MODULATION OF IMMUNE RESPONSES UPON PROGESTERONE ADMINISTRATION AND VACCINE POTENTIAL OF A 5-FLUOROURACIL RESISTANT MUTANT IN A MURINE RESPIRATORY INFECTION OF *CHLAMYDIA MURIDARUM*

By

KAMALA DEEPTHI MANDA

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

OF

MASTER OF SCIENCE

I dedicate this thesis to my parents

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ABSTRACT

Immune responses to chlamydial infections are poorly understood due to the complex intracellular lifestyle of the bacterium and the lack of genetic manipulation tools. A clear understanding of the role of immune response to infection is mandatory for generation of a successful chlamydial vaccine. My project focuses on two aspects (i) study the effect of progesterone administration on immune responses to chlamydial infection in mice and (ii) test the vaccine potential of a metabolic mutant of *C.muridarum.*

Progesterone, a steroidal sex hormone produced by the ovaries and the placenta, has major roles in the modulation of host immune responses both locally and systemically. This hormone has a well documented role in enhancing susceptibility of the host to the development of a wide range of infections by means of either immunosuppression or by tipping the balance towards a non-protective Th2 response. Mouse genital tract infection models of *C.muridarum* routinely employ progesterone pretreatment regimens to facilitate the establishment of infection.

Female, age matched, C57Bl/6 mice were injected subcutaneously with either one or two doses of Depo-Provera, a long acting progestational formulation, prior to infection with *C.muridarum* and immune response was studied to compare the dose effect on host susceptibility to infection. Immunity to chlamydial infection is dominated by a protective Th1 response typically characterized by the production of the cytokine, IFN-γ. Hence, cytokine production in culture supernatants of antigen stimulated spleen and lymph node cells and lung homogenates was determined by sandwich ELISA. We further investigated the bacterial burden in the lungs by *in vitro*

infectivity assays. Serum levels of anti-chlamydial antibody was quantitated to study the effect of the hormone on humoral immunity. In addition, histopathological changes in the lungs were examined to determine the severity of the disease.

IFN-γ expression was significantly decreased, IL-10 production was increased and the mice suffered greater bacterial loads in the lung together with severe pathological changes in the lung. Taken together, these results indicate that progesterone pretreatment at higher doses alters the immune response to chlamydial infection. Our data suggest that progesterone has marked immunosuppressive effect when administered in higher doses in comparison to a single dose pretreatment. These results have important implications for the development of a successful chlamydial vaccine which also utilize progesterone pretreatment regimens for vaccine efficacy studies in mice.

In the second part of the study, a metabolic mutant of *C.muridarum* was analyzed for its potential as a vaccine. Chlamydiae are obligate intracellular parasites and hence are auxotrophic for three of the four ribonucleoside triphosphates. Among the few differences that exist between *C.muridarum*, a mouse pathogen and *C.trachomatis*, a human pathogen is that the former synthesizes an enzyme called Uracil phosphoribosyltransferase (UPRT) encoded by the *upp* gene that is necessary for the mouse pathogen to maintain its UTP pools. UPRT catalyzes the synthesis of Uracilmonophoshpate (UMP) and also catalyzes the formation of the cytotoxic 5-Fluorouracil monophosphate (5-FUMP) from 5-Fluorouracil (5-FU). Mutations in the *upp* gene were generated by growing the bacterium in the presence of increasing concentrations of 5-FU, in a mammalian cell line (CHO UrdC-) that is resistant to the

toxic effects of 5-FU. Only Chlamydiae that have an inactivating mutation in the *upp* gene can survive the toxic effects of 5-FU. One such particular mutant that has a point mutation in its upp gene was isolated and was studied *in vivo* in a mouse respiratory infection model.

Body weight loss, bacterial load in lungs, humoral and cell mediated immune responses and histopathological changes in the lungs of mice were studied in the WT verus *upp* mutant infection. Interestingly, the *upp* mutant was found to be significantly attenuated in mice with minimal damage to the host. On the other hand, the mutant grew equally well in the mammalian HeLa229 cell line. These results prompted us to study the vaccination efficiency of this mutant in the mouse respiratory model. For this purpose we immunized young female, BALB/c mice with a small dose of either the mutant or WT chlamydia and all mice were challenged with a high dose of WT *C.muridarum* following complete recovery from primary infection. Impressively, all mice that were challenged developed sterilizing immunity and exhibited resistance to reinfection. Collectively, these results indicate that mutations in the metabolic pathways of chlamydial species can attenuate the bacterium and therefore a live attenuated vaccine is likely an achievable goal in human chlamydial vaccine development.

INTRODUCTION

General

Chlamydia spp are gram-negative, obligate intracellular eubacteria (1). Unlike viruses, they have both RNA and DNA, ribosomes, a cell wall, and divide by binary fission. They differ from most true bacteria in that they do not have peptidoglycan in their cell wall and lack the ability to produce their own Adenosine triphosphate (ATP), which is therefore obtained from the host cell. Hence, they are called "energy parasites". The family *Chlamydiacaeae* is divided into two genera *Chlamydia* and *Chlamydophila* (2). In humans, disease is primarily caused by *Chlamydia trachomatis* and *Chlamydophila pneumoniae. C. psittaci* is a pathogen of birds and many other animal species. *C. pecorum* mainly infects ruminants. *C. pneumoniae* causes acute respiratory disorders and is the causative agent for 10% of community acquired pneumonia. A chronic infection with *C. pnuemoniae* has been strongly associated with the development of cardiovascular diseases (3, 4). *C. muridarum* (earlier identified as *C. trachomatis* biovar mouse pneumonitis) was originally isolated from the lungs of mice (5, 6) and is widely used in genital tract infection models of mice to study the innate and adaptive immune responses to infections with *C. trachomatis*. *C. muridarum* is not a human pathogen. *C. trachomatis* primarily causes ocular and genital infections in humans. The mucosae of the conjunctiva and genital tract are the sites of invasion. Infections are largely associated with tissue inflammation and pathology. Although infected individuals can effectively be treated with antibiotics

Species	Disease	Host
Family: *Chlamydiacaeae*		
Genera: *Chlamydia*		
Species: *Chlamydia trachomatis*	trachoma, lymphogranuloma venereum, genital tract infection pelvic inflammatory disease	humans
C. suis	conjunctivitis, enteritis, pneumonia, genital tract infection	ruminants and swine
C. muridarum	pneumonia, experimental respiratory, ocular and genital tract infections in animal models	mice, hamsters and guinea pigs
Genera: *Chlamydophila*		
Species: *Chlamydophila pneumoniae*	acute respiratory infections, COPD, asthma, atherosclerosis	humans
C. abortus	ovine enzootic abortion, spontaneous abortion or still births in pregnant women	sheep, cattle, horses, pigs and swine
C. pecorum	pneumonia, polyarthritis, conjunctivitis, enteritis, metritis, mastitis and encephalomyelitis	ruminants, swine and marsupials
C. felis	feline chlamydiosis	cats
C. psittaci	psittacosis;respiratory distress, sinusitis, rhinitis & conjunctivitis	birds and poultry
C. caviae	experimental respiratory, ocular and genital tract infections in animal models	guinea pigs

Table 1: List of species belonging to the two genera Chlamydia and Chlamydophila, diseases they cause and their hosts.

like tetracycline derivatives and macrolides, more than 80% of infections are asymptomatic. Hence, untreated persistent infections of the genital tract in women result in the development of post-infection complications such as pelvic inflammatory disease (PID), ectopic pregnancy and tubal occlusion which ultimately leads to infertility and chronic pelvic pain. In men, urethritis and epididymitis can lead to infertility when left untreated. *C. trachomatis* also causes trachoma which affects the upper inner eyelid and the cornea. Trachoma is the leading cause of preventable blindness in the developing world especially in African countries. Neonates generally contract the disease from infected mothers, while passing through the birth canal at the time of delivery. In such infants, *C. trachomatis* causes eye infection, 'ophthalmia neonatorum', and severe pneumonia. A number of serovars have been identified and isolated from humans based on their serological responses to the major outer membrane protein (MOMP). Serovars A, B, Ba and C have been associated with endemic trachoma and serovars D through K are predominantly associated with urogenital infections. Significant mortality and morbidity is associated with diseases caused by Chlamydiae. According to the World Health Organization, 90 million of the 500 million new sexually transmitted diseases occurring worldwide annually are caused by *C. trachomatis* alone (7).

Developmental Cycle of Chlamydiae

Chlamydial species are among many pathogenic bacteria and parasites that grow within a membrane bound vacuole inside the host cell after infection. Among the others are *Mycobacterium tuberculosis, Coxiella burnetii, Legionella pneumophilua,*

Salmonella species, Leishmania donovani, *Plasmodium lophuhrae*, and *Toxoplasma gondii* (1). One of the most characteristic features of Chlamydia spp is its biphasic developmental cycle. Chlamydial life cycle consists of two distinctive forms- extracellular, osmotically stable, metabolically inert, infectious Elementary Bodies (EB) and intracellular, osmotically sensitive, metabolically active, non-infectious Reticulate Bodies (RB). The EBs are small (300-350nm) and have a rigid cell wall consisting of extensively cross linked cysteine-rich outer membrane proteins found in the cell envelope. The elementary body upon attachment to its host cell is phagocytosed. It remains within the phagosome and prevents fusion with lysosomes by an unknown mechanism and thereby evades degradation by the lysosomal contents. Subsequently, 6-8 hrs after entry into the host cell, the EB differentiates into a much bigger RB (800-1000nm). Early in the cycle many ribosomes are seen followed by reorganization of DNA into cores for the progeny EBs. RB divides by binary fission resulting in about 100-1000 RBs in an individual inclusion. At this point, the inclusion occupies the majority of the host cell cytoplasm. Binary fission is followed by the RBs being differentiated back into EB, which are ultimately released by lysis of the infected host cell. The cycle is asynchronous as both EBs and RBs exist together at any one point of the cycle (Figure 1). In the presence of anti-chlamydial factors, such as antibiotics or IFN-γ and under nutrient deprivation, chlamydiae assumes an aberrant, non-replicating and persistent form which can later be reactivated in response to unknown stimuli (8, 9). This strategy is similar to the lysogenic state of viruses except that chlamydial DNA is not incorporated into the host chromosome. Chlamydia lacks the ability to carry out energy metabolism and was initially

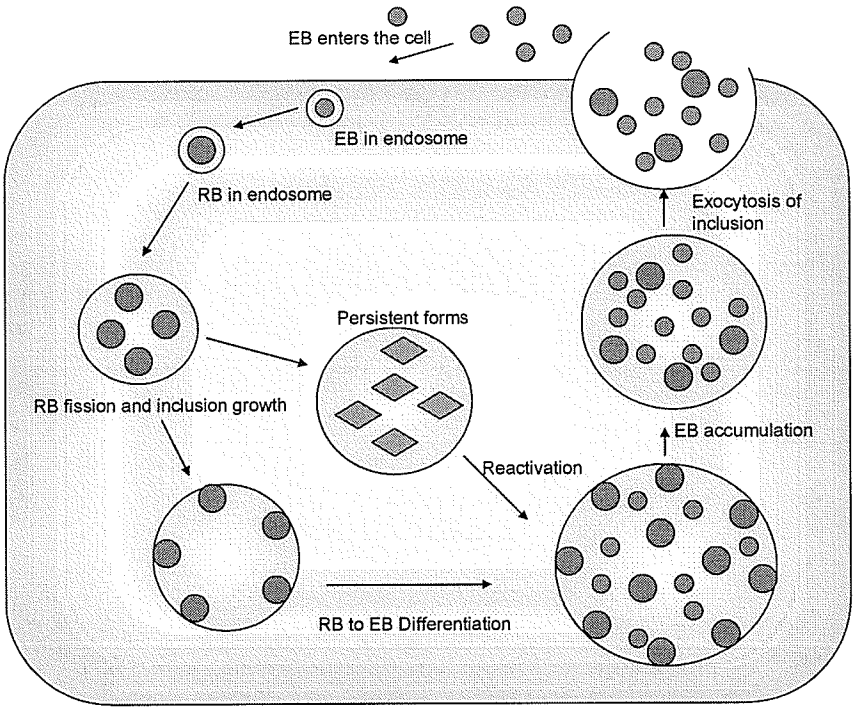


Figure 1: Development cycle of Chlamydial species.

The replication cycle of Chlamydia involves two developmental forms EBs and RBs. EBs are extracellular, metabolically inactive form that attach to the host cell and are endocytosed. Within the endosome, the EB is differentiated into a metabolically active RB. RB undergoes binary fission and the DNA is organized into cores which are further differentiated back into the infectious EBs. In the presence of anti-chlamydial factors such as antibiotics, IFN-g or nutrient deprivation, chlamydia assume an aberrant persistent form which may reactivate to known stimuli.

thought to be devoid of many biosynthetic pathways and that they are majorly dependant on the host cell to supply them with ATP and other high-energy intermediates. *C. trachomatis* is auxotrophic for at least three out of four ribonucleoside phosphates (10). Nevertheless, several enzymes of energy producing pathways such as pyruvate kinase, phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase and glucose-6-phosphate were identified and found to be functional when cloned in *E.coli* indicating that *C. trachomatis* is capable of carrying out ATP synthesis and generating its own reducing power (11). The presence of functional enzymes in the bacterium offers a possibility for the generation of attenuated metabolic mutants.

Th1/ Th2 hypothesis

In an adaptive immune response, CD4+ and CD8+ T cells are the major cell types involved in mediating cellular immunity against pathogens via the elaboration of a wide range of cytokines which have immediate effects on the resolution of infection. After an encounter with the antigen, following activation, CD4+ T helper lymphocytes undergo differentiation into either a Th1 type or a Th2 type, depending upon the cytokine milieu in the micro environment (12, 13). Other important factors which also determine the differentiation pattern are type of antigen presenting cell (APC), dose of antigen (14, 15), co-stimulatory molecules expressed, affinity, duration of exposure and the nature of the peptide:MHC class II complexes . Both Th1 and Th2 cells differ in cytokine production and hence in their function. Th1 cells are primarily involved in combating viruses and other intracellular pathogens, eliminating cancerous cells, and

stimulating delayed type hypersensitivity (DTH) reactions. Moreover, Th1 cells activate the macrophages and induce the B- cells to produce opsonizing antibodies such as IgG. On the other hand, Th2 cells upregulate the production of neutralizing antibodies which are important in fighting extracellular organisms. IFN-γ, Tumor necrosis factor- β (TNF- β), IL-2 and IL-12 are typical of a type 1 response. Whereas, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 are typical of a type 2 response. Moreover, over-activation of either the Th1 response or a Th2 response can cause disease. In autoimmune diseases like insulin-dependent diabetes mellitus, Th1 cytokines are attributed to the expression of the disease whereas Th2 cell and their cytokines have pathological effects in allergic disorders, systemic lupus erythematosus etc.,. Th2 cytokines enhance antibody production, particularly IgE, and also promote eosinophil proliferation and function.

Although, the Th1 and Th2 cytokine patterns are well documented, other cytokine patterns were observed. Cells expressing high amounts of TGF- β are termed as Th3 cells and those cells producing excessive amounts of IL-10, exhibit regulatory activities are termed as Tr1 cells. Th0 cells are described as those which produce both Th1 and Th2 cytokines without a predilection towards one or other.

Apart from Th1 and Th2 cells which express their respective set of cytokines, other cells of the immune system also follow the Th1 and Th2 like cytokine pattern. For instance, mast cell, B-cells, basophils, and CD3+ CD4+ NK1.1 cells produce IL-4 and possibly other Th2 like cytokines. Furthermore, NK cells produce IFN-γ and TNF-α, which are Th1 like cytokines. Though the cells retain their cytokine pattern *in vivo*, certain environmental cues can cause transient alterations in this pattern. Several

nutrients and hormones measurably influence the Th1/Th2 balance. Among them are plant sterols, melatonin, probiotics, progesterone and the minerals selenium and Zinc. Similarly, depletion of intracellular glutathione polarizes towards a Th2 dominance. Both type 1 and type 2 responses reciprocally regulate each other and the outcome of the disease unequivocally depends on the appropriate type of response elicited during the infection. Certain genetic factors of an individual may influence the ability to mount appropriate cellular responses against infections (16).

Immunity to Chlamydia

In a human genital tract infection with *C. trachomatis*, the disease is self-limiting and is mostly asymptomatic though the course of infection can last for many months (17). In majority of infected individuals, development of persistence and reinfections is very common and often contributes to the pathological changes observed in the host (18). However, immunity induced by a single exposure to Chlamydia induces protection that is only short lived and also does not protect from reinfections (19). Chlamydial infection in mice resolves naturally in about 3-4 weeks with the development of long-lived adaptive immunity to reinfection. Genital tract and respiratory tract infections in mice with *C. muridarum* serve as excellent models for the study of disease pathology and immunobiological features of immunity to *C. trachomatis*. In the respiratory model of *C. muridarum*, infection is mostly limited to the lung but can disseminate to other major organs such as heart, liver and kidney. The persistent nature of the chlamydial infections is attributed to the fact that the organism

Table 2: The roles of immune cell types in protection against chlamydial infections.

Cells of the immune system	Role in Chlamydia Immunity
CD4+ T Cells	Produce pro-inflammatory Th1 cytokines (IFN- γ, IL-2,TNF-a) that induce anti microbial systems
CD8+ T Cells	IFN- γ, required but not sufficient
Dendritic Cells	Primary antigen presenting cells, produce IL-12, enhanced Costimulation via ICAM-1, LFA-3, CD40 and B7 molecules
Macrophages	Phagocytosis of infected cells, generation of toxic NO intermediates, ADCC
Epithelial Cells	Pro-inflammatory chemokines such as CXCL1, CXCL8, CXCL16, GM-CSF, IL-1a, IL-6 and TNF.
B-Cells	Antibody ADCC, neutralization, FcR- mediated rapid Th1 activation, APCs, important in resistance against reinfection, local IgA
NK cells	Cytokines like IFN-γ , induction of antimicrobial agents, ADCC

has developed a number of immune evasion strategies to thrive in the host cell. Chlamydia survives inside the host cell by secluding itself in an inclusion and thus inhibiting access to antibodies and the host defense apparatus. The bacterium has evolved to minimize recognition by the inflammatory and adaptive immune effector mechanisms induced by the host in response to infection, persist as alternative intracellular forms and express genes that suppress the growth inhibitory effect of IFN-γ (20).

