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The Role of the Inflammasome During Chlamydia Infection

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ABSTRACT

BIOLOGICAL SCIENCES

McKEITHEN, DANIELLE N. B.S. COLUMBIA COLLEGE, 2003
M.P.H. UNIVERSITY OF PITTSBURGH, 2006

THE ROLE OF THE INFLAMMASOME DURING CHLAMYDIA INFECTION

Committee Chair: Godwin A. Ananaba, Ph.D.
Dissertation dated July 2016

Chlamydia trachomatis (C. trachomatis) is the most prevalent sexually transmitted bacteria with devastating reproductive consequences that lead to tubal factor infertility (TFI). Recent studies have implicated apoptosis – associated speck – like protein containing a caspase recruitment domain (ASC) as an adaptor of inflammasomes that stimulate IL – 1β and IL – 18 secretion, pro – inflammatory cytokines with critical functions in host defense against a variety of pathogens. Therefore, for the first time, we are reporting the use of ASC⁻/⁻ mice in a mouse model of Chlamydia infection that might provide some information on the role of inflammasomes in the pathogenesis of Chlamydia infection. In this study, wild type (WT) and ASC⁻/⁻ mice were infected with Chlamydia. Infectivity, pathology of the upper genital tract (UGT), and, fertility were evaluated. In addition, expression of ASC – dependent inflammasomes and the activation of immune cells within the genital tract (GT) were studied. Results showed
that *Chlamydia* infectivity in ASC−/− mice was significantly higher (p<0.05) compared to WT mice. There was an observed increase in infertility within ASC−/− mice which, when compared to infected WT mice, was exhibited by decrease in average number of pups and percent pregnancy. There was also severe UGT damage in ASC−/− mice compared to WT mice, correlating with the higher number of hydrosalpinx observed on the UGT of *Chlamydia* infected ASC−/− mice. Furthermore, IL−1β and IL−18 production as well as immune cell activation were down regulated in the GT of *Chlamydia* infected ASC−/− mice. This finding indicates that in absence of ASC, host innate and adaptive immunity is impaired. Results imply that ASC plays a protective role in the mucosal immunity against GT *Chlamydia* infection.
THE ROLE OF THE INFLAMMASOME DURING CHLAMYDIA INFECTION

A DISSERTATION
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
DANIELLE NICOLE McKEITHEN

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

JULY 2016
ACKNOWLEDGEMENTS

First and foremost I would like to thank God; without him and his continual blessings none of my accomplishments would be possible. This whole experience has increased my faith in Him and I now know that there can be nothing put in front of me that I cannot or will not handle.

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Last, but certainly not least, I would like to take a moment and thank the people who mean the most to me–my family and friends. You guys have been through the verbiage of “HIV, Cancer, and now, CHLAMYDIA” with me. I certainly hope you enjoyed the ride and learned some fascinating facts along the way. Without you guys I would be lost, just another soul passing in the breeze.
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<th>Description</th>
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<tbody>
<tr>
<td>AIM2</td>
<td>Absent in melanoma 2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC-</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis – associated speck – like protein containing a CARD</td>
</tr>
<tr>
<td>ASC-/-</td>
<td>ASC knockout</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow dendritic cell</td>
</tr>
<tr>
<td>C. muridarum</td>
<td>Chlamydia muridarum</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td>Chlamydia trachomatis</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation recruitment domain</td>
</tr>
<tr>
<td>CASP</td>
<td>Caspase</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>EB</td>
<td>Elementary body</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>FITC-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3 – phosphate dehydrogenase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GM–CSF</td>
<td>Granulocyte–Macrophage colony–stimulating factor</td>
</tr>
<tr>
<td>GT</td>
<td>Genital tract</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HEK II</td>
<td>Hexokinase II</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IFU</td>
<td>Inclusion forming unit</td>
</tr>
<tr>
<td>IL–10−/−</td>
<td>IL–10 knockout</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LGV</td>
<td>Lymphogranuloma venereum</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic–activated cell sorting</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>NBD–LRR</td>
<td>Nucleotide–binding domain–leucine–rich repeat</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa–light–chain–enhancer of activated B cells</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD–like receptor</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR–Pyrin domain containing protein 3</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PDHA1</td>
<td>Pyruvate dehydrogenase E1 component subunit alpha</td>
</tr>
<tr>
<td>PE-R</td>
<td>R–phycoerythrin</td>
</tr>
<tr>
<td>PGR</td>
<td>Proline and glycine–rich</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin–PAAD–DAPIN domain</td>
</tr>
<tr>
<td>RB</td>
<td>Reticulate body</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygenated species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmitted infection</td>
</tr>
<tr>
<td>TFI</td>
<td>Tubal factor infertility</td>
</tr>
<tr>
<td>TIM</td>
<td>Triosephosphate</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll – like receptor</td>
</tr>
<tr>
<td>TMS1</td>
<td>Target of methylation – mediated silencing</td>
</tr>
<tr>
<td>UGT</td>
<td>Upper genital tract</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
CHAPTER I

CHLAMYDIA

*Chlamydia trachomatis* (*C. trachomatis*) is the most frequently reported sexually transmitted bacterial infection within the United States. In 2014 there were over 1.44 million reported cases, a 2.8% increase from the previous year, however, it is estimated that well over 2.86 million infections occur annually (1). The discrepancy between the reported and the estimated statistics lies with the asymptomatic nature of the pathogen. Most infected do not seek treatment because their bodies are not sick. Most notably is the fact that *Chlamydia* infections are most prevalent among young people, with 1 in 15 sexually active females within 14–19 year age group (Figure 1) (1). Interestingly though, *Chlamydia* diagnosis rates have gradually increased over the years. From 2006–2010 diagnoses were up 27%; plus, screening rates have more than doubled since 2000 (1). Despite the rise in awareness of screening and treatments, chlamydia still remains a health concern that disproportionately affect African Americans nine times higher than whites and three times higher than Hispanics (Figure 2). More specifically, the population of young black women are hit the hardest; 7,719.1 per 100,000, which likely reflects a combination of factors including biological differences that place females at greater risk for sexually transmitted infections (STIs) than males as well as higher STI screening
rates among young women (1). Notably, one of the leading global causes of tubal factor infertility (TFI) is thought to be asymptomatic infection (up to 70%) of the female reproductive tract with *C. trachomatis* (2). Taken together, these statistics highlight the impact of reoccurring infections on the upper genital tract (UGT), beginning from such an early age.

