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Evaluating whether pregnancy has an influence on the course of *Chlamydia muridarum* infection in murine models

in

Quantitative Systems Biology

by

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Chair

University of California, Merced

2009

DEDICATION

I dedicate my thesis to my mother and father, Joanna Tsai and Richard Johnson, for their support through my years of schooling. As immigrants, they left their homeland, Vietnam, to start a new beginning on a long road of struggles. They worked long hours to provide for me and allow me opportunities they never received. My parent's determination to succeed in the United States has been instilled into me. Their sacrifice has inspired me to continually persevere and fulfill my dreams.

TABLE OF CONTENTS

ABSTRACT OF THE THESIS

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Chlamydia trachomatis is the most common bacterial sexually transmitted infection amongst individuals between the ages of 14-39 within the United States [1]. The immune system responds to the recognition of *Chlamydia* infection by inducing an inflammatory response and recruiting T cells to fight the pathogen. The proposed experiment evaluates whether pregnancy has an influence on the course of *Chlamydia*

muridarum infection in a murine model. Previous studies show production of type 1 helper T cells (T_h1) are important for aiding in the clearance of *Chlamydia*. However, pregnancy elicits type 2 helper T cells (T_h2) to protect the fetus, which aren't effective in fighting an infection.[2] In my experiment, pregnant and nonpregnant C57BL/6 mice were infected with *Chlamydia muridarum*. Vaginal secretion and vaginal swabs were collected everyday for eleven days. It is hypothesized a T_h2 cytokine production will not protect against *Chlamydia* infection, but may contribute to the development of persistent infection. In my study, infection can be tracked without sacrificing mice at different time points. This allows for minimal usage of mice. It will also illustrate a real-time course of infection throughout pregnancy. The findings provide a murine model that can be applied in illustrating the possible outcomes of infection in humans during pregnancy.

INTRODUCTION

The Center for Disease Control (CDC) reports a cost of two billion dollars annually to deal with *Chlamydia* within the United States. Over a million cases of *Chlamydia* infection were reported to the CDC in 2006. Individuals showing symptoms of infection are prescribed azithromycin or doxycycline as treatment. [1] However, the World Health Organization states about 70%-75% of women infected with the bacterium are asymptomatic. Therefore, many more cases may not have been reported because an individual unknowingly was infected. Untreated or chronic *Chlamydia* infections may lead to problems with infertility, ectopic pregnancy, or pelvic inflammatory disease (PID).[3]

The immune system is important for protecting the body from disease by eliminating pathogens or cancerous cells. The human body has both physical and physiological barriers as a first line of defense. Skin and mucosal surfaces act as its physical barriers, while maintaining temperature and acid pH play a role as physiological barriers. Host defense initiates with the recognition of a foreign invader, and is followed by an immune response. Recognition occurs with complement proteins binding to pathogens. These proteins can either attack the invader or be used as a marker for effector cells. Effector cells, such as phagocytic macrophages or neutrophils, bind to the complement with surface receptors and induce a signal to engulf the pathogen. Two fates can occur after phagocytosis of the microorganism. It is either digested within the cell and released as degraded particles, or the foreign antigen is presented on the cell surface bound to a major histocompatibility complex (MHC) for activation of other specialized immune response cells. Activated macrophages release cytokines, including IL-1β, and

chemokines to induce inflammation at the site of infection, which are distinguished by heat, pain, redness, and swelling. IL-1β is particularly interesting for this project because it contributes to local tissue damage when persistent secretion of the cytokine occurs. The collective processes beginning from the recognition of a foreign invader to inflammation of the infected site illustrates the innate immune response.[4]

The innate immune response is the first line of defense for the body, but it is not always sufficient in clearing an infection. As a result, the adaptive immune system is the next step in responding to persistent infections. Antigen-presenting cells (APCs) are recruited to the site of infection, engulf the pathogen and degrade it, and then present its antigens to lymphocytes. These APCs include macrophages, dendritic cells, and B cells. Activated dendritic cells are important in presenting nonself antigens to lymphocytes. However, problems arise within the immune system when it is unable to distinguish between self and nonself; this a characteristic of autoimmune disease, where the immune system attacks its own healthy cells.[4]

After presentation of antigens to lymphocytes, two adaptive immune responses can occur. One response is a humoral-mediated response with B cells secreting antibodies to neutralize the threat. The B cell receptor and the antigen interaction promote release of antibodies from the B cell. Antibodies aid in clearing infection through processes of neutralization, opsonization, or complement activation. These mechanisms destroy the pathogen directly, mark the pathogen for other immune cells to phagocytose, or inhibit pathogen toxins from binding to receptors. Some antibodies are developed from previous encounters with antigens. In these cases, antibodies can clear these infection more quickly and effectively.[4]

The other adaptive immune response is a cell-mediated response of activated T cells within the lymph nodes. Either CD4 T cells or CD8 T cells are activated depending on the peptide presented on the MHCs. CD8 T cell receptors bind to MHC class I, which present peptides from the cytosol; CD4 T cell receptors bind to MHC class II, which present peptides from endocytic vesicles. Upon recognition of peptides, CD8 T cells differentiate into cytotoxic T cells. The cytotoxic T cells use granzymes and perforins to destroy the pathogen. Granzymes signal infected cells to induce apoptosis. Perforins create holes on the membrane of infected cells to eliminate the pathogen. [4]

