The role of innate immune responses in resistance/ susceptibility to chlamydial lung infection

A thesis submitted to the Faculty of Graduate Study of the University of Manitoba

In partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

By

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ABBREVIATIONS

ADCC- antibody-dependent cell-mediated cytotoxicity

AG- aminoguanidin

APC- antigen presenting cell

APC- allophycocyanin

BAL- bronchoalveolar lavage

BMDM- bond marrow derived macrophage

C3H- C3H/HeN

CMI- cell mediated immunity

C. muridarum MoPn- *Chlamydia muridarum* mouse pneumonitis

CL_2MDP- dichloromethylene diphosphonate

Daxx- Fax death domain associated protein

DC- dendritic cell

DTH- delayed type hypersensitivity

EB- elementary body

eIF2α- eukaryotic initiation factor 2α

ELISA- enzyme-linked immunosorbent assay

FACS- fluorescent activated cell sorting

FcR- Fc receptor

FITC- fluorescein isothiocyanate

H&E- hematoxylin and eosin

HBSS- Hank's balanced salt solution

HLA- human leukocyte antigen

hsp- heat shock protein

ICAM-1 - intercellular adhesion molecule 1

IDO- indoleamine dioxygenase

IFNAR-/-- IFNα/β receptor knock out

IFN- interferon

IFNIs- type I interferons

IFU- inclusion-forming unit

Ig- immunoglobulin

IL- interleukin

i.n.- intranasally

iNOS- induced nitric oxide synthetase

i.p. - intraperitoneally

IRF9- IFN regulatory factor 9

ISCOM- immunostimulating complexes

ISGF3- IFN stimulated gene factor 3

ISRE- IFN-stimulated response elements

KO- knock out

LGV- lymphogranuloma venereum biovar

LRR- leucine rich repeat

mAb- monoclonal antibody

MCP - monocyte chemoattractant protein

MEM- minimum essential media

MHC- major histocompatibility complex

MIP- macrophage inflammatory protein

MOI- multiplicity of infection

MOMP- major out membrane protein

NK cell- natural killer cell

NKT cell- natural killer T cell

NO- nitric oxide

PAMP- pathogen associated molecule pattern

PBS- Phosphate Buffered Saline

pDC- plasmacytoid dendritic cell

PE- phycoerythrin

PerCP- Peridinin-chlorophyll-protein Complex

PFP- pore forming protein

PID- pelvic inflammatory disease

PKR- RNA dependent protein kinase

PMA - phorbol myristate acetate

POMP- polymorphic outer membrane proteins

PRR- pattern recognition receptors

RAG- recombination activating gene

RB- reticulated body

ROS- reactive oxygen species

RT-PCR- reverse transcription polymerase chain reaction

sIgA- secreted IgA

SPG- sucrose phosphate glutamic acid

STAT- signal transducer and activator of transcription

STD- sexually transmitted diseases

Th- T helper

TLR- Toll-like receptor

TNF- Tumor necrosis factor

TRAIL- TNF-related apoptosis-inducing ligand

TUNEL- TdT-mediated dUTP nick end labeling

UV- ultraviolet

VCAM-1- vascular cell adhesion molecule-1

VLA-4- Very Late Antigen-4 (CD49d/CD29)

WT- wild type

FIGURES AND TABLES

ABSTRACT

Chlamydia trachomatis is the causative agent for many important human diseases including sexually transmitted diseases, trachoma and infant pneumonia. Despite the availability of effective antimicrobial therapy, *C. trachomatis* remains a significant cause for human morbidity worldwide. Owing to the lack of a clear understanding of the immunological mechanisms underlying the protective and pathological responses to chlamydial infection, human vaccines to the important pathogen and related diseases are unavailable at the present time. In the present study, we examined the immunological mechanisms of chlamydial infection by using mice with different genetic backgrounds and gene knock-out mice.

First, we compared the susceptibility of C3H/HeN mice and C57BL/6 mice to *C. trachomatis* mouse pneumonitis (MoPn, more recently called *C. muridarum*) lung infection. We found that C3H mice exhibited a more severe disease with higher mortality, greater organism growth and more severe pathological changes in the lung compared to C57BL/6 mice subjected to the same infection. However, the pattern of adaptive immune responses including organism-specific delayed type hypersensitivity (DTH) responses, antibody and cytokine (IFN-γ, IL-12, IL-4, IL-5, IL-10 and TNF-α) production by spleen and local draining lymph node cells in these two strains of mice appeared comparable over the course of the infection. We further analyzed local inflammation of the infected lungs and found that C3H mice exhibited significantly more neutrophil infiltration after infection. It is in line with a greater increased expression of neutrophil chemokines MIP-2, LIX, KC and chemokine receptor CXCR-2. However, the increased infiltration of neutrophils is not protective against infection, but may contribute to tissue damage and

more severe inflammation. We further tested the kinetics of organism growth after infection and found that as early as two days post infection (p.i.), the lungs of C3H mice showed significant higher levels of *C. muridaurm* compared to those of C57BL/6 mice. We then tested *in vivo* expression of cell cytotoxicity-related molecules including FAS, FASL, perforin, granzymes A and B; IFN-γ dependent IDO and p47GTPases by RT-PCR and found no significant difference between the two strains of mice. Interestingly, we found that the macrophages in C3H mice are less efficient in clearing chlamydial infection following exogenous IFN-γ treatment compared to those of C57BL/6 mice. The data suggested that the cellular events downstream of cytokine production in *Chlamydia*-infected cells may be important in determining the differential susceptibility of hosts to chlamydial infection.

Second, we examined the role of major histocompatibility complex (MHC) genes and non-MHC genes on host resistance to chlamydial infection. Given that C3H/HeN (H-2^k) mice were more susceptible to *C. muridarum* infection compared to C57BL/6 (H-2^b) mice, we compared the susceptibility to *C. muridarum* infection of MHC congenic mice, B6.H2k [C57BL/6 background, C3H MHC (H-2^k)] and C3H.H2b [C3H/HeN background, C57BL/6 MHC (H-2^b)] and their corresponding wild type C57BL/6 mice and C3H/HeN mice, respectively. The data revealed that B6.H2k, C3H.H2b and C3H/HeN mice were more susceptible to chlamydial lung infection compared to the wild type C57BL/6. Congenic B6.H2k mice showed significantly lower levels of IL-12 and IFN-γ production compared to C57BL/6, C3H/HeN as well as C3H.H2b mice. On the other hand, although congenic C3H.H2b mice displayed a similar cytokine response to C57BL/6 mice, they were highly susceptible to *C. muridarum* infection. Overall, the

results suggest that protection against chlamydial lung infection is both MHC and non-MHC gene dependent, and that the interaction between MHC and non-MHC elements may contribute to host resistance to chlamydial infection.

Third, we studied the role of type I interferon in host susceptibility to chlamydial infection. We used IFN-α/β receptor knock-out mice (IFNAR-/-) and wild type (WT) mice to compare their resistance to *C. muridarum* lung infection. Surprisingly, WT mice were significantly more susceptible to chlamydial infection than IFNAR-/- mice, as evidenced by more organism growth, more bodyweight loss after infection, and more severe lung inflammation. Both WT and IFNAR-/- mice showed strong Th1-like immune responses post-infection with high IFN-γ, and low IL-4, IL-5 and IL-10 from draining lymph node and spleen cell cultures, and both groups of mice showed higher serum IgG2a than IgG1 following *C. muridaurm* infection. WT mice produced more IFN-γ in the infected lungs than IFNAR-/- mice. Histological analysis indicated that WT mice have much less resident macrophages in the lungs after infection. Depletion of lung macrophages with CL_2MDP-lipsome rendered the otherwise resistant IFNAR-/- mice highly susceptible to *C. muridarum* infection. The results also showed that WT mice had weaker VCAM-1 expression in the lungs, and VLA-4 expression in peripheral blood monocytes. Apoptosis of macrophages was also more pronounced in the infected WT mice. The type I interferon-induced apoptotic factors TRAIL, Daxx, and PKR were also found to be highly expressed in WT mice than in IFNAR-/- mice. In addition, we found significantly more neutrophils in the lungs of WT mice, which was correlated with IFN-α/β dependent inflammatory cytokine and chemokine expression. This may also contribute to an increased susceptibility to chlamydial infection. Interestingly, we

observed that there were more activated dendritic cells (DC) (*e.g.*, CD80-, CD86-, CD40-positive) in the spleen of WT mice. This study indicates that: 1) type I interferons enhance host susceptibility to chlamydial infection, which is dependent on decreased macrophage infiltration and increased macrophage apoptosis, and 2) type I interferon-regulated innate immune responses play a critical role in host susceptibility to chlamydial infection. Although type I interferons can activate DC and may promote their function in antigen presentation, they are not protective against chlamydial primary infection.

Our study further highlights the complexity of the mechanisms underlying host defence against chlamydial infection. It indicates that both innate and adaptive immune responses are important in host defence against chlamydial infection. Specifically, it suggests that: 1) cellular events downstream of Th1 cytokine production play an important role in the host defence and/or disease development to chlamydial infection; 2) macrophages, as an executioner of the host immune responses, are extremely important against chlamydial infection. As such, any factors that may influence the macrophage population such as macrophage infiltration and macrophage apoptosis may change host's susceptibility to chlamydial infection; 3) type I interferons, although enhance IFN-γ production and activate DC, may suppress host resistance to chlamydial infection through modulating the infiltration and survival of macrophages.

INTRODUCTION

1. *Chlamydia trachomatis*: Biology and Pathogenesis

1.1 General history and classification

Chlamydia trachomatis, are a group of gram-negative bacteria of the *Chlamydiae* genus which can only grow inside host cells. *Chlamydiae* have been found broadly existing in eukaryotic cells from amoebae to vertebrates. As obligate intracellular pathogens, *C. trachomatis* can infect humans, live stocks and birds. The records of *C. trachomatis*-induced disease can be traced back to thousands years ago when 'trachoma' was described in ancient Egypt and Chinese books. But it was not until 1957 when live *Chlamydia* was successfully isolated and cultured [1].

The members of the *Chlamydiae* family have a specific biphasic replication life cycle. They share similar genetic and biological characters, and are phylogenetically distinct from the other bacterial divisions. For a long time, *Chlamydiae* order was classified to contain only one *Chlamydia* genus and four species: *C. trachomatis*, *C. psittaci*, *C. pneumonia* and *C. pecourm*. Following the progress of molecular biological techniques, nine species of *Chlamydiaceae* were arguably re-classified based on their sequences of certain surface and intracellular proteins as well as structures of the 16S and 23S rRNA, LPS and chromosomes [2-4]. In particular, a biovar of *C. trachomatis*, the mouse pneumonitis, was reclassified as an independent species, *C. muridarum*. According to the new classification, in the *Chlamydiaceae* family, *C. trachomatis*, *C. muridarum*, and *C. suis* are evolutionarily the closest relatives and belong to the same *Chlamydia* genus and species [2, 5]; *C. pneumonia*, *C. pecourm* and *C. psittaci* are re-ascribed to *Chlamydophila* genus and species. *C. trachomatis* contains 15 biovars that

can induce eye trachoma, sexually transmitted diseases, arthritis and neonatal pneumonia. At present, two strains of *C. muridium,* the MoPn (mouse pneumonitis) and SFPD isolated from mouse and hamster, respectively have been discovered [2, 5, 6]. Because *C. muridarum* MoPn is a natural murine pathogen and the pathological changes induced as a result of *C. muridarum* infection in mice are similar to those manifested in human *C. trachomatis* infection [7], *C. muridarum* MoPn mouse models have been widely used in investigations of host immune responses to chlamydial infection. Here, in my project, the *C. muridarum* lung infection mouse model was chosen to study the interactions between host and *Chlamydia*.

1.2 Biology of *Chlamydia*

Originally, *Chlamydiae* were thought to be virus. It was not until significant progress made in the areas of electron microscopy and biochemical technology that C*hlamydiae* were found to possess all properties of bacteria. The rRNA sequences of *Chlamydia* are more closely related to those of eubacteria than to the archaebacteria, which confirmed that *chlamydiae* are eubacteria [8]. Unlike other extracellular and intracellular bacteria, *Chlamydiae* display a unique biphasic development cycle [9]. A complete development cycle of *Chlamydiae* is composed of: 1) an invasion of a host cell by infectious elementary body (EB) and transformation of EB to reticulated body (RB); 2) a replication in the form of RB in inclusion bodies inside host cells; 3) transformation of RB back to EB again and 4) release of EB to initiate a brand-new development cycle on neighbouring cells [10-12]. EBs are small (0.3 μm diameter), extracellular infectious forms that can attach to and invade susceptible host cells. To survive the harsh extracellular environment, *Chlamydiae* have adapted a similar survival strategy to that

used by bacterial spores which include possession of a hard cell surface and inactive metabolism. In so doing, extra-cellular EBs would not need to exchange energy and nutrients with the outer environment. Unlike the cell wall of the other bacteria, the *Chlamydiae* EB cell wall contains very little or no peptidoglycan [13], but many cysteine-rich proteins including OmpA, OmpB and OmpC [14]. The disulfide bonds between these proteins form a rigid structure that maintains the integrity of the *Chlamydiae* EB cell wall and resists outside pressure [15]. To initiate a successful infection in non-phagocytic cells, *Chlamydiae* attach and enter host cells through elaborate mechanisms [16]. Although it is not very clearly understood, the initial step is thought to be through a receptor-ligand interaction. The possible ligands on the surface of *Chlamydiae* that have been studied include heparan sulfate-like glycosaminoglycan (GAG) [17], chlamydial major out membrane protein (MOMP) and polymorphic outer membrane proteins (POMP) [20]. The candidate cellular receptors include heparan sulfate proteoglycans [19], mannose-receptor [21], estrogen receptor complex [22], and insulin-like growth factor 2 receptor [23]. Following the initial reversible interaction between EB and host cell, *Chlamydiae* is endocytosed into host cell, a process which is dependent on chlamydial type III secretion system translocated phosphorprotein [24]. After the endocytosis, EBs remain in a membrane-bound inclusion and differentiate into RBs. RBs are non-infectious and replicating forms of *Chlamydia* which are metabolically active and can not survive outside the host cells. RB (approximately 1 μm in diameter) is larger than EB and contains diffused nucleic acids instead of condensed nucleic acids as EB, for the purpose of active metabolism. About 8 hours after infection, RBs undergo repeated binary fission similar to the other bacteria until there are about 500-1000 progeny in one

inclusion. Approximately 48-72h post-infection, RBs differentiate back to EBs [25] and released through a burst of host cells. To escape from the normal endocytic or autophagic trafficking pathway that may digest and destroy pathogens, *Chlamydia* have developed a set of mechanisms to dissociate inclusions from late endosomes, lysosomes and the autophagysomes [26, 27]. This process needs *de novo* protein synthesis because it can be blocked by antibiotics which inhibit transcription or translation [28].

1.3 Pathogenesis and epidemiology of *Chlamydia* infection

As a major cause of trachoma and sexually transmitted diseases (STDs) in human, *C. trachomatis* infections result in huge financial burdens to global public health systems [25]. The annual cost of treatment for chlamydial infections and associated complications in USA is about 2-6 billion dollars [29]. According to their specific target tissues, the total 15 serovars of human *C. trachomatis* can be ascribed to 2 biovariants: the lymphogranuloma venereum biovar (LGV) and trachoma biovar. LGV biovar includes the serovars L1, L2, and L3 that can invade and grow in lymph nodes and result in necrotic granulomas [30]. Trachoma biovar normally invade and grow in epithelial cells which lead to ocular or urogenital infection.

The ocular infection-associated serotypes include serovars A, B, Ba and C, while serovars D to K induce urogenital infection. Eye trachoma is a chronic follicular inflammation induced by ocular serovars which, if left untreated, may cause corneal opacity and eventual blindness. This infection is normally transmitted by direct contact or through flies in some endemic area [31]. Trachoma is a very old disease that was described in the Ebers Papyrus 3,500 years ago [32]. Currently, *C. trachomatis* has already induced blindness in 6 million of the infected patients [33], which account for

15% of all blindness. As the major cause of preventable blindness and the second cause of total blindness (after cataracts) [32-34], trachoma is a potential threat to at least 500 million people who are in close contact with 150 million people already infected with the pathogen [35-37]. Genital serotypes may also cause neonatal trachoma through infected genital tract during birth [32, 35]. The prevalence of trachoma is inversely correlated with the socio-economical development of an area. Once an epidemic disease worldwide, trachoma is now rare in developed countries, while it is still an endemic problem in many developing countries in Asia, Latin America, Middle East and Africa [36, 38].

In contrast to trachoma as an endemic disease in developing countries, *C. trachomatis*-induced STD remains a global epidemic problem. Because most genital tract *C. trachomatis* infection is asymptomatic [39], it can be transmitted easily and unknowingly through sexual activity. As the most common bacterial pathogen of STDs, *C. trachomatis* causes approximately 90 million new episodes a year through sexual activity [30, 40], of which, about 49 million cases occur in southern and southeastern Asia, while 15 million in Africa [40]. In industrialized countries, the incidence is also disturbingly high and the pathogen mainly affects young sexually active adults. For example, the prevalence rate is about 2.1% in people of 20-64 years old in Britain [41] but can be as high as 8.1% in those under 20 years old [42]. Similarly in the US, the incidence is approximately 4.2% for young adults at the age between 18-26 years old [30, 43]. Although chlamydial infection is mostly not life-threatening, it can result in infertility if left untreated or infected multiple times [30, 44]. Chlamydial genital tract infection also facilitates the transmission of HIV and other STD [45, 46]. In women, chlamydial infection can cause pelvic inflammatory disease, chronic pelvic pain,

salpingitis, ectopic pregnancy and infertility. Although *C. trachomatis*-induced STDs occur more frequently in female, it also causes a severe disease in males [40]. The infection in the latter group can lead to urethritis, epididymitis [47, 48], prostatitis [47], and infertility [49]. A positivity for anti-*Chlamydia* IgG antibody in men has been shown to be associated with a lower rate of pregnancy [49]. It is also reported that *Chlamydia* can give rise to pathological changes, or even death, to the sperms, hence, male infertility. Except trachoma and STD, *C. trachomatis* infection can also lead to manifestation of other diseases including reactive arthritis [51, 52], ankylosing spondylitis [51, 53], and likely Alzheimer's diseases [54].

In recent years, some *Chlamydia* species especially *C. pneumonia* and *C. trachomatis*, have been reported to be associated with atherosclerosis, myocarditis and endocarditis [55, 56]. Animal models of atherosclerosis with chlamydial infection were also successfully reported in rabbits and mice [57-61]. Lung infection with *C. muridarum* may directly induce cardiovascular pathological change [61]. Moreover, chlamydial hsp60 is correlated with myocarditis. It has been shown that chlamydial hsp60 regulates tumor necrosis factor-α (TNF-α) and matrix metalloproteinase expression [62], which are involved in immune pathology [63]. The 60 KDa cysteine-rich out membrane proteins of *Chlamydia* can also cause atherosclerosis through antigen mimicry with mouse heart muscle α-myosin [64].

Because of the severe outcomes of chlamydial infection, quick and early diagnosis and treatment are necessary to control the infection. However, since it is asymptomatic in 85% infected women and 45% infected men [65, 66], the early identification of the infection is difficult. Further, it is estimated that around 10 to 15%

women treated with antibiotics display a recurrent *C. trachomatis* infection [67]. Clinical studies also show that antibiotic-resistant strains of *C. trachomatis* will induce treatment failure [68-70]. For these reasons, it is highly necessary to study and understand the pathogenesis of chlamydial infection and host immune responses, in order to rationally develop an efficient vaccine to prevent chlamydial diseases.

2. Immune responses to *Chlamydia* infection

Both innate and adaptive immune responses are necessary to protect host against chlamydial infection. The Th1-like immune response is thought to be the most important players in host resistance to chlamydial infection and has been extensively studied. The central mechanism of Th1-like immune response is the production of IFN-γ, an important cytokine that activates many bactericidal pathways to eliminate pathogens. Natural killer cells (NK) and NKT cells, DC cells, CD4+ and CD8+ T cells, and macrophages are among the major players which control the production of IFN-γ and elimination of *Chlamydia*.

2.1 Sense of *Chlamydia* infection and innate resistance mechanisms

Mammalian immune system is composed of innate and adaptive immunity. Innate immune responses are initiated through the recognition of the pathogen-associated molecular patterns (PAMPs) by the germline encoded pattern recognition receptors (PRR) that are expressed on the cell surface or in cytosol [71, 72]. PAMPs are conserved in different microbes during billions years of evolution and they are necessary for microorganism survival. The most well known PRRs are Toll-like receptors (TLRs). Twelve different TLRs have been identified in mammals, which are expressed unevenly

in different tissues and cell types [71, 73]. TLRs are trans-membrane glycoproteins which contain an extracellular leucine rich repeat (LRR) domains and a cytoplasmic Toll/IL-R homology domain (TIR domain). The LRR domain participates in the binding and recognition of PAMPs, and the TIR domain transmits the PAMP-TLR signal downstream, which ultimately induces cell activation. TLRs recognize pathogens extracellularly or intracellularly via endosome/lysosome. To sense the pathogens residing in the cytosol, the cells also develop some other PRRs inside the cytoplasm such as NODs [71, 74]. NODs also contain the LRR domain to recognize PAMPs, and some other domains to transmit the signal to the nucleus. During the infection, PRRs interact with PAMPs and the activating signals are transmitted through MyD88-dependent and/or -independent pathways to activate host cells. Activated cells secret interferon (IFN)-α/β and other proinflammatory cytokines such as interleukin (IL)-1, IL-6, TNF-α and IL-12 [75, 76]. These cytokines subsequently induce the production of microbicidal molecules such as nitric oxide and reactive oxygen intermediates; depletion of necessary nutrients for microbe growth such as tryptophan and Fe^{2+}; phagocytosis of pathogens by macrophages and DC; enhanced expression of MHC molecules that promote presentation of pathogen antigens and induce adaptive immune responses [71, 72]. It has been found that PRRs participate in the sensing of *Chlamydia* and regulate immune responses. When stimulated with sonicated *Chlamydia*, peripheral blood mononuclear cells (PBMCs) produce proinflammatory cytokines through TLR2-dependent pathways [77]. Chlamydial heat shock protein 60 (hsp60) activates macrophages through TLR4 [78]. When co-cultured with live *Chlamydia*, DC activation mostly depends on TLR2 but not TLR4 [79]. Correspondingly, TLR2-/- mice produce significantly lower TNF-α and MIP-2 and

develop less oviduct pathology during chlamydial genital tract infection compared to WT mice [80]. Except TLR2 and TLR4, some other TLRs may be also involved in *Chlamydia* recognition and cell activation, because the production of IFN-β and IP-10 is not changed in TLR2-/-, TLR4-/- cells relative to that in WT cells, but significantly decreased in MyD88-/- cells [81]. As intracellular bacteria, *Chlamydia* may release some of their components into the cytosol during its replication thus cytosolic PRRs may also be involved in the sensing of *Chlamydiae.* A recent study has shown that Nod1 can sense the existence of trace peptidoglycan of *Chlamyida* and induce the production of proinflammatory cytokines [82].

2.2 Small bactericidal molecules

In addition to nitric oxide (NO), indoleamine dioxygenase (IDO), and peroxide, some other consistently expressed molecules can act as the first line of defense against chlamydial infection as well. Mucosal antimicrobial peptides such as β-defesin and surfactants, have been found to play a role in host resistance against chlamydial infection [83]. For example, surfactant A and D, the colletins that participate in innate immunity, were found to promote the phagocytosis of *C. trachomatis* and *C. pneumonia* by THP-1 cell, a macrophage cell line [84]. The cysteine-rich β-sheet anti-microbial peptides such as HNP-2 and PG-1 can inhibit the infection of *C. trachomatis* through direct damage of the membrane and killing of the bacterial EBs [85]. Another leukocyte derived anti-microbial peptide is the cathelicidin peptide, which is also capable of suppressing chlamydial infection [86]. Except being inhibited by anti-microbial peptides and other cell surface secreted molecules, chlamydial infections are suppressed largely by phagocytosis of leukocytes.

2.3 Neutrophils and chlamydial infection

Neutrophils are professional phagocytes that can efficiently kill ingested microbes during infection, which are critical innate immune cells involved in host protection against many pathogens. The microbicides produced by neutrophils include toxic reactive oxygen species (ROS)- O_2^- and H_2O_2 (NADPH oxidase catalyzed), HOCl (produced by MPO), proteases and antimicrobial peptides such as α-defensins [87]. The role of neutrophils in host resistance against chlamydial infection appears controversial. It was reported that during chlamydial intra-peritoneal or genital tract infection, complete depletion of neutrophils with antibodies largely enhanced chlamydial growth and transmission [88]. However, in this study, 2E6, a monoclonal antibody (mAb) against mouse CD18 which was used to deplete neutrophils, also inadvertently remove monocytes. Similarily, the use of another antibody RB6-8C5 depletes both neutrophils and plasmacytoid DC [89]. In consequence, such cross reactivity effectively remove neutrophils and other cells which may be important in the host immune responses during the infection. A later study using CXCR2 KO mice that had a deficiency in neutrophil infiltration showed similar resistance to *C. muridarum* to WT mice [90]. The data indicated that over-infiltration of neutrophils is not necessary to combat chlamydial infection. Interestingly, *C. pneumonia* can multiply inside neutrophils and infect neighboring epithelial cells, and depletion of neutrophils with RB6-8C5 decreased organism burden in the lungs [91, 92]. Therefore, it seems that neutrophils may be beneficial or detrimental to hosts depending on the type of the infecting *Chlamydia*. In this regard, further work is needed to resolve the interdependences between neutrophil infiltration and chlamydial infection.

2.4 Macrophages and *Chlamydia* infection

Macrophages are recruited to infection area later than neutrophils. They can not only phagocytose microbes, but also remove dead or apoptotic cells to suppress inflammatory responses [87]. Compared with neutrophils, macrophages are longer lived, and may differentiate to become DC to help regulate adaptive immune responses. The fate of *Chlamydia* in macrophages is determined by the activation status of macrophages, which in turn is modulated by cytokines secreted by the macrophage itself or by other cells [93-99]. *In vitro* cell culture analysis showed that NO was important in the inhibition of *Chlamydia* growth by macrophages. It was further revealed that suppression of the NO production via interference with the function of inducible nitric oxide synthase (iNOS) in cultured peritoneal macrophages enhanced *Chlamydia* survival [93, 94]. A study with iNOS KO mice also indicated that iNOS was necessary in the control of *Chlamydia* dissemination to the other organs in a genital tract infection model [95]. Along the same line, IFN-γ and TNF-α were also found to be required in macrophages to control chlamydial growth, probably through among others, the activation iNOS synthesis. While administration of rIFN-γ suppressed chlamydial growth [93, 96], neutralization of TNF-α was proposed to enhance the growth of *Chlamydia* [94, 97, 98]. Moreover, adoptive transferring of WT bone marrow-derived macrophages protected RAG-1(-/-)/IFN-γ(-/-) mice against chlamydial infection, while the transferring of macrophages from IFN-γ KO mice did not. In addition to killing *Chlamydia* directly, macrophages were found to be able to present chlamydial antigen to T cells [99]. Although macrophages are important for inhibiting chlamydial growth, *Chlamydia* have also developed a set of mechanisms to evade the destruction or even hijack macrophages.

For example, *C. trachomatis*-infected macrophages can secret TNF-α that can induce apoptosis of activated T-cells [100, 101], which may be a mechanism of persistent chlamydial infection. If infected *Chlamydia* can not be cleared, they may migrate with macrophages to other areas [102, 103].

2.5 NK, NKT cells and *Chlamydia* infection

NK cells are important in host resistance to viral [104, 105], intracellular bacterial (salmonella and listeria) [106, 107], and intracellular protozoan infections [108]. NK cells can directly lyse target cells when they are activated. NK cells express both activating and inhibitory receptors on their surface. The interaction of the receptors with the target cells and the subsequent transmitted signals will dictate the final outcome of NK cell activation. The main inhibitory signals are mediated through the interaction between NK cell inhibitory receptor (KIR) family molecules on the NK cell surface with the MHC molecules on the target cells. Chlamydial infection can degrade the host transcription factor regulatory factor X5 (RFX5) and upstream stimulatory factor-1 (USF-1), thus downregulate MHC class I and class II expression on the infected cells [109, 110]. Although this would help *Chlamydia* evade host adaptive immune response by decreasing pathogen antigen presentation to CD4+ and CD8+ T cell, it also increases the possibility to activate NK cells to lyse *Chlamydia* infected host cells [111].

During the early phase of chlamydial infection, NK cells are responsible for a surge in IFN-γ production. As such, depletion of NK cells will exacerbate the course of infection and skew the adaptive response towards that typical of a Th2 like response (*i.e.*, low IFN-γ, high IL-4, high IgG1) [112]. Recent data have documented that human NK cells express TLRs that can recognize PAMPs [113].

NKT cells contain both NK cell markers and the T cell receptor (TCR). Similar to typical CD4+ and CD8+ T cells, NKT cells develop in the thymus. They contain only a limited TCR repertoire, which recognize lipids and glycolipids presented by CD1- an atypical MHC class I-related molecule [114-116]. NKT cells play an important role in the regulation of both Th1 and Th2 immune responses. Moreover, NKT cells can modulate host resistance to many different pathogens including virus, bacteria and protozoan [114, 115, 117-119]. Recent studies with CD-1 KO mice and NKT KO mice showed that NKT cells enhanced *C. trachomatis* infection through increased IL-4, IgE, and decreased DTH responses [120]. Interestingly, in *C. pneumonia* infection model, it was found that CD1 and NKT KO mice were more susceptible to *C. pneumonia* infection. In line with these findings, KO mice showed significantly less IFN-γ production during *C. pneumoniae* infection [121]. The different roles of NKT cells in *C. trachomatis* and *C. pneumoniae* infection were correlated with the activation of different NKT subsets that produced IFN-γ and IL-4, respectively [122, 123]. The results indicated that the antigens from different pathogens may regulate the specific cytokine secretion by different NKT cell subtypes.

2.6 Cytokines and *Chlamydia* infection

2.6.1 IFN-γ and *Chlamydia* infection

IFN-γ is probably the most important cytokine in controlling chlamydial infection and also probably the most extensively studied cytokine in the research on *Chlamydia* [7, 61, 124-130]. Studies using genital tract or lung infection models with IFN-γ receptor KO mice clearly showed that IFN-γ was absolutely essential to the host resistance to chlamydial infection as well as to prevent dissemination of the organism to the other organs [61, 126, 131-133]. The lack of IFN-γ signalling results in delayed resolution of

genital tract infection, increased death post respiratory tract infection, and severe dissemination of organisms following the infection [61, 127, 128, 131]. IFN-γ has pleiotropic functions which can activate many immune responses. IFN-γ can activate NADPH to induce ROS; iNOS to produce nitric oxide; IDO - a tryptophan-decyclizing enzyme to deplete tryptophan, an essential amino acid for chlamydial growth; restrict ion supply; enhance MHC molecule expression and antigen presentation to promote adaptive immune responses.

Induction of iNOS, IDO and p47 GTPase are three important pathways that attract the attention of scientists in recent years. Although NO is an important bactericide, its function in chlamydial infection is controversial. Some studies with iNOS-deficient mice showed that KO mice resolve chlamydial infections as fast as those of the WT mice [95, 134, 135], while the other *in vivo* and *in vitro* studies indicated NO could inhibit the dissemination of chlamydial infection [95], and suppression of iNOS would promote chlamydial growth in cells [93, 94, 136, 137]. As stated above, IFN-γ-triggered IDO degrades intracellular tryptophan and inhibit chlamydial growth [129, 138, 139]. *In vivo* regulation of tryptophan by DC may influence the adaptive immune responses and resistance to chlamydial infection [140]. For a long time, it was observed that human strain *C. trachomatis* are more susceptible to IFN-γ compared to *C. muridarum*, especially in mouse genital tract infection models. Recently, comprehensive study using different human and mouse cell lines has revealed possible mechanisms underlying the innate cellular resistance to the infection with mouse and human *C. trachomatis* strains (MoPn and L2). It was found that the bactericides induced by IFN-γ were different in human and mouse cells and that while human cell lines could produce IDO but not NO,

the reverse was true for mouse cell lines. IDO inhibited the growth of both L2 and *C. muridarum*, while L2 was more greatly suppressed than *C. muridarum* in IFN-γ treated mouse cell lines [141]. These results indicated that *C. trachomatis* is adaptive to its natural host during evolution, and IDO is an important factor for human against chlamydial infection. Although IFN-γ induced NO was correlated with *C. muridarum* growth inhibition, blocking NO production could not rescue IFNγ-suppressed *C. muridarum* completely, suggesting that other factors/mechanisms were involved in the host immune response against *Chlamydia* [141]. Recently, it was revealed that IFN-γ gives rise to the expression of p47GTPase in murine epithelial cells, which may inhibit chlamydial growth [142]. p47GTPase, having a molecular weight of 47Kda, are intracellular membrane binding proteins, which help regulate trafficking and maturation of phagosomes that contain phagocytosed pathogens [143]. Probably for this reason, P47GTPases have been found to be critical to the host resistance against many intracellular bacteria infections [144-146]. Most recently, p47GTPase has been proposed to be able to modulate host susceptibility to chlamydial infection. Attenuated or abolished expression of specific members of p47GTPase resulted in an increased susceptibility to *C. trachomatis* [147].

2.6.2 IL-4 and *Chlamydia* infection

IL-4 is the characteristic Th2 cytokine secreted by NKT cells and Th2-like T cells. IL-4 can suppress T-bet triggered IFN-γ production and Th1 like responses through Stat6 signalling pathway [148, 149], and induce Th2 like responses. Studies indicated that increased IL-4 production was correlated with an increased susceptibility to chlamydial infections [120, 150] or with a persistent chlamydial infection, which could potentially

lead to pathological responses and trachoma formation [151]. It remains unclear whether IL-4 has a direct influence on *Chlamydia* growth or replication in host cells.

2.6.3 IL-10 and chlamydial infection

IL-10 was originally recognized as a Th2 cytokine but found later as an anti-inflammatory cytokine secreted by T cells and macrophages. IL-10 can suppress both Th1 and Th2 responses by inhibiting antigen presentation and inflammatory cytokine secretion. For this reason, IL-10 is associated with suppressed Th1 responses and increased susceptibility to chlamydial infection. For example, BALB/c mice were observed to be more susceptible to *C. muridarum* infection compared to C57BL6 mice and this was accompanied by higher production of organism-specific IL-10 [152]. IL-10 KO mice were also found to be more resistant to *C. muridarum* infection compared to WT mice. In those IL-10 KO mice which did develop the infection, they typically endured a shorter duration of disease, less organism growth and less server pathological changes including granulomas and fibrosis formation [153]. It is postulated that the mechanisms for the enhanced resistance of IL-10 KO mice could probably be due to the fact that antigen presenting cells (APC) from the IL-10 KO mice are more potent in presenting antigens to T cells and illicit Th1 responses. In contrast, increased production of IL-10 is also associated with persistent infection and followed by chronic inflammation and scar formation (trachoma) [154].