Figure 1: Most reported *Chlamydia* Infections Occur among 15–24-Year-Olds (1). Surveillance data shows both the numbers and rates of reported cases of chlamydia continues to be highest among young people aged 15-24.

1.1 Life Cycle

*Chlamydia* is an obligate intercellular Gram–negative bacterium that infects both humans and animal species, including birds, pigs, and, cats. Originally characterized as protozoan, as research progressed, their classification changed various times, and, by late 1960s the pathogen came to be recognized as a bacterium (3-5). The pathogen resides within an intracellular vacuole, which has been identified as an inclusion body, and is vital to the survival of the bacteria during replication (6). The development cycle of *Chlamydia* is biphasic, adding to its unique ability to differentiate between an infectious and replicative stage (Figure 4). The infectious particles, also known as elementary bodies (EBs), are released from the cells at the end of the cycle. Though small in nature,
only about 0.3µm, the EBs contain condensed DNA and are responsible for infecting new cells to continue the replicative cycle. Entry of the EB into the mucosal epithelial cell is facilitated by a type III secretion system, which releases effector proteins to aid in infection as well as in the ability of the bacteria to evade the host’s immune system (7). Upon entry, the EB transforms into its metabolically active replicative form, the reticulate body (RB), and increases its inclusion body size (to approximately 1µm) to accommodate the multiplying RBs (7). Within 48h post infection, the RBs differentiate back into EBs and by 72h post infection EBs are released through either cell lysis or exocytosis (7). Throughout the developmental cycle, *Chlamydia* is continually modifying its inclusion membrane to avoid an endocytic pathway via the effector proteins secreted early in infection (7). Interestingly, persistent infections occur when the RBs are prevented from EB differentiation, which results in aberrant nucleated RBs. This has been shown to be triggered through antibiotic usage, limitations on available nutrients, and, the presence of cytokines (7). Persistence of *Chlamydia* infections result in continued damaged to host tissues and is one of the main causes of infertility observed in *C. trachomatis* patients (2).
Figure 3: The life cycle of genital serovars of C. trachomatis. The chlamydial growth cycle involves transformation between distinct forms: the elementary body (EB) and the reticulate body (RB). The highly infectious EB attaches to nonciliated columnar or cuboidal epithelial cells and induces ingestion by the host cell. EB are metabolically inactive and represent the extracellular C. trachomatis growth form. Once ingested into a phagosome, fusion of the phagosome with the host lysosome is prevented, a highly unusual occurrence that ensures EB survival. The EB reorganizes within the phagosome into a metabolically active RB. RBs are noninfectious but can replicate and do so by binary fission. The RB reorganizes back into EB, which will be released from the host cell to infect surrounding epithelial cells (23).

1.2 Disease

C. trachomatis can be classified into three distinctive serovars: A–C, D–K, and, L1–L3. The ocular disease, trachoma, is caused by serovars A–C, both neonatal pneumonia and conjunctivitis are caused by serovars D–K, and, the systemic disease lymphogranuloma venereum (LGV) is caused by serovars L1–L3. Of most importance are serovars D–K due to the fact that this group is associated with STIs, and when left untreated, results in urethritis, ectopic pregnancy, pelvic inflammatory disease (PID), and, TFI (8). As previously mentioned, persistence of a Chlamydia infection causes not
only continual damage to the host tissues, but results in infertility. This *Chlamydia* persistence is localized to the female UGT, specifically the fallopian tubes (9). The pathology following infection of non-immune cells results in the production of pro-inflammatory cytokines (Interleukin (IL)–1, TNF–α, etc.) and chemokines (IL–8, etc.). This in turns leads to the recruitment and activation of both innate and adaptive immunity in an effort to resolve the infection (8). Studies have shown that both protective immunity and eradication are dependent upon cell-mediated immunity, leading to the newly adopted immunological paradigm for chlamydial pathogenesis based upon T–cell responses that are essential to host defense but may also lead to collateral tissue damage (8).

Figure 4: The effects of Chlamydia on the Female reproductive tract (11).
CHAPTER II

INFLAMMASOME

The cytotoxic effects of *Chlamydia* on the UGT begin with identification of EBs, also referred to as pathogen associated molecular patterns (PAMPs). Since the EB is internalized, the pattern recognition receptor (PRR) that recognizes the pathogen has been observed to be the cytosolic NOD–like receptors (NLRs) (9). The NLRs are a large family of intracellular proteins observed to be involved primarily in the activation of innate immunity in response to microbial pathogens. Recently *Chlamydia* has been observed to yield an increase in the expression of the NLR–Pyrin domain containing Protein 3 (NLRP3) inflammasome activation. Inflammasomes are large cytosolic protein complexes in which pro–inflammatory caspases undergo autocatalytic activation in response to infectious and noxious insults (10). Inflammasomes are also responsible for the inflammatory process activation and linked to pyroptosis, which is a form of programmed cell death associated with antimicrobial responses, acting as a defense mechanism against infection by inducing inflammation (11). The formation of an inflammasome and the activity of caspase-1 (CASP–1) determine the balance between pathogen resolution and disease pathology. It should be noted that NLRP3 is not constitutively expressed in most resting cells and
its activation relies on two factors: (1) nuclear factor kappa–light–chain–enhancer of activated B cells (NF-κB)–dependent transcriptional induction downstream of NOD ligands or inflammatory cytokines and (2) an agonist to induce oligomerization (12). The formation of the NLRP3 inflammasome begins once these factors are present and involves the assembly of platform proteins (NLRP3), effector proteins (CASP–1), and, adaptor proteins (apoptosis–associated speck–like protein containing a caspase recruitment domain (ASC)) (Figure 3). The NLRP3 contains a nucleotide-binding domain–leucine-rich repeat (NBD-LRR), while the monomeric CASP–1 contains the caspase domain required for the processing of pro–IL-1β and pro–IL–18 (Figure 5).

