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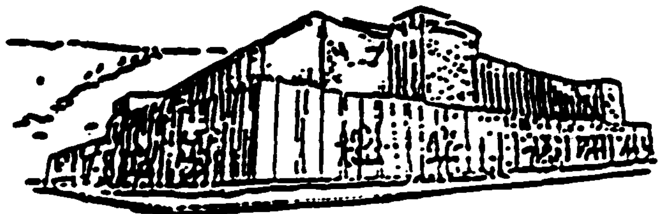

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IMMUNIZATION AGAINST *CHLAMYDIA TRACHOMATIS* INFECTION OF THE GENITAL TRACT WITH ANTIGEN PULSED DENDRITIC CELLS

by

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B.S. Florida State University, 1995

presented in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
Department of Pharmaceutical Sciences
The University of Montana
December 2000

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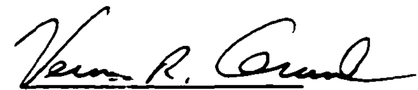

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Shaw, Jennifer Hughes, Ph.D. December, 2000 Pharmaceutical Sciences

Immunization Against Chlamydia trachomatis Infection of the Genital Tract with Antigen Pulsed Dendritic Cells

VRG HC
Research Advisors: Vernon R. Grund, Ph.D. and Harlan D. Caldwell, Ph.D.

Chlamydia trachomatis is an obligate intracellular bacterium that infects epithelial cells of the ocular and genital mucosa. *C. trachomatis* infection of the genital mucosa are a leading cause of sexually transmitted disease (STD). Chlamydial genital infection in women results in infertility and is a major risk factor for the transmission of HIV. A vaccine to prevent chlamydial infection is needed but has proven difficult to produce using conventional vaccination approaches. Potent protective immunity to vaginal re-challenge in a murine model of chlamydial genital infection has only been achieved by infection or by immunization with dendritic cells (DC) pulsed *ex vivo* with whole inactivated organisms. This immunity correlates with a chlamydial specific CD4 Th1 immune response although the precise effector mechanisms of this immunity are not known. To better understand the potent immunizing properties of chlamydial pulsed DC the expression of DC surface antigens (Ags), chemokines, chemokine receptors and cytokines were investigated. Inactivated chlamydial organisms were found to have the capacity to stimulate upregulation of Ag presentation and costimulatory molecules, chemokines and chemokine receptors important for cellular migration, and cytokines that mediate a Th1 immune response. Because of the potent anti-chlamydial immunizing properties of DC, it was hypothesized that DC could be a powerful vehicle for the delivery of individual chlamydial Ags that are thought to be targets for more conventional vaccine approaches. The chlamydial major outer membrane protein (MOMP) was investigated as a target Ag. The results demonstrate that recombinant MOMP (rMOMP) pulsed DC secrete large amounts of IL-12 and stimulate infection sensitized CD4+ T cells to proliferate and secrete high levels of IFN-γ. Therefore, the rMOMP pulsed DC were primed to drive a MOMP-specific Th1 immune response. Interestingly, adoptive immunization with rMOMP pulsed DC generated a Th2 biased immune response and immunized mice were not protected following infectious challenge. It was concluded that the delivery of MOMP to DC for immunization is a plausible approach to determine the protective capabilities of MOMP; however, the plasticity of DC and the nature of the immunogen will require additional measures to stimulate the appropriate type of immune response.

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GLOSSARY OF ABBREVIATIONS

Ag = antigen
APC = antigen presenting cell
BCA = B cell attracting chemokine
BCR = B cell receptor
CC, CXC = chemokine
CD = cellular differentiation marker
CD3 = cellular differentiation marker 3 (T cell receptor complex)
CD11b = cellular differentiation marker 11b (macrophage, dendritic cell marker)
CD19 = cellular differentiation marker 19 (B cell marker)
CD28 = cellular differentiation marker 28 (costimulation)
CD40 = cellular differentiation marker 40 (costimulation)
CD86 = cellular differentiation marker 86 (B72 molecule, costimulation)
CCR = chemokine receptor (CCR1 –8)
CXCR = chemokine receptor (CXCR1-5)
D = *C. trachomatis* serovar D
DC = dendritic cell
DMEM = Dulbecco's Modified Eagles Medium
EB = elementary body
ELISA = enzyme linked immunosorbent assay
FACS = fluorescence activated cell sorter
GM-CSF = granulocyte/macrophage-colony stimulating factor
HK EB = heat killed elementary bodies
I-A^b = major histocompatibility complex Class II murine gene loci
IFN-γ = interferon-gamma (Th1 cytokine)
IFU = infection forming units
Ig = immunoglobulin
IgG1 = immunoglobulin gamma-isotype 1 (Th2 antibody)
IgG2a = immunoglobulin gamma-isotype 2a (Th1 antibody)
IL-1 = interleukin-1
IL-1RA = interleukin-1 receptor antagonist
IL-2 = interleukin-2 (Th1 cytokine)
IL-4 = interleukin-4 (Th2 cytokine)
IL-6 = interleukin-6
IL-10 = interleukin-10 (Th2 cytokine)
IL-12 = interlekin-12 (Th1 cytokine)
IL-12p35 = interleukin-12 subunit 35kD
IL-12p40 = interleukin-12 subunit 40kD
IMDM = Iscove's Modified Dulbecco's Medium
iNOS = inducible nitric oxide synthase
i.p. = intra-peritoneal
IP-10 = interferon induced protein-10
i.v. = intravenous

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KO = gene knock out
LB = latex beads
LC = Langerhans cells (skin DC)
LN = lymph nodes
Ltn = lymphotactin
LPS = lipopolysaccharide (endotoxin)
MAb = monoclonal antibody
MBP = maltose binding protein
MCP = monocyte chemotactic protein
M-CSF = macrophage-colony stimulating factor
μH-/- = μ heavy chain knock out (B cell knock out mice)
MHC = major histocompatibility complex (antigen presentation)
MHC I β_2 -/- = major histocompatibility complex β_2 knock out (blocks CD8 expansion)
MHC II β -/- = major histocompatibility complex β chain knock out (blocks CD4 expansion)
MIF = macrophage inhibitory factor
MIP = macrophage inflammatory protein
MOMP = major outer membrane protein
MoPn = mouse pneumonitis (mouse adopted strain of *C. trachomatis*)
NK cell = natural killer cell
Nonprofessional APC = non-professional antigen presenting cells (i.e., epithelial cells)
Professional APC = professional antigen presenting cell (i.e., DC, macophages)
RANTES = regulated on activation, normal T cell expressed and secreted
RB = reticulate body
rMOMP = recombinant MOMP
RPA = RNase Protection Assay
s.c. = subcutaneous
STD = sexually transmitted disease
TCA-3 = T cell activation gene
TCR = T cell receptor
TCR α -/- = T cell receptor α chain knock out (αβ T cell knock out mice)
TCR δ -/- = T cell receptor δ chain knock out (γδ T cell knock out mice)
TEM = transmission electron microscopy
Th1 response = T helper type 1 immune response (IFN-γ, IL-12, IgG2a)
Th2 response = T helper type 2 immune response (IL-4, IL-10, IgG1)
TNF-α = tumor necrosis factor-alpha (Th1 cytokine)

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Chapter 1: Background and Introduction

The genus *Chlamydia* includes four species: *C. trachomatis, C. psittaci, C. pneumoniae* and *C. pecorum* (1, 2). *C. trachomatis* and *C. pneumonia* are predominantly human pathogens while *C. psittaci* and *C. pecorum* typically infect birds and large animals. *C. trachomatis* is the leading cause of preventable blindness in developing countries and a major cause of sexually transmitted disease (STD) worldwide (3). Chlamydial infection in the genital tract of women can ascend to the upper regions causing salpingitis that potentially leads to ectopic pregnancy or tubal infertility (4-6). Other significant health problems include urethritis and epididymitis. There is a correlation between genital infection and the increased transmission of human immunodeficiency virus (HIV) indicating *C. trachomatis* as a risk factor (2). *C. pneumoniea,* also a human pathogen, causes acute respiratory diseases such as pneumonia, bronchitis and sinusitis. Recently, *C. pneumoniae* infection has been correlated with coronary heart disease (7, 8).

C. psittaci is a common pathogen to all bird species and, although rare, can infect humans who work with the organism or handle birds. Transmission occurs via inhalation of aerosolized droplets. Psittacosis has a wide range of severity from asymptomatic infection to flu-like symptoms to lethal respiratory complications (2). *C. pecorum* also rarely infects humans and mainly leads to disease in animals.

Human *C. trachomatis* infections are a major cause of STD. Various ideas for preventing and controlling STD have been generated by the medical and political

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communities. Behavioral changes have been suggested yet implementing social change is very difficult. Physicians strive for better diagnosis and subsequent treatment with antibiotics but unfortunately 50% of infected persons are asymptomatic thus the disease goes unrecognized and untreated (2). Research scientists striving to develop topical microbicides have demonstrated cytotoxicity to various organisms such as *Chlamydia trachomatis*, herpes simplex virus, *Neisseria gonorrhoeae* and HIV in cell culture; however, prevention of infection in animal models has yet to be achieved (9).

Although antibiotic treatment effectively eradicates chlamydial infection, the preponderance of asymptomatic infection somewhat precludes this mode of therapy for all infected persons. In addition, antibiotics do not necessarily eliminate an infection that has already had the opportunity to progress to chronic disease. Development of an alternative pharmacological tool, such as a vaccine, is an attractive avenue for control and prevention of sexually transmitted chlamydial diseases.

Life Cycle of *Chlamydia*

Chlamydia are defined as Gram-negative bacteria due to the presence of an inner cytoplasmic membrane and an outer membrane although they are devoid of the characteristic peptidoglycan layer between these membranes of the cellular envelope (2). These bacteria are obligate intracellular parasites that potentially lead to a variety of chronic diseases. *Chlamydia* are successful and complex parasites due to their unique developmental life cycle which is comprised of two metabolically and functionally

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distinct stages. The small, infectious and metabolically inactive elementary bodies (EBs) are able to survive in the extracellular environment long enough to transmit infection from one host cell to another. EBs bind to and are rapidly internalized (10) by eukaryotic cells into membrane-bound inclusions whereupon they transform during the first two hours postinfection into the larger and metabolically active form termed reticulate bodies (RBs). At 8 to 12 hours postinfection the RBs begin to increase in size and beyond 12 hours they undergo replication by binary fission within inclusions. After 18 hours, the expanded RBs differentiate into new infectious EBs leading to lysis of the host cell (between 36 and 72 hours) and release of infectious organisms to surrounding cells thereby facilitating the spread of infection (11).

Chlamydia and Host Cell Interactions

Binding and internalization of EBs is an extremely efficient process (10) and years of extensive studies suggest that chlamydial protein components enable the EBs to bind to host cells and initiate phagocytosis by typically non-phagocytic cells such as mucosal epithelial cells. Progress towards identifying such chlamydial proteins has been hindered by the fact that there are no genetic systems available for chlamydia (12, 13). Despite these challenges, the major outer membrane protein (MOMP) which makes up approximately 60 % of the chlamydial cell wall (14) has come forward as a strong candidate involved in EB and host cell interactions. MOMP has been suggested to play a vital role in adherence of chlamydia to host cells (15), therefore it is intimately involved in

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pathogenesis, and it is an immunodominant antigen recognized by the host during chlamydial infection (16).

Antisera to EBs neutralize infectivity in a serotype-specific fashion (2, 17) and MOMP is reported to be the predominant serotyping antigen (18). MOMP contains eight transmembrane (outer membrane) domains with four extracellular loops termed variable domain (VD) I through IV (19). Specific monoclonal antibodies to negatively charged VD I or II and IV block attachment of EBs to cells suggesting MOMP's involvement in electrostatic interactions necessary for the chlamydial organism to bind to the host cell. It has been shown that VD IV contains a conserved hydrophobic domain that is inaccessible to antibody on native EBs suggesting it lies within a structural pocket. Thermal disruption of this domain inhibits attachment of EBs implying that MOMP VD IV might modulate essential hydrophobic interactions with the host cell during the infection process (19).

Studies using a recombinant maltose-binding protein (MBP)-MOMP fusion show MBP-MOMP can competitively inhibit EB binding to host cells (15). Additionally, MBP-MOMP binding to cells is inhibited by heparin or heparin sulfate suggesting that MOMP binds to heparin sulfate receptors on host cells (15). However, MBP-MOMP is not internalized by the host cells. It is plausible that MOMP mediates chlamydial binding to host cells while a separate, unknown component of the EB may initiate internalization. Nonetheless, it is widely believed that MOMP plays an important role

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as a cytoadhesion and is one of possibly many surface components that initiate chlamydial pathogenesis.

C. trachomatis

The *C. trachomatis* biovariants are made up of two human pathogens, trachoma and lymphogranuloma venereum (LGV) strains, and a mouse pathogen, mouse pneumonitis (MoPn) strain (2). Trachoma strains infect human squamocolumnar epithileal cells (serovars A to K) with infection typically occurring at and localized to the genital or ocular mucosa (17). LGV is comprised of serovars L1, L2 and L3 and leads to systemic illness (17). LGV strains colonize epithelial cells but invade submucosal tissues such as lymph node cells. MoPn infects mouse mucosal cells and remains localized, therefore it is similar to trachoma biovars. The mouse model utilizing MoPn has been the most thoroughly studied and characterized of the various animal models for respiratory and reproductive tract *C. trachomatis* infections. MoPn shares 95% sequence homology with the human *C. trachomatis* serovar D (20). The mouse model for chlamydial infection and disease of the female genital tract closely parallels human genital infection (21-24). As a result of the availability of inbred strains of mice, genetically defined KO mice and numerous mouse immunological reagents, valuable knowledge has come forth regarding infection and immunity.

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Immunology of chlamydial infection

Immunity is the state of protection from infectious disease and is comprised of innate and acquired immunity. Innate immunity is inborn, nonspecific and incapable of memory responses (25). It is the first line of defense to resist infection until either infection is quickly resolved or acquired immunity develops. Innate immunity consists of four types of defensive barriers: anatomic (skin, mucosa), physiologic (temperature, pH, chemical mediators), endocytic/phagocytic (neutrophils, macrophages/monocytes, NK cells) and inflammatory barriers (vasodilation, edema, influx of phagocytes) (25). These nonspecific, innate mechanisms rarely eradicate infection alone but they are essential for orchestrating the adaptive response by recruiting lymphocytes and antigen presenting cells. An intact immune system that can both specifically recognize and selectively eliminate infection is essential for acquired immunity. Acquired immune responses, unlike innate immunity, are adaptive and have the following characteristics: antigenic specificity, diversity, immunologic memory and self/nonself recognition (25). Due to these adaptive attributes, acquired immunity is the target for development of vaccines and consists of two major components, humoral and cell-mediated immunity.

Humoral Immunity

Humoral immunity is driven by antibodies that specifically bind unprocessed antigens and possess multiple functions for controlling infection. Antibodies are immunoglobulin proteins consisting of two identical heavy chains and two identical light

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chains that recognize a specific epitope on antigen (26). Immunoglobulins are either present on the B cell membrane and/or secreted by plasma cells. Immunoglobulins (Ig) are divided into five isotypes based on their heavy chain: IgM, IgD, IgG, IgA and IgE (26). Initial antibody-mediated responses are typically of the IgM isotype which can bind multiple epitopes due to its ten antigen binding sites. IgM antibodies can destroy or opsonize target antigens by efficient complement fixation. IgM precedes production of the other isotypes that are predominantly involved in a memory response. IgD secretion is believed to induce TCR expression and augment T helper function. IgG (IgG-1,-2a,-2b,-3), the most abundant isotype in the serum, functions primarily by complement fixation. IgA antibodies are secreted into bodily fluids and capable of eliciting immunity at the mucosal surface. IgE antibodies bind receptors on basophils and mast cells leading to allergic inflammatory responses (26).

Critical events during B cell differentiation occur between the pro- and pre- B cell stages whereupon genetic rearrangement produces the IgM heavy (μH) chain allowing formation of cell-surface IgM receptors and development of the pre- B cell (27). Without the intact μH chain, IgM cannot form and B cell differentiation is halted at the pro- B cell stage (27). The essential nature of the μH chain has been exploited to study the role of antibody-mediated immunity to both primary and secondary *C. trachomatis* infections. Neonatal mice treated with anti-μ antibodies, thus suppressing B cells, were able to resolve chlamydial infection comparable to controls as well as show protection upon

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reinfection indicating that antibody-mediated immunity is not a key player in protective immunity to chlamydia (28). This type of B cell suppression is incomplete hence a small portion of B cells could theoretically function in immunity. To more thoroughly investigate the role of antibody-mediated immunity, knock out (KO) mice lacking the μ gene (μMT/μMT mice) were studied. It was observed that μ gene KO mice are also able to clear chlamydial infection comparable to wild type mice and exhibit complete protective immunity upon vaginal re-challenge (29).

Mice (μMT/μMT) that lack chlamydial-specific antibodies, however, did show an increased susceptibility to re-infection suggesting that specific antibodies at the genital tract may be involved with preventing colonization (29). Although local chlamydial antibodies such as secretory immunoglobulin A (sIgA) have been associated with decreased shedding of *Chlamydia* in the gene knockout work and other related work, they do not appear to be essential for clearance of a primary or secondary infection (30, 31). Thus sIgA appears to have a subordinate role in acquired protective immunity to genital chlamydial infection.

Cell-Mediated Immunity

T cells are the key players in cellular immunity. Unlike B cells, T cells have antigen receptors expressed on the cell surface specific for recognition of processed antigenic peptides on antigen presenting cells (25). Most T cells (~90%) have a T cell

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receptor (TCR) comprised of a disulfide-linked heterodimer consisting of an α and a β chain. These chains are considered members of the Ig superfamily and undergo genetic rearrangements of variable (V), joining (J) and diversity (D) genes to provide multiple antigenic specificities (25). A small subset of T cells (~10%) have γ and δ chains which are structurally similar to the αβ TCR; however, the γδ T cells have fewer germ-line gene elements which limit their antigen recognition repertoire (25). Although the γδ T cells are few in number, they predominate at mucosal sites such as the gut epithelium. This led to an investigation of whether the γδ T cells are involved in chlamydial infection and immunity as compared to αβ T cells. Mice with the inactivated gene for the TCR α chain do not clear chlamydial infection while KO mice for the δ chain resolved infection with similar kinetics of normal animals indicating a critical role for αβ T cells in the immune response (32).

T cells (αβ) function as either regulatory or effector cells depending upon their phenotype. CD4+ T helper (Th) cells regulate immune responses in several ways including secretion of cytokines and the activation of B cells to produce antibodies (25) (33). CD8+ cytotoxic T cells (Tc) act as effectors by killing antigen bearing cells through either perforin or Fas-mediated pathways. Some activated CD4+ Th1 cells can also exercise cytotoxic capabilities through Fas ligand/Fas receptor interactions with target cells (25).

To assess the necessary T cell phenotype to resolve and protect against chalmydial infection, monoclonal antibodies were produced to either CD4+ or CD8+ T cells. Results

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from these types of studies prove to be highly variable thus compounding any definitive conclusions (34-36). Other approaches employing adoptively transferred T cell clones to athymic mice illustrate the importance of both CD4+ and CD8+ T cells in protective immunity, however higher efficiency was observed in mice receiving transfer of CD4+ cells (34). In further investigation of a CD4+ versus a CD8+ T cell response, polyclonal CD4+ and CD8+ T cells isolated from the spleens of mice post primary infection with *C. trachomatis* were adoptively transferred to naive mice. Those administered polyclonal CD4+ T cells, but not those given CD8+ T cells, expressed protective immunity (37). Therefore, it has been demonstrated that CD4+ T helper cells play a major role in expediting protection while CD8+ cytotoxic T cells carry a minimal role.

Alternative approaches that block clonal expansion of either CD4+ or CD8+ T cells have also been utilized to characterize the T cell phenotype involved in *C. trachomatis* infection. It is well established that CD8+ cytotoxic T cells respond to antigenic peptides presented on MHC I molecules (MHC I-restricted) on antigen presenting cells (APC). Recognition of antigenic peptides leads to engagement of the TCR-antigen complex driving clonal expansion of CD8+ T cells (38, 39). Alternatively, CD4+ Th cells are activated by MHC II-presented Ags (MHC II-restricted) on APC with subsequent CD4+ T cell expansion. KO mice with inactivated gene encoding the β_2-microglobulin subunit of the MHC I molecule cannot stabilize the necessary interaction between MHC I at the APC cell surface with the antigenic peptide on the TCR of CD8+ cells and cannot expand CD8+ T cells. MHC II KO mice are produced by inactivating the

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I-A_{β} gene which is responsible for expression of the β chain, an essential component of the MHC II molecule for stabilizing the MHC II-antigen-TCR sandwich. Without this stabilized interaction, CD4+ T cells will not expand. Studies in mice lacking either properly functioning MHC I (Class I) or MHC II (Class II) revealed that resolution of and subsequent protection against chlamydial re-infection requires MHC II-restricted CD4+ T cell responses while mice lacking a CD8+ response ($\beta_2^{-/-}$) are able to clear genital infection similar to normal mice (40).

