Suppression of MHC Class II but not ICAM-1 Molecules by Chlamydial infection

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Suppression of MHC Class II but not ICAM-1 Molecules by Chlamydial infection

BY

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Abbreviations:

CMI: cell-mediated immune response

DAB: 3, 3'-Diaminobenzidine tetrahydrochloride liquid substrate system

DEPC: diethylpyrocarbonate

DMEM: Dulbecco's Modified Eagle Medium

EB: elementary body

EB: esodium bromide

EDTA: ethylenediamne tertra-acetic acid

ELAM-1: E-selectin, CD62E

ELISA: Enzyme-Linked Immunosorbent Assay

ES cell: embryonic stem cell

FACS: fluorescence-activated cell sorter

FITC: fluorescein isothiocyanate

HBSS: Hanks buffered salt solution

HRP: Horseradish perioxidase

IB: Inclusion body

ICAM-1: Intracellular adhesion molecule-1

IDO: indoleamine 2, 3-dioxygenase

IL: interleukin

i-NOS: inducible nitric acid synthase

ip injection: intra-peritoneal injection

KO: knockout

LFA-1: leukocyte function antigen-1

LGV: lymphogranuloma venereum

LPS: lipopolysaccharide

MAdCAM-1: belong to mucin-like vacular addressins

MHC: major histocompatibility complex

MOI: Multiplicity of Infection

MOMP: major outer membrane protein

MoPn: Mouse pneumonitis

NCM: Nitracellulose membrane

NK cell: natural killer cell

NO: nitric acid

PBS: phosphate buffered saline

PI: propidium iodine

PID: pelvic inflammatory disease

Qd: once a day

RB: reticular body

RT-PCR: Reverse transcriptase-polymerase chain reaction

SCID mice: severe combined immunodeficiency mice

SDS PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

Th: T helper

TNF-α: tumor necrosis factor-α

VCAM-1: CD106, belong to immunoglobulin superfamily

WB: western blot

I. ABSTRACT

Chlamydia, an obligate intracellular bacterial pathogen, can successfully infect a wide range of host species and persist in the infected hosts for a long period of time, suggesting that *chlamydia* may have evolved strategies for escaping host defense mechanisms. We have found that *chlamydia* indeed possesses the ability to evade host immune recognition. Immune recognition is a requirement for hosts to develop an effective immunity against microbial infections. It has been shown that MHC class II-mediated immune responses often play a critical role in controlling intracellular pathogen infection. We hypothesized that *chlamydia* may suppress MHC class II expression in order to escape MHC class II-mediated immune responses. We compared the IFN-γ-inducible MHC class II expression between epithelial cells with or without chlamydial infection. We found that chlamydial infection selectively suppressed the IFN-γ inducible MHC class II expression by several measurements. First, cell surface expression of MHC class II molecules was inhibited in chlamydia-infected cells as analyzed with flow cytometry. Second, the total amount of MHC class II molecules was significantly reduced in chlamydia-infected cells as detected on Western Blots. Third, the MHC class II mRNA expression was also inhibited in chlamydia-infected cells as measured with RT-PCR. Together, these observations suggest that chlamydia-induced inhibition of MHC class II expression occurs at the transcription level. Stimulation of epithelial cells with IFN-γ may confer the infected epithelial cells the ability to process and present chlamydial antigens for T cells to recognize the infected epithelial cells. Since epithelial cells are the primary targets for chlamydial infection, chlamydial inhibition of IFN-γ-inducible MHC class II

expression may represent a unique immune evasion mechanism that can facilitate chlamydial persistence in the infected hosts.

Despite the significant suppression of MHC class II molecules, we found that IFN-γ induction of ICAM-1 expression was not altered in chlamydia-infected cells, suggesting that ICAM-1 may not be essential for controlling chlamydial infections. ICAM-1 is a non-specific adhesion molecule that participates in lymphocyte activation and trafficking. It has been suggested that ICAM-1 may play some role in host defense against intracellular infections. To evaluate the role of ICAM-1 in host defense against chlamydial infection, we compared the resistance of mice with or without ICAM-1 deficiency to chlamydial lung infection. We found that although ICAM-1-/- mice had a significantly impaired IFN-γ and IL-2 production, both the wild type and ICAM-1-/- mice displayed a similar resistance to chlamydial lung infection.

In conclusion, the selective inhibition of IFN-γ-inducible MHC class II, but not ICAM-1, by chlamydial infection may be driven by host immune selective pressure.

II. INTRODUCTION

Chlamydia is an obligate intracellular bacterium. Because of its intracellular growth and unique life cycle, *chlamydia* was initially mistaken as protoza and later as a virus. There are four species in the genus chlamydia. Two of them are human pathogens and thus attract most research interest. They are *C. trachomatis* (Moulder *et al.*, 1984) and *C. pneumonia* (Grayston *et al.*, 1989). Another two, namely *C. psittaci* (Moulder *et al.*, 1984) and *C. pecorum* (Fukushi and Hirai, 1992), are primarily animal pathogens.

Chlamydia trachomatis is an important human pathogen in both developing and industrialized countries. Infection of the ocular mucosa with *C. trachomatis*, a disease called trachoma, is the leading cause of preventable blindness in developing countries. Infection in the genital with *C. trachomatis* tract is the second leading cause of sexually transmitted diseases in industrialized countries, ranking only after human immunodeficiency virus (HIV) (Jones *et al.*, 1982; Schachter J, 1978a).

C. pneumonia causes mild or asymptomatic chronic pulmonary infection in humans. Antibodies against *C. pneumonia* exist in as high as 50% of world population (Camm and Fox, 2000). Infection with *C. pneumonia* is implicated in the pathogenesis of atherosclerosis, which is a chronic inflammation of the blood vessels leading to occlusion of vessels and loss of blood supply to the tissues.

1. Chlamydial intracellular growth

Chlamydia has a unique intracellular biphasic life cycle that consists of a series of metabolic changes. *Chlamydia* exists in two forms: an elementary body (EB) and a reticular body (RB). Both EB and RB are bound by an outer membrane similar to that found in Gram negative bacteria, except that they do not contain peptidoglycan. A typical chlamydial infection starts with the attachment and internalization of an infectious but metabolically inactive elementary body (EB) into host cells. Once internalized, an EB loses its infectivity and differentiates into a metabolically active reticular body (RB), which multiplies and differentiates back to EBs. The vacuolar EBs are finally released extracellularly and spread to other potential host target cells.

Morphological changes are observed under the light microscope. Multiple EBs can be phagocytozed into the endosomes of one host cell. The endosomes may or may not fuse to form a single endosome, depending on the strain. *Chalmydia* restricts its intracellular growth to the endosome(s). As reticular bodies continue to replicate, the endosomes continue to expand. When the endosome is big enough to be observed under light microscope, we define it as a chlamydial inclusion body (IB). A mature inclusion body can occupy one-third to one-half of the host cell cytosol volume. The entire intracellular growth cycle *in vitro* takes about 48 to 72 hours. It is thought that the chlamydial growth cycle during natural infection is much longer.

To complete a life cycle, it is essential for *chlamydia* to maintain the integrity of the infected host cells since RBs are structurally fragile and not infectious. In natural

infections, in order to achieve a long-term infection in its hosts, it is important for

chlamydia to protect the infected cells from immune recognition and immune attack.

2. Chlamydial persistence and chlamydial diseases

Chlamydia-induced diseases in humans are largely due to the persistent infection.

"Persistence" describes a long-term association between *chlamydia* and its hosts, in

which the organisms remain in a viable but culture-negative state. When the environment

is not favorable for growth *chlamydia* goes through an incomplete life cycle, in which

reticular bodies remain alive inside the inclusions within host cell but do not differentiate

back to elementary bodies. Thus chlamydial infection can't be detected by culturing

suspected tissue isolates with cells *in vitro* to expand live organisms, because there are no

infectious elementary bodies. Later, when the environment changes favorably for

chlamydial growth, reticular bodies recover their ability to produce EB and complete the

life cycle. By going through an incomplete life cycle, *chlamydia* could be latent in an

immune-competent individual and re-flourish at the immune suppressive state. The

evidence for chlamydial persistence are well established in cell culture system, animal

models and epidemiological studies (Beatty et al., 1994b).

3. Chlamydial evasion of host defense responses

The obligate intracellular pathogen *chlamydia* is able to maintain a long-term infection in

immune competent hosts. Careful analysis of chlamydial intracellular growth and its

interactions with host cells has allowed us to conclude that *chlamydia* has evolved various strategies for evading host defense mechanisms.

A. *Intracellular sequestration*

Extracellular pathogens can easily be attacked by antibody neutralization or phagocyte ingestion. *Chlamydia*, as well as other successful intracellular pathogens, can hide in dedicated intracellular compartments and thus are invisible to these immune components.

B. *Avoidance of endo-lysosomal processing*

Chlamydia restricts itself within a vacuole (inclusion) in the cytoplasm of eukaryotic cells. The chlamydial inclusions neither become acidic, nor fuse with host cell lysosomes, which makes it difficult for host major histocompatibility complex (MHC) class II molecules to capture peptides derived from live chlamydial organisms (Eissenberg *et al.*, 1983).

However, only live chlamydia-burdened vacuoles inhibit lysosomal fusion because chlamydial early protein synthesis is required (Van Ooij *et al.*, 1998). Since a large portion of the chlamydial mass released from infected cells are not live EBs, there is a high probability for the infected cells to simultaneously internalize dead chlamydial antigens. Therefore, the chlamydia-infected cells are still able to carry out lysosomal antigen processing from dead chlamydial organisms. Moreover, chlamydial inclusions

can fuse with lysosomes in the presence of chlamydia-sensitive antibiotics (Van Ooij *et al.*, 1997; Escalante-Ochoa *et al.*, 1998), suggesting that chlamydial organisms during a non-productive infection can still be processed in the endocytic pathway.

C. *Inhibition of host cell apoptosis*

Apoptosis, also called programmed cell death or "cell suicide", plays an important role in a wide variety of normal and pathological processes. Intracellular bacteria or virus stimulates infected cells to undergo apoptosis. Infected host cells undergo a series of designated cytoplasmic and nuclear changes and die. Phagocytes then eliminate the dead cells. That dead cells are disposed with membrane-bound contents minimizes damage to uninfected neighboring cells, and discriminates apoptosis from necrosis, in which cell death leads to the production and release of noxious stimuli that cause extensive tissue damage.

During apoptosis, chromosomal DNA is fragmented as a result of cleavage between nucleosomes. This produces a large number of pieces of DNA about 200 nucleotides long. The chromatin condenses and the nucleus breaks up into small fragments. The cell as a whole shrinks and breaks up into membrane-enclosed fragments that have been termed apoptotic bodies.

Early apoptosis can be identified by the presence of DNA fragments detected on polyacrylamide gel electrophoresis, or using a technique called TUNEL (TdT-mediated

d-UTP-biotin nick end labeling). The mechanism of TUNEL is that the single- or double-stranded breaks introduced into the DNA as part of the apoptotic process can be labelled, and therefore the cells undergoing apoptosis can be recognized.

Intracellular pathogens have evolved a variety of strategies to evade eliciting infected cell apoptosis. Many viruses counteract host cell apoptosis and their apoptosis inhibitors have been identified. Examples include the caspase inhibitor CrmA in the cowpox virus (Renatus *et al.*, 2000), p35 in baculovirus (Crook *et al.*, 1993), viral Bcl-2 homologues (Yasuda *et al.*, 1999), and viral homologues of mammalian death receptors (Thome *et al.*, 1997).

Chlamydia requires several days to replicate and differentiate intracellularly to produce sufficient progeny to spread infection. Thus it is critical for *chlamydia* to prevent infected cells from undergoing apoptosis. Tao et al. (1998) demonstrated that *chlamydia* has developed antiapototic activity. By direct DNA staining with Hoechst dye, TUNEL and DNA ladder gel assays, they proved that chlamydia-infected cells resist apoptosis induced by several stimuli including: the kinase inhibitor staurosporine; the DNA-damaging agent etoposide; tumor necrosis factor-α; Fas antibody, and granzyme B/perforin. Since chlamydial antiapoptotic activity depends on chlamydial, but not host cell protein synthesis, *chlamydia* may secrete factors that are transported into the cytosol. The existence of chlamydial factors inside cytosol may provide a means for *chamydia* to actively manipulate antigen processing and presentation pathways of host cells. The exact factor, however, has not been identified.

D. *Potential Evasion of CD4+ T cell recognition by intracellular pathogens*

Intracellular pathogens are processed and presented to T cells by antigen presenting cells (APC) like macrophages or dendritic cells. Pathogens cleaved by lysosome enzymes are presented as a peptide fragment bound to a major histocompatibilty complex (MHC) molecule and expressed on the surface of the APC. There are two types of MHC molecules, namely MHC-class-I and MHC-class-II. They differ in subtle ways, but share some overall structural features.

MHC-class-I molecules collect peptides derived from proteins synthesized in the cytosol, and are thus able to display fragments of nascent viral proteins on the cell surface. CD8+ T cells recognize the MHC-class-I: peptide complex and eventually kill the infected cells. MHC class II molecules bind peptides derived from proteins in intracellular membrane-bound vesicles, and thus display peptides derived from pathogens living in target cell vesicles or antigens internalized by phagocytic cells. CD4+ T cells recognize the MHC-class-II: peptide complex and eventually activate macrophages to eliminate the infected cells.

In chlamydial infection, dead EB enters antigen presenting cells and MHC-class-II: peptide complexes are expressed on cell surfaces. CD4+ T cells then recognize the complex and differentiate into armed effector T cells. Effector T cells recognize the MHC-class-II: peptide complex on the chlmaydial infected host cell surface and the recognition triggers the CD4+ T cells to release powerful cytokines that are either

inhibitory or lethal to intracellular pathogens. The most important among these cytokines is interferon-gamma (IFN-γ). IFN-γ directly inhibits chlamydial intracellular growth (Daubener and MacKenzie, 1999). It also upregulates MHC-class-II expression on target cell surface and changes them to antigen presenting cells, since MHC-class-II molecules are not constitutively expressed on the target cells. (Harton and Ting, 2000)

MHC-class-II: peptide antigen presentation by both antigen presenting cells and infected target cells is essential for T cell mediated immunity to mount a specific immune attack against chlamydial infection. Conversely, T cell immunity may pose a selective pressure on *chlamydia*. Organisms that suppress MHC-class-II expression on antigen presenting cells or infected cells remain invisible to the host immune system and are selected to survive. In this research, we hypothesized that *chlamydia* had evolved strategies to inhibit the IFN-γ induced upregulation of MHC-class-II expression on infected host cells. Without MHC-class-II, these infected cells cannot present chlamydial antigens to their cell surface, and thus T cells cannot target and launch an effective attack against them.

4. The role of ICAM-1 in host defense against intracellular infection

A. Adhesion molecules

The migration of naïve T cells through the lymph nodes and their initial interactions with antigen-presenting cells involves non-specific binding to other cells. Similar non-specific binding eventually guides the effector T cells into the peripheral tissues and plays an important part in their interactions with target cells. Non-specific binding of T cells to

other cells is controlled by an array of adhesion molecules on the surface of the T lymphocyte. These cell-surface proteins recognize a complementary array of adhesion molecules on the surfaces of cells with which the T cell interacts. Four main classes of adhesion molecules are involved in lymphocyte interactions. They are the selectins, the integrins, the immunoglobulin superfamily and some mucin-like molecules.

B. ICAM-1

Intercellular adhesion molecules (ICAMs) belong to the immunoglobulin superfamily. They consist of three very similar members--ICAM-1, ICAM-2 and ICAM-3. All of these proteins bind to the same ligand; the lymphocyte function-associated antigen-1 (LFA-1), a member of the integrin family.

All T cells express LFA-1. It is thought to be the most important adhesion molecule for lymphocyte activation, as antibodies against LFA-1 effectively inhibit the activation of both naïve and armed effector T cells.

ICAM-1 and -2 are expressed on endothelial cells as well as on antigen-presenting cells. Binding to these molecules enables effector T cells to migrate through blood vessel walls and contributes to naïve T cells' immune recognition. ICAM-3 is expressed only on leukocytes and is thought to play an important part in adhesion between T cell and antigen presenting cells. The interaction of LFA-1 with ICAM-1 and ICAM-2 synergizes with a second adhesive interaction involving the immunoglobulin superfamily member

CD2 and lymphocyte function-associated antigen-3 (LFA-3). CD2 is expressed on the T cell surface and LFA-3 is expressed on the antigen-presenting cell.

ICAM-1 is the most important member in the ICAMs family. It is not constitutively expressed on endothelial cells. However, inflammatory stimuli such as IFN-γ, tumor necrosis factor-α (TNF-α) and lipopolysacchride (LPS) up-regulate its expression (Adams et al., 1989; Faull et al., 1989). ICAM-1 actively participates in naïve T cell activation and homing, leukocyte extravasation and armed effector T cell homing. Monoclonal antibodies to either ICAM-1 or its ligand LFA-1 show therapeutic effect in graft rejection, asthma, graft versus host disease and local or systemic reperfusion injury (Wegner et al., 1990).

ICAM-1 not only facilitates non-specific cell-cell interaction in all immune response processes, but also is misused by pathogens to invade host cells (Staunton et al., 1989, Roberts et al., 1992). It is not surprising that one study demonstrated that ICAM-1 provided a protective role in infection (Bendjelloul et al., 2000).

Mice deficient of ICAM-1 show impaired neutrophil emigration in chemical peritonitis and decreased contact hypersensitivity. Inactivated ICAM-1deficient T lymphocytes provided negligible stimulation in the mixed lymphocyte reaction when acting as antigen-presenting cell. However, these cells proliferate normally as responder-cells (Sligh et al., 1993). Naïve T cells require ICAM-1 more than memory T cells (Parra et al., 1993; Igietseme et al., 1999).

