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The Role of Non-Neuronal Acetylcholine in Urogenital Chlamydial Infection

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ABSTRACT

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Chlamydia trachomatis causes a bacterial sexually transmitted infection, Chlamydia, that is often chronic and causes reproductive complications in women. We hypothesized that Chlamydia infection increases local acetylcholine (ACh) production, which regulates the host's inflammatory response to the infection. Female mice infected with *C. muridarum* were sacrificed at days 3, 9, 15, and 21 post-infection, genital tract tissues harvested, and immunohistochemistry performed to enumerate ACh-producing cells. Infection increased the number of ACh-producing cells in cervical tissue at days 3, 15 and 21 post-infection (pi), uterine tissue at days 3 and 9 pi, and ovarian tissue at days 3, 15 and 21 pi. These findings suggest that *C. trachomatis* increases ACh production, which may suppress host's immunity and aid in establishing chronic infection.

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CHAPTER 1

INTRODUCTION

Chlamydia trachomatis is the organism responsible for the most prevalent bacterial sexually transmitted infection in the United States, known as Chlamydia or chlamydial infection. In 2016, there were approximately 1.6 million cases reported to the Centers for Disease Control and Prevention (CDC) and an estimated 2.86 million new cases of infection in the US alone.[1] The large discrepancy in reported cases and estimated new cases of chlamydial infection can be attributed to the often-asymptomatic nature of the infection.[2] While chlamydial infection may be asymptomatic, it can still cause inflammation and tissue damage, which may result in potentially life-altering complications. Chlamydial infection is not restricted to the United States; in 2012, there were an estimated 131 million new chlamydial infections worldwide,[3, 4] indicative of a global problem.

The genus *Chlamydia* is part of the family Chlamydiaceae, of the order Chlamydiales, of the phylum Chlamydiae, belonging to the Bacteria kingdom.[5] All species in the *Chlamydia* genus are Gram-negative obligate intracellular bacteria. Within the unique species *Chlamydia trachomatis*, there are multiple serovars. The serovars A, B, Ba, and C are most commonly associated with trachoma, an infection affecting the eyes that is the most common infectious cause of blindness worldwide.[6] The serovars D through K are those typically associated with non-invasive genital tract infections.[7, 8.]

Non-invasive urogenital chlamydial infection is associated with a number of complications and sequelae. However, one of the confounding factors with identifying infected individuals and initiating treatment corresponds with the findings of a 2003 study. This study found that, of those with urogenital chlamydial infection, only about 10% of men and 30% of

women show symptoms.[9] As a result, individuals without symptoms who have current infections due to the *C. trachomatis* bacteria are at risk of spreading the disease unknowingly. In addition, they are also at increased risk of HIV acquisition if they have sexual contact with individuals who are HIV infected.[10] In addition, there are a number of possible symptoms and complications of chlamydial infection related to the inflammatory processes. Chlamydial infection-associated inflammation can result in urethritis in both males and females and proctitis in males. It can also result in cervicitis and pelvic inflammatory disease (PID) in females. Long-term effects of PID may include tubo-ovarian abscess, tubal factor infertility, ectopic pregnancy, and chronic pelvic pain. Antibiotics can eliminate infection and cure PID; however, they cannot alter lasting damage that may have occurred as a result of the infection, such as genital tract scarring that results in infertility.

Significance of the Issue

Sexually active females between the ages of fifteen and twenty-four years of age have the highest rate of chlamydial infection, with an estimated rate of 1 in every 20 women in this age range is infected with *Chlamydia trachomatis* in the United States.[1] A study published in 2011 found that seven years after a clinical PID diagnosis, 21.3% of women in the study had recurring PID, 19.0% had developed infertility, and 42.7% of women reported having chronic pelvic pain.[11] In addition, women that experience recurrent PID are 1.8 times more likely to have trouble conceiving in a twelve month period with little or no contraception use.[12] This means that many young women, who are infected with Chlamydia but do not receive appropriate and timely medical care, may be at risk of developing PID, which can greatly impact their reproductive futures.

The Arrested Immunity Hypothesis

In chlamydial biology, the arrested immunity hypothesis has been proposed to explain persistent or recurrent chlamydial infections. The hypothesis states that, while treatment can halt progression of genital tract infection and subsequent disease, early treatment may eliminate the organism but can also reduce development of Chlamydia-specific adaptive immune responses. Thus, individuals who are infected and then treated may not develop sufficient anti-chlamydial T cell responses and the antibodies necessary to resist re-infection. Stimulation of innate immune responses, which would be expected to occur before treatment, is likely insufficient to protect against re-infection.[13] Ultimately, if these individuals are re-exposed, they will not have sufficient pre-existing immunity to protect them from re-infection. Unless lifestyle changes are made in regard to sexual activity, previously infected individuals who receive treatment but do not develop the necessary adaptive immune response to combat the infection are likely to acquire the infection again from an infected sexual partner. In addition, if the first or a subsequent infection remains asymptomatic and is not treated, this could lead to chronic infection and increased risk of future adverse reproductive consequences.

Chlamydial Genomics

Stephens et al. published the first genome sequence report for a human urogenital pathogenic chlamydial strain in 1998. This genome sequence and subsequent analysis for *C. trachomatis* serovar D has been one of the most productive advancements in the field of Chlamydia research. Chlamydiae are known to have genome sequences that are comparatively smaller than many other bacteria. *C. trachomatis* has a 1.04Mb genome sequence,[14, 15] which is approximately half the size of the average extracellular urogenital bacteria, at 2.11Mb,[16] and they

have limited abilities when it comes to performing many metabolic functions. It has been suggested that the relatively small genome of the species within the genus *Chlamydia* is related to their obligate intracellular parasitic relationship with their hosts.[7, 8, 14] *C. muridarum,* which is an oft-used organism for modeling infection with *C. trachomatis*, has largely conserved and highly similar genomes and biosynthetic functions.[17]

Chlamydial Developmental Cycle

C. trachomatis is a Gram-negative, obligate intracellular bacterium. It has a unique developmental cycle (Figure 1), existing in one of two forms. In the smaller, elementary body (EB) form, the Chlamydiae are infectious and capable of entering the host. EB use small endosome-like vacuoles to enter the host epithelial cells. The EB form of *C. trachomatis,* similar to other bacterial pathogens, can alter the host's functions and signaling pathways to make the environment conducive to survival, which helps prevent endosome acidification. This allows the Chlamydiae to avoid being killed by the host's lysosome contents.[18] Once the EB form enters the host cell through receptor-mediated endocytosis, [19] it can form a membrane-bound protective environment, called an inclusion. In order for chlamydial cells to reproduce, they must convert into the reticulate body (RB) form.[7] The reticulate body form is the non-infectious, intracellular form in which the chlamydiae divide and benefit from the host's natural resources and processes. Thus, the RB is the replicative form of the organism, and the reticulate bodies divide by binary fission. Once the reticulate body cells have successfully reproduced, they are then able to reorganize into the elementary body form once again. The elementary body cells are released from the host cell and are now able to go out and infect other cells.[20, 21]

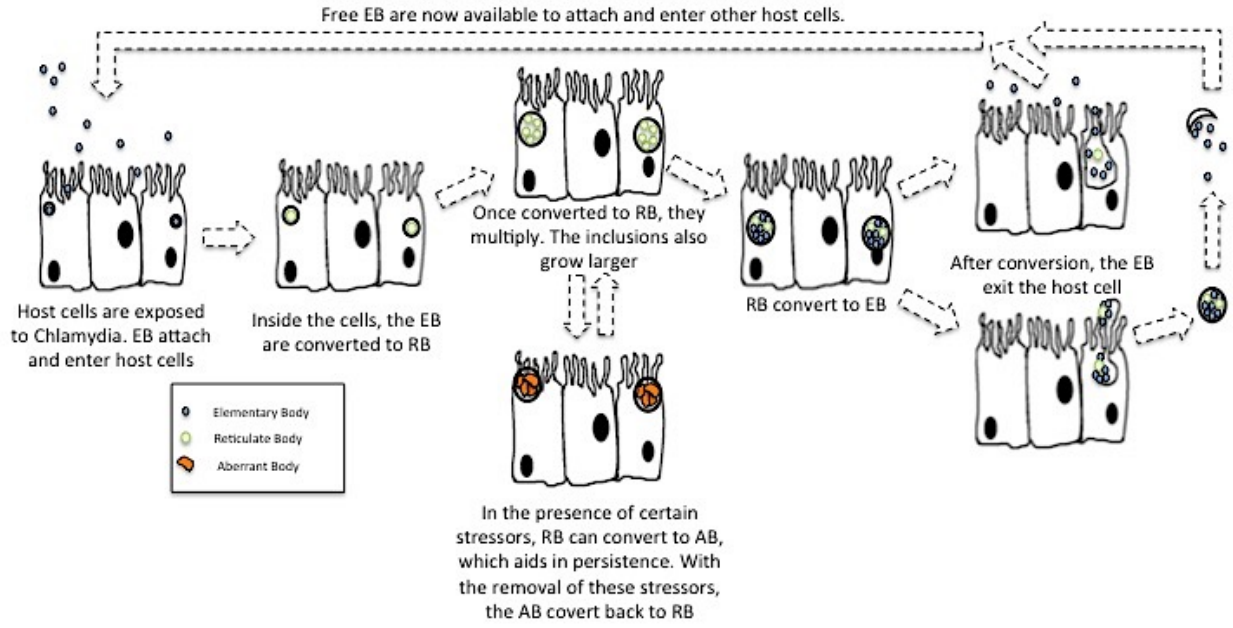

Figure 1. The Chlamydial Developmental Cycle. The host cells are infected by the elementary body (EB) form. The elementary bodies enter the host cells and are converted to reticulate bodies (RB). In the RB form, they are more metabolically active and undergo multiplication. The inclusions also increase in size. In the presence of stressors such as β-Lactam antibiotics, lack of necessary nutrients, and heat shock, the RB can convert into aberrant bodies (AB), which may aid in the persistence of chlamydial cells in the host. In the absence of these stressors, the AB can return to the RB form. RB convert to the EB form, and they exit the cell through extrusion or lysis. In the case of extrusion, the inclusion can rupture, which releases EB and allows the EB to infect other cells.