CD4 T cells are significant in cytokine secretion and recruitment of other cells as they differentiate into two subsets of T cells, T helper 1 cells (T_h1) and T helper 2 (T_h2) cells. Their differentiation is determined by the APCs, and the secretion of specific cytokines. Each T helper cytokine profile is specialized in clearance of different types of pathogens. [4] T_h1 cells are effective in clearing intracellular pathogens, while T_h2 cells are more successful in eliminating extracellular pathogens. [5]

To further understand a T_h1 response, there are key cytokines secreted. Interleukin-12 (IL-12) promotes T_h1 cell differentiation and activates natural killer (NK) cells. NK cells target virus-infected and tumor cells. Another cytokine is tumor-necrosis factor α, which induces local inflammation to recruit other immune cells. Macrophage activation occurs with the secretion of interferon-γ (IFN-γ). This cytokine is also important in suppressing T_h2 differentiation. The activation of NK cells and macrophages characterize a T_h1 response as cell-mediated immunity.[4]

On the other hand, a Th2 response characterizes a humoral immunity. This response stimulates B cell differentiation into plasma cells to secrete antibodies for

clearance of infection. Some important cytokines for this response include: interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10). IL- 4 promotes T_h2 cell differentiation and activates B cells. IL-6 also contributes to B cell activation and T cell activation. As T_h2 was suppressed by IFN-γ during a T_h1 response, IL-10 suppresses T_h1 cell differentiation. IL-10 also inhibits macrophage function. [4]

A T_h2 immunity aids successful pregnancy. [5] Raj Raghupathy reviews previous studies on the relationship between T cell immunity and pregnancy. [5] Abortions have been observed in pregnant mice administered TNF-α, IFN-γ, and IL-2 cytokines. These cytokines create an unfit environment for the fetus to develop due to the inflammation . Another group of investigators have reported IL-4 production is significantly increased in comparison to IL-2, which induces differentiation of NK cells, cytokines from activated lymphocytes in pregnant mice. This reveals a shift towards a T_h2 response. High levels of IL-10 cytokines have been found to contribute to maintaining a T_h2 immunity. These cytokines are released by the placenta as a protective shield.[5]

Hormones also contribute to switching from one T helper cell response to another. Two important hormones regulating these immunities are relaxin and progesterone. They contribute to the outcome of naïve T cells. Relaxin is responsible for differentiation of T_h1 cells, while progesterone regulates the switching from a T_h1 to a T_h2 response. Females produce progesterone to protect the fetus by down-regulating T_h1 cell differentiation. [5]

With a brief insight into the immune system and factors aiding in pregnancy, an introduction into *Chlamydia* will provide a better understanding of the project. Zdrodowska-Stefanow et al. describes the mechanisms for *C. trachomatis* infection and

its development. [6] The bacterium is an obligate intracellular pathogen existing in two forms. It can exist in its infectious form as elementary bodies (EBs), or its noninfectious form as reticulate bodies (RBs). *Chlamydia* infection begins with the interaction of a host cell and EBs. The EBs adhere to the cell surface and signal the host to phagocytose the small, 0.3μm diameter, pathogen. Once inside the cell, *Chlamydia* differentiates from elementary bodies into reticulate bodies within a membrane-enclosed vacuole called an inclusion. These reticulate bodies, about 1μm in diameter, remain in the inclusion as the pathogen replicates and divides. After *Chlamydia* replicates through several cycles of development, it differentiates back into elementary bodies and induces apoptosis or lysis the cell to spread infection. [6]

Chlamydia has evolved to modify normal cell function to its advantage for survival. One of the modifications is inhibiting programmed cell death. Apoptosis is important in maintaining homeostasis through the destruction of abnormal cells, such as cancerous or infected cells. Intrinsic and extrinsic are two pathways to signal apoptosis. The intrinsic pathway can be initiated through intracellular metabolic or physical stress; whereas, the extrinsic pathway can be activated with the binding of ligands to the death receptor on the cell surface to produce a danger signal. *Chlamydia* prevents intrinsic apoptosis by inhibiting release of cytochrome *c* from the mitochondria. Without release of cytochrome *c*, a cascade of caspase activations does not occur and inhibits programmed cell death. To modulate apoptosis of the cell by the death receptor, the bacterium produces a *Chlamydia* protein associating with death domain (CADD) proteins. CADD is beneficial to the pathogen in both inhibiting apoptosis during

replication and triggering cell death after completing reproduction. By controlling these pathways, the microbe decides when to lyse the cell.[7]

Another way *Chlamydia* utilizes the host cell for its own benefits is acquiring nutrients from the cell to proliferate. Heuer et al. describes a mechanism for *C. trachomatis* diverting sphingolipids and cholesterol destined for host cell membrane to be transported to the inclusion.[8] The Golgi apparatus sorts and exports proteins and lipids produced by the cell. Its structure is comprised of laterally stacked cisternae. A Golgi matrix protein, golgin-84, also contributes to the stability of the Golgi apparatus. This protein is normally phosphorylated during mitosis or cleaved during apoptosis. Cleavage of golgin-84 results in the disassembly of the Golgi apparatus. In the findings of Heuer et al, *C. trachomatis* infection cleaves golgin-84, while inhibiting programmed cell death. The resulting fragmentation of the Golgi apparatus creates Golgi ministacks surrounding the inclusion and providing lipids for the pathogen.[8]

