INDUCTION OF PROTECTIVE IMMUNITY AGAINST CHLAMYDIAL INFECTION WITH ANTIGEN-PULSED DENDRITIC CELLS

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Induction of Protective Immunity Against Chlamydial Infection with Antigen-Pulsed Dendritic Cells

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of Master of Science

TABLE OF CONTENTS

ABSTRACT

Chlamydia trachomatis species are important causative agents of sexually transmitted diseases and preventable trachoma in the world. Immune intervention-based approaches are badly needed for preventing and controlling chlamydia diseases. However, despite considerable effort, no significant progress has been made in developing an effective vaccine. This is largely due to an inadequate understanding of host immunity to chlamydial infection. The goal of this project is to develop a strategy for modulating protective immunity against chlamydia infection and to determine the protective parameters. The information generated from these studies may be useful for designing efficacious vaccines. Immunization with dendritic cells pulsed ex vivo with antigens has been successfully used to elicit primary antigen-specific immune responses. Using a murine model of pneumonia induced by intranasal inoculation with *Chlamydia trachomatis*, we found that mouse bone marrow-derived dendritic cells pulsed with inactivated chlamydial organisms induced a strong protection against live chlamydial infection. Either the dendritic cells or chlamydial organisms alone or macrophages similarly pulsed with inactivated chlamydial organisms failed to induce any significant protection. These observations suggest that dendritic cells can efficiently process and present chlamydial antigens to naive T cells in vivo. Mice immunized with the chlamydia-pulsed dendritic cells preferentially developed a Th1 cell dominant immune response with high levels of Ag-specifc IgG2a Ab and IFN-γ production but minimal IL-4 production, while mice immunized with the other immunogens failed to do so, suggesting a correlation between a Th1 cell dominant response and immune protection against chlamydial infection. We also found that dendritic cells produced large amounts of IL-12 upon ex vivo pulsing with inactivated chlamydial organisms, which may allow these cells

to direct a Th1 cell dominant response in vivo. Dendritic cells from mice deficient in IL-12p40 gene failed to produce IL-12 after a similar ex vivo pulse with inactivated chlamydial organisms and more importantly, immunization with these pulsed dendritic cells failed to induce a Th1 cell dominant response and did not induce a strong protection against chlamydial infection. Thus, the ability of dendritic cells to efficiently process and present chlamydial antigens and to produce IL-12 upon chlamydial organism stimulation is required for the induction of protection against chlamydial infection. This information may be used for the design of effective chlamydial vaccines.

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LIST OF FIGURES

Page

ABBREVIATIONS

Ab: antibody

Ag: antigen

APC: antigen-presenting cell

CFA: complete Freund's adjuvant

CTL: cytotoxic T lymphocyte

DC: dendritic cell

DEAE: diethylaminoethyl

DMEM: Dulbecco's modified Eagle medium

DTH: delayed-type hypersensitivity

EB: elementary body

ELISA: Enzyme-linked immunosorbent assay

FACS®: registered trademark of Beckton Dickinson and Company for a fluorescence activated cell sorter

FITC: fluorescein isothiocyanate

g: unit of gravity

GM-CSF: granulocyte-macrophage colony stimulating factor

HBSS: Hank's balanced salt solution

HEL: hen egg lysozyme

HRP: horseradish peroxidase

IFA: incomplete Freund's adjuvant

IFN: interferon

IFU: inclusion forming unit

i.p.: intraperitoneal

KO: knock out

LPS: lipopolysaccharide

2-Me: 2-mercaptoethanol

MHC: major histocompatibility complex

MOI: multiplicity of inoculation

MOMP: major outer membrane protein

MoPn: mouse pneumonitis

Mφ: macrophage

OD: optical density

P: probability

PE: phycoerythrin

PI: propidium iodide

PID: pelvic inflammatory disease

RB: reticular body

rpm: rotations per minute

SEM: standard error of the mean

SPG: sucrose phosphate glutamic acid

STD: sexually transmitted disease

TCR: T cell receptor for antigen

TGF: transforming growth factor

TNF: tumor necrosis factor

UV: Ultraviolet

1. INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterial pathogen that causes various human diseases (Darville, 1998; Groseclose et al., 1999; Munoz and West, 1997; Stokes, 1997). The estimated annual incidence of *C. trachomatis* in the United States was approximately 3 million people in 1996 among women age 14 to 44 (Groseclose et al., 1999). Ascending infection with *C. trachomatis* of the genital tract is the leading cause of many important sexually transmitted diseases. These urogenital tract infections are frequently asymptomatic in their acute phase, but can progress to chronic pelvic pain, salpingitis and pelvic inflammatory disease that often result in sterility and life-threatening ectopic pregnancy, which are the major public health burdens of this infection in North American. In addition, ocular infection with *C. trachomatis* can result in trachoma that is the major cause of preventable blindness in many developing countries (Munoz and West, 1997). Infants born to mothers with chlamydial cervicitis are at high risk for developing pneumonia (Darville, 1998). Therefore, efficient intervention strategies are badly needed for the prevention of chlamydial infection. The immunization with whole organisms or their detergent extracts were capable of inducing humoral responses but they only offered short-lived and limited protection (MacDonald et al., 1984). To date, no successful chlamydial vaccine is available despite the tremendous amount of effort that has been made by many research groups (Fan and Stephens, 1997; MacDonald et al., 1984; Su and Caldwell, 1995; Zhong et al., 1993).

Dendritic cells (DCs) are characterized by their potent capacity to activate

immunologically naïve T cells and by their high constitutive expression of MHC class II molecules. DCs are a family of bone marrow derived APCs and are found in most nonlymphoid organs, including epithelia, dermis and the interstitia of heart and kidneys. DCs are also found in blood, lymph and all lymphoid organs. The DC network is a specialized system for bringing antigens from peripheral sites to secondary lymphoid organs to present antigen to naive T cells (Steinman, 1991). DCs have distinct developmental stages ranging from immature to mature (Banchereau and Steinman, 1998; Pierre et al., 1997). The immature dendritic cells are highly active in phagocytizing and processing microbial antigens. Maturation of DCs can be triggered in vivo by exposure to microbial agents and/or inflammatory cytokines and coincidentally, DCs can migrate to a draining lymph node. The matured DCs express high levels of costimulatory and MHC molecules that may allow the mature DCs to efficiently present antigens and to induce primary antigen specific CD4 or CD8 T cell-mediated responses. The unique capacities of DCs recruited to invade sites of pathogen entry, responding to microbial signals and further activating naïve T cells suggest a critical role for these cells in initiating and maintaining antimicrobial immunity (Inaba et al., 1993; Reis e Sousa and Austyn, 1993).

As the generation of relatively pure and large quantity of DCs became possible (Inaba et al., 1992), DCs have been successfully used to induce antigen specific immune responses and protective immunity against various cancers and infectious diseases (Ludewig et al., 1998; Ossevoort et al., 1995; Schuler and Steinman, 1997). In many of these studies, DCs

were pulsed ex vivo with either peptides or whole protein antigens and delivered in vivo to syngeneic hosts. The protection thus induced often correlated with a strong, antigen specific T cell response (Inaba et al., 1990). Dendritic cells were found to produce large amounts of IL-12 in both CD40 ligand-dependent and -independent manner (Cella et al., 1996; Sousa et al., 1997). This IL-12 production by DCs may allow the DCs to direct the development of Th1 cells from naive CD4+ T cells (Macatonia et al., 1995) since antigen-specific CD4 Th cell responses can be divided into two types, type 1 and type 2 based upon cytokine secretion and effector function. Type 1 responses involve Th1 cells, whose differentiation is driven by IL-12 and IFN-γ (Mosmann and Coffman, 1989). A Th1 cell dominant immune response is often required for controlling many intracellular infections (Geginat et al., 1998; Igietseme et al., 1993). Thus, DCs not only possess the ability to prime an antigen specific response but are also able to direct a protective immune response against infections.

Since it has been demonstrated that a Th1 cell-mediated immune response played a critical role in controlling the intracellular chlamydial infection (Perry et al., 1997; Su and Caldwell, 1995; Yang et al., 1996; Zhong et al., 1988), we have used the DC-based immunization approach for inducing protective immunity to chlamydial infection in the present study. We found that mouse bone marrow-derived DCs pulsed ex vivo with inactivated chlamydial organisms induced a strong protection against chlamydial infection in a mouse lung infection model. Either DCs or chlamydial organisms alone or bone marrow-derived Mϕs similarly pulsed with inactivated chlamydial organisms failed to induce any

significant protection. The protection induced by the chlamydia-pulsed DCs was dependent on donor DCs production of IL-12 and required to the development of a Th1 cell dominant immune response.

2. LITERATURE REVIEW

2.1. Taxonomy

Chlamydiae are small obligate intracellular parasites that are classified into bacteria because of the composition of their cell wall, bacterial organelles, DNA, RNA and growth pattern of binary division (Stephens, 1992). Molecular analysis of rRNA sequences confirms that chlamydiae are eubacteria although there is a very distant relationship with other eubacteriae (Weisburg et al., 1986).

Due to their unique obligate intracellular biology, chlamydiae are recognized in their own order, chlamydiaes, with one family chlamydiaceae and a single genus chlamydia, including *C. trachomatis*, *C. pneumonia*, *C. psittaci* and *C. pecorum* (Fukushi and Hirai, 1992; Grayston, 1989; Pudjiatmoko et al., 1997). They share many biological and biochemical properties with high homology of protein sequences (Weisburg et al., 1986). Both *C. trachomatis* and *C. pneumonia* are causative agents of human diseases.

With the use of mAb, the species *C. trachomatis* has been further divided into three biovariants (biovars): trachoma, lymphogranuloma venereum (LGV) and mouse pneumonitis (MoPn), whereas there is still no complete typing system for either *C. psittaci* or *C. pnemoniae* species (Kuo et al., 1983; Wang et al., 1977). Of the three biovars of *C. trachomatis*, trachoma and LGV biovars have almost identical genomic DNA and share a very high level of sequence homology of their proteins, but the murine biovar only exhibits 30 to 60% of DNA homology with other two biovars [Peterson, 1988 #80].

The trachoma biovars currently have been further separated by indirect microimmunofluorescence into 15 closely related serovars designated by the letters A through K, including Ba, Da, and Ia (Grayston and Wang, 1975; Moulder, 1991; Wang et al., 1977). Chlamydiae display a broad diversity in tissue tropisms and disease expression. The trachoma biovar is a parasite of the squamocolumnar cells of mucous membranes. Its transmission involves in the two distinct routes. Serovars A, B, Ba and C are predominately involved in ocular trachoma, a follicular conjunctivitis spread by close contact. Infection with serovars D through K mainly occurs in the genital tract and is associated with sexually transmitted diseases.

LGV biovar, named originally by its infectivity on lymphoid cells, exists in 4 serovars including L1, L2, L2a and L3. Trachoma and LGV biovars are known to only infect humans, but they show distinct features in their behaviors in cell culture, animal model and significant differences in their clinical features. Within each biovar of trachoma and LGV, there is little variation among the different strains. LGV biovar strains are more invasive and often cause systemic infections. In contrast, trachoma biovar is much less invasive and appears to only growth on the mucosal surfaces (Moulder, 1991).

The analysis of genomic DNA and biological characteristics, including growth and morphological features, suggest that another species *C. psittaci* is more diverse compared to *C. trachomatis.* They have remained classified because of the lack of biological markers to distinguish between these groups of organisms (Fukushi and Hirai, 1989; Perez-Martinez and

Storz, 1985; Spears and Storz, 1979). However, *C. psittaci* is primarily a pathogen of animals (Schachter and Meyer, 1969).

A new chlamydia agent, *C. pneumoniae*, has been characterized as a common cause of acute respiratory infection (Grayston et al., 1989) and more importantly it has been associated with atherosclerosis (Kuo et al., 1995; Kuo et al., 1993). *C. pneumonia* can infect various host cells, including endothelial cells, cause macrophage foam cell formation and lipid oxidation which contribute to the tissue damage and allow the LDL deposition, finally may progress to atherosclerosis (Byrne and Kalayoglu, 1999). Various *C. pneumonia* isolates have 94% to 100% DNA homology (Cox et al., 1991). Recently Kalman Sk, et al compared the genomes of *C. pneumonia* and *C. trachomatis* and found there is about 80 % of *C. pneumonia* and *C. trachomatis* serovar D predicted coding sequences are orthologues. There is highly functional conservation between *C. pneumonia* and *C. trachomatis* (Kalman et al., 1999).

2.2.Immune responses during chlamydial infection

Following the invasion of microbial pathogens, the innate immune response operates immediately, which is based on the pre-existing elements and the non-specific induction of the innate immune system. If an infection can not be aborted by these early defense mechanisms, adaptive immune responses are invoked and immune memory may develop.

2.2.1.Innate immunity to chlamydia infection

The epithelial cell layers that line the various mucosal surfaces of the body are both a physical barrier against the entry of pathogenic organism and also the initial cellular target for microbial invasion. Importantly, epithelial cells serve as sensitive indicators of mucosal infection by actively initiating a host defense response through the secretion of chemokines and proinflammatory cytokines. Endogenous chemokines and proinflammatory cytokines produced by epithelial cells at early stages of infection not only recruit immune cells to the epithelial/mucosal sites from the circulation but also help in the activation of macrophages and dendritic cells and the differentiation of effector lymphocytes. Chlamydial infection of the mucosa is the initial site of chlamydia infection, therefore, mucosa cytokine response during the early stage of infection undoubtedly play an important role in host defense. IL-8, IL-1α, IL-6, GM-CSF, and other proinflammatory cytokines are induced from epithelial cell lines and primary endocervical epithelial cells following the chlamydia infection (Rasmussen et al., 1997). IL-8 is a potent chemotactic factor for neutrophils. Human neutrophils migrated to chlamydia infected epithelial cells in vitro culture, which coincided with the upregulation of chemokine mRNA expression (Wyrick et al., 1999). Chlamydia infection also induces the expression of adhesion molecules. Kaukoranta-Tolvanen et al found that *C. Pneumonia* infection of human endothelial cells resulted in the expression of endothelial-leukocyte adhesion (ELAM-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) on those cells within a few hours after infection (Kaukoranta-Tolvanen et al., 1996). This raised the possibility that the proinflammatory cytokines may be secreted at

focal sites in the initial stage of chlamydial infection and recruit the inflammatory cells to the site of infection. These cytokines and increased expression of adhesion molecules may further initiate the innate immune response or inflammatory reaction, followed by the development of adaptive immune response. This was partly supported by the increased amount of MHC class II antigen-presenting cells or dendritic cell-like cells accompanied by the increased local GM-CSF levels in sites of chlamydial infection in mouse models (Stagg et al., 1998; Zhang et al., 1999).

Neutrophils are the most predominant effector cells of the innate immune system and they are recruited to the infectious site during the early stage of chlamydial infection (Brunham et al., 1984; Pal et al., 1993). Neutrophils kill most internalized chlamydial organisms. After phagocytosis, *C. trachomatis*-containing phagosomes fused with neutrophil lysosome, followed by progressive degradation of chlamydiae (Yong et al., 1986). Mice depleted of neutrophils with the specific Ab in vivo delayed recovery of *C. trachomatis* from the spleen, liver, and lungs postinfection, but eventually controlled the infection as did isotype Ig treated mice. These studies suggest that neutrophils play an important, but not essential in restricting chlamydial infection. Once neutralizing Ab was catabolized or other immune mechanisms developed in vivo, mice were capable of blocking the spread of chlamydial infection (Barteneva et al., 1996).

The focal inflammation of chlamydia infection is initially dominated by an infiltrate of neutrophils, followed by mononuclear cells recruited to the site of infection. Mononuclear

phagocytes are another pivotal component of innate immune system and are important in diverse ways for controlling chlamydial infection. It appears that the clearance of chlamydial infection related to the participation of macrophages. On the other hand, macrophages may not immediately kill the ingested chlamydia organisms. Viable organisms can persist within macrophage and other host cells. Thus, macrophages may contribute to both the control of acute infection and the persistency of chlamydial infection (Byrne et al., 1989). The intracellular fate of chlamydiae in macrophage is related to the multiplicity of infection (MOI), i.e. the chlamydiae-to-macrophage ratio; and the capacity of the EB to inhibit the fusion of phagosome and lysosome. At a low MOI (1:1), internalized chlamydial EBs survived, differentiated, and multiplied in macrophages. In contrast, at the high MOI (100:1), chlamydial EBs resulted in direct damage to macrophage causing cell lysis (Wyrick and Brownridge, 1978).