Further study of the developmental cycle and persistence of chlamydial infection indicates that there is an additional form, the aberrant body (AB; Figure 1). Reports of a chlamydial body that fits neither the RB nor the EB form date back to at least 1950, when Weiss noticed that *Chlamydia psittaci* transitioned into a new developmental form when exposed to certain stressors.[22] Examples of stressors that can induce transition into this new form include environments that do not provide the necessary nutrients, as well as exposure to β-Lactam antibiotics.[23] After induction into these AB, the chlamydial cells can persist until these stressors are no longer present, as long as they have a healthy host cell. Once the stressor is removed, the AB revert back into RB, which continue to develop into infectious EB as described above.

Conversion to these aberrant bodies gives the infectious cells a method to escape conditions that might otherwise result in death of the chlamydial cells. It should be noted that review of published work by Bavoil cautions against using terminology such as aberrant bodies or persistent bodies, as it implies that these abnormal chlamydiae are universal in features.[24] There are a number of characteristics and details about aberrant bodies that are yet to be discovered and understood, including whether the aberrant bodies have standard features.

Chlamydial Infection and Inflammation

Inflammation is part of the innate immune response and the body's response to injury or invader. It initiates suddenly when a pathogen or other insult is detected by cell surface receptors.[25] Inflammation can be either local or systemic and acute or chronic. Inflammation at the local level is often characterized by 4 main signs- pain, erythema, increased warmth, and swelling at the site of infection or injury. There are also changes to vascular permeability and expression of cell adhesion proteins, which can aid in the recruitment of infection-fighting white blood cells, including neutrophils, lymphocytes, and monocytes.[26] Systemic inflammation can result in leukocytosis, an elevated number of circulating white blood cells, and it can also result in fever. Local inflammation is primarily mediated by cytokines, while control of systemic inflammation can be neuroendocrine-mediated.[27] Acute inflammation is also part of the immune response to pathogen, invader, or injury; however, chronic inflammation is typically not a part of the initial immune response to a pathogen. Chronic inflammation occurs when the body continues to demonstrate these inflammatory responses for an extended period of time, which suggests that either the body's system of inflammatory checks and balances is not maintained or that there is a persistent infection that is initiating the inflammation.[28]

The inflammatory process is typically regarded as part of the innate immune response and begins with pattern recognition receptions (PRR), which recognize pathogen-associated molecular patterns (PAMPs) and/or danger-associated molecular patterns (DAMPs). [28] In the case of *C. trachomatis* infection, inflammation begins with detection of a PAMP by the toll like receptors (TLRs) on host epithelial cells or on tissue-resident macrophages. Lipopolysaccharide (LPS) is a component of the outer layer of the cell wall of Gram-negative bacteria that is detected by TLR4 and is an example of a chlamydial PAMP.[29] Chlamydiae have also been recently demonstrated to produce peptidoglycan, another PAMP, which is detected by TLR2.[30] Once cell surface or cytoplasmic TLRs bind to their target PAMP or DAMP, they activate a complex series of intracellular signaling events that result in the translocation of nuclear factor kappa beta (NFκB). Once in the host cell nucleus, NFκB increases transcription of many genes related to the inflammatory response, including those encoding inflammatory cytokines.[31] Following activation of NFκB, macrophages release cytokines, such as tumor necrosis factor (TNF), interleukin-1 beta (IL-1β), IL-6, and IL-8. These pro-inflammatory cytokines are associated with the start of local inflammation. TNF and IL- 1β increase vascular permeability to allow immune cells to reach the affected area and have also been shown to significantly increase the expression of cell adhesion molecules (CAM), resulting in increased leukocyte adhesion, activation, and migration into tissues[25, 32, 33] These neutrophils then participate in the immune response by phagocytosis. After the neutrophils have been recruited, IL-6 can also function to recruit monocytes.[34] Chlamydial infection has been observed to result in significantly increased levels of IL-8 [35], which is responsible for the recruitment of neutrophils to the site of the chlamydial infection. It is important to point out that the influx of neutrophils is critical for

controlling chlamydial infection.[36] Neutrophils can also function to attract other types of immune cells, so the neutrophils can phagosytize the bacteria, as well as call on other cells to help fight off infection by phagocytosis or releasing cytokines.[37] The production of these pro-inflammatory cytokines is essential for resolution of infection, but an excessive response of these immune cells may be the cause of the inflammation-related injuries that can cause long-term damage.

The Cholinergic Anti-Inflammatory Pathway

When PAMPs, DAMPs, or pro-inflammatory cytokines are released, they can bind to neuronal PRR or cytokine receptors and stimulate vagus nerve fibers capable of sending information towards the brain stem. Following this communication with the brain, efferent vagal nerve impulses can then trigger norepinephrine (NE) release from nerve terminals in the spleen, and perhaps in the mucosal tissues of the gastrointestinal tract and lungs. This NE then binds to β-adrenergic receptor 2, which stimulates the expression of the choline acetyl-transferase (ChAT) enzyme and promotes synthesis of acetylcholine (ACh) by a sub-set of CD4+ T cells. This secreted ACh binds α7-nicotinic acetylcholine (α7nAChR) on nearby macrophages, and stimulates these receptors, reducing the activity of NFκB, which corresponds with decreased production of pro-inflammatory cytokines (Figure 2).[38] Thus, activation of the cholinergic anti-inflammatory pathway (CAP) is one of several regulatory pathways that function to reduce inflammatory responses in peripheral tissues.[39]

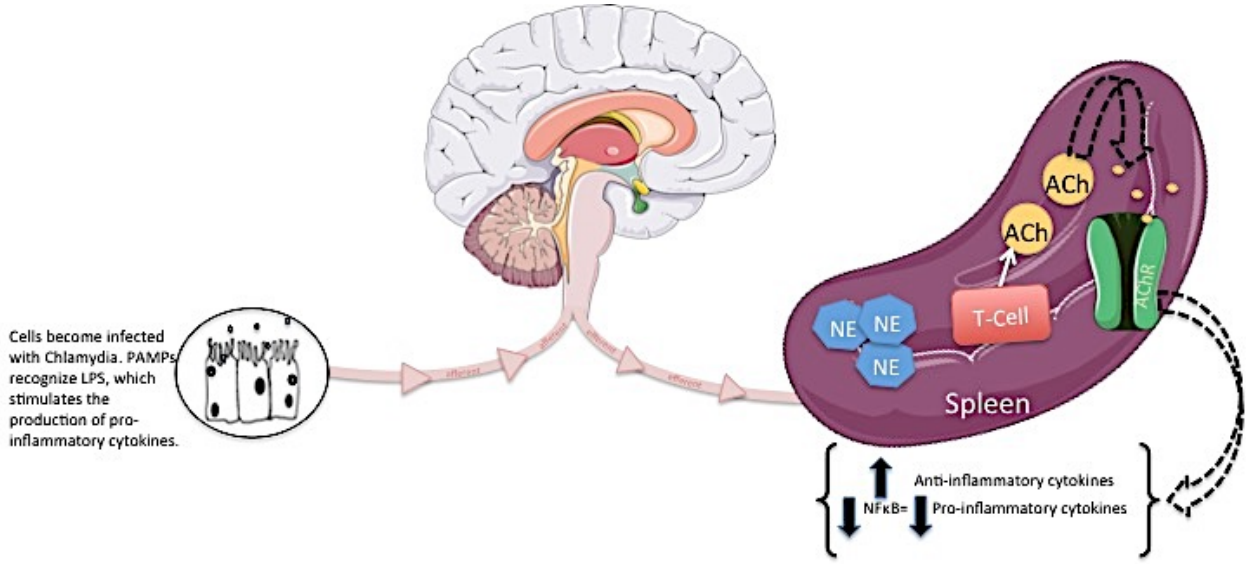

Figure 2. The Cholinergic Anti-Inflammatory Pathway. Upon recognition of PAMP or DAMP, such as Lipopolysaccharide in the case of chlamydial infection, cytokines are released. These cytokines signal to the brain using the afferent vagal nerve. The efferent vagal nerve signals to the spleen to release norepinephrine, which binds to a β-adrenergic receptor. CD4+ T-cells are stimulated to release acetylcholine, and ACh binds to a nAChR, stimulating an increase in production of anti-inflammatory cytokines and decreasing the production of NFκB, which results in a decrease of the production of pro-inflammatory cytokines.[*93]

Cytokines such as IL-10 and IL-4, antagonists for IL-1 receptors, and transforming growth factor beta (TGF-β) also all participate in counteracting inflammation. TNF and IL-1β also work as mediators of inflammation in the brain on top of their local influence.[40, 41, 42] In addition to these pathways, when the vagus nerve is activated, afferent sensory neurons signal to the brain that inflammation is occurring.[27, 28, 43] The vagus nerve, part of the Autonomic Parasympathetic Nervous System, stimulates production of anti-inflammatory responses in the hypothalamus, pituitary gland, and adrenal gland.[44] The vagus nerve can function as a link between the immune system and the brain. This mechanism, which is involved in modulating immune response, is termed the "cholinergic anti-inflammatory pathway."

Vagal Nerve Involvement

The tenth cranial nerve, the vagus nerve, is the longest nerve in the human body, starting at the brain and traveling down to a number of organ systems. It is involved in a multitude of processes and functions, as it is the nerve with most complex and expansive distribution.[45] It is composed of mixed nerve fibers and is involved in both sensory and motor function.[46] Recent research has focused on the relationship between the vagus nerve and immune function. Specifically, attention has been focused on the parasympathetic nervous system.[27] In a 2002 review, Tracey outlined the role of vagus nerve stimulation and the inflammatory process, termed "the inflammatory reflex."[47] In essence, the inflammatory reflex provides information about the links between local inflammation and the central nervous system. Stimulation of the immune system initiates processes, such as the release of cytokines and chemokines that allow the peripheral tissues to communicate with the brain through the vagus nerve. Subsequently, immune function can be regulated through a neural pathway.