The strain chosen to infect the mice for this project is mouse pneumonitis (MoPn). It is one of two *Chlamydia muridarum* strains. MoPn has diverged from *C. trachomatis* on the *Chlamydiae* evolutionary tree. However, Read et al. describe MoPn and serovar D, a *C. trachomatis* species, containing highly conserved genes when comparing genomes. [9] There are also a couple significant differences to these strains. *C. trachomatis* has been shown to infect humans, while MoPn infects the family Muridae. This species within this family include: mice, rats, and hamsters. Another difference is serovar D contains tryptophan biosynthesis genes, while MoPn lacks them. As an immune response to *Chlamydia* infection, pro-inflammatory cytokine IFN-γ decreases intracellular tryptophan availability for the bacterium. Serovar D has the advantage to

continue proliferating and survive expressing its genes as an alternative mechanism to produce tryptophan. Toxin genes also differ between both strains. Serovar D has mutations in its toxin gene. This decreases its toxicity and can contribute to a less persistent and virulent infection. On the other hand, MoPn's fully functional toxin gene may allow it to be more infective. Accounting for the similarities and differences between *C. trachomatis* and *C. muridarum* MoPn when evaluating infection, MoPn is a likely candidate to be used in murine models of *Chlamydia* infection. [9]

Recent studies have shown a T_h1 immune response contributes to clearance of *Chlamydia muridarum*. Toni Darville et al. revealed a comparison of T_h1 immune responses between three different types of mice: C57BL/6, BALB/c, and C3H/HeN. These mice were injected with progesterone to synchronize their reproduction cycle in anestrus and infected with 10^7 inclusion-forming units (IFU) a week later. Their results show IFN-γ secretion increased after infection for all three mice strains. High levels of IFN-γ for C57BL/6 mice lasted longer than BALB/c and C3H/HeN. Secretion of IL-10 and IL-4 were low for all mice strains. The findings from these experiments show C57BL/6 mice elicit a higher T_h1 cytokine profile to aid in clearance of *Chlamydial* genital tract infection compared to BALB/c and C3H/HeN mice. [10]

Sukumar Pal et al. conducted an experiment to determine the pregnancy outcome of *Chlamydia* infected mice. Pregnant BALB/c mice were inoculated with different doses of MoPn on day five of gestation. Another group of pregnant mice was used as a control with mock-infected HeLa 299 extracts. Control pregnant mice carried their babies to full term. On the other hand, mice infected with higher than 10^5 IFUs prematurely gave birth.

It is concluded, pregnancy is unsuccessful with the occurrence of *Chlamydia* infection early in gestation.[11]

After describing a brief background on the immune system and *Chlamydia*, there is still a gap of knowledge to be filled about whether infectivity of *Chlamydia* remains for a longer period of time in pregnant mice than nonpregnant mice. This study is significant in understanding the time-course of infection. If pregnant mice show inability to clear infection, it may explain why they abort their fetuses as shown previously by Pal et al. In this project, the relationship of *Chlamydia* infection and secretion of IL-1β, a pro-inflammatory cytokine, will also be more closely investigated.

MATERIALS AND METHODS

Animals

Female C57/Bl6 mice, 5 to 6 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were given food and water ad libitum. They were allowed to acclimate in an environmentally controlled room with a 12 h light and 12 h darkness cycle.

Mating

One male and one female were placed in a cage overnight. The presence of a vaginal plug the following morning indicated successful mating and was considered to be day 0 of gestation. Pregnant mice were weighed and moved to a separate cage.

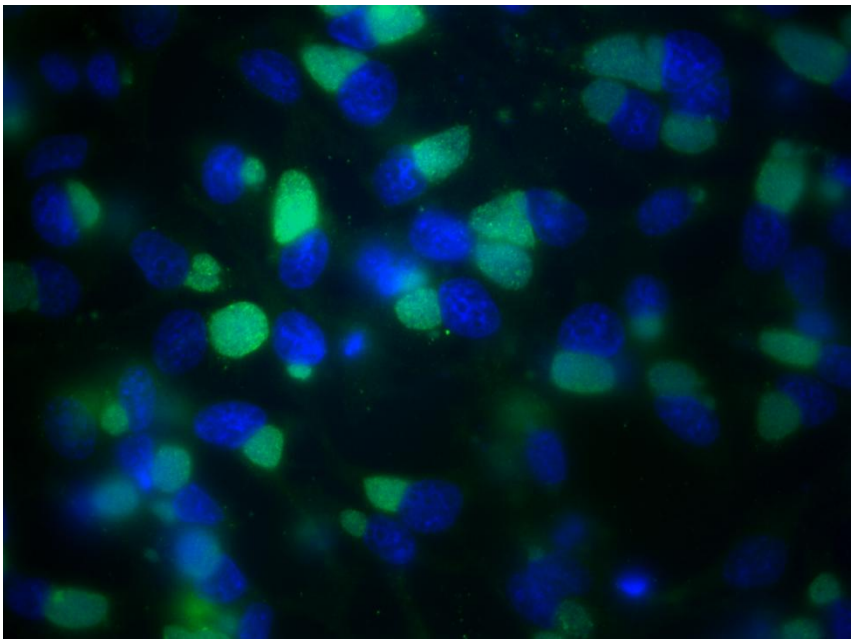
Expansion of *C. trachomatis* MoPn

The *C. trachomatis* MoPn was grown in HeLa-229 cells (American Type Tissue Culture Collection) and elementary bodies (EB) were purified. HeLa cells were grown in a tissue culture dishes to a 70%-80% confluency in Dulbecco's Modified Eagle Medium (DMEM) . It contained 10% fetal bovine serum and 0.5% gentimiacin. The media was changed prior to infection. Infected cells were allowed replicate and infect through two cycles. The plates were swirled 30 hours post-infection to spread the EBs. After 50 hours post-infection, cells were scrapped using a police scraper and aliquotted into 15 ml tubes with about 7 ml of cells per tube. Then the tubes were frozen in the -80°C for about 20 minutes. The tubes were thawed and vortexed. All contents of tubes were distributed into 50 ml conical tubes and spun down in the low speed centrifuge at 1000 revolutions per minute (rpm) for 5 minutes at 4°C. Supernatant from each tube was collected into

