DC is closely associated with type-1 T cell responses in chlamydial infection(191). Therefore, the enhanced Th17 response may enhance type-1 T cell responses through modulating DC function. The enhancing effect of Th17 on Th1 response has been reported in another intracellular pathogen model, mycobacterial infection (228). It was found that the generation and persistence of Th17 is preceded IFN- $\gamma$  recall response and is important for initiation of Th1 protective response(228). Our finding on the modulating effect of Th17 on DC function in the context of IL-10 deficiency provides new insights on the mechanism by which Th17 cells modulate host responses to infections especially intracellular bacterial infections.

In summary, we found enhanced Th17 response in IL-10 KO mice. The enhanced Th17 response was dependent on higher ICOS-L expression on DC. ICOS/ICOS-L interaction is important for creating a microenvironment suitable for Th17 cell development/maintenance including enhancing the production of Th17-driving cytokines such as TGF $\beta$  and IL-23. The enhanced Th17 response in IL-10 KO mice can contribute significantly to protective immunity to chlamydial infection through promoting type-1 T cells via modulating DC function.

## **PART V General discussion, Conclusions and Future work**

### **5.1 Summary of major findings**

Allergy and asthma are the most frequent immunologic disorders at all ages in the world, and have received a great international attention. In contrast, infectious diseases have been largely controlled in many developed countries due to widely used antibiotics and vaccination. Many studies suggested that exposure to microbial infections or microbial products might inhibit the development or pathogenesis of allergic diseases. Our previous study confirmed that DC is critical in chlamydia/BCG infection-mediated the inhibition of the development of allergic reactions. The major focus of this thesis was on the mechanisms of the modulating effects of these intracellular bacterial infections on allergic reactions, especially on the role of DC/DC subsets and the related molecular basis. We also examined some aspects of the immune responses in BCG and *C.m* infection, especially those related to DC immunobiology.

In part III *chapter 1 and chapter 2*, we studied the protective role of DC subsets in BCG infection per se and in mediating the modulatory effect of BCG infection on allergic responses. We purified DC subpopulations based on their surface markers (CD11c, CD8) expression using MACS system or Flow Cytometry sorting and directly examined their ability to modulate CD4 T cell differentiation to protect challenge infection or prevent allergic reactions using *in vitro* (co-culture system) and *in vivo* (transfer of BCG-educated DC subsets into syngeneic mice) experiments. This work not only characterizes the particular DC subset, which is imprinted by BCG, but

also identifies the molecular basis for the changes in these DCs, which are responsible for altering CD4 T cell differentiation. We found that BCG immunization led to a preferential expansion of CD8<sup>+</sup>DCs, which showed different co-stimulatory molecule expression and cytokine profile from CD8<sup>-</sup> DC. BCG modified CD8<sup>+</sup>DC exhibited higher levels of mRNA and protein for IL-12, while CD8<sup>-</sup> DC showed higher IL-10 production. Adoptive transfer of CD8<sup>+</sup>DC, but not CD8<sup>-</sup>DC, from BCG immunized mice enhanced bacterial clearance and reduced pathological reactions in the infected tissues following BCG challenge. Both DC subsets from BCG-infected mice inhibited the OVA-induced mucus over-production and pulmonary eosinophilia inflammation, but involving different mechanisms. CD8<sup>+</sup> DC changed the balance of Th1/Th2 to allergen leading to reduced Th2 response through promoting Th1 response. However, the CD8<sup>-</sup> DC enhanced Treg activity, thereby inhibiting Th2 response.

In **Part III chapter 3**, we used *C.m*, another intracellular bacterial infection model, to examine the role of DC subsets in modulating the allergic reactions. In particular, we focused on the role of IL-10 in DC mediated inhibition of allergic reaction, because previous studies in our lab have demonstrated that *C.m*-infected CD8<sup>+</sup>DC produce higher IL-10, which is more inhibitory to allergic reactions. Purified DC subsets from *C.m* infected IL-10 KO mice were transferred to naive mice followed by ragweed sensitization/challenge. We monitored the allergic reactions in the mice treated with different DC subsets and found that Chlamydia infection imprinted DC subsets were also able to decrease ragweed-induced serum IgE level and eosinophil infiltration in airway. Only the inhibition of CD8<sup>+</sup>DC on allergy

depended on IL-10, but IL-10 deficient CD8<sup>+</sup>DC failed to prevent the development of allergic responses. (The expansion of TGF- $\beta$  producing Treg and CTLA-4 positive CD4 T cells in IL-10 deficient CD8<sup>+</sup>DC treated mice were dramatically dampened compared to recipient mice pretreated with WT-CD8<sup>+</sup>DC, which implied that inhibition of CD8<sup>+</sup>DC from *C.m* infected mice on allergic responses might through the TGF- $\beta$  or CTLA-4 mediated pathway.)