Although ICAM-1 is an important non-specific adhesion molecule, its importance varies at different body sites. For instance, ICAM-1 deficiency does not affect acute neutrophil emigration to the pulmonary alveoli, although emigration to peritoneum is completely absent (Bullard *et al.*, 1995).

C. ICAM-1 in host defense against chlamydial infection

The role of ICAM-1 in chlamydial infection is complicated because ICAM-1 not only facilitates the interaction of host immune components, but may also help *chlamydia* to attach and enter host cells (Staunton *et al.*, 1989, Roberts *et al.*, 1992). One of the early-stage events in chlamydial infection is ICAM-1's upregulated expression on epithelial cells (Kol *et al.*, 1999). Kelly and Rank (1997) have also found an increased expression of $\alpha4\beta7$ and LFA-1 in the genital tract in chlamydial infection. Although the ICAM-1 and its ligands' upregulated expression are part of host immune response to better enable T cell adhesion, they may also provide the organisms a useful tool to attach to the host epithelial cells.

Studies of ICAM-1 function in host defense against chlamydial infection are in disagreement. Some studies suggest ICAM-1 may be required to mount an effective immune attack. Igietseme (1996a, 1996b) found that adding ICAM-1 to co-cultured chlamydial infected epithelial cells co-cultured with IFN-γ-producing T cell clones lead to more profound inhibition of chlamydial growth than not adding ICAM-1 to the co-culture.

However, studies in our lab suggest ICAM-1 is not required to mount an effective immune response against chlamydial infection. Both MHC-class-II and ICAM-1 are surface molecules expressed on chlamydial infected epithelial cells upon IFN-γ stimulation, since they are not constitutively expressed. In a flow cytometry study, we added IFN-γ to cells with or without chlamydial infection, and found that chlamydial infection inhibited IFN-γ induced MHC-class-II but not ICAM-1 expression. *Chlamydia* selected inhibition of MHC-class-II but not ICAM-1 makes sense because MHC-class-II molecule is critical to chlamydial infection. It presents chlamydial antigens to CD4+ T cells and thus threatens chlamydial survival. On the other hand, ICAM-1 is not as big a threat to chlamydial survival as MHC-class-II and thus *chlamydia* does not need to develop a strategy to evade ICAM-1 upregulation on the target cell surface upon IFN-γ stimulation. This *in vitro* finding leads us to design an in *vivo* experiment to test ICAM-1 function in natural infection.

III. LITERATURE REVIEW

1. Introduction of chlamydia

A. *Taxonomy*

Chlamydiae, originally viewed as a protoza and later as a virus, are actually obligate intracellular eubacteria (Moulder, 1964). *Chlamydiae* now have been placed in their own order, *Chlamydiales*, with one family, *Chlamydiaceae*, and a single genus, *chlamydia* (Moulder *et al.*, 1984). They are responsible for a wide variety of important human and animal infections. There are four species in the genus *chlamydia*, namely *C. trachomatis* (Moulder *et al.*, 1984), *C. pneumonia* (Grayston *et al.*, 1989), *C. psittaci* (Moulder *et al.*, 1984) and *C. pecorum* (Fukushi and Hirai, 1992). The first two species are human pathogens while the last two are primarily causative agents of animal diseases.

C. trachomatis has been subdivided into three biovars: trachoma, LGV (lymphogranuloma venereum), and mouse pneumonitis agent (Mouse pneumonitis agent; abbreviated as MoPn). The human trachoma and LGV biovars are further divided into 15 serovars on the basis of antigenic differences in the major outer membrane protein (MOMP). The LGV biovar consists of three serovars (L1, L2, L3), with the remaining 12 serovars (A, B, Ba, C to K) in the trachoma biovar.

A disease called trachoma, caused by *C. trachomatis* infection, is the number one cause

of blindness in developing countries. Moreover, *C. trachmatis* infection in the genital

tract is the second leading cause of sexually transmitted disease throughout the world

(Grayston and Wang, 1975; Jones *et al.,* 1982). The consequences of *C. trachomatis*

infections in women include pelvic inflammatory disease (PID), infertility, and ectopic

pregnancy. They are the most costly outcome of any STD except human

immunodeficiency virus (HIV/AIDS), resulting in an estimated 4 billion dollars in health

care per annum in the United States (Water, 1999). *C. pneumonia* is recognized as a

major cause of sinusitis, pharyngitis, bronchitis, and pneumonia (Kuo *et al.,* 1993a and

b). Studies indicate that it is by far the most common chlamydial infection, affecting at

least 50% of the population worldwide. Moreover, coronary heart disease and myocardial

infarction have been associated with *C. pneumonia* infection, supported by sero-

prevalence studies and by direct detection of the organism within artheromatous plaques

(Thom *et al.,* 1992; Jackson *et al.,* 1997). *C. psittaci* causes psittacosis (parrot fever) that

can occasionally be transferred to humans.

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B. *Unique life cycle*

Chlamydiae invade hosts through mucosal epithelial cells. They have successfully

developed an obligate intracellular lifecycle, by alternating between an elementary body

(EB) and a reticular body (RB). EB is an infectious form and RB is a large metabolically

active form. EB is responsible for attaching and promoting chlamydial entry into the

target host cell, while RB replicates (Ward, 1988). The growth cycle is initiated when an

infectious EB attaches to and enters the endosome of a susceptible host cell. The metabolically inert EB then undergoes morphological changes and differentiates to the larger RB. The resulting RB then divides by binary fission within the expanding endosome. When big enough, the endosome becomes visible under a microscope and represents a unique compartment inside the host cell. This is referred to as the chlamydial inclusion body (IB). After a period of growth and division, RBs reorganize and condense to form infectious EBs. The developmental cycle is complete when host cells lyse and the Ebs are released, allowing the *chlamydia* to start a new infectious cycle. The entire intracellular growth cycle takes about 48 to 72 hours *in vitro* and varies as a function of the infecting strain, host cell, and environmental conditions.

2. Persistence of chlamydial infection

Chlamydial infections tend to be asymptomatic in the mild form, but can progress to chronic. Prolonged infections lead to severe damage if treated improperly. Several factors contribute to the persistent infection. Firstly, *chlamydia* exists in the environment and causes repeated episodes of infections. Secondly, the chlamydial major outer membrane protein MOMP gene is genetically variable. MOMP is a porin with an important structure. It forms a trimer enclosing a pore in the outer membrane, and binds to proteoglycans on the epithelial surface during chlamydial attachment to the host cell. After entry into epithelial cells, the environment in the endosome triggers an increase its pore diameter, thus initiating the differentiation from the elementary body to the reticular body. Because of the antigenic variability of MOMP, effector immune cells stimulated by

old antigens can't recognize newly generated antigens and thus host immunity must constantly catch up with the changes of antigen. Thus it is difficult to generate a sufficient number of effector cells to attack a specific antigen (Peeling and Brunham, 1996; Lampe *et al.*, 1997). Thridly, chlamydial persistence is a major factor in the pathogenesis of chlamydial disease (Meyer and Eddie, 1933).

"Persistence" describes a long-term association between *chlamydia* and their host cells, in which these organisms remain in a viable, but culture-negative state. All four chlamydial species are capable of causing persistent infection. Persistent chlamydial infection is associated with incomplete chlamydial development, with only sporadic production of EBs. Thus traditional methods to reactivate and amplify the organism in cell culture failed to detect *chlamydia*. This concept started from work of Moulder and *et al.* (1980, 1981, 1982) and has dominated in this area for nearly 2 decades. Persistent chlamydial infections have been observed in cell culture, animal models and during natural infection in humans.

A. Persistent infection in cell culture

In the early 1960s when *chlamydia* was viewed as a virus, chronic chlamydial infection in cultured cells could be maintained for longer than one year with proper conditions (Galasso and Manire, 1961; Officer and Brown, 1961). In 1980's, Moulder and colleagues (1980, 1981, 1982) infected mouse fibroblasts (L cells) with C. psittaci. The infection at high multiplicity of infection (MOI) resulted in alternate periods of host cell

destruction by chlamydial multiplication and periods of chlamydial proliferation inside L cells without destruction of host cells. The coexistence of L cell and *C. psittaci* can be maintained indefinitely in the absence of typical chlamydial inclusions. This form of *chlamydia* was defined as a cryptic body.

Since then, conditions that alter the chlamydial developmental cycle in cell cultures have been thoroughly investigated. Some studies found persistent chlamydial infection can be induced in nutrient-deficient medium (Bader and Morgan, 1958, 1961; Morgan 1956). Supplementation of nutrients to the medium restored productive chlamydial growth (Bader and Morgan, 1958, 1961). Coles and colleagues (1993) mapped amino acid deficiency in the chlamydial persistence induction. Medium lacking any of the 13 essential amino acids with the exception of valine induces aberrant chlamydial development and leads to persistent infection. Whether the amino acid-deficiency-induced persistence occurs *in vivo* during natural infection, however, is unknown.

A vast number of antibiotics have been shown to inhibit the intracellular growth of *chlamydia*. The effectiveness of antibiotic inhibition depends not only on the mechanism, but also on the ability of a specific antibiotic to reach effective concentrations inside inclusion bodies. Penicillin (Storey and Chopra, 2001; Clark *et al.*, 1982a), ampicillin (Beale *et al.*, 1991) and D-cycloserine (Moulder *et al.*, 1963) can inhibit the synthesis of peptidoglycan without affecting protein biosynthesis. Treatment with these antibiotics often induces an atypical form of *chlamydia* and leads to persistent infection. The phenomenon is unusual because *chlamydia* is deficient in peptidoglycan (Barbour *et al.*,

1982; Garrett *et al.*, 1974). The inhibition is actually associated with a cysteine-rich 60-kDa-envelope protein (Moulder 1993).

Chloramphenicol, chlorotetracycline (Tribby *et al.*, 1973) and erythromycin (Clark *et al.*, 1982b) effectively inhibit prokaryotic protein synthesis. They often clear chlamydial infection if the treatment is early and thorough enough. However, the established inclusion bodies are very resistant to antibiotic treatment since the matured EBs are no longer metabolically active. Some researchers have observed that the inclusion bodies keep expanding regardless of the inhibition of protein synthesis by antibiotics. Therefore, a prolonged antibiotic treatment is required in chlamydial infection.

Immunological factors such as IFN-γ that inhibits chlamydial growth may also induce persistent chlamydial infection. IFN-γ inhibits chlamydial growth *in vitro* (Byrne *et al.*, 1989; De la Maza *et al.*, 1985; Kazar *et al.*, 1971; Rothermel *et al.*, 1983a) as well as *in vivo* (Williams *et al.*, 1988). In a cell culture system, IFN-γ inhibits chlamydial growth in various types of cells including mononuclear phagocytes (Rothermel *et al.*, 1983b), fibroblasts (Byrne and Krueger, 1983) and epithelial cells (Byrne *et al.*, 1986). Once IFN-γ is removed, productive chlamydial growth is often restored.

IFN-γ-induced chlamydial inhibition is time and dose dependent. Pretreatment of the cells with high levels of IFN-γ completely inhibits chlamydial growth. Delayed addition or low levels of IFN-γ often induces persistent infection, characterized by the development of morphologically aberrant bodies (Shemer and Sarov, 1985). Even in the

presence of a very high concentration of IFN-γ, EBs enter host cells and remain viable for a period of time.

The complexity of the influence of IFN-γ on chlamydial growth has triggered intensive investigation. A deficiency of tryptophan caused by the IFN-γ-activated indoleamine 2,3-dioxygenase (IDO) is a major mechanism for IFN-γ–induced chlamydial inhibition (Byrne et al., 1986; Thomas et al., 1993). This is because the IFN-γ–induced chlamydial inhibition correlated with an increased IDO activity and can be reversed by addition of tryptophan. Up-regulation of macrophage-derived nitric oxide synthase (iNOS) (Byrne et al., 1992), an enzyme engaged in the generation of cytotoxic reactive nitrogen intermediates (Mayer et al., 1993; Igietseme et al., 1996c) provided an alternative mechanism. Blocking of nitric oxide in vitro, by adding the nitric acid (NO) synthase inhibitor N^G-monomethyl-l-arginine monoacetate to the culture solution, reversed the effect of interferon. In vivo studies of iNOS, however, suggested that iNOS may not play any significant role in host defense against chlamydial infection (Igietseme et al., 1996c; Ramsey et al., 1998; Igietseme et al., 1998a).

Other immunological factors like TNF-α (tumor necrosis factor) have also been studied and found to induce an altered form of chlamydia (Shemer-Avni et al., 1989).

B. *Persistent chlamydial infection in animal model*

Different mouse models are established to study the persistent infection of *chlamydia*.

Taiwan monkeys, infected through their eyes with trachoma isolates of *C. trachomatis*,

resulted in intermittent shedding of viable *chlamydia* (Wang *et al.*, 1967). Immuno-

suppression with a corticosteroid frequently reactivated shedding. Immuno-suppression

also lengthens the shedding of viable chlamydia in mice lungs infected with *C.*

trachomatis or *C. pneumonia* (Latitinen *et al.*, 1996; Malinverni *et al.*, 1995) and mice

genital tracts infected with *C. trachomatis* (Cotter *et al.*, 1997a). When progesterone was

used to treat C57BL/6 mice intravaginally infected with the mouse pneumonitis agent,

dissemination of chlamydiae in small numbers to the lymph nodes, peritoneum, spleen,

kidney, liver and lungs was observed (Cotter *et al.*, 1997b).

C. *Epidemiological studies of Persistent infection with Chlamydia*

Lysis of one host cell in culture 48 to 72 hrs following infection can result in the release

of hundreds of EBs. Each viable EB is capable of eliciting a new round of infection.

Culturing L2 strain of C. trachomatis in Hela cells, we expand EB infectivity titer as high

as 50-100 times per passage generation, measured by inclusion body forming unit (IFU).

It is believed that the chlamydial developmental cycle is similar in natural infection as in

cell culture. Rapid expansion of *chlamydia* would result in rapid progression of the

infection. In the natural condition, however, chlamydial infection exhibits a prolonged

course, and is often insidious and asymptomatic. This suggests incomplete or altered

chlamydial development cycles may exist following natural infection, although there is

no direct evidence. It is expected that chlamydial growth, during natural infections, is

more complex, with interruptions in chlamydial differentiation and production of

infectious progeny similar to persistence infectious events defined in cell culture models.

i. Ocular chlamydial infection

Although a clear correlation between trachoma and infection with *C. trachomatis* has

been established, chlamydia organisms cannot be identified by tissue culture in over 20%

of the cases, even in the presence of severe abnormalities (Taylor *et al., 1989*).

Progressive scarring disease with a clinically unapparent, nonproductive (culture-

negative) infection indicates that these individuals may bear a cryptic form of *chlamydia*

in the infected tissue (Schachter, 1978). Therefore, viable *chlamydiae* may be present in a

latent, non-replicating form, inconspicuously contributing to the progression of disease

toward blinding trachoma. Other evidence for the continued presence of *C. trachomatis*

in a culture-negative state comes from studies of trachoma patients and a primate model

for ocular disease. They developed a hybridization screening system directed toward

chlamydial ribosomal RNA (rRNA). The presence of un-amplified chlamydial r-RNA in

conjunctive swabs was detected long after live EB can be revived from infected tissues

(Cheema *et al.,* 1991; Holland *et al.,* 1992).

ii. Genital tract infection

Several studies indicate a strong association between serological evidence of a previous

C. trachomatis infection and obstructive infertility (Brunham *et al.,* 1985; Henry-Suchet

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et al., 1987; Shepard and Jones, 1989). However, recovery of viable *chlamydiae* from tubal biopsy specimens is rare. Although *chlamydia* is difficult to culture, especially when the disease process has become chronic, the presence of chlamydial antigens and nucleic acid is indicative of persistent organisms. Although reticular bodies may replicate and are mildly active inside inclusions, they do not differentiate into elementary bodies and thus can't be detected by cell culture. A number of studies have identified *C. trachomatis* antigens and chlamydia-specific DNA in endometrial and tubal specimens of culture-negative infertile women (Soong *et al.,* 1990; Theijis *et al.,* 1991).

3. Chlamydia and immunity

A. Initiation of immunity

The sites of *C. trachomatis* introduction into the host are epithelium-lined mucosa in the genital tract, eye, or respiratory tract. Each of these sites is unique and has a big impact on the nature, speed, pace of progression, and intensity of local host immune system and pathogen interactions. Different sites may also have different capacities to bind pathogens and thus chlamydial infection disseminates differently.

A chain of events occurs after chlamydial elementary bodies are internalized into the epithelial cells. In a cell culture study, IL-8 and IL-1 produced by HeLa and human endocervical cells were the earliest response to infection by *C. trachomatis* strain L2 infection (Rusmussen *et al.,* 1997). Viable organism is necessary for the cytokine

production. Adhesion molecules like ELAM-1, ICAM-1, and VCAM-1 are induced

within a few hours on human epithelial cells after infection by *C. pneumonia*

(Kaukoranta-Tolvanen *et al.*, 1996). Tumor necrosis factor alpha (TNF-α) is released

from human peripheral blood leukocytes after cells are stimulated *in vitro* by EBs and

LPS from *chlamydia* (Ingalls *et al.*, 1995). LPS may be a major factor in the release of

TNF-α in a natural infection because specific inhibitors of LPS can block TNF-α. The

production of adhesion molecules and cytokines in the early stage of chlamydial infection

initiates the inflammation and immune response.

B. Dominant Cell-mediated immune response

Host immunity against chlamydial infection consists of both humoral and cell-mediated

immune response. The effector cells of the humoral response are plasma cells

differentiated from B-lymphocytes. They secrete specific antibodies directed against

extracellular pathogens. The effector cells of cell-mediated immune response are T

lymphocytes. They kill infected cells or activate other cells of the immune system. The

relative contribution of each to the resolution of the infection has long been the subject of

debate, and is further complicated by the site of infection, the animal species, and even

the strain of the animal.