Importantly for our study, it has been shown that the vagus nerve indirectly innervates the genital tract. Gerendai and Halasz performed a small study that shows a connection between the vagus nerve and ovarian function. Specifically, they found that ovarian regulation was affected by interference with the physiology and function of the vagus nerve.[48] Furthermore, there is evidence that the vagal nerve branches extend to the uterus[49] and can receive information from the lower genital tract. Current research shows that the lung and gut, which are innervated more directly by the vagus than the genital tract, demonstrate similar methods to regulate the mucosal immunity process.[26, 27] Thus, the inflammatory reflex described initially by Tracey may also contribute to regulation of immune process initiated by sexually transmitted infections such as chlamydial infection in the genital tract.

Importance of Anti-Inflammatory Responses

The inflammatory reflex's role is significant because it serves to regulate the body's immune response. With this regulation, the body attempts to protect itself from the detrimental effects of inflammation as a function of the immune response. There is potential therapeutic importance behind this phenomenon, because it may be a method that can be employed to prevent the effects of conditions related to excess inflammation within the body.[46, 50, 51] For example, Pavlov *et al.* demonstrated that treatment with the alpha 7 nAChR agonist GTS-21 protects mice from the lethal effects of sepsis, a disease the lethal effects of which are partially mediated by over-production of inflammatory cytokines and systemic inflammation. The authors proposed that, as would be predicted by the CAP model illustrated in Figure 2, GTS-21 acts by reducing inflammatory cytokine production much like ACh acts during CAP activation.[52] These experiments, and many others, illustrate how CAP function might be modulated to reduce the severity of inflammatory diseases in humans. A 2017 review by Hoover summarizes recent research developments about the CAP. [50] Recent data suggests that vagal nerve stimulation can result in anti-inflammatory effects. Hoover also discussed the possibility that α7nAChRs may provide a new mechanism that may be employed to approach treatment of conditions that can cause damage as a result of excess inflammation.[53] Specifically, it may be possible to repurpose current therapies, such as Memantine, which will be further addressed in a subsequent section. The idea behind this is that the manipulation of the innate α7nAChRs may aid in controlling inflammation and, thus, it may prevent some of the down-stream effects of chlamydial infection – many of which are elicited by chronic inflammation.[50]

Acetylcholine and its Function

Acetylcholine (ACh) is a neurotransmitter of high importance in the body. It is found in numerous places throughout the CNS, is involved in ganglion synaptic transmission in the sympathetic and parasympathetic neurons, and is also a major component of postganglionic efferent neurons. Acetylcholine is not limited to the nervous system, however. For example, research published in 1982 indicates the acetylcholine is present in denervated skeletal muscles, indicating the presence of ACh is peripheral tissues.[54] Research has also shown that ACh is present in the circulating blood of mammals, as well as the spleen.[55] This information confirms the possibility that ACh may have effects that have remained undiscovered, such as involvement in immune function.

Synthesis. ACh is formed from acetyl coenzyme A (acetyl CoA) and choline. This reaction is made possible by catalysis from choline acetyltransferase (ChAT) as well as carnitine acetyltransferase (CarAT). A review analysis by Kawashima et al. in 2015 found that, in different cell lines, ChAT but not CarAT activity corresponded with the amount of ACh found in the cells. From this, they concluded that ChAT is responsible for the synthesis of ACh in lymphocytes. These observations also indicate that ChAT levels can be used as an indirect indicator of ACh production in various cell types. In fact, ChAT expression is a commonly used experimental marker for ACh production.[56, 57] Both non-neuronal cells, such as T cells, and neurons produce ACh. This ACh then binds to receptors on non-neuronal cells, such as endothelial cells, and immune cells such as macrophages.

Receptors. ACh exerts its biologic effects by binding to two main types of receptors—muscarinic, G-protein signal transduction receptors, and nicotinic, ion channel pore receptors. Muscarinic receptors are associated with biological processes such as heart rate, smooth muscle contraction, and neurotransmitter released, while nicotinic receptors are more closely related to signaling and transmission. [43, 58] Non-neuronal ACh and the AChR (acetylcholine receptors) have a number of functions in immune regulation. α7nAChRs have been of special interest in the investigation into cholinergic regulation of immune processes.[58, 59] Once activated, α7nAChRs results in depolarization of the membrane of the immune cells and excitation due to a change in ion permeability. Immune cells such as macrophages express the α7 subunit of the nicotinic acetylcholine receptor. When ACh binds to an α7nAChR, it prevents the release of TNF, which is secreted by macrophages in response to an immune threat.[42] Thus, the inflammatory process is attenuated. These findings indicate that acetylcholine is part of a specialized circuit involving the efferent vagus nerve, because it is capable of taking advantage of α7 receptors for signaling and decreasing pro-inflammatory cytokines.[60] Importantly, Yamaguchi et al. showed that acetylcholine, as well as other nicotinic acetylcholine receptor (nAChR) agonists, enhanced the growth of *Chlamydia pneumoniae* infection *in vitro*; these findings indicate that nAChRs may also act directly on developing Chlamydiae, in addition to their role in neurotransmission and immunologic regulation.[61]

Regulation of Mucosal Immunity by the CAP

Mucosal Immunity

Once an injury or infectious agent has surmounted the mechanical and chemical barriers that are part of the natural defense system, another line of defense is employed to protect the body from these insults. This line of defense is the innate immune system.[25] Phagocytes are a significant part of the innate immune response, and they function at the site of the invader or insult. The word phagocyte translates as "a cell that eats," which gives insight into its function. Phagocytes engulf particles that are detected as foreign, such as pathogens or other types of small cells and then travel to the lymph nodes.[62] Innate immune cells, including macrophages, neutrophils, and dendritic cells, demonstrate phagocytic activity. These cells also have Toll-like receptors (TLRs). These TLRs can detect characteristics that are often associated with pathogens and invaders, sometimes termed PAMPs. Following detection of a pathogen-associated characteristic by their TLRs, these dendritic cells and macrophages can secrete cytokines and chemokines, which allow for the recruitment of other immune cells. Activated dendritic cells can then enter lymphoid tissues and activate pathogen-specific T-cells within the lymphoid system. Following activation of the T-cells, they can either migrate to the site of the infection or stay in the lymphoid system, where they can help activate other T-cells, as well as B-cells.[25]

The CAP is a significant part of the regulation of mucosal immunity. This regulation is achieved by affecting the amount of inflammation, part of the innate immune response, at the site of an invader or injury. The CAP begins with detection of an insult to the cell through recognition of LPS or other pro-inflammatory cytokines with the help of PAMPs or DAMPs. Ultimately, this process results in reduced inflammation overall. Since the genital tract is innervated by the vagus nerve, albeit indirectly, it is possible that an infection in the genital tract

could activate the CAP. Similar phenomena have been observed in the lung and digestive mucosa.[51]

Lymphocytes

T cells are thought to have a significant regulatory effect on the CAP. One of the first suggestions of T cell involvement occurred when Fuji et al.[60] observed that a T cell activator, phytohemagglutinin (PHA), corresponds to an increase in ACh. Specifically, they were able to determine that PHA affects ChAT, but not CarAT, activity, suggestive that only the ChAT enzyme corresponds with T cell involvement.[61] The finding that T-cell involvement is correlated with the presence of the enzyme ChAT is of importance, because it supports the use of genetically engineered $ChAT^{gfp}$ mice to investigate possible effects of chlamydial function on immune response. Since infections result in activation of T-cells and T-cells correspond with the production of acetylcholine, it may be possible to achieve better understanding of the immune response as a result of chlamydial infection by further investigating how lymphocyte production changes.

Activation

Once the dendritic cells and macrophages are activated following the detection of a PAMP, such as LPS, T-cells are activated, which can result in ACh synthesis. This synthesized ACh then acts to regulate immune processes. It does so by reducing the production of pro-inflammatory cytokines, such as TNF and interleukins. With the knowledge that ACh synthesis is dependent on T-cells, there could be therapeutic potential to regulate the expression of ChAT by blocking antigen presentation between CD4+ T cells and APCs. [57, 63] If the APCs are no

longer able to present to the the T-cells, the inflammation would be reduced and initiation of adaptive immune responses halted.

Strategies of Infection

One of the treatment challenges associated with urogenital chlamydial infection is that it has a tendency towards recurrence.[64] Persistence is characterized by the ability for the bacteria to exist in a non-culturable state. During the persistent state, the hosts do not show signs of infection, and it is often undetectable. It is also worth considering that many of the individuals that do have a detectable *C. trachomatis* infection receive treatment but do not make lifestyle changes, leading to a greater likelihood of reinfection. More recent studies have suggested that IFN-ϒ treatment, as well as antibiotic treatment can lead to the aberrant state. [7, 65, 66] In addition, iron deficiencies and other nutrient limitations may also lead to formation of AB.[67, 68] These AB may be allowing the chlamydial inclusions to remain in the human body, while avoiding degradation. As discussed previously, this is thought to be a method of establishing long-term infection in the host, because it allows the chlamydial cells to remain alive despite stressful conditions. After the removal of these stressors, the AB can convert back to RB so that they can then multiply or convert to the EB form and go on to infect additional cells.[69]

