Determinants of Upper Genital Tract Complications in a Chlamydial Urogenital Mouse Model

Enitra N. Jones

University of Tennessee Health Science Center

Follow this and additional works at: http://dc.uthsc.edu/dissertations

Part of the Medical Sciences Commons

Recommended Citation

Determinants of Upper Genital Tract Complications in a Chlamydial Urogenital Mouse Model

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Enitra N. Jones
December 2013
DEDICATION

This work is lovingly dedicated to my mother, Evelyn T. Jones. You believed in me when I was filled with self-doubt. You encouraged me when others said I could not accomplish the goal. Your faith never wavered and you fervently prayed. For this and more I am eternally grateful.
ACKNOWLEDGEMENTS

*It takes a village . . . .*

–African Proverb

First, I would like to thank my research advisor, Dr. Gerald I. Byrne, for the opportunity to carry out the enclosed research in his laboratory. His passion for science and enthusiasm for learning have been both inspiring and motivational throughout my doctoral journey. Dr. Byrne has also allowed me the freedom to integrate my interest in public health with my academic pursuits and for that I am grateful.

The guidance offered by each member of my graduate committee was essential. I would like to thank Drs. Robert Belland, B. Keith English, Elizabeth Fitzpatrick, and P. David Rogers for providing scientific insight throughout this process and for being genuinely invested in my success while matriculating at the University of Tennessee Health Science Center and beyond. Whether it was help with a laboratory technique, a recommendation letter, or an encouraging word, each member was more than willing to lend a helping hand. Thank you for sharing your expertise and offering thoughtful critiques on my work.

I would also like to acknowledge past and present members of the Byrne Laboratory. My first teachers in the laboratory were Drs. O. Sadia Mahdi and Isao Miyairi who patiently taught me the “ins and outs” of cell culture and chlamydial growth. I am thankful for Vijaya Onguri’s contributions to early vaccination studies and her ongoing friendship. The technical help throughout the years provided by Dr. Jan Peters and Jonathan Laxton, now members of the RBL staff, is greatly appreciated. The histological studies discussed in this dissertation would not have been possible without Xeofei Wang. Conversations with Dr. Yin Su concerning animal studies were invaluable. I also want to acknowledge the RBL staff and the many summer students that have been active participants in laboratory discussions throughout the years. Thank you all!

My time at the University of Tennessee Health Science Center has been enriched by several members of the faculty/staff and student body. I was truly blessed to have made the acquaintance of my “UT Mom”, Mrs. Ruby McNeal. Your unwavering support and encouragement will never be forgotten and is appreciated beyond words. Thank you to Mrs. Devonia Cage, Mrs. Carolyn Fields, and Mrs. Evelyn Lewis for your prayer and encouragement. The guidance and mentorship offered by Executive Vice Chancellor and Chief Operations Officer, Dr. Kennard Brown, was invaluable and greatly appreciated. I also want to thank the Microbiology, Immunology, and Biochemistry Department, the members of the Black Graduate Student Association, and many of my fellow graduate students for their help throughout the years.

I would like to thank my immediate and extended family for their love and support. To my parents, Ezra and Evelyn Jones, who prayed without ceasing, dried my tears when things seemed too difficult to continue, and continue to love me
unconditionally. I love you to life forever. To my grandmother, Annie Sue Gibbs, thank you for reminding me that God doesn’t always “move the mountains”, sometimes He gives us the strength to climb them! To my aunts, uncles, siblings, cousins, friends, and sorority sisters- thank you for the comedic relief and unwavering support. To my best friends (outside of my parents), Tron Foster, Jason Hughes and Luviska Nicholas, thank you for nourishing my spirit and believing in my dreams. Sincere thanks to two of my ongoing mentors and friends, Drs. Oswald D’Auvergne and Ivory Toldson. Last but certainly not least, I thank the Lord for granting me the serenity to accept the things I can not change, the courage to change the things I can, and the wisdom to know the difference.
Genital *Chlamydia trachomatis* infection is a major public health concern. *Chlamydia* is the most commonly reported infection in the United States and the most common bacterial sexually transmitted infection worldwide. Unrecognized infection endangers female reproductive health by serious complications such as Pelvic Inflammatory Disease, ectopic pregnancy, and involuntary infertility. Widespread *Chlamydia* control programs were implemented more than two decades ago to improve women’s reproductive health but, despite initial success, the number of chlamydial infections reported have increased.

One of the hypotheses put forth to explain increased chlamydial reporting suggests that a long-term caveat of control initiatives is interference with the development of natural occurring immunity as a result of mass screening and rapid treatment. It is proposed that human cohorts are more susceptible to subsequent chlamydiae infection and their increased susceptibility drive the current increase in sexually transmitted chlamydiae case notifications.

In these studies we describe a comprehensive approach to assessing the role of early anti-chlamydial intervention, an integral component of control initiatives, on the subsequent development and severity of upper genital tract sequelae in a murine model of recurrent chlamydiae urogenital infection. The development of an in vivo model of urogenital *Chlamydia trachomatis* infection is central to defining the risk of developing long term reproductive complications, delineating potential biomarkers for chlamydial-induced genital tract disease, interrogating host factors that may contribute to the development of adverse complications, and anti-chlamydial vaccine development.
# Chapter 1. Chlamydiae

**Historical Perspective** ................................................................. 1
**Taxonomy** ..................................................................................... 1
**Developmental Cycle** ................................................................. 2
**Chlamydiae Clinical Significance** ................................................. 4
  - *Chlamydia pneumoniae* ................................................................. 4
  - *Chlamydia psittaci* ..................................................................... 4
  - *Chlamydia trachomatis* ............................................................... 6
  - Ocular *Chlamydia trachomatis* ...................................................... 6
  - Genital *Chlamydia trachomatis* .................................................... 6
**Mouse Model of Genital Infection** .............................................. 7
**Murine Genital Tract Pathology** ............................................... 9
**Murine Model Limitations** .......................................................... 9
**Immunological Response to Genital Infection** ....................... 11
**Intracellular Immune Response Overview** ............................ 11
  - *Chlamydia* Innate Response ....................................................... 11
  - *Chlamydia* Adaptive Response .................................................. 12
**Vaccination** .................................................................................. 13
**Control Measures** ....................................................................... 14
  - Sexually Transmitted *Chlamydia* Epidemiology ......................... 15
  - Hypotheses for Rebounding *Chlamydia* Rates ......................... 16
  - Arrested Immunity Hypothesis ..................................................... 18
**Dissertation Rationale** ................................................................. 19

# Chapter 2. Arrested Immunity: Impact of Early Treatment on Upper Genital Tract Sequelae

**Introduction** .................................................................................. 20
**Materials and Methods** .............................................................. 21
  - *Mice* ......................................................................................... 21
  - *Chlamydia* Strain and Titration ............................................... 21
  - Mouse Infection ....................................................................... 21
  - Antibiotic Treatment ................................................................. 21
  - Bacterial Shedding ................................................................... 21
  - Fluorescence-Linked Immunosorbent Assay (FLISA) Antibody Analysis ...................................................... 22
  - Pathology .................................................................................. 22
  - Organ-Total Body Weight Ratio Analysis ................................ 22
  - Statistical Analysis .................................................................. 23
**Results** ........................................................................................ 23
  - Kinetics of Genital Tract Infection in Antibiotic-Treated Mice .......... 23
  - Immune Arrest and Pathology Following Primary Infection of Antibiotic- Treated Mice ............................... 23
  - Disease Severity Following Reinfection in Antibiotic-Treated Mice ................................................................. 26
IL-4 Is Detectable Late Following Reinfection and Positively Correlates with Disease Severity in Antibiotic-Treated Mice ........................................31
Chlamydia and Cellular Infiltrates Following Reinfection in Antibiotic-Treated Mice .................................................................31
Discussion ...........................................................................................36

CHAPTER 3. CHLAMYDIAL GENITAL TRACT SEQUELAE IS AGE AND STRAIN-DEPENDENT .................................................................42

Introduction .........................................................................................42
Materials and Methods .......................................................................42
Mice .....................................................................................................42
Chlamydia Strain ................................................................................43
Chlamydia muridarum Infection ..........................................................43
Pathology Assessment .........................................................................43
Results ................................................................................................44
C57BL/6J Exhibit Age-Dependent Upper Genital Tract Sequelae Post-Primary Intravaginal Infection .........................................................44
Secondary Intravaginal Infection Exacerbates Chlamydial-Induced Upper Genital Tract Complications ....................................................44
C57BL/6 Mice Are More Susceptible to Chlamydial-Induced Upper Genital Tract Complications When Compared to DBA/2J Mice ........44
Discussion ..........................................................................................47

CHAPTER 4. IMMUNIZATION WITH C. MURIDARUM OUTER MEMBRANE COMPLEX FAILS TO PROTECT AGAINST UPPER GENITAL TRACT COMPLICATIONS IN A MURINE MODEL OF GENITAL INFECTION ........................................................................50

Introduction .........................................................................................50
Materials and Methods (Systemic Lethal Model) ..................................51
Mice .....................................................................................................51
Chlamydiae ........................................................................................51
COMC Extraction ...............................................................................51
Immunization and Infection .................................................................52
Results (Systemic Lethal Model) ..........................................................52
Materials and Methods (Urogenital Model) .........................................55
Mice .....................................................................................................55
Chlamydiae ........................................................................................55
COMC Extraction ...............................................................................55
Immunization and Infection .................................................................55
Results (Urogenital Model) .................................................................56
Materials and Methods (Alum Study) ................................................56
Mice .....................................................................................................56
Chlamydiae ........................................................................................56
Immunization and Infection .................................................................59
Pathology Assessment .........................................................................59
Results (Alum Study)........................................................................................................59
  Immunogens without Intravaginal *Chlamydia muridarum* Challenge Do Not
  Induce Severe Upper Genital Tract Sequelae Formation.............................................59
  Alum Alone Does Not Negate the Development of Severe Upper Genital Tract
  Complications.............................................................................................................63
Discussion......................................................................................................................63

**CHAPTER 5. CONCLUSIONS**.......................................................................................68
  Testing the IL-4 Hypothesis.........................................................................................68
  Identifying Genetic Link to Age-Dependent Disease Severity.................................68
  Implications for Vaccine Development .....................................................................69

**LIST OF REFERENCES**..............................................................................................70

**APPENDIX A. MORBIDITY AND PATHOLOGY SEVERITY POST-**
**PRIMARY INFECTION IN DEFERRED TREATMENT STUDIES**.........................91

**APPENDIX B. MORBIDITY AND PATHOLOGY SEVERITY POST-**
**SECONDARY INFECTION IN DEFERRED TREATMENT STUDIES**.....................92

**APPENDIX C. TH1-RELATED CYTOKINES POST RECURRENT**
**INFECTION IN ARRESTED IMMUNITY MODEL**.....................................................93

**APPENDIX D. TH2-RELATED CYTOKINES POST RECURRENT**
**INFECTION IN ARRESTED IMMUNITY MODEL**.....................................................94

VITA................................................................................................................................95
LIST OF TABLES

Table 1-1. Chlamydiae that cause human disease. .................................................................5
Table 1-2. Increased chlamydial rate hypotheses. ..................................................................17
LIST OF FIGURES

Figure 1-1. Chlamydiae developmental cycle. .................................................................3
Figure 2-1. Shedding of viable C. muridarum following intravaginal challenge ..........24
Figure 2-2. C57BL/6 pathology severity scores 49 days post-primary C. muridarum intravaginal infection. ............................................................25
Figure 2-3. Total Immunoglobulin G (IgG) antibody response 49 days post-primary C. muridarum intravaginal infection. ..............................................27
Figure 2-4. Immunoglobulin G (IgG) isotype responses 49 days post C. muridarum intravaginal infection in mice intraperitoneally treated with doxycycline..28
Figure 2-5. Incidence and severity of upper genital tract sequelae in antibiotic-treated mice 28 days post-secondary infection. ............................................29
Figure 2-6. Incidence and severity of uterine horn and oviduct sequelae in antibiotic-treated mice 28 days post-secondary infection. ..................30
Figure 2-7. Weight ratio verses total severity score correlative analysis. ..................32
Figure 2-8. Comparative analysis of weight ratios post-primary and secondary sacrifice in untreated intravaginally infected C57BL/6 mice. ........33
Figure 2-9. Systemic IL-4 post recurrent infection. .......................................................34
Figure 2-10. Neutrophil infiltrates post-secondary infection. ...........................................35
Figure 2-11. Mast cell infiltrate post-secondary infection.............................................37
Figure 2-12. Mast cell infiltrate versus systemic IL-4 correlative analysis.................38
Figure 2-13. Eosinophil infiltrate post-secondary infection........................................39
Figure 3-1. Upper genital tract disease incidence and severity post-primary infection ...........................................................................................................45
Figure 3-2. Upper genital tract disease incidence and severity post-secondary infection ........................................................................................................46
Figure 3-3. Weight ratio disease severity validation post-primary and secondary intravaginal infection. .................................................................48
Figure 4-1. High dose 6BC C. psittaci systemic infection survival curve. ..............53
Figure 4-2. Low dose 6BC C. psittaci systemic infection survival curve. ..........54
Figure 4-3. Comassie stained SDS-Page gel of *C. muridarum* (MoPn)-derived COMC and *C. psittaci* (6BC)-derived COMC preparations. ..........................57

Figure 4-4. *C. muridarum*-derived COMC immunization study gross pathology. ..........58

Figure 4-5. Alum study experimental timeline.................................................................60

Figure 4-6. Oviduct pathology severity of non-challenged immunized groups. ..........61

Figure 4-7. Uterine horn pathology severity of immunized, non-challenged groups......62

Figure 4-8. Oviduct pathology severity of immunized and challenged groups..........64

Figure 4-9. Uterine horn pathology severity of immunized and challenged groups. ......65
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>Cpn</td>
<td><em>Chlamydia pneumoniae</em></td>
</tr>
<tr>
<td>Ct</td>
<td><em>Chlamydia trachomatis</em></td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EB</td>
<td>Elementary bodies</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FLISA</td>
<td>Fluorescent-linked immunosorbent assay</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin stain</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFU</td>
<td>Inclusion-forming units</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal dose, 50%</td>
</tr>
<tr>
<td>LGV</td>
<td>Lymphogranuloma venereum</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOMP</td>
<td>Major outer membrane protein</td>
</tr>
<tr>
<td>MoPn</td>
<td><em>Chlamydia muridarum</em></td>
</tr>
<tr>
<td>NAAT</td>
<td>Nucleic acid amplification testing</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PID</td>
<td>Pelvic inflammatory disease</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RB</td>
<td>Reticulate bodies</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>STD</td>
<td>Sexually transmitted disease</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmitted infection</td>
</tr>
<tr>
<td>TH0</td>
<td>Naive T Cells</td>
</tr>
<tr>
<td>TH1</td>
<td>T Helper 1 Cells</td>
</tr>
<tr>
<td>TH2</td>
<td>T Helper 2 Cells</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UGT</td>
<td>Upper genital tract</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
CHAPTER 1. CHLAMYDIAE

Historical Perspective

*Tales as old as time . . . .*
-Howard Ashman and Alan Menken

Descriptions of chlamydial disease date back to ancient Chinese and Egyptian texts detailing trachoma, a blinding chlamydial ocular disease. In 2002 Dimitrakov reviewed the expedition in which Ludwig Halberstaedter and Stanislaus von Prowazek discovered the causative agent of trachoma [1]. In 1907, while on the island of Java, Halberstaedter and von Prowazek pioneered early chlamydial etiology studies by inoculating orangutans with material obtained from trachoma patients. They were able to demonstrate the infectious nature of the pathogen by describing intracellular vacuoles in Giemsa-stained epithelial cells derived from conjunctival scrapings of the infected animals but incorrectly characterized the agent as protozoan.

In 1910, Linder reported finding the same type of intracytoplasmic inclusions in the eyes of neonates and linked their neonatal conjunctivitis to intrapartum exposure in women with unrecognized and untreated infection. Linder went on to speculate that the infection was transmitted sexually after identifying inclusion bodies in the mother’s cervical scrapings, urethral cells from the fathers, and in individuals with non-gonococcal urethritis [2] [3] [4].