high speed centrifuge tubes. Then they were spun down in the high speed centrifuge at 16,500 rpm for 45 minutes at 4°C. Supernatant was discarded. Pellets were resuspended in DMEM and aliquotted into 30µl of the expansion per tube.

Quantification of *Chlamydia* expansion

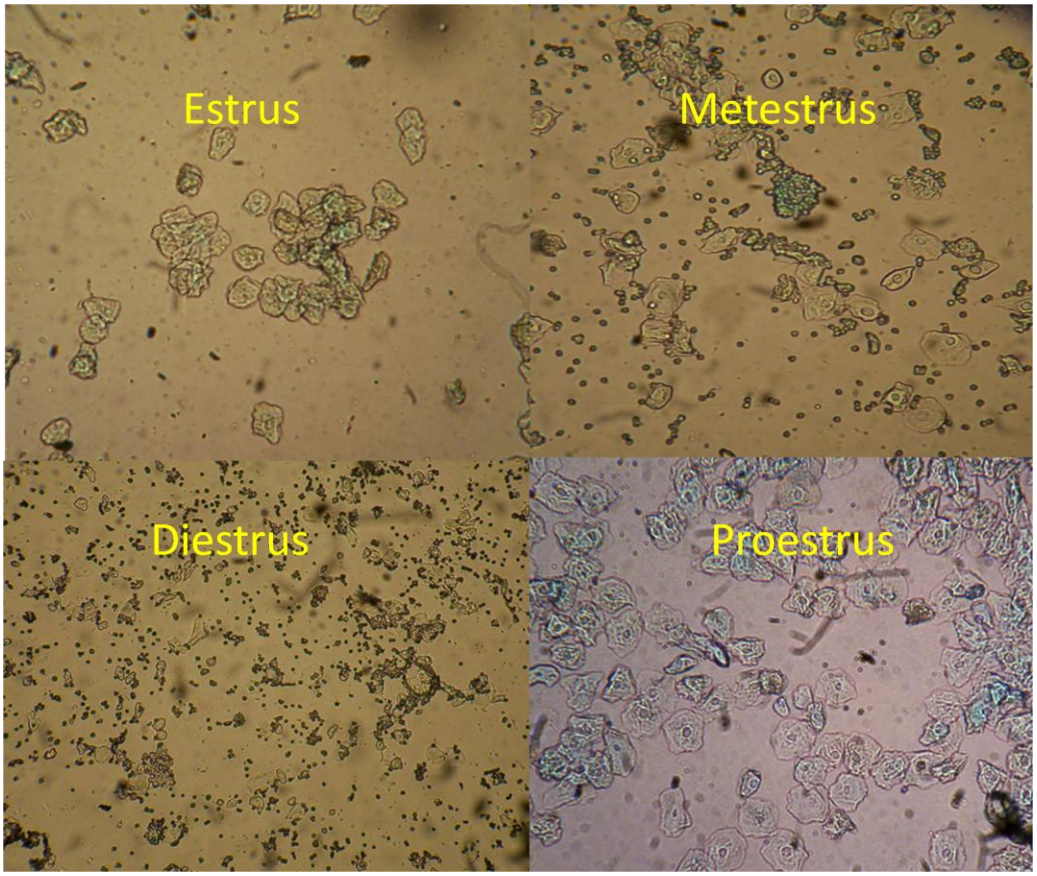
HeLa-229 cells are grown in a 24-well plate with coverslips in each well. At 40% confluency of cells, the aliquots from the *Chlamydia* expansion are titrated with dilutions ranging from .1µl to 50 µl. After 24 hours of infection, cells were fixed in cold methanol for 10 minutes. Coverslips were removed from the wells and washed with phosphate buffered solution (PBS). Then cells were stained for 15 minutes with an antibody mixture containing Anti-*Chlamydia* antibody, PBS, 0.2% Triton-X, and Hoechst stain. The coverslips are washed again with PBS and mounted onto microscope slides. Slides were observed under an immunofluorescence microscope. Infection was quantified by calculating the ratio of non-infected versus infected HeLa cells.



Shown above are HeLa cells infected with Chlamydia MoPn after 30 hours. Hoechst stain marks the nucleus in blue, while Chlamydial-antibody reveals Chlamydial inclusions in green.

Vaginal cytology

20 µl of sterile PBS is pipetted into each female mouse's vaginal vault. A sample is collected from the vagina in the pipette tip and smeared onto a microscope slide. The sample is left to air dry. Then the cells are fixed in 100% cold methanol for 10 minutes. Once fixed, the slide is stained using 0.1% Giemsa Stain in ethanol for 10 minutes. Using a light microscope, epithelial cells are observed under a microscope to determine the mouse's reproduction stage.