Both cellular immunity mediated by CD4+ Th cells and humoral immunity mediated by antibodies are necessary for immunity against chlamydial infection (21-23). Initial inflammatory response involves the infiltration of polymorphonuclear neutrophils, whereas in the later stages of infection, lymphocytes and macrophages are the major infiltrators in the sub epithelial mucosae. Studies have shown that early inflammatory mediators such as IL-1α, IL-6, TNF-α, GM-CSF, IL-8 and chemokines like CXCL-1 and CXCL-16 are produced by epithelial and other immune cells upon infection with live chlamydia (24). These pro-inflammatory cytokines are necessary for eliciting an appropriate immune response against the bacteria. CD4+ Th cell responses are critical for host defense in Chlamydia infection. These cells mediate their protection by means of producing cytokines, mainly IFN-γ and IL-12. IL-12 is one of the crucial cytokines for polarizing the immune response towards a Th1 response. In the presence of IFN- γ, Chlamydiae assume aberrant persistent forms which have also been observed when clinical infections were treated with antibiotics. The effects of IFN-γ can be mediated by (i) inducing the production of indoleamine 2,3-dioxygenase (IDO) in human epithelial cells and thereby depleting the host

tryptophan pools; (ii) inducing the expression of inducible nitric oxide synthase (iNOS), which catalyzes the production of toxic reactive nitrogen species like nitric oxide, in murine cells; and (iii) enhancing phagocytosis by macrophages and induces the expression of MHC class I and class II maturation markers on antigen presenting cells like dendritic cells. IFN-γ and IFN-γ receptor knock out mice displayed a more severe disease and inability to resolve the infection (25). Of note, production of NO by macrophages is an important non-specific immune defense mechanism against intracellular organisms like *Toxoplasma gondii, Chlamydia trachomatis* and *Listeria monocytogenes* (26).

Major outer membrane protein (MOMP) is the dominant protein of chlamydia constituting about 60% of the dry weight of the organism. MOMP is the major antigenic determinant of Chlamydia and serovars or serotypes can be distinguished based on variation in the MOMP. Antibodies directed towards the MOMP participate in mediating protection during a reinfection. Of greater importance is the mucosal IgA. Antibody dependant defense mechanisms include (i) neutralization of EBs in the extracellular environment and prevention from their attachment to the host cell, (ii) antibody dependant cell mediated cytotoxicity and (iii) opsonization and thereby enhancement of phagocytosis by macrophages. On the contrary, CD8+ T cells are neither sufficient nor necessary to confer protection to *C. muridarum* infection in the mouse pneumonia model (27, 28).

Progesterone

Progesterone (pregn-4-ene-3, 20-dione), is a steroidal sex hormone produced by the corpus luteum of the ovary and the placenta (during pregnancy). Smaller

amounts of the hormone are also produced by adrenal glands and by testes in males. During pregnancy, high levels of progesterone are essential for survival of the embryo and for exerting an immunosuppressive effect towards tolerating the semi-allogenic fetus. Progesterone is the precursor for other sex hormones, estrogen and testosterone and all other important adreno-cortical hormones. It is required for bone growth and maintenance of secretory endometrium. In combination with estrogen, it is used for hormone replacement therapy and contraception.

To date, there is considerable evidence linking progesterone with an increased susceptibility to infection. In women, use of progesterone based contraceptives such as depot medroxy progesteroneacetate (Depo-Provera, an injectable progestational formulation is a synthetic analog of progesterone) has increased their risk of acquiring sexually transmitted diseases like *HIV, SIV, Chlamydia* and *gonorrhoea* (29-33) when compared with women not using contraceptives. Similar trends in enhanced susceptibility to infection has been observed, based on a number of clinical and epidemiological data, in pregnant women who were otherwise resistant to Th1 dominated infections like *Mycobacterium tuberculosis*, *M. leprae*, *Listeria monocytogenes* and *Toxoplasma gondii* (34, 35). A decrease in the Th1/Th2 cytokine ratio in pregnant women best explains these differences in disease expression (36). The levels of sex hormones, estrogen and progesterone, vary greatly during different phases of the menstrual cycle such as ovulation and menstruation, and these processes are in turn influenced by the immune system (37). In addition, progesterone is required for the establishment and maintenance of pregnancy including, uterine and mammary gland development (38). It would be worthwhile to note that sexual dimorphism is

observed in immune responses between males and females. In fact, cellular and humoral responses in females are more vigorous than in males. Other studies have demonstrated that sex hormones contributed to gender related differences in normal and pathological immune responses via modulating the cytokine production *in vivo* (39). Also, females are relatively more resistant to certain bacterial infections and susceptible to the development of autoimmune diseases when compared to males.

Progesterone exerts its effect on the cells/tissue of action by binding to its receptors. Receptors for progesterone (PR) are intracellular and occur in two main isoforms, A and B, expressed by the same gene. Progesterone receptors were found to be expressed in rat lung and are differentially regulated at the mRNA and protein levels by treatment with progesterone (40). In addition, BALB/c mice expressed progesterone receptors in pre and post-natal lungs suggesting their role in the maturation and development of lungs (41).Furthermore, progesterone can significantly cross-react with glucocorticoid receptors (42). NF-kappa B, an inducible transcription factor, positively regulates the expression of various cytokines especially those involved in a pro-inflammatory response. The steroid-receptor complex is capable of binding to NF-kappa B and can thus inhibit the activity of this protein. Progesterone binding sites have been observed on peripheral blood lymphocytes during pregnancy and suggests their possible role in affecting lymphocyte function (43-46).

Studies in the past have reported a number of effects of progesterone on the components and cells of the immune system (47). A normal human pregnancy, with elevated levels of progesterone, is typically characterized by a Th2 cytokine bias, low peripheral NK activity and low percentage of blocking antibodies (48). This

suppression is thought to be mediated by a protein called, progesterone induced blocking factor (PIBF) (49). Moreover, the presence of either decreased levels of progesterone or an increased Th1 activity was associated with fetal loss. Incubation of PBMC derived immature dendritic cells (iDC) or mature dendritic cells (mDC) with progesterone produced elevated amounts of cytokine IL-10 (50). However, Huck et al found that the phenotype of DCs was not altered by progesterone (50). In contrast, mouse bone marrow derived LPS matured DCs displayed reduced expression of accessory molecules like MHC-II, CD40, CD54, and CD80 when treated with increasing doses of progesterone (51). In *in vitro* studies, the hormone has also been shown to have inhibitory effect on the expression of inducible Nitric Oxide Synthase (iNOS) and thereby the production of toxic nitric oxide (NO) intermediates (52).

In a mouse model of *Chlamydia muridarum* genital tract infection, progesterone is routinely given as a subcutaneous injection prior to infection. The hormone is given either as a single dose of 2.5mg (53-59) or as two doses of 2.5 mg each (60-66) prior to infection with Chlamydia. The hormone injections are used to stabilize the mouse in an anestrus condition, reduce the epithelial turnover in the genital tract and thus establish a successful infection (67) (68). Since, *C. muridarum* does not naturally cause genital tract infections in mice and human serovars of Chlamydia poorly infect the epithelial layer of the genital tract, mice are pretreated with progesterone before infection. In view of the immunosuppressive role of progesterone during pregnancy and its ability to tip the balance towards a Th2 type of immune response (69), the effect of the hormone in modulating the immune response to infection with *C. muridarum* was studied in our respiratory model.

Vaccine

In humans, particularly women, infections caused by *C. trachomatis* pose a serious threat to both developed and developing nations. The morbidity of chlamydial infections is rather high and the generation of a successful vaccine remains a top priority of chlamydial research. Currently, there are no vaccines available for the prevention of chlamydial infections and researchers believe that understanding the molecular basis of the pathogenesis of the infection might better facilitate the development of vaccine. With the sequencing of the chlamydial genome, new antigenic determinants can be identified and might enable the development of a successful vaccine.

Several important considerations for vaccine development follow from our knowledge of the interactions between Chlamydia and its host. The unique lifestyle of the organism, its ability to cause persistent and chronic infections, complexity of the environment in the genital tract and strict tropism for mucosal epithelial cells present as key challenges to the development of an appropriate vaccine. The lack of tools for genetic manipulation and poor knowledge of the immune response to infection have hampered the progress in vaccine generation. Of equal importance is the development of effective delivery vehicles and adjuvants that can induce long-lasting protective immunity.

Attempts at generating a vaccine involved the use of live attenuated strains and adjuvants. Several qualities of MOMP such as its abundance, high antigenicity and immunoaccessibility has generated interest in the protein as a potential target for vaccine. However, vaccines that utilized MOMP alone generated only partial

protection. Nonetheless, MOMP DNA vaccines which employed adjuvants induced sufficient protection (70). Successful protection was observed only in a lung infection model and not in a genital tract infection model. Several other proteins such as HSP60, polymorphic outer membrane protein (POMP), polymorphic membrane protein D (PmpD), PorB family proteins, ADP/ATP translocase (in *C. pneumoniae*) have been identified as potential vaccine candidates.

Dendritic cells (DCs), the most efficient antigen presenting cells (APCs), were identified as useful targets for cell based immunotherapeutic purposes. Antigen primed DCs are remarkable in their ability to process the antigen and thus elicit a strong Th1 cytokine response. Adoptive transfer of DCs pulsed with whole inactivated bacteria *ex vivo* induced an efficient protective response during a vaginal challenge in a genital tract infection in mice. In contrast, experiments done by Shaw et al (71) have shown that DCs pulsed *ex vivo* with recombinant MOMP protein when adoptively transferred generated a deleterious Th2 response. Therefore, these results suggest that the *in vivo* response might be influenced by the nature of antigen used to pulse DCs.

C. psittaci induces spontaneous abortion in ewes. Temperature sensitive live attenuated strains were first isolated in *C. psittaci*. Interestingly, vaccination with live attenuated strains of *C. psittaci* protected ewes from this infection-induced abortion (72-74) demonstrating that immunization with live Chlamydia induced strong immune responses which were not observed when dead organisms were utilized for immunization (73).

Despite unsatisfactory results so far, vaccination is still feasible as there is evidence for short term protection in genital tract infection and no antigenic variation.

However, an efficacious vaccine candidate should be able to induce both systemic and mucosal Th1 immune responses followed by a humoral response that augments protection during reinfection. In addition to eliciting strong Th1 immune responses, a putative vaccine should be non toxic, induce heterotypic protection across species and serovars and possess sufficient antigenic and molecular conservation (75).

Uracil Phosphoribosyltransferase Mutant

Nucleotides are the building blocks of DNA & RNA in growing cells. Nucleotides can be obtained by *de novo* synthesis, utilization of preformed nucleotides or nucleotide intermediates, or by the uptake of nucleotide precursors like uracil, uridine, cytidine, hypoxanthine etc., available from the growth medium. Human and mouse strains of Chlamydiae lack the machinery for the *de novo* synthesis of purine and pyrimidine nucleotides. Hence, Chlamydiae are auxotrophic for ribonucleoside triphosphates (10). This need for NTPs can be met by the Npt2 transporter, assisted import from the host cell cytoplasm (Figure 2). Subsequent conversion to their respective reduced forms, deoxyribonucleotide phosphates, is catalyzed by the bacterium's own ribonucleotide reductase.

Many microorganisms, including *C. muridarum*, utilize the Uracil phosphoribosyl transferase (UPRTase) to catalyze the key reaction in the uracil salvage pathways. The enzyme, UPRTase is encoded by the *upp* gene. Though *C. muridarum* is very similar to *C. trachomatis* in its virulence factors and mechanism of pathogenesis, several differences exist between both the species. UPRTase is synthesized only in *C. muridarum* and absent in all other chlamydial species. The

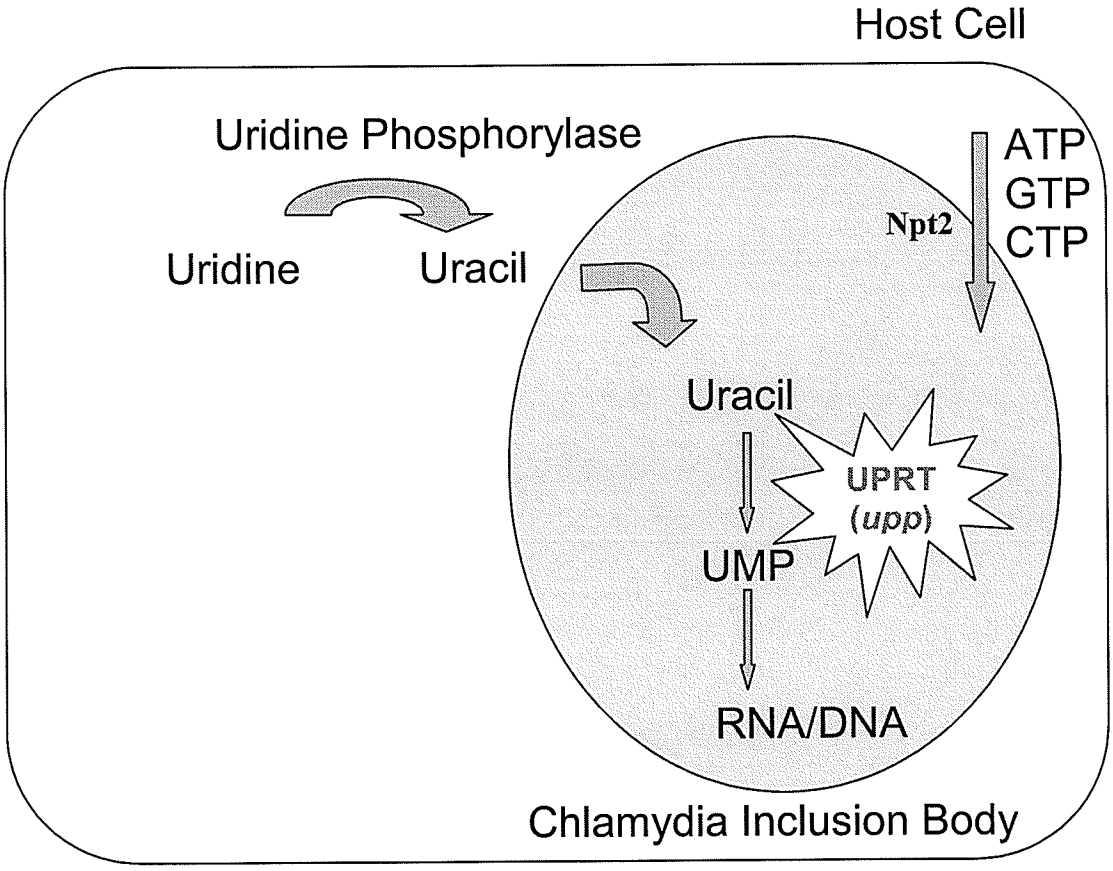


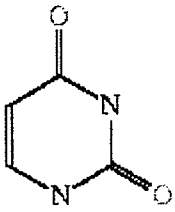
Figure 2: Pictorial representation of the mutant within the host cell.

Uracil phosphoribosyltransferase (UPRT) catalyzes the synthesis of UMP from uracil. This enzyme is the product of the *upp* gene. UPRT compensates for the reduced affinity of the Npt2 transporter for the import of UTP from the host cell. Chlamydia acquire the ribonucleotide triphosphates from the host cell cytoplasm.

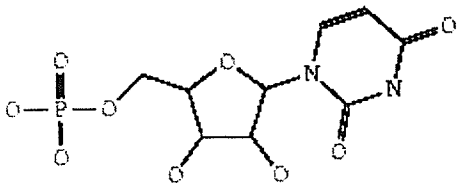
presence of this enzyme apparently compensates for the decreased affinity of the Npt2 transporter for UTP in this particular species. In *C. muridarum*, UPRTase catalyzes the formation of uracil monophosphate (UMP) by the transfer of a ribose 5- phosphate from D-5-phosphoribosyl pyrophosphates (PRPP) to uracil, up taken from the host cell cytoplasm (Figure 3A). In addition, UPRTase also catalyzes the conversion of the prodrug, 5-FU to 5-FUMP, which is cytotoxic when incorporated into DNA or RNA (Figure 3B). The *upp* mutant strain used in my study was isolated by step wise selection for resistance to increasing doses of 5-fluorouracil. The principle for the generation of this mutant is based on the fact that 5-FU is toxic to the replicating cells and thus only bacteria which have undergone mutation in the *upp* gene and thereby have lost the ability to form 5-FUTP can survive. The mammalian counterpart of this UPRT enzyme is orotate phosphoribosyltransferase (OPRT), which can also catalyze the conversion of 5-FU to the toxic 5-FUTP. Hence, Chinese hamster ovary (CHO Urd C^-) cell line which is deficient in OPRT is used for the selection of the mutant. The cell line itself cannot catalyze the formation of the toxic 5-FUTP thereby enabling the growth of Chlamydia which have undergone mutation only in the *upp* gene. In the absence of uridine or uracil supplementation, the *upp* mutant grows equally well as the WT chlamydial strains.