Current Treatment with Antibiotics

The Center for Disease Control (CDC)-recommended treatment guidelines suggest Azithromycin or Doxycycline to treat chlamydial infection. However, alternative treatments include Erythromycin, Levofloxacin, and Ofloxacin.[70] Treatment challenges arise when an individual has asymptomatic infection but is treated with a beta-lactam antibiotic for another

reason, as this may help induce a persistent state of the bacteria.[23] There are also numerous studies focusing on the development of a vaccine to inoculate against *C. trachomatis*. Some scientists believe that a vaccine may be an optimal management strategy due to the nature of urogenital chlamydial infections, because *C. trachomatis* can employ unique strategies, such as its genetic change capabilities, to evade the innate infection-fighting immune response and treatment by antibiotics. [71, 72] Nonetheless, unless the disease is eradicated, there will still be inflammation related sequelae if the inflammation deregulation process is not remedied. Current studies, such as the one we are conducting, are looking more specifically at ways to manipulate what we know about current immune response and chlamydial pathogenesis. A 2015 research article by Yu et al. focuses on repurposing a current Alzheimer's Disease treatment, Memantine, to treat bacterial infections through manipulating the cholinergic anti-inflammatory pathway using nAChR. They found that Memantine is capable of modulating both inflammatory and anti-inflammatory pathways within the host. Combined with the use of appropriate antibiotics, it may be an effective therapy to treat sexually transmitted diseases or other bacterial infections. However, it is important to note that Memantine alone would not be a sufficient treatment for chlamydial infection, as the potential utility of Memantine is focused on reducing inflammation and disrupting the inflammatory response. [72, 73] Rather, it offers a possible mechanism of treatment for the potential side effects of chlamydial infection, such as inflammation-related injuries.[73]

Challenges

Despite these future possibilities and advancements, we continue to strive to develop alternative infection treatment and management strategies. One commonly faced issue in

healthcare is the increased risk of antibiotic-resistant bacteria due to overprescribing of antibiotics. Even with multiple effective pharmaceutical treatments for chlamydial infections, there can still be significant negative effects, both medically and economically. It is in our best interest to continue seeking treatment options that are more effective and to continue learning about host-bacteria interaction. Thus, we hypothesized that urogenital *C. trachomatis* infection results in increased levels of ACh within the host cells and that the ACh enhances urogenital chlamydial infection.

CHAPTER 2

MATERIALS AND METHODS

Overview

To determine whether chlamydia infection regulates the CAP, we examined production of ChAT in vaginally infected ChAT^{gfp} transgenic mice. These mice contain a transgene composed of the ChAT-promoter driven expression of a green fluorescent protein (GFP) gene. Since the ChAT enzyme is required for ACh production, ChAT promoter activity is a commonly used experimental marker to determine if cells are producing ACh.[56, 63] Thus, we determined how many host genital tract cells in this ChAT^{gfp} mouse line were producing ACh by staining tissue sections with an anti-GFP antibody.[57] In addition, we determined if the ChAT-expressing cells are at the same tissue location as the chlamydia inclusion-containing cells using anti-chlamydia antibody staining. The comparison between the number of ChAT-expressing cells and cells containing chlamydial inclusions was determined using immunohistochemistry (IHC) of mouse tissues. The mice were infected with *C. muridarum* Weiss strain (a Chlamydia species that infects mice) and sacrificed at days 3, 9, 15, and 21 post-infection.[75, 76] Tissues from the cervix, uterine horn, and ovaries were harvested for the immunohistochemistry assays. Chlamydial titer assays were also performed as a positive control to confirm that chlamydia-infected mice were shedding chlamydiae. The numbers of ChAT-GFP producing cells were counted via a microscope with a 63x oil-immersion objective lens, equating to 630x magnification. For each slide, an average of twelve counts from three tissue sections was determined, using two non-consecutive sections. Data were averaged and the standard error of the mean values calculated and compared using an independent T test.

Murine Modeling

A review by De Clerq et al.[77] explores animal modeling in chlamydial research. The similarity between human urogenital chlamydial infections and murine modeling systems is dependent on the strain of mouse and the chlamydial species used. For example, *C. trachomatis* infection is typically less severe in most non-human animal models than in humans. It does not typically ascend up the genital tract, and it often resolves on its own.[74, 77] However, *C. muridarum* cervicovaginal infection in mice ascends to the upper genital tract from the cervical tissues, as observed with *C. trachomatis* in humans. *C. muridarum* genital tract infection also causes hydro- and pyo-salpinyx and reproductive complications similar to those observed in *C. trachomatis* infected humans.[17] For these reasons, *C. muridarum* is the most widely used animal model for chlamydial genital tract infection. Therefore, we chose the *C. muridarum* cervicovaginal infection model to use in our studies. Notably, murine genital infection is also affected by the stage of the estrous cycle at the time of infection. Likewise, the estrogen and progesterone levels within the organism being studied affect *C. trachomatis* infection significantly.[75] Thus, the estrous cycles of all mice used in these studies were synchronized by hormone treatment prior to infection, as described below.

Animal Handling and Infections

ChAT^{BAC}-eGFP mice (MGI ref ID J:114554; PubMed ID:1694043) from Jackson Laboratories were used for all experiments.[76] Male and female homozygous founder mice were obtained from Jackson Laboratories and used to establish a breeding colony in the Quillen College of Medicine DLAR. All mice were provided food and water *ad libitum* and kept on a standard 12-hour light/dark cycle. After 1-week acclimation period, 9-week-old mice were

subcutaneously injected with 2.5 mg Depo-Provera (Greenstone LLC, Peapack, NJ). Mice were vaginally infected using a micropipette at 10 weeks of age with 10^6 inclusion-forming units (IFU) of *C. muridarum* Weiss strain in 2SPG on day 0, represented by the star in Figure 1. *C. muridarum* Weiss strain was obtained from Kyle Ramsey (Midwestern University). Mock-infected animals were included in each experiment and received 10µl of 2SPG alone on the infection day. A total of 6 mock and 5 infected animals from at least 2 separate infection experiments were analyzed at each time interval described in these studies. Mice were sacrificed at days 3, 9, 15, or 21 post-infection by cervical dislocation. To determine pathogen shedding, vaginal swabbing and titers were performed every 3 days, as described by Phillips-Campbell et al.[77] (Figure 3) All animal experiments in this study were conducted in strict accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals." The University Committee on Animal Care at East Tennessee State University approved the animal protocol under the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, US Department of Agriculture, and in compliance with the Public Health Service Policy on Human Care and Use of Laboratory Animals.

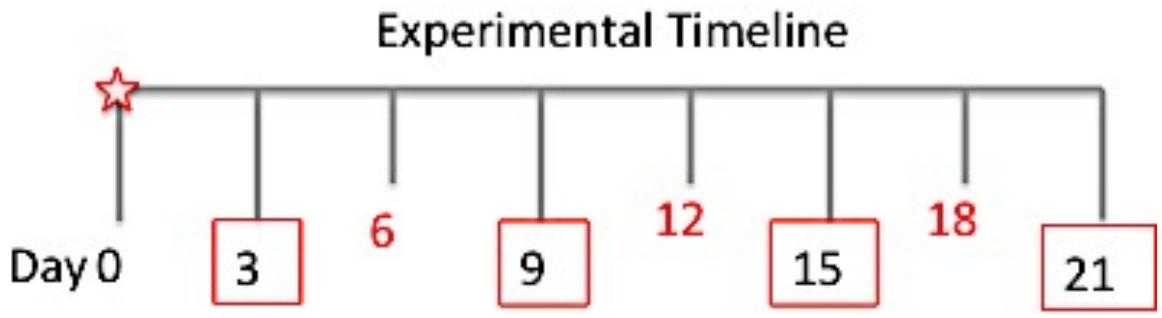

Figure 3. Experimental timeline. Star at Day 0 indicates day of infection. Titers were taken every three days. Mice were sacrificed on days 3, 9, 15, and 21 post-infection.

Tissue Collection, Fixation, and Embedding

Genital tract tissues were removed and immersed in 10% unbuffered formalin made from 10ml formaldehyde 37-40% (Fisher Scientific, Waltham, MA) and 90ml distilled H_2O for 48-72 hours. Tissues were then transferred to 70% EtOH and stored at 4°C for 24-72 hours until they were embedded in paraffin. Paraffin blocks were stored at ~20-25°C with a desiccant for a period of up to two years until being cut. They were cut into 5μm slices using a microtome and placed on positively charged microscope slides (Fisherbrand Superfrost Plus, catalog #12-550-15, lot #010515-9). The slides were allowed to dry overnight. Then, IHC was started the next day or they were stored for future use (at room temperature). ABC immunohistochemistry was used to stain for ChAT-producing cells within the tissue using anti-GFP antibody-ChIP Grade (ab290, lots GR278073-1 and GR196874-1, rabbit polyclonal, Abcam, Cambridge, UK).

In addition to anti-GFP staining to look at the presence of ChAT within the tissues, staining using an antibody to *C. trachomatis* was performed using the same method as the anti-GFP staining. The only differences were the antibody used at the primary antibody stage of immunostaining (Rb pAB to *C. trachomatis*, catalog ab31131, lot GR235293-2) and the length of time that the VIP substrate was on the tissues (20 minutes). Slides were analyzed at 40x magnification for a total of 400x magnification. The number of inclusion-containing cells was not quantified. Instead, they were analyzed qualitatively to look for the presence or absence of chlamydial inclusions.

Immunohistochemistry

Rehydration

Microscope slides with tissues sections were placed into a glass slide rack and placed in 60°C oven for 30 minutes to melt paraffin and desiccate the samples. After 30 minutes, the slides were removed in the slide rack, and the slide rack was placed on the bench surface for approximately 30 minutes to allow the slides to cool to room temperature. After the slides were brought to room temperature, they were rehydrated. The slides were rehydrated with xylene for two 5-minute increments, 100% EtOH for two 2-minute increments, 95% EtOH for two 2-minute increments, 75% EtOH for one 2-minute increment, 50% EtOH for one 2-minute increment, distilled H_2O for two 5-minute increments, and 0.1M OBS for one 5-minute increment.

Antigen Retrieval

Slides were then placed in Tissue-Tek slide holder. The container was then filled with Antigen Retrieval Solution (Appendix A) and microwaved at full power for 3.5 minutes and then using the defrost setting for 6 minutes. The slides in the Tissue-Tek slide holder were removed and placed on bench to allow them to cool to room temperature (45 minutes). After cooling to room temperature, the slides were washed in distilled H_2O three times for 5 minutes per wash. Following the dH_2O wash, the slides either started in the immunostaining protocol or were stored in IHC PBS for 24-72 hours at room temperature until immunostaining.

