A study on the role of lung dendritic cells and their interaction with innate lymphocytes in host defense against a bacterial lung infection

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By

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THESIS ABSTRACT

Chlamydia is an obligate intracellular bacterial pathogen that causes a wide spectrum of diseases worldwide. At present, there are no vaccines to prevent chlamydial infections due to poor understanding of how anti-chlamydial immunity ensues. In this study, we employed a variety of *in vitro* and *in vivo* systems, including knockout (KO) mice and adoptive transfer, to investigate the role of lung dendritic cells (LDCs) and their relationship with innate lymphocytes, natural killer (NK) and invariant NKT (iNKT) cells, in host defense against chlamydial lung infections in mice. We found that iNKT cells altered the phenotype and cytokine production pattern of LDCs following *C. pneumoniae* infection. Adoptive transfer of LDCs from infected Jα18-KO mice, which lack iNKT cells, into naïve wild-type (WT) mice promoted Th2 (IL-4) immunity following infection challenge, whereas the transfer of LDCs from the infected WT mice induced protective Th1/Tc1 (IFN-γ) immunity. On the other hand, upon adoptive transfer, LDCs from *C. muridarum*-infected NK-cell-depleted mice (NK-LDCs) conferred reduced protection after chlamydial challenge than the recipients of LDCs from infected sham-treated mice (NK+LDCs). NK+LDC recipients exhibited an enhanced Th1/Th17, in contrast to Th2, response compared to the NK-LDC recipients. In coculture experiments, NK cells isolated from the infected mice promoted IL-12p70, IL-6, and IL-23 production by LDCs through NKG2D receptor signaling. These findings indicate that iNKT and NK cells condition LDCs to confer protective Th1/Tc1/Th17 immunity against chlamydial lung infection.

We also analyzed the contribution of major LDC subsets, CD103+ and $CD11b^{hi}$

LDCs, in host defense against *C. muridarum* infection. We found that CD103+ and CD11b^{hi} LDC subsets expanded following chlamydial infection. CD103+ LDCs showed higher expression of costimulatory molecules and greater production of Th1- and Th17-inducing cytokines (IL-12, IL-6 and IL-23) than CD11b^{hi} LDCs. Coculture of *Chlamydia*-specific CD4+ T cells with LDC subsets revealed that the T cells cultured with CD103+ LDCs produced larger amounts of IFN-γ and IL-17 compared to those with CD11b^{hi} LDCs. To test their function *in vivo*, we isolated CD103+ and CD11b^{hi} LDC subsets from infected mice and transferred them into naïve syngeneic mice that received chlamydial challenge. CD103+ LDC-recipients showed better protection, as evidenced by their reduced body weight loss, bacterial burden and lung pathology, than CD11b^{hi} LDC recipients. Mice that received CD103+, compared to CD11b^{hi}, LDCs produced enhanced Th1/Th17 cytokines (IFN-γ and IL-17) in the lung and the MLNs. In conclusion, these findings demonstrate that CD103+ LDCs are more efficient in inducing Th1/Th17 immunity to chlamydial infection than CD11b^{hi} LDCs.

Taken together, our findings have provided direct *in vivo* evidence on the role of LDCs and their conditioning by iNKT and NK cells in generating mucosal T-cell immunity against a bacterial lung infection. The findings have added new knowledge to the field of lung immunology, which have implications for developing prophylactic and/or therapeutic strategies against respiratory diseases.

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DEDICATION

I dedicate this thesis to the memory of my brother, Mr. Himanshu Shekhar, MA, LLB.

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Figure 1: Dendritic cell (DC) subsets in the airways. (Reprinted with permission from Macmillan Publishers Ltd: Mucosal Immunology, volume 1 (6): 442-450. © 2008)

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL FORM
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
APC	Allophycocyanin
APCs	Antigen-presenting cells
BSA	Bovine serum albumin
CCR	Chemokine Receptor
CD	Cluster of differentiation
DCs	Dendritic cells
DC-SIGN	DC-specific intracellular adhesive molecule-3-grabbing non-integrin
EB	Elementary body
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent activated cell sorting
FcR	Immunoglobulin Fc receptors
FITC	Fluorescein isothiocyanate
HE	Hematoxylin and eosin
IFN	Interferon
IL	Interleukin

i.n.	Intranasal
iNKT	Invariant natural killer T
i.p.	Intraperitoneal
i.v.	Intravenous
iLDCs	Inflammatory LDCs
Lung DCs	LDCs
LNs	Lymph nodes
MHC	Major histocompatibility complex
MLNs	Mesenteric lymph nodes
NK	Natural killer
NKT	Natural killer T
PPRs	Pattern recognition receptors
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy7
PerCP	Peridinin chlorophyll
pDCs	Plasmacytoid DCs
pLDCs	Plasmacytoid LDCs
RB	Reticulate body
SPG	Sucrose-phosphate-glutamic acid buffer
TCR	T cell receptor
Tc	T cytotoxic

Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tfh	T follicular helper
Treg	Regulatory T cell
UV	Ultraviolet

1.0. CHAPTER 1

GENERAL INTRODUCTION

1.1.0. CHLAMYDIAL DISEASES

1.1.1. Etiology and Epidemiology

Chlamydia is an obligate intracellular bacterium. It causes a wide spectrum of diseases in humans (1). These diseases are referred to as chlamydial diseases or chlamydiosis. Chlamydial species, which are pathogenic to humans, include *C. trachomatis* and *C. pneumoniae* (2-9). Chlamydial infections afflict humans with various diseases, posing a menace to the public health worldwide. *C. trachomatis* is the most common cause of bacterial STDs. It causes 90 million cases of STDs occurring each year across the globe, with approximately 3 million cases alone in the United States (10). *C. trachomatis* can be transmitted during sexual intercourse and from infected mother to the baby during parturition. The clinical manifestations of its genital infection in women include cervicitis, pelvic inflammatory disease and ectopic pregnancy, whereas prostatitis and epididymitis in men. Some strains of *C. trachomatis* cause lymphogranuloma venereum, which is a venereal disease with genital lesions. Upon exposure to chlamydial infection, women are more likely to become infected with Human Immunodeficiency virus and human papilloma virus-induced cervical neoplasia (11-12). Apart from genital tract infection, *C. trachomatis* causes trachoma, which is an important cause of infectious blindness worldwide that affects about 84 million people (13-14). Pathologic lesions in trachoma include the development of follicles and inflamed conjunctivae that lead to cloudy and vascularized cornea. Transmission of *C. trachomatis* for trachoma takes

place by contaminated fingers or fomites or through placenta in infected mothers (5).

C. pneumoniae causes a variety of respiratory diseases, including sinusitis, pharyngitis, bronchitis and community-acquired pneumonia, which are common throughout the world (15). A higher prevalence of chlamydial infection is however noted in third world countries compared to the developed ones. In recent years, there are various reports based on epidemiological, immunological, and pharmacological studies that indicate a relationship between *C. pneumoniae* infection and causation of cardiovascular and neurodegenerative diseases, such as atherosclerosis, Alzheimer's disease, and multiple sclerosis (7-9). A significantly higher titer of antibodies specific to *C. pneumoniae* was found in the patients with atherosclerosis compared with control patients. Furthermore, Chlamydiae were significantly found to be present in the brain of Alzheimer's patients compared to the controls (7,8). Humans are the only known reservoir for *C. pneumoniae*. Transmission of *C. pneumoniae* occurs from person to person by inhaling aerosols containing the bacteria. These bacteria in aerosol can survive at room temperature, but their infectivity is diminished with passage of time. Seroepidemiological studies have shown that antibodies specific for *C. pneumoniae* are found in a large proportion of the population (16-18).

To treat chlamydial infections in humans, a range of broad spectrum antibiotics such as erythromycin and tetracycline are available. Although antibiotics are effective against *Chlamydia,* an accurate and timely diagnosis of chlamydial infections presents a challenge to the clinician due to their diverse clinical manifestations. Population-based

screening and antibiotic treatment for chlamydial diseases are not a cost-effective and pragmatic option for medical care. The widely accepted medical opinion is that the effective vaccination would be the most efficient strategy to confer protection against chlamydial diseases (5). **Despite a number of attempts, it still remains a challenge to develop a safe and effective chlamydial vaccine due to inadequate knowledge of protective immunity and immunopathology of chlamydial infections.**

1.1.2. Chlamydial life cycle

The life cycle of *Chlamydia* is biphasic consisting of two distinct forms, elementary body (EB) and reticulate body (RB) (5). EB is an extracellular and metabolically inactive form, which is responsible for dissemination of infection from one person to another. On the contrary, RB is an intracellular and metabolically active form. EBs attach and enter the epithelial cells through endocytosis. Following their entry into the cell, the EBs undergo germination to give rise to RBs. The RBs so formed multiply by binary fission. These RBs are also called as inclusion bodies because of their presence within chlamydial inclusion in the cell. Some of the RBs change into EBs, which are released by the cells to infect more cells (Fig. 1).

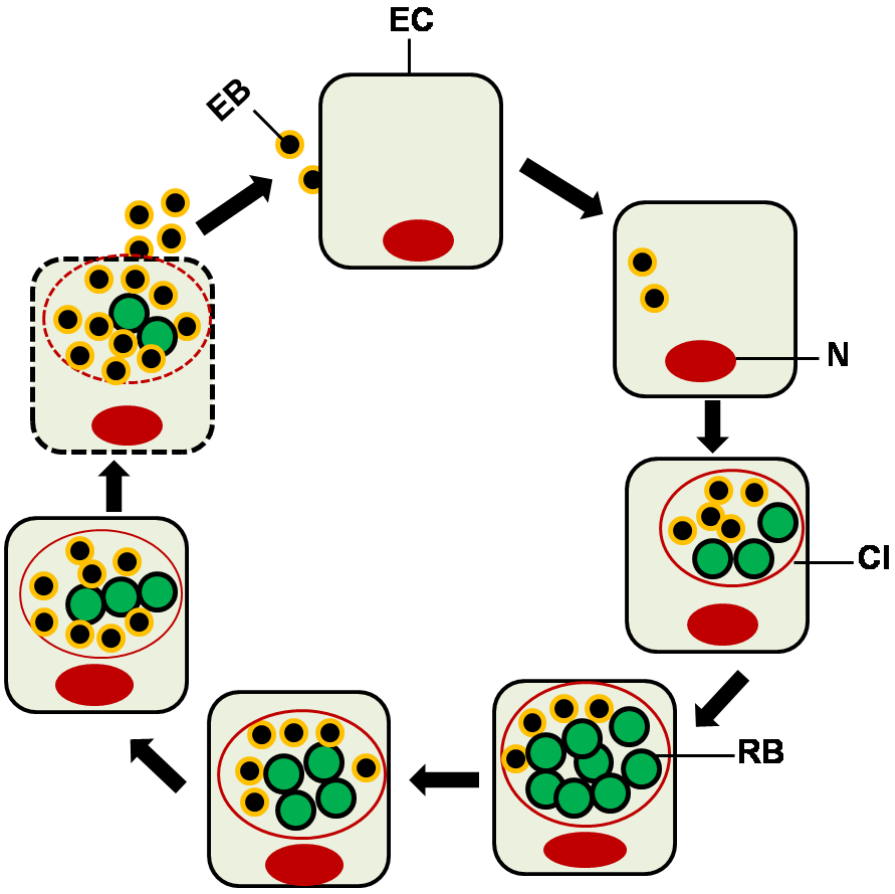

Figure 1. Chlamydial biphasic developmental cycle. *Chlamydia* completes its developmental cycle in two different forms – EB and RB. EBs infect epithelial cells. After entering the cell, a vacuole, referred to as inclusion, is formed around the EB. Inside the inclusion, EBs change into larger metabolically active RBs. RBs then undergo multiplication to give rise to EBs, which are subsequently released to infect the other epithelial cells. Abbreviation: N – Nucleus; EC – Epithelial cell; Cl – Chlamydial inclusion

1.1.3. Pathogenesis of chlamydial diseases

Coexistence of *Chlamydia* and its host imposes an evolutionary pressure on both of them. The host's immune system has developed to defend the body from chlamydial infections, whereas *Chlamydia* is equipped with various evasion mechanisms to escape the host's immune system. The mechanism of how *Chlamydia*-host interactions result in the development of disease and tissue damage remains poorly understood. Data from human and animal studies demonstrate that infection with *Chlamydia* leads to the activation of mucosal epithelial cells. Activation of epithelial cells induces secretion of multiple cytokines and chemokines, such as IL-1, TNF-α, IL-8, GM-CSF, and IL-6, which cause infiltration of immune cells at the primary site of infection (19,20). These immune cells include, but not limited to, neutrophils, monocytes, natural killer (NK) cells, and T cells. Infected epithelial cells and neutrophils secrete potent proteolytic enzymes like elastase and MMPs to cause tissue damage (19-21). **Persistence of chlamydial infection can lead to the continuous release of proinflammatory cytokines from the epithelial cells which results in tissue damage.**

Immune responses have also been held responsible for tissue damage. Although IFN-γ+CD4+ T cells induce immunity to chlamydial infection, they might have detrimental effects on the primary site of infection resulting in collateral damage (22). CD4+ T cells producing IL-4 can elicit immunopathology via suppression of protective responses (23). Autoreactive T cells specific for *Chlamydia* and host proteins such as heat-shock protein 60 have also been described, although the mechanism of their development can be assigned to the phenomenon of molecular mimicry (24). Molecular

mimicry refers to the similarities between self and foreign antigens that cause cross-activation of immune cells by microbial antigens. A reduced pathology in IL-10 knockout mice, compared to the wild-type (WT), during *Chlamydia*-infected mice suggests a detrimental role for IL-10 in this infection model (25-26). **Therefore, overt responses by immune cells can culminate into pathology during chlamydial infection.**

1.1.4. Animal models of chlamydial infection

A variety of animal models have been used to understand the immunobiology of chlamydial infections. These animals include, but not limited to, nonhuman primates, rabbits, guinea pigs and mice (27-32). Mice are, however, the most preferred animal model due to the fact that they show many similarities with human chlamydial diseases. In addition, various reagents and transgenic varieties are available to study chlamydial infections in mice. In particular, murine models have been extensively used to better understand the protective immunity and pathogenesis of chlamydial respiratory diseases caused by *C. pneumoniae* and *C. muridarum* (a mouse biovar of *C. trachomatis*). *Chlamydia* infects mice through their respiratory route and run a prolonged course. Disease development in *Chlamydia*-infected mice can be determined by bacterial burden, lung pathology and body weight loss. Although mice start losing their body weight following *C. muridarum* infection, they would gain the body weight after 2-3 weeks of the infection. The main chlamydial target happens to be the pulmonary epithelial cells and macrophages. Infection induces severe inflammatory and pathologic changes in the lungs as reflected by patchy interstitial pneumonitis, heavy infiltration of inflammatory cells and loss of tissue texture. Aggregation of lymphoid cells

in the perivascular and peribronchial area is a pathognomonic microscopic lesion of chlamydial pathology (5, 32).

1.1.5. Host immunity to chlamydial infection

Emerging evidence demonstrates an important role for innate and adaptive immune responses against chlamydial infections. Innate immunity elicits an early and rapid immune response, acting as the first line of defense. Chlamydial infections activate the components of innate immunity, such as dendritic cells (DCs), macrophages, NK cells, NK T (NKT) cells, γδ T cells, and pattern recognition receptors (PRRs). These cells not only induce innate but also influence adaptive immune responses (5). On the other hand, adaptive or acquired immunity consists of humoral and cell-mediated immunity. Humoral immunity involves B cells that generate antibodies to neutralize microbial antigens, whereas cell-mediated immunity involves T cells that interact with peptide antigens presented on major histocompatibility complex (MHC) molecules to elicit antigen-specific responses against intracellular pathogens. **In murine models of *C. trachomatis* infection, there was no difference between B cell-knockout (KO) mice that lack antibody production and WT mice for protection against challenge infection, suggesting that humoral immune responses do not play a significant role in protection (33-34).** In contrast, many studies of animal models have demonstrated that T-cell immunity is critical for resolution of chlamydial infections. Chlamydial infections however induce differential T-cell responses depending upon various factors, most notably the site of infection and bacterial strain. In genital infection of *C. trachomatis,* CD4+, but not CD8+, T cells are indispensable for resolution of

primary as well as secondary infections (35-38). Although both CD4+ and CD8+ T cells contribute to immunity to *C. pneumoniae* lung infection, the predominant role is played by CD8+ T cells (39-41). The predominant role of CD4+ and CD8+ T cells in *C. muridarum* and *C. pneumoniae* infection appears to be due to differences in nature of pathogens. The type of T-cell immunity has a **profound effect on whether the infection is contained or culminated into pathology. T-cell immunity, which is characterized by IFN-γ production by CD4+ and CD8+ T cells that induce Th1 and Tc1 responses respectively, promotes protective immunity (23, 40-45).** It is also shown that IL-17/Th17, in cooperation with Th1, responses exert anti-chlamydial adaptive immunity. However, some studies suggest a pathogenic role for Th17 in chlamydial infections (46-48). On the other hand, Th2 immunity characterized by secretion of cytokines such as IL-4 and IL-5 leads to inflammatory and pathologic changes (49). IL-10 has also been annexed with a pathologic response (25-26). **In summary, Th1/Tc1 and Th17 responses are important for resolution of chlamydial infection, whilst Th2 responses result in pathologic changes.**

1.2.0. DCs

1.2.1. What are DCs?

DCs represent one of the most important immune cells. They are so named because of their morphological resemblance to the branches of trees (Greek, dendron, tree). Dr Paul Langerhans (1868), a German physician and anatomist, for the first time described DCs in the skin, also known as Langerhans cells, but he mistakenly identified the cells

as part of the nervous system. **The credit goes to Prof Ralph Steinman (1973) for establishing DCs as a new class of immune cells with distinct morphological and physiological properties (50)**. In recognition of his work, Prof Steinman was awarded Nobel Prize in Physiology or Medicine in 2011. The hallmark of DC function is to process and present foreign antigens to naïve T cells to generate an adaptive immune response, thus acting as the most potent antigen-presenting cells (APCs). Since DCs not only play a role in innate immunity but also orchestrate adaptive immunity, they are considered as a bridge between innate and adaptive immunity (51).

1.2.2. Phenotype and function of DCs

DCs originate in the bone marrow and then move to peripheral tissues like the lung and intestine. In these tissues, DCs remain in an immature state characterized by lower expression of MHC-II molecules, and costimulatory molecules (CD40, CD80, and CD86) (50). Upon sensing ‘danger signals’, the immature DCs turn into mature DCs, thereby upregulating MHC-II and costimulatory molecules, and secreting various cytokines (e.g. IL-12, IL-6, and IL-10) (50). Recognition of microbes by DCs is done by PRRs such as Toll like receptors (TLRs) and then internalized by phagocytosis/pinocytosis. The ligands for TLRs are conserved among various microbes, e.g. lipopolysaccharides (LPS), lipoproteins, etc. Mature DCs then home to the tissue-draining lymph nodes (LNs), mainly through the expression of CCR7, a chemokine receptor for which CCL21 is the cognate ligand on the endothelial cells (52-53). In the LNs, DCs process and present the antigens on MHC molecules to naïve T cells to induce an adaptive immune response. Priming of T cells by DCs involves the interaction

of CD80 (B7-1)/CD86 (B7.2) and CD40 with CD28/CTLA4 (CD152) and CD40L on T cells, respectively. In addition to the costimulation, secretion of cytokines by DCs is a key to the differentiation of naïve T cells into different Th cells. For example, IL-12 production induces T cell differentiation into Th1 cells, while IL-4 and IL-10 promote Th2 cell differentiation (50).

DCs are heterogeneous consisting of various subsets. Broadly, DCs can be divided into plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDCs represent a small subset of DCs that enter the lymph nodes through the blood circulation. These DCs express lower levels of CD11c, MHC-II, and costimulatory molecules on their surface, but higher levels of TLR-7 and TLR-9 inside the cell. An important functional characteristic of pDCs is spontaneous production of IFN-α (54). On the other hand, cDCs can be classified into two groups, lymphoid and non-lymphoid/tissue DCs. Depending upon the expression of CD8α on their surface, cDCs residing in the lymphoid organs such as the spleen and the LNs can be divided into CD8α+ and CD8α- DCs. In addition, cDCs in the non-lymphoid tissues such as the intestine and the lung consist of two major subsets, CD103+ and $CD11b^{hi}$ DCs. Functionally, CD8α+ and CD8α- DCs are distinct. While CD8α+ DCs induce Th0 cells to elicit a Th1 response (IFN-γ and IL-2), CD8α- DCs induce a Th2 response (IL-4 and IL-10). A growing body of evidence points out that CD8α+ DCs are quite potent at cross-presenting and cross-dressing antigens to generate an effective CD8+ T cell response (55). In cross-presentation, DCs internalize and present exogenous antigens onto MHC-I molecules, whereas cross-dressing stands for the transfer of unaltered MHC-I/peptide complexes

between cells (56-57).

1.2.3. Heterogeneity of LDCs

With recent advancements in LDC biology, it is now possible to characterize different LDC subsets in mice (Fig. 2). Analogous to lymphoid DCs, LDCs can be broadly classified into conventional LDCs (cLDCs) and plasmacytoid LDCs (pLDCs). pLDCs express lower levels (dim) of CD11c but higher levels of Siglec-H, bone marrow stromal antigen-2 (BST-2), Ly6C, but not CD11b or SIRPα. They are present in the airways (58-59). On the other hand, cLDCs express higher levels of CD11c and consist of two subsets, CD103+ and $CD11b^{hi}$ LDCs. CD103+ LDCs line the airways, and are phenotypically $CD11c^{hi}$CD103+CD207 (langerin)+MHC-II+XCR1+Clec9a/DNGR-1+CD64-CD11b- (60). Of note, both CD103+ and $CD11b^{hi}$ LDCs express zbtb46 that is specific for classical LDCs (61). Below the airways, $CD11b^{hi}$ LDCs reside in the lamina propria. They are characterized as $CD11c^{hi}CD11b^{hi}$ CX3CR1+SIRPα+CD64-CD103-XCR1-CD207-MHC-II+ (62). In the light of recent findings that CD8α+ and CD103+ DCs share similar ontological and physiological properties, LDCs can also be divided into CD8α-type and CD11b-type DCs. CD8α-type DCs are XCR1+, including CD8α+ and CD103+ DCs, whereas CD11b-type DCs do not express XCR1, e.g. $CD11b^{hi}$ and CD8α- DCs (63-64). In humans, CD141+ DCs are considered to be a member of CD8α-type DCs because of their XCR1 expression (59). In inflammatory conditions, a population of monocyte-derived inflammatory LDCs (iLDCs) is recruited to the lung that express CD11c, CD11b, MHC-II, Ly6C, CX3CR, CD64, Mar-1, SIRPα, DC-SIGN (DC-specific intracellular adhesive molecule-3-grabbing non-integrin), E-cadherin, and BST-

2 (60). In addition, a huge population of alveolar macrophages (AMs) resides in pulmonary alveoli. AMs are $CD11c^{hi}$CD11b-CD64+Singlec-F+Autofluorescent+MHC-II+F4/80+SIRPα+. It is to be noted that AMs express high levels of CD11c and therefore can contaminate LDCs during flow cytometric analysis. A reliable strategy to distinguish AMs from LDCs is to use FL-1/FITC empty flow cytometric channel to exclude highly autofluorescent AMs (59).

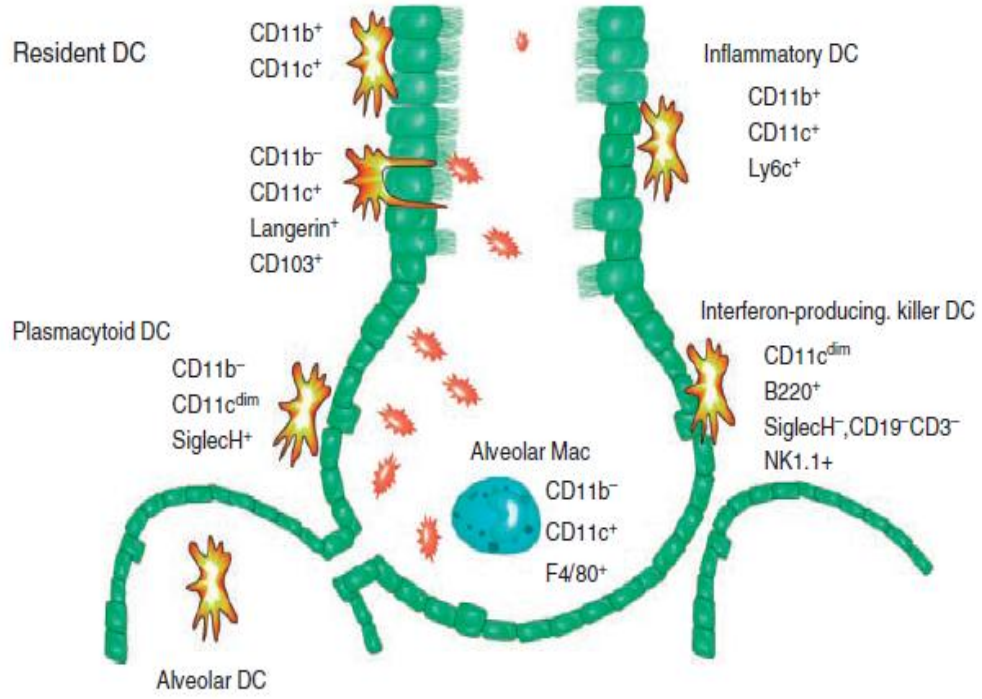

Figure 2. LDC subsets in the airways. The conducting airways are composed of airway epithelial cells, which act as a molecular sieve excluding inhaled antigens and pathogens. Two populations of resident LDCs are located underneath the epithelial layer. Mucosal CD11b- LDCs situated in the basolateral space and can extend their processes between epithelial cells directly into the airway lumen. This 'periscope' function provides a mechanism for continuous immune surveillance of the airway luminal surface. CD11b+ resident LDCs and pLDCs are located underneath the basal membrane. In the alveolar space, alveolar LDCs and macrophages are located. On inflammatory conditions like respiratory viral infections, an activated population of inflammatory LDCs expressing CD11b and Ly6C can be found in the lung tissue as well as the interferon-producing killer LDCs. (Adapted from Ref. 73)

CD103+ and CD11b^{hi} LDC subsets reflect distinct functional responses. CD103+ LDCs are mainly involved with the cross-presenting of antigens, whereas CD11b^{hi} LDCs are responsible for producing various cytokines (65-66). Upon stimulation with TLR ligands, CD11b^{hi} LDCs produced significantly large quantities of IL-6 and TNF-α, whereas CD103+ LDCs secreted higher levels of IL-12p70 (67). Furthermore, CD103+ and CD11b^{hi} LDCs have been found to induce differential T cell responses. When cultured with naïve DO11.10 CD4+ T cells, CD103+ and CD11b^{hi} LDCs mounted Th1/Th17 and Th2 responses, respectively (67). Recent studies have shown that mice lacking the transcription factors, Batf3 and Irf8, had a defect in development of CD8α+ DCs and CD103+ LDCs (68), suggesting that these DC subsets share similar developmental pattern. The relationship between the DC subsets is however not limited to their development. **Both CD8α+ DCs and CD103+ LDC subsets reflect similar function in that they are highly efficient in cross-presentation and cross-dressing of antigens (56-57)**. Cross-dressing stands for the transfer of unaltered MHC-I/peptide complexes between cells (57). On the other hand, the functional role of pLDCs and iLDCs is not clear. pLDCs are a major source of IFN-α during pulmonary viral infections (69-70). Accumulation of iLDCs in the lung is reported in many viral infections that depends upon type 1 IFN and CCR (chemokine receptor)-2 (59, 71-73).

1.2.4. Role of LDC/LDC subsets in immunity to respiratory bacterial infections

Studies have extensively explored the function of LDC/LDC subsets in immunity to respiratory viral infections (74). Following viral infections, LDCs not only show the expression of higher levels of costimulatory molecules and cytokines but also migrate to the lung-draining mediastinal LNs (MLNs) to confer T cell responses (75). Langerin-Diphtheria toxin (DT) mice treated with DT or Batf3$^{-/-}$ mice, which are deficient in CD103+ LDCs, failed to induce an optimal CD8+ T cell immunity to influenza infection, suggesting a protective role for CD103+ LDCs in this viral infection (76-77). In contrast, Ballesteros-Tato *et al*. showed a predominant role for CD11b^{hi} LDC subset in stimulating anti-influenza CD8+ T cell responses, which could be attributed to different experimental conditions, such as infective viral doses (78). These findings conclude that LDCs and their subsets particularly CD103+ and CD11b^{hi} LDCs are important for eliciting T-cell responses against viral infections.

In pulmonary bacterial infections, LDCs upregulate MHC-II and costimulatory molecules and secrete IL-12. After capturing the mycobacterial antigen, LDCs migrate to the MLNs to induce a CD4+ T-cell response that depends on IL-12p40 secretion by LDCs (79-82). Depletion of LDCs led to an increased bacterial burden following *Staphylococcus aureus* infection (83). In line with this, DC-deficient individuals are more susceptible to atypical mycobacterial infection (84). In chlamydial infections, studies have mainly focused on the functions of splenic DCs (SDCs). *C. muridarum* infection in the peritoneum induced maturation of DCs, and the DCs from *Chlamydia*-infected mice expressed higher levels of IL-12 than those from naïve mice (85-87). Using DC adoptive

transfer, CD8α+ and CD8α- DC subsets were examined for their functional role in immunity to chlamydial lung infection. When subjected to a chlamydial challenge, the mice that received CD8α+ DCs showed less severe body weight loss, chlamydial burden and pathology compared to those receiving CD8α- DCs. Cytokine analysis of the local tissues of CD8α+, compared to CD8α-, DC-recipients revealed an enhanced Th1 response (IFN-γ) but reduced Th2 response (IL-4) (88). These findings suggest that CD8α+ DCs are more potent in inducing Th1 immunity against chlamydial lung infection than CD8α- DCs. But the role of CD103+ LDCs, which are functionally related to CD8α+ DCs and reside at the primary site of bacterial lung infections, in host defense is not clear. Recently, Dunne *et al*. investigated the function of CD103+ LDC subset during a bacterial lung infection. Mice receiving anti-CD103 antibodies exhibited decreased resistance to *Bordetella pertussis* infection compared to the sham-treated mice (89). It is of importance to mention here that anti-CD103 antibodies not only block CD103+ LDCs but also CD103+ T cells in the lung. Therefore, these data cannot be applied to understand the function of CD103+ LDCs. On the other hand, mice depleted of pLDCs had greater morbidity and expressive inflammatory lesions compared to the control mice (90). In addition, the antibody-treated mice produced significantly larger quantities of proinflammatory cytokines (TNF-α) but lower immunoregulatory cytokines (IL-10). There results underscore a critical role for pLDCs in both protective immunity as well as immunoregulation (90). Overall, LDCs contribute to protective immunity to bacterial infections.

1.3.0. INNATE LYMPHOCYTES

1.3.1. What are innate lymphocytes?

Lymphocytes represent a critical component of the adaptive immune system. A subpopulation of lymphocytes referred to as innate lymphocytes is, however, distinct from conventional lymphocytes due to their ability to elicit early and rapid immune responses, thus acting as the first line of host defense. Accumulating evidence further suggests that the function of innate lymphocytes is more complex and wider than previously thought. The impact of innate lymphocytes on DCs is crucial for shaping T-cell responses, bridging innate and adaptive immunity (91).