In the 1930’s Samuel Bedson and colleagues characterized the developmental cycle of psittacosis particles, which at the time were thought to be viruses given their dependence on eukaryotic cells for replication. In 1957, fifty years after the Java excursion, *C. trachomatis* was successfully isolated and cultured in yolk sacs by Feifan T’ang et al [5] [6]. It was not until the mid 1960’s that chlamydiae would be defined as prokaryotic bacteria that possessed a non-infectious intracellular replication phase [7]. In 1969, Gordon et al described culturing chlamydiae in irradiated McCoy cells, a less time consuming technique alternative to isolating chlamydiae in yolk sac that would revolutionize diagnostic procedures for chlamydial infection [8].

Taxonomy

*What is in a name?*
–William Shakespeare

Chlamydiae are prokaryotic obligate intracellular bacteria that belong to the order Chlamydiales. Under the Chlamydiaceae umbrella are the families Chlamydiaceae, Parachlamydiaceae, Waddliaceae, and Simkaniaceae [9]. In 1999, it was recommended that the Chlamydiaceae family be subdivided into two genera, *Chlamydia* and *Chlamydophila*, based on the phylogenetic analysis of the 16s and 23s rRNA [10] but this recommendation was riddled with inconsistencies and controversial amongst those in the
chlamydial field. As reviewed by Stephens and colleagues [11], the overwhelming majority of publications (81% in the year 2006), continued to use the single genus name Chlamydia, despite the implementation of the two genera system. To that end, nine species are recognized in the Chlamydia genus: Chlamydia abortus, Chlamydia caviae, Chlamydia felis, Chlamydia muridarum, Chlamydia pecorum, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia suis, and Chlamydia trachomatis. Historically, chlamydiae serovars were based on conventional immunoeptiotope analysis by monoclonal antibody directed against the major outer membrane protein (MOMP) [12]. Today, chlamydial classification includes genovar sequence data of ompA, the gene that encodes MOMP [13] [14]. The ompA-based classifying system, when compared to the conventional sera-based analysis, more accurately reflects strain virulence and genotypic diversity on the population level [15].

**Developmental Cycle**

*In the circle, the circle of life.*
–Sir Timothy Miles Bendon Rice

Originally described in the 1930’s by Samuel Bedson et al [16], the unique biphasic developmental cycle of chlamydiae has been extensively reviewed [17] [18] [19] [20] [21] [22]. Two morphologically different forms characterize the chlamydial developmental cycle, the elementary body (EB) and the reticulate body (RB). The elementary body is the infectious form of the organism. Although small in size, about 0.2 to 0.4 microns in diameter, EBs are resistant to extracellular conditions and are able to attach and enter susceptible host cells. Once endocytosed into the host cell, EBs differentiate into RBs inside a membrane bound vacuole called an inclusion. Reticulate bodies are the metabolically active and non-infectious intracellular forms of chlamydiae which multiply via binary fusion within the inclusion. After repeated cycles of cell division, RBs undergo a second differentiation stage resulting in infectious chlamydial EBs. Depending on the Chlamydia species, infectious progeny exit the initially infected cell between 30-72 hours post infection and infect neighboring host cells (Figure 1-1).

For millions of years Chlamydia species have infected eukaryotic cells and many researchers believe that they have ensured their survival by deviating from their normal biphasic developmental cycle. Persistence, a reversible interruption in the productive intracellular chlamydial growth cycle by environmental factors, has been reviewed many times over [23] [24] [25] [26] [27] and is characterized by large aberrant non-replicating RBs which are unable to alternate between EB and RB morphological forms. In vitro inducers of this abnormal growth state include physiological changes in the host cell, gamma interferon treatment [28] [29] [30] [31] [32], beta lactam administration [33] [34] [35], nutrient restriction [36] [37] [38] [39] [40], and concurrent herpes infection [41] [42] [43]. By definition, restoration of normal chlamydial development occurs when the environmental stressor that induced the persistent state is removed. While the likelihood of persistence being involved in chlamydiae-induced pathogenesis has been documented in a variety of culture-based systems, particularly as it relates to antibiotic treatment and
Figure 1-1. Chlamydiae developmental cycle.

Note: Chlamydiae share a unique biphasic developmental cycle consisting of the following steps: 1) Attachment and entry of the infectious Elementary Body (EB) form 2) Primary differentiation from the infectious EB form to the metabolically active but non-infectious Reticulate Body (RB) form in a cytoplasmic vacuole called an inclusion 3) Cell division by binary fission 4) RB genomic replication 5) Secondary differentiation from RB to EB and 6) Cell lysis/endocytosis and release of infectious progeny. Chlamydiae may enter an abnormal state of growth called persistence under the selective pressure of a variety of inducers. Chlamydiae re-enter the cycle and resume normal development when the selective inducer is removed.
host immunological pressures, conclusive evidence of human chlamydial persistence remains to be demonstrated.

**Chlamydiae Clinical Significance**

Chlamydial infection is of global importance given its broad host tropism, the scope of its geographic distribution, and the risk of developing debilitating sequelae. Of the nine *Chlamydia* species, three are known to cause a diverse spectrum of diseases in human populations: *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia trachomatis* (Table 1-1).

**Chlamydia pneumoniae**

*So you’re telling me that you can catch Chlamydia by just breathing? There goes the neighborhood.*

–Corey Dannard

*Chlamydia pneumoniae* was introduced as a novel member of the genus *Chlamydia* by Grayston et al in 1989 and is commonly associated with upper respiratory infections. *C. pneumoniae* causes approximately ten percent of community-acquired pneumonia and five percent of pharyngitis, bronchitis, and sinusitis [44]. In addition, strong associations exist between *C. pneumoniae* infection and atherosclerosis, a chronic cardiovascular disease whose complications lead to half of the adult deaths in the United States and other parts of the western world (Reviewed by Belland et al [45]). It is important to note that the association between *Chlamydia pneumoniae* and atherosclerosis is a point of debate given the failure to improve clinical outcomes with the administration of antichlamydial antibiotics in large scale clinical trials including patients with cardiovascular disease [46] [47] [48].

**Chlamydia psittaci**

*You do know, of course, that zoonotic doesn’t mean the viruses came from the zoo.*

–Law and Order: Criminal Intent

*Chlamydia psittaci*, a zoonotic pathogen whose natural reservoir is avian, is recognized by the Centers for Disease Control and Prevention as a category B select agent due to the ease of respiratory dissemination and associated morbidity and mortality rates [49] [50]. As reviewed by Harkinezhad and colleagues, human *C. psittaci* infection is called psittacosis and is acquired by inhalation or, to a lesser extent, ingestion of bird excretions [51]. After inhalation, the organism infects the respiratory epithelium and remains latent for up to three weeks void of clinical symptoms. Following the incubation period those infected experience flu-like symptoms such as headaches, chills, fever, cough, and in rare instances, neurological and cardiac-related complications. In extreme, untreated cases the infection can be fatal.
<table>
<thead>
<tr>
<th>Species</th>
<th>Serovar</th>
<th>Acute Disease</th>
<th>Sequelae</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pneumoniae</em></td>
<td>Community-Acquired Pneumonia Sinusitis Bronchitis Pharyngitis</td>
<td>Arthritis Asthma Arthrosclerosis*</td>
<td></td>
</tr>
<tr>
<td><em>C. psittaci</em></td>
<td>Atypical Pneumonia Renal and Hepatic Complication</td>
<td>Potentially Fatal</td>
<td></td>
</tr>
<tr>
<td>LGV</td>
<td>Lymphogranuloma venereum (Bubonic)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: * indicates debated chlamydial-sequelae association.
Chlamydia trachomatis

Chlamydia trachomatis is composed of four biovars, biological strains, based on the target cells they infect and whole genome sequencing [52] [53] [54]. As implied by the names, the ocular trachoma lineage commonly infects eye mucosa while urogenital linages most commonly infect the genital epithelia. Lymphogranuloma venereum (LGV) is a disseminating biovar that thrives within the lymphatic niche. Trachoma biovars are subdivided into fifteen serovars based on ompA antigenic variation encoding the major outer membrane protein (MOMP). Loosely, serovars A, B, Ba, and C serve as the causative agent of Trachoma, the leading cause of infectious blindness throughout the world. Serovars D-K are commonly associated with sexually transmitted infection. While L1, L2, L3 are the three main LGV serovars, the newly identified L2b serotype has emerged as the causative agent of the current European and North America epidemic [54].

Ocular Chlamydia trachomatis

The earliest documented historical accounts of chlamydial infection detail trachoma, a contagious disease of the conjunctiva, the outside covering of the eye, and cornea caused by Chlamydia trachomatis. While trachoma rarely occurs in western societies, ocular serovars of C. trachomatis are epidemic in parts of Africa, Asia, South America, Australia, and the Middle East making it the world’s leading cause of preventable infectious blindness.

Active infection, a self-limiting inflammation of the conjunctiva, is primarily seen in children and is transmitted by direct contact with mucosal secretions, poor sanitation, inanimate objects such as towels and clothing that have infected secretions on them, and natural vectors such as flies (Reviewed by [55] [56]). Recurrent infection, which is common in developing countries, leads to the development of scarred tissue and, eventually, trichiasis, inversion of the eyelid. Inwardly turned eyelashes cause physical damage to the cornea. This results in the blindness seen primarily in endemic adult populations. The World Health Organization endeavors to eliminate trachoma by the year 2020 with the implementation of the S.A.F.E. campaign which includes surgery, antibiotics, facial cleanliness, and environmental improvements [57] [58].

Genital Chlamydia trachomatis

Sexually transmitted diseases are hidden epidemics of tremendous health and economic consequence in the United States...the scope, impact and consequences of STDs are under recognized by the public and health care professionals.

–Institute of Medicine, 1997

Sexually transmitted Chlamydia trachomatis has a significant impact on human health given its adverse effects on reproduction. The World Health Organization
estimates that ninety million cases of chlamydial infection occur worldwide each year and an estimated four million cases are reported annually in the United States. Many believe these estimates are much lower than the actual incidence due to the fact that infection is largely asymptomatic. While individuals who are infected may not experience symptoms, it is important to note that they are still at risk of developing long term sequelae.

In men, *C. trachomatis* primarily infects the urethra making it the most common cause of non-gonococcal urethritis. In some instances, the infection spreads from the urethra to the epididymis resulting in epididymitis, a condition primarily associated with sexually active males under the age of thirty-five. Infection may also result in Reiter’s Syndrome in male and female populations. Whether or not *Chlamydia* infection plays a direct role in male infertility is still controversial despite the fact that chlamydial DNA can be recovered from a substantial number of male partners in infertile couples [59] [60] and has been recovered attached to spermatozoa from the peritoneal fluid of women with salpingitis [61].

Genital serovars of chlamydiae are of particular importance to women due to the irreversible reproductive sequelae that may result post infection. Infection of the cervix can ascend causing endometritis, inflammation of the endometrium, and salpingitis, inflammation of the fallopian tubes. Untreated *C. trachomatis* infection has been linked to chronic complications such as Pelvic Inflammatory Disease (PID), involuntary infertility and ectopic pregnancy. Moreover, chlamydial infection increases the risk of contracting human immunodeficiency virus (HIV) and has been implicated in the development of human papilloma virus (HPV)-induced cervical neoplasia [62] [63]. Although rare, *C. trachomatis*-induced salpingitis spreading beyond the upper genital tract into the peritoneum has been documented. The resulting peritonitis and perihepatitis is called Fitz-Hugh-Curtis Syndrome and may be accompanied by upper quadrant abdominal pain and the development of adhesions that resemble the strings of a violin [64] [65].

**Mouse Model of Genital Infection**

*Life is hard for insects. And don’t think mice are having any fun either.*

—Woody Allen

Humans are not the only hosts in which chlamydiae can establish an infection and cause disease. Guinea pigs, turkeys, sheep, and higher order primates have been used to study chlamydiae-associated disease but an extensive amount of data has been extracted from mouse models. Mice infected with either *C. muridarum* or human biovars of *C. trachomatis* are most frequently used as models of chlamydial genital infection due to the ease of reproducibility afforded by inbred strains, commercial ability of reagents, and genetically engineered animals that allow for immunological interrogation [66].

While nonhuman primate [67] [68] [69] and guinea pig [70] [71] models of infection were established, Barron and colleagues [72] developed a novel system in
which researchers intravaginally infected mice with *Chlamydia muridarum*, previously known as *C. trachomatis* mouse pneumonitis strain or MoPn, in 1981. In the Barron study, chlamydial inclusions were identified by examining Giemsa stained vaginal smear preparations and chlamydiae-specific immunofluorescent cervical scrapings and epithelial tissue. These findings were significant because they identified a convenient model using a natural mouse pathogen that induced pathologies remarkable similar to those observed in humans infected with *C. trachomatis* serovar D [73].

Mouse models of chlamydial infection have been used to evaluate the role a host’s genetic background may play in chlamydiae infection resistance and *Chlamydia*-associated outcomes. De la Maza and colleagues intravaginally infected mice with varying H-2 complexes to determine its effect on chlamydial-related infertility [74]. The H-2 complex defines the major histocompatibility complex (MHC) in mice and is homologous to *HLA* in humans. Six weeks after challenge BALB/c, C57BL/6, and C3H mice were mated with male breeders and the embryos were counted. Seventy-five percent (N=20) of C57BL/6 mice became pregnant and had a mean of 4.5 embryos per mouse. Forty percent (N=20) of BALB/c mice became pregnant and had a mean score of 1.5 embryos per mouse. Thirty percent (N=20) of C3H animals became pregnant and had a mean score of 1.7 embryos per mouse. From these studies the researchers concluded that the genetic makeup of the host modulates the degree of chlamydial-induced infertility. In 1997, Darville et al expanded the study by comparing C3H/HeN mice with C57BL/6 mice using varying strains of *Chlamydia* [75]. When intravaginally infected with *C. trachomatis*, serovar E or *C. muridarum*, C3H mice had an increased incidence of hydrosalpinx, increased chlamydial shedding, and prolonged infection course when compared to C57BL/6 animals. This suggested that genetic factors played a role in chlamydial resistance and that the murine model could be used to understand the mechanisms responsible for resistance variability in the human population.

Advances in human genetics, such as the human genome project, have revolutionized our understanding of the host’s role in human health and disease by allowing for inter- and intra-species genetic comparisons [76] [77]. Indeed, whole genome association studies would allow researchers to interrogate genes and their associated disease phenotypes relatively quickly when compared to the previous method of “knocking out” genes in *in vivo* models and looking for changes in the initially observed response. As one would expect, identifying conserved genetic sequences across diverse species would require vast amounts of genetic information. This requirement grants the mouse model a significant advantage over other animal models given the fact that genetic sequences and many gene function relationships have been identified and are readily available using informatics tools like the Mouse Genome Database (http://www.informatics.jax.org/).

Although chlamydial comparative studies are still in their infancy, high-throughput genomic analyses have the potential to transform how we currently identify and therapeutically treat those infected. As proof of principle, Miyairi and colleagues recently sought to predict outcomes of systemic chlamydial infection using recombinant inbred mice (BXD) and computational modeling [78]. Infection of parental strains,
C57BL/6 and DBA/2J, an extensive panel of B (C57BL/6) x D (DBA/2J) mice, in conjunction with gene mapping and computational Bayesian network modeling were used to define underlying pathways contributing to variations in disease severity. The researchers validated predictions that Ctrq3 or polymorphisms in immunological relevant GTPases conferred resistance in B6 dominant genetic backgrounds, whereas, susceptibility was heightened in D2 dominant backgrounds as a function of neutrophilic influx modulation. While there are no homologs of interferon-inducible p47 GTPases in humans, Miyairi’s findings implicate neutrophils as a tentative therapeutic target, validate computational chlamydiae-related modeling as a way of predicting disease outcomes, and highlight recombinant inbred strains as a way of elucidating previously unknown host-derived pathways contributing to disease.

Murine Genital Tract Pathology

_C. muridarum_, although originally isolated from the murine respiratory tract [79] [80], closely mimicked human sexually transmitted chlamydiae disease when used to infect the genital tracts of mice. In 1983, Swenson and colleagues reported _Chlamydia_-induced genital pathology mirrored that seen in human populations [81]. In these experiments mice were inoculated with _C. muridarum_ in the ovarian bursa, a thin membrane that encapsulates the ovary and separates it from the interperitoneal cavity. Hydrosalpinx, blockage of the fallopian tube(s) with serous fluid, was observed in mice between 25-30 days post infection. Salpingitis and hydrosalpinx formation have been linked to involuntary infertility in human female populations by irreversible scarring in the reproductive system and similar outcomes were demonstrated in the model. Although the natural route of infection was not used in this study, it validated that the mouse model could be used to explore mechanisms associated with the development and severity of chlamydiae-induced upper tract complications. Since, several studies, including those outlined in this body of work, have demonstrated vaginal inoculation of the mouse results in reproducible upper genital tract pathology.