If chlamydial organisms overcome the phagocytic barrier, host adaptive immune responses are required to control the chlamydial infection.

2.2.2. Chlamydial antigens

Chlamydiae have a unique biphasic life cycle with two cell types: infectious elementary body (EB) and metabolically active reticulate body (RB). EB is adapted to extracellular survival, whereas RB is non-infectious and labile and only exists in intracellular environment.

Chlamydial cells either EB or RB forms are enclosed by an envelope structure like

that of gram-negative bacteria. The envelope is composed of two trilaminar membranes, an outer membrane (OM) and an inner cytoplasmic membrane (IM) (Caldwell et al., 1981). However, unlike other gram-negative bacteria, there is no peptidoglycan in the pericytoplasmic space of chlamydial cell. Chlamydial cells contain 40-50% of lipids and 35% of protein. Both RNA and DNA exist in chlamydia (Manire and Tamura, 1967). Chlamydial antigens can be classified as genus, species, subspecies and serotype-specific.

Chlamydial LPS has lipid A and 2-keto-3deoxyctanoic acid among all members in the chlamydial genus. The lipid A from chlamydial LPS is pentaacyl and has five fatty acids with a phosphate in the distal segment. Studies with immune electron microscopy demonstrated that LPS was poorly exposed on the surface of chlamydia EBs. Chlamydial LPS is capable of inducing cytokine release from phagocytic cells although with ~100 fold less potency than other bacterial LPS. The reduced biological activity of chlamydial LPS may be due to the acylation of lipid A (Brade et al., 1987; Brade et al., 1986; Caldwell and Hitchcock, 1984) and/or unusual fatty acid composition of lipid A moiety (Qureshi et al., 1997). Antibodies to LPS were usually elicited during natural infection although such antibody is neither neutralizing nor protective roles in vitro or in animal model (Brunham et al., 1987; Caldwell and Hitchcock, 1984). The results do not support that chlamydial LPS is the main factor involved in the immune pathogenesis, although more studies need be done.

Caldwell H, et al identified a major outer membrane protein (MOMP) with a molecule weight of 39.5 kDa from chlamydial EB, which is analogous to porin of other gram-negative

bacteria. MOMP is composed of approximately 61% of outer membrane complexes. Through extensive disulfide bonding, MOMP is involved in maintaining the structural integrity of EB (Caldwell et al., 1981). Anti-MOMP sera raised to one species do not necessarily recognize the organisms of other chlamydial species, indicating the antigenic heterogeneity of MOMP (Caldwell and Perry, 1982). Monoclonal Ab staining by microimmunofluorescence of EB preparations from 18 serotypes of chlamydiae showed that there were genus-, species-, subspecies- and type-specific distributions of MOMP antigens. The genus-specific epitopes were heat-stable, pronase resistance and relative periodate sensitive, while the species-, subspecies- and type-specific antigenic epitopes were also heat stable, but pronase sensitive and periodate resistant (Stephens, 1992). However, studies with radiolabelling, SDS-gel electrophoresis and chromatographic approaches demonstrated considerable structural homology of MOMP among the various strains of chlamydia (Caldwell and Schachter, 1982).

MOMP is the principal target of neutralizing antibodies and may be the target of protective immunity. Progress has been limited in part because of the absence of the knowledge regarding the confirmation and the antigenic variation of the protein structure (Brunham and Peeling, 1994). Besides MOMP and LPS, two heat shock proteins (hsp), including hsp60 and hsp70, are also major antigens which perhaps are important to the immune pathogenesis of human infection (Bachmaier et al., 1999; Brunham and Peeling, 1994). The genes encoding hsp70 and hsp60 are constitutively expressed and transcription is

upregulated upon heat stress. Chlamydia hsp60 shares nearly 50% homology with human hsp60. Studies have shown that continuous B cell epitopes exist in chlamydia hsp60 and there is about 50% cross-reaction between human and chlamydia hsp60 (Yi et al., 1993). Individuals with severe chlamydia disease often display immune responses to chlamydia hsp60 antigens. The presence of antibodies to chlamydial hsp60 correlated significantly as a risk factor for PID (i.e., tubal infertility and occluded fallopian tubes), but not for acute infection without PID (Eckert et al., 1997; Peeling et al., 1999). More recently, Bachmaier, et al demonstrated that endogenous cardiac muscle specific myosin heavy chain and chlamydial-derived peptide have sequence homology of hsp and both of them can induce cardiac inflammation with associated T and B cell reactivity (Bachmaier et al., 1999). Therefore it is speculated that molecular mimicry may be the mechanism in chlamydial induction of autoimmune inflammatory responses that further cause chlamydia disease sequelae (Bachmaier et al., 1999; Brunham and Peeling, 1994).

Collectively, it remains unclear which chlamydial component(s) containing dominant epitope(s). MOMP is the major vaccine target since it induces relatively high immune response. However, we can not rule out the other chlamydial components as vaccine target(s) since the vaccination with MOMP protein and gene only provides partial protection against infection.

2.2.3.Adaptive immunity to chlamydia infection

Adaptive immunity is triggered when an infectious agent eludes the innate defense

system and generates a threshold dose of antigens. These antigens, thereafter, subsequently initiate a specific acquired immune response, which becomes effective after several days following the proliferation and differentiation into chlamydia specific cells. These effector cells and molecules, combined with innate immune mechanisms, can lead to clearance of the infection and establishment of a state of protective immunity.

Dendritic cells, macrophages and B cells are professional antigen-processing cells, although other cells can also process antigens upon upregulation of MHC and costimulatory molecules. However, only dendritic cells and macrophages have a high capacity of processing particular antigens. Furthermore, these APCs present microbial antigens, in the form of complexes with MHC molecules, to the immune cells, thereafter initiate the specific-T cell proliferation.

2.2.3.1. Humoral immune response

Adaptive immunity can be divided into two major components: humoral immunity mediated by antibodies, and cell-mediated immunity.

Humoral immunity is mediated through antibodies to specific antigens. Chlamydia have numerous antigens, which can elicit strong local and systemic antibody responses including the production of IgG, IgM, IgA and SIgA in human and various animal models(Cotter et al., 1997; Grayston and Wang, 1975; Johansson et al., 1997; Pal et al., 1993; Rank et al., 1990). Either polyclonal antibodies raised against EBs or monoclonal antibodies to epitopes in variable domain (VD) II or IV of the MOMP can block the attachment and/or ingestion of *C.*

trachomatis in cultured cell lines (Su et al., 1990; Ward and Murray, 1984; Wenman and Meuser, 1986). Some antibodies to MOMP neutralized the infectivity of *C. trachomatis* by inhibiting inclusion formation or interfering ATP transportation rather than by affecting the attachment (Caldwell and Perry, 1982; Peeling et al., 1984). These results suggested that different antibodies might neutralize chlamydia infectivity at various stages in their growth cycle.

Passive transfer of anti-chlamydia antibody or of the hybridoma secreting specific antibodies to naive animals showed partial protection against chlamydial challenge (Rank et al., 1979; Williams et al., 1984; Zhang et al., 1987). It was found that Igh-/- B cell deficient mice were more susceptible to chlamydial infection than wild type mice, but much less susceptible to infection than mice with MHC class II deficiency (Williams et al., 1997). B cell deficient mice showed significant higher mortality rate and chlamydia growth as well as reduced DTH response than wild type mice following chlamydial lung infection, although mice finally resolve the infection (Yang and Brunham, 1998). These results suggested that B cells also play an important, but not essential role on the development of anti-chlamydia immunity. B cell may function in the initiation of T cell response during chlamydia infection.

2.2.3.2. Cellular immune response

To participate in an adaptive immune response, naive T cells including CD4 and CD8 T cells must be induced to proliferate and differentiate into effector cells. Athymic nude mice (nu-/nu-) were much more susceptible to *C. trachomatis* infection than their heterozygous

(nu-/+) littermates. In contrast, transfer of immune sera to nude mice did not significantly reduce mortality (Williams et al., 1981; Williams et al., 1984). Without immune intervention, athymic nude mice developed chronic infection which persists much longer than in heterozygous control mice (265 v.s. 20 days) following the genital infection (Rank et al., 1985). These experiments indicated that the murine protective immune response is more dependent on T cells than on B cells.

Recently studies from several groups have demonstrated the distinct roles of T cell subpopulations, CD4 and CD8 T cells, in the resolution of chlamydia infections. Naive CD8 T cells can differentiate into cytotoxic cells. Cytotoxic cells can kill target cells by releasing cytotoxic granules and/or cytokines. In vivo experiments have revealed that CD8 T cells or CD8 lines derived from mice recovered from chlamydial infection were able to specifically lyse chlamydia infected target cells (Beatty and Stephens, 1994; Igietseme et al., 1994). This cytotoxic activity was cross reactive between *C. trachomatis* and *C. psittaci* infection (Beatty et al., 1997) or among various serovars of *C. trachomatis* (Starnbach et al., 1995). More importantly, adoptive transfer of Ag-specific CTL lines or CD8 T cell clones provided infected mice with IFN-γ dependent protection (Starnbach et al., 1994) or resulted in the clearance of chlamydia organisms from nude mice (Igietseme et al., 1994). However, when mice with beta2-microblobulin deficiency (MHC class I knock out) were used to directly address the role of CD8 T cells in host defense against chlamydial infection. These MHC class I KO mice displayed a slightly impaired resistance to chlamydia lung infection

(Williams et al., 1997). Therefore, the results indicated that CD8 T cell-mediated immune response is important, but not essential in the induction of resistance to chlamydia.

Adoptive transfer of antigen-specific T lymphocytes further revealed a vital role of CD4 T cells in anti-chlamydial infection. Adoptive Ag-specific CD4 T cell transfer provided significant protection for naive mice (Su and Caldwell, 1995), nude mice (Igietseme et al., 1993; Ramsey and Rank, 1991) or mice with severe combined immunodeficiency (Magee et al., 1995). Furthermore, CD4 T cell depleted mice showed a significant increase in the number of organisms recovered from the oviducts of infected cells and the exacerbated salpingitis (Landers et al., 1991). Those CD4 T cell depleted mice also had much higher mortality following the lung infection (Magee et al., 1995). Studies based on MHC gene knock out mice further demonstrated that class II-restricted immune responses are more important than MHC class I (Morrison et al., 1995; Williams et al., 1997). Transfer of splenic CD4 but not CD8 T cells from mice recovered from genital infection to naive mice can confer resistance to chlamydial genital infection (Su and Caldwell, 1995). Collectively, the results clearly suggest that CD4 T cell-mediated immunity is essential for the protective immunity during chlamydia infection.

More recently, various groups have addressed the relative roles of CD4 Th1 cells in the resolution of chlamydia infection. IFN-γ, a typical Th1 type of cytokine, KO mice showed significant impaired immune response and eventually develop dissemination of bacteria in the genital tract and lung infection models (Perry et al., 1997; Wang et al., 1999).

IL-12 is the potent stimulator of IFN-γ production and the key regulator to skew the Th1-mediated immunity. IL-12 KO mice were unable to clear genital infections and mice were characterized by the significantly reduced levels of IFN-γ and low IgG2a, but an increase in IgG1 levels in vivo (Perry et al., 1997). Those data support the necessity of Th1 cell-mediated immune protection in the resolution of infection.

2.3.Vaccine development

Vaccination is one of the most effective means for controlling and/or preventing many human infectious diseases. Chlamydial infection can cause many human diseases including infertility, life-threating ectopic pregnancy and blindness. Treatment of chlamydial infection is costly although effective. Recent studies suggest that *C. pneumonia* infection can exacerbate the development of atherosclerosis, which is the major cause of many cardiovascular diseases in human. Therefore, developing a vaccine capable of protecting against chlamydial infection may provide an effective and long-term solution for controlling chlamydial diseases.

There are many types of vaccines including dead or attenuated whole pathogen organisms, components of pathogen, and genes encoding microbial components (DNA vaccine).

2.3.1.Attenuated vaccines

Various animal models have been used in chlamydia vaccine research, such as mice, guinea pigs, monkeys and cattle. Guinea pig immunized with living *C. psittaci* had less severe disease and lower percentages of infected mucosal cells following the conjunctival or the vaginal challenge (Nichols et al., 1978). Studies later confirmed Nichols' results and further showed that immunized guinea pig with *C. psittaci* by either intravenous, subcutaneous, or ocular routes displayed a significantly reduced infection course. In contrast, orally immunized animals did not. Guinea pigs immunized with viable organisms were more likely to develop resistance to challenge infection (Rank et al., 1990). However, the accumulated evidence indicated that the hypersensitivity reaction induced by the whole organism as vaccine and the inability of systemic immunization failed to produce sufficient local antibody levels to afford protection. The protection induced by these attenuated organisms was usually shorted-lived and strain-specific (Grayston and Wang, 1975; Taylor et al., 1987). These results indicated that chlamydial organism may contain not only protective but also immunopathogenic antigens, which may enhance the serious sequelae of chlamydial infection. The owl monkey immunized with inactivated *C. trachomatis* showed a longer disease course and more ocular discharge than did controls although there were 10 fold higher of antibody levels in serum or tears in the immunized animals (MacDonald et al., 1984). Therefore, it is important to develop the non-toxic and simple approaches that may avoid chlamydial-immunopathological responses and focus on the protective immunity (Su and Caldwell, 1992).

2.3.2.Subunit vaccines

The advances in molecular biology, immunology and peptide biochemistry have allowed the development of subunit vaccines based on microbial recombinants, peptides and plasmid vectors. Theoretically, these vaccine candidates only contain protective Ags. Therefore, this ideal replacement for living or attenuated-vaccine could impart protective efficacy as great as whole organism but without its drawbacks, such as toxicity and the presence of infectious agents, which make them attractive vaccine candidates (Su and Caldwell, 1992; Su et al., 1990).

MOMP is a surface-exposed, integral membrane protein and primary chlamydial-serotyping antigen. The conserved proteins are interspersed four short variable VDs. The sequences of VDs vary among different MOMP (Stephens et al., 1987). Three of the four VDs have be identified as antigenic sites on the surface of the whole organism (Baehr et al., 1988; Stephens et al., 1987; Stephens et al., 1988). Zhong et al synthesized two peptides from either the VD IV of serovar B or VD I of serovar C and evaluated their immunogenicity. They found that polymerization of the peptides to form a lipid core structure significantly enhanced immunogenicity in terms of the binding ability of the induced antibodies to native antigens (Zhong et al., 1993). Su et al synthesized the overlapping peptides (25mers) representing the entire primary sequence of serovar A MOMP and screened the T cell antigenic determinants among these peptides by their abilities to stimulate the specific T cell

proliferation. They found that eight of twenty-five of these peptides could stimulate the antigen-specific T cell proliferation and therefore may function as Th cell antigens. Immunization with these peptides induced IgG response specific to native MOMP and intact organism (Su et al., 1990). This group also synthesized the chimeric peptides containing a conserved Th cell epitope(s) and serovar A specific neutralizing epitopes in VDI region. These chimeric peptides induced neutralizing Abs in mice with different MHC backgrounds (Su and Caldwell, 1992). This is important because immunogens possessing defined T cell and B cell epitopes should be recognized by multiple MHC alleles in a genetically diverse population, such as human. However they could not demonstrate that these peptides can induce protective role in mouse model. Those results suggested that the chimeric peptides containing both Th cell and B cell epitopes were potential candidates, but need further study for a trachoma vaccine.

MOMP of *C. trachomatis* has been considered to be the most likely candidate for an acellular vaccine (Fitch et al., 1993; Stephens et al., 1987). Recently Pal et al made various MOMPs and outer membrane complex (COMC) with various types of detergents including SDS, α-octyl-β-D-glucopyranoside, and Zwittergent 3-14. Immunization with these MOMPs or COMC produced significant humoral and cell-mediated immune responses, but only COMC induced a vaginal IgA response. More importantly, immunization with COMC reduced the intensity and duration of vaginal shedding and provided comparable rate of fertility compared to control animals. It was concluded that only the preparation of COMC

protected mice against chlamydial infection and infertility. Western blot analysis of these MOMP and COMC preparations revealed that there were more components in COMC, such as LPS, 60kDa crp, etc (Pal et al., 1997). These results suggested that either the conformation of MOMP epitopes or additional chlamydial antigens were important for inducing the protective response. Therefore, it is possible to develop an acellular vaccine against chlamydial infection.