Figure 5: Domain architecture of NLRP3 Inflammasome. NLRs are characterized by the combined presence of a NACHT domain followed by a variable number of LRRs. The PYD domain of NLRP3 recruits the bipartite adaptor protein ASC in order to interact with the CARD domain of the effector protein (11).
2.1 ASC Adaptor Protein

Originally named for its ability to induce apoptosis when over expressed in HL–60 human leukemia cells, ASC is now considered a key mediator in apoptosis and inflammation (13). The ASC mRNA gene is encoded by three exons: 1) PYRIN–PAAD–DAPIN (PYD) domain 1–90 amino acids long, 2) proline and glycine–rich (PGR) domain, and, 3) caspase–recruitment (CARD) domain 107–195 amino acids long (16). The PYD and CARD domains are members of the six–helix bundle death domain–fold superfamily that mediates assembly of large signaling complexes within the inflammatory (innate immunity) and apoptotic signaling pathways, both through the activation of caspase–8 (17). Of special note, two transcript variants encoding different isoforms have been identified, and ASC is one of the few proteins in the human genome that contains both an N–Terminal and a C–Terminal CARD, which allow it to bridge PYD–containing proteins with the CARD of caspase–1 (18). The location of the protein is primarily in the nucleus of resting monocytes and macrophages, but ASC rapidly redistributes to the cytoplasm upon pathogen infection (16). The ASC protein is widely expressed at low levels in various organs and peripheral blood leukocytes, however, very low levels are detected within the heart, brain, and, skeletal muscle (18). Interestingly, proteins containing the death domain fold motifs control the delicate balance between survival and death through regulation of NF–κB and caspase activity (18). The ASC protein promotes caspase–mediated apoptosis predominantly involving caspase–8 and –9, the main components of the ASC pyroptosome (19). As previously
mentioned, the innate immunity response acts as an integral adapter in the NLRP3 inflammasome, which leads to the activation of caspase–1 and the processing and secretions of downstream pro–inflammatory cytokines IL–β and IL–18 (19). The ASC protein has been observed facilitating the translocation of Bax to the mitochondria, inadvertently mediating the p53–Bax mitochondrial apoptotic pathway, thusly implicating itself in both tumor suppression and apoptosis (19, 21). Within the research, ASC is also known as Target of Methylation-mediated Silencing (TMS1) due to the suppression of its expression in human tumors via methylation of CpG islands within the gene (10, 19). More recently, ASC knockout studies have suggested a role for ASC in adaptive immunity that is independent of the NLRP3 inflammasome (20).
CHAPTER III
PUBLIC HEALTH CONCERN

3.1 Infertility

Fertility in women is overwhelmingly affected by unresolved or untreated infection of the reproductive tract with *C. trachomatis* (2). Consequences of prolonged exposure to the pathogen are observed within its target areas, which include the fallopian tubes, oviduct, and, ovaries—all components of the UGT and required for effective reproduction. The UGT plays an essential role in reproduction by way of gamete and zygote transportation. Specifically, gamete transport relies on ciliary activity, fallopian tube contractions, and, tubal secretions (2). Failures of these transport mechanisms, which can arise from chlamydial infections, are direct consequences of luminal architecture distortions (2). Studies have revealed that chlamydial infections of the UGT produce a mixture of patches of flattened cells along with loss of cilia or single elongated–cilium cells (2). It should be noted that women with TFI are defined as having either damaged or occluded fallopian tubes or a history of salpingectomy (2). Such tubal diseases resulting in TFI are generally due to the inflammatory process occurring either in or around the fallopian tube. In the case of *Chlamydia*, disease severity can range from complete occlusion with hydrosalpinx to mild intraluminal adhesions, dependent on both
severity and duration of the infection (2). Interestingly, the severity of the inflammatory response towards the infection is enhanced during re–infections and results in the known pathologies of inflammation, tissue damage, and, scarring.

Collectively, studies have shown that when recurrent chlamydial infections are left untreated, the results are traumatic tissue damage and a decrease in the ability to reproduce. These known *C. trachomatis* structural changes within the UGT are a result of the activation of the innate and adaptive immunity, which is a requirement to clear the infection. A better understanding of the activation of the immune system would highlight possible innate immunoregulators. It should be noted that ASC has previously been suggested as an important molecular component of innate immunity activated specifically by chlamydial and other parasitic infections (9, 21).

### 3.2 Significance

*Chlamydia trachomatis* (*C. trachomatis*) is a public health concern due to its prevalence and devastating reproductive consequences within developed and developing countries (7). As of 2014, 51,945 cases were reported in Georgia, with 6,575 of the state’s reported cases coming from Fulton County, which includes the city of Atlanta. Within the US, the rates of reported cases were most prevalent within the 15–24 year old female age group, while the male age group was 20–24 year old within the population (1). Known as the silent infection, the STI can persist within a host and results in devastating damage on the reproductive system. This is especially traumatic to the
female UGT, where in chronic infections chlamydiae have ascended and are known to cause pelvic inflammatory disease (PID), chronic pelvic pain, tubal factor infertility (TFI), and, ectopic pregnancy (8). The host relies on the adaptive immunity to begin the clearance of the infection; however the host response to the pathogen requires the initiation of the inflammatory process. And due to recurrent infections, the ending result is severe tissue damage, which leads to infertility, otherwise known as TFI. Recently, the NOD–like Receptor – Pryin domain containing 3 (NLRP3) Inflammasome has been identified as the inducer of the inflammatory process during C. trachomatis infections. And more importantly, its adapter protein ASC has emerged as a key mediator in both apoptosis and inflammation. Studies have implicated ASC as an important component of the innate immune system, specifically, citing the direct dependency of innate immunity for ASC following Chlamydia infections of the oviduct (9). Therefore, we hypothesize that the immunoregulatory effects of ASC affect the mucosal and systemic immunity following C. trachomatis infection.
CHAPTER IV
DISSERTATION AIMS

The research presented within the current dissertation is concentrated on acquiring a better understanding of the association between ASC and *C. trachomatis* on both the mucosal and systemic aspects of immunity following infection.

**Specific Aim 1:** To investigate a potential link between NLRP3 inflammasome adapter protein ASC and Chlamydia–induced infertility following infection.

It has previously been reported that a single chlamydial infection of the UGT does not result in tubal scarring; however, prolonged exposure to Chlamydia due to a chronic persistent infection or frequent reinfection has been associated with chronic inflammation associated with TFI (2). Inflammasome activation produces the essential pro-inflammatory cytokines, which in turn recruit an influx of immune cells necessary to begin the clearance of *C. trachomatis*. Therefore, we propose to examine the association between ASC and *C. trachomatis* on A) Infectivity and B) Fertility following recurrent infection.