Murine Model Limitations

Although the murine model has been advantageous, there are some caveats. For instance, there is great variability in chlamydiae strains, inoculum doses used, and the inbred strain used. The efficiency of infection using human strains of _C. trachomatis_ is significantly lessened when compared to the strain isolated from the murine respiratory tract, _C. muridarum_ [82]. As a result, investigators commonly use higher doses of _Chlamydia trachomatis_ to establish murine infections. Inoculating doses have also been a point of debate in the chlamydial field. In 2004, Maxion and colleagues evaluated differences in BALB/c cell infiltration and pathology formation as a result of inoculum doses ranging from 10^4 to 10^7 inclusion-forming units (IFUs). They found that dose variation altered immune cell representation in the genital tract noting increases in PMN and DC infiltrates in the lower genital tract as chlamydial dose increased [83]. Carey et al investigated the effects of inoculum dose on pathology development in BALB/c female
mice [84]. They concluded higher doses of *Chlamydia muridarum* lead to greater oviduct infection.

Lastly, and perhaps the most relevant argument, is that *C. trachomatis* is transmitted by oral, vaginal, and anal sexual contact with an infected individual. The likelihood of large amounts of infectious organisms being transmitted by the routes mentioned is low. In support of this argument, the infectious chlamydial load in humans is low with a median IFU of 72 from male-derived urethral swabs and 450 IFU from cervical swabs taken from women [85]. Collectively, these studies underscore the need to standardize infection parameters in chlamydial models across the board to reflect the likely transmission inoculums seen in human scenarios of infection.

Another caveat of the mouse model is the five day estrous cycle. The frequency of epithelial uterine sloughing proved to be a problem in establishing chlamydial infection in mice because the target population (epithelial cells) was turning over prior to the completion of the chlamydial developmental cycle. Tuffery et al performed experiments that showed the female genital tract epithelium could be stabilized by subcutaneously injecting progesterone one to two weeks prior to intravaginal infection (Tuffrey, and Taylor-Robinson, 1981). Hormone treatment stabilized murine menses and enhanced chlamydiae genital infection by preventing the normal renewal of genital epithelium. As a result, the mouse model can be used to study the natural course of infection and chlamydiae-induced pathology with increased reproducibility. Currently, the use of progesterone in animal models is debated because sex hormones have been shown to affect susceptibility to a number of sexually transmitted infections in human and animal studies [86] [87] [88].

Another concern is the availability of a persistent murine model. In human female populations, persistent infections defined by chronic asymptomatic chlamydial genital tract infections, may give way to the development of reproductive complications such as PID, chronic abdominal pain, and tubal infertility. As mentioned previously, in vitro persistent states have been induced by environmental stresses such as the interferon gamma inducible tryptophan decyclizing enzyme 2, 3-indoleamine dioxygenase, iron depletion, and by treatment with penicillin. In efforts to reproduce persistence in vivo, Ramsey et al used iNOS knockout mice [89]. When NOS2<sup>−/−</sup> mice were infected with *C. muridarum*, they exhibited higher rates of upper genital tract sequelae but culture-based resolution was comparable to that observed in wild-type mice. In 1997, Cotter et al intravaginally infected wild-type immunocompetent mice and were able to reactivate chlamydiae shedding after initial clearance, evidenced by the inability to recover viable organisms from the vaginal vault, with the immunosuppressive drug cyclophosphamid [90]. While these studies illustrate persistence in the mouse, the mouse model may not be a useful tool for exploring mechanisms of persistent infection because it difficult to get reactivation. Further investigation for a suitable animal model is warranted given the lack of conclusive evidence for persistent infection in the human population.
Immunological Response to Genital Infection

Before discussing the host immune response to sexually transmitted chlamydial infection, it is important to note key distinctions of the female genital mucosa when compared to other mucosal sites of the body. The reproductive environment is unique in that it must exhibit a certain level of tolerance for commensal flora that colonize the lower genital tract and withstand the presence of an immunologically foreign fetus in the uterus during pregnancy, all the while maintaining its ability to mount a response to pathogenic organisms. In addition, the genital environment differs from other mucosal sites like the proximal intestinal system in that many of its effector functions, including immunological properties, are hormonally regulated [91]. These distinctions and the lack of local concentrations of lymphoid tissue such as the gut-associated lymphoid tissue (GALT) component Peyer’s Patches in the intestine or bronchial-associated lymphoid tissue (BALT) in the lung contribute to the complexity of chlamydiae infection in the genital tract.

Intracellular Immune Response Overview

After “self verses non-self” discrimination, the host orchestrates appropriate immunological responses based on the niche in which pathogens thrive [92]. Bacteria such as Mycobacterium tuberculosis[93] [94], species associated with the genus Rickettsia [95] [96], chlamydiae species [82] [97] [98] [99] [100] [101], and viruses like HIV [102] [103] and influenza [104] are hallmark intracellular pathogens that require specialized approaches to achieve clearance. Antigen-presenting cells (APCs) and T-cells are crucial to intracellular pathogen elimination. For instance, pattern recognition receptors (PRRs) associated with APCs like dendritic cells or macrophages recognize pathogen-associated molecular patterns (PAMPs) which, in turn, initiate antimicrobial compounds such as interferon-gamma, tumor necrosis factor-alpha, and interleukin-two [105]. These cytokines assist in the activation of other APCs and push naïve T-cells (Th0) toward an appropriate TH1 pathogen-specific lineage. This cascade culminates in B-cell activation and the development of plasma cells, antibody-producing B-cells that ready the host for subsequent encounters.

Chlamydia Innate Response

Induction of the innate immune response is central to mounting an effective attack against chlamydiae genital pathogens. The process begins with the recognition of pathogen associated molecular patterns (PAMPS) by Pattern Recognition Receptors (PRRs) of Antigen Presenting Cells (APCs) or host cell membranes. Various chlamydiae components or the entire organism may serve as ligands for toll-like receptors (TLRs), a membrane bound family of PRRs. For example, chlamydiae-derived lipopeptide was shown to stimulate TLRs 2, 1, and 6 in macrophages [106]. Although less stimulatory than E. coli lipopolysaccharide (LPS), chlamydial LPS may serve as a ligand for TLR4 [107] and to a lesser extent TLR2 [108]. Moreover, whole organism was used to determine differences in chlamydiae-induced oviduct pathology after discriminatory
stimulation of TLRs 2 and 4 [109]. Stimulation of TLRs give way to activation of the NF-κ B pathway which, in turn, results in production of pro-inflammatory cytokines and chemokines.

Almost immediately after infection, a cascade of proinflammatory cytokines are secreted by the target epithelium. This was first reported by Rasmussen and colleagues in 1997 [110]. Using the in vitro HeLa 229 epithelial cell line, these studies showed that interleukin-8, growth-related oncogene- alpha (GRO-alpha), neutrophil-derived granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-6, and interleukin-1alpha were secreted following infection. Experiments using mouse derived oviduct epithelial cells performed by Johnson et al showed that proinflammatory cytokines and chemokines like tumor necrosis factor alpha, GM-CSF, interleukin-6, MIP-2, KC, MCP-1 and MCP-5 were produced following C. muridarum infection [111]. The secretion of these effector molecules lead to the massive influx of innate immune cells. Darville and colleagues, by flow cytometry, and Morrison and Morrison, by in situ immunohistochemistry, characterized the primary infiltrate and found that monocytes, natural killer cells, and neutrophils were prevalent in these cell populations [75] [112].

The newly recruited cells expanded the repertoire of effector molecules being produced and, depending on the cytokines and chemokines released, recruited pathogen-specific lymphocytes to the site of infection.

**Chlamydia Adaptive Response**

Effective host responses require the induction of, both, the innate and acquired arms of the immune system. Indeed, resident and recruited innate immune cells such as neutrophils and professional antigen presenting cells such as macrophages and dendritic cells begin producing tumor necrosis factor-alpha, interleukin-12, and interferon-gamma. This stimulates naïve T cells toward CD4 T-helper 1 lineages which are necessary for chlamydial clearance in the genital tract. Studies in interferon gamma [113] and interferon gamma receptor knockout mice [114] illustrate a prominent role for the cytokine in the resolution of primary chlamydial infection. Rank and colleagues first demonstrated an important role for CD4 T cells in the genital tract by vaginally infecting athymic nude mice [115]. Nude animals had an active infection evidenced by chlamydial shedding for more than 200 days post infection, whereas animals with intact T cell populations resolved infection within 21 days. Landers et al reinforced this finding in 1991 when they used CD4 antigen specific anti-L2T4 antibodies to deplete CD4 in mice vaginally infected with C. muridarum [116]. These depletion studies resulted in increased vaginally shedding of Chlamydia and an increased number of organisms recovered from the oviduct. Su et al later transferred CD4 and CD8-enriched spleen cells from immune mice to naïve mice and found that adoptive transfer of CD4 lymphocytes conferred immunity in C. muridarum genital tract infection [117]. Kelly and colleagues published work illustrating that CD4+ cells were abundantly recruited to the genital tract following intravaginal infection due to interaction between CD4+ cell home receptor alpha-4-beta-7 and adhesion molecules, ICAM-1, VCAM-1, and MadCAM-1 [118] [119]. Most recently, Gondek and colleagues reported that CD4+ T cells were necessary
and sufficient to clear genital tract infection [120]. Using transcervical inoculation, a method that directly infects the uterine lining of mice, the researchers demonstrated *C. trachomatis*, LGV and *C. muridarum* infected mice are protected from infection and reinfection when treated with pathogen-specific CD4+ lymphocytes. Furthermore, when animals were treated with anti-CD4 antibody, *Chlamydia* 16S DNA levels where comparable to those observed in naïve mice. Collectively, these studies demonstrate the importance of T helper 1 type cytokines and CD4 T cells in the resolution and protection of chlamydial-induced genital infection.

The humoral response has also been implicated in chlamydial immunity. Plasma cells, commonly known as antibody producing B cells, are thought to play a leading role in protecting against reinfection, while playing a secondary role to CD4-mediated resolution of primary infection [99]. One mechanism by which B cells may control subsequent infection is by antibody driven neutralization, which is plausible given the biphasic developmental cycle of chlamydiae. Data from Peeling et al using serum from guinea pigs to neutralize *C. trachomatis* with UM-4, a strain specific monoclonal antibody in an in vitro neutralization assay support this theory [121]. Furthermore, Morrison and colleagues showed that B-cell deficient mice, when depleted of CD4+ T cells during secondary infection, where unable to clear infection [98] [99]. Although the experiments by Morrison et al did not rule out the feasibility of direct neutralization, they implicated a more collaborative mechanism between CD4 lymphocytes and plasma cells, perhaps by the enhancement of antigen presentation during recurrent infection episodes. Although the exact mechanism remains unclear, antibody, particularly IgG given it is the predominant antibody produced in the genital mucosa, is thought to play a collaborative role in chlamydial immunity.

**Vaccination**

*The prevention of disease today is one of the most important factors in the line of human endeavor.*

–Charles H. Mayo, M.D.

The immune response is the host’s natural defense mechanism to foreign antigens but the system can be manipulated to respond to foreign antigens quicker and more efficiently than if it were the first encounter. The previously mentioned form of manipulation serves as the basis for vaccination or, what some refer to as immunization.

Although current anti-chlamydial therapies effectively clear the organism from the lower genital tract, they do not address the potential consequences of reinfection. Batteiger and colleagues reported that almost one third of previously infected individuals are reinfected with the same serovar due to sexual interaction with the same sexual partner [122], potentially increasing the likelihood of irreversible tissue damage. One way of addressing reoccurring infection is through the development of an effective and, most importantly, safe vaccine.
Perhaps the most well-known *C. trachomatis* vaccination studies were the trachoma human trials performed in the 1960’s [123] [124]. During these trials children were intramuscularly vaccinated with formalin-fixed whole organisms and traced for three years. Unfortunately, a portion of those immunized developed severe disease upon exposure to chlamydiae while others developed partial, serovar-specific immunity when compared to unvaccinated controls. Since then, vaccination studies have been exclusively performed in animal models and frequently exploit components of chlamydiae for vaccination.

First purified by Caldwell and colleagues [125], the major outer membrane protein (MOMP) has been the focal point of chlamydiae subunit vaccination studies for the past thirty years. Recently reviewed by Farris and Morrison, native or recombinant protein, DNA, plasmid, and outer membrane complexed MOMP have been the most frequently studied MOMP-derived antigens [126]. At best, these studies have only elicited partial immunity by either failing to reduce bacterial burden, failing to protect animals from reinfection, and/or failing to protect against sequelae formation. Interestingly, recombinant vault nanoparticle delivery of MOMP immunogens has renewed hope in MOMP’s potential as an effective vaccine candidate. Intranasal immunization with rMOMP nanoparticles results in a T-helper 1 driven immune response and significantly reduces chlamydial vaginal shedding when compared to animals immunized with live *C. muridarum* [127].

Additional antigens have been identified based, mainly, on human seroactivity. Some of these include chlamydial protease-like activity factor (CPAF), other outer membrane proteins such as OmcB and Pmps, porin protein B (PorB), and the type III secretion protein, Tarp. Of the previously mentioned chlamydial components, CPAF was thought to have the most promise. Intranasal immunization with recombinant CPAF (rCPAF) plus interleukin-12 reduced bacterial shedding and sequelae formation in BALB/c intravaginally challenged with *C. muridarum* [128]. In addition, rCPAF and Cpg-1826 was used to intranasally immunize BALB/c mice prior to multiple rounds of *C. muridarum* intravaginal challenge [129]. rCPAF plus CpG vaccination resulted in a significantly greater number of pregnancies when compared to mock immunized controls indicating protection post primary and secondary exposures to genital chlamydial infection. In 2012, Chen et al called into question the entire body of work done using CPAF and its proposed substrates after reporting CPAF lost its ability to cleave or degrade 11 of the 16 previously reported host proteins when a CPAF-specific inhibitor was used prior to cell lysis [130]. These results suggest that the proteolysis activity observed in all previous CPAF studies was likely due to the way in which the preparations were prepared and discredit, at least for now, the idea that CPAF protease activity is a virulence factor important in chlamydial pathogenesis.

**Control Measures**

*Chlamydia trachomatis* infection is the most commonly reported sexually transmitted disease in the United States and a significant threat to public health worldwide. *Chlamydia* control programs were implemented to alleviate the public health
burden of chlamydial infection by improving detection mechanisms, shortening the duration of infection, and tracking source-associated sexual networks [131]. These programs ushered in an initial lag phase denoted by a decline in chlamydial cases. However, over the last two decades, case notifications of Chlamydia-associated infection have steadily increased and, in many countries, exceed rates recorded prior to the implementation of intervention strategies [132] [133] [134]. Norway, Finland, Sweden, and Canada have documented similar trends with an initial decline following the introduction of Chlamydia control programs [132] [135] [136] [137]. In recent years, these countries have witnessed chlamydial rates exceeding those recorded prior to the establishment of surveillance systems. Given the magnitude and scope of current epidemiological trends, researchers and policy makers alike are interested in pinpointing the causal agent(s) for increased chlamydial case notifications.

Sexually Transmitted Chlamydia Epidemiology

The insidious nature of Chlamydia trachomatis infection has made it the world’s most common cause of curable sexually transmitted disease. In 2010, over a million cases (1,307,893) of chlamydial genital tract infection were reported to the Centers for Disease Control and Prevention from fifty states and the District of Columbia [63]. This was the largest number of cases reported to the CDC for any reportable condition with a case rate of 426.0 per 100,000 people, representing a 5.1% increase in reported cases over the previous year. Although these rates are staggering, the actual number is more than likely higher than that reported seeing as many cases remain undiagnosed.

Gender disparities have consistently been reported among individuals infected with sexually transmitted C. trachomatis. According to the National Sexually Transmitted Disease Surveillance Report 2010, women bear a heavier Chlamydia burden than men with reported rates two and half times more than their male counterparts despite the fact that Quinn and colleagues reported a nearly identical frequency of transmission among 494 male and female sexually transmitted disease clinic patients [138]. This discrepancy may be attributed to the fact that women are more likely than men to be screened for chlamydial infection [139]. However, the switch from urethral swabbing to urine-based non-invasive nucleic acid amplification testing as the main detection method may result in more men opting to get tested [140] [141]. This is supported, at least in part, by the 36% increase in chlamydiae case notification since the year 2006. A 19.5% increase was observed in the female population between 2006 and 2010, indicating the increase in male reporting was not solely because of an increase in population-based chlamydiae prevalence [63].