Although strong evidence exists for T helper activity, a role for CD8+ cytotoxic T cells was further examined in cytolysis or apoptosis deficient mice produced by gene targeting. Perforin is a pore-forming protein secreted by cytotoxic T cells that perforates target cell membranes and permits entrance of granzyme proteases that degrade DNA leading to apoptotic death of the target cell (25). Another mechanism for cytotoxicity is through Fas ligand (CD95L) found on activated CD8+ and CD4+ T cells (25). Fas ligand binds Fas receptors (CD95/TNFα-receptor family) on target cells transducing a signal that results in apoptotic cell death. Mice lacking genes for either perforin, Fas, Fas ligand or both perforin and Fas ligand each demonstrated complete resolution of chlamydial infection at the genital tract and protective immunity (41). These results clearly rule out any necessity of either a CD4+ or CD8+ mediated cytotoxic response. Studies that contribute to our overall understanding of the immunology of chlamydial infection are summarized in Figure 1.1.

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Cytokines

Antigen recognition by and subsequent clonal activation of CD4+ Th cells results in their expansion, producing cells that are all specific for a particular antigen-MHC II complex. These $CD4^+$ Th cells then secrete intercellular signals (proteins) termed cytokines. It is the cytokine profile of these cells that dictate bias towards a Th1 (type 1-T helper) or Th2 (type 2-T helper) response (33). A Th1 response is typically characterized by the secretion of IFN-γ, IL-2, IL-12 and TNF-α with activation of macrophages, cytotoxic T cells and IgG2a production (Figure 1.2) (33). High levels of IFN-γ, IL-12 and IgG2a are found in the murine model for *C. trachomatis* infection hence indicate a Th1 polarized response and is correlated with clearance of *C. trachomatis* and offering protection against an infectious challenge (32, 42). The secretion of IFN-γ by Th1 cells also has an inhibitory effect on the Th2 pathway by suppressing IgG1 secretion (33). Th2 cells secrete IL-3, IL-4, IL-5, IL-10 and activate B cells, eosinophils and mast cells. IL-4 and IL-10 not only activate Th2 but also inhibit the Th1 response (33). For example, IL-4 activates Th2 cells by priming B cells to undergo class switching from IgM to IgG1 and concomitantly blocks IgG2a production.

Cytokine production upon chlamydial infection has been extensively studied to further elucidate the specific T helper pathway. Secretion of IL-6, IL-10, IL-12, TNF-α and IFN-γ by splenic CD4+ T cells isolated from mice post resolution of chlamydial

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infection has been consistently reported (37, 43). Studies employing various strains of KO mice that are deficient in these cytokines have been useful in assessing the importance of individual cytokines in resolution of, and protection against, chlamydial genital infection.

IL-6 is a major immune and inflammatory mediator that targets B lymphoblasts as well as stimulates T cell proliferation. IL-6 has also been reported to regulate IgA (an antibody to many mucosal antigens) production that led to studies of the potential role of IL-6 in mucosal immunity to chlamydia. Data from IL-6 KO mice demonstrate that IL-6 is not required for production of IgA at the genital mucosa that target chlamydial antigens nor is IL-6 required for resolution of chalmydial infection (44).

IL-10 is a regulatory cytokine produced upon chlamydial infection and serves to dampen the Th1 response, while IL-12 production enhances a Th1 response (32, 33). Anti-IL-12 treatment in mice slows clearance of chlamydial infections at the genital tract suggesting that IL-12 is a key cytokine although these mice eventually clear infection (32). Production of TNF-α stimulates T cells to produce IFN-γ and drives both B cell proliferation and IgG2a production. It has been shown that the TNF-α cytotoxic pathway inhibits chlamydial growth both *in vitro* and *in vivo* (45, 46). However, TNF-α is not required in resolution of chlamydial infection (44).

IFN-γ secretion activates macrophage function and blocks cytokine dependent B cell differentiation and proliferation. IFN-γ drives a strong preference to the Th1 pathway

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by both activating Th1 cells and simultaneously inhibiting Th2 cells. IFN-γ is a key cytokine in host defense due to its inhibition of indoleamine 2,3-dioxygenase (47-49) that depletes tryptophan, an essential amino acid for chlamydial replication. However, IFN-γ KO mice surprisingly clear 99.9% of genital chlamydia by 3 weeks postinfection but develop systemic disease in several organs (32). This suggests that IFN-γ is an essential factor for preventing dissemination of chlamydial organisms and persistent infection. IFN-γ also induces nitric oxide (NO) production that provides an anti-bacterial effect via damage to cell membranes, proteins and nucleic acids by free radicals. Although inducible nitric oxide synthase (iNOS) is detected locally and systemically during chlamydial infection, studies with mice deficient in the iNOS gene show that iNOS is not needed to resolve infection (32).

Overall, the data suggest that genital infection is controlled by CD4+ Th cells in an IL-12-dependent and IFN-γ-independent manner. Despite this essential immunological information and years of scientific research towards vaccine development against *C. trachomatis*, an efficacious vaccine against genital infection has not been developed.

Development of Chlamydial Vaccines

Zhang, et al. demonstrated marginal success using DNA immunization with the *C. trachomatis* MOMP gene (*OmpA*) in a mouse lung model (50). This group reported that intramuscular immunization with the gene encoding MOMP elicited a Th1 polarized

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response and IgG2a production with significant but incomplete protection upon lung challenge with infectious MoPn (50). In contrast, it was recently reported that this vaccination strategy applied to a genital tract model evoked a somewhat weak cellular and humoral immune response and offered no protection upon genital challenge (51). The reasons underlying the differential success in the lung versus the genital tract utilizing a DNA based vaccine is yet to be understood. Do these two anatomically different sites require tissue specific immune mechanisms or different protective antigens?

The first successful vaccination against chlamydial genital tract infection in a mouse model was carried out by Su et al (52). The immunization strategy employed murine bone marrow-derived dendritic cells (DC) pulsed *ex vivo* with heat killed chlamydial EBs that were adoptively transferred to naive mice. This vaccination elicited an immune response that was nearly identical to mice that had cleared a primary infection with viable organisms and are known to be highly resistant to secondary re-challenge. The potent chlamydial-specific CD4+ immune response favored the Th1 pathway (high levels of IL-12, IFN-γ, IgG2a) and for the first time demonstrated protection against a primary genital tract challenge with MoPn (52). The observed protection was equal to that of postinfected mice. These findings demonstrate that viable organisms are not necessary to evoke anti-chlamvdial protective immunity at the genital mucosa, although delivery of killed chlamydiae alone (without using DC as a delivery system) does not elicit protection. These results strongly suggest that the DC vehicle for antigen is critical for protection against chlamydiae at the genital mucosa. This DC based vaccine approach

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was utilized by a separate lab studying a murine model for chlamydial respiratory infection and was able to successfully produce protective immunity against *C. trachomatis* in the lung (53).

Dendritic cell vaccination is early in its development and it is currently an impractical approach to prevent infectious diseases in humans. However, more conventional methods of vaccination against chlamydia have failed to offer protection at the genital mucosa, while succeeding in the lung, lead us to believe the dendritic cell is a potentially unique vehicle and instrument for development of genital immunity. The underlying molecular mechanisms that enable chlamydial pulsed DC to successfully drive potent protection at the genital tract site is not understood and has yet to be extensively characterized. Further pursuit to unravel the DC biology and the elicited immune response to the chlamydial organism or its protective antigenic components is justified.

Exploiting Dendritic Cells for Immunotherapy

Dendritic cells (DC) are sentinels of the immune system and professional antigen presenting cells (APC) that efficiently initiate an immune response upon antigenic insult (54). Proliferating DC progenitors originate in the bone marrow and are released into the bloodstream as non-proliferating DC precursors. These precursors then differentiate into immature/interstitial DC and reside in the peripheral tissues as highly efficient phagocytes (54). Immature DC are known to readily capture Ag by phagocytosis of particulate matter such as latex beads (55), bacteria , viruses (56), apoptotic bodies and necrotic

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cellular debris (57). DC capture these antigens through either macropinocytosis, Fc receptors (FcRε and FcRγ), mannose receptors, C-type lectin receptors (DEC-205) or engulfment of apoptotic bodies and necrotic cellular debris. The characteristic cytoplasmic projections extending from the DC surface are thought to play a role in both phagocytosis of particles as well as migration through the lymphatic system. The immature DC phagocytize and process antigens but are inefficient at antigen presentation. However, upon antigen uptake the DC begin to mature and migrate out of the peripheral tissues via the afferent lymphatics to the lymphoid organs whereupon they down-regulate their capacity for antigen uptake and up-regulate expression of class II (MHC II) and costimulatory molecules (B7-1, B7-2, CD40) enabling efficient antigen presentation and subsequent activation of Ag-specific T cells (54, 58). Activation of these Ag-specific T cells is carried out by the mature DC, termed interdigitating DC, which display Ag within the lymph nodes and spleen (59).

Antigens processed through the exogenous pathway typically lead to presentation of antigenic peptides onto MHC II while antigens originating in the cytosol are processed and presented onto MHC I molecules (38, 39). Presentation on MHC II, accompanied by costimulation, leads to the activation of Ag-specific T helper cells; likewise, presentation on MHC I can activate cytotoxic lymphocytes (25, 38). DC and macrophages have been shown to take up exogenous Ag and present antigenic peptides on both MHC II and MHC I which is known as 'cross priming' (60). This unique crossover capacity of both DC and macrophages is not fully understood but studies by Shen et al

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(61) demonstrate that ovalbumin protein (OVA) pulsed DC present OVA peptides onto MHC I by traveling through the following pathway: phagosome -> cytosol -> proteasome -> ER -> Golgi -> cell surface presentation -> activation of T-T hybridomas specific for OVA peptides on MHC I. From a teleological perspective, it would make sense that more arms of the immune system could be alerted by this 'cross priming' within APC, such as DC and macrophages, which play such a critical role in regulating the immune response. This unique capacity also allows presentation of peptides derived from engulfed apoptotic bodies onto MHC I for recognition by cytotoxic T lymphocytes (CTL). Although DC and macrophages have many similar capabilities, DC are set apart from other professional APC because they are (i) the only APC that can activate naïve T cells and, (ii) are able to drive T cell activation with a much higher potency (50-100X).

The ability of DC to traffic to the lymphoid organs is key to their function as initiators of the immune response, therefore it is essential to understand for optimization of their use in vaccination strategies. Inflammatory signals such as LPS or TNF-α induce DC migration to the lymphoid organs *in vivo* (58) and in chemokine gradient microchambers *in vitro* (62). Several chemokines have been shown to control DC trafficking from the periphery to the lymph nodes such as MIP-1α, MCP and RANTES (63-65). Chemokines are small proteins containing four conserved cysteines which form critical disulphide bonds. The chemokine family is categorized according to the position of these cysteines: the first two cysteines are adjacent in the "CC" chemokine subfamily

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while the cysteines are separated by one amino acid in the "CXC" chemokines (66). Responses to chemokines are mediated through seven transmembrane-spanning G protein coupled receptors reported to be on DC and various other leukocytes (66). DC have been shown *in vitro* to express the chemokine receptors CCR1, CCR2, CCR5, CCR6, CCR7, CXCR1, CXCR2, CXCR4 and C5aR (64).

DC at various stages of development have the ability to migrate throughout the body in a highly regulated and organized fashion. As DC precursors, the cells migrate out of the bone marrow and circulate through the bloodstream to the peripheral, nonlymphoid tissues. Upon reaching their destination, typically epithelial and skin surfaces, immature DC are encouraged by chemokines to stay localized to the periphery where they become "resident DC" or "interstitial DC." The predominant chemokine responsible for localizing the DC to the periphery is MIP-3α (macrophage inflammatory protein) which binds to the chemokine receptor CCR6 on immature DC (62). DC can produce their own chemokines such as MIP-3α which, along with several other pro-inflammatory mediators (ie., IL-1, TNF-α), have been suggested to recruit circulating DC out of the bloodstream upon exposure to Ag (67). Studies at the bronchial epithelium have shown a drastic accumulation of DC within one hour from the bloodstream to the site of Ag exposure (68). CCR6 is also expressed by memory T cells and γδ T cells. Both cell types have been shown to respond to MIP-3α (69) through CCR6, thereby enabling the recruitment of additional cellular players from the bloodstream to the site of infection.

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The levels of CCR6 begin to decline and/or receptor desensitization occurs as immature DC phagocytose Ag (62, 70). Concomitantly, an upregulation of CCR7 occurs which responds to the ligands 6Ckine (secondary lymphoid-tissue chemokine, SLC) and MIP-3β (62, 71). The chemokine 6Ckine is expressed along lymphatic vessels and facilitates migration through the lymphatics to the secondary lymphoid tissue (72). MIP-3β is produced within the lymph nodes at the T cell zone area and not only attracts maturing DC but also naïve T cells and B cells (73). Collectively, the decreased responsiveness to MIP-3α allows resident DC to escape the peripheral tissues, meanwhile the increased responsiveness to 6Ckine and MIP-3β attracts the DC through the lymphatics to the nodes and spleen (Fig. 1.3). Once in the lymph nodes MIP-3β further attracts DC through the paracortical region to increase the likelihood of DC-T cell interactions (62). The critical nature of CCR7 and 6Ckine for cellular trafficking to the nodes is demonstrated by CCR7 loss of function studies revealing deficiencies in both DC and T cell homing to the secondary lymphoid tissues (74, 75).

DC not only respond to chemokines from other cell sources via their ligand specific receptors, but they also produce chemokines to attract subsets of T cells and monocytes (66). For example, RANTES is a very potent chemoattractant for memory T cells while MIP-1α and MIP-1β attract CD8+ and CD4+ T cells, respectively (66). Thus, the identification of chemokines, chemokine receptors and cytokines expressed by chlamydial pulsed DC that potentially enhance DC migration to the lymphatics and drive

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Th1-mediated protective immunity may offer the needed direction for vaccine development.

The mechanism by which dead chlamydial organisms are able to initiate DC mediated responses has not been extensively characterized. Furthermore, if dead chlamydial organisms can provide protection, what about chlamydial proteins or DNA? This prompts the consideration of chlamydial DNA and/or recombinant protein vaccines delivered by DC. **The hypothesis to be tested is that the use of *ex vivo* activated bone marrow-derived dendritic cells will serve as a vaccine delivery system to assess potential protective antigens against chlamydial genital infection in a mouse model.** One of the most likely candidates to begin these studies is the major component of the chlamydial cell wall, MOMP, due to its antigenic immunodominance and cytoadhesion capabilities. A more complete understanding of the potential capabilities of chlamydial pulsed DC for antigen delivery could result in new information regarding vaccine approaches against other infectious diseases and malignancies that do not respond to a classical vaccination strategy. In addition, such work could contribute knowledge regarding potential protective antigens as well as enhance our understanding of effector immune mechanisms at the genital mucosa.

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The specific aims of this study are to:

A. Extensively characterize chlamydial pulsed DC

1. Define culturing conditions to establish enriched (>95%) immature and mature DC as determined by phenotypic markers utilizing FACS analysis and examine shifts in CD antigens necessary for costimulation and presentation post treatment with heat killed (HK) MoPn EBs
2. Determine differential gene expression of selected chemokines, chemokine receptors and cytokines by DC in the presence of HK MoPn EBs by RNase Protection Assay (RPA).
3. Evaluate DC trafficking upon HK MoPn treatment as determined by an *in vivo* migration assay.

B. Establish delivery of MOMP fusion protein versus MOMP DNA to DC.

1. Employ immunofluorescence microscopy to characterize phagocytic uptake and processing of recombinant MOMP (rMOMP) fusion protein.
2. Deliver MOMP plasmid DNA to DC (electroporation, cationic liposomes or viral vectors) then assay expression of MOMP by immunofluorescence.
3. Determine whether MOMP antigens presented on the DC surface are able to stimulate proliferation of infection sensitized T cells and cytokine production.

C. Immunize mice with DC pulsed with rMOMP or MOMP DNA.

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1. Characterize subsequent immune response by ELISA, T cell assays, and Western Blot analysis.
2. Administer infectious challenge to immunized animals to assess protection.

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Figure 1.1. A summary of collective studies in the literature describing the immune response required for the clearance of chlamydial infection in a murine model. This diagram summarizes the relevance of individual cell types necessary for the clearance of chlamydial infection as shown by knock out (KO) and monoclonal antibody (MAb) studies in mice. See glossary for definitions of listed abbreviations.

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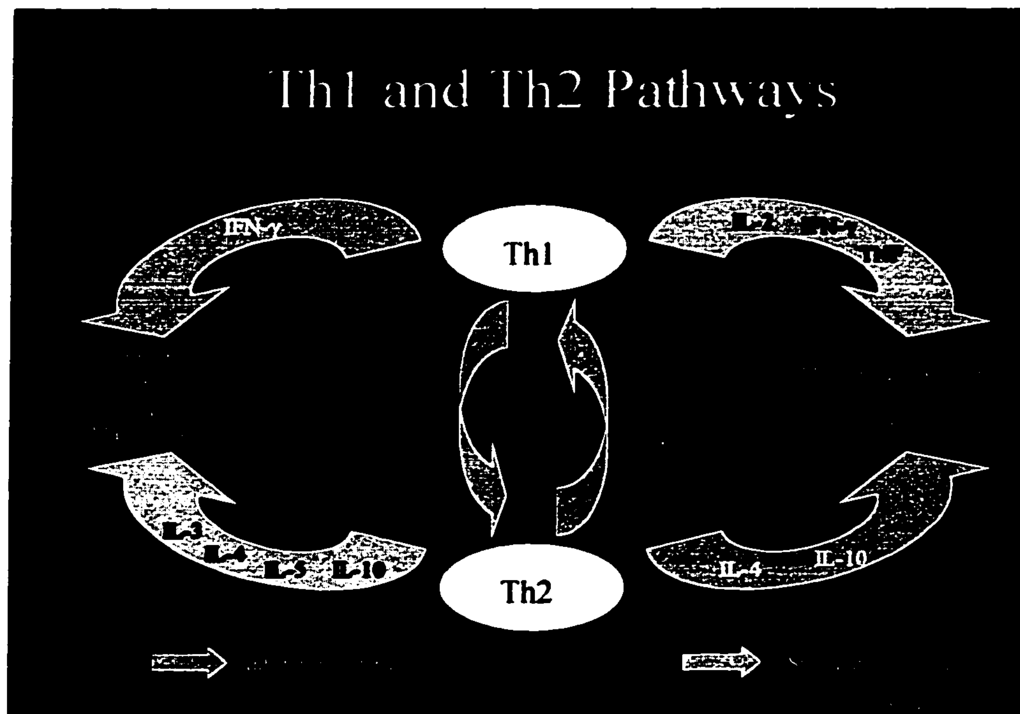

Figure 1.2. Summary of Th1 and Th2 pathways. This diagram summarizes the key cytokines secreted by T helper type 1 (Th1) and T helper type 2 (Th2) cells and the overall cellular events that follow. Note that red arrows indicate inhibitory interactions and green arrows indicate stimulatory interactions. For the purposes of this work, please note that Th1 responses are typically indicated by T cell secretion of IFN-γ and B cell production (T helper mediated) of IgG2a antibodies. Th2 responses are indicated by T cell secretion of IL-4 and IL-10 and B cell production (T helper mediated) of IgG1 antibodies.

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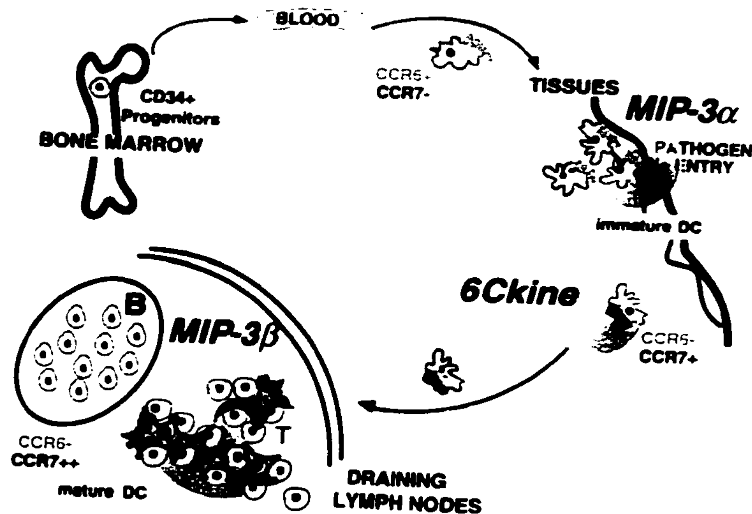

Figure 1.3. The critical role of chemokines in DC migration and function. This illustration shows the origination and migration of DC precursors, localization of immature DC to the peripheral tissues, and DC maturation and migration following immunostimulus. Chemokines and chemokine receptors play a critical role in differentiation, maturation and migration of DC. Diagram from Banchereau, et al (Annu. Rev. Immunol. 2000. 18: 767-811.