**Specific Aim 2:** To study the regulatory effects of ASC on Chlamydia induced UGT pathophysiology.
Successful fertility relies on intact, functioning, and secreting cells within the UGT. As a consequence of recurrent infections, *C. trachomatis* histopathology results in the influx of immune cells to the site of infection for clearance, and ciliated cells become damaged and tissue is scarred (2, 9). Hence, the cells can no longer transport the gamete through the UGT (9). Therefore, we propose to investigate the immunoregulatory effects of ASC on the known A) Histology and B) Pathology of *C. trachomatis* infected UGT post infection.

**Specific Aim 3: To analyze the regulatory effects of ASC on the maturation of DCs following Chlamydia stimulation.**

The inflammasome is important in activating the innate immunity and the ensuing adaptive immunity response (9). Signaling for these downstream effects relies on the capability of antigen–presenting cells (APCs) to function properly and in rapid succession for clearance. Dendritic Cells (DCs) are important when it comes to antigen presentation; they are the link between the innate and adaptive immune systems post *C. trachomatis* infection. Therefore, we propose to investigate the immunoregulatory role of ASC on A) The maturation of DCs and B) The DCs antigen–presenting function of priming naïve T–cells.
5.1 Mice.

Six week old female wild type (WT) mice with C57BL/6 J background were purchased from The Jackson Laboratory, and female ASC−/− mice were provided by Dr. Dixit at Genentech. Mice were fed food and provided water ad libitum, and maintained in laminar-flow racks under pathogen-free conditions with a 12-hr light and a 12-hr dark cycle in an accredited animal facility at Morehouse School of Medicine. The protocols involving mice were performed in compliance with Morehouse School of Medicine-Institutional Animal Care and Use Committee (MSM-IACUC) and prescribed federal guidelines.

5.2 Chlamydia Stocks.

Stocks of *Chlamydia muridarum* (*C. muridarum*), the agent of mouse pneumonitis, (Division of Scientific Research, Centers for Disease Control and Prevention) used for infections were prepared by propagating elementary bodies (EBs) in McCoy or HeLa cells (Division of Scientific Research, Centers for Disease Control and Prevention), according to standard procedures. The cell lines were maintained in Minimum Essential Medium (MEM) supplemented with Earle’s with 2mM L-glutamine, 10% heat-inactivated FBS, 1mM sodium pyruvate, 0.5% fungizone, and, 1.0%
penicillin/streptomycin (100U/mL; 100µg/mL) in a humidified atmosphere under 5% CO2, at 37°C. *Chlamydia* stock titers were expressed as inclusion forming units (IFU) per milliliter.

5.3 Bone Marrow DC (BMDC) Extraction.

Immature DCs were isolated from the bone marrow of WT and ASC−/− mice (n = 5 per group) by the standard method and differentiated *in vitro* by culturing with IL–4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), as described by Inaba. (28). Briefly, bone marrow progenitor cells from mice femurs were removed, washed three times with RPMI 1640 supplemented with 10% FBS, and, incubated in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 200 mM glutamine, 100 mM sodium pyruvate, 0.5% HEPES, 5ng/mL IL–4, and, 10ng/mL GM–CSF at 37C for 6 days, replacing with fresh media on day 3 and transferring cells to new dishes on day 5. After 5 days in culture, the cells were characterized as loosely adherent mononuclear cells and further purified as CD11c expressing DCs by using the Pan Dendritic Cell Isolation Kit from Miltenyi Biotec.

5.4 Infectivity.

The WT and ASC−/− mice (n = 8 per group) were anesthetized with sodium pentobarbital (30µg/body weight) (Sigma-Aldrich) and infected intravaginally with 20µl of 1 X 10^5 IFUs of *C. muriduram* approximately 5 days after intramuscular administration of 2.5 microgram/mouse Depo Provera (Pfizer Inc.). Infections were administered three times, four weeks apart. After the initial infection, the mice were swabbed vaginally every three days for two weeks and once a week for two weeks. The course of infection
was monitored by isolation of *Chlamydia* from the collected cervicovaginal swabs cultured in 24–well plates with McCoy cells (Division of Scientific Research, Centers for Disease Control and Prevention) at 37°C, 5% CO₂ for 1hr. Culture media was replaced with MEM media supplemented with 2mM L-glutamine, 10% heat-inactivated FBS, 1 mM sodium pyruvate, and, 0.5% fungizone and incubated at 37°C, 5 %CO₂ for an additional 48hrs. The Pathfinder, Chlamydia Culture Confirmation System (Bio-Rad) manufacture’s protocol was followed for immunofluorescence staining and IFU counting.

**5.5 Fertility Assay.**

Two weeks post the 3rd infection infected and uninfected WT and ASC⁻/⁻ mice (n = 8 per group) were transferred to cages with a male mouse of the same strain and maintained as previously stated. Each cage contained no more than two female mice per group for mating purposes. The mice were checked twice weekly for weight gain as a sign of fertility. Fifteen days post mating, female mice from both experimental groups were euthanized, and the number of pregnant mice and the mean number of pups in the different groups were enumerated and calculated.

**5.6 Histopathology.**

**5.6.1 Assessment of Chlamydia Induced Infertility.**

The UGTs of uninfected and infected infertile WT and ASC⁻/⁻ mice (n = 4 per group) from the fertility assay were harvested fifteen days post mating and observed grossly for the presence of *Chlamydia* induced pathologies, including hydrosalpinx and uterine abnormalities. Following examination, the UGTs were placed flat on cardboard squares, fixed in 4% buffered formalin (Thermo Fisher Scientific Inc.) for 1hr at room
temperature, and then stored at 4°C.

5.6.2 Assessment of Persistent *Chlamydia* Induced Inflammation.

Two weeks post the 3rd infection the UGTs of non–mated uninfected and infected WT and ASC−/− mice were harvested and fixed in 4% buffered formalin and embedded in paraffin. Briefly, formalin fixed tissues were washed in 1X PBS for 10min and dehydrated with increasing ethyl alcohol (Thermo Fisher Scientific Inc.) concentrations for 2hrs each at room temperature. Tissues were then treated with an ethyl alcohol/xylene mixture beginning with a 3:1 and ending with 1:3 for 2hrs each at room temperature followed by and overnight incubation in pure xylene (VWR) at room temperature. After 2 days of 3hr pure xylene incubations, tissues were transferred to disposable molds with pure xylene, incubated 56–58°C three times for 3hrs, and, left incubating overnight. On day three pure xylene was replaced with precision cut paraffin wax (Thermo Fisher Scientific, Inc.) and blocks were left on the chiller overnight. Solidified wax blocks were then stored at room temperature. Longitudinal sections measuring 4µm were cut, processed, and, stained with either hematoxylin and eosin or Masson's trichrome (Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol. Evaluations of the stained sections were blinded and each anatomic site (ovary, oviduct, and, uterine horn) analyzed for the presence of inflammation, edema, immune cells, fibrosis, stromal vacuoles, ectasia, and, apoptotic necrosis.