Age has been implicated as a risk factor for urogenital chlamydial infection [142] [143] [144] [145] [146] [147] [148]. The highest notification rates correspond to sexually active female adolescents between the ages of 15-19. The second highest rate among females is young adults between the ages of 20-24. Age-specific rates among males are higher among 20-24 year olds with 1,187 cases per 100,000 people reported in the year 2010. These numbers are particularly troubling considering chronic chlamydial
Complications have been linked to infertility in both genders and the age groups greatest affected represent peak reproductive years [63].

Significant ethnic and racial disparities exist among reported sexually transmitted infections, specifically *Chlamydia* cases. For instance, African-Americans only represent 14% of the national demographic, but they account for approximately half of all reported syphilis and chlamydia cases and 75% of all reported gonorrhea infections [63]. With an incidence rate 1,167.5 per 100,000 persons, African-American chlamydial rates are eight times higher than white Americans. American Indian/Alaska Natives chlamydial rates are 4.3 times higher than white Americans while Hispanic American chlamydial rates are 2.7 times greater than those reported among Caucasian-Americans. While it would be easy for researchers and epidemiologists to dismiss these health inequalities based on the idea that these communities are more susceptible to infection and/or are more likely to participate in risky behaviors, the reasons are more complex [149]. While individual behaviors can influence health, surveillance trends are, in large part, influenced by cultural, economical, environmental, educational, and social factors [150] [151] [152].

For instance, in a study conducted by Kaplan and colleagues in 77 different Chicago communities, sexually transmitted infections were higher in neighborhoods that had high poverty rates, high unemployment rates, and a low percentage of high school graduates [153]. In a cross-sectional analysis of over 12,000 young adults, Nguyen et al concluded that geographical location and economic status were factors associated with chlamydial reporting with study participants living in the southern region and no functional income being more likely to report chlamydial infection [154]. While the studies cited highlight findings seen in relatively small participant groups, they are representative of health determinants influencing national surveillance profiles.

**Hypotheses for Rebounding Chlamydia Rates**

Seven hypotheses were put forth to explain this phenomena and were published by Rekart and Brunham [133] and are adapted in Table 1-2. Hypotheses one through four relate to the use of nucleic acid amplification testing for detecting genital infection with chlamydiae. Hypothesis one speaks to an increased probability of “false positive” tests because of a loss of specificity in detection methods. Watson and colleagues performed a meta-analysis including the following diagnostic methods for urogenital chlamydia: nucleic acid amplification testing (NAAT), gene probe, enzyme immunoassay (EIA), direct immunofluorescence (DFA), and cell culture [141]. The study concluded that NAAT testing was the best option for massive screening programs because the results were comparable to those observed using the “gold standard”, cell culture, and urine samples did not constitute a gender bias. Men and women would be willing to undergo screening using this non-invasive technique.

Hypotheses two through four argue increased sensitivity, improved screening amongst high-risk populations, and greater screening among the male population are the basis for surveillance data trends. In 2006, Burckhardt et al assessed the impact of changing from culture-based chlamydial detection to nucleic acid amplification testing in
Table 1-2. Increased chlamydial rate hypotheses.

<table>
<thead>
<tr>
<th>Hypothesis Number</th>
<th>Hypothesis Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>False positive increase due to lower specificity of NAAT methods compared to culture methods</td>
</tr>
<tr>
<td>H2</td>
<td>Increase in case detection due to increased sensitivity of NAAT testing compared to non-NAAT testing</td>
</tr>
<tr>
<td>H3</td>
<td>Higher testing rates among men due to non-invasive NAT urine-based testing</td>
</tr>
<tr>
<td>H4</td>
<td>Targeted screening of high-risk populations and NAAT self-collected sampling among female population(s)</td>
</tr>
<tr>
<td>H5</td>
<td>Development of antibiotic resistance</td>
</tr>
<tr>
<td>H6</td>
<td>Increase in unsafe sexual behaviors</td>
</tr>
<tr>
<td>H7</td>
<td>Chlamydial arrested immunity</td>
</tr>
</tbody>
</table>
a genitourinary medicine clinic cohort [155]. Although an initial increase in positive testing occurred in the two and half years immediately following the implementation of NAAT testing, positive test percentages returned to those observed prior to the detection method change. The researchers concluded that the upturn of chlamydial incidence was not exclusively due to the improvement of detection methods.

Hypothesis five suggests antimicrobial resistance is responsible for the rebounding rates. Indeed, antibiotic resistance is a serious issue, particularly for individuals infected with syphilis and/or gonorrhea [156] [157] [133] [158], two major sexually transmitted infections. Although tetracycline resistance has been documented in chlamydiae serovars that infect livestock [159], resistance among human isolates are rarely observed.

Hypothesis six implicates the safe sex practices, or lack thereof, as a potential cause. Undoubtedly, the behavioral choices made by individuals influence population-based surveillance data. Perhaps the ideal example is that of HIV/AIDS. After the initial recognition of HIV in the early 1980’s, huge awareness campaigns were implemented that led to a decline in STI transmission across the board [160] [161]. After effective treatment was introduced and the general population no longer viewed HIV/AIDS as a “death sentence”, STI incidence rates rebounded. This demonstrates that effect of human behavior on modulating infection cases and confirms the likelihood of current trends being a result of several factors.

The final hypothesis is the arrested immunity hypothesis. Although it is important to remember that none of the previously mentioned postulates are likely to be mutually exclusive, the arrested immunity hypothesis is the cornerstone of my dissertation research and the primary theme hereafter.

Arrested Immunity Hypothesis

The arrested immunity hypothesis asserts that prompt treatment of genital tract infection is coupled with a reduction in naturally acquired immunity, resulting in increased incidence and prevalence of chlamydial infection [132]. Interference with the development of host immunity to chlamydiae genital infection has been shown using the urogenital murine model by the Caldwell research group [162]. Su and colleagues treated C57BL/10 female mice with doxycycline at different timepoints postinfection. No IgG antibody was detected in sera from animals that received treatment at day zero, the onset of infection. This demonstrated the effects of early treatment on the development of anti-chlamydial immune responses.

Brunham et al applied the Cox proportional hazards model to evaluate the effects of antibiotic treatment on the development of population-based immunity [163]. The Cox model is a statistical survival model developed by Sir David R. Cox that relates the time between an “event” occurring to one or more covariates that make also occur within that time frame. The Brunham model is hinged on findings by Molano and colleagues [164] suggesting immunity in human populations may take years to develop. These findings,
when considered with the early treatment component of control programs, suggest that human hosts don’t develop an adequate immunological response to chlamydiae due to the truncated exposure time with chlamydial antigen(s) in the genital tract. Using the Cox model, Brunham’s groups concluded that early treatment renders the general population more susceptible to reinfection.

**Dissertation Rationale**

Despite the implementation of wide-spread control initiatives, sexually transmitted *Chlamydia* rates continue to rise and are coupled with a decline in chlamydiae-associated chronic complications such as PID, ectopic pregnancy, and involuntary infertility [131]. Several rebounding hypotheses have been put forth to explain current surveillance trends and, optimally, humans studies would provide the most insight as to why intensive screening and treatment strategies are, seemingly, ineffective. For obvious reasons, these studies are impractical given the ethical concerns surrounding trials undertaken with the potential of chlamydial-induced upper genital tract complications. Taking into account current therapeutic intervention, immune arrest and infection history, we used the chlamydiae urogenital mouse model to determine the effects of antibiotic treatment on duration of infection, immunological parameters and, most importantly, chlamydial-induced upper tract complications in primary and reoccurring cycles of infection. Moreover, we documented the effects of host factors, such as age and genetic make-up, and immunization on the development of severe chlamydial-induced genital tract disease.
CHAPTER 2. ARRESTED IMMUNITY: IMPACT OF EARLY TREATMENT ON UPPER GENITAL TRACT SEQUELAES

Introduction

Sexually transmitted *Chlamydia trachomatis* is the most commonly reported notifiable infection in the United States [147]. Although up to 75% of women and 50% of men with lower tract chlamydial infections are asymptomatic [165] [166] [167], the potential to develop genital tract sequelae remains unchanged when compared to individuals that experience symptoms such as postcoital bleeding, fever, abdominal pain, and mucopurulent vaginal discharge [168]. This is of particular concern given the reported rate of chlamydial infection among women is over two and half times greater than the reported rates for men [147] and *C. trachomatis* infection can ascend into the female upper genital tract causing long term reproductive complications such as ectopic pregnancy, involuntary infertility, miscarriage, and increased risk of coinfection and transmission of other pathogenic agents [169] [170] [171] [172]. As a result, in the absence of a protective chlamydial vaccine, *Chlamydia* prevention efforts mainly focus on increasing the rate of screening and treating infected individuals [134] [170] [173].

While current antibiotic regimens are highly effective in clearing the organism during uncomplicated lower genital tract infection [174] [175], it has been reported that doxycycline treatment prevents the development of host immune responses in a murine model of chlamydial genital infection [162]. Furthermore, Brunham and Rekart hypothesized that populations were susceptible to reinfection due to a shortened duration of *C. trachomatis* encounters which may limit natural immunity [132] [133]. Indeed, repeated chlamydial exposure has been identified as a risk factor for worse disease in animal and human studies when compared to reference groups that have only been exposed once or are considered low-risk sexually transmitted disease populations [176] [177] [178]. Among a high-risk population of sex workers, reoccurring *C. trachomatis* infection was associated with Pelvic Inflammatory Disease [179]. Similarly, in a retrospective study of 11,000 Wisconsin women, ≥3 positive chlamydial tests increased the chances of Pelvic Inflammatory Disease diagnosis by a factor of six [177]. Although these studies indicate an increased risk for severe disease following multiple positive *Chlamydia* tests, they fail to distinguish between prolonged exposure and reinfection, or the role antibiotic intervention played, if any, in the development of disease. This is further complicated by the initial decline in reported chlamydial infection rates following the introduction of screening and treatment control programs which have since rebounded to levels comparable or exceeding those observed prior to the implementation of intervention strategies.

In this study, we sought to determine if data derived from population surveillance, human studies, and *in vivo* experiments could be recapitulated using the urogenital mouse model as a framework to determine how treatment at various time-points throughout the course of an active primary infection affects disease outcomes during repeated infection cycles.
Materials and Methods

Mice

7 to 8 week old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at our facility, which is fully accredited by the Association for Accreditation and Assessment of Laboratory Animal Care International. The Animal Care and Use Committee at the University of Tennessee Health Science Center (Memphis, TN) approved all animal studies.

Chlamydia Strain and Titration

The Chlamydia muridarum Weiss strain was propagated in HeLa 229 cells and stored at -80°C. Chlamydial titer was evaluated by infecting HeLa 229 cells and counting Inclusion Forming Units (IFUs) by fluorescence microscopy as previously described [113].

Mouse Infection

Mice were subcutaneously treated with 2.5 mg of medroxyprogesterone acetate (Sicor or Depo-Provera, Pfizer, New York, NY) in 0.1 mL of PBS at fourteen and seven days prior to vaginal infection. Mice were infected by vaginal inoculation with 2 x 10^3 IFU of C. muridarum in 10 uL of sucrose phosphate glutamic acid (SPG) buffer. For rechallenge experiments, mice were treated with progesterone and vaginally challenged, as previously described, fifty-six days post primary infection unless otherwise specified.

Antibiotic Treatment

Mice were treated daily with 0.3 mg of doxycycline hyclate (APP Pharmaceuticals, Schaumburg, IL) diluted in distilled, endotoxin-free water (HyClone, Logan, Ut) by interperitoneal injection during the primary infection course only. All treatment groups received antibiotic for fourteen days consecutively except for uninfected, negative control mice treated with progesterone. Treatment groups included treatment from 0-14 d, 14-28 d, 28-42 d, 35-49 d, and Untreated-(UN).

Bacterial Shedding

Vaginal swabs were taken on interval days throughout infection and collected in 2-mL microcentrifuge tubes that contained 0.5 mL of SPG (with three 4mm diameter round glass beads) and stored at -80°C. Swab samples were thawed and vortexed. Infectivity was quantified by inoculating Hela 229 monolayers seeded in 48-well plates with swab supernatant fluid. After 44 h incubation at 37°C in an atmosphere of 5% CO_2,
plates were washed with PBS-Azide and methanol fixed. Chlamydial inclusions were visualized and counted by fluorescence microscopy.

**Fluorescence-Linked Immunosorbent Assay (FLISA) Antibody Analysis**

For total immunoglobulin G (IgG) and IgG isotype studies, HeLa 229 cells were seeded in 48-well plates (CoStar, Corning, NY) at a cell number of $10^5$ cells/well overnight. HeLa 229 monolayers were infected with *C. muridarum* at a multiplicity of infection (MOI) of two for 2 hours with consistent rocking at 37°C. The inoculum was immediately aspirated and plates were incubated in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, California) complete containing 2ug/mL of cycloheximide for 36 hours at 37°C in an atmosphere of 5% CO₂. Following incubation, plates were washed 3 times with PBS-Azide and fixed with 500 uL of methanol per well for 10 minutes. After fixation, plates were washed 3 times with PBS-Azide and incubated at 37°C with a 1:500 dilution of murine sera, pooled based on antibiotic treatment group, in PBS/0.1% grade IV ovalbumin/0.05% Tween 20. After incubation, plates were washed 3 times and incubated with FitC-conjugated rat anti-mouse IgG, IgG1, IgG2a, IgG2b (Southern Biotech, Birmingham, AL), FitC-conjugated goat anti-mouse IgG2c, or Alexa Fluor ® 488 -conjugated goat anti-mouse IgG3 (Invitrogen by Life Technologies, Carlsbad, California) for thirty minutes. Plates were washed 3 times with PBS-Azide and fluorescence intensity was immediately read as fluorescent light units (FLUs) at sensitivity 70 using a Biotex Synergy 2 Microplate Reader. Results represent the mean of triplicate values for each pooled sample group while the solid horizontal bar denotes background intensity as measured by normal sera from uninfected untreated animals.

**Pathology**

Immediately following sacrifice, individual mice were grossly examined for genital tract pathology abnormalities and photographs were taken. The uterine horns and oviducts were collectively and separately scored based on dilation and visual inflammation (reddening and blockage) of the aseptically removed urogenital tissue. Scoring for dilation of uterine horn and oviduct tissues was as follows: 0, normal/no significant dilation; 1, mild dilation of a single cross-section; 2, one to three dilated cross-sections; 3, more than three dilated cross-sections; 4, confluent dilation. Excised tissues were methanol fixed and paraffin-embedded. Longitudinal sections were later stained with one of the following: hematoxylin and eosin (H&E), NIMP-R14 (neutrophil identifying marker), CD68 (macrophage identifying marker), CD4 (T-cell identifying marker), and Toluidine Blue (mast cell identifying stain).

**Organ-Total Body Weight Ratio Analysis**

Mice were individually weighed to obtain the total body weight in grams. Immediately following sacrifice, the genital tract was aseptically removed and weighed in
milligrams. Organ-total body weights were expressed as a function of organ weight divided by total body weight to generate weight ratios. Weight ratios for each animal were averaged by corresponding antibiotic treatment group.

**Statistical Analysis**

Statistical significance of differences between treatment groups was analyzed with GraphPad Prism 4 software (La Jolla, CA) using one-way analysis of variance (ANOVA) with post hoc Tukey testing comparing all possible pairs of means unless otherwise noted. The strength of linear correlations was determined using Pearson product moment correlation coefficient unless otherwise noted. All analyses were performed using two-tailed testing at a 95% confidence interval of difference.

**Results**

**Kinetics of Genital Tract Infection in Antibiotic-Treated Mice**

To evaluate the effect of antibiotic treatment on the duration of chlamydial infection, cervicovaginal swabs were collected throughout the primary course of infection. Progesterone treated mice were vaginally infected with $2 \times 10^3$ IFU chlamydiae, treated with doxycycline and swabbed as described in Materials and Methods. Mice infected but not treated displayed kinetics of an active infection shedding $3-5 \log_{10}$ of viable organisms within the first 3 weeks (*Figure 2-1*). These untreated animals were culture negative, indicating a natural resolution of lower genital tract infection, by approximately week four. We were unable to recover infectious organisms at day four after inoculation in early treated animals, the earliest time point analyzed. This suggests that antibiotic treatment prevented intravaginal chlamydial colonization, negating the establishment of an active urogenital tract infection. Mice that started treatment at an intermediate time point (14-28 d) exhibited a rapid reduction in recoverable organisms after the onset of treatment, whereas, groups treated late during the infection cycle (treatment initiation at day 28 or 35) paralleled untreated clearance rates.