Different stages of the oestrus cycle in mice are shown above. Slides are prepared using vaginal cytology.

Weight gain

Weight is measured for both pregnant and nonpregnant mice daily from the beginning of the experimental run. By tracking weight gain, pregnant female mice show a difference of about 2 grams compared to nonpregnant female by day 11 of gestation. Observing the weight gain also allows the removal of pregnant mice from the experiment, where vaginal plugs were missed.

Progesterone injection

Nonpregnant female mice are given an intramuscular injection of 2.5 mg progesterone (Depo-Provera) one week prior to infection.

Sedation of mice

Mice were subcutaneously injected with an anesthesia cocktail prior to infection and on days of sample collection. The mixture contained 85% saline, 5% xylazine, and 10% ketamine.

Animal challenge

Each pregnant female mice was inoculated intravaginally with 10^7 inclusion-forming units (IFU) of *C. trachomatis* MoPn, in 30µl DMEM, on day 5 of gestation. Nonpregnant mice were infected with the same dosage when vaginal cytology revealed they were in estrus.

Collection of genital tract secretion and extraction of cytokines for analysis

Aseptic surgical sponges, manufactured by DeRoyal Surgical Accessories, placed into pregnant and nonpregnant vaginal vaults to collect cytokine secretion. Samples were obtained prior to and after infection. These sponges were frozen in -80^{o}C until used for enzyme-linked immunosorbent assay (ELISA). In preparation for the ELISA, the samples were extracted from the sponges as previously described by Darville et al.[10]

Collection of vaginal epithelial cells

Sterile aluminum applicator swabs (Fisher brand) were lubricated with sterile PBS. Infected mice were swabbed in their vaginas, and the swab was frozen in -80^{o}C in TRIzol reagent (Invitrogen).

RNA extraction of vaginal swabs and analysis of infection intensity using quantitative polymerase chain reaction (qPCR)

Samples from days 1, 2, 5, and 10 post-infection were used in the analysis of infection. Glassballs were placed into eppendorfs containing the swabs and vortexed for a minute. The TRIzol reagent was transferred from each tube to a new eppendorf. Chloroform was added and incubated. Eppendorfs were spun down and the clear upper phases are transferred to new tubes. RNA is precipitated using isopropyl alcohol and purified using ethanol. The resulting pellet is resuspended in molecular grade water and measurement of RNA is performed on the nanospectrometer. RNA is reverse transcribed into cDNA. Measurement of infection is determined by qPCR.

Polymerase chain reaction gel

Samples reverse transcribed were prepped with QIAGEN fast cycling PCR master mix and the amplicon, *Chlamydia trachomatis* L2. L2 primers were used because genes are highly conserved between L2 and MoPn. PCR samples are loaded into PCR machine to be amplified. Then samples are added in a 1% agarose gel with ethidium bromide. Gel pictures were taken on an Chemidoc imaging system with an ethidium bromide filter.

RESULTS

Weight comparision of pregnant and nonpregnant mice

Figure 1 illustrates the distinguishable weight difference between nonpregnant and pregnant mice by day 11 of gestation. Pregnant mice are euthanized a few days prior to the end of pregnancy and are observed to be carrying four to eight fetuses.

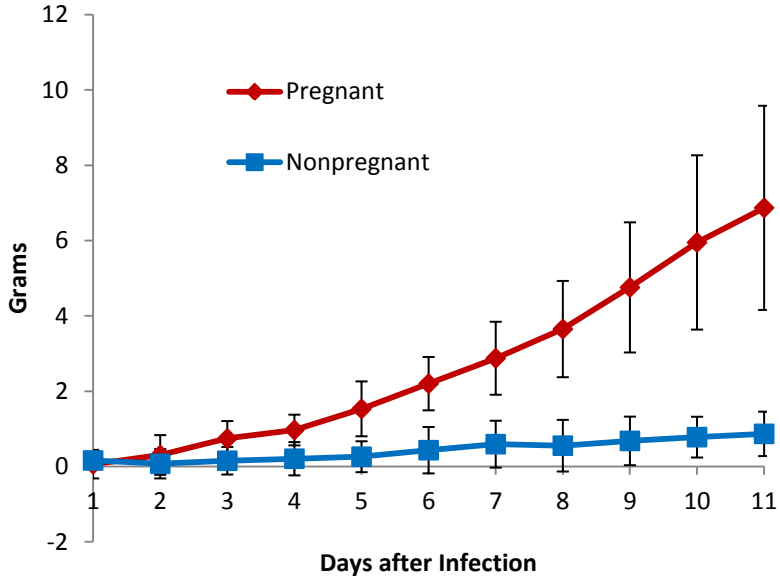


Figure 1- A comparison of weight gain for pregnant and nonpregnant mice over a course of 11 days post-infection. Results are a representation of an average of 5 mice from 5 separate experiments. Error bars are standard deviations of each day.

Nonpregnant progesterone treated mice

To optimize methods for cytokine secretion and ELISA, an experiment previously published was repeated for measurements of IL-1β and IFN-γ in *Chlamydia* infected mice. Progesterone treated mice show a large increase in IL-1β secretion 1 day after infection compared to collections prior to infection (Fig.2). The levels of IL-1β remain elevated for 5 days and begin to decrease for the remaining five days measured. When looking at cytokine IFN-γ, progesterone treated mice had low levels of IFN-γ until day 4 (Fig.3). Then secretion of IFN-γ triples on day 5 post-infection, followed by a decrease through the remainder of the experiment.