5.7 Immunofluorescence Microscopy.

Two weeks post the 3rd infection the UGTs of non–mated uninfected and infected WT and ASC−/− mice (n = 4 per group) were harvested and fixed in 4% buffered formalin
and embedded in paraffin. Longitudinal sections measuring 4µm were cut and deparaffinized. Tissue slides were blocked with normal goat serum and labeled with primary (Santa Cruz) and fluorescent–conjugated secondary antibodies (Jackson ImmunoResearch Labs) specific for DCs, CD4+ T–cells, and, Macrophages, while the DNA was stained with DRAQ5 (Abcam), and, mounted in Fluorogel with Tris Buffer (Electron Microscopy Sciences). Images were observed separately from different fields within the ovary, oviduct, and, uterine horn with the Carl Zeiss 510 Vis confocal microscope. The total number of positively stained immune cells were counted and graphed. Statistical significance was judged at p<0.05 using a Student t–test and ANOVA.

5.8 T – cell Co – culture.

5.8.1 Enrichment of GT DCs.

Whole genital tracts (GTs) from uninfected WT and ASC+/− mice (n = 10 per group) were harvested and pooled per group. Single cell suspension was obtained through tissue digestion. Briefly, the tissue was minced, treated with 5mg/ml collagenase (Atlanta Biologicals) in Hanks' balanced salt solution (Mediatech), and, 1mg DNAse (Sigma–Aldrich) in 5ml calcium/magnesium-free Hanks' (CMF) buffer (Sigma–Aldrich) at 37 °C for 20m. After incubation, 1 ml of 180 mm EDTA (Boston BioProducts, Ashland) was added to each sample, followed by tubular rotation at room temperature for 5m. Single cell suspensions were prepared by expressing the digests through a 70µm Falcom™ Cell Strainer in CMF Hanks' (Fisher Scientific).
5.8.2 Enrichment of GT T–cells.

Whole GT from *Chlamydia*–infected WT and ASC<sup>+</sup> mice (n = 10 per group) were harvested and pooled per group. Single cell suspension was obtained through time digestion. Briefly, the tissue was minced, treated with 0.6mg collagenase (Atlanta Biologicals) in 5% FBS (HyClone<sup>TM</sup> Fetal Bovine Serum), and, incubated at 37 °C for 45m. The digest were passed through a 40μm Falcon<sup>TM</sup> Cell Strainer in CMF, centrifuged twice and resuspend with 1%HEPES/5%FBS (Mediatech).

5.8.3 T–cell Proliferation.

Dendritic cells (DCs) were isolated from the single cell suspension of uninfected GT from WT and ASC<sup>+</sup> mice per MACS Miltenyi Biotec’s Pan Dendritic Cell Isolation Kit protocol and γ–irradiated (2,000 rad, 3m 10s). In a round bottom 96–well plate γ–irradiated DCs (50μl; final concentration 1 X 10<sup>5</sup>) and UV–*Chlamydia* (50μl; final concentration 25 X 10<sup>5</sup>) were incubated at 37 °C for 30m. T–cells were isolated from the single cell suspension of *Chlamydia* infected GT from WT and ASC<sup>+</sup> mice per MACS Miltenyi Biotec’s Pan T Cell Isolation Kit (mouse) protocol. T–cells (50μl; final concentration 5 X 10<sup>5</sup>) were added to the round bottom 96 – well plate containing γ–irradiated DCs and UV–*Chlamydia* (final volume of 250μl) and incubated at 37 °C for 5 days. T–cell proliferation was detected per Cell Signaling XTT Cell Viability Kit protocol. The mean and SD of all replicate cultures were calculated.

5.9 Cytokine Profile 5.9.1 Genital Tract DC Antigen Presentation.

Cultured supernatants (150μl) from Day 5 of the UGT T–cell co–culture assay
were collected, pooled, and, analyzed by Bio-Plex Pro Mouse Cytokine 23-Plex multiplex array according to manufacturer’s guidelines (Bio-Rad). The concentration of cytokines in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. The mean and SD of all replicate cultures were calculated. The experiment was repeated three times.

5.9.2 BMDC Antigen Presentation.

Splenocytes were obtained from spleens of *Chlamydia* infected WT and ASC−/− mice using a 40μm filter and syringe plunger and resuspended in PBS. CD4+ T cells were then purified using the MACS mouse Pan T Cell Isolation Kit (mouse) (Miltenyi Biotec, Auburn, CA). To assess the antigen-presenting function of DCs from either ASC−/− or WT mice, 1 × 10^5 DCs were co-cultured with 2 × 10^5 purified T–cells in the presence or absence of UV-inactivated *C. muriduram* in 96-well tissue culture plates for 5 days. The amounts of IL-2, -5, -9, -12, -13, -17A, IFN-γ, MIP-1β, RANTES, and TNF-α in the culture supernatants were measured using the Bio-Plex Pro Mouse Cytokine 23-Plex multiplex array according to the manufacturer’s guidelines (Bio-Rad, Hercules, CA). T–cell proliferation was detected using Cell Signaling XTT Cell Viability Kit protocol (Danvers, MA). The concentration of cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. The mean and SD of all replicate cultures were calculated. The experiment was repeated two times.
5.10 Immunoblotting.