1.3.2. Types of innate lymphocytes

The most widely studied subsets of innate lymphocytes include NK, NKT, and γδ T cells. NK cells are large granular lymphocytes that do not express T or B-cell receptor. The function of NK cells is decided by the activating and inhibitory signals from various receptors (92). In contrast to αβ T cells, NKT and γδ T cells express the invariant αβ and oligoclonal γδ T-cell receptors (TCRs), respectively. Although murine and human NKT cells recognize glycolipid antigens presented to them by non-classical MHC-I CD1d molecules, whereas human γδ T cells recognize phosphate molecules via non-MHC-restriction (93-94). Despite these differences, NK, NKT and γδ T cells possess many functional similarities, such as rapid production of multiple cytokines and cytotoxic activities, and have been shown to play a crucial role in various disease settings (92-94).

1.3.3. Bridging innate and adaptive immunity

DCs are considered to be the orchestrators of adaptive immunity. Increasing evidence has however proposed a new thesis that the actions of DCs are orchestrated by innate lymphocytes (91). Impact of innate lymphocytes on DC function directs the outcome of adaptive immunity. The communication between innate lymphocytes and DCs is two way (95). Innate lymphocytes induce maturation of DCs *in vivo*. The maturing DCs upregulate costimulatory molecules and secrete cytokines such as IL-12, regulating T cell responses (96). On the other hand, DCs produce multiple cytokines to modulate the effector function of innate lymphocytes (97). Thus, crosstalk between innate lymphocytes and DCs manipulates the adaptive immunity.

1.4.0. iNKT CELLS

1.4.1. What are iNKT cells?

NKT cells constitute an unconventional population of lymphocytes that share the marker of both αβ T and NK cells. NKT cells express αβ TCR. They also express NK1.1 molecules on their surface like NK cells (93). These cells undergo differentiation through somatic recombination and thymus selection. NKT cells possess the ability to recognize glycolipid and lipid antigens presented to them by CD1d molecules (98). NKT cells are of two types: type 1 or classical/invariant NKT (iNKT) and type 2 or non-classical NKT cells. The best known type of NKT cells are iNKT cells. These cells express the invariant αβ TCR using Vα14-Jα18 in mice and Vα24-Jα18 in humans. Because of their invariant TCR, iNKT cells recognize glycolipid and lipid antigens presented on CD1d

molecules. An important signature of iNKT cells is to rapidly produce large quantities of a range of cytokines, and have been found to play a crucial role in diverse disease settings, including infectious diseases, autoimmunity and malignancy (98). Type 2 NKT cells are a scare and heterogeneous cell population that has oligoclonal αβ TCR repertoire. These cells can recognize sulfated galactosylceramides compounds, which are hydrophobic in nature (93). Although emerging evidence has begun to highlight the physiological properties of type 2 NKT cells, their role in immunity is poorly understood.

1.4.2. Activation of iNKT cells

Activation of iNKT cells is achieved through two mechanisms, CD1d-dependent and CD1d-independent. In CD1d-independent mechanism, iNKT cells are activated without involvement of CD1d molecules. This type of activation is mediated through innate or inflammatory stimuli regardless of the foreign antigens (99). Recent data further illustrate that the innate stimuli such as cytokines are the important means of iNKT-cell activation, even with bacteria that carry iNKT-cell agonists (99). In CD1d-dependent mechanism, antigens are presented by CD1d molecules expressed on DCs for interaction with iTCR that possesses a conformation, which is able to recognize glycolipid and lipid antigens processed and presented to them by CD1d molecules. Interaction between iTCR and its cognate ligand leads to the activation of iNKT cells, as evidenced by massive production of multiple cytokines, such as Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17) cytokines. The biochemical and physiological nature of iNKT-cell-specific ligands has been deciphered by many recent studies. Kawano *et al.* for the first time identified a lipid antigen specific for iTCR, α-galactosylceramide (α-GalCer). α-

GalCer was originally extracted from marine sponges (100). Since α-GalCer is a potent ligand for iNKT-cell activation and has been instrumental in understanding the biological properties of iNKT cells, it is referred to as a prototypic antigen for these cells. The ability to activate iNKT cells is however not limited to α-GalCer. A variety of microbial antigens have been shown to activate iNKT cells, such as α-glucuronosylceramide from *Sphingomonas* spp. (101-103), α-galactosyldiacylglycerol from *Borrelia burgdorferi* (104), and phosphatidylinositol-manosidase from *Mycobacterium bovis* BCG (105-106). More recently, we identified a glycolipid exoantigen from *C. muridarum* (GLXA) that induced activation of iNKT cells (107). Intravenous injection of GLXA, compared to cell mock, into WT mice led to an enhanced production of IFN-γ and IL-4 in sera, whereas there was no difference between GLAX and cell-mock treatments in Jα18-KO mice that lack iNKT cells. Flow cytometric analysis further showed that iNKT cells from GLXA-treated WT mice expressed higher levels of CD69, an activation marker, and cytokines, IFN-γ and IL-4, compared to those from cell-mock treated mice (107). These findings suggest that GLXA act as a specific ligand for iNKT-cell activation. In line with these findings, another group, using an APC-free culture system, has shown that both iNKT as well as type 2 NKT-cell hybridomas were activated when cultured with killed *C. muridarum* (108). It is known that iNKT and type 2 NKT cells are activated by different ligands. For example, lipid/glycolipids and hydrophobic antigens such as sulfatides induce specific activation of iNKT and type 2 NKT cells, respectively. Since *C. muridarum* activated iNKT and type 2 NKT-cell hybridomas, it is likely that there are different chlamydial antigens for activating these cell types (108). Future studies should focus on the identification and purification of different chlamydial antigens for NKT-cell

activation, which may be crucial for chlamydial vaccine development. **It is thus reasonable to conclude that iTCR is involved in activation of iNKT cells by interacting with chlamydial antigens.**

1.4.3. Role of iNKT cells in protection and pathology to chlamydial infections

Recent studies in mice have provided significant evidence on the role of iNKT cells in protection and pathology to various infections, including chlamydial infections (109). *In vivo* stimulation of iNKT cells by α-GalCer mounted a strong protective immunity to intranasal *C. pneumoniae*, intra-articular *C. trachomatis* and intravaginal *C. muridarum* infection (110-113). Mice receiving α-GalCer treatment exhibited reduced pathology and bacterial burden following challenge infection compared to sham-treated control mice. Interestingly, although the treatment with α-GalCer induced protection against intravaginal *C. muridarum*, it promoted pathology against intranasal *C. muridarum* infection, suggesting that different routes of infection even with the same bacterial species can alter the outcome of iNKT-cell-mediated immunity. To better understand the dynamics of NKT-cell function *in vivo* during chlamydial infections, we and other groups have used various experimental approaches, including transgenic/KO mice. During *C. trachomatis* intra-articular infection, CD1d-KO mice, which lack both iNKT and type 2 NKT cells, resulted in an enhanced body weight loss, higher bacterial burden and more severe lung pathology compared to the WT mice, which indicated a protective role for NKT cells in this infection model (113). In contrast, CD1d-KO showed increased resistance against pulmonary as well as genital tract infection with *C. muridarum* (108,110). To directly examine the contribution of iNKT cells in chlamydial infections, we

used Jα18-KO mice and infected them with *C. muridarum* and *C. pneumoniae* (111). With *C. muridarum*, in contrast to *C. pneumoniae*, lung infection, Jα18-KO mice, which lack iNKT cells, exhibited reduced weight loss, bacterial burden and pathology compared to the WT mice. These findings indicated that although iNKT cells exert a detrimental role in *C. muridarum* infection, they confer protective immunity against *C. pneumoniae*. Cytokine analysis further demonstrated that the WT mice, compared to the KO, revealed a robust Th1/Tc1 response, characterized by IFN-γ production, in *C. pneumoniae* infection. On the contrary, a predominant Th2 response (IL-4) was observed in case of *C. muridarum* infection (111). The reason behind why iNKT cells act differently in the outcome of infection with these two related chlamydial species is still unclear and so warrants further investigation. Since Chlamydiae may have various antigens for iNKT cells, the antigenic variability among the antigens from different chlamydial species might explain the differential iNKT-cell responses.

1.4.4. Mechanism of iNKT-cell-mediated immune responses

To induce immune responses, iNKT cells follow the pathways of direct and/or indirect actions (109). During the pathway of direct action, iNKT cells perform their function without involvement of other cell types. The direct action of iNKT cells on bacteria-infected cells is mediated through cytokine production, FASL-FAS and iTCR-antigen-CD1d interaction. IFN-γ produced by iNKT cells activates the infected cells that lead to the secretion of nitric oxide (NO), a bactericidal agent, intracellularly for killing the bacteria. On the other hand, iTCR on iNKT cells interact with their specific antigens presented on CD1d molecules expressed on infected cells (109).

It is becoming clearer that innate and adaptive immune systems do not work in isolation, rather interact with each other to give rise to an optimal immune response against infections. A significant example in this context is of innate lymphocytes that have been shown to bridge innate and adaptive immunity by modulating DCs (91). To study the impact of iNKT cells on DC function, α-GalCer as a model antigen has been widely used. Whether this is true in case of real infections has been addressed by some recent studies (109, 114-119). Our recent studies using *C. pneumoniae* infection have done an in-depth analysis of the impact of iNKT cell on DC function in antibacterial T cell immunity (118-119). When transferred to naïve recipient mice, SDCs from *Chlamydia*-infected Jα18-KO, in contrast to WT, mice promoted infection and pathology upon challenge with chlamydial infection, suggesting that iNKT cells are crucial for DCs to confer protective Th1 immunity (118). Since DCs demonstrate a high degree of heterogeneity consisting of various subsets, we wondered whether this modulating effect of iNKT cells was biased to a DC subset. CD8α+ and CD8α- DCs are important DC subsets residing in the lymphoid tissues such as the spleen. While CD8α+ DCs induce Th1 responses, CD8α- DCs skew Th2 responses (120). These DC subsets were purified from Jα18-KO and WT mice following chlamydial infection and then adoptively transferred to naïve recipient mice, which subsequently received chlamydial challenge. Mice receiving CD8α+ and CD8α- DCs from WT mice showed significant resistance to infection compared to those from Jα18-KO mice, although the protection was better in CD8α+ DC recipients (119). Therefore, CD8α+ DCs are the main DC subset that interact with iNKT cells. In addition, we have shown that the expression of

CD1d molecules is higher on DCs from WT mice than the DCs from KO mice following chlamydial infection (118). Since the local pulmonary immune responses may not be similar to the splenic, we also examined the iNKT cell-DC interaction in the lung, which is the primary site of infection where maximal inflammatory changes occur (109). These findings, however, were not different from what we observed in splenic DC studies, suggesting that although residing in different anatomical compartments, DCs induce similar immune responses during chlamydial lung infection. Apart from DCs, AMs are also a critical immune cell in the lung that regulates immune responses against pulmonary pathogens. **Altogether, these findings suggest that iNKT cells exert protective T-cell immunity to *C. pneumoniae* by modulating the function of DCs.**

How do iNKT cells modulate the DC function? In *C. pneumoniae* infection, the expression of CD40L and IFN-γ by iNKT cells was found to be upregulated (118). To evaluate the contribution of CD40L and IFN-γ in the modulating effect of iNKT cells on DC, iNKT cells was cultured with DCs and then blocking antibodies against these molecules were used. Blockade of either CD40L or IFN-γ significantly reduced the enhancing effect of iNKT cells on IL-12 production by DCs. But the increased IL-12 production was virtually abolished when physical contact between these cells was prevented (118). These data conclude that CD40-CD40L interaction, IFN-γ production and cell-to-cell contact are critical for iNKT cells to modulate DC function (Fig. 3).

(The section 1.4.0. has been adapted from Ref. 282)

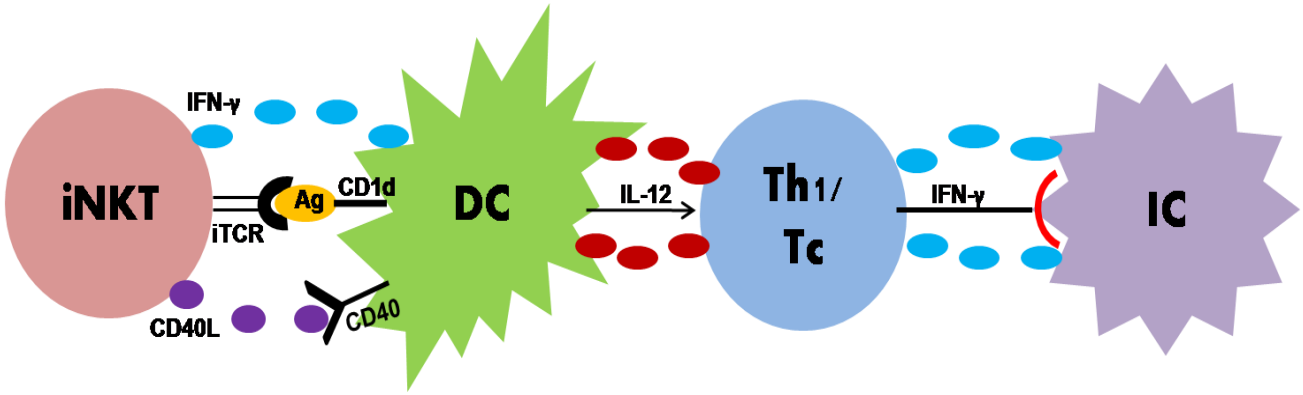

Figure 3. Induction of anti-chlamydial T cell responses by iNKT cells through DC modulation. During chlamydial infections, iNKT cells induce DC maturation through IFN-γ, CD40-CD40L binding, iTCR-Ag interaction and cell-to-cell contact. Once matured, DCs induce enhanced production of IL-12 that skews Th1/Tc responses. Th1/Tc responses characterized by IFN-γ production lead to the killing of intracellular Chlamydiae. iNKT – Invariant natural killer T cell; iTCR – Invariant T cell receptor; CD40L – CD40 Ligand; DC – Dendritic cell; IL-12 – Interleukin-12; Th1 – T helper cell; Tc – T cytotoxic cell; IC – Infected cell; C – *Chlamydia*. (Adapted from Ref. 282)

1.5.0. NK CELLS

1.5.1. What are NK cells?

NK cells are a major subset of innate lymphocytes conserved among various taxa of animal kingdom, including aves and mammalia (92). They were first identified for their potential to kill tumor cells *in vitro* without premeditated activation and subsequently found to be able to recognize and kill the virus-infected cells that lacked the expression of MHC-I antigens (121-123). Early and rapid secretion of various cytokines and robust cytotoxic activity are hallmark of NK cells. A growing body of evidence suggests that the function of NK cells is much more sophisticated and broader than previously thought. They play a crucial role in autoimmunity, tumor rejection and host defense against various pathogens. While substantial progress has been made toward understanding the biology of NK cells in viral infections, their contribution to defense against bacterial pathogens is still unclear.

1.5.2. Phenotypic and functional characteristics

NK cells are large in size with granular cytoplasm. They are present in various tissues, including the peripheral blood and lymphoid tissues. In contrast to T and B cells, NK cells do not express T- and B-cell receptors. NK cells are endowed with various receptor molecules, with either inhibitory or activating signaling function, which are important for performing their functions. These receptors may be of two types, 1) type-II transmembrane proteins with a C-terminal domain, also called as killer cell lectin-like receptors (KLR), e.g. CD94, NKG2D, NKR-P1and Ly49, and 2) type-I transmembrane

proteins with extracellular immunoglobulin superfamily domains, e.g. LILR, KIR and NKp46 (124). NKG2D is expressed on NK, NKT, and T cell subsets. Its ligands are expressed induced proteins that include MICA, MICB, ULBP 1, 2, and 3 in humans and RAE1, Mult1, and H60 in mice (124). Of note, Ly49 is of multigenic presence in mice, while humans have only one pseudogenic Ly49 (124). Phenotypically, NK cells are defined as CD3-CD56+ lymphocytes in humans, while these cells do not express CD56 and can be identified by the expression of CD11b in mice (124). These lymphocytes possess distinct functional properties, such as rapid secretion of a variety of cytokines and chemokines, and robust cytotoxic activity (125-127). Upon activation, NK cells secrete large quantities of multiple cytokines and chemokines, such as IFN-γ, TNF-α, GM-CSF, CCL3, CCL4 and CCL5 (128). Interestingly, NK cells have been found to exhibit memory responses against specific antigens (129-131). A new role for NK cells has also been assigned in reproductive physiology where they contribute to remodeling of vasculature during fetal implantation (132-133).

1.5.3. Mechanism of NK-cell-mediated immune responses

To perform their function, NK cells require an effective stimulus that can be generated through the upregulation of ligands for NK-cell-activating receptors or the down-regulation of ligands for NK cell inhibitory receptors (134-135). NK cells are able to recognize and kill the virus-infected cells that lacked the expression of MHC-I antigens, often termed as missing self-hypothesis (121-123). In addition to their cytotoxic potential, NK cells also function as producers of cytokines such as IFN-γ and TNF-α that contribute to the activation of macrophages for expression of antimicrobial killing

mechanisms, e.g. inducible or type-2 nitric oxide synthase (iNOS or NOS_2) (136-139). Another important way by which NK cells perform their cytotoxic activities against pathogens is antibody-dependent cell-mediated cytotoxicity (ADCC). In ADCC, specific antibodies bind to their cognate antigens recognized by FcYRIII (CD16) receptors expressed on NK cells, resulting in release of cytolytic granules, such as perforin and granzyme, and consequent cell apoptosis of target cell via caspase-dependent and –independent mechanisms. Caspase-dependent apoptosis of target cells can also take place by interaction of death receptors (Fas) on target cells with their cognate ligands (FasL) on NK cells (123, 133). More importantly, NK cells not only induce innate immunity but also shape the outcome of adaptive immunity through modulating DCs, bridging innate and adaptive immunity (91). Specifically, NK cells induce DC maturation that depends upon cell-to-cell contact, cytokine production (IFN-γ and TNF-α) and receptor-ligand interactions. The mature DCs then orchestrate the polarization of T cells to elicit immunity against various pathogens (129,140). This NK cell-DC talk is not unidirectional. Mature DCs can also activate NK cells by producing IL-12, IL-15, and IFN-α/β (129).

1.5.4. Role of NK cells in immunity to chlamydial infections

The role of NK cells in host defense against viral infections is well-documented (141-142). What role these cells play in protective immunity and immunopathology to bacterial infections is relatively less understood. Hall *et al*. demonstrated an expansion and recruitment of NK cells following *Citrobacter rodentium* infection. *In vivo* depletion of NK cells in mice led to a higher bacterial load following bacterial challenge compared to

sham-treated mice, suggesting a role for NK cells in inducing protective immunity (143). In Rickettsial infection, NK cells contributed to the innate phase of host protection, most likely through production of IFN-γ, and limited infection-induced tissue damage (144). In line with these findings, a protective role for NK cells has been demonstrated against many other bacterial pathogens such as *M. tuberculosis*, *L. monocytogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* (145-153).

In chlamydial infections, the contribution of NK cells in host defense has been controversial. Williams *et al*. showed that the treatment of mice with antibodies to deplete NK cells did not have any significant effect on the bacterial burden, pathology and survival when subjected to a *C. muridarum* lung infection (154). In addition, *in vivo* depletion of NK cells in nu/nu mice failed to decrease the production of IFN-γ in the lung (155). When challenged with *C. pneumoniae*, RAG-$1^{-/-}/\gamma_c R^{-/-}$ mice that lack T, B, and NK cells exhibited no significant difference in their lung bacterial burden and pathologic changes compared to RAG-$1^{-/-}$ mice deficient in T and B cells (156). All these results pointed toward an insignificant role for NK cells in protective immunity to chlamydial infections. On the other hand, we recently examined the role and mechanism of NK cells in protection and pathology to *C. muridarum* lung infection (140). Compared to the sham-treated, NK cell-depleted mice exhibited a higher bacterial load, pulmonary pathology and body weight loss following chlamydial infection. Cytokine analysis of the LNs and the lung tissues demonstrated that NK-cell-depleted mice exhibited a reduced Th1, but enhanced Th2, response compared to the sham-treated mice (140). Similar to these findings, NK-cell-depleted mice exhibited an exacerbated genital tract infection

with *C. muridarum* by inducing a Th2 response (157). A pertinent question arises here why there are discrepancies in the findings of our study with the findings of some other studies. This could be attributed to the different conditions under which these experiments were carried out such as the use of different chlamydial strains, mouse models and routes of infection. To decipher how NK cells confer protective Th1 immunity, we isolated splenic DCs from infected NK cell-depleted and sham-treated mice and transferred them to naïve recipient mice which subsequently received chlamydial challenge. Mice that received DCs from NK-cell-depleted mice promoted infection and exacerbated the disease, whereas those receiving DCs from sham-treated mice showed greater protective Th1 immunity. This suggests that NK cells modulate the function of DCs to generate protective immune responses to chlamydial infection. We further demonstrated that the modulation of DCs by NK cells was dependent upon IFN-γ production, NKG2D receptor signaling and cell-to-cell contact (140). Studies have also demonstrated that NK cells lyse immature DCs *in vitro*. Although the low NK cell/DC ratios promotes DC maturation, higher NK cell/DC ratios results in lysis of DCs by NK cells that involves the signaling by NKp30 expressed on NK cells (141-142).

Recent reports demonstrated a modulating effect of NKT cells on the function of NK cells. *In vivo* administration of α-GalCer in mice induced NK cells to produce IFN-γ as well as cause cytotoxicity (158-159). Since NKT and NK cells have been shown to contribute to immunity against chlamydial infections, we focused on whether NKT cells influence the functional role of NK cells during infection (160). We found a reduced expansion of NK cells Jα18-KO mice following *C. muridarum* infection. A lower

percentage of IFN-γ-producing, but higher CD107a+ degranulating, NK cells were observed in Jα18-KO than in WT mice. These data suggest that NKT cells have a differential effect on NK cell functions. They enhance IFN-γ production by NK cells but inhibit their cytotoxic activities during chlamydial infection (160).

1.6.0. T-CELL RESPONSES TO CHLAMYDIAL INFECTION

T cells occupy a central position in mediating adaptive immunity. Their functions include killing of intracellular pathogens as well as helping B cells for humoral immunity. CD4+ T cells interact with antigens presented to MHC-II molecules to induce effector cytokines for specific cell-mediated immunity, whereas CD8+ T cells recognize peptides on MHC-I to elicit effector cytokine and cytotoxic responses (161).

1.6.1. Th cell differentiation

Naïve CD4+ T (Th0) cells can differentiate into various effector subsets (162-163). These subsets include, but not limited to, Th1, Th2 and Th17 cells. Th1 cells are characterized by the production of IFN-γ, IL-2 and TNF-α/β. Similar to Th1 cells, CD8+ T cells that produce IFN-γ and possess cytotoxic activities are termed as Tc1 cells (164). Differentiation of Th0 into different Th subsets is controlled by their cytokine milieu and TCR-Ag interactions (163). More importantly, the cytokines produced by DCs play a pivotal role in skewing Th differentiation. In the presence of IL-12 and IFN-γ, Th0 cells differentiate into Th1 cells through the signal transducer and activator of transcription 4 (STAT-4), STAT-1, and T box transcription factor T-bet (165-166). Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. The milieu rich in IL-4 favor the bias

toward differentiation of Th2 cells that require the help of GATA-3 and STAT-6 (167). A combination of cytokines such as IL-6, IL-21, IL-23, and TGF-β and transcription factor retinoid-related orphan receptor (ROR)γt is essential for Th17 differentiation (168).

In addition to Th1, Th2, and Th17 cells, many other Th cell subsets such as Th9, Th22, follicular helper T (Tfh), regulatory T (Treg) cells have been described that have distinct lineages. Of these, the widely studied subsets are Treg and Tfh cells. Treg cells are so named because of their regulatory functions, such as maintenance of peripheral tolerance and suppression of autoimmunity. They are characterized as CD4+CD25+Foxp3+ cells and produce copious amounts of IL-10 and TGF-β (169). On the other hand, Tfh cells require IL-21 and Bcl-6 for their differentiation and provide help to B cells to mediate humoral immunity (170).

1.6.2. Th1/Th2 responses

Th1 cells do not kill infected cells and pathogens directly, but rather provide assistance to other effector immune cells to confer cell-mediated immunity. IFN-γ production by Th1 cells induces macrophages and B cells to enhance their bactericidal activities and opsonizing and complement fixing antibodies, respectively (163). Activation of Tc1 cells is also assisted by cytokine signals from Th1 cells. On the other hand, Th2 responses characterized mainly by IL-4 production promote antibody-mediated immunity and eosinophilic accumulation but inhibit the function of macrophages. In various mouse infection models, Th1/Th2 responses have been examined in great detail. Th2 responses are more effective in dealing with extracellular pathogens, such as helminthic

parasites and extracellular bacteria (171). In contrast, Th1 responses are mainly effective against intracellular pathogens, including *Chlamydiae*. Analogous to many intracellular pathogens, **Th1/Tc1 cells play a critical role in clearance of chlamydial infections, whilst Th2 responses promote infection and pathology (172-173).**

1.6.3. IL-17/Th17 responses

Th17 cells are so named because of their predominant production of IL-17 (174-175). Production of IL-17 is however not limited to NK cells. Activation of γδ T and αβ-TCR+ CD4-CD8- T cells also leads to the secretion of large quantities of IL-17 during early stages of microbial infection (176-179). The main antimicrobial effector mechanisms of IL-17/Th17 responses include the production of inflammatory cytokines and promotion of the functional activities of neutrophils. In particular, Th17 cells secrete large quantities of IL-17 and IL-22 that act on epithelial cells to induce production of multiple antimicrobial peptides such as β-defensin-2 and lipocalin-2 (180-184). IL-17 not only causes the upregulation of chemokines such as CXCL8 for neutrophil recruitment to the site of infection but also promotes the formation, phagocytosis, and cytotoxicity of neutrophils (185-189). Although the primary function of IL-17/Th17 cells appears to be controlling intracellular bacterial infections, their definitive role in immunity to intracellular bacterial pathogens awaits further exploration (186-187, 190-191).

Some previous studies suggested that IL-17/Th17 cells do not have a significant role in protective immunity to intracellular bacterial infections (192-194). In contrast, recent studies have shown a protective role for IL-17/Th17 cells in immunity to

intracellular bacteria. IL-17-KO mice had decreased resistance to *Francisella tularensis* infection, indicating the contribution of IL-17 in protection to this bacterium (179). In accordance with these results, we found that *in vivo* neutralization of IL-17 in mice resulted in increased susceptibility to *C. muridarum* lung infection compared to the control mice. Furthermore, IL-17-depleted mice showed a reduced Th1, in contrast to Th2, response (195). Similar observations were recorded in other studies using chlamydial lung infections (196-197). These data suggest an important role for IL-17/Th17 responses in defense to intracellular bacterial infections. Zhang *et al.* reported a synergistic relationship between IL-17/Th17 and Th1 responses to inhibit chlamydial growth (198). To understand the mechanism of how IL-17 regulates adaptive immunity, we did an adoptive transfer experiment with DCs isolated from *C. muridarum*-infected IL-17-depleted mice. When subjected to a chlamydial challenge, naïve mice receiving DCs from infected IL-17-depleted mice demonstrated reduced Th1 immunity compared to the recipients of DCs from control mice, suggesting that IL-17/Th17 responses modulate DCs to confer protective Th1 immunity (195); however some studies indicate a pathogenic role for Th17/IL-17 responses. In addition, Th17 responses can contain intracellular bacterial infection through enhanced recruitment of neutrophils at the primary site of pulmonary tularemia and chlamydial infections (196,199). **In conclusion, IL-17/Th17, in synergy with Th1, responses mainly elicit protective immunity through DC modulation and neutrophil recruitment.**

1.7.0. SUMMARY OF LITERATURE

The salient points of the background literature are *infra dictus*:

- *Chlamydia* is an intracellular obligate bacterial pathogen that causes a wide range of diseases worldwide. *C. muridarum* is the cause of bacterial STDs, trachoma, and pneumonia, whereas *C. pneumoniae* infection may result in bronchitis, sinusitis, and community acquired pneumonia, and is also associated with chronic inflammatory diseases. However, there are no effective vaccines to control chlamydial infections in humans due to limited knowledge of chlamydial immunobiology.
- Th1/Tc1 and Th17 responses are critical for protective immunity to chlamydial infections, whereas Th2 responses may result in pathology.
- DCs are professional APCs that consist of various subsets. Lymphoid DCs are of two types, CD8α+ and CD8α- DCs, whereas non-lymphoid/tissues DCs can be classified into CD103+ and $CD11b^{hi}$ DCs.
- CD8α+ and CD103+ DCs are developmentally and functionally related. They share the same transcription factors (e.g. Batf) and are efficient at cross-presenting and cross-dressing antigens to naïve CD8+ T cells.
- CD8α+ DCs are more potent in conferring Th1 protective immunity against C. muridarum infection that CD8α- DCs.

- Although the role of CD103+ and CD11b^{hi} LDCs in host defense to viral infections is well-documented, how these LDC subsets contribute to immunity in bacterial lung infections is still unclear.
- Innate lymphocytes are an unconventional subpopulation of lymphocytes that induce rapid and early immune responses. The major subsets of innate lymphocytes include NK, iNKT and γδ T cells that have been shown to play a crucial role in autoimmunity, tumor and pathogen defense.
- Innate lymphocytes shape the adaptive immunity through modulating DC function, thus acting as a connecting link between innate and adaptive immunity.
- iNKT cells express the marker of both NK and αβ T cells, such as NK1.1 and a semi-invariant TCR, and are activated by endogenous as well as exogenous ligands. The prototypic antigen for their activation is α-GalCer, which is derived from marine sponges.
- iNKT cells confer Th1/Tc1 protective responses against *C. pneumoniae* infection, but promote Th2 pathologic responses to *C. muridarum* infection.
- iNKT cells induce protection against *C. pneumoniae* lung infection by modulating the function of SDCs. iNKT cell-mediated modulation of SDC is dependent upon IFN-γ production, CD40-CD40L, and cell-to-cell contact.
- iNKT cells preferentially modulate the function of CD8α+ SDC subset to generate protective T-cell immunity to *C. pneumoniae* infection.

- NK cells are critical for protective Th1 immunity to *C. muridarum* infection. The mechanism by which NK cells induce the immunity is through SDC modulation. iNKT cell modulate SDCs via IFN-γ, NKG2D receptor signaling and cell-to-cell contact.

2.0 CHAPTER 2

RESEARCH AIMS, HYPOTHESES AND RATIONALE

2.1.0. SPECIFIC AIMS

The overall goal of our study was to assess the role of LDCs and their relationship with innate lymphocytes in host defense against chlamydial lung infection in mice. The specific aims of the study were: **1)** to investigate the role of iNKT cells in eliciting protective immunity against *C. pneumoniae* infection by modulating LDC function (Chapter-4), **2)** to examine the role and mechanism of NK cells in modulating the function of LDCs to induce Th1/Th17 immunity to *C. muridarum* infection (Chapter-5), and **3)** to analyze the contribution of CD103+ and $CD11b^{hi}$ LDC subsets in host defense against *C. muridarum* infection (Chapter-6).

2.2.0. GLOBAL HYPOTHESIS

Innate lymphocytes may modulate LDC function to induce protection against chlamydial infection, and that CD103+ and $CD11b^{hi}$ LDCs may have differential capacity in conferring protective immunity.

2.3.0. SPECIFIC HYPOTHESES

Specific hypotheses were; **1)** iNKT cells may induce protective immunity to chlamydial infection by modulating LDC function; **2)** NK cells may modulate LDC function to promote protective Th1/Th17 immunity to chlamydial infection; **3)** CD103+ and $CD11b^{hi}$ LDCs may have differential abilities in eliciting protection against chlamydial infection.