2.3.3.DNA vaccines

DNA vaccination is an important and flexible vaccine strategy that delivers DNA constructs encoding specific antigen(s) into the host. These expression cassettes transfect the host cells that become the in vivo protein source for the production of antigen(s), which initiate and drive the resulting immune responses. There are many potential advantages to DNA vaccination, such as the avoidance of possible reversion of attenuated strains, improved cross strain protection, simplified combined vaccines, more effective quality control and lower costs, which make these developments important and of great potential value to public health (Chattergoon et al., 1997).

A large number of papers have described long lasting humoral and cellular immune responses following in vivo administration of plasmids encoding foreign antigens. These immune responses were acquired by either intramuscular, intradermal, or intravenous injection of naked DNA. Studies showed that direct gene transfer could be enhanced in

various tissues and cells using different approaches. One of the most surprising and important features of DNA vaccination was that naked DNA appears to be taken up and expressed by host cells with much greater efficiency than would have been predicted based on DNA transfection in tissue culture (Pardoll and Beckerleg, 1995). However, the underlying mechanisms of antigen presentation followed by humoral and/or cellular immune responses remain unclear. Studies have shown that factors influencing DNA vaccine immunogenicity are the antigen-encoded by the gene and the naked DNA element itself (Pardoll and Beckerleg, 1995; Sato et al., 1996). The immunization with naked DNA through various routes can reproducibly induce cellular and/or humoral immune responses against encoded Ags (Casares et al., 1997; Chattergoon et al., 1997; Pardoll and Beckerleg, 1995; Sato et al., 1996). Induction of cellular immune response requires Ag processing and presentation by antigen presenting cells (APCs). Recently, Casares et al presented evidence that myocytes and MHC-II negative dermal cells can be transfected following intramuscular or subcutaneous injection of DNA plasmid and these transfected cells can secret the protein encoded by the foreign gene. The secreted protein can then be processed and presented by dendritic cells. In addition, dendritic cells can directly take up the plasmid and express vaccine epitopes directly. It was concluded that dendritic cells play a vital role on the induction of immune response by DNA vaccination (Casares et al., 1997). Once the desired proteins are synthesized within the host cell, cellular immune response can be induced without the inherent risk of certain viral vectors or of reversion of certain attenuated bacteria

and viruses. The study on hepatitis B and influenza hemagglutinin DNA vaccines showed the specific immunity could be maintained for at least 1-2 years, although the reporter gene expression reached a maximum at the 6 months following the DNA injection and declined thereafter (Deck et al., 1997). DNA vaccination is a very effective means of generating polyclonal antibodies without the need for purified protein and these antibodies have the cross-strain protection against influenza infection, which has the great potential in that the identity of future drifted strains can not be predicted (Ulmer et al., 1996). Raz et al demonstrated that plasmid DNA immunization can induce Th1 responses, whereas protein immunization induces Th2 response to the same antigen (Raz, 1997). Later, the studies from various laboratories highlighted an interesting immunomodulatory property of DNA vaccination itself, which is termed as short immunostimulatory sequences (ISS). ISS shares a CpG motif, containing a central unmethylated CpG dinucleotide in a particular base context. CpG dinucleotides are present in bacterial DNA at the expected frequency of 1/16 bases, but three- to fourfold less frequent in mammalian DNA. Elimination of the CpG sequence or methylation of the cytosine abrogates the immune stimulatory activity of ISS (Sato et al., 1996).

In spite of the obvious advantages to the approach, the limited immunogenicity of many DNA vaccines has hindered their development. Therefore, it is critical to explore the approaches to specifically engineer immune responses in order to improve the first generation of DNA vaccines. Co-immunization with cytokine genes as molecular adjuvants for DNA

vaccine constructs can modulate Ag-specific immune responses. Such molecular adjuvants include immunologically relevant cytokines as well as co-stimulatory molecules. In an attempt to enhance the cellular immune response, the researchers found that coadministration of an IL-12 expression vector with DNA vaccine resulted in a dramatic increase in specific CTL responses compared with DNA vaccine alone (Kim et al., 1997). The efficacy of a DNA vaccine could also be greatly improved by simultaneous expression of IL-2. The study revealed the co-expression of IL-2 and hepatitis B envelope protein significantly enhanced cellular and humoral immune responses against infection in vivo. In terms of dosage used in experiment, the efficacy was increased at least 100-fold by co-expression of IL-2 (Chow et al., 1997). A recent study showed another novel approach that dendritic cells transfected with plasmid DNA induced protective immunity against herpes simplex virus(Manickan et al., 1997). Thus, it is practical to attempt to evoke an immune response by selective modulation of the cytokines, adjuvants, antigen presenting cells and DNA delivery system in the DNA vaccination.

The immunization of a plasmid encoding the MOMP gene could induce significant delayed-type hypersensitivity and serum specific antibodies to chlamydia MoPn biovar. More importantly the DNA vaccination partially reduced the chlamydial lung growth following the intranasal challenge. This data suggested that DNA immunization might be a new approach against human chlamydial infection (Zhang et al., 1997). It is desirable to increase the potency of these saline-formulated DNA vaccines as much as possible. The partial immune

protection provided by the MOMP DNA vaccination indicates that it is necessary to modify the existing strategies and develop new cocktail regimens as well as continue to explore new vaccine candidate(s). The DNA vaccine strategy to prevent chlamydial infection has a great potential to control the prevalent STDs, trachoma and to reduce the formation of atherosclosis if chlamydial infection is a definite cofactor for the generation of atherosclosis.

3. MATERIALS AND METHODS

3.1. Mice:

Female six- to eight-week old C57 BL/6 (H-2^b), IL-12 p40 knock out (KO) mice on C57BL background and CBA mice (H-2^k) were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were housed at the University of Manitoba animal facility in filter top cages and provided with food and water ad libitum. All animal procedures performed were approved by the Protocol Review Committee of University of Manitoba.

3.2. Growth of chlamydia strains and purification of elementary bodies (EBs):

A *Chlamydia trachomatis* murine strain designated as mouse pneumonitis (MoPn) was used in this study. Hela 229 cells {American Type Culture Collection (ATCC), Rockville, MD} were grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO, GrandIsland, NY) containing 10% FCS (Intergen Inc., Purchase, NY). The MoPn strain was grown in Hela 229 cells and EBs were purified on discontinuous gradients of RENO grafin-76 as following steps (Zhong et al., 1990):

- split Hela cells and culture in the 175 cm^2 flask (Corning Inc., Corning, NY) the day before infection
- remove the medium from a confluent monolayers
- add 5 ml of Hank's balanced salt solution (HBSS) with 30μg/ml of DEAE-dextran (Pharmacia, Dorval, Quebec) at 37^0C for 30 minutes.

- remove HBSS with DEAE-dextran solution and add 1ml of MoPn stock (for preparation of stock see below)
- roll the flask every 20 minutes during the 2 hour culture at 37^0C, make sure the solution is just sufficient to cover the Hela cell monolayer.
- discard the inoculum and add 20 ml of DMEM with 5% FCS and 1μg/ml cycloheximide.
- culture at 37^0C for 24 to 36 hours and remove the growth medium from flask
- add 30 glass beads with diameter of 2 mm in 10 ml of cold DMEM to flask
- roll cells gently off flask and rinse with 10 ml cold DMEM and collect to 50 ml polycarbonate centrifuge tube (Corning Inc.)
- sonicate cell suspension for 20 seconds at a setting of three (Microprobe, Branson Sonifier, Danbury, CT)
- centrifuge at 500*g* for 15 minutes at 4^0C and discard the pellet containing cell debris
- layer supernatant over 8 ml 35% renograffin (Squibb Canada Inc., Montreal, Canada) in HEPES buffer {0.01 M hydroxyethylpiperazine-N1-2-ethane sulfonic acid (HEPES) in 0.15M NaCl, pH 7.2} in a 50 ml ultra-clear tube (Nalge Nunc International, Rochester, NY)
- centrifuge at 43,000*g* (Beckman ultracentrifuger 16,000 rpm) for 60 minutes at 4^0C in a SW 28 rotor
- resuspend pellet containing EBs and RBs in 10 ml cold SPG (sucrose phosphate glutamic acid, pH 7.4)

- layer over a discontinuous renograffin gradient containing: 13 ml 40% (top), 8 ml 44% (middle), 5 ml 52% (bottom) renograffin in HEPES buffer
- centrifuge at 16,000 rpm for 90 minutes at 4^0C in a SW 28 rotor
- collect band at the interface of 44-52% renograffin as EBs
- wash EBs with cold SPG and spin down at 30,000g (Beckman 20 rotor) for 30 minutes at 4^0C
- resuspend pellet in SPG and aliquot as desired, store at -70^0. The same seed stock of MoPn was used throughout the study

3.3. Titration of chlamydia organisms on Hela 229 cells

The infectivity of the purified EB organisms was titrated by counting chlamydial inclusion-forming units (IFUs) on the monolayers of Hela 229 cells grown in 96 well plates (Zhong et al., 1990) as described below.

- add $4X10^4$ Hela 229 cells to each well of the 96 well plate and culture overnight to confluency
- treat monolayer with DEAE-Dextran for 20 minutes
- add 50µl serial diluted EB stock in SPG buffer for 2 hours at 37^0C to allow EBs attach to monolayer
- discard inoculum and culture in 200µl of DMEM with 5% FCS for 36 hours
- discard supernatant and fix the Hela 229 cells with 2 % paraformaldehyde { (PF), BDH

Inc., Toronto, ON, Canada } in PBS (pH 7.4) for 30 minutes

- permeabilize the fixed cells with 0.5% saponin (Sigma, St.Louis, MI) in PBS for 30 minutes
- add mouse anti-chlamydia LPS Ab supernatant (mouse IgG, kindly provided by Dr.H Caldwell from NIH) for 1 hour
- wash with PBS and probe with 1:200 diluted Goat anti-mouse IgG conjugated horseradish peroxidase (HRP) for 1 hour
- wash with PBS and add 50μl of HRP-substrate (PBS:1%4-chloro1-naphthol in methonal:hydrogen peroxide = 1000:100:1) for 10 to 30 minutes
- count stained EBs and get average in 5 fields with magnification of 20.

The infectivity of EBs was calculated based on the following equation:

Titer (IFU/ml) = average count of each field in five fields X dilution X 20

3.4.Inactivation of chlamydia organisms by UV light:

Portions of the purified EBs were inactivated by UV-light (G15T8 UV lamp, D.William Fuller Inc., Chicago, IL) irradiation at a distance of 5 cm for 1 hour at room temperature. For convenience, the number of UV-inactivated EBs (UV-EBs) used in all experiments was calculated based on the number of IFUs of the corresponding live EBs prior to UV-light treatment. UV-EBs were checked for infectivity. No chlamydial growth was detected when the UV-EBs were inoculated onto Hela monolayers at a dose of equivalent to

1X 10^8 IFUs per 1X10^6 Hela cells.

3.5.Generation of bone marrow-derived dendritic cell (BM-DC) and macrophage (BM-Mφ):

Bone marrow-derived DC and Mφ were generated by culture of bone marrow cells as described by Inaba et al (Inaba et al., 1990). Briefly, mouse bone marrow was collected from tibias and femurs. Erythrocytes were lysed by the incubation with 0.7% of ammonium chloride for 2 minutes. Lymphocytes, granulocytes, Ia-positive cells were killed with a cocktail of mAbs and followed by the lysis of rabbit complement (Sigma, St Louis, MI). The mAbs were GK1.5 anti-CD4, 2.43 anti-CD8, RA3-3A1/6.1 anti-B220/CD45R and 25-9-17s11 anti-Ia (TIB 207, 210, 146 and HB26 respectively; ATCC, Rockville, MD) and anti-GR1 (PharMingen, San Diego, CA). The remaining bone marrow cells, 1×10^6 cells/ml, were plated in 6-well culture plate in RPMI-1640 medium (GIBCO) supplemented with 10% FCS, 5mM 2-Me and 5 ng/ml mouse rGM-CSF (PharMingen). At day 3 of culture, floating cells were gently removed and fresh medium was added. Thereafter, cells were refed with same concentration of rGM-CSF every 2 days. At day 6 or 8 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were collected, designated as DCs, and replated for additional day, and further used for pulsing or analysis. After harvesting DCs, the culture dishes were vigorously washed to remove non-adherent or loosely adherent cells and then incubated with 1×trypsin EDTA (GIBCO) at 37^{0}C for 30 min. The residual adherent cells were then dislodged by policeman after incubation with 1×trypsin-EDTA solution and

collected as bone marrow derived-Mφ. The DC fraction contained 74-90% DCs based on the culture characteristics, cell morphology and MHC class II, CD11C phenotype. More than 90% cells in the Mφ fraction were $CD11b^+$ and $CD11C^-$ based on the FACS analysis.

3.6.Generation of peritoneal macrophage

The peritoneal cavities of naive mice were lavaged with 5ml of cold steriled-PBS. The lavage was withdrawn with 27-gauge needle slowly and gently, further washed twice with RPMI-1640 medium and centrifuged by 1,000*g* at 4^0C. The pellet was resuspended and cultured at 2×10^5 per well in 200μl of RPMI-1640 medium with 10%FCS, 50mM 2-Me and 12μg/ml Gentamycin (Sigma). After 2 hours, the floating cells were removed. The adherent cells were cultured in the above medium as unstimulated peritoneal Mφ. The cell viability was 100% by trypan blue exclusion test.

3.7.Flow Cytometry analysis:

Cell surface staining was performed by directly staining with PE- or/and FITC-conjugated Ab or matched Isotype Igs. The following Abs or matched isotype Igs were purchased from PharMingen: anti-mouse $I\text{-}A^k$-PE (Catlog#6005A) or $I\text{-}A^b$-PE (6045A), hamster IgG anti-mouse CD11C-PE (9714D) and rat IgG2a anti-mouse CD11C-FITC (9704D) or CD86 (B7.2)-FITC (9274D), rat IgG2b anti-mouse CD11b-FITC (1714D), hamster IgG anti-mouse CD80 (B7.1)-FITC (9604D), rat anti-mouse CD45R/B220-FITC

(1124D) or Gr-1-FITC (1214A), mouse IgG2a-PE (3025), hamster IgG-FITC (11144L), rat IgG2b-FITC (11034), rat IgG2a-FITC (11024C) and mouse IgG2a-FITC (3024C). Anti-mouse CD4-FITC (CL012F) and anti-mouse CD8-FITC (CL169F) were purchased from Cedarlane Lab. Limited (Hornby, ON, Canada). The staining procedures were described as following:

- Collect 3 to 5 $X10^5$ cells into tube (Falcon Becton Dickinson Labware, Lincoln Park, NJ) and centrifuge at 1,000*g* at 4^0C
- Cell pellet is resuspended and incubated with PE- and/or FITC-conjugated Ab or isotype Ig (1μg Ab or isotype Ig per $1X10^6$ cells) in 30 μl of PBS containing 1% BSA (Sigma) for 30 to 60 minutes on ice, avoid light
- Directly add 0.4 ml PBS with 10μl of 100μg/ml of propidium iodide (PI) to tube and ready for analysis
- Stained cells were analyzed on FACScan™ cytometry with CellQuest™ software (Becton Dickinson, Mountain View, CA). PI staining and scatter gating were used to exclude dead cells and debris.

3.8. In vitro pulse of dendritic cells and macrophages with inactivated chlamydia organisms

BM-DCs or Mφs were cultured with UV-inactivated chlamydial organisms in RPMI-1640 medium with 5% FCS at the indicated ratio in each experiment for 18 hours when DCs can efficiently stimulate antigen specific T cell proliferation and produce IL-12 in vitro. Cell

density in culture was 1×10^6/ml. After 18 hours pulsing, those pulsed cells were harvested and washed twice with PBS as Ag-pulsed DCs or Mφs for experiments.

3.9.Mouse immunization protocol

On day 6 or 8 of culture, DCs were collected and recultured in $1X10^6$/ml of same fresh medium containing UV-EB for 18 hours. These pulsed cells were washed twice with PBS. $1X10^5$ cells per mouse in 50μl of sterile PBS were injected subcutaneously into the hind footpads. As a control, the same number of Mφs pulsed with UV-EB (MOI =1), or 1 X 10^5 IFU of UV-EB or PBS were injected into mice of each group respectively. Trypan blue exclusion and propidium iodide staining analyzed by FACS showed the viability of Ag-pulsed DCs or Mφs prior to footpad injection was above 90%.