**Immune Arrest and Pathology Following Primary Infection of Antibiotic-Treated Mice**

Antibiotic treatment has been shown to prevent the onset of host immune responses and the development of upper tract pathology post primary infection [162] [180]. To assess whether an immunological response had occurred we measured systemic chlamydial specific IgG antibody titers post primary infection by FLISA in addition to scoring the severity of disease. As hypothesized, pathology following primary challenge was significantly reduced in early treatment groups when compared to later treatment groups or untreated controls (*Figure 2-2*). Animals that began treatment at 14 days post
Figure 2-1. Shedding of viable *C. muridarum* following intravaginal challenge.

Note: Solid lines represent various antibiotic treatment groups. Blue: Untreated control, Red: (0-14 d), Green: (14-28 d), Purple: (28-42 d), Aqua: (35-49 d). Symbols represent the mean inclusion forming units (IFUs) per milliliter recovered at each time point. Error bars denote standard deviation (SD) on a log<sub>10</sub> scale.
Figure 2-2. C57BL/6 pathology severity scores 49 days post-primary C. muridarum intravaginal infection.

Note: Solid bars represent the mean severity score for each treatment group. Symbols represent individual animals. Differences between treatment groups were observed by one-way analysis of variance (ANOVA) with post-hoc analysis using a Tukey-Kramer Multiple Comparison test. * denotes p < 0.05 at 95% confidence interval of difference. ** denotes p < 0.01 at 95% confidence interval of difference. No difference was observed between untreated animals and animals treated at 14-28d, 28-42d, and 35-49d. These data are representative of multiple experiments.
primary infection displayed reduced disease scores (mean of 1, mild disease) when compared to moderately diseased untreated animals and late treatment groups. In addition, the severity of upper genital tract complications followed the same trend established with the development of chlamydiae-specific immune responses (Figure 2-3). In treatment groups where *Chlamydia*-specific antibody titer were observed as determined by FLISA analysis, chlamydiae-related genital pathology was more severe. Total anti-chlamydia IgG antibody titer was undetectable in early treated animals and was significantly different in comparison to untreated animals and animals treated at 14-28d, 28-42d, and 35-49d. Anti-chlamydia IgG levels resembling those of untreated controls suggested a role for immune-mediated upper tract pathology in the development of adverse disease outcomes.

Given the importance of anti-chlamydial Th1-dominant adaptive responses in the resolution of chlamydiae genital infection [181] [182] [183] [184], we evaluated the effects of antibiotic intervention on isotype switching as a marker for Th1 verses Th2 biased responses in a FLISA assay with *C. muridarum*-infected HeLa 229 cells as antigen. IgG isotypes were undetectable following early treatment but data for untreated and late treated animals strongly suggested a Th1 bias, evidenced by IgG2 dominance, regardless of the onset of antibiotic intervention (Figure 2-4). Collectively, these data implicate a bias for Th1 immunity irrespective of severe disease development or the initiation of anti-chlamydial drug intervention.

**Disease Severity Following Reinfection in Antibiotic-Treated Mice**

Repeated infections are common in women and early treatment and have been postulated to increase reinfection susceptibility on the population level [163] [185] [186]. Therefore, it was important to assess whether immune arrest during primary infection contributed to disease severity post-secondary infection in our model. As a result, mice were rechallenged 56 days after primary vaginal encounter to assess disease severity following repeated infection. The animal groups are reflective of the onset of antibiotic treatment during primary infection as animals did not receive treatment during the secondary infection course. Gross examination of early treatment samples showed significant differences in the magnitude and localization of *Chlamydia*-induced pathology. Data presented in (Figure 2-5) show that reinfected animals treated at day zero during primary infection had a mean disease severity score of 1.6, indicating mild to no disease. Conversely, animals treated at days fourteen, twenty-eight, and thirty-five displayed moderate to severe disease, with mean severity scores ranging from 2-3.1. To ascertain differences in the localization of disease, we separately scored uterine horn (Figure 2-6A) and oviduct (Figure 2-6B) pathology. Early treatment resulted in significantly less endometrial disease when compared to untreated controls or animals treated at fourteen, twenty-eight, and thirty-five days post primary infection. Gross evaluation of the oviduct pathology revealed mild disease irrespective of the onset of treatment.

In contrast to human isolates, *C. muridarum* may result in pathology and
Figure 2-3. Total Immunoglobulin G (IgG) antibody response 49 days post-primary *C. muridarum* intravaginal infection.

Note: Bars represent mean fluorescent light units (FLUs) for each treatment group. Differences between treatment groups were observed by one-way analysis of variance (ANOVA) with post-hoc analysis using a Tukey-Kramer Multiple Comparison test. ** denotes $p = 0.001$ at 95% confidence interval of difference.
Figure 2-4. Immunoglobulin G (IgG) isotype responses 49 days post *C. muridarum* intravaginal infection in mice intraperitoneally treated with doxycycline.
Figure 2-5. Incidence and severity of upper genital tract sequelae in antibiotic-treated mice 28 days post-secondary infection.

Note: Solid bars represent the mean severity score for each treatment group. Symbols represent individual animals. Data depicted here represents trends seen in multiple experiments.
Figure 2-6. Incidence and severity of uterine horn and oviduct sequelae in antibiotic-treated mice 28 days post-secondary infection.

Note: Solid bars represent the mean severity score for each treatment group. Symbols represent individual animals. Data depicted here represents trends seen in multiple experiments.
infertility after a single infection in mice [187]. Consequently, we questioned whether the increase in disease severity following secondary infection was a result of primary disease worsening over time or if repeated infection compounded sequelae severity. To determine whether secondary challenge resulted in more severe disease when compared to disease post primary infection, we compared weight ratios as a measure of objective disease severity for three independent untreated groups as weight positively correlated with disease severity score (Figure 2-7). As depicted in (Figure 2-8), untreated C. muridarum challenged animals were sacrificed fifty-six days post primary infection (Prim56) and were compared to untreated C. muridarum challenged mice sacrificed one hundred and twelve days post primary infection (Prim112). Prim56 and Prim112 mg/g ratio means remained stable despite a fifty-six day gap between sacrifice dates. Albeit not statistically significant, untreated C. muridarum challenged animals sacrificed fifty-six days post-secondary infection (Sec56), displayed an increase in mg/g ratio when compared to animals that only received primary infection. These data suggest severe disease is compounded with repeated chlamydial exposure as opposed to residual primary disease progressively worsening over time.

IL-4 Is Detectable Late Following Reinfection and Positively Correlates with Disease Severity in Antibiotic-Treated Mice

Little is known about the cytokine milieu in the late stages of recurrent chlamydial infection. To address the question of which, if any, cytokines were detectable systemically during reinfection cycles, IFN-gamma, TNF-alpha, GM-CSF, IL-1B, IL-2, IL-4, IL-6, IL-8, IL-10, and IL-12 were simultaneously analyzed from individual sera samples collected from mice sacrificed 56 days post-secondary infection. Our results implicate a relatively short-lived TH1 response as suggested by a dominant IgG2 antibody response (Figure 2-2) and unremarkable detection of IFN-gamma, TNF-alpha, GM-CSF, IL-1B, IL-2, and IL-12 post recurrent infection (See appendices). Interestingly, IL-4, a potent inducer of TH2 immunity and a key defensive player against extracellular pathogens, was detected in the circulation and positively correlated with an objective measure of disease severity, weight ratios (Figure 2-9). Moreover, IL-4 detection in the observed sera analytes gradually increased with the deferred treatment of primary infection suggesting a TH2-independent role for IL-4 in this model.

Chlamydia and Cellular Infiltrates Following Reinfection in Antibiotic-Treated Mice

Genital tract sections were stained with H&E, NIMP-R14, CD68, CD4, or Toluidine to identify abundant cell types present in the genital tract after recurrent infection, including potential populations of known IL-4 secreting cells. Late treated tissues stained with neutrophil-specific marker, NIMP-R14, revealed dense polymorphonuclear populations when compared to the residential populations observed in uninfected, untreated controls. Tissues excised from early treated animals (Figure 2-10B) displayed far fewer neutrophils than late treated groups (Figure 2-10A). Levels of T-cells expressing CD4 and macrophages expressing CD68 were comparable to
Figure 2-7. Weight ratio verses total severity score correlative analysis.

Note: Pearson’s correlation was used to determine whether a relationship existed between severity scoring and weight ratio variables. Solid line represents the line of best fit. Blue circles represent individual mice (N=30). Red box highlights a significant positive correlation between the two variables, weight ratios and total disease severity, using a two-tailed analysis at 95% confidence interval of difference.
Figure 2-8. Comparative analysis of weight ratios post-primary and secondary sacrifice in untreated intravaginally infected C57BL/6 mice.

Note: Disease severity was assessed by dividing individual genital tract weights (in milligrams) by the corresponding total weight of the animal (grams) as described in Materials and Methods. Bars represent the mean weight ratio from three independent studies for each infection cycle/time group while error bars correspond to the standard error of the mean (SEM). Prim56 animals (N=3) were sacrificed 56 days post primary infection. Prim 112 animals (N=8) were sacrificed 112 days post-primary infection. Sec30 animals (N=9) were sacrificed 56 days post-secondary infection, totaling 112 days post initial chlamydial exposure.
Figure 2-9.  Systemic IL-4 post recurrent infection.

Note: Sera collected by submandibular bleed post-secondary infection was subjected to Luminex 10-Plex analysis to determine the cytokine milieu during late stages of recurrent infection. Individual samples were plated in duplicate and bars represent the mean IL-4 concentration for each group (Panel A). To determine whether IL-4 correlated with disease, Pearson’s correlation was used (Panel B). A significant positive correlation (p = 0.0462) was observed when IL-4 was plotted against the weight ratios of corresponding animals (N=30). Solid black line represents the line of best fit.
Figure 2-10. Neutrophil infiltrates post-secondary infection.

Note: Immunohistochemistry evaluation using NIMP-R14, a neutrophil-specific marker, of genital tract samples taken 56 days post-secondary *C. muridarum* infection revealed marked increases in neutrophil influx in untreated controls (Panel A, x20) when compared to early treatment groups (Panel B, x20) and normal, non-infected tissue (Panel C, x20). Representative samples from each group are shown.
levels observed in untreated, uninfected controls (data not shown) assessed at the same time-point.

As previously mentioned, we detected unexpected concentrations of systemic IL-4 (Figure 2-9) despite seeing the antibody surrogate marker for TH1-related cytokines, IgG2 (Figure 2-4). As a result we sought to histologically identify known IL-4 producing cell populations. Using Toluidine Blue stain we identified a treatment dependent increase in genital tract mast cell populations, the number of mast cells increased the longer treatment was deferred (Figure 2-11). Furthermore, there was a significant correlation (p= 0.0090) between the severity of disease as assessed by weight ratios and mast cell density (Figure 2-12). We also identified eosinophil infiltrates in H&E stained genital tissue preparations but no statistically significant association was observed (Figure 2-13).

Discussion

In this study, the main objective was to integrate various Chlamydia observations into a single, comprehensive mouse model of chlamydial genital infection. The reproductive health of women is of particular concern with current CDC Chlamydia surveillance data indicating increases in reported chlamydial infections, perhaps, in part, as a result of rapid antibiotic intervention. In contrast, rising infection rates are coupled with reduced complication rates in screened populations [131] [134] [188] despite evidence of adverse complications being linked to multiple or prolonged chlamydial exposures in human epidemiological studies [176] [177] [179]. Collectively, these observations underscore the importance of determining the impact of antibiotic intervention on the development of severe upper tract disease following repeated infection.

In this C. muridarum model of genital infection, we showed that mice intravaginally infected and treated early during primary infection displayed rapid bacterial clearance kinetics in the lower genital tract, significantly reduced anti-chlamydial IgG titer, and virtually no upper genital tract disease. These results verify and validate a previous antibiotic study in C57BL/10 mice that concluded early antibiotic treatment reduced the development of natural immunity [162]. Our study expands on this model by extending observation on disease severity to post-secondary infection and by defining differences in disease localization. Mice treated early during primary infection displayed mild disease severity scores (mean score of 1) in both uterine horn dilation and hydrosalpinx formation. Conversely, untreated infected mice and mice treated later during primary infection displayed moderate to severe disease severity scores in the uterine horn (mean range of 2 to 3.3). However, in the oviduct, significant differences were not observed between treatment groups suggesting either chlamydiae-related responses differ between these two tissues or severe endometrial disease limits the likelihood of oviduct disease. Interestingly, and perhaps not logically, mice that received treatment midway through the natural course of an active primary infection (14-28d) displayed elevated disease severity scores in both the uterine horn and the oviduct, albeit not significantly different from other treatment groups. So far, we have not studied this phenomenon extensively but eradication of the organism soon after the initiation of
Figure 2-11. Mast cell infiltrate post-secondary infection.

Note: Immunohistochemistry evaluation using Toluidine Blue, a mast cell identifying stain, of genital tract samples taken 56 days post-secondary *C. muridarum* infection revealed mast cells in all of the groups assessed. Organ specific mast cell density was determined by manually counting positively stained granulated mast cells in the genital tissue. Bars represent mean mast cell counts for each treatment group.
### Figure 2-12. Mast cell infiltrate verses systemic IL-4 correlative analysis.

Note: Organ specific mast cell density was determined by manually counting positively stained granulated mast cells in the genital tissue. A strong positive correlation between systemic IL-4 and mast cell infiltrate ($r = 0.6682$, $p = 0.0090$) was observed using Pearson’s correlation two-tailed testing at a 95% confidence interval.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of XY Pairs</td>
<td>14</td>
</tr>
<tr>
<td>Pearson r</td>
<td>0.6682</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.2130 to 0.8850</td>
</tr>
<tr>
<td>P value (two-tailed)</td>
<td>0.0090</td>
</tr>
<tr>
<td>P value summary</td>
<td>**</td>
</tr>
<tr>
<td>Is the correlation significant? (alpha=0.05)</td>
<td>Yes</td>
</tr>
<tr>
<td>R squared</td>
<td>0.4465</td>
</tr>
</tbody>
</table>
Figure 2-13. Eosinophil infiltrate post-secondary infection.

Note: Immunohistochemistry H&E staining of genital tract samples taken from mice 56 days post-secondary infection with *C. muridarum* revealed eosinophils in untreated, uninfected animals (A, x40), early treatment groups (B, x20), and late treatment groups (C, x30). Thirty-six fields were randomly selected and counted (12 cervical fields, 12 uterine horn fields, and 12 oviduct fields.) for each sample. No significant correlation between the number of eosinophils present in the genital tract and systemic IL-4 was found (D). Representative samples from each group are shown.
antibiotic treatment and detectable humoral antibody responses fail to explain the observation. Future characterization of the model, including but not limited to the cellular immune response, is of interest and should be investigated to elucidate the underlying immune mechanism involved.

Histological analyses of various cellular populations suggest an influx of polymorphonuclear cells during late stages of recurrent urogenital infection. Using NIMP-R14, a neutrophil–specific marker, we observed an increase in neutrophils among late treated animals when compared to uninfected controls and early treated groups. This follows previous observations by our group [189] [190] and others [191] [192] [193] that cellular infiltrates composed of neutrophils correlate with the development of more severe disease. Data from our studies illustrate the sustained presence of neutrophils during late stages of recurrent infection (fifty six days post-secondary infection) and suggest that disease severity may be a function of sustained innate reactivity rather than the TH1-biased response that was observed in all animals, irrespective of disease severity. This expands the current paradigm of neutrophil influx as an early marker of infection into a potential mediator of disease outcomes and implies that modulation of neutrophil influx may improve chlamydiae-related disease severity and may be investigated as a potential way to improve reproductive health.