5.10.1 Genital Tract DC Protein Expression.

Two weeks post the 3\textsuperscript{rd} infection, the UGTs of uninfected and infected WT and ASC\textsuperscript{−/−} mice (n = 4 per group) were harvested and homogenized RIPA lysis buffer supplemented with 1mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Promega) using the gentle MACS Dissociator for total protein extraction per MACS Miltenyi Biotec protocol. UGT protein levels, consisting of the ovary, oviduct, and, the upper half of the uterine horn, were equalized by Pierce\textsuperscript{TM} BCA Protein Assay Kit, loaded resolved by 4\%–20\% TGX gel (Bio-Rad) gradient gel, and run for 1hour. Proteins (40µg) were then transferred onto nitrocellulose paper (Bio-Rad). After 1hour, blots were washed, blocked with 5\% milk, then incubated with primary antibody for NLRP3, CARD12, AIM2, pro–caspase–1, IL-1β, IL-18, and, GAPDH (Santa Cruz) overnight at 4°C. Horseradish peroxidase (HRP) – conjugated secondary antibodies (R&D Systems) were added for 1 hour at room temperature, blots were then developed using Clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad) and viewed with ImageQuant LAS 4000 (GE Healthcare). The mean and SD of all replicate cultures were calculated.

5.10.2 BMDC Protein Expression.

Lysates from \textit{Chlamydia} stimulated WT and ASC\textsuperscript{−/−} BMDCs were prepared by homogenization in lysis buffer supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail on ice. 20µg of WT and ASC\textsuperscript{−/−} DC lysates and supernatants from cell cultures were loaded onto the same 4-20\% TGX gradient gel (Bio-
Rad, Hercules, CA) and run for 1 hour. Proteins were then transferred onto nitrocellulose paper (Bio-Rad, Hercules, CA). After 1 hour, the blots were washed, blocked with 5% milk, and then incubated with primary antibody for ASC, ENO1, hexokinase, pyruvate dehydrogenase E1 component subunit alpha (PDHA1), triosephosphate isomerase (TIM), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, Dallas, TX) overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (R&D Systems) were added for 1 hour at room temperature, and then the blots were developed using Clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, CA) and viewed with ImageQuant LAS 4000 (GE Healthcare, Pittsburgh, PA). The mean and SD of all replicate cultures were calculated. The experiment was repeated three times.

5.11 Adoptive Transfer.

Isolated BMDCs from WT and ASC\(^{-/-}\) mice were stimulated with C. muridarum EBs for 2hrs and adoptively transferred into 6-8 weeks old female WT mice (2.5 \(\times\) 10\(^7\) cells/mouse) by intravenous infection into the tail vein in 0.2mL of 1X PBS (n = 4). Treated mice were infected intravaginally with 1\(\times\)10\(^5\) IFU/mouse of live C. muridarum 1 week after adoptive transfer. The status of the infection was monitored by periodic cervicovaginal swabbing of individual animal and isolation of Chlamydiae in tissue culture as previously described.

5.12 Flow Cytometry and Cytokine Expression.

Isolated BMDCs from the bone marrow of WT and ASC\(^{-/-}\) mice (n = 5 per group) were stimulated at a ratio of 1:5 with C. muridarum for 2 hours at 37°C and washed with
FACS Buffer at 4°C and stained with FITC-, PE-, or APC-conjugated antibodies against CD80, CD103, MHC II, CD40, CD14, TLR4, and CD11c (BD Pharmingen). Cultured supernatants were collected and analyzed by Bio-Plex Pro Mouse Cytokine 23-Plex multiplex array according to manufacturer’s guidelines (Bio-Rad, Hercules, CA). The concentration of cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. The mean and SD of all replicate cultures were calculated. The experiment was repeated three times.

5.13 Pyruvate Assay.

Isolated WT and ASC−/− BMDCs were stimulated with C. muriduram in 24-well tissue culture plates at a ratio of 1:5 for 1and 2hours. The BMDC supernatants were collected and analyzed for pyruvate using the Eton Bioscience Pyruvate Assay Kit (San Diego, CA). Each sample was incubated with the Pyruvate Assay Kit Reaction Solution in a 96-well flat bottom reader plate for 30min at 37°C and measured at 570 nm. Included in the OD570 reading were unstimulated WT and ASC−/− BMDCs and culture media, both control groups. The concentration of pyruvate from each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. The mean and SD of all replicate cultures were calculated. The experiment was repeated three times.

5.14 Transmission Electron Microscopy (TEM).

Isolated WT and ASC−/− BMDCs were stimulated with C. muriduram at a ratio of 1:5 for 2hours in 24-well tissue culture plates. The BMDCs were washed and fixed on a glass slide with 2.5% (w/v) glutaraldehyde–0.1 M cacodylate buffer for 2–6hours at room temperature. After fixation, BMDCs were mounted, cut into 200-mm slices with a
Vibratome (EM Corp., Chestnut Hill, MA), and post fixed in aqueous osmium tetroxide. Fixed BMDC slices were dehydrated through graded ethanol and propylene oxide, and embedded in Polybed 812 resin (Polysciences, Inc., Warrington, PA). Thin sections (80 nm) were then cut with a diamond knife and stained with 5% uranyl acetate and Reynold’s lead citrate. A JEOL 1200EX transmission electron microscope was used to examine mitochondrial changes. The experiment was repeated three times.
CHAPTER VI

RESULTS

6.1 ASC deficiency results in higher number of IFUs following an acute *Chlamydia* infection.

We first wanted to assess a link between ASC deficiency and the initial or acute chlamydial infection. This was accomplished through comparing the course of genital chlamydial infections between WT and ASC\(^{-/-}\) mice (Figure 6). Our infectivity data confirmed the presence of infection though the observance of chlamydial IFUs. As with our lab’s previously published WT mice infectivity data \((22)\), our current data shows a decreasing trend in IFUs, with the infection nearly cleared 28 Days post the initial infection. Conversely, the number of chlamydial IFUs within ASC\(^{-/-}\) mice were significantly higher \((p<0.05)\) beginning on Day 15 post the initial infection, and remained significantly higher throughout Day 28 in comparison to the WT mice. This heavy bacterial load and long duration of infection within the ASC\(^{-/-}\) mice may in fact increase the risk of *Chlamydia* induced pathology of the UGT.
Figure 6: Course of genital *Chlamydia* infection in WT and ASC\(^{-/}\) mice. Mice were infected intravaginally with \(1 \times 10^5\) IFU/mouse of *C. muridarum*. The status of the infection was monitored by periodic cervicovaginal swabbing for the isolation of *chlamydiae* in tissue culture. ASC\(^{-/}\) mice exhibit an increase in the average number of IFUs compared to WT. (p<0.05)

6.2 Histological Assessment of the UGT pathology and fertility in *Chlamydia* infected ASC\(^{-/}\) mice

It has previously been shown that activation of inflammasomes is required for *Chlamydia*-induced inflammatory pathology of the UGT. We therefore assessed the role of ASC in inflammatory tubal pathologies and fertility in mice infected multiple times with *C. muridarum*. Significant changes in tissue morphology were detected in the H&E stained ovaries, oviducts, and uterine horn sections of ASC\(^{-/}\) mice. Six out of 10 ASC\(^{-/}\) mice exhibited known *Chlamydia* infection characteristic trait; hydrosalpinx, only
2 out of 10 *Chlamydia* infected WT mice (p<0.005) (Figure 7A).