The humoral immune response appears to not be required for the resolution of both

primary infection and seconday infection in the murine genital tract infection with

chlamydia. B-cell-deficient mice of both the Balb/c and B57BL/6 background resolved

the infection the same way, as did the immunological normal mice in the first infection. In addition, all mice were immune to the rechallenge (Su et al., 1997; Ramsey et al., 1988).

Williams and colleagues (1987b) studied the role of B cells in the immune response against chlamydial infection in the lung. They treated Balb/c mice with an anti-IgM antibody for several months to create IgM deficient mice, and then challenged these mice intranasally with mouse pnemonitis strain (MoPn) of C. trachomatis. IgM deficient mice recovered from the pulmonary infection as efficiently as immunologically normal mice. Williams and colleagues (1997) reported that IgH-/- B cell deficient mice resolved primary lung infection as well as control mice. However, these mice had a slight but significant increase of chlamydial burden following secondary infection, compared to immune competent mice.

Humoral immunity is accepted generally as not essential, or mildly required in the host defense against chlamydial infection.

Cell-mediated immunity is very important in host defense against chlamydial infection. Host resistance to primary infection is strongly T cell dependent since nude mice were much more susceptible to infection with chlamydia than normal mice and often failed to clear the infection (Coalson et al., 1987; Williams et al., 1981; Magee et al., 1993). Athymic mice on a BALB/c background remained susceptible to chlamydial rechallenge, whereas euthymic animals developed resistance (Williams et al., 1982).

T cells are classified as CD4+ T cells or CD8+ T cells according to their cell-surface molecules. The CD nomenclature reflects the characterization of leukocyte cell-surface molecules as recognized by monoclonal antibodies. CD4+T cells are also called T helper cells. They recognize pathogens and their products in the vesicular compartments that are presented by major histocompatibility complex (MHC) class II; then they activate other cells. CD8+ T cells are also called cytotoxic T cells. They recognize viruses and bacteria that live in the cytosol and are presented by MHC-class-I; then they kill infected cells.

In murine MoPn infection in the genital tract, CD4+ T cells played a more important role than CD8+ T cells in host defense against chlamydial infection in mice with a BALB/c or C57BL/6 background (Magee et al., 1995; Morrison et al., 1994; Igietseme et al., 1993; Stagg et al., 1998). Adoptive transfer of donor CD4 T cells, but not CD8 T cells, obtained from mice following resolution of primary challenge or during secondary genital tract infection, transferred antichlamydial immunity to severe combined immune deficiency (SCID) mice (Magee et al., 1993). It was reported that there is an influx of major histocompatibility complex (MHC) class II-bearing cells into the genital tract. These cells may be important for antigen processing and activation of CD4 cells (Stagg et al., 1998). However, the exact function of these cells in the mouse genital tract is still unknown.

CD8+ T cells may not play any significant role in clearing chlamydial infection. Perry and colleagues have shown that clearance of chlamydia trachomatis from the murine genital tract did not require perforin-mediated cytolysis or Fas-mediated apoptosis

(1999a), demonstrating that the cytolysis function of CD8+ T cells is not required for resolving chlamydial infection.

However, in a single lung infection, mice depleted of either CD4 or CD8 T cells had a significant increase in chlamydial burden on day 14 postinfection, although the mortality rate was significantly higher in mouse depleted of CD4 cells than in those with CD8 cell depletion (Magee *et al.*, 1995). Upon reinfection, β2m-/- mice (MHC class I deficient and thus functional CD8 T cell deficient) resolved infection simultaneously with the control C57BL/6 mice. MHC class II-/- mice failed to resolve infection (Williams *et al.*, 1997). The result suggested a more important role of CD4+ T cell in secondary lung infection.

In conclusion, CD4+ T cells are critical to mediate an acquired immune response against chlamydial infection both in the lung and the genital tract.

C. Dominant Th1 response

CD4+ cells can be further divided into two functional groups: Th1 and Th2 cells. *Chlamydia* and many other intracellular bacteria survive because the vesicles they occupy do not fuse with the lysosomes. Th1 cells, after differentiation into effector cells, secrete cytokines like IL-12, IL-2, and IFN-γ. These cytokines activate macrophages, inducing the fusion of their lysosomes with the vesicles containing the bacteria and at the same time stimulating other antibacterial mechanisms of the phagocyte. Th1 cells also release cytokines that attract macrophages to the site of infection. Th2 cells, upon activation,

secrete IL-4 instead, and help B cells to proliferate and differentiate into effector plasma cells. Most extracellular antigens require an accompanying signal from Th2 cells before they can stimulate B cells. Thus, Th2 cells mediate humoral immune response.

Since humoral response is proved to be not required in efficient immunity against chlamydial infection, Th2 cells may not be required either. Neutralization of IL-4 had no detectable effect on host immunity or on bacterial clearance in mouse genital tract and pulmonary infections (Trinchieri 1995).

Th1 immune response is critical against chlamydial infection. Mice depleted of IFN-γ by treatment with anti-IFN-γ antibody had a higher mortality rate than wild type mice when challenged intranasally with mouse pneumonitis agent (MoPn) (Williams *et al.*, 1988). Mice with IFN-γ or IFN-γ receptor gene knockouts showed decreased ability to control chlamydial infections (Perry *et al.*, 1997; Cotter *et al.*, 1997b; Johansson *et al.*, 1997). Th1 cytokines were predominantly detected in infected animals (Cain and Rank, 1995; VAN Voorhis *et al.*, 1997; Perry *et al.*, 1997; Williams *et al.*, 1997). Neutralization of IL-12, the cytokine ultimately responsible for the induction of Th1 type immune response, was associated with an apparent decrease in the infiltration of CD4+ T cells into infected tissues (Trinchieri 1995).

Studies in our lab found that chlamydia-pulsed dendritic cells from wild type mice could elicit a protective Th1 immune response to chlamydial infection. However, dendritic cells from IL-12 p40 gene deficient mice failed to do so, suggesting that the ability of dendritic

cells to produce IL-12 is required for the induction of protection against chlamydial
infection (Lu and Zhong, 1999).

4. Adhesion molecules and Chlamydial infection

A. Introduction of ICAM-1

The ICAM family consists of ICAM-1, 2, and 3. They are all intercellular adhesion
molecules that non-specifically facilitate migration and adhesion of immune cells. All
ICAMs bind to the same ligand LFA-1, a member of another intracellular adhesion
molecule integrin family. ICAM-1 and –2 are endothelial ligands for leukocytes. ICAM-3
plays a role in adhesion between T cells and antigen presenting cells.

ICAM-1 is the most important member of the ICAM family; it is involved in many
physiological processes, including naïve T cell activation and homing, leukocyte
extravasation and armed effector T cell homing (Hayflick et al., 1998; Hubbard and
Rothlein 2000). However, it is not constitutively expressed on non-APCs. Expression of
ICAM-1 can be drastically increased on epithelial cells upon IFN-γ, TNF-α or
lipopolysaccharide (LPS) stimulation (Adams et al., 1989, Faull et al., 1989).

B. ICAM-1 and chlamydial infection

Whether ICAM-1 is essential to launch an effective immune response against chlamydial infection is in debate.

Some studies indicate that ICAM-1 plays a significant role in host defense against chlamydial infection. Igietseme and colleagues (1996c) co-cultured chlamydial infected epithelial cells with IFN-γ-producing T cell clones. Adding ICAM-1 to the co-cultured medium lead to more profound inhibition of chlamydial growth than co-culture alone. Monoclonal antibodies against either ICAM-1 or LFA-1 partially reversed the inhibition. They concluded that adhesion molecules are required for the enhanced chlamydial inhibition in epithelial-T cell interaction.

In an *in vivo* experiment, Kelly and Rank (1997) infected mice with chlamydia in the genital tract. They found local CD4+ T cells had increased expression of $\alpha 4\beta 7$ and LFA-1. Although these cells may not be chlamydia-specific, it suggested that LFA-ICAM-1 interaction promoted T cell trafficking in the local inflammatory response.

Igietseme and colleagues (1999) further tested the role of ICAM-1 in chlamydial infection in a genital tract infection model. They challenged both ICAM-1 deficient mice and wild type mice with live EBs. Reactivated and amplified organism reovered from cell culture and cytokine production, as parameters of resistance against chlamydial infection were compared in the two groups. ICAM-1 deficient mice had significantly greater chlamydial burden and decreased IFN-γ, TNF-α and IL-12 level during the first 2 weeks of infection compared to wild type mice. However, the organism burden quickly reached

a similar level between wild type and ICAM-1 deficient mice two weeks after the infection. All mice completely recovered from the infection. They concluded that ICAM-1 may be required for early T cell recruitment and activation.

Our study suggests otherwise. Both MHC-class-II and ICAM-1 are surface molecules expressed on epithelial cells upon IFN-γ stimulation. Both molecules are not expressed constitutively. In an analysis using flow cytometry, we added IFN-γ to the culture supernatant of cells with or without chlamydial infection, and found that chlamydial infection inhibited IFN-γ induced MHC-class-II, but not ICAM-1 expression. The selected inhibition of MHC-class-II but not ICAM-1 makes sense, because the MHC-class-II molecule is critical to chlamydial infection in that it presents chlamydial antigen to CD4+ T cells and thus threatens chlamydial survival. Conversely, ICAM-1 isn't as big a threat to chlamydial survival, and thus chlamydia does not need to develop a strategy to evade ICAM-1 upregulation on the target cell surface. We concluded that ICAM-1 is not important in the immune response against chlamydial infection.

In summary, the hypotheses to be tested in this study are two-fold: first, *chlamydia* has evolved a strategy to inhibit IFN-γ induced major histocompatibility complex II (MHC-class-II) molecule expression in order to evade immune recognition. Second, intercellular adhesion molecule-1 (ICAM-1) is not important in host immune response against chlamydial infection.

The goal of this research is to explore the mechanism of chlamydial persistence under host immune monitoring. Until now, little is known about chlamydia-host cell interactions, as the organism is very difficult to work with: it cannot be cultivated in cell-free media, there are no well-characterized mutants available and genetic manipulation techniques have not been devised for the organism. By studying chlamydial manipulation of host cell surface protein MHC-class-II and ICAM-1 expression, this research provides useful information of the chlamydia-host interaction. The findings may lead to isolation of functional chlamydial components responsible for persistent infection, and thus contribute to the development of an efficient vaccine against chlamydial infection.

IV. Methods and Materials

1. Cell line and cell culture

The following human cell lines were used in the thesis studies:

MCF-7: a mammary epithelium line, provided by Dr. Arnold Greenberg of the Manitoba Institute of Cell Biology

MRC-5: fibroblast, American Type Culture Collection [ATCC, Manassas, VA]

2C4: fibroblast, provided by Dr. George Stark of the Cleveland Clinic Foundation

HeLa: Human cervical epithelium, ATCC

Cell culture procedures:

➤ Remove medium from an established cell culture flask with a conflent monolayer (VWR, 150cm^2 Mississauga, Ont).

➤ Rinse the cells with 5-10ml phosphate buffered saline (PBS) 1×.

➤ Rinse the cells with 3ml 0.1% trypsin solution (GIBCO, Burlingto, Ont)

➤ Add enough trypsin to cover monolayer (1-2 ml for 150cm^2 flask)

➤ Incubate at 37°C until the cell layer starts to detach.

➤ Pat the flask.

➤ Quickly add 10ml Dulbecco's Modified Eagle Medium (Eagle's DMEM) with 10% fetal calf serum (DMEM-10) (GIBCO) and pipet vigorously to disperse the cells.

> Split the cell suspension from one into two flasks if cells are required in 24 hours. Otherwise, the cell suspension from one flask seeds three flasks. For maintenance, discard 2/3 of cell suspension and put remaining 1/3 back into the original flask.

> Change to new flasks after passing 3 culture generations.

> Add 20ml DMEM-10 in each flask and incubate at 37°C, 5% CO_2.

2. Chlamydia strains and culture conditions:

The following strains of chlamydia are used:

L2: belongs to LGV biovar, *C. trachomatis* species

MoPn (Weiss strain): belongs to mouse peumonitis biovar, *C. trachomatis* species

Procedures for growing L2 and MoPn stock in Hela cells:

> Prepare a confluent 24-hour monolayer of HeLa cells in $150cm^2$ flasks.

> Discard the old medium.

> Add 10 ml HBSS-EDTA (Hanks balanced salt solution - ethylenediamne tetra-acetic acid) to each flask, and let them stand at room temperature (rt) for 15-20 minutes.

> Prepare L2 or MoPn inoculum: 5ml DMEM-10 + L2 or MoPn stock with 3×10^7 IFU/flask to make the MOI (Multiplicity of Infection) a little higher than 1.

> Remove HBSS-EDTA from each flask, and add inocula. Incubate at 37°C, 5% CO2 for 2 hours.

➤ Remove the inocula.

➤ Add 20 ml DMEM-10 to each flask and incubate in 37°C, 5%CO2.

➤ 42 hours after L2 infection or 36 hours after MoPn infection, harvest chlamydia.

➤ Harvest the infected cells as a cell suspension in 50-ml centrifuge tube (Fisher, Nepean, Ont) on ice.

➤ Centrifuge at 4°C, 300 × g 10 mins to get cell pellets. Discard the supernatant.

➤ Resuspend L2 and MoPn cell pellets in cold SPG (sucrose 60g, KH_2PO_4 0.415G, Na_2HPO_4 0.976g, Glutamic acid 0.576g Diluted in 800ml ddH2O) and set the suspension on ice.

➤ Sonicate cell suspension 15 sec × 3 times, to release EBs from the inclusion bodies. Set the tube on ice for 5-minute intervals, to cool down the cells. (Sonics and materials, Inc., Danbury, Connecticut, USA)

➤ Centrifuge the suspension 300× g 10min × 3 times, each time removing the cell debris and saving the supernatant.

➤ Divide the 3 × purified supernatant (EB stock) in 500ul aliquots and store at −80°C.

➤ The stock is used in cell culture studies, so all the procedures are performed under condition of strict sterility.

➤ *L2 and MoPn stock titer determination*

➤ Prepare fresh (prepared within 24 hours) HeLa cell monolayer in 24-well-plate (Fisher), 2×10^5 cells/well.

➤ Infect cells with 5-fold serial diluted L2 or MoPn stock in cold SPG.

- 24 hrs after incubation, perform DAB (3, 3'-Diaminobenzidine tetrahydrochloride liquid substrate system) staining to visualize the inclusion bodies.

- Fix infected cells with 300ul/well 100% methanol, 30mins rt.

- Block the cells with 500ul/well 1% BSA/PBS (Bovine serum albumin/phosphate buffered saline), 30mins rt.

- Add monoclonal antibody Mc22 of MOMP (major outer membrane protein, made in our lab) 200ul/well, 30min, rt.

- Wash the cells with PBS × 3 times to remove the remaining antibodies.

- Dilute Rabbit anti Mouse IgG conjugated with HRP, (Horse Radish perioxidase Capnel 3211-0082) 1:200 in 1% BSA/PBS, and add 200ul diluted antibody into each well, incubate 30min rt.

- Wash with PBS × 3 times to remove the remaining antibodies.

- Apply 1×DAB 250ul/well (10×concentrated stock + DAB buffer, kit from BOEHRINGER MANNHEIM, Laval, Quebec), and monitor closely under the light microscope.

- When the inclusions stain dark brown, stop the reaction with 1ml PBS in each well.

IFU (Inclusion body forming unit) calculation

- Choose the wells with infection rate between 10% and 90%.

- Count inclusion bodies under five 32× high power-fields per well.

- IFU calculated from each individual well: Average inclusions per field × 1500 × dilution × 4.

- Average calculated IFU from each well.

IFU is defined as the amount of inclusion bodies formed after 1ml EB stock passes one generation in cell culture, in order to estimate the infectivity of a particular stock preparation. Because the area of 1 field at 32x high-power under microscope is 1 mm^2, and the area of 1 well in 24-well plate is 1.5 cm^2, so the area of 1500 fields at 32x high-power equals one well of 24-well-plate. Thus, we calculate the amount of inclusions forming in a well of 24-well-plate by multiplying IBs counted × 1500. Because each well was infected with a differently diluted EB stock concentration, we also multiply by the fold diluted. Finally, because we infected each well of 24-well-plate with 250 ul inocula, and IFU is the estimation of 1ml stock, we also multiply by 4 in the formula to bring the calculation to 1 ml.

3. <u>Preparing cell samples for flow cytometry, WB and RT-PCR: chlamydia infection and interferon-gamma stimulation</u>

Prepare 4 samples per set for FACS, WB and RT-PCR:

- Sample A: Normal cells,

- Sample B: Normal cells + interferon-gamma,

- Sample C: L2 infected cells,

- Sample D: L2 infected cells + interferon-gamma.

➢ Set up 24 hrs fresh sets of four 25cm^2-flasks, each set consisting of 2 × 10^6 MCF-7, 2C4 or HeLa cells in each flask, or 4 × 10^6 MRC-5 cells per flask, for a total of 16 flasks per experiment.

> Infect samples C and D with 5×10^6 IFU L2 stock, to make the MOI (Multiplicity of infection) 1.2, and add 5ml DMEM-10 each flask after infection.

> Incubate the flasks at 37°C with 5% CO2 for 22 hours.

> Add into samples B and D 5ul of human IFN-γ (Pharmingen, San Diego, CA [stock concentration 2×10^5 unit/ml]) to make the final concentration in the flask (5ml DMEM-10) 400unit/ml.

> Incubate at 37°C with 5% CO_2 for another 22-24 hours.

> Harvest cells.

> Use freshly harvested cells in flow cytometry.

> Freeze cell pellets at –80°C for WB and RT-PCR.