2.4.0. RATIONALE OF STUDY

Previous studies have examined the role of innate lymphocytes and their impact on DC function in inducing adaptive immune responses in the splenic environment during chlamydial lung infections (111, 118, 140). In murine models of chlamydial lung infection, the primary site of infection is the lung. Since chlamydiae are mainly contained in the lung tissues, the host-pathogen interactions take place within the pulmonary micromilieu to inflict inflammatory and pathologic changes (29, 31-32). Therefore, immune responses in the spleen may not reflect the true image of the pathogen-specific immune responses induced at the local site of infection (the lung). In addition, a large proportion of effector T cells, which are specific to *Chlamydia*, are recruited to the lung following their priming in the MLNs (28, 29). This further emphasizes that there are differences in the immune mechanisms in these anatomical compartments. Recent studies have demonstrated that DCs residing in the spleen and the lung exhibit phenotypic and functional differences. In the steady-state condition, LDCs showed enhanced expression of costimulatory molecules (CD80, CD86 and CD40) compared to the SDCs. More importantly, LDCs produce cytokines such as IL-6 and IL-10 and preferentially polarize T cells toward a Th2 response, whereas SDCs produce IL-12 and skew toward a Th1 response (200-204). During respiratory infections, LDCs can however generate different immune responses depending upon the type of pathogen. For example, LDCs induce Th1 responses characterized by IFN-γ production against intracellular bacteria and viruses, while exposure to extracellular pathogens such as parasites mounts Th2 responses that lead to eosinophilia and IgE antibody production (209-2011). In addition, different functions of LDCs and SDCs have been demonstrated

in various infection models (78, 203-204). These functional differences between LDCs and SDCs therefore could give rise to differential T-cell responses. Emerging evidence further suggests that innate lymphocytes interact with DCs in the lung during pulmonary viral infections (116, 205-206). Paget *et al*. showed that iNKT cell deficiency significantly influences both the migration as well as maturation of LDCs, which might be responsible for impaired CD8+ T cell function in Jα18-KO mice during influenza infection (116). In line with these findings, NK-cell depletion impaired LDC recruitment to the MLNs following influenza infection, and that the recruitment was dependent upon IFN-γ production by NK cells (Ge et al., J Immunol, 2012). However, the impact of iNKT and NK cells on LDC function to confer protective mucosal T-cell immunity against lung infections, especially bacterial infections, is poorly understood. **Taking into account of these facts, it is critical to study the innate lymphocyte-LDC interactions at the local site of chlamydial lung infection, in particular the mechanisms of how NK and iNKT cells condition LDCs to induce Th1/Tc1/Th17 immunity.** This would provide new information on how the pathogen-specific local immune responses contribute to the outcome of a pulmonary infection, and how these responses are different from the lymphoid responses.

CD103+ and CD11b^{hi} LDC subsets are phenotypically and functionally distinct. CD103+ LDCs cross-present exogenous antigens to naïve CD8+ T cells, while CD11b^{hi} LDCs produce various chemokines and cytokines (65-66). The contribution of CD103+ and CD11b^{hi} LDC subsets in immunity to respiratory viral infections is well-documented. CD103+ LDCs are the primary LDC subset for cross-presenting exogenous antigens to

CD8+ T cells in response to influenza infection (78), although a recent study suggested a predominant role for CD103+ LDCs in cross-presenting influenza antigens. Furuhashi *et al*. examined the ability of CD103+ and CD11b^{hi} LDCs to preferentially induce distinct Th responses under steady state conditions (67). Upon coculture of naïve DO11.10 CD4+ T cells with CD103+ or CD11b^{hi} LDCs obtained from normal BALB/c mice, the CD4+ T cells primed with CD103+ LDCs produced significantly large quantities of Th1 (IFN-γ) and Th17 (IL-17) cytokines, while those primed with CD11b^{hi} LDCs secreted higher levels of Th2 cytokines (IL-4 and IL-10), suggesting that CD103+ and CD11b^{hi} LDCs predominantly generate Th1/Th17 and Th2 responses *in vitro*, respectively (67). Recently, Dunne *et al*. investigated the function of CD103+ LDC subset during a bacterial lung infection. Mice that received anti-CD103 antibodies exhibited decreased resistance to *Bordetella pertussis* infection compared to the sham-treated mice (89). Since anti-CD103 antibodies not only block CD103+ LDCs but also CD103+ T cells in the lung, these data cannot be applied to the function of CD103+ LDCs. **Given that there are limited studies, the *in vivo* function of CD103+ and CD11b^{hi} LDC subsets in eliciting Th1/Th17 responses against bacterial infections remains largely unclear.**

3.0. CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1.0. ETHICS STATEMENT

All experiments were done in compliance with the guidelines issued by the Canadian Council of Animal Care, and the animal protocol was approved by the institutional ethical committee.

3.2.0. CHLAMYDIA

The culture and preparation of *C. muridarum* and *C. pneumoniae* (AR-39) were performed as described previously (118, 140). In brief, *Chlamydia* was cultured in a HeLa 229 cells in Eagle's MEM/DMEM containing 10% FBS and 2 mM L-glutamine for 48 h. For inoculum preparation, infected cells were harvested with sterile glass beads and purified by successive 15-min 500 X g and 30-min 30,000 X g centrifugations. The glass beads were used to detach the infected cells attached to the culture flask. The purified organisms were resuspended in sucrose-phosphate-glutamic acid (SPG) buffer, and frozen at -80^{0}C until used. The same seed stock of *C. muridarum* and *C. pneumoniae* was used throughout the study. Of note, our previous studies used *C. muridarum* and *C. pneumoniae* infection models to investigate NK cell-SDC and iNKT cell-SDC interactions, respectively. To extend these studies to the level of innate lymphocyte-LDC relationship, we used *C. pneumoniae* for iNKT cell-LDC and *C. muridarum* for NK cell-LDC interactions.

3.3.0. MICE INFECTION AND QUANTIFICATION OF IN VIVO BACTERIAL LOAD

Mice were mildly sedated with isoflurane and inoculated intranasally with 1 X 10^3 inclusion-forming units (IFUs) of *C. muridarum*, or 3 X 10^6 IFUs of *C. pneumoniae*, in 40 µl PBS buffer. Unlike *C. muridarum*, which is a mouse biovar of *C. trachomatis*, *C. pneumoniae* is a human pathogen. To infect mice and produce a pulmonary disease, a much higher dose of *C. pneumoniae* is required compared to *C. muridarum*. Mice were sacrificed at different time points and the lungs were aseptically isolated and homogenized using a cell grinder in SPG buffer. The tissue homogenates were centrifuged at 1900 X g for 30 minutes at 4^0C, and the supernatants were stored at -80^0C until tested. For assessment of chlamydial growth *in vivo*, 100 µl of serially organ tissue supernatants was inoculated onto HeLa-229 monolayers in 96-well flat-bottom microtiter plates. After 2 hours of incubation, the cell layers were washed, and 200 µl MEM containing cycloheximide (1.5 g/ml), gentamicin (20 g/ml), and vancomycin (25 g/ml) was added to each well. The plates were incubated for 48 hours at 37^0C in 5% CO_2. Then, the culture medium was removed, and cell monolayers were fixed with methanol and subsequently stained with *Chlamydia*-specific murine mAb and HRP-conjugated goat anti-mouse IgG secondary Abs and developed with substrate (4-chloro-1-naphthol; Sigma-Aldrich). The number of inclusions was counted under a microscope at X200 magnification. Five fields through the midline of each well were counted. Chlamydial titers in the organs were calculated based on dilution titers of the original inoculum. For *in vitro* antigenic restimulation assays and cytokine production in culture, an ultraviolet-killed preparation of elementary bodies (UK-EB) was used.

3.4.0. MICE

C57BL/6 mice were bred at the facility of the University of Manitoba animal care facility. Pairs of Jα18-KO mice that are deficient in iNKT cells were provided by Dr. Masaru Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). All mice used in this study were males of 6-8 weeks of age, and bred and maintained at a pathogen-free animal care facility at the University of Manitoba.

3.5.0 ISOLATION OF LDC/LDC SUBSETS

Mice were killed at predetermined days (day 3 or 8) after intranasal inoculation with *C. muridarum/C. pneumoniae*, and the lungs were aseptically removed and processed into single-cell suspensions. The lungs were digested in 10 mg/ml collagenase XI (Roche Diagnostics, Meylan, France) in RPMI 1640 for 1 hour at 37^{o}C. EDTA at 5mM was added during the last 5 minutes, and the cell suspension was then pipetted up and down several times and filtered. The complete RPMI medium included 10% heat-inactivated FBS, 25 µg/ml gentamicin, 2 mM L-glutamine, and 5 X 10^{-5} 2-ME (Kodak, Rochester, NY). Total lung cells after RBC lysis with ACK lysis buffer (150 mM NH4Cl, 10mM KHCO3, 0.1 mM EDTA) were incubated with CD11c microbeads (Miltenyi Biotec) for 15 minutes at 4^{o}C. The cells were then washed, resuspended in cell separation buffer (Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca^{2+} and Mg^{2+} (Sigma) containing 0.5% BSA and 5mM EDTA, and passed through magnetic columns for positive selection of CD11c+ lung cells. For LDC isolation, the CD11c+ lung cells were stained with anti-CD11c antibodies and subjected to sorting on a flow cytometric cell sorter. CD11c^{hi}autofluorescence- lung cells were gated as LDCs and sorted with high

purity (>97%), as shown using anti-F4/80 antibodies. For LDC subset isolation, the CD11c+ lung cells were stained with anti-CD11c, anti-MHC-II, anti-CD103 and anti-CD11b^{hi} antibodies. CD11c^{hi}MHC-II^{hi}autofluorescence- lung cells were gated as LDCs and then displayed in terms of CD103+ and CD11b^{hi} LDC subsets for a flow cytometric sorting (purity >99%). A BD FACSAria II Cell Sorter (BD Biosciences, San Diego, CA) was used to sort LDC/LDC subsets. Hemocytometer and trypan blue were used to count viable cells. Of note, for different studies, LDCs were isolated from KO or NK cell-depleted mice following infection. For NK-cell depletion *in vivo*, anti-asialo-GM1 antibodies were used as described previously (140). Our previous study has shown that anti-asialo-GM1 antibodies deplete NK but not NKT cells during chlamydial infection (140).

3.5.1. Adoptive transfer of LDC/LDC subsets and challenge infection

For adoptive transfer, LDC/LDC subsets isolated from the lungs of *Chlamydia*-infected mice were first washed in protein-free PBS and then inoculated intranasally into naïve recipient mice at the rate of 2 X 10^5 LDCs or 1 X 10^5 CD103+/CD11b^{hi} LDCs per mouse. Two hours after the adoptive transfer, mice were intranasally inoculated with 1 X 10^3 IFUs of *C. muridarum* or 3 X 10^6 IFUs of *C. pneumoniae* in 40 µl of PBS. Body weights of mice before (day 0) and after inoculation were recorded daily. The mice were sacrificed at different time points (day 7 or 8) following infection challenge, and the lung tissues were aseptically isolated and processed for quantitative evaluation of chlamydial *in vivo* growth as described previously (140).

3.5.2. Analysis of cytokine response by LDC/LDC subsets

To test spontaneous cytokine production by the freshly isolated LDC/LDC subsets from *Chlamydia*-infected or uninfected mice, the LDC/LDC subsets were cultured with complete medium using 96-well culture plates at 2 X 10^5 LDCs or 1 X 10^5 LDC subset/well for 72 hours. The levels of various cytokines such as IL-12p70, IL-12p40, IL-6, IL-23 and IL-10 in the culture supernatants were measured by ELISA.

3.6.0. FLOW CYTOMETRIC ANALYSIS

To analyze LDC/LDC subsets, single cell suspensions were prepared from the freshly isolated lung. CD11c+ lung cells were isolated from the single cell suspension using CD11c microbeads (Miltenyi Botec). The CD11c+ cells were incubated with anti-mouse CD16/32 antibodies for 15 minutes to block Fc receptors. Then, the single cell suspension was stained with specific antibodies at 4^0C for 30 minutes in dark. After washing with a staining buffer (Dulbecco's PBS containing 0.5% BSA and 1mM EDTA), the cells were fixed and analyzed. Sample data were collected using a BD LSR II flow cytometer (BD Biosciences, San Diego, CA), and the data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA). To analyze surface markers on LDC/LDC subsets, multicolor staining was performed. For LDCs, anti-CD11c-APC, anti-F4/80-PE-Cy7, anti-MHC-II-PE (I-A/I-E), anti-CD40-PE, anti-CD80-PE, anti-CD86-PE, anti-CD103-PE, anti-RAE1-PE antibodies or their respective isotype controls were used. For LDC subsets, anti-CD11c-APC-Cy7, anti-MHC-PECy7, anti-CD11b-APC, anti-CD103-PE, anti-CD40-PerCP, anti-CD80-PerCP, anti-CD86-PerCP antibodies or their respective isotype controls were used. To analyze NK cells, anti-NK1.1-PE, anti-

CD3-PE-Cy7 antibodies or their respective isotype controls were used. All antibodies were purchased from eBioscience, San Diego, CA.

3.7.0. INTRACELLULAR CYTOKINE STAINING

Lung cells were incubated at 5 X 10^6 cells per ml of complete RPMI medium in 48-well plates at 37^{o}C, and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma Chemical) and 1 µg/ml ionomycin (Sigma). After 3 h of incubation, 20 µg/ml brefeldin A (sigma) was added to the culture for another 3 hours. After 3 hours, the cells from the culture were washed and incubated with FcR blocking Abs (anti-16/32; eBioscience) for 15 min at 4^{o}C. The cell surface markers were first stained with anti-CD3-PE-Cy7, anti-CD4-PE and anti-CD8-FITC or respective isotype control Abs (eBioscience, San Diego, CA). The cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen). Then, the cells were subjected to the intracellular staining with anti-IFN-γ-APC, anti-IL-17-APC, anti-IL-4-APC or isotype control Abs (eBioscience, San Diego, CA). Finally, the cells were washed, resuspended in Dulbecco's PBS containing 0.5% BSA and 1mM EDTA, and analyzed by flow cytomtery. Sample data were collected using a BD LSR II flow cytometer (BD Biosciences, San Diego, CA), and the data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

3.8.0. HISTOPATHOLOGICAL ANALYSIS

The histopathologic analysis was performed as described previously (118, 140), In brief, the lung tissues were fixed in 10% buffered formalin. The fixed tissues were embedded in paraffin, and then cut into sections for staining with hematoxylin and eosin (HE).

Pathologic/histologic changes were assessed by light microscopy.

3.9.0. *IN VITRO* RESTIMULATION ASSAYS AND CYTOKINE MEASUREMENTS

MLNs were aseptically removed from mice and processed into single-cell suspensions as described previously (118). MLN cells were cultured at a concentration of 5 X 10^6 cells per ml RPMI complete medium with UK-EB (1 X 10^4 IFUs/ml). After incubation for 72 hours in a 5% CO_2 atmosphere, the supernatants were collected, frozen at -80^0C, and later analyzed for cytokines by ELISA. To test cytokine levels in the lung, the lung tissues were homogenized in 2 ml cold PBS. The supernatants were collected after centrifugation, and analyzed for various cytokines by ELISA. ELISA plates (96-well plate) were coated with anti-cytokine antibodies dissolved in coating buffer (0.2 M sodium carbonate) (50 µl/well), and incubated over night at 4^0C. The plate was washed 3 times, and 200 µl of blocking buffer (2% BSA) was added to each well and left at room temperature for 1-2 hours. After removing the blocker, the samples were added to each well in duplicate, and incubated at room temperature for 3 hours. Biotinylated detection antibodies were mixed with dilution buffer. 50 µl of the solution was added to each well, and incubated at 4^0C overnight. After washing the plate, 100 µl of streptavidin conjugated alkaline phosphatase (1:6000) was added to each well and incubated at room temperature for 45 minutes. The plate was washed 6 times with washing buffer, and 100 µl of substrate solution was added to each well and read values at 30 minutes. Of note, the details of antibodies used in ELISA have been mentioned in Table 1. All antibodies were purchased from eBioscience, San Diego, CA.

Table 1. Details of antibodies used in ELISA.

Cytokine	Coating purified antibodies/ Clones	Coating antibody dilution	Sample dilution	Biotinylated antibody/ Clones	Biotinylated antibody dilution
IL-4	Anti-mouse-IL-4/11B11	1 µg/ml	1:2	Anti-mouse-IL-4Biotin/ BVD6-24G2	1 µg/ml
IL-6	Anti-mouse-IL-6 /MP5-20F3	1 µg/ml	1:4	Anti-mouse-IL-6 Biotin/ MP5-32C11	1 µg/ml
IL-10	Anti-mouse-IL-10/ JES5-16E3	1 µg/ml	1:4	Anti-mouse-IL-10 Biotin/ JES5-2A5	1 µg/ml
IL-12p40	Anti-mouse-IL-12p40/C15.6	2 µg/ml	1:2	Anti-mouse-IL-12p40 Biotin/C17.8	1 µg/ml
IL-12p70	Anti-mouse-IL-12p70/C18.2	1 µg/ml	1:2	Anti-mouse-ILp70Biotin/C18.3	1 µg/ml
IFN-γ	Anti-mouse-IFN-γ/R46A2	1 µg/ml	1:4	Anti-mouse-IFN-γ Biotin/ XMG1.2	1 µg/ml
TNF-α	Anti-mouse-TNF-α/ IF3F3D4	1 µg/ml	1:2	Anti-mouse-TNF-α Biotin/ XT3/XT22	1 µg/ml
IL-17	Anti-mouse-IL-17/ 17CK15A5	1 µg/ml	1:2	Anti-mouse-IL-17 Biotin/ 17B7	1 µg/ml

3.10.0. LDC:T CELL COCULTURE

For immunization of mice with *C. pneumoniae*, mice were infected with *C. pneumoniae* (3 X 10^6 IFUs/mouse) intranasally and boosted two weeks later with the same dose through the same route. For immunization with *C. muridarum*, mice were injected with 1 X 10^3 IFUs of *C. muridarum* intraperitoneally, and two weeks later boosted with the same dose of infection. One week after the challenge, T/CD4+ T cells were isolated from the spleen of *Chlamydia*-immunized mice using a cocktail of antibodies attached to magnetic beads (Miltenyi Biotec, Auburn, CA) and MACS columns as described previously (118). The purity of the T/CD4+ T cells was more than 97% as determined by flow cytometric analysis. LDC/LDC subsets isolated from *Chlamydia*-infected mice were cocultured with purified T/CD4+ T cells (LDC/T cell ratio, 1:5) in 200 µl complete RPMI medium, with UK-EB (1 X 10^4 IFUs/ml) in 96-well plates for 72 hours, and the concentrations of cytokines in the supernatants was measured by ELISA.

3.11.0. NK CELL:LDC COCULTURE

NK cells were isolated from the lungs of mice at day 3 following *C. muridarum* infection as described previously (140). In brief, NK cells were isolated from the lung using anti-CD3 and anti-DX5 microbeads for negative and positive selection, respectively. As determined by the flow cytometric analysis, the purity of the sorted NK cells was more than 96%. LDCs isolated from syngeneic naïve mice were cultured with the NK cells (NK cell/LDC ratio, 1:5) in 200 µl complete RPMI medium in 96-well plates with or without 50 µl/ml anti-IFN-γ, 40 µl/ml anti-TNF-α, 3 µl/ml anti-NKG2D or a combination of both anti-IFN-γ and anti-NKG2D antibodies (Armenian Hamster IgG purchased from e-

Bioscience) in the presence of UK-EB (1 X 10^4 IFUs/ml) for 48 hours. The concentrations of IL-12p70, IL-6 and IL-23 in the supernatants were measured by ELISA.

3.12.0. STATISTICAL ANALYSIS

Unpaired Student's *t* test was used to analyze the data from two groups of mice. One-way analysis of variance (ANOVA) was used to determine statistical significance among three or more groups of mice (GraphPad Prism software, version 4, Graph Pad, San Diego, CA). A p value less than 0.05 was considered significant.

4.0. CHAPTER 4

INVARIANT NKT CELLS PROMOTE T CELL IMMUNITY BY MODULATING THE FUNCTION OF LUNG DENDRITIC CELLS DURING CHLAMYDIA PNEUMONIAE INFECTION

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Sudhanshu Shekhar drafted and contributed to 70% of data collection and analysis for the realization of this manuscript.

4.1.0. INTRODUCTION AND RATIONALE

The lung is a vital organ that facilitates gaseous exchange during respiration. In doing so, the lung is continually exposed to a plethora of environmental pathogens, including bacteria and viruses. LDCs are the chief orchestrators of immune responses in the lung because of their unique ability to process and present antigens in the most efficient way. They express various surface molecules like CD11c and MHC-II and are located within the epithelium and interstitium of the lung, functioning as a cellular interface between the external and internal lung environment (207). Upon acquiring microbial antigens, LDCs undergo a coordinated maturation program that enables them not only to upregulate MHC-II and costimulatory molecules and induce enhanced cytokine production, but also to migrate to MLNs to prime naïve T cells to generate adaptive immune responses (208). In the steady-state condition, LDCs produce cytokines such as IL-6 and IL-10 and preferentially polarize T cells toward a Th2 response (200-201). During respiratory infections, LDCs can, however, generate different immune responses, depending upon the type of pathogen. For example, LDCs induce Th1 responses characterized by IFN-γ production against intracellular bacteria and viruses, while exposure to extracellular pathogens such as parasites mounts Th2 responses that lead to eosinophilia and IgE antibody production (209-2011).

iNKT cells represent a subset of innate lymphocytes that possess the characteristics of both NK and αβ T cells. They express the αβ TCR with the use of chain Vα14-Jα18 in mice and Vα24-Jα18 in humans. They also express markers of NK cells, such as NK1.1 (98). Unlike conventional T cells, iNKT cells recognize glycolipid

and lipid antigens presented to them by non-classical MHC-I CD1d molecules. The prototypic antigen used in experimental studies for iNKT cell activation is α-GalCer which is, biochemically, a glycolipid derived from marine sponges. Upon stimulation with specific antigens, iNKT cells promptly produce a variety of cytokines, most notably IFN-γ and IL-4 (106). Many glycolipid and lipid antigens specific for the activation of iNKT cells have also been derived from several bacterial species (106). Recently, we identified a glycolipid exoantigen (GLXA) from *C. muridarum* that induced iNKT cells to express higher levels of an activation marker, CD69, and to produce cytokines like IFN-γ and IL-4 (107). These findings suggest a direct role of the invariant TCR of iNKT cells in the induction of immune responses. In addition to the direct recognition of antigens, iNKT cells can be indirectly activated through innate or inflammatory cytokine-driven stimuli during microbial infections. This indirect activation of iNKT cells is an important pathway that is mediated through the secretion of IL-12 by APCs following TLR activation (115). iNKT cells have been shown to play a crucial role in tumor rejection, maintenance of tolerance, and protective immunity against many pathogens (106). They not only contribute to innate immune responses but also influence the outcome of adaptive immune responses. The major cellular mechanism by which iNKT cells manipulate adaptive immune responses is by modulating the function of DCs. Thus, these cells act as a connecting link between innate and adaptive immunity (98).

C. pneumoniae, an obligate intracellular bacterial pathogen, causes various diseases in humans, thus posing a threat to public health worldwide. These diseases include bronchitis, sinusitis, and community acquired pneumonia (6). Serological studies

have shown that most people have been infected with *C. pneumoniae* at least once during their lifetime (18). Recently, *C. pneumoniae* has been implicated in the pathogenesis of atherosclerosis, Alzheimer's disease, multiple sclerosis, arthritis, and asthma (15). At present, no vaccine is available for chlamydial diseases. Efforts to develop a safe and rational *C. pneumoniae* vaccine have been hindered by the lack of adequate understanding of the protective and pathologic immune responses to chlamydial infection. Emerging evidence suggests that Th1/Tc1 responses play a pivotal role in the clearance of *C. pneumoniae* infection, while Th2 responses may result in immunopathology (15). In addition, unlike *C. trachomatis* infection, CD8+, relative to CD4+, T cell responses play a dominant role in *C. pneumoniae* infection.

We previously reported a significant modulating effect of iNKT cells on the function of DCs in the spleen to induce protective immunity against *C. pneumoniae* lung infection (118-119). How iNKT cells interact with LDCs, which reside at the primary site of *C. pneumoniae* infection, i.e. the lung, where maximal inflammatory and pathologic changes occur, is an important question yet to be addressed. The question is further emphasized by emerging evidence that LDCs and SDCs reflect phenotypic and functional distinctions, which may be attributed to the influence of their local tissue environment (200-202). In addition, functional differences between LDCs and SDCs have been reported in various infection models (78, 203-204). Recent studies further demonstrate that iNKT cells interact with LDCs in the lung microenvironment to induce local immune responses during respiratory infections (116, 205). Taking into consideration the facts that **1)** phenotypic and functional distinctions between LDCs and

SDCs could differentially contribute to the outcome of T cell responses, **2)** iNKT cells elicit immune responses to respiratory pathogens by interacting with local DCs, and **3)** the events occurring at the secondary lymphoid organs such as the spleen may not necessarily mirror what happens in the primary organ of infection (the lung), we directly examined the influence of iNKT cells on the functional competence of LDCs in inducing protective immune responses against *C. pneumoniae* lung infection. We found that LDCs from Jα18-KO mice, which lack iNKT cells, showed reduced expansion, lower MHC-II and costimulatory molecule expression, and decreased IL-12 production when compared with those from WT mice p.i. Furthermore, LDCs from *C. pneumoniae*-infected KO mice (KO-LDCs) were less efficient in directing antigen-specific Th1/Tc1 responses than LDCs from WT mice (WT-LDCs); this is supported by coculture data. Moreover, upon adoptive transfer, KO-LDCs enhanced Th2 responses and increased the severity of *C. pneumoniae* infection, in sharp contrast to the effect with the transfer of WT-LDCs that promoted protective Th1/Tc1 immunity against *C. pneumoniae* challenge. Together, our findings have shed light on the role played by iNKT cells in modulating the function of LDCs for generating T-cell immunity against chlamydial infection. To our knowledge, this is the first report demonstrating the effect of iNKT cells on LDC function in the lung microenvironment during a clinically relevant intracellular bacterial infection.

4.2.0. RESULTS

4.2.1. iNKT-cell deficiency alters the number, surface molecule expression and cytokine production pattern of LDCs following *C. pneumoniae* infection

In order to assess the modulating effect of iNKT cells on LDCs, we first analyzed the number and surface molecule expression of the LDCs from KO and WT mice after *C. pneumoniae* infection. To characterize LDCs by flow cytometry, we used a combination of CD11c-expression and autofluorescence (212). The flow cytometric FL-1/FITC-detection channel was kept empty, i.e. there were no fluorochrome-conjugated antibodies in the sample for this channel, to measure the autofluorescence. $CD11c^{hi}$ non-autofluorescent LDCs were defined as LDCs and distinguished from the highly autofluorescent AMs based on their autofluorescence emission. We also used an antibody against the pan-macrophage marker F4/80 to confirm if $CD11c^{hi}$ non-autofluorescent lung cells are indeed LDCs. Most $CD11c^{hi}$ non-autofluorescent lung cells were F4/80- (Fig. 4A). We found that the frequency and absolute number of LDCs in the KO mice were lower than in the WT mice after infection; however, these numbers in the uninfected WT and KO mice were similar (Fig. 4B & C). Although there was no difference in the frequency of AMs between uninfected WT and KO mice, it increased in the WT mice (compared to in the KO mice) at day 3 p.i. (Fig. 4D). We also found a reduced accumulation of DCs in the MLNs of KO mice compared with WT mice p.i. (Fig. 4E & F). We further analyzed the surface molecule expression on the LDCs. The LDCs from KO mice showed a reduced expression of MHC-II, CD80 and CD40 molecules compared to WT mice (Fig. 5, Table 1). On the other hand, the LDCs from KO mice

showed a higher expression of CD86 compared to WT mice. Of note, the levels of surface marker expression on LDCs revealed no significant difference between uninfected WT and KO mice (Table 3).

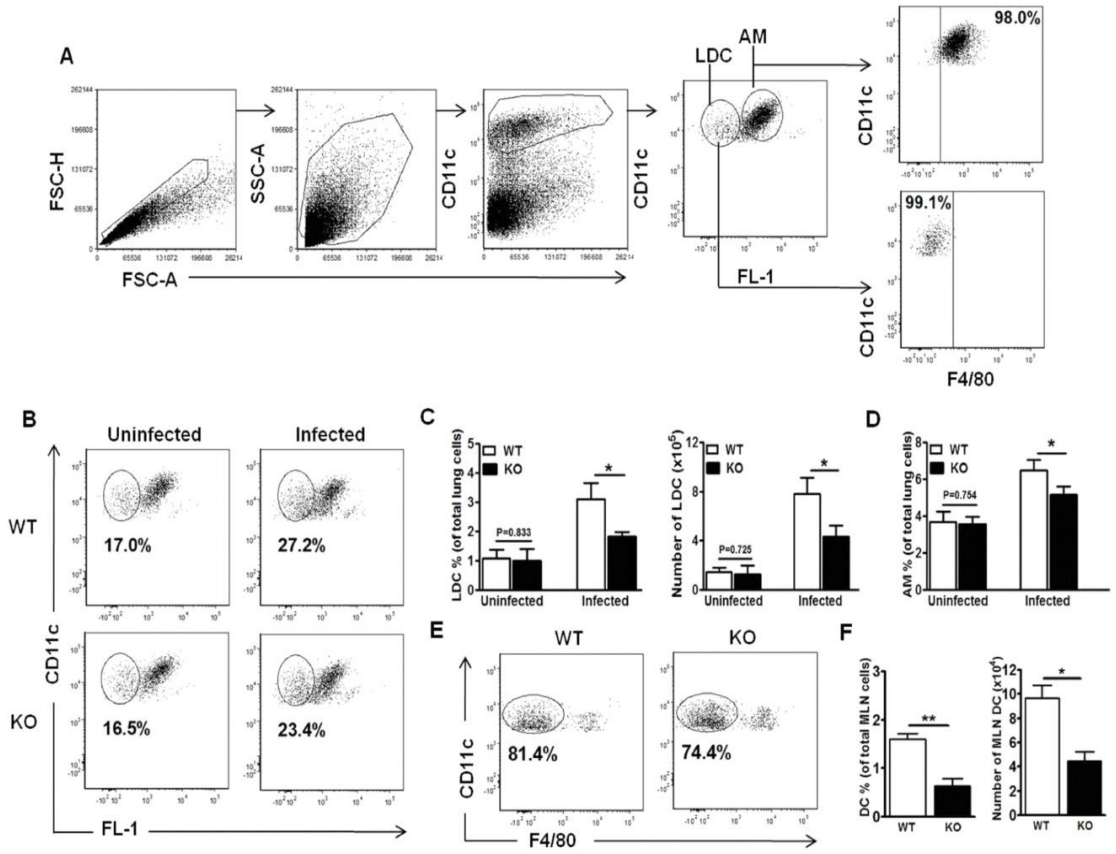

Figure 4. LDCs in KO mice show reduced expansion after *C. pneumoniae* infection. KO and WT mice (3 in each group) were intranasally infected with *C. pneumoniae* (3 X 10^6 IFUs/mouse) and sacrificed at day 3 p.i. Lungs and MLNs were harvested from the mice and processed into single-cell suspensions. (a) Flow cytometric gating strategy for analysis of LDCs. $CD11c^{hi}$ non-autofluorescent and $CD11c^{low/-}$ autofluorescent lung cells were gated and analyzed as LDCs and AMs, respectively. Analysis was performed on gated $CD11c^{hi}$ non-autofluorescent lung cells. F4/80 was used to confirm whether $CD11c^{hi}$ non-autofluorescent represent LDCs. (b) Representative flow cytometric images showing the frequency of LDCs after infection. (c) Graphs representing the percentage and absolute numbers of LDCs. The absolute numbers of LDCs were calculated as the percentage of $CD11c^{hi}$ non-autofluorescent lung cells X total number of lung cells/100. (d) Graphs representing the percentage of AMs of all the lung cells. (e) CD11c+F4/80- cells in the MLNs were analyzed as DCs. Flow cytometric images are shown. (f) Graphs representing the percentage and absolute numbers of DCs in the MLNs. Data are shown as mean ± SD. One of the 2 independent experiments with similar results is shown. *$p < 0.05$; **$p < 0.01$.