3.10. Protection assay

Ten days after the immunization, mice were intranasally challenged by $2X10^4$ IFU MoPn in 50μl of SPG buffer. Body weight was measured daily and mortality was monitored. Mice were sacrificed following 10 days of challenging. The lungs were aseptically removed and homogenized with a cell grinder in 4ml cold SPG buffer. Tissue suspensions were centrifuged at 500*g* for 10 min at 4^0C to remove coarse tissue debris and were frozen at -80^0C until tested. For quantitation of MoPn, Hela 229 monolayers were inoculated with serial diluted lung tissue supernatants for 2 hours on a rock platform. The plates were washed and

cultured with DMEM containing 5%FCS, 1.5μg/ml cycloheximide, 100μg/ml of vancomycin and 12μg/ml of gentamycin (Sigma) for 36 hours. The cell monolayer was then fixed with 2% paraformaldehyde and followed by permeabilization of 0.5% saponin. The cells having chlamydial inclusions were detected by the staining with anti-chlamydial LPS Ab followed by rabbit anti-mouse IgG-horseradish peroxidase. The number of inclusions was counted in five fields under a microscope. The chlamydial infectivity in each lung was further calculated based on the number of inclusions in one field, dilution titers and magnification of microscope as described detail in section 3.3 (Yang et al., 1996).

3.11. Determination of MoPn specific-Ab levels

ELISA was used for the determination of MoPn-specific Ab response as described by Yang (Yang et al., 1996). In brief, a solid phase of MoPn particles was used to capture anti-MoPn specific Abs in the sera at day 10 following the immunization but prior to challenge. A 96 well ELISA plate (Corning Costar) was coated with 50μl of $1X10^5$ IFU MoPn EBs in SPG buffer at 4^0C overnight. Non-specific binding sites were blocked with 1% BSA in PBS for 2 hours at room temperature. Fivefold serial dilutions of serum starting at 1:100 were then added to the appropriate wells and incubated for 4 hours at room temperature. Furthermore, MoPn-specific IgG1 and IgG2a responses were detected by adding biotin-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc., Birmingham, AL) or goat anti-mouse IgG2a (Caltag, Burlingaame, CA) at dilution of 1:500. After overnight incubation,

1:5000 alkalinephosphatase (AKP)-conjugated streptavidin (Sigma, St Louis, MO) was added to plates. Plates were developed by the addition of p-nitro phenyl phosphate as the AKP substrate (Sigma) and read at 405 nm. The plates were extensively washed with PBS containing 0.05% Tween-20 solution between steps.

3.12. Purification of T lymphocyte

In this method, cells are negatively selected by their inabilities to bind antibody coated on plates. The procedure is described as below:

- Dilute the donkey anti-mouse IgM (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) to 50μg/ml in PBS
- Add 5 ml diluted Ab to a non-tissue culture petri-dish (Fisher Scientific Co., Pittsburgh, PA) and incubate overnight on rolling shaker at 4^0C
- Remove unbound antibody from the dish, wash with PBS twice, block the plate with 5 ml 2% FCS in PBS at room temperature for 30 minutes
- Resuspend spleen cell pellet in 1×10^7/ ml of RPMI and apply to dish for 2 hours at 37^0C
- Collect non-adherent cells by gently pipetting the fluid from the dish after gently rocking the dish
- Wash with RPMI and resuspend as T cell-enriched population
- The purity of the population is determined by FACS with anti-CD45R/B220, CD4 and CD8.

The strategy allowed us to obtain populations >80% T cells from spleen suspension.

3.13. Measurement of chlamydia specific T cells in vitro and in vivo

The spleens from the immunized mice were removed for in vitro stimulation. After purification of T cells as indicated in section 3.12, the proliferation of chlamydia-specific T cells was measured by the IL-2 production in the culture supernatant as following procedures. The IL-2 level in the culture supernatant reflected the magnitude of Ag-specific T cell proliferation from the immunized mice.

3.13.1.In vitro stimulation of in vivo sensitized T cells with UV-EB pulsed DC

To determine whether the DC was capable of presenting chlamydia-derived antigens to sensitized T cells, DC were incubated with UV-EB and T cells from mice recovered from chlamydia infection. T cell activation was monitored by measuring IL-2 production in the culture supernatant. T cells from mice immunized with HEL (hen egg lysozyme) was used as control. The IL-2 level can help determining whether UV-EB pulsed DC can specifically activate the corresponding T cells.

- immunize the mouse with intraperitoneal injection of 0.2 ml of 10^7 IFU MoPn EBs in PBS emulsified 1:1 in complete Freund's adjuvant {(CFA) Difco Laboratories, Inc., Detroit, MI}
- boost mouse with intranasal challenge of 10^5IFU viable MoPn EBs after 2 weeks of the first immunization

- sacrifice the mouse at the day 12 to 14 following the boost and collect the spleen from the mouse
- purify the T cell as described in section 3.12
- culture $1X10^5$ DCs with 10^6 IFU of UV-EBs for 18 h and followed by adding $5X10^5$ purified T cells in 200μl of RPMI-5
- culture for 1, 3, 5 days and harvest supernatant for IL-2 assay as described in section 3.14

The immunization protocol of HEL was similar to these of MoPn. Simply, the mice were i.p. injected with 240μl of 133 mM HEL (Sigma) mixed with 240μl of CFA following the sonication. The mice were boosted in a similar fashion 2 weeks later. After another 3 weeks, the mice were sacrificed and splenic T cells were collected and assayed as HEL-specific T cells similar to those of MoPn-specific T cells as described in section 3.12.

3.13.2. The immunization by UV-EB pulsed DC sensitized T cells in vivo

To determine whether immunization with UV-EB pulsed DC can induce specific immune response in vivo, we measured the supernatant IL-2 level in the immunized spleen cells in the presence of UV-EB in vitro as following.

- pulse the DCs with UV-EBs for 18 hours as described in section 3.8.
- immunize the mouse with footpad injection of $1X10^5$ ex vivo pulsed DCs (MOI= 1)
- collect spleen at day 10 following the immunization and suspend as spleen cells as described in section 3.12

- seed $5X10^5$ spleen cells with $1X10^6$ IFU UV-EBs in 200µl of RPMI-10 for culture
- harvest supernatants at various days of culture for IL-2, IL-4 and INF-γ assay by ELISA

3.14. Cytokine determination

Mice were sacrificed 10 days postimmunization. Spleen cell suspensions were washed and resuspended in RPMI-1640 medium containing 10% FCS. Cells ($7.5X10^6$/ml) were cultured in 24 well-plates for 24, 48 and 96 h. Supernatants were assayed for IL-2, IL-4, INF-γ, and p40 chain of IL-12 heterodimer by a sandwich ELISA as per the PharMingen protocol. The capture Abs were R4-6A2, JES6-1A12, BVD4-1D11 and C15.6, the detection Abs were XMG1.2, JES6-5H4, BVD6-24G2 and C17.8 for INF-γ, IL-2, IL-4, IL-12p40/p70 ELISA assays respectively. Briefly, 96 well ELISA plates (Fisher) were coated with capture Ab in 0.1 M $NaHCO_3$ (pH 8.2) overnight at 4°C. Non-specific binding sites were blocked overnight with 1% BSA in PBS. Samples or standards were added to the plates and incubated overnight at 4^0C. The plates were incubated with detection Ab followed by HRP conjugated-streptavidin and substrate p-nitro phenyl phosphate, read at 405. The plates were washed extensively with 0.05% Tween-20 in PBS between steps. Recombinant mouse cytokines (IFN-γ, IL-2, IL-4, IL-12) were used as standards. All Abs and recombinant cytokines were purchased from PharMingen.

3.15. Statistics

The data were expressed as X ± SEM and Student′ s *t* test was used for analysis of statistical significance (*p* value).

4. RESULTS

4.1.BM-derived DCs but not Mφs pulsed ex vivo with inactivated chlamydial organisms induced protection against chlamydia infection

4.1.1.DC pulsed with inactivated chlamydial organisms can stimulate specific T cell response in vitro

Since Inaba et al (Inaba et al., 1992) developed a method to generate relatively pure and large quantities of DCs, DCs have been successfully used to induce antigen specific immune responses and protective immunity against various cancers and infectious diseases (Ahuja et al., 1998). DC-based immunotherapy requires pure and large quantity of DCs, therefore we initially made efforts to optimize culture condition.

At day 3 or 4 of bone marrow culture, there were cellular aggregates attached to a layer of adherent cells. Some of cells in the aggregates had the veil- or sheet-like proceeds of dendritic cells. At day 5 or 7 of culture, we harvested the aggregates and replated. After another day of culture, these adherent cells came off the plate surface and many typical dendritic cells were floating in the culture medium. Flow cytometry analysis showed 74 to 90% of these floating cells designated as DC-enriched cell population was MHC class II+ CD11C+ (Fig 1 D, E and G). Less than 8% of these floating cells showed Gr-1+ of granulocyte marker (Fig 1H). About 89% or 36% of these DC-enriched cells expressed B7.1 or B7.1 molecules respectively (Fig.1E and G). The optimal yield was obtained when the aggregates were harvested on day 6 and then replated overnight.

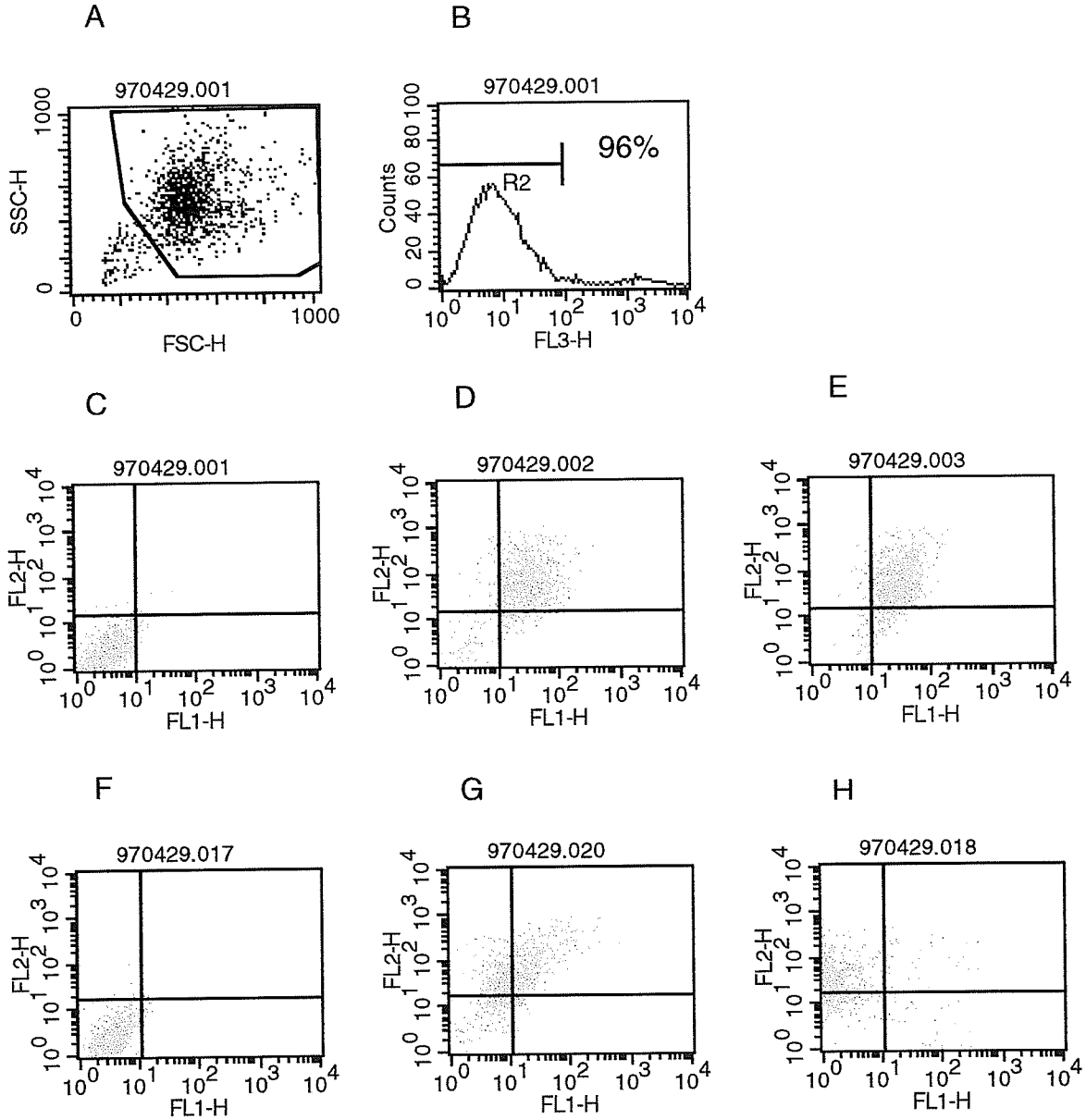

A
970429.001
SSC-H
FSC-H
0
1000
B
970429.001
96%
R2
Counts
FL3-H
C
970429.001
D
970429.002
E
970429.003
F
970429.017
G
970429.020
H
970429.018
FL2-H
FL1-H

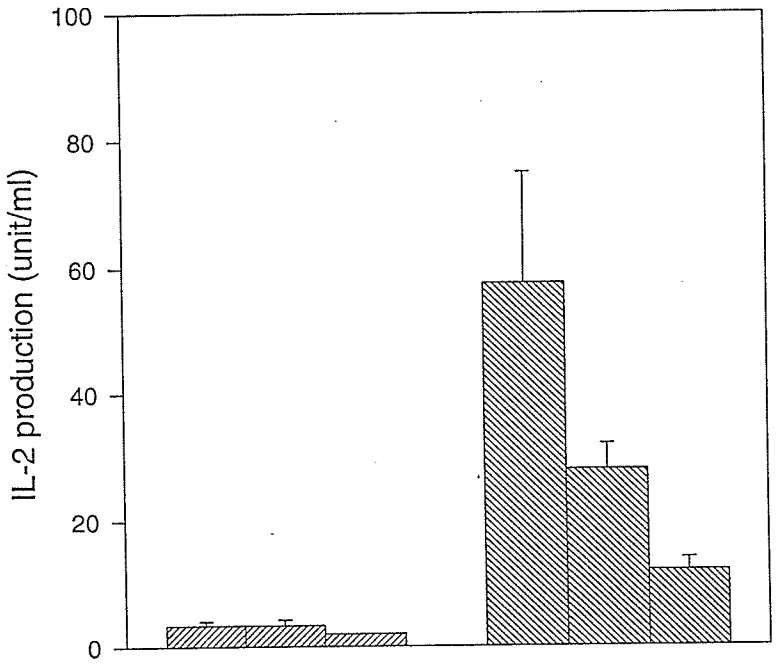
Figure 1. The phenotypes of DC-enriched cells from bone marrow cell culture. Mouse bone marrow cells, after the deletion of Ia+ cells, B cells, CD4+ cells and CD8+ cells, were cultured in the presence of 5ng/ml GM-CSF. After 6 days of culturing, DC-enriched cells from aggregates were harvested and replated for another day. The DCs were then harvested for staining with conjugated mAb or isotype Ig as described in Materials and Methods. Quadrants were set using appropriate irrelevant control Ig. Ten thousand cells in R1 gate were analyzed. A. histogram of DC-enriched cells; B. PI staining of DC-enriched cells and 96% PI^- or viable cells; C. mouse IgG2a-PE and hamster IgG-FITC staining as control for D and E; D. anti-A^k-PE and anti-CD11C-FITC staining; E. anti-A^k-PE and anti-CD80-FITC staining; F. mouse IgG2a-PE and rat IgG2a-FITC staining as control for G to H, G. anti-A^k-PE and anti-CD86-FITC staining; H. anti-A^k-PE and anti-Gr 1-FITC staining. The number of the quadrants of B to H represented the percentage of positive cells in each quadrant. The data represents one of four experiments with similar results.