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Ligand	Receptor	Function
MIP-1α	CCR1, CCR5	chemoattract immature DC and activated CD8 T cells
MIP-1β	CCR5, CCR8*	chemoattract activated CD4+ T cells, activate macrophages
MIP-2	CCR8*	chemoattract CD4+, CD8+ T cells
MIP-3α	CCR6	chemoattract immature DC, memory T cells, γδ T cells
MIP-3β	CCR7	chemoattract mature DC through nodes, attract naïve T cells and B cells
MCP-1	CCR2	regulate expression of integrins, chemoattracts and activates monocytes
IP-10	CXCR3*	chemoattract IL-2 activated T cells
RANTES	CCR1, CCR3, CCR4, CCR5	chemoattract immature DC and memory T cells

Figure 1.4. Summary of chemokine ligands, their corresponding receptors and functions. *Receptors only found on T cells.

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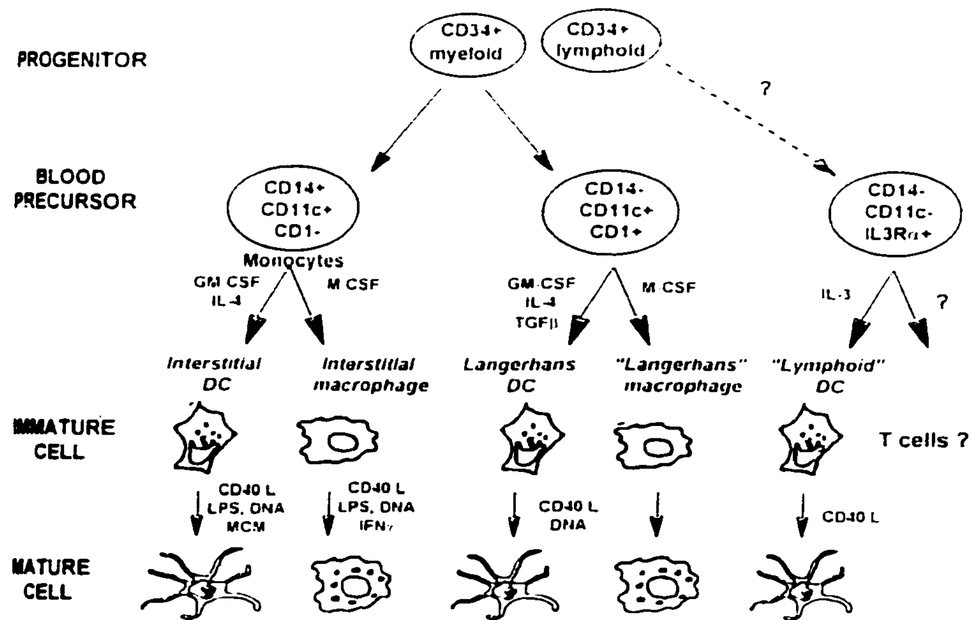

Figure 1.5. DC lineage pathways. Differentiation of DC precursors to various types of DC according to the cytokine environment and presence of immunostimulus. Diagram from Banchereau, et al (Annu. Rev. Immunol. 2000, 18: 767-811.)

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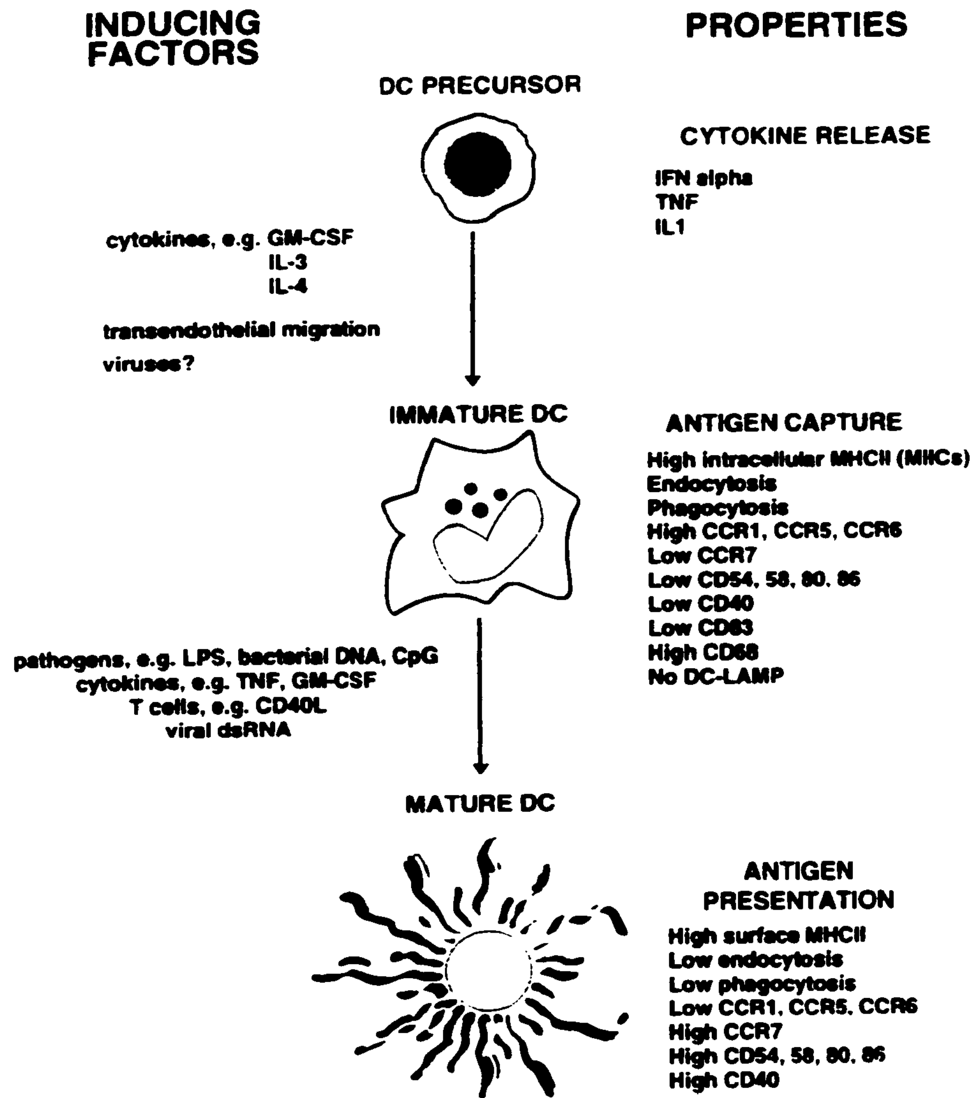

Figure 1.6. Inducing factors and functional properties of DC maturation. A summary of the inducing factors and cellular events during DC differentiation and maturation. Diagram from Banchereau, et al (Annu. Rev. Immunol. 2000. 18: 767-811).

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Chapter 2: Materials and Methods

Generation, Culture, Enrichment and FACS of primary BmDC

Bone marrow-derived dendritic cells (BmDC) were generated from C57BL/10 female mice (6-12 weeks old) using a modified version of the Inaba technique (76). Briefly, mice were sacrificed and femurs were removed, bone marrow cells were flushed from femurs and cultured in IMDM (GIBCO BRL) supplemented with 10% FBS, 10 ug/ml gentamicin sulfate, 10 ng/ml GM-CSF and 1 X 10^3 U/ml IL-4 (PharMingen) at 2 X 10^6 cells/ml in 100mm tissue culture dishes at 37° C with 5% CO_2. On day 3 of culture, nonadherent cells were removed and fresh medium containing GM-CSF and IL-4 was added. On day 5 of culture, DC were panned by incubation at 37° C for at least 2 h then repeated for another 2h to remove adherent macrophages. The DC were further purified (>95% purity) by density gradient centrifugation with 14.5% metrizamide solution (Sigma) in cell culture medium. DC purity was characterized by cell size, dendritic morphology by phase microscopy, and viability assessed by trypan blue exclusion. DC populations were further characterized phenotypically for purity by FACS analysis after staining with anti-I-A^b, anti-CD86, anti-CD40 and anti-CD11b mAbs for resolution of DC. DC cultures were also analyzed after staining with anti-Gr1, anti-CD3, anti-CD19 and anti-Pan NK MAbs for detection of any contaminating granulocytes, T cells, B cells, NK cells, respectively.

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Pulsing DC

Day 5 DC were enriched by density gradient centrifugation to >95% purity based upon cell size, morphology and FACS analysis. Enriched DC were plated in 4 ml of IMDM-10 supplemented with 10 ng/ml GM-CSF at 2.5 X 10^6 DC/well in 6 well tissue culture plates. Treatment groups are as follows: (1) IMDM alone, (2) 10 ng/ml LPS (*E. coli* strain 026:B6, Sigma), (3) Latex beads (2.5% solids-latex, 0.585 microns diameter, Polysciences, Inc.), (4) Heat killed MoPn chlamydial EBs (heat inactivated at 56° C for 30 min) at MOI of 25. Treatments were added directly to the cells, mixed well, and incubated at 37° C for 2h, 12h, 24h, or 48h.

RNase Protection Assay for Chemokines/Receptors and Cytokines

DC total RNA was harvested by the TriZol method at 2h, 12h, 24h and 48h post treatment (Life Technologies RNA Isolation Protocols). Multi probe RNase Protection Assay (RPA) System (PharMingen) was employed to detect DC expression of MIP-1α, MIP-1β, MIP-2, MIP-3α, MIP-3β, MCP-1, Ltn (lymphotactin), eotaxin, RANTES, IP-10, TCA-3, CCR1, CCR1β, CCR3, CCR4, CCR5, CCR6, CCR7, IL-1α, IL-1β, IL-1RA, IL-6, IL-10, IL-12p35, IL-12p40, IL-18, IFN-γ, TNF-α, MIF and constitutive genes (internal controls) L32, GAPDH. Briefly, DNA templates (encoding exonic sequences of the genes of interest) fused to a T7 promoter were used for T7 RNA polymerase-directed

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synthesis of highly specific ^{32}P- labeled antisense RNA probes. Labeled RNA probes were hybridized in excess overnight at 56° C with 4 µg target DC mRNA. The following day, free probe and other single-stranded RNA molecules were digested with RNases T1 and A. The remaining "RNase-protected" probes were purified, resolved on denaturing polyacrylamide gels based upon size and imaged by autoradiography. Protected probes at the appropriate sizes represent specific DC mRNA.

In Vivo DC Trafficking Assays

Day 5 DC were panned 2X (2h), enriched by density gradient centrifugation to >95% and labeled with a fluorescent tracer (PKH2-2, Sigma) that inserts into cellular membranes. Briefly, 2 X 10^7 DC were extensively washed with IMDM to remove FBS and stained for 5 minutes in PKH2-2 staining solution. The staining reaction was stopped by adding 100% serum and the cells were washed 4X with IMDM-10 to remove unbound dye. The cells were then viewed by immunofluorescence microscopy for approximate percent and pattern of DC staining and viability was assessed by trypan blue exclusion. Upon establishment of DC staining with PKH2-2, day 5 enriched and stained DC were either (1) irradiated (3,000 Rad), (2) pulsed with IMDM or (3) pulsed with heat-killed MoPn EBs (MOI=25) overnight then 5 X 10^5 DC in 40µl of PBS was injected into the hind footpad of C57BL/10 mice. Mice were sacrificed 48h post injection and popliteal nodes were harvested. A single cell suspension of lymph node (LN) cells was made and density

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gradient centrifugation of the LN cells was performed to enrich for DC. The DC were then analyzed by immunofluorescence microscopy for number of fluorescent cells indicating DC migration to the lymph nodes.

Pulsing DC with Recombinant MOMP

Day 5 DC were panned 2X (2h) and enriched by density gradient centrifugation. Predominantly immature (highly phagocytic) DC populations (5 X 10^6 DC in 1 ml IMDM-10) were incubated in the presence of 0, 0.06 μg, 0.6 μg , 6 μg and 60 μg of MBP-MOMP colloid spheres (30-40nm) for 1h at 37° C with shaking every 15 min. The cells were then centrifuged, washed gently 2X with IMDM-10 and plated in 4 ml IMDM-10 + GM-CSF (10 ng/ml) overnight. The following day, the cells were fixed in methanol and stained with murine 33b MAb (MoPn specific MAb to MOMP) then stained with a secondary FITC conjugated goat anti-mouse IgG antibody and viewed by immunofluorescence and confocal microscopy for internalization of MOMP spheres.

Electroporation of DC with MOMP DNA

Enriched DC were electroporated at 150 volts to 450 volts to generate a killing curve to assess the necessary voltage to produce 40-60% killing (trypan blue exclusion) which is optimal for successful transfection of cells with DNA (BioRad Gene Pulser II Instruction Manual). Briefly, cells are placed on ice for 10 min, pulsed with a given voltage, placed on ice for another 10 min, pulsed again at the same voltage then allowed to recover on ice

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for >10 min. Between 300 and 400 volts, approximately 50% of the DC were killed. Live DC were enriched by density centrifugation while dead cells were removed. The cells were then monitored over the next 24h for survival to determine whether this method is practical for transfection purposes.

Transfection of DC with MOMP DNA

DMRIE-C Reagent (GIBCO-BRL) is a 1:1 (M/M) liposome formulation of the cationic lipid DMRIE (1,2 –dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol in membrane-filtered water. The DMRIE-C cationic lipid reagent interacts spontaneously with negatively charged DNA to form lipid-DNA complexes that penetrate cell membranes for efficient, transient transfection of suspension cells. The protocol is as follows: 6 well plates were prepared with 500 µl OPTI-MEM I (GIBCO-BRL) per well plus either 0, 2, 4, 8 or 12µl of DMRIE-C reagent and mixed by swirling the plate. 4 µg of plasmid (containing SV40 and CMV promoters) DNA containing *OmpA* gene (encoding MOMP) was added to each well. Samples were incubated at room temperature for 30 min then cationic liposome-DNA complexes were allowed to form. 2 X 10^6 DC in 200 µl IMDM (serum-free) were added to each well and incubate at 37° C CO_2 incubator for 4 h. After 4 h, 2 ml of IMDM supplemented with 15% FBS was added. Cells were fixed in methanol at 4, 12, 24 and 48 h and stained with murine 33b MAb and stained with a secondary FITC conjugated goat anti-mouse IgG antibody and

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viewed by immunofluorescence. In addition, lysates from transfected DC were run on SDS-PAGE for Western blot analysis using the murine 33b MAb to detect expression of MOMP by the transfected cells.

Infecting and culturing female C57BL/10 mice

At ten days and seven days before infecting mice, 2.5 mg/mouse of Depo-Provera (Pharmacia & Upjohn) was injected by s.c. to synchronize estrous cycles. To infect, 5 µl sucrose phosphate glutamate (SPG) containing chlamydial EBs was injected into the vaginal vault. For infecting C57BL/10 mice, either 150, 1,500 or 15,000 infection forming units (IFU) of MoPn EBs were injected. At three days post infection, mice were cultured by swabbing the vaginal vault (Calgi-swab, 8 rotations left/right). Swabs were placed into the corresponding tubes, on ice, containing 600 µl SPG and 2 sterile glass beads. Samples were vortexed vigorously and then 200 µl was removed from each sample and transferred into a 96 well "dilution plate". Serial dilutions (1:10) were then made from each sample. HeLa cells (in 96 well flat bottom plates) were inoculated in triplicate for all dilutions. After the inoculum was been added to the HeLa cells, the plates were centrifuged for 1h at 700 x g then rocked at 37° C for 30 min. Cells were washed with Hanks Balanced Salt Solution (HBSS) 3X and fed 100 µl Minimal Essentials Medium supplemented with 10% FBS (MEM-10) and cyclohexamide (10 ng/ml) per well. Cultures were incubated for 24 hours (MoPn) then fixed in methanol. Primary Ab

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staining was carried out with murine MAb EVI H1 (specific for chlamydial LPS) and secondary staining with FITC conjugated goat anti-mouse IgG. Stained cultures were viewed by immunofluorescence and recoverable IFU/sample were determined. Culturing was performed at intervals postinfection (days 3, 5, 7, 14, 21, 28) until the infection was resolved. In parallel with culturing time points, mice were sacrificed, genital tracts removed and fixed in formalin, and stained for histopathology to determine infiltrating cells/inflammation (Histo-Path of America, Millersville, MD). Ten days after the mice had cleared the infection, vaginal secretions and sera were obtained for Ab analysis by ELISA. Additionally, at 60 days postinfection, the genital tracts were removed for gross pathological evaluation and scored for the presence of hydrosalpinx (Histo-Path of America, Millersville, MD).

Indirect Enzyme Linked Immunosorbent Assay (ELISA)

Round bottom 96 well microplates (Immulon 2, Dynatech) were coated with 10 μg/ml of formalin fixed EBs in 50 mM Tris buffer, .15M NaCl (pH 7.5) and placed at 4° C overnight. The following day, the plates were washed with Tris buffer containing .05% Tween 20 to remove unbound antigens. Wells were blocked using 2% BSA in Tris buffer containing .05% Tween 20 for 2h at 37° C. Serial dilutions of mouse sera and vaginal washes were made in buffer (2% BSA in Tris buffer, .05% Tween 20). The diluted sera and vaginal washes were added to the Ag coated plates for 1.5h at 37° C. The plates were washed extensively with PBS-Tween buffer and alkaline-phosphatase (AP) secondary Ab

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was added for an incubation period of 1h and 15 min at 37° C. The secondary Abs employed were the following: AP rabbit anti-mouse IgG-γ (Zymed), AP goat anti-mouse IgG2a (Southern Biotech Associates), AP goat anti-mouse IgG1 (Southern Biotech Associates), AP-goat anti-mouse IgA (Southern Biotech Associates). The plates were again washed extensively and 100 μl of substrate (5 mg o P-nitrophenyl phosphate (Sigma)/10 ml buffer), was added. The plates were incubated 15-45 min and optical density read at 405 nm.

Direct (Cytokine) Enzyme Linked Immunosorbent Assay (ELISA)

Purified anti-cytokine "capture antibodies" were diluted 1:250 (2 ug/ml) in .05M Tris buffer, .15M NaCl (pH 7.5) and 100 μl used to coat 96 well microplates overnight at 4° C. The plates were then washed to remove unbound antibodies. Blocking solution (2% BSA in TBS-Tween 20) was added for 2 h at 37° C. Next, cytokine standards for IL-4, IL-6, IL-10, IL-12 and IFN-γ (PharMingen) and supernatants (from APC + T cell cultures) were added to the plates overnight at 4° C. The next day, 100 μl of biotin-labeled anti-cytokine "detection antibodies" (1:250, 2 μg/ml) were added and incubated at 37° C for 1 h. The plates were washed extensively and 100 μl of alkaline phosphatase-avidin (2 μg/ml) added for 45 min at 37° C. Plates were washed again and 100 μl of substrate (5 mg o P-nitrophenyl phosphate (Sigma)/10 ml buffer) was added. The plates were incubated 15-45 min and optical density read at 405nm.

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T cell Proliferation Assays

Spleens from infection sensitized mice were harvested and a single cell suspension made in PBBS-5% FBS. Magnetic CD90 (Thy 1.2), CD4 (L3T4) or CD8a (Ly-2) microbeads (Miltenyi Biotec) were utilized for positive selection of total T cells, CD4+ T cells or CD8+ T cells, respectively. T cells from mice that have resolved infection and are protective following adoptive transfer were plated in round bottom 96 well plates at 3 X 10^5 cells/well. Day 5 enriched DC that had been pulsed overnight (ie., with rMOMP) were then harvested and incubated with the infection sensitized T cells at 1 X 10^4 DC per well thus giving a 1:30 ratio of DC to infection sensitized T cells. 48 h later 1 µCi of ^{3}H-thymidine (78.4 Ci/mmol, LIFESCIENCE-NEN) was added to each well. Radioactive cultures were incubated overnight at 37° C and counts per minute (cpm) measured (Top Count plate reader) to assess thymidine uptake/incorporation. Note: standard Antigen Presentation Assay controls were used (T cells alone; APC alone; T cells + APC; T cells + APC + antigen); differentiated DC serve as the antigen presenting cells (APC) and are non-proliferating cells.

Adoptive Immunization with pulsed DC

Female C57BL/10 mice (6-12 wks) were used as bone marrow donors, DC were propagated and enriched by the modified Inaba (76) procedure discussed above. Mice (5 -

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8 per treatment group) were adoptively immunized by s.c., i.p. or i.v. (1-7 X 10^6 DC in HBSS/mouse) with either of the following: (1) no treatment = naïve group, (2) unpulsed DC, (3) DC pulsed with HK MoPn EB (MOI=25) for 12, 24 or 48 h, (4) HK MoPn EB in HBSS (given the same number of HK EB as group 3), (5) DC pulsed with rMOMP and (6) DC pulsed with MBP alone. A booster immunization was administered 14 days after the initial immunization. Serum IgG1, IgG2a and IgA levels were measured by ELISA to ascertain the IgG isotype of the immune response elicited by the immunized mice. IgA and total IgG (γ) levels in vaginal secretions were also measured by ELISA. One week following the booster immunization mice were injected by s.c. with 2.5mg Depo-Provera per mouse at day -10 and day -7 to synchronize their estrous cycles prior to an infectious challenge. One week following the second Depo-Provera injection, mice were administered an infectious, vaginal challenge with chlamydial MoPn (150 or 1,500 IFU, ID_{50} = 10 or 100 respectively). Protection was assessed by performing cervico-vaginal swab cultures. Briefly, the vagina was swabbed, samples were placed in SPG, samples were vortexed, serial dilutions of samples were used to infect monolayers of HeLa cells to determine the number of infectious organisms being shed on days 3, 5, 7, 14, 21, 28 (and each following week until infection was resolved). Cultures were incubated at 37 C for 24 h, fixed with methanol, and stained with the murine MAb EVI H1 specific to chlamydial LPS and IFU counts determined by immunofluorescence. The total recoverable IFU per sample was determined by the following equation: IFU/sample = (# of fields)(dilution factor)(original sample volume/inoculum volume).