In **Part III chapter 4**, we investigated the role of IL-10 produced by DC in modulating Th cell responses in *C.m* infection. We demonstrated that IL-10 produced by DC can inhibit Th1 and Th17 responses in *C.m* infection. Th17 response was enhanced in IL-10 deficient mice, which contributed to host resistance to the infection through modulation on Th1 responses. More interestingly, we showed that ICOSL expression on DC is critical for Th17, but not Th1, development. Specifically, we found that IL-10 deficient mice showed higher percentage of ICOSL expressing DCs in the lung and spleen. Correspondingly, ICOS expression on Th17 cells was higher than that on Th1 cells. The data suggest that the ICOS signaling is very important for development of Th17, rather than Th1 immune responses in *C.m* infection.

## **5.2 The mechanism of DC subsets educated by BCG/*C.m* infection in inhibition of allergy.**

### **5.2.1 The role of different DC subsets from infected mice in inhibiting allergy**

In present study, two intracellular infection models (BCG and *C.m*) and two allergens (OVA and ragweed) were used to better understand the role of DC subsets in

infection modulating allergic responses and more generally of the influence of microbial agents on DC function. We have shown that both BCG and *Chlamydia* infections can modulate DC subsets which have a significant imprinting effect on the inhibition of allergy/asthma induced by natural or model allergens, ragweed or ovalbumin (OVA), although with different mechanisms. We found DC subsets showed multiple differences after BCG or *C.m* infection, which may be the molecular basis for DC subsets in generating protective immunity to challenge infection and modulation of allergic reaction. First of all, we found DC subsets were different in cytokine production. CD8<sup>+</sup>DC from BCG-infected (CD8<sup>+</sup>DC (BDG)) preferentially produced IL-12, whereas CD8<sup>-</sup>DC BCG-infected (CD8<sup>-</sup>DC (BCG)) mice produced higher IL-10. In contrast, *C.m* infection-modulated CD8<sup>+</sup>DC showed increased production of both IL-12 and IL-10 cytokines. The difference in cytokine production by DC subsets in these two infection models were consistent with mechanisms they implied to inhibit the Th2 immune responses. We demonstrated that both CD8<sup>+</sup>DC and CD8<sup>-</sup>DCs from BCG-infected mice can inhibit OVA-induced Th2 immune responses, although their capacity of inhibition and mechanisms used for the inhibition were different. Specifically, CD8<sup>+</sup>DC(BCG) pretreatment dramatically prevented OVA-induced airway eosinophil inflammation by redirecting the allergen-specific Th2 immune response and promoting Th1 response. In contrast, less pronounced but significant inhibitory effects were observed in recipient mice pretreated with CD8<sup>-</sup>DC(BCG). CD8<sup>-</sup>DC(BCG) efficiently inhibited allergic inflammations, but failed to trigger significant production of IFN- $\gamma$ . It implied that

immune deviation is not the major mechanism in CD8<sup>-</sup>DC(BCG)-mediated suppression of allergic response. The increased production of IL-10 in dLN of iCD8<sup>-</sup> DC treated mice indicated regulatory net induced in recipient mice.

Interestingly, in *C.m.*, another intracellular bacteria, infection model, we found significant difference in DC subsets for the modulatory effects of infection on allergy. We previously showed that the CD8<sup>+</sup>DC produced both higher IL-12 and IL-10 than CD8<sup>-</sup>DC in Chlamydia infection. In **Part III Chapter III**, we found that the adoptive transfer of CD8<sup>+</sup>DC(*C.m.*,WT), compared to CD8<sup>-</sup>DC(*C.m.*,WT), are much more capable of transferring inhibition against Rg-induced allergy. However, adoptive transfer of CD8<sup>+</sup>DC from IL-10 KO mice failed to show the same suppression on RW-induced airway eosinophilia inflammation, mucus over-production and Th2 related cytokine production as did CD8<sup>+</sup>DC(WT). These results suggested, in the Chlamydia infection model, CD8<sup>+</sup>DC mediated inhibition of allergy mainly depends on the IL-10, which is different from what was in BCG model.