Purine auxotrophic mutants of *Salmonella typhimurium* were found to be highly attenuated and deficient in replication though they retained the ability to invade host epithelial monolayers and survive inside mouse macrophages (76). Furthermore, uracil auxotrophs of *Toxoplasma gondii* with a mutation in the enzyme, carbamoyl phosphate synthetase II, required for *de novo* pyrimidine biosynthesis were completely

A



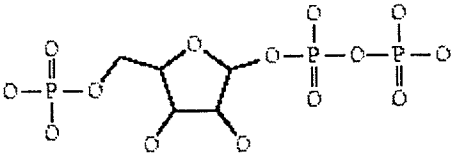
Uracil



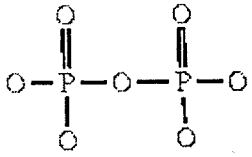
Uracil monophosphate (UMP)

UPRTase

\+ ----------> +



5-phospho-alpha-D-ribose 1-diphosphate (PRPP)



Diphosphate

B

UPRT

5-Fluorouracil ----------> 5-Fluoro- deoxyuridine triphosphate (5-dFUTP)

Figure 3: Reactions catalyzed by Uracil phosphoribosyltransferase

In *Chlamydia muridarum*, the enzyme, Uracil phosphoribosyltransferase is coded for by the *upp* gene. UPRT belongs to the class of enzymes that catalyze the transfer of phosphoribosyl groups between molecules. UPRTase catalyzes the formation of Uracil monophosphate from uracil and PRPP, **A**. UPRT also catalyzes the formation of cytotoxic 5-FUTP from 5-Fluorouracil, **B**. In humans, the counterpart for UPRT is OPRT or Orotate phosphoribosyl transferase.

attenuated in both immunocompetent and immunodefecient IFN-γ knock out mice (77). Thus far, identifying targets that limit the availability of essential nutrients to the bacterium and thereby enabling attenuation can be interesting in the design of vaccines.

The first part of the project concentrates on investigating the effect of progesterone pretreatment on infection and immunity to *Chlamydia muridarum.* We hypothesize that progesterone exerts an immunosuppressive action on the generation of protective Th1 responses by shifting the balance of a type 1 to a type 2 response, and thereby leading to a more severe disease phenotype. Secondly, the metabolic *upp* mutant of *C. muridarum* generated by 5-Fluorouracil mutagenesis, is studied for its potential as a vaccine candidate in the mouse respiratory model. Since, the enzyme UPRT, coded for by the *upp* gene, is required for the efficient replication of the bacterium within the host cell, we hypothesize that the mutation in this particular gene will attenuate the bacterium inside the host and may be capable of generating a immunity to reinfection.

MATERIALS AND METHODS

1. Mice

Seven to eight week old, female C57BL/6 mice were used for all experiments involving progesterone. Five to seven week old, female BALB/c mice were used for all experiments utilizing the upp mutant of *C. muridarum*. Mice were purchased from Genetic model center (GMC) (University of Manitoba) or from Charles River Canada (St. Constant, Quebec, Canada) and were maintained at the central animal care facility at the University of Manitoba. Mice were used in accordance with guidelines issued by the Canadian Council on Animal Care.

2. Materials

MEM medium for HeLa229 cells and RPMI1640 medium for primary cell culture were purchased from Invitrogen, Burlington, ON. Fetal bovine serum was purchased from Hyclone, Mississauga, ON. All chemicals were obtained from Fischer Scientific, Ottawa, ON. Depo-provera was purchased from Pharmacia & UPJohn Company, Pharmacia Canada Inc, Mississauga, ON. All purified and biotinylated antibodies for cytokines were purchased from e-biosciences, San Diego, CA. Biotinylated antibodies for immunoglobulins were purchased from Southern biotechnology associates, Birmingham, Alabama. All cell culture plates and sterile centrifuge tunes were purchased from Corning Incorporated, NY.

3. Propagation of *C.muridarum*

C. muridarum was propagated in HeLa229 cell monolayers in Eagle's MEM containing 10% fetal bovine serum and 2mM glutamine and 25μM gentamycin. HeLa cell monolayers were inoculated with purified EBs and infected cultures were incubated for 30-36 hrs at 37°C in 5% CO_2. Infected cells were harvested by glass beads and lysed by sonication. For *in vitro* mutant growth studies, Chinese Hamster Ovary (CHO) Urd C- cell monolayers were maintained in Eagle's MEM supplemented with the same as above and further 30μM uridine and 300μM proline were added.

4. Isolation and purification of elementary bodies by density gradient centrifugation

Confluent HeLa 229 cells in the exponential phase were infected with Chlamydia and allowed to grow for 26- 28 hours. Infected flasks (4 × 175 cm^2) were observed under the microscope to confirm the infection and monitored until the inclusions grow to their maximum size thereby occupying most of the cell cytoplasm. Then, the medium was discarded from the infected culture flasks. Ten ml of cold HBSS was pipetted into the flask and approximately 30 glass beads were added to the flask and was rolled gently to scrape the cells off the bottom. Cell suspension was transferred to a centrifuge tube kept on ice. The flask was rinsed with 10 ml more of HBSS and added to the centrifuge tube. The cell suspension was sonicated at a probe intensity of 35 for 20 seconds. Initially, the cells were centrifuged at 500×g for 10 min at 4°C. The pellet containing cell debris was discarded and the supernatant was transferred to a fresh centrifuge tube and layered with 8 ml of 35% renograffin. Tubes were centrifuged at

43,000×g in a Beckman ultracentrifuge at 16,000 rpm for 60 minutes at 4°C in a SW27 rotor. The band obtained was collected and resuspended in 10 ml of SPG. The suspension was layered over with a discontinuous renograffin gradient containing 13 ml of 40% renograffin in Hepes, 8ml of 44% renograffin and 5 ml of 52% renograffin. The tubes were then centrifuged at 17,000 rpm for 90 minutes at 4°C in a SW27 rotor. Elementary bodies were collected at the 44-52% gradient interface and diluted with three volumes of SPG or HEPES. This suspension was further centrifuged at 30,000×g at 16,000 rpm (Sorvall) for 30 minutes at 4°C. This step was repeated and the pellet containing purified EBs was finally suspended in 1ml of SPG per initial flask used. 1ml aliquots of the suspension were made and stored at -80°C until further use.

5. Chlamydial infectivity assay

In cases in which only a part of the organ was homogenized counts were corrected for the whole organ weights. HeLa cells were plated at 4×10^5 cells/ml (100µl/well) in a 96 well culture plate and incubated for 16 hrs at 37C in 5% CO2. HeLa cells were incubated at room temperature for at least 30 minutes with HBSS containing DEAE-dextran at a final concentration of 30µM. Several dilutions of infected samples were made, inoculated onto HeLa cell monolayers in triplicate and incubated for 2hrs. For all infectivity assay studies, inoculum was removed and the infected cells were grown in MEM containing 1µg/ml of cycloheximide for 26-28 hours. Cells were then fixed with absolute methanol for 10 min and stained with genus specific monoclonal antibody and secondary goat anti-mouse IgG (Fab specific) peroxidase (Sigma). Color develops 2 to 4 min after the addition of the substrate, 4- alpha chloronapthol with

10ul of Hydrogen peroxidase. Excess substrate is removed and replaced with 200µl of 1× phosphate buffered saline (PBS). Chlamydial inclusions were counted in five fields at 200× magnification under a light microscope. The average of five counts was multiplied with the dilution factor, $\log_{10}$ transformed and the mean values were graphed as IFUs/lung or IFUs/ml on a logarithmic scale.

6. Infection of mice

Mice were anesthetized by exposing to vaporized isoflurone (Baxter Corporation) in a closed chamber. Mice were placed on their back and 1×10^3 IFU of C.muridarum in 40µl of SPG was administered intranasally using a pipette. Mice were sacrificed on days 7, 10 or 15 post-infection as indicated.

7. Progesterone treatment of mice

Depo-Provera (medroxy progesterone acetate) was purchased from shopper's drug mart under a doctor's prescription. Progesterone was administered to the mice by a subcutaneous injection. 2.5 mg/mouse/dose of depo-provera was injected either on day 7 (one dose) or on days 10 and 3 (two doses) prior to infection with *C.muridarum*. Hormone untreated, infected mice served as controls. The dosage and timing regimen of Depo-Provera that is widely used in genital tract infection models has been selected for my experiments (Figure 4).

8. Preparation of spleen and lymph node cell culture

Mice were sacrificed and spleen and mediastinal lymph nodes were removed aseptically. Glass homogenizers were used to prepare a single cell suspension in RPMI 1640 medium. Cells were washed once by centrifuging at 1000 rpm for 10 min. Cells were resuspended in complete RPMI 1640 medium containing 10% heat-inactivated FBS, 25 μg/ml gentamycin, 2 mM L-glutamine, and 50μM 2-ME (Kodak) and counted in a hemocytometer after dilution in trypan blue and acetic acid. Cells were further adjusted to the required concentration.

9. Ex-vivo stimulation of splenocytes and lymph node cells

Spleen cells were cultured at 7.5×10^6 cells/ml alone or restimulated with UV killed chlamydia or plate-bound anti-CD3 mAb in 48 well culture plates. Lymph node cells were cultured at 5×10^5 cells/ml in a 48 well culture plate and were stimulated similarly to spleen cells. Cells from three or four mice/group were cultured and analyzed independently. Culture supernatants were harvested at 72 hours and stored at -20°C until cytokine analysis was performed. On occasion, duplicate culture supernatants were harvested at 120 hours to determine IL-10 production.

10. HeLa cell culture conditions

HeLa 229 cell line was purchased from American Type Culture Collection (ATCC) and was continuously maintained in our laboratory. HeLa 229 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine at 37°C in 5% CO_2.

11. Immunization and challenge protocol

5 week old, female BALB/c mice were immunized intranasally with a small dose of 500 IFU of either the *upp* mutant or wild type *C.muridarum* after anesthetizing with pentobarbital. Mice were allowed to recover completely for 30 days and a challenge dose of 1×10^4 IFU was given intranasally. Body weight changes were recorded throughout the infection period. Mice were sacrificed 10 days post challenge. Mice that did not receive immunization served as controls.

12. Cytokine determinations

A two-mAb sandwich ELISA was used to determine cytokine concentrations in culture supernatants or lung homogenates. Briefly, 96 well ELISA plates were coated with the respective capture mAb in coating buffer (0.1M NaHCO3, pH 8.2). After overnight coating at 4°C, plates were blocked with blocking buffer for 2 hrs at room temperature and washed four times. Cytokine standards were serially diluted in the wells. Culture supernatants were diluted depending upon the cytokine being tested for. The plates were incubated at 37°C for 3 hrs and then washed four times. Biotinylated anti-cytokine secondary mAb was added and plates were kept at 4°C overnight. The following day, plates were washed and incubated with alkaline phosphatase conjugated streptavidin (Jackson Immunoresearch Laboratories) at a 1:6000 dilution and incubated at 37°C for 45min. Plates were then washed for six times before the substrate, p-nitrophenyl phosphate (Sigma) is added. Plates were read at 405nm at different time points depending upon the cytokine being measured. A basic endpoint protocol was used for graph construction.

Paired antibodies for ELISA analysis of IFN-γ were purchased from e-Biosciences. Plates were coated overnight with 1μg/ml of capture mAb and detected with 1μg/ml biotinylated mAb. Samples were diluted five times in dilution buffer. Plates were read 10 min after substrate addition. Paired antibodies for ELISA analysis of IL-10 were purchased from BD Pharmingen.

13. Quantification of chlamydia specific antibodies in serum

Blood was collected from the tail vein before the mice were sacrificed. Blood in microfuge tubes was kept at 4°C overnight and the following day blood clots were removed from the samples before spinning them down at 3000×g for 10 minutes. Three to four aliquots of the serum samples were made and stored at -80°C until further analysis. Chlamydia specific antibody titers in serum were determined by enzyme-linked immunosorbent assay. ELISA plates (Corning, NY) were coated with UV killed EBs of *C.muridarum* in bicarbonate buffer (0.05M, pH 9.6) and kept overnight at 4°C. The following day, plates were blocked with a blocking buffer containing 2% BSA, 0.05% Tween-20 solution (pH 7.4) for two hours at room temperature. Plates were then washed for four times before samples were added. Serial dilutions of the sample were made in the plates and they were incubated at 37°C for 3 hours. Following washing, biotinylated Ab subclass specific goat anti-mouse antibodies were added to the respective plates and kept overnight at 4°C. Alkaline phosphatase conjugated streptavidin was added and incubated at 37°C for 45 minutes. After washing the plates for six times, alternating between ddH_2O and 1X PBS containing tween-20, *p*-nitrophenyl phosphate (in 05.mM MgCl2, 10%

diethanolamine, pH 9.8) was added and the plates were kept in dark for 60 min. The plates were read with a microplate reader (BioRad 3550, Bio-Rad laboratories, CA) at 405nm using a basic end point protocol. Results were expressed as logarithmic values calculated from a standard graph. The cutoff point used was an O.D of 0.5.

14. Histopathology: Hematoxylin & Eosin staining

Lungs from mice were removed aseptically and fixed in formalin. Fixed tissues were embedded in paraffin and sectioned into 5μM thick and stained with Hematoxylin and Eosin. Sections were observed under a light microscope (Olympus 1X51). The imaging system used to acquire the images was Olympus Micro DP70.

15. Histopathology: Periodic Acid Schiff staining

One lung was snap frozen in liq N2 and samples were later transferred and stored in a -80°C freezer until processed for immunofluorescence staining. Frozen tissues were cryosectioned to 10μm thickness and were treated with blocking buffer before incubating the sections with mouse anti-chlamydia major outer membrane protein Ab (Chemicon). The sections were then incubated with FITC- conjugated goat anti-mouse IgG Ab (Sigma-Aldrich). To stain epithelial cells in the lung, sections were incubated with mouse anti-pan-cytokeratin Ab (Calbiochem- Novabiochem, CA). The sections were then washed, incubated with biotinylated rabbit anti-mouse Ab (Dako Corp, CA) and further developed using Texas Red Avidin D (Vector Laboratories). Olumpus AX70 fluorescent microscope was used to observe the slides. Images were acquired using a Image Proplus Imaging software.

16. Statistical analysis

Statistical analysis was performed using GraphPad prism software (GraphPad, SanDiego, CA). Unpaired Student's t test was used to determine statistical significance between the groups. A p value of less than 0.05 was considered significant. Data are presented as mean $\pm$ SEM.

RESULTS

A. Regulation of immune response to *Chlamydia muridarum* infection upon progesterone administration.

1. Introduction

The major focus of this project is to examine the effect of progesterone pretreatment on immune response to a mouse respiratory infection model of *Chlamydia muridarum*. Steroidal hormones like progesterone have strong immune regulatory properties which can be crucial in protection against an infection. In addition, this study will have important implications in the evaluation of vaccine efficiency as testing of vaccine candidates in mice also employs the progesterone pretreatment regimens similar to those practiced in experimental genital tract infection models.

2. Effect of progesterone pretreatment on resolution of chlamydial infection in the lung

Studies done by other groups have shown that infection is aggravated in the presence of high levels of progesterone. In the lung infection model of *C. muridarum*, the number of recoverable IFUs from the infected organs is directly proportional to the severity of the disease. In this study, mice were subcutaneously injected with different doses of progesterone prior to infection with *C. muridarum* as described in *Materials and Methods* (Figure 4). Mice were sacrificed on either day 7 or on day 15 and lungs were collected aseptically. The ability of the hormone to modulate the severity of the disease in terms of bacterial burden in the lung was assessed. Mice that did not receive

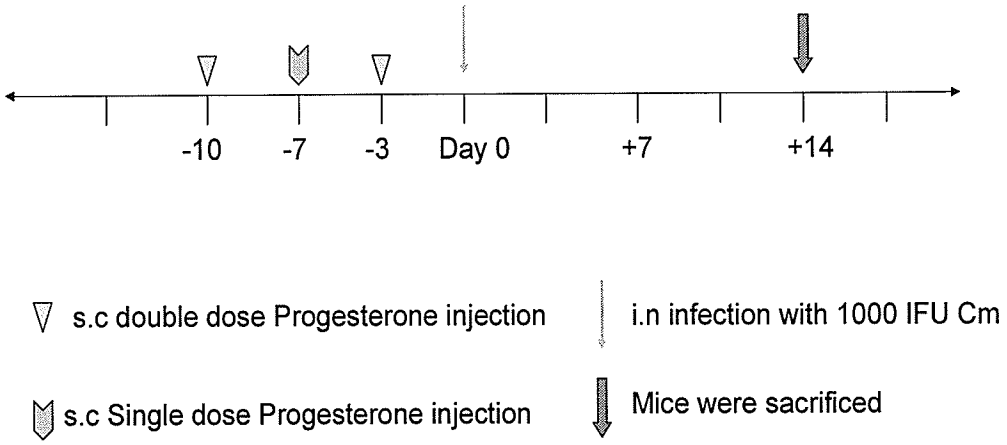


Figure 4: Progesterone pretreatment protocol

One group of mice were sub-cutaneously given two injections of depo-provera (medroxy progestereone acetate), a synthetic analog of progesterone, 2.5mg each dose per mouse once on day 10 and on day 3 before infection. A second group of mice received one sub-cutaneous injection of progesterone on day 7 before infection with *C. muridarum*. A third group that received no injections but infected alone served as control. Four mice were used in each group to study the effect of progesterone on the modulation of immune response to *C. muridarum* infection.

progesterone before being infected served as controls. Lung homogenates were used to infect the HeLa cell monolayers and the numbers of IFUs in the original sample were enumerated by staining the monolayers with genus specific antibody. The inclusions were counted under a light microscope and plotted as logarithmic graphs.