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Western Slot Blot Analysis

MoPn EB lysates (100 μg total protein) were electrophoresed on a SDS-PAGE mini-gel using BioRad SDS Running Buffer for 1h at 180 V. Proteins were transferred to nitrocellulose membrane (NCM) using 350mA (constant) for 1 h in 1:5 diluted phosphate transfer buffer (60.35 gm/L sodium phosphate dibasic, anhydrous, and 10.33 gm/L sodium phosphate monobasic, monohydrate). The NCM was blocked with PBS + 3% BSA, .05% Tween-20 for 2 h to overnight. The NCM was placed in the Mini-Protean II apparatus (BioRad, multichannel apparatus) with the antigen side facing upward. Sera from immune, normal, DC + HK EB immunized, DC + rMOMP immunized or DC + MBP immunized mice were added for 2 h to overnight at 37° C. An anti-mouse MAb (33b) specific to MOMP was included as a positive control for localizing the MOMP. The NCM was washed several times and secondary antibodies (goat anti mouse IgG2a-AP, IgG1-AP or IgG3-AP) were diluted 1:200 and added for 2 h. The NCM was washed and developed using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

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Chapter 3: Characterization of Chlamydial Pulsed DC.

FACS Analysis of Chlamydial Pulsed DC:

A modified version of the Inaba (76) technique was utilized to culture murine bone marrow-derived DC followed by panning and density gradient centrifugation in 14.5% metrizamide. This procedure produced enriched populations of >95% DC. Bone marrow cultures (myeloid progenitors) treated with GM-CSF and IL-4 for 5 days differentiate into a mixture of cell types including macrophages, T cells, B cells, granulocytes, NK cells, DC precursors and immature DC. Enriched DC cultures showed positive staining for surface markers I-A^b (MHC II), CD86 (B7-2) and CD11b by FACS analysis (Fig. 3.1). Based upon the adherent property of macrophages in culture with GM-CSF, the panning procedure effectively separated macrophages away from non-adherent/loosely adherent DC. Enriched DC cultures did not show staining above background for CD3 or CD19 indicating an absence of contaminating T and B cells, respectively. DC cultures showed negligible staining (<5%) of NK cells (Fig. 3.1).

Upon establishment of culturing techniques that provided highly enriched populations of DC, transmission electron microscopy (TEM) and immunofluorescence microscopy was used to detect the uptake of chlamydial EBs by enriched DC. At a ratio of 25:1 (25 beads/EBs per one DC) efficient phagocytosis of latex beads (Fig. 3.2B) and nonviable chlamydial EBs (Fig. 3.2C) by DC was observed by TEM. In addition, DC phagocytosis of EBs is shown by immunofluorescence microscopy (Fig 3.3). The

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immunofluorescence experiments revealed 100% uptake of chlamydiae as shown by positive staining for chlamydial LPS within every DC. Cytoplasmic projections extending from the DC surface, the hallmark DC morphology, is shown by phase microscopy (Fig. 3.2D). The highly granular appearance common to all professional phagocytes is also demonstrated by TEM (Fig. 3.2A).

Next, changes in cell surface antigens were investigated after pulsing DC with heat killed chlamydiae. Control unpulsed DC showed two populations for I-A^b (MHC II) staining; indicating immature (lower fluorescence) and mature (increased fluorescence) DC populations (Fig. 3.4). Thus, the culturing method produced mixed DC populations in regard to DC maturity. DC maturation was enhanced upon treatment with either the positive control (10 ng/ml LPS, *E. coli*) or killed chlamydiae, but not with latex beads. DC maturation following uptake of chlamydiae is shown by a log increase in fluorescence for MHC II staining and a $^1/_2$ log increase for CD86 and CD40 staining (Fig. 3.4). There was also a marked increase in the number of cells expressing the costimulatory molecules, CD86 and CD40 following uptake of chlamydiae and treatment with LPS (Fig. 3.4).

RNase Protection Analysis of Chlamydial Pulsed DC:

The ability of DC to traffic from peripheral tissues to the draining region lymph nodes is critical to their immunizing capacities. This crucial function is regulated by chemokines, chemokine receptors and cytokines. The kinetics of DC chemokine, chemokine receptor and cytokine gene expression following endocytosis of chlamydiae

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was studied by using an RNase Protection Assay (RPA). The data are summarized in Figure 3.9. The RPA procedure is shown in Figure 3.8. The data show that MIP-1α, MIP-2, TNF-α, IL-1α, IL-1β and IL-1RA (IL-1 Receptor Antagonist) were expressed by 2 h post treatment with chlamydiae, but not latex beads, and remained continuously expressed for 48 h (Fig. 3.5, 3.6A, 3.6B). MIP-3α and IL-12p40 were expressed by 12 h and showed similar continuous expression (Fig. 3.5, 3.6A). In contrast, IP-10 (interferon induced protein) and IL-6 were transiently expressed being first detected at 12 h, but not at 48 h (Fig. 3.5, 3.6A). High levels of CCR7 expression by DC were observed under all conditions at 2 and 24 h post treatment, however only chlamydial pulsed DC expression remained high at 48 h (Fig. 3.7).

In vivo migration assays

To investigate DC migration capacities *in vivo* fluorescent chlamydial pulsed DC or fluorescent unpulsed DC were injected into the hind footpad of mice. Two days later the popliteal nodes were harvested, total lymph node cells were collected, and DC were isolated by metrizamide density centrifugation. The number of fluorescent DC detected by immunofluorescence microscopy was ≤1% of the DC injected therefore it was extremely difficult to recover reasonable numbers of DC by this method. Six chlamydial pulsed DC were detected by immunofluorescence microscopy demonstrating DC migration *in vivo* to the pooled popliteal nodes. However, no fluorescent unpulsed DC

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were observed by immunofluorescence and therefore unactivated DC did not migrate (Fig. 10).

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FIGURES

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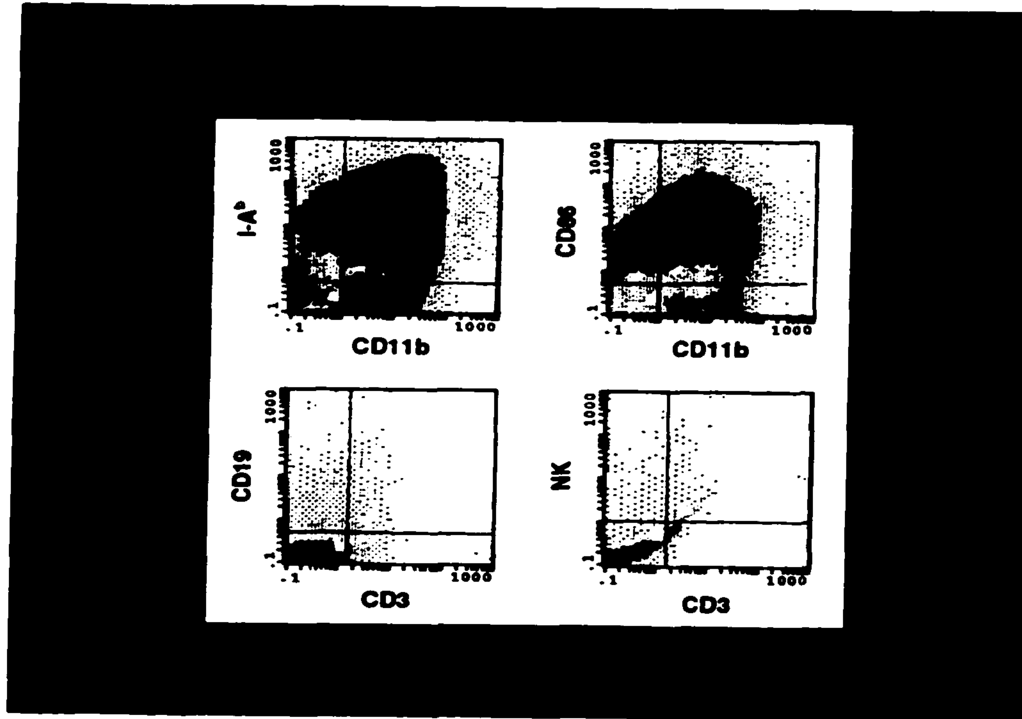

Figure 3.1. Establishing Purity of BmDC Population by FACS Analysis. DC purity was characterized by FACS analysis after staining with anti-I-A^b (MHC II), anti-CD86 (B7-2), anti-CD11b for resolution of DC. Isotype matched controls were used to determine background staining. DC cultures were also analyzed after staining for anti-CD3, anti-CD19, and anti-Pan NK for resolution of any contaminating T cells, B cells or NK cells, respectively. The cultures are >95% DC as demonstrated by positive staining for all DC markers and negligible staining for potential contaminating cell types. Based upon the adherent property of activated macrophages, 2X-2 h panning procedures separate the macrophage population from the DC population.

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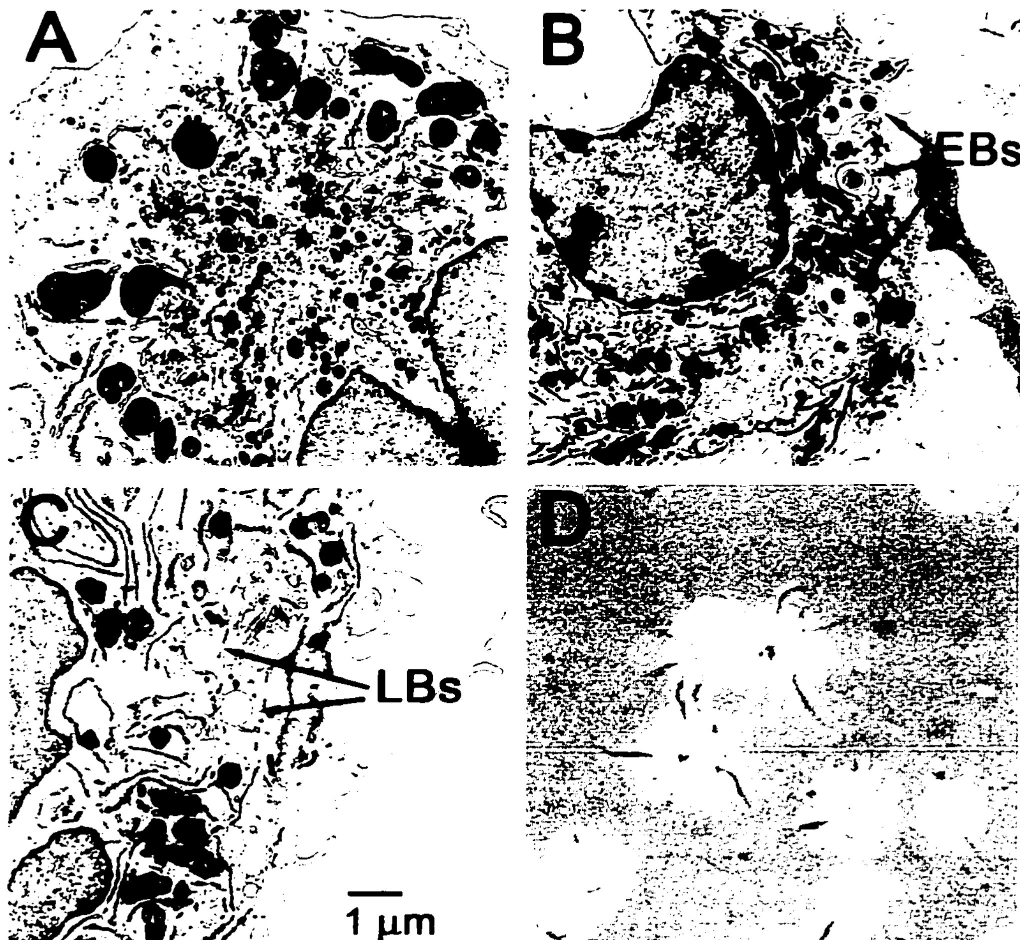

Figure 3.2. Transmission Electron Microscopy (TEM) and Phase Microscopy of DC. (A) TEM of unpulsed DC. (B) TEM of DC pulsed with heat killed chlamydial EBs at 25:1 EB to DC ratio. (C) TEM of DC pulsed with latex beads (LBs) at 25:1 beads to DC ratio. (D) phase microscopy of DC revealing the characteristic cytoplasmic projections.

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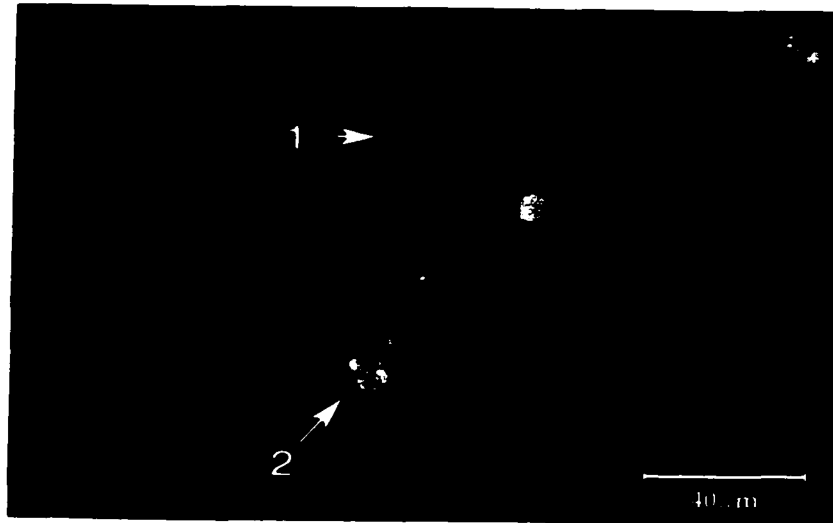

Figure 3.3. DC phagocytize EBs efficiently. DC were pulsed overnight with chlamydial EBs, fixed in methanol, and stained for chlamydial LPS (EVH1 MAb). Individual DC show two staining patterns: **(1)** fine punctate, and **(2)** aggregate immunofluorescence staining throughout the cytoplasm. The **#1** punctate staining pattern was observed more frequently than the large aggregate **#2** staining. 100% of the observed DC stained positively for the presence of chlamydiae. This staining pattern is consistent with previously published immunofluorescence micrographs (52). Control unpulsed DC did not exhibit fluorescent staining (data not shown). Size bar = 40 µm.

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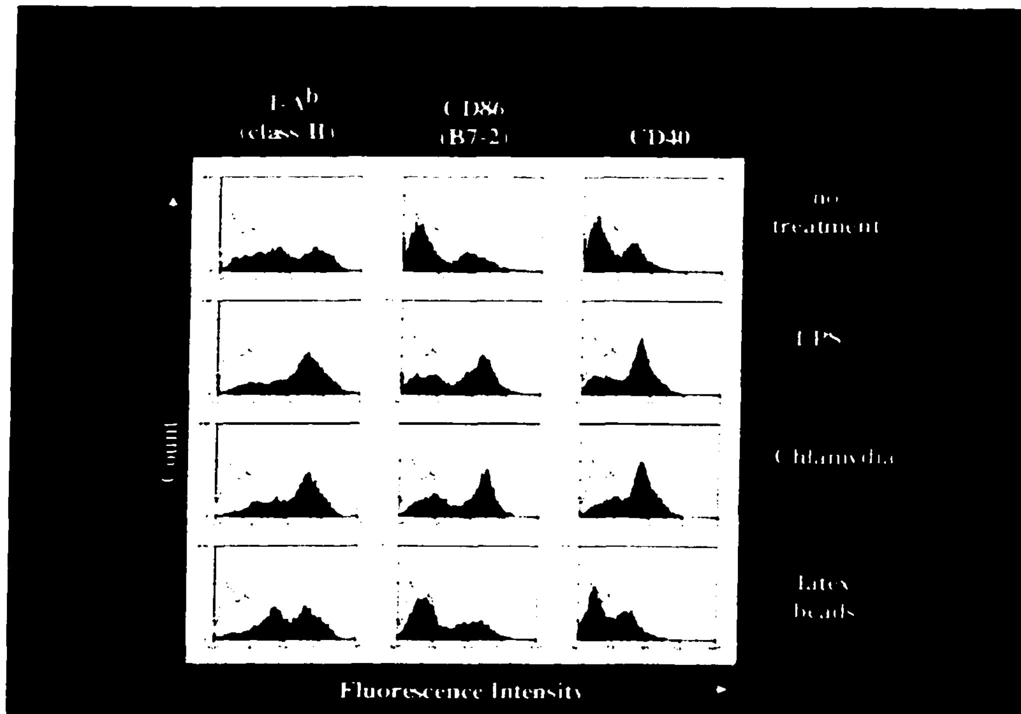

Figure 3.4. FACS Analysis of Chlamydial Pulsed DC. Enriched unpulsed DC populations show two populations of Class II (I-A^b) staining indicating immature (less fluorescence) and mature (increased fluorescence) DC populations. DC maturation is enhanced after pulsing with either LPS (10 ng/ml, *E.coli* strain) as the standard positive control or with nonviable chlamydiae (25: 1 EB to DC ratio) illustrated by a log shift in fluorescence intensity for Class II staining. Staining for CD86 and CD40 costimulatory molecules increases in the presence of LPS or chlamydiae as compared to negative controls. Latex beads were employed as a control for phagocytosis alone and demonstrate similar staining as the no treatment group.

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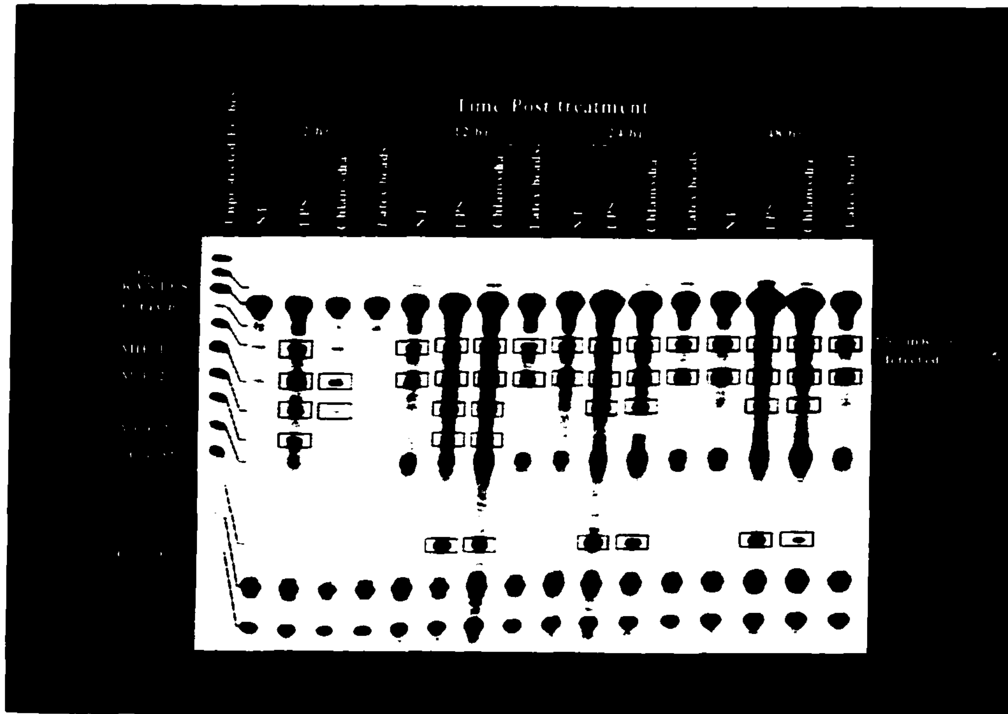

Figure 3.5. RPA Anlaysis of DC Expression of chemokines. A kinetic analysis of chemokine RNA expression by DC subjected to NT (no treatment; lanes 2, 6, 10, 14), LPS (10 ng/ml; lanes 3, 7, 11, 15), heat killed MoPn EBs (25: 1 EB to DC ratio; lanes 4, 8, 12, 16) and latex beads (25:1 beads to DC ratio; lanes 5, 9, 13, 17). The NT group likely reflects spontaneous maturation resulting from cell culture alone. LPS serves as a positive control for immunostimulation. HK EB treatment is the experimental group and latex beads control for the effects of phagocytosis alone. Boxed bands represent differentially expressed genes.

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A.