2.6.4 Inflammatory cytokines and *Chlamydia* infection

Infection of epithelial cells can quickly lead to large secretion of inflammatory cytokines such as IL-1, IL-6, IL-12, IL-8, TNF-α [75, 76, 155, 156]. These cytokines promote host immune responses to inhibit *Chlamydia* growth, through attracting and

activating neutrophils, monocytes and T cells [155, 157]. For example, deficiency of IL-6 cytokines enhanced host susceptibility to chlamydial infection [158], while IL-1 would potentiate the function of IFN-γ in inhibiting *Chlamydia* growth [159]. Further, lymphotoxin can suppress *C. pneumoniae* growth *in vitro* [160, 161]. However, this response is a double-edged sword as these very same proinflammatory cytokines can also cause significant tissue damage and pathological changes, which may be correlated with the clinically evident chlamydial infection-induced scarring trachoma, infertility and atherosclerosis [150, 155, 156]. Whether cytokine production will result in protection or tissue damage to the host essentially relies on the interactions between the host and pathogen. If the invading organism can not be cleared swiftly and completely and the infection persists, secretion of a large amount of inflammatory cytokines followed by infiltration of the immune cells may inevitably lead to tissue damage, fibrosis and scar formation.

2.6.5 Dendritic cells and chlamydial infection

Dendritic cells are the most important APC that phagocytose, process and present antigen to MHC-restricted T cells to illicit adaptive immunity. During chlamydial infection, local resident DC will be activated through a direct interaction of their surface or cytosol PRRs with chlamydial PAMPs, or through cytokine receptor-ligand binding via autocrine and paracrine fashion. In consequence, activated immature DC phagocytose *Chlamydia*, express CCR7 and some other chemokine receptors and adhesion molecules and migrate to local draining lymph nodes, where maturation of DC renders them the ability to present processed antigens to T cells through MHC-antigen-TCR interaction. With the help of co-stimulatory factors and cytokines such as IL-12, T cells and B cells

will be activated and proliferate, eventually migrate back to the infected tissue where secretion of cytokines and antibodies ensues. It was found that during chlamydial genital tract infection, MHC class II-positive APC infiltrated to infected organs of resistant strain (BALB/c) mice but not of susceptible strain (C3H) mice, which may be associated with protection against infection [162]. Infection or immunized with *Chlamydia* can induce a release of local granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-12 as well as trigger DC maturation [163]. How would DC participate in the immune responses to chlamydial infection? Studies on *in vitro* culturing of DC with *Chlamydia* have provided some clues. One study suggested that live *Chlamydia* were internalized by DC through nonspecific macropinocytosis because heparin, a reagent reported to inhibit *Chlamydia* binding to cell, failed to suppress the internalization of *Chlamydia* by DC [164]. In contrast, in another study with *C. trachomatis* L2, it was found that L2 entered DC through heparin-inhibited pathway but not by macropinocytosis [165]. In both cases, *Chlamydia* showed a similar fate after entering DC. Chlamydial inclusions in DC will not develop normally like they would be in epithelial cells. Rather, the pathogen fuses with lysosome and subsequently degraded and presented to both CD4+ or CD8+ T cells to initiate the development of adaptive immune responses [164, 165]. At the same time, the pulsed DC will express a complex profile of immuno-modulatory molecules including CCR7, IL-12, IP-10, for their homing and stimulation of the protective immune response [166]. It was found that activated DC upregulated MHC, CD40, CD80 and CD86, and secreted a large amount of IL-12 and TNF-α. Further, DC activation (*e.g.,* secretion of cytokines, expression of surface activation markers and co-stimulatory molecules) was both TLR2- and TLR4-dependent [79]. These activated DC were able to initiate host

immune responses and rendered the host resistant to pathogen challenge. When bone marrow-derived DC were pulsed with killed *Chlamydia*, they were not only able to secret IL-12 and activate *Chlamydia*-specific CD4+ T cells *in vitro*, but also protect mice against chlamydial genital tract challenge after being adoptively transferred to naïve mice [167]. Immunized mice displayed a typical Th1 like response with strong IFN-γ and weak IL-4 production. The Th1-like response was IL-12-dependent because IL-12p40 KO DC would not induce IFN-γ production and strong protection [168]. As mentioned before, depletion of IL-10 could enhance host immune responses to chlamydial infection [153, 169]. *Chlamydia*-pulsed IL-10 KO APC or antisense IL-10 oligonucleotide-treated WT APC acquired capability to promote anti-*Chlamydia* infection upon adoptive transfer [169]. Further, pulsed IL-10 deficient DC were found to express higher levels of CD11c, CD40, co-stimulatory molecules and IL-12, which collectively enhanced their ability to activate T cells [170]. A recent study comparing live and UV-killed *C. muridarum* as antigen found that live *C. muridarum* induced stronger DC activation as determined by higher expression of CD40, CD86 as well as higher levels of IL-12 and TNF-α compared to UV-killed MoPn [171]. After adoptively transferred into mice, live *C. muridarum* - pulsed DC exhibited better protection against chlamydial infection than UV-killed organism. When DC were co-cultured with UV-EBs plus CpG, they displayed stronger protection compared to those cultured with only UV-EBs suggesting that TLR may be important in activating DC functions [171]. Further, chlamydial proteins were also used as antigens to stimulate DC for vaccination study. DC pulsed with recombinant chlamydial MOMP will stimulate CD4+ T cell proliferation, but the T cells induced Th2-like immune responses after adoptively transferred to mice, possibly owing to lack of the

stimulatory signals from TLR [172]. Human monocyte-derived DC pulsed with chlamydial hsp60 promote a Th1-type immune response through IL-12/IL-23 production *in vitro* [173]. Recently, it was documented that different subtypes of DC showed different abilities to initiate protective Th1-like immune response to chlamydial infection. In an adoptive transfer experiment, CD8α+ DC isolated from mice which had been infected with *C. muridarum* were found to be more effective in protecting the host against *C. muridarum* infection compared to CD8α- DC highlighting the importance of CD8α+ DC in host resistance to chlamydial infection.[174].

2.7 Adaptive immune responses

Innate immunity is important but it may not be sufficient to control the invasion of *Chlamydia*. In fact, it has been reported that severe combined immunodeficiency (SCID) mice, which lack adaptive immunity, display a severe course of disease after chlamydial infection as shown by significant organism growth and high mortality [175-177]. In this regard, adaptive immunity, especially Th1-like DTH response, is essential to the resolution of infection.

2.7.1 MHC and *Chlamydia* infection

Before an adaptive immune response can be mounted, proteins from pathogens either extracellularly or from inside an infected cell, have to be broken down into short peptides, displayed on the cell surface of APC or target cells, in the context of MHC molecules and recognized by T cells. In addition to the classical MHC molecules (*i.e.*, class I and class II), there are also non-classical MHC molecules such as CD1, which may involved in antigen processing and NKT cell activation. MHC class I, expressed on all nucleated cells, delivers a 8 to 10 amino acid long peptide from the cytosol to cell

surface, where the peptide-MHC I complex is recognized by CD8+ T cells [178]. Extracellular antigens processed intracellularly to antigenic peptides (13-20 amino acids in length) are displayed in the context of MHC II molecules on the surface of APC and recognized by CD4+ T cells [179].

MHC gene is polygenic, and highly polymorphic, the sequence of each type of MHC gene may be different in each individual. As such, different MHC exhibit variable binding affinity to different antigenic peptides, and thus in turn contributes to inherent variations among individuals in the recognition of T cells to the particular antigenic peptide. Because of the crucial role of MHC in T cell responses, one can predicate the importance of MHC polymorphism in host-pathogen interactions. In a study with B10 H2 congenic mice, B10.M (H-2f) and B10.S (H-2s) were observed to be more resistant to *Leishmania amazonesis* induced cutaneous lesions [180]. The B10 congenic mice with different MHC genes also demonstrated different degree of resistance to the infection of *Cryptococcus neoformans* [181]. In addition to its role in the initiation of T cell response, MHC polymorphism were also reported to influence the antibody responses [182, 183]. For example, different MHC congenic B10 mice showed variable antigen-specific IgG antibody responses after immunization with influenza virus hemagglutinin [182].

Some studies have reported the relationship between different MHC genes and the susceptibility to chlamydial infection. Using MHC gene KO, Morrison *et al* demonstrated that MHC class II- restricted response was important in the host defense against *C. trachomatis* genital tract infection and that MHC class $II^{-/-}$ mice failed to resolve chlamydial genital infection, producing low titers of IgG2a and failing to mount positive DTH responses [184]. On the other hand, certain MHC class genes are involved in *C.*

trachomatis-induced immunopathologic responses. Based on a molecular epidemiological investigation, Kimani *et al* found that women expressing a specific HLA type I molecule, HLA-A31 is a risk factor for *C. trachomatis* pelvic inflammatory disease [185]. HLA-B27-restricted peptides with possible relevance to HLA-B27-associated diseases have been identified from *C. trachomatis* proteome [186].

2.7.2 Cell-mediated immunity (CMI) and *Chlamdyia* infection

Many different mouse models have been utilized to study the role of lymphocytes in chlamydial infection including specific lymphocyte deficient mice [176, 184, 187, 188], adoptive transfer of functional lymphocytes or T cell clones to naïve mice [175, 189-191], and depletion of specific subpopulation of lymphocytes *in vivo* with antibodies [192-194].

A study using nude mice (*i.e.*, deficient of T cells) in a genital tract infection model revealed the importance of CMI in chlamydial infection [195]. A subsequent investigation revealed that the resistance was attributed to the presence of αβ T cells since αβ T cell deficient mice displayed the same susceptibility to chlamydial infection as RAG-/- mice, which lack both T and B cells, while γδ T cells appeared to have no direct in governing host resistance to infection [196].

Evidence is available to support the notion that IFN-γ may be one of the most efficient factors secreted by T cells after infection, that both CD4+ T cell and CD8+ T cells participate in the protection against chlamydial infection, and that CD4+ T cells may be more important than CD8+ T cells [187]. Specifically, depletion of CD4+ T cells rendered mice susceptible to infection, while adoptive transfer of CD4+ T cells from previously chlamydial infected mice showed protection to naïve mice [190]. Transferring of CD4+ Th2 clone could not protect mice against infection [197]. Similarly, a lack of

IL-12, IFN-γ or IFN-γ receptor rendered hosts susceptible to chlamydial primary infection [128, 133]. Taken together, these studies highlighted the importance of CD4+ T cells in chlamydial infection.

During infection, CD8+ T cells also secret IFN-γ which may inhibit *Chlamydia* growth. But as mentioned above, the importance of CD8+ T cells may not be as great as that of CD4+ T cells. Although CD8+ T cells can lyse *Chlamydia* infected cells *in vitro* [198, 199], cytotoxicity may not be a major effect of CD8+ T cells in chlamydial infection. For example, in mice deficient of perforin, Fas or Fas ligand, or of both perforin and Fas, although CD8+ T cell-dependent killing activity was compromised, their resistance to chlamydial infection was comparable to that of WT mice [200]. Further, in genital infection models, β_2 microglobulin gene KO mice (*i.e.*, CD8+ T cell deficient) cleared the infection as fast as WT mice [184]. In contrast, in lung infection models, absence of CD8+ T cells rendered mice highly susceptible to chlamydial infection as evidenced by organism growth and increased mortality, although such increase was not as pronounced as that seen in CD4+ T cell deficient mice [187]. Because CD8+ T cells are activated earlier than CD4+ T cells, they may be more important at an early stage of infection via secretion of IFN-γ and initiation of Th1-like responses [177]. In this instance, the role of IFN-γ is supported by the finding that adoptive transferring of IFNγ-producing CD8+ T cell but not of IFNγ-deficient CD8+ T cells showed protection [191].

2.7.3 B cells and antibody responses in *Chlamydia* infection

B cells are antibody-producing cells as well as APC in recall immunity. The function of B cells during chlamydial infection has been studied for about twenty years. In genital tract infection models, μMT gene KO mice (B cells deficient) were as resistant

as WT mice to chlamydial infection [201]. However, following recovery from a primary infection, B cell-deficient mice showed stronger susceptibility to secondary infection than WT mice, as shown by more *Chlamydia* growth and delayed clearance of the pathogen suggesting that humoral immunity was more important in secondary infection than in primary infection [202, 203]. In contrast, in the lung infection model, B cell deficiency resulted in stronger susceptibiliy to chlamyidal primary as well as secondary infections [188]. After lung infection with *Chlamydia*, B cell-deficient mice not only lacked the presence of antibodies, but also failed to mount a typical DTH response and induce Th1 cytokine production. The observation indicates that B cells are critical to the development of humoral immunity and antigen presentation for CMI responses, and lung infection models maybe more sensitive compared to genital tract infection models [188].

The role of antibodies in chlamydial infection has been studied for over 30 years but is still not completely elucidated. A study of antibody function is appealing since its role in neutralization, antibody-induced cell cytotoxicity (ADCC), activation of complement, and enhancing antigen presentation through the FcR on APC [204, 205], all of which are important features in the host resistance to chlamydial infection. Early studies with human and monkeys suggested an association between locally secreted *Chlamydia*-specific antibodies and protection [206-208]. For example, ocular antibodies from *Chlamydia*-infected children can neutralize the infectivity of the organism [209]. Later, it was found that locally secreted IgA (sIgA) was strongly inversely correlated with the shedding of organisms from human endocevix [210], *i.e.*, female patients with highest levels of sIgA harboring lowest numbers of organisms recovered [210]. Early studies with guinea pig models showed that chlamydial infection induced strong humoral

immune responses against several chlamydial antigens [211]. Guinea pigs with suppressed humoral immune response by cyclophosphamide treatment but with intact CMI showed increased susceptibility to chlamydial infection [212], indicating that humoral immunity was important in acquiring the ability to resist *Chlamydia* re-challenge.

In addition, Sukumar *et al.* [213] developed two IgA monoclonal antibodies (mAbs) which could inhibit *Chlamydia*-induced infertility when the organisms were preincubated with the mAbs before infection. *In vivo* protection was also shown by less organism shedding and shorter duration of disease after passive transfer of the mAbs to naïve mice [213]. Using IgA-/- mice, Murthy *et al.* found KO mice exhibited more severe pathological changes after infection, suggesting a regulatory function of IgA during chlamdyial infection and maintaining mucosal homeostasis although it was not absolutely required for primary defense against pulmonary chlamydial infection [214]. Further, it was demonstrated that IgA was not necessary for the resolution of secondary infection in the genital tract infection mouse model [215].

In the mouse genital infection model, it has been reported that infection of mice through different routes (intravaginal, intranasal, subcutaneous, or oral) showed different degree of protection against re-challenge [216]. Specifically, mice immunized intranasally developed the strongest resistance against chlamydial re-infection, which was correlated with approximately 6-fold higher in the level of IgG2a in the genital tract compared to those infected by other routes, although there was no difference in IgA titers and mucosal CMI between the groups [216].

The availability of Fc receptor KO mice provides another useful tool to assess the role of antibodies in chlamydial infection since many important functions of antibodies

including ADCC and enhancing antigen presentation are mediated through the Fc receptors. Similar to IgA-deficient mice, FcR-/- mice possessed the same resistance as WT mice to chlamydial infection. However, the FcR-/- mice cleared the organisms more slowly than did the FcR+/+ mice which was correlated with suppressed macrophage killing of *Chlamydia*, and removing of *Chlamydia*-infected epithelial cells by ADCC [217]. Moreover, the ability of APC from the FcR-/- mice to uptake and present antigens to T cells is compromised in the presence of anti-*Chlamydia* antibodies [217]. It was later found that it is the IgG2a and IgA which mediated the enhanced Th1 responses by antibodies [218]. Taken together, the induction of mucosal antibodies (IgA and IgG) could be critical because these antibodies are the first line of barriers which can neutralize and block the invasion of *Chlamydia*, thus preventing development of downstream inflammatory responses and in effect, avoiding future complications of such responses [219, 220].

Overall, these data indicate that humoral immunity is strongly mounted after *Chlamydia* infection and antibodies can neutralize organisms, but the particular roles of antibody response in host defense against chlamydial infection remain controversial. In general, antibody responses may not be as important as CMI or IFN-γ in the protection against primary or secondary infection. However, antibodies may contribute to the optimal protection against the chlamydial infection.

2.8 Genetically determined resistance to *Chlamydia* infection

As mentioned above, many immune factors are involved in host resistance against chlamydial infection. Genetic determinants which control the expression of the immune factors can potentially regulate host immune responses to chlamydial infection. In human,

it has been observed that single-nucleotide polymorphisms (SNPs) of certain cytokines have been associated with chronicity in chlamydial infection or repeated exposure to the pathogen. For example, IL-10 polymorphism was correlated with alteration in IL-10 production, lymphocyte proliferation and IFN-γ responses, which may regulate host cell-mediated immune resistance to *C. trachomatis* [221]. Further, it has also been suggested that polymorphisms within the HLA and IL-10 gene was correlated with recurrent *C. trachomatis* infection [222]. Matrix metalloproteinase 9 (MMP9) gene polymorphism was found to be related to ocular *C. trachomatis*- induced scar formation [223]. In mice, different strains of inbred mice show different susceptibility to chlamydial lung or genital tract infection. C57BL/6 mice were more resistant to chlamydial lung infection than BALB/c mice, owing to different Th1/Th2 cytokine productions. Compared to BALB/c mice, C57BL/6 mice produced more IFN-γ and less IL-10 as well as more IgG2a and less IgG1, and showed stronger DTH responses [152]. C57BL/6 mice were more resistant to genital tract infection with *Chlamydia* compared to C3H mice, which produced nearly comparable levels of IFN-γ. It was suggested C3H produced less TNF-α [224], but the finding was not reproducible in the lung infection model, and TNF-α blocked mice showed similar resistance to chlamydial infection [225]. Studies on the susceptibility of different strains of mice to chlamydial infection may provide an insight on resistance-determining factors.

3. *Chlamydia* Vaccine Development

As mentioned above, *C. trachomatis* is an important human pathogen because of its broad transmission, severe clinical complications and the cost for treatment. To

control or prevent *C. trachomatis* infection efficiently, implementation of procedures combining vaccination, early diagnosis, antibiotic treatment are necessary. Because most chlamydial infections are asymptomatic [226], new diagnosis is often missed even with the development of sophisticated diagnosic tools such as polymerase chain reaction (PCR). In consequence, a large number of patients will not be treated until symptoms are manifested. Although antibiotic treatment is generally efficient, emergence of mutations to the WT *C. trachomatis* genomic sequence would often lead to resistance to treatment [227] or persistence of the infection [220]. In this regard, a prophylaxic vaccine would be the most cost-effective approach to prevent new infections.

For several decades, scientists have tried many ways to develop a safe and effective human chlamydial vaccine, but with limited success. Earliest attempts using inactivated whole organisms (*i.e.*, EBs) as antigens showed disappointing results. Although a study from one field trial in India showed that the vaccine exhibited short-term protection [228, 229], another trial in Gambia documented that the vaccine not only gave little protection, but also led to a more serious ocular infection in many vaccinated volunteers than controls [230, 231]. Similar results were observed in monkey models [232]. Later investigations suggested that some of the chlamydial components such as hsp60 were involved in the immunopathological responses and induced hypersensitivity responses to the organisms [29, 233, 234]. In consequence, subsequent vaccine studies focused on the use of subcomponents of *Chlamydia*. Among others, chlamydial MOMP, the major surface component of the bacteria, is by far the best understood candidate antigen and has long been a target of various vaccine studies [229]. Purified and re-folded MOMP were found to induce strong protection [220, 235, 236], while recombinant

MOMP (rMOMP) could only induce partial protection [29]. A possible reason for the less protection of rMOMP could be due to a conformational change of rMOMP compared to its native form displayed on the cell surface, or to a lack of an effective adjuvant [220, 236]. Recent DNA vaccination studies using MOMP along with cholera holotoxin and CpG ODN as adjuvants have demonstrated increased protection against *C. trachomatis* infection [219, 220, 237]. Further studies with other antigens including chlamydial type III secretion system structural proteins and chlamydial protease/proteasome-like activate factor (CPAF) are in progress and may prove to be promising candidate vaccines in the near future [238, 239].

For chlamydial vaccine development, several specific biological characteristics of *C. trachomatis* have to be considered for choosing the antigen. As previously discussed, since protective immunity to chlamydial infection is a cell-mediated Th1 response, an effective vaccine needs to stimulate vigorous but desirable this kind of immune response. Because *C. trachomatis* mainly infects host mucosal cells, the induction of mucosal immunity, especially the production of local mucosal antibodies (sIgA), is also an important feature of a good vaccine [29]. Vaccines using live, attenuated organisms, particularly the whole organism, have been shown to induce strongest protection [163, 227, 240, 241] as they would elicit immune responses to many different antigens of the pathogen such that mutations of several antigens within the pathogen may not effectively exert a negative influence on the effectiveness of the vaccine. Having said that, owing to the presence of potential immune pathological responses induced by the whole organism, these vaccines may not be practical. Because of the specific development cycle of *C. trachomatis*, RBs are always inside inclusions and to a great degree, avoid the

surveillance of the host immune system. The use of RBs as a vaccine was proven to be not protective to mice compared to that with EBs [242]. Thus, the subunit vaccines based on EB components or secreted molecules of RBs, which can be processed and presented to T cells, may be more efficacious to the host. In this regard, the subcomponents from EBs may give rise to the production of mucosal neutralizing antibody, which can prevent *C. trachomatis* entry to host epithelial cells. In consequence, this would help block a release of proinflammatory cytokines and avoid inflammation, which is often associated with development of trachoma and infertility [155, 156]. Recently, the sequencing of the whole genome of *C. trachomatis* serovar D and technical advances in the area of proteomics has paved a new approach to the development of chlamydial vaccines. The *C. trachomatis* genome encodes about 900 proteins, and through analysis of the expression and the location of chlamydial proteins at specific time, scientists can rationally determine a candidate vaccine which may elicit protective immunity [229]. Further, based on the sequence of specific antigens of different serovars, it is possible to elucidate a general vaccine which can provide sterilizing protection against infections with many different serovars.

In addition to choice of antigen, development of the adjuvant and delivery systems are also important to elicit strong immune responses. New adjuvant such as immunostimulating complexes (ISCOM), CpG and cholera toxin were proven to be efficient in stimulating immune responses especially mucosal immunity [219, 220, 243-245]. Cytokines such as IL-12 and GM-CSF have also been used as adjuvant in chlamyidal vaccine study because of their role in activation of DC [239, 246]. Probably, adjuvant which induces balanced Th1 and mucosal immune responses when

administrated intramuscularly may be an ideal standard for a good chlamydial vaccine in the future.

4. Animal Models of chlamydial infection

Animal models are very useful for studies on host-*Chlamydia* interactions, pathological changes, immune responses, vaccine development, and anti-*Chlamydia* chemotherapy. Many animal models have been utilized to study the chlamydial infection from different angles. The available animal models include primates such as monkeys, guinea pigs, mice, rats and pigs. While monkeys are genetically closer to humans and the pathological and physiological changes are most resemble to human chlamydial disease, the source is limited. For trachoma study, the developed animal models include monkeys and guinea pigs. Non-human primate (Macaque) and pigs were normally used to study genital and/or rectal infections and test topical microbicide function [247]. Rat and guinea pig are used to study genital tract infection and *Chlamydia*-induced arthritis. Among all animal models, mice are the most widely used model in chlamydial research because of their wide-spread availability, including that of transgenic mice. As such, majority of the scientific advances made in regard to mechanisms pertinent to host immune responses to chlamydial infection comes from studies using mouse models. Mouse models for chlamydial infection include genital tract infection, respiratory tract infection and intraperitoneal route [7, 88, 248]. The genital tract and respiratory tract infection models are the most widely used.

Genital tract infection of female mice has been reported to closely mimic acute infection, pathological responses, and disease outcome in human (*e.g.*, infertility) [7]. In

the genital tract infection model, mice or rats need to be pre-treated with progesterone to successfully establish infection. It has been reported that progesterone could not only affect the susceptibility to chlamydial infection, but also enhanced the susceptibility of mice to some other pathogens such as herpes virus and simian immunodeficiency virus [249, 250]. There is a possibility that the administration of progesterone may affect host immune responses to chlamydial infection.

Respiratory tract infection model is also widely used and has provided useful information about the host immune factors that regulate susceptibility to chlamydial infections. One advantage of this model is that it avoids possible artificial influence on the immune system caused by administration of progesterone. In this article, all the studies were performed by using respiratory tract infected mouse model.

5. Type I interferons (IFNIs)

IFNIs are the first group of cytokines discovered (1957) and named after their special function as "interfering" with virus replication [251, 252]. Quickly produced by cells of the innate immunity in response to pathogen invasion, IFNIs are known to play pleiotropic roles in regulating host immune responses, mediating inflammation, and inducing or inhibiting autoimmune diseases [253, 254].

5.1 Biology of type I interferons

IFNIs belong to the α-helical cytokine class II family, which also includes IL-10 and IL-10 homology cytokines such as IL-19 and IL-20 [254-256]. As a multiple member family, human IFNIs contain 12 IFN-α subtypes, 1 IFN-β, 1 IFN-ω, and some other members including IFN–ε and -κ. IFN-ξ, IFN-δ and IFN-τ were found in non-human

animals such as mice and pigs [254, 257]. It is not clear why there are so many subtypes of IFN-α, but all of them are capable of inhibiting viral infectivity and replication as well as cell proliferation [258, 259]. Although sharing some degree of similarity in sequence, structural differences of IFNIs were discovered and found critical to their interaction with interferon receptors, which may influence the biological activity of different IFNIs [252, 260-262]. Recently, new members of the type I IFN family known as IFN-λ1, -λ2 and -λ3, or IL-28A, IL-28B and IL-29 were discovered [263, 264]. INF-λ exerts its effects on host cells through receptors which are different from the traditional type I IFN receptors used by the other IFNIs. But similar to the other IFNIs, INF-λ can activate STAT2 and suppress viral infection. [265]. Except IFN-λ, almost all IFNIs act as monomer on the target cells through the same receptor called the IFNα/β receptor (IFNAR) and signal through the Jak-Stat pathway [266, 267]. The IFNAR complex is composed of a heterodimer, IFNAR1 and IFNAR2, which are expressed ubiquitously in all cells. Both subunits are necessary for initiation of signalling events triggered by IFNIs. Similar to the other enzyme-linked cell surface receptors, the high affinity binding between IFNIs and IFNAR leads to receptor dimerization and phosphorylation of the associated kinases Tyk2 and Jak1, followed by phosphorylation of the IFNAR2, and associated STAT2 and STAT1. Phosphorylated STAT1/STAT2 dimers will be released and interact with IFN regulatory factor 9 (IRF9) to form IFN-stimulated gene factor 3 (ISGF3), a trimeric complex that is translocated to the nucleus and binds to IFN-stimulated response elements (ISRE) - the regulator consensus sequences of IFN-α/β inducible genes initiating the transcription of IFNI activated genes [252, 254, 268]. Further, STAT-3, -4 and -5 have also been reported to participate in the IFNIs-mediated signaling pathway. T-

bet, an important transcription factor promoting Th1 response is expressed in IFN-α treated T cells through activation of STAT1 and STAT 3, which in turn, upregulates expression of surface IL-12 receptor β2. Increased IL-12R promotes naïve T cells to differentiate into IFN-γ producing Th1 cells [269]. IFN-α is also able to induce IFN-γ production through STAT4, which is observed in host responses to bacterial, protozoan and viral infections [270-272].

5.2 Production of type I interferons

Under the stimulation of mitogen or pathogens, many cell types, which include monocyte/macrophage, T cell, NK cell, neutrophil and different DC subtypes can produce type I IFN (IFNI) [273-275]. Among all of them, plasmacytoid DC (pDC), previously recognized as a natural IFN-producing cell (NIPC), remains the most potent source of IFNI producing 1000-fold higher in the levels of IFNIs than any other cell types stimulated under the same condition [274, 276-279]. Unlike other DC, immature pDC contain less dendrits compared to typical DC. pDCs can induce tolerance but not activation [280]. TLR expression is also different between common DC and pDC in that the former express TLR3, TLR8, TLR2 and TLR4, while the latter are strongly positive for TLR7 and TLR9 present in their intracellular endosome to sense the single-stranded RNA and unmethylated DNA from pathogens. Upon activated by IL-3, CD40L, unmethylated CpG-DNA, or viruses, pDC undergoes maturation and gains function and morphology of common DC expressing high-level surface MHC and co-stimulatory molecules [281, 282].

5.3 Mechanisms for type I interferon induction

As mentioned before, mammalian hosts depend on PRR to sense the presence of pathogens and subsequently, activate the immune system. Because of the importance of type I IFN in host resistance to viral infection, mammalians have evolved a set of sensors and redundant pathways to induce quickly production of IFNIs during infection or upon activation. Two classes of receptors, which differ in their localization and cognate ligands, have been characterized. The two different localizations of these receptors ensure both infected cells and neighboring uninfected cells sense the products of pathogens and initiate immune resistance status, with the latter one being important as some pathogens may produce factors, which can block the production of IFNIs.

5.3.1 Induction of type I Interferons by Toll-like receptor signaling

As alluded to earlier, TLRs are a group of membrane-bound proteins with leucine- rich repeat domains with each member of the superfamily recognizing a specific PAMP [283]. For example, TLR3 binds to dsRNA, TLR4 to lipopolysaccharide (LPS), TLR7 and TLR8 to single-strand RNA, and TLR9 to unmethylated CpG DNA. Since expressed on the cell surface, TLR4 can detect extracellular bacteria directly. However, where TLR3, TLR7, TLR8 and TLR 9 normally localize in cell endosome, these TLRs can not readily interact with extracellular pathogens directly. Rather, the pathogen is phagocytosed and processed in the endosome, where the products recognized by the specific TLR. Binding of the TLR to its ligand initiates specific downstream signaling cascades leading to activation of molecules required to elicit a targeted immune response. For example, signal transmission resulting from the dsRNA- TLR3 interaction requires an adaptor molecule known as Toll/interleukin-1 receptor domain-containing adapter protein (TRIF) and the kinase TBK1, which phosphorylates IFN regulatory factor 3

(IRF3). Subsequent translocation of the activated IRF3 dimer to the nucleus initiates transcription of the IFNI genes. Along the same line, through activation of NF-κB and AP-1, TRIF can also activate I*k*B kinase and induce IFNI production [284]. Similarly, in a cooperation with TRIF-related adaptor molecule (TRAM), TRIF also helps activate IRF3 following binding of TLR4 to LPS and CD14 [284].

In contrast, the production of IFNIs triggered by binding of TLR-7, -8 and -9 to their respective ligands is thought to be initiated by the activation of IRF7, a transcription factor having a similar function as IFR3. The signalling pathway initiated by IRF7 depends on the presence of MyD88, IRAK1, 4 and TRAF6, which lead to the activation of MAPK cascades. Interestingly, IRF7 and TLR-7, -8 and -9 are found constitutively expressed in pDC, which may be correlated with high levels of IFNI usually produced by pDC. In the case of other cell types which weakly express TLR-7, -9 and IRF7, if at all, activation of IRF3 is first required to initiate production of low-level IFN-β and IFN-α4, which upregulates transcription of the IRF7 gene through IFNAR signals (ISGF3). Subsequent activation of IRF7 allows for "amplification" synthesis of all types of IFNIs [285].

5.3.2 Induction of IFNIs through the cytoplasmic pathway

During virus infection, two RNA helicases retinoic acid-inducible gene I (RIG I) and melanoma differentiation associated protein-5 (MDA-5) have been observed to be responsible for sensing the presence of cytoplasmic dsRNA and production of IFNIs [284, 286]. Further, it has been reported that intracellular bacteria can also induce activation of the TBK1-IRF3 pathway and secretion of IFNIs [71].

5.4 Functions of type I interferons

5.4.1 Cell activation

IFNIs can activate macrophage to express iNOS, which catalyzes the production of NO production - an important antimicrobial molecule. It has been reported that the iNOS induction by IFNIs requires artificial stimulation with LPS or a true bacterial infection [287]. In contrast, others have documented that IFNIs is necessary for iNOS induction by LPS or virus [288, 289]. However, it should be mentioned that new findings with *Chlamydia pneumonia*-infected bone marrow derived macrophage (BMDM) have disputed this notion in suggesting that IFNγ, but not IFNα, is responsible for the production of iNOS by *C. pneumonia*-infected cells [290]. When pretreated with IFN-α or IFN-β, killing of tumor cells by macrophages via ADCC has been observed to be greatly enhanced [291]. IFNIs also activate NK cells to promote their cytotoxic activity, which is important in controlling viral infection and tumor immunity [292, 293]. Although expression of granzymes A, B and perforin, which all have a role in promoting cell death, is not increased, NK cell capability to lyse target cells was significantly higher in WT mice relative those in IFNAR-/- mice or STAT-/- mice [294, 295]. Interestingly, evidence also suggests that IFNIs can downregulate the expression of NKG2D ligand H60 on tumor cells, which may also activate the lytic function of NK cells [296].

In addition, IFNIs can efficiently stimulate the expression of proinflammatory and/or anti-inflammatory cytokines including IL-1, -2, -6, -10, TNF-α and chemokines [297]. Poly I:C, which stimulates production of IFN-α, and in turn induces a release of high mobility group protein 1 (HMGB1), which exerts inflammatory cytokine-like activity [298]. Along the same line, IFN-β was found necessary for synthesis of IL-6,

chemokine CCL5 and CXCL10 by murine and human alveolar macrophages following binding of TLR2 to lipopeptides [299].

Interestingly, although IFN-α/β has been shown to partially suppress expression of anti-inflammatory cytokine IL-10, thus, aggravate the inflammation [300], IFN-α, in certain instances, can reverse the prototype anti-inflammatory function of IL-10 such that the latter can give rise to expression of STAT1-dependent genes including IRF-1, IP-10 and other chemokines [301]. Further, IFNIs have been reported to induce IL-15, which is necessary for NK cell activation and survival [295] and enhance expression of IL-21 and its receptor by T cells [302].

With respect to a role of IFNIs as anti-inflammatory modulators, it is thought that IFN-β induces SOCS-1 expression which leads to suppression of STAT-1 activation and CD40 expression [303]. By attenuating TNF-α production, and IFNIs might inhibit infiltration of T cells or macrophages [304], which would be beneficial in the treatment of autoimmune and inflammatory diseases [305].

In addition to their role in controlling innate immune response, IFNIs are also equally important in the control of adaptive immune response [306, 307]. First, IFNIs have been shown to induce differentiation of monocytes into DC, which are the most important professional APC [308]. Second, it was found that during immunization with chicken gamma globulin (CGG), IFN-α/β exerted a strong adjuvant function by enhancing antibody secretion in primary as well as secondary immune responses. Especially, the adjuvant function of complete Freund's adjuvant (CFA) is largely dependent on the endogenous production of type I IFNs post administration [309]. Third, IFNIs can modulate immature DCs to express MHC molecules and co-stimulatory

molecules including CD80, CD86 and CD40; secret cytokines and chemokines; home to secondary lymphoid organs and prime the activation of antigen-specific T cells [310-313]. In fact, type I IFN receptor deficient DCs have a suppressed ability to stimulate naïve T cell proliferation compared to normal DCs [313]. Along the same line, IFNI signals activate the maturation of DCs to cross-prime CD8+ T cells with viral antigens, a potentially important mechanism in the host resistance to viral infection [314].