As expected, mice which received two doses of progesterone exhibited higher levels of IFUs (Figure 5B) when compared to control mice on day 15 p.i during which time the mice should have cleared most of the Chlamydia from the lung. However, there was no significant difference in the number of recoverable IFUs from mice which received only one dose of progesterone. On day 7 p.i, all three groups displayed similar levels of bacterial load (Figure 5A) which indicates that progesterone did not enhance the susceptibility of the host to Chlamydia rather interfered with the clearance of bacteria from the lung as evidenced by the presence of high numbers of recoverable IFUs even on day 15 post infection from mice that received two doses of progesterone. These results suggest that the hormone might have immune modulating effects on the course of infection with *C. muridarum.*

3. Modulation of cytokine production in progesterone treated mice

Th1 cytokines like IFN-γ and IL-12 are critical for the protection of host in infection with many intracellular pathogens including *C. muridarum* (78, 79). Studies done in our own lab and others have demonstrated the important role of IFN-γ in the clearance of the organism from infected tissues. Since progesterone has a strong bias towards inducing a Th2 like response, we hypothesized that Th1 cytokine responses would be suppressed when mice were treated with progesterone. In this study, we

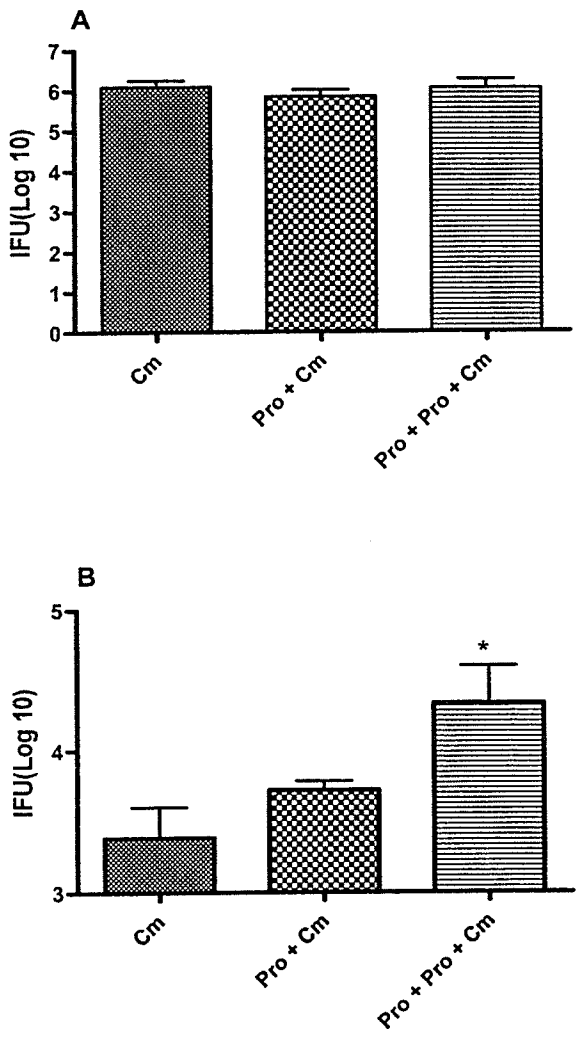


Figure 5: Resolution of infection in progesterone treated versus untreated mice. Female C57Bl/6 mice were administered subcutaneously either as a single dose (2.5mg) or two doses (2.5 mg each) before infection with 1000 Inclusion Forming Units (IFU) of *C. muridarum*. Untreated infected mice served as controls. Mice were sacrificed on day 7(A) or on day 15(B) post-infection and lung homogenates were tested for *C. muridarum* infectivity as described in Materials and Methods. * $P < 0.05$. Data represent one of two independent experiments with similar results.

determined the levels of *C. muridarum* specific IFN-γ produced by stimulating spleen and lymph node cells with UV killed chlamydia.

Early immune responses mounted towards infection are crucial in determining the course of the disease. Cytokine production early in the infection was examined to predict the outcome of the infection and thus test the ability of the host to resolve the infection. On day 7 p.i, IFN-γ production was significantly reduced in culture supernatants of spleen ($p<0.05$) and lymph node cells ($p<0.01$) from mice which received two doses (2.5mg + 2.5mg) of progesterone (Figure 6A). Though the reduction in IFN-γ was not significant, mice that received a single dose of progesterone produced smaller amounts of IFN-γ when compared to the control group. Also, a trend for reduced IFN-γ production was observed locally in the lung. IFN-γ in the control medium was not measurable indicating that there was no spontaneous production of the cytokine in unstimulated cultures (data not shown). IFN-γ production was reduced by day 15 p.i and no significant differences were found among the three groups but a trend towards decreased production of IFN- γ was observed in antigen driven lymph node cells (Figure 6B) Since IFN-γ levels early in the infection are crucial for protection, it is evident from Figure 6, that a decreased IFN-g protection on day 7 p.i in progesterone treated mice could have contributed to the impaired clearance of Chlamydia from the lungs when sacrificed on day 15 p.i. From the results in Figure 7A, it is clear that not only local IFN-γ production but IFN-γ produced systemically (i.e spleen) is necessary for clearance of the bacteria in the lung during the recovery phase.

However, it is interesting to note that though IFN-γ levels were suppressed,

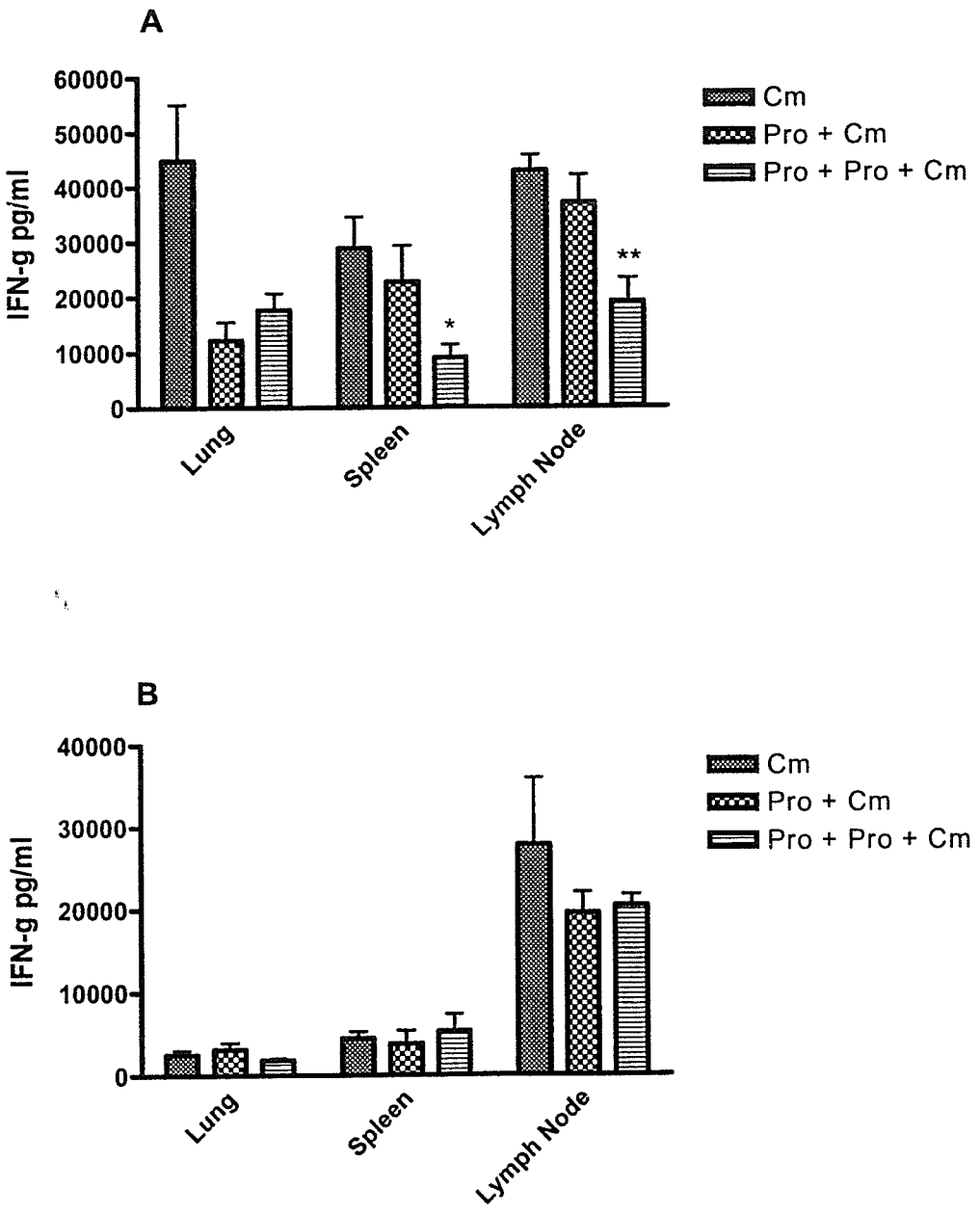


Figure 6: Effect of progesterone pretreatment on the production of IFN-γ in infected mice.

Mice (n=4) were treated as described in the legend to Fig 6. Lung homogenates, Spleen cell and Lymph node cell culture (72hrs) supernatants were analyzed for IFN-γ, on day 7, A and on day 15, B, following infection by Enzyme Linked Immuno Sorbent Assay (ELISA). Data are represented as mean ± SEM. Results shown is one representative of two individual experiments with similar results. * $P < 0.05$ and ** $P < 0.01$.

Il-12 produced by antigen driven lymph node cells and splenocytes did not alter among the hormone treated and untreated mice (data not shown). Nevertheless, a trend towards decreased production of IL-12 in the progesterone treated groups was observed locally in the lung homogenates both on day 7 and day 15 following infection, though the decrease was not significant (Figure 7). It is unclear as to why IL-12 levels did not change. The data presented here indicate that progesterone has the ability to profoundly suppress the production of IFN-γ by antigen driven splenocytes and lymph node cells. Furthermore, IFN-γ expression in the lung, possibly produced by cells in the mucosal inductive sites, was suppressed as well in mice that received progesterone. Thus, in agreement with earlier reports (80), progesterone can abrogate IFN-γ dependant protection of the host against infection with *C.muridarum*.

4. Influence of progesterone on Th2 cytokine production

It is well known that IL-10 inhibits the production of inflammatory cytokines such as IL-12 and IFN-γ (81, 82). We hypothesized that a decrease seen in the elaboration of Th1 cytokines, IFN-γ and IL-12, could result from an enhanced production of Th2 cytokines in mice that were treated with progesterone. To test this hypothesis, we examined the production of Th2 cytokines, IL-4, IL-5 and IL-10 levels in antigen driven spleen and lymph node cell culture supernatants and in lung homogenates.

On day 7 following infection, there was a significant enhancement of IL-10 levels in lung homogenates ($p<0.05$) from mice that were pretreated with two doses of

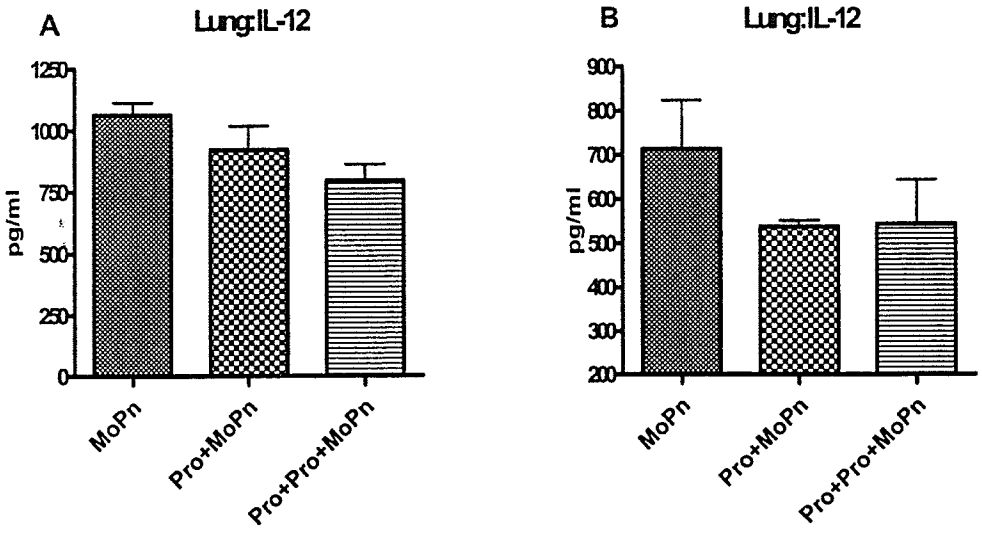


Figure 7: Local production of IL-12 in the lung following infection of progesterone treated versus untreated mice.
C57Bl/6 mice (four mice per group) were injected with either two doses or one dose of progesterone and all mice were infected with 1×10^3 IFU of *C. muridarum*. Day 7 p.i (A) and day 15 p.i (B) lung homogenates were analyzed for IL-12 expression by ELISA.

progesterone (Figure 8A). A trend towards increased production of IL-10 was observed in antigen driven splenic and lymph node cell culture supernatants in all mice that received progesterone. However, there was not significant difference between untreated mice and mice that received a single dose of progesterone. Similar to the responses that were observed on day 7 p.i , IL-10 production in spleen, lymph node cells and lung homogenates on day 15 p.i were significantly elevated ($p<0.05$). Though there was no significant difference between the control group and the group that received only one dose of progesterone (Figure 8B), the levels of IL-10 were increased with one progesterone injection. IL-4 was not detected in cultures from either hormone treated or untreated mice on both days 7 and 15 p.i. Altogether, the data presented here indicate that there was an enhanced Th2 response as evidenced by the increased production of IL-10 in the culture supernatants as is also found in the lung homogenates.

5. Humoral responses in progesterone administered mice

Both cellular and humoral responses by the host immune system participates in protection against *C. muridarum* infection though the relative contribution of the humoral immunity in primary infection is not clearly understood (22, 83). In addition to investigating the effect of progesterone on cytokine responses, we also examined antigen specific antibody production in the serum. Since progesterone is known to induce Th2 type of responses, antibody class switching can occur in hormone treated mice. Hence, chlamydia specific IgG2a, IgG1 and IgA titers in the serum on day 15 p.i were quantitated by ELISA. Interestingly, the levels of all three antibodies among the

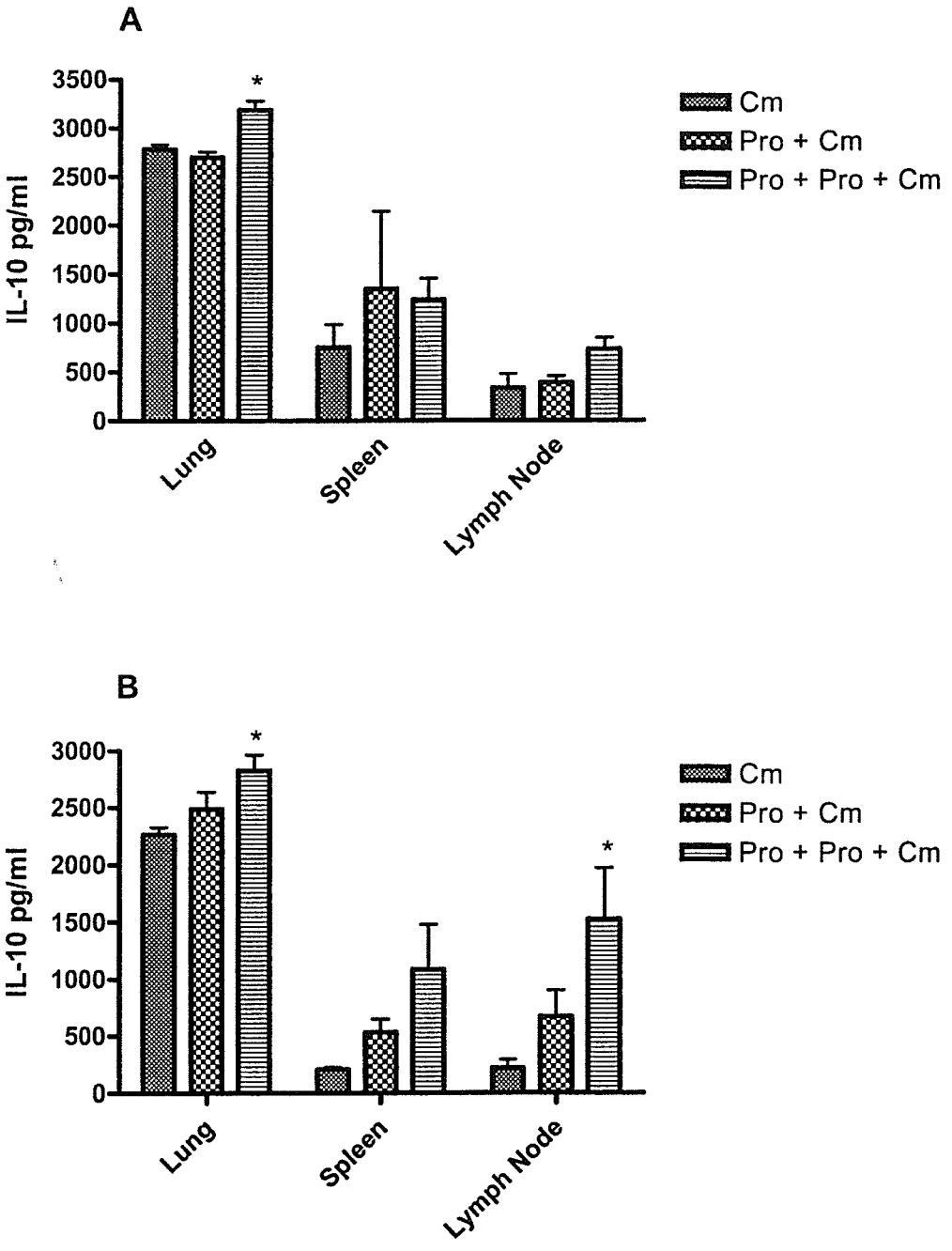


Figure 8: IL-10 production in mice that received progesterone prior to infection
Mice were given progesterone and infected with C.muridarum as described in Materials and Methods. Briefly, spleen and lymph node cells from infected mice were cultured in the presence of UV killed *C. muridarum* and 72hr supernatants were analyzed for IL-10 by ELISA. Lung homogenates were frozen and stored at – 80°C until analyzed together with cell culture supernatants. Day 7 p.i (A) and day 15 p.i (B)
* $P<0.05$ mice that received progesterone.

three groups of mice were comparable and did not have significant differences (Figure 9). This contradicts what is known about the hormonal effects on antibody production, but the differences could be due to pathogen specific responses.