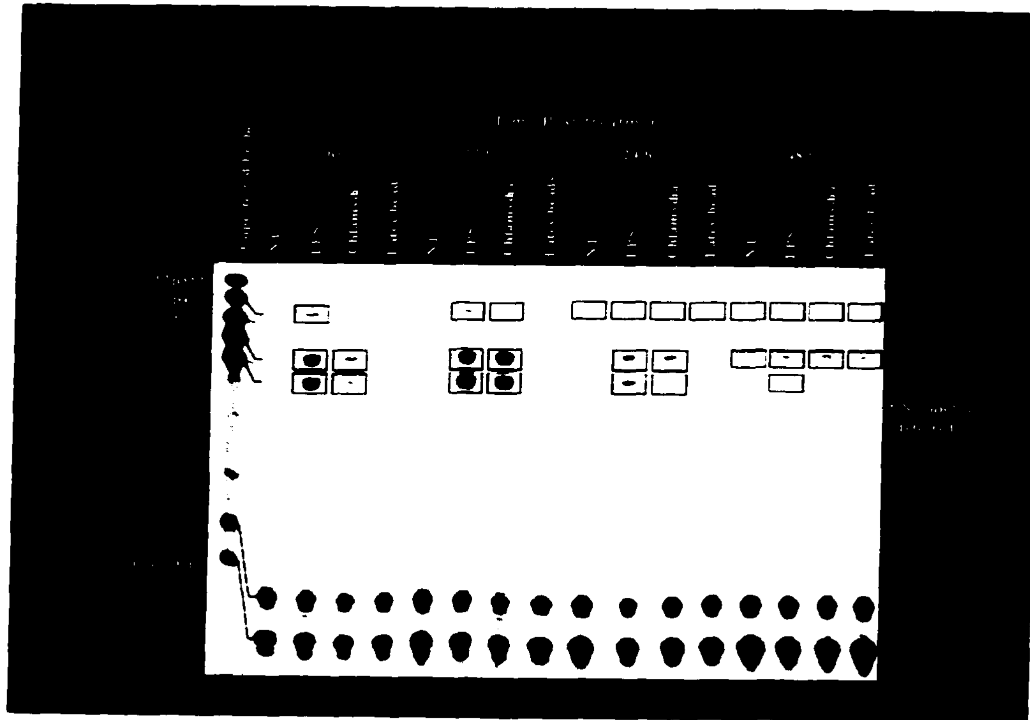

Figure 3.6A, B. RPA Analysis of DC Expression of Cytokines. A kinetic analysis of cytokine RNA expression by DC subjected to NT (no treatment: lanes 2, 6, 10, 14), LPS (10 ng/ml: lanes 3, 7, 11, 15), heat killed EBs (25:1 EB to DC ratio: lanes 4, 8, 12, 16) and latex beads (25:1 beads to DC ratio: lanes 5, 9, 13, 17). Boxed bands represent differentially expressed genes.

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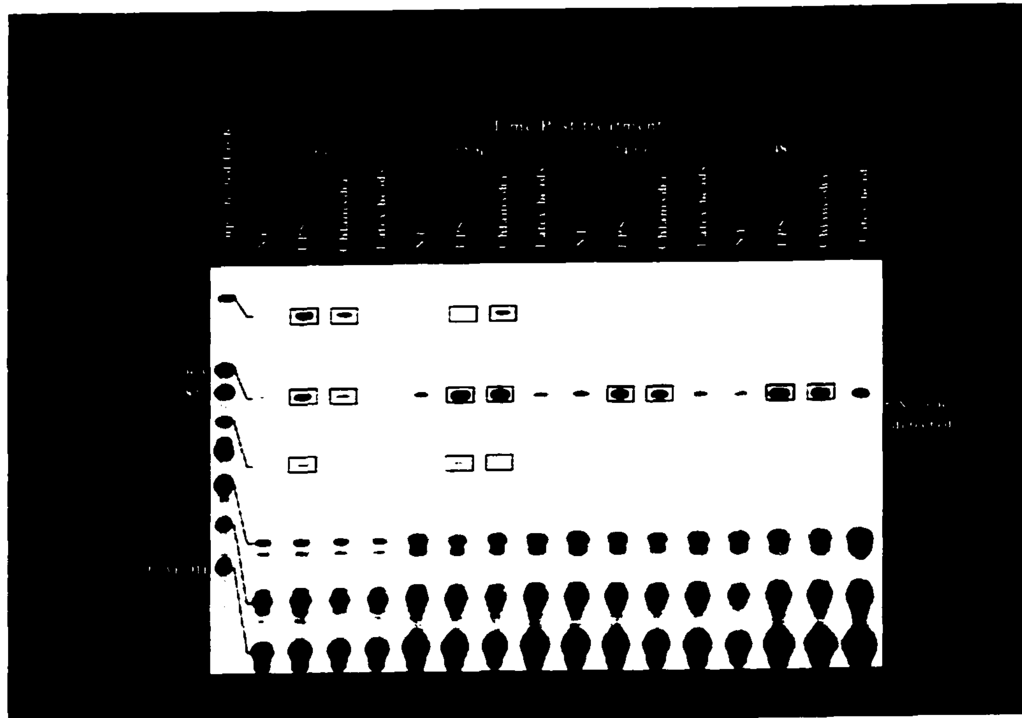
B.



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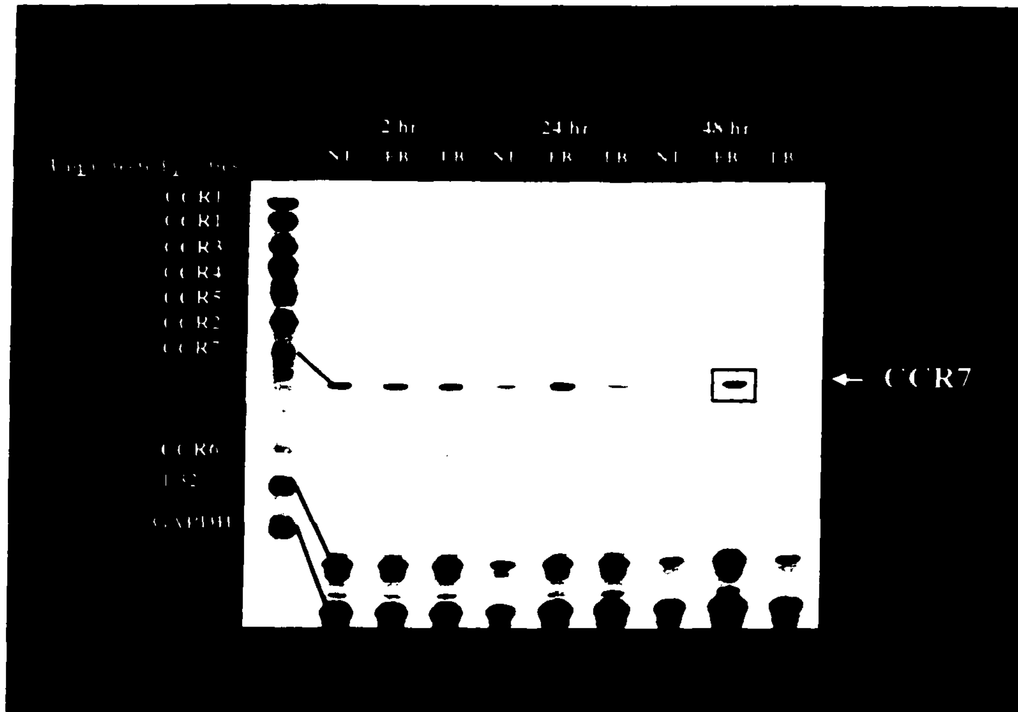

Figure 3.7. RPA Analysis of DC Expression of Chemokine Receptors. A kinetic analysis of DC treated with NT (no treatment, lanes 1, 4, 7), MoPn HK EB (25:1 EB to DC ratio: lanes 2, 5, 8) and LB (25:1 latex beads to DC ratio: lanes 3, 6, 9). The increased longevity of CCR7 mRNA in chalmydial pulsed DC is indicated by the boxed band.

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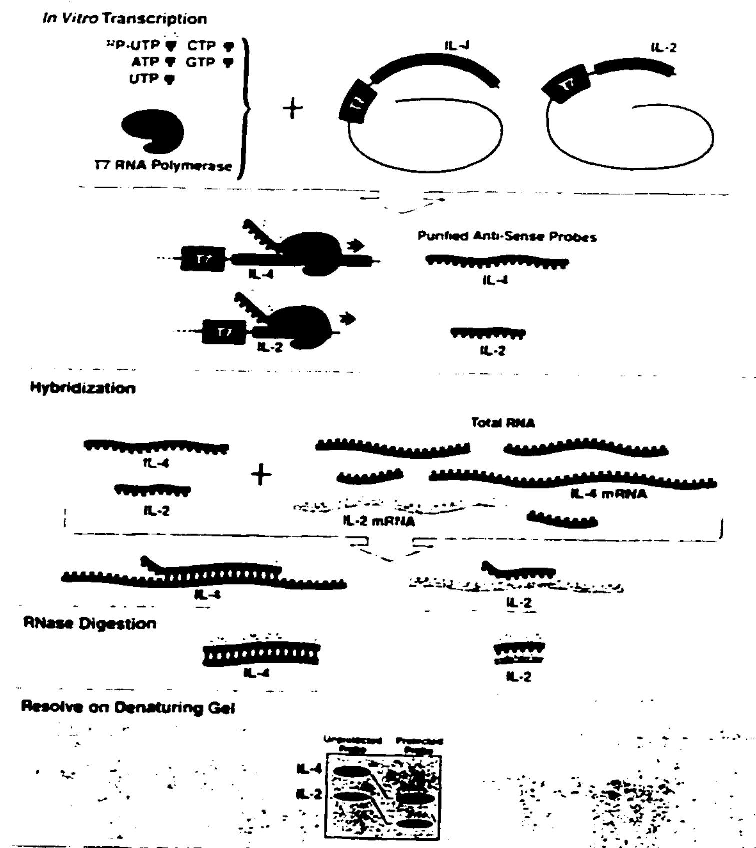

Figure 3.8. RNase Protection Assay (RPA) procedure. *In vitro* transcription in the presence of radioactively labeled UTP produced labeled RNA probes which were used to hybridize with DC sample mRNA. RNase digestion degraded single stranded (non-hybridized) RNA. The remaining double stranded RNA was resolved on denaturing gels and visualized by autoradiography. This outlined method is from the BD PharMingen manual.

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2hrs -> 48 hrs	**12 hrs -> 24 hrs**	**12 hrs -> 48 hrs**
MIP-1α	IP-10	MIP-3α
MIP-2	IL-6	IL-12p40
TNF-α		
IL-1α		
IL-1β		
IL-1RA		
CCR7		

Figure 3.9. Summary of the DC kinetic expression profile of chemokines, chemokine receptors and cytokines. DC pulsed with nonviable chlamydiae differentially express the chemokines and cytokines listed above as shown by RPA. Activated transcription was temporally regulated as indicated by the columns for DC chemokine/cytokine expression during 2 to 48h, 12 to 24h, and 12 to 48 h.

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Treatment	# of mice	fluorescent DC
Irradiated DC	4	0
Unpulsed DC	4	0
DC + HK EB	4	6

Figure 3.10. Summary of data from an *in vivo* DC migration assay. Mice were injected with fluorescently labeled DC into the hind footpad. After 48 h, the popliteal nodes were harvested and pooled within each treatment group. Density centrifugation (14.5% metrizamide) of lymph node cells was used to enrich for DC. The recoverable DC were plated in tissue culture wells and viewed by direct fluorescence microscopy to detect injected fluorescent DC that had migrated to the popliteal nodes. This method resulted in an extremely low yield of recoverable DC.

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Chapter 4: Delivery of rMOMP to DC for Antigen Presentation

DC Readily Phagocytose Recombinant MOMP and Secrete Large Amounts of IL-12:

Bone marrow-derived DC were pulsed with a range of concentrations (0 to 60 μg) of particulate recombinant MOMP (rMOMP) for 15 min, 30 min, 1 h, 4 h, 14 h and 22 h to optimize uptake and detection of MOMP within the DC cytoplasm. DC phagocytosis of recombinant protein was determined after methanol fixation and staining with an mouse MAb (33b) to MOMP. Positive staining was observed by immunofluorescence microscopy for 5 X 10^4 DC pulsed with 0.6 μg of rMOMP for 1 h (Fig. 4.1). To determine whether the rMOMP was internalized by DC, confocal microscopy was utilized to detect MOMP in the cytoplasm. Z- series of images were digitally stacked and revealed positive staining for MOMP throughout the cytoplasm of DC (Fig. 4.2). Increasing the number of DC and rMOMP proportionally resulted in similar staining by immunofluorescence and confocal microscopy (data not shown).

As a result of expressing rMOMP the purified MOMP preparation contained high levels of bacterial endotoxin (~300,000 U/ml LPS). Due to the potent immunostimulatory effect of LPS on DC maturation (see Chapter 3 data figures), the contaminating LPS was not removed from the rMOMP preparations. DC pulsed with rMOMP produced large amounts of IL-12, a potent Th1 cytokine (Fig. 4.3). In contrast, pulsed DC did not produce either IL-10 or IL-4, important Th2 cytokines (Fig. 4.3).

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DC Presentation of MOMP Antigen(s) to Infection Sensitized CD4+ T Cells:

Upon establishment of efficient DC phagocytosis of rMOMP, presentation of MOMP antigens (Ags) by DC was investigated next. A summary of the data is diagrammed in Figure 4.7. DC pulsed with rMOMP were incubated in the presence of infection sensitized T cells for 48 h, then pulsed with ^{3}H-thymidine overnight. Infection sensitized T cells proliferated 2.5 times more in the presence of DC pulsed with rMOMP than unpulsed DC (Fig. 4.4). To determine whether MOMP antigens were presented by MHC I, MHC II or both, CD8+ T cells and CD4+ T cells were incubated with pulsed DC. CD4+ but not CD8+ T cells proliferated in response to rMOMP pulsed DC (Fig. 4.5). CD4+ T cell proliferation also occurred following incubation with MBP (maltose binding protein) pulsed DC, although at a significantly lower level than the response to rMOMP (Fig 4.5).

To determine the type of T cells (Th1 or Th2) that were being activated in response to the MOMP pulsed DC, CD4+ T cells were incubated in the presence of DC for 48 h and the supernatants were collected and assayed for cytokine production. DC pulsed with rMOMP, but not with MBP, induced CD4+ T cells to produce IFN-γ, a Th1 cytokine (Fig. 4.6). Low levels of IL-10 were detected in the presence of either DC + MOMP or MBP. IL-4, another Th2 cytokine, was not detected in the supernatants (Fig. 4.6). It was concluded from these results that rMOMP (i) is efficiently phagocytosed by DC, (ii) stimulates DC to secrete IL-12 (iii) is processed and presented onto MHC II

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molecules, (iv) stimulates CD4+, but not CD8+, T cells to proliferate, and; (v) stimulates CD4+ T cells to differentially produce high levels of IFN-γ.

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FIGURES

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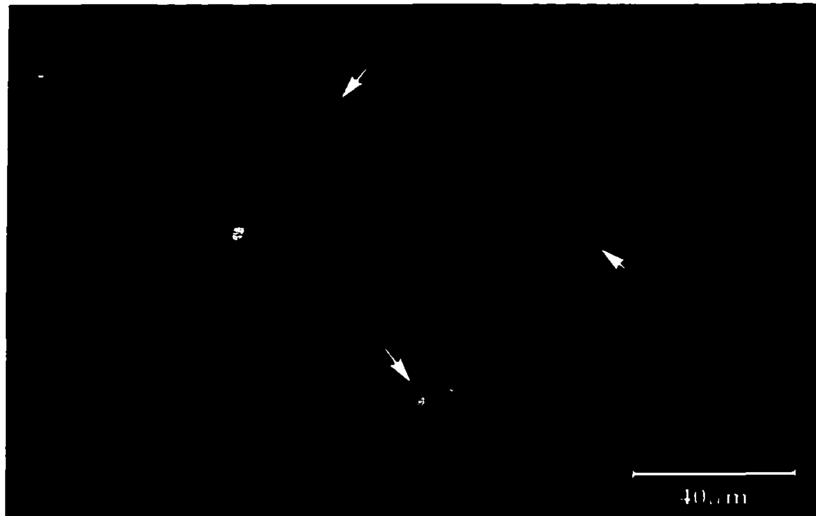

Figure 4.1. DC phagocytosis of rMOMP as shown by immunofluorescence microscopy. 5 X 10^4 DC were pulsed with 0.6 µg rMOMP for 1 h at 37° C, washed and incubated overnight at 37° C with 10 ng/ml GM-CSF. DC were fixed in methanol and stained with an anti mouse MAb to MoPn MOMP and viewed by immunofluorescence microscopy. Arrows indicate a punctate positive staining pattern for MOMP epitopes along the perimeter of the DC cell surface. Negative controls were (i) unpulsed DC and (ii) pulsed DC stained with an isotype matched primary Ab to a serovar L2 MOMP and the same secondary Ab as the experimental group. Both controls were negative for MOMP staining (data not shown). Size bar = 40 µm.

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Figure 4.2. DC phagocytosis of rMOMP as shown by confocal microscopy. 5 X 10^4 DC were pulsed with 0.6 µg rMOMP for 1 h at 37° C, washed and incubated overnight at 37° C with 10 ng/ml GM-CSF. DC were fixed and stained with an anti mouse MAb to MOMP. Arrows indicate each individual DC represented as a stacked Z series of images indicating positive staining (green) for MOMP within numerous planes (Z axis) through the cytoplasm of the rMOMP pulsed DC. Unpulsed DC did not show staining for MOMP (data not shown).

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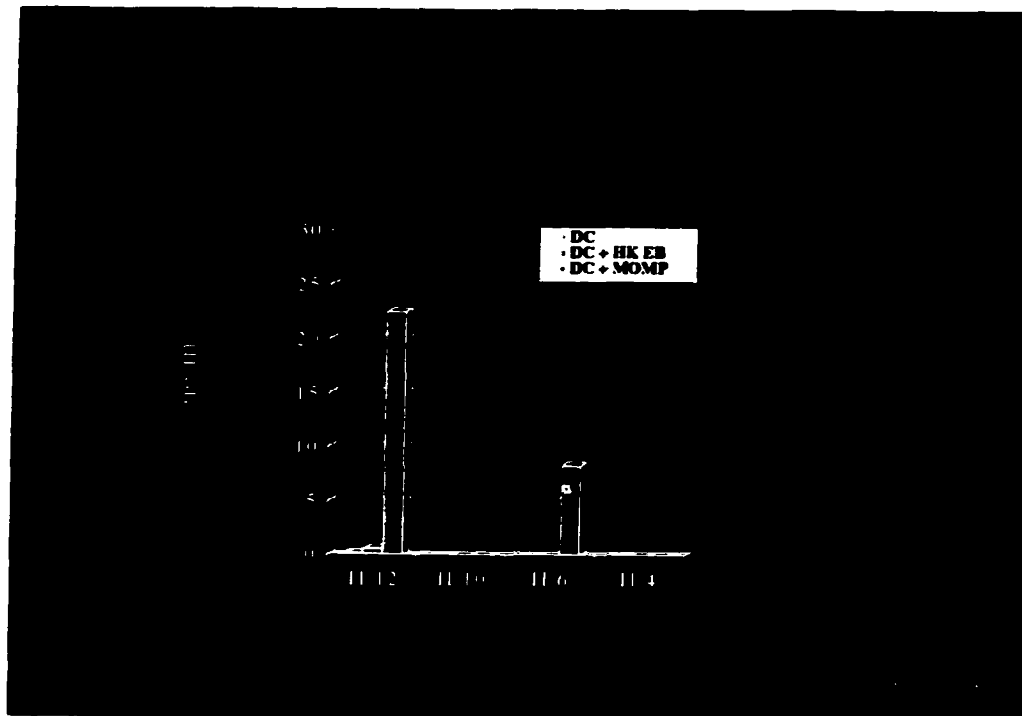

Figure 4.3. DC pulsed with rMOMP secrete large amounts of IL-12. DC supernatants were collected 24 h after pulsing with rMOMP. Large amounts (~22 ng/ml) of IL-12 (Th1) was detected by ELISA. IL-10 and IL-4, Th2 cytokines, were not detected in the DC supernatants. Two separate experiments were each done in duplicate and are expressed as ng/ml +/- SD.