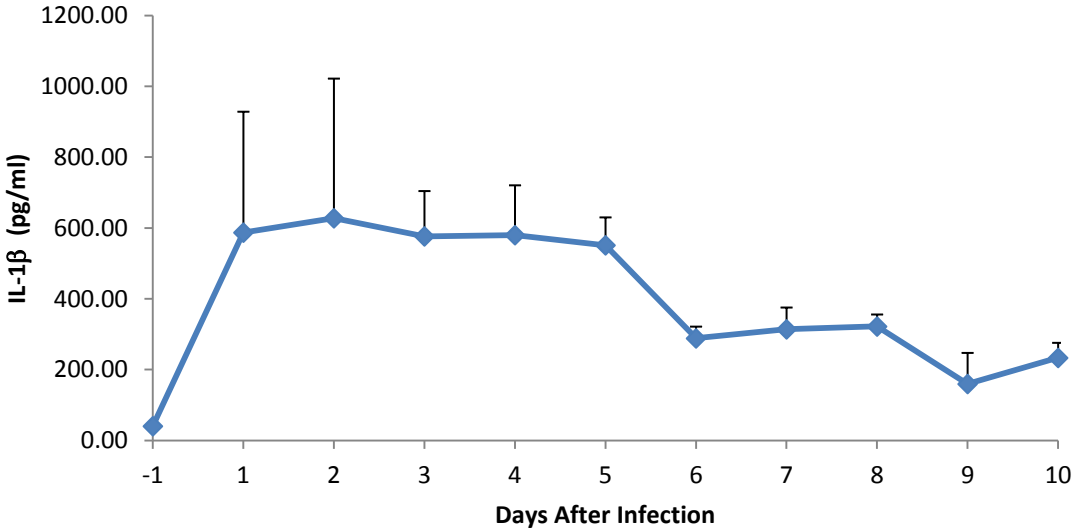


Figure 2- Measurement of IL-1β secretion for infected nonpregnant mice treated with progesterone using an ELISA kit. Results are averages plus standard errors of the means. The graph is a representation of five mice.

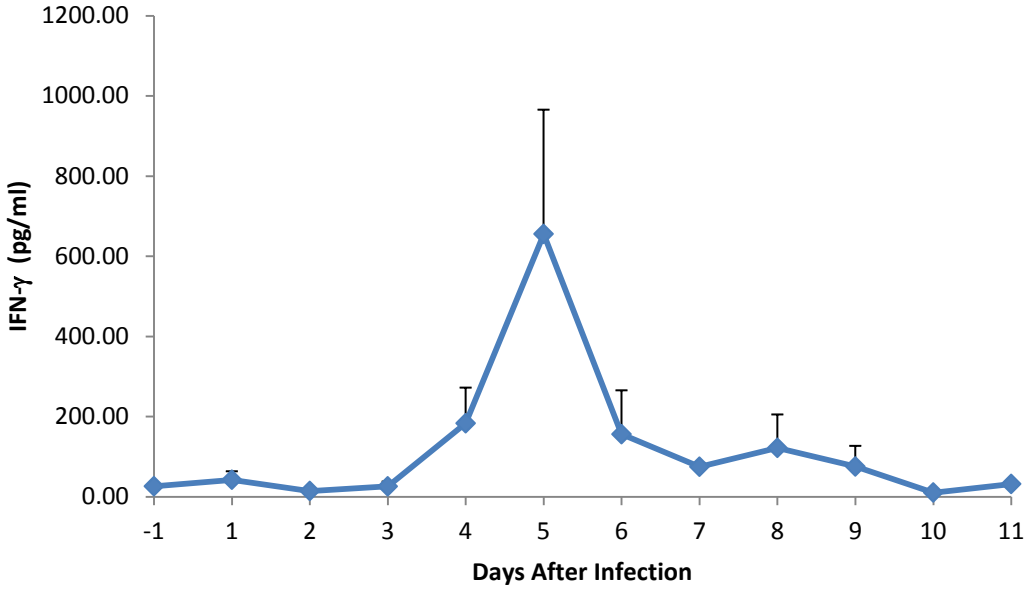


Figure 3- IFN-γ levels are measured by ELISA and are an average of five mice with standard error of means. Nonpregnant mice are treated with progesterone and infected with Chlamydia trachomatis MoPn.

Quantification of *Chlamydia trachomatis* MoPn

It is hypothesized pregnant mice will show a persistent infection compared to nonpregnant mice. Primers used for qPCR are specific to the binding of *Chlamydia trachomatis* MoPn 16S rRNA (Fig.4). Uninfected HeLa cells show no band, while infected HeLa reveal a large band at about 200 base pairs. Samples from days 1, 2, 5, and 10 post-infection are measured with qPCR (Fig.5). Pregnant mice show an increase of infection from day 1 to day 5 post-infection. Nonpregnant mice decrease in infection on day 2, but slightly increases 5 days after infection. Then the nonpregnant mice drop in fold increase by day 10.

Progesterone treated mice reveal a 2-fold increase for infection on day 2, but decreases through day 5 and day 10 measurements.