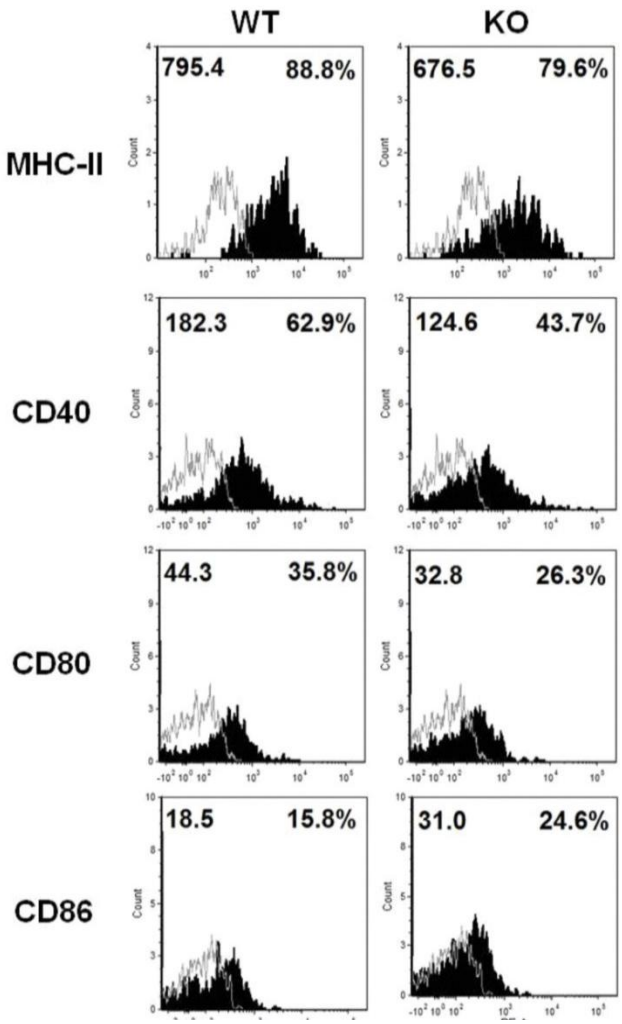

Figure 5. iNKT cells alter LDC surface molecule expression after infection. Both KO as well as WT mice (3 in each group) were sacrificed at day 3 after intranasal infection (3 X 10^6 IFUs/mouse). Lungs were harvested and processed into single-cell suspensions. CD11c+ lung cells were isolated by using CD11c-magnetic microbead selection. $CD11c^{hi}$ non-autofluorescent lung cells were gated as LDCs as shown in figure 1a. The LDCs were analyzed for the expression of MHC-II and costimulatory molecules by flow cytometry. Flow cytometric dotplots are shown. Expression of CD80, CD86, CD40 and MHC-II on LDCs (shaded histogram) and isotype control (line) were shown. The mean fluorescence intensity (left) and percentages of positive cells (right) were indicated. One of the 2 independent experiments with similar results is shown.

Table 2. Summary of surface marker expression on LDCs from WT and KO mice following *C. pneumoniae* infection

Surface Markers	WT	KO
CD40		
%	57.2 ± 2.9	39.1 ± 3.1*
MFI	178.7 ± 2.5	115.9 ± 2.0***
CD80		
%	29.6 ± 2.3	21.4 ± 1.6*
MFI	36.8 ± 2.1	27.9 ± 2.3
CD86		
%	14.9 ± 1.0	22.5 ± 1.0**
MFI	15.3 ± 1.3	23.9 ± 2.4*
MHC-II		
%	91.0 ± 1.1	86.9 ± 1.0*
MFI	571.8 ± 13.5	500.7 ± 8.8*

Three mice from each group were sacrificed at day 3 after *C. pneumoniae* infection. CD11c+ lung cells were isolated by using CD11c-magnetic microbead selection. $CD11c^{hi}$ non-autofluorescent lung cells were gated as LDCs as described in figure 1a. The surface molecule expression on LDCs was analyzed by flow cytometry. Statistical analyses were performed using a Student's t test. Data are shown as mean ± SD of three mice in each group. One of the 2 independent experiments with similar results is shown. MFI = Mean fluorescence intensity. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. Summary of surface marker expression on LDCs from WT and KO mice in naïve mice

Surface Markers	WT	KO
CD40		
%	20.1 ± 3.1	18.9 ± 4.8
CD80		
%	14.9 ± 2.4	15.7 ± 3.9
CD86		
%	10.3 ± 3.7	9.6 ± 3.2
MHC-II		
%	34.5 ± 6.5	31.3 ± 8.9

Three naïve mice from each group were sacrificed. CD11c+ lung cells were isolated by using CD11c-magnetic microbead selection. $CD11c^{hi}$ non-autofluorescent lung cells were gated as LDCs as described in figure 1a. The surface molecule expression on LDCs was analyzed by flow cytometry. Statistical analyses were performed using a Student's t test. Data are shown as mean ± SD of three mice in each group. One of the 2 independent experiments with similar results is shown.

The cytokine production pattern of DCs plays a critical role in skewing T cell responses. In particular, IL-12 production by DCs has been found to promote Th1/Tc1 response important for protection against chlamydial infection. Therefore, to further test the functional implication of iNKT cells on the expansion and surface molecule expression of LDCs, we isolated KO-LDCs and WT-LDCs mice with purity >97%, excluding AMs (Fig. 6a). Upon culture, we found that KO-LDCs produced significantly lower levels of IL-12 (IL-12p40 and IL-12p70) than WT-LDCs. Conversely, IL-10 levels were significantly higher in the supernatants of cultured KO-LDCs, compared with those of WT-LDCs (Fig. 6b). Taken together, these data suggest that iNKT cells can significantly modulate both the phenotype as well as the cytokine production pattern of LDCs after *C. pneumoniae* infection, particularly promoting IL-12 but regulating IL-10 production.

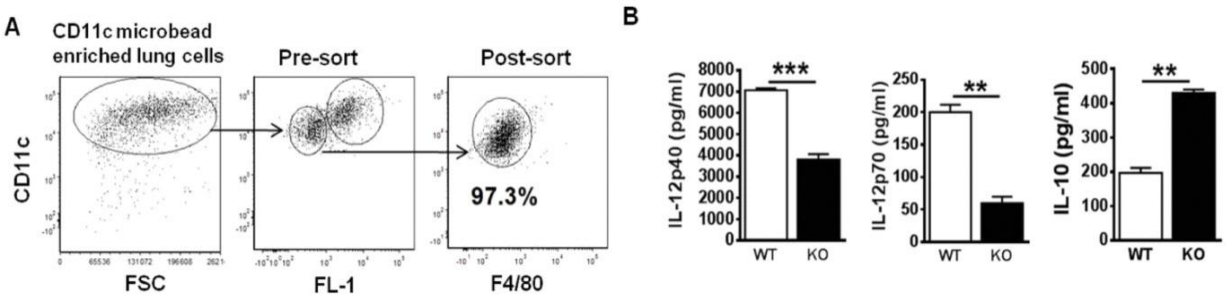

Figure 6. Analysis of purity and cytokine profile of sorted LDCs following infection. Lungs were harvested from mice at day 3 p.i., processed into single cell suspensions and enriched with CD11c magnetic beads using an MACS column. The CD11c-enriched lung cells were stained with APC-conjugated-anti-CD11c or isotype control antibodies. Flow cytometric analysis was performed on gated CD11c+ cells. $CD11c^{hi}$ non-autofluorescent cells were considered as LDCs and sorted by flow cytometry as described in materials and methods. (a) Flow cytometric dot plots showing the gating strategy and the purity of sorted LDCs using antibodies for CD11c and F4/80. (b) LDCs purified from KO or WT mice were placed in culture for 72 h, and the concentrations of IL-12p40, IL-12p70 and IL-10 in the supernatants were measured by ELISA. Data are shown as mean ± SD. One of the 2 independent experiments (3 mice in each group) with similar results is shown. $^{**}p < 0.01$; $^{***}p < 0.001$.

4.2.2. iNKT cells modulate the functional ability of LDCs to direct T-cell responses

To directly examine the modulating effect of iNKT cells on LDC function in directing *C. pneumoniae*-specific T-cell responses, we cultured T cells from *Chlamydia*-immunized WT mice with KO-LDCs or WT-LDCs in the presence of UK-EB stimulation. T cells cultured alone with UK-EB were used as controls. Upon analysis of culture supernatants by ELISA, we found that IFN-γ production by T cells was significantly lower and IL-4 production significantly higher in KO-LDCs than in WT-LDCs (Fig. 7a). Furthermore, we performed intracellular cytokine staining to specifically analyze the cytokine profile of CD4+ and CD8+ T cells following coculture with WT-LDCs or KO-LDCs. Flow cytometric analysis showed that WT-LDCs induced CD8+ and CD4+ T cells to produce large quantities of IFN-γ, which was significantly higher than that induced by KO-LDCs (Fig. 7b). Overall, these *in vitro* results demonstrate that iNKT cells modulate the functional ability of LDCs in polarizing CD4+ and CD8+ T cells toward a Th1/Tc1 response that is necessary for immune protection against *C. pneumoniae* infection.

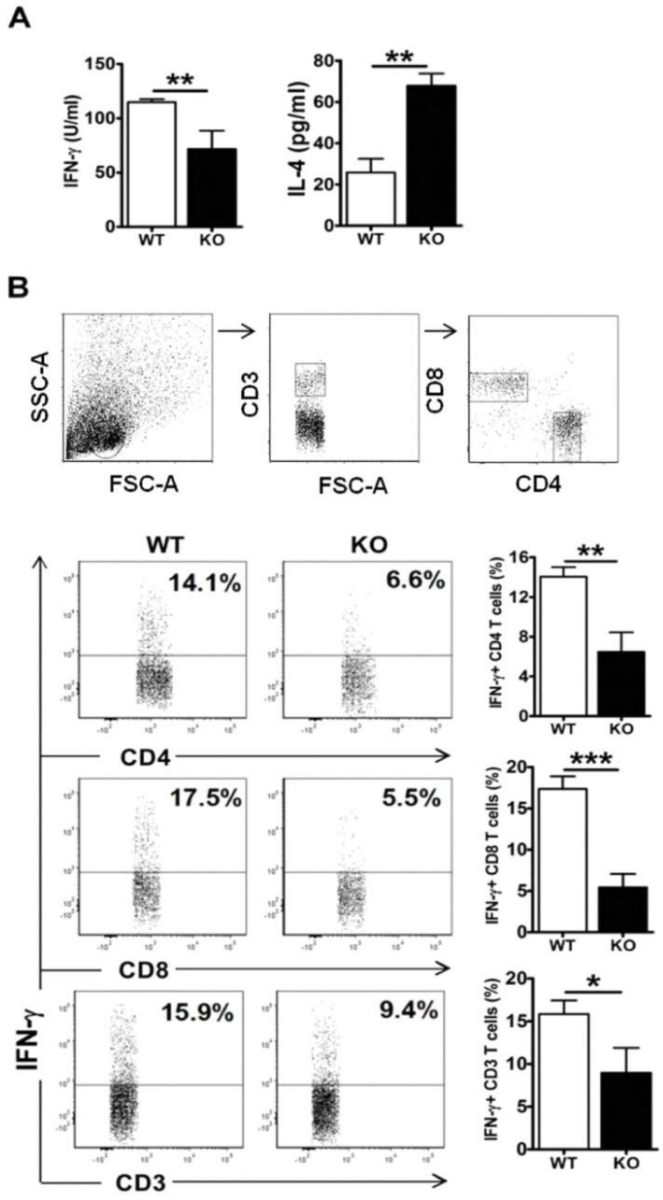

Figure 7. iNKT cells modulate LDC function in polarizing T cells toward Th1/Tc1 responses. T cells from *C. pneumoniae*-immunized mice were isolated and cocultured with KO-LDCs or WT-LDCs in the presence of UK-EB stimulation. (a) The concentrations of Th1/Tc1 (IFN-γ) and Th2 (IL-4) cytokines in the culture supernatants were measured by ELISA. (b) Intracellular cytokine production by T cells in LDC:T-cell coculture. Analysis was performed on gated CD3+ cells. Representative flow cytometric images (left) and the graphs (right) representing the summary of the percentage of IFN-γ+ CD4+, CD8+, and CD3+ T cells. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and 1 representative experiment is shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.2.3. Adoptive transfer of LDCs from KO mice increases the severity of *C. pneumoniae* infection

We further used the adoptive transfer approach to directly compare the functional capacity of WT-LDCs and KO-LDCs to induce protective immunity against *C. pneumoniae* challenge infection. We intranasally transferred WT-LDCs or KO-LDCs to naïve recipient mice and then challenged them with *C. pneumoniae* infection. Mice receiving PBS alone with the same challenge infection were used as controls. We found that the mice receiving KO-LDCs exhibited the most significant body weight loss compared with the WT-LDC recipients or even the PBS controls. On the other hand, WT-LDC-recipient mice lost less body weight and showed signs of earlier recovery (Fig. 8Aa). The mice were sacrificed at day 7 p.i. and their lungs were harvested for the analysis of *in vivo* chlamydial growth and lung histopathology. We found that the *C. pneumoniae* loads in the lungs of KO-LDC recipients were significantly higher than the WT-LDC recipients and controls (Fig. 8Ab). Of note, no viable chlamydial organisms were found in the WT-LDCs and KO-LDCs, and therefore the increased infection in KO-LDC recipients was unlikely due to the potential trace amount of *C. pneumoniae* organisms carried by the transferred LDCs. Furthermore, we found the most severe inflammatory and pathologic changes of all the groups occurred in the lungs of the KO-LDC recipients (Fig. 8Ac). To rule out the possibility of whether LDCs from uninfected mice have any impact on the outcome of T-cell immunity, we pooled the LDCs from uninfected WT or KO mice and transferred them to naïve recipient mice. Upon challenge infection, the mice receiving LDCs from uninfected WT or KO mice and PBS recipients showed no significant difference in their loss of body weight, chlamydial lung

burden, and lung pathology (Fig. 8Ba, Bb, Bc). These *in vivo* data indicate that iNKT cells are critically important for enhancing LDC function to induce protective immunity against *C. pneumoniae* infection. Without iNKT cell modulation, LDCs not only fail to promote protective immunity but also increase the infection and the pathology.

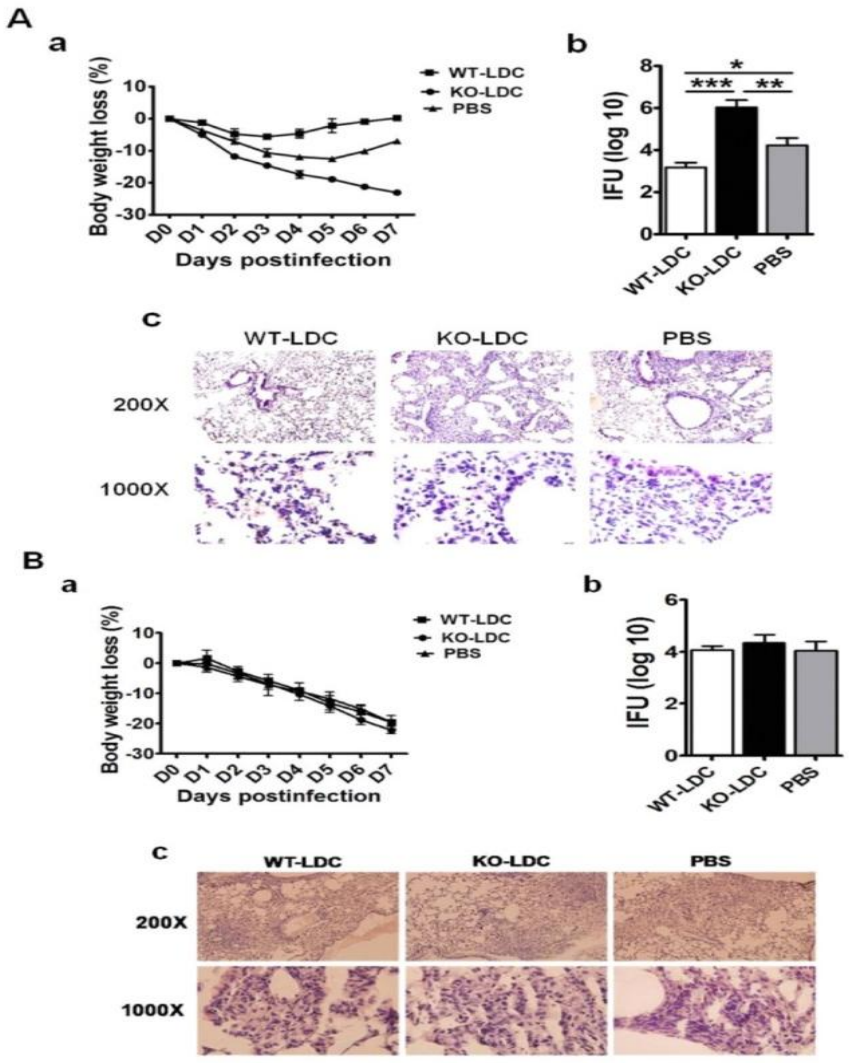

Figure 8. Adoptive transfer of KO-LDCs and WT-LDCs to test their function *in vivo*. Naïve mice were intranasally delivered KO-LDCs or WT-LDCs and then challenged with *C. pneumoniae* infection (3 X 10^6 IFUs/mouse). Mice receiving PBS only with the same challenge infection were considered as controls. All mice were sacrificed at day 7 p.i. Lungs from the recipient mice homogenized and tested for chlamydial loads. The mean of the $\log_{10}$ transformed IFUs/lung is shown. Lungs from the recipient mice were examined for pathology by HE staining and analyzed under light microscope. (A) Transfer of KO-LDCs and WT-LDCs isolated from *C. pneumoniae*-infected mice. (a) Body weight loss. (b) *In vivo* chlamydial growth. (c) Lung pathology. (B) Transfer of KO-LDCs and WT-LDCs isolated from uninfected mice. (a) Loss of body weight. (b) Chlamydial burden. (c) Lung pathology. Data are shown as mean ± SD. Two independent experiments with 3-4 mice in each group were performed, and 1 representative experiment is shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.2.4. Adoptive transfer of LDCs from KO and WT mice generates differential cytokine production patterns in recipient mice after challenge infection

To examine the immunological basis for the distinct effect of adoptively transferred KO-LDCs and WT-LDCs, we analyzed the cytokine production pattern in the lung tissues and MLNs of the LDC-recipient mice at day 7 following chlamydial challenge. We found that the mice receiving KO-LDCs showed an increased Th2 response with significantly higher levels of IL-4 and IL-10, whereas those receiving WT-LDCs showed increased Th1/Tc1 response with enhanced levels of IFN-γ, IL-12p40, and IL-12p70 (Fig. 9, 10). In addition, the KO-LDC-recipient mice had lower levels of TNF-α in their lungs than in the WT-LDC recipients or PBS control mice (Fig. 10).

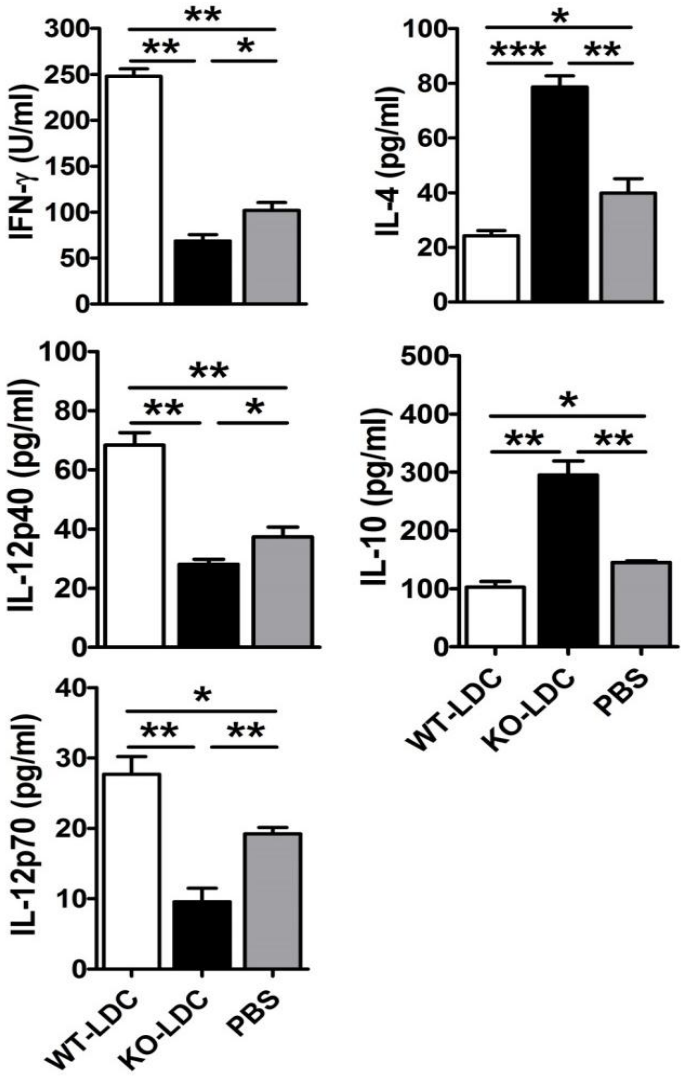

Figure 9. Adoptive transfer of KO-LDCs induces a Th2 response in the MLNs of recipient mice. Recipient mice were sacrificed at day 7 p.i. MLNs were harvested from the mice and processed into single-cell suspensions. MLN cells were cultured with UK-EB for 72 h. The cytokine levels in the culture supernatants were examined by ELISA. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and 1 representative experiment is shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

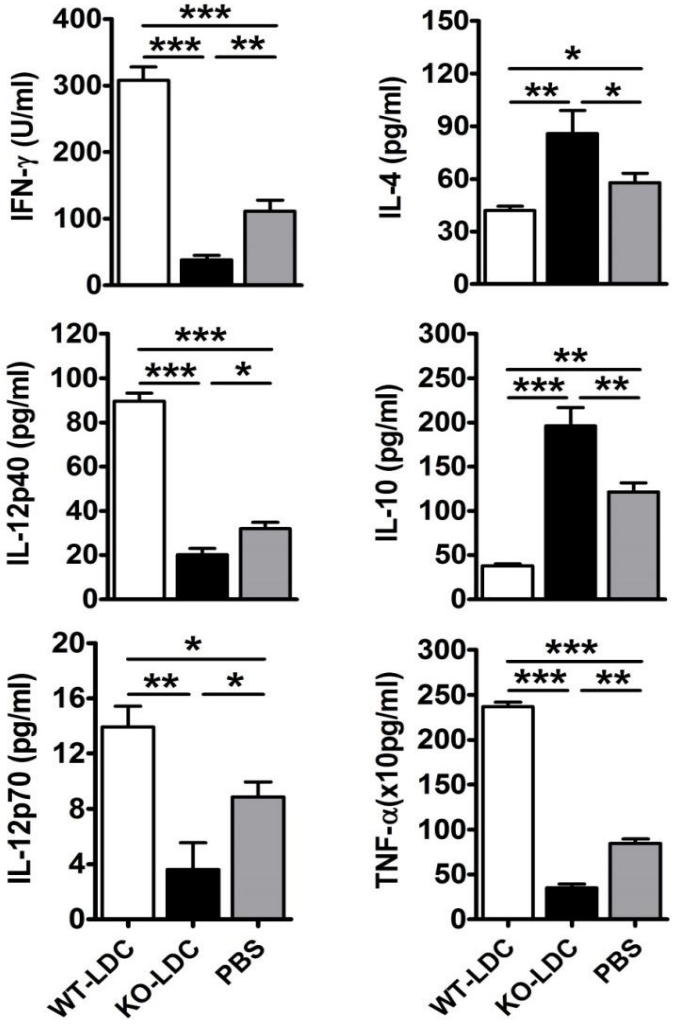

Figure 10. Effect of LDC adoptive transfer on the local cytokine environment in the lungs of recipient mice. Mice receiving LDCs or PBS were sacrificed at day 7 p.i. Lungs were harvested from the mice, homogenized in cold, protein-free PBS, and centrifuged. Levels of different cytokines in the supernatants were measured by ELISA. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and 1 representative experiment is shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

To further investigate the specific T-cell cytokine profile, we performed intracellular cytokine analysis of the lung T cells by flow cytometry. A greater number of IFN-γ+ T cells were found in the recipients of WT-LDC than in KO-LDC recipients or controls (Fig. 11). Together, these data suggest that WT-LDCs, in contrast to KO-LDCs, significantly induce a Th1/Tc1 polarized immune response *in vivo.*

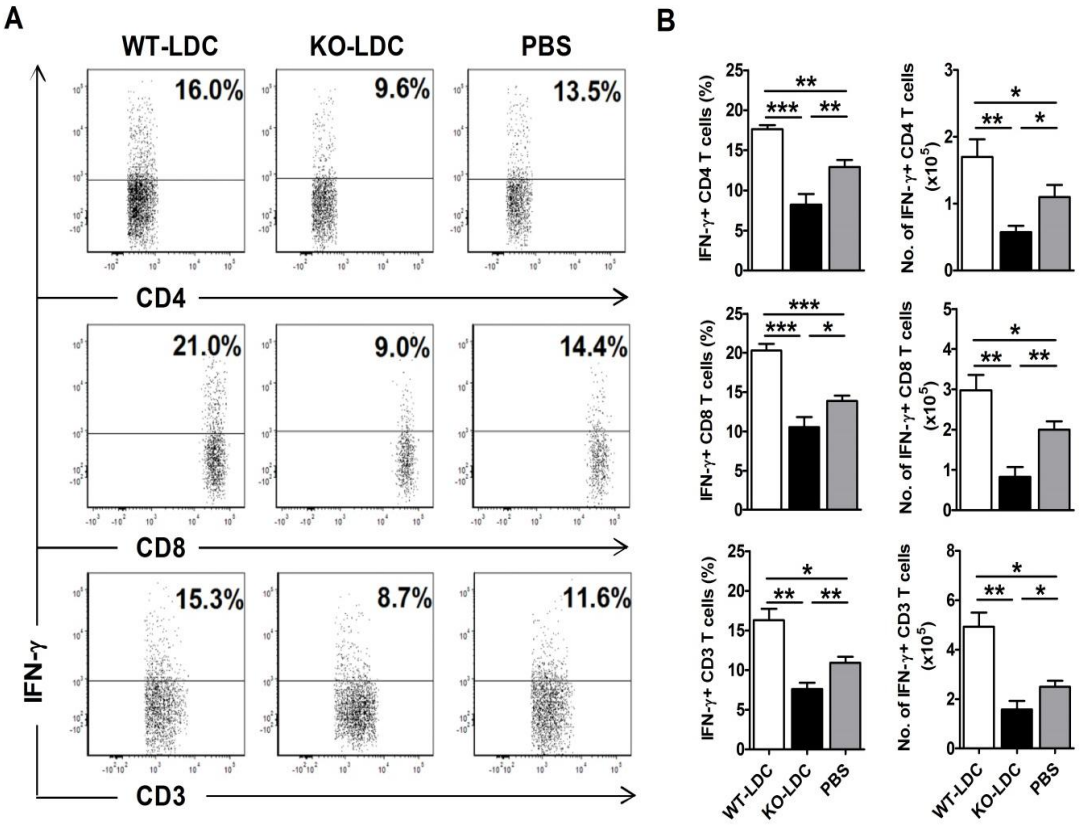

Figure 11. Local T-cell cytokine production in the lungs of recipient mice. Mice receiving KO-LDCs, WT-LDCs or PBS were sacrificed at day 7 p.i. Lung cells from the recipient mice were cultured at the concentration of 5 X 10^6 cells in complete RPMI medium. The intracellular staining was in response to PMA and ionomycin stimulation. The cytokine production pattern in the T cells was analyzed by intracellular cytokine staining. Analysis was performed on gated CD3+ cells as described in figure 7B. (a) Representative flow cytometric images showing the percentages of IFN-γ+ CD4+, CD8+, and CD3+ T cells. (b) Graphical summary of the percentages and absolute numbers of IFN-γ+ T cells. The absolute numbers of IFN-γ+ T cells were calculated as percentage of IFN-γ+ T cells X total number of cells per lung/100. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and 1 representative experiment is shown. *p < 0.05; **p < 0.01; ***p < 0.001.

4.3.0. DISCUSSION

In the present study, we demonstrate a promoting effect of iNKT cells on LDC function in generating protective T-cell immunity to *C. pneumoniae* infection using various experimental approaches. Specifically, we found that iNKT cells induced the expansion of LDCs following *C. pneumoniae* lung infection and, more importantly, enhanced the expression of costimulatory molecules and cytokine production by LDCs. Furthermore, we found that WT-LDCs promoted protective Th1/Tc1 responses, whereas KO-LDCs augmented Th2 response as assessed in *in vitro* LDC:T cell coculture experiments. Moreover, upon adoptive transfer, WT-LDCs enhanced the clearance of lung infection, while KO-LDCs increased the severity of the infection. This was associated with a significant differential effect on the lung cytokine pattern in the corresponding recipient mice; KO-LDC-recipient mice exhibited increased Th2 cytokine production, while WT-LDC recipients reflected a Th1/Tc1 cytokine profile with enhanced IFN-γ production. Altogether, the *in vitro* and *in vivo* data suggest that iNKT cells play a critical role in conferring protective immunity against *C. pneumoniae* infection through modulating the function of LDCs.

The key aspect derived from the results of this study is the modulating effect of iNKT cells on the function of major APCs residing at the local site during a respiratory tract infection with *C. pneumoniae*, a clinically relevant human pathogen known to cause a wide variety of respiratory disorders. Our previous findings demonstrated that the KO mice showed exacerbated susceptibility to primary *C. pneumoniae* infection (111). Following *C. pneumoniae* infection, these mice had greater body weight loss,

higher lung bacterial loads, and more intense inflammatory and pathologic changes in their lungs compared to the WT controls. Moreover, the KO mice compared to WT mice showed dramatically reduced Th1/Tc1 responses, showing that iNKT cells influence the development of protective adaptive immune responses. Overall, the findings suggested that the lack of iNKT cell activity in the KO mice significantly altered the pulmonary environment and immune responses following *C. pneumoniae* infection. These changes can alter the infiltration/differentiation/activation status of LDCs which play a key role in the development of protective immune responses. Indeed, the present findings showed that iNKT cell deficiency induced phenotypic and functional changes in LDCs following *C. pneumoniae* infection. Furthermore, we found that the intranasal adoptive transfer of WT-LDCs in naïve recipient mice conferred strong protective immunity, whereas transfer of KO-LDCs promoted pathology after chlamydial challenge infection. However, the transfer of LDCs from uninfected KO and WT mice did not have any impact on the outcome of infection. Altogether, these findings highlight the significance of *in vivo* interactions between iNKT cells and LDCs for the development of protective immunity against an intracellular bacterial infection. In particular, the differential effects observed following transfer of LDCs from infected mice compared to uninfected mice demonstrate iNKT cell responses playing an important role in programing LDCs during the primary infection in determining the outcome of a secondary infection. These findings have important implications in vaccine development. In order to execute their protective role in host defense against chlamydial infection, LDCs need the help from iNKT cells which can tune the function of LDC to polarize T cells toward a protective Th1/Tc1 response. In the absence of this help, the LDCs switch to a “DC2” mode that leads to the

generation of Th2 responses and pathology. The functional implication of the iNKT cell-LDC interaction is that it confers protective immunity as well as preventing pathologic changes. The mechanism of how iNKT cells impact the SDC function during *C. pneumoniae* infection has been investigated in our previous study, where we found that iNKT cells modulate DC responses in a CD40L-, IFN-γ-, and cell-cell contact-dependent fashion (118). It should be noted that this study has examined LDCs only at one time point following *C. pneumoniae* infection. It is still possible that the effect of iNKT cells on LDCs and SDCs could be quantitatively and qualitatively different at different stages of infection.