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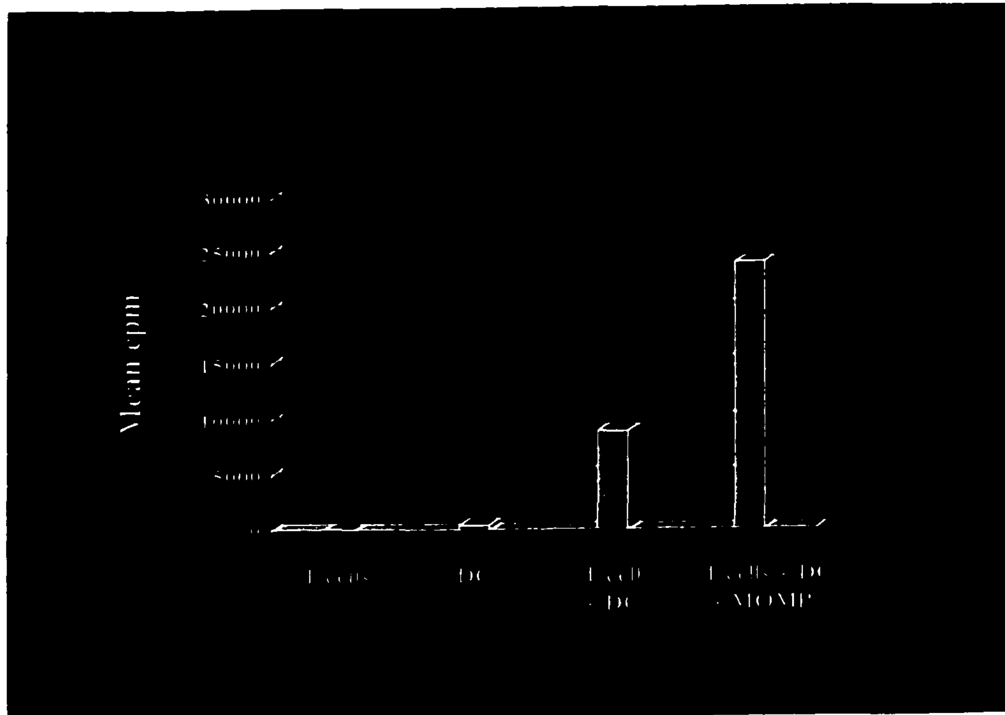

Figure 4.4. Infection sensitized T cells proliferate in response to DC pulsed with rMOMP. Spleens from infection sensitized mice were harvested and total T cells collected. Infection sensitized T cells were incubated in the presence of unpulsed DC and rMOMP pulsed DC for 48 h. Next, ^{3}H-thymidine was added overnight and cpm counts were used to assess T cell proliferation. DC are non-proliferative cells, therefore only activated T cell proliferation is observed by this type of APC assay (see Chapter 2). Experiments were done in triplicate and are expressed as mean cpm +/- SD.

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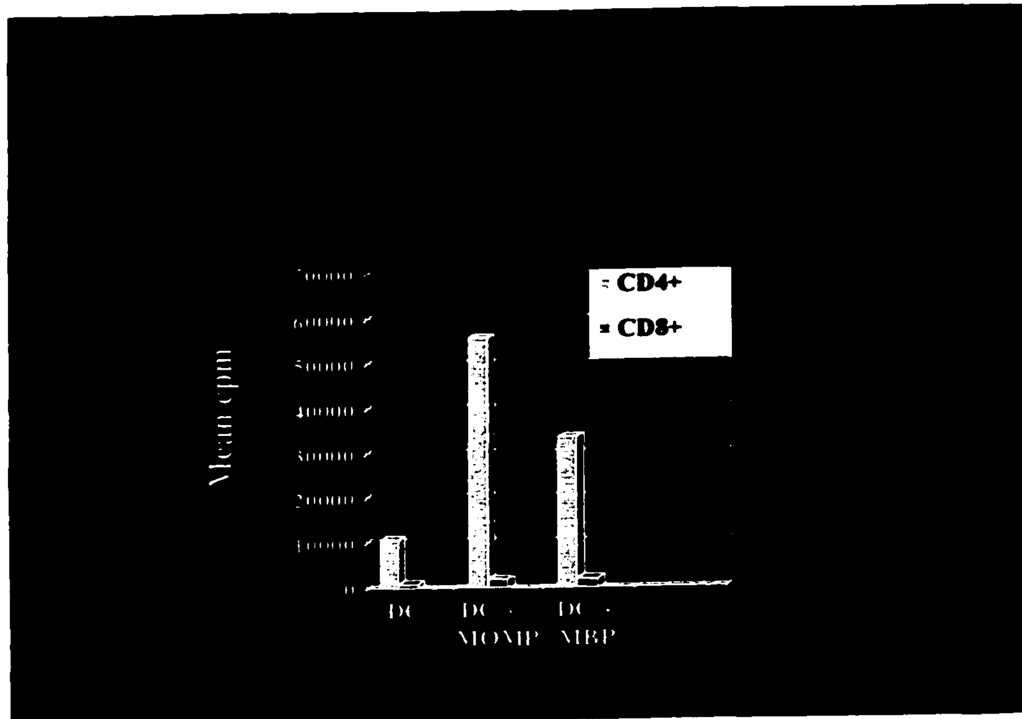

Figure 4.5. Infection sensitized CD4+ but not CD8+ T cells proliferate in response to DC pulsed with rMOMP. Infection sensitized CD4+ and CD8+ T cells were collected and incubated for 48 h in the presence of unpulsed DC, DC pulsed with rMOMP or DC pulsed with MBP. Next, ^{3}H-thymidine was added overnight and cpm counts used to determine T cell proliferation. Experiments were done in triplicate and expressed as mean cpm +/- SD.

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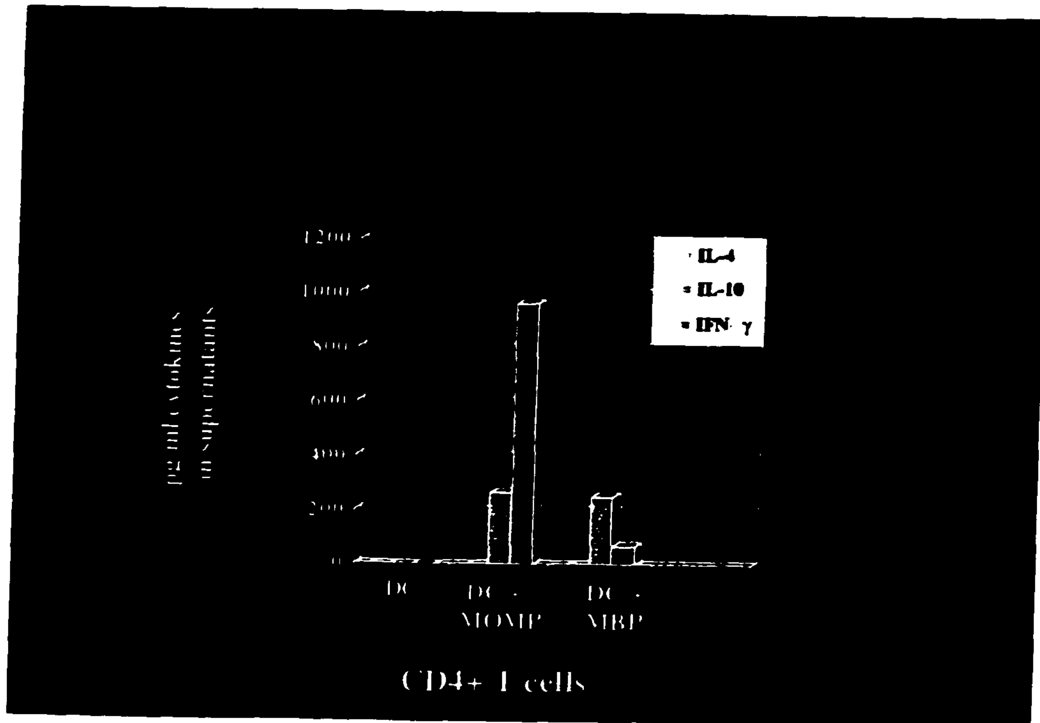

Figure 4.6. DC pulsed with rMOMP induce infection sensitized CD4+ T cells to differentially produce IFN-γ. Infection sensitized CD4+ T cells were incubated in the presence of DC + rMOMP or MBP for 48 h then supernatants were collected. Cytokine levels in supernatants were determined by indirect ELISA. Experiments were done in triplicate and are expressed as mean pg/ml +/- SD.

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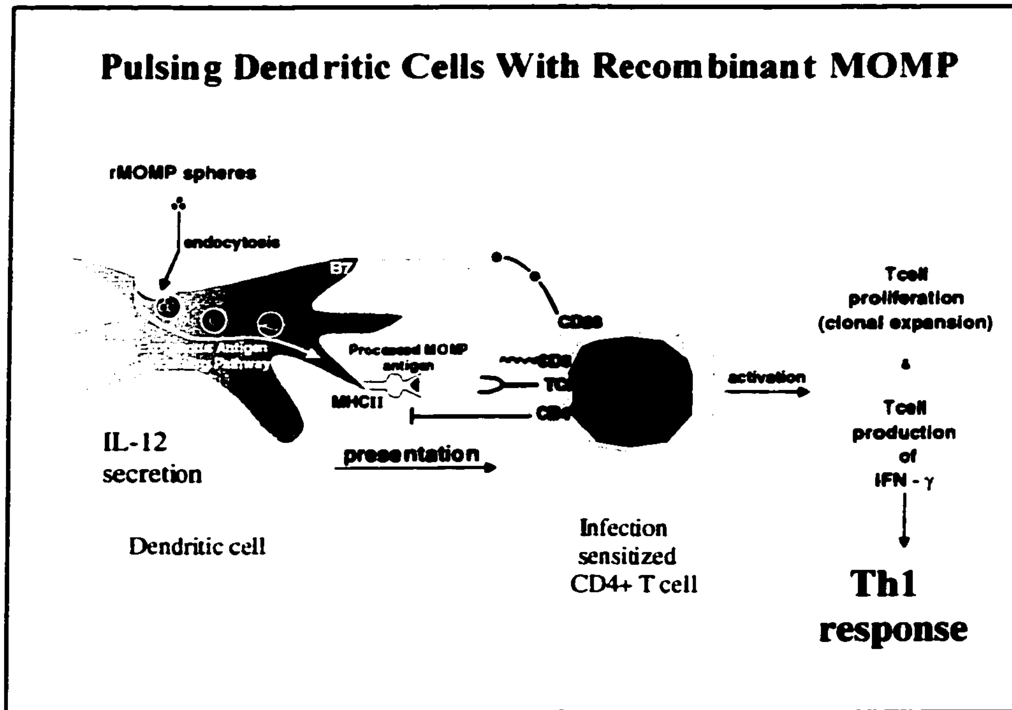

Figure 4.7. Endocytosis, processing and presentation of MOMP by DC. This is a schematic summary of the data found in Chapter 4. This figure illustrates DC endocytosis of particulate rMOMP spheres, processing and presentation of MOMP Ags onto MHC II molecules and subsequent DC secretion of IL-12. Infection sensitized T cells recognize the presented MOMP Ags on MHC II via their T Cell Receptor (TCR). This interaction with the DC MHC II is stabilized by the T cell CD4 molecule. Proper recognition of MOMP epitopes accompanied by costimulation (B7: CD28) leads to the activation of the $CD4^+$ T cells to proliferate (clonally expand) and secrete IFN-γ. Both DC secretion of IL-12 and T cell secretion of IFN-γ indicate a potential to drive a Th1 immune response *in vivo*. Therefore, the rMOMP pulsed DC were primed *ex vivo* to drive a potent MOMP specific Th1 immune response upon adoptive immunization in mice.

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Chapter 5: Immunization of mice using DC pulsed with rMOMP

Optimizing the DC Immunization:

Prior to immunization studies utilizing DC pulsed with rMOMP, optimization of the delivery and overall efficacy of the positive control vaccine (DC pulsed with HK EB) was performed. Intra-peritoneal (i.p.), subcutaneous (s.c.) and intra-venous (i.v.) routes of administration were investigated for immunizing against chlamydial infection using DC pulsed for 12, 24 or 48 h with HK EB. The i.v. immunization (via the orbital sinus) with 12 h pulsed DC proved to be the superior route of administration as shown by powerful protection (5.6 logs) following intravaginal challenge (Fig. 5.1). There was very little variability among the i.v. immunized mice between days 3 and 7 (Fig. 5.2A, B), however some variability was observed between days 10 to 14 post-challenge (Fig. 5.2C, D). The majority of the i.v. immunized mice exhibited equal or higher levels of protection than that of infected and re-challenged mice (Fig. 5.2A-D).

Adoptive immunization by i.p., but not s.c., elicited strong protection (~3 logs) against chlamydiae in 3 of the 5 mice (Fig. 5.3A) on the fourth day following intravaginal challenge . In contrast, mice immunized by s.c. shed similar numbers of organisms as the naive mice at 4 days post challenge although 15 days post-challenge these mice had resolved infection to the same degree as the i.p. immunized group (Fig. 5.3B). Equivalent numbers of unpulsed DC or HK EB administered alone did not yield protection (Fig. 5.3A, B). In attempt to understand the large variability in the i.p. immunization group,

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the presence of antibody isotypic differences in pre-challenge sera from the i.p. immunized mice was tested. Protected mice produced elevated levels of chlamydial specific IgG2a antibodies (Th1) compared to the unprotected mice (Fig. 5.4). All mice in the i.p. immunization group produced low levels of chlamydial specific IgG1 antibodies. All mice in the i.v. immunized group produced both IgG2a and IgG1 at approximately equivalent levels (Fig. 5.5A, B).

The course of infection in naïve mice infected with 15,000 IFU, 1,500 IFU or 150 IFU is shown in Fig. 5.6. The results show that 100% of the mice became infected at each challenge dose, shed similar numbers of organisms and resolved infection with similar kinetics (Fig. 5.6). Therefore, the lower infectious load (150 IFU) was selected for challenging the immunized mice to avoid overwhelming the immune system and possibly masking any low levels of resistance post challenge. It is on this basis that the infectious challenge was lowered from 1500 IFU/mouse to 150 IFU/mouse for the rMOMP immunization experiments. In further efforts to optimize the DC vaccine approach, the number of DC administered was increased from 1×10^6 DC to 7×10^6 DC.

Immune Response in Mice Following Immunization using DC Pulsed with rMOMP:

Mice were immunized twice using 7×10^6 DC pulsed with rMOMP or MBP and intravaginally challenged with 150 IFU 14 days after immunization. Previous results (see Chapter 4) demonstrated that DC pulsed *in vitro* with rMOMP secreted large amounts of IL-12 (Fig. 4.3) and stimulated infection sensitized CD4+ T cells to strongly proliferate

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and secrete large amounts of IFN-γ (Fig. 4.4, 4.5). However adoptive immunization (i.v.) using DC pulsed with rMOMP generated a Th2 biased immune response illustrated by secretion of Th2 cytokines by rMOMP vaccine sensitized CD4+ T cells (Fig. 5.7) and a predominance of IgG1 antibodies reactive with the EB surface as detected by ELISA (Fig. 5.5B). IgG1 and IgG2a antibodies to denatured MOMP were detected in pre-challenge sera as shown by Western blot analysis (Fig. 5.5A).

Challenging rMOMP Immunized Mice

Following intravaginal challenge, rMOMP immunized mice were not protected (Fig. 5.8). The MBP control group resolved infection with similar kinetics as the naïve animals (Fig. 5.8). Adoptive immunization by i.p. elicited marginal (1-2 logs) and very transient (< 7 days) protection in 3 of the 6 mice immunized. The onset of infection in the i.p. immunized mice was delayed by three to six days. All i.p. immunized mice became similarly infected by day 7 with infectious burdens comparable to naïve mice (Fig. 5.9).

Although this DC vaccine delivery system was designed to theoretically drive a MOMP specific Th1 immune response, a Th2 response was generated following adoptive immunization using DC pulsed with rMOMP. It was concluded that; (i) infusion of pulsed DC into the retro-orbital sinus (i.v.) is the most effective route of pulsed DC vaccine administration , (ii) MOMP immunized mice produce predominantly IgG1 antibodies reactive with the EB surface, (iii) CD4 + T cells from MOMP

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immunized mice respond to EB Ag by the secretion of Th2 cytokines IL-4 and IL-10 and (iv) MOMP immunized mice are not protected following intravaginal challenge with chlamydia.

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FIGURES

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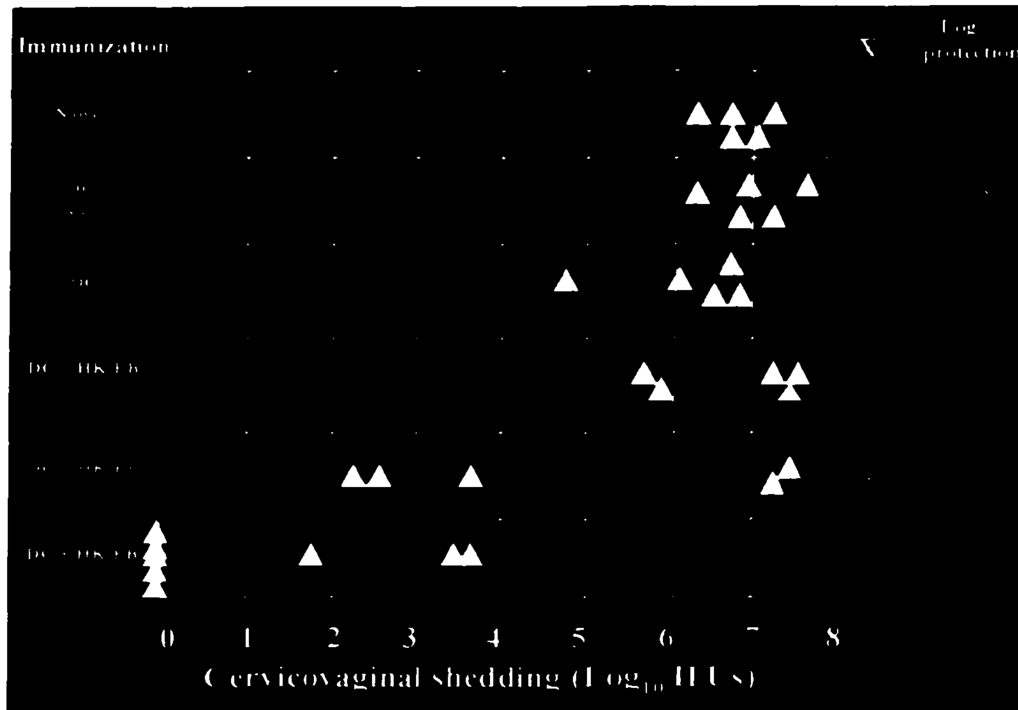

Figure 5.1. Intravenous injection (i.v.) is the superior route of DC vaccine administration. Mice received either no immunization (naïve) or 2X immunization (s.c., i.p., i.v.) with unpulsed DC or DC pulsed with HK EB, and were challenged intravaginally with chlamydiae. Protective immunity was determined by quantifying the number of chlamydial IFU recovered from cervicovaginal swabs. Day 4 post-challenge is shown here. Triangles represent individual mice. The number of recoverable IFU is shown along the x axis (log scale). The mean recoverable $\log_{10}$ IFU and the $\log_{10}$ protection are shown to the right of the graph.

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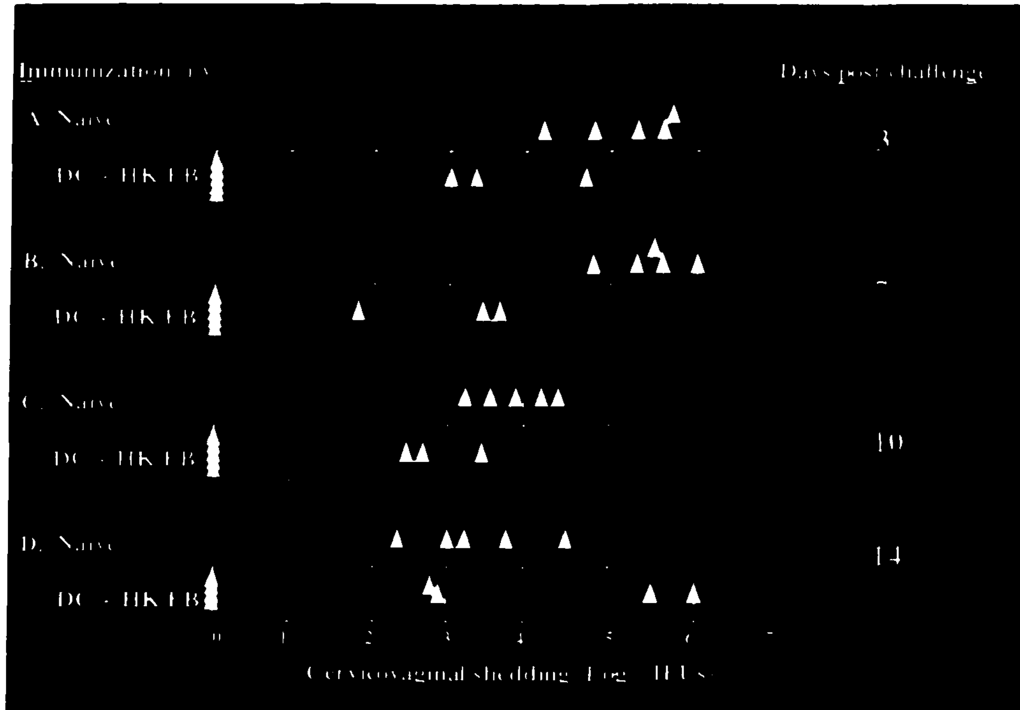

Figure 5.2. Mice immunized by intravenous injection (i.v.) with chlamydial pulsed DC are strongly protected following intravaginal challenge with chlamydiae. Naïve = no immunization. DC + HK EB = DC pulsed with heat killed chlamydial EBs. (A) day 3 post challenge, (B) day 7 post challenge, (C) day 10 post challenge and (D) day 14 post challenge. Triangles represent individual mice.

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A.