6. Histopathological changes following infection of progesterone treated mice.

Persistent inflammation and infiltration of neutrophils were observed in a genital herpes simplex virus type2 infection in mice that were treated with progesterone. (84). During a chlamydial infection in the genital tract, severe pathological changes in the infected tissue, such as fallopian tubes, can lead to scarring and fibrosis which ultimately leads to tubal blocking and results in infertility. Pathological changes in the infected tissue were examined in order to determine the effect of progesterone in modulating the inflammatory response. Lungs from infected mice were fixed in formalin and stained with hematoxylin and eosin and histopathological analysis was performed. On day 15 p.i., lymphocytes were the major cell types in the infected tissue. In mice treated with two doses of progesterone, a marked increase in inflammatory cell infiltration obliterating most of the air spaces in the lung sections was observed (Figure 10). The cellular changes in the lung sections did not alter between the control mice and the single dose progesterone group. Early in the infection, neutrophils and macrophages were the major cell types visible under pathological examination. However, there were no visible differences in the types of inflammatory cells and the intensity of cellular infiltration in lung sections among all three groups early (day 7) in the infection. Also, immunofluorescent staining was done on liq N2 frozen lung tissues to directly stain the chlamydial inclusions inside the lung

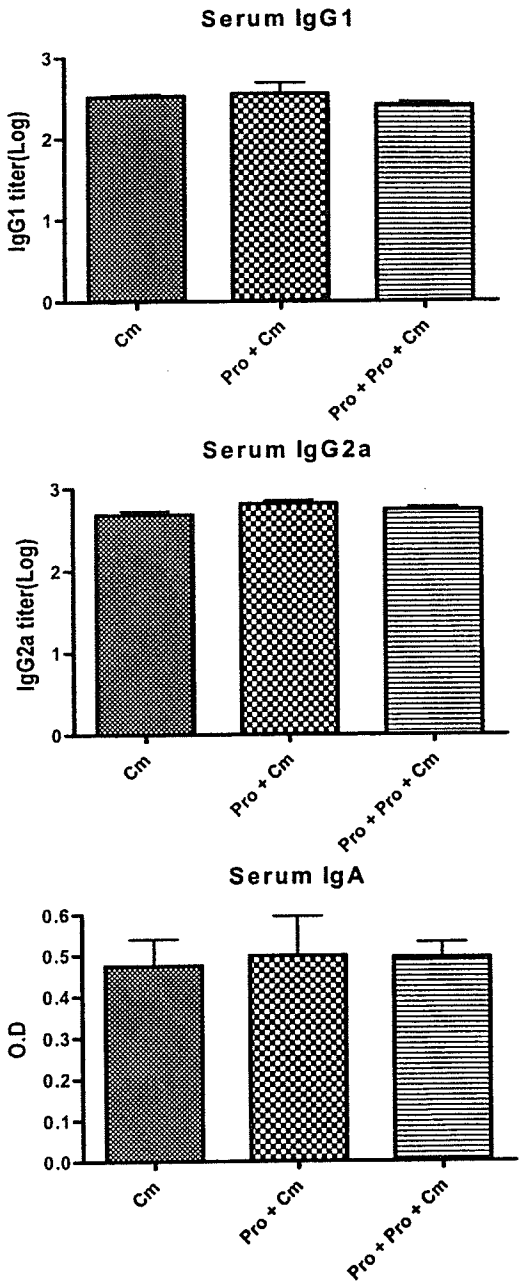


Figure 9: Humoral immune responses in progesterone pretreated Vs untreated mice during infection with *C. muridarum*

Mice (n=4) were treated as described in the legend to Fig 1. Day 15 post-infection, serum *C. muridarum* specific IgG2a, IgG1 and IgA antibodies were quantitated by Enzyme Linked Immuno Sorbent Assay (ELISA).End point used is 0.5 O.D at 120" for IgG1 and 60" for IgG2a. Data are represented as mean ± SD.

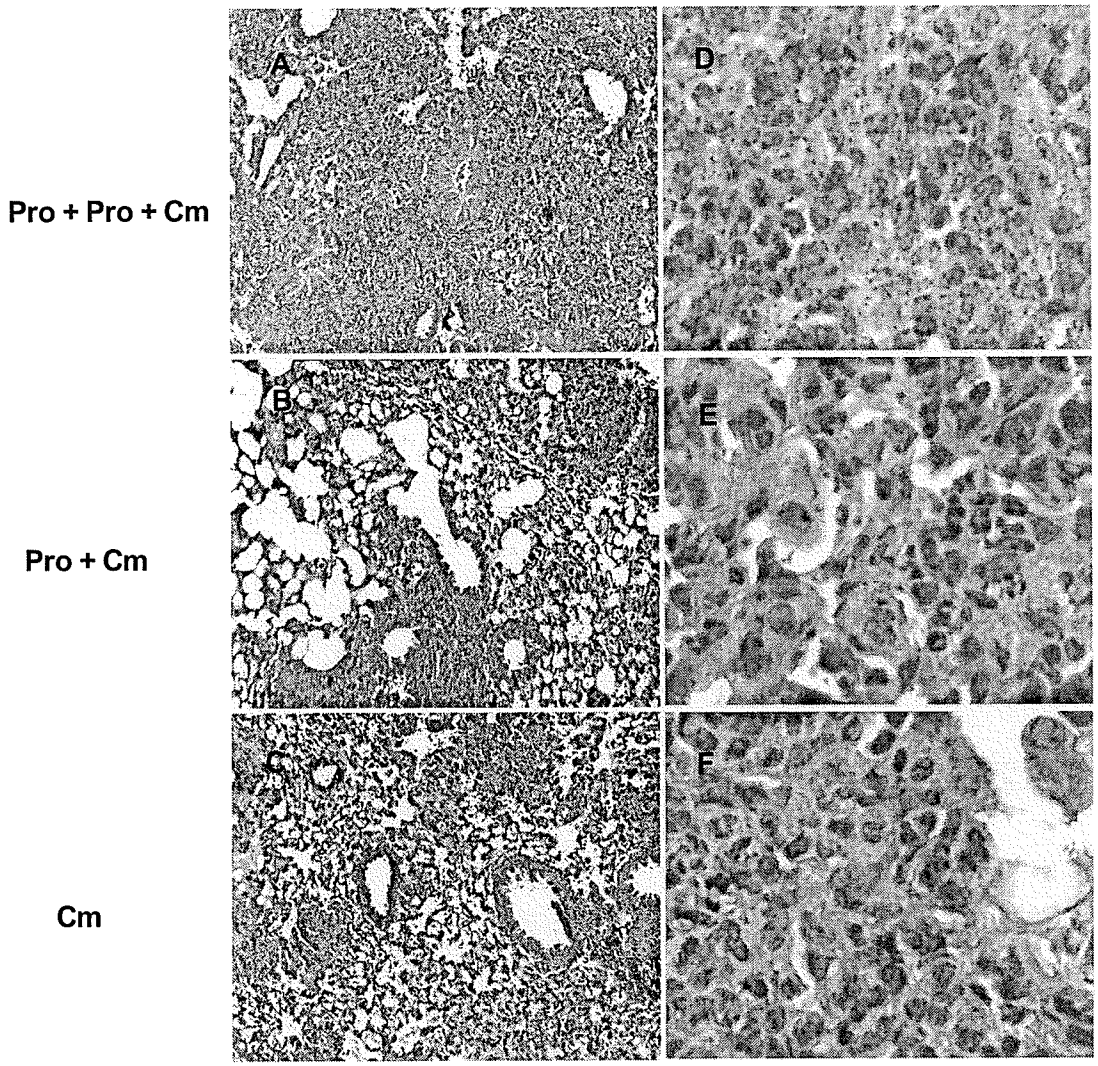
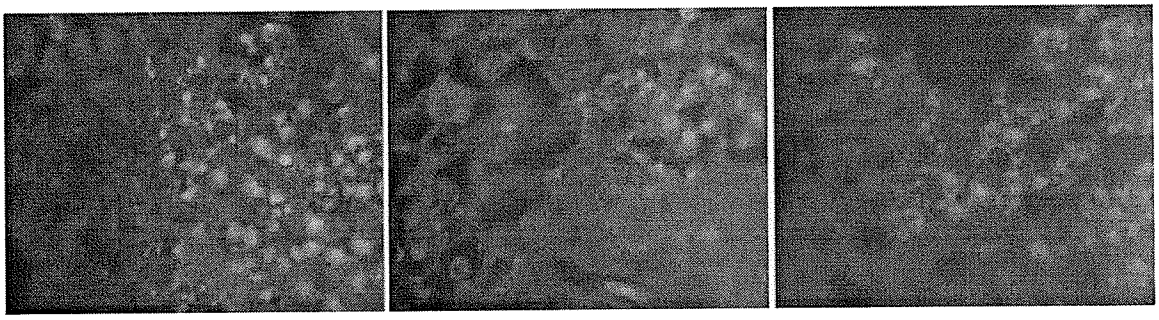


Figure 10: Histopathology of infected lung tissues in progesterone treated Vs untreated mice

Hormone treatments and infections were given as described in the legend to Fig 1.Untreated infected mice served as the control group. All mice were sacrificed on day 15 post-infection and lung sections were stained with Hematoxylin & Eosin. Magnification A, B, C- ×200 D, E, F - ×400



Pro + Pro + Cm **Pro + Cm** **Cm**

Figure 11: Immunofluorescent staining of lung tissues from progesterone pretreated, infected C57Bl/6 mice.

Lungs from mice were snap frozen in liq N2, cryosectioned and stained using periodic acid schiff staining. Chlamydial inclusions were stained using *C. muridarum* (Cm) major outer membrane protein specific mAb as described in materials and methods. Orange color represents chlamydial inclusions in the lung sections. Slides were photographed at ×400 magnification

tissue (Figure 11). These results have also confirmed what was observed when lung homogenates were analyzed for recoverable IFUs. This observation indicates that progesterone treated mice suppressed the resolution of the infection when two injections of the hormone were given instead of one.

Taken together, these studies suggest that progesterone is capable of modulating the immune responses by means of suppressing the protective IFN-γ production, enhancing the IL-10, and thus promoting pathological changes in the host.

B. Growth characteristics of the *upp* mutant of *C. muridarum* .

1. Introduction

Live attenuated vaccines of Chlamydia are difficult to generate due to the complex nature of the disease and the organism in itself. The *C. muridarum upp* mutant was generated by a step-wise selection for resistance to increased concentrations of 5-Fluorouracil in CHO Urd C^- cell line. This was possible because, the CHO Urd C^- cell line is deficient in the enzyme OPRT and therefore cannot catalyze the conversion of 5-FU to the cytotoxic 5-FUTP. It is worthwhile to test the in vitro and in vivo growth properties of the organism before assessing its potential as a vaccine candidate.

2. *In vitro* growth in mammalian cell lines, CHO Urd C- and HeLa 229.

In order to observe the effect of the mutation in the *upp* gene, on its growth in CHO Urd C^- cell line and the HeLa 229 cells, both the WT and mutant *C. muridarum* were inoculated onto the cell lines at a MOI of 1. Culture medium supplemented with

either 30μM uridine or 30μM uracil was used as controls for the growth of WT and mutant strains. In the presence of uracil, growth of mutant was similar to that observed in starved conditions, suggesting that the enzyme UPRT is inactivated in the mutant and was thus unable to utilize exogenously supplied uracil. In contrast, 1 $\log_{10}$ unit higher IFUs were seen when uracil was added to the WT strain indicating a preference for growth when supplemented with uracil. When incubated with varying concentrations of 5-FU, the growth of WT strain was inhibited by 2 $\log_{10}$ recoverable IFUs when compared to the mutant strain (Figure 12) in the CHO UrdC$^-$ cell line .The dysfunctional OPRT enzyme in the cell line rendered them resistant to the toxic effects of the drug while the WT *C. muridarum* with a functional UPRT was susceptible to the drug. The *upp* mutant displayed complete resistance to 5-FU at all three concentrations (5μM, 10μM & 25μM) of the drug used in the experiment. During starved conditions, the growth levels were almost similar for the WT and mutant strains.

In the HeLa 229 cells, in starved conditions, similar levels of growth occurred for both WT and the mutant strain (Figure 13). In contrast, both WT and mutant strains exhibited an inhibition of growth of 2 Log_{10} units of recoverable IFUs, when incubated in the presence of increased concentrations of 5-FU. Since the enzyme OPRT is functional in the HeLa 229 cells, and can thus convert the drug to its toxic form, the observed growth inhibition was expected despite the absence of UPRT activity in the mutant. The *in vitro* growth experiments confirmed the attenuated nature of the mutant.

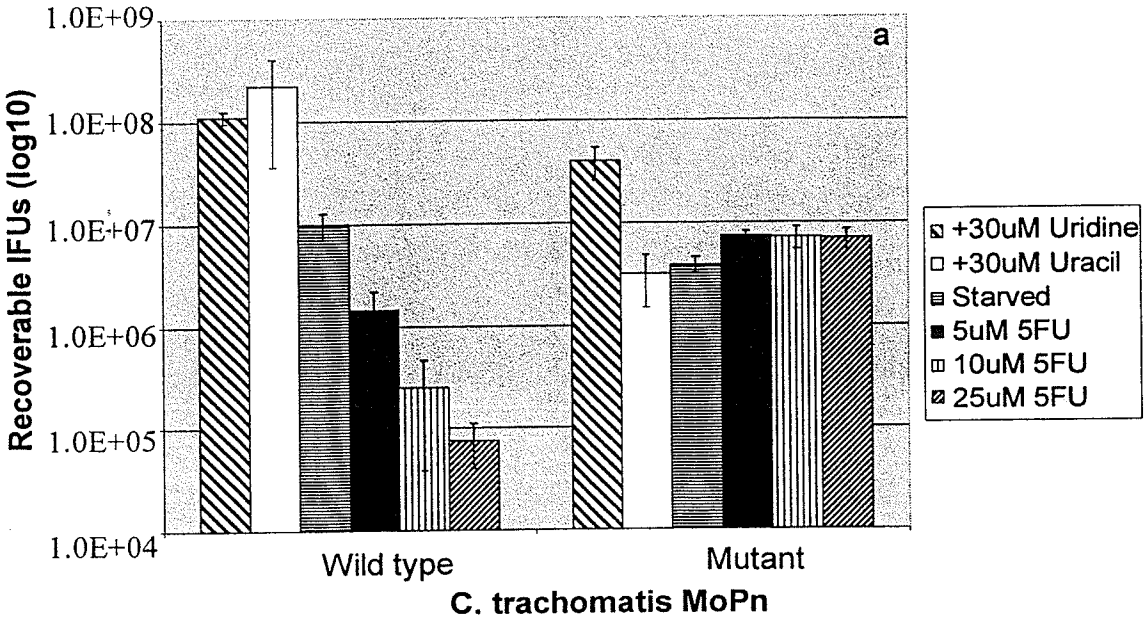


Figure 12: Effect of 5-Fluorouracil on the growth of WT and *upp* mutant *C. muridarum* in CHO Urd C⁻ cell line.

CHO Urd C⁻ cell line is inoculated with either the WT or *upp* mutant of *C. muridarum* at a MOI of 1 and cells were incubated in the presence of three different concentrations of 5-Fluoruracil. Media supplemented with 30μM uridine or 30μM uracil served as controls. Recoverable IFUs were enumerated as described in Materials and Methods.

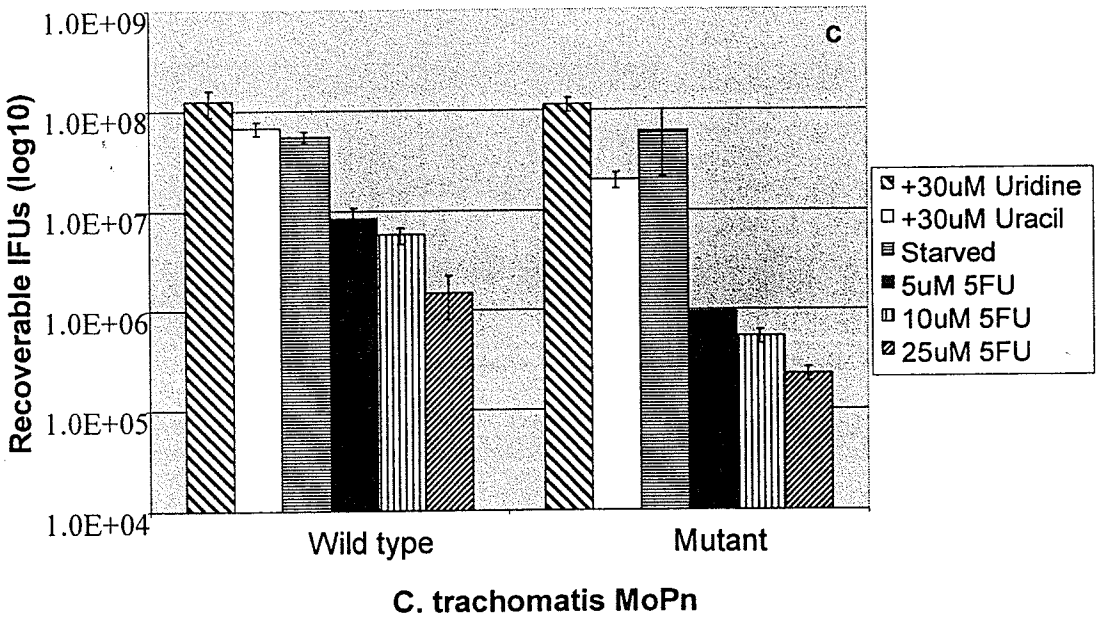


Figure 13: Effect of 5-Fluorouracil on the growth of WT and *upp* mutant *C. muridarum* in HeLa 299 cell line.
HeLa229 cell line is inoculated with either the WT or *upp* mutant of C.muridarum at a MOI of 1 and cells were incubated in the presence of increasing concentrations of 5-Fluoruracil. Media supplemented with 30μM uridine or 30μM uracil served as controls. Recoverable IFUs were enumerated as described in Materials and Methods.