The effect of iNKT cells on LDCs is reflected by the cell number, surface markers and cytokine pattern. We found a markedly reduced expansion of DCs in the lung and the MLNs of KO mice following lung infection, as shown by the lower percentage and number of DCs in these tissues. However, it should be noted that the number of LDCs in KO mice also increased following chlamydial infection, suggesting that the expansion of LDCs was not dependent on the iNKT cells, although these cells could promote LDC function. Furthermore, KO-LDCs exhibited lower expression of MHC-II, CD40, and CD80 molecules compared with that of WT-LDCs. These findings are largely consistent with those of a recent study by Paget *et al.* (116), where a major population of LDCs, CD103+ LDCs, was significantly reduced in number in KO mice, and showed reduced expression of the costimulatory molecule, CD40, after influenza A virus infection. They also demonstrated reduced expression of CD86 following viral infection as opposed to the increased expression in our study. This indicates that different pathogens may

modulate the expression of some costimulatory molecules in diverse ways. Although the role of costimulatory molecules on DCs in promoting a Th1 response is widely appreciated, some studies suggest that their function may overlap in modulating Th1/Th2 differentiation, particularly in the case of CD86 (213-214). Moreover, it has been reported that CD86 expression is important for Th2 responses; this matches the data from our study which showed an elevated Th2 response in the KO-LDC-recipient mice. It is also possible that the impact of elevated CD86 expression on LDCs in KO mice is a reflection of the reduced expression of CD80, CD40, and MHC-II molecules on the final outcome of T-cell responses. The cytokine production pattern of DCs is more relevant to their function of directing T-cell responses. Our data on the cytokine pattern of LDCs further support the importance of iNKT cells on LDC function. KO-LDCs produced significantly lower levels of IL-12 (IL-12p70 and IL-12p40), which is a Th1 promoting cytokine. In contrast, the IL-10 production by LDCs was significantly higher in the KO mice than in the WT mice. The detrimental role of IL-10 in host defense against chlamydial infections has been demonstrated by many studies (25, 215). Thus, our overall findings demonstrate that iNKT cells activated by local (the lung) infection can comprehensively influence LDCs in their expansion, phenotype, and cytokine profile.

Importantly, our data provided direct *in vivo* evidence on the effect of iNKT cells on LDC function. Given the critical role of cytokines in generating protective responses against chlamydial infections, we examined the effect of iNKT cells on the modulation of LDC function in influencing the local cytokine environment and thus the outcome of the infection. Intranasal adoptive transfer of WT-LDCs and KO-LDCs showed a differential

effect on the cytokine production pattern in the local tissues, particularly the lungs and MLNs of the recipient mice after the challenge infection. The WT-LDC recipients had higher levels than the KO-LDC recipients of the cytokines that induce Th1/Tc1 immunity. Of note, our results showed an enhanced production of TNF-α in the lungs of mice receiving WT-LDCs compared to the mice that received KO-LDCs or PBS; this is in line with the finding that TNF-α inhibits *C. pneumoniae* growth (216). Furthermore, we analyzed both CD4+ and CD8+ T cell responses. Previous studies suggest that CD8+ T-cell responses are crucial for controlling *C. pneumoniae* infection, although its role in *C. trachomatis* is still debatable (40, 217). Accordingly, KO-LDC-recipient mice exhibited significantly lower levels of lung IFN-γ+ CD8+ T cells than recipients of WT-LDCs or the PBS controls. In correlation with the *in vivo* findings, our *in vitro* data also showed reduced IFN-γ production by CD4+ and CD8+ T cells upon coculture with KO-LDCs compared to WT-LDCs.

In summary, our study illustrates the critical role of iNKT cells in conferring protective immunity against *C. pneumoniae* infection by modulating the function of LDCs which are specialized to act primarily at the local site of infection and initiate effective mucosal immunity against respiratory pathogens. Our findings may well have implications for exploring the immune mechanisms that enhance host immunity to chlamydial lung infections and the control of the long-term complications of these infections, such as atherosclerosis and Alzheimer's disease.

4.4.0. ACKNOWLEDGEMENTS

This work was supported by grants from the Canadian Institutes of Health Research (CIHR), Manitoba Health Research Council (MHRC) and Manitoba Institute of Child Health (MICH) to X.Y., who is the Canada Research Chair in Infection and Immunity. S.S. is the recipient of a MHRC/MICH graduate studentship.

5.0. CHAPTER 5

NK CELLS MODULATE THE FUNCTION OF LUNG DENDRITIC CELLS IN INDUCING PROTECTIVE TH1/TH17 IMMUNITY AGAINST AN INTRACELLULAR BACTERIAL INFECTION THROUGH NKG2D SIGNALING

This work is in revision for European Journal of Immunology

(Shekhar et al., European Journal of Immunology, In revision)

5.1.0. INTRODUCTION AND RATIONALE

Chlamydia is an obligate intracellular bacterial pathogen that afflicts humans with a variety of diseases, posing a menace to public health worldwide. Clinically significant chlamydial species are *C. trachomatis* and *C. pneumoniae* that cause various diseases such as trachoma, pneumonia, and sexually transmitted diseases, and are also associated with chronic inflammatory diseases (218-225). Thus far there is no vaccine to prevent chlamydial diseases in humans. Rising public health concerns with chlamydial diseases underscores the development of an effective vaccine; however, limited understanding of the immunobiology of chlamydial infections is the main constraint in achieving this goal (5). In recent years, significant advances have been made in this field, largely due to experimental studies using mouse infection model of *C. muridarum* and epidemiological studies in humans (226-227). Accumulating evidence indicates that the induction of Th1 responses, and in particular IFN-γ production, is a key to protective immunity against chlamydial lung infections. Initial studies have further acknowledged that Th17 responses are also protective in chlamydial lung infections (195-196, 228), and that both Th1 and Th17 responses act in synergy to generate the protective immunity (198, 229-230). In contrast, Th2 responses characterized by IL-4 production appear to have a role in inducing immunopathology and susceptibility to infection (49).

DCs are the most potent APCs. On the basis of anatomical location, DCs can be classified into two subsets, lymphoid and non-lymphoid/tissue DCs. Lymphoid DCs reside in the lymphoid tissues such as the spleen and the lymph nodes, while non-

lymphoid/tissue DCs are present in the non-lymphoid tissues, such as the intestine and the lung (231). LDCs are specialized immune cells that have evolved to sense microbial antigens and orchestrate local immune responses in the lung (232). On sensing and capturing the invading pathogens, LDCs transition from an immature to a mature stage characterized by upregulation of surface molecules (MHC-II and costimulatory molecules) and secretion of cytokines (IL-12 and IL-10). Mature LDCs efficiently migrate to the MLNs to present captured microbial antigens to T cells for generation of cell-mediated immunity (233). A crucial role for LDCs has been described in protecting the host from a variety of respiratory pathogens, including viruses and bacteria (59, 74, 207, 209, 234-235). LDCs initiate antiviral CD8+ cytotoxic T-cell responses that lead to the clearance of viruses from the lung of mice (59, 74). In *Mycobacterium bovis* BCG lung infection, LDCs not only internalized bacteria and secreted IL-12 but also migrated to the MLNs to induce strong Th1 immunity (209, 235).

NK cells represent a unique innate lymphocyte population that provides a first line of host defense (128, 236). They constitute about 10% of resident lymphocytes in the lung, in numbers second only to those in the spleen, and are suited to play a role in the host response to respiratory infections because of their rapidity of response and bridging role in between innate and adaptive immunity (206, 237-239). In chlamydial lung infections, the role of NK cells in inducing protective immunity has been inconsistent. Previous studies suggest a negligible role for NK cells in host defense (154-156). Recently, we showed that NK-cell depletion in mice led to their reduced ability to clear *C. muridarum* infection, more severe lung pathology and decreased Th1

response, suggesting that these cells are essential for induction of protective immunity (140). Growing evidence further indicates that NK cells do not act in isolation but coordinate with DCs in the regulation of adaptive immune responses (240-241). *In vitro* experiments have shown that activation of NK cells induce DC maturation, which is dependent on both TNF-α/IFN-γ secretion and a cell-cell contact. The mature DCs further polarize T cells toward a Th1 response via secretion of IL-12 (242-244). *In vivo* NK cell/DC interactions have also been studied under various infectious conditions (140, 245-247). Depletion of NK cells in mice resulted in impaired DC maturation and IL-12 production after *Plasmodium chabaudi* parasitic infection, which further led to significantly lower CD4+ T-cell proliferation and IFN-γ production compared to those without NK-cell depletion (247). In murine cytomegalovirus infection, NK cells are necessary for maintenance of CD8α+ DCs, whereas these DCs play a role in expansion of NK cells through IL-12 and IL-18 (245-246). In line with these studies, we using SDCs also showed that NK cells contribute to the enhancement of Th1 immunity against *C. muridarum* lung infection by modulating SDC function (140).

Recent studies have shown that NK cells and LDCs play a crucial role in mucosal immunity to various lung infections, and that these cells interact with each other in the lung to modulate immune responses during inflammation and infection (59, 206, 237-239). It remains however unclear how these cells interact in the lung microenvironment and, more importantly, what are the consequences of their interaction on the outcome of T cell immunity to respiratory infections. In the present study, we addressed these questions by examining the modulating effect of NK cells on LDC function in inducing

protective immunity using various experimental approaches, including *in vivo* NK-cell depletion in mice and a respiratory intracellular bacterial infection model of *C. muridarum.* Our findings from this study have provided direct *in vivo* evidence on the critical role of NK cells in generating Th1/Th17 responses through modulating LDC function during an intracellular bacterial infection.

5.2.0. RESULTS

5.2.1. Adoptive transfer of LDCs from *C. muridarum*-infected NK cell depleted mice (NK-LDCs) was less potent in inducing protective immunity to chlamydial infection than the LDCs from infected sham-treated mice (NK+LDCs)

To directly test the role of NK cells in modulating LDC function *in vivo*, we conducted adoptive transfer experiments with NK+LDCs and NK-LDCs, and evaluated their ability to confer protective immunity against chlamydial lung infection. Firstly, we intravenously administered anti-asialo GM1 antibodies in mice to deplete NK cells (Fig. 12A). Of note, our previous study has shown that anti-asialo GM1 antibodies do not deplete NKT cells following chlamydial infection (140). Then, we isolated LDCs from *Chlamydia*-infected mice, which received anti-asialo GM1 antibodies or isotype control antibodies (sham). Since LDCs and AMs share some common markers, including CD11c, it is important to differentiate LDCs from these macrophages. An authentic way to identify LDCs is to exclude the AMs from LDCs on the basis of autofluorescence emission by the former (212). We therefore defined LDCs as $CD11c^{hi}$non-autofluorescent lung cells using flow cytometric analysis. The purity of the isolated

LDCs (>97%) was determined using the antibodies against the macrophage marker F4/80 (Fig. 12B). NK-LDCs or NK+LDCs were intranasally transferred to naïve recipient mice, which were challenged with intranasal chlamydial infection after the adoptive transfer. Mice receiving only PBS with the same challenge infection were taken as controls. We found that the transfer of NK+LDCs or NK-LDCs enhanced protection, shown by the faster recovery of body weight loss and lower bacterial loads (IFUs) in the recipients. However, the mice receiving NK-LDCs were less protected because their body weight loss was significantly higher than those receiving NK+LDCs (Fig. 13A). Consistently, chlamydial loads in the lungs of NK-LDC recipients were significantly higher than the mice receiving NK+LDCs (Fig. 13B). The histopathologic analysis showed the least intense pathologic changes in the lungs of NK+LDC recipients than the other groups, although the pathological changes in NK-LDC recipients were also milder than sham-treated mice (Fig. 13C). Of note, the intranasal transfer of LDCs from uninfected NK cell depleted or sham-treated mice did not induce protective immunity against chlamydial challenge (Fig. 14A, B, C). These results indicate that the adoptive transfer of NK+LDCs and NK-LDCs confer protective immunity, but NK+LDCs are more potent in generating the immunity than NK-LDCs.

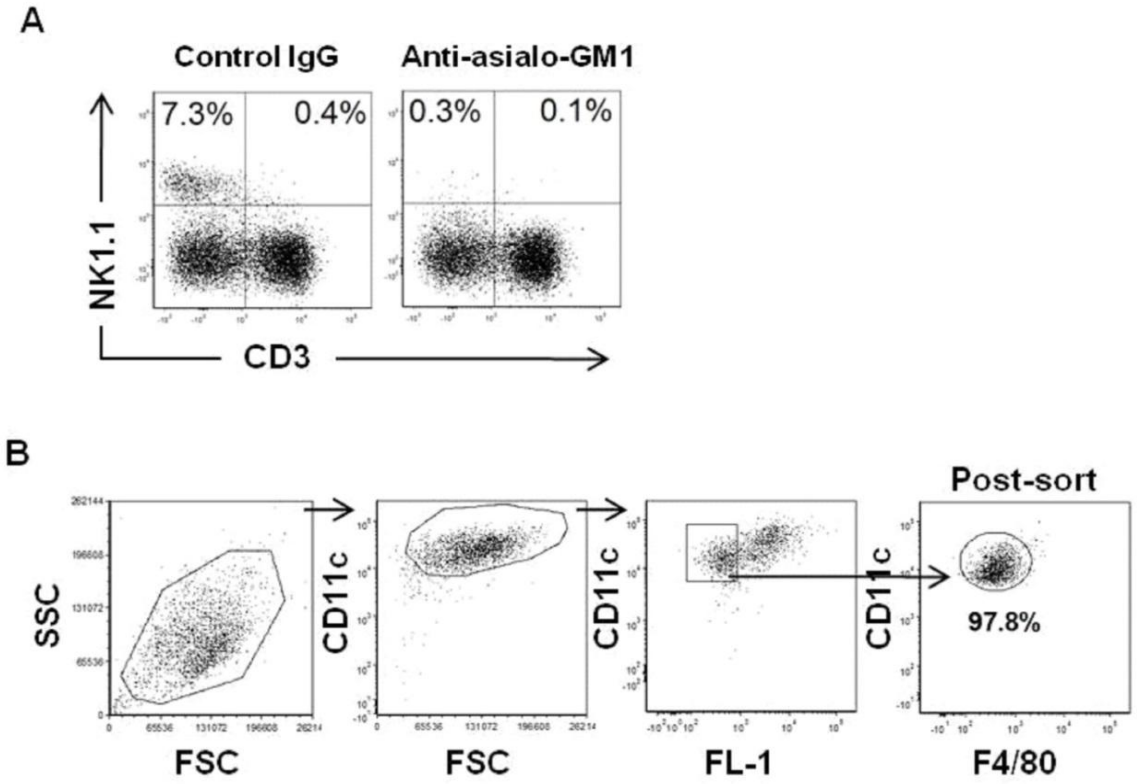

Figure 12. Depletion of NK cells in the lung by antibody treatment, and the sorting and purity analysis of LDCs by flow cytometry. (A) NK cell depletion by anti-asialo-GM1 antibodies. Mice infected with *C. muridarum* were intravenously injected with anti-asialo-GM1 or isotype control IgG antibodies (sham). Lungs were harvested from the mice sacrificed at day 8 p.i., and processed into single-cell suspensions. The lung cells were stained with anti-NK1.1-PE and anti-CD3-PE-Cy7 or respective isotype control antibodies, and analyzed. Cells were gated on living lymphocytes based on forward- and side-scatter characteristics. NK1.1+CD3- cells were gated and identified as NK cells. Flow cytometry images showing NK cell depletion in the lungs. (B) Flow cytometric gating strategy for LDC sorting, and the post-sort purity of LDCs. Mice with or without NK cell depletion were sacrificed at day 8 after intranasal infection with *C. muridarum*. The single-cell suspensions from the lung were enriched for CD11c-magnetic beads using MACS column. The CD11c-enriched lung cells were stained with APC-conjugated-anti-CD11c or isotype control antibodies, and then subjected to flow cytometric analysis. $CD11c^{hi}$non-autofluorescent lung cells were gated and analyzed as LDCs, and accordingly sorted by flow cytometry. The antibodies against F4/80 were used to test the purity of the sorted LDCs. Representative flow cytometric images are shown.

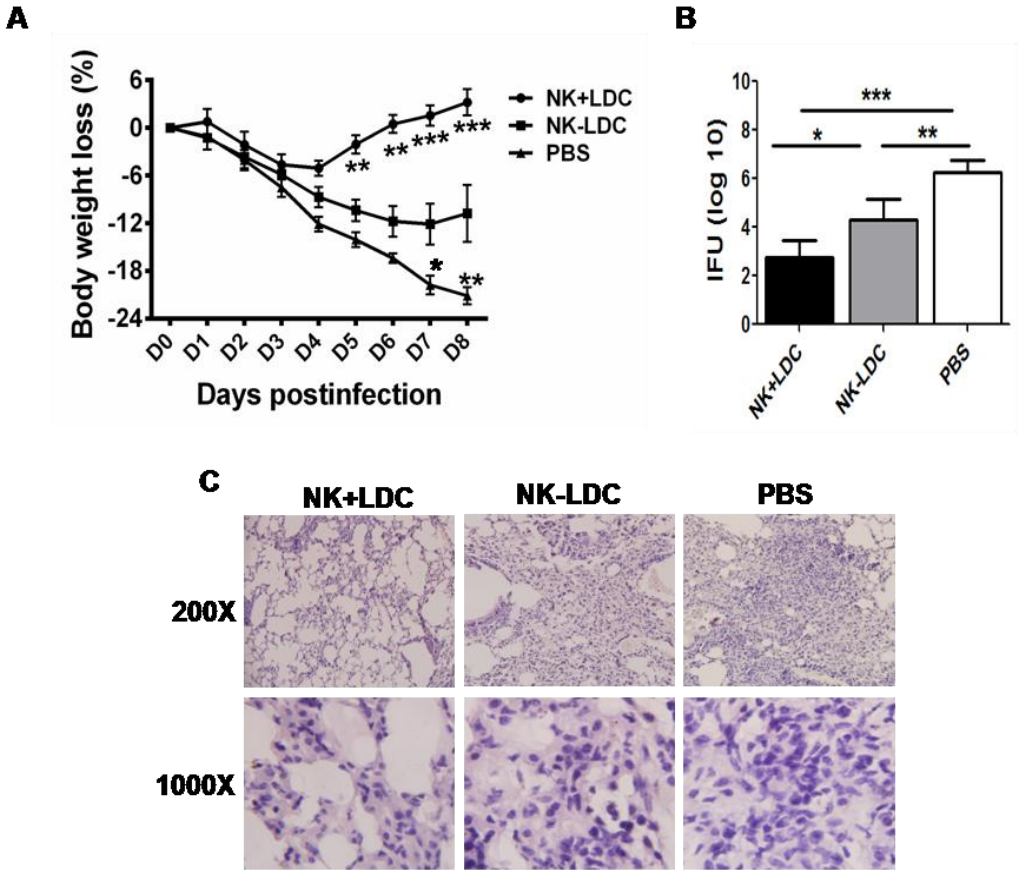

Figure 13. Adoptive transfer of NK+LDCs, compared to NK-LDCs, shows enhanced protective immunity against chlamydial infection. NK+LDCs or NK-LDCs were intranasally transferred to naïve recipient mice (4 in each group) and challenged with *C. muridarum* infection (1 X 10^3 IFUs/mouse). The body weight changes after the infection were monitored daily. Mice were sacrificed at day 8 p.i., and the lung bacterial loads and histopathology were analyzed. (A) Body weight loss. (B) Chlamydial growth in the lung. (C) Lung histopathology. The lungs were subjected to hematoxylin and eosin (H & E) staining. Representative histopathologic images of the lung tissues were shown at low (200X) and high (1000X) magnification. Data are shown as mean ± SD. One of the three independent experiments with similar results is shown; *p < 0.05; **p < 0.01; ***p < 0.001.

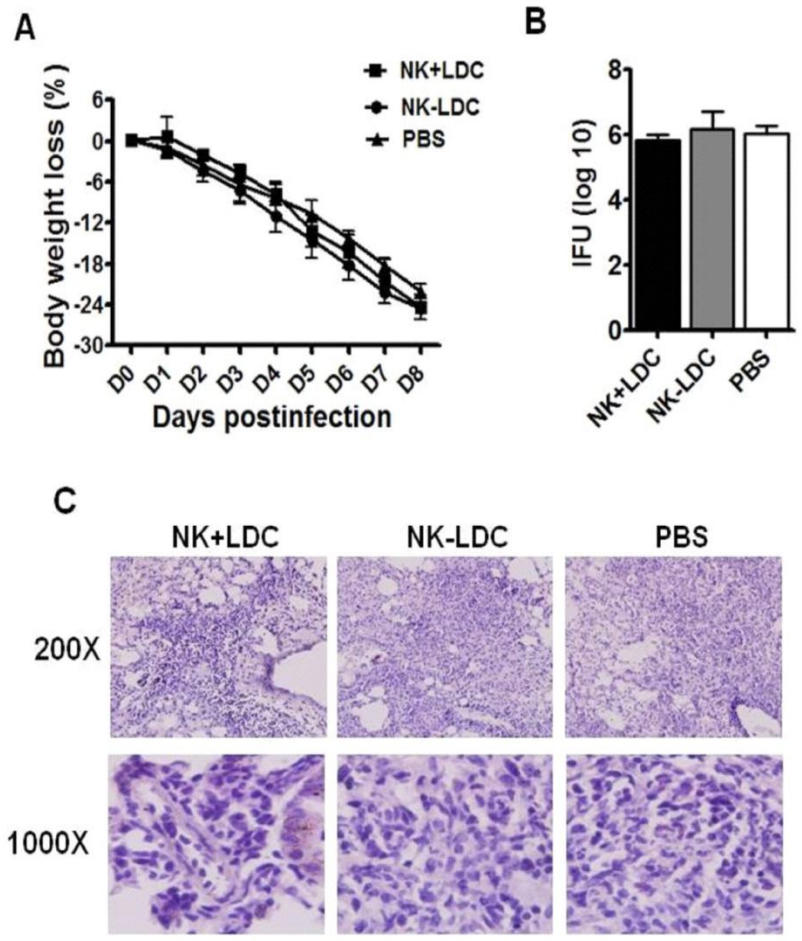

Figure 14. LDCs from uninfected NK cell depleted and sham-treated mice confer no protection against chlamydial challenge. The uninfected LDCs from NK-cell-depleted (NK-LDCs) or sham-treated (NK+LDCs) mice were intranasally transferred to naïve recipient mice (3 in each group) and challenged with *C. muridarum* infection (1 X 10^3 IFUs/mouse). All mice were sacrificed at day 8 p.i., and the lung tissues were analyzed for chlamydial growth and pathology as described in material and methods. (A) Body weight loss. (B) Chlamydial growth. (C) Lung histopathology. Representative histopathologic (HE staining) images of the lung tissues were shown at low (200X) and high (1000X) magnification. Data are shown as mean ± SD. One of the two independent experiments with similar results is shown.

5.2.2. Mice receiving NK+LDCs, compared to NK-LDCs, showed enhanced protective Th1/Th17, but reduced Th2, cytokine responses at the local site of infection

In order to examine the mechanisms involved in the protection induced by LDCs following chlamydial challenge, we investigated the effect of LDC transfer on the local cytokine environment of the infection site, i.e. the lung and the MLNs in the recipient mice. Analysis of cytokine production by MLN cells showed that the transfer of both NK+LDCs and NK-LDCs enhanced IFN-γ and IL-17 responses, with the reduction of IL-4, thus leading to predominant Th1/Th17 immunity, although the capacity of NK-LDCs to do so was significantly weaker than NK+LDCs (Fig. 15A). Consistently, the lung tissues from the mice receiving NK+LDCs exhibited a stronger bias toward Th1/Th17 cytokine pattern shown by higher levels of IFN-γ, IL-17 and IL-12 than those receiving NK-LDCs. In contrast, NK+LDC recipient mice had lower Th2 cytokine, IL-4, production in the lung than the mice receiving NK-LDCs (Fig. 15B). To have a clearer understanding of T-cell specific cytokine production at single-cell level, we performed intracellular cytokine staining of CD4+ and CD8+ T cells in the lung after challenge infection. The intracellular cytokine analysis showed a smaller number of IFN-γ and IL-17-producing CD4+ and CD8+ T cells in the lung of recipients of NK-LDCs than that in mice receiving NK+LDCs, although the recipients of either type of LDCs showed higher percentage of IFN-γ and IL-17-producing T cells compared to sham-treated mice (Fig. 16). Furthermore, NK+LDC transfer not only had a significant effect on the polarization of T cells but also on their number in the lung after challenge infection (Fig. 17). Together, these findings from the adoptive transfer experiments suggested NK cell -

mediated effect on LDCs to direct Th1/Th17 responses against chlamydial infection.

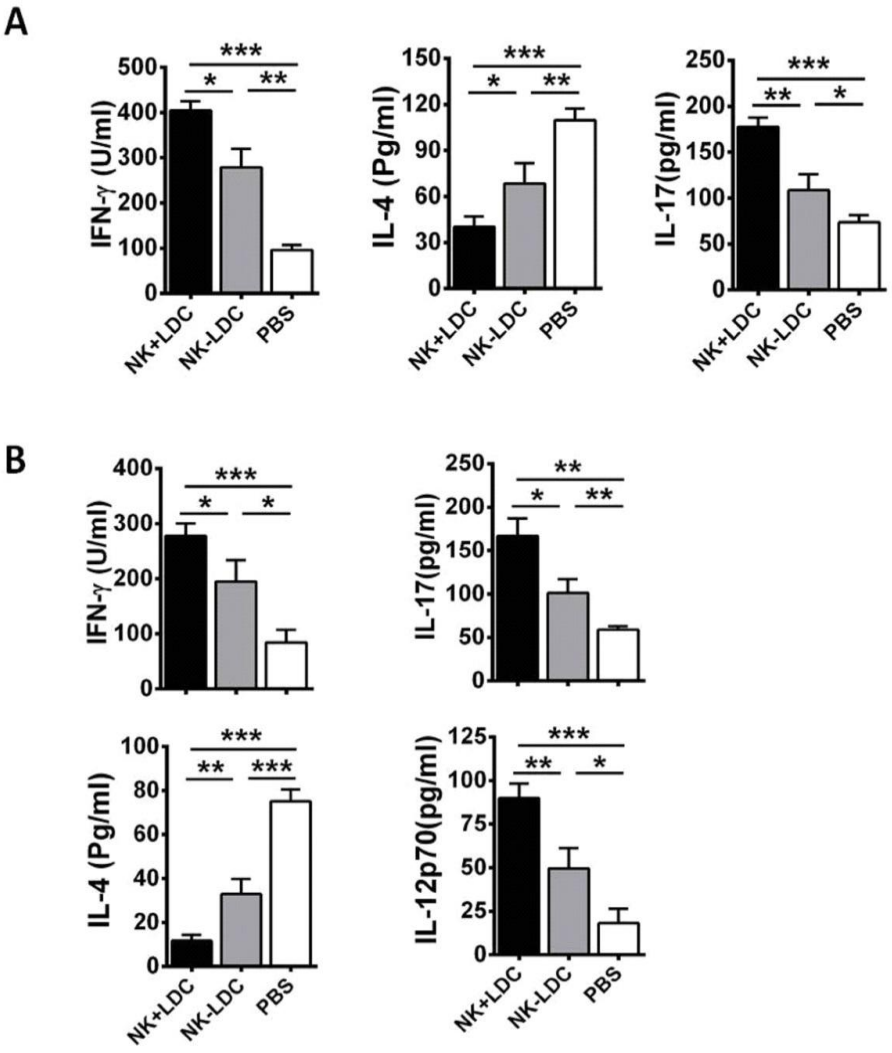

Figure 15. Adoptive transfer of NK+LDCs and NK-LDCs affects the cytokine production pattern differently in the recipient mice after challenge infection. Mice (4 in each group) receiving NK+LDCs or NK-LDCs were sacrificed at day 8 after challenge infection. Lungs and MLNs were isolated from the mice and processed into single-cell suspensions. (A) Cytokine production by MLN cells. MLN cells were cultured with UK-EB and the 72 h culture supernatants were examined by ELISA. (B) Lung tissues from the mice recipient of NK+LDCs or NK-LDC were homogenized in protein free PBS and centrifuged. The cytokine levels in the lung tissue supernatants were measured by ELISA. Data are shown as mean ± SD. One of the 3 independent experiments with similar results is shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

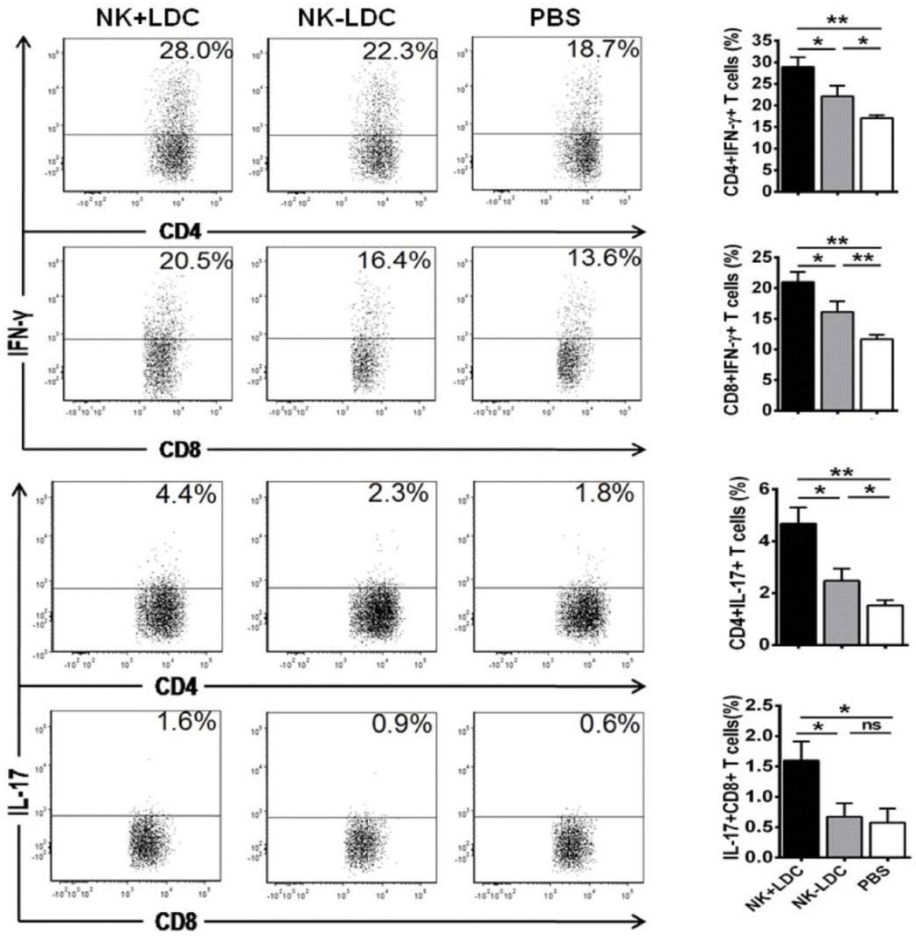

Figure 16. Recipients of NK+LDCs exhibited increased Th1/Th17 cytokine pattern in lung T cells compared to NK-LDC recipients. NK+LDCs or NK-LDCs were intranasally transferred to naïve recipient mice (4 in each group) prior to challenge infection with *C. muridarum* and then sacrificed at day 8 p.i. Lung cells from the mice recipient of NK+LDCs or NK-LDCs were cultured at the concentration of 5 X 10^6 cells/ml. The cytokine production pattern in CD4+ and CD8+ T cells was analyzed by flow cytometric intracellular cytokine staining. Cells were gated on CD3+ cells. Gating cut off was determined using isotype control antibodies. Representative flow cytometric images (left) and a summary of the percentages of IFN-γ- or IL-17-producing CD4+ and CD8+ T cells (right) are shown. Data are shown as mean ± SD. One of the three independent experiments with similar results is shown; *$p < 0.05$; **$p < 0.01$.