In addition to its role in activating APC, type I IFNs are also able to activate B and T lymphocytes [316, 317]. In fact, both cross priming of T cells as well as antibody production (and isotype switching) by B cells are enhanced by the presence of T cells [315]. Further, IFNIs are also needed to prolong proliferation and longevity of activated T cells either directly or through secretion of IL-2, IL-15 by DC and NK cells as well as up-regulation of the corresponding receptors on antigen- specific T cells [316-318].

5.4.2 The roles of type I interferons in infections

5.4.2.1 Viral infections

Many *in vitro* and *in vivo* studies have demonstrated an absolute requirement of IFNIs in the resistance to viral infections [285, 319]. The basic mechanism of resistance is that IFNIs activate host cells to produce factors important in the suppression of viral replication and enhance adaptive immune response to viral infection. Further, IFNIs have also been attributed to apoptosis of virus-infected cells. It has been established that binding of IFNIs to their receptor initiates activation of signaling cascades leading to expression of a number of IFN-stimulated genes (ISG), which help induce anti-virus state within the cell. Some of the most well known ISGs are 2'-5'-oligoadenylate synthetase (2'-5'- OAS), dsRNA-activated protein serine/theronine kinase (PKR), MxA and

guanylate-binding proteins (GBP). While OAS activates the riboendonuclease RNaseL leading to degradation of viral RNA, PKR phosphorylates eukaryotic initiation factor 2α (eIF2α) to suppress viral protein translation [253]. Although less characterized, MxA and GBP have also been documented to contribute to inhibition of viral replication. In addition, IFN-α is thought to augment APOBEC3G (A3G) expression in macrophages and liver cells [320]. Although the mechanism remains unclear, A3G is a factor with broad antiviral functions, especially to retrovirus and HBV [320-323]. With respect to the role of IFNIs in apoptosis, RNaseL, PKR, p53 have all been shown to be associated with apoptosis of virally infected cells [324-326].

Besides directly targeting the virus within the infected cells, IFNIs are strong modulators of the immune response. They contribute to the activation of macrophages, NK cells and cytotoxic T cells, thus enhancing the adaptive arm of immunity, a necessity in the host ability to recover from a viral infection.

5.4.2.2 Protozoan infections

IFNIs activate macrophages and induce iNOS, promote Th1-like responses via production of IFN-γ. Thus, IFNIs have a protective role to the host in protozoan infections. For *Leishmania* infection, IFNIs are required for the expression of iNOS and subsequent production of NO in macrophages - a major host target for *Leishmania*. It should be noted that NO is highly important in the clearance of *Leishmania* [272, 327]. In *Trypanosoma cruzi* infection, IFNα/β can suppress parasite growth, while IFNAR-/- mice exhibited 3-fold higher in the level of parasite growth post-infection compared to WT mice. Interestingly, this higher number of parasite present was correlated with lower production of NO [273, 328]. With respect to infections with other parasites including

Toxoplasmosis gondii and *Plasmodiu*, IFNIs have been reported to help enhance Th1-type response and NK cell functions [273].

5.4.2.3 Bacterial infections

Functions of IFNIs are complex in bacterial infection in that they can enhance or suppress host immune responses. The role of IFNIs on host resistance has been studied on about 10 different bacteria [259, 273]. Although some studies have supported the notion of IFNIs being beneficial to the host, others have contradicted it in indicating that IFNIs actually enhanced susceptibility to the infection. For example, pretreatment of cells with IFNIs have been shown to reduce the number of *Shigella flexneri* and *Salmonella typhimurium* in infected cells [329, 330] or protect macrophages from *Bacillua anthracis*-induced death and inhibited the growth of the bacteria [331]. Along the same line, it has been documented that exogenously provided IFNIs significantly reduce *S. typhimurium*-induced lethality in infant mice [332]. Heat-killed M protein WT (M+) *S. pyogenes*-pretreated mice showed increased resistance to organism challenge, which is correlated with enhanced IFN-α/β production [333]. Similarly, exogenous IFNIs inhibited *Legionella pneumophila* replication in macrophages through an IFNγ-independent pathway [334].

In some human studies, administration with type I IFN (*e.g.*, IFN-α) improves host resistance to pulmonary tuberculosis [335, 336]. IFN-β was also found protecting mice from systemic *M. avium* infection [337], while IFNAR-/- mice showed increased susceptibility to BCG pulmonary infection, which was correlated with low levels of NO production [338]. However, this positive effect of IFNα and IFNβ was not observed in other studies. In fact, administration of IFNα/β to human monocytes/macrophages

promoted BCG growth *in vitro* [339]. Especially, one highly virulent strain of *M. tuberculosis* found to induce high production of IFNα/β in mice, which was associated with lower *M. tuberculosis*-specific Th1 responses and early death of mice, compared to those infected with other strains. Intranasal administration of purified IFNIs also enhanced the susceptibility to *M. tuberculosis* [340]. Such contradictory findings of these studies may be partially due to inherent differences of the pathogen, the host as well as the time and dosage of administrated IFNIs. Further work is prudent to tease out the impact of these factors. In recent studies, comparing the susceptibility of IFNIR-/- mice with WT mice in *Listeria monocytogenes* infection [341-343], it has been found that KO mice were more resistant to *L. monocytogenes* infection compared to WT mice. It is possible that type I IFN signaling pathway promotes apoptosis of lymphocytes and decreases IL-12 production in the spleen, which collectively gives rise to weaker resistance to listeria in the WT mice. In fact, Listeriolysin O (LLO) of *Listeria* has been documented to induce apoptosis of monocytes in WT but not in IFNAR KO mice [344]. In chlamydial infection, an early study using Newcastle disease virus or poly I:C as IFNI-stimulating agents has indicated that IFNIs may be protective during the infection [345]. Later investigations supported this finding in that depletion of tryptophan via IFNIs was shown to protect macrophages against chlamydial infection [159, 346, 347].

Although the study using IFNIR-/- macrophages showed that IFNI dependent IFN-γ and iNOS can inhibit *Chlamydia pneumonia* growth [290]. But this observation was not reproducible in the mouse model and no significant difference in *C. pneumonia* growth between WT and IFNIR-/- mice could be found in another study [348].

6. Rational and Hypothesis

Previously, we and others have proven that genetic backgrounds influence host resistance to chlamydial infection, and a Th1-like response is an important determinant of host susceptibility to chlamydial infection. The objectives of this project are: 1) To elucidate molecular mechanisms which govern the differential susceptibility to *C. muridarum* lung infection by C3H/HeN and C57BL/6 mice; 2) To investigate whether only MHC, or both MHC and non-MHC genes participate in defence against chlamydial infection, and 3) To assess the role of type I IFN in host resistance to chlamydial infection.

Hypothesis 1: Differential susceptibility between C3H and C57BL/6 mice to chlamydial infection is due to different patterns of cytokine production

de la Maza et al and Darville et al reported dramatically different susceptibility to genital tract chlamydial infection between C57BL/6 and C3H/HeN (C3H) mice [224, 349-351]. However, whether this holds true in a lung infection and, more importantly, the molecular mechanism underlying this phenomenon are largely unknown. In the present study, we intended to further analyze the susceptibility and immune responses of C57BL/6 and C3H mice to chlamydial infection using a respiratory tract infection model. Since studies in other laboratories and ours in recent years have demonstrated a particularly important role of Th1-type cytokines such as IFNγ and IL-12 and of T cell-mediated immune responses in host defense against chlamydial infection [130, 152, 153, 176, 188, 190, 352-354], we hypothesize that the different resistance between C3H and C57BL/6 mice may be attributed to different patterns of cytokine production.

Hypothesis 2: MHC dictates host susceptibility to chlamydial infection

Previous studies documented that C3H/HeN and C57BL/6 mice exhibited a high degree of variability in their susceptibility to *C. trachomatis* lung and genital tract infections [93, 224, 350, 351]. Because C57BL/6 (H-2^b) mice and C3H/HeN (H-2^k) mice possess different MHC molecules and that MHC is essential to the development of host adaptive immunity, we hypothesize that MHC haplotypes determine the host susceptibility to chlamydial infection.

Hypothesis 3: Type I IFNs regulate both innate and adaptive immune responses to chlamydial infection

Type I IFNs, IFNα/β are cytokines with pleiotropic functions regulating both innate and adaptive immunity. On one hand, the cytokines stimulate expression of iNOS, IDO, and IFN-γ and may also participate in the inhibition of bacterial infection as well [259, 273, 319, 355]. On the other hand, type I IFNs can enhance apoptosis of lymphocytes and macrophages, which may be detrimental to host resistance to bacterial infection [341-343]. IFNIs are also strong activators for DC by promoting expression of MHC, co-stimulatory molecules, cytokines and chemokines leading to eventual activation of T and B cells [309, 356-358]. We hypothesize that during chlamydial infection, type I IFNs may influence host susceptibility by regulating both innate and adaptive immunity.

MATERIALS AND METHODS

Animals

Female age matched C57BL/6 mice and C3H/HeN mice were purchased from Charles River Canada. Female C3H. SW-$H2^b$/SnJ mice (defined as C3H.H2b that is $H\text{-}2^b$ in C3H background) and B6. AKR-$H2^k$/FlaEg mice (defined as B6. H2k that is $H\text{-}2^k$ in C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). Female CXCR2-/- mice were from Jackson Lab, corresponding Balb/c were from GMC (University of Manitoba). Breeding pairs of IFNAR-/- mice (interferon α/β receptor knock out mice) [359] were from B&K Universal (Grimston, England), the corresponding breeding pairs of wild type control mice (129Sv/Ev) were from Taconic (Hudson, NY, USA). All animals were maintained under specific pathogen-free conditions in the central animal facilities of the University of Manitoba and utilized under the guidance of Canadian Council on Animal Care. Age-matched mice at 8-12 week were used for studies.

Culture and purification of *C. muridarum*

Chlamydia muridarum biovar MoPn (mouse pneumonitis, previously called as *C. trachomatis* mouse pneumonitis) was used throughout all studies. The procedures for *C. muridarum* culture, harvest and purification were performed as previously described [152]. Briefly, HeLa229 cells were cultured in Eagle's MEM medium with 10% heat-inactivated FBS and 2 mM L-glutamine to form confluent monolayers and treated with Hanks' balanced salt solution (HBSS) (with 30 μg/ml DEAE-dextran) for 30 min. *C. muridarum* EBs were inoculated onto cell layers at MOI 1:1. About 48 h after infection, infected cells were harvested with sterile glass beads and disrupted by sonication to

release EBs from the cells. Organisms were purified by 15 min centrifugation at 500 x *g* (debride cell debris) followed by 30 min spin at 30,000 x *g* and then by discontinuous density gradient centrifugation with 35% Renografin (Princeton, NJ). Purified EBs were resuspended in SPG (sucrose phosphate glutamic acid) and aliquots were stored at –80 ^{0}C until used.

Infectivity test of EBs

HeLa cells were cultured in 96-well flat-bottom plates and treated with HBSS-DEAE. Cells were then inoculated in triplicate with 50 µl serially diluted *C. muridarum* MoPn stock at 37^{0}C for 2 h. Washed cells were cultured in MEM complete medium containing 25 µg/ml gentamycine and 1 µg/ml cycloheximide. After 40-hour incubation, cells were fixed with methanol and processed for inclusion staining. Inclusions were stained with Chlamydia genus-specific anti-lipopolysaccharide (anti-LPS) monoclonal antibody (mAb) and secondary goat anti-mouse IgG conjugated with horseradish peroxidase. After substrate (4-chloro-1-naphthol) addition and color development, inclusions were counted under microscope and *C. muridarum* titer was calculated based on serial dilutions of the original inoculum.

Respiratory tract infection

Following anaesthesia with isoflurane in an anaesthetic vaporizer, age matched mice were inoculated intranasally with various doses (specified in different experiments) of *C. muridarum* in 40 µl of SPG buffer. The mice were monitored daily for morbidity (*e.g.*, body weight loss, ruffled fur, piloerection, and difficulty in breathing) and mortality following infection.

Organism growth in organs

Mice were killed on selected days after infection as specified in particular experiments. To analyze the *in vivo* growth of the organism, the major organs: lungs, hearts, livers and kidneys (as depicted for each experiment) from each mouse were aseptically isolated and homogenized in 3 ml SPG buffer. Tissue homogenates were spun down at 1,900 x *g* for 30 min at 4^0C, and the supernatant aliquots (1 ml/vial) were kept at -80^0C until tested. The infectivity of MoPn in the organ homogenates was assayed as described above. For each experiment, all samples were tested at the same time. For repeat testing, only fresh aliquots were used to avoid repeated freezing- thawing cycles and loss of viability of the organisms.

Determination of DTH responses

Eleven days after infection, mice were injected subcutaneously (s.c). with 25 µl ultraviolet light (UV)-inactivated MoPn EBs (5 x 10^4 IFUs) on the left footpad and the same volume of SPG buffer on the right side. The difference in thickness between the two footpads at 24, 48 and 72 h post injection was measured with micrometer caliper and used for comparison of the DTH response. Footpads were decalcified in 10% formic acid overnight and processed for histological analysis to compare the DTH responses in different groups of mice.

Histopathological and immunohistochemical analysis

The lungs and footpads were collected and fixed in 10% neutral buffered formalin, embedded in paraffin and six-micron sections were cut. Tissues were stained with hematoxylin and eosin (H&E), and examined by light microscopy. The examiner was blinded as to the samples were collected from which groups of mice.

For immunohistochemical analysis, lung tissues were frozen in liquid nitrogen and stored at -80^0C until analysis. Frozen tissues were imbedded in frozen tissue embedding media (Fisher Scientific, Canada) and 10 micron sections were cut. Tissues were fixed with acetone and specific antigens were stained with corresponding antibodies as listed in Table 1. Single, double or tricolor staining was performed for tissues as specified in particular experiments and the stained tissue sections were examined with a fluorescent microscope (Olympus AX70) or confocal microscope (Olympus IX70).

Table 1 Reagents for Immunohistochemistry

Antigens	1st antibodies	2nd antibodies(Jackson ImmunoResearch)	Fluorescent
Macrophage	Rat α mouse F4/80 (BM8), eBioscience	Goat α Rat IgG	FITC, Texas- Red
Epithelial cells	Mouse Anti-pan-cytokeratin (B311.1), Calbiochem	Goat α mouse IgG	FITC
MoPn	Mouse anti- chlamydia LPS[1]	Biotinylated rabbit anti-mouse IgG[2]	AMCA-avidin D[3]
VCAM-1	Rat α mouse VCAM-1 (429), eBioscience	Goat α Rat IgG	FITC
Cleaved caspase-3	Rabbit α cleaved caspase-3 mAb, Cell Signalling Technology	Goat α rabbit IgG	FITC
Neutrophil	Rat α mouse Ly-6G (RB6-8C5), eBioscience	Goat α Rat IgG	Texas-Red
lymphocyte	Rat α mouse CD2(RM2-5), eBioscience	Goat α Rat IgG	Texas-Red

1. Kind gift from Dr. Jody Berry (PHAC, NML Canada)
2. Chemicon International (Temecula, CA)
3. Vecter Laboratories Inc (Burlingame, CA).

Spleen and local draining lymph node cell culture

Infected mice were euthanized at selected days following infection. Spleens and draining (mediastinum) lymph nodes were aseptically removed and homogenized to release single cells. After erythrocyte lysis and filtration through 70 μm cell strainer (BD Falcon), cells were resuspended in RPMI-1640 complete medium (10% heat-inactivated FBS, 20μg/ml 2-β-mercaptoethanol, 25 μg/ml gentamycin). Spleen cells were cultured at

7.5 x 10^6 cells/ml, lymph node cells were cultured at 5 x 10^6 cells/ml (with or without the stimulation of UV-inactivated *C. muridarum* EBs at 5 x 10^5 IFU/ml). Cultured supernatants were harvested at 72 h or 120h for cytokine determination.

Cytokine and antibody determination and corresponding reagents

Cytokines in cultured supernatants and homogenized lung supernatants were measured by using two-mAb sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well ELISA plates were coated with purified capture anti-cytokine mAbs overnight, incubated with diluted samples followed by incubation with biotin conjugated secondary antibodies and alkaline phosphatase-conjugated streptavidin. After substrate (Sigma-Aldrich) addition, results were determined using a microplate reader (Molecular Devices) at absorbance of 405 nm. Purified capture and biotinylated detection antibodies and recombinant cytokine standards were purchased from BD PharMingen (San Diego, CA), eBioscience (San Diego, CA), and R&D systems (Minneapolis, MN), respectively.

Serum IgM, IgG1, IgG2a and IgA antibodies specific to *C. muridarum* EBs were determined by ELISA as previously described [152]. 96-well ELISA plates were incubated with 10^5 IFU UV-killed *C. muridarum* EBs/100 μl/well. Serially diluted sera were incubated with coated antigen followed by incubation with the corresponding biotin-conjugated anti-mouse immunoglobulin secondary antibodies. Results were expressed as ELISA titers at 60 min using the end-point (cutoff at optical density at 405nm, 0.5) of the titration curves.

In vitro* infection of macrophages with *C. muridarum

Peritoneal macrophages

Peritoneal macrophages were collected from C3H/HeN and C57BL/6 mice following intra-peritoneal (i.p) injection with 4% thioglycollate (1.5 ml/mouse) as described [360]. Peritoneal cells were harvested by lavaging the peritoneal cavity with HBSS containing 1U/ml heparin followed by 10 min centrifugation of the lavage fluids at 210 x *g*. Following red blood cells (RBCs) lysis with 0.85% NH_4Cl, peritoneal cells (mostly macrophages) were resuspended at 2 x 10^6 cells/ml with RPMI-1640 complete medium and seeded at a concentration of 2 x 10^5 cells/100 µl/well in the 96-well cell culture plates. After two-hour incubation at 37^0C, unattached cells were removed by washing once with HBSS. Fresh complete medium (200 µl/well) was added and the plates were further incubated overnight to form cell monolayers. The macrophage monolayers were then inoculated with *C. muridarum* EBs and incubated for 2 h followed by a washing step with HBSS. Subsequently, fresh complete culture medium with or without recombinant IFN-γ (1 or 10 U/ml) or LPS (1 µg/ml) was added to the wells at 200 µl/well. In some experiments, aminoguanidin (AG) at a final concentration of 100 µM was added to the wells along with IFN-γ. Cycloheximide was not added in macrophage culture for the purpose of analysis of NO production. Chlamydial inclusions in the macrophage monolayers were stained and counted after 48-hour inoculation, in the same way as described previously [85].

Bone marrow-derived macrophages (BMDM)

Leg bones from naïve IFNAR-/- and wild type 129Sv/Ev mice were treated with 70% ethanol for 5 min to remove fibroblasts and muscle cells. Connective tissues were completely removed and the bone marrow aseptically flushed out with DMEM. The cell

suspension was treated with ACK lysis buffer to lyse RBCs. Resuspended cells were cultured in complete L929 conditioned medium (DMEM with 10% FBS, 30% L929 conditioned media, penicillin and streptomycin) at 0.5 x 10^6 cells/ml in a petridish. Medium was changed every 2 or 3 days. Cells were harvested 10 days later with a scraper, counted and resuspended at 2 x 10^6 cells/ml in DMEM complete medium as described before. The purity of macrophages was analyzed by flow cytometry. Cells were infected with *C. muridarum* and chlamydial inclusions were stained and counted using a software (Olympus DP Controller) under microscopy (Olympous IX51).

Measurement of Nitric Oxide (NO)

The levels of NO produced by *ex vivo* cultured macrophages following *C. muridarum* infection in the presence or absence of IFN-γ stimulation were measured by Griess assay. Briefly, culture supernatants were collected at 48 h post-infection and serially diluted in phosphate buffered saline (PBS). The samples were added to 96-well microplates at 150 μl/well. Following the addition of Griess reagent (50 μl/well), plates were incubated at RT for 10 min. The absorbance was measured using a microplate reader at 570 nm. Serial dilutions of known concentrations of $NaNO_2$ were used to generate a standard curve from which NO levels in test samples were to be extrapolated.

RT-PCR

Lungs of naïve mice and infected mice were harvested and frozen immediately in liquid nitrogen, and stored at –80 ^{0}C until used. Total RNA from frozen lungs was isolated with TRIzol reagent (Invitrogen Canada) according to the manufacturer's protocol. Total RNA was quantified and treated with TURBO DNase (Ambion) to remove contaminating chromosomal DNA and reversely transcribed with Superscript III

reverse transcriptase (Invitrogen). To confirm the efficiency of DNase treatment, 1 μg treated RNA from infected mouse lung was tested for DNA amplification of *C. muridarum* 23sRNA by PCR and no amplification was detected (data not shown). cDNAs were amplified (30 cycles) using primers as listed in Table 2. Amplification of test cDNAs using primers a housekeeping gene (β-actin) as a control. PCR products were resolved on an agarose gel by electrophoresis and band density of samples was determined using SCION IMAGE software analysis.

Table 2 Primers for RT-PCR

β-actin: sense 5'-GTGGGGCGCCCCAGGCACCA-3', **Anti-sense: 5'-CTCCTTAATGTCACGCACGATTTC-3'**	**550bps**
IL-1β: sense 5'-GCAACTGTTCCTGAACTCA-3' **Anti-sense: 5'-CTCGGAGCCTGTAGTGCAG-3'**	**363bps**
MIP-2: sense: 5'-ACCCTGCCAAGGGTTGACTTC-3' **Anti-sense: 5'-GGCACATCAGGTACGATTCCAG-3'**	**285bps**
LIX: snese: 5'- AGCTCGCCATTCATGCGGATG-3' **Anti-sense: 5'- CTATTGAACACTGGCCGTTCT-3'**	**344bps**
KC: sense: 5'- AACGGAGAAAGAAGACAGACTG-3' **Anti-sense: 5'- GACGAGACCAGGAGAAACAG-3'**	**530bps**
CXCR2: sense: 5'- CTCCTTGGTGATGCTGGTCA-3' **Anti-sense: 5'- AGAATTAAGATGGGCAGGGC -3'**	**331bps**
gp91: sense: 5'-CTTTGTCATTCTGGTGTGGTTGG-3' **anti-sense: 5'-CCCCATTCTTCGATTTTGTCTGC-3'**	**720bps**
IDO: sense: 5'-GTACATCACCATGGCGTATG-3' **Anti-sense: 5'-GCTTTCGTCAAGTCTTCATTG-3'**	**741bps**
FAS: sense: 5'-AGCAATACAAACTGCAGGAAAC-3' **Anti-sense:5'-TCTCCTCTCTTCATGGCTGG-3'**	**531bps**

FASL: sense:5'-GAGTTCTGTCCTTGACACCTGA-3' Anti-sense: 5'-CATGAGGTCTTTGTGGCTCATGT-3'	1038bps
Perforin: sense-5'-GATGTGAACCCTAGGCCAGAG-3' Anti-sense:5'-GTCTCCTACCTCATCAGCTGTGA-3'	369bps
GarnzymeA: sense-5'-ACACGGTTGTTCCTCACTCAA-3' Anti-sense: 5'-CTACTCGGCATCTGGTTCCTG-3'	351bps
GranzymeB: sense: 5'-TACTGCTGACCTTGTCTCTGG-3' Anti-sense: 5'-CCTCACAGCTCTAGTCCTCTT-3'	359bps
GTPI: sense: 5'-ACTTCTCCGACGCTGTATTCAT-3' Anti-sense: 5'-GAACTGCTCAGAAGCTACGATG-3'	422bps
IGTP: sense: 5'-TCTATGACTGCTGGTGAGTCAC-3' Anti-sense: 5'-ATGTGCTGATCTGCATCTCCT-3'	385bps
IIGP: sense: 5'-CTCTTCACCTAAGAGTGATGAG-3' Anti-sense: 5'-CAGGGTATTGATGAAGCTGGAC-3'	253bps
IRG47: sense: 5'-GCTAAGGAGTTTCTGCCTCAGT-3' Anti-sense:5'-CTTGGTCTAGATAAGCGTCTGA-3'	485bps
LRG47: sense: 5'-ACCATCACACAGTTCCTGCTA-3' Anti-sense: 5'-CTGCTCAGAGGCAATGATGATGA-3'	493bps
TGTP: sense: 5'-GTACTGAGAGACATCGAGAGTG-3' Anti-sense: 5'-CGCTGTCTATCTTGGTTCTGA-3'	382bps
TRAIL: sense: 5'-ACCTCAGCTTCAGTCAGCACTTCA-3' Anti-sense: 5'-AAGCTGAGTTGCTTCTCCGAGTGA-3'	396bps
PKR: sense: 5'-GTGGACATCTTTGCTTTGGGCCTT-3' Anti-sense: 5'-TGTTCCTCCATTCAGCCAAGGTCT-3'	223bps
DAXX: sense: 5'-TGAACTTAGCTCCTGCAGCCTCAA-3' Anti-sense: 5'-TTAATGAGCCGTTCAATGCGCCTG-3'	400bps

Flowcytmetry analysis

Surface marker expression

For adhesion molecule analysis, mice were infected with 1,000 IFUs of *C. muridarum*. Four days post-infection, blood samples were collected in centrifuge tubes containing EDTA-K_3 as an anticoagulant. The whole blood was stained with anti-CD45 (PerCP), CD11b (APC), anti-mouse CD11a or ICAM-1 (FITC), anti-mouse CD49d, or CD18, or CD29 (PE) for 20 min. After which time, erythrocytes were lysed with a lysis buffer, samples were washed, fixed, washed again and analyzed with BD Calibur (BD Bioscience) and CellQuest software.

For phenotyping of DC surface, 2 x 10^5 affinity-purified DCs (MACS beads, Miltenyi Biotec) were incubated with a Fc blocker (anti-Cd16/32) and double stained with anti-CD11c (FITC) and anti- CD80/86/40/I-K^b (PE) antibodies for 20 min. The cells were subsequently washed with FACS buffer (0.5% BSA in PBS), fixed (with 2% paraformaldehyde) and analyzed with EPICS Altra flow cytometer (Beckman Coulter, CA).

Intracellular cytokine staining

Spleen cells were cultured as mentioned before. After 72 h, supernatants were harvested for ELISA analysis. Left-over cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 3 h, and with brefeldin A (20 μg/ml) incubation for another 4 h. Cells were harvested, incubated with anti-CD16/32 and stained with anti-CD3e (PE-Cy7), anti-CD4 (PE), anti-CD8 (FITC) antibodies. The cells were fixed and permeabilized (freshly prepared 0.1% Saponin in flow buffer), and stained with APC-labeled anti-IFNγ, anti-IL4 or anti-IL10 antibodies. After washing, the cells were analyzed with BD Calibur (flow cytometer)/Cell Quest (software) (BD Bioscience).

Identification of Bronchoalveolar lavage (BAL) and blood leukocytes

Euthanized mice were intra-trachea cannulated and the lungs were lavaged twice with 1 ml PBS. After centrifugation, BAL fluid cells were resuspended immediately for cell counting and cell smear. Air-dried BAL smear slides were then fixed with acetone and stained with Hema 3 Stain Set (Fisher Scientific, Ontario, Canada). Five hundred cells of each slide were counted and the number of macrophages, neutrophils, and lymphocytes were counted based on the staining characteristics of the cell, cytoplasma and nuclei. Blood smear from mice were stained and counted similarly.

Lung macrophage depletion and susceptibility test

Pulmonary macrophages were depleted by intranasal administration with liposome encapsulated dichloromethylene diphosphonate (clodronate, or CL_2MDP), a kind gift from Roche Diagnostics (Mannheim, Germany). Liposome containing CL_2MDP and liposome encapsulating PBS were prepared and provided by Dr. Van Rooijen Nico (Free University Medical Center, Amsterdam, the Netherland) as mentioned previously [361]. IFNAR-/- mice were administrated with 90 μl of CL_2MDP liposome or PBS liposome, or PBS by intranasal route at 3 days before infection with 200 IFUs of *C. muridarum*. Four days post-infection, liposome administration was repeated once again. Mice were euthanized at 7 days post-infection. *Chlamydia* yields and pathological changes were analyzed as described above.

Apoptosis analysis

Wild type 129Sv/Ev and IFNAR-/- mice were intranasally infected with 1,000 IFUs of *C. muridarum*, and 4 days post-infection, mice were euthanized and lungs were fixed with 10% neutral buffered formalin. Apoptosis was tested using TUNEL *in situ* cell

death detection kit- POD (Roche Diagnostic Canada) as mentioned before [341]. Briefly, paraffin-embedded tissue sections were dewaxed with xylene and subsequently processed through ethanol/water mixture with gradually increasing the ratio of water to ethanol to finally just water. Hydrated sections were treated with protease K (Sigma-Aldrich, Canada) and blocked with blocking buffer (Dako, Canada). Tissue section were incubated with 50 µl TUNEL reaction mixture for 60 min at 37 ^{0}C in darkness, then labeled with converter-POD in 37 ^{0}C for 30 min. After thoroughly washed with PBS, tissue sections were treated with 100 µl DAB substrate and observed under microscope. POD reaction was stopped by washing with PBS and counter staining was done with hemoxylin. Tissue sections were then analyzed under microscope.

Dendritic cell isolation and analysis

DC from mouse spleens were isolated with MACS Technology as described in manufacture's protocol (Miltenyi Biotec). Briefly, mouse spleens were treated with collagenase D (1 µg/ml) for 45min and filtered through 70-µm cell strainers to release single cells. The cell were labeled with CD11c microbeads and passed through columns. Isolated CD11c-positive cells were used for cell surface markers analysis by flow cytometry.

Statistical analysis

The difference in the cytokine levels and RT-PCR band density were analyzed by Student's *t*-test. The IFU counts and antibody titers were transformed to base 10 logarithms and analyzed by Student's *t*-test.

RESULTS AND DISCUSSIONS

PART I Genetically Determined Susceptibility to *Chlamydia muridarum* Lung Infection between C3H and C57BL/6 Mice

As mentioned above, studies by numerous laboratories in recent years have demonstrated the particularly important role of Th1 type cytokines such as IFN-γ and IL-12 and T cell-mediated immune responses in host defense against chlamydial infection [130, 152, 153, 176, 188, 190, 196, 352-354]. Mice with higher IFN-γ and IL-12 production and stronger cell-mediated immune responses such as DTH appear to be more resistant to chlamydial infection [152, 153, 188, 196]. IFN-γ gene and IFN-γ receptor gene knockout mice showed more serious diseases and higher chlamydial growth *in vivo* [127, 133, 362]. IL-12 knockout mice displayed delayed clearance of *C. trachomatis* and more serious pathological responses [363]. Conversely, supplement of recombinant IL-12 before and after infection with *Chlamydia psittaci* reduces the severity of chlamydial pneumonia and the chlamydial load in the lung [364]. Similarly, some human studies showed that individuals with severe scarring trachoma exhibited higher Th2 cytokine production and impaired cell-mediated immune responses compared with the controls [151, 365]. All of these reports support a significant protective role of Th1 cytokine production and cell-mediated immune responses in host defense against chlamydial infection.

Genetically determined difference to chlamydial infection has been demonstrated in humans as well as animal models. Persistent infection and severe sequelae of ocular and genital tract chlamydial infection appear to occur only in certain individuals [366]. It has been reported that C57BL/6 and BALB/c mice are different in their susceptibility to

respiratory tract chlamydial infection, which is associated with different patterns of cytokine production and types of immune responses [152]. More recently, de la Maza *et al.* and Darville *et al.* reported dramatically different susceptibility between C57BL/6 and C3H/HeN (C3H) mice to genital tract chlamydial infection [177, 224, 349-351]. However, the mechanism for this difference is largely unknown.

In this part of the study, we analyzed the susceptibility and immune responses of C57BL/6 and C3H mice to chlamydial infection using a respiratory tract infection model. We found that C3H mice mounted as strong Th1 type (*i.e.*, IFN-γ and DTH) responses as C57BL/6 mice following chlamydial lung infection, but suffered a much more serious disease and higher organism growth than C57BL/6 mice. Comparative analysis of chlamydial growth in cultured *ex vivo* macrophages from different strains of mice revealed that the inhibitory effect of exogenous IFN-γ on chlamydial growth in the infected cells was significantly less in C3H mice than in C57BL/6 mice. The lower inhibition of IFN-γ on chlamydial growth in cultured *ex vivo* macrophages from C3H mice was associated with significantly lower NO production by these cells following IFN-γ stimulation. Our study further highlights the complexity of the mechanisms underlying host defense against chlamydial infection, which may include both innate and adaptive immune responses, and suggests that the cellular events in host cells downstream of Th1 cytokine production play an important role in host defense and/or disease development to *C. trachomatis* infection.

RESULT 1 C. muridarum-infected C3H mice showed significantly higher mortality, pulmonary organism growth, and more severe pathological change than C57BL/6 mice

To test the susceptibility to chlamydial infection, C3H and C57BL/6 mice were intranasally inoculated with 1 x 10^4 IFUs of *C. muridarum* and mouse survival was monitored daily for 14 days post-infection. As shown in Figure 1A, C3H mice started to die at day 5 post-infection. 14 days after inoculation, 80% of C3H mice died of infection while all C57BL/6 mice survived. At a lower dose (3 x 10^3 IFUs), C3H mice started to die at day 9 post-infection with a mortality rate of 50% at day 14 post-infection (Fig 1B). When severity of disease was assessed based on body weight loss following exposure to a lower dose (1.5 x 10^3 IFUs) infection, C3H mice showed dramatic and continuous body weight loss following infection, unlike C57BL/6 mice which exhibited mild body weight loss that started to recover within 2 weeks (Fig 2).

To further compare the susceptibility to *C. muridarum* infection between C3H and C57BL/6 mice, we examined the growth of *Chlamydia* organisms in the infected lungs. Mice were infected with *C. muridarum* (1.5 x 10^3 IFUs) and sacrificed at day 8 and day 14 post-infection, respectively. As shown in Figure 3, C3H mice displayed significantly higher (> 10- fold) *in vivo* growth of *Chlamydia* than C57BL/6 mice at both early (day 8) and late (day 14) stages of infection. Similarly, when mice were infected with a higher dose (1 x 10^4 IFUs) of the organism, C3H mice showed more than 10-fold higher *C. muridarum* growth in the lungs compared to C57BL/6 mice (2.5 x10^6 IFUs vs 1.9 x 10^5 IFUs) at day 4 post-infection.

Histological analysis of the lungs also showed significant difference in pathological responses between C3H and C57BL/6 mice. On day 8, all C3H mice showed severe inflammatory cell infiltration and broad lung consolidation (Fig. 4). The infiltrates included mononuclear cells, lymphocytes and polymorphonuclear cells, especially more

neutrophils. In contrast, *C. muridarum* infected lungs of C57BL/6 mice showed less severe inflammation and consolidation at the same stage (Fig. 4). On day 14 post-infection, the difference between C3H and C57BL/6 mice was even more pronounced, with the former showing serious consolidation while the latter nearly resolved the inflammation (Fig. 4). Taken together, the data indicate that C3H mice are much more susceptible to *C. muridarum* infection and suffer more severe diseases than C57BL/6 mice following chlamydial lung infection.