3. Growth of chlamydia *in vivo* in the lungs of BALB/c mice.

Previous studies done in our lab clearly showed a direct correlation between the loss of body weight and the severity of the disease. For *in vivo* studies, five weeks old, female BALB/c mice were intranasally infected with 1×10^3 IFUs of the *upp* mutant. An equal number of wild type *C. muridarum* IFUs were used to infect a second group of mice to study the difference in growth, if any, between the two groups of mice. Mice were weighed regularly during the course of infection and the changes in body weight post infection were plotted as a graph (Figure 14). Wild type *C. muridarum* infected mice lost an average of 2.5 gms and started regaining their body weight later in the course of infection at about day 11. In contrast, mice that were infected with the *upp* mutant Chlamydia lost very little body weight (1gm) when compared to the wild type infected mice and infact started to regain their weight on day 5. Furthermore, mice were given a higher infectious dose to examine if the observed difference in weight loss between the two groups of mice persisted at increased bacterial doses. Hence, mice were intranasally infected with 4000 IFU of either the mutant or WT Chlamydia and were monitored daily for body weight changes. Interestingly, higher infectious dose generated a similar effect as was observed when lower doses were used (Figure 15). However, the body weight loss was proportional to the dose they received as WT infected mice lost an average of 6gms of their body weight while mutant infected mice lost as less as 2.5 gms. This was further supported by the observation that WT infected mice developed symptoms like ruffled hair, hunched poster and decreased mobility which was not observed in the *upp* mutant infected mice.

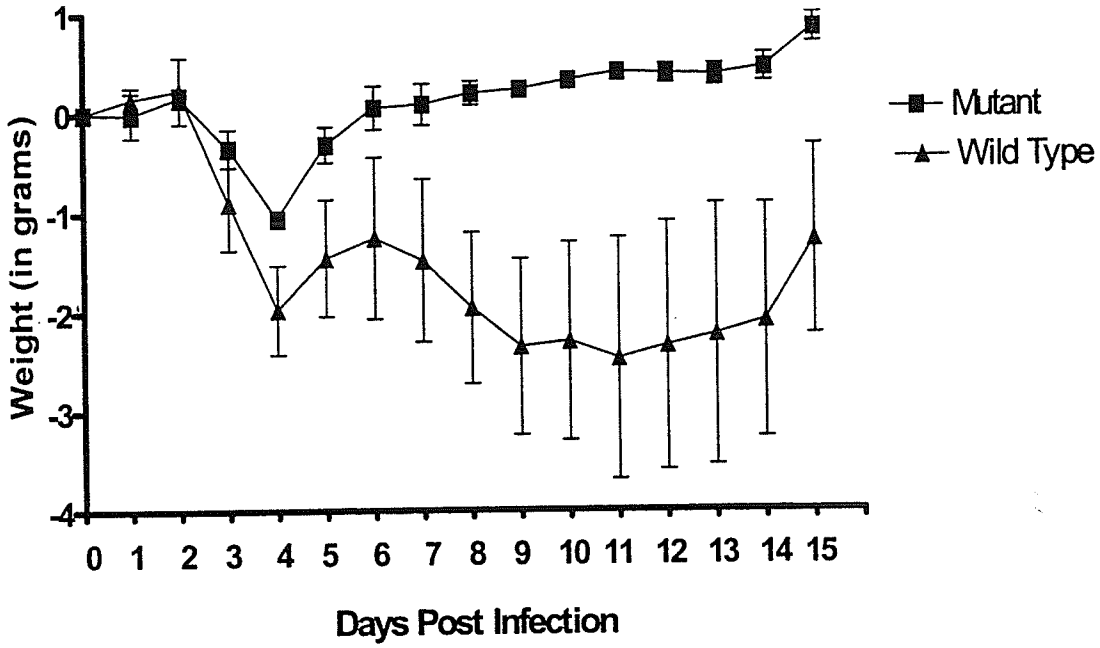


Figure 14 : Body weight changes in BALB/c mice following infection with 1×10^3 IFU of upp mutant Vs wild type *C. muridarum*

Infection with the chlamydial mutant results in comparatively less body weight loss. 8 week old BALB/c mice (n=4) were intranasally infected with 1000 IFU of either Wild Type or *upp* mutant of *C. muridarum*. Body weights were recorded daily following infection until sacrificed on day 15 post infection.

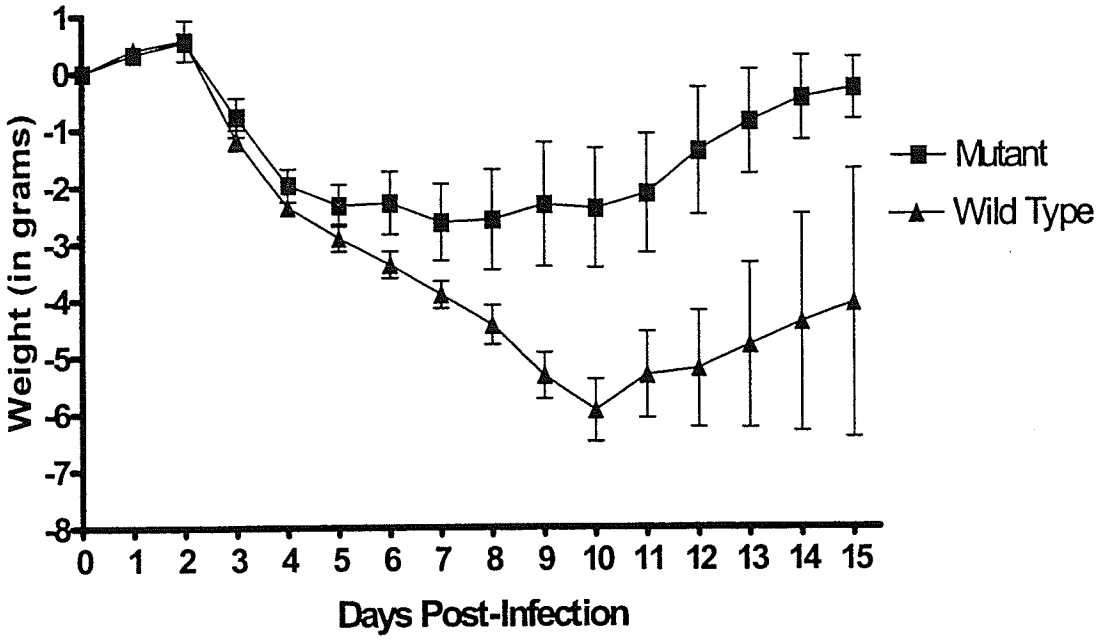


Figure 15: Body weight changes in BALB/c mice following infection with 4×10^3 IFU of upp mutant Vs wild type *C. muridarum*

Infection with the Chlamydial mutant results in comparatively less body weight loss. 8 week old BALB/c mice (n=4) were intranasally infected with 4000 IFU of either Wild Type or *upp* mutant of *C. muridarum*. Body weights were recorded daily following infection until sacrificed on day 15.

Previously, Yang and colleagues showed that bacterial burdens in the infected lungs are directly proportional to and reflective of the disease severity in the mice (73, 85). Hence, lungs from infected mice were isolated aseptically in sucrose phosphate glutamate and homogenized. Supernatants were used to infect HeLa cell monolayers and recoverable IFUs were enumerated in both the groups of mice. There was a significant difference ($p< 0.005$) in the number of Chlamydia between the *upp* mutant infected mice and those infected with WT Chlamydia. Far less chlamydia were recovered from the mutant infected mice when compared to those of WT infected mice (Figure 16). It is not understood why the mutant was unable to grow in mice but repeated experiments showed similar results.

Pathological changes in the lung tissue were studied after staining the tissues with Hematoxylin and Eosin. In the *upp* mutant infected mice, the inflammation was mostly cleared with little or no cellular infiltration and slides appeared similar to those that were taken from naïve mice (Figure 17). On the other hand, WT infected mice showed massive infiltration of lymphocytes thus confirming the results obtained in the lung IFU analysis. These results further confirm the attenuation of the *upp* mutant in a mouse respiratory infection model.

C. Analysis of *upp* mutant of *Chlamydia muridarum* as live attenuated vaccine candidate

The immune status of the *upp* mutant and wild type immunized hosts was monitored before and after challenge to assess the degree of protection conferred by the mutant as compares to the WT strain immunization. Mice were immunized once

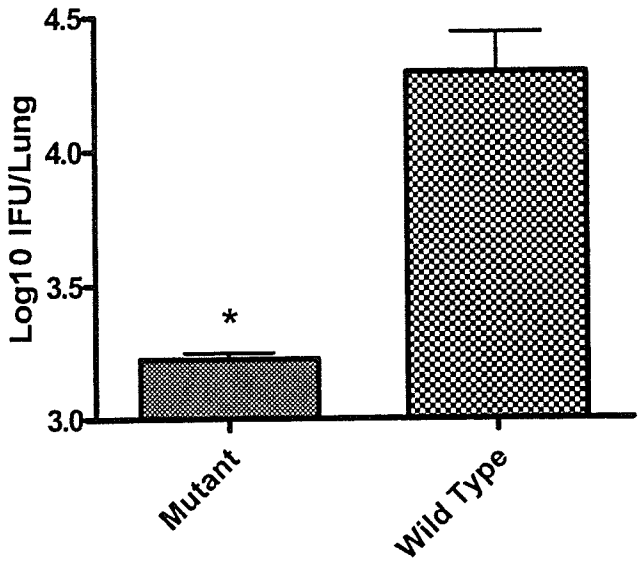


Figure 16: Detection of *in vivo* growth in *upp* mutant versus WT *C. muridarum* infection

Mutant infected mice cleared the organism from the lung to a greater extent than the wild type infected mice. Mice were sacrificed on Day 15 and lung homogenates were used to infect Hela-229 monolayers for quantification. Statistical analysis showed significant differences at P values of < 0.005 between the two groups.

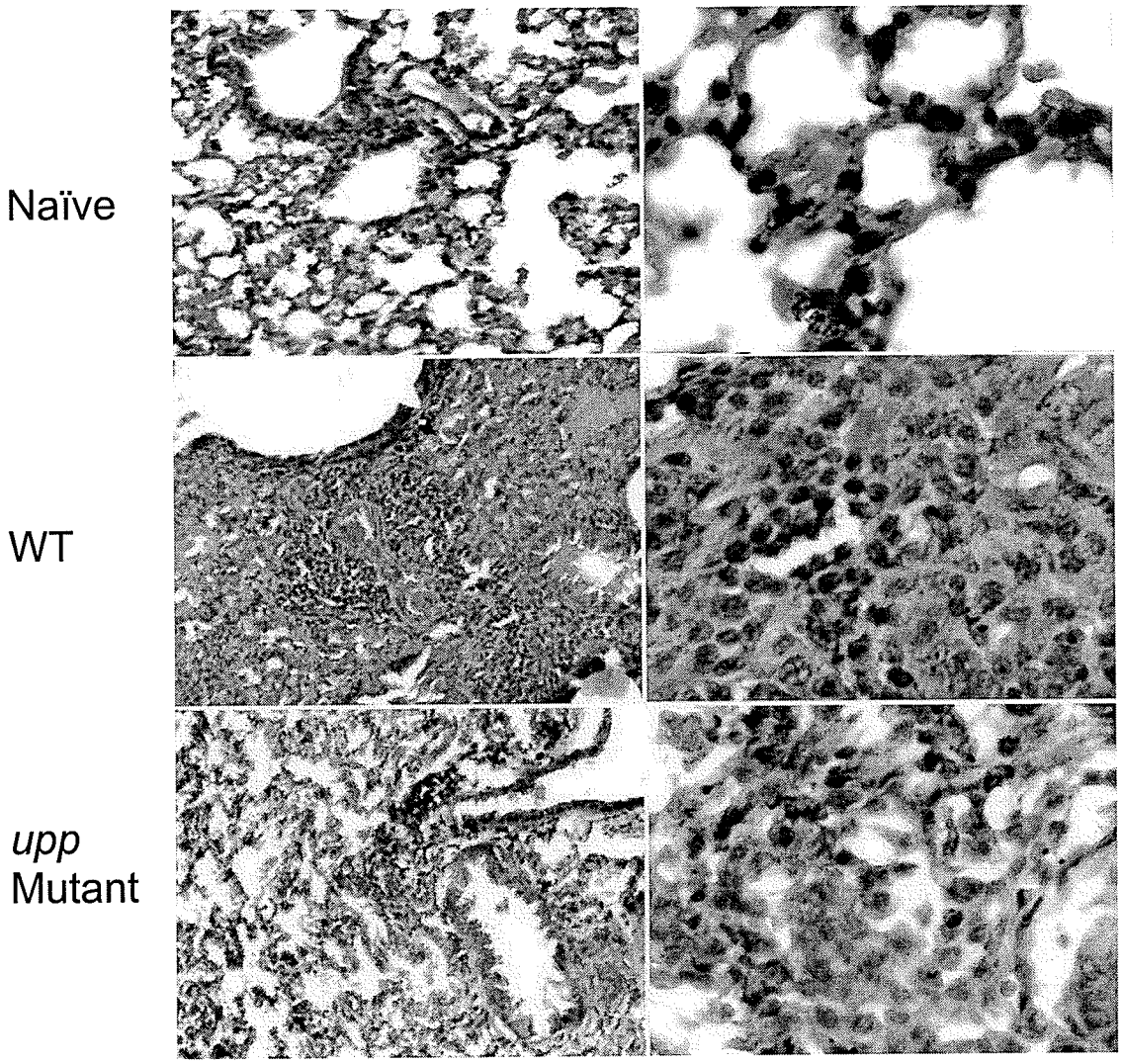


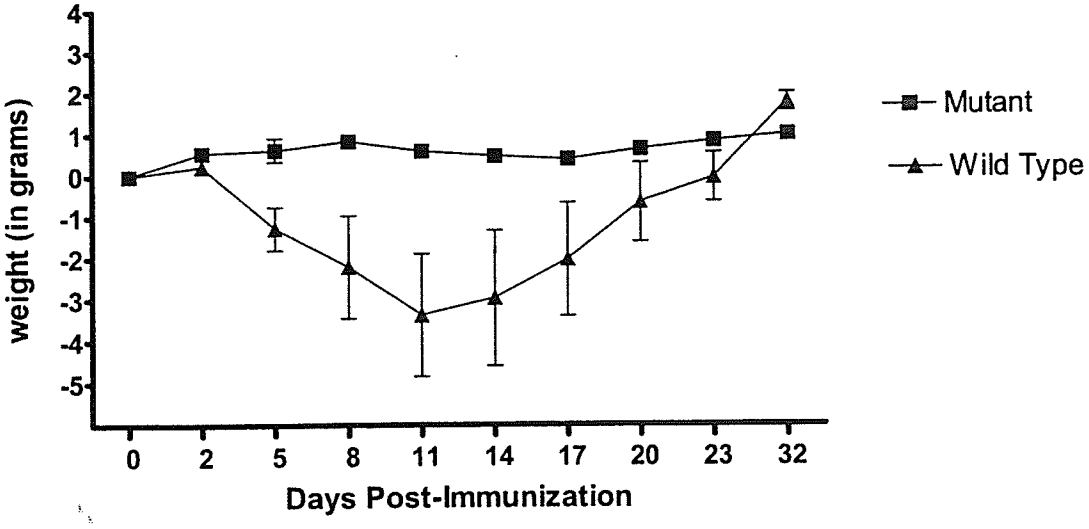
Figure 17: Pathological changes in the lung following infection with *upp* mutant versus WT *C. muridarum* infection.

After euthanasia, lungs were removed from mice and fixed in 10% buffered formalin, embedded and sectioned (6μm). Slides were stained with Hematoxylin & Eosin and were observed under a light microscope. Mice were infected intranasally with 1×10^3 IFU of either the mutant or wild type *C. muridarum* and sacrificed on Day 15. Uninfected mice served as controls. Pictures were taken at magnifications of ×400 and ×1000.

with a small dose of 500 IFU and were allowed to recover completely. During this period mice were continuously monitored and changes in body weight were recorded during a period of 30days. Following immunization, the mutant infected mice did not loose any body weight whereas the WT strain infected mice lost an average of 3gms in total (Figure 18A). Mice were further challenged with a higher dose of 1×10^4 IFU of WT *C. muridarum* and body weight was recorded daily up to 10 days post-infection. Unimmunized mice were challenged to serve as controls. Mice that were immunized with either the mutant or WT chlamydia did not display any loss of body weight (Figure 18B) but control mice lost an average of 7gms by day10 p.i and did not show any signs of recovery. Lung homogenates from challenged mice were analyzed for recoverable IFUs as described in materials and methods. Surprisingly, *upp* mutant vaccinated mice were protected against an otherwise lethal intranasal challenge with 1×10^4 IFU of the wild type chlamydia given 30 days after immunization. The protection was invariably accompanied with elimination of the bacteria and the development of complete sterile immunity. Control mice that did not receive any immunization exhibited a very high bacterial load in their lungs amounting to 6 $\log_{10}$ recoverable IFUs (Figure 19).

IL-12 and IFN-γ are essential factors for vaccine induced protective responses. Earlier studies in our laboratory have reported that vaccination with UV killed EBs is less effective at inducing anti-chlamydia immunity. In contrast, protection was achieved when live EBs were used for immunization. The essential reason for the difference could be due to secretion of pro-inflammatory cytokines like IL-1β, TNF-α and IL-6 by infected epithelial cells while killed EBs cannot infect cells and difference

A



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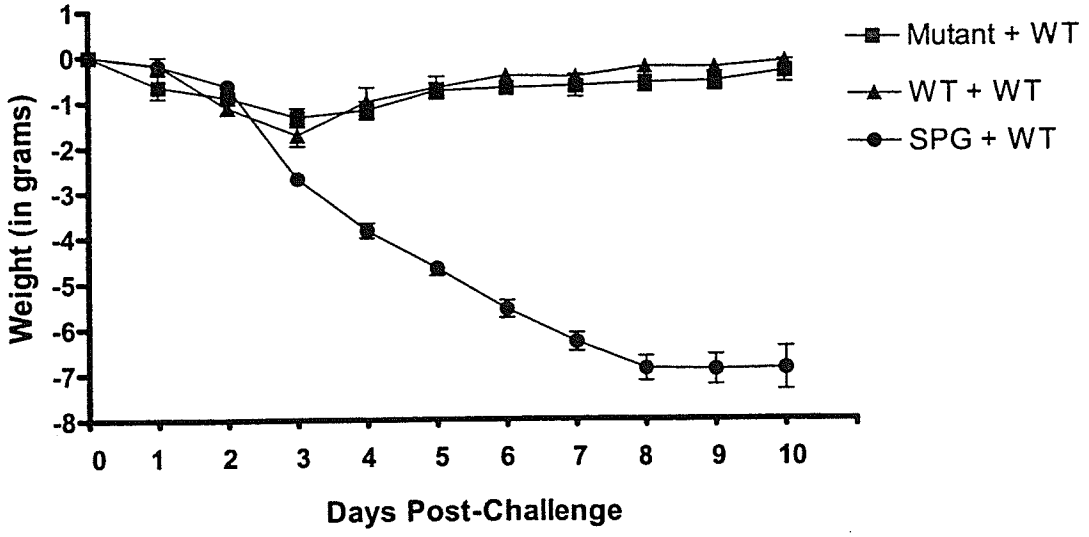

Figure 18: Body weight changes in Balb/C mice following immunization with upp mutant Vs wild type *C. muridarum*

A) 5-6 wk old Balb/C mice (n=4) were immunized intranasally with 500 IFU of either WT or Mutant and were allowed to recover completely for 30-40 days. The control group received SPG alone. B) All mice were challenged with 1×10^4 IFU of *C. muridarum* and were daily monitored for survival and body weight changes. Body weight changes following infection, immunization or challenge were recorded daily.