Perhaps the most interesting finding in this investigation was the systemic detection of TH2-linked immune regulator, IL-4 (Figure 2-9), with greater detection of IL-4 corresponding with late treatment groups displaying the most severe disease. While our data support the current dogma of TH1-type responses playing a role in the resolution of infection as evidenced by a detectable IgG2-biased chlamydial-specific antibody response (Figure 2-4), they also suggest an alternative TH2 independent role for IL-4 production during recurrent chlamydiae infection. Our data illustrate the development of chlamydial-induced genital tract pathology, with the severity of disease increasing the longer you defer antichlamydial treatment and with multiple rounds of chlamydial exposure. Following secondary exposure, a scenario in which we report exacerbated genital tissue damage, large amounts of IL-4 were detectable and gradually increased as the magnitude of disease increased. Our data compliments observations made by Holland et al of increased TH2-related cytokine production in patients with more severe disease [194]. Miquel et al reported that women with a history of Chlamydia trachomatis infection secrete cytokines consistent with TH2 immunity, specifically significant levels of IL-4, in response to ex vivo stimulation [195]. Collectively, these observations lead us to hypothesis a role for IL-4 in tissue repair. Recent reports have linked IL-4 to tissue repair and wound healing by alternatively activating macrophages and stimulating the expansion of IL-4 producing cells [196] [197] [198] [199] [200]. In line with these reports, we were able to detect both eosinophils and mast cells, known IL-4 producing cells, in our model. Furthermore, it is important to keep in mind that we detected a TH1 response, at least early on during infection, as evidenced by an IgG2 biased antibody response. This suggests that the production of IL-4 occurs later in the immunological cascade (following the production and secretion of chlamydial-specific antibody), which decreases the likelihood of IL-4 initiating the genital tract tissue damage observed (the
idea of IL-4 being an injury promoter) and points to the possibility of IL-4 playing a role in “injury response.”

While we present data in support of the IL-4-driven injury response hypothesis, further study is necessary for hypothesis validation and mechanism delineation. For instance, we proposed the transient production of TH1-related cytokines led to the detection of IgG2 isotypes during early infection cycles. Time course studies following the onset and dissipation of TH1-linked cytokines and IL-4 are key to verifying that proposal. While we know eosinophils and mast cells are capable of producing IL-4 and their presence in the genital tract implicates them as IL-4 sources, it would be useful to know if they are actually responsible for producing the detected IL-4. This can be accomplished by microarray analysis of IL-4 gene expression and subsequent ELISA assay for IL-4 production ex vivo. Additional experiments may also include looking at the upregulation of genes known to particulate in repair signaling pathways in genital tract-derived tissue and dispelling the possibility of IL-4 participating in the initial stages of disease development using IL-4/- knockout mice.

Rapid antibiotic treatment is a key component of current Chlamydia control initiatives that may influence infection rates and, more importantly, disease outcome trends on the population level, but assessing the impact of antibiotic intervention on severe disease development is difficult to address due to ethical concerns associated with delaying or withholding treatment in the human population. To this end, we interrogated the murine model for its potential to serve as a translational bridge between population-based epidemiology and previous animal and human studies. Our results reaffirm the benefit of rapid antibiotic intervention in Chlamydia control efforts and suggest an added benefit during repeated infection even in the absence of antibiotic treatment. Although early-treated mice developed some upper tract disease after re-challenge, it was substantially less severe than the disease observed in groups where treatment was delayed. This is of particular interest since previously screened and treated women commonly re-enter susceptible sexual networks, acquire a secondary infection, and fail to seek treatment due to the asymptomatic nature of Chlamydia trachomatis infection. Most importantly, we provide support for IL-4 dependent tissue damage response following the induction of chlamydial-induced genital tract complications. We conclude that the integrated murine model is useful for studying the efficacy of antibiotic intervention at varying time-points with respect to disease severity but requires further characterization to elucidate potential mechanisms by which chlamydial pathogenesis occurs and is regulated, particularly as it pertains to the induction of IL-4. The dichotomy between cell-mediated chlamydiae-related immune responses may not be as straightforward as we previously thought.
CHAPTER 3. CHLAMYDIAL GENITAL TRACT SEQUELAE IS AGE AND STRAIN-DEPENDENT

Introduction

Sexually transmitted Chlamydia trachomatis is the causative agent of the most commonly reportable disease in the United States [147] and a small percentage of infected women develop reproductive and gynecologic complications such as chronic pelvic pain [170, 201-204], pelvic inflammatory disease (PID) [170] [201] [205] [206], involuntary infertility [170] [172] [201] [203] [206], and ectopic pregnancy [170] [172] [176] [201] [204] [206]. While many factors contribute to individual disease variability to chlamydiae [74] [75] [100] [207], the effects of biological determinants such as genetic predisposition and host age at the time of infection [208] [209] are largely unknown. Identification of host genetic factors is central to improving the quality of current disease animal models and understanding chlamydial-induced disease heterogeneity. Moreover, murine genetic studies may implicate novel pathway targets for individualized therapeutic intervention in humans.

Inbred mouse models have been extensively used to study host contributions to chlamydiae infection susceptibility [74] [75] [210] [211] [212] [213] [214] [215]. Cytokine induction and expression, H2 haplotypes, chlamydial species, and Th-1-mediated responses have all been identified as factors that contribute to varying murine susceptibility. More recently, unbiased genome-wide approaches have been used to identify p47 GTPase genes encoded on chromosome 11 that confer resistance in mice to systemic models of C. trachomatis and C. psittaci infection, respectively [190] [216] [217]. Since, Miyairi et al have combined the high throughput forward genetic approach of BxD recombinant inbred strains with Bayesian network modeling to identify host pathways that modulate susceptibility to systemic chlamydial infection [78].

In the present investigation, we considered the age of the animal at time of infection and the genetic composition of the host when identifying differences in upper genital tract disease severity. We conclude that age and host genetic make-up are both important determinants of disease.

Materials and Methods

Mice

Seven week old C57BL/6J (H-2\textsuperscript{b}) and DBA/2J (H-2\textsuperscript{d}) female mice (referred to as \leq 9wk animals) were commissioned from The Jackson Laboratory (Bar Harbor, ME, USA). Fourteen to 32-week old C57BL/6J and DBA/2J female mice (referred to as >14wks) were bred and maintained at our facility, which is fully accredited by the Association for Accreditation and Assessment of Laboratory Animal Care International.
The Animal Care and Use Committee at The University of Tennessee Health Science Center (Memphis, TN) approved all animal studies.

**Chlamydia Strain**

The *Chlamydia muridarum* (Weiss) strain was propagated in HeLa 229 cells and elementary bodies (EBs) were purified from infected cells by 30% density gradient centrifugation in Renograffin (E.R. Squibb and Sons, Princeton, NJ). Chlamydial titer was evaluated by infecting HeLa 229 cells and enumerating by indirect fluorescence microscopy as previously described. Stocks were stored at -80°C until use.

**Chlamydia muridarum Infection**

DBA/2J, C57BL/6J, and BXD mice were pretreated with progesterone to synchronize the estrous cycle and stabilize the target genital epithelium. Pretreatment consisted of two subcutaneous injections of 2.5 mg medroxyprogesterone acetate injectable suspension (Sicor, Irvine, CA) in 0.1 mL phosphate buffered saline (PBS), given at 10 and 3 days prior to infection. Mice were inoculated with 5000 inclusion-forming units (IFUs) contained in 5 μL sucrose phosphate-buffered glutamic acid (SPG) via the vaginal vault with a filtered micropipettor on day 0. Five animals from each group (DBA/2J-≤9wks, DBA/2J->14wks, C57BL/6J-≤9wks, and C57BL/6-2J->14wks) were sacrificed 40-days post infection. For rechallenge studies, five animals from each group were progesterone treated at day 30 and intravaginally challenged with 5000 IFU at day 40. All rechallenged animals were euthanized 30 days post reinfection, seventy days post-primary infection.

**Pathology Assessment**

Individual mice were weighed to obtain total body weight in grams. In situ macroscopic examinations were performed for evidence of upper genital tract abnormalities immediately following sacrifice. Genital tissues were then aseptically removed, photographed, and weighed to obtain organ weight in micrograms. Severity scores were determined for the uterine horn and oviducts separately: 0, no significant dilatation; 1, mild dilatation of a single cross-section; 2, one to three dilated cross-sections; 3, greater than three dilated cross-sections; 4, confluent pronounced dilation. Scores assigned to individual mice were averaged to obtain the mean severity score for each group of animals. Weight ratios (organ weight-milligrams/ total body weight-grams) were used as an objective validation tool for upper genital tract severity scoring.
Results

C57BL/6J Exhibit Age-Dependent Upper Genital Tract Sequelae Post-Primary Intravaginal Infection

DBA/2 and C57BL/6 female animals of varying ages were intravaginally infected with *C. muridarum* as described in materials and methods. Individual mice were scored according to the localization of disease 40 days post infection. Nine out of 20 animals (45%) assessed developed hydrosalpinx with B6≤9wk animals displaying significantly more oviduct disease (p<0.001) when compared to all other animal groups (**Figure 3-1A**). Twenty percent of DBA/2, regardless of age, displayed mild oviduct dilatation indicated by a mean severity score of 0.23. Conversely, discordant oviduct severity scores were observed among C57BL/6J≤9wk and C57BL/6J>14wk groups. A moderate oviduct severity score of 2.2 was observed in B6≤9wk animals while B6>14wk animals displayed mild oviduct involvement with a mean score of 0.4. No significant differences were observed in endometrial dilatation among groups, irrespective of inbred strain or age (**Figure 3-1B**).

Secondary Intravaginal Infection Exacerbates Chlamydial-Induced Upper Genital Tract Complications

We hypothesized that recurrent chlamydial exposures would result in worse disease when compared to animals that received primary infection alone. To test this hypothesis, DBA and B6 female mice were intravaginally infected at day 0 and rechallenged at day 40 as described in materials and methods. Thirty days after rechallenge, genital tissues were aseptically harvested and assessed for oviduct and uterine horn dilatation. As we hypothesized, secondary disease exacerbated negative disease outcomes in both strains and age groups (**Figure 3-2**) when compared to the corresponding groups that only received a primary infection (**Figure 3-1**). Sixteen out of 21 animals (76%) examined after secondary infection developed oviduct pathology as opposed to 45% of primarily infected animals. Fourteen out of 21 animals (66.6%) examined after secondary infection developed uterine horn pathology as opposed to 30% of primarily infected animals.

C57BL/6 Mice Are More Susceptible to Chlamydial-Induced Upper Genital Tract Complications When Compared to DBA/2J Mice

DBA and B6 female mice were intravaginally infected at day 0 and rechallenged at day 40 as described in materials and methods. Thirty days after rechallenge, genital tissues were aseptically harvested and assessed for oviduct and uterine horn dilatation. Oviduct disease observed in C57BL/6J≤9wk animals was significantly worse (mean severity score 3.6) than that observed in DBA/2≤9wk and DBA/2>14wk animals with mean scores of 0.8 and 1.2, respectively. Additively, C57BL/6J>14wk animals displayed significantly more severe oviduct disease than that seen in DBA/2≤9wk or DBA/2
Figure 3-1. Upper genital tract disease incidence and severity post-primary infection.

Note: DBA/2 and C57BL/6J female mice were intravaginally infected with *Chlamydia muridarum* as described in materials and methods. Forty days after infection, mice were sacrificed and pathology was assessed grossly. The left figure (Panel A) displays oviduct disease severity and disease incidence by inbred strain and age group. The right figure (Panel B) displays uterine horn disease severity and disease incidence by inbred strain and age group. 5 animals were assessed per group and bars indicate mean severity scores. * and ** indicate significant differences (p ≤ 0.01 and p ≤ 0.001) as determined by one-way ANOVA with *post hoc* Newman-Keuls Multiple Comparison Test. Data are representative of two independent studies.
Figure 3-2. **Upper genital tract disease incidence and severity post-secondary infection.**

Note: DBA/2 and C57BL6 female mice were intravaginally infected and rechallenged with *Chlamydia muridarum* as described in materials and methods. Thirty days post-secondary infection, mice were sacrificed and pathology was assessed grossly. The left figure (Panel A) displays oviduct disease severity and incidence by inbred strain and age group. The right figure (Panel B) displays uterine horn disease severity and incidence by inbred strain and age group. 5-6 animals were assessed per group and bars indicate mean severity scores. * indicate significant differences (p<0.01) as determined by one-way ANOVA with post hoc Newman-Keuls Multiple Comparison Test. Data are representative of two independent studies.
>14wk animals. Although uterine horn severity assessments did not result in statistically significant differences, B6≤ 9wk animals consistently displayed moderate disease severity as indicated by a mean score of 2 while B6->14wk endometrial involvement was comparable to that observed in DBA/2 animals.

To remove any unintentional disease bias imposed by using the quantitative severity scoring system, we assessed disease severity as a function of organ weight in milligrams divided by total body weight in grams (Figure 3-3). We have previously observed a direct correlation between severe disease and increased weight ratios given genital abnormalities that involve dilatation of murine oviduct and uterine horn tissues increase the ratio numerator value-organ weight in milligrams (Figure 2-7). Weight ratios compliment severity score data in that secondary infection results in worse disease when compared to animals of the same strain and age group. Moreover, C57BL/6≤ 9wk animals are most susceptible to chlamydial-induced upper genital tract complications evidenced by significant differences observed in recurrent infection oviduct severity scores and objective weight ratio analysis.

**Discussion**

Individual differences in disease susceptibility as a result of infectious agents exist in the population. For example only a small subset of women with uncomplicated chlamydial genital infection go on to develop severe upper genital tract complications[170]. Furthermore, adolescent females are more likely to contract *Chlamydia* than any reported age group [185] [218] [219]. In the United States alone, the reported 2011 rates of sexually transmitted chlamydial infection for women between the ages of 15-19 and 20-24 were 3416.5 and 3722.5 per 100,000 population, respectively [147]. The reported rate for adolescents between the ages of 15-19 yrs is over five times the national rate of 648.9 per 100,000 population and almost six times the national rate for young women between the ages of 20-24 yrs. These rates are particularly alarming considering the rate for women in their mid-forties to early fifties is only 35.8 per 100,000 population [147]. Taken together, current epidemiological trends highlight the need for chlamydial models to account for biological factors such as host age when assessing incidence and severity of chlamydial-induced reproductive complications.

In this study, chlamydial-induced disease outcomes were largely dependent on the mouse age and strain used to model urogenital infection. C57BL/6≤ 9wk mice were more susceptible to oviduct pathology when compared to DBA/2 mice as evidenced by mean severity score post primary (Figure 3-1A) and secondary infection cycles (Figure 3-2A). In addition to extra-strain disease variation, intra-strain disease variation among B6 was shown to be age-dependent in the oviduct post primary infection and, albeit not statistically significant, in the oviduct and uterine horn post-secondary infection. In addition to the four-tiered semi-quantitative severity scoring system, these results were objectively validated with weight ratio analysis (Figure 3-3). BL/6≤ 9wk animals display significantly more disease as evidenced by weight ratios when compared to
Figure 3-3. Weight ratio disease severity validation post-primary and secondary intravaginal infection.

Note: DBA/2 and C57BL6 female mice were intravaginally infected and rechallenged with *Chlamydia muridarum* as described in materials and methods. Weight ratios were determined by dividing individual organ weights (milligrams) by total body weight (grams). Ratios were averaged to calculate the mean value for each group (indicated by bar). Error bars depict the standard error of the mean. * indicate significant differences (p< 0.05) as determined by one-way ANOVA with *post hoc* Newman-Keuls Multiple Comparison Test. ** indicate significant differences (p< 0.01) as determined by one-way ANOVA with *post hoc* Newman-Keuls Multiple Comparison Test. Data are representative of two independent studies.
> 14wk animals with the same genetic background. Interestingly, disease severity as a function of weight observed in DBA/2 animals remained consistent irrespective of age or disease localization.

While inter-strain susceptibility variations have been appreciated for some time [74] [75] [210] [220], our studies suggest C57BL/6 animals are susceptible to chlamydial-induced genital tract complications when compared to DBA/2 mice as determined by semi-quantitative severity scoring (Figures 3-1 and 3-2) and weight ratios (Figures 3-3). Kaltenboeck et al also found that C57BL/6 animals were susceptible to chlamydial-induced disease using a respiratory murine model of infection [221]. In their studies C57BL/6 mice developed severe pneumonia while BALB/C mice were completely void of symptoms following intranasal priming with C. psittaci and subsequent challenge. Interestingly, our results and those seen in the Kaltenboeck model conflict with genital studies proposing a resistant phenotype for C57BL/6 when compared to C3H and BALB inbred strains, both of which are commonly referred to as susceptible strains given their increased chlamydial shedding and longer courses of infection when compared to C57BL/6 mice. Although we have not delineated a mechanism for the results seen in our study, immunological validation is warranted and currently underway. The discrepancies observed between our results and those published by others may also be attributed, at least in part, to differences in the strains of Chlamydia used, inoculum dosage, murine strains, multiple rounds of infection, and variation in what is defined as resistance or susceptibility (I. E. reduced shedding, shorter infection course, infertility, eradication of the infectious agent in the lower and upper genital tract, or the development of upper genital tract sequelae).