T cells recognize the complexes of antigenic peptide and MHC molecules on the surface of different types of APCs. DCs are the potent professional APC that can be used to present exogenous Ags to stimulate Ag-specific T cell proliferation in vitro. We observed the efficacy of the MoPn-specific T cell proliferation stimulated by the inactivated chlamydia organisms pulsed DCs in vitro. The response of HEL-specific T cell to the same pulsed DCs were used as control. The T cell proliferation was assessed by the supernatant IL-2 level determined by ELISA.

As shown in Fig.2 there was significant higher level of IL-2 production in wells containing UV-EB pulsed DC and MoPn-specific T cells while no obvious IL-2 production in wells containing UV-EB pulsed DCs plus HEL-specific T cells, indicating that IL-2 production is Ag-specific. T cell-enriched population itself did not produce significant amount of IL-2 in the presence of UV-EB (MOI=10). DCs alone did not stimulate the non-specific IL-2 production from MoPn-specific T cells in the absence of Ag of UV-EB. The results indicate that UV-EB pulsed DCs can stimulate Ag-specific T cell proliferation in vitro.

4.1.2. The development of DC- and Mφ-based immunization

DCs are potent professional APC that play a central role in the induction of T cell mediated immunity in vivo. It is well documented that ex vivo Ag pulsed DCs are effective inducers of tumor-specific protective immunity. These DC-based immune therapies have not been extensively studied in the field of infectious agents. Since DC pulsed inactivated EB can



DC	+	-	+	+	+	+
UV-EB (moi)	-	4	10	10	1	0.1
MoPn-T cells	+	+	-	+	+	+
HEL- T cells	-	-	+	-	-	-

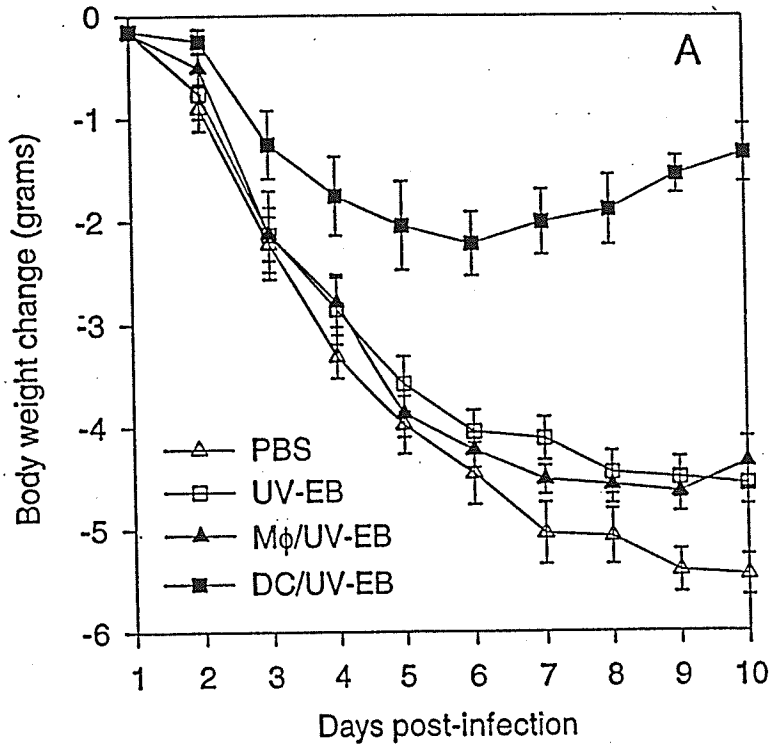
FIGURE 2. Stimulation of Ag-specific T cell proliferation in vitro by UV-EB pulsed DCs. Bone marrow-derived DCs from naive mice were pulsed with UV-EB (moi=1) for 18 hours and cultured with Ag specific T cells recovered from chlamydia organism intranasally challenged mice. After 3 days of culturing, supernatants were harvested for the determination of IL-2 levels by ELISA as described in Materials and Methods. Supernatants from either the same culture except T cells from HEL immunized mouse or the same culture except without T cells were used as negative controls. The data is expressed as mean ± SEM and the representative of three experiments with similar results.

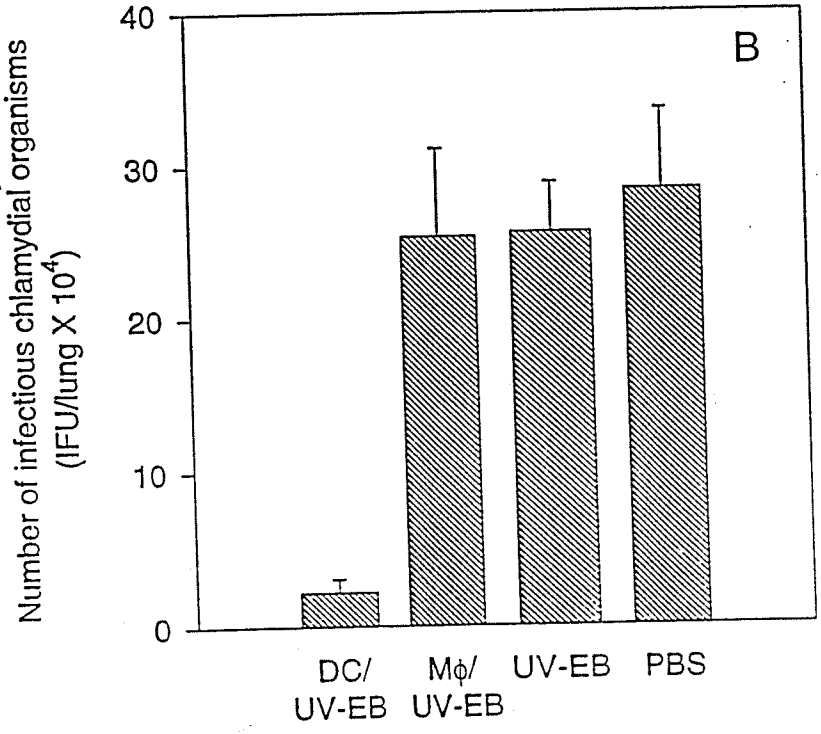
stimulate the MoPn-specifc T cell proliferation in vitro, we further evaluated the ability of the antigen-pulsed DCs to induce protective immune responses against chlamydial infection in a mouse lung infection model. The protection was evaluated by monitoring mouse body weight loss and measuring the infectious chlamydial organism recovery from mouse lung tissues. Mouse body weight reduction reflects the systemic toxicity of intranasal infection of MoPn (Yang et al., 1996).

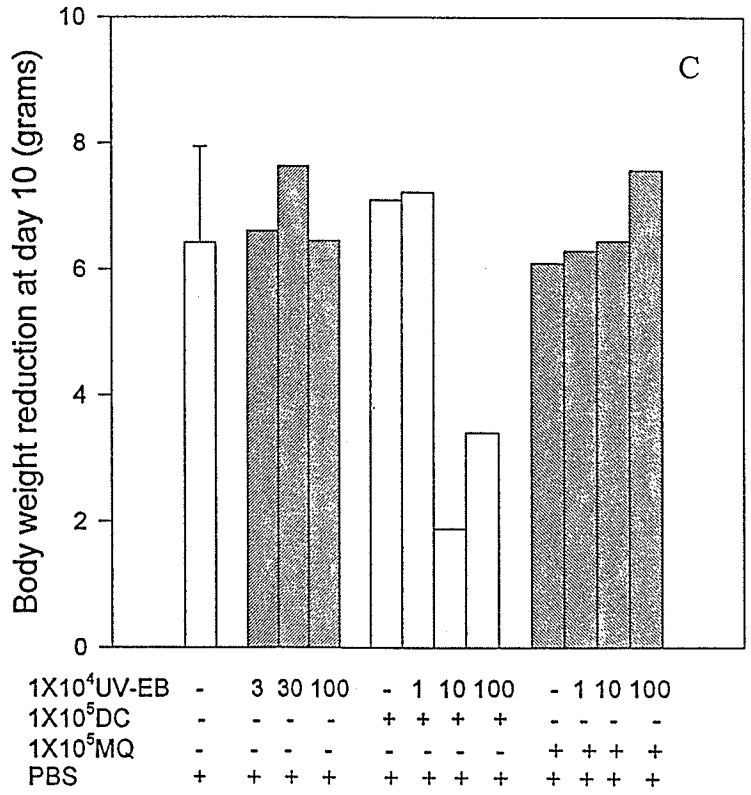
In initial experiments, we found that immunization with $4X10^5$ BM-derived DC induced a non-specific protection following the intranasal challenge of $1.5X10^4$ IFU of MoPn EB, regardless of DC pulsed or non-pulsed with inactivated chlamydial organisms (Fig.3A and B). Careful titration experiments showed that footpad injection of $1X10^5$ DCs pulsed ex vivo inactivated chlamydia organisms could induce protection while same amount of unpulsed DC could not (Fig 3A and B).

To obtain the maximal protective efficacy of Ag-pulsed DC immunization, we defined the optimal conditions where Ag-pulsed DCs can most efficiently induce immune response and protection against chlamydia infection. We found that DC can efficiently stimulate the antigen-specific T cell response following 18 hours pulsing in vitro as measured by IL-2 production in supernatant (Fig.2). Based on weight reduction and lung chlamydia growth, UV-EB pulsed DC at MOI of 1 or 10 was determined to be the most appropriate (Fig.3C and D).

To exclude the possibility that the protection may be induced by UV-EB adherent to


A
Body weight change (grams)
0
-1
-2
-3
-4
-5
-6
PBS
UV-EB
Mϕ/UV-EB
DC/UV-EB
1
2
3
4
5
6
7
8
9
10
Days post-infection


B
Number of infectious chlamydial organisms
(IFU/lung X 10⁴)
40
30
20
10
0
DC/
UV-EB
Mϕ/
UV-EB
UV-EB
PBS


C
Body weight reduction at day 10 (grams)
10
8
6
4
2
0
1X10⁴UV-EB - 3 30 100 - 1 10 100 - 1 10 100
1X10⁵DC - - - - + + + + - - - -
1X10⁵MQ - - - - - - - - + + + +
PBS + + + + + + + + + + + +

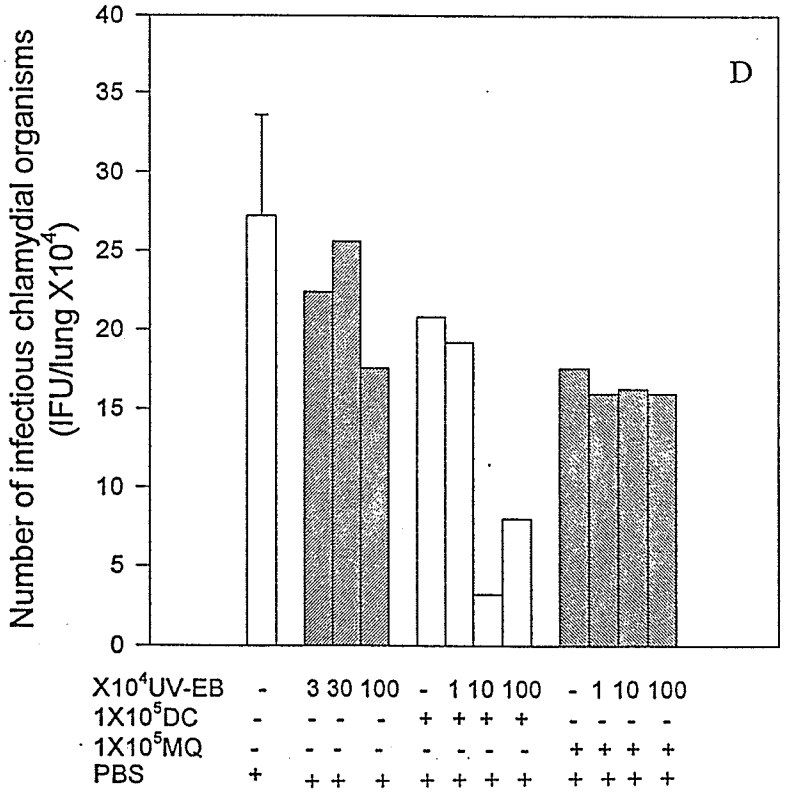

D
Number of infectious chlamydial organisms (IFU/lung X10⁴)
40
35
30
25
20
15
10
5
0
X10⁴UV-EB - 3 30 100 - 1 10 100 - 1 10 100
1X10⁵DC - - - - + + + + - - - -
1X10⁵MQ - - - - - - - - + + + +
PBS + + + + + + + + + + + +

FIGURE 3. Optimization of immunization with UV-EB pulsed DCs. Mice were immunized by footpad injection with PBS or $1X10^5$DCs, $4X10^5$DCs pulsed with or without UV-EB (MOI= 1). Ten days later, mice were intranasally challenged by $1.5X10^4$ IFU MoPn EB. The body weight change was measured daily (A) and the mice were sacrificed at day 10 following the challenge. The lung tissues were analyzed for enumerating infectious chlamydial organisms (B) as described in Materials and Methods.

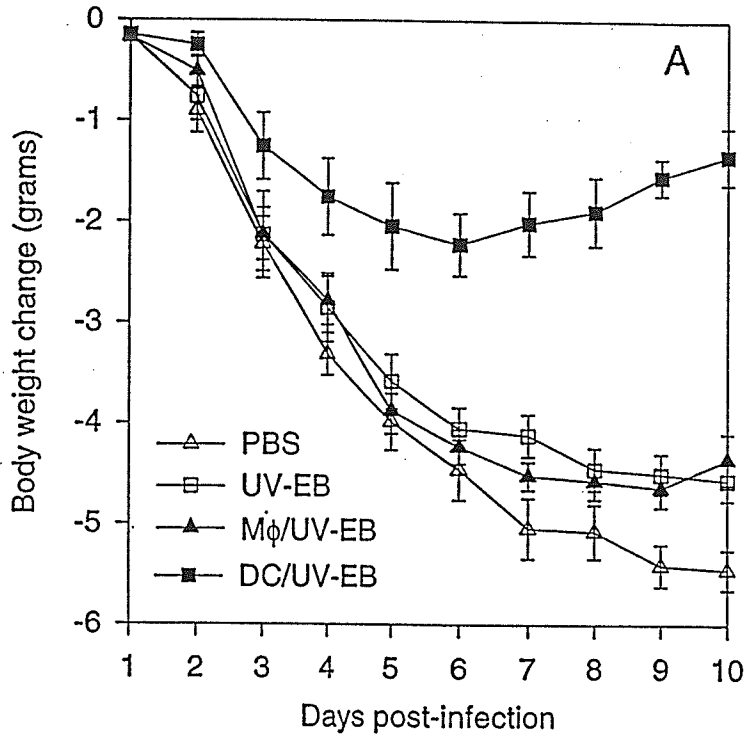
Similarly, the mice were immunized by a footpad injection with PBS, various amount of UV inactivated chlamydial organisms or $1X10^5$DCs or Mφ pulsed with or without UV-EB. These mice were intranasal challenged with $2X10^4$ MoPn EB after 10 days of immunization. The body weight change was measured and body weights at day 10 were compared to those measured prior to challenge as shown in C. All mice were sacrificed at day 10 following challenge. Lung tissues were isolated and analyzed for chlamydial titers (D).

DCs following footpad injection, we measured the effect of various amount of UV-EB induced immune responses on chlamydia infection. Fig.3C and D showed that the immunization with UV-EB alone did not induce any protection against chlamydia infection. The amount of UV-EB used for immunization was comparable to those used in DC pulsing culture.

4.1.3. Bone marrow-DCs but not Mφ pulsed ex vivo with inactivated chlamydial organisms induced protection against chlamydia infection in vivo

We further observed the dynamic change of mouse body weight following infection and found that mice immunized with DCs pulsed with UV-EBs displayed significantly less body weight reduction comparing to other treatment groups (Fig.4A). The DC/UV-EB immunized mice only experienced a minimal body weight loss and started to regain body weight on day 7 after the chlamydia challenge infection. One mouse died from the PBS treatment group on day 7. Since mouse body weight reduction has been used to evaluate the systemic toxicity following a chlamydial intranasal challenge [Yang, 1996 #134]. This observation suggests that immunization with the DC/UV-EBs greatly improved the overall resistance of the animals to the toxicity of the chlamydial challenge infection while immunization with the Mφ/UV-EBs failed to do so.