4. Flow cytometry

The technique of flow cytometry is similar to the visual assessment of the intensity of fluorescence of cells stained with a fluorescein-conjugated antibody under an Ultra-violet (UV) light microscope. In flow cytometry, the fluorescence intensity for each cell from different angles is determined in a machine called a fluorescence-activated cell sorter (FACS) and the distribution of the cell population in a sample is presented grophically. A certain population can also be sorted and separated from the rest of the sample. Measurement on the average of 10,000 cells per sample can be made in a few minutes.

The laser beam interrogates each individual cell, and the scattered light from cells is analyzed in the forward and 90° angles. The intensity of the forward angle light scatter

(FALS) signal is directly proportional to the volume of the cell being analyzed. The 90-degree light scatter (90°LS) not only is proportional to the volume of the cell, but also is affected by other parameters such as granularity, surface topography, etc.

In practice, all 4 sets (A-D) of cells are labeled by each of the following first antibodies, namely anti-HLA-DRα (L243; ATCC), mouse anti-human intracellular adhesion molecule-1 (ICAM-1) (HA58; Pharmingen), and normal mouse IgG (Zymed Labs, Inc, South San Francisco, CA). Primary antibody binding was detected using goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Caltag Labs, Burlingame, CA) and analyzed with a FACSCalibur ™ equipped with CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Dead cells were excluded by propidium iodine (PI) staining.

Electronic gates were set prior to analyzing the data. Even though cell debris or cell clusters were present, they were "gated out" from further analysis. Quantitation of fluorescence is performed to evaluate the MHC class II molecule and ICAM-1 expression on the cell surface.

5. Western Blot

Cell samples are lysed with a RIPA buffer (50mM Tris-HCL, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 ug/ml Aprotinin, 10ug/ml leupeptin, 1ug/ml pepstatin A, and 1 mM sodium orthovanadate) and

centrifuged at 13,000g at 4°C for 15mins to obtain the postnuclear supernatants that contain the whole cell protein. The protein concentrations are then calculated based on a standard curve derived from serial diluted BSA.

Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins with different molecular weights. In electrophoresis, the migration of proteins is dependent upon the charge, size and shape of the molecules. However, in the presence of SDS, proteins bind the SDS and become negatively charged with similar charge: weight ratios. When SDS-coated proteins are placed in an electric field, their spatial separation will depend only on their size and shape. By varying the concentration of the polyacrylamide gels used as the medium for the electrophoretic separation, different resolution ranges of molecule weights may be obtained.

Following electrophoresis, a process called electroblotting transfers the proteins to a nitrocellulose membrane (NCM, Bio-Rad, .45 um, Mississauga, Ont). Assemble the transferring sandwich within the blotting cassette taking in order: filter paper, NCM, polyacrylamide gel, filter paper. A TRANS BLOT apparatus (Bio-Rad) was used for the electroblotting.

After the transfer, the NCM with the bound proteins are blocked and stained with first antibodies, namely ∝HLA-DRα (DA6.147; provided by Dr. Peter Cresswell, Yale University) and ∝MOMP: clone Mc22. Then second antibodies [anti-mouse or anti-

rabbit IgG conjugated with alkaline phosphatase or horseradish perioxidase (HRP)] are used to detect first antibody.

Finally, the blot is developed on X ray films for 2-3 minutes with the ECL kit (Amersham Corp, Buckinghamshire, UK).

6. RT-PCR

Polymerase chain reaction is a basic molecular technique to detect a particular gene if the DNA sequence is known. Two short oligonucletide primers are designed that are complementary to either end of the gene. The sample with the genome, the primers and DNA polymerase are heated to separate the double-stranded DNA to single-stranded DNA, and then the sample is cooled down. In the presence of primer, and the particular gene in the genome as the template, and DNA polymerase, the particular gene sequence goes through semi-conservative replication and expands a single copy of the gene to 2 copies. This is called a PCR cycle. With the repetition of 20-30 cycles, in which the newly synthesized gene copy can also be used as template, a single copy of the gene is expanded to thousands of copies that can be detected in an agrose gel under UV light.

Reverse transcriptase, a retroviral enzyme that synthesizes complementary DNA (cDNA) from RNA template, has allowed the development of RT-PCR to identify a gene expressed specifically under *in vivo* conditions. RT-PCR differs from PCR in that the RT step uses RNA as template. Only when a protein is actively expressed in a cell, is its gene

transcribed into mRNA. Thus RT-PCR detects the expression of mRNA for a particular gene within a cell.

In our study, total RNA was first extracted using a Qiagen RNeasy minikit. DNAase was then applied to the total RNA to further remove DNA from the sample. The first strand of cDNA was synthesized using total RNA as the template and avian myeloblastosis virus (AMV) reverse transcriptase (Amersham pharmacia Biotech kit). PCR was performed to expand the cDNA. PCR products from Sample A to D, namely normal cells, normal cells with IFN-γ stimulation, infected cells and infected cells with IFN-γ stimulation, were run in parallel on an agrose gel. Finally, the product amounts as the indication of the level of gene expression were visually compared from a picture taken under UV light.

The primers used in PCR are:

DRα (Miller, et al, 1998) forward 5'-AAAGCGCTCCAACTATACTCCGA-3'

Reverse 5'-ACCCTGCAGTCGTAAACGTCC-3'

DMα: (Albanesi et al, 1998) forward 5'-ACCTACTGTGTGGCAAGAAGGTATG-3'

Reverse 5'-GCTGGCATCAAACTCTGGTCTGGAA-3'

Ip41: (Albanesi et al, 1998)

Forward 5'-CAGACCCTGCAGCTGGAGAACCTGCGCATG-3'

Reverse 5'-GCAGTTATGGTGCCCGCGGCTTCTGGTGTT-3'

β-Actin: forward 5'-GTGGGGCGCCCCAGGCACCA-3'

Reverse 5'-CTCCTTAAGTCACGCACGATTTC-3'

7. Mouse strains and housing

20 wild type and 20 ICAM-1 deficient female mice (5-6 weeks old) with the C57BL/6 background were obtained from Jackson (Bar Harbor, Maine), and were maintained under standard environmental conditions in the central facilities of the University of Manitoba.

8. Mouse infection

Respiratory MoPn infection procedure (Nigg, C 1942; Williams et al., 1981; Cho-chou et al., 1980)

➤ Anesthetize each mouse with Aerrane (isoflurane, JANSSEN), and apply 20ul inocula to each mouse intranasally.

➤ For the first infection, the concentration of the inoculum: 10^4 IFU/mouse; the dilution process was: 8.5ul stock (6×10^8 IFU/ml) + 10 ml PBS.

➤ For the second infection, the concentration of the inoculum: 2×10^6 IFU/mouse; the dilution process was: 166ul stock (6×10^8 IFU/ml) + 834ul PBS. The second infection was 1 month after the first infection. By the time of second infection, the body weights of all the mice had recovered to the original level.

➤ Monitor mouse body weights on a daily basis for 10 successive days after the first infection and 4 days after the second infection before the mice were sacrificed.

9. Lung titration

a. Prepare MoPn inocula from the lung:

➢ Weigh each lung, and set each lung in 4ml SPG on ice.

➢ Homogenize the lung on ice to release free EBs in the lung tissue.

➢ Centrifuge the homogenized tissue 300 × g 10 mins and remove the coarse tissue

pellet.

➢ Divide the supernatant into 500ul aliquots and freeze them at –80°C.

b. Lung titration:

Infect HeLa cells with the MoPn inocula prepared above. EB titer of each lung is then

determined by DAB (3, 3'-Diaminobenzidine tetrahydrochloride liquid substrate system)

staining of the inclusion bodies and calculation of IFU/g lung, following the procedure

described in section 2 in Methods and Materials.

10. Spleen cell stimulation *in vitro*

Mice were sacrificed and their lungs and spleens were extracted. Lung samples were

immediately freshly used as inocula to infect HeLa cells in order to quantify the number

of live organism and assess the severity of infection in the mice. T lymphocytes were

extracted from spleen samples, and then were stimulated with dead EB antigen for

several days. Cytokine production in the supernatant of the stimulated T cells was quantified by ELISA (enzyme-linked immunosorbent assay) to assess the magnitude of immune response against chlamydial infection in the mice.

Procedure of spleen cell stimulation in vitro is:

- ➢ Heat MoPn elementary bodies at 56°C for 30 min to inactivate EB.

- ➢ Dilute dead EB in RPMI with 5% fetal calf serum and 2-ME (10uM) to achieve a final concentration 10^7 IFU/ ml.

- ➢ Smash spleen in PBS to release cells.

- ➢ Pipet cell suspension through a nylon filter to remove the coarse tissue.

- ➢ Determine white blood cell concentration under light microscope, excluding the dead cells by trypan blue.

- ➢ Take 3×10^7 cells from each mouse for spleen cell stimulation.

- ➢ Wash the cells once with PBS in 50-ml centrifuge tube.

- ➢ Resuspend the cells in 3ml DMEM-10.

- ➢ Distribute the cells in 24-well plates with 10^7 cells per well, with triplicate wells for each mouse. Identify the spleen from each mouse with a number.

- ➢ Add 1ml prepared dead EB solution to each well.

- ➢ Following 24 hours incubation take 0.5ml supernatant from each well and freeze at – 20°C.

- ➢ 72 hours after incubation (another 48 hrs) seal and freeze the whole plates at –20°C.

11. ELISA

The enzyme-linked immunosorbent assay (ELISA) is a serological assay in which bound antigens or antibodies are detected by an enzyme that converts a colorless substrate into a colored product. The ELISA assay is widely used in biology and medicine as well as immunology. We used a kit from Pharmingen in our study to detect IL-2, IFN-γ and IL-4 production levels.

Procedure:

> Coat plate: coat capture antibodies at 100ul / well at 4° C overnight. Discard coating solution before blocking (no wash).

> Block plate: add 200ul blocking buffer (1% BSA/PBS) to each well and leave it at room temperature for 1-2 hours.

> Discard the solution from plate and wash plate 4 times with washing buffer.

> Add samples and standard: samples and standard should be serially diluted in dilution buffer in the plate in an extra plate. 100ul diluted samples or standard will be added to each well.

> Incubate the plate for 2-3 hours.

> Wash the plate 4 times with washing buffer.

> Add diluted streptavidin conjugated alkaline phosphatase (1:6000) at 100ul / well.

> Incubate the plate at 37° C for 45 min.

> Wash the plate 6 times with washing buffer.

➢ Add substrate (solved in substrate buffer immediately before use) at 100 ul /well

➢ Read plate at 30, 60 and 120 min.

Capture Abs:

IL-2 purified rat ∝ mouse IL-2 0.25mg/ml, final concentration 2.5ug/ml

IL-4 purified ∝ mouse IL-4 0.5mg/ml, final concentration 2.5ug/ml

IFN-γ Purified ∝ mouse interferon-gamma 0.5mg/ml, final concentration

    1.25ug/ml.

Standard:

IL-2 final dilution: 300u/ml

IL-4 final dilution: 5ng/ml

IFN-γ final dilution: 5ng/ml

Detection Abs:

IL-2 Biotin Rat ∝ mouse IL-2, final concentration: 2ug/ml

IL-4 Biotin ∝ mouse IL-4 final concentration: 2ug/ml

IFN-γ Biotin ∝ mouse IFN-γ, final concentration: 2ug/ml

12. Statistics used in the study

$$Sc = \{[\Sigma X_1^2 - (\Sigma X_1)^2 / n_1] + [\Sigma X_2^2 - (\Sigma X_2)^2 / n_2]\} / (n_1 + n_2 - 2)$$

$$S\ x_1\text{-}x_2 = [Sc^2\ (n_1 + n_2)\ / n_1 n_2]^{1/2}$$

$$T = (\text{mean } X1 - \text{mean } X2) / S\ x_1\text{-}x_2$$

V. Results:

Part A: Chlamydia growth inhibits IFN-γ inducible MHC class II gene expression in epithelial cells at the level of gene transcription

1. <u>Chlamydia growth inhibits IFN-γ inducible MHC class II gene expression on the cell surface as detected by flow cytometry. (Fig 1)</u>

To investigate whether chlamydia possesses the ability to evade the IFN-γ induced immune recognition, we first used flow cytometry to evaluate IFN-γ inducible expression of MHC class II antigens on the surface of cells in the presence or absence of chlamydial infection.

Four cell samples were prepared in parallel: MCF-7 cells alone, MCF-7 stimulated by IFN-γ, MCF-7 infected with chlamydial strain L2, and MCF-7 infected with L2 and stimulated by IFN-γ. Briefly, we set up 4 flasks of 24-hour fresh MCF-7 cell monolayers and infected two of them with *C. trachomatis* strain L2 at an MOI=1.2. MOI 1.2 was chosen to make sure most cells were infected. 24 hours later, human IFN-γ was added to one flask of normal MCF-7 cells and one flask of L2 infected cells. Another 24 hours later, all 4 flasks of cells were harvested at the same time and used immediately for analysis by flow cytometry. Intact cells were labeled with mouse antibodies against human MHC-class-II and ICAM-1 molecules, then the first antibodies were detected by a secondary antibody against mouse immunoglobulin conjugated with fluorescein. Thus

MHC-class-II and ICAM-1 expression levels change into fluorescence signals and are recognized and quantitated by the FACS machine. The effects of IFN-γ and chlamydial infection on MHC-class-II and ICAM-1 expression levels were then evaluated by comparison of the above four samples. The same gates were established to exclude cell debris or cell clusters in both normal cells and chlamydial infected cells, so that similar cell populations are evaluated.

Mouse IgG anti-DRα (L243; ATCC) was used to detect human MHC-class-II molecule expression on the four different samples. The MHC-class-II molecule is composed of two transsmembrane glycoprotein chains, α (34,000 Da) and β (29,000 Da). Each chain has two domains, and the two chains together form a cleft on the surface of the molecule that is the site of peptide binding. There are three MHC-class-II α- and β- chain genes, called HLA-DR, -DP and –DQ.

Mouse IgG was applied as a control to exclude non-specific binding of the variation of Mouse IgG anti-DRα signal observed in the four different samples (see below). As demonstrated in Figure 1C, there was a similar background in all cells since the four curves that represent the four samples stained with Mouse IgG are almost superimpoable.

Comparing MHC-class-II molecule expression on cells stimulated by IFN-γ to normal cells gave us ideas of how much MHC-class-II expression was upregulated upon IFN-γ stimulation, and thus provides us a baseline to assess the effect of chlamydial infection. As seen by the great distance between the curves of IFN-γ stimulated cells and the curve

of normal cells in Fig 1A, IFN-γ greatly increases the expression of MHC-class-II molecules. The distance offers a clear window to further test the effect of chlamydial infection on IFN-γ induced MHC-class-II expression.

The curve that represents the level of MHC-class-II expression in cells with chlamydial infection falls between the curves of normal cells and the cells stimulated with IFN-γ, but closer to the former. The slight shift in the curves probably represents background. Excluding the background factor, we assumed that chlamydial infected cells had the same level of MHC-class-II expression as normal cells.

Importantly, the curve of MCF-7 cells infected with L2 and stimulated by IFN-γ locates to the left side and is distant from the curve of MCF-7 cells stimulated with IFN-γ alone. That means chlamydial infection effectively inhibits the expression of IFN-γ inducible MHC-class-II on MCF-7 cells. Also the curve of MCF-7 cells infected with L2 and stimulated by IFN-γ locates slightly at the right side of the curve of MCF-7 cells infected with L2. Since the same shift happens with the anti IgG antibody in Figure 1C, the shift most likely represents the background. Excluding background noise, we concluded that the inhibition of chlamydial infection on IFN-γ inducible MHC-class-II expression was complete.

To clarify that chlamydial inhibition was specific for MHC-class-II, another IFN-γ inducible cell surface protein ICAM-1 was designed as a control. ICAM-1 expression on the four cell samples was evaluated to show the effect of chlamydial infection on the

level of IFN-γ inducible ICAM-1 expression, as presented in Figure 1B. IFN-γ greatly

increased the expression level of ICAM-1. The curve of MCF-7 cells infected with L2

and stimulated with IFN-γ, however, overlaps curve of MCF-7 cells stimulated with IFN-

γ. That means that chlamydial infection did not inhibit IFN-γ inducible ICAM-1

expression on MCF-7 cells and thus proved that the chlamydial inhibition of MHC-class-

II expression was selective.

During the flow cytometry study, chlamyida-infected cells were found to be more fragile

than uninfected cells, since a large proportion of infected cells died during the procedure,

while almost all uninfected cells remained alive. This phenomenon is consistent with the

visual observation of cells stained with trypan blue under light microscope. The

chlmayida-infected cells exclude trypan blue less efficiently.

This phenomenon is also well demonstrated in Fig 1(a)-(d) where we measured the size

distribution and PI staining of all the cell samples. PI enters cells when cell membrane

permeability is high, such as in the case of dead cells. The distribution in the chlamydial

infected cells was much more scattered than uninfected cells, which suggests that

chlamydial infected cells vary in size and density, or that the infected cells more easily

form cell clusters and debris. Also, when dead cells are excluded by PI staining, it was

noticed that not only was a large proportion of infected cells dead, as demonstrated in Fig

1 (d) with two peaks, but also that the PI intensity was higher in live chlamydial infected

cells than uninfected cells. This suggests that the chlamydia-infected cells may have a

higher cell membrane permeability than uninfected cells.