As many researchers developing infectious disease models, we are interested in identifying human correlates of phenotypic differences seen in our model. Studies are currently underway using B (C57BL/6) x D (DBA/2J) recombinant inbred mouse strains to identify genetic factors that may modulate disease susceptibility in an age-dependent manner. By interrogating the GeneNetwork database using a forward genomic approach, we hope to identify genes associated with the modulation of oviduct and uterine horn disease severity in an age and strain dependent manner. More importantly, we are interested in identifying human correlates for the potential murine-derived phenotypic-genetic links observed in our model.

While studies aimed at delineating causal mechanisms for the age and strain dependence reported here are currently underway, these initial observations have immediate implications for the use of mouse models in understanding chlamydial-induced disease. Furthermore, these data suggest that the host genetic contribution to genital pathogenesis may be more complex than previously appreciated.
CHAPTER 4. IMMUNIZATION WITH C. MURIDARUM OUTER MEMBRANE COMPLEX FAILS TO PROTECT AGAINST UPPER GENITAL TRACT COMPLICATIONS IN A MURINE MODEL OF GENITAL INFECTION

Introduction

*Chlamydia trachomatis* is the causative agent of the most commonly reported sexually transmitted disease in the United States, Chlamydia, and a public health threat worldwide [56] [63] [147] [222]. Despite the overwhelming global presence of chlamydial infection, many cases are undiagnosed due to asymptomatic infection and may serve as a reservoir for infection [175] [223]. Regardless of whether *Chlamydia trachomatis* infection is symptomatic or asymptomatic, women with genital infections may develop long-term sequelae like pelvic inflammatory disease (PID), ectopic pregnancy, involuntary infertility, and miscarriage [169] [170]. Antibiotic therapy is currently effective in clearing the organism from the lower genital tract but the emergence of antibiotic resistance, although rare, is a viable concern [224] [225] [226] [227]. Currently, rapid antibiotic intervention is a key component of *Chlamydia* control programs [173] [175] and may serve as the selective pressure necessary for the evolution of antibiotic resistant human strains. Taken together, the high rate of asymptomatic infection and the potential for developing antibiotic resistance despite the current availability of effective antimicrobial therapies underscore the need for a safe and efficacious vaccine.

Trachoma vaccine trials using whole organisms, both inactivated and live, were performed decades ago and are perhaps the most notable vaccination studies to date. Unfortunately, a subset of individuals who were vaccinated and reexposed to *Chlamydia* experienced significantly more severe disease when compared to non-vaccinated cohorts [124] [228] [229]. While no human trials have been undertaken since the trachoma study, researchers have continued the quest to identify safe chlamydial antigens that elicit immunological memory - the basis of all vaccines.

A number of *Chlamydia trachomatis* - associated antigenic proteins have been described, particularly proteins associated with the cell surface. Many studies have focused on developing a subunit-based vaccine using *Chlamydia trachomatis* major outer membrane protein (MOMP) as the antigen [230] [231]. MOMP has been an attractive candidate because it constitutes an approximated 60% of the total outer membrane mass and is immune-assessable due to its location on the chlamydial surface [21] [125]. Although MOMP seemed promising, its ability to induce protection has been sketchy at best, displaying varying protection in small animal models and higher order primates [232] [233] [234] [235] [236] [237] [238] [239] [240] [241]. There may be several reasons for the variation seen in the previously mentioned studies including but not limited to the animal model and strain(s) used, immunization site, challenge route, definition of protection (I.E. reduction in shedding, fertility, cytokine production, antibody titer, or sequelae formation), and antigen preparation. Indeed, one of the main lessons taken from previous MOMP studies is that conformational integrity of the chlamydial membrane may increase protection following chlamydiae challenge. As a
result, we elected to use detergent-extracted chlamydial outer membrane complexes (COMCs) as described by Caldwell et al [125] in the preliminary studies outlined later in this Chapter. Sarkosyl-Insoluble COMC was found to be structurally intact and maintained the shape observed in protein-rich infectious chlamydial particles, elementary bodies (EBs).

In the pages to follow, I outline experiments evaluating the efficacy of COMC as a potential antigen in both systemic and genital models of chlamydiae infection. As a disclaimer, these studies are in their infancy at best and should not be interpreted as complete studies. Several gaps exist in the experiments outlined hereafter but these data do provide interesting tangents that may serve as the basis for future experimental direction. To that end, our initial results in the systemic model showed promise by negating the effects of lethal interperitoneal chlamydial doses. Conclusions originating from studies in the urogenital murine model were more muddled when compared to the lethal model. Despite the inconclusive nature of the genital tract model results, we examined the potential benefits of alum, an adjuvant known to generate a TH2 cell-mediated response, in the prevention of severe genital sequelae.

Materials and Methods (Systemic Lethal Model)

Mice

Female DBA/2J mice were purchased from Jackson Laboratories at 7-8 weeks of age. They were housed in the University of Tennessee Health Science Center animal facility and animal protocols were performed in compliance with institutional IACUC and federal mandates.

Chlamydiae

*Chlamydia psittaci* 6BC was propagated in the murine fibroblast cell line L929 (ATCC; Manassas, VA) and stored at -80°C. Titers were determined by infecting L929 cells plated at a density of 2 x 10⁵ cells/well in 24-well plates (Corning Costar Corporation, Cambridge, MA), with tenfold dilutions of thawed stock. Infected monolayers were incubated for 48 hours, methanol fixed, and chlamydial inclusions were enumerated using fluorescent staining with FITC-conjugated anti-chlamydial lipopolysaccharide antibody (Fitzgerald Industries Internation Incorporated, Concord, MA).

COMC Extraction

*Chlamydia psittaci* 6BC COMC was purified as described by Caldwell et al [125]. In brief, *C. psittaci* 6BC stock was thawed at room temperature and suspended in 5mL of pH 8 phosphate buffered saline (PBS) containing 2% dipolar ionic Zwittergonic detergent and 1.5 mm EDTA. A dry ice bath was used to precipitate proteins and the resulting
suspension was sonicated, in series, 3 times with 1 minute iced incubation periods between each round of sonication. The cell suspension was incubated for 3 hours at 37°C and ultracentrifuged at 32,700 rotations per minute (RPM) for 1 hour.

**Immunization and Infection**

DBA/2J mice were intramuscularly mock immunized with 100mL of sterile Dulbecco’s phosphate buffered saline (PBS), viable whole elementary bodies (EBs) of 6BC (5 x 10^2 IFU in 100mL of sterile PBS), or 6BC-derived COMC (40mg total) in 100mL of PBS, respectively. Mice received a total of two immunizations at days -14 and -7. On day 0, mice were intraperitoneally challenged with a low dose (1 x 10^3 IFU, equivalent to 1000 LD_{50}) or a high dose (1 x 10^5 IFU, equivalent to 100000 LD_{50}) and monitored for survival. Surviving animals were sacrificed 15 days post infection irrespective of immunization regimen.

**Results (Systemic Lethal Model)**

Our laboratory was instrumental in developing a systemic interperitoneal model of chlamydial disease using *C. psittaci* 6BC, a zoonotic to which mice are highly susceptible. Using the systemic model Byrne and colleagues were able to elucidate basic concepts concerning interferon-gamma and *Chlamydia psittaci* clearance [242] [243] [244]. Unpublished data from our laboratory showed that doses as low as 1 EB can cause mice to succumb to infection within 10 days of initial *C. psittaci* challenge. These data provided the basis for using the systemic model of chlamydiae infection to elucidate COMC immunogenicity. One hundred percent of mice immunized with viable 6BC *Chlamydia psittaci* elementary bodies (EBs) survived challenge and recovered from infection irrespective of the challenge dose. Conversely, all mock PBS immunized mice succumbed to infection within 11 days of interperitoneal challenge. 20% of COMC antigen immunized mice survived an inordinately high dose challenge and went on to recover from infection (**Figure 4-1**). Moreover, 60% of mice immunized with 6BC *Chlamydia psittaci*-derived antigen survived and recovered from lethal challenge (**Figure 4-2**).

Despite the rigor associated with the systemic chlamydial intraperitoneal murine model, COMC preparations were capable of eliciting partial protection against lethal challenge. Both immunizing doses, 10^2 and 10^5 IFU, supersede the LD_{50} and highlight the sensitivity of DBA/2J inbred mice to 6BC *Chlamydia psittaci* intraperitoneal infection as previously illustrated by the Byrne laboratory. Collectively, these data suggested COMC-induced partial protection with homologous challenge and justified transitioning these studies into the urogenital model which would display more subtle read-outs.
Figure 4-1. High dose 6BC *C. psittaci* systemic infection survival curve.
Figure 4-2. Low dose 6BC C. psittaci systemic infection survival curve.
Materials and Methods (Urogenital Model)

Mice

Female DBA/2J mice were purchased from Jackson Laboratories at 7-8 weeks of age. They were housed in the University of Tennessee Health Science Center animal facility and animal protocols were performed in compliance with institutional IACUC and federal mandates.

Chlamydiae

Chlamydia muridarum (Weiss) was propagated in the human epithelial cell line HeLa 229 (ATCC; Manassas, VA) and stored at -80°C. Titers were determined by infecting HeLa 229 cells seeded at a density of 2 x 10^5 cells/well in 24-well plates (Corning Costar Corporation, Cambridge, MA), with tenfold dilutions of thawed stock. Infected monolayers were incubated for 48 hours, methanol fixed, and chlamydial inclusions were enumerated using fluorescent staining with FITC-conjugated anti-chlamydial lipopolysaccharide antibody (Fitzgerald Industries Internation Incorporated, Concord, MA).

COMC Extraction

Chlamydia muridarum COMC was purified as described by Caldwell et al [125]. In brief, C. muridarum stock was thawed at room temperature and suspended in 5mL of pH 8 phosphate buffered saline (PBS) containing 2% dipolar ionic Zwittergonic detergent and 1.5 mm EDTA. A dry ice bath was used to precipitate proteins and the resulting suspension was sonicated, in series, 3 times with 1 minute iced incubation periods between each round of sonication. The cell suspension was incubated for 3 hours at 37°C and ultracentrifuged at 32, 700 rotations per minute (RPM) for 1 hour.

Immunization and Infection

DBA/2J mice were intramuscularly immunized with 100mL of sterile Dulbecco’s phosphate buffered saline (PBS), 100mL of sterile Dulbecco’s phosphate buffered saline (PBS) and 25ug of alum, live C. muridarum elementary bodies (5 x 10^2 IFU in 100mL of sterile PBS), 25ug of Chlamydia muridarum-derived COMC in 100mL of PBS, or 25ug of Chlamydia muridarum-derived COMC in 100mL of PBS and 25ug of alum. Mice received a total of two immunizations at days -14 and -7. One week prior to challenge, the estrous cycle was synchronized by subcutaneous injection of 2.5mg of medroxyprogesterone acetate (Sicor, CA). On day 0, mice were intravaginally challenged with 2 x 10^3 IFU, a non-lethal dose of C. muridarum.
Results (Urogenital Model)

*C. muridarum* and *C. psittaci*-derived COMC preparations were compared by commassie-stained SDS-Page gel and displayed comparable banding patterns (Figure 4-3). As expected, animals intramuscularly immunized with viable whole *C. muridarum* elementary bodies and intravaginally challenged with 5 x 10^3 IFU displayed the most severe disease when compared to other immunization groups as evidenced by dilation of the uterine horns and reddening of the genital tract (Figure 4-4A). Mock immunized animals (Figure 4-4B) also displayed fluid accumulation in the uterine horn, although to a lesser extent when compared to the live immunized group. COMC immunized animals displayed less severe disease when compared to live immunized groups but still displayed mild to moderate dilation and reddening (Figure 4-4D). While animals immunized with COMC-alum displayed mild reddening, dilation was not observed (Figure 4-4E). Lastly but most intriguingly, animals immunized with the PBS-alum combination displayed no dilation or substantial reddening (Figure 4-4C).

While we hypothesized that alum would augment protection elicited by COMC antigen immunization despite its polarity toward a Th2-type response, we were perplexed by the observation seen in PBS-alum immunized groups. Unexpectedly, the alum adjuvant seemed to negate the development of severe genital tract sequelae induced by exposure to *C. muridarum* intravaginal infection. The study described below followed as an attempt to assess whether alum alone was capable of negating the development of upper genital tract sequelae following *C. muridarum* intravaginal infection.

Materials and Methods (Alum Study)

Mice

Female C57BL/6J mice were purchased from Jackson Laboratories at 7-8 weeks of age. They were housed in the University of Tennessee Health Science Center animal facility and animal protocols were performed in compliance with institutional IACUC and federal mandates.

Chlamydiae

*Chlamydia muridarum* (Weiss) was propagated in the human epithelial cell line HeLa 229 (ATCC; Manassas, VA) and stored at -80°C. Titors were determined by infecting HeLa 229 cells seeded at a density of 2 x 10^5 cells/well in 24- well plates (Corning Costar Corporation, Cambridge, MA), with tenfold dilutions of thawed stock. Infected monolayers were incubated for 48 hours, methanol fixed, and chlamydial inclusions were enumerated using fluorescent staining with FITC-conjugated anti-chlamydial lipopolysaccride antibody (Fitzgerald Industries Internation Incorporated, Concord, MA).
Figure 4-3.  Comassie stained SDS-Page gel of *C. muridarum* (MoPn)-derived COMC and *C. psittaci* (6BC)-derived COMC preparations.

Note: Chlamydiae COMC preparations were extracted as previously described in Chapter 4. Protein concentrations were determined by Modified Lowry Protein Assay and visualized by SDS-Page gel. Molecular weight protein standard (shown on left) were used to approximate the molecular weight of visualized bands.
Figure 4-4. *C. muridarum*-derived COMC immunization study gross pathology.

Note: Representative photos of genital gross pathology post homologous *C. muridarum* immunization and challenge. Photo (A) is representative of live immunizations. Photo (B) is representative of mock immunized animals. Photo (C) is representative of mock-alum immunized animals. Photo (D) is representative of COMC immunized animals and photo (E) represents animal cohorts immunized with COMC-alum. Black arrows point out selected areas of dilatation, reddening, and hydrosalpinx formation.
Immunization and Infection

C57BL/6J mice were intramuscularly immunized with 100mL of sterile Dulbecco’s phosphate buffered saline, 100mL of sterile Dulbecco’s phosphate buffered saline and 25ug of alum (Thermo Scientific), live C. muridarum elementary bodies (5 x 10^2 IFU in 100mL of sterile PBS), or a mixture of live C. muridarum (5 x 10^2 IFU in 100mL of sterile PBS) and 25ug of alum. Mice received two immunizations one week apart and were treated subcutaneously with 2.5mg of medroxyprogesterone acetate (Sicor, CA) at days -9 and -3 to synchronize the esterous cycle and enhance mouse susceptibility to chlamydial infection. On day 0, mice were intravaginally challenged with 2 x 10^3 IFU Chlamydia muridarum in 10uL of PBS. Animals that were immunized and challenged were sacrificed 35 days post infection. Immunized animals that did not receive an infection were sacrificed 21 days after day 0.

Pathology Assessment

Before removing the genital tissue, an in situ gross examination was performed for evidence of chlamydial-induced abnormalities such as uterine horn dilation and hydrosalpinx formation. Genital tracts were aseptically removed, photographed, subjected to macroscopic inspection, and assigned a severity score on the day of sacrifice.

Results (Alum Study)

Immunogens without Intravaginal Chlamydia muridarum Challenge Do Not Induce Severe Upper Genital Tract Sequelae Formation

Female C57BL/6J mice were intramuscularly immunized with alum adjuvant, viable C. muridarum and alum adjuvant, viable C. muridarum, or mock immunized with sterile PBS according to the timeline displayed in (Figure 4-5). Genital tracts from each animal were aseptically removed, weighed, photographed, and grossly assessed for upper genital tract sequelae formation three weeks after day 0. Two non-immunized C57BL/6 mice, of the same age and gender, were used as a reference. As hypothesized, hydrosalpinx formation was not observed in any of the immunization groups (Figure 4-6). Negligible increases in dilation of the uterine horns were observed in mock, C. muridarum and alum adjuvant, and C. muridarum alone immunized animals. Although these increases were minimal, they were documented by granting the lowest severity score possible (Figure 4-7). Overall, genital tract tissues, regardless of immunization group were comparable to that observed in the two non-immunized, non-challenged reference samples.
Figure 4-5. Alum study experimental timeline.
Figure 4-6. Oviduct pathology severity of non-challenged immunized groups.