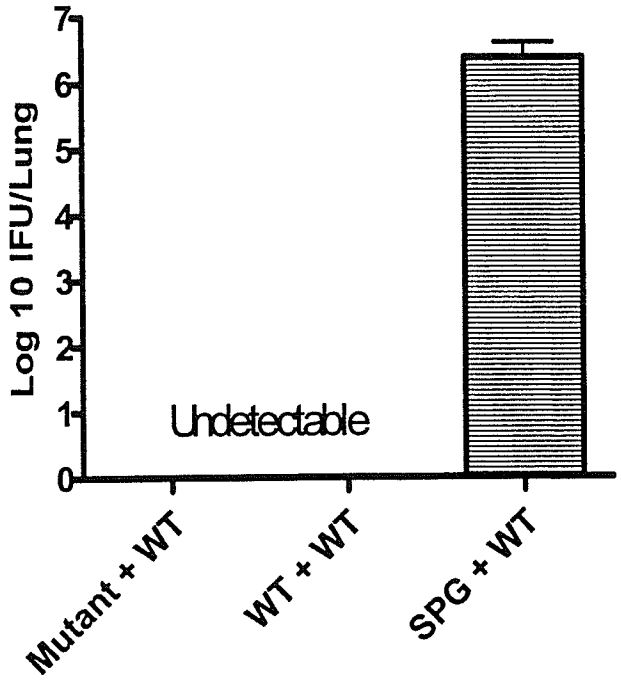

Figure 19: Detection of *in vivo* chlamydial growth in mutant immunized mice after challenge with WT *C. muridarum*

Mice were immunized with either 500 IFUs of the *upp* mutant or the WT *C.muridarum*. 30 days following immunization, all mice were challenged with 1×10^4 IFUs of WT *C. muridarum* . Mice were sacrificed on day 10 post challenge and lungs were removed to detect chlamydial growth. Inclusion Forming Units (IFU) were undetectable in mice immunized with either the Mutant or the Wild type *C. muridarum*. Unimmunized controls displayed 6 Log_{10} units of IFUs in the lung.

in the antigen concentration that is being presented by the dendritic cells. These cytokines are responsible for inducing an efficient Th1 response thus producing robust amounts of IL-12 and IFN-γ which are required for the successful clearance of the bacteria. We investigated whether the mechanism of protection upon *upp* mutant vaccination was similar to what was earlier known about the protection generated by WT immunization. Expression of both the Th1 cytokines, IFN-γ and IL-12, was comparable (Figure 20).

The role of B-cell mediated, antibody responses in primary infection with *C. muridarum* are less clearly understood (83, 86). In contrast, the involvement of humoral immune responses in reinfection or secondary infection is crucial in protection against infection with *C. muridarum* (22). These antibodies are required for the neutralization of EBs in the extracellular environment and thus inhibit their attachment to the host epithelial cells. In order to determine the humoral responses in secondary infection, mice were bled before sacrificing them on day 10 post challenge and the serum was analyzed for antigen specific IgG2a, IgG1 and IgA antibody titers. Two weeks after vaccination with the *upp* mutant, all mice in the group had high antigen specific IgG2a titer (Figure 21), in serum, which is the major antibody involved in protection against *C. trachomatis*. Following challenge with wild type *C. muridarum*, all immunized mice produced significant amounts of IgG2a and IgG1 but lesser amounts of IgA and very little IgM. IgG2a is clearly the dominant isotype in this *upp* mutant immunized mice as it always is in wild type induced protection. SPG immunized mice produced little, if any, measurable levels of Chlamydia specific antibodies at day 10 post challenge. Thus, the *upp* mutant immunized mice

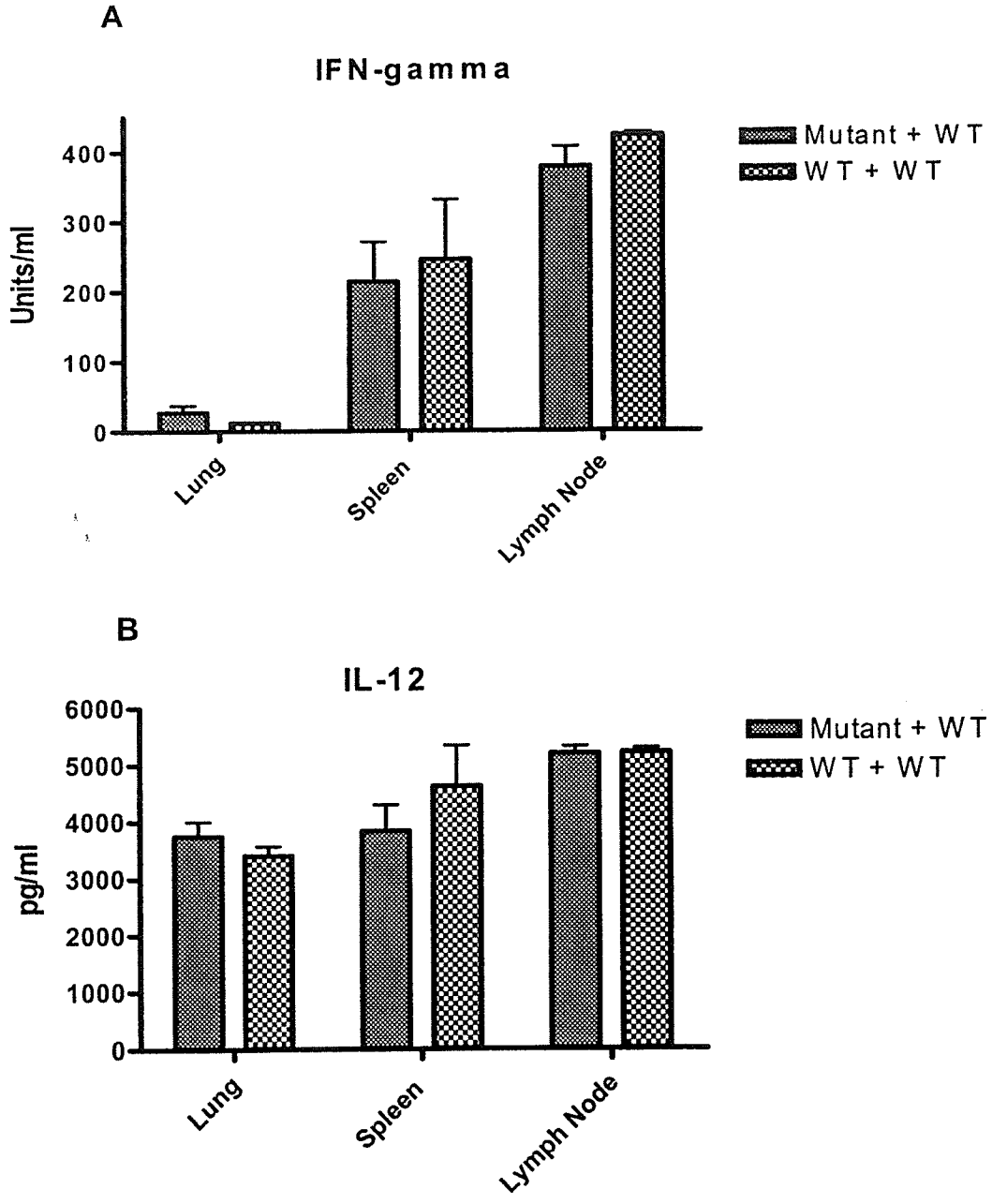

Figure 20: Expression of protective cytokines, IL-12 and IFN-γ, in lungs, spleen and lymph node cell cultures in *upp* mutant Vs WT infected mice.
Mice were sacrificed day 10 post challenge. Cytokines in lung homogenates, splenocytes culture supernatants or draining lymph node cell culture supernatants were assayed using ELISA. Values are the means and SD is derived from four mice in each group.

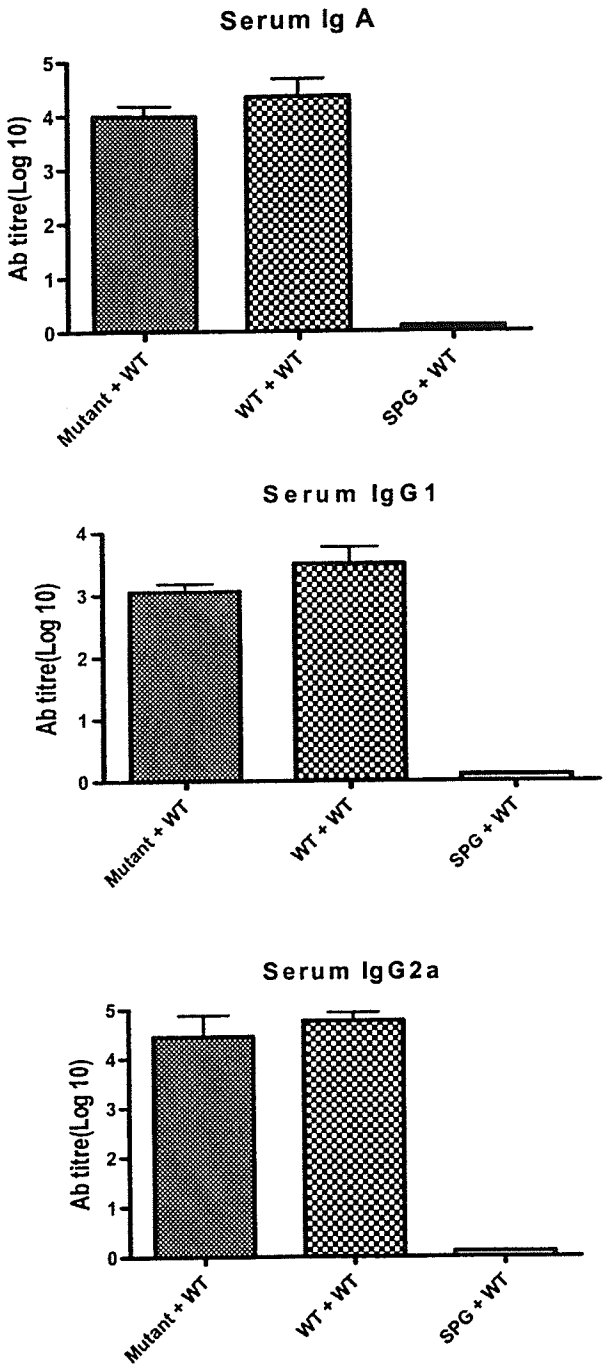

Figure 21: Antibody production in *upp* mutant immunized mice following challenge with WT *C. muridarum*

Serum was collected day 10 post-challenge and anti-chlamydial antibodies in individual mice were quantitated by ELISA. 0.5 O.D at 405 nm is used as an endpoint and plates were read at 30min for IgG2a and IgG1 and at 1hr for IgA.

spontaneously cleared the infection and were resistant to challenge with the WT chlamydial strain.

Intranasal inoculation of *C. muridarum* induces a strong inflammatory reaction in the lung. To study the mouse lung histopathology, infected tissues were formalin fixed and stained with H&E. Stained tissues were blindly observed under a light microscope for pathological changes such as inflammatory cell infiltration which gives an indication of the severity of the disease. In line with the results obtained in cytokine and antibody production, the lung architecture was completely normal in mice that were immunized with either the *upp* mutant or the WT *C. muridarum.* The alveoli were clearly visible with very little inflammatory cell infiltration (Figure 22). In contrast, lung sections from mice that received SPG alone had immense inflammatory cell infiltration completely occupying the airspaces.

These results collectively suggest that vaccination with the *upp* mutant is capable of inducing protection as effectively as has been observed when WT chlamydia were used for vaccination. The cellular and humoral mediated immune mechanisms of protection are identical too. However, the exact mechanism by which the growth of the *upp* mutant is inhibited in mice is unknown remains to be determined. the mutant grows equally well in HeLa cells as does the WT without supplementation of either uracil or uridine. The attenuation in the growth of the mutant could be due to the effect of IFN-γ, produced by the mice in response to infection. This cytokine can induce the enzyme, uridine phosphorylase, which converts uridine to uracil and could have decreased the availability of UTP in the lung. Uridine phosphorylase is the most important enzyme in pyrimidine metabolism and is

involved in the regulation of uridine homeostasis (87). Hence, it is possible that the lack of UPRT activity coupled with the limitation for UTPs was responsible for the attenuation of the mutant strain.

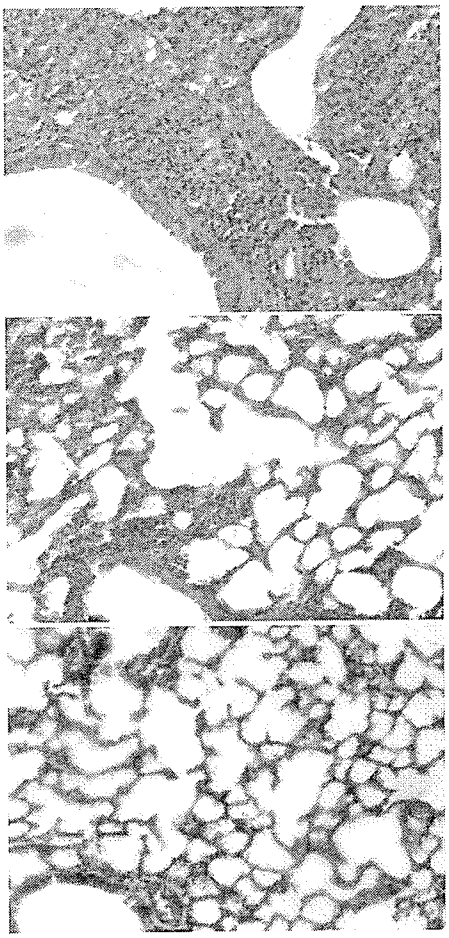

Figure 22: Histopathology of lungs from mutant Vs wild type infected mice following challenge infection.
Mice were intranasally immunized with 500 IFU of either the upp mutant or WT *C. muridarum*. 30days following infection, all mice were challenged with 1×10^4 IFU of WT *C. muridarum.* Mice were sacrificed 10 days post challenge. Lungs were fixed in formalin and sections were stained with Hematoxylin & Eosin and slides were analyzed for histological changes. Magnification is ×200.

DISCUSSION

A. Modulation of immune responses to *C. muridarum* infection upon progesterone administration

A mouse model of the genital tract infection induced by *C. muridarum* has been well studied to understand the immunological parameters of infection and immunity (88). In this study we have investigated the effect of progesterone on host susceptibility and its ability to modulate protective immune responses in a respiratory tract infection with *C. muridarum*. For this purpose, mice were subcutaneously injected with Depo-provera, a long acting progestational formulation, which is routinely used to render mice susceptible to a genital tract infection with *C. muridarum*. In this model, progesterone is supposed to synchronize all mice into an anestrus condition and further reduce the epithelial turn over in the genital tract. Some experimenters believe that, thinning of the epithelial layer under the influence of progesterone will have facilitated the establishment of an infection which is other wise not possible (89). *C. muridarum* was initially isolated from the lungs of mice and does not cause disease in humans (6). Hence, *C. muridarum* poorly infects the genital tract in mice. Therefore, mice were pretreated with subcutaneous injection(s) of progesterone in order to establish a successful infection. Moreover, human serovars do not induce an infection in mice unless the Chlamydiae are directly injected into the uterine horns or a very high dose of the inoculum is used.

Kausic et al studied the effect of progesterone on genital herpes infection in mice (90). In their experiments, Depo treatment significantly enhanced the susceptibility of the mice to genital HSV-2 both at estrus and diestrus, decreased the

local antibody production and failed to protect mice from challenge after immunization with HSV-2 (90). Furthermore, studies done by the same group showed that ovariectomized mice, when treated with progesterone succumbed to the genital HSV-2 infection accompanied by persistent inflammation and neutrophil infiltration (91, 92). In our experiments, we did not ovariectomize mice in order to closely replicate the Chlamydial genital tract infection model protocols. In order to control for estrus cycle changes, sex and age matched mice were used in all the experiments. Since our model is a respiratory tract infection, the immune modulation by progesterone can be studied both locally and systemically without the necessary influence of the progesterone present in the genital tract.

In my experiments, mice were treated with either one s.c injection or two injections of Depo-provera before infection with 1×10^3 IFU of *C. muridarum*. Body weight loss is a significant clinical feature of the disease severity in mice. Following infection, body weight loss was comparable among all three groups of mice irrespective of the treatment they received (Data not shown). Progesterone belongs to the steroidal group of hormones and these hormones have known anabolic effects that could have masked the body weight loss. The growth kinetics of *C. muridarum* in progesterone treated mice was monitored in order to determine the effect of hormone pretreatment on organism burden in the lungs. Early in the course of infection, on day 7, mice in all three groups did not show any difference in their chlamydial burdens in the lungs irrespective of hormone treatment (Figure 5A). However, on day 15 p.i, chlamydial titers from lung homogenates of mice which received two doses of progesterone were 7 fold higher when compared to the control, untreated mice

indicating the hormone interferes with the clearance of Chlamydia from the lungs. It is well known that progesterone increases susceptibility to infection both in experimental studies in animals (33, 93-95) and epidemiological studies in users of injectable progesterone (Depo-Provera) for contraceptive purposes (29, 31, 32). Also, persistent infections of *C. muridarum* were observed when ovariectomized rats were pretreated with progesterone (96).