No visible signs of tissue damage were observed within the UGT of uninfected WT and ASC−/− mice. The ovary of the infected WT mice exhibited vacuoles within the epithelia lining, whereas the clear vacuoles observed within the stromal cells and epithelia cell of infected ASC−/− mice were similar to those observed within uninfected ASC−/− mice. The oviduct of the infected WT mice exhibited mild vacuoles and cellular degeneration, whereas moderate ectasia occurred within ASC−/− mice. The uterine of the infected WT mice exhibited less cellular degeneration compared to ASC−/− mice, whereas necrosis, ectasia, and, edema occurred within ASC−/− mice (Figure 7B). We also evaluated the UGT for fibrosis with Masson’s trichrome stain. Analysis in Figure 7C shows no observed difference in the presence of fibrosis between infected ASC−/− and WT mice.

Results from the fertility assay showed that non – infected WT and ASC−/− mice had 100% and 79% pregnancy respectively compared to infected WT mice (60%) and ASC−/− mice (38%) (p = 0.00008). There was also a significantly higher number of pups in non–infected WT and ASC−/− mice compared to Chlamydia infected WT and ASC−/− mice (p = 0.01) (Figure 7D).
Figure 7: *Chlamydia* induced histopathological and fertility changes. A. WT and ASC−/− mice exhibiting known *Chlamydia* infection characteristic trait: hydrosalpinx. The graph depicts the average number of mice hydrosalpinx occurrences within the multi–infected *Chlamydia* infected/infertile group. B. Histopathological data indicates no visible signs of tissue damage within the GT of uninfected WT and ASC−/− mice. C. Masson’s Trichrome analysis showed no difference in the presence of fibrosis between infected ASC−/− and WT mice GT. D. Infected ASC−/− mice exhibit an increased risk of infertility post *Chlamydia* infection. (p<0.05)

6.3 ASC deficiency decreases mucosal immune cell population

To delineate the influence of chronic *Chlamydia* infection on the population of
immune cells, the UGT of uninfected and infected WT and ASC−/− mice were analyzed by high-resolution, immunofluorescence confocal microscopy. Figure 8A shows the population distribution differences for DCs, CD4+ T−cells, and, Macrophages present within the uninfected and Chlamydia infected WT and ASC−/− mice ovary, oviduct, and, uterine horn. The difference in immune cell distribution observed between the Chlamydia infected WT and ASC−/− mice GTs suggest a possible explanation for the tissue damage previously observed within the UGT of infected ASC−/− mice (Figure 8B).

The total population of immune cells observed within uninfected UGT of ASC−/− mice was similar in comparison to uninfected UGT WT mice for positively stained DCs, CD4+ T−cells, and, Macrophages (Figure 8B). This similarity correlated with our observed pathology data (Figure 8A-B), indicating that ASC deficiency does not on its own trigger an increase in the number of immune cells within the female reproductive tract. The population of CD4+ T−cells and Macrophages were significantly decreased (p<0.04 and p<0.01 respectively) within the UGT of infected ASC−/− mice when compared to the UGT of infected WT mice. Interestingly, there was no significant decrease in the presence of DCs within the UGT of infected WT and ASC−/− mice.
Figure 8: *Chlamydia* infected GT from WT and ASC−/− mice labeled for DCs, CD4+ T-cells and, Macrophages. A. Immunofluorescence of immune cell distribution within the ovary, oviduct, and, uterine horn. Immune cells labeled green and nucleus labeled blue. B. Graph depicting total cell count ± SD of positively stained DCs, CD4+ T−cells and, Macrophages. ASC−/− mice exhibit a decrease in the presence of Dendritic cells, CD4+ T−cells, and, Macrophages post chronic *Chlamydia* infection when compared to WT mice (p<0.05)

6.4 ASC regulates DC function and T – cell integrity

The DC is the most potent APC and appears the sole cell type capable of inducing primary T−cell responses. Following antigen uptake, DCs mature, costimulatory molecules such as CD40, CD80, and, CD86 are up-regulated, and, the DCs migrate to the lymphoid tissues where they activate effector T−cells (24). To further characterize the effect of ASC deficiency on DC function, *Chlamydia* infected WT and ASC−/− mice UGT T−cells were co−cultured with WT or ASC−/− mice UGT DCs from infected (Figure 9). The ASC−/− DCs yielded a significant decrease in WT T−cell activation when compared to the control (OD450 value 1.62 vs 2.08), signifying a loss of antigen presentation when
compared to WT DCs. Interestingly, ASC\(^{−/−}\) T–cell activation was drastically reduced when co–culture with WT DCs compared to the control (OD\(_{450}\) value 0.37 vs 2.08), demonstrating a loss in the integrity of T-cells. Taken together, the findings suggest ASC deficiency affects the ability of DCs to optimally initiate an adequate immune response.