Secondly, we found difference in surface molecular expression on the DC subsets. Specifically, we found that both infections induced higher expression of costimulatory surface markers (CD80, CD86, CD40) on iCD8<sup>+</sup>DC compared with iCD8<sup>-</sup>DC.

### **5.2.2 The mechanism of DC subsets in inducing immune deviation and Treg.**

It is generally accepted that some microbial infections may modulate Th2-like allergic responses by promoting immune deviation (toward Th1) and/or enhancing immune regulation, depending on the nature of the infectious agents, the time and duration of exposure to infection, the extent and stages of the infection, and the

genetic background of the hosts. Previous studies in our lab suggested that DCs play a critical role in *C.m* and BCG infection-mediated inhibition of allergy. DCs isolated from infected mice showed significant modulating effect on allergen-specific Th2 cell responses in in vitro and in vivo systems. The studies in the present thesis further explored and provided experimental evidence for the different mechanisms of DC subsets educated by infections in regulating allergic reactions.

In BCG infection model, we found CD8<sup>+</sup>DC(BCG) produced significantly higher levels of IL-12 than CD8<sup>-</sup>DC(BCG) or naïve DC subsets. Blockade of IL-12 dramatically reduced the Th1 enhancing effect, which was associated with a reversal of the inhibitory effect on Th2 cytokines responses in vitro (coculture of DC subsets with CD4 T cells). Therefore, CD8<sup>+</sup>DC from BCG infected mice can modulate allergic responses through immune deviation.

In recent years, it has become accepted that peripheral tolerance is mediated, at least partly, by various subtypes of regulatory T cells. One type of Treg expresses CD4 and CD25,  $\alpha$  chain of IL-2R, which presents 5-10% of unstimulated CD4 T cells in peripheral blood. One possible mechanism of immunosuppression by CD25<sup>+</sup>CD4<sup>+</sup> T cells is that these cells can secrete or cause secretion of TGF- $\beta$  and/or IL-10. Another mechanism is the cytotoxic T lymphocyte associated antigen 4 (CTLA-4) mediated inhibition, which is a potent negative regulator of T cell immune response. A recent studies have confirmed that CTLA-4 is essential for Treg to maintain immune homeostasis(334). (However, it was also reported that Treg have acquired compensatory suppressive mechanisms in the absence of CTLA-4, enhanced TGF- $\beta$

expression *in vitro*(365) and more IL-10 dependent inhibition *in vivo*(366).) In **chapter II and chapter III**, we further detected different subtypes of Treg in recipient with CD8<sup>-</sup>DC or CD8<sup>+</sup>DC from BCG or *C.m* infected mice. We found that high IL-10 producing DC subsets (CD8<sup>-</sup>DC in BCG infection and CD8<sup>+</sup>DC in *C.m* infected mice) are efficiently able to increase FoxP3<sup>+</sup> CD4 T cells, CTLA-4<sup>+</sup> CD4 T cells and TGFβ-expressing CD4 T cells in subsequent? allergic mice after adoptive transfer.

In BCG infection, the adoptive transfer of iCD8<sup>-</sup>DC(BCG) induced the expansion of surface TGFβ expressing CD4 T cells in local? lung, which may imply that TGFβ-mediated cell contact dependent mechanisms are important for the immunosuppressive ability of CD4 T cells in the recipient of iCD8<sup>-</sup>DC(BCG). Membrane-tethered TGF-β can mediate and amplify regulatory function of Treg in a cell-cell contact-dependent manner (367). Heme oxygenase-1 attenuated OVA-induced airway inflammation through membrane-tethered TGFβ and IL-10 secretion by Treg cells(310). Another study also showed that surface TGFβ was vital in the immune-suppression of CD4 T cells in the allergic tolerance induced by repeated exposure to a low dose of aerosolized antigen(367). In our study, iCD8<sup>-</sup> DC(BCG) preferentially expanded both IL-10 positive regulatory T cell and TGFβ positive regulatory T cells *in vivo* and *in vitro*. Both may contribute to the iCD8<sup>-</sup>DC-mediated inhibition of allergic inflammation. (The extent to which they are used to inhibition of allergic response and whether their function is overlap in this process need further refinement.)