To evaluate whether this overall resistance improvement was due to the decreased chlamydial infection as a result of the DC/UV-EB immunization, we further quantitated the infectious chlamydia organisms recovered from mouse lung tissues. As shown in Fig.4B, mice


A
Body weight change (grams)
0
-1
-2
-3
-4
-5
-6
PBS
UV-EB
Mϕ/UV-EB
DC/UV-EB
1
2
3
4
5
6
7
8
9
10
Days post-infection

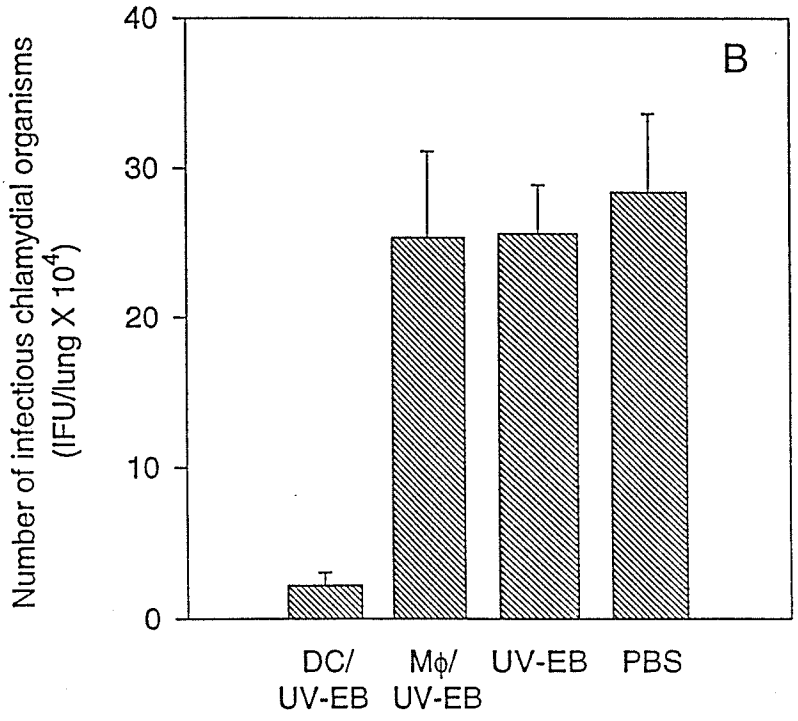

B
Number of infectious chlamydial organisms
(IFU/lung X 10^4)
40
30
20
10
0
DC/
UV-EB
Mϕ/
UV-EB
UV-EB
PBS

FIGURE 4. Protection induced by immunization with UV-inactivated MoPn pulsed DC. Seven to eight mice of each group were injected via footpads with $1X10^5$ UV-EB pulsed DC, similarly pulsed Mφ, UV-EB, or PBS alone respectively. Ten days later, mice were challenged intranasally with $2X10^4$ IFU infectious MoPn. (A) Body weight of mice was measured daily following challenge. Each point represents mean ± SEM of body weight change of 7 to 8 mice. *$p< 0.05$, UV-EB pulsed DC group v.s. other groups. (B) Mice were sacrificed at day 10 following challenge and MoPn growth in lung was quantitated in vitro as described in Materials and Methods. Data are mean±SEM of IFU X 10^4 /lung for 7 to 8 mice each group. *$p< 0.01$, UV-EB pulsed DC group v.s. other groups.

DCs following footpad injection, we measured the effect of various amount of UV-EB induced DC/UV-EBs preparation was washed before injection. More importantly, immunization with UV-EB alone was not protective (Fig.3C, D and 4A, B). The DC/UV-EB immunization induced protection may not be caused by the non-specific cellular particle effect either since Mφ similarly pulsed with the UV-EB did not induce any protection (Fig.3C, D and 4A, B). These observations suggested that the DCs but not Mφ pulsed ex vivo with inactivated chlamydial organisms induced a protective response against intranasal chlamydial infection.

4.2.DCs pulsed ex vivo with inactivated chlamydial organisms induced Th1 dominated response in vivo

It has been demonstrated that Th1 dominated immune responses play a significant role in the development of protection against chlamydia infection. To further explore the mechanisms of immune protection against chlamydia lung infection induced by UV-EB pulsed DC immunization, we evaluated the phenotypes of immune responses induced by UV-EB pulsed DC injection. The Th1 and Th2 phenotypes can be assessed by the measurements of cytokine profiles and IgG isotype patterns. We then determined the chlamydial Ag-specific IgG subclass distribution in sera and cytokine profiles from spleen cells of mice immunized with UV-EB pulsed DC, but prior to infection.

4.2.1.DC but not Mϕ pulsed ex vivo with inactivated chlamydial organisms induced a Th1

cell dominated Ig isotype responses in vivo

Mice were injected F.P. with UV-EB pulsed DCs, UV-EB pulsed Mφ, UV-EB alone or PBS alone respectively. Ten days later, the blood was collected and Ag-specific IgG2a and IgG1 levels were measured using a modified ELISA. As shown in Fig.5, no specific responses were detected in mice injected with PBS alone and a minimal IgG2a production in mice injected with UV-EB alone or UV-EB pulsed Mφ. Either UV-EB pulsed DC or Mφ induced Ag-specific IgG1 responses. However, immunization with UV-EB pulsed DCs induced a high IgG2a production, which is one of the features of Th1-mediated immune response.

4.2.2.DCs but not Mφs pulsed ex vivo with inactivated chlamydial organisms induced a Th1 cytokine response

We also collected the spleens from 6 mice immunized with UV-EB pulsed DC for the determination of cytokine production in response to UV-EB restimulation in vitro. We found that spleen cells from naive mice were unresponsive to UV-EB while spleen cells from mice immunized with UV-EB pulsed DCs produced a large amount of IL-2 in response to UV-EB re-stimulation in vitro (Fig.6A). These observations suggest that ex vivo Ag-pulsed DC but not Mφ can direct specific immune responses in vivo.

Since the ratio of IL-4 v.s. IFN-γ levels can be used to determine whether a given Ag-specific immune response is Th1 or Th2 dominant, we further assessed Ag-specific IFN-γ and IL-4 levels in mice immunized with UV-EB pulsed DCs. Ten days after immunization,

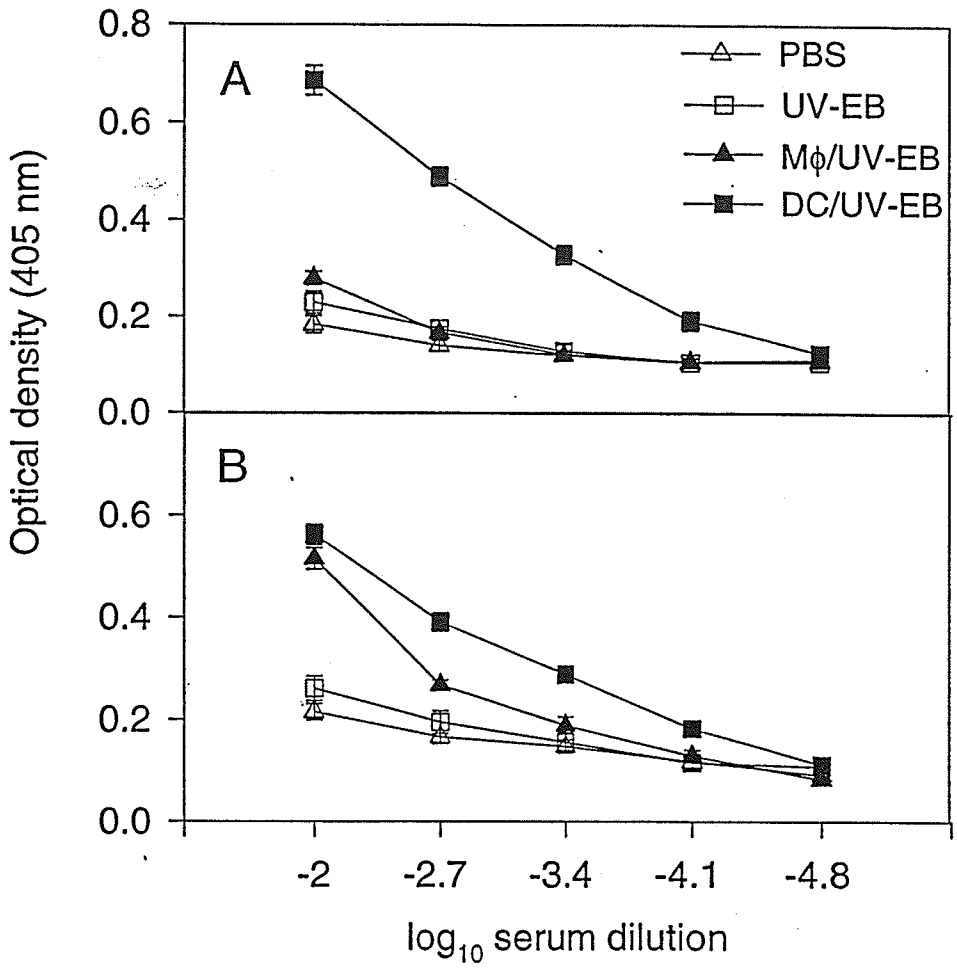
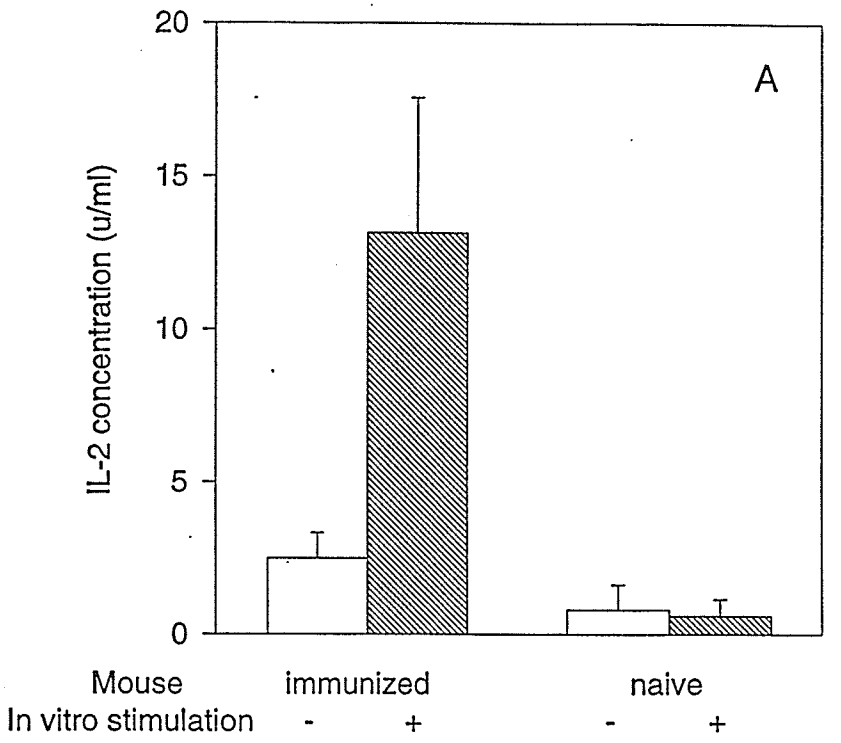


FIGURE 5. Antigen specific IgG isotypes in mice immunized with various Ag preparations. Eight to ten mice in each group were injected into footpads by 1 X 10^5 UV-inactivated MoPn pulsed DC or Mφ, UV-inactivated MoPn, or PBS respectively. Sera were collected from mice 10 days post-infection and assayed by ELISA for (A) anti-MoPn IgG2a and (B) anti-MoPn IgG1 as described in Materials and Methods. Data are expressed as mean ± SEM of OD at 405 nm.


A
IL-2 concentration (u/ml)
20
15
10
5
0
Mouse
immunized
naive
In vitro stimulation
-
+
-
+

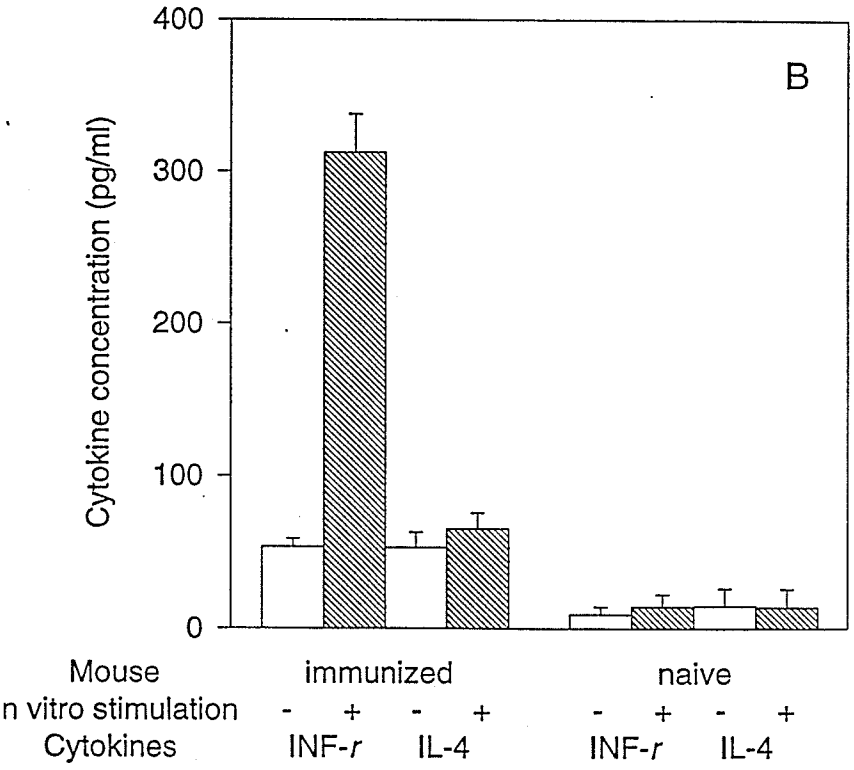
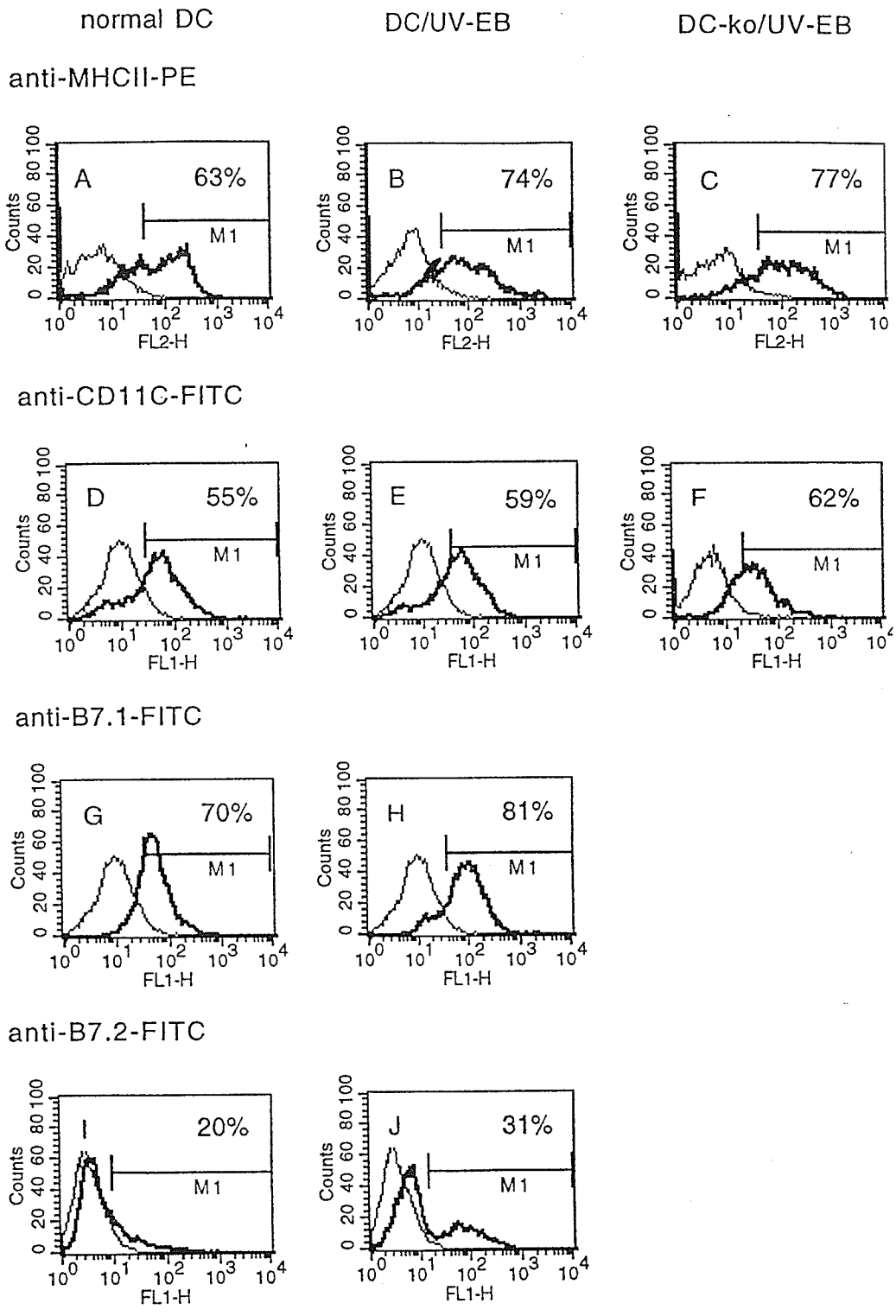