The high cell membrane permeability in chlamydial infected cell has not been reported

before. It is a very interesting finding because essentially this will affect material

exchange between the extracellular environment and infected cells, and leads to further

understanding of the difference between chlamydial infected and normal cells. The

finding will be tested further in the future.
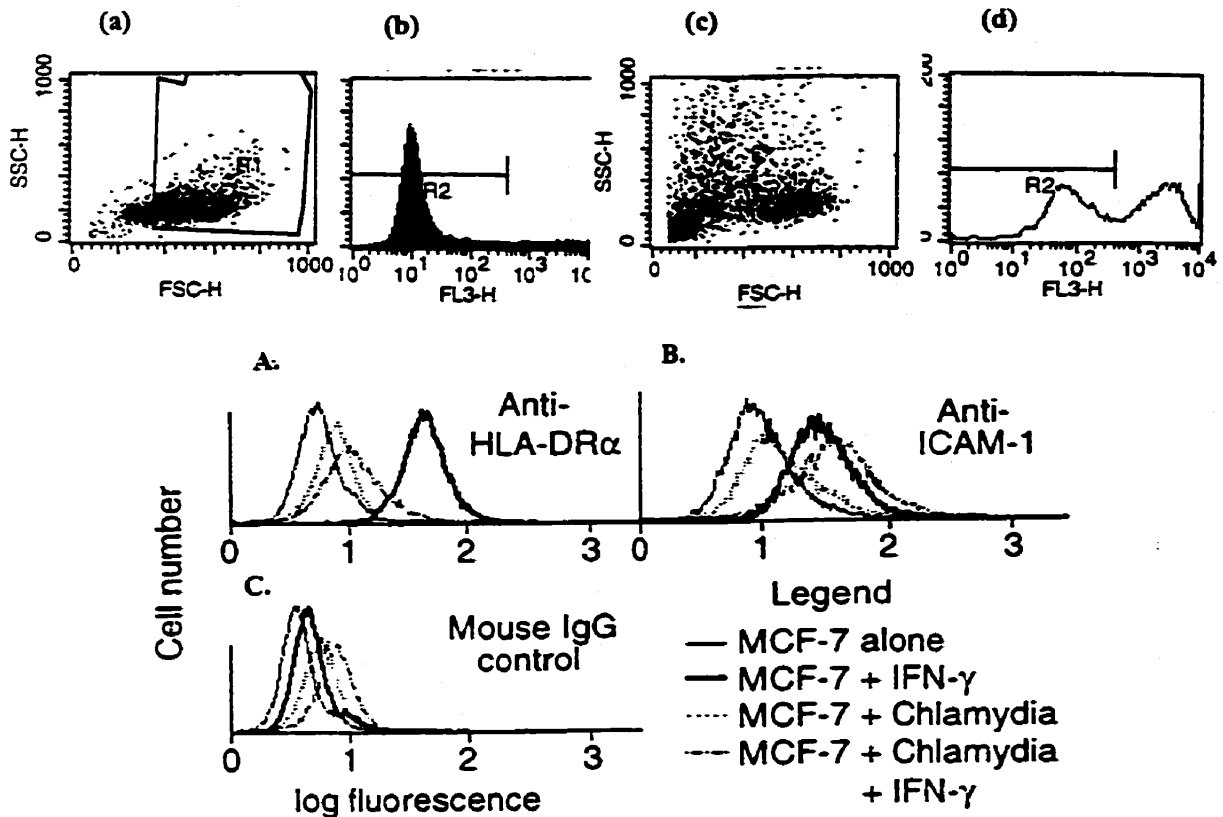
Fig 1. Chlamydia infection inhibits IFN-gamma inducible MHC-class-II but not ICAM-1 expression on cell surface

MCF-7 cells with or without chlamydial infection were stimulated with IFN-gamma and collected for flow cytometry. (a). distribution of non-infected cells in forward angle light scatter (horizontal axis) and 90-degree light scatter (vertical axis); (b): PI (propidium iodine) staining of non-infected cells; (c): distribution of chlamydia-infected cells; (d): PI staining of chlamydia-infected cells. A: HLA-DRα expression is detected on cell surface; B: ICAM-1 expression is detected; C: mouse IgG background is detected.

2. Chlamydia growth inhibits IFN-γ inducible MHC class II gene expression on epithelium cells at total protein level, detected by WB. (Fig 2)

Because previous results (Figure 1) indicated that IFN-γ inducible MHC-class-II molecule expression decreased at its functional site, the cell surface, it was important to determine the mechanism of this decrease. The MHC-class-II gene, like other genes, is first transcribed into messenger RNA in the nucleus, then transported into the cytosol and translated into proteins by ribosomes on the rough endoplasmic reticulum (RER). Proteins destined to function in the cytosol are released from ribosomes directly to the cytosol. Plasma-membrane proteins, like MHC-class-II, which are localized to the cell surface or extracellular space, enter the lumen of the RER upon synthesis. After being properly trimmed and glycosylated in the Golgi complex, they finally bud off from the Golgi complex and are transported to the cell surface.

Based on the protein biosynthetic pathway, inhibition of IFN-γ inducible MHC-class-II molecule expression on cell surface could be caused by a decreased production of MHC-class-II molecules, an increased degradation of synthesized protein, or an inhibition of transport of newly synthesized protein from the RER to cell surface. To investigate this, the total cellular level of IFN-gamma inducible HLA-DRα protein was evaluated by western-bloting, in the same set of four samples as described in Fig1.

Cell samples stored at −80°C were used in the western blot assays. Cells were first thawed gradually on ice to avoid possible protein degradation, and then were lysed with

RIPA buffer to release everything inside the cells. The protein concentrations were then

calculated from a standard curve derived from serial diluted BSA. Equivalent amounts of

proteins in the four samples were seperated by SDS-PAGE and a mouse antibody against

human MHC-class-II was used to detect the protein. The primary antibody binding was

detected with horseradish peroxidase-conjugated goat anti-mouse IgG, and visualized

using an ECL kit. The relative amounts of MHC-class-II protein in the four samples were

compared by inspection of the band intensities in each sample.

Manipulations of the cell samples are shown at the top of Figure 2. Normal cells are

chlamydia -, and IFN-γ -; chlamydial strain L2 infected cells are chlamydia +, and IFN-γ

-; cells stimulated by IFN-γ are chlamydia -, and IFN-γ +; cells infected with chlamydial

as well as stimulated by IFN-γ are marked by chlamydia +, and IFN-γ +. The different

cell lines used: MCF-7, MRC-5, 2C4, and Hela cells are indicated at the left. The four

cell lines were used to visualize the relative amount of MHC-class-II protein following

the four treatments.

For example, in MCF-7 cells, there is almost no MHC-class-II protein detectable in

normal cells (first lane) and chlamydia-infected cells (third lane). However, when normal

cells are stimulated by IFN-γ (second lane), the level of MHC-class-II protein was greatly

increased. Interestingly, there is almost no MHC-class-II protein detectable in the cells

infected with L2 and stimulated by IFN-γ (fourth lane), similar to normal cells without

IFN-γ stimulation (first lane). That indicates that chlamydial infection suppresses the

IFN-γ inducible expression of MHC-class-II protein.

To summerize, chlamydial infection was found to suppress IFN-γ inducible MHC-class-II

expression at both the cell surface and within the cell. Similar results were obtained in

each of four different cell lines. These results infer that chlamydia prevents the IFN-γ

induced synthesis of MHC-class-II, and excludes the possible trafficking problem that

newly synthesized MHC-class-II molecules localized inside the cells.

MCF-7 and HeLa cells are mammary epithelial cell lines; MRC-5 and 2C4 cells are

mammary fibroblast cell lines. Epithelial cells and fibroblasts are non-professional

antigen-presenting cells that change into professional antigen presenting cells upon IFN-γ

stimulation. The pattern of MHC-class-II expression in Figure.2 was similar in all four

cell-lines, although there were variations in the amount of protein in each cell line. These

results suggest that chlamydial inhibition of IFN-γ inducible MHC-class-II expression is
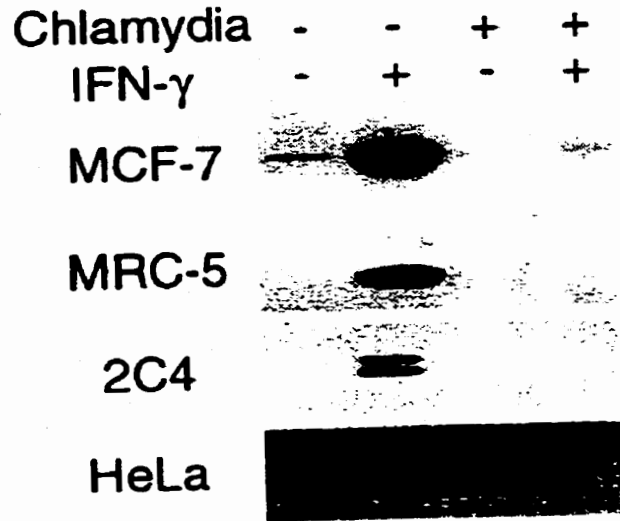
not cell-line specific.

Fig2. Chlamyial infection selectively inhibits IFN-gamma inducible MHC-class-II expression at the total protein level

Chlamydial infection suppresses the total cellular level of HLA-DRα in various human cell lines, MRC-5, 2C4, MCF-7 and HeLa cells were stimulated with IFN-gamma at 400u/ml. HLA-DRα were detected in a western blot assay.

3. Chlamydia growth inhibits IFN-γ inducible MHC class II gene expression in epithelial cells at the m-RNA level as detected by RT-PCR. (Fig 3)

The FACS and western blot studies indicate that chlamydia inhibits IFN-γ inducible MHC-class-II expression by decreasing protein synthesis. We next wanted to determine whether the inhibition of synthesis occurs at the level of translation or transcription. To do this, we measured the level of IFN-γ inducible MHC class II messenger RNA by semi-quantitative RT-PCR in the four cell samples.

Cell samples stored at −80°C were gradually thawed on ice. Total RNA was extracted, and then DNAase was applied to eliminate any residual DNA present in the samples. The concentration of RNA was determined by measuring OD260 and OD280, and then equivalent amounts of RNA were removed from the four samples for later procedures. First strand complementary DNA (cDNA) was synthesized using total RNA as the template, various gene specific primers (see page 37) and avian myeloblastosis virus (AMV) reverse transcriptase. PCR was then performed to amplify the cDNA. PCR products from the four samples were run in parallel on an agrose gel. The amount of product (the band intensities) was used as an indication of the level of gene expression. Amounts were compared visually on a picture of the gel exposed to UV light.

β-Actin was chosen as a positive control for the RT-PCR procedure. Also, because it is expressed constitutively, all four samples should have same amount of β-actin, so the intensity of its product can be used to monitor consistency between the samples.

We examined the level of the mRNA encoding DRα, DMα and the invariant chain p41 (Ip41) in the four samples. DMα and the invariant chain p41 (Ip41) are molecules involved in the MHC-class-II secretion pathway. They are not constitutively expressed in the non-professional antigen presenting cells but are IFN-γ inducible, similar to MHC-class-II proteins. Moreover, the genes encoding the invariant chain and the DM molecules are located in the MHC-class-II region of the genome.

The results of RT-PCR study are shown in Figure.3. The different treatments of the four cell samples are shown at the top, and the DRα, DMα and invariant chain p41 (Ip41) PCR products are shown on the left. No bands corresponding to the three molecules showed up in normal cells and chlamydial infected cells (lane 1 and lane 3). However, in normal cell upon IFN-γ stimulation, mRNA products of all three molecules were greatly up regulated. Importantly, the intensity of all three products was greatly decreased in chlamydial infected cells stimulated with IFN-γ, indicating that the chlamydial infection prevented the IFN-γ inducible transcription of DRα, DMα and invariant chain p41 (Ip41). In another words, inhibition of IFN-γ inducible MHC-class-II expression by chlamydia occurs at the level of gene transcription.
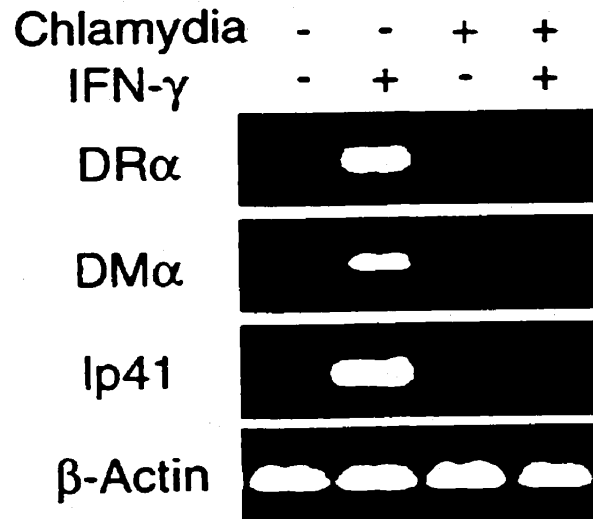
Fig 3. Chlamyial infection selectively inhibits IFN-gamma inducible MHC-class-II expression at the gene transcription level

In MCF-7 cells, DRα, DMα, Ip41 primers were used to amplify and detected in an RT-PCR assay, and β-actin was used as control of the method.

Part B: ICAM-1 is not required in clearance of mice lung MoPn infection.

1. Resistance to primary chlamydial infection is similar in mice with or without ICAM-1 deficiency, although CD4 Th1 cytokine production was significantly impaired.

To evaluate the resistance of ICAM-1 deficient mice against chlamydial infection, mice with or without ICAM-1 deficiency were infected intranasally with the mouse pnumonitis agent (MoPn) of chlamydia trachomatis. Because MoPn infection is air-borne, cages of mice were separated with plastic filters to avoid cross-infection.

Body weight changes were monitored every 24 hours within the 10-day period after the first infection. After sacrificing the mice, the level of infection in lung tissue was indicative of the invasive ability of the chlamydial pathogen. This was calculated based on titer obtained from culturing extracted inocula with HeLa cells to reactivate and amplify the organism. Cytokines IL2, IL-4 and IFN-γ production levels were indicative of the strength of the host immune response and were measured in the supernatants of EB stimulated spleen cells.

A. Body weight change following the first infection

During the first challenge, all mice survived the 10-day period following infection. Although the average starting body weight of ICAM-1 deficient mice was about 1.5g less than that of wild type mice, both groups' body weight change exhibited similar patterns

(Figure 4). All mice, including the ICAM-1 knockout and the wild type mice, were about 6 weeks old at first challenge. The reason why the ICAM-1 mice weigh less is unknown. There was a mild increase of body weight during the first 2 days and then a sharp drop from day 3, which reached the lowest level at day 6 or 7 and was then followed by a bounce-back. Mice that were severely sick had not only a sharp drop in body weight, but also lacked energy and responsiveness, were inactive, and lost their normal hair gloss.

B. Infectivity determined by Lung titration

Intact lungs were extracted quickly from sacrificed mice, weighed and set on ice. Lungs from very sick mice appeared bloody red, indicating extensive blood cell infiltration. Some of these lungs were necrotic, and parts of their lobes were destroyed. Lungs from relatively healthy mice, however, showed a normal reddish-white appearance and normal size. Severely sick lungs usually weighed more than relatively healthy lungs, maybe because of water and blood cell infiltration.

After smashing the lungs in equivalent volume of cold SPG to release free EBs from inside the lung tissue, each sample was centrifuged to remove the coarse tissue in the pellets. Free EBs in the supernatant then were diluted serially and used as inocula to infect the prepared HeLa cell monolayer. 24 hours later, infected HeLa cells were fixed and stained with an antibody against the chlamyidal major outer membrane protien (MOMP). Inclusion bodies were then counted and inclusion body forming unit per gram lung tissue was determined.

Infectivity of the lung tissue was used as an indicator of the severity of the chlamydial

infection. The average Log IFU/g lung tissue for wild type mice was 6.27+/- 0.55, and for

ICAM-1 deficient mice was 6.28+/- 0.47. The two averages (Log IFU/g lung tissue)

show no statistically significant difference. The results are summarized in Figure.5. As

the severity of infection was similar in mice with or without ICAM-1, it suggests that the

two groups of mice had a similar immune response to the chlamyidal infection.

C. Cytokine production level in first infection

Diluted dead EBs inactivated by treatment at 56°C for 30 min were used as the stimulant

for cytokine production study. Freshly extracted spleens were smashed to release

lymphocytes, and then the cell suspension was pipeted through a nylon filter to remove

the coarse tissue. The white blood cell concentration was determined under a light

microscope, and equal amounts of white blood cells were taken from each mouse for

spleen cell stimulation. The cells were distributed in 24-well plates and same IFU of

prepared dead EB solution were added to each well, and incubated 37°C with 5% CO_2.

Following 24 hours incubation 0.5ml supernatant were taken from each well and frozen

at –20°C for IL2 measurements. Following 72 hours incubation (another 48hrs) another

0.5ml supernatant were taken from each well and frozen at –20°C for the determination

of IL-4 and IFN-γ.

The cytokine profile derived from the supernatant of *in vitro* stimulated spleen cell

cultures are indicators of the strength of the host immune response against chlamydial

infection. The cytokines reflect immunity side of the chlamydia-host immunity interaction. IL-2 and IFN-γ are CD4+ Th1 cell cytokines, and IL-4 is a CD4+ Th2 cytokine. IL2 is secreted from Th1 cells early in a immune response. It acts as a paracrine factor to stimulate neighboring T cell to proliferate and differentiate into Th1 cells. IFN-γ is secreted late in the response, and it acts as effector to activate macrophages to attack pathogens.

All samples showed abundant IFN-γ and IL-2, but negligible level of IL-4, consistent with the knowledge that host immunity responsible for clearance of chlamydial infection lies in CD4+ Th1, but not Th2 cell groups. Surprisingly, markedly lower level of IFN-γ (p< 0.001) and IL-2 (p<0.001) were found in the ICAM-1 deficient mice when compared to the wild type mice. As demonstrated in Figure 6 (IFN-γ) and Figure 7 (IL-2), the average IFN-γ (ng/ml) production from wild type mice was 17.52+/- 2.21, and from ICAM-1 deficient mice was 13.79+/- 1.66. The average IL-2 (u/ml) level of wild type was 5.78+/- 4.08, and of ICAM-1 deficient mice were 1.38+/- 3.31. Obviously there are huge standard deviations in the readings of IL-2 cytokine level, but because the difference is huge and there is seldom overlaping readings in two groups of mice, it is considered that the statistical significant difference is reliable.

2. Resistance to secondary chlamydial infection was also similar in mice with or without ICAM-1 deficiency, although IL-2 production was still significantly impaired.