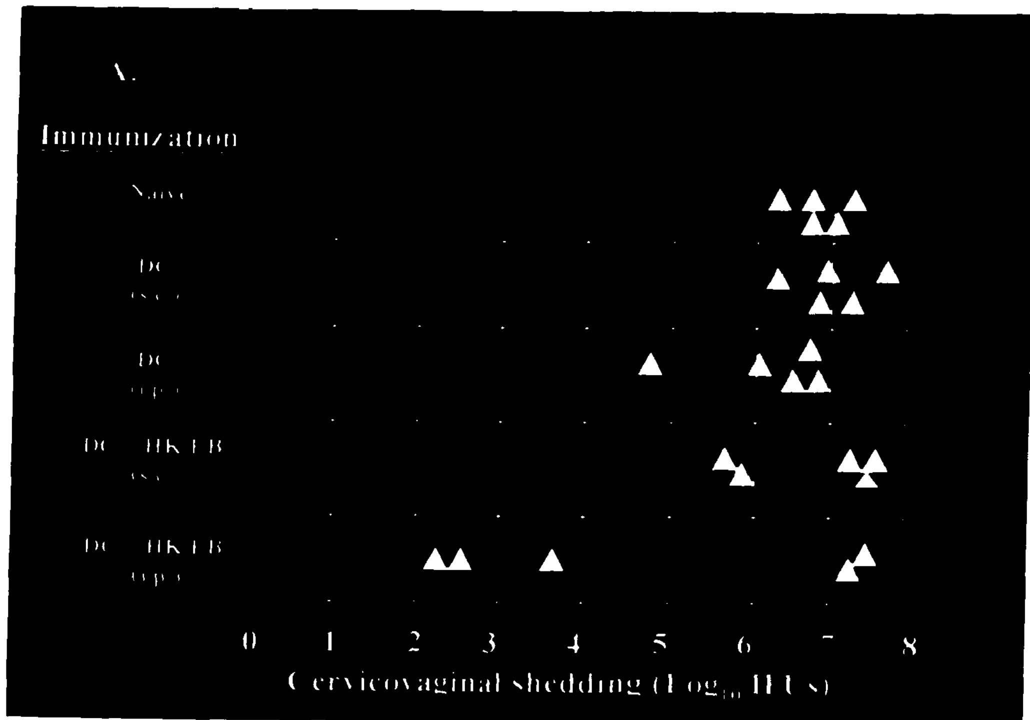

Figure 5.3. The level of protection following chlamydial challenge was variable in mice that received intra-peritoneal (i.p.) immunization using DC pulsed with HK EB. Naïve = no immunization. DC = unpulsed DC. DC + HK EB = DC pulsed with heat killed chlamydial EBs. (A) day 4 post challenge and (B) day 15 post challenge. Triangles represent individual mice. Subcutaneous injection = s.c.

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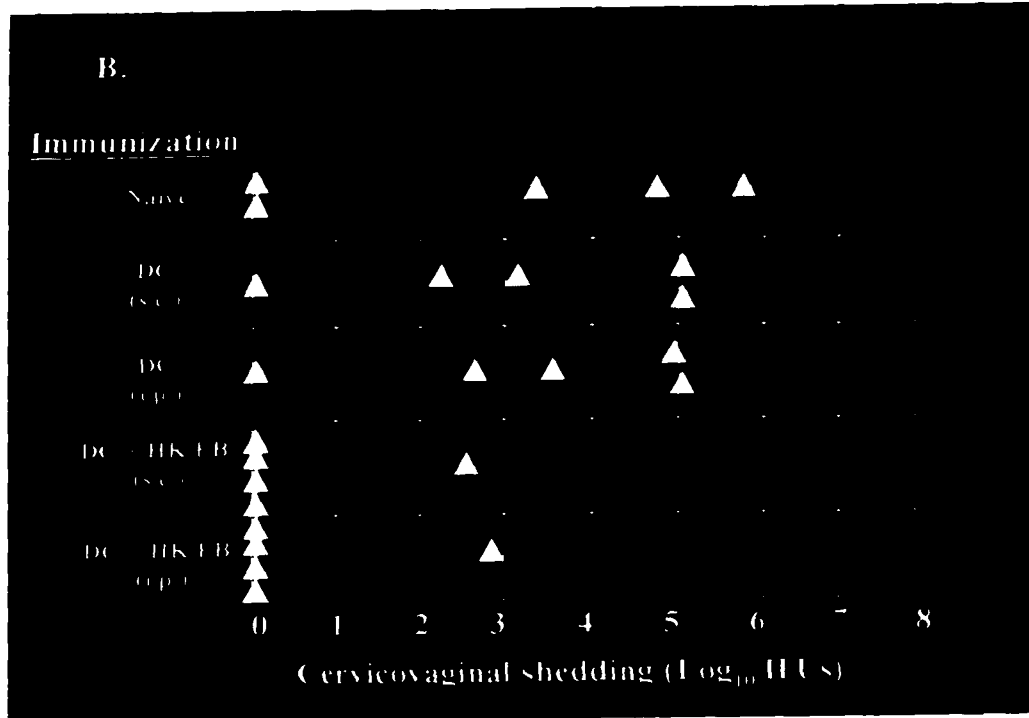
B.



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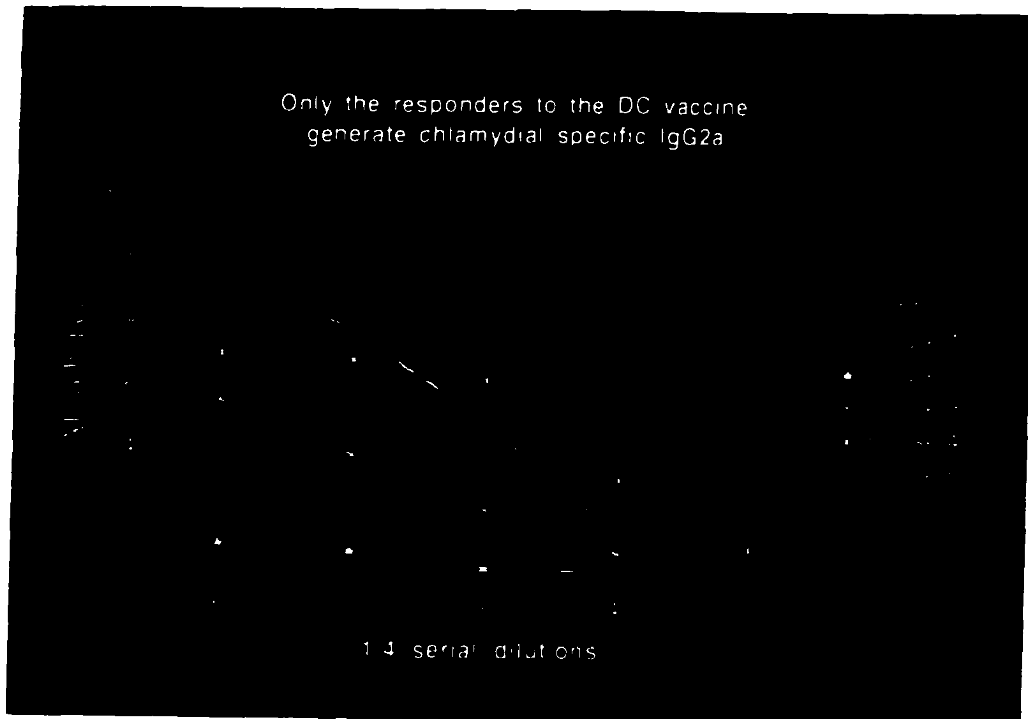

Figure 5.4. Protection correlates with the production of chlamydial specific serum IgG2a antibodies in intra-peritoneal (i.p.) immunized mice. Measurement of IgG2a serum antibodies by ELISA in mice immunized (i.p.) with chlamydial pulsed DC prior to infectious challenge. Positive control = sera from infection sensitized mice. Mice # 2 and # 5 did not produce chlamydial specific serum IgG2a antibodies (above) and were not protected following intravaginal challenge with chlamydiae (Fig. 5.1). Mice #1, #3, and #4 produced serum IgG2a antibodies (above) and showed 2.1 logs protection following intravaginal challenge (Fig. 5.1).

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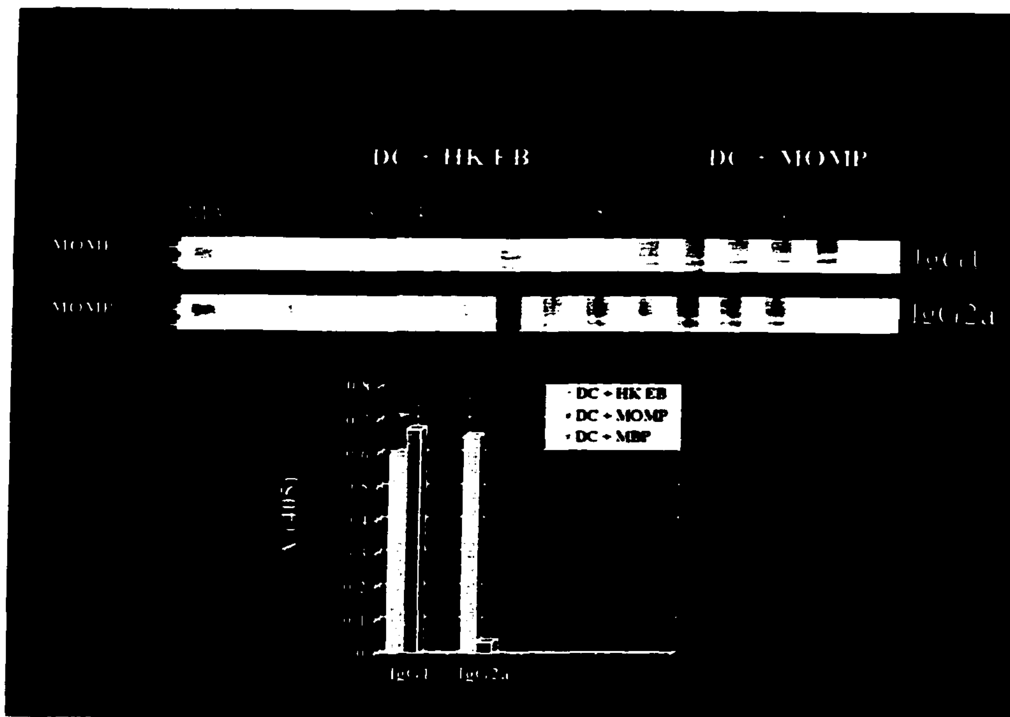

Figure 5.5. Antibody responses to immunization via i.v. with DC pulsed with HK EB or rMOMP . (A) IgG1 and IgG2a antibodies specific for denatured MOMP were detected in pre-challenge sera from all immunized mice as shown by Western blot analysis and (B) IgG1, but not IgG2a, antibodies that are specific to the EB surface were detected in pre-challenge sera from mice immunized using DC pulsed with rMOMP as shown by ELISA. Mice immunized using DC pulsed with HK EB produce equivalent amounts of both IgG2a and IgG1 to the EB surface. Negative controls were mice immunized with HK EB alone (no DC vehicle) and unpulsed DC: neither group produced chlamydial specific IgG2a or IgG1 antibodies (data not shown). Experiments were done in triplicate. MAb to MOMP shows that MOMP runs as a doublet of 40 kD and 38 kD.

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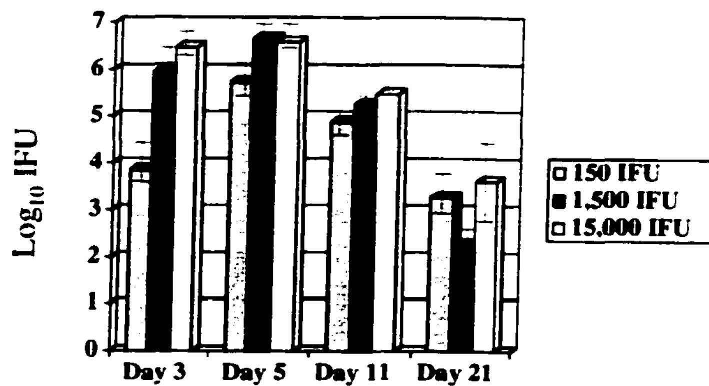

Figure 5.6. Course of infection in naïve mice infected with three different amounts of IFU of chlamydiae. Mice were infected with 15,000, 1,500 or 150 IFU and cultured for 28 days by cervico-vaginal swabbing (day 3, 5, 11, and 21 shown above). Recoverable chlamydial IFU is expressed as $\log_{10}$ IFU on the y axis. All mice resolved infection after 21 days.

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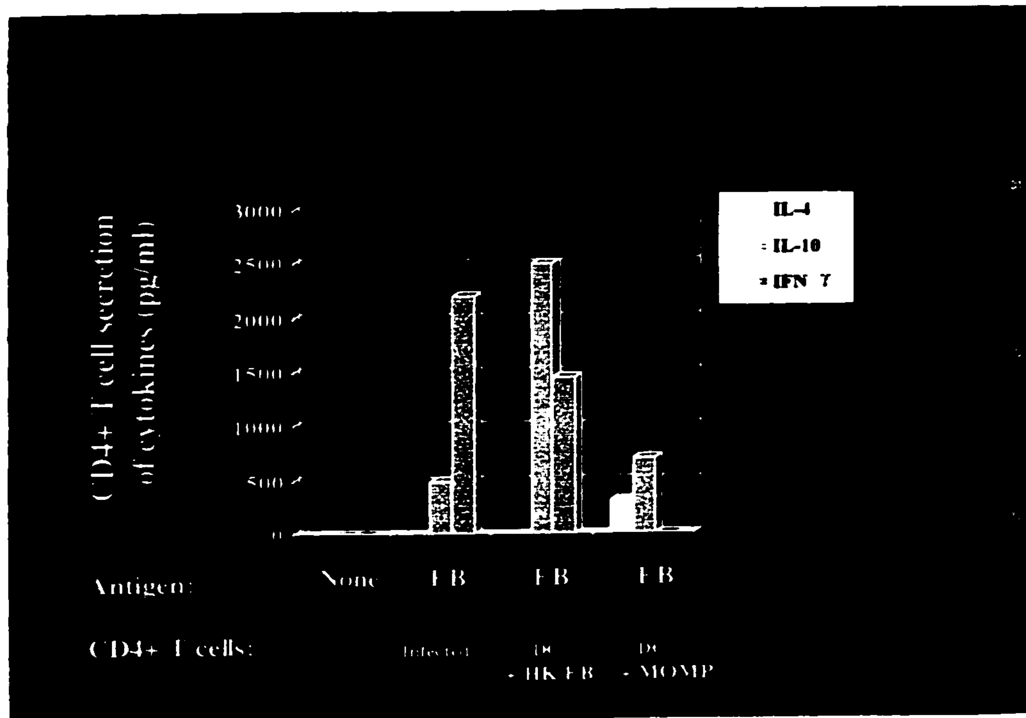

Figure 5.7. Immunization of mice with rMOMP pulsed DC elicits a predominant Th2 response. $CD4^+$ T cells were harvested from infection sensitized mice, mice immunized 2X chlamydial pulsed DC, and mice immunized 2X with rMOMP pulsed DC. $CD4^+$ T cells were incubated in the presence of either antigen presenting cells (APC) alone (no Ag) or APC pulsed with whole EB Ag and supernatants were collected at 24, 48 and 72 h. Supernatants were analyzed by ELISA to detect levels of IL-4 (Th2), IL-10 (Th2) and IFN-γ (Th1). Results shown here are from 72 h supernatants. Experiments were done in duplicate and are expressed as pg/ml +/- SD.

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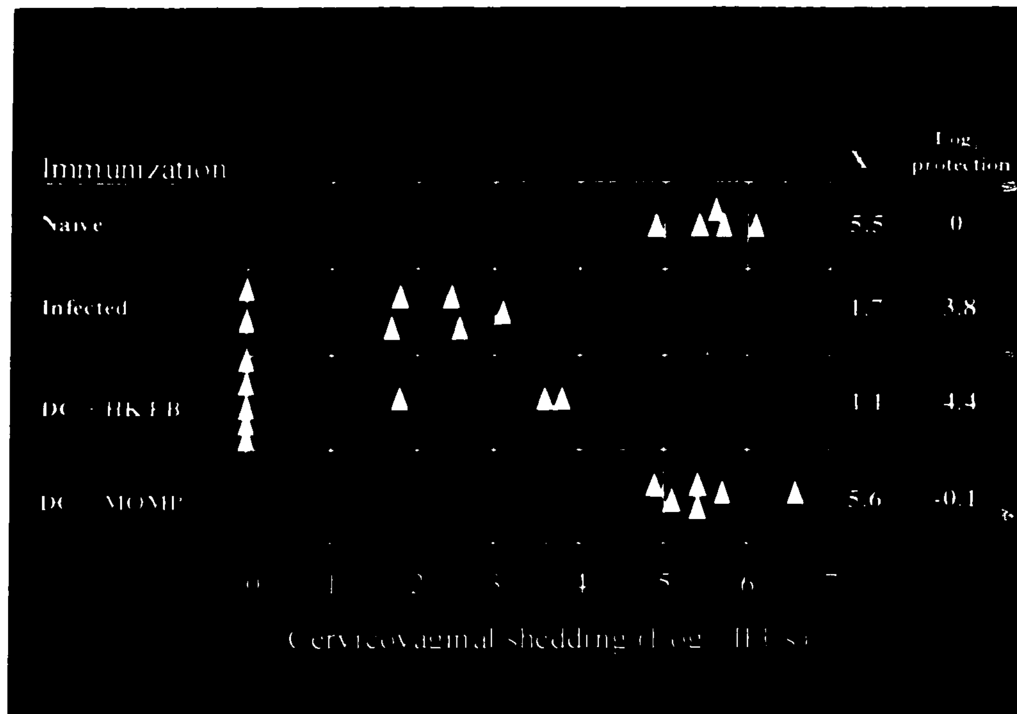

Figure 5.8. Mice immunized using DC pulsed with rMOMP were not protected following intravaginal challenge. Mice were given no immunization (naïve), infected, immunized using DCpulsed with heat killed EBs (DC + HK EB), and immunized with DC pulsed with rMOMP (DC + MOMP) then challenged with 150 IFU. The number of organisms shed ($\log_{10}$ scale) from the cervico-vagina 7 days post challenge is shown on the x axis above. Triangles represent individual mice.

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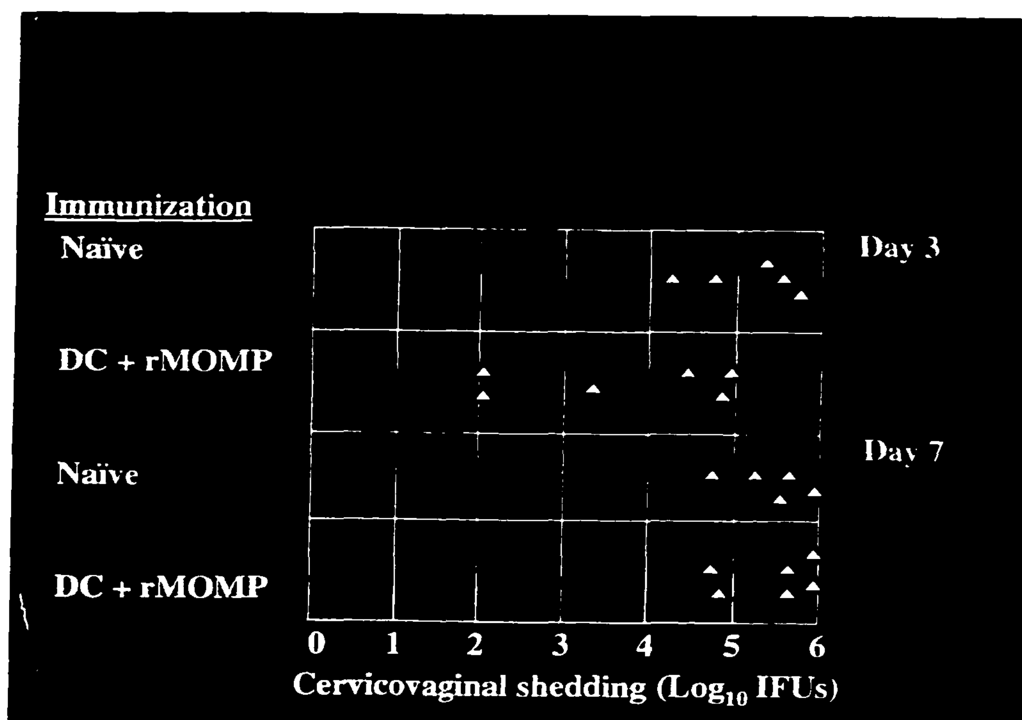

Figure 5.9. Immunization via i.p. using DC pulsed with rMOMP elicited marginal and transient protection in some mice following intavaginal challenge. Mice received no immunization (naïve) or immunized (i.p.) using DC pulsed with rMOMP (DC + rMOMP) and challenged with 150 IFU. Recoverable chlamydial IFU (log_{10} scale) is quantified at 3 and 7 days following intravaginal challenge and shown on the x axis. Triangles represent individual mice.

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Chapter 6: Discussion

A vaccine to prevent chlamydial infection is needed but has proven difficult to produce using conventional vaccination approaches. Potent protective immunity to vaginal re-challenge in a murine model of chlamydial infection has only been achieved by infection or by immunization with DC pulsed ex vivo with whole inactivated organisms. Immunity generated by infection or ex vivo antigen (Ag) pulsed DC correlates with a chlamydial specific CD4+ Th1 immune response although the precise effector mechanisms of this immunity are not known. Because of the potent anti-chlamydial immunizing properties of DC it was hypothesized that DC could be a powerful vehicle for the delivery of individual chlamydial Ags that are thought to be potential targets for more conventional vaccine approaches. Here, the chlamydial major outer membrane protein (MOMP) was investigated as a target Ag. The overall objectives were to extensively characterize chlamydial pulsed DC, deliver chlamydial MOMP to DC and then immunize mice with DC bearing MOMP Ags to assess protection.