B
Cytokine concentration (pg/ml)
400
300
200
100
0
Mouse
immunized
naive
In vitro stimulation
-
+
-
+
-
+
-
+
Cytokines
INF-r
IL-4
INF-r
IL-4

FIGURE 6. Cytokine patterns analysis in mice immunized with UV-EB pulsed DCs. Six mice were injected via footpads with 1 X 10^5 UV-inactivated MoPn pulsed DC. Ten days later, mice were sacrificed and 7.5 X 10^6/ml spleen cells were cultured for 3 days in the presence or absence of UV-inactivated MoPn. The culture supernatants were measured by ELISA for (A) IL-2 and (B) IFN-γ and IL-4 production respectively. Data are representative of triplicate results of ELISA (mean $\pm$ SEM). *$p < 0.01$, UV-inactivated MoPn pulsed DC group v.s. other groups.

splenocytes were collected and incubated with or without UV-EB. INF-γ and IL-4 production were assayed using an ELISA (Fig.6B). Upon stimulation with UV-EB, splenocytes from naive mice showed little or no Ag-specific production of IFN-γ and IL-4. In contrast, splenocytes from mice immunized with UV-EB pulsed DCs showed Ag-specific production of IFN-γ, while only margin levels of IL-4 production. Combined with the observations on Ag-specific Ab isotype responses, the cytokine profile results strongly suggested that immunization with UV-EB pulsed DCs can preferentially induce a Th1-dominant response in vivo.

4.3. IL-12 production by pulsed DCs is required for the induction of optimal protection

We further explored the mechanisms that immunization with ex vivo Ag-pulsd DCs, but not Mφs, provides immune protection in vivo. It is known that Mφ expressed lower levels of MHC class II, B7.1 and B7.2 molecules compared to those on DCs. We similarly found that UV-EB pulsed Mφ displayed much lower levels of both MHC class II and B7 molecules than those on similarly pulsed DCs (Fig.7A and B) which may be one of the mechanisms that immunization of UV-EB pulsed Mφ can not efficiently induce the immune protection. However, it has been demonstrated that bone marrow derived-Mφ pulsed with heat-killed chlamydia, followed by fixation with paraformaldehyde, could specifically stimulate chlamydial Ag-specific T cell proliferation in vitro, suggesting that Mφ can efficiently process and present chlamydial Ags, at least, in vitro (Su and Caldwell, 1995). The


normal DC
DC/UV-EB
DC-ko/UV-EB
anti-MHCII-PE
A
63%
B
74%
C
77%
anti-CD11C-FITC
D
55%
E
59%
F
62%
anti-B7.1-FITC
G
70%
H
81%
anti-B7.2-FITC
I
20%
J
31%
M1
Counts
FL2-H
FL1-H

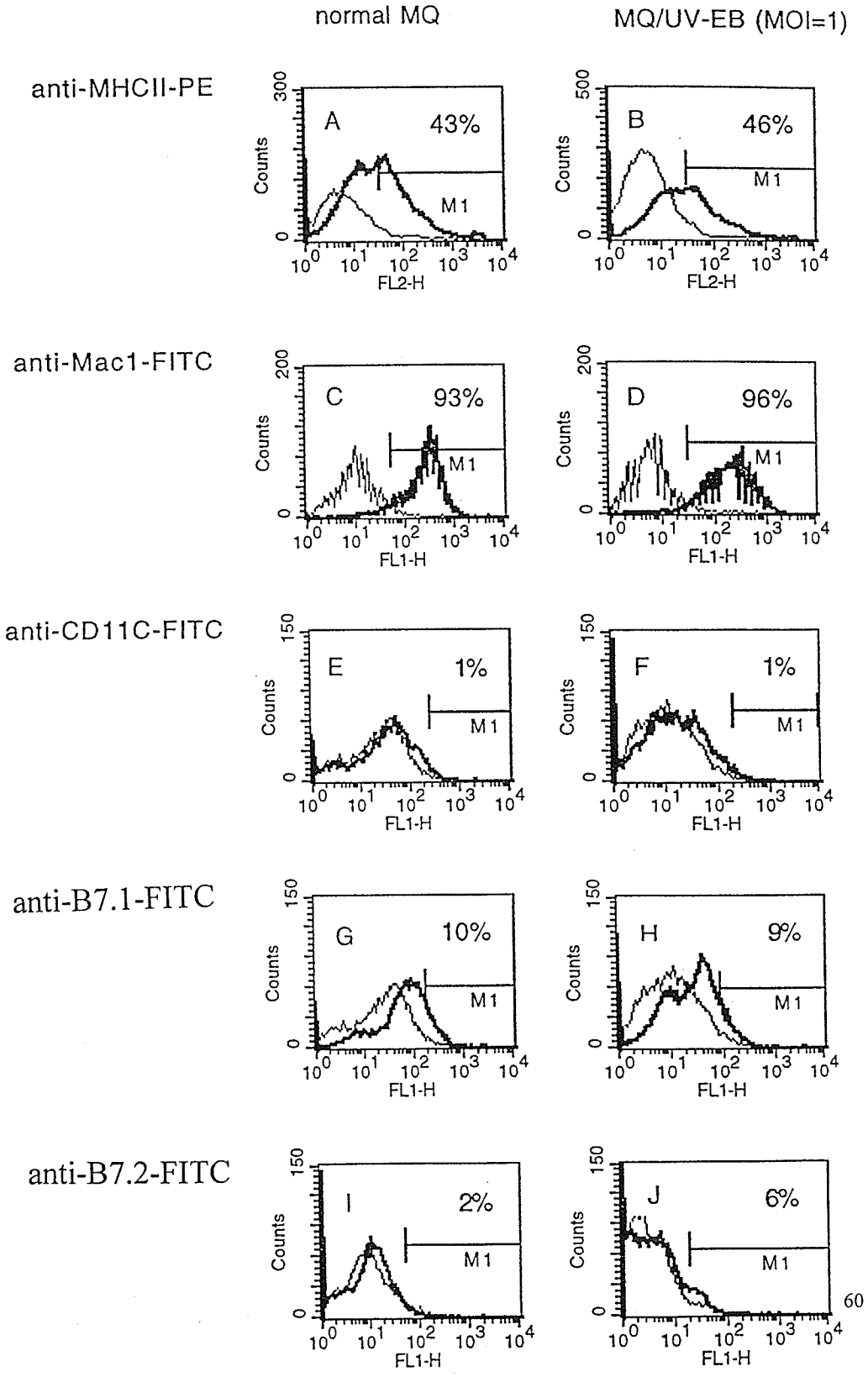
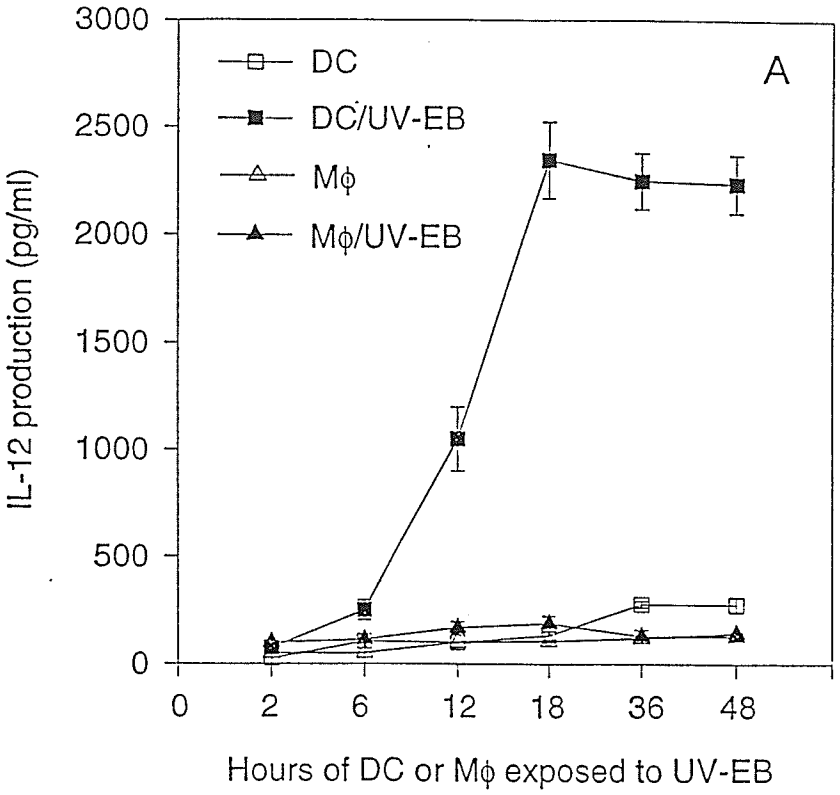

normal MQ
MQ/UV-EB (MOI=1)
anti-MHCII-PE
anti-Mac1-FITC
anti-CD11C-FITC
anti-B7.1-FITC
anti-B7.2-FITC
A
43%
B
46%
C
93%
D
96%
E
1%
F
1%
G
10%
H
9%
I
2%
J
6%
M1
Counts
FL2-H
FL1-H

FIGURE 7. FACS analysis of cell surface molecule expression on chlamydial organism pulsed DCs and Mφs. The bone marrow cells from wild or IL-12 p40 KO mice were cultured in the presence of 5 ng/ml of GM-CSF. DC or Mφ-enriched cells were collected at day 6 and replated with UV-EB (MOI: 1) for additional 18 hours. Cells were collected and stained by anti-I-A^b-PE, anti-CD11C-FITC, anti-B7.1-FITC or anti-B7.2-FITC respectively as described in Materials and Methods. The percentage of cells with certain surface marker positive (dark curve) was determined based on matched isotype control (light curve) and shown above each plot. A. surface markers of DCs, B. surface markers of Mφs.

selective differentiation of naive CD4+ T cells into Th1 and Th2 cells is established during the initial priming of these cells and is influenced by factors such as cytokines and co-stimulation molecules. It has been documented that the presence of IL-12 and IFN-γ during the activation drives the differentiation of naive CD4 T cells into Th1 cells, whereas the presence of IL-4 promotes differentiating into Th2 cells. Since IL-12 is mainly produced by antigen-presenting cells and can be present during initial contact between APC and naive T cells, IL-12 production by APC may be critical for determining the phenotypes of activated T cells. We then measured IL-12 production by APCs pulsed with UV-EB.

4.3.1. DCs produced a large amount of IL-12 upon the pulsing of inactivated chlamydial organisms

We compared the ability of DC or Mφ to produce IL-12 following UV-EB stimulation. Figure 8A showed that DC produced a high level of IL-12 following pulsing with UV-EB. The peak production time appeared to be 18 hours after pulsing. To assess the duration of IL-12 production, DCs were washed after 18 hours pulsing of antigen. Some of these washed DCs were injected into footpads for immunization, while the rest of the washed DCs were replated for another 1 to 6 days culture. At various time points, supernatants were sampled for IL-12 production and at the same time, DCs were washed and continue to culture in the same but fresh medium until the next time point. We found that the pulsed DCs can continue produce a large amount of IL-12 even in the absence of additional Ags for 6 days


A
DC
DC/UV-EB
Mϕ
Mϕ/UV-EB
IL-12 production (pg/ml)
3000
2500
2000
1500
1000
500
0
0
2
6
12
18
36
48
Hours of DC or Mϕ exposed to UV-EB

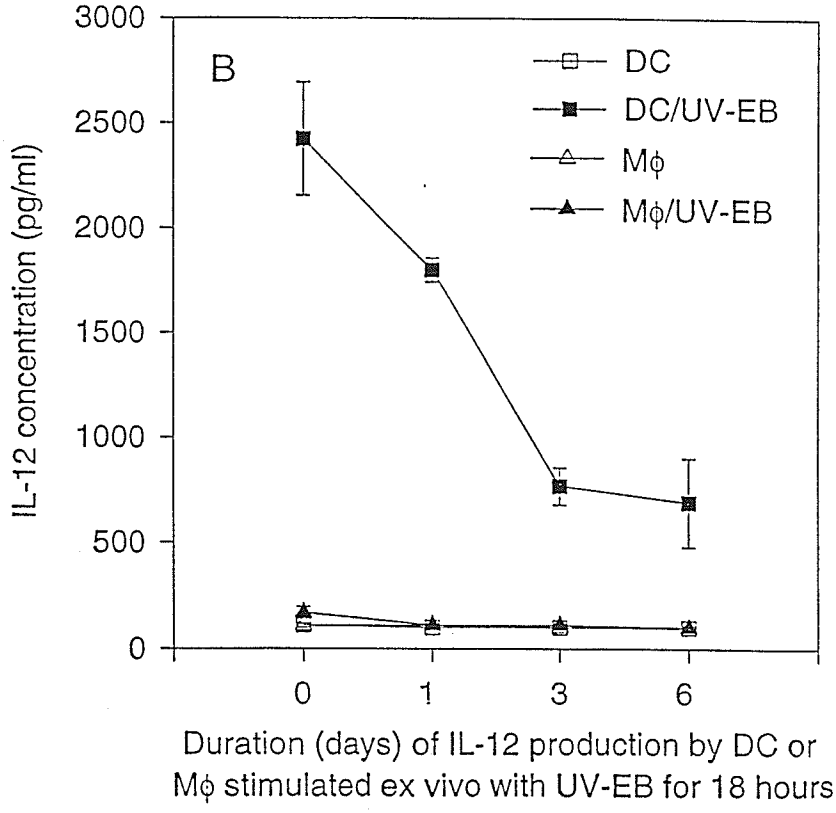

B
DC
DC/UV-EB
Mϕ
Mϕ/UV-EB
IL-12 concentration (pg/ml)
3000
2500
2000
1500
1000
500
0
0
1
3
6
Duration (days) of IL-12 production by DC or
Mϕ stimulated ex vivo with UV-EB for 18 hours

FIGURE 8. Production of IL-12 by DCs upon exposure to UV-inactivated MoPn. (A) 1 X 10^5 DCs or Mφs were cultured with UV-EB at an MOI of 1. Supernatants at the indicated time points were collected for IL-12 level determination with an ELISA. (B) 1 X 10^5 DCs or Mφs were cultured in the presence or absence of UV-EB for 18 hours (designated as day 0). Then these pulsed cells were washed away free Ags and replated with fresh medium for 1 day (day 1). After harvesting supernatant at day 1, fresh medium was added and culture for another 2 days (day 3). Day 6 culture supernatants were harvested at 3rd day following the change of day 3 culture medium. Data are expressed as mean ± SEM from 3 to 6 separate experiments with similar results.