Immunostaining

Slides were washed in IHC PBS for four washes, each lasting 5 minutes. Then they were washed once in 0.5% BSA (Appendix A) for 10 minutes, once in 1% H_2O_2 for 15 minutes, four times in IHC PBS for 5 minutes each, and finally, once in 0.5% BSA again for 10 minutes. Slides were removed one at a time, and the area surrounding the tissue was dried using a Kimwipe. ImmEdge hydrophobic barrier pens (Vector Labs, Burlingame, CA, catalog # H4000) were used to draw water barrier circle around each section of tissue on the slide. The barriers were given one minute to dry before slides were placed into a humidity box and covered with blocking buffer (Appendix A). After 2 hours of incubation at room temperature, the blocking buffer was poured off of each slide and replaced with primary antibody solution (Appendix A). The slides were then allowed to incubate overnight (at least 2.5 hours but not more than 36 hours) at room temperature.

After primary antibody staining, slides were washed four times in IHC PBS at 5 minutes/wash, and then, washed with 0.5% BSA once time for ten minutes. Slides were removed from BSA and gently dried using Kimwipes, carefully avoiding the tissue. Biotinylated secondary antibody solution (Appendix A) was placed on the tissue sections and allowed to incubate at room temperature for two hours. Slides were rinsed in IHC PBS, washed four times in IHC PBS for 5-minutes/wash and, finally, subjected to one 10-minute wash in 0.5% BSA. The tissues were again removed from the slide holder and gently dried, before being covered with ABC reagent and incubated for 1.5 hours. The ABC reagent was poured off of each slide before the slides were washed in TBS four times at 5-minutes/wash. Slides were removed from slide holder, gently dried, and then placed on a light-colored background. The VIP Substrate (Vector Laboratories, Burlingame, CA, catalog SK-4600) solution was applied to each tissue section and

allowed to develop for 2 minutes for ChAT^{gfp} staining or 20 minutes for chlamydial staining. Slides were rinsed in dH_2O and then washed for two 2-minute periods. Then, slides were counterstained using Methyl Green (Vector Laboratories, Burlingame, CA, catalog # H-3402) for 2 minutes at room temperature (~23°C). Slides were rinsed in ddH_2O and then washed twice for 2-minutes/wash in ddH_2O Slides were dehydrated using the opposite order of the rehydration protocol, starting at 50% EtOH, up to xylene. Following the last soak in xylene, the tissues remained in the last xylene-staining dish until being removed one by one for coverslip placement. Cytoseal 60 (catalog # 327734, lot #8310-4) was used to mount coverslips over the tissue sections. After applying coverslips, the slides were allowed to lie flat at room temperature until they were viewed under the microscope. In the event of water under the coverslip, the slides were soaked in xylene for no more than 30 minutes so that the coverslip came off without disturbing the tissue, and a new coverslip was mounted on the slide.[79, 80, 81]

Data Collection and Analysis

Data was collected from IHC tissues using 630x magnification oil immersion microscopy to count the number of ChAT-producing cells as described previously.[74, 75] Counts were taken from at least 2 non-consecutive tissue slices per slide for each tissue. Slides contained approximately 4 tissue sections each. Two slices or sections of tissue were counted per slide, and there were twelve fields within each slice/section. Microscopic fields were non-consecutive. Tissue counting started at the top left of the tissue and moved down and to the right in the progressive fields. Every effort was made to ensure that fields did not overlap and that fields were not all directly beside each other. Averages were determined for each tissue section; the

averages were combined for each tissue type at each time point.[82, 83] Independent T-test analysis was used to determine significance ($p<0.05$), and standard error was calculated.

CHAPTER 3

RESULTS

As the most common bacterial sexually transmitted infection in the world, *Chlamydia* imposes significant financial burdens and can impose long-term consequences on infected individuals that are not treated in a timely manner. Chlamydial infection presents unique treatment and management challenges due to its ability to manipulate host cells to make a more favorable intracecllular environment for survival and proliferation. Thus, it often goes undetected. At this point, current treatment methodologies can be effective, but are not optimal due to the ability of the organism to persist despite stressors. Additionally, antibiotic treatment is associated with financial burden and possible contraindiations.

To better understand the pathogenicity mechansims behind infection with *C. trachomatis*, we would ultimately like to understand how the organism interacts with the host's immune response. So far, a majority of the work investigating CAP activity and its effects on disease have been limited to study of non-infectious disease processes, such as cancer[87] and autoimmune conditions.[89, 90] In these studies, the non-infectious disease often benefits from nAChR stimulation, which results in reduced inflammation. However, in infectious disease, the significance of pro-inflammatory cholinergic responses is not as clear. Previous studies show that it is possible for CAP activation to protect the infected host, as evidenced by reduced mortality in sepsis and viral myocarditis with CAP stimulation.[85, 91] Nonetheless, it is also possible for CAP activation to be detrimental to the host, as demonstrated by the increased mortality observed in *Escherichia coli* infected nAChR knockout mice that cannot activate the CAP due to the absence of α7nAChR.[87] In addition, the use of an α7nAChR agonist, such as nicotine, to prevent initiation of the CAP has been found to increase the amount of inflammation

in *Staphylococcus pneumoniae* infected mice, and increased bacterial load was observed.[92] These findings suggest that, in some ways, the CAP may aid pathogenicity, because it can reduce the body's innate immune response to infection. This raises the possibility that some organisms may specifically activate the CAP by inducing host cellular ACh synthesis in order to evade host responses and help establish infection. Based on this knowledge of CAP activity, we hypothesized that *C. muridarum*-infection causes an increase in ACh production within genital tract tissues. To begin to test this hypothesis, IHC using anti-GFP antibody was performed on tissues extracted from ChAT-eGFP mice that were sacrificed on days 3, 9, 15, or 21 from the time of inoculation with *C. muridarum,* allowing for the identification and enumeration of the number of cells producing ChAT. An increase in the number of ChAT-expressing cells in *C. muridarum*-infected genital tissue compared to that in mock-infected mice would suggest that chlamydial infection does result in an increase of ACh, which would be consistent with our hypothesis.

To start, we *C. muridarum* infected or mock-infected female ChATBAC-eGFP mice as described in the methods. Neither *C. muridarum* nor mock-infected showed any sign of distress and all mice survived for the duration experiment until the sacrifice date (Figure 3). There was no detectable chlamydial shedding in mock-infected mice. All chlamydial-infected mice shed infectious *C. muridarum* EB, with maximum shedding observed at day 3 post-infection (data not shown).

Effect of Chlamydial Infection on ChAT Production within Cervical Tissue

Table 1 shows the average number of ChAT positive cells/microscopic field obtained from cervical tissue isolated from *C. muridarum*- and mock-infected mice. At all three time points, we observed an increase in the average number of ChAT-producing cells in infected tissues as compared to genital tissues from mock-infected mice. Statistically, the number of ChAT-producing cells was significantly increased on days 3 and 15, and 21post-infection (pi). On day 21pi, there was a large difference in the averages of the mock and infected tissues (Table 1), but there was also a large degree of variability between different samples. (Figure 4). These findings suggest that *C. muridarum* increases ACh production in the cervix within infected mice. This is inferred from the increased number of cells that produce ChAT, which, as stated previously, is necessary for ACh production and is a commonly used experimental marker for ACh synthesis.

Table 1. Summary of counts of ChAT-gfp producing cells within cervical tissue samples harvested at time points 3, 15, and 21 days post-infection. $p<0.05$

Cervical Tissue				
Day	Mock	Infected	Standard Error	Independent T-Test
3	0.0656746	2.093402778	1.013864087	0.007636731**
15	0.353703704	5.84380787	2.745052083	0.011713378*
21	0.581018519	4.831597222	2.125289352	0.047643868*

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.

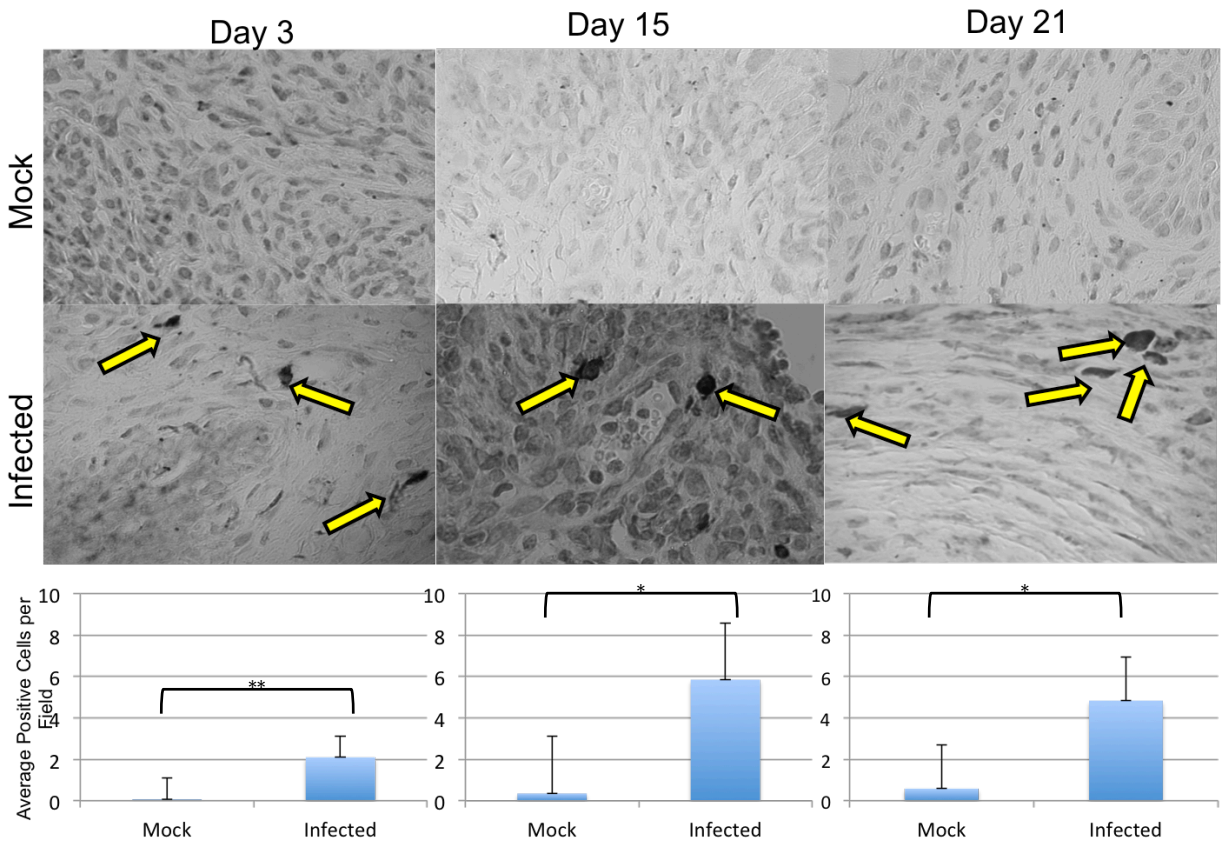

Figure 4. ChAT^{gfp} Cervical Tissue Data. A. Representative images from each time point- days 3, 15, and 21 in both mock- and Cm-infected groups. Yellow arrows indicate ChAT positive cells. B. Comparison of average counts for mock vs. infected tissues. * indicate those values that are significantly different at P=0.05 or P=0.01. Images taken with 630x maginification.