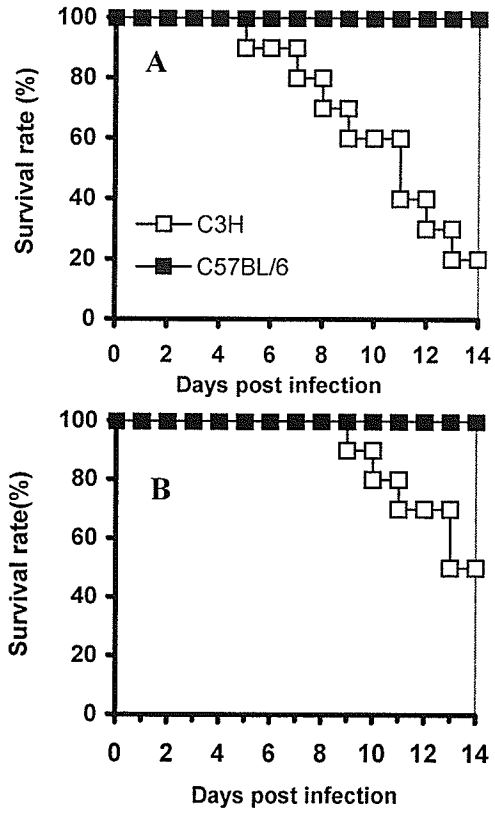


Figure 1. C3H mice showed higher mortality following intranasal *C. muridarum* infection. A: Age-matched female C3H and C57BL/6mice (10 mice/group) were intranasally infected with 1 x 10^4 IFUs *C. muridarum* and monitored daily for survival. The data are representative of four independent experiments that showed similar results. B: Age-matched female C3H and C57BL/6 mice (12 mice/group) were intranasally infected with 3 x 10^3 IFUs *C. muridarum* and monitored daily for survival. The data are representative of two independent experiments that showed similar results.

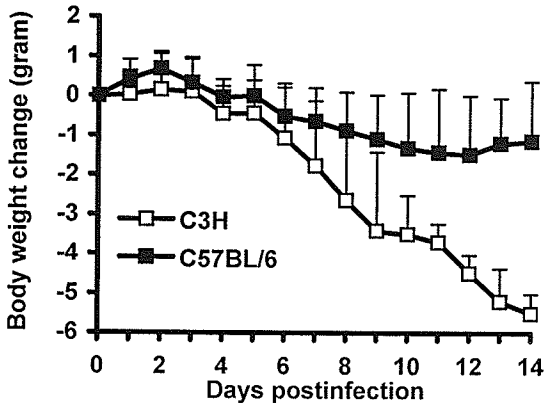


Figure 2. C3H mice showed more bodyweight loss following chlamydial infection. Age-matched female C3H and C57BL/6 mice (11 mice/group) were intranasally infected with 1.5 x 10^3 IFUs *C. muridarum* and the body weight changes of the mice were measured daily following infection. The initial body weights before infection were similar between the two strains of mice. The data show the net body weight change (mean $\pm$ SD) following infection. The data are representative of three independent experiments that showed similar results.

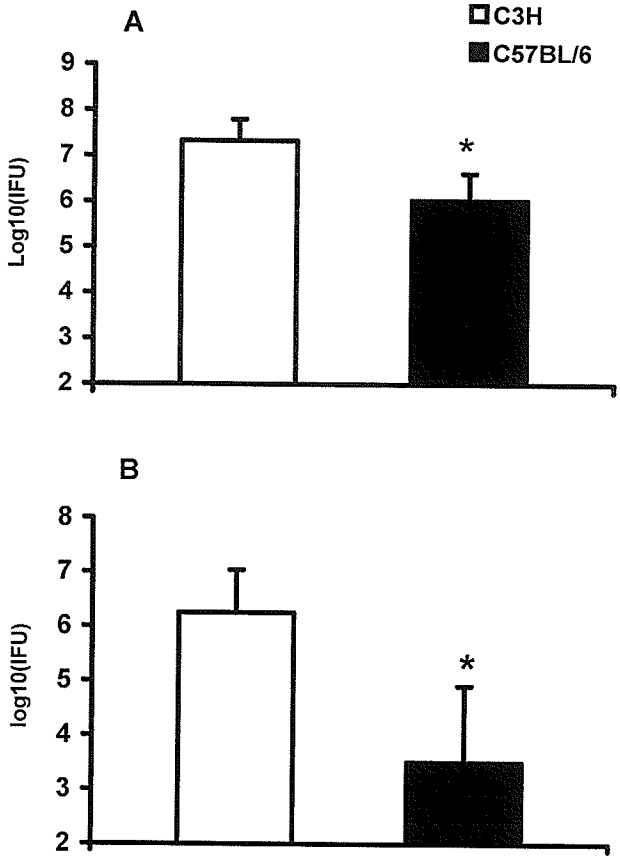


Figure 3. C3H mice showed more organism growth in lungs at early and late stages of infection. Mice were intranasally infected with *C. muridarum* at 1.5 x 10^3 IFUs and sacrificed at day 8 (Panel A) and day 14 (Panel B) post-infection. Chlamydial infectivity in the lung tissues was determined as described in *Materials and Methods*. Each column represents the mean $\pm$ SD of IFUs (log10) for seven mice. *: $p<0.05$. The experiment was repeated three times and similar results were obtained.

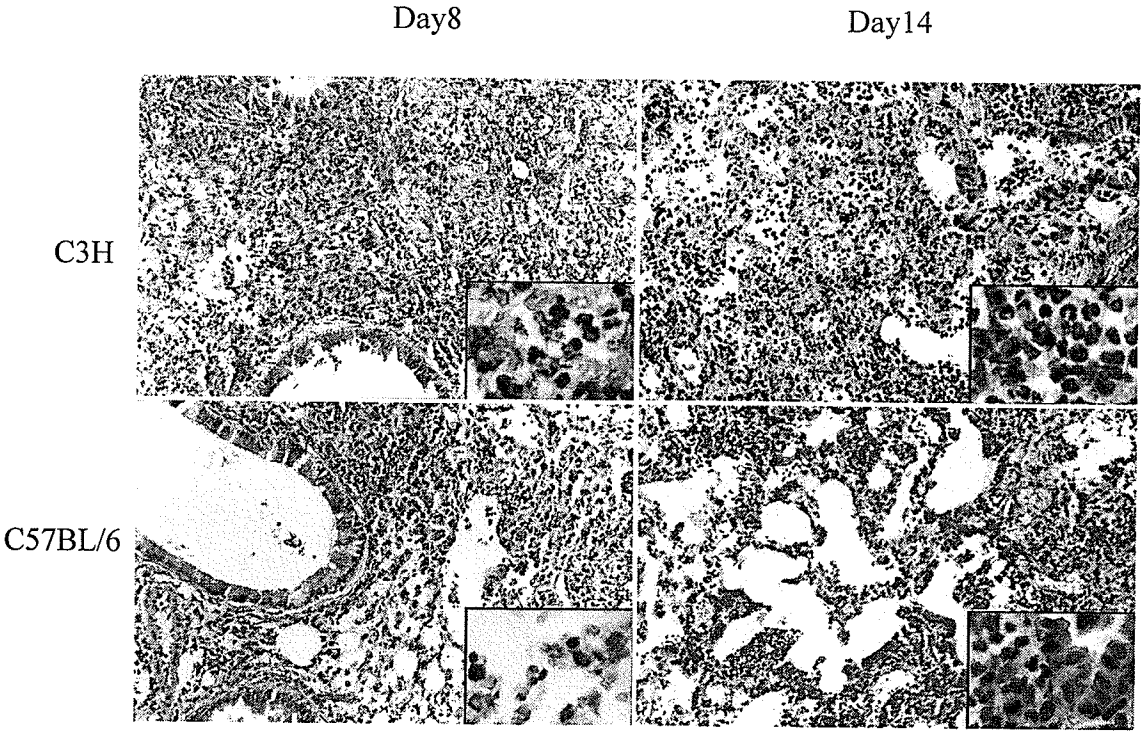


Figure 4 C3H mice show more severe inflammation and consolidation in the lungs following chlamydial infection. C3H and C57BL/6 mice were infected with *C. muridarum* MoPn as described in the legend of Figure 2. Mice were sacrificed at 8 or 14 days post infection. Lung sections were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy.

RESULT 2 C3H mice and C57BL/6 mice showed similar Th1 like responses following C. muridarum infection

Previous studies have suggested that the difference in susceptibility to *C. muridarum* infection in different mouse strains may be associated with different types of immune response induced by infection [152, 352]. We therefore compared DTH and antibody responses elicited by *C. muridarum* infection in C3H and C57BL/6 mice. Surprisingly, both strains of mice after infection exhibited strong and similar levels of DTH reaction shown by similar footpad changes in thickness following local dead EB challenge (Fig. 5A). Histological analysis of the footpad also showed similar patterns and types of cellular infiltration in the two strains of mice (Fig. 5B), confirming the finding obtained from the measurement of footpad thickness. In addition, for serum anti-*Chlamydia* antibodies, both strains of infected mice consistently demonstrated significantly higher titers of IgG2a subclass than IgG1 subclass, but no significant difference was found between C3H and C57BL/6 mice (Table 3). Similarly, when mice were infected with a higher dose of *C. muridarum* (1×10^4 IFUs), C3H survivors showed comparable levels of antibodies (IgG1 and IgG2a) and DTH response to C57BL/6 mice (Table 4). Moreover, there was no significant difference in IgA production between the two groups of mice.

Since T cell cytokine patterns have been found to be highly associated with the types of immune response and host susceptibility/resistance to chlamydial infection, we further analyzed cytokine production by spleen and local lymph node cells collected from *C. muridarum*-infected C3H and C57BL/6 mice following organism-specific *in vitro* re-stimulation. Notably, spleen cells from C3H and C57BL/6 mice produced similar levels

of IFN-γ in 8 and 14 days post lung infection. Furthermore, for Th2-type cytokine production, C3H and C57BL/6 synthesized comparable levels of IL-10 and IL-4 on day 14 post-infection (Table 5). An analysis of *Chlamydia*-driven cytokine production by local draining lymph node cells also demonstrated that both strains of mice secreted similar levels of IFN-γ, IL-4 and IL-10 but C3H mice made less IL-5 in the spleen and draining lymph nodes than C57BL/6 mice (Table 5). There was no significant difference in the production of proinflammatory cytokines IL-12 and TNF-α at these sites in the two strains of mice at day 14 post-infection, while at day 8 post-infection, C3H mice appeared to make higher levels of IL-12p40 in the lymph nodes (Table 5). These data collectively demonstrate that C3H, like C57BL/6 mice, mount strong Th1-like cytokine production following respiratory tract chlamydial infection. We also analyzed cytokine concentrations in the lung tissues after infection. Interestingly, there was a significantly greater amount of IFN-γ in the lungs of C3H mice (Fig 6), which may be correlated with higher *C. muridarum* growth and increased antigenic stimulation. Taken together, these data indicate that although both C3H and C57BL/6 mice mount strong cell mediated immunity to *C. muridarum* infection, the host immune responses fail to protect C3H mice from the infection.

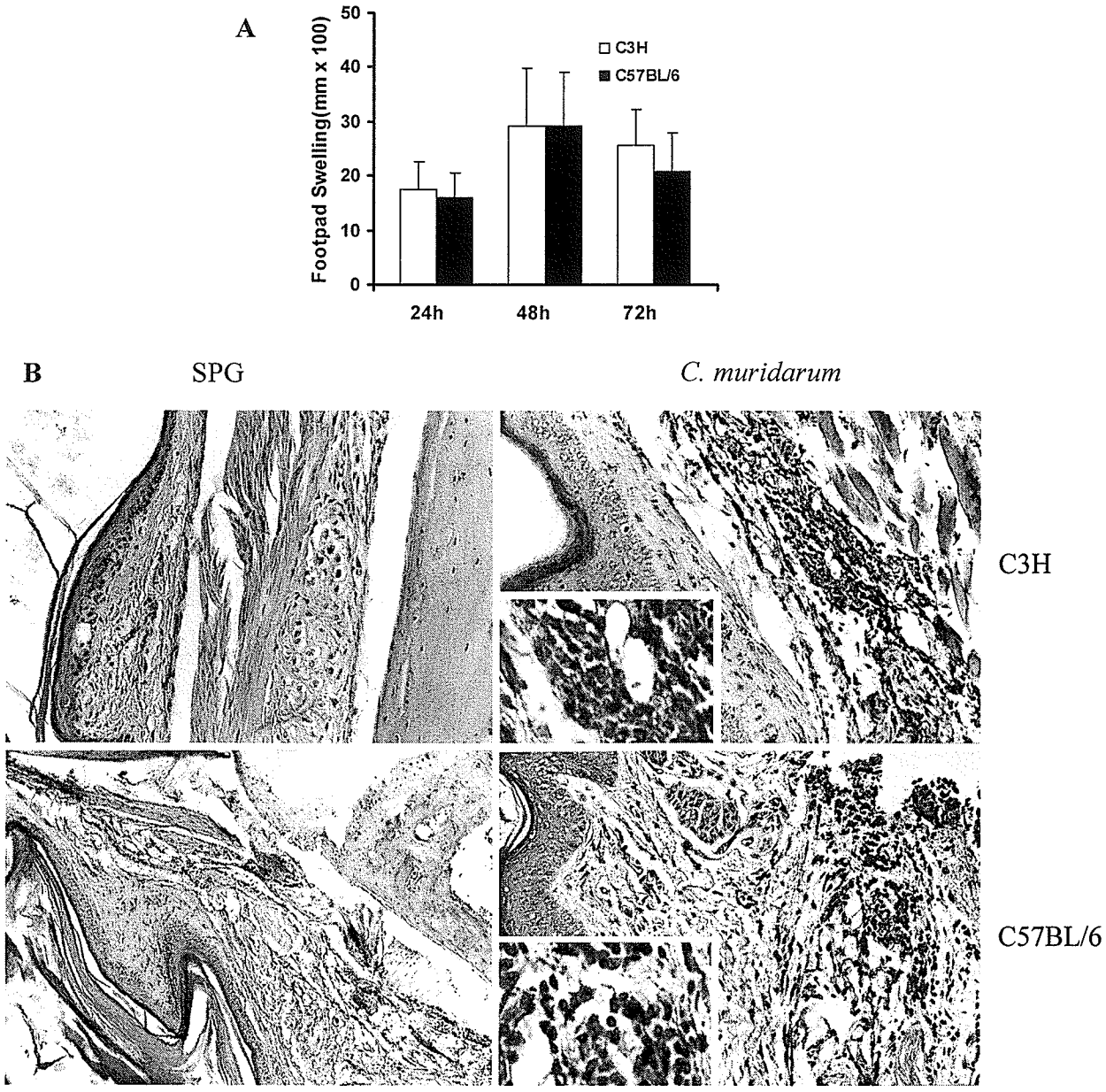


Figure 5. Comparable DTH responses between C3H and C57BL/6 mice. 5A. Mice (7-10 mice/group) were infected intranasally with $1.5X10^3$ IFUs *C. muridarum* MoPn and delayed type hypersensitivity (DTH) was determined at 11 days following infection as described in *Materials and Methods*. Changes in the footpad thickness at various times following local dead EB challenge were shown as mean $\pm$ SD. 5B. Histological photo shows comparable DTH responses (inflammatory cell infiltration). C3H and C57BL/6 mice were infected with MoPn and DTH response determined as described in *Materials and Methods*. The picture shows footpad DTH at 48 h following dead EB challenge. Pictures were taken under 200x and 400x magnifications

Table 3 Serum *Chlamydia*-specific antibody production following intranasal *C. muridarum* infection

IgG1[a]		IgG2a[a]		IgA[b]	
C3H/HeN	C57BL/6	C3H/HeN	C57BL/6	C3H/HeN	C57BL/6
1.5± 0.5	1.5± 0.4	3.6±0.4	3.1± 0.4	5.1± 1.5	4.2± 1.4

C3H/HeN and C57BL/6 mice (9 mice/group) were infected with *C. muridarum* ($1.5X10^3$) and bled at day 14 following infection. Sera were collected individually and test for MoPn specific antibody production using ELISA as described in *Materials and Methods*. a, Antibody titers were transformed into log10. b. Antibody tires were transformed into natural log (Ln).

Table 4 Antibody production and DTH responses following high dose of *C. muriduram* infection

	IgG1 (Ln)	IgG2a (Ln)	DTH (mmx100, 48h)
C3H	4.18±0.31	7.62±0.56	31±12
C57BL/6	4.55±0.39	6.93±0.60	33±9

C3H/HeN and C57BL/6 mice were infected with *C. muridarum* ($1X10^4$) and bled at day 14 following infection. Sera titer and DTH responses were assayed as described in *Materials and Methods*.

Table 5. Organism-driven cytokine production by spleen and draining lymph node cells (pg/ml)

			IFN-γ	IL-12	TNF-α	IL-4	IL-5	IL-10
Spleen cells	Day 8	C3H	5100±1300	2975±880	977±280	<8	67±64	402±17
		C57BL/6	6500±2700	2667±790	1053±354	<8	531±262*	496±125
	Day 14	C3H	1500±500	1427±699	714±336	25±3	29±11	469+121
		C57BL/6	1500±600	969±561	564±365	24±11	201±55*	361±124
Lymph node cells	Day 8	C3H	20800±7200	3571±312*	846±226	< 8	31±28	297±143
		C57BL/6	24900±6800	2666±317	971±293	<8	146±70*	212±85
	Day 14	C3H	23500±8700	2564±885	972±196	38±12	74+40	168±32
		C57BL/6	18400±8300	2361±1326	841±450	31±6	251+ 71*	15±11

Cytokine production by spleen and draining (mediastinum) lymph node cells following organism-specific re-stimulation. C3H and C57BL/6 mice (4 mice/group) were infected with *C. muridarum* at 1.5 x 10^3 IFUs and sacrificed on day 8 or day 14 post-infection. Spleen and lymph node cells were cultured separately in the presence of UV-inactivated EBs for 72 h or 120 h (IL-10) and cytokine levels in the culture supernatants were measured by ELISA. Data are shown as mean ± SD in each group of mice. A representative experiment of three independent experiments with similar results is shown. *, $p < 0.01$

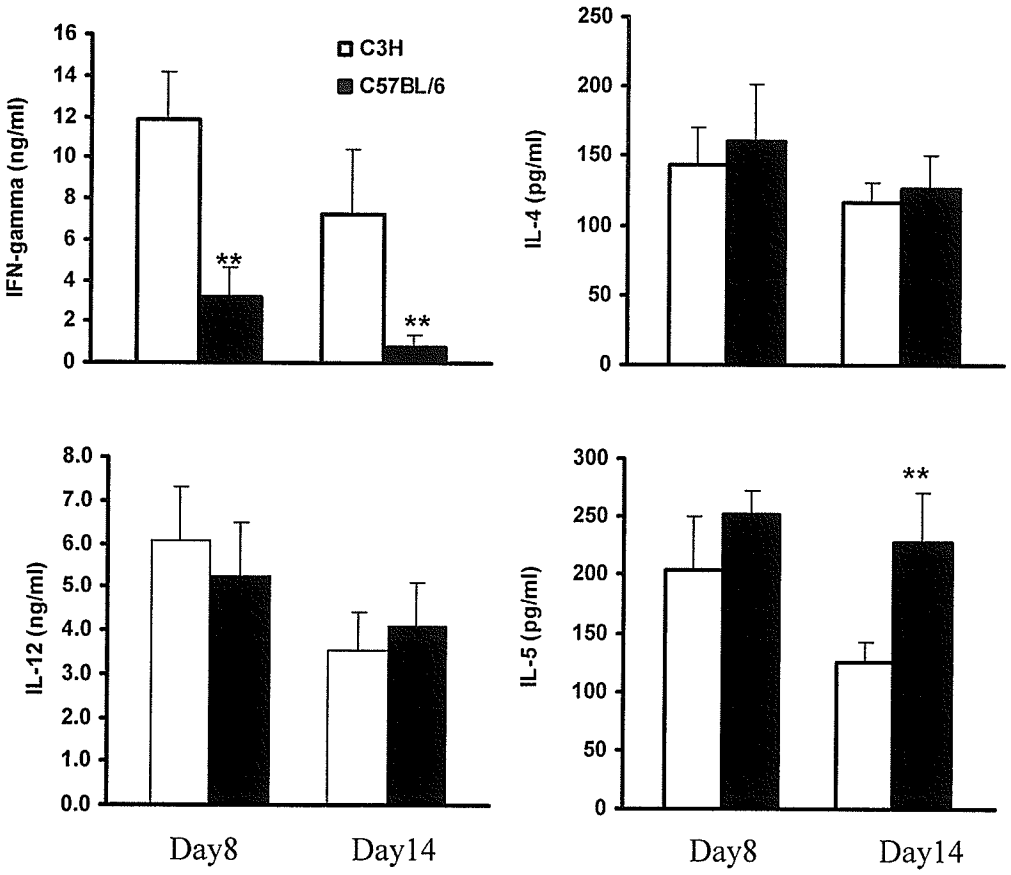


Figure 6. C3H mice showed higher IFN-γ and lower IL-5 in infected lungs post-infection. C3H and C57BL/6 mice (4 mice/group) were infected with *C. muridarum* at 1.5 x 10^3 IFUs and were sacrificed on day 8 or day 14 post-infection. Supernatants from homogenized lungs were used for cytokine measurement by ELISA. Data are shown as mean± SD in each group of mice. A representative experiment of three independent experiments with similar results is shown. *, $p < 0.05$, **, $p < 0.01$

RESULT 3 The lung tissues of C3H mice showed more severe inflammation and neutrophil infiltration after infection, which is not associated with better protection against chlamydial infection

Replication of *Chlamydia* in infected lungs causes extensive inflammation as a result of infiltration by neutrophils, macrophages and lymphocytes and of increased inflammatory cytokine and chemokine production in local tissues [127, 152]. Here, we found that the inflammation is correlated with the severity of infection. As shown in Figure 4, both in early (day 8) and late stages (day 14) of infection, *Chlamydia* susceptible C3H mice showed significantly more lung inflammatory cell infiltration. Correspondingly, significantly greater proinflammatory cytokines IL-6 and TNF-α were found in the lungs of C3H mice than in those of C57BL/6 mice (Fig. 7). There was no difference in the production of anti-inflammatory cytokine IL-10. Both C3H and C57BL/6 mice showed increased expression of IL-1β after infection compared to naïve mice, although only in C3H mice did the augmented IL-1β expression persisted to later stages of the infection (day 14). This was correlated with the load of *Chlamydia* in different strains of mice, with C57BL/6 mice already cleared most of the pathogens and a stimulation from the organism was decreased at a later stage of infection. Also shown in Figure 4, C3H mice contained significantly more neutrophils in the infected lungs at day 8 and day 14. We further confirmed the pathological result using cytological approaches. We examined the percentage and the absolute numbers of infiltrated neutrophils in the lungs post *C. muridarum* infection through lung digestion and cell counting (Fig. 8). In both strains of mice, *C. muridarum* infection promoted greatly neutrophil infiltration in the lungs. However, it was found that the lungs of C3H mice contained more neutrophils,

both in the absolute number and the percentage, than in those of C57BL/6 mice. The results indicate that chlamydial infection induces significant inflammatory cell infiltration, and that the recruitment of different patterns of the inflammatory cells depends on the genetic background of the host. Next, we asked why the neutrophil infiltration differs in different strains of mice during infection. Because the proinflammatory cytokines IL-1β, IL-6 and TNF-α are able to induce chemokine production and trigger inflammatory cell infiltration, we analyzed the expression of particular CXC chemokines which are important to the recruitment of neutrophils. As illustrated in Figure 9, although the lungs of both strains of naïve mice expressed very low mRNA of MIP-2, LIX and KC, expression of these chemokines was up-regulated significantly 2 days after infection. In line with the infiltration of neutrophils, the expression of these chemokines was higher in C3H mice than in C57BL/6 mice. Particularly, the expression of MIP-2 in C3H mice was consistently higher than that in C57BL/6 mice throughout the infection period, while that of LIX and KC was not significantly different between these two strains of mice after 7 days post infection. Generally, CXC chemokine expression in the infected mice was correlated with the neutrophil infiltration, which suggested that CXC chemokines participate in the recruitment of neutrophils into the lungs of infected mice. Given that CXCR2 is an important receptor of CXC chemokines that attract neutrophils during inflammatory responses, we also analyzed the level of CXCR2 mRNA in the lungs and found that although it was very low in naïve mice, transcription of the CXCR2 gene was greatly up-regulated after infection in both strains of mice (Fig9). However, the expression of CXCR2 is much higher in C3H mice than in C57BL/6 mice during infection, and the expression pattern of CXCR2 is similar to that of MIP-2. The increased

expression of chemokine receptor as well as enhanced levels of corresponding chemokines, might have contributed to the constant higher neutrophil infiltration in C3H mice following infection.

Although C3H mice showed extensive neutrophil infiltration after chlamydial infection, a significantly higher bacterial load was found in the lungs of C3H mice compared to those of C57BL/6 mice. To investigate the role of neutrophils in chlamydial infection, we used CXCR2-/- mice (Balb/c background) and examined neutrophil activity. As shown in Figure 10, although both CXCR2-/- and wild type (WT) Balb/c mice showed similar titers of *C. muridarum* in the lungs following infection, there was much less neutrophil activity detected in the lungs of CXCR2-/- mice (Figure 10). These results indicate that neutrophils are not critical for host resistance in Chlamydial infection.

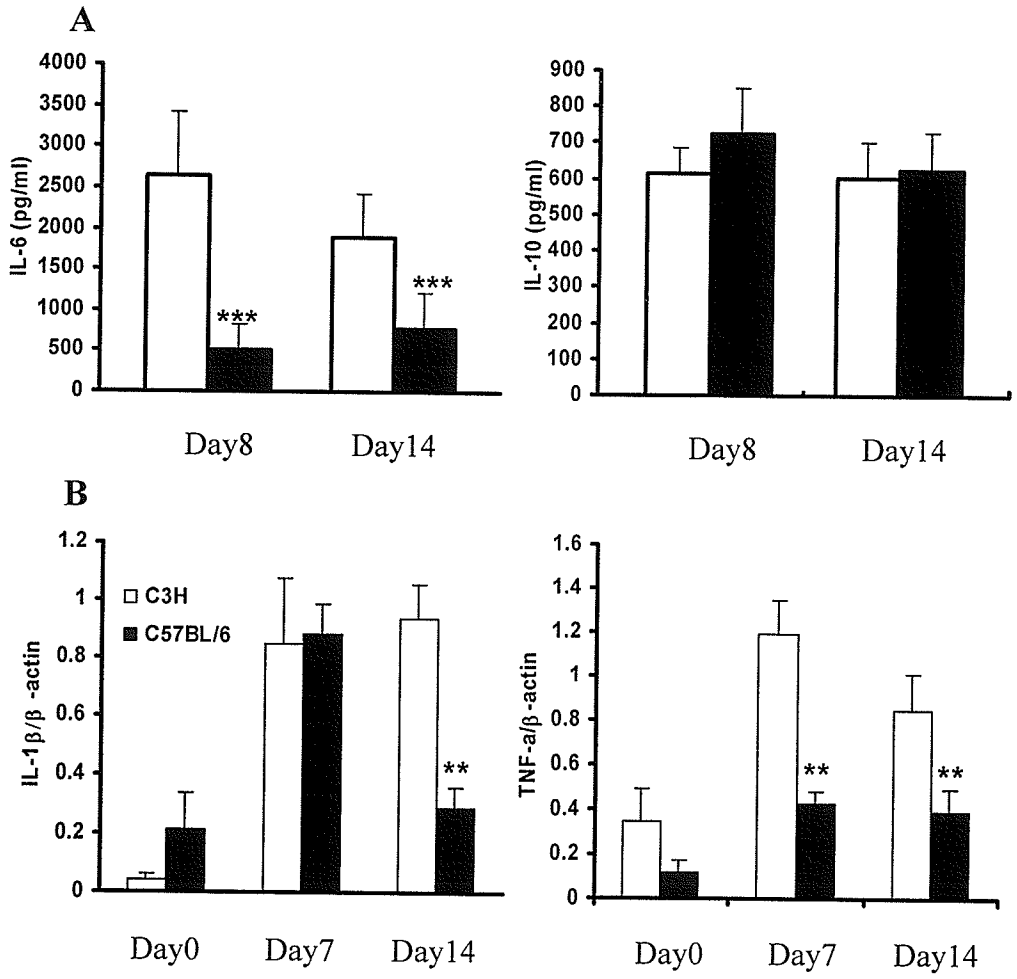


Figure 7. C3H mice showed higher proinflammatory cytokine expression in the lung following *C. muridarum* infection. A. Mice were infected with 1.5 x 10^3 IFUs *C. muridarum* and homogenized lungs were subjected to cytokine ELISA. B. Mice were infected with 3 x 10^3 IFUs *C. muridarum* and fresh lung tissues were collected at 7 and 14 days post-infection and snap frozen in liquid nitrogen. Total RNA from frozen lungs was isolated, RT-PCR of cytokine mRNA was performed as described in the *Materials and Methods*. The data are presented as mean± SEM of the band density of test samples as a percentage of that given by β-actin. * $p < 0.05$ and ** $p < 0.01$: comparison between C3H and C57BL/6 mice.

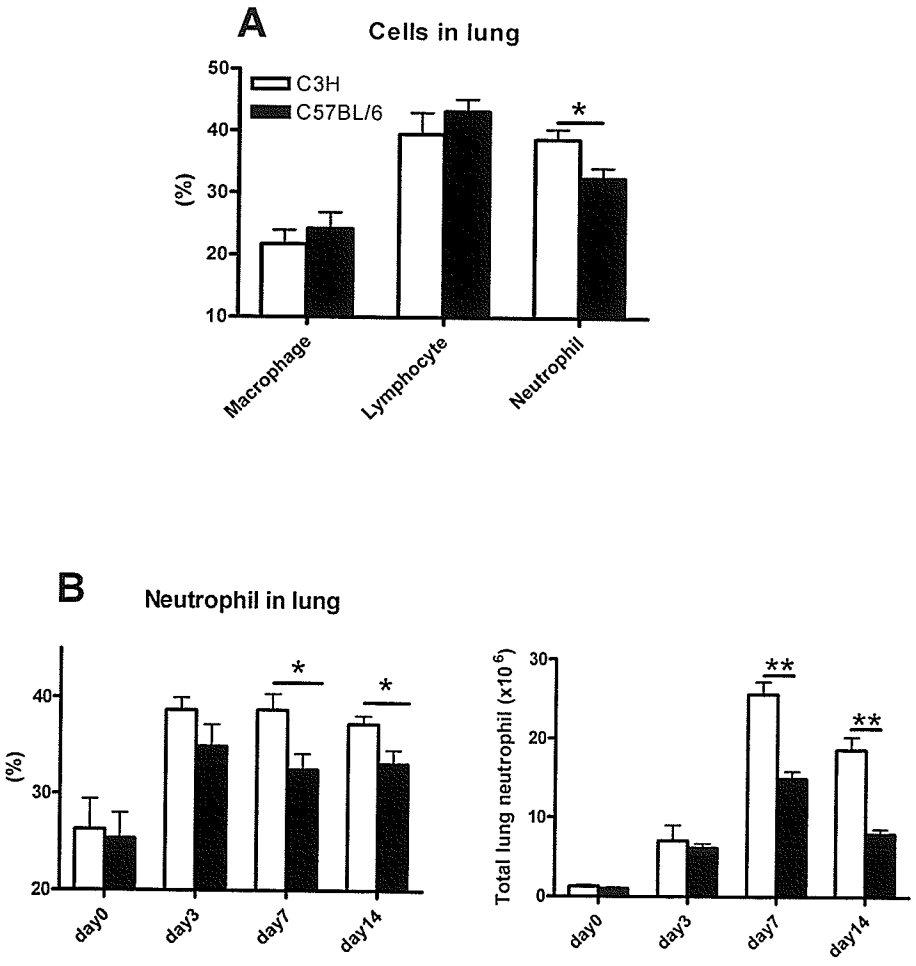


Figure 8. Significantly higher neutrophil infiltration in the lungs of C3H than in those of C57BL/6 mice after chlamydial infection. C3H and C57BL/6 mice (14 mice per group) were infected with 3,000 IFUs *C. muridarum* MoPn and killed at specific days following infection. The lungs were digested with collagenase and the recovered cells were enumerated post staining. A. Different cell populations in the lungs at 7 days post-infection. B. Neutrophils in the recovered lung cells. Data are presented as mean $\pm$ SEM. * $p < 0.05$: comparison between C3H and C57BL/6 mice.

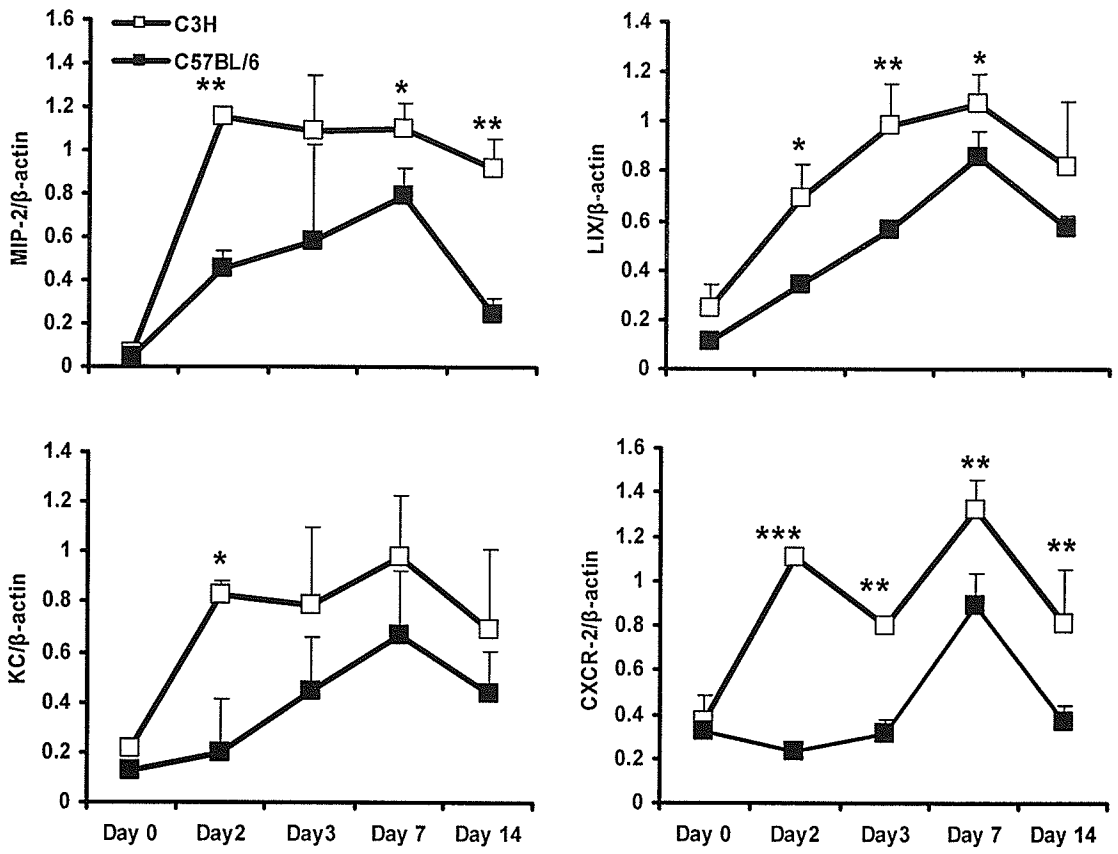


Figure 9. C3H mice displayed more chemokine/chemokine receptor expression in the lungs following infection. C3H and C57BL/6 mice were infected with 3, 000 IFUs *C. muridarum*. Fresh lung tissues were collected and frozen in liquid nitrogen. Total RNA was isolated with TRIzol Reagent. Chemokine and chemokine receptor mRNA expression were analyzed by RT-PCR as described in *Material and Methods*. The data are presented as mean ± SEM of the band density as a percentage of that given by β-actin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

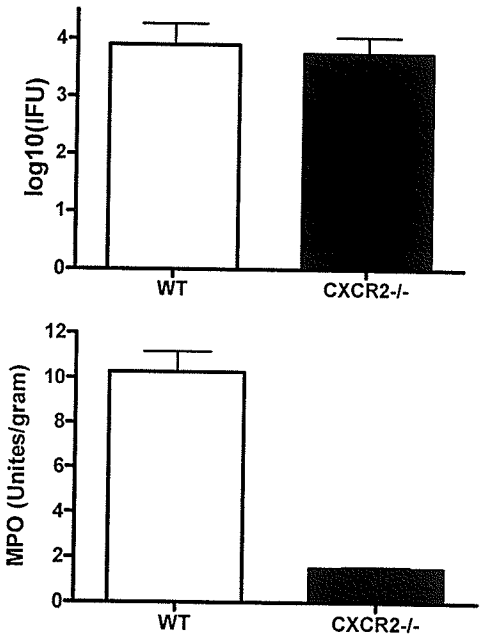


Figure 10. CXCR2-/- mice exhibited comparable levels of infection to wild type mice but less neutrophil. CXCR2-/- mice and control wild type BALB/c mice (4 mice/group) were infected intranasally with 1 x 10^3 IFUs of *C. muridarum* and killed on day 7 post-infection. The levels of chlamydial infection in the lungs and MPO activities were analyzed as described in the *Materials and Methods*.