Note: Solid line represents the mean of each immunogen group. Symbols represent individual mice (N=5 per group except for normal control).
Figure 4-7. Uterine horn pathology severity of immunized, non-challenged groups.

Note: Solid bars represent the mean severity score for each immunogen group. Symbols represent individual animals (N=5 per group except for normal controls).
Alum Alone Does Not Negate the Development of Severe Upper Genital Tract Complications

Female C57BL/6J mice were intramuscularly immunized with alum adjuvant, viable \textit{C. muridarum} and alum adjuvant, viable viable \textit{C. muridarum}, or mock immunized with sterile PBS according to the timeline displayed in (Figure 4-5). All immunization groups were intravaginally challenged with $2 \times 10^3$ IFU of \textit{Chlamydia muridarum}. Genital tracts from each animal were aseptically removed, weighed, photographed, and grossly assessed for upper genital tract sequelae formation and severity 35 days post infection. Three female C57BL/6 mice which were not immunized or challenged were used as a negative reference.

Unlike their non-challenged counterparts (Figure 4-6), all immunization groups displayed some level of oviduct disease (Figure 4-8). 40% of mock immunized animals displayed unilateral hydrosalphinx formation while 20% displayed bilateral oviduct involvement. 20% of animals immunized with \textit{C. muridarum} or \textit{C. muridarum} and alum adjuvant exhibited unilateral oviduct disease. Interestingly, 20% of animals immunized with alum adjuvant alone displayed bilateral hydrosalpinx while unilateral oviduct pathology was observed in 40% of the same immunization group.

Uterine horn dilation was seen in all groups immunized (Figure 4-9). 60% of animals immunized with \textit{C. muridarum} alone displayed mild dilation with a mean severity score of approximately 1. Eighty percent of animals immunized with \textit{C. muridarum} and alum adjuvant were found to have dilated uterine horns with 60% of those being mildly dilated and 20% displaying moderate dilation. Only 20% of alum adjuvant immunized animals displayed uterine involvement, whereas, 60% of mock immunized animals experienced uterine horn dilation.

Discussion

Although \textit{C. trachomatis} lower genital tract infections may resolve without adverse reproductive complications [164] [245] [246], a subset of those infected may develop upper genital tract sequelae. Presently, \textit{Chlamydia} control initiatives focus on identifying and treating individuals before, at least in humans, irreversible tissue damage occurs. This approach is complicated by the fact that most human chlamydial infections are often asymptomatic. As a result, the most effective way of controlling chlamydiae infections would be to develop a vaccine. Although the studies outlined in this Chapter fail to provide definitive answers pertaining to protection against chlamydial infection, they do highlight the need for ongoing vaccine development initiatives to consider the pathogenesis approach given the overarching goal of any \textit{Chlamydia trachomatis} vaccination effort would be to preserve reproductive health by preventing the development of severe disease manifestations.

In the systemic model of chlamydial infection we showed that a detergent-extracted preparation of \textit{C. psittaci}-derived COMC protected mice from lethal intraperitoneal challenge. To our knowledge, this is the first study of its kind and the
Figure 4-8. Oviduct pathology severity of immunized and challenged groups.

Note: Solid bars represent the mean severity score for each immunogen group. Symbols represent individual animals (N= 5 per group except for normal control).
Figure 4-9. Uterine horn pathology severity of immunized and challenged groups.

Note: Solid bar represents the mean for each immunogen group. Symbols represent individual animals (N = 5 per group except for normal control).
results inspired us to examine the efficacy of COMC in the *C. muridarum* urogenital model given the fact that mice intravaginally infected with *Chlamydia muridarum* develop genital sequelae similar to that observed in human populations. Seeing as the development of genital morbidities such as hydrosalpinx and endometritis are more subtle than the mortality (survival vs death) end point observed in the systemic model, we opted to co-administer alum adjuvant in subsequent genital tract experiments as a way of enhancing the responses observed with homologous COMC antigen. Additionally, aluminum hydroxide, aluminum phosphate, and aluminum sulfate are the only vaccine adjuvant formations licensed in the United States and are currently being used in vaccines directed against the intracellular pathogens, hepatitis B virus (HBV) and human papilloma virus (HPV) [247].

In our studies, COMC-alum immunized groups (Figure 4-1E), as hypothesized, fared better than mock immunized groups (Figure 4-1B) and live immunized groups (Figure 4-1A) displaying no increases in uterine horn or hydrosalpinx formation. As we hypothesized, DBA/2J mice immunized with live *C. muridarum* and challenged with *C. muridarum* displayed the most severe pathology with both endometrial dilation and oviduct inflammation (Figure 4-1A). These findings complement our studies and the studies of others that suggest chlamydial infection recurrence increases the risk for reproductive sequelae [177] [179] [248].

By far the most interesting observation was the fact that alum adjuvant and PBS immunized animals displayed no signs of chlamydial-induced pathology or reddening which is indicative of inflammation. While Th2-driven responses such as those prompted by the alum adjuvant have been shown to promote immunopathology in chronic diseases such as schistosomiasis [249] [250], autoimmune diseases, and allergies, more recent studies have implicated TH2-linked responses to tissue repair and wound healing. As a result, we hypothesized that a Th2-type response may play a protective role in upper genital tract disease formation.

To experimentally address the effects of alum adjuvant on chlamydial-induced genital tract pathology we immunized female C57BL/6J mice with alum adjuvant, alum and whole organism *C. muridarum*, or intact *C. muridarum* organisms alone. Based on the observations seen in the COMC urogenital studies, we hypothesized that alum only severity scores would be on par with severity scores associated with non-immunized, non-challenges animals (negative controls). Interestingly, animals immunized with alum adjuvant alone displayed severity scores comparable to those observed in alum/*C. muridarum* and *C. muridarum* alone immunization groups (Figure 4-9). While the alum alone observation was unexpected, we were encouraged by the fact that all immunization groups exhibited less severe disease, as indicated by mean severity score, than the mock-immunized PBS group which complemented the results seen in the preliminary COMC urogenital studies (Figure 4-4 and Figure 4-9).

There are at least two, not mutually exclusive, explanations for the discrepant results seen in the alum adjuvant alone group. One is the mouse inbred strain we used in the follow-up studies. We substituted the DBA/2J mouse strain originally used in the
COMC studies for the C57BL/6J inbred strain because B6 animals used in ongoing, unrelated studies in our laboratory routinely exhibit more severe genital tract disease when compared to DBA/2J animals. We hypothesized that by using the ‘more severe’ B6 background we would stratify the results seen between groups, making the potential benefits of alum immunization easier to identify. We know that the enhancement effects of alum are less pronounced than that of other adjuvants like Freud’s complete adjuvant (FCA) and are optimal when used in conjunction with an immunogenic compound. Assuming the upper genital tract sequelae observed in our model is mediated, at least in part, by the host immune response, it is possible that the B6 TH_1-bias was too much for alum adjuvant to overcome which manifested as mild disease. Secondly, but not less important, is the chlamydial preparation used in the study. Chlamydiae-derived outer membrane complex preparations were used as immunogen in the preliminary studies while whole viable organisms were used in the alum efficacy studies. This is important in that the alum alone immunized group scores (Figure 4-9) are indistinguishable from Chlamydia muridarum-alum and Chlamydia muridarum alone group scores. In using concentrated COMC preparations, we directed the host response against known immunogenic proteins associated with the chlamydial membrane. Perhaps statistical differences were not observed amongst immunization groups, including alum alone, because we diversified the anti-chlamydial antibody pool by using whole organisms over purified COMC preparations. While it is conceivable that either or both of these explanations contributed to differences seen in our alum efficacy experiments, further study is needed to draw definitive conclusions.

While data presented in this Chapter would, seemingly, negate the hypothesis put forth in Chapter 2 relating to the potential benefits of having TH_2-related IL-4 present late during recurrent infection that is not the case. Our hypothesis hinges on the idea that IL-4 is detectable late during recurrent infection cycles as a way for chlamydial hosts to repair severe tissue damage. In our studies, alum adjuvant was administered concurrently with chlamydial immunogen, prior to the development of severe upper genital tract complications. It is logically to hypothesize that a portion of time in which the host perceives ‘self-damage’ is necessary for the gearing up of potentially protective TH_2-related responses. As stated previously, this is only a hypothesis albeit formed from data derived from ongoing chlamydial genital studies in our laboratory. The continuation and translational relevance of the studies reported in this Chapter are currently being investigated in our laboratory and are supported, at least in part, by the recent report published by Rodolfo et al suggesting an evolutionary genital tract bias for Th_2 responses as a mechanism to prevent chlamydial-induced genital tract complications in humans [195].
CHAPTER 5. CONCLUSIONS

The reproductive health of women is of particular concern with current CDC *Chlamydia* surveillance data indicating increases in reported chlamydial infections, perhaps, in part, as a result of rapid antibiotic intervention. To this end, we interrogated the murine model for its potential to serve as a translational bridge between population-based epidemiology and previous animal and human studies. Our studies reaffirm the benefit of rapid antibiotic intervention in *Chlamydia* control efforts and suggest an added benefit during repeated infection even in the absence of antibiotic treatment. Moreover, our results suggest a TH2-independent role for IL-4 in genital tract tissue repair.

**Testing the IL-4 Hypothesis**

IL-4 as a mediator of TH2-driven immunity is well established but the role IL-4 may play in genital tract tissue repair is largely unknown. In our studies we show elevated levels of systemic IL-4 late during recurrent chlamydial genital infection and cellular infiltrates that are known to produce IL-4 in the genital tract. We also show a correlation between systemic IL-4 and disease severity. To assess the role of IL-4 in the arrested immunity model, longitudinal studies are necessary. In these studies we would measure the onset, duration, and resolution of TH1 and TH2-linked cytokines and disease progression. This is important given the fact that tissue repair mechanisms rely on the presence of tissue damage. If IL-4 is indeed a driver of in vivo tissue repair, at least in this model, it is plausible that the production and secretion of the cytokine would begin with or after the development of chlamydial-induced pathology. Future studies should also measure IL-4 transcription levels in implicated cell types (mast cells and eosinophils) to confirm that they are the primary source of IL-4. These studies would be followed by IL-4 knockout studies that examine the effect of IL-4 on collateral tissue damage during multiple rounds of chlamydiae infection.

**Identifying Genetic Link to Age-Dependent Disease Severity**

Studies are currently underway using B (C57BL/6) x D (DBA/2J) recombinant inbred mouse strains to identify genetic factors that may modulate disease susceptibility in an age-dependent manner. By interrogating the GeneNetwork database using a forward genomic approach, we hope to identify genes associated with the modulation of oviduct and uterine horn disease severity in an age and strain dependent manner. If disease severity is associated with specific genetic loci, human cell culture studies focused on RNA silencing will ensue. These studies will be followed with in vivo studies using knockout mice specific for the identified gene(s).
Implications for Vaccine Development

It is generally accepted that an effective anti-chlamydial vaccine would need to elicit a robust TH₁-type CD4+ T cell response but our studies suggest that prophylactic control of chlamydiae may be more complex than originally appreciated. If the goal is to clear the pathogen from the site of infection and minimize the likelihood of developing adverse reproductive upper genital tract complications, eliciting TH₁-linked immune responses may not be enough. It may be beneficial for the host to mount a mixed immunological response (I.E. TH₁/ TH₂). Our work highlights the need for additional studies aimed at addressing the role of mixed cell-mediated responses in chlamydial infection and immunopathological genital tract damage.
LIST OF REFERENCES


63. CDC. *Sexually Transmitted Disease Surveillance 2010*: Centers for Disease Control and Prevention 2011.


70. Mount DT, Bigazzi PE, Barron AL. Infection of genital tract and transmission of ocular infection to newborns by the agent of guinea pig inclusion conjunctivitis. Infect Immun 1972; 5:921-6.


117. Su H CH. CD4+ T cells play a significant role in adoptive immunity to Chlamydia trachomatis infection of the mouse genital tract. Infection and Immunity 1995; 63:3302-8.

118. Kelly KA, Rank RG. Identification of homing receptors that mediate the recruitment of CD4 T cells to the genital tract following intravaginal infection with Chlamydia trachomatis. Infection and Immunity 1997; 65:5198-208.


128. Murthy AK, Chambers JP, Meier PA, Zhong G, Arulanandam BP. Intranasal vaccination with a secreted chlamydial protein enhances resolution of genital Chlamydia muridarum infection, protects against oviduct pathology, and is highly dependent upon endogenous gamma interferon production. Infection and Immunity 2007; 75:666-76.


133. Rekart ML, Brunham RC. Epidemiology of chlamydial infection: are we losing ground? Sexually transmitted infections 2008; 84:87-91.


171. Fleming DT, Wasserheit JN. From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. Sexually transmitted infections 1999; 75:3-17.


183. Cain TK RR. Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis biovar of Chlamydia trachomatis. Infection and Immunity 1995; 63:1784-9.


188. Gottlieb SL, Martin DH, Xu F, Byrne GI, Brunham RC. Summary: The natural history and immunobiology of *Chlamydia trachomatis* genital infection and implications for *Chlamydia* control. The Journal of infectious diseases 2010; 201 Suppl 2:S190-204.


195. Vicetti Miguel RD, Harvey SA, LaFramboise WA, Reighard SD, Matthews DB, Cherpes TL. Human female genital tract infection by the obligate intracellular bacterium *Chlamydia trachomatis* elicits robust Type 2 immunity. PloS one 2013; 8:e58565.


Note: C57BL/6 female mice were purchased from Jackson Laboratories at 7-8 weeks of age. Animals were subcutaneously treated with progesterone at days 14 and 7 per intravaginal challenge. On day 0, animals were intravaginally challenged with $2 \times 10^3$ IFU C. muridarum. UN refers to animals that did not receive treatment during the course of the experiment. (0-14) represents animals that were intraperitoneally treated with doxycycline starting at day 0 for 14 days consecutively. (2-16) represents animals that were intraperitoneally treated with doxycycline starting 2 days post primary challenge for 14 days consecutively. (4-18) represents animals that were intraperitoneally treated with doxycycline starting on day 4 post primary challenge for 14 days consecutively. Normal represents negative the negative control group which neither received treatment nor intravaginal Chlamydia muridarum challenge. Animals were sacrificed 56 post-primary infection.
APPENDIX B. MORBIDITY AND PATHOLOGY SEVERITY POST-SECONDARY INFECTION IN DEFERRED TREATMENT STUDIES

Note: C57BL/6 female mice were purchased from Jackson Laboratories at 7-8 weeks of age. Animals were subcutaneously treated with progesterone at days 14 and 7 per intravaginal challenge. On day 0, animals were intravaginally challenged with $2 \times 10^3$ IFU *C. muridarum*. UN refers to animals that did not receive treatment during the course of the experiment. (0-14) represents animals that were intraperitoneally treated with doxycycline starting at day 0 for 14 days consecutively. (2-16) represents animals that were intraperitoneally treated with doxycycline starting 2 days post primary challenge for 14 days consecutively. (4-18) represents animals that were intraperitoneally treated with doxycycline starting on day 4 post primary challenge for 14 days consecutively. Normal represents negative the negative control group which neither received treatment nor intravaginal *Chlamydia muridarum* challenge. Animals were sacrificed 56 post-secondary infection.
APPENDIX C. TH₁-RELATED CYKINES POST RECURRENT INFECTION IN ARRESTED IMMUNITY MODEL

![Graphs showing cytokine levels after doxycycline treatment](image-url)
APPENDIX D.  TH2-RELATED CYTOKINES POST RECURRENT INFECTION IN ARRESTED IMMUNITY MODEL
VITA

Enitra N. Jones was born in Houston, Texas in 1983, the daughter of Ezra C. Jones and Evelyn T. Jones. In 2001, she graduated from West Jefferson High School of Harvey, Louisiana in the top three percent of her class. In July 2005, Enitra earned her Bachelor of Science degree in Biology with a concentration in Microbiology from the Honors College at Southern University and Agricultural and Mechanical College in Baton Rouge, Louisiana (Cum Laude Latin Honors). In 2006, Enitra enrolled in the Integrated Program of Biomedical Sciences at the University of Tennessee Health Science Center in Memphis, Tennessee. She graduated in 2013 with a Doctorate of Philosophy in Microbial Pathogenesis, Immunology, and Inflammation. Enitra is currently a two-year American Public Health Laboratories (APHL)/ Centers for Disease Control and Prevention (CDC) Emerging Infectious Diseases (EID) Postdoctoral Research Fellow in Atlanta, Georgia.