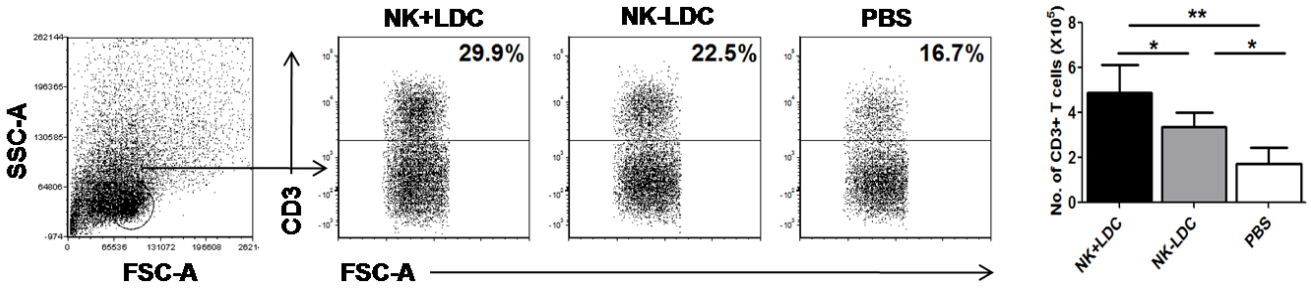

Figure 17. LDC transfer induces expansion of lung T cells. Mice receiving NK+LDCs or NK-LDCs were sacrificed at day 8 after chlamydial challenge. Lung CD3+ T cells were analyzed by flow cytometry. Representative flow cytometric images (left) and a summary of the numbers of CD3+ T cells (right) are shown. Data are shown as mean ± SD. One of the 2 independent experiments with 4 mice in each group with similar results is shown. *$p < 0.05$; **$p < 0.01$.

5.2.3. NK cells instructed LDCs to polarize T-cell responses

To evaluate the effect of NK cells on the functional ability of LDCs to activate *Chlamydia*-specific T-cell responses, we isolated CD4+ T cells from *Chlamydia*-immunized mice, and cocultured with NK+LDCs or NK-LDCs, which were isolated from the mice at day 3 and 8 p.i., in the presence of UK-EB stimulation. As a control, CD4+ T cells isolated from the immunized mice were cultured alone in the presence of UK-EB. We found that NK+LDCs promoted CD4+ T cells to produce more IFN-γ and IL-17, but less IL-4, than NK-LDCs (Fig. 18A,B). These *in vitro* data suggest that NK cells enhance the ability of LDCs to skew T cells toward a Th1 response.

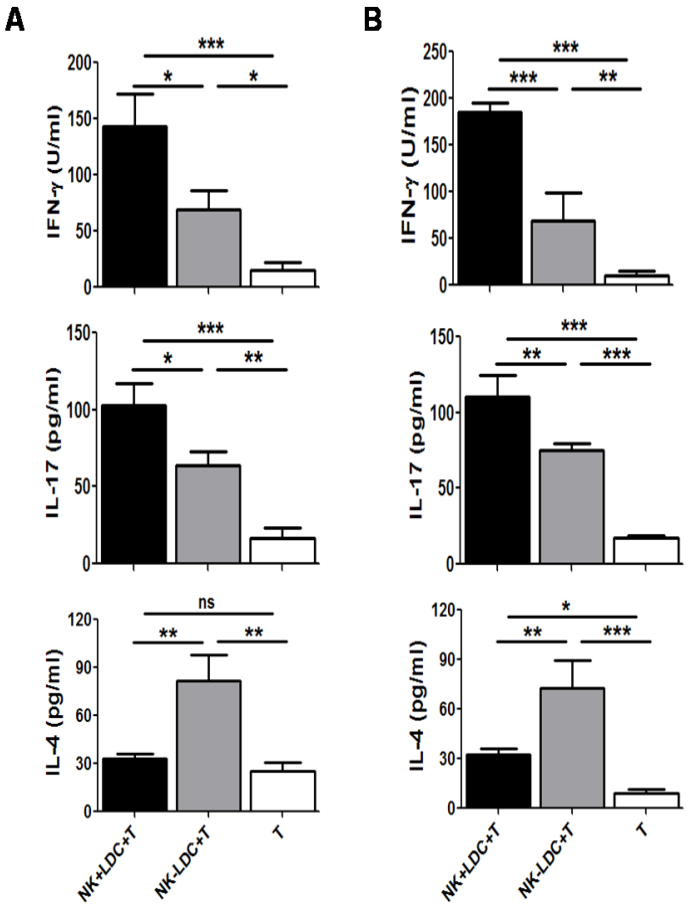

Figure 18. Differential ability of LDCs to direct T-cell responses. NK-LDCs or NK+LDCs isolated from infected-mice at day 8 p.i. and were cocultured with CD4+ T cells isolated from the spleen of *C. muridarum*-immunized mice in the presence of UK-EB. CD4+ T cells from the immunized mice were cultured alone in the presence of UK-EB and used as controls. The concentrations of various cytokines in the culture supernatants were measured by ELISA. Data are shown as mean ± SD. One of the 2 independent experiments with similar results is shown. *p < 0.05; **p < 0.01.

5.2.4. NK cells isolated from *Chlamydia*-infected mice accelerated IL-12p70 production by naïve LDCs through both IFN-γ production and NKG2D receptor signaling, but IL-6 and IL-23 production through NKG2D receptor signaling only

Our previous study has shown that chlamydial infection induces NK cell activation that mounts an enhancing effect on splenic DCs to produce IL-12p70 through IFN-γ and NKG2D receptor signaling (140). To dissect the mechanism by which NK cells modulate the ability of LDCs to produce Th1 (IL-12) as well as Th17 (IL-6 and IL-23)-inducing cytokines, we isolated NK cells from the lung of *Chlamydia*-infected mice and cocultured them with LDCs from uninfected mice in the presence of UK-EB. NK/DC ratio used in this experiment was 1:5. We found that the addition of either anti-IFN-γ or anti-NKG2D antibody in the coculture led to the partial reduction of IL-12p70 production by LDCs, whereas adding a combination of both these antibodies virtually abolished the IL-12p70 production (Fig. 19). On the other hand, the addition of anti-NKG2D, but not anti-IFN-γ, antibody caused a reduction in IL-6 and IL-23 production by LDCs (Fig. 19). Furthermore, the addition of anti-TNF-α antibody in the coculture led to a significant decrease in the production of IL-6 by LDCs compared to the controls (Fig. 19). Of note, the presence of naïve NK cells in the culture failed to have any significant impact on LDCs (data not shown). We also found an upregulation of NKG2D ligand RAE1 on LDCs after chlamydial infection (Fig. 20). These findings indicate that *Chlamydia*-infected NK cells modulate the function of LDCs through accelerating the production of IL-12p70, IL-6 and IL-23 cytokines. Although the modulating effect of NK cells on LDCs to enhance Th1 cytokine (IL-12p70) production depends on both IFN-γ and NKG2D, the production of Th17 (IL-6 and IL-23) cytokines is mediated via NKG2D receptor signaling

only. The data also suggest that the TNF-α production stimulates LDCs to produce IL-6.

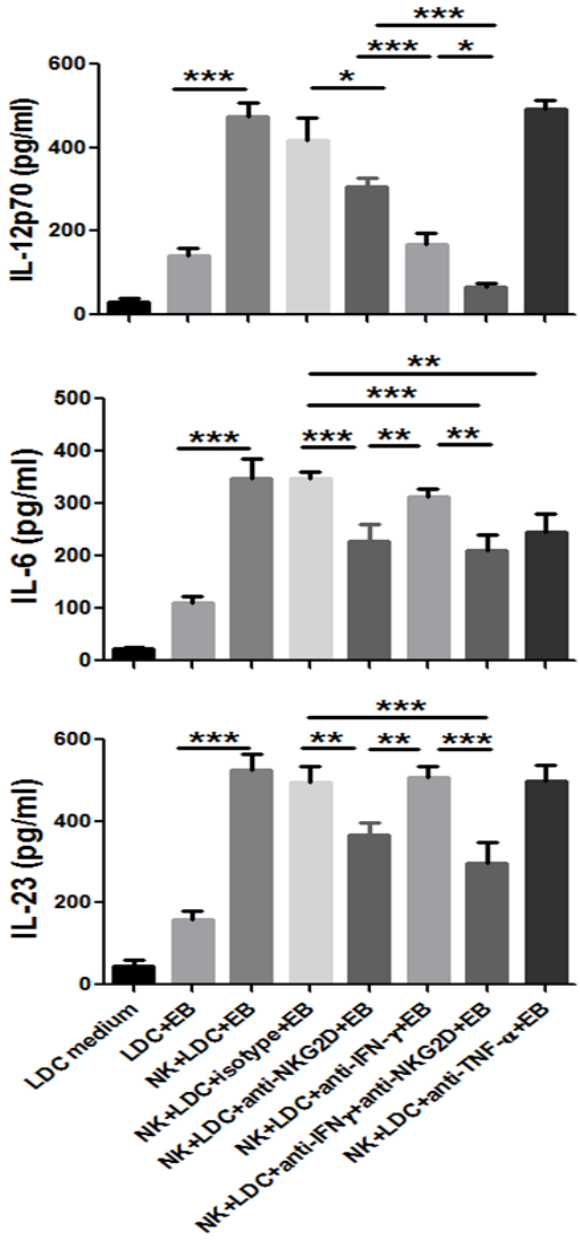

Figure 19. Modulation of LDC cytokine pattern by NK cells. NK cells were isolated from the lungs of mice at day 3 following chlamydial infection. These cells were cocultured with LDC from naïve mice in the presence of UK-EB with or without anti-NKG2D, anti-IFN-γ, TNF-α or isotype control antibodies for 72 hours. The culture supernatants were analyzed for the production of IL-12p70, IL-6 and IL-23 by ELISA. Data are shown as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

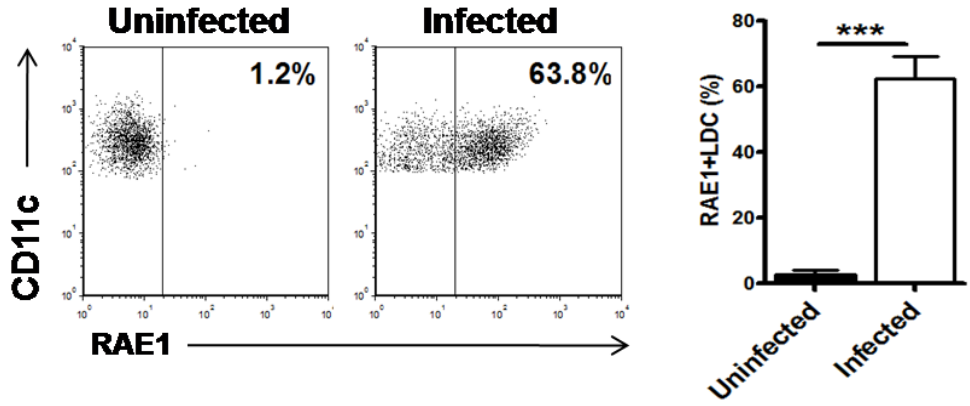

Figure 20. Expression of NKG2D ligand RAE1 on LDCs following *C. muridarum* infection. LDCs were isolated from mice (3 in each group) at day 3 p.i., and analyzed for the expression of RAE1 by flow cytometry. The analysis was performed on gated LDCs as described in Fig. 12B. Shown are flow cytometric images and a summary of the percentages of LDCs expressing RAE1. Data are shown as mean ± SD. One of the 2 independent experiments with similar results is shown. ***$p < 0.001$.

5.2.5. NK-LDCs reduced IL-12 production and surface molecule expression after *C. muridarum* infection

Since IL-12 production by DCs is critically important for their function to induce Th1 responses, we examined the IL-12 production by LDCs from *C. muridarum*-infected mice with NK-cell depletion, NK (-), or sham-treatment. The analysis of culture supernatants of sorted LDCs (Fig. 21B) showed that the spontaneous IL-12p70 and IL-12p40 production by LDCs from NK (-) mice was significantly lower than those from sham-treated mice (Fig. 21B). In contrast, the production of IL-10 by LDCs from NK (-) mice was higher than that of LDCs from sham-treated mice (Fig. 21B). In addition, we analyzed the expression of various surface molecules including costimulatory molecules on LDCs at day 8 p.i. Compared to those from sham-treated mice, a lower percentage of LDCs from NK (-) mice expressed MHC-II, CD103, CD80, CD86 and CD40 molecules on their surface following infection (Fig. 21A, Table - 2). Interestingly, we found that NK cells preferentially modulated the number of CD103+ LDCs (Fig. 22). Overall, these data support that NK cells enhance the expression of cell surface molecules on LDCs and the production of Th1 promoting cytokines (IL-12) by these cells.

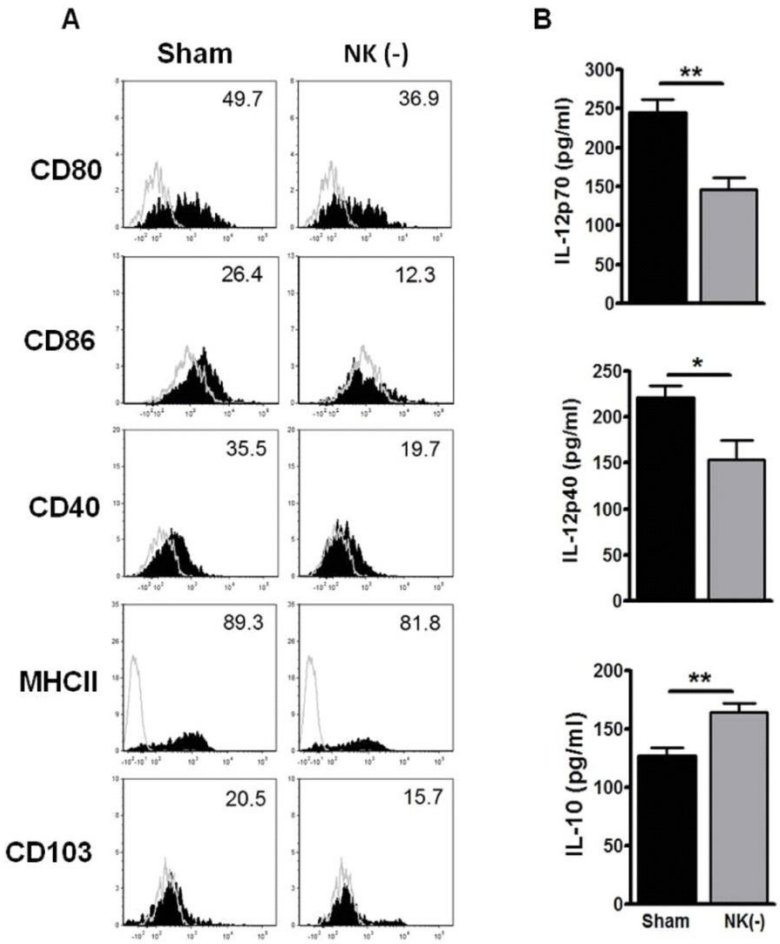

Figure 21. LDCs from NK (-) mice exhibit reduced surface molecule expression and IL-12 production after *C. muridarum* infection. Mice were infected and sacrificed at day 8 p.i. (A) Altered surface molecule expression on LDCs in NK (-) mice following infection. The lung cells from NK (-) and sham-treated mice (3 in each group) were stained for various surface molecules, and the expression of respective molecules was analyzed by flow cytometry. The analysis was performed on gated LDCs, which were defined as $CD11c^{hi}$non-autofluorescent lung cells, as described in Fig. 12B. Expression of CD80, CD86, CD40, MHC-II, and CD103 on LDCs (shaded histograms) and isotype control (line) were shown. Percentages of positive cells were indicated. One of the three independent experiments with similar results is shown. (B) LDC cytokine production. LDCs from NK (-) and sham-treated mice were purified, and placed in culture for 72 hours, and the cytokines in the supernatants were measured by ELISA. Data are shown as mean ± SD. One representative of the 2 independent experiments is shown. *$p < 0.05$; **$p < 0.01$. NK (-) = NK cell depletion.

Table 4. Summary of surface marker expression on LDC from NK cell depleted and sham-treated mice after *C. muridarum* infection

Surface Markers	Sham-treated	NK (-)
CD80		
%	48.7 ± 4.6	32.5 ± 2.4*
MFI	1662.0 ± 75.5	900.4 ± 107.8**
CD86		
%	24.1 ± 3.7	9.1 ± 1.8*
MFI	643.9 ± 198.9	451.7 ± 142.9
CD40		
%	33.1 ± 3.3	16.8 ± 2.1*
MFI	470.5 ± 35.5	254.5 ± 39.8*
MHC-II		
%	95.1 ± 3.9	72.8 ± 5.0*
MFI	753.8 ± 40.9	425.6 ± 30.9**
CD103		
%	20.4 ± 1.7	11.8 ± 2.1**
MFI	749.7 ± 64.0	490.7 ± 35.5*

Mice (3 in each group) were infected with *C. muridarum* (1 X 10^3 IFUs/mouse) and examined at day 8 after infection. The expression of surface molecules on LDCs was analyzed by flow cytometry. LDCs were characterized as $CD11c^{hi}$non-autofluorescent lung cells as described in Fig. 12B. Statistical analyses were performed using Student's *t* test. Data are shown as mean ± SD of three mice in each group. One of the three independent experiments with similar results is shown. * $p < 0.05$, NK (-) versus sham-treated mice. **$p < 0.01$, NK (-) versus sham-treated mice. MFI = mean fluorescence intensity. NK-cell-depleted: NK (-).

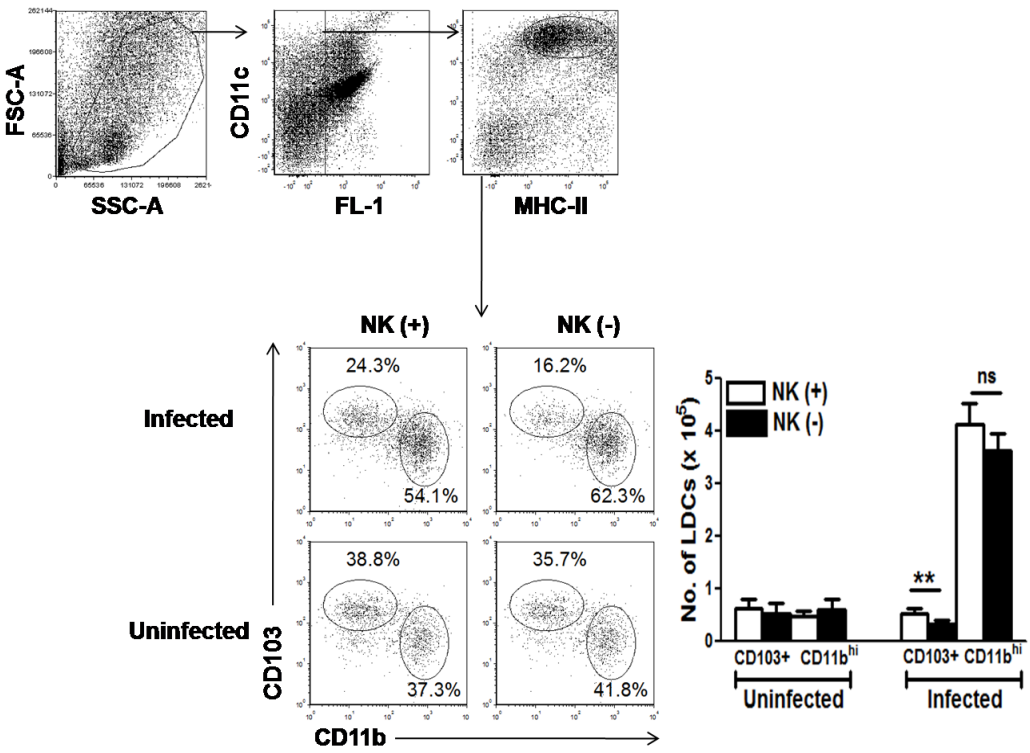

Figure 22. Reduced expansion of CD103+ LDCs in NK (-) mice after chlamydial infection. NK (-) or sham-treated mice were infected with *C. muridarum* and sacrificed at day 8 following infection. CD11c^{hi}MHC-II^{hi}non-autofluorescent lung cells were analyzed as LDCs by flow cytometry, and then displayed in terms of CD103+ and CD11b^{hi} LDC subsets. Shown are flow cytometric images and a summary of the numbers of LDC subsets. Data are shown as mean ± SD. One of the two independent experiments with 3 mice in each group was performed, and one representative experiment is shown. **p < 0.01.

5.3.0. DISCUSSION

In this study, we have examined the NK cell/DC interaction in the lung during a respiratory bacterial infection. Our data demonstrate a significant promoting effect of NK cells on the function of LDCs to direct Th1/Th17 protective immunity against the infection. This conclusion is supported by the findings showing; 1) *In vivo* adoptive transfer of NK+LDCs generated significantly stronger protection against challenge infection than NK-LDCs, which was associated with stronger IFN-γ and IL-17 but lower IL-4 responses in NK+LDCs recipients. 2) When cocultured with T cells, NK+LDCs directed T cells toward Th1/Th17 responses, whereas NK-LDCs polarized the T cells toward Th2 responses. 3) NK cells isolated from *Chlamydia*-infected mice accelerated IL-12p70 production by naïve LDCs through both IFN-γ production and NKG2D receptor signaling, but IL-6 and IL-23 production through NKG2D receptor signaling only. 4) LDCs from NK-cell-depleted mice exhibited reduced surface molecule expression and IL-12 production compared to LDCs from sham-treated mice. To our knowledge, this is the first study demonstrating the ability of NK cells to dictate the function of LDCs in eliciting local T-cell immunity against an intracellular bacterial lung infection.

Our previous study focusing on splenic DCs showed that the splenic DCs from NK cell depleted mice exhibited reduced expression of costimulatory molecules and lower levels of IL-12 and IL-10 than those from sham-treated mice following *C. muridarum* infection (140). These alterations fit well with the changes in LDCs observed in the present study with an exception of higher IL-10 production by LDCs in NK-cell depleted mice. These similarities are true for the observation in *in vitro* culture

experiments showing that the DCs from NK cell depleted mice induced much less IFN-γ production and more IL-4 production by antigen-specific $CD4^+$ T cells. In this study, in vivo experiments however showed remarkable differences. We found that both NK+LDCs and NK-LDCs, when adoptively transferred, enhanced protective immunity against infection challenge, although NK+LDCs were more efficient in inducing the protection than NK-LDCs. In contrast, our previous study using splenic DCs showed that the adoptive transfer of DCs from NK-cell-depleted mice, rather than promoting protective immunity, led to a more serious disease in the DC recipients, whereas recipients of DCs from sham-treated mice conferred protection, suggesting that NK cells are absolutely required for protection. This difference of LDCs and splenic DCs from NK cell depleted mice in generating protective immunity *in vivo* was associated with differences in cytokine patterns of the recipients of these DCs. Indeed, the transfer of splenic DCs from NK-cell-depleted mice mounted Th2 polarized response instead of Th1 response as shown in the recipients of DCs from sham-treated mice. Thus, the DCs from NK cell depleted mice were qualitatively different from those in normal mice. In contrast, the transfer of either NK+LDCs or NK-LDCs induced increased Th1, but reduced Th2, responses, while NK+LDCs were more efficient in doing so, showing a quantitative difference. These data suggest that the impact of NK cells on DCs in the lung might be less dramatic than observed in the spleen, although their role is very important. The study has further confirmed the importance to comprehensively examine the cellular responses in the lymphoid organs and local tissues. It is unclear why the influence of NK cells on DCs is less impactful in the lung from the lymphoid organs. One of the reasons could be that multiple cells are activated in the local tissues after

infection, and that many of them can modulate DC function, thus the effect of each type of cell may show some redundancy. Indeed, it has been reported that macrophages, neutrophils, NKT cells, γδ T, cells and epithelial cells can be activated after chlamydial infection. Although most of these cells also exist in the spleen, the density of these cells in the lung after infection becomes many folds higher than that in the spleen. In line with our previous findings on NK cell-splenic DC interaction (140), NK cells influenced the LDC function to produce Th1-inducing cytokine (IL-12p70) via IFN-γ and NKG2D signaling. However, the production of Th17-inducing cytokines (IL-6 and IL-23) by LDCs was dependent on TNF-α and NKG2D signaling, but not IFN-γ, suggesting that NK cells can exert their function through different molecules to skew different T-cell responses against chlamydial infection.

Unlike splenic conventional DCs, which include CD8α+ and CD8α- DC subpopulations, conventional DCs in the non-lymphoid organs such as the lung, gut and skin can be grouped into CD103+ and CD103- subsets (64, 68). The alteration of CD103+ LDCs the NK cell depleted mice (Fig. 19) following chlamydial infection is remarkable. Although detailed study is needed, the data suggest that NK cells may influence the development of DC subsets in the lung (Fig. 22). We previously reported that splenic CD8α+ DCs are more efficient in inducing protective immunity against chlamydial lung infection than CD8α- DCs (88). Indeed, the CD8α+ DCs from *C. muridarum*-infected mice show greater expression of costimulatory markers, higher production of IL-12 and induce stronger IFN-γ production than CD8α- DCs. Recently, the similarity between CD103+ LDCs and splenic CD8α+ DCs has been demonstrated

(64, 68), leading to the notion that they are a unified subset. This correlation is demonstrated by their shared dependence on certain transcriptional factors such as Batf3 and Irf8 and functional characteristics of antigen cross-presentation as well as chemokine receptor expression (64, 68). Obviously, further study on LDC subsets would be useful for understanding chlamydial immunobiology and for identifying manipulating targets to improve host defense against chlamydial infections.

Collectively, the findings from this study suggest that NK cells play a crucial role in enhancing physiological competence of LDCs in generation of protective host immune responses to *C. muridarum* lung infection, and provide a rationale for targeting NK cell-LDC cross-talk in developing therapeutic and/or prophylactic strategies against respiratory diseases.

5.4.0. ACKNOWLEDGEMENTS

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and Manitoba Health Research Council (MHRC) to XY. SS is the recipient of a MHRC graduate studentship.

6.0. CHAPTER 6

CD103+ LUNG DENDRITIC CELLS (LDCS) CONFER BETTER TH1/TH17 IMMUNITY TO A BACTERIAL LUNG INFECTION THAN CD11b^{hi} LDCS

This work is presented as a manuscript to be submitted

6.1.0. INTRODUCTION AND RATIONALE

C. trachomatis, an obligate intracellular bacterium, infection is a concern for global public health because it causes various human diseases, including STDs and trachoma (248). Although Th1 (IFN-γ) and Th17 (IL-17) responses are important for resolution of chlamydial infections (46, 49), a complete understanding of how anti-chlamydial immune responses ensue is still unclear. LDCs are the most potent pulmonary APCs that migrate to the MLNs to present antigens to naïve T cells for induction of a primary T cell response to respiratory pathogens (207). In mice, LDCs consist of two major subsets, CD103+ and $CD11b^{hi}$ LDCs, which are phenotypically and functionally distinct. CD103+ LDCs are efficient at cross-presenting exogenous antigens to CD8+ T cells. $CD11b^{hi}$ however LDCs produce large quantities of proinflammatory cytokines (65-66). Although both CD103+ and $CD11b^{hi}$ LDC subsets stimulated CD8+ T cells during influenza infection, CD103+ LDC was the primary subset for cross-presenting exogenous antigens to CD8+ T cells (76-77). In contrast, a study found $CD11b^{hi}$ LDCs to be the predominant subset for anti-influenza CD8+ T cell responses (78). Recently, Furuhashi *et al*. examined the ability of CD103+ and $CD11b^{hi}$ LDCs to exert distinct Th responses under steady state conditions (67). In coculture experiments, CD4+ T cells primed by CD103+ LDCs produced significantly large quantities of Th1 and Th17 cytokines, while those primed by $CD11b^{hi}$ LDCs secreted higher levels of Th2 cytokines, suggesting that CD103+ and $CD11b^{hi}$ LDCs preferentially induce Th1/Th17 and Th2 responses, respectively (67). It however remains to be determined the *in vivo* role of CD103+ and $CD11b^{hi}$ LDC subsets in eliciting protective Th1/Th17 responses against a real bacterial lung infection.

Here we show that CD103+ and CD11b^{hi} LDC subsets expand following *C. muridarum* lung infection. CD103+ LDCs showed higher expression of costimulatory molecules and greater production of Th1- and Th17-inducing cytokines (IL-12, IL-6 and IL-23) than CD11b^{hi} LDCs. Coculture of *Chlamydia*-specific CD4+ T cells with LDC subsets revealed that the T cells cultured with CD103+ LDCs produced large amounts of IFN-γ and IL-17 compared to those with CD11b^{hi} LDCs. To test their function *in vivo*, we isolated CD103+ and CD11b^{hi} LDC subsets from infected mice and transferred them to naïve syngeneic mice that subsequently received chlamydial challenge. CD103+ LDC recipients showed better protection, as evidenced by their reduced body weight loss, bacterial burden and lung pathology, than CD11b^{hi} LDC recipients. Mice that received CD103+, compared to CD11b^{hi}, LDCs produced enhanced Th1/Th17 cytokines (IFN-γ and IL-17) in the lung and the MLNs. In conclusion, these findings demonstrate that CD103+ LDCs confer more potent Th1/Th17 immunity to chlamydial infection than CD11b^{hi} LDCs.

6.2.0. RESULTS

6.2.1 Infection with *C. muridarum* led to a higher expression of surface molecules on CD103+ LDCs

Our LDC kinetic studies showed that both CD103+ and CD11b^{hi} LDC subsets expanded following chlamydial infection. Although we noticed a steady increase in the number of CD11b^{hi} LDCs, CD103+ LDCs showed a gradual decrease after day 3 p.i.

(Fig. 23 A&B). Due to their costimulatory role in influencing T cell responses, we examined the expression of various surface molecules on these LDC subsets at day 3 following infection. CD103+ LDCs showed higher expression of CD40, CD80 and CD86 than $CD11b^{hi}$ LDCs after chlamydial infection (Fig. 24). However, there was no statistically significant difference between CD103+ and $CD11b^{hi}$ LDCs under steady state condition (Fig. 24). Overall, chlamydial infection altered both the frequency and phenotype of LDC subsets.

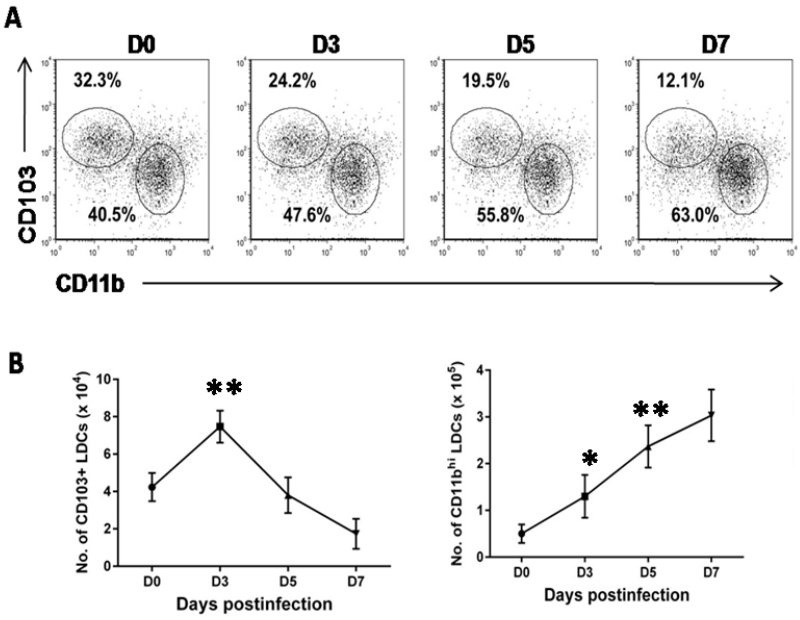

Figure 23. CD103+ and CD11b^{hi} LDC subsets expand following chlamydial infection. Mice were infected with *C. muridarum* and sacrificed at various time points following infection. Lungs were harvested from the mice and processed into single-cell suspensions. CD11c^{hi}MHC-II^{hi}non-autofluorescent lung cells were analyzed as LDCs by flow cytometry, and then displayed in terms of CD103+ and CD11b^{hi} LDC subsets (as described in Fig. 22). (A) Representative flow cytometric images showing the frequencies of LDC subsets at different time points following infection. (B) Graphs representing the absolute numbers of CD103+ and CD11b^{hi} LDC subsets. Data are shown as mean ± SD. One of the two independent experiments with 3 mice in each group was performed, and one representative experiment is shown.