RESULT 4 Susceptibility to C. muridarum lung infection in C3H mice is largely due to the innate immune responses

C3H mouse lymphocytes cultured with *C. muridarum in vitro* produced Th1 and Th2 cytokines, DTH responses and antibody responses which were similar to those of C57BL/6 mice. These results indicate both strains of mice mounted adaptive immune responses to an equal degree. However, since C3H mice suffered a more serious disease compared to C57BL/6 mice, it was prudent to ascertain whether both strains of mice possess inherent difference in innate immunity to chlamydial infection. In order to address this question, we tested the kinetics of *C. muridarum* growth *in vivo* following infection. As shown in Figure 11, 2 days after infection, C3H mice harboured significantly more organism in the lungs compared to C57BL/6 mice, and differences in the bacterial load were observed to be increased during subsequent days of the infection. For example, at day 6 post-infection, the lungs of C3H mice contained 10-fold higher in the amounts of *C. muridarum* than those of C57BL/6 mice. We also tested cytokine production at 3 days post- infection. As shown in Figure 12, except that C3H mice contained a higher titer of *C. muridarum* in the lungs, there was no significant difference in the levels of cytokines released between C3H and C57BL/6 mice. It should be pointed out that IFN-γ production in C3H mice was found to be higher than that in C57BL/6 mice, albeit not statistically significant. These data indicate that compared to C57BL/6 mice, C3H mice seem to acquire a deficiency in the innate immune system which may contribute to their greater susceptibility to chlamydial infection. The difference in cytokine production, especially the production of IFN-γ does not appear to the reason for the differential susceptibility.

Because there was no difference in IFN-γ production after infection, we tested other possible innate immune mechanisms that may regulate immune resistance to Chlamydial infection. gp91 is an important component of the NADPH oxidases in resident phagocytes and catalyze the production of superoxide ($O2^-$), a source of massive reactive oxidants (oxidized halogens, free radicals, and singlet oxygen), which is important in killing microorganisms. IDO is a critical enzyme in regulating the consumption of tryptophan, a necessary amino acid for *Chlamydia* growth. As shown in Figure 13, both naïve C3H and C57BL/6 mice produced comparably low levels of gp91 and IDO mRNA. However, 7 days post-infection, both strains of mice showed highly elevated expression of gp91 and IDO. There was no significant difference in the expression of gp91 and IDO between the lungs of C3H and of C57BL/6 mice. On day 14 post-infection, there was no difference in IDO expression, while that of gp91 was higher in C3H mice than in C57BL/6 mice, which was correlated with an increased number of *Chlamydia* growth in the former. As an obligate intracellular bacterium, *Chlamydia* needs living host cells for their replication. As such, host cell death would suppress the growth of *Chlamydia*. We tested the expression of apoptosis-related molecules. As evident in Figure 14, FAS and FASL were not differentially expressed in the both strains of mice at day 7 post-infection. Similarly, expression levels of perforin and granzymes A and B in C3H mice was similar to that of C57BL/6 mice. The expression of perforin and granzyme A was even higher in the lungs of C3H mice, which may correlate with higher levels of IFN-γ produced in C3H mice.

Since IFNγ-induced p47GTPase is important in suppressing the growth of many intracellular pathogens [143-146], we analyzed expression of all available p47GTPase

genes during the course of the infection. As shown in Figure 15A and B, at 7 days post-infection, the expression of 6 different p47GTPases in the lungs of C3H and C57BL/6 mice appeared similar, which may suggest that expression of the GTPases may not play a role on the differential susceptibility of C3H and C57BL/6 mice to chlamydial lung infection.

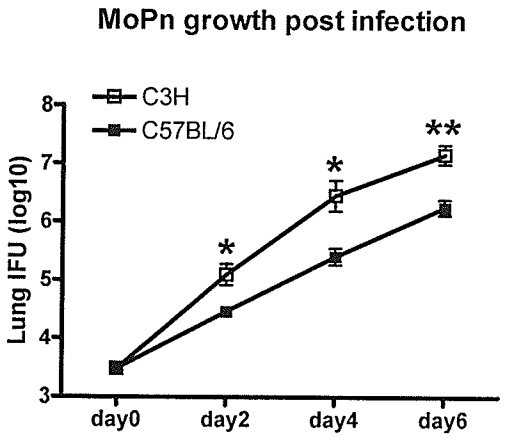


Figure 11. ***C. muridarum*** **growth at the early stages post infection.** 4-5mice /group / time were infected with 3,000 IFU and euthanized at 2, 4 and 6 days post-infection. Bacterial titers were assayed as mentioned in *Materials and Methods*. * $p < 0.05$; ** $p < 0.01$

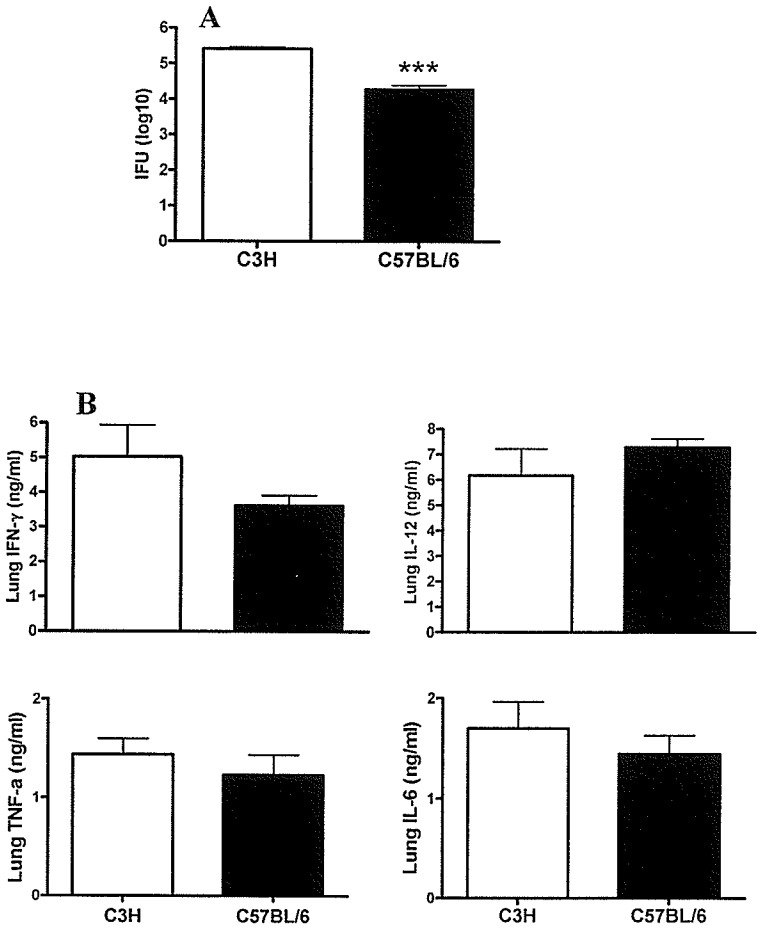


Figure 12. Differences in *Chlamydia* growth in C3H and C57BL/6 mice at 3 days post-infection despite comparable cytokine production in the lungs. Four mice/group were infected with 3,000 IFUs and killed at day 3 post infection. *C. muridarum* IFUs and lung cytokines were measured as mentioned in *Materials and Methods*. A. Chlamydial growth in the lungs. B. Cytokine production in the lungs. *** $p < 0.001$

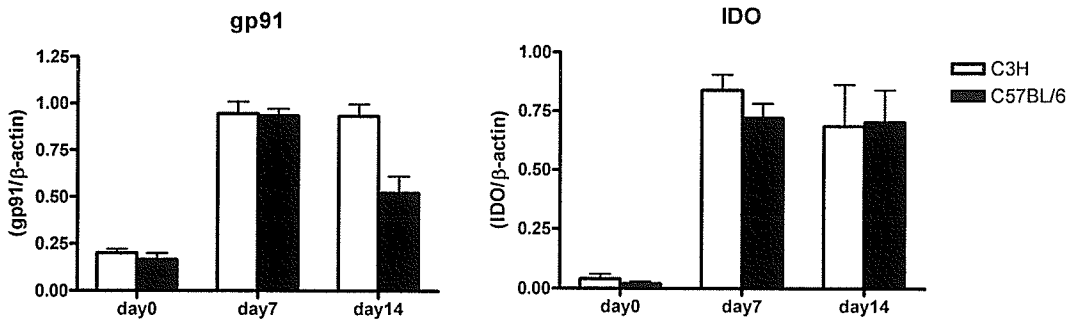


Figure 13. Similar expression of gp91 and IDO in the lungs of C3H mice and C57BL/6 mice. C3H and C57BL/6 mice were infected with 3,000 IFU *C. muridarum*. Fresh lung tissues from naïve mice and infected mice were collected and frozen in liquid nitrogen. Total RNA was isolated with TRIzol Reagent. mRNA expression were analyzed by RT-PCR as described in *Material and Methods*. The data are presented as mean ± SEM band density of gp91/IDO as a percentage of that given by β-actin.

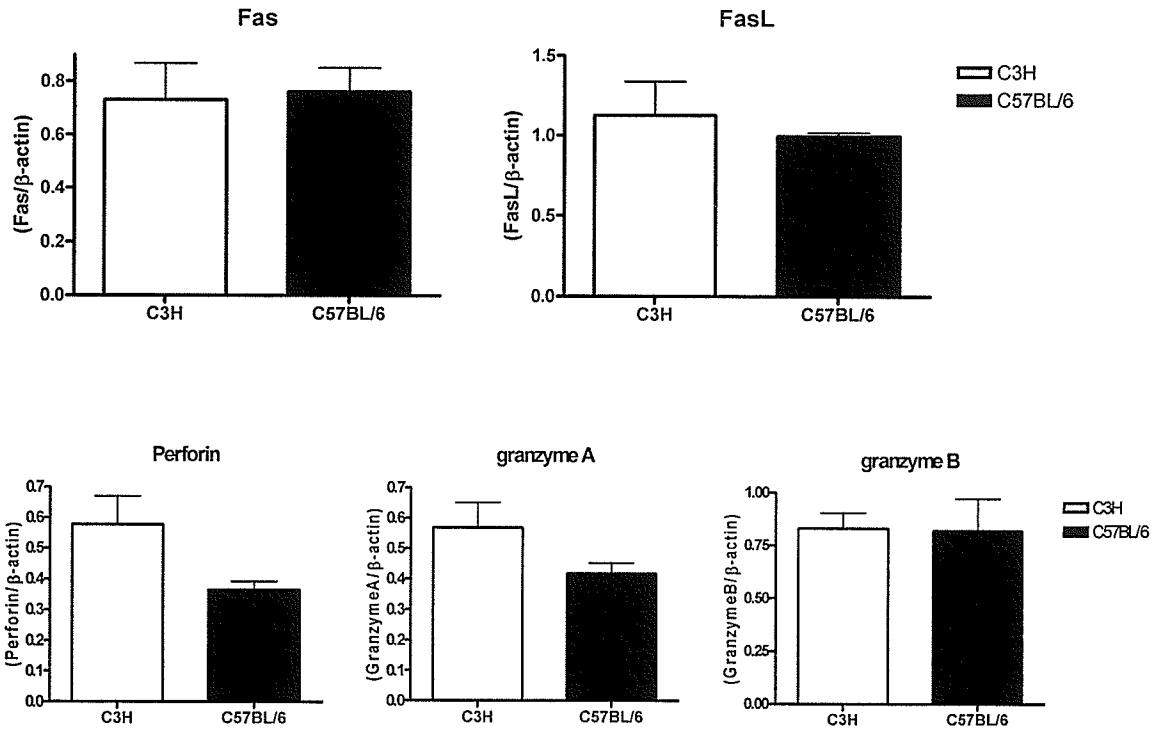


Figure 14. Similar mRNA expression of cytotoxic molecules in the lungs of C3H mice and C57BL/6 mice. C3H and C57BL/6 mice were infected with 3,000 IFU *C. muridarum*. Fresh lung tissues from naïve mice and infected mice were collected and frozen in liquid nitrogen. Total RNA was isolated with TRIzol Reagent. mRNA expression were analyzed by RT-PCR as described in *Material and Methods*. The data are presented as mean ± SEM band density of cytotoxicity molecules as a percentage of that given by β-actin.

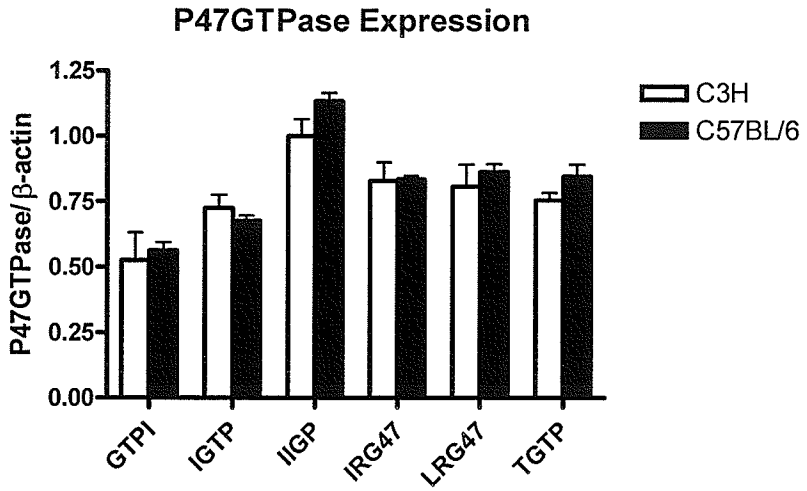


Figure 15. Similar expression of p47GTPases in the lungs of C3H mice and C57BL/6 mice. C3H and C57BL/6 mice were infected with 3,000 IFU *C. muridarum*. Fresh lung tissues obtained from the infected mice on day 7 post-infection were collected and frozen in liquid nitrogen. Total RNA was isolated with TRIzol Reagent. mRNA expression were determined by RT-PCR as described in *Material and Methods*. The data are expressed as mean ± SEM band density of p47GTPases as a percentage of that given by β-actin.

RESULT 5 Chlamydial growth in ex vivo cultured macrophages from C3H mice was less inhibited by exogenously provided IFN-γ

The finding that C3H mice exhibit similar Th1-type immune responses to C57BL/6 mice but suffer a more serious disease than the latter appeared contradictory to the protective role of Th1 responses, especially of IFN-γ, in chlamydial infection. Because the differential susceptibility appears to be due to a defect in innate immunity, it raised the question as to whether host cells such as macrophages from the two strains of mice respond equally to IFN-γ to suppress chlamydial growth. In an attempt to this question, we tested the *in vitro* growth of *C. muridarum* in *ex vivo* cultured macrophages from C3H and C57BL/6 mice in the absence or presence of exogenously provided recombinant IFN-γ (rIFN-γ). Peritoneal macrophages collected from the naïve mice of the two strains were infected *in vitro* with *C. muridarum* and various concentrations (1 or 10 U/ml) of rIFN-γ were added to the culture before or after inoculation. In the absence of rIFN-γ, macrophages from both C3H and C57BL/6 mice were found to be infected with *C. muridarum* and chlamydial inclusions were readily measurable using an immunocytochemical method as described in *Materials and Methods*. In the presence of rIFN-γ before or after infection, chlamydial inclusions in the macrophages from both strains of mice were still readily measurable, but the levels of chlamydial growth within the cells (*i.e.*, the number of chlamydial inclusions per well) showed a dramatic decrease in a dose-dependent manner, with pre-infection treatment of rIFN-γ showing stronger inhibition of organism growth. However, the degree of inhibition of rIFN-γ on *C. muridarum* growth in *ex vivo* cultured macrophages was significantly different in the two strains of mice. The difference was more significant when low levels of exogenous rIFN-

γ (1 U/ml) were present in the culture (Figure 16A and B). Thus, the results suggest that the macrophages from C3H mice were less responsive to the inhibitory effect of rIFN-γ on chlamydial growth compared to those from C57BL/6 mice.

To further elucidate the mechanism underlying the less effectiveness of rIFN-γ in inhibiting *C. muridarum* growth in C3H mice, we examined the production of NO by the infected macrophages from the two strains of mice following exogenous rIFN-γ stimulation. NO production has been shown to be important in host defense against microbial infection including *Chlamydia*. As shown in Figure 16C and D, *Chlamydia*-infected macrophages from C3H mice produced significantly lower levels of NO than C57BL/6 mice in the presence of various doses of rIFN-γ. To confirm the role of NO in the inhibition of chlamydial growth, we examined the effect of AG, an analogue of L-arginine, which is a potential inhibitor of iNOS [367, 368], on rIFN-γ-induced inhibition of *C. muridarum* growth in macrophages. As shown, in both C3H and C57BL/6 mice, following rIFN-γ stimulation, NO production in samples treated with AG was significantly lower than in those without (Fig. 17A). Also, the decreased NO production by AG was correlated with an increase in chlamydial growth (Fig. 17B). The results therefore imply that lower levels of NO produced by the infected cells following rIFN-γ stimulation might be one of the reasons for the less inhibition induced by rIFN-γ in chlamydial growth observed in C3H mice. Since LPS is a potent stimulator of macrophages, we also examined the role of LPS-mediated NO production on chlamydia-infected macrophages. Interestingly, although chlamydial growth *in vitro* was less in LPS-activated macrophages from C3H mice, we did not find a difference in LPS-triggered NO production in macrophages, lungs or sera from either strain of mice,

suggesting that factors other than NO play a role in suppressing chlamydial growth (Fig. 18). We also tested the production of NO in the sera and lungs of infected mice and there were no difference between those two strains of mice (Fig 19). Probably NO was produced not only by macrophages *in vivo*.

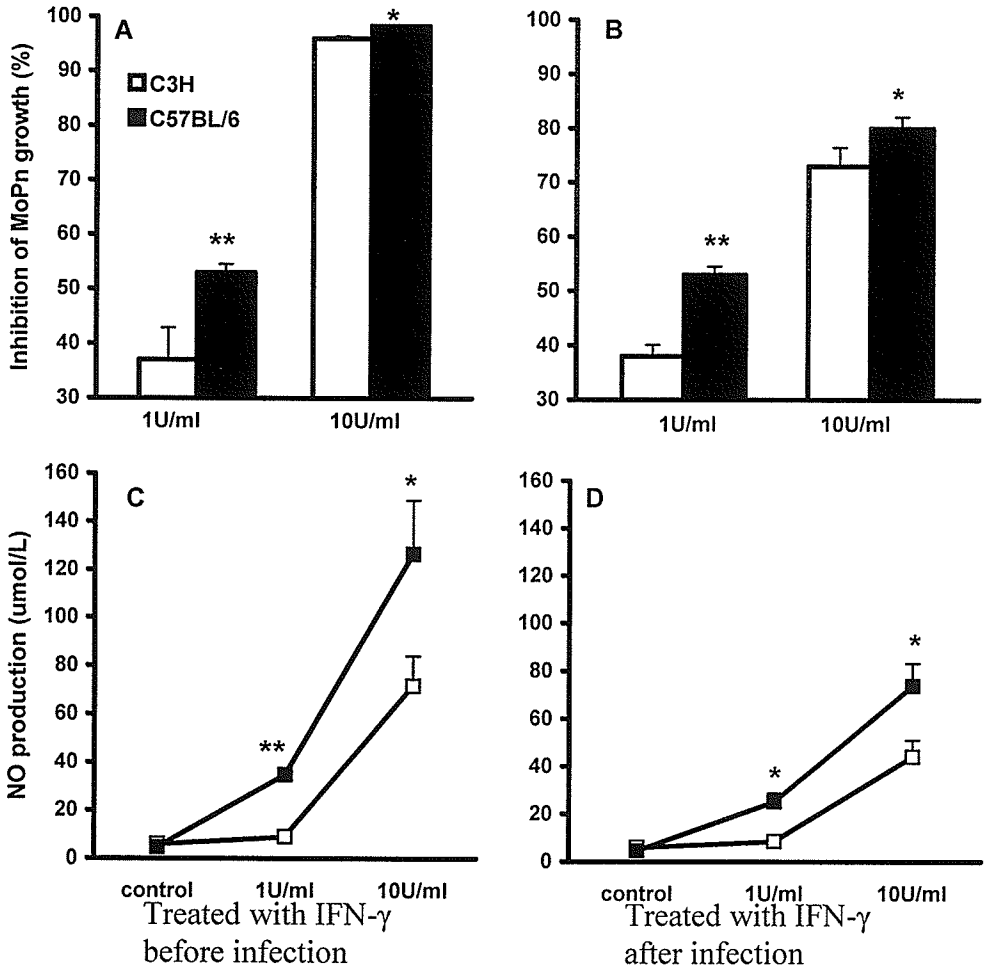


Figure 16. Inhibitory effect of rIFN-γ on Chlamydial growth in cultured peritoneal macrophages from C3H and C57BL/6 mice and its association with NO production. Peritoneal macrophages were collected from C3H and C57BL/6 mice following thiogycllate (TG) injection. Macrophage monolayers in 96-well culture plates were inoculated with *C. muridarum* EBs and cultured in the presence or absence of various concentrations of rIFN-γ for 48 h. Chlamydial inclusions in the macrophage monolayers were stained and counted the same way as those in cultured Hela 229 cells (*Materials and Methods*). Panels A and B. Inhibition of Chlamydial growth in macrophages from C3H and C57BL/6 mice which were treated *ex vivo* with various concentrations of rIFN-γ. on organism growth in macrophages. The percentage of inhibition in organism growth by rIFN-γ in *ex vivo* cultured macrophages was calculated based on the following formula: % = (the number of inclusions in the wells without rIFNγ - the number of inclusions in the wells with rIFN-γ)/ the number of inclusions in the wells without rIFN-γ x 100. Panels C and D. NO levels released in culture supernatants of macrophages infected *ex vivo* with *C. muridarum* which were treated with various concentrations of rIFN-γ at 48 h following infection.

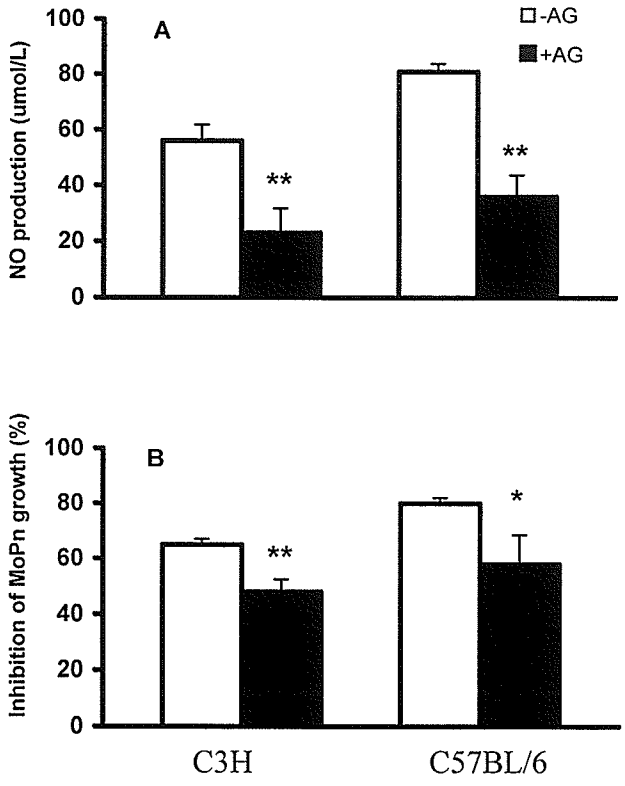


Figure 17. Inhibition of NO production decreases the inhibitory effect of rIFN-γ on chlamydial growth. Peritoneal macrophages collected from C3H and C57BL/6 mice following thioglycollate injection were inoculated with *C. muridarum* EBs and cultured in medium containing 10 U/ml rIFN-γ with or without AG (100 μM) for 48 h. Chlamydial inclusions in the macrophage monolayers were stained and counted as described in *Materials and Methods*. Panel A. NO levels in culture supernatants of *C. muridarum*-infected macrophages treated with rIFN-γ with or without the simultaneous presence of AG. Panel B. The percentage of inhibition of chlamydial growth by rIFN-γ in the presence or absence of AG. ** $p < 0.01$; * $p < 0.05$

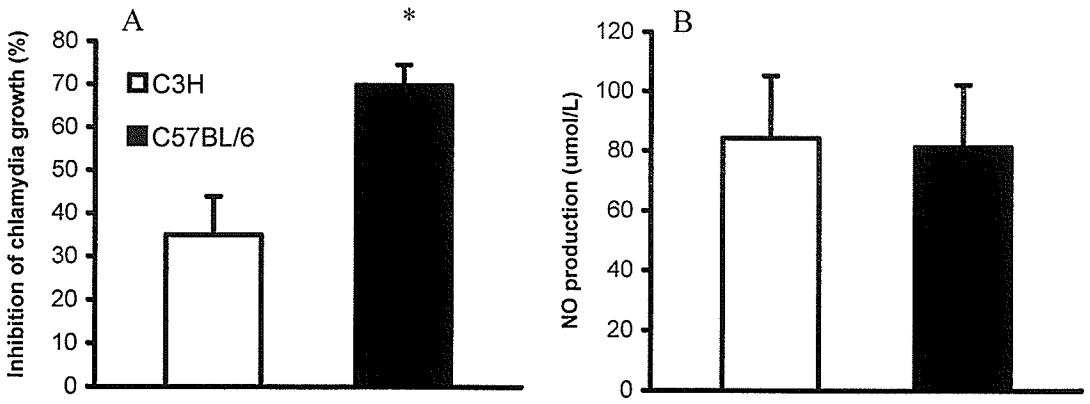


Figure 18. LPS-treated macrophages showed differential ability in inhibiting chlamydial growth. Peritoneal macrophages from C3H and C57BL/6 mice following TG injection were inoculated with *C. muridarum* EBs and cultured in the presence or absence of 1 μg/ml LPS for 48 h. Chlamydial inclusions in the macrophage monolayers were stained and counted the same way as those in cultured Hela 229 cells (*Materials and Methods*). Panel A. LPS-induced inhibition on *C. muridarum* growth in macrophages from C3H and C57BL/6 mice. The percentage of inhibition by LPS on chlamydial growth in *ex vivo* cultured macrophages was calculated based on the same formula described in legend to Figure 16. Panel B. NO levels in supernatants of macrophage cultures infected *ex vivo* with *C. muridarum* in the presence of LPS at 48 h following infection.

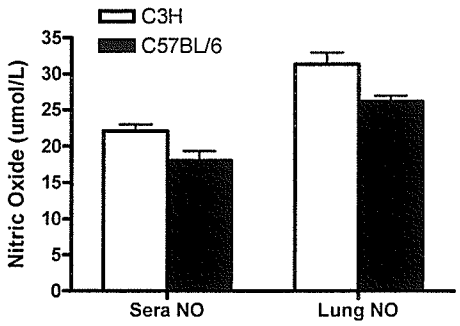


Figure 19. NO production in the sera and lungs of C3H and C57BL/6 mice. 5 mice/group were inoculated (i.n) with 3,000 IFUs *C. muridarum* and killed at day 7 post-infection. NO levels from sera and homogenized lung supernatants were measured by the Griess assay as described in *Materials and Methods*. The data are presented as mean $\pm$ SEM of NO concentrations.

DISCUSSION OF PART I

Here, we show that C3H mice are significantly more susceptible to *C. muridarum* lung infection compared to C57BL/6 mice. The higher susceptibility in C3H mice was demonstrated by higher mortality, greater body weight loss, more organism growth and more severe tissue damage in the lung. Although on one hand, our data, generated using a respiratory tract infection model, extend previous findings in the genital infection model [224, 349-351], they, on the other hand, also reveal the complexity of the mechanisms of host defense against Chlamydial infection. Largely consistent with the findings reported by Darville *et al.* [349-351], we found that C57BL/6 and C3H mice mounted similar levels of Th1-type cytokine production and cell-mediated immune responses following respiratory tract *C. muridarum* infection.

Although it remains unclear as to why C3H mice produce comparable levels of IFN-γ to C57BL/6 mice but suffer a much more serious infection and disease than the latter, our present study has shed some light on this issue. The experiments comparing *ex vivo* cultured macrophages from C3H and C57BL/6 mice showed significantly different degrees of inhibitory effect of IFN-γ on chlamydial growth in macrophages from the two strains of mice. In the presence of the same levels of exogenous IFN-γ, especially when the IFN-γ concentration was relatively low (1 U/ml), the chlamydial growth in the macrophages was significantly less inhibited in C3H compared to C57BL/6 mice (Fig. 16A and B). This lower inhibition by IFN-γ in C3H mice was correlated with lower levels of NO production by these *Chlamydia*-infected macrophages following IFN-γ stimulation (Fig. 16C and D). It has been found that IFN-γ can regulate NO production by macrophages via induction of transcriptional activating factors needed for the expression

of the iNOS gene [369] and that NO plays a critical role in immunity to various pathogens including *Chlamydia* [137, 370-374]. Igietseme *et al.* reported previously that co-culture of *Chlamydia*-specific T cell clones with infected epithelial cells induced NO production, which was correlated with inhibition of Chlamydial growth in the epithelial cell [137]. In addition, the same group documented that delivery of iNOS inhibitors *in vivo* and *in vitro* inhibited the clearance of *Chlamydia* [370]. Therefore, it is likely that the comparable levels IFN-γ produced by C3H and C57BL/6 mice may result in different levels of NO production or those of other inhibitory factors by the infected cells in the different strains of mice, leading to the variation in Chlamydial growth *in vitro* and *in vivo*. It should be noted, however, that our present study did not directly examine the inhibition of IFN-γ on Chlamydial growth in epithelial cells. Since epithelial cell is the major cell type infected by *Chlamydia in vivo*, analysis of this cell population to Chlamydial infection especially its response to IFN-γ in the two strains of mice will shed more light on the basis of the high susceptibility of C3H mice to Chlamydial infection.

Notably, previous studies using the genital infection model have shown some mild differences in host immune responses between C3H and C57BL/6 mice. Darville *et al.* reported a slight switch in the cytokine patterns at the late stage of infection in C3H mice following *C. muridarum* infection [351]. They found that although IFNγ production in local tissues was comparable between C3H and C57BL/6 mice at the early stage of infection (prior to day 10 post-infection), the latter produced significantly higher levels of IFN-γ than the former at the late stage of infection (after day 10 post-infection). In contrast, the production of IL-10, a Th2-related cytokine in mouse, increased at the late stage of infection in C3H, but not in C57BL/6, mice. The authors proposed that the

difference might contribute to the difference of these two strains of mice to genital tract chlamydial infection. Since C3H mice suffered a very serious disease with high mortality following respiratory tract infection even when low dose *C. muridarum* (1.5×10^3 IFUs) was used, the latest time point tested in the present study for cytokine production was day 14 post-infection. Unlike that observed in the reported genital infection model, the production of IFNγ and IL-10 in intranasally infected C3H mice at the late stage of infection (day 14) were still similar to those in C57BL/6 mice, as found at the early stage (day 8) of infection. Therefore, the more serious disease in C3H mice following respiratory tract *C. muridarum* infection can not be explained by a late cytokine pattern switch. Moreover, another study [224] previously showed significantly lower levels of TNFα production in local tissues in C3H mice compared to C57BL/6 mice following genital tract chlamydial infection. The authors argued that the lower TNFα production might be an important reason for the higher susceptibility of C3H mice to chlamydial genital tract infection. Our data from a respiratory tract infection model, however, failed to show this difference. We examined TNFα levels in lung tissues (Fig. 7) and its production by spleen and lymph node cells (Table 5). All of these experiments showed similar levels of TNFα production between these two strains of mice. The reason for the minor difference between our data obtained from the lung infection model and the previously reported data from the genital infection model remains to be determined. However, in addition to the possibility that local tissues of the genital and respiratory tract may respond differently to Chlamydial infection, some other apparent differences exist between the two models: (a) C3H mice in the former (lung) model virtually suffer lethal diseases even after being exposed to a relatively low dose of *Chlamydia*, but no

mortality has been reported in the latter model; (b) the Chlamydial strains used in these studies were different. The previous study used a human serovar, *C. trachomatis* serovar E, while our study used *C. muridarum*, a natural pathogen of mice.

Pro-inflammatory cytokines were found to participate in host resistance to chlamydial infection. By using IL-6 deficient mouse model, Williams *et al.* [158] reported that IL-6 is critical for the host to resist chlamydial lung infection. Neutralization of TNF-α with antibody also significantly increased chlamydial growth and mouse death rate following intranasal inoculation with *C. muridarum* [375]. On the other hand, some other studies argued against the protective role of proinflammatory cytokines in host defense against chlamydial infection [150, 225]. Moreover, TNF-α and IL-1 may also be involved in the pathological responses in chlamydial infection through mediating the production of collagenase and prostaglandin E2 [376], which will lead to tissue destruction and remodeling [377]. In this study, *C. muridarum* infection leads to a release of proinflammatory cytokines such as IL-6, IL-1β and TNF-α in both strains of mice, and that C3H mice expressed significantly higher levels of IL-6 and IL-1β than C57BL/6 mice. These data indicate that the proinflammatory cytokines may have only a marginal role for differential susceptibility to infection. On the contrary, the higher production of these proinflammatory cytokines in C3H mice supports the hypothesis that proinflammatory cytokines enhance lung pathological responses following *C. muridarum* infection.