In **chlamydia** infection, our results showed a lower Th2 cytokine production in iCD8<sup>+</sup> DC recipients. Since CD8<sup>+</sup>DC (*C.m*) produced higher IL-12 and IL-10 than CD8<sup>-</sup>DC (*C.m*), we further examined the role of IL-10 produced by CD8<sup>+</sup>DC from Chlamydia infected mice on allergic reactions, particularly on the Treg development in **chapter III**. (For Treg development, they may be induced by immature DCs or the DCs with altered maturation status.) Some specialized DCs, which, even in their fully mature state can induce Treg. Levings et al proposed a model of two-step differentiation of Treg (91). At the first step, naive T cells encounter antigen presented by DC in the presence of IL-10 and possibly TGF $\beta$ , becoming hyporesponsive to the antigen through a cell-cell contact dependent process. At the second step, following repeated antigen exposure, these T cells gain their ability to produce IL-10 and TGF $\beta$ , mediating suppression through a cytokine-dependent and cell-cell contact independent mechanism. The fully differentiated Treg is more potent in its suppressive activity than anergic T cells. Our data showed that FoxP3<sup>+</sup>, CTLA-4<sup>+</sup>, CD25<sup>+</sup> and CD4<sup>+</sup> Treg increased in the CD8<sup>+</sup>DC (*C.m*) treated mice, which demonstrated less airway allergic inflammation after allergen exposure. However, in the absence of IL-10, CD8<sup>+</sup>DC (IL-10KO, *C.m*) fail to increase these FoxP3<sup>+</sup>, CTLA-4<sup>+</sup>, CD25<sup>+</sup> and CD4<sup>+</sup> Treg in both lymphoid organ (spleen) and local inflammatory site (lung). CTLA-4 has been demonstrated to engage B7 family on APC cells and induce indoleamine 2,3-dioxygenase expression, leading to the degradation of tryptophan and resulting in inhibition of T cell proliferation (368, 369). It is possible that CTLA-4 may contribute to the regulatory network through sending a negative signal to DC or helping the

secretion of suppressive molecules in the local inflammatory environment or lymphoid organs. IL-10 facilitates the differentiation of CTLA-4 expressing cells in both spleen and lung, but it has a negative effect on TGF- $\beta$  production in the lung. This suggested that TGF- $\beta$  expression could be induced, at least partly, independently of CTLA-4 and IL-10.

We proposed that IL-10-producing DCs are central to the maintenance of tolerance by controlling allergic T cell activity. But what mechanisms were involved in iCD8<sup>-</sup>DC (BCG)-mediated inhibition of allergy needs more effort. Further understanding the activity of these molecules may help design more effective therapeutics for allergic diseases.

Interestingly, we found some differences in DC subset phenotypes in two different mouse strains (C57BL/6 and Balb/c). Specifically, we found the higher level of IL-10 and IL-12 production in DC subsets isolated from C57BL/6 mice compared to those from Balb/c mice. Moreover, we found that CD86 expression was enhanced on both CD8<sup>+</sup>DC and CD8<sup>-</sup>DC subsets after BCG infection in C57BL/6 mice, but the CD86 expression only significantly increased on CD8<sup>-</sup>DC in Balb/C mice. The significance and relevance of these differences in DC subsets between the two strains of mice remain to be further studied.

In summary, in BCG infection model, we showed that both immune deviation and immune regulation mechanisms can operate in a single type of infection, and this coordination can be done by DC subsets. More importantly, we have shown that the CD8<sup>+</sup> and CD8<sup>-</sup>DC subsets may be the basis for immune deviation and immune