The mechanisms that enable chlamydial pulsed DC to successfully drive a potent level of protective immunity at the genital mucosa are not understood. However, there are several possibilities that could account for this protective immunity including (i) increases in DC Ag presentation and costimulatory molecules for activating naïve and effector T cells against chlamydial Ags, (ii) alterations in chemokine and chemokine

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receptor profiles that potentially enhance DC migration and recruitment/localization of other important cellular players, (iii) cytokine production by DC, or by T cells activated by DC, that favor a Th1 response, and (iv) DC activation of B cells to produce antibodies specific to chlamydiae.

(i) Phenotypic and Transcriptional Analysis of Chlamydial Pulsed DC

An accumulation of literature demonstrates the importance of Ag presentation accompanied by costimulation , chemokines, chemokine receptors and cytokines in orchestrating dynamic and complex immune responses against bacteria, viruses and tumors. Chemokine/cytokine ligands along with their respective receptors and functions are summarized in Chapter 1. It was hypothesized that the extensive characterization of the immunomodulatory effects of killed chlamydiae on DC would potentially provide the necessary information for the design of more conventional methods for vaccination against sexually transmitted chlamydial infection. To better understand the immunizing properties of chlamydial pulsed DC the following was studied; (i) changes in cell surface antigen expression on DC by FACS analysis, (ii) the kinetics of chemokine, chemokine receptor and cytokine gene expression by RNase Protection Assay (RPA) and, (iii) DC migration *in vivo*.

For several years progress in the DC field was hindered by the lack of DC culturing and isolation techniques. However, it is now possible to culture large numbers of primary DC from bone marrow or peripheral blood by inducing growth and

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differentiation with GM-CSF. DC can be induced to differentiate from myeloid precursors upon treatment with GM-CSF (76) or Flt-3 Ligand (77). "Skin DC," known as Langerhans cells (LC), can be amplified with transforming growth factor (TGF)-β (78). Although methods have been developed to culture murine DC, it is still a challenge to purify DC because reliable, unique DC markers have yet to be identified. Many of the DC markers (MHC II, CD80, CD83, CD86, CD11a/b, CD40, LFA-3, ICAM, Fc receptors, scavenger receptors, receptors for cytokines and chemokines) are also found on macrophages, monocytes or other cell types. In addition, there are many subsets of DC and several lineages which further compounds efforts to enrich a single population of DC (72). The various DC lineage pathways are summarized in Chapter 1. The results show successful enrichment of DC populations to >95% as shown by positive staining for known DC markers CD11b, CD86 and MHC II by FACS analysis. Although these cell surface molecules are also found on macrophages, the panning procedure eliminates the macrophages based upon the adherent nature of GM-CSF activated macrophages.

It has been well established that following Ag capture by immature DC there is a downregulation of receptors (CD68) for phagocytosis and a loss of adhesion molecules(79). The DC then upregulate MHC molecules and costimulatory molecules for Ag presentation and interaction with both T and B cells (54). Inducing factors and functional properties of DC maturation are summarized in Chapter 1. The data show phagocytosis/uptake of nonviable chlamydial organisms by DCs upregulates the expression of MHC II, CD86 and CD40. Conversely, DC uptake of latex beads does not

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affect expression of these molecules. Thus, the act of phagocytosis alone cannot be responsible for the alterations in DC surface expression of these immunomodulatory molecules. Therefore, dead chlamydial organisms have the capacity to stimulate DC for amplified Ag presentation and costimulation thereby providing the necessary stimuli to initiate the immune response. This suggests that chlamydiae do not need to be viable or replicate to elicit a protective immune response. Moreover, these results imply that an EB structural component could be responsible for initiating protective immunity and may therefore be important in the development of an efficacious vaccine.

It is known that culturing alone or simple physical manipulation (pipetting) can lead to spontaneous maturation of DC. This explains the mixed population of immature (lower MHC II expression) and mature DC (higher MHC II expression) in the absence of immunostimuli such as LPS or pulsing with heat killed EBs. Additionally, the expression of CCR7 by untreated DC indicates the onset of maturation during *in vitro* culture/growth.

The *E.coli* LPS positive control employed herein is routinely used to drive DC maturation. It is not suspected that chlamydial LPS is functioning similarly due its dramatically less potency (100 fold) than *E. coli* LPS as defined by the capacity to activate mononuclear phagocytes (80) and induce fever. The reduced endotoxicity has been correlated with structural differences within the chlamydial LPS moiety. These differences are as follows: reduced number of fatty acids (5 rather than 6 fatty acyl groups), fatty acyl groups longer than C_{14}, normal fatty acyls groups instead of hydroxy

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fatty acids and an enlarged hydrophobic domain in the lipid A (81). The effects of chlamydial LPS on DC maturation were not tested because of the difficulty in purifying significant amounts of chlamydial LPS. It is currently unknown which chlamydial components are responsible for DC maturation and will require further studies. It was recently shown that chlamydial heat shock protein 60 (hsp60) activates monocytes/macrophages and endothelial cells (80). Perhaps chlamydial hsp60 also activates DC and potentially leads to DC maturation.

The ability of chlamydial pulsed DC to migrate to the nodes and spleen is crucial for localizing the DC to the appropriate cellular players (T and B cells) to generate a protective immune response against chlamydia. The data show that nonviable chlamydia induce the immunomodulatory genes that encode MIP-1α, MIP-2, MIP-3α, TNF-α, IL-1α, IL-1β, IL-1RA IP-10, IL-6, IL-12p40 and CCR7. These gene products have been shown in other systems to affect DC migration and interaction with other cells. It was found that nonviable EBs induce DC to migrate to the lymph nodes *in vivo*, are potent transcriptional activators of DC and the induced gene expression is temporally regulated. The kinetics of chemokine, chemokine receptor and cytokine gene expression are summarized in Chapter 3.

Knowledge of this gene expression profile may increase our understanding of the DC immunizing properties. The chemokine MIP-1α binds to CCR1 and CCR5, which are found on immature DC, monocytes, activated T cells and macrophages. MIP-1α is

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chemotactic for immature DC, monocytes and T cells while serving as an activation signal for macrophages to become cytotoxic against tumor targets. MIP-2, on the other hand, binds CCR8 which is only expressed on CD4+ and CD8+ T cells. DC expression of these two chemokines may allow recruitment of activated T cells to the site of infection or naïve T cells toward Ag bearing DC to aid in the response against chlamydiae. MIP-3α binds CCR6 to induce migration of CD34+ derived DC, memory T cells and γδ T cells from epithelial surfaces (69). Differential expression of MIP-3α by chlamydial pulsed mature DC may attract immature DC that have recently sampled Ag towards the nodes where the mature DC have already migrated. Also, memory T cells and γδ T cells could be drawn towards chlamydial Ag bearing DC for activation. The expression of IP-10 by chlamydial pulsed DC suggests an ability to chemoattract activated T cells. This chemokine binds the CXCR3 receptor which is found on IL-2 activated T cells. This chemokine has recently been reported to chemoattract B cells, monocytes and NK cells as well (82).

Immature DC have been shown in other systems to express the following chemokine receptors: high CCR1, CCR2, CCR4, high CCR5, high CCR6 (LC), CXCR1, CXCR4 and low CCR7 (refer to Chapter 1 for ligands to these receptors). Mature DC downregulate the aforementioned receptors and strongly upregulate CCR7 enabling DC to bind 6Ckine and MIP-3β which will induce migration toward the secondary tissues. High levels of CCR7 expression by DC were observed under all conditions at 2 and 24 h post

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treatment indicating that DC maturation is occurring with or without immunostimulus. High levels of CCR7 without immunostimulus likely reflect spontaneous maturation from cell culturing techniques. CCR7 expression remains high at 48 h for chlamydial pulsed DC however expression is no longer detected in unpulsed DC or DC pulsed with latex beads. This stable expression of CCR7 by chlamydial pulsed DC may extend the longevity of DC maturation thereby increasing DC migration into the T cell zone of the lymph nodes for eliciting an immune response against chlamydiae.

The cytokines IL-1α and IL-1β expressed by chlamydial pulsed DC are known mediators of inflammation and could therefore be responsible for initiating innate immune responses following antigenic insult. IL-1RA (which binds the IL-1 receptor with antagonist activity) expressed by chlamydial pulsed DC could serve to balance this innate inflammatory response thereby preventing an overreaction to chlamydial infection. DC expression of TNF-α, another pleiotropic cytokine, has been found to chemoattract immature DC as well as activate DC to maturity. It has also been implicated in the recruitment of DC from the bloodstream to the vaginal and cervical epithelium upon chlamydial infection . Immune responses to either chlamydial infection or vaccination with pulsed DC have been correlated with a strong Th1 bias thus the expression of IL-12p40 by chlamydial pulsed DC may function in skewing the immune response toward a Th1 phenotype.

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It is important to note that other chemokines such as RANTES and MCP are expressed without a stimulus other than continuous cell culture. This would suggest that there are different degrees or levels of DC maturity that are controlled according to the type of stimulus encountered. Transcripts that were not detected under any conditions were MIP-3β, TCA-3, IFN-γ, IL-18, IL-10 and IL-12p35 . The absence of the Th2 cytokine IL-10 expression by chlamydial pulsed DC may further facilitate a Th1 response.

Clearly, chlamydial pulsed DC differentially express several genes that encode proteins necessary for DC trafficking to the nodes, for recruitment of naïve and activated αβ T cells, γδ T cells, memory T cells, CD4+ T helper cells and CD8+ cytotoxic T cells. Chemical gradients provided by chlamydial pulsed DC allow for quick and efficient congregation of the necessary cell types to elicit protective immunity. This permits crucial DC-T cell interactions to occur that are responsible for initiating a cascade of cellular interactions whose products drive a predominant Th1 immune response against chlamydiae. From these studies it was concluded that; (i) killed chlamydiae are potent transcriptional activators of DC, (ii) gene expression by pulsed DC is temporally regulated, and (iii) transcriptionally activated genes encode proteins that promote DC migration and activation of naïve and effector T cells.

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(ii) Delivery of rMOMP to DC for Antigen Presentation

This work suggests that the DC is a crucial vehicle for the appropriate delivery of chlamydial Ags to elicit strong protective immunity against infection at the genital mucosa. Therefore it was hypothesized that the use of *ex vivo* activated bone marrow derived-DC could serve as a powerful vaccine delivery system to assess the immunizing properties of targeted protective Ags against chlamydial genital infection. The chlamydial major outer membrane protein (MOMP) was selected to investigate this working hypothesis. The rationale for using MOMP is: (i) outer membrane preparations have been shown to elicit partial protection at the genital mucosa (83), (ii) MOMP is an immunodominant Ag and target of neutralizing antibodies (16), (iii) MOMP plays a role as a cytoadhesion thus is intimately linked to chlamydial pathogenesis (15). Certainly there are other potential surface or secreted antigens to investigate and the DC vaccine procedure could be utilized to screen these other target Ags as well.

Recombinant MOMP was produced in *E.coli* using a maltose binding protein (MBP) fusion system and purified on amylose columns (15). The particulate colloid spheres (30-40 nm) of MBP-MOMP fusion protein were used to pulse DC. Immunofluorescence and confocal microscopy show that DC phagocytized recombinant MOMP (rMOMP) as illustrated by positive staining for MOMP within the cytoplasm. The rMOMP contained high levels (~300,000 U/ml) of *E.coli* endotoxin (LPS). It was decided that the contaminating LPS would not be removed from the rMOMP due to the ability of LPS to increase the adjuvanticity of rMOMP. This strategy is supported by

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the FACS and RPA data (see Chapter 3) showing that LPS strongly stimulates DC maturation. The DC expression profile for cell surface Ags, chemokines and cytokines are identical in DC pulsed with either LPS or HK EB. Therefore, the contaminating LPS will likely provide the necessary stimuli for DC maturation that otherwise may not have occurred in the presence of recombinant protein alone. It is also known that LPS drives DC to secrete IL-12, an important Th1 cytokine (72). DC pulsed with rMOMP secreted large amounts of IL-12 *in vitro* suggesting rMOMP pulsed DC have the potential to drive a strong Th1 immune response following adoptive immunization.

It has been shown that adoptive transfer of infection sensitized T cells confers protection against chlamydial intravaginal challenge (37). Therefore, whether DC could process rMOMP and present epitopes to infection sensitized T cells was investigated next. It was found that infection sensitized T cells recognized DC bearing MOMP Ag(s) and were induced to proliferate. This suggests that DC process rMOMP and present epitopes necessary to stimulate memory T cell expansion in response to chlamydial re-infection.

DC are known to phagocytose exogenous Ag with presentation onto MHC II. However, processed exogenous Ag can also cross over to the endogenous pathway for presentation onto MHC I. This process is termed 'cross priming.' The mechanisms that control this phenomenon are not understood. Infection sensitized CD4+ and CD8+ T cells were incubated in the presence of pulsed DC to determine whether DC uptake of exogenous rMOMP led to Ag presentation on MHC I, MHC II or both molecules. The

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data show that infection sensitized CD4+ but not CD8+ T cells proliferated in response to DC presenting rMOMP Ag(s). Therefore, it was concluded that rMOMP Ag(s) were presented onto MHC II molecules and drive a CD4+ T cell response. Proliferation of infection sensitized CD4+ T cells in response to DC pulsed with the MBP portion of rMOMP was observed although at a significantly lower level.

Next, supernatants were collected from infection sensitized CD4+ T cells in the presence of pulsed DC. The responding CD4 phenotype (Th1/Th2) was determined by measuring levels of selected cytokines produced by the activated T cells. DC pulsed with rMOMP, but not MBP, induced infection sensitized CD4+ T cells to produce high levels of IFN-γ, a known Th1 cytokine. Conversely, lower levels (5X) of the Th2 cytokine IL-10 were produced by T cells in the presence of DC pulsed with rMOMP or MBP. IL-4, another Th2 secreted cytokine, was not detected. Therefore, the infection sensitized CD4+ T cells respond to rMOMP pulsed DC in a MOMP-specific manner with respect to IFN-γ production. This MOMP specific response parallels the CD4 Th1 response necessary for the clearance of genital chlamydial infection. From these results, it was concluded that rMOMP; (i) is readily endocytosed by DC, (ii) drives DC to secrete IL-12 (iii) is processed and presented onto MHC II molecules, (iv) stimulates immune CD4+ but not CD8+ T cells to proliferate and, (v) promotes the production of IFN-γ by activated CD4+ T cells.

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(iii) Immunization using DC pulsed with rMOMP

The *in vitro* data demonstrate that DC pulsed with rMOMP secrete very large amounts of IL-12 and present the appropriate epitopes to infection sensitized CD4+ Th1 cells driving proliferation and secretion of IFN-γ. Collectively, these data suggest that DC pulsed with rMOMP have the capacity to drive a potent anti-MOMP Th1 immune response. Interestingly, adoptive immunization using DC pulsed with rMOMP elicited a Th2 immune response. CD4+ T cells from rMOMP immunized mice responded to EB Ag by secretion of IL-4 and IL-10, but not IFN-γ. These mice also generated a predominant IgG1 serum antibody response specific to the EB surface. This Th2 biased immune response failed to elicit protective immunity following chlamydial challenge.

In this work and as shown by others, *ex vivo* Ag pulsed DC used to adoptively immunize mice promote T cell production of IL-10 (52, 84). In the case of mice immunized with chlamydial pulsed DC, there is enhanced T cell secretion of IL-10 as compared to T cells obtained from chlamydial infected mice (52). However, T cells in both cases produce large amounts of IFN-γ and elicit an overall Th1 biased response that is protective following chlamydial challenge. IFN-γ drives Th1 responses and concomitantly inhibits Th2 cells (85). T cell production of IFN-γ in mice either infected or immunized with chlamydial pulsed DC may serve to override the presence of IL-10 resulting an overall Th1 response that is protective. T cell secretion of IL-10 in rMOMP

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immunized mice, in the absence of IFN-γ production, may be responsible for the observed Th2 response.

DC pulsed with rMOMP secrete large amounts of IL-12 suggesting these cells will potentially induce a Th1 response following adoptive immunization. However, immunization using rMOMP pulsed DC induced a Th2 response. Recent studies demonstrated that IL-12 secreting DC, which promote Th1 biased immune responses, were switched to DC that induced a Th2 response or T cell anergy (72). This observed switch is not currently understood, however it was shown to occur in response to IL-10, prostaglandin E2, or steroids. In the absence of IFN-γ production, the secretion of IL-10 in response to immunization using rMOMP pulsed DC may be sufficient to drive this switch and offers a possible explanation for the observed Th2 response.

T cells from infected mice or mice immunized with chlamydial pulsed DC did not secrete IL-4, a potent Th2 cytokine. In contrast, T cells obtained from mice immunized with DC pulsed with rMOMP secreted ~300 pg/ml of IL-4 and were not protected following chlamydial challenge. An IL-10 induced switch may possibly be responsible for DC activation of T cell differentiation into Th2 cells known to secrete cytokines such as IL-4. Perhaps, an IL-10 threshold has been met allowing the IL-12 secreting DC to switch to DC that induce differentiation into Th2 cells that secrete IL-4. T cell secretion of IL-4 was only observed in the mice immunized with DC pulsed with rMOMP lending support to the observed Th2 response in these mice.

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Although our rMOMP pulsed DC were primed *ex vivo* to drive a Th1 response, following adoptive immunization the rMOMP pulsed DC elicited a potent Th2 response that was not protective. The complex nature of DC may require additional steps such as co-administration of anti-IL10 receptor antibodies or anti-IL-4 receptor antibodies. Igietseme, et al ((84)) have shown increased efficacy of adoptive immunization using DC pulsed with HK EB in IL-10 KO mice. Abrogating an IL-10 response may allow DC pulsed with rMOMP to generate an appropriate Th1 response following adoptive immunization. Alternatively, cytokine therapy using IFN-γ may skew the immune response towards the needed Th1 phenotype. It was concluded that delivery of MOMP to DC for immunization and determination of protective capabilities is possible however the plasticity of DC, with regard to the ability to drive a Th1 versus Th2 response, will require additional measures to drive the appropriate response.

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Chapter 7: Summary and Conclusions

The need for a vaccine to control chlamydial sexually transmitted disease has been addressed in this work. The fact that conventional methods of vaccination have failed to elicit protective immunity at the genital mucosa led to the study of a less conventional approach. This approach used *ex vivo* activated DC to vaccinate against chlamydial infection. The DC approach is currently the only known means to develop potent protective immunity against *C. trachomatis* at the genital tract other than infection. Protective immunity generated by infection or *ex vivo* antigen pulsed DC in a murine model correlates with a CD4+ Th1, IL-12 dependent immune response, however the precise effector mechanisms of this immunity are not understood (32, 37, 40, 41, 44, 86). In this work, the DC approach was characterized and manipulated in order to test the hypothesis that the use of *ex vivo* antigen pulsed DC could serve as a powerful vehicle for the delivery of individual chlamydial antigens that are thought to be targets for more conventional vaccine approaches. Chlamydial MOMP was selected as a target antigen to investigate.

Specific aims:

1. To characterize DC expression of cell surface antigens, chemokines, chemokine receptors, and cytokines following pulsing with whole inactivated EBs
2. To deliver chlamydial MOMP to DC and elucidate the antigen processing/presentation pathways and the subsequent DC cytokine secretion profile.

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3. To immunize mice with MOMP pulsed DC and assess the protective capacities of chlamydial MOMP.

Conclusions:

1. DC pulsed with whole nonviable EBs upregulated cell surface expression of MHC II, CD86 and CD40.
2. DC pulsed with whole nonviable EBs differentially expressed MIP-1α, MIP-2, TNF-α, IL-1α, IL-1β, IL-1RA, CCR7 between 2 - 48 hours; IP-10 and IL-6 between 12 – 24 hours; MIP-3α and IL-12p40 between 12 – 48 hours.
3. DC efficiently phagocytized recombinant MOMP colloid spheres with subsequent secretion of large amounts of IL-12.
4. DC pulsed with recombinant MOMP presented MOMP antigens onto MHC II molecules and stimulated infection sensitized CD4+ T cells to proliferate and produce large amounts of IFN-γ.
5. The intravenous (infusion into the retro-orbital sinus) route of DC vaccine administration was found to be superior to the subcutaneous and intra-peritoneal routes.
6. Variability in the levels of protective immunity generated by DC pulsed with whole inactivated EBs by intra-peritoneal injection correlated with chlamydial specific serum IgG2a antibody production.

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7. Adoptive immunization of DC pulsed with recombinant MOMP elicited a Th2 immune response as shown by:

    (a) vaccine sensitized CD4+ T cell secretion of IL-4 and IL-10 in response to whole EB antigen

    (b) production of serum IgG1 antibodies specific to the EB surface

8. Adoptive immunization of DC pulsed with recombinant MOMP was not protective following intra-vaginal chlamydial challenge.

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