The second challenge with *chlamydia* was given at 31 days after the first challenge, when

all mice had recovered their lost body weight. Similar to the first challenge, body weight

change, lung titration, and cytokine production levels were determined after the second

challenge.

Body weight changes were monitored daily over a 5-day period after the second

infection. This period is sufficient to elicit a full adaptive immunity. Graphs of the body

weight change of mice with or without ICAM-1 deficiency after the second challenge

demonstrated a parallel pattern -- both groups lost weight from the first day after the

infection and the trend continued until they were sacrificed (Figure 8). Two out of ten

wild type mice died of heavy infection, while one out of ten ICAM-1 knockout mice

died.

In the infectivity study, the average (Log IFU/gram lung tissue) of wild type mice was

5.52+/- 0.60, and of ICAM-1 deficient mice was 6.00+/- 0.80. There was no statistically

significant difference between the two titer-averages. Together with the similar mortality

rate in both mice groups, it is concluded that ICAM-1 deficient mice have similar

immune resistance to the wild type mice in the second chlamydial infection. The result is

demonstrated in **Figure 5**.

The cytokine production profile illustrated an abundant production of IFN-γ (Figure 6)

and IL-2 (Figure 7), but negligible level of IL-4, similar to the results observed following

the first infection. The average IL-2 level of ICAM-1 deficient mice was still

significantly lower than in the wild type mice as in the first challenge ($p < 0.05$). The

level of IL-2 (u/ml) of wild type mice was 3.33+/- 1.65, and of ICAM-1 deficient mice

was 2.72+/- 1.60. Although the production of IFN-γ was found to be a little higher in wild

type mice than in ICAM-1 deficient mice, the difference did not have statistical

significance ($0.05 < p < 0.1$). The average IFN-γ level (ng/ml) of wild type mice was

16.54+/- 1.81, and of ICAM-1 deficient mice was 18.78+/- 2.96.

The data of individual mice, including Log IFU/gram lung tissue and the amount of both

IFN-γ and IL-2, are summarized in Figure 9. Mouse of identification number (ID) 12 to

20 and mouse ID 41 are wild type mice with first challenge; mice ID 21-31 are ICAM-1

deficient mice with first challenge. Mice ID 3-11 are wild type mice with second

infection; and mice ID 32-40 are ICAM-1 deficient mice with second challenge.
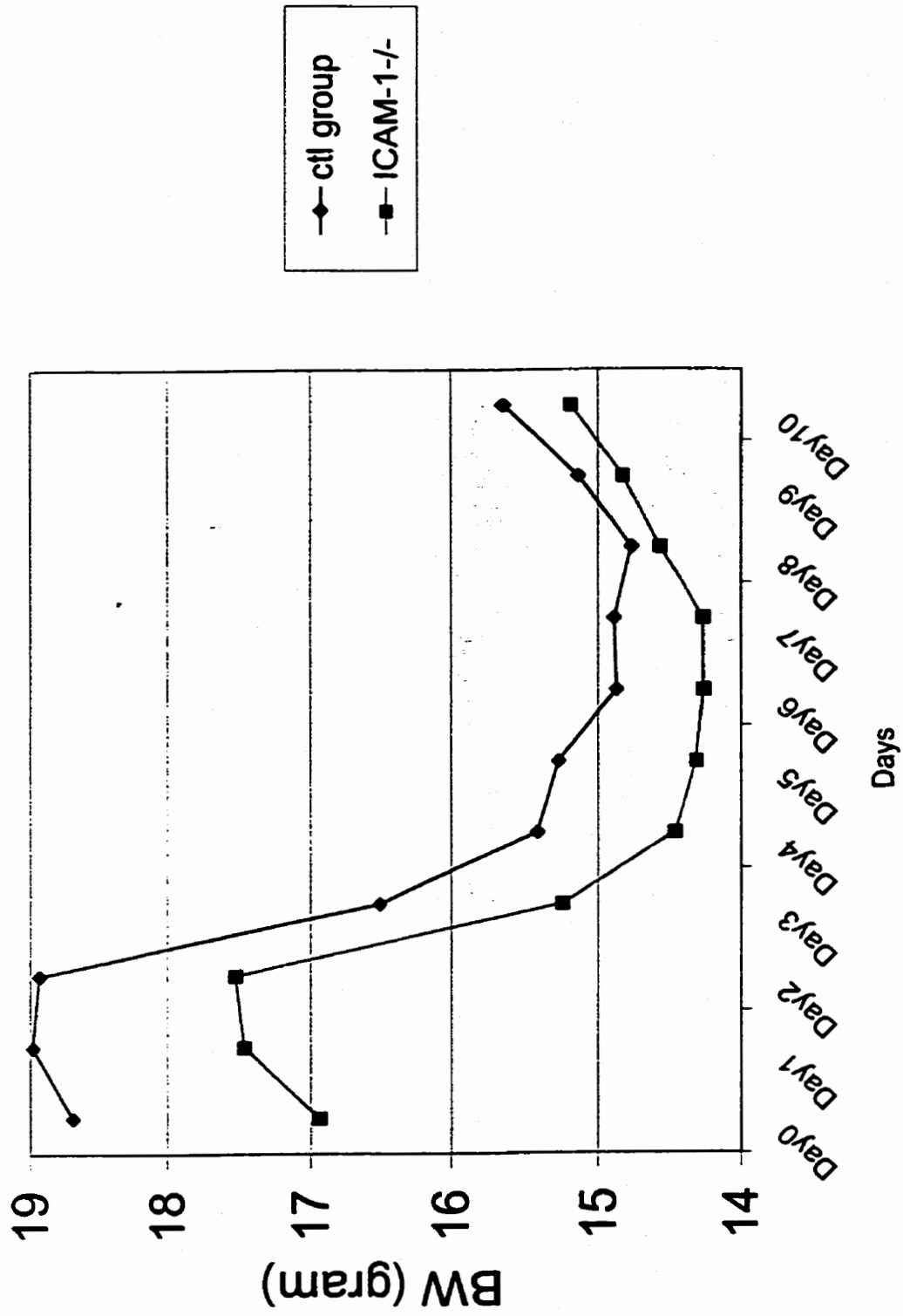
Fig 4. Primary Infection Body Weight Change

IFU/lung(log)

wild type 1st in| ICAM-1-/- 1st inf
6.27+/-0.55   6.28+/-0.47
wild type 2nd in ICAM-1-/- 2nd inf
5.52+/-0.60   6.00+/-0.80

IFU/lung(log)
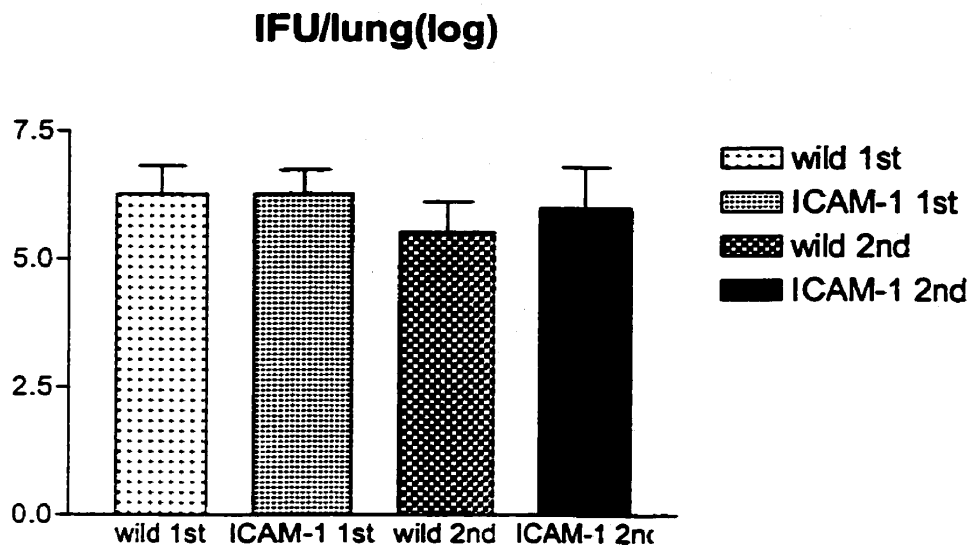
Fig 5. Summary of infectivity in mice with or without ICAM-1 deficiency in both primary and secondary chlamydial infections

Mice were sacrificed 10 days after the first infection or 5 days after the second infection, Free chlamydia EBs were extracted from the lungs and were used as inocula to infect HeLa cells. IFU (inclusion forming unit) of each lung was calculated and corrected by the lung weight.

IFN-gamma(ng/ml) wild type 1st in ICAM-1-/- 1st inf
13.79+/-1.66   17.52+/2.21
wild type 2nd in ICAM-1-/- 2nd inf
16.54+/-1.81   18.78+/-2.96
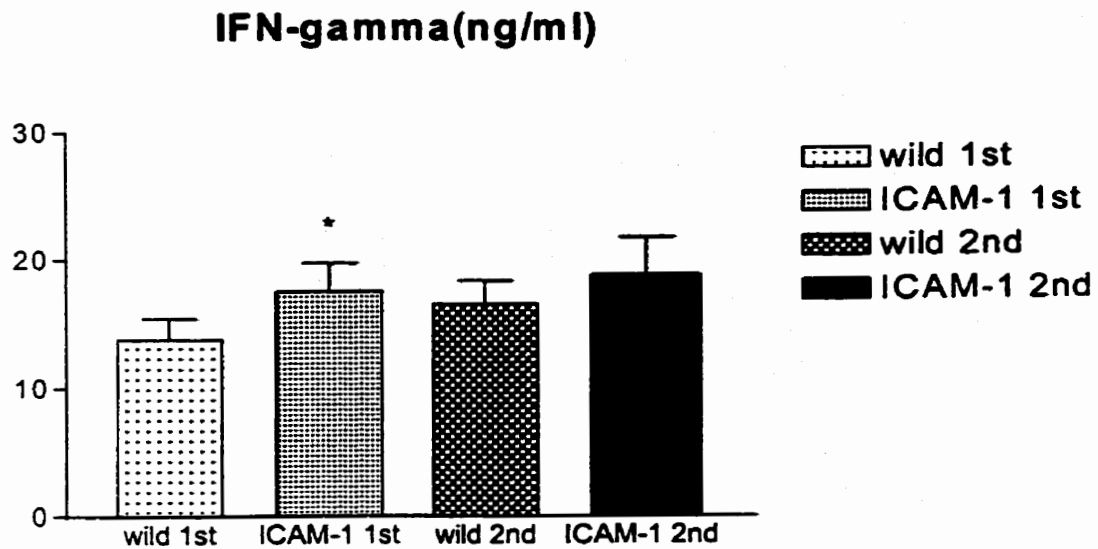
Fig 6. Summary of IFN-gamma production levels in mice with or without ICAM-1 deficiency in both primary and secondary infections

Free spleen cells of each mouse were obtained and were stimulated by dead chlamydia elementary bodies for 3 days. Supernatant was obtained and IFN-gamma production level was determined by ELISA.

IL2 (u/ml)

	wild type 1st inf	ICAM-1-/- 1st inf
	5.78+/-4.08	1.38+/-3.31
	wild type 2nd inf	ICAM-1-/- 2nd inf
	3.33+/-1.65	2.72+/-1.60

IL2 (u/ml)
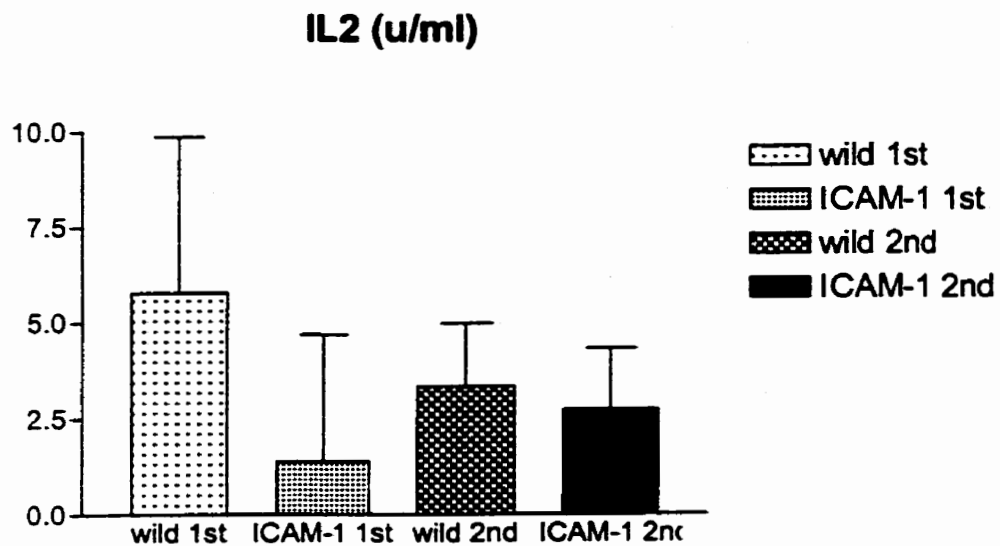
Fig 7. Summary of IL-2 production levels in mice with or without ICAM-1 deficiency in both primary and secondary infections

Free spleen cells of each mouse were obtained and were stimulated by dead chlamydia elementary bodies for 1 day. Supernatant was obtained and IL-2 production level was determined by ELISA.
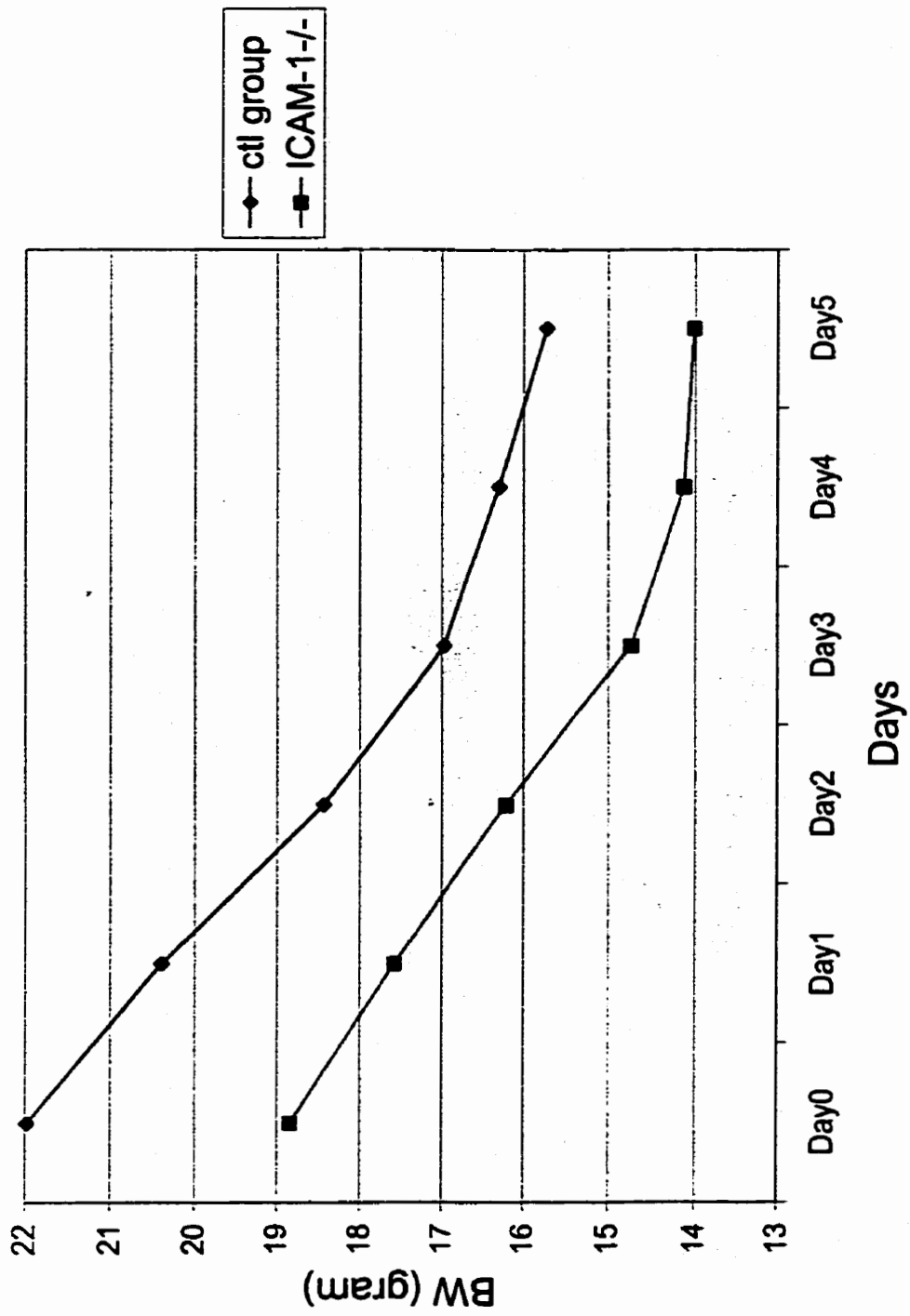
Fig 8. Secondary Infection Body Weight Change