Effect of Chlamydial Infection on ChAT Production within Uterine Horn Tissue

Table 2 shows the average number of ChAT positive cells/microscopic field obtained from uterine horn tissue isolated from *C. muridarum*- and mock-infected mice. In the uterine horn, the number of ChAT cells in infected tissues showed a statistically significant increase from the mock tissues on days 3 and 9 (Table 2). Again, infection appeared to increase the number of ChAT positive cells at all time points (Figure 5). Since chlamydial infection ascends up the genital tract, it is reasonable to expect that the amount of change between mock and infected averages may increase as the uterine horn tissues become more heavily infected at later

times post-infection. However, this is not reflected in the collected data. Further testing with more samples would help support or refute this possibility.

Table 2. Summary of counts of ChAT-gfp producing cells within uterine horn tissue samples harvested at time points 3, 9, 15, and 21 days post-infection. $p<0.05$

Uterine Horn Tissue				
Day	Mock	Infected	Standard Error	Paired Test
3	0.128472222	0.639583333	0.255555556	0.004934258**
9	0.261284722	0.859375	0.299045139	0.012250255*
15	0.258333334	1.354166667	0.547916667	0.144864044
21	0.131944444	2.615277778	1.241666667	0.104392292

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.

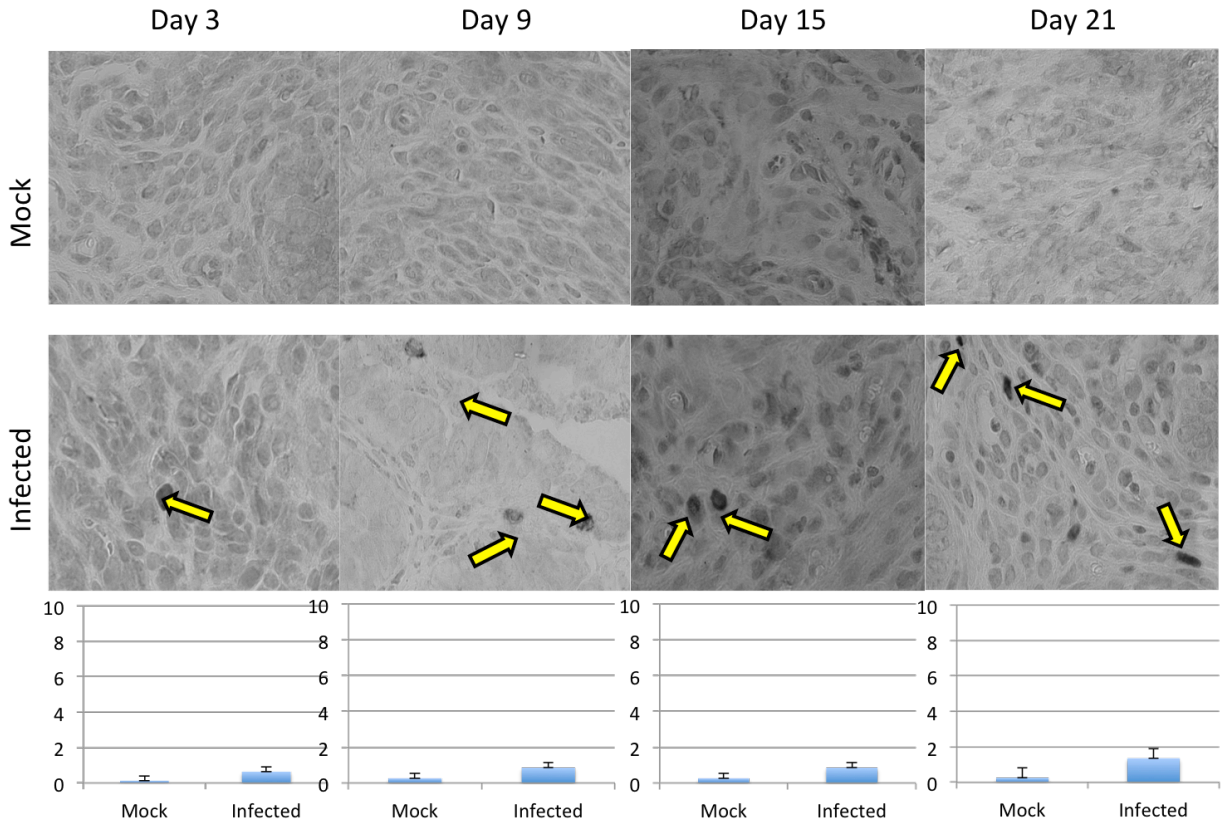

Figure 5. ChAT^{gfp} Uterine Horn Tissue Data. A. Representative images from each time point-days 3, 9, 15, and 21 in both mock- and Cm-infected groups. Yellow arrows indicate ChAT

positive cells. B. Comparison of average counts for mock vs. infected tissues. * indicate those values that are significantly different at P=0.05. Images taken with 630x magnification.

Effect of Chlamydial Infection on ChAT Production within Ovarian Tissue

Finally, Table 3 shows the average number of ChAT positive cells/microscopic field obtained from ovarian tissue isolated from *C. muridarum*- and mock-infected mice. Ovaries had significantly more ChAT cells in the infected tissue samples as compared to the mock tissue samples on days 3, 15, and 21 pi (Table 2). The largest amount of variation between mock and infected tissues samples at each time point was observed in ovarian tissues. As mentioned previously, if considered in conjunction with the ascending nature of urogenital chlamydial infection, there is the possibility of a correlation between later time points post-infection, ascension of infection, and the effect on ChAT production.

Table 3. Summary of counts of ChAT-gfp producing cells within ovarian tissue samples harvested at time points 3, 9, 15, and 21 days post-infection. $p<0.05$

Ovarian Tissue				
Day	Mock	Infected	Standard Error	Paired- T-Test
3	0	0.347177128	0.173588564	0.023981837*
9	0.117424242	2.700025252	1.291300505	0.191469334
15	0.15625	2.052083333	0.947916667	0.030374735*
21	0.013888889	3.550347222	1.768229167	0.02682588*

* Significant at the 0.05 probability level.

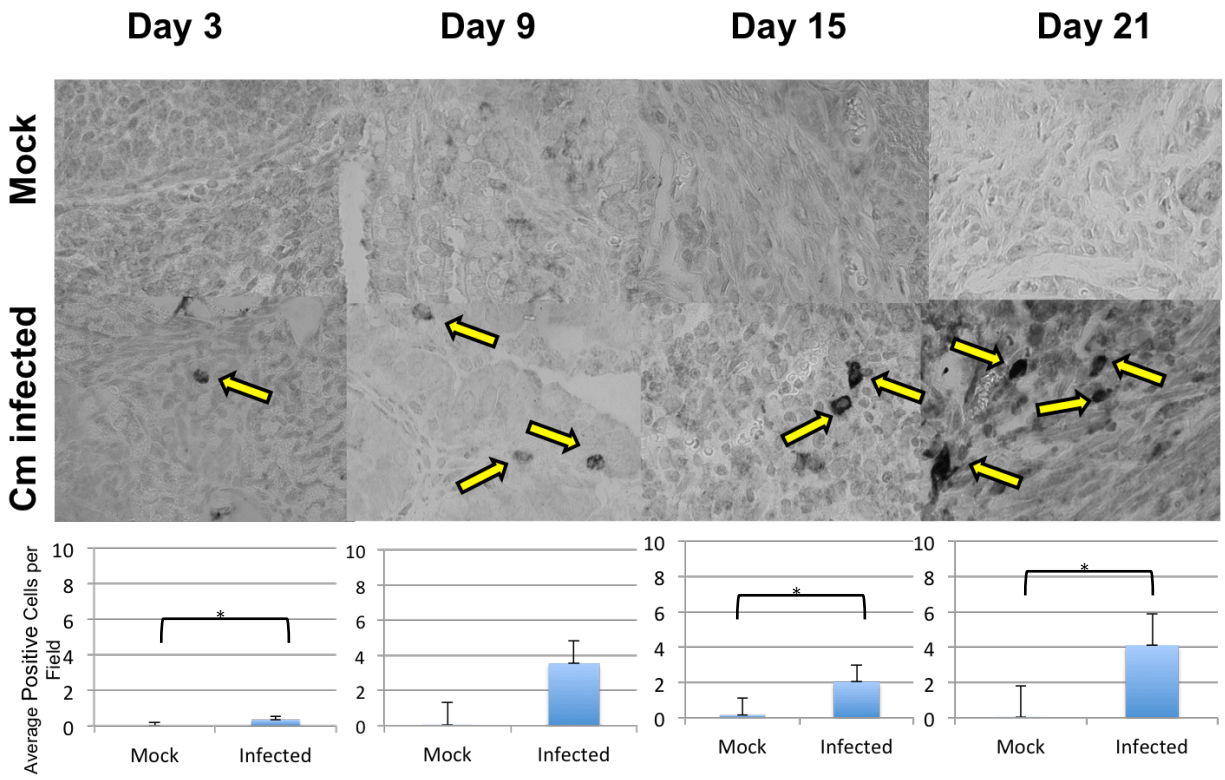

Figure 6. ChAT^{gfp} Ovarian Tissue Data. A. Representative images from each time point- days 3, 9, 15, and 21 in both mock- and Cm-infected groups. Yellow arrows indicate ChAT positive cells. B. Comparison of average counts for mock vs. infected tissues. * indicate those values that are significantly different at P=0.05. Images taken with 630x magnification.