Progesterone has widely accepted suppressive action on the development of Th1 response (97, 98) . However, the cellular and molecular mechanisms underlying this suppression are unknown. Both in human subjects and animal experiments, the difference in the cytokine patterns clearly correlated with the progression of the chlamydial disease. Particularly, Th1 type cytokines were involved in protection whereas, Th2 type cytokines were associated with severe immunopathology. By using IFN-γ gene or IFN-γ receptor gene knock out mice and the adoptive transfer of IFN-γ producing CD4+ helper T cells, studies done in our laboratory and others have confirmed the protective role of this cytokine in *C. muridarum* infection of mice (25, 66). IFN-γ is a pleiotropic cytokine that induces and modulates an array of immune responses. In mice, the *in vivo* inhibitory effect of IFN-γ on chlamydiae is mediated via the transcriptional regulation of inducible nitric oxide synthase (iNOS) gene in infected macrophages and epithelial cells (99). iNOS essentially catalyzes the production of NO intermediates that are toxic to the Chlamydiae (100). It has been reported that progesterone pretreatment of human intestinal epithelial cell lines inhibited cytokine induced iNOS mRNA expression (52) and iNOS gene activity was suppressed in LPS-activated mouse macrophages when exposed to pharmacological

levels of progesterone (101, 102). Because the cytokine IFN-γ is necessary for host control of *C. muridarum* infection, it is possible that progesterone could have induced persistent inflammation by down regulating the production of IFN-γ by a yet unknown mechanism. However, persistent inflammation was observed only in mice which received two injections (2.5mg each/mouse) of progesterone suggesting that the concentration of the hormone may determine the susceptibility of the mice to infection. Notably, the decreased production of IFN-γ in mice that received a single dose of progesterone was not significant in comparison to the control group. Altogether, these results indicate that progesterone pretreatment of mice significantly suppresses the protective IFN-γ production when higher doses of the hormone were administered.

IL-10 is an important anti-inflammatory cytokine and is primarily produced by the Th2 subset of CD4+ helper T cells. Dendritic cells, the major antigen presenting cells, and macrophages also produce significant amounts of IL-10 in response to exposure to antigen. IL-10 is known to inhibit the antimicrobial activity of IFN-γ against a variety of pathogens, and increases susceptibility to chlamydial infection (23, 85, 103). In particular, IL-10 knock out mice exhibited increased expression of IFN-γ, IL-12 and delayed type hypersensitive responses, and reduced expression of IL-5 without granulomatous pathologic changes in the lung (85). Multiple and distinct signaling pathways, including the JAK-STAT pathways, mediate the various functions of IL-10 (Table 3). The major effects of IL-10 are inhibition of cytokine production by macrophages and inhibition of T-cell activation by regulating the differentiation and maturation of APC. The latter function can be mediated by the reduced expression of

Table 3: General effects of IL-10 in different cell lineages

Cell lineage	Effects of IL-10 on cell type
T -Lymphocytes	Specifically inhibits Th1 cytokine production in mouse macrophages Exhibits inhibitory effects on T-cell proliferation (by suppressing IL-2 production), survival and cytokine production by T-cells of human origin. Inhibits IFN-γ synthesis by CD8+ T cells Inhibits T-cell function by down regulating expression of maturation markers such as MHC II on monocytes Prolongs the survival of T-cell by inhibiting apoptotic cell death via up-regulation of Bcl-2
Monocytes/ Macrophages	Inhibits macrophage activation by IFN- γ Interferes with the production of pro inflammatory cytokines like IL-1, IL-1b, TNF-a, IL-6, IL-8, GM-CSF by human monocytes upon activation Exerts a negative feedback loop on its own production by monocytes Inhibitory effect on the expression of maturation molecules like MHC II on the surface of monocytes
Natural Killer cells	Inhibits production of IFN- γ by NK cells via suppression of monocyte functions. Induces NK cytotoxic activity on NK resistant tumor cells
-lymphocytes	Induces the expression of MHC II molecules on resting B-cells Stimulates differentiation of B-cells into antibody producing cells Induces apoptosis in germinal center B-cells

MHC-II and co-stimulatory molecules such as B7. Since progesterone induces a switch in the immune response from a Th1 to a Th2 type, antigen driven IL-10 production in the spleen and lymph node cells was analyzed ($p<0.05$). As expected, lung homogenates from infected mice, both early (Figure 8A) and late (Figure 8B) in the infection, contained significantly enhanced amounts of IL-10. At least one study has reported that mouse bone marrow derived dendritic cells produced accelerated amounts of IL-10 when cultured in the presence of progesterone (51). The source of the increased IL-10 observed in my experiments could be either due to a shift of balance towards a Th2 type of immune response or due to an enhanced IL-10 production by DCs or both. Overall, the comparison of cytokine patterns among the different hormone treatment groups and the finding that IL-10 expression is elevated suggests that progesterone has deleterious effect on the host immune response to infection and promotes pathological changes caused by *C. muridarum* infection. It is well known that too weak a Th1 response or a response with a substantial Th2 component can lead to severe disease in intracellular pathogens other than Chlamydia, such as HIV, *Mycobacterium tuberculosis* (104) and *Leishmania* species (105).

A definitive role for antibodies in host defense against Chlamydia is not demonstrated in a primary infection. In our murine respiratory model, antibodies to chlamydia are detected in the serum and locally in the lung. Antibodies first appear about 10 days p.i and are detectable in the circulation for several weeks after infection. IL-10 can induce the production of IgG2a antibodies which are the major IgG isoforms involved in anti-chlamydial activity. Inspite of the increased amounts of IL-10.

The expression of IFN-γ by spleen and lymph node cells and locally in the lung correlated well with reduced pathology in the lung. The group which received two progesterone injections exhibited increased pathology in the lungs with massive infiltration of inflammatory cells.

Abel et al., studied the effect of progesterone administration before intravaginal challenge infection with pathogenic strains of simian immunodeficiency virus, on the ability of the non-pathogenic SHIV strain immunized mice, in the resolution of infection (106). Primates were otherwise protected from challenge with pathogenic SIV strains when immunized with non-pathogenic strains of SHIV. In progesterone administered primates, the rate of protection against challenge decreased significantly and loss of protection was also associated with increased plasma viral RNA levels. Recent studies done by other groups also demonstrated that exogenous administration of progesterone increased the susceptibility of rhesus macaques to intravaginal inoculation with simian-human immunodeficiency virus by suppressing the anti-viral cellular immune responses (107). Besides the immunosuppressive role of progesterone during pregnancy and infections, exogenously administered progesterone has been documented to have profound effects in the enhancement of skin allograft survival in rabbits and renal allograft survival in dogs (108).

In total, these results suggest that progesterone has a significant immunomodulatory role when administered in higher doses (2.5mg +2.5mg). The results of this study have important implications in the testing and development of vaccines using genital tract infection models.

B. Vaccination potential for the *upp* mutant of *C.muridarum*

Currently, intervention strategies for prevention of Chlamydial strains are aimed at improving the screening programs that might enable early antibiotic treatment of infected individuals and providing sex education. It is well understood that such screening programs do not protect from reinfections or the development of postinfection complications which follow persistent or recurrent infections (109, 110). In developing nations, people often have little or no access to adequate treatment and hygiene. However, reinfection of patients treated with antibiotics is very common and strong immunity is unlikely to develop without multiple exposures to the bacterium. The only plausible solution to combat the spread of the infection, and thereby reduce the morbidity associated with the infection is to develop an effective vaccine that induces a long lasting, robust heterotypic immune response.

Several strategies for the prevention of infection have been suggested by experts in the field of chlamydial research. They are as follows: (i) kill the bacterium immediately upon entering the host; a topical microbicide in the form of a cream, gel or foam. Similar products are in clinical trails to prevent the spread of HIV which is also a sexually transmitted disease. (ii) induce intracellular destruction of the bacterium; the bacterium's type III secretion apparatus may secrete proteins that prevent the fusion of the host cell lysosomes. Identification of such proteins and targeting them can be useful (iii) arrest the growth of bacteria within the host cell; Chlamydia imports lipids and other nutrients from the host cell by yet unidentified proteins. Vaccines can then be designed to immobilize or mutate these proteins when identified. Since the chlamydial genome has been sequenced recently, the

identification of such proteins is not impractical. (iv) prevent the spread of infection; triggering suicidal death in infected cells before the chlamydia start a second replication cycle.

Initial attempts to develop vaccines for Chlamydia employed killed EBs, inactivated EBs, adjuvant use, subunit vaccines containing MOMP, recombinant proteins and peptides, DNA and dendritic cells pulsed ex vivo with killed EBs etc., (70, 71, 111-113). The results of these studies were disappointing as there was only partial protection or the duration of the protection was short-lived. While results were encouraging in animal models (114), clinical trails in humans did not prove to be protective (115). Live attenuated vaccines have been generated and are being successfully used for immunization against several viruses like mumps, measles, rubella, polio and varicella zoster. Additionally, attenuated live vaccines are currently in human use for bacteria like *Salmonella typhi* (116), *Mycobacterium tuberculosis* and *Vibrio cholerae*. Most importantly, killed or live attenuated vaccines have been successfully generated for animal pathogens like *Chlamydophila abortus* (72) and *Chlamydophila* felis (117). Live attenuated strain of *C. abortus* was developed by chemical mutagenesis and this temperature sensitive strain can grow at 35°C but not at 39.5°C, the body temperature of sheep. The major advantages of live attenuated vaccines over the use of a killed vaccine are that they induce a more balanced immune response, the immunity generated is invariably long lasting and they can stimulate mucosal immune responses that are in turn capable of inducing the systemic humoral and cell-mediated immunity (118). Additionally, they are capable of eliciting a multifunctional and antigen specific response. Hence, the use of attenuated bacteria

that cannot cause clinical disease but trigger a self-limiting infection leading to the development of a strong adaptive protective immunity would be an impressive alternative to subunit and killed vaccines. However, some of the major concerns associated with the use of live attenuated vaccines include safety and stability.

A single intranasal dose of 500 IFU of WT *C. muridarum* is sufficient to induce sterile immunity upon challenge with a higher dose of 1×10^4 IFU. The *upp* mutant was tested *in vivo* in our mouse respiratory model for its ability to protect from challenge infection with WT, when mice were immunized with the mutant, this protection was compared to that induced by WT immunization. Surprisingly, both groups of mice which were immunized, cleared the bacteria from the lungs.

Immunity to a secondary or challenge infection with chlamydia is regulated by the adaptive immune responses, both Th1 cell dependant cellular and humoral effector mechanisms. Cell mediated immune responses to the bacterium were evaluated by measuring the antigen specific expression of IFN-γ in lymph node and spleen cells cultured in the presence of UV killed chlamydia. In addition, IFN-γ in the lung homogenates was also determined. Mucosal immunity is committed to combat pathogens entering the body through the mucosal surfaces. Again, all mice that were immunized produced comparable amounts of IFN-γ that is necessary for the resolution of infection. In humans, Chlamydia invades the body through the mucosal tissues of the eye and the genital tract. In mice, mucosal surfaces of the lung are the primary sites of entry. Organized lymphoid tissues in the lung are called BALT or Bronchial associated lymphoid tissues. BALT consists of germinal centers containing CD4+ and CD8+ T-cells, IgA secreting B-cells, and follicular dendritic cells (FDCs) that might

play an important role in picking up antigens from the mucosal surfaces. These T-cells and FDCs could be the source of the IFN-γ expressed in the lungs that positively contributes to the resistance of mice to challenge infection.

Anti-chlamydial antibodies play a major role in the resolution of a secondary infection. IgG2a which is involved in the neutralization of cell free EBs is critical for protection. Both, mutant strain and WT strain infected mice, produced comparable levels of antibody. However, it was interesting to learn that the mutant was equally effective in stimulating the immune response to an unknown threshold level that facilitated the development of immunity to re-infection despite causing minimal pathological changes in the host.

Uracil phosphoribosyltransferase is present in a number of microorganisms and is responsible for uracil salvage. UPRT is necessary for growth of *C.muridarum* in the host cells as the bacterium has a transport system that is insufficient for importing required UTP from the host cell cytoplasm. Hence, this enzyme served as an appropriate target for mutagenesis using 5-FU. The CHO Urd C^- cell line which lacks the ability to catalyze the formation of cytotoxic 5-FUTP, has served as an excellent host for mutagenesis. Often, bacteria resistant to the toxic effects of 5-FU have mutations in the *upp* gene. To date, the *upp* gene has been studied and characterized in *E.coli, Bacillus subtilis, Saccharomyces cervisiae, Lactococcus lactis* (119), *Mycoplasma genitalium etc,*. Earlier, mutants of the *upp* gene have been generated for *Toxoplasma gondii* (120) and *Salmonella typhimurium* (76). A uracil auxotrophic mutant of *T.gondii* failed to multiply in the absence of uracil supplementation and was attenuated inside the host. Nevertheless, it generated a robust long lived immunity in

WT as well as IFN-γ knockout mice. Similar purine and pyrimidine mutants were generated in the facultative intracellular bacterium, *S.typhimurium*. Some of these mutants also exhibited attenuated properties once inside the host and failed to replicate inspite of successfully entering and surviving in the host cell (76).

In our mutant, the *upp* gene was sequenced following mutagenesis. A point mutation, G to T transversion, was found at base 362. This transversion led to coding of the aminoacid Leu_{121}, instead of an Arg_{121} present in the WT strain (Figure 23). Conservation of this arginine among bacteria, archebacteria and plants partly signifies a major role for this amino acid in the function of the enzyme. Earlier, complementation studies and enzyme assay studies were done in Dr. McClarty's lab to confirm the loss of UPRT activity. *In vitro* growth characterization studies done in HeLa229 and CHO UrdC- cell lines also confirmed the loss of enzymatic activity to some extent. In *in vivo* experiments, the mutant was attenuated significantly and caused minimal damage to the host but was still able to grow within the lungs of mice (Figure 16). The reason for this attenuation was not yet clear but some thoughts have been put forward.

Microarray studies done in mouse primary epithelial cells upon IFN-γ treatment have shown the up-regulation of one particular enzyme, uridine phosphorylase. The function of this enzyme results in increased uracil pools which may then enable the growth of WT strains. On the contrary, the loss of UPRT activity in the mutant could have led to its attenuation in mice. The induction of the uridine phosphorylase enzyme in the mouse epithelial cells indicates an adaptation of the WT strains against the negative effects of IFN-γ on their growth. *C. trachomatis*

Sequence of wild type and 5-fuorouracil resistant *Chlamydia muridarum upp* genes:

```
Wild Type Cm      365  ATTCCGTGAATGCCTAAAAGAAATCTCTTTAGG 390
Mutant Cm         365  ATTCCTTGAATGCCTAAAAGAAATCTCTTTAGG 390
                       ***** ***************************
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Amino acid alignment:

```
Wild Type Cm       90  DSLSSKPLAAVYVLSHPLIQHKASLLRNKNTKSKIF 120
Mutant Cm          90  DSLSSKPLAAVYVLSHPLIQHKASLLRNKNTKSKIF 120
                       ************************************
Wild Type Cm      121  RECLKEISLGVCYEATRDLALKNISIQTPLMQAECP 157
Mutant Cm         121  LECLKEISLGVCYEATRDLALKNISIQTPLMQAECP 157
                        *********************************
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Figure 23: *upp* gene and amino acid sequence comparison

The wild type *upp* gene sequence acquired from the database is compared to the mutant sequence. A G to T transversion is revealed from the sequence above. Comparison of the aminoacid sequence reveals the coding for a hydrophobic aminoacid, Leucine at position 121 in the mutant instead of a basic aminoacid, Arginine 121 .

causes disease primarily in humans and its growth *in vivo* is inhibited by tryptophan degradation in the presence of IFN-γ inducing indoleamine 2,3-dioxygenase (9). It is now well understood that *C. trachomatis* has evolved to circumvent the inhibitory effects of IFN-γ, on tryptophan depletion, by expressing genes encoding tryptophan synthase (121). The tryptophan precursor, indole, synthesized by normal flora in the female genital tract is utilized as a substrate by Chlamydia expressed tryptophan synthase for the synthesis of tryptophan (122).

In summary, the results of this work demonstrate that a mutation in one of the genes required for the multiplication and successful growth in the host can significantly attenuate the bacterium. A more promising approach can be directed towards the development of double mutants. Because this auxotrophic mutant causes minimal damage to the host during a primary infection and can effectively protect mice from a reinfection, generation and selection of similar mutants of human serovars extends the possibility for the development of a futuristic human chlamydial vaccine.

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APPENDIX

Abbreviations

Ab	Antibody
Ag	Antigen
Cm	*Chlamydia muridarum*
EB	Elementary Body
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
IFN-γ	Interferon-γ
IFU	Inclusion Forming Unit
Ig	Immunoglobulin
iNOS	inducible nitric oxide synthase
IL	Interleukin
KO	Knockout
MEM	Minimum Essential Medium
MHC	Major Hisotcompatibility Complex
MOI	Multiplicity of infection
MOMP	Major Outer Membrane Protein
MoPn	*C.trachomatis* mouse pneumonitis
NK cells	Natural Killer cells
NTP	Nucleoside triphosphate
PID	Pelvic Inflammatory Disease

p.i.	Post infection
Pro	Progesterone
PRPP	Phosphoribosyl pyrophosphate
RB	Reticulate Body
rpm	revolutions per minute
SEM	Standard error of the mean
STD	sexually transmitted disease
UMP	uracil monophosphate
UPRTase	uracil phosphoribosyltransferase
upp	gene that encodes uracil phosphoribosyltransferase
WT	Wild Type