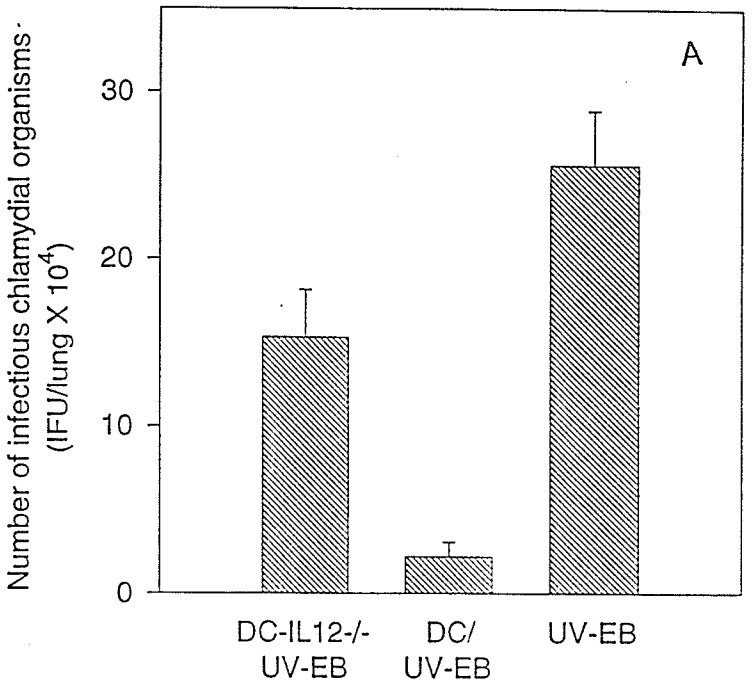
(Fig.8B).

4.3.2. Neither peritoneal nor BM-derived Mφ produced detectable level of IL-12 upon the similar stimulation of inactivated chlamydial organisms

In contrast, freshly isolated resident peritoneal Mφ did not produce any detectable IL 12, and bone marrow derived-Mφ produced only marginal levels of IL-12 following stimulation with UV-EB (Fig.8A and B). The low level of IL-12 in bone marrow derived Mφ culture could be produced by contaminating DCs since we can not exclude the possibility of DC in Mφ enriched population. FACS data showed that these Mφ-enriched populations were more than 90% of CD11b+ (Fig.7B panel C and D) and less than 1% of CD11C+ (Fig.7B panel E and F).

4.3.3. DCs from mice with IL-12 deficiency failed to provoke significant protection against chlamydial lung infection

We have demonstrated that DC but not Mφ pulsed with UV-EB can produce high levels of IL-12 which correlates with the ability of DCs to induce a Th1-dominant response and a strong protection against chlamydia infection. However, we have not demonstrated whether IL-12 production by the UV-EB pulsed DCs is necessary for the induced protection. To further explore the importance of DC derived IL-12 in the protective immunity, we performed the following studies using DCs from IL-12 p40 KO mice. Indeed, DCs with IL-


A
Number of infectious chlamydial organisms
(IFU/lung X 10^4)
30
20
10
0
DC-IL12-/-
UV-EB
DC/
UV-EB
UV-EB

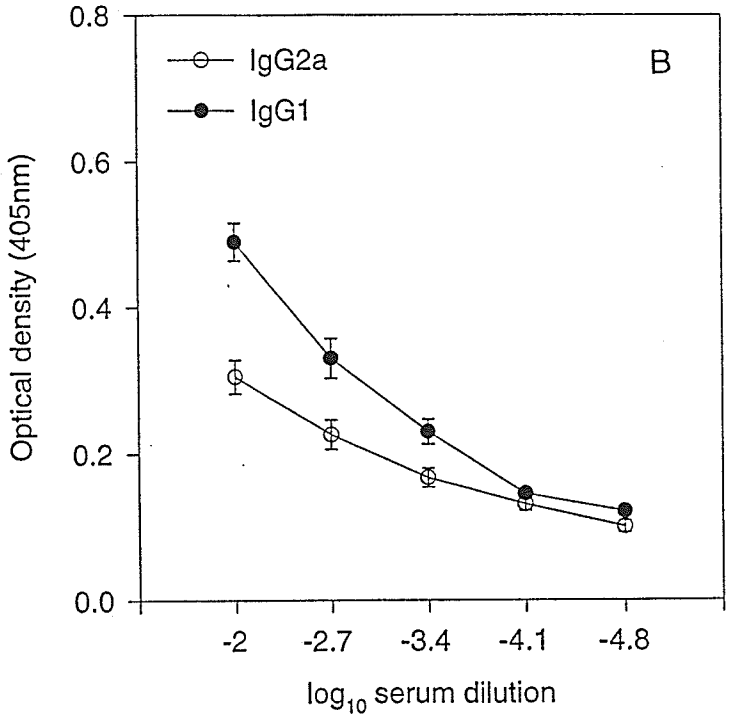

B
IgG2a
IgG1
Optical density (405nm)
0.8
0.6
0.4
0.2
0.0
-2
-2.7
-3.4
-4.1
-4.8
$\log_{10}$ serum dilution

Figure 9. Comparison of immune responses induced with IL-12 p40-/- DCs versus wild type DCs. (A) 7 to 8 mice were immunized by 1 X 10^5 UV-inactivated MoPn pulsed DC from wild or IL-12 p40 KO mice, or UV-inactivated MoPn alone. The mice were challenged intranasally with 2 X 10^4 IFU live MoPn at day 10 following the immunization. All the mice were sacrificed at day 10 after challenge infection. The lung tissues were analyzed for infectious chlamydial titers. Each point represents the mean ± SEM of IFU X 10^4/lung for 7 to 8 mice. (B) Sera were collected at day 10 following the immunization and anti-MoPn specific IgG2a and IgG1 Abs were assayed by ELISA. Data are expressed as mean ± SEM of OD at 405 nm from 7 mice.

12 p40-/- did not produce IL-12 regardless of the stimulation with UV-EB. Immunization with the UV-EB pulsed DC with IL-12 p40-/- induced a much lower level of protection compared to the immunization with the pulsed wide type DC (Fig.9A). Fig.9B showed that immunization with UV-EB pulsed DC with IL-12 p40-/- failed to induce Ag-specific IgG2a response in vivo. FACS results showed that there was no significant difference in MHC class II and CD11C levels between IL-12 p40-/- DC and wild type DC (Fig.7A). Nevertheless, the above observations can allow us to conclude that DC derived IL-12 plays an important role in the development of protective immunity against chlamydia infection.

5. DISCUSSION

5.1.The induction of protection against chlamydia lung infection by the immunization with Ag-pulsed DCs but not Ag-pulsed Mφ

The naive CD4+ Th cells recognize Ag specific peptide-MHC complexes on the APC via the TCR complex. In addition to TCR-mediated signals, costimulatory signals are provided by the ligation of B7 proteins on APC with CD28 molecules on the T cells. The combination of these two signals induces T cell clonal expansion. After 3 to 5 days of specific T cell proliferation, naive CD4+ T cells differentiate into armed effector Th1 or Th2 cells which mediate the specific immune responses and may provide immune protection against microbial infection or tumor. The rationale for using DCs to develop anti-infective strategies is based on the unique biologic features of DCs that make them ideally suited for taking up, processing, and presenting Ags to naive CD4+ T cells, further inducing specific immunity (Caux et al., 1995; Hsu et al., 1996; Inaba et al., 1993; Paglia et al., 1996; Zitvogel et al., 1996). Our preliminary study showed that DC in vitro can efficiently present chlamydia Ags to sensitized T cells from the mice following the resolution of chlamydia infection. These Ag pulsed DCs can greatly stimulate the Ag-specific T cell proliferation (Fig.6A) and increase their INF-γ production (Fig.6B). Therefore it is reasonable to hypothesize that chlamydial Ag pulsed DCs may induce a protective immune response in vivo.

We evaluated the role of the DC-based immunotherapy in a murine lung infection model. We demonstrated that a single immunization at the footpads with DCs pulsed ex vivo with inactivated chlamydiae were capable of conferring significant levels of protection against chlamydial respiratory infection. In contrast, the immunization of Mφ pulsed with the same Ag was not capable of inducing protection in this model. Using a similar approach with model antigens, Inaba et al (Inaba et al., 1990) shown that DCs pulsed ex vivo with various protein antigens can efficiently prime antigen specific T cell responses in draining lymph node while similarly pulsed other APCs such as peritoneal macrophages failed to do so. Liu et al collected DC-enriched cells from the thoracic duct lymph following the oral administration of Ag as pulsed APC. These DCs could prime politeal lymph node CD4+ T cells after footpad injection while the same source of DCs but fixed with glutaraldehyde or killed by dry ice, or live B cells from same rats did not which suggests that DCs can efficiently capture Ags in vivo (Liu and MacPherson, 1993). These results supported our observation that DCs have the unique ability to prime naive T cells in vivo. This approach has since been successfully used to evaluate the roles of DCs in inducing protective immunity against various tumors and microbial infections (Ludewig et al., 1998; Ossevoort et al., 1995; Schuler and Steinman, 1997). During the preparation of this thesis, Su et al (Su et al., 1998) reported that intravenous injection of DCs pulsed ex vivo with nonviable chlamydiae induced a profound protection against chlamydial genital tract infection, which is consistent with our finding made in the lung infection model. All of these results suggest that DCs are not only

able to present chlamydial antigens in vitro but also able to prime naive T cells in vivo. It is interesting to note that in the present study, a footpad injection of antigen-pulsed DCs induced a protective response against respiratory infection. This can be explained by a previous finding that footpad injection of antigen-pulsed DCs induced antigen-specific T cells not only in the immediate draining lymph node but also in brachial lymph node (Inaba et al., 1990). These observations suggest that a local immunization with DCs pulsed ex vivo with a defined antigen can induce a systemic protective response, which may be useful for developing the DC-based vaccines since in many cases, the infection sites may not be the sites convenient for delivering vaccines.

5.2. DCs pulsed ex vivo with inactivated chlamydial organisms induced a Th1 cell-mediated immunity

Two subsets of effector Th cells have been defined on the basis of their distinct cytokine secretion patterns and immune modulatory effects. Th1 cells secret INF-γ, TNF-α and IL-12, which are required for cell-mediated immunity. INF-γ activates Mφs and enhances Ig isotype switching to IgG2a, a hallmark of Th1 immunity. Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which mediate B cell activation and differentiation, further induce humoral immunity (Finkelman et al., 1990).

It is known that a Th1-like cell dominant response is usually beneficial in controlling many intracellular infections. The intracellular chlamydial infection is no exception. Studies

with gene knockout mice demonstrated that an MHC class II-restricted response (CD4 T cell function) was most critical for controlling chlamydial infection, while MHC class I-restricted response (CD8 T cell function) only made limited contribution to the control of mucosal chlamydia infection (Williams et al., 1997). Passive transfer of a chlamydia specific T cell clone that secretes Th1 type of cytokines offered protection against chlamydial challenge infection in the recipient mice (Igietseme et al., 1993). Following the chlamydial infection in the genital tract, Ag-specific lymphocytes were present in the genital mucosa. Most of the lymphocytes were IFN-γ secreting cells rather than IL-4 secreting cells, which indicated the Th1-like response occurred in the local infected tissue in vivo. The administration of exogenous IFN-γ into mice enhanced mouse resistance to chlamydial challenge infection (Zhong et al., 1989) while mice with IFN-γ knockout (Cotter et al., 1997) or treated with an anti-IFN-γ neutralizing antibody (Zhong et al., 1989) displayed more severe and disseminated chlamydial infection. In addition to IFN-γ, another potent Th1 promoting cytokine IL-12, was found to be important in limiting chlamydial infection. Neutralization of IL-12 was associated with an apparent reduction of infiltration of the CD4 T cells in the local infection site and prolonged shedding of high levels of organisms, while neutralization of IL-4 had undetectable effects on host immunity or chlamydia clearance (Perry et al., 1997). Finally, mouse strains that respond to chlamydial infection with preferential production of IFN-γ displayed significantly less severe infection than the strains with preferential production of IL-10, a Th2 phenotype promoting cytokine (Wyrick et al., 1999; Yang et al., 1996). All of

these studies demonstrated that Th1-mediated immunity plays a dominant role on the induction of the protective immune responses against chlamydia infection.

The present study demonstrated that Th1 immune responses can be efficiently induced by the immunization with inactivated EB pulsed DCs rather than similarly pulsed Mφs. The DC/UV-EB immunization preferentially induced a Th1 cell dominant response and conferred protection while the Mφ/UV-EB immunization failed to induce a Th1 response and did not provide protection against chlamydial infection. There was a correlation between a Th1 cell dominant response and protection against chlamydial infection.

5.3. DC-derived IL-12 upon the pulsing was required for the induction of protection against chlamydia lung infection

It has been illustrated that specialized bone marrow derived APC normally are required to initiate all T cell dependent immune responses (Steinman, 1991; Timares et al., 1998). We developed the procedure to immunize the mice with Ag-pulsed DCs or Mφs, but the responses were quite different. It is known that bone marrow derived Mφs can efficiently process the non-viable chlamydia Ags and, after fixation with paraformaldehyde, induce the Ag specific T cell proliferation in vitro (Su and Caldwell, 1995).

Why did the DC but not Mφ-based immunization induced a Th1 cell dominant responses and further provided protection in vivo? It is known that IL-12 is very efficient in directing Th1 cell development from Th0 precursors (Lamont and Adorini, 1996). Therefore,

the availability of sufficient IL-12 during the initial interactions between APCs and CD4+ T cells may determine the functional phenotypes of the involved T cells. Sousa et al have recently shown that DCs but not unprimed Mφs can produce IL-12 in a CD40 ligand-independent manner upon stimulation with microbial components such as LPS in the early stage, which may allow the DCs to direct the development of a Th1 phenotype from the Th0 precursors while the DCs presenting microbial antigens (Sousa et al., 1997). Chlamydial outer membrane contains LPS and furthermore, chlamydial organisms have been found to stimulate production of many other inflammatory cytokines. Indeed we found that ex vivo stimulation of DCs but not unprimed Mφs by inactivated chlamydial organisms can lead to a high level production of IL-12 in the culture supernatants. The present study further demonstrated that DCs exposed to the chlamydial organisms for 18 hours, followed by washing away chlamydia Ags, can continue to produce IL-12 for at least another 5 days. We therefore hypothesize that DCs pulsed ex vivo with chlamydial organisms may still be able to produce IL-12 when migrating to draining lymph node and presenting chlamydial antigens to lymph node T cells. Such direct IL-12 production may be both necessary and sufficient for promoting the involved T cells to develop towards a Th1 phenotype. Recently Ahuja (Ahuja et al., 1998) demonstrated that IL-12 transduced human DCs, following the pulsing of microbial Leishmania or histoplasma Ags, enhanced Ag specific proliferation of CD4+ T cells and inhibited their IL-4 production if compared to non-transduced or IFN-γ transduced DCs. This is consistent with our results and suggests that DC with its ability to secrete IL-12

may preferentially induce Ag-specific Th1 response but down modulate Th2 responses. Studies from cutaneous leishmania infection showed that skin-derived DCs could release IL-12 and unregulated MHC class II or CD80, CD86 surface molecule expression following infection, while Mϕs could not (von Stebut et al., 1998). It was suggested that both skin DCs and Mφs were infected sequentially in cutaneous leshmaniasis and played distinct roles in the immune responses initiated by *Leishmania*. Mφ may capture organisms and kill them in the early stage of infection but probably does not actively involve in T cell priming, while skin DC seems to initiate Th1 immune responses (Ahuja et al., 1998).

Consistent with the above observations, we have demonstrated that antigen-pulsed DCs from IL-12 KO mice failed to induce a Th1-dominant response and induced a much lower level of protection against infection, although there were no significant differences in the expression of MHC class II and CD11C between UV-EB pulsed wild type DC and IL-12p40-/- DC. Therefore, the local source of IL-12 during the interaction of Ag pulsed-APCs and naive T cells may be the key component in the induction of efficient immune response.

Since IL-12 can also effectively stimulate NK cells and macrophages to secrete IFN-γ (Lamont and Adorini, 1996), it is possible that the direct IFN-γ production stimulated by IL-12 secreted from DC/UV-EBs contributed to the protection against chlamydial infection in the DC/UV-EB-immunized mice. This innate immunity occurs sooner and may be important in controlling infection during the early stage of the live pathogen infections. It was demonstrated that acute infection with *Toxoplasma gondii* stimulated the early IFN-γ

synthesis, which enhanced host resistance to the infection (Gazzinelli et al., 1994). However, both the present study and the study by Su et al (Su et al., 1998) suggest that a DC-primed Th1 response rather than the direct early IFN-γ production was likely to be responsible for the observed protection, since mice were challenged 10 to 14 days after the final DC immunization. From the point of view of vaccination, induction of an antigen specific Th1 cell-dominant response is more important since only the antigen-specific response can produce a long-lasting and recallable protection.

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