ID No.	IFU	LungW(g)	log IFU	IFN-γ(ng/ml)	IL-2 (u/ml)
#12	$1.34*10^6$	0.54	6.39	17.20	8.91
#13	$2.92*10^6$	0.38	6.89	15.13	12.31
#14	$4.05*10^6$	0.41	6.99	15.70	4.21
#15	$4.03*10^5$	0.37	5.51	16.06	10.61
#16	$5.54*10^4$	0.17	7	15.25	9.11
#17	$4.5*10^6$	0.45	6.29	18.11	2.72
#18	$6.84*10^5$	0.35	6.17	18.84	1.16
#19	$6.16*10^5$	0.42	6.04	19.63	2.72
#20	$2.43*10^5$	0.35	5.84	22.11	4.63
#41	$1.6*10^5$	0.39	5.61	17.18	1.43
		Average	6.27	17.52	5.78
		SD	6.26	2.21	4.08
#21	$3.12*10^5$	0.29	6.03	15.00	7.31
#22	$1.81*10^5$	0.35	5.72	11.74	0
#23	$1.2*10^6$	0.37	6.51	13.73	3.27
#24	$1.64*10^6$	0.38	6.64	13.55	1.02
#25	$4.18*10^5$	0.37	6.05	12.15	0.81
#26	$1.22*10^6$	0.38	6.51	12.49	0
#27	$1.17*10^6$	0.43	6.43	14.34	0.20
#28	$3.95*10^5$	0.29	6.13	14.14	0.95
#29	$1.05*10^5$	0.26	5.61	17.52	0.20
#30	$6.32*10^6$	0.39	7.21	13.26	0
		Average	6.28	13.79	1.38
		SD	0.47	1.65	2.31

Fig 9. Data of infectivity and cytokine profile of individual mouse
Mouse ID 12-20 + 41 wild type 1st infection
Mouse ID 21-31   ICAM-1 deficient mice 1st infection

ID No.	IFU	LungW(g)	log IFU	IFN-γ(ng/ml)	IL-2 (u/ml)
#3	1.05^*10^5	0.5	5.32		
#4	7.27^*10^5	0.52	6.15	16.59	4.99
#5	2.85^*10^5	0.51	5.75	17.84	4.64
#6	1.74^*10^6	0.51	6.53	17.82	5.53
#7	2.38^*10^4	0.51	4.67	16.81	1.75
#9	1.2^*10^5	0.45	5.43	16.94	2.38
#10	4.75^*10^4	0.4	5.08	24.70	1.89
#11	8.74^*10^4	0.51	5.23	20.74	2.12
		Average	5.52	18.78	3.33
		SD	0.60	2.96	1.65
#32	1.14^*10^4	0.39	4.47	17.34	0.89
#33	1.71^*10^5	0.44	5.59	17.73	0.54
#34	2.39^*10^5	0.42	5.76	16.44	1.43
#35	3.78^*10^5	0.47	5.91	15.84	3.15
#36	5.87^*10^5	0.46	6.11	15.59	1.37
#37	6.41^*10^6	0.52	7.09	13.25	0.54
#39	8.21^*10^5	0.47	6.24	19.46	2.12
#40	2.95^*10^6	0.46	6.81	16.68	2.72
		Average	6.00	16.54	1.60
		SD	0.80	1.81	0.98

Fig 9. (Continued) Data of infectivity and cytokine profile of individual mouse
Mouse ID 3-11   wild type 2nd infection
Mouse ID 32-40   ICAM-1 deficient mice 2nd infection

VI. DISCUSSION

Part A: Chlamydia inhibits IFN-γ inducible MHC class II expression as described in a cell culture study

1. Development of Hypothesis

Chlamydia is an obligate intracellular bacterium that has a unique biphasic life cycle, switching from an EB to a RB, and back to an EB again. The elementary body (EB) is infectious but metabolically inactive, while reticular body (RB) is metabolically active and capable of replication, but is non-infectious. *Chlamydia trachomatis* is an important human pathogen that causes most cases of preventable blindness in developing countries and high incidence of sexually transmitted disease in industrialized countries. Chlamydial infection tends to be mild or asymptomatic in nature but usually lasts for a long time.

Three factors contribute to chronic chlamydial infection. First, repeated infections are very common. Second, chlamydial major outer membrane protein MOMP gene is highly variable, resulting in frequent antigenic variability. Third, *chlamydia* has developed an incomplete life cycle and causes "persistence" in host. In an incomplete life cycle, reticular bodies (RBs) form aberrantly. They remain alive in host cells but do not differentiate back into elementary bodies. Thus the traditional way to detect *chlamydia* (by culturing tissue isolates with tumor cell lines *in vitro* to replicate the organism) fails to recognize *chlamydia* in this state.

Therefore, "persistence" is defined as a long-term association between *chlamydia* and their host cells, in which these organisms remain in a viable but culture-negative state. The stimulants of persistent chlamydial infection include absence of certain kinds of nutrition in the growth environment, or presence of certain immunological factors and antibiotics.

Although the theory of chlamydial persistence explains why cell culture fails to detect the infection *in vitro*, the incomplete life cycle does not spare *chlamydia* from being attacked *in vivo*, by an immune competent host. In a natural infection, if chlamydial antigens continue to be presented to the host cell surface during an incomplete life cycle, immune competent hosts can still detect the existence of *chalmydia* and launch an effective attack against infected cells.

Long-term survival of *chlamydia* under host immunity involves active bacteria-host interaction. CD4+ T cell subgroup Th1 cells are responsible for the acquired immunity against chlamydial infection. Naïve T cells recognize MHC-class-II: peptide complexes on professional antigen-presenting cells like macrophages, then proliferate and differentiate into armed effector T cells. Again, armed effector T cells recognize MHC-class-II: peptide complexes on the target cells and signal the target cell to undergo programmed cell death (apoptosis). Effector T cells also release cytokines like IFN-γ to activate macrophages to engulf and kill *chlamydia*.

Thus, the capacity of MHC-class-II molecules to present chlamydial antigens is critical for the immune attack against chlamydial infection. Because epithelial cells that *chlamydia* target in a natural infection are non-professional antigen-presenting cells, they do not produce MHC-class-II molecules until they are stimulated by IFN-γ. Thus IFN-γ inducible MHC-class-II expression is very important in immune recognition in an effective host defense against chlamydial infection.

This study hypothesized that *chlamydia* has evolved a strategy to inhibit the IFN-γ inducible MHC-class-II expression on non-professional antigen-presenting cells. The inhibition enables *chlamydia* to hide in the target cells and remain invisible to the host immune system, and thus maintain long-term survival in an immune competent host.

Many other intracellular pathogens have developed the strategy of inhibiting IFN-γ inducible MHC expression on non-professional antigen presenting cells to help their survival in the host. Examples include *leishmania*, (Reiner *et al.,* 1988) *listeria*, (Schuller *et al.,* 1998) *cowdria*, (Vachiery *et al.,* 1998) and *cytomegalovirus* (Heise *et al.,* 1998a and b; Miller *et al.,* 1998). Since MHC presentation of antigens is important in host defense against these intracellular pathogens, inhibition of IFN-γ inducible MHC expression might be a universal strategy for intracellular pathogens to evade host immune recognition.

Until now most studies have focused on what the immune system does to *chlamydia* rather than what *chlamydia* does to the host cells. This study brings in a new perspective:

that *chlamydia* may actively manipulate the host immune recognition system to ensure they can establish a persistent infection. In addition, *chlamaydia* passively adapt to the unfavorable environment by changing developmental forms. Therefore, this study emphasizes and contributes to the knowledge of the active interaction between a host and pathogen.

2. Confirmation of Hypothesis

IFN-γ inducible MHC-class-II expression has been previously studied in a cell culture system (Sgagias et al, 1996). We added *chlamydia* to the system and tested whether chlamydial infection reverses IFN-γ inducible MHC-class-II expression. Thus, a set of four cell-samples were used in the study; normal cells, normal cells + IFN-γ, normal cells + chlamydial strain L2, and normal cells + IFN-γ + L2.

In most previous studies, cultured cells were stimulated by IFN-γ for 48-72 hours before MHC-class-II expression on cell surface as tested. However, a whole life cycle for *C. trachomatis* (strain L2) takes only 44-48 hours. Many cells lyse after 44 hours growth and the remaining intact cells are extremely fragile and difficult to handle. Moreover, our previous study (Tao *et al,* 1998) suggested that when the chlamydial inclusion body (IB) volume reached half the size of the host cell, *chlamydia* direct antiapoptotic activity against apoptotic stimulant. This phenomenon is also true in this study. This means that *chlamydia* need to grow until its inclusion body volume reaches half the size of the host cell before we apply IFN-γ stimulation to the infected cells. Therefore, if we cannot fit

the inclusion body growth time and IFN-γ stimulation time in the duration of a chlamydial life cycle, the method of cell culture system cannot be used to test our hypothesis.

In a pre-experiment we titrated dose and timing of IFN-γ to find the shortest possible duration of IFN-γ stimulation needed to induce a maximum MHC-class-II expression. Fortunately, we found that with an IFN-γ concentration of 400unit/ml, a great increase in MHC-class-II expression happened in 22 hours. *Chlamydia* needs to grow 22-24 hours before its inclusion volume reaches half the size of host cell when the multiplicity of infection (MOI) is 1-1.2. Thus, we infected epithelial cells with *chlamydia* for 22 hours and then stimulated cells with IFN-γ for another 22-24 hours to test the effect of both IFN-γ and chlamydial infection on MHC-class-II expression.

Other *C. trachmatis* strains like MoPn take only 36 hours to complete a life cycle and thus cannot be examined by this cell culture method. Also, the *C. trachomatis* strain was chosen over the *C. pneumonia* strain even though the life cycle of *C. Pneumonia* takes 3-5 days and seems suitable for the study. *C. Pneumonia* is very difficult to grow and huge variations in growth are seen in separate cultures. Until now the maximum *C. pneumonia* infections rate in cultured cells is 60-70%, thus it is difficult to get uniformly infected cells to compare with normal cells. Moreover, *C. pneumonia* inclusions are not visible under the light microscope and this makes observation of the inclusion body volume difficult. We believe the result from this study also applies to MoPn and *C. pneumonia*, except that different experimental methods need to be used to test these strains.

After preparation of the four samples, we performed flow cytometry, western bloting and RT-PCR to test our hypothesis. The flow cytometry study determines the level of protein expression on the cell-surface. Western bloting detects the total amount of a certain protein in the cell. RT-PCR evaluates the level of mRNA present in a cell for a certain gene product. The results suggested that chlamydial infection inhibited IFN-γ inducible MHC-class-II expression at all three levels. Considering the process from MHC-class-II synthesis to its functional expression on the cell surface, we conclude that chlamydia inhibit the IFN-γ induced transcription of MHC-class-II genes.

3. Inferences from the results

The expression of MHC-class-II moleculse and some proteins involved in MHC-class-II secretion pathway [invariant chain (Ip41) and DM molecules] were tested in this study.

Newly synthesized MHC-class-II proteins assemble into a protein complex with a MHC-class-II-associated invariant chain (Ip41 is one of them) to prevent MHC-class-II from binding to other peptides in the luman of the endoplasmic reticulum. The invariant chain (Ii) has a second function, which is to target the delivery of the MHC-class-II molecules from the endoplasmic reticulum to an appropriate, low pH endosomal compartment. The complex of MHC-class-II with invariant chain is retained for 2-4 hours in this compartment. During this time, the invariant chain is cleaved in several places. The cleavage removes most parts of the invariant chain but leaves a short peptide of Ii bound

to the MHC-class-II complex. Thus, at this stage, the MHC-class-II molecule still cannot bind to antigen peptides.

HLA- DM is a MHC-class-II-like molecule found predominantly in MHC-class-II compartments (MIIC), late endosomes that transport MHC-class-II molecules to the cell surface. DM also consists of α and β chains. It competes with MHC-class-II and binds to the peptide fragment from invariant chain that blocks the antigen-binding site, thus exposing the antigen binding site of MHC-class-II molecules and helping the inactive MHC-class-II to become functional.

Invariant chain and DM molecules were selected because their genes located in the MHC-class-II gene region. In addition, like MHC-class-II, these molecules are not constitutively expressed but are IFN-γ inducible in non-professional antigen presenting cells. IFN-γ induces the transcription of all the genes in the region, including MHC-class-II, DM and the invariant chain, through the production of a transcriptional activator known as the MHC class II transactivator (CIITA). Since it was shown in this study that chlamydial infection inhibits the transcription of MHC-class-II genes and related genes in the same region, it is highly probable that the inhibition is achieved by inhibiting the expression of CIITA.

CIITA is the key regulator in IFN-γ induced MHC-class-II gene transcription (Harton and Ting, 2000) Epithelial cells do not constitutively express CIITA, and therefore do not normally express MHC class II molecules. Transfection of epithelial cells with CIITA

can induce MHC class II expression. A deficiency of CIITA is responsible for some cases

of bare lymphocyte syndrome, in which the individual lacks expression of all MHC class

II gene products. These individuals suffer severe combined immunodeficiency,

illustrating the central importance of CIITA in the regulation of host immunity. These

observations suggest that CIITA is both necessary and sufficient for MHC class II gene

expression. Since CIITA is an obligate mediator of IFN-γ inducible MHC-class-II

expression, inhibition of CIITA, or the upstream factors that regulate CIITA activity,

might be the mechanism that chlamydia inhibits IFN-γ inducible MHC-class-II

expression.

4. Summary of known chlamydial pathways to evade host immunity

Five strategies will be discussed that *chlamydia* has developed to evade chlamydia-host

interaction. First, chlamydia growth alternates between EB and RB. EB is surrounded by

a very rigid structure that is resistant to most adverse environmental conditions, such as

heat, dessication, and a wide range of pH. RB grows in a membrane-bound vesicle inside

host cells and thus protects itself from detection and neutralization by the host's humoral

immune response. Moreover, RB appears to be devoid of any energy-generating system

and so it is an energy parasite, being totally dependent on ATP synthesized from the host

cells. The ATP translocase of these organisms takes in ATP and excretes ADP-the

opposite of the vast majority of other bacteria. Because chlamydia cannot be cultivated in

cell-free media, and because EB and RB have unique structures, there are no well-

characterized mutants available and genetic manipulation techniques have not been

developed for the organism. Thus compared to other bacteria, little is known about chlamydia.

Second, although chlamydia is a prokaryotic organism with a relatively simple structure, its major outer membrane protein (MOMP) is antigenically variable, a phenomenon known as antigen polymorphism. Host immunity has to catch up with the newly synthesized antigen in order to generate armed effector T cells specific for the new antigens. If MOMP mutation is too frequent and host immunity fails to recognize new antigen, these chlamydial organisms start to spread in the host.

Third, after EB enters target host cells, the endosome it resides in provides a favorable environment for EB to start differentiation and it does not fuse with lysosomes. The lack of fusion protects *chlamydia* from exposure to an acidic environment and proteolytic enzymes. There is considerable evidence that the vacuole containing newly internalized EBs does not precede along the normal phagosome route (Eissenberg *et al*, 1983). The mannose 6-phosphate receptor (a late endosomal-prelysosomal marker) cannot be detected in the vacuole containing internalized chlamydia. Acid phosphatase and cathepsin D (lysosomal enzymes) are absent and lysosomal glycoproteins LAMP-1 and LAMP-2 are also not found (Heinzen *et al*, 1996). The endosome is not acidified and does not fuse with lysosomes, but is transported to the Golgi apparatus, where it fuses with sphingomyelin-containing exocytic vesicles. The sphingomyelin is incorporated into the outer membrane of the bacteria.

Fourth, *chlamydia* has evolved an antiapoptotic activity, as discovered by Tao *et al.* (1998). By direct DNA staining with Hoechst dye, TUNEL and DNA ladder gel assays, they proved that chlamydia-infected cells resist apoptosis induced by a variety of stimuli. The stimuli include not only chemicals that cause direct damage to cell structures like the DNA-damaging agent etoposide and the kinase inhibitor staurosporine, but also immunological factors that are involved in pathogen-host interactions such as tumor necrosis factor-α (TNF-α), Fas antibody, granzyme B, and perforin.

The antiapoptotic activity against physiological stimuli significantly increases chlamydial survival in a natural infection. TNF-α is a cytokine that is produced by macrophages and T cells. It has multiple functions in both innate and acquired immune response. The Fas gene is another member of the TNF gene family and it is expressed on certain target cells. These cells are susceptible to be triggered to undergo programmed cell death by lymphocytes expressing the Fas ligand. Granzymes are serine esterases found in the granules of cytotoxic lymphocytes including CD8+ T cells and natural killer cells. When granzymes enter the cytosol of target cells it induces them to undergo apoptosis. Perforin is a protein that can polymerize to form membrane pores that are an important part of the killing mechanism in cell-mediated cytotoxicity. It is produced by, and stored in the granules of cytotoxic T cells and NK cells. It is released upon meeting target cells. Since infected cells usually undergo apoptosis, the ultimate mechanism of both innate and acquired host immune attack against intracellular pathogens. The antiapoptotic activity is a very powerful tool for chlamydia to disarm host immunity.

Fifth, in this study we demonstrated that *chlamydia* inhibits IFN-γ inducible MHC-class-II expression on non-professional antigen-presenting cells such as epithelial cells and fibroblasts. Since epithelial cells are the initial target chlamydial infection and first line of host immune defense, inhibition of IFN-γ inducible MHC-class-II expression allows *chlamydia* to be invisible to the host immunity and maintain long term survival in the host.

5. Future exploration of the mechanism behind the Inhibition of IFN-γ inducible MHC-class-II expression

A. Factors from Chlmayia may be responsible for the inhibitory activity

The mechanisms evolved by *chlamydia* to inhibit IFN-γ inducible MHC-class-II expression needs to be further explored. Questions that need to be addressed include 1) what is the factor responsible for the inhibition; 2) does the factor comes from the host or *chlamydia*; 3) what and; 4) where are the targets; 5) how does the factor acts upon the targets need to be asked and investigated.

We noticed in these experiments that the inhibition of IFN-γ inducible MHC-class-II expression occurred only after the volume of the chlamydial inclusion body reached half the size of the cell. That gave us a hint that a critical amount of *chlamydia* is associated with the inhibitory ability, and thus, we further hypothesize that a chlamydial protein but not host protein, might be responsible for the inhibition.

We can design future experiments to test this hypothesis. First the association of a specific amount of *chlamydia* and the inhibition of IFN-γ inducible MHC-class-II expression needs to be confirmed. This could be done in two ways --time course and dose response experiments. Then, antibiotics could be used to help us determine the origin of the protein responsible for MHC-class-II inhibition.