Some previous studies with intraperitoneal or intravaginal infection mouse models showed that *in vivo* depletion of neutrophils or blocking neutrophil migration promoted chlamydial growth at the early stage of infection, which suggested that neutrophils are

beneficial in chlamydial infection [88]. In our study, employing the lung infection model, we found that C3H mice could not control the growth of *C. muridarum* though they showed significantly greater neutrophil infiltration compared to C57BL/6 mice, in both the absolute numbers and the percentage. Moreover, CXCR2 KO mice showed similar resistance to chlamydial infection as that of wild type mice, though the KO mice exhibited significantly weak neutrophil activity in the lungs. The results suggested that during the lung infection, neutrophils are not efficient in controlling chlamydial infection but may contribute to lung tissue damage via the production of high concentrations of neutrophil proteases and reactive oxygen intermediates. These molecules can destroy structural proteins, which may enhance pathological responses in C3H mice and lead to a more serious disease.

In chlamydial infection, it was reported that MIP-2 presence is correlated with neutrophil infiltration [350, 364]. To further elucidate the mechanism of neutrophil infiltration to infected tissue, we studied the association between neutrophil recruitment and the expression of chemokines. Our data suggested that neutrophil infiltration is highly associated with the expression of chemokines MIP-2, KC and LIX and chemokine receptor CXCR-2. In mouse, both MIP-2 and LIX bind to CXCR-2 and lead to neutrophil chemotaxis and exocytosis [378]. In this study, all chemokines and chemokine receptors were significantly higher in C3H mice than in C57BL/6 mice at the early stage of infection. 7 days post-infection, expression of LIX and KC was comparable in both strains of mice, while MIP-2 and CXCR-2 were still higher in C3H mice than in C57BL/6 mice. The data indicates that the consistent expression of MIP-2 and CXCR-2 help regulate different neutrophil recruitment between the two strains of mice over the

course of infection, while KC and LIX only contribute to the greater neutrophil infiltration in C3H mice at the early stage of infection (day 7).

The data in the present study provide one explanation for the sharp difference between C3H and C57BL/6 mice in their susceptibility to chlamydial infection by showing the difference in inhibition by IFN-γ to Chlamydial growth in the infected cells. This suggests that IFN-γ production and Th1 type immune responses alone may not be sufficient in controlling chlamydial infection. The responsiveness of *Chlamydia*-infected cells to the inhibitory cytokines such as IFN-γ may be important in determining the outcome of infection. The cellular events downstream of Th1 cytokine production such as NO synthesis by *Chlamydia*-infected cells may be important in the genetically determined difference in susceptibility to chlamydial infection. It should be noted, however, that the variation in NO production may not be the only reason for the difference observed in the two strains of mice in the inhibition of chlamydial growth following IFN-γ stimulation. This is because IFN-γ can activate multiple cellular events which may inhibit chlamydial growth [379]. Further studies on these cellular events downstream of IFN-γ stimulation are required to elucidate the mechanisms determining the variation of host susceptibility to chlamydial infection. Recently, Bernstein-Hanley *et al.* reported that the p47GTPase Irgb10 is likely correlated with the susceptibility to chlamydial infection in C3H mice [147]. By using an i.v *C. trachomatis L2* infection model, the authors identified three quantitative trait loci (QTL) - Ctrq-1, 2, and 3, which are correlated with the susceptibility to Chlamydial infection. They mapped Ctrq-3 in mouse chromosome 11 and identified two p47GTPase Igtp and Irgb10 in Ctrq-3 with both p47GTPases regulating *in vitro* embryonic fibroblasts resistance to chlamydial

infection. Because the expression of Irgb10 was differential and much less in C3H mice, it is possible that Irgb10 regulates host susceptibility to chlamydial infection [147, 380]. We also tested the expression of a series of p47GTPase in the lungs after chlamydial infection. Our previous analysis showed no difference in the mRNA expression of 6 p47GTPases including Igtp. Because Irgb10 was recently found to be a member of p47GTPase [147, 381], we have not tested the expression of this gene in mice. It will be interesting to test the *in vivo* level of Irgb10 in both C3H and C57BL/6 mice after chlamydial infection and IFN-γ stimulation.

In general, our present study further emphasizes the complexity of the mechanisms underlying host defense against chlamydial infection and in the development of related diseases. The mechanisms likely involve both the innate and adaptive arms of immunity. Further studies focusing on the both arms will be needed if elucidation of the mechanisms by which *Chlamydia* cause human diseases were to be realized.

PART II

Resistance to Chlamydial lung infection is dependent on MHC as well as non-MHC determinants

Our study shown in Part I and those of others found that C3H/HeN and C57BL/6 were highly variable in their susceptibility patterns to *C. muridarum* lung and genital tract infections [93, 142, 224, 350, 351]. In particular, the results in Part I have shown that, even though both strains of mice displayed similar Th1-like responses, C3H/HeN mice were more susceptible to *C. muridarum* lung infection, as determined by more severe morbidity and higher mortality compared to C57BL/6 mice [93].

As mentioned in introduction, both innate and adaptive immune responses are important in controlling chlamydial infection. Our work and those of others have demonstrated that innate immunity is necessary in the enhanced resistance to chlamydial infection in C57BL/6 mice compared to C3H mice and as such, this may involve NO production and p47GTPase expression [93, 147]. Local synthesis of cytokines and chemokines may also have a role in the protective responses to chlamydial infection [224, 350, 351]. But the role of adaptive immune responses in such differential susceptibility to chlamydial infection is still not clear. Because C57BL/6 (H-2^b) mice and C3H/HeN (H-2^k) mice possess different MHC molecules and that MHC is essential to the initiation of host adaptive immunity, we aimed to further analyze whether different MHC backgrounds influence host susceptibility to chlamydial infection. In the present study, we compared MHC congenic mice, B6.H2K [C57BL/6 background, C3H MHC (H-2^k)] and C3H.H2b [C3H/HeN background, C57BL/6 MHC (H-2^b)] and their corresponding wild type C57BL/6 mice and C3H/HeN mice, respectively, in their susceptibility to *C. muridarum* infection.

RESULT 1 C. muridarum MoPn-infected MHC congenic B6.H2k and C3H.H2b mice showed significantly higher in vivo chlamydial growth, body weight loss and more severe pathological change than C57BL/6 mice

To determine the contribution of MHC and non-MHC genes in host defense against chlamydial infection, we first compared the resistant C57BL/6 and susceptible C3H/HeN mice and their respective MHC congenic mice (B6.H2k and C3H.H2b) in susceptibility to chlamydial lung infection. Body weight changes were evaluated daily following *C. muridarum* MoPn (3×10^3 IFUs) intranasal infection. It was observed that although all strains of mice lost body weight following infection, the degree of weight loss in C57BL/6 mice was significantly less than that endured by both strains of congenic mice, one with background from resistant C57BL/6 but MHC from susceptible C3H/HeN (B6.H2k) and the other with background from susceptible C3H/HeN but MHC from resistant C57BL/6 (C3H.H2b) (Fig. 20). Although all C57BL/6 mice can completely recover within three weeks following infection as we previously reported [93], the congenic mice started to die about 10 days after inoculation (data not shown). On day 8 post- infection, while the average body weight loss in C57BL/6 mice was less than 15%, the congenic mice showed body weight loss of about 25% (Fig. 20). In addition, relative to C57BL/6 mice, the congenic mice also experienced more severe symptoms including shortness of breath, ruffled fur and slow movement. Severity of disease in the congenic mice was similar to that of susceptible C3H/HeN mice. The quantitative analysis of the level of *in vivo* chlamydial growth showed a similar pattern to that of body weight loss. As shown in Figure 21, C57BL/6 mice harboured significantly lower organism load (> 10-fold) in the lungs when compared to the other three strains of mice. Similarly,

C57BL/6 mice also displayed significantly lower bacterial burden in the heart compared to the congenic mice, suggesting higher chlamydial dissemination in the congenic mice (Fig. 21). Interestingly, although the levels of chlamydial growth in the lung were comparable among B6.H2k, C3H and C3H.H2b mice, the former strain showed significantly higher chlamydial growth in the heart than the two latter strains ($p < 0.05$) (Fig. 21).

Histopathological analysis of the lung tissues showed the most severe inflammatory and pathological changes in the lungs of the B6.H2k mice on day 8 post-infection among the four strains of mice. The lung tissues showed dense cellular infiltration and broader areas of lung consolidation under microscopy. The inflammatory cells were seen to infiltrate into interstitial tissue and most alveoli were filled with tissue exudates (Fig. 22A), indicating almost complete lung dysfunction. Consistent with our previous findings [93], the C3H/HeN mice also displayed very severe lung inflammation. The cellular infiltrates mainly comprised of polymorphnuclear cells especially neutrophils but also included mononuclear cells including lymphocytes (Fig. 22C). The congenic C3H.H2b mice showed pathological changes like the wild-type C3H/HeN mice (Fig. 22D). In contrast, lung histology of C57BL/6 mice showed only a narrow area of inflammation and consolidation under microscope (Fig. 22B) with much less polymorphnuclear cells. Collectively, the results showed that the two types of MHC congenic mice, one with MHC (C3H.H2b) and one with non-MHC (B6.H2k) of C57BL/6 mice, were more susceptible to *C. muridarum* infection than wild-type C57BL/6 mice, suggesting the importance of the co-existence of "right" MHC and non-MHC elements in host defense against chlamydial lung infection.

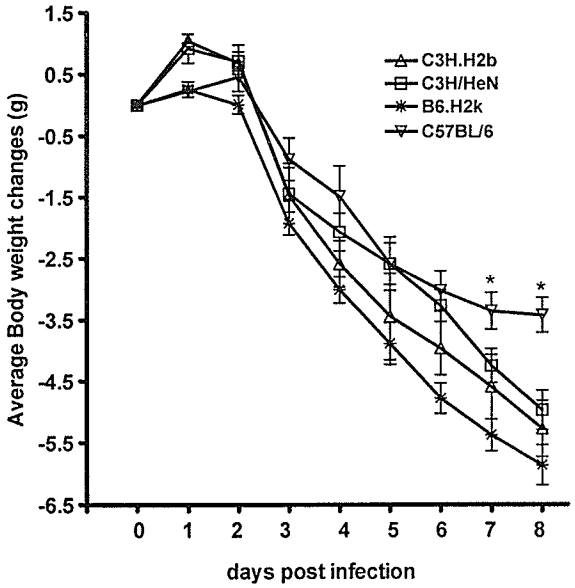


Figure 20. C57BL/6 mice show significantly less body weight loss than MHC congenic (B6.H2k and C3H.H2b) and C3H/HeN mice following *C. muridarum* mouse pneumonitis (MoPn) intranasal infection. Mice were infected intranasally with 3 x 10^3 IFU *C.muridarum*, and measured for body weight changes daily. The data (mean ± SEM) represent pooled results from three independent experiments with similar results (total mice number is 10-12 mice in each group). * $p < 0.05$: C57BL/6 mice versus B6.H2k mice, C3H/NeH and C3H.H2b mice.

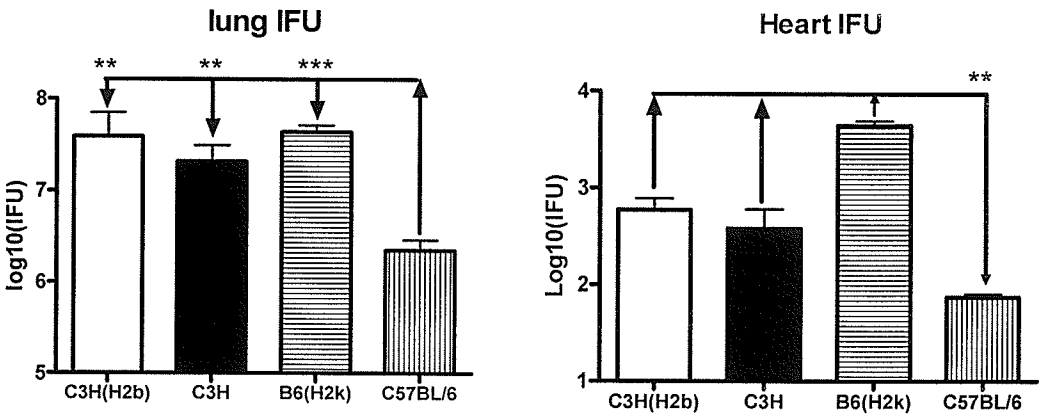


Figure 21. MHC congenic mice show significantly higher organism growth *in vivo* than C57BL/6 mice following intranasal *C. muridarum* infection. Mice (3 to 4 per group) were intranasally infected with 3 x 10^3 IFU *C. muridarum* and sacrificed at day 8 post-infection. The lungs and hearts were collected aseptically from the mice. The levels of *C. muridarum* infectivity in the supernatants of tissue homogenates were measured as described in *Material and Methods*. ** $p < 0.01$, *** $p < 0.001$, the comparison between C57BL/6 mice and B6.H2k or C3H.H2b or C3H/HeN mice. The data (mean ± SEM) is a representative of three independent experiments with similar results.

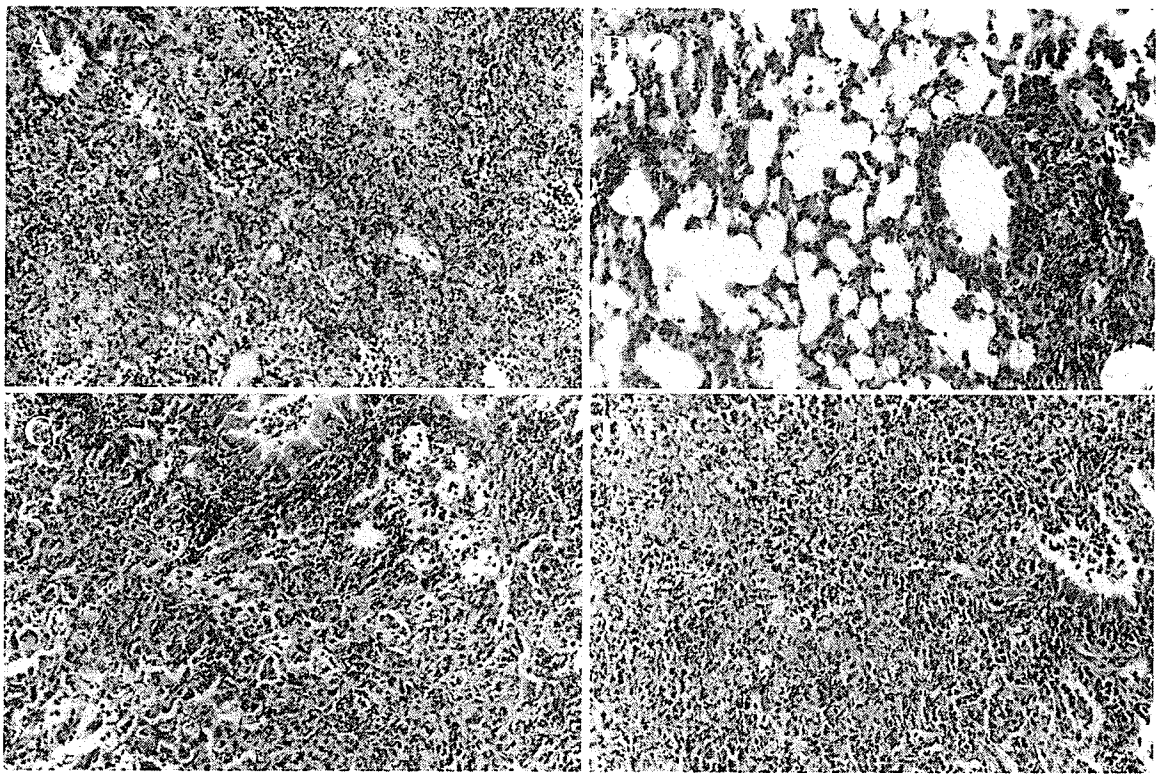


Figure 22. MHC congenic mice showed much more severe inflammation in the lungs than C57BL/6 mice following *C. muridarum* infection. Mice were infected intranasally as described in Figure 19. Lung tissues from infected mice at day 8 post-infection were fixed, embedded, sectioned and stained (H & E) and analyzed under light microscope (200x). Panel A. B6.H2k; Panel B, C57BL/6; Panel C, C3H.H2b; and Panel D, C3H/HeN.

RESULT 2 B6.H2k congenic mice showed lower organism-driven IFN-γ, IL-12, and serum IgG2a response compared to C57BL/6 ($H2^b$) mice following C. muridarum infection

Because congenic B6.H2k mice showed considerably higher *in vivo* organism growth, we compared cytokine production in the draining lymph nodes upon antigenic re-stimulation. As shown in Figure 23, B6.H2k mice produced significantly lower IFN-γ (about 10-fold) and IL-12 (about 3-fold) compared to the wild type C57BL/6 mice. Because the only genetic difference between B6.H2k (H-2^k) and C57BL/6 (H-2^b) mice is the MHC, the results suggest that MHC plays a critical role in determining IFN-γ and IL-12 responses to chlamydial infection. On the other hand, the MHC congenic C3H.H2b mice produced comparable levels of IFN-γ and IL-12 as wild type C3H/HeN mice (Fig. 23). Because C3H.H2b (H-2^b) and C3H/HeN (H-2^k) mice are different in MHC but sharing the same genetic background (non-MHC element), the results suggest that MHC is not the only element which determines production of IFN-γ and IL-12. Conversely, the congenic B6.H2k and wild-type C3H/HeN mice possessed the same MHC but showed significantly different IFNγ and IL-12 production, suggesting a critical interaction between MHC and non-MHC elements in H-2^b mice. Similarly, serum antibody testing also revealed that congenic B6.H2k mice made less IgG2a compared to B6.H2b mice (Fig. 24). However, the titer of serum IgG1 was similar in both strains of mice (Fig. 23). Antibody titers of both C3H.H2b and C3H.H2k mice were similar to that of wild type B6.H2b mice (data not shown). These results indicate that congenic B6.H2k mice produce less Th1-like responses compared to the other three strains of mice.

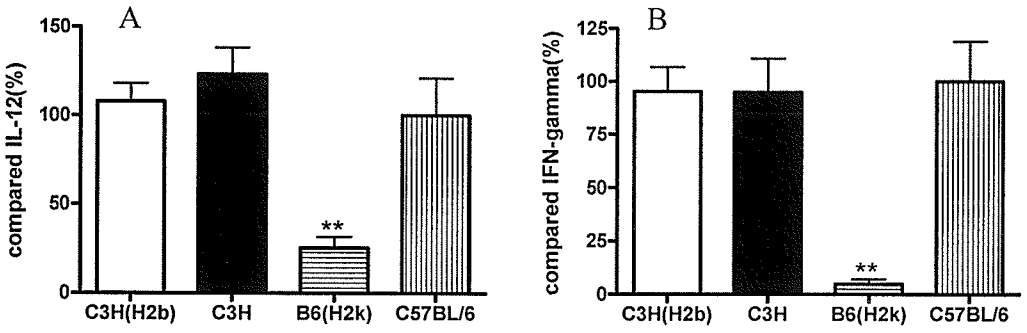


Figure 23. B6.H2k mice showed significantly lower IFN-γ and IL-12 production post infection compared to C57BL/6 and other strains of mice. Mice were infected as described in Figure 19 and single-cell suspensions from the draining lymph nodes were cultured in the presence of UV-killed chlamydia elementary bodies (EB) for 72 h as described in *Materials and Methods*. Cytokine levels of each culture sample were compared to the mean level of C57BL/6 mice cell culture (reference set at 100%) and expressed as the percentage of the reference. The data represent pooled data from three independent experiments with similar results (mean ±SEM). ** $p < 0.01$, C57BL/6 versus B6.H2k, C3H.H2k, or C3H/HeN mice. A. Average concentration of IL-12 in C57BL/6 mice is 1956 pg/ml. B. Average concentration of IFN-γ of C57BL/6 mice is 63.9 ng/ml.

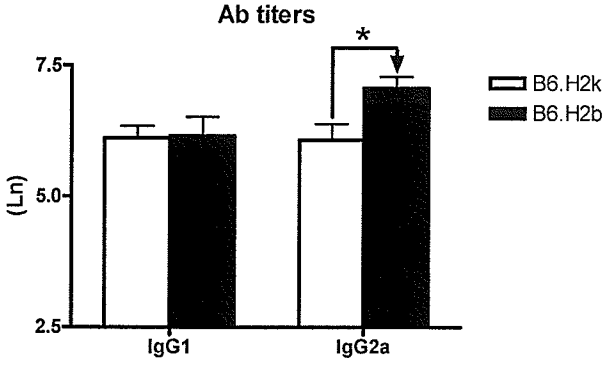


Figure 24. B6.H2k mice showed significantly lower serum IgG2a post-infection compared to wild-type B6.H2b mice. Mice were infected as described in *Materials and Methods* and serum antibody titers were assayed as described in *Materials and Methods*. The data represent pooled data from three independent experiments with similar results (mean ±SEM). * $p < 0.05$

RESULT 3 Congenic and wild-type mice showed similar organism-driven IL-4 and IL-10 production

IL-10 is a regulatory cytokine secreted by macrophages, DC and T cells. IL-10 has been shown to interfere with the antimicrobial activity of protective IFN-γ responses against various pathogens, and is associated with increased susceptibility to chlamydial infection [152, 153, 382]. IL-4 and IL-5 are prototypic Th2 cytokines with capability to inhibit Th1 responses. Because the congenic B6.H2k mice displayed much lower levels of IFN-γ and IL-12, we further analyzed IL-10, IL-4 and IL-5 production to ascertain whether the lower IL-12 and IFN-γ production is caused by a higher synthesis of these Th2-related cytokines. As shown in Figure 25, the 4 strains of mice had similar levels of IL-10 and IL-4 in the draining lymph nodes. Moreover, although the production of IL-5 in C3H mice appeared to be lower than the other three strains of mice (Fig. 25). These results suggest that the change in the MHC gene from $H\text{-}2^b$ to $H\text{-}2^k$ in mice with C57BL/6 background does not have a significant effect on IL-10, IL-4 and IL-5 production following chlamydial lung infection.

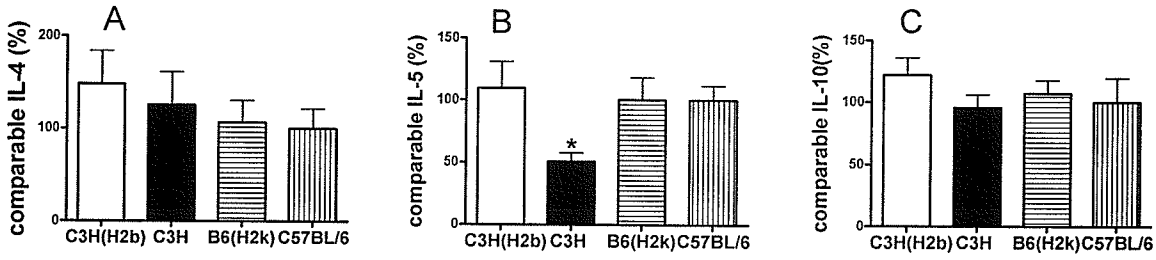


Figure 25. B6.H2k mice show similar levels of IL-4 and IL-10 production post-infection compared to C57BL/6 and other strains of mice. IL-4, IL-5 and IL-10 levels in the cells of draining lymph nodes cultured in the presence of *C.muridarum* EBs as described in Figure 23 were measured by ELISA. Cytokine concentration of each culture sample was compared with the average amount of C57BL/6 mice cell culture (reference set at 100%) and expressed as percentage of the reference. The data represent pooled results from three independent experiments with similar results (mean ± SEM). A. Average concentration of IL-4 in C57BL/6 mice is 6.4 pg/ml. B. Mean concentration of IL-10 of C57BL/6 mice is 576 pg/ml. C. Average concentration of IL-5 of C57BL/6 mice is 249pg/ml. * $p < 0.05$: C3H/HeN versus C57BL/6, B6.H2k, or C3H.H2b mice.

DSICUSSION OF PART II

Our previous study showed that C57BL/6 and C3H/HeN mice displayed highly differential susceptibility to *C. muridarum* lung infection [93] in Part I. Differences in the susceptibility to other infectious organisms has also been reported in these two strains of mice. Because the MHC complex is essential to the initiation and maintenance of host adaptive immunity and in consequence, to the control of progression of infectious diseases, we analyzed the immune responses in different inbred and MHC congenic strains of mice following lung infection with *C. muridarum.* Our results showed that *in vivo* chlamydial growth was significantly higher in congenic B6.H2k mice, which have C57BL/6 background with MHC from the susceptible strain ($H\text{-}2^k$), than in C57BL/6 mice. At the same time, C3H.H2b mice which acquire the same MHC genes as the resistant C57BL/6 mice ($H\text{-}2^b$) failed to show an increase in resistance to *C. muridarum* infection compared to wild-type C3H/HeN mice. Overall, the results suggest that both MHC and non-MHC genes are equally important in host defense against chlamydial lung infection.

While the codependence of MHC and non-MHC linked genes on the determination of cytokine responses and resistance to infection was not previously studied in respiratory tract chlamydial infection previously, it had been examined in other infection models [180, 181, 383-385]. For example, it has been shown that different genetic factors contribute to susceptibility to *Leishmania* infection by different mechanisms mediated by H-2 genes as well as non-H-2 genes [180, 384, 385]. Moreover, using the genital infection model, Tuffrey *et al.* found that some, but not all, tested inbred mouse strains developed salpingitis following genital infection with human

C. trachomatis strain [386]. In particular, they found that both BALB/c (H2d) and congenic BALB/k (H2k) mice showed intact fertility following the infection while infected C3H mice (H2k) were less fertile than naïve mice [386]. Furthermore, using an intravenous infection model, Busonit-Gatel *et al.* reported that both non-H-2 and H-2 gene complex were responsible for the differences in the resistance of various mouse strains to *C. psittaci* infection [387]. In the current study, in sharp contrast to findings in inbred C57BL/6 (H-2^b) and congenic B6.H2k (H-2^k) mice where the MHC appeared to have a prominent role in determining host susceptibility to chlamydial infection, we found no such phenomenon between the inbred C3H/NeH (H-2^k) and congenic C3H.H2b (H-2^b) mice. Specifically, it was evident that an alteration in the MHC gene status ($H2^k$ to $H2^b$) in the C3H/HeN genetic background in congenic C3H.H2b mice can not confer resistance to chlamydial infection, suggesting that there might exist some non-MHC determinants that contribute to the differential susceptibility between C3H/HeN and C57BL/6 mice. Our previous study demonstrated that IFN-γ production alone may not be sufficient and that cellular events downstream of IFN-γ cytokine production such as NO production or other possible microbicidal mechanisms are also important in controlling chlamydial infection [93]. It is likely that these downstream cellular events are determined, at least in part, by non-MHC genes. Moreover, since the association of H-2^b with resistance to chlamydial infection was obvious in C57BL/6, but not in C3H/NeH background, it is likely interdependences of both MHC or non-MHC genes are prudent in dictating the resistance phenotype to chlamydial infection. This notion is further supported by our finding that although B6.H2k mice have non-MHC genes from the resistant mice (C57BL/6), they showed higher dissemination of *Chlamydia* to the heart

than C3H and C3H.H2b mice (Fig. 21). In addition, from the cytokine pattern point of view, the finding that C3H mice produced less IL-5 than other strains of mice (Fig. 25) also suggests the importance of both MHC and non-MHC genes in modulating cytokine production, although the levels of IL-5 did not appear to be correlated with susceptibility to chlamydial infection in this model. Clearly, further studies are required to identify and elucidate the role of MHC and non-MHC determinants, and more importantly, the interaction between MHC and non-MHC genes in regulating immune responses and host susceptibility to chlamydial infection.

To our knowledge, this is the first report on the use of congenic mice to explore the role of MHC and non-MHC elements on host susceptibility to respiratory tract chlamydial infection. Our results suggest that resistance to chlamydial infection is dependent on MHC as well as non-MHC genetic determinants. These data, based on mouse models, may have implications on human studies which aim to elucidate the impact of HLA or other genes, if any, on susceptibility to chlamydial infection. Further work is needed to identify specific genes responsible for these differences. Initially, one may use microsatellite markers and linkage-mapping techniques which have been shown to be useful in the identification of specific loci responsible for resistance to microbial infections [388, 389]. Moreover, as the susceptibility pattern with respect to MHC in chlamydial infection can vary depending on the genetic background of the host, an investigation on the interaction between MHC and non-MHC linked genes is also critical in understanding host resistance to chlamydial infection.

PART III

Type I interferons enhance susceptibility to *Chlamydia muridarum* lung infection by reducing local infiltration, and enhancing apoptosis, of macrophages

IFNIs were named after their role in "interfering" with viral replication in mammalian cells, an essential function for host coping with viral infections. As mentioned before, IFNIs belong to a multiple-member family [254]. IFN-α and IFN-β are the major components of IFNIs in human and rodents, and they share the same receptor known as the IFNα/β receptor (IFNAR), to initiate downstream STAT signal transduction [254, 319]. Physiological levels of IFNIs are very low, but upon stimulation, they are produced in great quantities [254, 309, 390]. IFNα/β are among the earliest cytokines which are induced during infection and play an important regulatory role in both innate and adaptive immune responses. Previous reports have shown that IFNα/β are able to activate cytolytic activity of NK cells [282], activate macrophages [273], promote differentiation of Th1 cells and IFN-γ secretion and [282]. In particular, IFNα/β can activate and modulate the function of DCs, the most important professional APC, via promoting expression of MHC, co-stimulator molecules and secretion of cytokines and chemokines, thus modulating adaptive T cell immune responses [309, 356-358].

Both viral and bacterial infections can promptly and strongly induce release of IFNIs through their specific genomic components or metabolic products. Although the function of IFNs in viral infection has been well documented, the role of IFNs in bacterial infections appears complicated and varies in different infection models [259]. Studies on *Legionella pneumonphila* [334], *Salmonella typhimurium* [271], *Shigella flexneri* [330], *Bacillus anthrasis* [331] and *Mycobacterium tuberculosis* [338] showed that IFNIs are

protective for the host, at least partially through enhancing production of IFN-γ and NO as well as suppressing bacterial invasion. IFN-γ and IL-12 have been suggested to be required for host resistance to chlamydial infection [30, 127, 128, 363, 364]. Previous *in vitro* and *in vivo* studies have demonstrated that IFNIs can inhibit the growth and infectivity of *C. trachomatis* in different cell types [290, 391]. On the other hand, findings from other studies have suggested that IFNIs are detrimental to the infected host. For example, it has been reported that IFNIs can promote infection with intracellular bacteria *Listeria monocytogenes* through increasing listeriolysin O-dependent apoptosis of macrophages and lymphocytes [344]. Studies using IFNα/β receptor KO (IFNAR-/-) mice showed that these mice were more resistant to *L. monocytogenes* infection that WT mice, which was correlated with less lymphocyte apoptosis, and elevated IL-12p70 production in IFNAR-/- mice [341-343]. Along the same line, it has been also shown that a highly virulent strain of *Mycobacterium tuberculosis* can promote IFNI production in the host cells, which enhanced susceptibility to the infection in a mouse model [340]. Further, in certain instances, poly I:C-mediated IFNI production through administration of Poly I:C during *C. trachomatis* lung infection, can accelerate mouse death following infection [345]. Administration of exogenous IFNα has also been shown to enhance hosts' susceptibility to *M. tuberculosis* infection [340].

In the case of *C. pneumonia* infection, bone marrow derived macrophage (BMDMs) from IFNAR-/- mice were found to be more susceptible to *C. pneumonia* infection than those from WT mice, which was correlated with lower IFN-γ and iNOS production [290]. In contrast, IFNAR-/- and WT mice were suggested in another study to possess similar susceptibility to *C. pneumonia* infection [348]. Such controversial results

in chlamydial and other infection studies indicate an absolute necessity to further investigate the role of IFNIs in host resistance or susceptibility to chlamydial infection using especially *in vivo* models.

In the current study, using the mouse model of respiratory tract Chlamydial infection, we examined the susceptibility of IFNAR-/- mice to *C. muridarum*, and possible mechanisms underlying IFNI-regulated immune responses to chlamaydial infection.

RESULT 1 IFNAR-/- mice showed enhanced resistance to C. muridarum lung infection

Following intranasal infection with *C. muridarum*, WT mice displayed continuous body weight loss without signs of recovery when they were infected with relatively higher (1000 IFUs) or lower doses (200 IFUs). On the contrary, IFNAR-/- mice showed much less body weight loss at high dose infection (Fig. 26A) and marginal body weight loss at low dose infection (Fig. 26B). The IFNAR-/- mice regained their original body weight within 7 days after infection (Fig. 26B). In line with the body weight loss, the IFNAR-/- mice showed significantly lower *in vivo* organism growth at day 6 post-infection, when higher dose was used (Fig. 26C). The bacterial loads were similar between the WT and IFNAR-/- mice at days 2 and 4 post-infection. When a lower dose was used, at 14 days post-infection, while the infection in IFNAR-/- mice was close to being resolved (less than 10^3 IFUs in the whole lung), the WT mice showed increase in bacterial burden in their lungs, 1000-fold higher than that in the IFNAR-/- mice (Fig. 26D). We also analyzed the titers of *C. muridaurm* in the other organs and found that

both groups of mice had very few organisms in the hearts, livers and kidneys (Fig. 27). The data overall indicates that *C. muridarum* is restricted in the lungs after i.n. infection of 129 mice. Histological analysis (Fig. 28) showed more intense lung inflammation in WT mice than IFNAR-/- mice since early on in the infection (day 2). A large portion of the infiltrating inflammatory cells of WT mice were neutrophils and a more severe inflammation in WT mice became more evident on days 6 and 7 post-infection. Most of the interstitial tissues were infiltrated with inflammatory cells and most alveoli were filled with inflammatory exudates in WT mice. In contrast, IFNAR-/- mice showed only mild inflammation, with minimal cellular infiltration around some bronchi (Fig. 28). At 14 days post-infection, the IFNAR-/- mice showed close to normal lung structure while the WT mice still exhibited extensive lung tissue damage and diffused cellular infiltration, which was correlated with continuous bacterial growth (Fig. 26D). These results indicate that IFNIs play a promoting role in host susceptibility to *C.muridarum* infection.