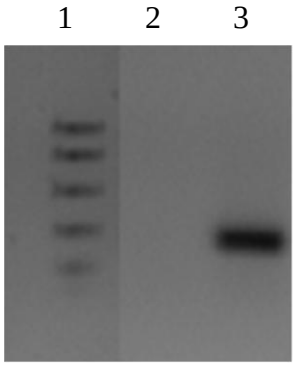


Figure 4- Primer specificity for Chlamydia. Above is a PCR gel of uninfected and infected HeLa cells. Lane 1 is the marker. Lane 2 illustrates uninfected HeLa (epithelial) cells. Lane 3 contains HeLa infected with Chlamydia trachomatis MoPn.

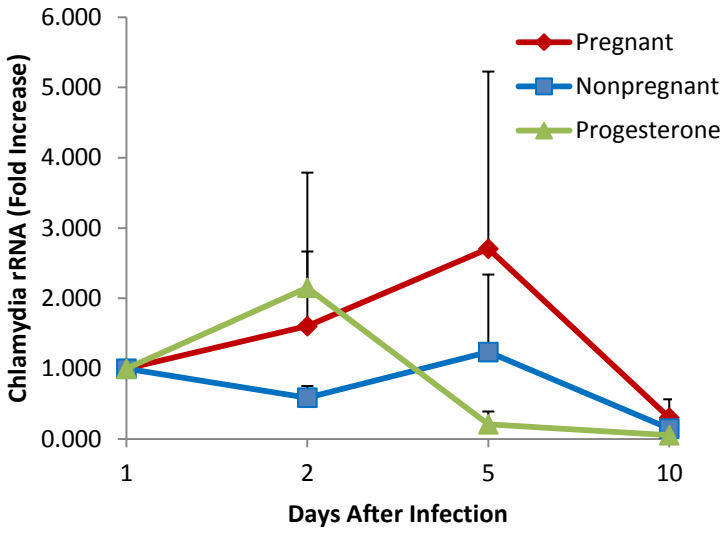


Figure 5- Quantification of Chlamydia infection for three conditions of mice. Primers specific for C. trachomatis MoPn. Data points are averages of three mice with standard error of means.

Comparisons of IL-1β secretion among pregnant and nonpregnant mice

Pregnant mice secrete an average of 175 pg/ml on day 1 post-infection (Fig.6). On the other hand, nonpregnant mice secrete an average of 20 pg/ml ($P<0.05$ by t-test). Infected pregnant mice maintain a high level of IL-1β for 5 days and show low levels through the last days. There isn't a large increase for infected nonpregnant mice throughout the course of collection. Their secretion of IL-1β rise after infection, but don't surpass pregnant measurements significantly.

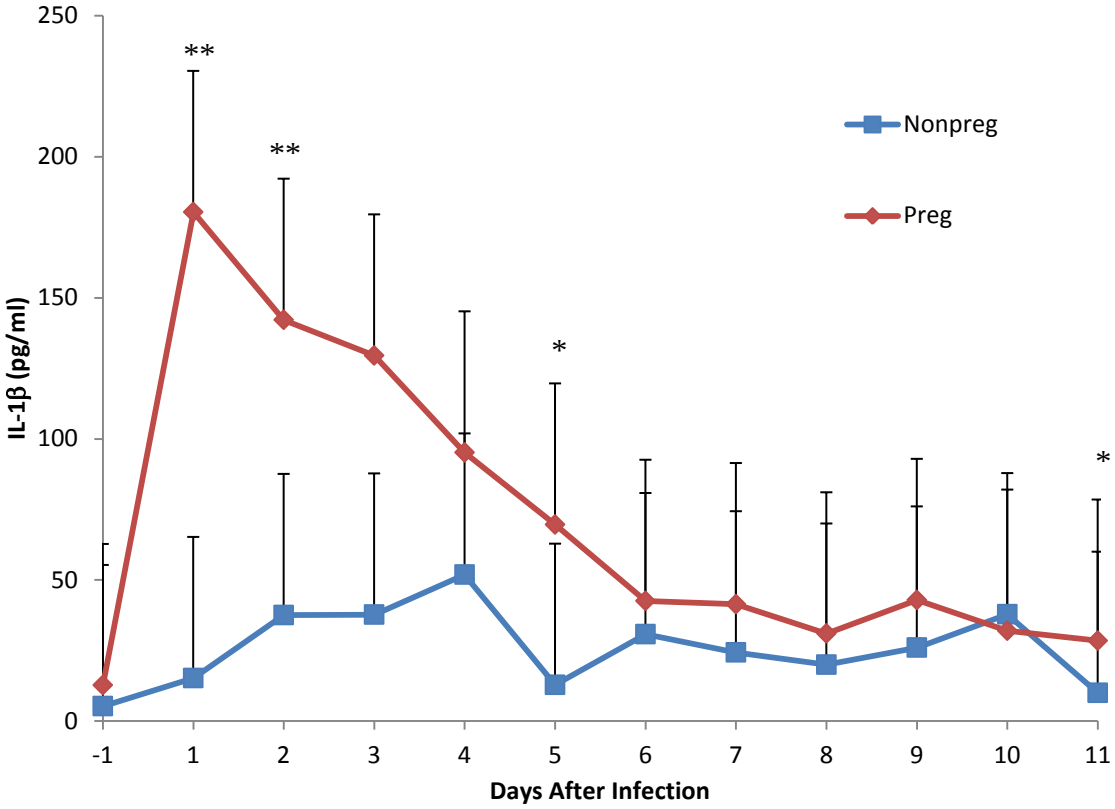


Figure 6- IL-1β secretion measured by ELISA. Nonpregnant and pregnant mice are infected with MoPn. Results are averages of four mice from four separate experiments plus standard error of means. *, *P* <0.05 for nonpregnant versus pregnant; **, P<0.01 for nonpregnant versus pregnant. N=16 mice per treatment group.