regulation mechanisms, respectively. However, in this particular infection, immune deviation appears to be the more dominant mechanisms. CD8<sup>+</sup>DC(BCG), which produce more IL-12 may prove to be a potential ideal candidate for vaccine development against allergy. In contrast, in the *C.m* infection model, CD8<sup>+</sup> DC (*C.m*) secreted IL-12 and IL-10 at significantly higher levels than CD8<sup>-</sup> DC (*C.m*) or either subset taken from naïve mice. (The lower Th2 cytokine production in iCD8<sup>+</sup> DC(*C.m*) recipients can be a combinational outcome of higher IL-12 production by this DC subset, thus inducing higher IFN $\gamma$  production by T cells and the inhibitory effect of IL-10 produced by DC which inhibit Th2 cell development via direct suppression of Th2 cells and/or the development of regulatory T cells.) Our study on the role of IL-10 in **Chapter III** confirmed that immune regulation is a dominant mechanism in the iCD8<sup>+</sup>DC (*C.m*) mediated inhibition of allergy. The finding that different DC subsets from different infected mice are different in their capacity to inhibit allergic responses with different mechanisms may be helpful in understanding the previous reports on the role of DC in immunobiology in relation to allergic disease mechanisms, opening the door for further allergy-DC-related studies. Because DCs are exquisitely sensitive to environmental signals, there is considerable interest in the development of therapeutic interventions targeting specifically at DC. Since DC subsets from infected mice are different in modulating allergic responses, simply targeting DC without discriminating subsets may not be efficient enough to achieve the goal. There is clearly a need to develop a greater understanding the mechanism by which DC and their subsets are activated/induced and more importantly manipulated

to be more capable of inhibiting allergic responses to allergens, in order to either prevent and/or treat allergic diseases.

### **5.3 The role of DC subsets in generating protection against mycobacterial infections**

To further understand that how DC subsets were manipulated by infectious component exposure, we also detected capacity of infection-modulated DC/DC subsets to direct Th cell development in bacterial challenge. Firstly, we found the functional difference of the DC subsets in protection against subsequent BCG challenge. We demonstrated remarkable differences in protective efficacy of different DC subsets. CD8<sup>+</sup> DC from BCG-infected mice can initiate the protective immunity demonstrated as less bacteria load and limited inflammation in the lung. However, CD8<sup>-</sup> DC (BCG) could not protect the subsequent challenge infection. CD8<sup>-</sup> DC(BCG) pretreated mice demonstrated more diffused inflammation, instead of granuloma formation. The difference in protection was associated with the frequency and amount of different cytokines production and the pattern of costimulatory molecule expression on DC subsets. IFN- $\gamma$  is the principal macrophage-activating cytokine and stimulates the synthesis of reactive oxygen intermediates, nitric oxide reactive nitrogen intermediates, which are important mechanisms against the mycobacterial tuberculosis (291, 370). The early presence of IL-12 from CD8<sup>+</sup>DC treated mice initiate the development of protective Th1 immune response at the very early stage of tuberculosis infection is likely the molecular basis for the higher Th1 response and better protection in the recipient of this DC subset. In another intracellular bacterial,

*Listeria monocytogenes*, infection model, the CD8<sup>+</sup> DCs are principal population that initiate CD8<sup>+</sup>T cell immunity (371). Sher et al. have found that CD8<sup>+</sup> DCs produce IL-12 in order to induce protective CD4<sup>+</sup> T cell responses in *Toxoplasma* infection(372). Our data have demonstrated the critical role for CD8<sup>+</sup>DC(BCG) in protecting host against BCG infection, which is associated with higher IL-12 production by this DC subset.

#### **5.4 The role of IL-10 produced by DC/DC subsets in the induction of Th17 response in Chlamydia infection.**

After identifying that IL-10 production is increased by the DC after Chlamydia infection, we further explored the role of IL-10 in DC mediated immune regulation in Chlamydia infection in **Part III chapter IV**. Although the distinct function and differentiation of different CD4 T cells (Th1/Th2/Th17/Treg) have been well-documented, the molecular basis determining the skewing remains unclear. The data showed that IL-10 KO mice were more resistant to *C.m* infection and had an enhanced Th17 immunity in conjunction with increased Th1 response. Less body weight loss, less bacterial load and milder lung inflammation were found in IL-10 KO mice, which was associated with dramatical increase of IL-17 production. (The better protection in IL-10 KO mice lost when the mice were treated with IL-17 neutralization antibody.) Further experiments showed that Th17 differentiation-related cytokines were increased in spleen, dLN and lung of IL-10 KO mice. It is widely accepted that TGF- $\beta$  plus IL-6 induce Th17 cell differentiation, while IL-23 terminally stabilizes the Th17 phenotype (216). Our data from both *in vivo* and *in*

*vitro* studies supported that DC from IL-10 KO mice produced more of these Th17-differentiation related cytokines in the microenvironment. Moreover, we found a critical role of ICOS/ICOS-L signaling for Th17 response in IL-10 KO mice. (Out data demonstrated that ICOS pathway dedicated to the Th17 response in chlamydia infection). More importantly, most IL-17 producing CD4 T cells were ICOS expressing cells. When ICOS-L was blocked by its specific antibody, enhanced Th17 response was attenuated *in vitro*. ICOS/ICOS-L interaction is important for Th17 responses. ICOS ligand KO mice have less Th17 than their wild-type counterparts (373), and ICOS promotes IL-17 synthesis on colonic intraepithelial lymphocytes(374). Altogether, these findings indicate that ICOS is a contributing factor in enhancing Th17 responses. (The effect is speculated through the direct interaction of ICOSL<sup>+</sup> DC with ICOS on the effector T cells, then promoting the cytokines production favoring Th17 differentiation.)