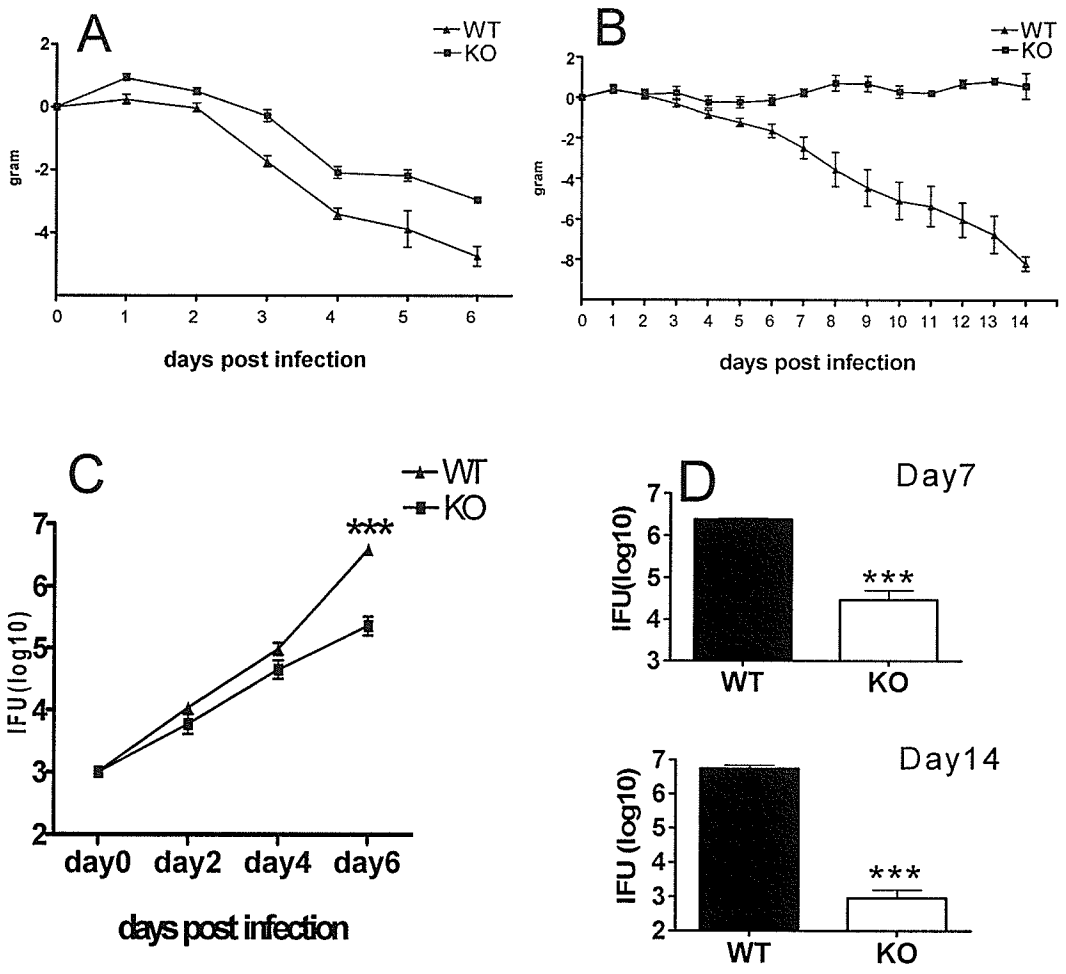


Figure 26. Wild-type mice showed more severe disease after infection. Mice were intranasally inoculated with a designated dose of *C. muridarum*, body weights were monitored daily and compared with the initial weight before the infection. Organism growth in the infected lungs at each time point was assayed as mentioned (*Materials and Methods*). **A.** Body weight changes of 20 mice in each group after infected with 1,000 IFU *C. muridarum*. The initial body weight before infection were comparable between WT and KO mice. **B.** Body weight changes of 8-10 mice of each group after infected with 200 IFU MoPn. This data are representative of three independent experiments that showed similar results. **C.** 6-8 mice of each group at each time point were infected intranasally with 1,000 IFU *C. muridarum*, and Chlamydia growth in the lungs assayed as described (*Materials and Methods*). **D.** 4-5 mice per group were infected with 200 IFU *C. muridarum* and euthanized at day 7 and day 14 separately and Chlamydia growth assayed as above. Each column represents the mean $\pm$ SEM of IFUs (log 10). *** $p < 0.001$

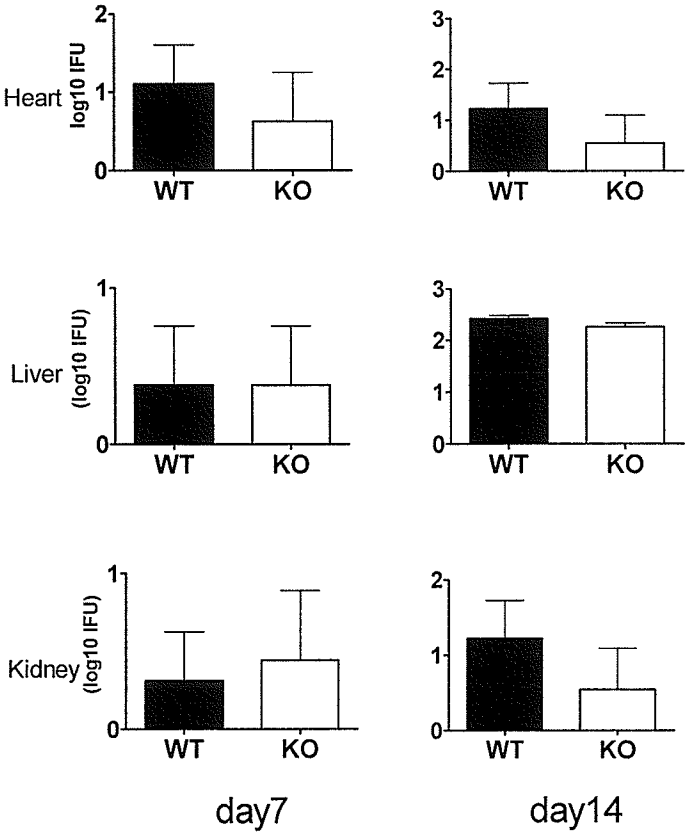


Figure 27. *C. muridarum* infection is not highly transmittable in 129 mice. Organism growth in hearts, livers and kidneys. Infectivity of Chlamydia measured as described (*Materials and Methods*). Each column represents the mean ± SEM of IFUs (log 10).

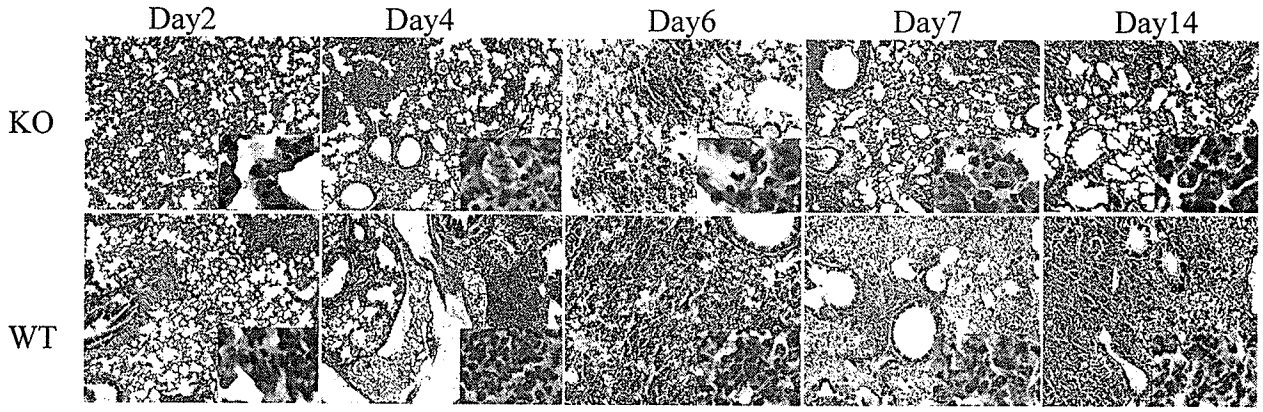


Figure 28. Wild-type mice showed more severe inflammation post infection. Wild-type and IFNAR-/- mice were infected with MoPn as described (*Materials and Methods*). Mice were killed at days 2, 4, 6, 7 and day 14 post-infection. Lung sections were stained with hematoxylin and eosin and analyzed under light microscopy. Slides were photographed at 100x and 400x magnifications.

RESULT 2 Cytokine and antibody responses after infection

Since cytokine/chemokine production patterns have been shown to be important the development of in inflammation and host defense against chlamydial infection [7, 30], we examined cytokine release in the lung tissues of the infected mice (Table 6). Consistent with a more severe inflammation observed in WT mice (Fig. 28), the levels of pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and IFN-γ, were significantly higher in the lungs of WT mice than in IFNAR-/- mice at early stage of infection (Table 6). In addition, the WT mice showed significantly higher expression of MCP-1 and MIP-2, the important chemokines for neutrophil infiltration [90, 392, 393] than IFNAR-/- mice. The results suggest that WT mice produce significantly higher local pro-inflammatory cytokines and chemokines than KO mice, which may be the basis for the more profound inflammation observed in the lung of WT mice.

To further elucidate the *Chlamydia*-driven T cell responses in the infected WT and IFNAR-/ mice, we examined *ex vivo* cytokine production in spleen and draining lymph node (LN) cells from infected mice upon organism-specific re-stimulation. As shown in Table 7, the WT and IFNAR-/- mice showed similar levels of Th1-type (*i.e.*, IFN-γ, IL-12p70 and TNF-α) and Th2-type (*i.e.*, IL-4, IL-5 and IL-10) cytokine production in the spleen at both early (day 7) and late (day 14) stages of infection. Similar pattern was observed when analyzing cytokine synthesis by draining lymph node cells at day 7 post-infection. In contrast, at late stages of infection (day 14) the lymph node cells in WT mice produced significantly higher IFN-γ and TNF-α and lower IL-10 than IFNAR-/- mice. We also tested intracellular production of IFN-γ, IL-4 and IL-10 in T cells from draining lymph nodes and spleens at day 7 post-infection. The results

revealed a similar pattern in both groups of mice (Fig. 29). Noticeably, IFN-γ positive T cells in lymph node cell culture was higher than in spleen cell culture indicating that the immune responses mostly happened in the draining lymph nodes but not in the spleen (Fig. 29C). Intracellular cytokine staining also indicated that both CD4+ and CD8+ positive T cells can produce IFN-γ although most IFNγ-positive T cells were CD8+ T cells in this infection model. The similar *Chlamydia*-driven Th1- and Th2- related cytokine production by spleen and draining lymph node lymphocytes in the WT and IFNAR-/- groups at the early stage of infection suggests a comparable pattern of T cell responses to the infection in these two groups. The persistently high level IFN-γ and TNF-α production by WT mice at a late stage of infection may reflect persistent infection in these mice.

Antibody production in the infected mice at 14 days post-infection is shown in Fig. 30. There was no significant difference in the levels of *C. muridarum*-specific serum IgM, IgG1 and IgG2a between WT and IFNAR-/- mice. Both WT and IFNAR-/- mice displayed higher titers of IgG2a than IgG1, which is in line with the cytokine patterns (*i.e.*, higher IFNγ production), indicating that both groups of mice showed Th1-like response following infection. Interestingly, we found that IFNAR-/- mice produced significantly higher levels of serum IgA compared to WT mice (Fig. 30). Overall, the analyses of organism-driven cytokine patterns and IgG1/IgG2a responses did not show significant differences in the adaptive immune responses in the WT and IFNAR-/- mice, suggesting that IFNIs are more important in mounting innate immunity to *C.muridarum* infection.

Table 6 lung cytokine and chemokine production post infection

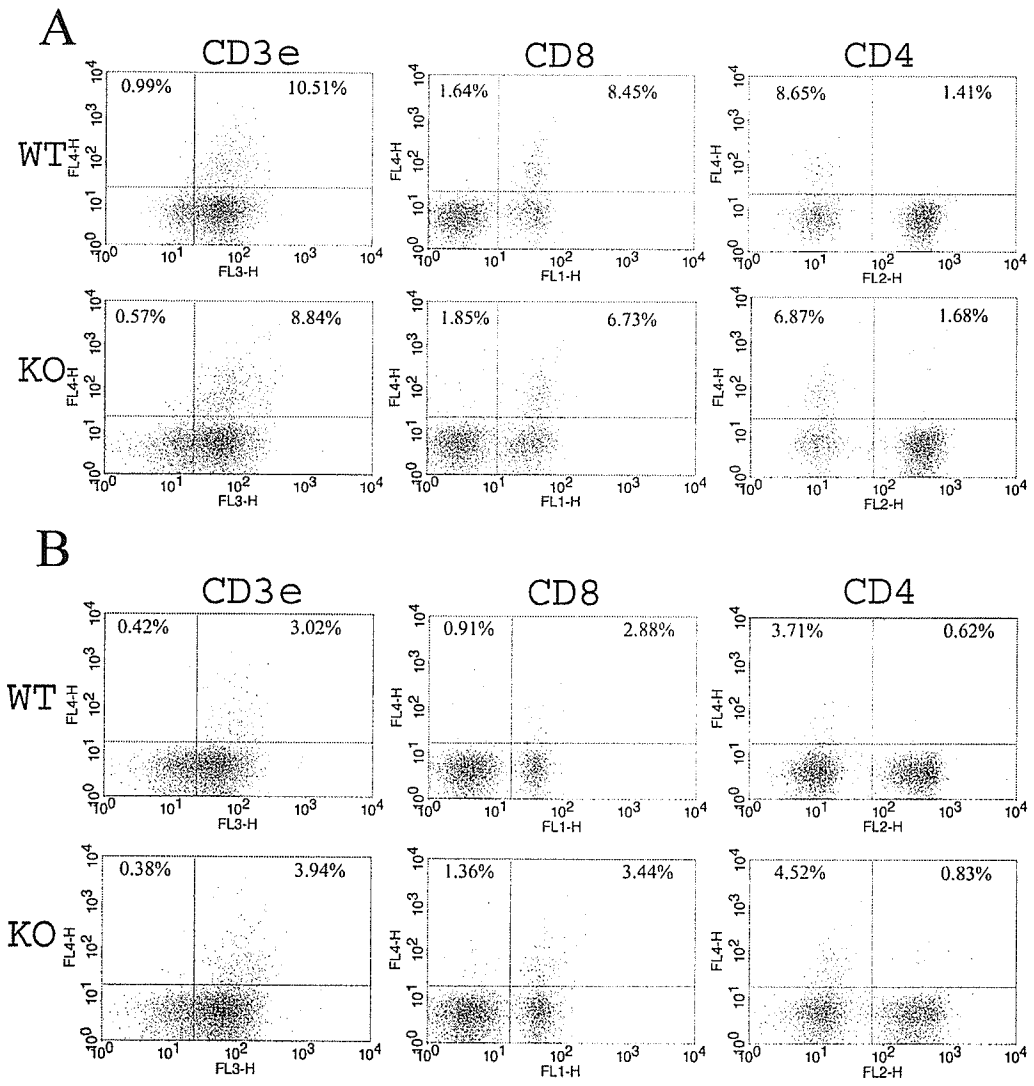
		IL-1α	IL-1β	IL-6	IFN-γ	MCP-1	MIP-2
Day 2	WT	444+75*	114+31*	902+246**	650+310	2298+264	0.54+0.04
	KO	232+41	54+15	260+132	325+50	2014+566	0.57+0.057
Day 4	WT	2281+300**	426+78*	4347+1364***	5256+1471***	4010+966***	0.8+0.04*
	KO	1503+213	310+58	1918+613	1218+409	2332+226	0.64+0.03
Day 6	WT	3393+320	554+89	6390+1276*	14000+3200*	6449+405***	1.45+0.25*
	KO	3942+450	662+93	4023+799.5	5575+2076	2218+142	0.85+0.04

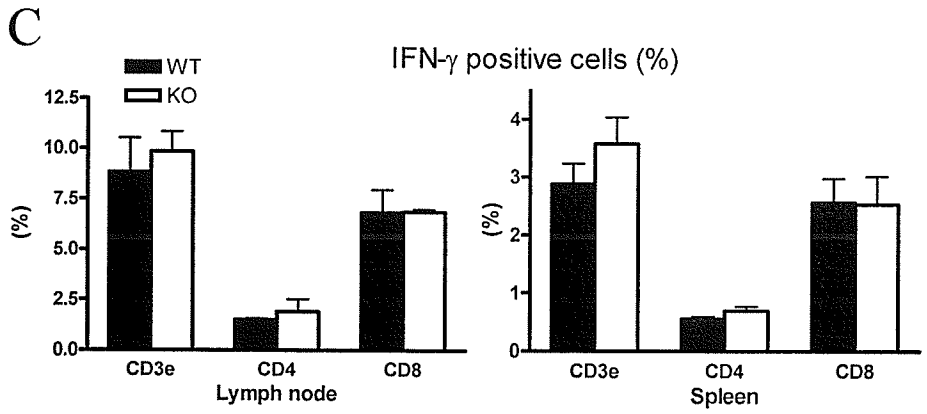
[1] Whole lungs were homogenized, supernatants harvested and assayed for IL-1α, IL-1β, IL-6, IFN-γ and MCP-1 by ELISA and for [2] MIP-2 by RT-PCR as described in *Materials and Methods.* Shown are mean ± SD protein concentrations as determined by ELISA assay or band density given by PCR amplicon from RT-PCR analysis.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 7 cytokine production by spleen and draining lymph node cells

			IFN-γ	IL-12p70	TNF-α	IL-4	IL-5	IL-10
LN	Day 7	WT	32420±2930	12±9	129±69	1.6±1.3	148±74	3238±1000
		KO	31200±4880	12±8	82±50	2.2±1.6	190±140	2641±698
	Day 14	WT	14200±8720*	51±20	148±45*	9.1±8.4	156±71	2401+971*
		KO	4433±1347	53±23	40±19	8.5±7	207±132	4915±1579
SP	Day 7	WT	20730±12600	41±29	94±39	47±15	146±90	1742±565
		KO	20240±12170	29±22	104±15	45±9	121±70	1417±82
	Day 14	WT	5214±2005	66±16	221±63	50±24	100+ 71	603±75
		KO	3654±2551	71±32	94±64	18±15	98+77	352±88

[1] 4-5 mice/group were infected intranasally with 200 IFU MoPn and euthanized at day 7 and day 14 post-infection. Supernatant from the infected lungs and that from 72-hour cultures of draining lymph node and spleen mononuclear cells were harvested and assayed for cytokines (*Materials and Methods*). Shown is a representative of three independent experiments that showed similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$


A
CD3e
CD8
CD4
WT
KO
0.99%
10.51%
1.64%
8.45%
8.65%
1.41%
0.57%
8.84%
1.85%
6.73%
6.87%
1.68%
FL4-H
FL3-H
FL1-H
FL2-H
B
CD3e
CD8
CD4
WT
KO
0.42%
3.02%
0.91%
2.88%
3.71%
0.62%
0.38%
3.94%
1.36%
3.44%
4.52%
0.83%
FL4-H
FL3-H
FL1-H
FL2-H


C
IFN-γ positive cells (%)
WT
KO
(%)
12.5
10.0
7.5
5.0
2.5
0.0
CD3e
CD4
CD8
Lymph node
4
3
2
1
0
CD3e
CD4
CD8
Spleen

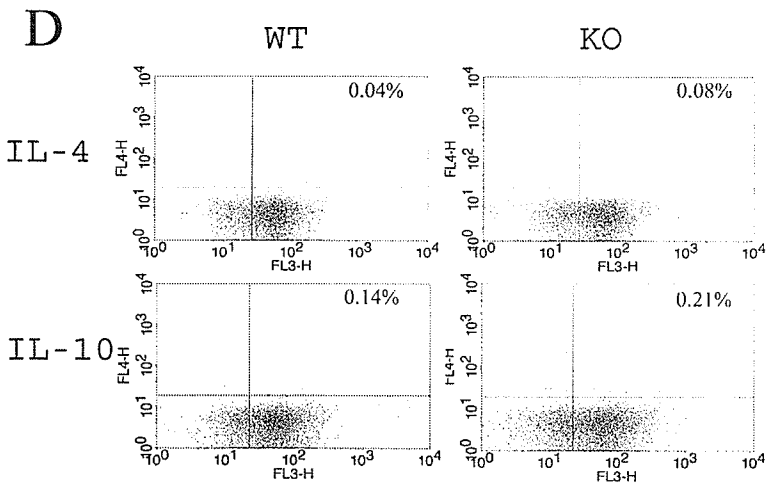


Figure 29. Intracellular cytokine staining showed similar patterns of T cells producing IFN-γ, IL-4 and IL-10 in WT and KO mice. 7 days post-infection, spleen and draining lymph node cells were harvested and cultured for 3 days with antigenic stimulation. Cells were then treated with PMA, ionomycin and Brefeldin A, and intracellular were stained for different cytokines as mentioned in *Materials and Methods*. A. IFN-γ staining in draining lymph node cells. B. IFN-γ staining in the spleen. C. Statistical analysis of intracellular staining patterns (4 mice per group). D. Staining for IL-10 and IL-14 in the lymph nodes. FL1: FITC-CD8; FL2: PE-CD4; FL3: PE-Cy7-CD3e and FL4: APC-cytokines.

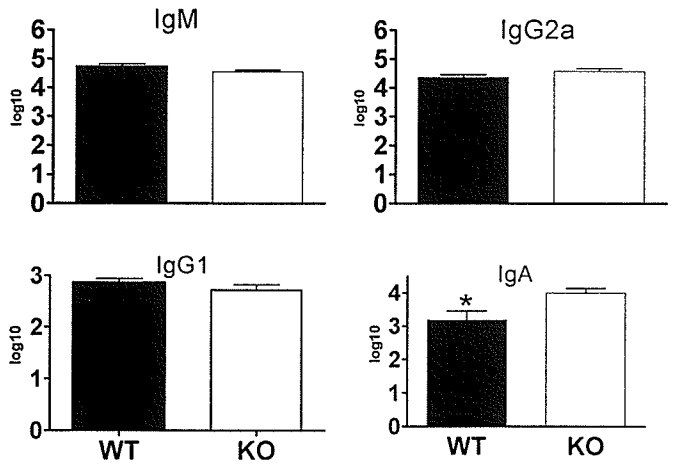


Figure 30. Antibody production in response to *C. muridarum* infection. 10 mice per group were infected intranasally with 200 IFU *C. muridarum* and bled at day 14 post-infection. Sera were used for measurement of Chlamydia-specific antibodies as described in *Materials and Methods*. The titers of antibodies were transformed into log 10. * $p <$ 0.05.

RESULT 3 Higher filtration of macrophages in the lungs of IFNAR-/- mice after C. muridarum infection

We further analyzed the cellular composition of infiltrating cells in the lungs following infection. As shown in Figure 31, the lung tissues of *IFNAR-/-* mice contained significantly higher number of macrophages compared to that in the WT controls, albeit the inflammation was much heavier in the WT mice. Similarly, analysis of BAL cells also revealed a much higher infiltration of macrophages in the lungs of *IFNAR-/-* mice, while those in WT mice had significantly higher number of neutrophils (Fig. 31A). Furthermore, we examined the kinetics of macrophage infiltration in the lungs following *C. muridarum* infection by immunohistochemical staining analysis using an anti-macrophage antibody. As shown in Figure 31B, at days 2, 4, and 7 post-infection, macrophages were consistently present in a higher density in the lungs of *IFNAR-/-* mice compared those of WT mice seen starting from day 4 p.i. These results suggested a beneficial role of macrophages infiltration in respiratory tract *C. muridarum* infection.

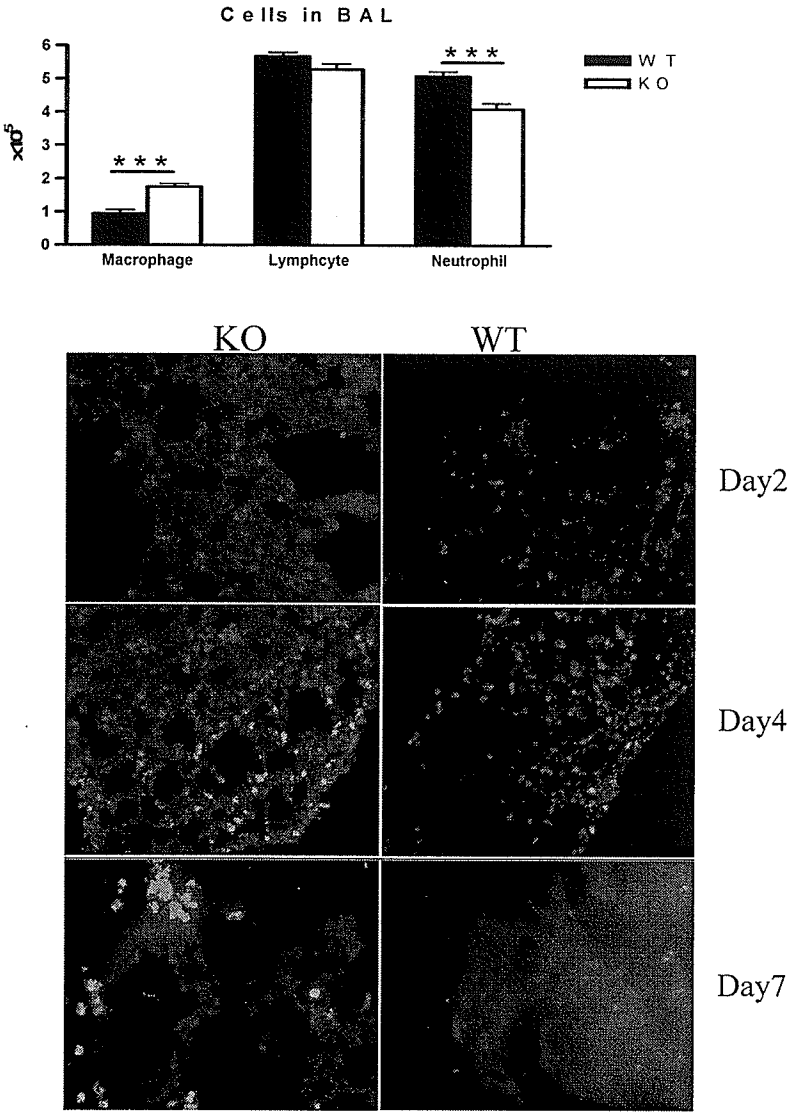


Figure 31. Higher macrophage infiltration in the lungs of *IFNAR-/-* mice after *C. muridarum* infection. A. Presence of inflammatory cells in BAL. *IFNAR-/-* and WT mice were infected with 1,000 IFU *C. muridarum* and killed at day 4 p.i. Lungs were washed, counted and stained for infiltrating inflammatory cells with hematoxylin and eosin. 500 infiltrated inflammatory cells were counted, the percentage of each kind of inflammatory cells were calculated, and the absolute number were expressed based on total BAL cell numbers. ** $p < 0.01$, *** $p < 0.001$. B. Kinetics of resident macrophages in the infected lungs. *IFNAR-/-* and WT mice were infected intranasally with 1,000 IFU of *C. muridarum* and killed at day 2, 4, 7 (infected with 200 IFU) post-infection. Macrophages were assessed as mentioned in *Materials and Methods.*

RESULT 4 Macrophages are an important host defense component in C. muridarum infection

To determine whether macrophages contribute to host resistance to *C.muridarum* infection in this infection model, we used Cl_2MDP-liposome treatment to deplete pulmonary macrophages as described before [361]. As shown, Cl_2MDP-liposome-treated *IFNAR-/-* mice exhibited greater body weight loss (Fig. 32A), increased organism growth (Fig. 32B) and more severe lung inflammation (Fig. 32C) compared to the two control mice groups treated with PBS only or with PBS-liposome. Macrophage depletion in the lungs was confirmed by H&E staining (Fig. 32C) and by immunohistochemical staining (data not shown). Most of the infiltrating inflammatory cells in CL_2MDP-lipsome treated mice were neutrophils following *C. muridarum* infection (Fig. 32C). Notably, PBS-liposome treated mice also showed slightly higher bacterial load and more pathological changes compared to the PBS-treated mice. This is probably due to the non-specific suppression of liposome on macrophages. The macrophage depletion experiments were also performed using another mouse strain, C57BL/6, which is more resistant to *C. muridarum* infection [93, 152] and a similar protective effect of macrophages was observed (Fig. 32D). The data suggests that local macrophages are important in host defense against *C. muridarum* infection.

As described in Result I, macrophages from different strains of mice may have different resistance characteristics to *C. muridarum* MoPn infection. It was found that wild-type BMDMs are more resistant to *C. pneumonia* infection compared to those from IFNAR-/- mice [290]. Thus, we tested *C. muridarum* MoPn growth in BMDMs from the both groups of mice. As illustrated in Fig. 33A, a vast majority of bone-marrow cells

cultured in L929-conditioned media for 10 days were of the macrophage phenotype (*e.g.*, positive for both F4/80 and CD11b). After infected with *C. muridarum* MoPn, cells were fixed at various times and stained with *Chlamydia*-specific antibody. No significant difference in chlamydial growth was observed in BMDMs from WT and *IFNAR-/-* mice with respect to both the number and the area of inclusion bodies as determined microscopically (Fig. 33B). We further assessed the growth of *C. muridarum* and NO production in cultured peritoneal macrophages and found no difference in the two groups (data not shown). Taken together, the data suggest that macrophages from WT and *IFNAR-/-* have the same ability to restrict chlamydial infection.

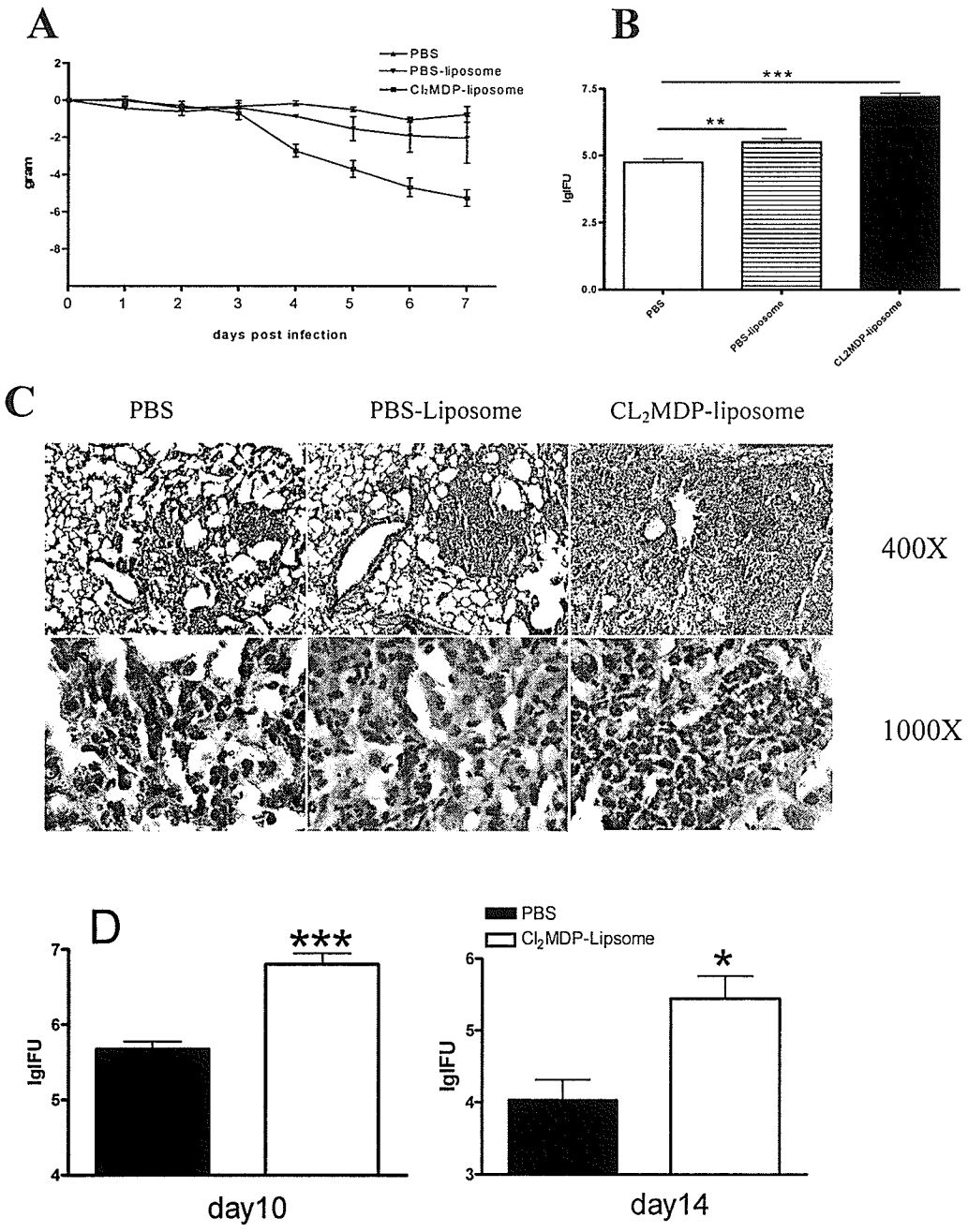


Figure 32. Depletion of lung macrophages enhanced chlamydial infectivity in IFNAR-/- mice. 4 mice per group were treated with PBS, PBS-Liposome and CL_2MDP-Liposome separately and infected with 200 IFU *C. muridarum* as mentioned in *Materials and Methods*. Body weight change, MoPn infectivity and lung pathological changes were assess as mentioned in *Material and Methods.* Shown is representative data of two independent experiments with similar results. **A.** Body weight change following lung infection with *C. muridarum* (200 IFU). Body weight changes were measured and analyzed as mentioned before. **B.** Chlamydia growth post-infection. 7 days post-infection, lungs were aseptically cut and homogenized. Supernatants were harvested after

centrifugation. MoPn IFUs were measured as mentioned in *Materials and Methods*. **C.** Lung histological change. 7 days after infection, lungs were collected and analyzed as described in *Materials and Methods*. **D.** C57BL/6 mice were treated with PBS or Cl2MDP as described in *Materials and Methods* and infected with 200 IFU *C. muridarum*, organism titers in the lungs were assayed.** $p < 0.01$, *** $p < 0.001$.

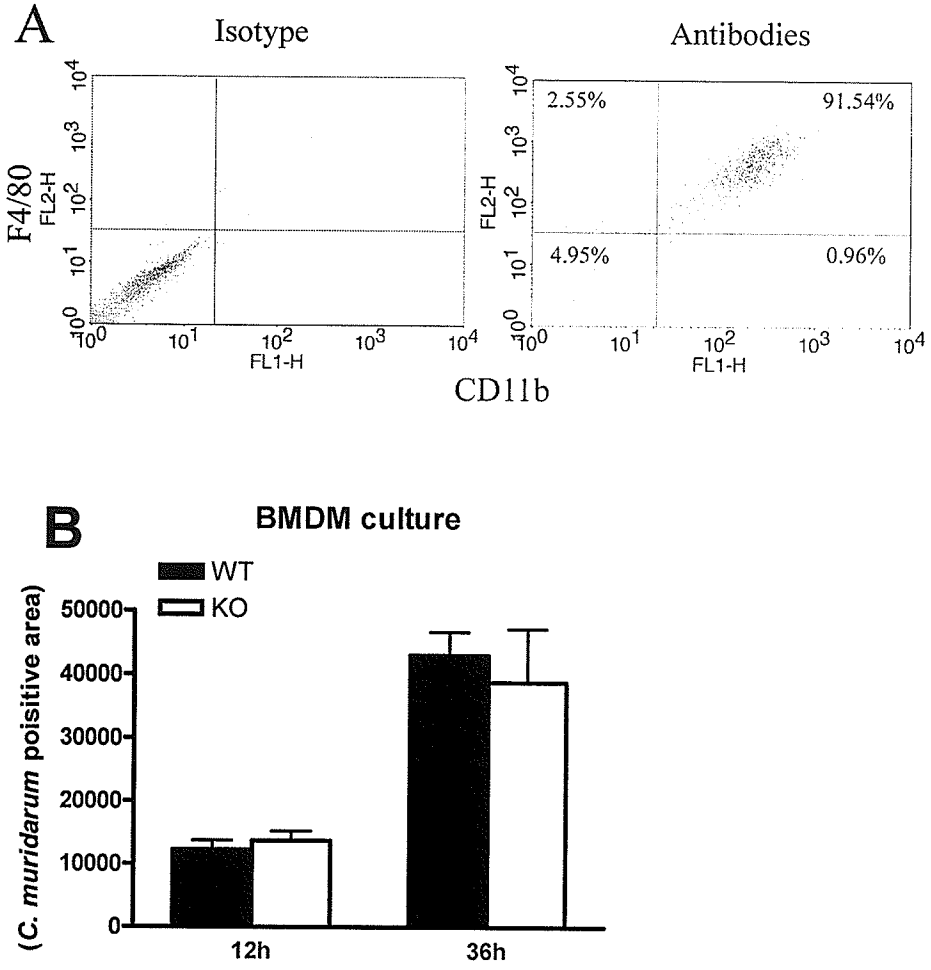


Figure 33. Similar *C. muridarum* growth rate in BMDM from wild-type and IFNAR-/- mice. A. Bone-marrow cells from WT and KO mice were cultured in L929 conditioned media for 10 days (*Materials and Methods*). Attached cells were harvested and stained with fluorescent antibodies and analyzed by FACS. Left graph is of cells stained an isotype control antibody. **B.** BMDM cultured in 96-well plate were infected with 5 x 10^4 IFU *C. muridarum MoPn* and harvested at various time, stained and counted as detailed in *Materials and Methods*. The inclusion body positive area was calculated automatically and compared as specified (*Materials and Methods*).