DISCUSSION

We hypothesized a T_h2 environment during pregnancy will not protect against *Chlamydia* infection, but may contribute to development of persistent infection. Before testing this hypothesis, preliminary experiments were conducted to optimize techniques and methods previously observed in the laboratory of Dr. Luis de la Maza, University of California, Irvine. As a pioneer graduate student at University of California, Merced, I was the first to work with *Chlamydia* infected mice. My training from Dr. de la Maza's laboratory was limited to learning to handle the mice and mainly observations. Therefore, I performed a couple trial experiments utilizing my experiences.

Many publications of *Chlamydia* experiments with mice use Depo-Provera. It is a form of birth control used by humans. The drug is administered to female mice to synchronize reproduction cycle, prevent release of eggs from the ovaries, and prevent epithelial shedding. This increases the likelihood of retaining *Chlamydia* infection. However, pregnant and nonpregnant mice in this project were not injected with Depo-Provera. Nonpregnant mice were infected during estrus to mimic similar physiological conditions, such as hormones, in pregnant mice.

Methods and techniques were improved for the project because of the problems found in the trial experiments. Measurements of infection intensity for the first couple experimental runs, using 10^6 IFUs per mouse, yielded no significant results (data not shown). The problem was thought to be the mice were not retaining the infection, so mice were infected twice on consecutive days in a separate trial. However, all the infected pregnant mice aborted and effects of infection by pregnancy could not be tracked

(data not shown). Another experiment performed increased the amount of Chlamydia administered to 10^7 IFUs per mouse, and mice were only infected once. In this test, pregnant infected mice carried their babies for 16 days of gestation and were euthanized prior to giving birth (Fig.1). This run showed the best conditions for the amount of infection and collection period to test the hypothesis.

A separate experiment with infected nonpregnant mice treated with progesterone was performed to ensure proper collection of vaginal secretion and vaginal swabs. Figures 2 and 3 reveal similar results previously published by Darville et al. [10, 12] Progesterone treated mice reveal a high inflammatory response with the secretion of IL-1β post-infection (Fig. 2). Results show an increase in infection for the first two days of infection and a clearing of infection on day five (Fig. 5). As previously mentioned, a T_h1 immune response has been shown to clear *Chlamydia* infection. The increased release of IFN-γ cytokine beginning day 4 post-infection may contribute to the decrease in infection (Fig. 3). From these findings, collection techniques and analyses portrayed reproducible data.

After adjustments from previous experiments, the hypothesis was tested. Results from the qPCR reveal infected pregnant mice increasing in infection up to day 5 (Fig 5.). It is a possibility the pregnant mice are using the antibodies, involved for protection of the fetus, to fight this infection, but are not succeeding. Infected nonpregnant mice illustrate a fluctuation in infection through the course of the experiment. These results weren't expected because the nonpregnant mice were thought to elicit a T_h1 immune response, which is useful in clearing this bacterial infection. The findings reveal

pregnant mice are unable to fight *Chlamydia* infection as effectively as nonpregnant mice. To improve results of tracking infection, more replications can be performed.

Pregnant mice have a higher inflammatory response during *Chlamydia* infection than nonpregnant mice. When comparing the qPCR and IL-1β results for each day, the cytokine secretion is likely an immune response to *Chlamydia* infection. The levels of IL-1β decrease along with infection 5 days post-infection. However, this inflammatory period may have contributed to stillbirths in pregnant mice for one experiment because the environment was unfit for the fetuses to develop properly (data not shown).

The overall results don't completely support the hypothesis, but opens up new perspectives. An observation for infected pregnant mice shows increase in the infectivity of *Chlamydia*, but not persistent infection when comparing to nonpregnant mice. Moreover, there is a significant difference in fold increase for IL-1β secretion with infected pregnant mice compared to infected nonpregnant mice. It is interesting the pregnant mice, which had a more robust IL-1β response, still showed higher *Chlamydia* replication. The ELISA results illustrate high levels and prolonged exposure to the inflammatory cytokine for the pregnant mice. It will be intriguing to further investigate the effects of the inflammation by studying the pathology of the uterus, ovary, and oviduct from these infected mice. The removal and observation of these tissues may reveal involvement of IL-1β contributing to local inflammation and tissue damage.

Because IL-1β secretion leads to activation of lymphocytes, it would be interesting to look into T helper cell cytokine profile to understand whether the cytokines present during pregnancy are ineffective to fight infection. IL-10 and IFN-γ are two

likely candidates to test because they suppress either a T_h1 or T_h2 immune response. These cytokines can be measured using similar methods from this project. Vaginal secretions can be collected and measured using ELISA. Another idea is to explore cytokine gene transcription in the placenta. This experiment can be conducted by removing the placenta of pregnant mice. Then RNA is extracted from the tissue, where samples are ran on the real-time PCR for T_h1 or T_h2 cytokines. This experiment may provide information with the specific cytokines involved with pregnancy. However, to conclusively determine the role of T_h1 and T_h2 cytokines and IL-1β in the increased persistence or infectivity of *Chlamydia* during pregnancy will require the use of transgenic and knockout animal models.

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