In this part of study, we also found that both IFN- $\gamma$ -producing cells and IL-17-producing cells were enhanced in the KO mice, which were correlated with the cessation of bacterial growth. Further investigation of the relationship of Th17 and Th1 showed that the enhanced Th1 immune response in IL-10 KO mice dramatically recuperated after IL-17 neutralization. In Mycobacterial infection model, the generation and persistence of Th17 precedes the IFN- $\gamma$  recall response, and Th17 is important for initiation of Th1 protective response(228).

### **5.5 Future work:**

1) Further exploration of the molecular mechanism of immune regulation in DC mediated inhibition of allergy is required. We have found that both BCG and *C.m*

infection can modulate DC/DC subsets in their cytokines production and surface molecular expression and can induce different types of T cells responses. However, little is known about how these different molecules work synergistically to promote immune regulation. Some studies showed that at least three molecular characteristics may be relevant to the ability of tolerogenic DCs: the production of cytokines (especially IL-10, TGF $\beta$  and IFN $\alpha$ ), the expression of intracellular enzyme (IDO) and the presence of surface markers (ICOSL, OX-2, PD-1). Indeed, all these factors, individually or in combination, are particularly important for inducing Tregs (263, 375-377). Further study is necessary to explore the molecular mechanisms behind infection-mediated inhibition of allergy.

2) The relationship between DC phenotype/lineage and the role of DCs in immune regulation is a topical and controversial issue. The heterogenous DCs can be grouped into plasmacytoid and myloid DC, in addition to CD8<sup>+</sup> and DC8<sup>-</sup> DC. We have demonstrated significant modulating effects of chlamydial and mycobacterial infections on DC function and significant expansion of CD8<sup>+</sup> DCs, but little is known about how chlamydial and mycobacterial infections modulate mDC/pDC and whether they play any roles in host defense against the infection and allergen.

3) We have shown in thesis that the mechanisms by which CD8<sup>-</sup>DC subsets in BCG infection and CD8<sup>+</sup>DC in *C.m* infection can induce Treg. However, the characteristics of Tregs in BCG or *C.m* infection-mediated inhibition of allergy have not been studied in detail. Different types of Treg may be involved in the bacterial infection-mediated modulation of allergy. The inducible Tregs include Tr1s (which mainly secrete IL-10), Th3s (which mainly secrete TGF $\beta$ ), converted FoxP3<sup>+</sup> Tregs and possibly others. Recently, a Th1-like Treg that produces both IL-10 and IFN $\gamma$  was reported(305), and more interestingly, a highly suppressive antigen-specific T cell

sharing features of Th17/Th1 and regulatory T cells was also reported (378). Further exploration of the nature of Treg in these models will deepen our understanding on hygiene hypothesis.

4) The Th17 had significant influence on allergy. Our data showed that *C.m* infection could induce Th17 responses, which was enhanced in IL-10 deficient mice. However, the role of the Th17 response in modulating allergic reaction remains to be tested. In particular, the role of Th17 in allergic responses is currently controversial. Some studies showed that neutralization of IL-17 prior to OVA challenge increased the Th2 cytokine levels, suggesting inhibitory role of Th17 in allergic responses(218, 243). Conversely, other studies showed that IL-17 enhanced eotaxin production and IL17 neutralization inhibited Th2 diseases (379, 380). In our infection model, IL-10 deficient DCs preferentially promote Th17 responses. What is the role of Th17 responses in infection-mediated inhibition of allergy needs further investigation.

Altogether, our novel findings address critical unresolved questions regarding hygiene hypothesis and provide significant insights into better understanding the mechanisms underlying the modulatory effects of infection on allergy/asthma, especially the relevant molecular and cellular basis. Our studies facilitate the rational development of new preventive/therapeutic approaches to both allergic and infectious diseases.

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