In time course experiment we harvest host cells at several time points after chlamydial infection and observed when the inhibitory activity emerges. Because the amount of chlamydia in the cell accumulates with time, the association of time with chlamydial inhibitory activity will reflect the association of the total amount of *chlamydia* with its inhibitory activity. We will also be able to observe that the chlamydial inhibitory activity continues to increase with time, until it reaches a plateau, when RB changes back to EB and no more new protein is synthesized.

In the dose-response experiment, at the same time point after infection a higher MOI (ratio of number of organisms versus numbers of host cells) should lead to more chlamydial protein being produced, as compared to lower MOI. If we infect cells with serially diluted chlamydial inocula and find that the chlamydial inhibition of IFN-γ inducible MHC-class-II proportionately increases as the MOI increases, we will be able to conclude that the inhibition is dependent on the amount of *chlamydia* newly synthesized protein.

Whether a chlamydia or host protein is responsible for inhibiting IFN-γ inducible MHC-class-II expression could be further examined using antibiotics that specifically inhibit either prokaryotic or eukaryotic protein synthesis. If the inhibition depends on chlamydial but not host protein, antibiotics that work by suppressing prokaryotic transcription, like rifampin, or suppressing prokaryotic translation, like chloramphenicol, would reverse the inhibition. On the other hand, antibiotics like cycloheximide that completely blocks new eukaryotic (host) protein synthesis would have no effect on the inhibition.

B. Exploration of upstream factors in the IFN-γ stimulation pathway

The genes encoding the MHC-class-II complex and the related molecules DRα, DMα and Ip41 share similar promoter structures that are resulted by the MHC class II transactivator (CIITA). Therefore, CIITA is a master regulator for the expression of these genes. We thus hypothesize that chlamydia may inhibit IFN-γ inducible MHC-class-II by suppressing CIITA function or CIITA gene expression. In the future we could test this hypothesis using western bloting and RT-PCR as used in this study to find out whether CIITA is suppressed and at what level is the suppression.

The Jak/Stat (Jak stands for Janus tyrosine kinase and Stat stands for signal transducers and activator of transcriptions) pathway IFN-γ uses to induce MHC class II expression is already known (Lian et al, 1999). Briefly, IFN-γ affects its target cells by binding to specific receptors that are composed of two chains. IFN-γ binding activates one of the receptor chains and induces IFN-γ receptor aggregation, and then the aggregated

receptors deliver signals to the inside of the cell. The cytoplasmic protein kinase Jak-1, a member of Janus family of kinases that is associated with the cytoplasmic domain of IFN-γ receptors, becomes active and then phosphorylates and activates other Jak-1 molecules to amplify the signal. In turn, Jak-1 phosphorylates and activates Signal Transducers and Activators of Transcription-1 (STAT-1), a member of STAT family that acts as gene-regulatory proteins. Upon activation, STAT proteins form a complex with each other, enter the nucleus, and bind to specific sequences in a gene to activate its transcription. A wide variety of genes such as MHC-class-II, IDO (indoleamine 2,3-dioxygenase), ICAM-1, and others are activated in this manner.

During IFN-γ inducible MHC-class-II expression, the STAT-1 complex activates two factors, namely upstream stimulatory factor-1 (USF-1) and interferon regulatory factor-1 (IRF-1). USF-1 and IRF-1 in turn activate CIITA expression, and eventually lead to the transcription of genes located in the MHC-class-II region. In our future study, if we find that CIITA is also suppressed, we could then test other upstream regulatory factors like USF-1 and IKF-1 until we determine the origin of the inhibition.

6. IFN-γ inducible ICAM-1 expression is not inhibited by chlamydial infection

ICAM-1 is a cell surface protein whose expression depends on IFN-γ stimulation through the same Jak/Stat signaling pathways as MHC-class-II molecules until the stage when the STAT-1 complex enters the nucleus. In IFN-γ induced ICAM-1 expression, a separate set of gene regulators other than USF-1, IRF-1 and CIITA are activated by the STAT-1

complex. Therefore, suppression of STAT-1 affects both IFN-γ inducible MHC-class-II and ICAM-1 expression, while changes in USF-1, IRF-1 and CIITA would only affect MHC-class-II expression. In the flow cytometry study, we discovered that chlamydial infection inhibits IFN-γ inducible MHC-class-II expression, but not ICAM-1 expression. Thus, it is highly probable that the chlamydial inhibitory activity targets one or more of the intra-nuclear gene regulators USF-1, IRF-1 or CIITA.

The discovery that chlamydial infection inhibits IFN-γ inducible MHC-class-II expression but not ICAM-1 expression, demonstrated the specificity of the inhibition, and also led to the second hypothesis of this study –that ICAM-1 is not important in the host immune response against chlamydial infection. *Chlamydia* selectively manipulates MHC-class-II function because disruption of the MHC-class-II response is critical to chlamydial survival. Conversely, ICAM-1 is not selected because it is not as important as MHC-class-II for chlamydial survival, and thus chlamydia can ignore its up-regulation on host cell surfaces.

Part B: ICAM-1 is not required for a host immune response against chlamydial infection as tested in a gene knockout mouse model

1. The gene knockout mouse model:

To test the hypothesis that ICAM-1 is not essential for a host immune response against chlamydial infection, we compared lung infection with *C. trachomatis* strain Mopn to test the immune resistence of ICAM-1 gene knockout and wild type mice with the same background.

In knockout mice, specific genes are inactivated and thus the gene product is not expressed at all. The knockout mouse is created following homologous recombination in tissue culture cells known as embryonic stem (ES) cells. On implantation into a blastocyst, stem cells give rise to all cell lineages in a chimeric mouse, in which cells derived from normal stem cells posess the gene of interest, whereas cells derived from the mutant stem cells lack this gene. If the mutant ES cells give rise to germ cells in the resulting chimeric mice, then the mutant gene can be transferred to their offspring. By breeding the mice to homozygosity, we can determine the effect of this gene deletion on the whole organism.

The ICAM-1 knockout mouse does not express the ICAM-1 molecule in any cell. These mice display impaired neutrophil emigration in chemical peritonitis and decreased contact hypersensitivity (Sligh *et al.*, 1993). Inactivated spleen cells derived from

infected knockout mice as antigen-presenting cells provided only a minimal stimulation in the mixed lymphocyte reaction. However, these cells proliferated normally as responder cells. Naïve T cells require ICAM-1 more than memory T cells (Parra *et al.*, 1993; Igietseme *et al.*, 1999) to carry out their functions. These findings not only confirm that ICAM-1 facilitates non-specific binding of immune components, but also suggests that there are subtle differences in the importance of ICAM-1 function in different immune processes.

Studies also suggest that the function of ICAM-1 in local immunity varies at different body sites. Mice with ICAM-1 deficiency displayed a normal neutrophil emigration to pulmonary alveoli although the neutrophil emigration to the peritoneum was completely absent (Bullard *et al.*, 1995).

We chose ICAM-1 deficient (knockout) mice to test our hypothesis because this mouse model has several advantages. Firstly, it is an *in vivo* study that mimics the natural infection. This *in vivo* study is better than an *in vitro* study because an immunological factor is tested in the context of the whole immune response, rather than as an isolated factor. Secondly, the gene knockout mouse model is better than other *in vivo* methods, such as partial functional depletion by antibody blocking and adoptive transfer experiments. Because model demonstrates not only the changes of immune function that occur in a host that lacks a specific factor, but also the compensatory mechanisms that the host develops when a specific factor is absent. Therefore, it is not surprising that studies using this model sometimes reach different conclusions than studies done by other

methods. For instance, up-regulation of macrophage-derived nitric oxide synthase (iNOS) (Byrne et al., 1992), an enzyme engaged in the generation of cytotoxic reactive nitrogen intermediates (Mayer et al., 1993; Igietseme 1996a, 1996b) used to be viewed as the mechanism of IFN-γ inhibition of chlamydial growth. Blocking nitric oxide *in vitro*, by adding the nitric acid (NO) synthase inhibitor N^G-monomethyl-l-arginine monoacetate in the culture solution, reversed the effect of interferon. However, a study done using the iNOS knockout mice, suggesting that iNOS did not play any significant role in host defense against chlamydial infection (Ramsey *et al.*, 1998; Igietseme *et al.*, 1998a).

2. Experimental Design

Four groups of mice were used in this study. They are:

- Wild type mice infected once with chlamydia;

- ICAM-1 deficient mice infected once with chlamydia;

- Wild mice with infected twice with chlamydia;

- ICAM-1 deficient mice infected twice with chlamydia.

Chlamydia trachomatis mouse pneumonitis strain (MoPn) was used in an established lung infection model. Mice are very sensitive to this strain of *chlamydia*. The infection tends to be severe in nature and has a short duration. Normal mice clear the MoPn infection in 14 days.

Near lethal-doses of chlamydia were used in both the first and second infections. By doing this, minor vulnerabilities in immune resistance show up easily and are revealed by a higher mortality rate in groups of mice. Thus, small discrepancies in immune function in wild type and ICAM-1 deficient mice may be more easily identified.

Body weight change, a sensitive parameter to the severity of chlamydial infection, was monitored every 24 hours for a 10-day period after the first infection, and for a 5-day period after second infection before mice were sacrificed. After sacrificing the mice, the infectivity of lung tissue was calculated based on the titers obtained from infecting cultured HeLa cells with lung isolates, and the levels of the IL2, IL-4 and IFN-γ cytokine produced were determined.

Groups of wide type and ICAM-1 deficient mice were each challenged twice. In first challenge, host innate immunity is initiated and lasts for 4-7 days before acquired immunity against the specific pathogen is produced. Thus the function of ICAM-1 is evaluated in both innate and acquired immunity in the first challenge. In contrast, in the second challenge, armed memory T cells respond and launch an efficient acquired immune response against the pathogen faster than in the first infection. Thus the function of ICAM-1 in acquired immunity is main parameter tested in second challenge. In addition, the function of ICAM-1 in naïve T cell activation and migration is emphasized in the first challenge, whereas the function of ICAM-1 in armed T cell activation and migration is more specifically tested in the second challenge.

3. Results and explanation

In this study, ICAM-1 deficient mice and wild type mice showed a similar immune resistance to both chlamydial infections, despite significantly impaired IL-2 and IFN-γ production in ICAM-1 deficient mice in first infection, and significantly impaired IL-2 production in ICAM-1 deficient mice in second infection. IFN-γ production levels were the same in both mice groups during the second infection.

Based on the results, we concluded that ICAM-1 is not required in an effective immune attack against chlamydial infection. This is not surprising because the ICAM family has powerful compensatory mechanisms. They compensate for each other within the family as well as compensated by members of other adhesion molecule families. For instance, the interaction of LFA-1 with ICAM-1 and ICAM-2 synergizes with a second adhesive interaction involving the immunoglobulin superfamily member CD2 and lymphocyte function-associated antigen-3 (LFA-3). CD2 is expressed on the T cell surface, and LFA-3 is expressed on the antigen-presenting cell. In the absence of LFA-1/ICAM-1, ICAM-2 could be compensated for by LFA-3/CD2, and vice versa. The powerful compensatory function of adhesion molecules originates not only from the synergistic activity of non-related molecules, but also from the relative redundancy of adhesion molecules. Maintaining a much higher potential than needed is a universal phenomenon in almost all organ systems; the immune system is not an exception.

There is evidence of powerful compensation among adhesion molecules. ICAM-1, -2 and -3 all bind to the same ligand LFA-1. Thus, LFA-1 deficient individuals must demonstrate functional impairment of the ICAM-1 family. However, such patients have

normal T cell response, despite being found to have dysfunctioning neutrophils and macrophages. Since cell medicated immunity is responsible for protection of the host against chlamydial infection, it is reasonable to infer that a deficiency of either LFA-1 or ICAM-1 cannot affect the immune resistance against chlamydial infection.

Many studies of ICAM-1 function in response to infection with other intracellular bacteria using the ICAM-1 deficient mouse-model have reported similar results to ours. ICAM-1 had only a minor or no influence on systemic and regional host defense in Listeria monocytogenes and Mycobacterium bovis infections despite distinct alterations of the gut lymphoid environment (Ulrich Steinhoff, 1998; Saunders, 1999).

Impaired cytokine production noted in our study following the first and second infections did not lead to impaired immune resistance against chlamydial infection. This result suggests that other immune components quickly and effectively compensated for CD4+ Th1 and IFN-γ function in both infections. During the second infection, the gap in IFN-γ production between wild type and ICAM-1 deficient mice narrowed, which may suggest a broader scale of compensation mechanisms took effect and produced more IFN-γ.

Interestingly, studies of other intracellular bacteria, such as Listeria monocytogenes and Mycobacterium bovis infections, in which ICAM-1 only has minor function in host immunity also showed a separation of cytokine production and the outcome of infection (Ulrich Steinhoff, 1998; Saunders, 1999).

4. Explanation of the debates in studies of ICAM-1 function in chlamydial infection

A. Introduction of other views about ICAM-1 in chlamydial infection

The function of ICAM-1 in host immunity against chlamydial infection has long been in debate. Some studies agree that ICAM-1 plays a significant role in host defense against chlamydial infection. Igietseme and colleagues (1996c) co-cultured chlamydial infected epithelial cells with IFN-γ-producing T cell clones. Adding ICAM-1 in the co-culture supernatant lead to more profound inhibition of chlamydial growth than co-culture alone. Monoclonal antibodies against either ICAM-1 or LFA-1 partially reversed the inhibition. In an *in vivo* experiment, Kelly and Rank (1997) infected mice with chlamydia via the genital tract. They found local CD4+ T cells had increased expression of $\alpha4\beta7$ and LFA-1.

Igietseme and colleagues (1999) further tested the role of ICAM-1 in chlamydial genital tract infection in a ICAM-1 knockout mouse model. They challenged both ICAM-1 deficient mice and wild type mice with live EBs. Reactivated and amplified organism amount and cytokine productions, as parameters of resistance against chlamydial infection, were compared in the two groups. ICAM-1 deficient mice had significantly greater chlamydial burden and decreased IFN-γ, TNF-α and IL-12 levels during the first 2 weeks of infection compared to wild type mice. However, the organism burden quickly reached a similar level in both wild type and ICAM-1 deficient mice two weeks after the

infection. All mice completely recovered from the infection. They concluded that ICAM-1 may be required for early T cell recruitment and activation.

B. Comparison of two gene knockout mice study

Both the Igietseme *et al.* (1999) study and this study are based on comparison of ICAM-1 gene knockout and wild type mice. Igietseme found ICAM-1 may be required for early T cell recruitment and activation, while our study did not find any difference in immune resistance between the groups of mice in two sequential infections. The two studies seemed to have reached conflicting conclusions. Why?

The two studies are not as contradictory as they initially appear; they share several similar results. First, both studies confirmed that cytokine IL2 and IFN-γ production levels are impaired in ICAM-1 deficient mice. Second, mice with or without ICAM-1 deficiency eventually all cleared infection at the same rate in both studies, except that Igietsemse found a lag of host immunity development in ICAM-1 deficient mice at the early stage of infection.

However, this difference may lie in the different experimental design. Igietsemse *et al.* studied chlamydial genital tract infection, whereas this study used a lung infection model. Just as the route of infection can elicit different types of immune response (Igietseme *et al.*, 1998b); each body part has its unique local immunity. Thus, different regions of the

body likely have a different course and intensity of immunity and different immune effector profiles.

Mice clear a pulmonary MoPn infection within 14 days, but clear a genital tract infection in twice the time. Mice start to die at a chlamydial organism infection concentration of 10^4 IFU/mouse in the lung but at a concentration of 10^7 IFU/mouse in the genital tract. Strong and acute host immunity in lung infection causes severe tissue damages that lead to a sharp body weight drop. Immunity in chlamydial genital tract infection however, tends to be chronic and mild. Genitally infected mice shed a great amount of viable chlamydia without a sign of sickness for a long duration. The evidence suggests mouse launch much more vigorous local immunity and resolve infection much more quickly in lung than genital tract.

Stronger local immunity in a lung infection may be caused by its central location in the body and a better blood supply. More immune components are recruited in a lung infection than in a genital tract, and they migrate to the lung more quickly. Evidence of stronger and faster lung immunity was found by Bullard and colleagues (1995). In that study, mice with ICAM-1 deficiency displayed a normal neutrophil emigration to pulmonary alveoli although the neutrophil emigration to peritoneum was completely absent. Therefore, the period of early naïve T cell recruitment and trafficking detected in the Igietseme et al. study may not exist in chlamydial lung infection.

Compared to the Igietseme study, this study has an advantage because it consists of two sequential infections and thus takes into account the ICAM-1 function and compensatory mechanisms developed in the memory immune response. Moreover, by combining the results of Igietseme et al and ours, we conclude that ICAM-1 only plays a marginal role in host immunity against chlamydial infection.

C. Conclusion

We found that ICAM-1 is not essential in host immune response against chlamydial infection. Total depletion of ICAM-1 could not alter the course and outcome of chlamydial pulmonary infection. One of the main goals of an immunology study is to understand the host-pathogen interaction in order to develop an effective vaccine; this ICAM-1 study is no exception. The results help direct future investigators to be focus on more important immune components in host immunity against chlamydial infection, like MHC molecules rather than subordinate factors like ICAM-1.

VII. FUTURE RESEARCH DIRECTIONs

Two directions are identified from this study:

1. Identify the factors that inhibit IFN-γ inducible MHC-class-II expression. Since the factors would be responsible for an efficient chlamydial strategy to evade host immunity, identifying or even purifying the factor may contribute to treatment and future vaccine development.

2. We noticed chlamydial infected cells possess higher cell-membrane permeability. It suggests possible structural changes on the cell membrane. Also, since the cell membrane carries out important exchange functions between the inside and outside of the cells, small size molecular weight molecules might leak in and out of chlamydial infected cells. Further research in this direction deepens the understanding of chlamydial influence on host cells.

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