Anti-Chlamydia Staining

As an additional control that genital tissues were infected, IHC staining for *C. muridarum* inclusions was also performed (Figure 7). There were no quantitative methods applied to these tissues following immunohistochemistry. Instead, the tissues were reviewed qualitatively to look for the presence of chlamydial inclusions. In all tissue types and time-points, there were *C. muridarum*-containing cells in the Cm-infected tissues, and there were no *C.muridarum*-containing cells detected within the mock-infected group, which would have suggested a false positive result.

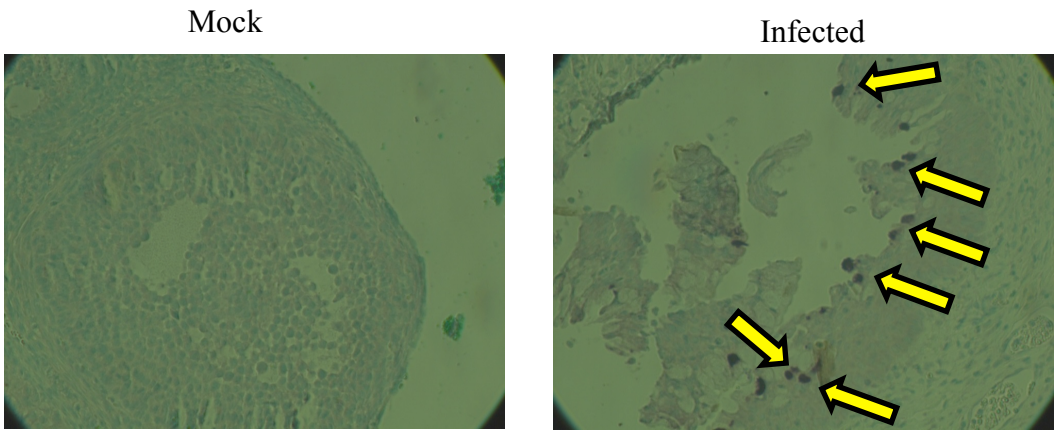

Figure 7. Representative anti-Cm staining. Cervical tissue samples taken from a mock-infected and Cm-infected mouse sacrificed at day 3pi. Yellow arrows indicate *C. muridarum* positive cells.

CHAPTER 4

DISCUSSION

Implications for Chlamydial Pathogenesis

The deactivation of an inflammation management mechanism, such as the CAP, could result in deleterious effects and increase the likelihood of the host suffering inflammation-related injury. Our findings suggest that this could be a potential method of pathogenesis for *C. trachomatis*. When chlamydial infection causes the production of acetylcholine to increase, the CAP will be activated. This would cause an increase in anti-inflammatory cytokines, as well as a decrease in pro-inflammatory cytokines. The decreased in pro-inflammatory cytokines would then diminish leukocyte attraction to the infection site and subsequent activation. When the the recruitment of immune cells is reduced, the likelihood that the bacterium is phagocytized or killed by the host's immune response is decreased. Furthermore, if the T-cells and B-cells are not recruited or recruited only minimally, this would prevent the host from developing or cause underdevelopment of adaptive immunity to *C. trachomatis*. Since *C. trachomatis* evades much of the innate immune response, it would be expected to reproduce rapidly. If this happens, there is such a great amount of bacteria present that, if the CAP is turned off, the body cannot fight off the infection alone. This may result in more severe disease, more rapid genital tract ascension, or increased transmission to new hosts.

Interestingly, *C. trachomatis* and other members of the Chlamydiae family have a unique relationship with neutrophils. As mentioned previously, neutrophils are some of the first responders from the immune system in response to an infection. They are able to phagocytize the infectious agents, and this is done by ingesting the organism and then initiating apoptosis shortly thereafter. However, *C. trachomatis* is actually able to delay the apoptosis by exposing the

neutrophils to pro-inflammatory cytokines.[93, 94] Since neutrophils express pro-inflammatory activity and *C. trachomatis* can postpone apoptosis, the result is increased inflammation at the site of the infection due the the combined activity of the neutrophils. [93] The effect of *C. trachomatis* infection on neutrophils is one of the mechanisms through which chlamydial infection can cause lasting damage to the tissues due to excessive inflammatory response.

For an individual that is infected with *C. trachomatis*, there is an increased likelihood of developing other STIs.[2, 9] The pathogenic mechanism of evading the immune response may be one of the reasons behind this. For example, studies have shown that individuals with Chlamydia are at increased risk of contracting HIV if exposed.[10] Though the mechanisms by which chlamydial infection promotes other STI is unknown, it is possible that chlamydial infection-induced down-regulation of the immune response by activation of the CAP could increase host susceptibility to other genital tract pathogens.

Conversely, if the CAP was downregulated or prevented from initiating, there would be an increased amount of pro-inflammatory cytokines present and a decrease in the release of anti-inflammatory cytokines. With the increase of the pro-inflammatory cytokines comes the risk of an inflammatory response that is so strong that it results in inflammation-related injuries, such as pelvic inflammatory disease and urethritis. However, if we understand that the CAP can reduce inflammation and that the CAP can be initiated through vagal stimulation, there is potential application for treatment of inflammation-related conditions by vagal stimulation. So, in the case of a hyper-response of the immune system, it may be possible to reduce inflammation and tissue damage after an inflammatory stimulus. In chlamydial infection, increased activation of the CAP may have utility in treating young females that still wish to reproduce, especially if they are already at higher risk of inflammatory conditions, such as PID.

Implications for Treating the Inflammatory Damage Caused by Chlamydial Infection

With increasing concern about antibiotic-resistant bacteria, there is more and more pressure to develop alternative treatment methods for bacterial infections. Fortunately, *C. trachomatis* poses less risk of developing antibiotic resistance.[81] However, there is an increased risk of individuals infected with Chlamydia contracting other STIs, so chlamydia-infected individuals may actually have concurrent infections with other bacterial species that cause STIs. When antibiotics are administered to treat the chlamydial infection, the other organism responsible for a STI, such as *Neisseria gonorrhoeae*, may develop this antibiotic resistance. Specifically, there has been a decline in susceptibility of *N. gonorrhoeae* to Azithromycin, which the CDC recommends as part of a dual therapy.[86] However, *N. gonorrhoeae* has been proven to rapidly evolve, especially in terms of antibiotic resistance. Therefore, reducing antibiotic usage by combining antibiotics with immunomodulators to treat STIs could be beneficial.

Understanding the effects of chlamydial infection on the inflammatory response can contribute to potential therapeutic strategy to prevent and treat inflammation-related injury. In the case of chlamydial infection, this may allow for significant decrease in the number of lasting complications from urogenital chlamydial infection, such as chronic pelvic pain, PID, and infertility. Understanding the infection's potential effects on the host's immune defenses, such as down regulation of pro-inflammatory processes and cytokine release, may also help develop another approach to treat other infectious diseases that similarly manipulate host physiological function to proliferate. If the inflammatory process can be manipulated to allow the body to fight off infection as needed but to not have so much inflammation that there are lasting consequences, it may allow for a revolutionary new method of treatment. This would require a delicate balance between activation and down regulation of CAP activity. For example, there is potential for

supplementation to antiviral or antibiotic therapies, such as in the case of infection. It will be particularly advantageous if these concepts can be applied using previously approved therapeutic agents so that new treatments can be developed more effectively, such as Memantine, as previously discussed.

Chlamydial infection poses a unique challenge since it is capable of affecting immune response in two extreme manners-- either by attenuating the inflammatory response or over-activating it. There are already a number of studies and investigators that are researching potential treatments for infections that affect the host's immune response and can harm the host through dysregulation of the inflammatory process. Our findings suggest that urogenital chlamydial infection does increase the production of acetylcholine, part of the cholinergic anti-inflammatory pathway, so it is possible that managing the inflammatory response could be beneficial in the treatment of chlamydial infections. Treatment by immunomodulation through administration of pro-inflammatory or anti-inflammatory cytokines, stimulation of the CAP, altering chemokine levels, and affecting the activation of T-cells, are all possible methods to control inflammation.[84] In the case of infected individuals with a weakened immune response, it may be helpful to down-regulate the CAP. There is also therapeutic potential in dampening the inflammatory response, which may be achieved through stimulation of the CAP, administration of the anti-inflammatory cytokines, or activating T-cells to increase ACh production. Therapeutically decreasing the inflammatory response could prove helpful in managing conditions such as sepsis. Nonetheless, immunomodulation does pose significant challenges, because global effects on inflammation may result in unintended consequences. Specifically, dampening the immune response could increase susceptibility to infection. In the case of preventing the activation of the CAP, there is risk that an inflammatory process may be robust

enough to cause inflammation-related injury. Thus, for many immunomodulation-based therapies to be effective and appropriately safe, further research is necessary to improve specificity. If immunomodulation proves to be an effective treatment strategy, it may be used independently or with the use of antibiotic and antiviral therapies to manage infection and reduce the likelihood of inflammation-related injuries.