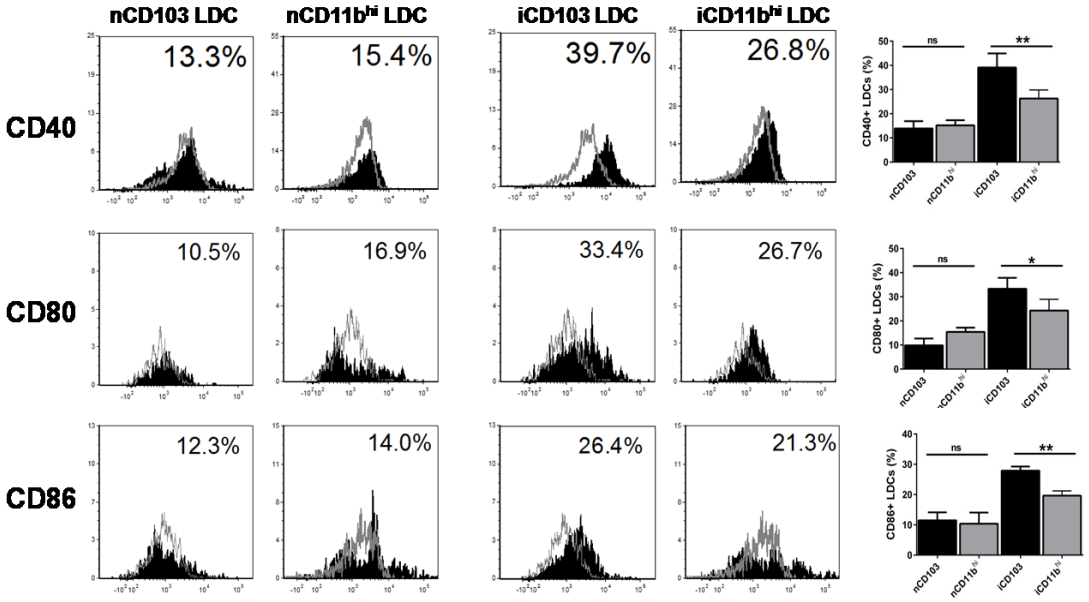

Figure 24. Surface molecule expression on CD103+ and CD11b^{hi} LDCs after infection. Mice were sacrificed at day 3 after infection, and CD11c+ cells were isolated from their lungs by using CD11c-microbead selection. Flow cytometric analysis was performed on gated CD11c^{hi} lung cells. CD11c^{hi}MHC-II^{hi} non-autofluorescent lung cells were considered as LDCs and CD103+ and CD11b^{hi} LDC subsets were analyzed by flow cytometry, as shown in Fig. 22. The LDC subsets were analyzed for the expression of costimulatory molecules. Flow cytometric dotplots are shown. Expression of CD80, CD86 and CD40 on LDC subsets (shaded histogram) and isotype control (line) were shown. The percentages of positive cells were indicated. Graphs right to the dotplots showing a summary of the percentages of costimulatory molecule expression on LDC subsets. Data are shown as mean ± SD. One of the two independent experiments with 3 mice in each group was performed, and one representative experiment is shown. *$p < 0.05$; **$p < 0.01$.

6.2.2. CD103+ LDCs produced more Th1- and Th17-inducing cytokines than CD11b^{hi} LDCs

The ability of DCs to influences T-cell responses largely depends upon their cytokine production pattern. IL-12 production by DCs induces Th1 responses (IFN-γ), whilst IL-6 and IL-23 production skews Th17 responses (IL-17) (174, 249). Therefore, we isolated CD103+ and CD11b^{hi} LDCs from *C. muridarum*-infected mice with high purity (Fig. 25A), and cultured them for cytokine analysis. CD103+ LDCs produced more IL-12, IL-6 and IL-23 than the CD11b^{hi} LDCs (Fig. 25B). IL-10 was secreted more by CD103+ LDCs than the CD11b^{hi} subset. However, there was no significant difference between uninfected CD103+ and CD11b^{hi} for their cytokine production (Fig. 25B). Collectively, chlamydial infection directed an enhanced production of Th1- and Th17-inducing cytokines by CD103+ LDCs compared to the CD11b^{hi} LDCs.

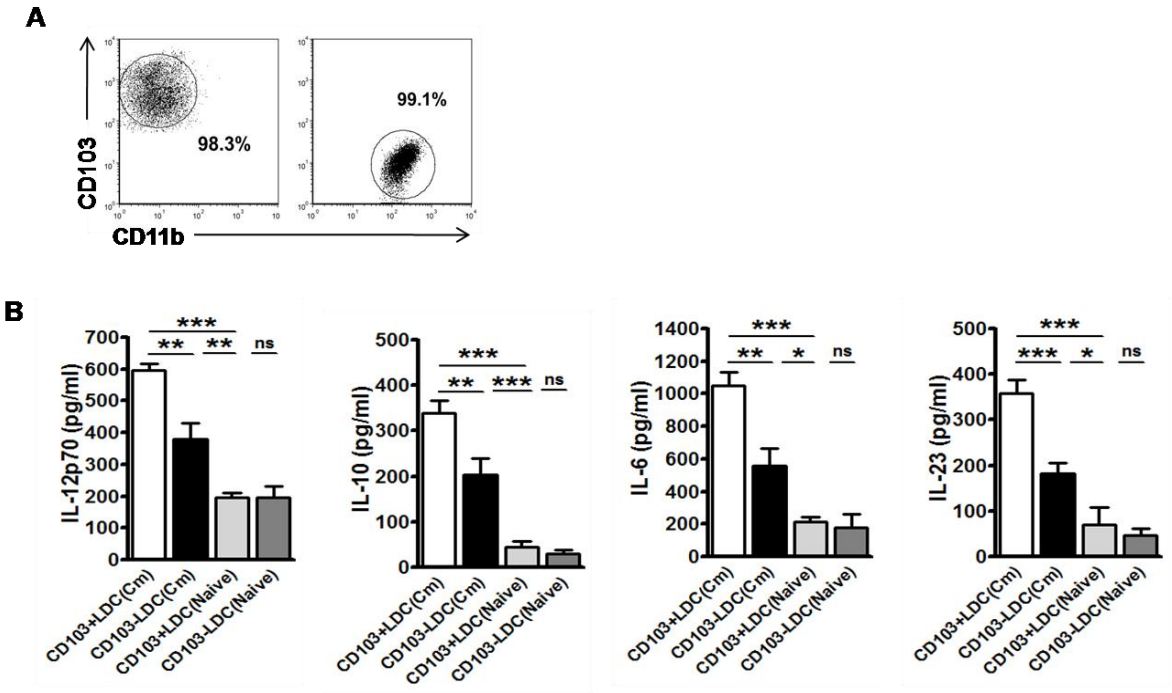

Figure 25. Cytokine profile of CD103+ and CD11b^{hi} LDC subsets. Lungs were harvested from mice at day 3 p.i., processed into single-cell suspensions and enriched with CD11c magnetic beads using MACS column. Flow cytometric analysis was performed on gated CD11c^{hi} cells. CD11c^{hi}MHC-II^{hi}non-autofluorescent lung cells were considered as LDCs and then displayed as CD103+ and CD11b^{hi} LDCs (as described in Fig. 22). The LDC subsets were sorted by flow cytometry. (A) Flow cytometric dot plots showing the purity of LDC subsets. (B) CD103+ and CD11b^{hi} LDCs purified from mice were placed in culture for 48 hours, and the concentrations of various cytokines in the supernatants were measured by ELISA. Data are shown as mean ± SD. One of the two independent experiments (4 mice in each group) with similar results is shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

6.2.3. CD103+ LDCs activate T cells to induce an enhanced Th1/Th17 response

The hallmark of DC function is to skew T-cell responses. To assess how CD103+ and CD11b^{hi} LDCs direct T-cell responses, we cocultured these LDC subsets isolated from *C. muridarum*-infected mice with CD4+ T cells from immunized mice in the presence of UK-EB stimulation and analyzed the cytokine production by the T cells. Culture of T cell alone was considered as a control. We found that CD103+ LDCs induced CD4+ T cells to produce higher levels of IFN-γ and IL-17 compared to the CD11b^{hi} LDCs (Fig. 26). These *in vitro* findings demonstrate that CD103+ and CD11b^{hi} LDC subsets are functionally different in directing *Chlamydia*-specific T-cell responses.

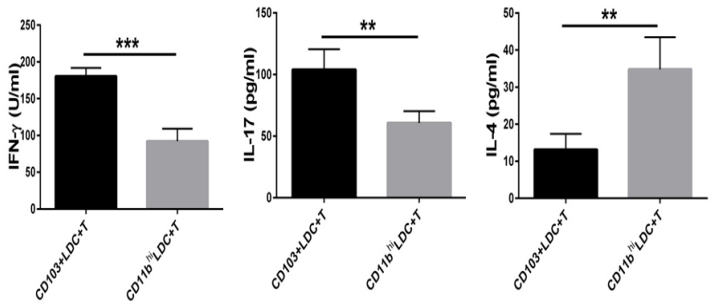

Figure 26. LDC:T-cell coculture to determine the ability of LDC subsets to activate T-cell responses. CD4 T cells from *C. muridarum*-immunized mice were isolated and cocultured with LDC subsets in the presence of UK-EB stimulation. The concentrations of cytokines in the culture supernatants were measured by ELISA. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and one representative experiment is shown. **p < 0.01; ***p < 0.001.

6.2.4. Transfer of CD103+ LDCs isolated from infected mice into uninfected mice conferred better protection against chlamydial challenge than CD11b^{hi} LDCs

To evaluate the relative contribution of LDC subsets in protection and pathology, we intranasally transferred CD103+ and CD11b^{hi} LDCs isolated from *C. muridarum*-infected mice into naïve syngeneic mice that subsequently received a challenge with chlamydial infection. We found that CD103+ LDC-recipients lost less body weight than the recipients of CD11b^{hi} LDCs following infection challenge (Fig. 27A). Consistently, the mice receiving CD11b^{hi} LDCs demonstrated a higher bacterial load and more severe lung pathology than CD103+ LDC-recipients (Fig. 27 B & C). A group of mice receiving PBS, subjected to the same infection challenge, were used as controls. These findings conclude that both CD103+ and CD11b^{hi} LDC subsets induce protection, but the protection induced by CD103+ LDCs is more potent than the CD11b^{hi} LDCs.

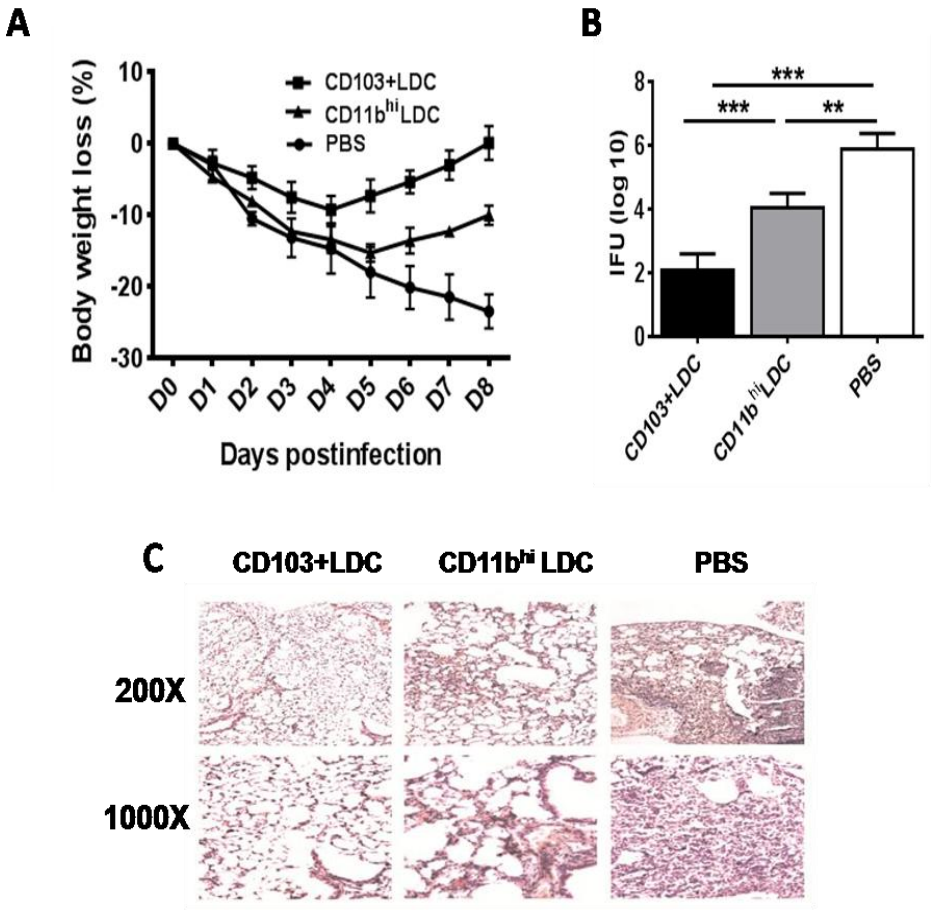

Figure 27. Adoptive transfer of CD103+ and CD11b^{hi} LDCs into naïve recipient mice. CD103+ and CD11b^{hi} LDCs isolated from *C. muridarum*-infected mice were transferred to naïve recipient mice and then challenged with chlamydial infection. A group of mice received PBS with the same challenge infection and taken as controls. All mice were sacrificed at day 8 following the challenge. (A) Body weight loss. (B) Chlamydial burden in the lung. (C) Lung histopathology. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and one representative experiment is shown. **p < 0.01; ***p < 0.001.

6.2.5. Adoptive transfer of CD103+, compared to CD11b^{hi}, LDCs showed superior Th1/Th17 responses following challenge infection

Since the quality of immune responses plays a crucial role in determining the outcome of infection, we further examined the local cytokine environment in the recipients of LDC subsets to dissect the mechanisms underlying the differences in protection. Firstly, we analyzed the lung tissues and lymph node cells for their cytokine production. The analysis showed that the recipient of CD103+ LDCs had higher levels of IFN-γ and IL-17 than the CD11b^{hi} recipients and sham controls. In contrast, Th2 cytokine (IL-4) production was lower in CD103+ LDC recipients than the recipients of CD11b^{hi} LDCs (Fig. 28 A&B). Then, we looked at the cytokine production at the level of T cells in the lung by intracellular cytokine staining. CD103+ LDC recipients showed a large number of CD4+ T cells to be producing Th1 (IFN-γ) and Th17 (IL-17) cytokines compared to the CD11b^{hi} LDC recipients (Fig. 29). Thus, these findings suggest that CD103+ LDCs are more potent in inducing Th1 and Th17, but less in promoting Th2, responses against the challenge infection than the CD11b^{hi} LDCs.

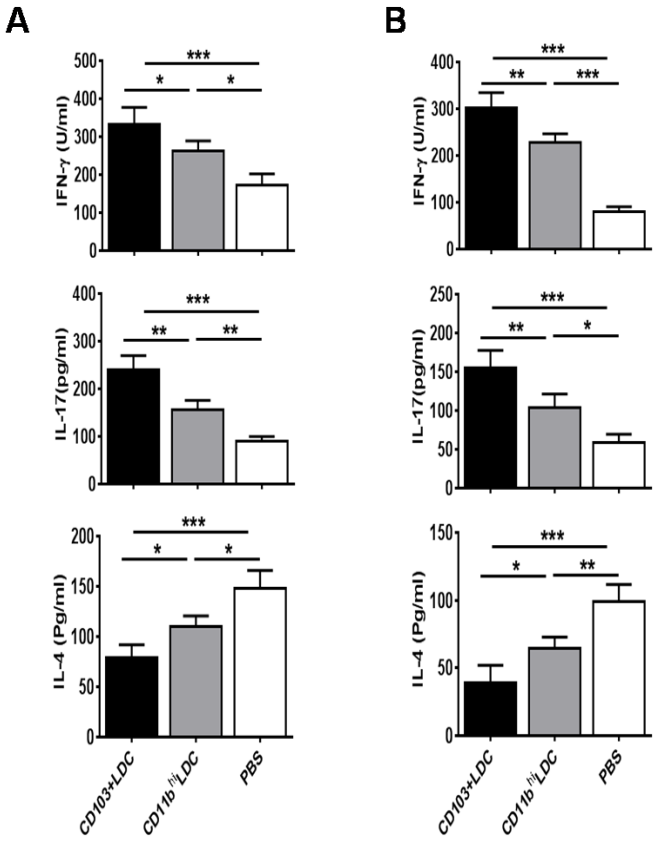

Figure 28. The impact of LDC subset transfer on the local cytokine environment in recipient mice. Mice receiving LDC subsets or PBS were sacrificed at day 8 following chlamydial challenge. The lungs and MLNs were harvested from the mice and processed into single-cell suspensions. (A) MLN cells were cultured with UK-EB for 72 hours. Various cytokine levels in the culture supernatants were examined by ELISA. (B) Lung tissues were homogenized in cold PBS and the supernatants were analyzed for cytokines by ELISA. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and one representative experiment is shown. *p < 0.05; **p < 0.01; ***p < 0.001.

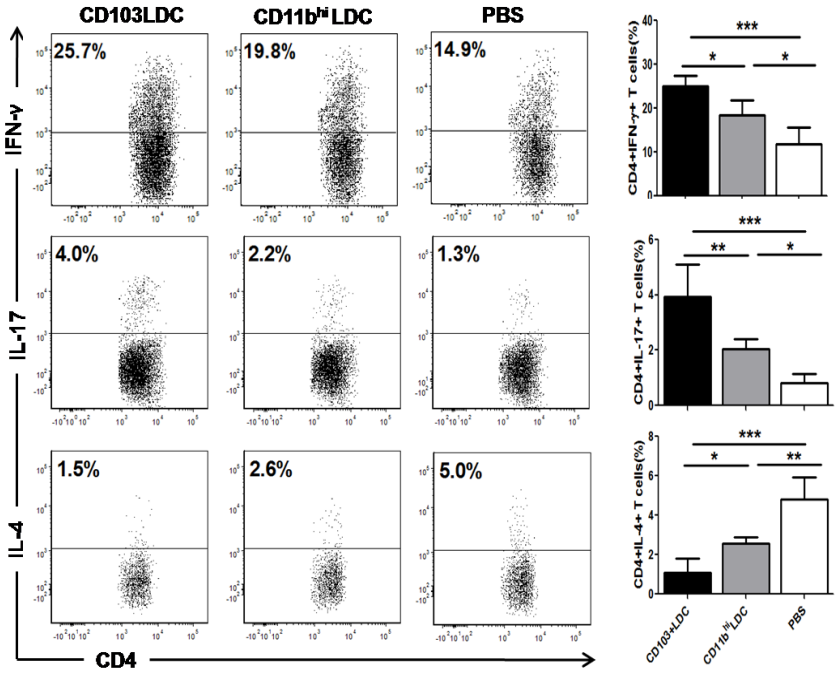

Figure 29. Cytokine production by T cells in recipient mice following challenge infection. Cytokine production pattern in the lung CD4+ T cells by intracellular cytokine analysis by flow cytometry. The flow cytometric dotplots (left) and the graphs showing the percentages of CD4+ T cells expressing various cytokines (right) are shown. Data are shown as mean ± SD. Two independent experiments with 4 mice in each group were performed, and one representative experiment is shown. *p < 0.05; **p < 0.01; ***p < 0.001.

6.3.0. DISCUSSION

The significance of this study lies in the fact that it sheds light on the role of major LDC subsets, CD103+ and CD11b^{hi} LDCs, in protective immunity to a bacterial lung infection. Our results demonstrate that the transfer of CD103+ LDCs into naïve mice induced better protection against *C. muridarum* infection than CD11b^{hi} recipients. Analysis of cytokines in the CD103+ LDC recipients clearly showed a stronger bias toward Th1 (IFN- γ) and Th17 (IL-17) responses than those receiving CD11b^{hi} LDCs. Similar findings were observed in LDC:T cell coculture experiments where CD103+, in response to CD11b^{hi}, LDCs activated CD4+ T cells to produce larger amounts of IFN-γ and IL-17. These data suggest that CD103+ LDC subset is more potent in conferring protective Th1/Th17 immunity to chlamydial infection than CD11b^{hi} LDCs. It is notable here that the LDC subsets were immediately transferred to naïve mice without any further manipulation such as cell culture following isolation from the infected mice. Therefore, this adoptive transfer approach to test the function of LDC subsets is near physiological. A recent discovery in the field of DC biology is that CD103+ and lymphoid CD8α+ DCs are functionally and developmentally related because they share the same transcription factor, Batf, and are very efficient in cross-presenting antigens to CD8+ T cells (68). Recent reports further illustrate the expression of a unique chemokine receptor, XCR1, on these DC subsets (64, 250). Our previous studies, using CD8α+ and CD8α- SDCs, demonstrated that CD8α+ SDCs conferred a stronger Th1 immunity to *C. muridarum* infection than CD8α- SDCs (88). In line with these findings, the presented study showed that CD103+ LDCs induced similar Th1 protective immunity, as of CD8α+ SDCs, in response to C. muridarum infection, suggesting that these DC

subsets not only share developmental and physiological properties but also exert similar immune responses during chlamydial infection. This emphasizes the concept of CD8α+ and CD103+ DCs as a unified and conserved subset of DCs among mammalian species (250). Despite their differences in phenotype and anatomical location, it appears that CD103+ LDCs and CD8α+ SDCs begin to elicit similar immune responses once they sense 'danger signals'. However, this is not always the case. Analogous to CD8α+ DCs, $CD11b^{hi}$, but not CD103+, LDCs mainly cross-present exogenous antigens to CD8+ T cells during influenza infection (78). It is possible that that these DC subsets induce differential immune responses in different infections. Therefore, it is important to directly analyze the immune responses in different infection models without extrapolating the findings from one infection model to the other. Moreover, our findings also showed an enhanced Th17, in addition to Th1, response induced by CD103+ LDCs in recipient mice compared to $CD11b^{hi}$ recipients. This is in accordance with the recent *in vitro* findings where CD103+ LDCs were found to be able to induce more efficient Th1/Th17 responses than $CD11b^{hi}$ LDCs (67). Studies have functionally characterized CD8α+ and CD8α- SDCs as DC-1 like cells, which preferentially induce Th1 responses and DC-2 like cells, which preferentially elicit Th2 responses, respectively (120, 251). In case of chlamydial infection, CD8α-, similar to CD8α+, SDCs induced Th1 immunity (88), which is in contrast to the DC-2 like activities (Th2) shown by CD8α- SDCs. Similar functions, as performed by CD8α- SDCs, were observed for $CD11b^{hi}$ subsets in our present study. In addition to CD103+, $CD11b^{hi}$ LDCs also conferred Th1 immunity to chlamydial infection, indicating that not only CD8α+ SDCs and CD103+ LDCs but also CD8α- SDCs and $CD11b^{hi}$ LDCs show similarity in their immune functions.

In conclusion, our findings from this study demonstrate that CD103+ and CD11b^{hi} LDCs are crucial for protection against chlamydial infection. Both these subsets induce Th1/Th17 immune responses, although the immune responses by CD103+ LDCs are more potent than the CD11b^{hi} LDCs. These findings have implications for developing strategies for control and treatment of respiratory infections.

6.4.0. ACKNOWLEDGEMENTS

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7.0. CHAPTER 7

OVERALL CONCLUSIONS AND GENERAL DISCUSSION

7.1.0. OVERALL CONCLUSIONS

The present work throws light on the role and mechanism of innate lymphocytes, NK and iNKT cells, in eliciting mucosal T-cell immunity to chlamydial lung infections. In addition, it examines the contribution of major LDC subsets, CD103+ and CD11b^{hi} LDCs, in inducing Th1/Th17 immunity. Taken together, these findings presented in this thesis provide direct evidence on how innate immune cells shape the direction and magnitude of adaptive immunity at the primary site of a bacterial lung infection (Figure - 30).

7.1.1. iNKT cells induce protective Th1/Tc1 immunity to *C. pneumoniae* infection through modulation of LDC function

Here we examined the effect of iNKT cells on the function of LDCs to elicit protective immunity against *C. pneumoniae* lung infection using a combination of approaches, including the use of Jα18-KO mice and LDC adoptive transfer. Our data suggest that iNKT cells can significantly influence the expression of surface molecules and cytokine profile of LDCs following *C. pneumoniae* infection. The findings from LDC:T cell coculture experiments suggested that iNKT cells promote the functional ability of LDCs in polarizing CD4+/CD8+ T cells toward Th1/Tc1 responses. To directly examine the function of WT-LDCs and KO-LDCs *in vivo*, we transferred WT-LDCs or KO-LDCs to naïve mice subjected to *C. pneumoniae* infection challenge. These

adoptive transfer experiments indicated that iNKT cells are critically important for enhancing LDC function to induce protective immunity against *C. pneumoniae* infection. In the absence of iNKT-cell modulation, LDCs not only fail to promote protective immunity but also increase the infection and pathology. Furthermore, cytokine analysis of the lung and the MLNs demonstrated that the mice that received KO-LDCs showed an increased Th2 response with significantly higher levels of IL-4 and IL-10, whereas those receiving WT-LDCs showed increased Th1 response with enhanced IFN-γ, IL-12p40 and IL-12p70, which suggested that WT-LDCs, in contrast to KO-LDCs, significantly induce a Th1 immune response *in vivo*.

7.1.2. NK cells modulate LDC function to confer Th1/Th17 immunity to *C. muridarum* infection through NKG2D receptor signaling

In this study, we investigated the effect and mechanism of NK cells on the function of LDCs in a murine model of *C. muridarum* lung infection. Our findings taken together suggested that NK cells confer protective Th1/Th17 responses against chlamydial infection by modulating the function of LDCs. To further dissect the underlying mechanism by which NK cells condition LDC function, we conducted NK cell: LDC coculture experiments. We found that the addition of anti-IFN-γ or anti-NKG2D antibodies in the coculture led to the partial reduction of IL-12p70 production by LDCs, whereas the IL-12p70 production was virtually abolished upon addition of both the antibodies. On the other hand, the addition of anti-NKG2D, but not anti-IFN-γ, antibodies caused a reduction in IL-6 and IL-23 production by LDCs. These results indicated that although the modulating effect of NK cells on LDCs to enhance Th1

cytokine (IL-12p70) production depends on both IFN-γ and NKG2D, the production of Th17 (IL-6 and IL-23) cytokines is mediated via NKG2D receptor signaling. In addition, NK cells not only upregulated the expression of costimulatory molecules on LDCs but also enhanced the production of Th1 promoting cytokines (IL-12), thus altering the phenotype as well as cytokine production pattern of LDCs.

7.1.3. CD103+ LDCs are more potent in eliciting protective immunity to *C. muridarum* infection than CD11b^{hi} LDCs

Here we used in vitro and in vivo systems to investigate the contribution of CD103+ and CD11b^{hi} LDCs in protective immunity to an intracellular bacterial infection of *C. muridarum* in mice. Our LDC kinetic studies concluded that chlamydial infection altered both the frequency as well as maturation of CD103+ and CD11b^{hi} LDC subsets. In particular CD103+ LDCs expressed higher levels of costimulatory molecules and Th1- (IL-12) and Th17-inducing (IL-6 & IL-23) cytokines than the CD11b^{hi} LDCs. Our *in vitro* findings demonstrated that CD103+ and CD11b^{hi} LDC subsets are functionally different in directing *Chlamydia*-specific T-cell responses. CD103+ LDCs were more potent in skewing Th1 and Th17 responses than the CD11b^{hi} LDCs. To evaluate the relative contribution of LDC subsets in protection and pathology, we intranasally transferred CD103+ and CD11b^{hi} LDCs isolated from *C. muridarum*-infected mice into naïve syngeneic mice that subsequently received a challenge with chlamydial infection. We found that CD103+ LDC-recipients lost less body weight than the recipients of CD11b^{hi} LDCs following infection challenge. Consistently, the mice receiving CD11b^{hi} LDCs demonstrated a higher bacterial load and more severe lung pathology than

CD103+ LDC-recipients. These findings conclude that both CD103+ and CD11b^{hi} LDC subsets induce protection, but the protection induced by CD103+ LDCs is more potent than that of CD11b^{hi} LDCs. In addition, CD103+ LDCs are more potent in inducing Th1 and Th17, but less in promoting Th2, responses against the challenge infection than the CD11b^{hi} LDCs.

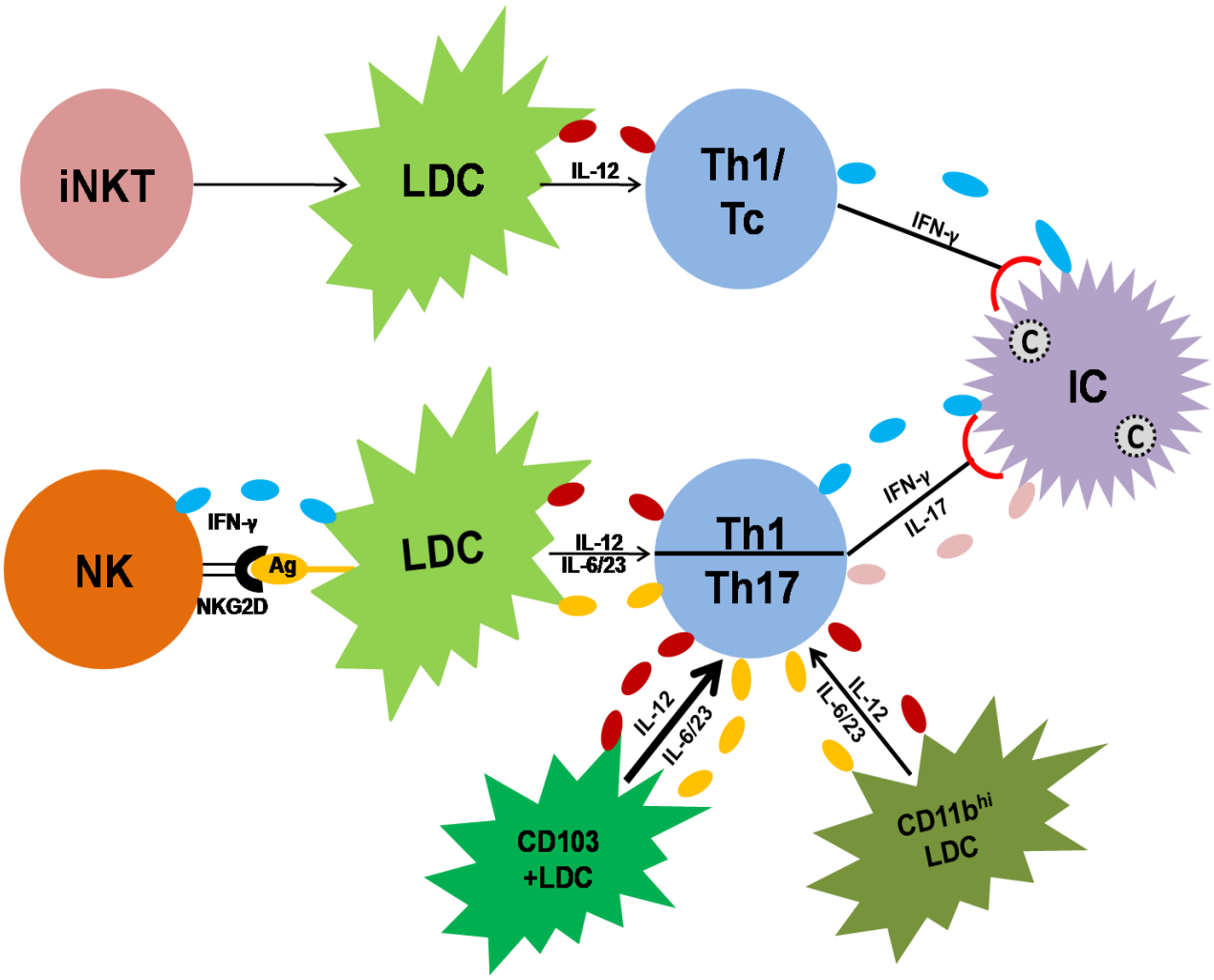

Figure 30. Role of LDC subsets and the interaction of innate lymphocytes with LDCs in host defense to chlamydial lung infection. Following chlamydial infection, iNKT cells stimulate LDCs to produce larger quantities of IL-12, a Th1-inducing cytokine, which in turn skews CD4+/CD8+ T cells toward Th1/Tc responses characterized by IFN-γ secretion. On the other hand, NK cells enhance IL-12 production by LDCs through both IFN-γ production and NKG2D receptor signaling, but IL-6 and IL-23 (Th17-inducing cytokines) production through NKG2D receptor signaling only. These mature LDCs polarize CD4+ T cells to induce Th1/Th17 responses (IFN-γ and IL-17). CD103+ LDCs are more potent in inducing protective Th1/Th17 responses than CD11b^{hi} LDCs through enhanced production of IL-12, IL-6 and IL-23. Thus, Th1/Tc and Th17 responses so generated can finally lead to the killing of intracellular *Chlamydiae*. Abbreviations: iNKT – Invariant natural killer T cell; NK – Natural killer cell; LDC – Lung dendritic cell; IL- Interleukin; Th – T helper cell; IC – Infected cell; C – *Chlamydia*; Ag – Antigen.