RESULT 5 Adhesion molecule expression in WT and IFNAR-/- mice

In an attempt to elucidate the molecular basis underlying the difference in lung macrophage infiltration between the WT and IFNAR-/- mice, we examined VCAM-1/VLA-4 expression in the lung tissues and peripheral blood monocytes to assess migration of monocytes/macrophages to the lung following infection. The analysis was performed at a very early stage of infection (day 4 post-infection) before significant difference in chlamydial growth between the WT and IFNAR-/- mice occurred (Fig. 26C). The data showed that the latter group exhibited significantly higher VCAM-1 expression in the lung tissues compared to the WT mice (Fig. 34A). Given that VCAM-1 binds to CD49d and CD29 which are two components of VLA-4 on macrophages, we analyzed expression of CD29 and CD49d on monocytes in peripheral blood. Monocytes were gated as SSC^{int} $CD45^{low}$ $CD11b^{hi}$ cells [394]. As shown in Figure 34B and C, monocytes from the IFNAR-/- mice showed higher expression of CD49d, and to a lesser extent, CD18 relative to those of WT mice following infection. There was no difference in the expression of CD29 and CD11a. These results indicate that IFNIs may down-regulate expression of certain adhesion molecules which are involved in macrophage infiltration during the early phase of chlamydial infection, thereby affecting monocyte/macrophage recruitment to the infection site.

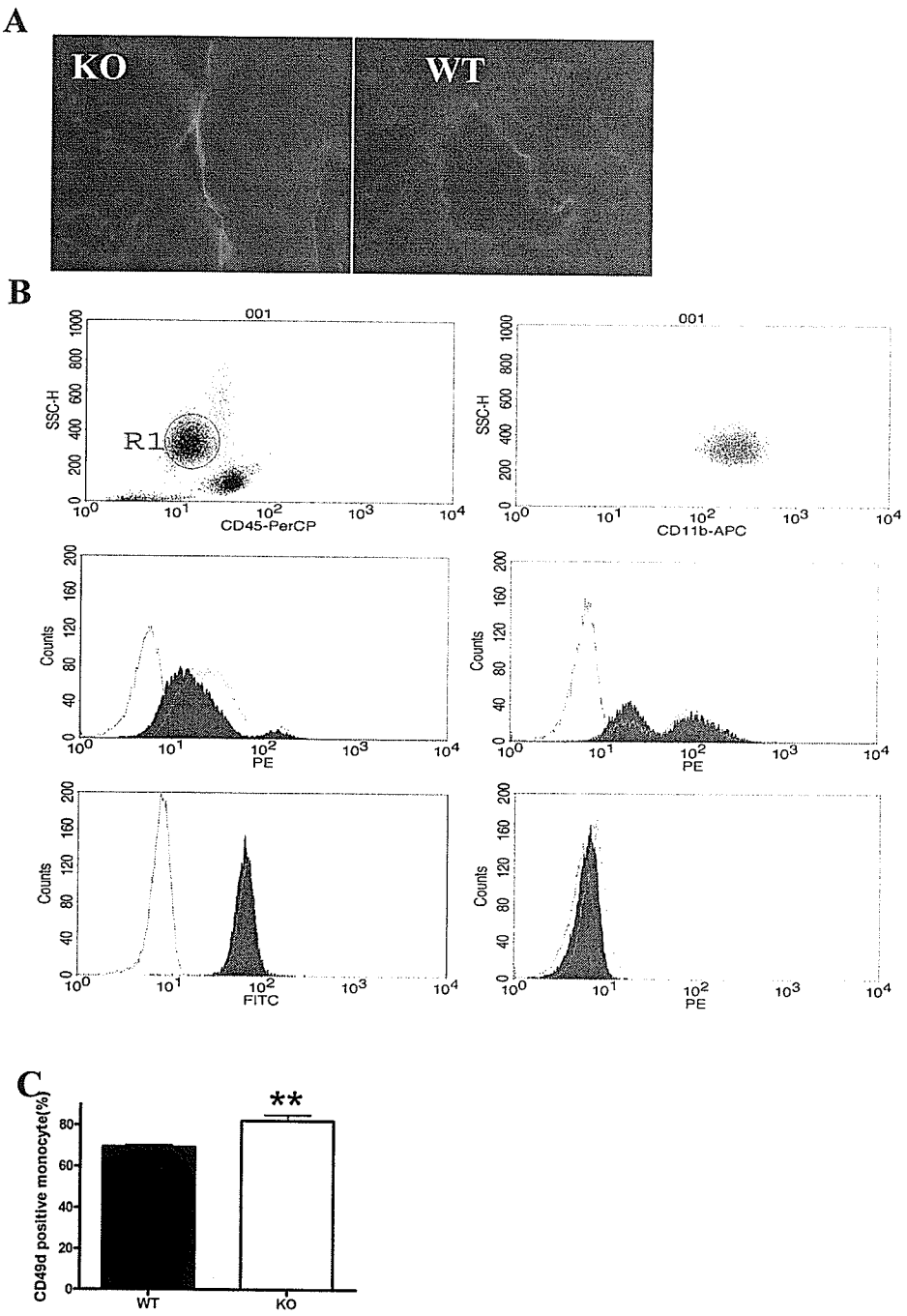


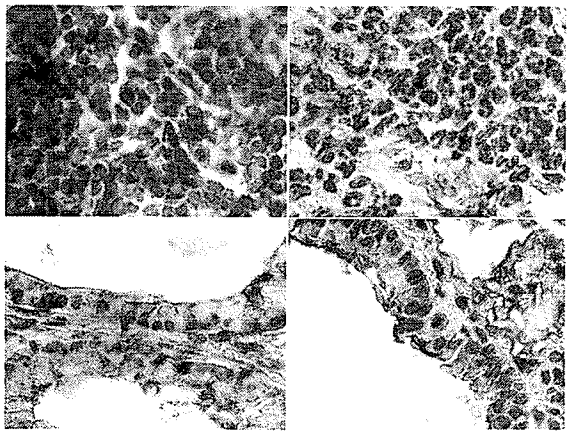
Figure 34. Adhesion molecule expression after infection. 4 days after infection with 1,000 IFU *C. muridarum*, lungs and peripheral blood of WT and IFNAR-/- mice were used for analysis. **A.** Cryogenic sections of lungs were stained with anti-mouse VCAM-1 antibody as mentioned in *Materials and Methods* and photographed with fluorescent microscopy. **B.** Peripheral blood was collected and treated (*Materials and Methods*). Histogram graphs show gated $CD45^{low}CD11b^{mid}$ monocytes/macrophages. **C.** Statistical analysis of CD49d expression on blood monocytes.

RESULT 6 Macrophage apoptosis was more pronounced in WT mice than in IFNAR-/- mice

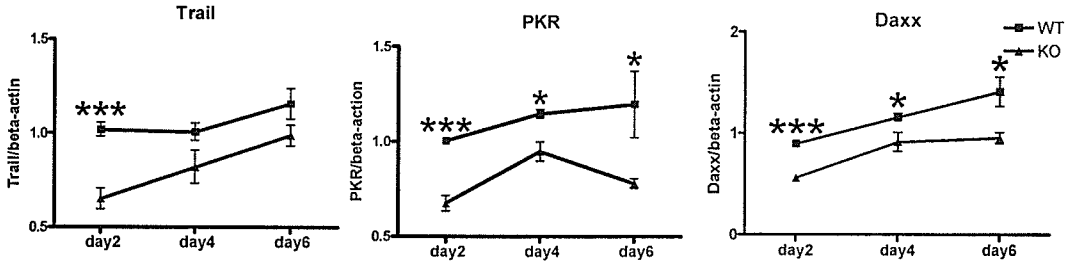
It has been suggested that IFNα/β-dependent apoptosis of lymphocytes and macrophages may be a mechanism contributing to increased susceptibility to infection in the mouse models of *L. monocytogenesis* [341, 343]. We therefore performed a TUNEL assay to analyze apoptosis in the lung following *C. muridarum* infection. As shown in Fig. 35A, the lung tissues of WT mice contained more apoptotic inflammatory cells, as indicated by the presence of cells with DAB stained nuclei, than IFNAR-/- mice as early as 4 days post-infection. However, no differences in the level of epithelial cell apoptosis observed between the WT and IFNAR-/- mice (Fig. 35A).

To confirm this finding, we further tested early expression in the infected lungs of PKR, Daxx, and Trail, factors related to IFNα/β-induced apoptosis [341]. As shown in Figure 35B, lung tissue of WT mice consistently showed significantly higher expression of Trail, Daxx and PKR compared to that of IFNAR-/- mice at as early as 2 days after infection and persisted up to day 6 post-infection when the difference in the bacterial loads in the 2 groups of mice became noticeable. In addition, a significantly higher number of activated caspase 3-positive inflammatory cells, with most of them being F4/80 positive macrophages, was observed in the lungs of WT mice suggesting a higher level of apoptosis in these mice following *C.muridarum* infection and a promoting role of IFNIs in apoptosis of macrophages (Fig. 35C, 35D).

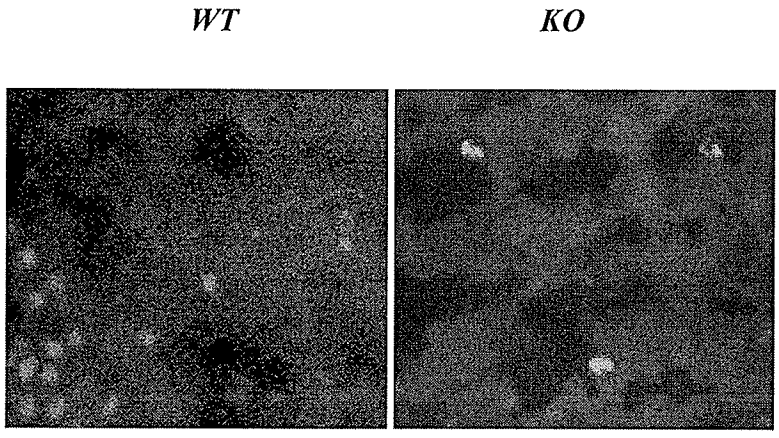
A


WT
KO
Infiltrated Inflammatory cells
Bronchi epithelial cells

B


Trail
PKR
Daxx
WT
KO
Trail/beta-actin
PKR/beta-action
Daxx/beta-actin
day2
day4
day6
***
*

C


WT
KO

D

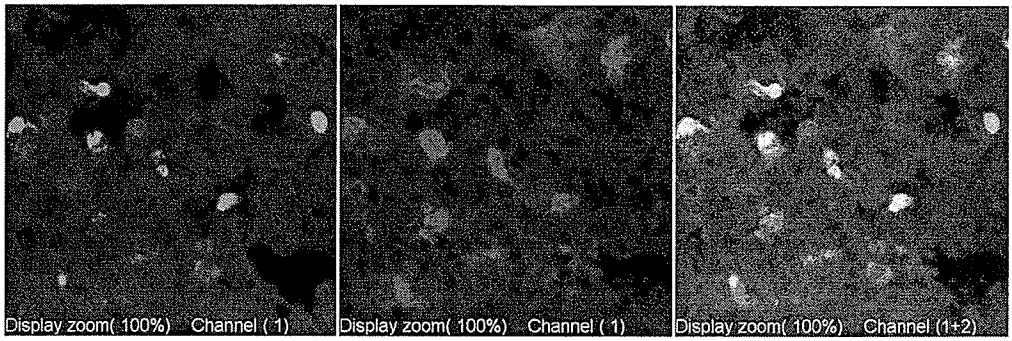


Figure 35. WT mice showed more inflammatory cell apoptosis post-infection. A. Lungs from WT and KO mice were harvested 4 days post- infection and analyzed for the presence of cell apoptosis using TUNEL assay (*Materials and Methods*). Slides were photographed under light microscopy and shown is representative pictures of at least 5 mice per group. **B.** Whole lungs from 4 mice/group were harvested and frozen by liquid nitrogen. Total RNA were isolated and analyzed by RT-PCR for mRNA expression of IFN-α/β-stimulated apoptotic factors (*Materials and Methods*). The data are presented as mean ± SD of band density expressed as percentage of that given by β-actin. * $p < 0.05$ and *** $p < 0.001$: comparison between WT and IFNAR-/- mice. **C.** Immunohistochemistry analysis of active caspase 3-positive cells post-infection. Lungs from WT and IFNAR-/- mice collected 4 days post-infection were stained with anti-cleaved caspase 3 as described (*Materials and Methods*). Shown is a representation of at least 5 mice/group. **D.** Immunohistochemistry analysis of active caspase 3-positive inflammatory cells post infection. Lungs harvested at 4 days post-infecition from WT mice were stained with anti-cleaved caspase 3 and anti-F4/80 mAbs, as mentioned in *Materials and Methods*. Green: cleaved caspase 3 and Red: F4/80.

RESULT 7 Type I interferons changed dendritic cell responses after chlamydial infection

Earlier results showed that induction of IFNIs early on after infection enhanced host susceptibility to *C. muridarum* via modulating innate immune response. Since DCs are among the most important cells linking innate and adaptive immune immunity, we assessed changes in the phenotype and function of DC after infection. As shown in Figure 36, at 7 days post-infection, spleen DC from WT mice contained significantly higher percentage of not only CD8+ DC but also of activated phenotype (*e.g.*, CD40+, CD80+, CD86+ and MHC-II+) compared to those from IFNAR-/- mice. Further, supernatants from affinity-purified DC cultures from WT mice were also found to contain significantly lower levels of IL-12p40, but higher levels of TNF-α relative to those from IFNAR-/- mice (Fig. 38). Practical consequences of such a change in the activation and functions of DC require a separate study.

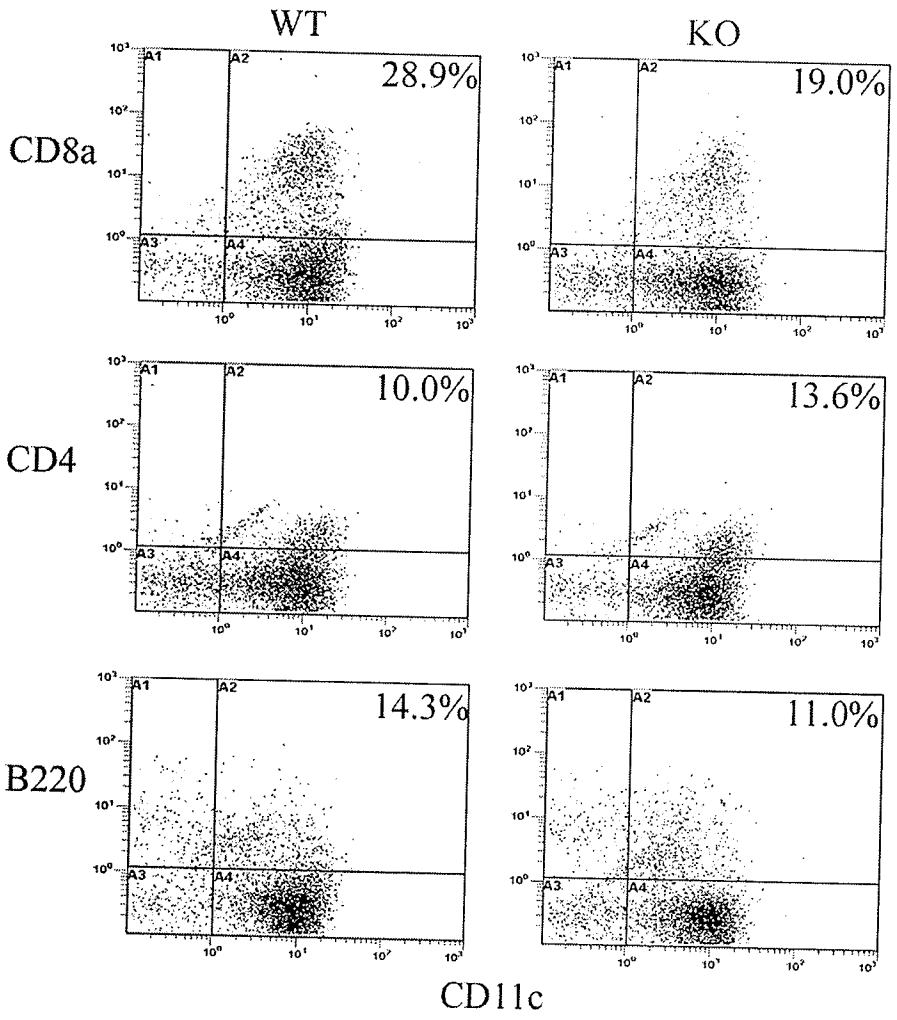


Figure 36. Higher percentage of CD8+ dendritic cells in wild-type than in IFNAR-/- mice. 7 days after infection with 1,000 IFU *C. muridarum* MoPn, DC were isolated from the spleens and phenotypes by FACS analysis as described *Materials and Methods*. Populations containing different subtypes of DCs are shown in dot plot.

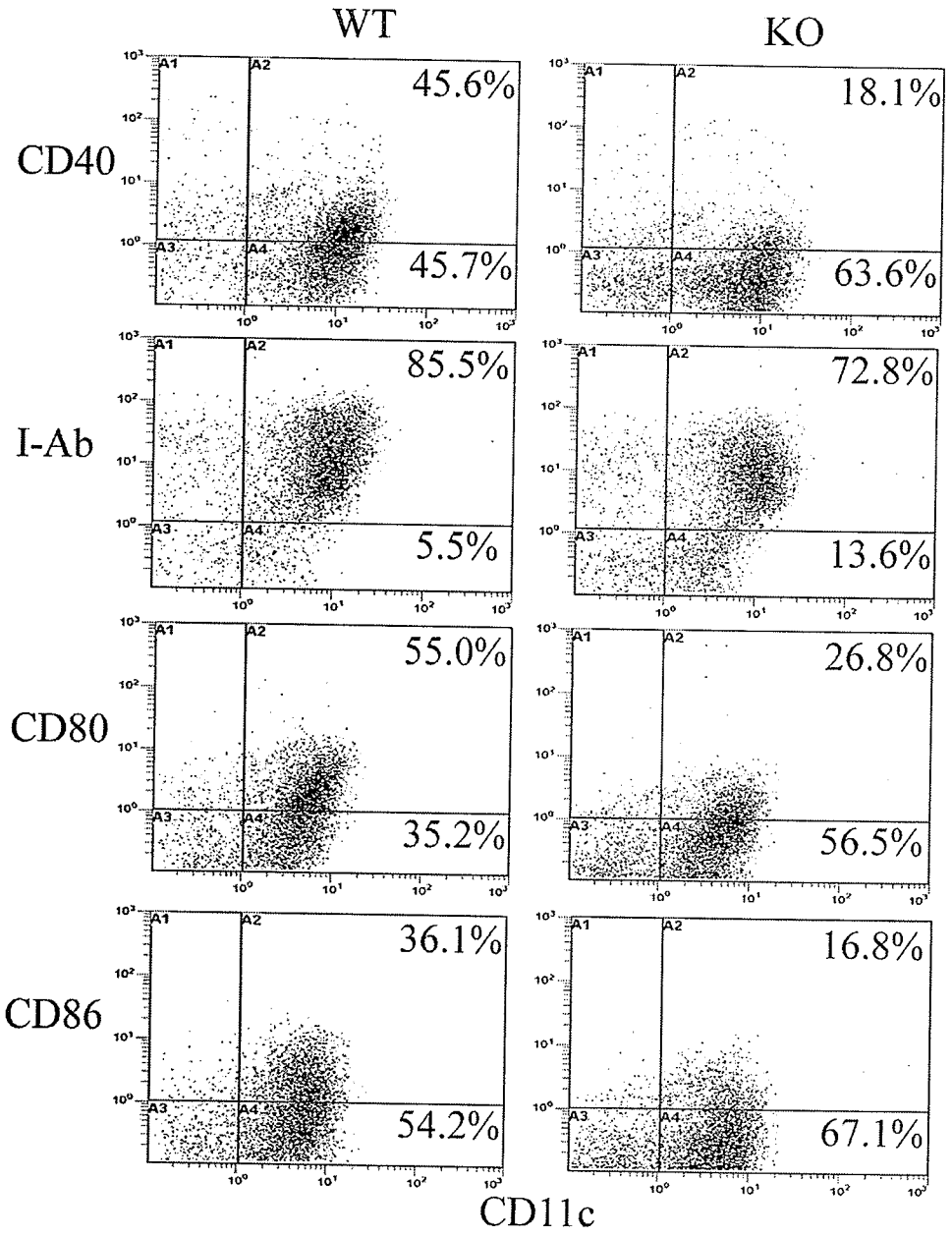


Figure 37. Wild-type mice contained higher number of activated dendritic cells than IFNAR-/- mice following *C. muridarum* infection. 7 days after infection with 1,000 IFU *C. muridarum* MoPn, affinity-purified DC were examined by flow cytometry for the presence of activation markers as mentioned in *Materials and Methods*. DC populations positive for different activation markers are shown in dot plot.

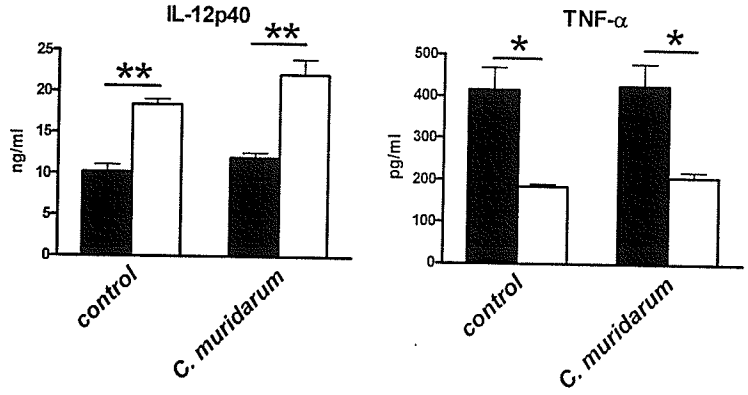


Figure 38. Cytokine secretion profiles by dendritic cells from wild-type and IFNAR-/- mice. Affinity-purified DC were cultured with or without antigenic stimulation and cytokines in the supernatant assayed by ELISA as described in *Materials and Methods*. * $p < 0.05$; ** $p < 0.01$.

DISCUSSION of PART III

Previous reports have shown that IFNIs are beneficial to *ex vivo* cultured host cells in their resistance to chlamydial infection via enhancing IFN-γ and NO production [290]. However, in the current study, we have demonstrated that IFNIs actually promote *C. muridarum* infection *in vivo* and that following respiratory tract *C. muridarum* infection, IFNAR-/- (KO) mice displayed much less body weight loss, lower organism burden and milder pathological changes in the lungs than WT mice. More importantly, we observed not only significantly less macrophage infiltration in the lungs but also less apoptosis of the infiltrating macrophages in KO mice than in WT mice. However, analysis of Th1- and Th2-types cytokine patterns in the two groups of mice failed to show a clear correlation between cytokine expression, organism clearance and severity of disease, especially at the early stage of infection. The higher production of IFN-γ, TNF-α and lower secretion of IL-10 in WT, relative to KO, mice at 14 days of infection may not be directly attributed to IFN-α/β, but rather, due to the uncontrolled chlamydial growth and continuous stimulation of the host immune system (Table 7). Taken together, the results lend support to the notion that IFNIs are critical in the innate immunity in chlamydial lung infection.

One interesting finding in this study is the particularly important role of IFNIs in the effector function of macrophages in chlamydial infection. Significant differences between WT and KO mice in susceptibility to *C. muridarum* was observed starting from the early stage of infection indicating that the functional effects of IFNIs occur in the innate phase of infection. The data showed that KO mice had less inflammation at both early and late stages of infection, but the number of infiltrating macrophages in the lungs

of the KO mice was higher than that in WT mice, which experienced more neutrophil influx. Notably, the difference in bacterial loads between the WT and KO mice occurred after the time when the difference in macrophage infiltration and survival was observed in these two groups of mice, suggesting a positive correlation between macrophage infiltration and bacterial clearance. The role of macrophages in restricting *C. muridarum* infection was confirmed by the macrophage depletion experiment using liposome-encapsulated Cl_2MDP. This finding is consistent with that from a previous study which illustrated an important role of macrophages in host resistance to *C. pneumoniae,* another chlamydial species, infection through an adoptive transfer approach [191].

What then contributes to the reduction in macrophages in the lungs of WT mice after chlamydial infection? Based on our data, less recruitment of the cells to the lungs and a higher in apoptosis of the infiltrating macrophages may be responsible for the decrease in the cell number. An important factor that regulates leukocyte infiltration is the expression of adhesion molecules which are involved in cell-matrix interaction and binding. We found significantly higher expression of VCAM-1 and CD49d (a component of VLA-4) in the lungs of KO mice than in those of WT mice. Previous studies have demonstrated the importance of VCAM-1 and VLA-4 interaction in macrophage migration and recruitment [395-397]. As such, reduced expression of VCAM-1 in the lung blood vessel endothelial cells and CD49d on blood monocytes in the WT mice may result in a weaker/diminished interaction between peripheral monocytes and endothelial cells lining lung blood vessels, which ultimately leads to less monocyte/macrophage infiltration. It remains unclear how IFNIs regulate the expression of VCAM-1 and VLA-4. However, some *in vivo* and *in vitro* studies have reported that IFN-β can convert

membrane-bound VCAM-1 to soluble VCAM-1 (sVCAM-1), thus reducing VCAM-1 expression on the cell surface. The increased level of sVCAM-1 can also down-regulate VLA-4 expression on lymphocytes resulting in the inhibition of interaction between lymphocytes and blood vessel endothelial cells [304].

The presence of more apoptosis in lung macrophages from WT than in those of KO mice was evidenced by significantly increased TUNEL-positive cells in the former, which was correlated with markedly higher gene expression of IFNI-dependent apoptotic factors including TRAIL, PKR and Daxx. Also observed was a high number of cleaved caspase 3-positive macrophages in the lungs of WT mice than in those of KO mice. Caspase 3 is a central executioner of apoptosis and inactive zymogen caspase 3 must be cleaved into activated caspase 3 (18 KDa) to initiate apoptosis [398, 399]. Therefore, our finding suggested that fewer macrophages in the lungs of WT mice may be due to a combination of reduced recruitment and increased local apoptosis after *C. muridarum* infection. Since higher macrophage apoptosis in the WT mice was seen at day 4 post-infection before the appearance of a differential bacterial load, it is reasonable to speculate that the less recruitment and more apoptosis of macrophages contribute significantly to the higher chlamydial growth in the lungs of WT mice. This notion is supported by the data from the macrophage depletion experiment with Cl_2MDP-lipsome (Fig. 32). Although the reason is not clear as to why without macrophage depletion mice treated with PBS-liposome alone also showed more chlamydial growth *in vivo* than PBS-treated mice (Fig 32), it is likely that the phagocytosis of liposome by macrophages can non-specifically suppress, to a certain degree, the killing capability of macrophages [343].

Macrophages from mice with different genetic backgrounds may exhibit differential resistance to chlamydial infection [93]. Specifically, recent studies with macrophages from wild-type or IFNα/βR-/- mice revealed that the former was more resistant to *C. pneumonia* infection *in vitro* than the latter [290]. However, we found no difference in the growth of *C. muridarum* in macrophages isolated from wild-type or IFNα/βR-/- mice suggesting that the killing ability of macrophages may not have a direct contribution to the difference in susceptibility to *C. muridarum* infection of the 2 groups of mice.

It has been shown that, during respiratory tract viral or fungal infections, IFNα/βR-/- mice produced more Th2-type cytokines and chemokines in the lungs compared to WT mice, which is correlated with enhanced eosinophil infiltration [400, 401]. In our chlamydial infection model, we found lower levels of inflammatory cytokines IL-1α, IL-1β and IL-6 and of chemokines MCP-1 and MIP-2 in the lungs of IFNα/βR-/- mice, which was associated with less severe inflammatory responses post-infection. MIP-2 has recently been reported to be involved in neutrophil chemotaxis and infiltration [392, 393], which can contribute to significant tissue damage and pathological responses [30].

Also evidenced from our study is that IL-6 and IFN-γ were produced at significantly higher levels in the lungs of WT mice starting at day 4 post-infection and persisted to day 14 (data not shown). Our data showing a higher level of apoptosis by neutrophils in WT mice lend support to the hypothesis that an interplay between IL-6 and IFN-γ enhances neutrophil infiltration and apoptosis [402]. Further, our findings imply that the higher number of apoptotic neutrophils in WT mice may ultimately contribute to

the overall loss of protective function of macrophages since the more apoptotic neutrophils are in the lungs, the more macrophages are needed to remove the dead cells. In consequence, bacterial clearance by macrophages may be compromised.

Although no difference in the titer of serum IgM, IgG1 and IgG2a between WT and KO mice, we found the titer of serum IgA of KO mice is statistically higher that that of WT mice. It is interesting to continue study whether the difference is correlated with higher susceptibility of WT mice. Because IFNIs can influence the activation and proliferation of B cell, it is also possible that IFNIs may suppress IgA production at the later stage of infection.

In summary, our study has demonstrated that IFNIs responses are detrimental to the host defense against primary respiratory tract *C. muridarum* infection. Our study has also revealed a particularly important role of IFNIs in hindering macrophage recruitment and function. Given that IFNIs are pleotropic in their functions *in vivo*, more in-depth studies are needed to fully elucidate the interdependences of different factors underlying the immunological responses.

GENERAL DISCUSSIONS AND FUTURE DIRECTIONS

The present research, by using C3H mice, C57BL/6 mice, MHC congenic mice, and IFNAR-/-mice, studied the genetic background determined susceptibility to *C. muridarum* respiratory tract infection, and the possible mechanisms for the differential susceptibility. Our work, has not only confirmed that adaptive Th1-type response is important for the host to resist chlamydial infection, but also further provided strong evidence to support the notion that innate immunity is highly essential for the host to restrict chlamydial infection.

By comparing the susceptibility of B6.H2^k congenic mice to that of wild-type C57BL/6 mice, we found that the former, with the C57BL/6 genetic background but altered MHC gene, exhibited increased susceptibility to chlamydial infection, which is correlated with suppressed Th1-like cytokine production (*i.e.*, IFN-γ and IL-12). This data, suggested that the MHC can efficiently influence the adaptive T cell responses, and Th1-like responses are important in the host resistance to chlamydial infection.

However, it is also evident from this work that although C3H. H2^b congenic mice possess the MHC from the resistant strain C57BL/6, they exhibited similar susceptibility to chlamydial infection relative to C3H/HeN mice, and both C3H mice were much more susceptible to chlamydial infection compared to C57BL/6 mice. Interestingly, in all of these three groups of mice, similar Th1-like responses after chlamydial infection were observed implying that non-MHC determinants could also influence the susceptibility to chlamydial infection, and that strong Th1-like immune response was not always associated with increased resistance to chlamydial infection.

Our subsequent findings have shown that significantly more *C. muridarum* grew in the lungs of C3H mice as early as 2 days after chlamydial infection, compared to those of C57BL/6 mice. Given that adaptive immune responses are not yet elicited 2 days post-infection, the data clearly suggested that inadequate innate immune response in C3H mice is likely a contributing factor. For this reason, we analyzed in the lungs the expression of cytotoxicity-related molecules including Fas, FasL, perforin, granzymes A and B, IFNγ-induced IDO and p47GTPases but could not find significant differences in the levels of these molecules between C3H and C57BL/6 mice. However, when we compared the *in vitro* resistance of peritoneal macrophages to *C. muridarum* infection, it was evident that macrophages from C3H mice were less efficient in inhibiting Chlamydial growth compared to those from C57BL/6 mice after being stimulated with IFN-γ and this was attributed to lower NO production in these cells from C3H mice. The observation thus suggests a possible mechanism underlying the difference in susceptibility to chlamydial infection between C3H and C57BL/6 mice, and in consequence, supports a protective role of innate immunity.

Similarly, by comparing the susceptibility of IFNAR-/- and wild-type mice, we have revealed a mechanism which once again lends support to the notion that innate immune responses are important in chlamydial infection. Although both IFNAR-/- and wild-type mice exhibited similar Th1-type responses following *C. muridarum* infection, wild-type mice were indeed much more susceptible to the infection as evidenced by significantly greater organism growth, body weight loss, and manifestation of inflammation. Although the adaptive immune responses were similar between the 2 groups of mice with respect to antibody production and cytokine secretion, we found them differed in: 1) the amount of

chlamydial growth observed at as early as 6 days post-infection, when adaptive immunity is not completely induced and 2) the number of macrophages at 4 days post-infection. In fact, depletion of lung macrophages with Cl_2MDP-liposome confirmed a critical role of macrophages in restricting *C. muridarum* over the course of infection.

We have found that IFNIs enhanced host susceptibility to Chlamydial infection by suppressing macrophage infiltration, which is in turn probably through inhibiting the expression of adhesion molecules VCAM-1 and VLA-4, and enhancing macrophage apoptosis in the lungs. Further, although we did not find a different pattern of cytokine secretion by lymphocytes from the spleen and draining lymph nodes of the infected wild-type and knockout mice, we did observe a change in the DC phenotype between these two groups of mice in response to infection. To fully understand how IFNIs regulate adaptive immunity, it is prudent to study the adaptive immune responses and DC functions using mice first immunized with dead *Chlamydia* and then challenge them with the pathogen and assess their resistance to chlamydial infection.

Overall, our study strongly supports the importance of innate immune responses during Chlamydial infection. Especially, the function of macrophage migration, activation and surviving largely influence host's resistance to chlamydial infection. Further studies focusing on down-stream bactericidal mechanisms activated by IFN-γ and on macrophage functions would provide new information on how the host can resist chlamydial infection.

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