Limitations of the Study

Sample Size

The primary limitation of the study pertains to sample size. The study will benefit from having more animals for each group—mock versus infected—at each time point. Increasing the number of animals at each time point will allow us to better account for variations in technique and to pinpoint outlying data. This is consistent with the data presented in Figures 4, 5, and 6. Though increased ChAT + cells are observed at days 3, 15 and 21 post-infection (pi) for cervical tissues and all four time intervals in uterine horn and ovarian tissues, the increase is only statistically significant in cervical tissue at days 3, 15, and 21pi, uterine horn tissue at days 3 and 9 pi, and ovarian tissue at days 3, 15, and 21pi . It seems likely, therefore, that analysis of samples from a greater number of mice would reduce the observed variability and demonstrate significant differences at a greater number of sampling times for each tissue. Furthermore, due to the nature of IHC experiments, especially the amount of labor required for each slide, analyzing more samples would be beneficial. Ideally, another technique can be adapted that will allow for larger sample sizes. There is increased likelihood in error due to the number of IHC experiments performed, as there may be unknown variations in technique and conditions despite the effort devoted to uniformity in each IHC experiment repeat. Currently, consideration is being given to

enumerating ChAT positive cells by intracellular anti-GFP staining followed by flow cytometry. Ultimately, though the data set needs to be expanded, the observation of a significant increase in Chat + cells in infected animals versus uninfected controls in the cervix on days 3, 15, and 21pi, the uterine horn on days 3 and 9pi, and ovary on days 3, 15 and 21pi provides compelling support for the hypothesis and for future studies. Though lower in number than in *C. muridarum* infected tissues, $ChAT^{gfp}$-producing cells were detected in mock tissues. Since acetylcholine is a part of the innate immune response and is expressed in non-neuronal cells, it is not wholly unexpected for some ChAT to be detected in the tissues in the absence of infection. However, as mentioned above, an increased number of repetitions, as well as confirmatory assays will be helpful in ruling out whether this may be due to experimental error.

Specificity

IHC was used to detect expression of ChAT, which is an enzyme involved in the production of ACh. Methods to test specifically for the presence of ACh, such as ELISA or Mass Spectrometry, will confirm that there is a positive correlation between the expression of ChAT and increased presence of ACh. To further investigate the application of current findings, it will also be beneficial to do α7nAChR inhibitor studies that investigate how a change in the availability and/or activity of ACh receptors affects the chlamydial infection. If α7nAChR inhibitors or agonists were administered to chlamydia-infected mice, the CAP would not be initiated by infection, because the ACh produced would not be able to bind to its receptors, which is the initiating step for decreased inflammatory cytokine production (Figure 2). In this case, the inflammatory response would lose a mechanism of modulation, so it would be expected that there might be an increased risk of inflammation-related injury during or after infection.

Despite years of investigation, there is still a great deal of information yet to be discovered about chlamydial infections and pathogenesis. Since *C. trachomatis* operates as an obligate intracellular parasite, it presents unique experimental challenges, but advancements in genetic analysis are providing novel insight about how the organism operates and potential methods for management and treatment.

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APPENDICES

APPENDIX A

IHC Reagent Recipes

IHC PBS (0.1 M PBS, pH 7.3)

Na2HPO4 (FW=141.96)	29.6 g
KH2PO4 (FW=136.09)	8.6 g
NaCl	14.4 g

- For 0.1 M: Adjust pH by adding drops of 1.0 or 5.0 N NaOH. Total volume should be 2L.
- For 1.0 M: Adjust pH by adding ~100mL of 5 NaOH. Autoclave 20 min, store at RT. Total volume should be 2L. Use heat if it isn't going into solution. Then leave to cool to RT before adjusting pH). Autoclave to sterilize. (Liquid 20-minute cycle)

0.5% BSA Solution

BSA	5.0 g
Triton-X100	4.0 mL

Add BSA to IHC PBS in beaker and stir until dissolved. Add Triton-X100 and stir until dissolved. Total volume should be 1L.

1.0% BSA Solution

BSA	1.0 g
Triton-X100	400 µL

Add BSA to IHC PBS in beaker and stir until dissolved. Add Triton-X100 and stir until dissolved. Total volume should be 100 mL.

Antigen Retrieval Solution

dH2O	300mL	900mL	1.5 L
Sodium Citrate	0.882 g	2.646g	4.41g

Adjust pH to 7 or slightly lower by adding drops of 1 N HCl. Filter Sterilize.

IHC TBS

Tris-HCl	6.35 g	12.7 g
Tris Base	1.18 g	2.36 g
NaCl	8.77 g	17.54 g
dH2O	1 L	2L

Adjust pH to 7.5 using NaOH or HCl. Autoclave or filter sterilize.

1.0% H_2O_2 Solution

2 mL of 30% H_2O_2+ 58 mL IHC PBS = 60 mL total volume. H_2O_2 is stored at 4°C.

For 3 times the amount: 6 mL 30% H_2O_2 + 174 mL IHC PBS = 180 mL total volume.

APPENDIX B

Data Tables

Cervical				
Tissue Sample	Mock or Infected	Time	Average	Mouse Study
15A	Mock	Day 3	0.059523809	Chat 3
16A	Mock	Day 3	0.0125	Chat 3
1A	Mock	Day 3	0.125	Chat 1
17A	Infected	Day 3	2.530208333	Chat 3
18 A	Infected	Day 3	2.46875	Chat 3
9A	Infected	Day 3	1.28125	Chat 1
19A	Mock	Day 15	0.125	Chat 3
20A	Mock	Day 15	0.186111111	Chat 3
5A	Mock	Day 15	0.75	Chat 1
21A	Infected	Day 15	2.898148148	Chat 3
22A	Infected	Day 15	4.927083333	Chat 3
6A	Infected	Day 15	7.8	Chat 1
11A	Infected	Day 15	7.75	Chat 1
23A	Mock	Day 21	0.083333334	Chat 3
24A	Mock	Day 21	1.597222222	Chat 3
7A	Mock	Day 21	0.0625	Chat 1
25A	Infected	Day 21	1.833333334	Chat 3
8A	Infected	Day 21	3.645833333	Chat 1
12A	Infected	Day 21	5.916666667	Chat 1
42A	Infected	Day 21	7.930555556	Chat 4

Table 4. Averages for Each Cervical Tissue Sample. Average number of ChAT-containing inclusions observed in cervical tissue at each time point, including mouse study and whether the animal was mock-infected or infected with *C. muridarum.*

Uterine Horn				
Tissue Sample	Mock or Infected	Time	Average	Mouse Study
15B	Mock	Day 3	0	Chat 3
16B	Mock	Day 3	0.013888889	Chat 3
1B	Mock	Day 3	0.375	Chat 1
2B	Mock	Day 3	0.125	Chat 1
17B	Infected	Day 3	0.833333333	Chat 3
18B	Infected	Day 3	0.516666667	Chat 3
9B	Infected	Day 3	0.708333333	Chat 1
1B	Mock	Day 9	0.294270833	Chat 2
2B	Mock	Day 9	0.1875	Chat 2
3B	Mock	Day 9	0.302083333	Chat 1 & 2
4B	Infected	Day 9	0.916666667	Chat 2
5B	Infected	Day 9	0.479166667	Chat 2
6B	Infected	Day 9	1	Chat 2
10B	Infected	Day 9	1.041666667	Chat 1
19B	Mock	Day 15	0.066666667	Chat 3
20B	Mock	Day 15	0.166666667	Chat 3
5B	Mock	Day 15	0.541666667	Chat 1
21B	Infected	Day 15	2.541666667	Chat 3
22B	Infected	Day 15	1.916666667	Chat 3
6B	Infected	Day 15	0.666666667	Chat 1
11B	Infected	Day 15	0.291666667	Chat 1
23B	Mock	Day 21	0	Chat 3
24B	Mock	Day 21	0.104166667	Chat 3
7B	Mock	Day 21	0.291666667	Chat 1
25B	Infected	Day 21	2.6	Chat 3
8B	Infected	Day 21	1.083333333	Chat 1
12B	Infected	Day 21	1.166666667	Chat 1
42B	Infected	Day 21	5.611111111	Chat 4

Table 5. Averages for Each Uterine Horn Tissue Sample. Average number of ChAT-containing inclusions observed in uterine horn tissue at each time point, including mouse study and whether the animal was mock-infected or infected with *C. muridarum*.

Ovary				
Tissue Sample	Mock or Infected	Time	Average	Mouse Study
15C	Mock	Day 3	0	Chat 3
16C	Mock	Day 3	0	Chat 3
1C	Mock	Day 3	0	Chat 1
17C	Infected	Day 3	0.531565657	Chat 3
18C	Infected	Day 3	0.520833333	Chat 3
2C	Infected	Day 3	0.0625	Chat 1
9C	Infected	Day 3	0.273809524	Chat 1
1C	Mock	Day 9	0	Chat 2
2C	Mock	Day 9	0.041666667	Chat 2
3C	Mock	Day 9	0.310606061	Chat 1& 2
4C	Infected	Day 9	5.095959596	Chat 2
5C	Infected	Day 9	0.604166667	Chat 2
6C	Infected	Day 9	0.666666667	Chat 2
10C	Infected	Day 9	6.633333333	Chat 1
19C	Mock	Day 15	0.052083333	Chat 3
20C	Mock	Day 15	0	Chat 3
5C	Mock	Day 15	0.416666667	Chat 1
21C	Infected	Day 15	2.354166667	Chat 3
22C	Infected	Day 15	3.395833333	Chat 3
6C	Infected	Day 15	1.4375	Chat 1
11C	Infected	Day 15	1.020833333	Chat 1
23C	Mock	Day 21	0	Chat 3
24C	Mock	Day 21	0.041666667	Chat 3
7C	Mock	Day 21	0	Chat 1
25C	Infected	Day 21	3.645833333	Chat 3
8C	Infected	Day 21	2.458333333	Chat 1
12C	Infected	Day 21	1.875	Chat 1
42C	Infected	Day 21	6.222222222	Chat 4

Table 6. Averages for Each Ovarian Tissue Sample. Average number of ChAT-containing inclusions observed in ovarian tissue at each time point, including mouse study and whether the animal was mock-infected or infected with *C. muridarum.*

VITA

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