7.2.0. GENERAL DISCUSSION

In this thesis, we have presented data focusing on the mechanisms of mucosal pulmonary immune responses at the primary site of a bacterial lung infection. In particular, it has been explored as how innate immune cells such as NK, iNKT cells and LDCs perform their functions to regulate adaptive immune responses. These findings highlight the cellular/molecular mechanisms behind the generation of local T-cell responses in the lung.

7.2.1. Adoptive transfer approach to test the function of LDC/LDC subsets *in vivo*

Understanding the functional biology of LDCs is a key to manipulate mucosal T-cell responses in the lung. To study the function of LDC/LDC subsets *in vivo*, various methodologies - antibody depletion, DTR transgenic mice/diphtheria toxin treatment, genetically engineered mice, and LDC adoptive transfer – have been employed. Using antibodies against specific surface markers constitutes a common method to deplete a particular LDC population, e.g. pLDCs and CD103+ LDCs (89-90). For example, we used anti-mPDCA antibodies to deplete pLDCs in vivo to examine their functional role in immunity to *C. pneumoniae* infection (89). Similarly, Dunne *et al*. explored the role of CD103+ LDCs in protection against *Bordetella pertussis* infection using anti-CD103 antibodies (90). The major constraint in antibody-mediated LDC deletion is that it also targets the other cell populations that express similar markers. For instance, in addition to CD103+ LDCs, anti-CD103 antibodies target a large proportion of T cells in the lung that express CD103. Another question remains about what should be the exact dose of antibodies to induce optimal depletion. This is important because calibrating the

antibody doses can result in different immune responses. Apart from antibody-mediated LDC-depletion, mice heterozygous for CD11c/langerin-C-DTR transgene and DT treatment have been widely used to study the *in vivo* contribution of LDCs/LDC subsets in host defense (205, 252). CD11c- and langerin-C-DTR transgenic mice after DT treatment are deficient in LDCs and CD103+ LDCs, respectively (252), but there are no DTR transgenic mice available that can be used to deplete of other LDC subsets, such as $CD11b^{hi}$ or iLDCs. It is also crucial to note that CD11c-DTR mice/diphtheria toxin depletes not only CD103+ LDCs but also CD103-expressing DCs in other anatomical compartments. Another point to be taken into account is that that the treatment of DTR transgenic mice with DT might alter the function of LDCs because they are fragile and sensitive to the environmental factors such as toxins. Recently, $Batf^{-/-}$ mice, which lack CD103+ DCs, were used to study the development and function of CD103+ DCs (68). In addition to CD103+ LDCs, these mice are also deficient in lymphoid CD8α+ DCs and tissue resident CD103+ DCs. Due to poor knowledge of the ontogeny of LDCs, there is unavailability of transgenic mice required for more specific deletion of a particular LDC subset. Although the experimental approaches described above are commonly used to study LDC function, they may not elucidate the definitive function of LDC subsets, particularly the relative contribution of different LDC subsets in immunity to pulmonary infections.

In immunological research, adoptive transfer approach has been traditionally used to assess the function of an immune cell population. There are many studies including ours that involved the adoptive transfer of splenic DCs and their subsets (88,

118-119, 253-254). Since the functional analysis of splenic DCs in a pulmonary infection model, where the primary site of infection is the lung, would not depict a true picture of the specific immune responses. It is therefore desirable to evaluate DC responses in the lung micromilieu. A few studies have thus far used the adoptive transfer of LDCs (255-256). However, the strategy to characterize LDCs was not appropriate in these studies. For example, Rey-Ladino *et al*. using CD11c microbeads isolated LDCs from *C. muridarum* infected mice for intranasal adoptive transfer (256). Since the majority of CD11c+ lung cells are AMs, the isolated LDCs did not represent LDCs. With advancement of technology in the field of DC biology, the characterization/isolation of LDC/LDC subsets is becoming more accurate, although it requires a high level of technical expertise in cell sorting as well as multicolor flow cytometric analysis. Because LDCs are a scarce population, many more mice are required to isolate sufficient number of viable LDCs for functional assays. In this study, we employed an adoptive transfer of LDCs/LDC subsets to test their function during a bacterial lung infection. We used standard techniques and procedures to isolate LDCs with high purity. The most crucial constraint in LDC characterization/isolation is the contamination of LDCs with AMs because these two cell types share common markers, such as CD11c. The widely used technique to distinguish AMs from LDCs is to exclude highly autofluorescent AMs using empty FL-1/FITC channel during flow cytometric analysis. Following this protocol and staining LDCs with various surface markers such as CD11c, MHC-II, CD103, CD11b^{hi}, and F4/80, we purified the LDCs and their subsets and immediately transferred them to naïve mice without further manipulation. In line with this, different LDC subsets – pLDCs, iLDCs, CD103+, and CD11b^{hi} LDCs – can be sorted and transferred into mice

to assess their relative contribution in protective immunity to a lung infection. The route of LDC transfer opted in this study deserves discussion as well. In our previous studies, SDCs were intravenously transferred into recipient mice that received a subsequent challenge with chlamydial lung infection (88, 118-119). Tracking experiments showed that a large proportion of the transferred SDCs migrated to the organs like spleen, with only few cells in the lung (unpublished observation). Intranasal transfer of SDCs induced better protection against BCG infection than the transfer of SDCs through intravenous route (257). Furthermore, intranasal transfer of a lesser number of SDCs (2 x 10^5/mouse) was more potent in inducing immunity than the intravenous transfer of a greater number of SDCs (5 x 10^5/mouse) (257). This shows the superiority of the intranasal route for inducing mucosal immune responses over the intravenous in a BCG lung infection model. It is possible that DCs from the primary site of an infection are more efficient in exerting their effect than the DCs from the lymphoid organs. LDCs isolated from the lung are well attuned to the pulmonary microenvironment. Despite the advantages of LDC adoptive transfer approach, there is a limitation to LDC transfer approach that deserves a mention. Isolation of LDC/LDC subsets from the lung requires multistep separation procedure, including enzymatic digestion of the lung. DCs are highly sensitive to their environment and the stress such as enzymatic digestion and sorting may induce prompt changes in gene expression of isolated DCs (258). The ideal situation however would be to obtain *ex vivo* LDCs from the mice with minimal external intervention. In large animals, many more immunologically active DCs can be directly collected from the afferent lymph vessels that drain the peripheral and mucosal surfaces (259-260). Although thoracic duct cannulation in rodents such as rats and mice has

been used to study intestinal DCs, there are no reports of isolating LDCs from the lymph vessels that carry LDCs from the lung to the MLNs because of their very narrow caliber (258, 261). Taking into account of these points, the adoptive transfer approach to isolate and test the function of LDC/LDC subsets appears to be the most pragmatic one at present. This approach is useful for researchers to assess the functions of LDC/LDC subsets from the local site of infection/inflammation. **This will provide new knowledge on how these cells shape the local immune responses at non-lymphoid tissues such as the lung and how the immune responses induced by them are different from the lymphoid DC responses,** which may have implications for vaccine development and diagnosis of diseases.

7.2.2. Bridging innate and adaptive immunity by innate lymphocytes at the primary site of a bacterial lung infection

Cooperation between innate and adaptive immune systems is critical for an optimal immune response against infectious agents. Innate lymphocytes, although being a component of innate immune system, have earned much credit as a bridge between innate and adaptive immunity. Crosstalk between these lymphocytes, such as NK, NKT and γδ T cells, and APCs particularly DCs has been reported to shape the adaptive immunity. Majority of studies on how innate lymphocytes interact with DCs have focused on the lymphoid organs such as the spleen during various infections. Analysis of immune responses in the spleen however may not depict the real image of how immune responses ensue at the local site of infection where predominant immunologic/inflammatory changes occur. In addition, a large proportion of pathogen-

specific effector T cells are recruited to the local site of infection compared to the blood or lymphoid organs. In respiratory infections, multiple immunologic mechanisms work in a delicate coordinated fashion to develop and shape T-cell immunity in the lung. To better understand the dynamics of pulmonary immune mechanisms, we focused on how innate lymphocytes, NK and iNKT cells, interact with LDCs to mount protective mucosal T-cell immunity to a bacterial lung infection. **Our findings pointed out some interesting similarities as well as differences with the findings from previous studies on innate lymphocyte-DC relationship in the spleen**. We found that the intranasal transfer of WT-LDCs in naïve recipient mice conferred strong protective Tc1/Th1 immunity, whereas the transfer of KO-LDCs promoted Th2 immunity that resulted in pathology following *C. pneumoniae* challenge (Chapter-4). These findings are similar to the findings of our previous study that analyzed the effect of iNKT cell on SDCs (118), suggesting an absolute requirement for iNKT cells in generating protective T-cell immunity to chlamydial infection through DC modulation. **Thus, iNKT cell-DC interaction resulted in similar immune responses in the lung as well the spleen**. On the other hand, we found that NK+LDCs and NK-LDCs conferred protective Th1/Th17 immunity, when transferred to recipient mice, against challenge with *C. muridarum* infection, but the protection induced by NK+LDCs was much more effective than the NK-LDCs (Chapter - 5). However, our previous studies using SDCs showed that although the transfer of SDCs from sham-treated mice conferred protective immunity to challenge, the SDCs from NK-cell-depleted mice led to more serious inflammatory and pathologic changes (140). Thus, LDCs and SDCs from NK-cell-depleted mice promoted protection and pathology, respectively, indicating that NK cell-

DC interaction is much more critical for protection in the spleen than that of in the lung. Indeed, the impact of NK cell on LDC function is different in the spleen and the lung. This is in line with studies demonstrating a tissue-specific response conferred by innate lymphocyte-DC interaction during viral infections (117). Overall, our findings highlight the importance of a critical analysis of immune responses at the lymphoid and the local tissues. **A pertinent question arises as to why NK cell-DC, but not iNKT cell-DC, interaction leads to different immune responses to chlamydial lung infections in the spleen and the lung.** One of the reasons could be that multiple cells are activated in the local tissues after infection, and that many of them can modulate DC function, thus the effect of each type of cell may show some redundancy. Indeed, it has been reported that macrophages, neutrophils, γδ T cells, and epithelial cells, etc. can be activated after chlamydial infection. Although most of these cells also exist in the spleen, the density of these cells in the lungs after infection becomes many folds higher than that in the spleen. To understand the mechanism of this discrepancy, we can isolate DCs from the spleen and the lung of *Chlamydia*-infected mice and cocultured them with Chlamydia-specific T cells and then block various markers (e.g., PD1-PD1L, ICOS-ICOSL, and CD40-CD40L), which are important for DC-T cell interaction.

Whilst our findings describe the impact of innate lymphocytes on LDCs to induce mucosal T-cell immunity at the site of a bacterial infection, the overall mechanism of generation of adaptive immune responses may not be as simple as it appears. Many reports have indicated a functional relationship between iNKT and NK cells *in vivo* (262-263). The activation of NK cells by NKT cells is referred to as transactivation (262). In

chlamydial infection, iNKT cells significantly altered the cytokine profile and cytotoxic activities of NK cells via production of IFN-γ (160). Furthermore, there is increasing evidence that DCs have a profound effect on the immune functions of NK and iNKT cells (97, 264-267). These findings put forward some critical questions in context of pulmonary infections. Do iNKT cells target DCs via modulation of NK cells, apart from their direct action on DCs? In turn, can NK cells influence iNKT cells to modulate DCs? **On contemplating these crucial points, it seems that a multifaceted communication system exists between DC, NK and iNKT cells, forming a 'trio' in the lung, and that the outcome of lung infection is decided by combined efforts of these cells types.** Future studies need to unravel the mechanisms by which these cells interact with each other to direct the quality and quantity of adaptive immunity during lung infections. To perform these studies, we will coculture LDCs and NK/iNKT cells and block various markers such as CD40-CD40L, OX40-OX40L, IFN-γ, TNF-α, and ICOS-ICOSL and analyze the production of different cytokines by LDCs.

7.2.3. Mechanism of NK-cell-mediated modulation of LDC function during chlamydial lung infection

One of our key findings has elucidated the underlying mechanism by which NK cells modulate LDC function during *C. muridarum* lung infection. We found that NK-LDCs produced lower levels of IL-12 than the NK+LDCs. We further observed that the addition of anti-IFN-γ or anti-NKG2D antibodies in NK cell:LDC coculture led to the partial reduction of IL-12p70 production by LDCs, whereas the IL-12p70 production was virtually abolished upon addition of both the antibodies (Fig. 19). On the other hand, the

addition of anti-NKG2D, but not anti-IFN-γ, antibodies caused a reduction in IL-6 and IL-23 production by LDCs (Fig. 19). These data suggest that although the modulating effect of NK cells on LDCs to enhance Th1-inducing cytokine (IL-12p70) production depends on both IFN-γ and NKG2D, the production of Th17-inducing cytokines (IL-6 and IL-23) is mediated via NKG2D receptor signaling. Following chlamydial infection, we also found an upregulation of RAE1, an NKG2D ligand, on LDCs. Previous study has shown that DCs need at least two signals, IFN-γ and NKG2D signaling, from NK cells during *C. muridarum* infection, which leads to the production of IL-12 that is crucial for generating Th1 responses (140). Similar role is played by CD40L on NKT and T cells in terms of IL-12 production. Whether these signals condition DCs to secrete IL-6 and IL-23 has been addressed in our study, where we observed that NK-cell-mediated modulation of LDCs for these cytokines was achieved through NKG2D signaling but not IFN-γ production. It appears that NK cells communicate with LDCs through different molecules to skew different T-cell responses against chlamydial infection.

7.2.4. Division of labor among major LDC subsets in a bacterial lung infection

The lung is a unique organ because of its various critical functions. Although the lung is an essential respiratory organ, it plays an important role in providing defense against a variety of respiratory pathogens. LDCs occupy a central position in the pulmonary immune system. This is because they are the most potent APCs in the lung. To prime naïve T cells to induce a primary adaptive immune response makes LDCs indispensable for local anti-microbial immunity to pathogens. Analogous to DCs in the secondary lymphoid organs, LDCs exhibit a high level of heterogeneity consisting of

various subsets, of which CD103+ and CD11b^{hi} LDCs are the major subsets. The LDC subsets in the lung are characterized by their phenotype, anatomical location and physiological functions. Accumulating evidence on LDC biology sheds light on how LDC subsets perform specific functions reflecting a 'division of labor' (73). This division of labor is not always rigid. It can be flexible depending upon the fluctuations in the lung micromilieu. To maintain pulmonary homeostasis, different LDC subsets work within their niche with defined functional roles. Upon confrontation with infectious agents, the functions of LDC subsets are drastically changed, acquiring specific roles in response to different respiratory pathogens. In addition, monocyte-derived DCs are continually recruited to the lung during inflammatory conditions. In recent years, to study the LDC subsets has become easier due to the availability of tools that allow the analysis of their phenotypic and functional characteristics. To dissect their role in pathogen defense, LDC subsets have been mainly studied using respiratory viral infection models in mice (59, 268-269). Although some previous studies have focused on the function of different LDC subsets in bacterial lung infections, their role in protective immunity is largely unknown. We recently investigated the role of pLDCs in immunity to *C. pneumoniae* infection, and found a protective role for these cells in chlamydial infection (90). What specific roles conventional LDC subsets, CD103+ and CD11b^{hi} LDCs, play in immunity to bacterial lung infections is poorly understood. Leepiyasakulchai *et al*. showed that CD103+ LDCs are mainly monocyte-derived during *M. tuberculosis* infection and contain the highest frequency of IL-12-producing cells among the myeloid cell subsets in the lung (270). In this study, we focused on the role of LDC subsets, CD103+ and CD11b^{hi} LDCs, in protective immunity to *C. muridarum* infection. Upon adoptive transfer

of CD103+, compared to CD11b^{hi}, LDCs isolated from infected mice into naïve mice showed reduced body weight loss, bacterial burden and pulmonary pathology when subjected to chlamydial infection. Furthermore, the CD103+ LDC recipient mice revealed an enhanced Th1 and Th17 response compared to the mice that received CD11b^{hi} LDCs. **These findings suggest that CD103+ LDCs confer better Th1/Th17 protective immunity to a bacterial lung infection than CD11b^{hi} LDCs** (Chapter- 6). Our findings are in line with previous studies suggesting an important role for CD103+ LDCs in control of respiratory viral infections. Langerin-C-DT transgenic mice treated with diphtheria toxin or Batf3$^{-/-}$ mice, which are deficient in CD103+ LDCs, failed to induce an optimal CD8+ T cell immunity to influenza infection (76-77). In *C. muridarum* infection, CD103+ LDCs elicited a robust CD4+ T cell response, characterized by IFN-γ and IL-17 production (Th1/Th17), although they are shown to be important for CD8+ T cell responses in viral infections. **It appears that the type of T-cell response induced by CD103+ LDCs is specific to a pathogen**. In this study, we also found that CD11b^{hi}, similar to CD103+, LDCs contributed to protection against chlamydial infection. However, it was not clear whether CD11b^{hi} LDCs were contaminated with iLDCs. Following infection, a sudden influx of iLDCs, which express high levels of CD11b, has been reported in many studies (59, 74). To distinguish iLDCs from CD11b^{hi} LDCs, it is crucial to use antibodies against SIRPα, a receptor expressed by iLDCs but not CD11b^{hi} LDCs, for flow cytometric analysis and sorting. It is important to note that there are differential requirements for CD8+ T cells with *C. pneumoniae* and *C. muridarum*. CD8+ T cells are more important for *C. pneumoniae* than *C. muridarum*. Since CD103+ LDCs are quite potent in cross-presenting antigens to CD8+ T cells, it is possible that CD103+

LDCs play a critical role in CD8+ T-cell immunity to *C. pneumoniae*. However, recent studies demonstrate that $CD11b^{hi}$ LDCs can cross-present antigens to CD8+ T cells during influenza lung infection. Therefore, the function of these LDC subsets might differ in relation to *C. pneumoniae* and *C. muridarum*.

Increasing knowledge on DC biology points out a relationship between DC subsets that reside in different anatomical locations. A selective loss of lymphoid CD8α+ and non-lymphoid CD103+ DCs in Batf-deficient mice suggested a developmental relationship between these DC subsets (271). Subsequent studies showed that CD8α+ and CD103+ DCs are very efficient at cross-presenting as well as cross-dressing antigens to CD8+ T cells (68, 272). In addition, a chemokine receptor, XCR1, is specifically expressed on CD103+ and CD8α+ DCs in mice and CD141+ DCs in humans, accentuating the notion that these DCs constitute a unified subset (64, 273). These findings taken together indicate that XCR1+ DCs such as CD103+ and CD8α+ DCs are developmentally and functionally related. Growing evidence further demonstrates that there are also similarities between the functional roles of CD103+ and CD8α+ DCs in response to pulmonary viral infections (68, 272, 274). Do these DC subsets induce similar immune responses to bacterial infections has been unclear. Our previous study showed a predominant role for CD8α+ DCs in resolution of chlamydial lung infection. CD8α+, compared to CD8α-, DCs not only produced higher levels of IL-12 but also induced an enhanced Th1 immunity (88). In line, our present findings demonstrated that CD103+ LDCs conferred a better protective Th1 immunity than the $CD11b^{hi}$ LDCs (Chapter – 6). This implies that both CD8α+ and CD103+ exert similar

immune responses to a bacterial lung infection irrespective of their anatomical location. **In a broader prospective, our findings add new information to the field of DC immunology that CD103+ and CD8α+ DCs reflect functional similarities in response to not only viral but also bacterial infections.**

8.0. CHAPTER 8

SIGNIFICANCE, LIMITATIONS AND FUTURE WORK

8.1.0. SIGNIFICANCE

In this study, we focused on the role of LDC subsets and the interaction of innate lymphocytes with LDCs in immunity to chlamydial infection. Our findings highlight the mechanisms of how NK, iNKT cells and LDCs manipulate the adaptive immunity at the local site of a bacterial lung infection. In addition, these findings point out potential targets that may be exploited to develop therapeutic strategies.

1) DCs represent a promising target for vaccine development against infections due to their crucial role in initiating the immune response to microbial antigens. Adjuvants also exert their effect to enhance the immune responses, primarily through DC activation. Being a heterogeneous cell population, DCs consist of various subsets that possess differential abilities to induce T-cell responses. It is therefore of interest to identify the DC subset that plays the most potent role in protective immunity to a microbial infection. In this study, we showed that CD103+ LDCs confer better protection against chlamydial lung infection than $CD11b^{hi}$ LDCs. The finding that CD103+ LDCs are major LDC subset at the site of infection that is involved in protective immunity may have implications for developing vaccines against chlamydial as well as other respiratory infections. An important novel approach to induce T-cell immunity is to directly target DCs *in vivo* via antibodies against endocytic receptors (DEC-205) expressed by DCs

(275). For instance, intranasal administration of *Yersinia pestis*, a bacterial lung pathogen that causes plague, LcrV (V) protein fused to anti-DEC-205 antibodies together with adjuvants induced enhanced mucosal Th1 responses in the lung (276). Recent evidence that CD103+ LDCs express DEC-205 and have a human equivalent, CD141+ DCs, highlights the suitability and relevance of CD103+ LDC subset to be targeted through anti-DEC-205 antibodies fused with pulmonary microbial antigens for vaccine development (277-278). It is however notable that precaution should be taken while extrapolating our findings to the other lung infection models as LDC subsets may function differently in different infection models.

2) We also demonstrated that iNKT cells confer protective Th1 immunity against *C. pneumoniae* infection through functional modulation of LDCs. Indeed, iNKT cell-LDC interaction results in effective mucosal immunity at the local site of infection. These findings may have implications for exploring the immune mechanisms that enhance host immunity against respiratory infections, and also for the control of long-term complications of *C. pneumoniae* infections, such as atherosclerosis and Alzheimer's disease. It should be noted that glycolipid agonists, such as α-GalCer, for iNKT cells may be used to enhance iNKT-cell-mediated immune responses that are critical for vaccine development (279). However, an important limitation of α-GalCer treatment is their repeated doses can lead to development of anergy or unresponsiveness of iNKT cells.

3) We also showed that NK cells enhance IL-12p70 (Th1-inducing cytokine), and IL-6 and IL-23 (Th17-inducing cytokines) production by LDCs through NKG2D receptor signaling. Therefore, NKG2D could be a potential target to augment protective immunity to curb infections.

In summary, our findings provide a better understanding of the immune mechanisms that bridge innate and adaptive immunity at the local site of a lung infection. Exploiting this knowledge can be useful for developing the strategies on how to control respiratory diseases.

8.2.0. LIMITATIONS

Although our data presented in this thesis throw light on the role of LDC subsets and the impact of NK/iNKT cells on LDC function in immunity to a pulmonary bacterial infection, there are some limitations that need to be discussed to critically ascertain the quality and soundness of the data.

1) During our studies, we isolated LDC/LDC subsets from the lung that requires complex digestion and multistep separation procedures. DCs are extremely sensitive to their environment. The lengthy procedure to isolate LDCs, which involves enzymatic digestion and sorting, may alter their functional profile. A genuine concern therefore arises as to whether the LDCs we isolated represent the physiological properties of LDCs *in toto* (described in Chapter- 8). Despite this limitation, the adoptive transfer of LDCs to test their function appears to be

the most practical approach, particularly to examine the relative contribution of LDCs in host defense against lung infection.

2) Another limitation stems from our LDC:T cell coculture experiments. We used T cells isolated from the mice immunized with *Chlamydia* to examine the ability of LDC/LDC subsets to activate *Chlamydia*-specific T-cell responses. Whether the isolated T cells were indeed *Chlamydia*-specific could not be fully ascertained. This is because transgenic mice having TCR specific for chlamydial antigens are currently not available to us. However, it is relevant to mention that when the LDC/LDC subsets were transferred in mice, they induced Tc1/Th1 responses *in vivo* similar to the immune responses elicited by T cells from immunized mice in the coculture.

3) We could not analyze the base level immune responses in the MLNs. The reason behind this is that the MLNs are too small to be seen with naked eye in uninfected mice and therefore cannot be taken out. However, we did analyze the MLN immune responses at different time points after infection and also compared the responses between infected-KO/NK-cell-depleted and control mice.

8.3.0. FUTURE WORK

Our findings have provided significant insights into the contribution of innate lymphocytes and LDC subsets in orchestrating the direction and magnitude of adaptive

immune responses during chlamydial lung infections, while some pertinent questions remain unanswered that need to be addressed in future studies (Figure 31).

1) Our findings have demonstrated that NK and iNKT cells modulate the function of LDCs to exert T-cell immunity to chlamydial infections. Since LDCs consist of two major subsets, CD103+ and CD11b^{hi} LDCs, it would be of interest to explore whether the modulating effect of NK and iNKT cells on LDC function to induce Th1/Tc1 and Th17 immunity is biased to a LDC subset.

2) iNKT cells promote protective immunity and pathology to *C. pneumoniae* and *C. muridarum* lung infection, respectively (111). Our findings demonstrated that iNKT cells confer protective immunity to *C. pneumoniae* infection by modulating the function of LDCs. How iNKT cells interact with LDCs during *C. muridarum* infection is an interesting area that merits further exploration.

3) Apart from impacting DC function, innate lymphocytes trigger the activation of macrophages that play a critical role in host immunity (280-281). Our preliminary data also showed that iNKT cells influence the phenotype and cytokine profile of AMs during chlamydial infection. To directly examine how the interaction of iNKT cells with AMs can shape the outcome of chlamydial infection would be an important point to be addressed in times to come. For this study, we can isolate AMs from KO mice and analyze them for their function using adoptive transfer approach.

4) We previously showed that iNKT cells influence the cytotoxic properties and cytokine profile of NK cells during chlamydial infection (160). Whether iNKT cells communicate with NK cells to regulate LDC function to elicit T-cell responses holds promise for further investigation. To sort out this mechanism, we would deplete NK cells in KO mice and isolate LDCs from them following infection. These LDCs will be transferred to the naïve recipient mice subjected to a challenge infection to assess their protective effect.

5) Our previous and present studies showed that pLDCs, CD103+, and $CD11b^{hi}$ LDCs play a protective role in chlamydial infections (90, Chapter – 6). Future studies can focus on the relative contribution of pLDCs, CD103+, $CD11b^{hi}$, and iLDCs in inducing protective immunity to chlamydial lung infections. These studies can be done by isolating different LDC subsets and transferring them to naive syngeneic mice that subsequently receive chlamydial challenge.

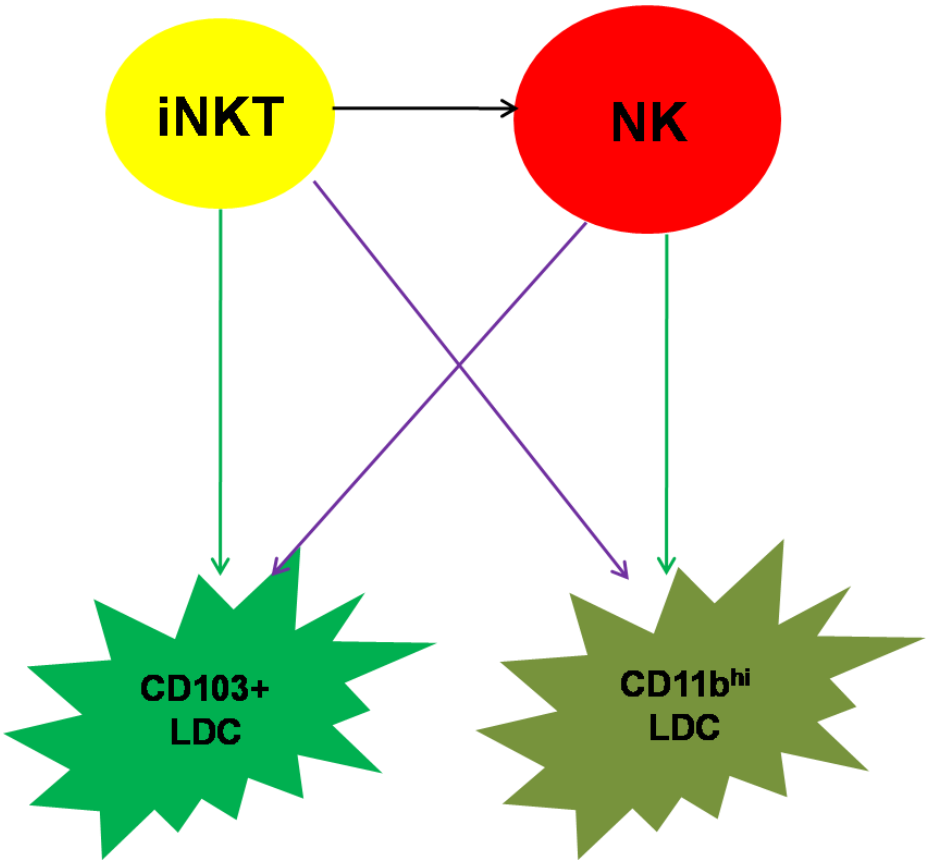

Figure 31. Effect of NK/iNKT cells on LDC subsets. It will be important to examine whether the modulating effect of NK/iNKT cells on LDCs is biased to a LDC subset, such as CD103+ or CD11b^{hi} LDCs. In addition, do iNKT cells modulate LDC function through influencing NK cells merits further exploration.

If the future studies as suggested above are taken into account, we would have a better and deeper understanding of the holistic relationship between innate lymphocytes and LDCs/AMs in generating adaptive immunity to pulmonary infections. The findings from these studies would have implications for designing prophylactics and therapeutics against respiratory diseases.

9.0. CHAPTER 9

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