Since an adaptive response is necessary for limiting the spread of the infection, and providing protection against recurrent infections (25), we next we analyzed the cytokine profiles of the WT/ASC\(^{−/−}\) DC T–cell co-culture. We found that IL–12, IFN–\(\gamma\), and, TNF–\(\alpha\) (Th1) cytokine production decreased, while IL–10 and IL–17A (Th2) cytokine production increased for ASC\(^{−/−}\) T–cells when compared to WT T–cell cytokine profile. It is important to note that in response to a Chlamydia infection, T–cells exhibit a characteristic Th1 response by secreting large amounts of IFN–\(\gamma\) and secretion of IL–12 and TNF–\(\alpha\) induces protection, all of which are required to aid in clearing the bacterial infection (26). Conversely, secretion of pro–inflammatory cytokine IL–10 along with the IL–17A cytokine following Chlamydia infection is associated with an increase in disease pathology (25). In our study, the observed decrease of Th1 and overexpression of Th2 cytokine secretion validates the inability of ASC\(^{−/−}\) T–cells to clear chronic Chlamydia infections.
Figure 9: *Chlamydia* clearance is decreased in the absence of ASC. A. ASC<sup>−/−</sup> mice T–cell activation was significantly decreased when compared to WT mice. In addition, ASC<sup>−/−</sup> mice DCs lost their antigen presenting function to WT T–cells when compared to WT mice (p<0.05). B. ASC<sup>−/−</sup> mice T–cells exhibit a change in secretion profile by decreasing IFN–γ, IL–12, TNF–α (Th1) and increasing IL–10 and IL–17A (Th2) cytokine secretion when compared to WT mice.
6.5 ASC deficiency inhibits IL-1β expression

In the absence of the ASC adaptor protein, ASC – dependent inflammasome assembly is expected to be abolished; therefore we want to investigate the effects on the individual inflammasome proteins, as well as the activation of the downstream pro–inflammatory cytokines, specifically IL-1β (Figure 10). We observed that the expression of NLRP3, CARD12, and AIM2 in the UGT of ASC<sup>−/−</sup> mice was comparable to expression in the UGT of WT mice, indicating that ASC–dependent inflammasome proteins are not affected by the absence of the adaptor protein ASC.

As expected, the effector pro–caspase–1 protein, which is not ASC–dependent, was only detected in the UGT of WT and ASC<sup>−/−</sup> mice that were infected with *Chlamydia*. Pro–inflammatory cytokine IL–18 expressions remained unchanged when the UGT of infected WT and ASC<sup>−/−</sup> mice were compared. Nagarajan and colleagues have identified that IL-1β secretion is dependent upon caspase–1 activation and ASC–dependent inflammasomes during *Chlamydia* GT infection (8). Our current study corroborates with their findings in that we show the expression of IL-1β was eliminated within the UGT of *Chlamydia* infected ASC<sup>−/−</sup> mice, and only detectable within the UGT of *Chlamydia* infected WT mice.
Figure 10: Expression of UGT ASC-dependent inflammasome proteins and pro-inflammatory cytokines post chronic *Chlamydia* infection. Western blot analysis showed no change in ASC-dependent inflammasome expression along with decreased IL-1β expression within the ASC−/− mice when compared to WT mice.

### 6.6 Deficiency of ASC induces changes in BMDC maturation and cytokine profile

In order to determine if the immunometabolic damage within ASC−/− BMDCs has an effect on DC activation, we measured the expression of activation markers and cytokine profile on *Chlamydia* stimulated WT and ASC−/− BMDCs. Figure 11A shows that following 2 hours of stimulation, the expression of DC activation and maturation surface markers CD80 (WT 24.15% vs ASC−/− 2.39%), TLR4 (WT 3.97% vs ASC−/− 3.12%), CD40 (WT 81.79% vs ASC−/− 0.72%), and MHC II (WT 90.42% vs ASC−/− 9.78%) were downregulated in ASC−/− BMDCs whereas, CD 103 (WT 0.03% vs ASC−/− 1.04%) and CD14 (WT 0.61% vs ASC−/− 41.30%) were upregulated. ASC−/− BMDCs
exhibited a different cytokine profile compared to WT BMDCs. The cytokine profile showed that there was a significant decrease in $\text{T}_h1$ cytokine secretions of IL-3, -9, -12, GM-CSF, and IFN-γ; and a significant increase in the $\text{T}_h2$ cytokines IL-5, -10 and -17A in ASC$^+$ BMDCs (Figure 11B). Interestingly, IL-β expression was doubled between unstimulated and stimulated ASC$^+$ BMDCs (13.3 vs 26.52), which might be due to an upregulation of casp-11 expression.
Figure 11: Effect of targeted inhibition of ASC deficiency on DC expression of maturation and co-stimulatory molecules. A. *Chlamydia* stimulated WT and ASC−/− BMDCs were stained with FITC-, APC- and PE-conjugated mAbs against the indicated surface markers and analyzed by flow cytometry after gating for DCs according to scatter criteria. The results are representative plots for 3 independent experiments. Bar graph (11A) shows mean percentages and SD of markers were derived from those 3 experiments. B. The concentration of cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously (p<0.05).
6.7 Evidence for ASC regulation of antigen-processing events in BMDCs

Since activated DCs produce the cytokines that initiate inflammation and help modify the direction of the T-cell response, we determined the effect of ASC deficiency on the *Chlamydia*-induced T-cell cytokine profile. Results revealed a decrease in secretions of IL-12, IFN-α, MIP-1β and TNF-α in the ASC−/− BMDC+T cell co-culture, while IL-2,-5, and -13 secretions increased compared to the WT BMDC +T cell culture co-culture (Figure 12A). We next examined the antigen presenting function of BMDCs in the absence of ASC. Data showed a significant impairment in the ability of ASC−/− BMDCs to induce Ag-specific proliferation of T-cells (p< 0.012) (Figure 12 B). This suggests that ASC deficiency in DCs abrogates their capacity to activate elevated T-cell responses against *Chlamydia* stimulation.
Figure 12: Effect of ASC deficiency on antigen presentation for T-cell activation and proliferation by DCs. A. ASC<sup>−/−</sup> BMDC T–cell activation was significantly decreased compared to WT BMDCs. B. ASC<sup>−/−</sup> BMDC cytokine profile exhibits a decrease in secretions compared to WT BMDCs (p<0.05).
6.8 Cotransfer of ASC−/− BMDCs promotes *Chlamydia* infection

For further support of our hypothesis on the immunoregulatory effects of ASC following *Chlamydia* infection, the course of infection in adoptively transferred mice was monitored (Figure 13). *Chlamydia* infection within the WT mice mimics the trend that we previously observed where there is a gradual steady decrease of chlamydial shedding through Day 28 (27). Figure 13 shows that the IFUs from the group of *Chlamydia* stimulated WT BMDCs transferred into WT mice began to decrease by Day 9 with the infection cleared by Day 21, indicating an end to the infection one week earlier than the group of unstimulated WT BMDCs transferred into WT mice.

Here, our results revealed that the mice that received the ASC−/− BMDCs were more vulnerable to increased chlamydial shedding. WT mice that received unstimulated ASC−/− BMDCs experienced an immediate increase of chlamydial shedding between Days 3–9, a gradual shedding decrease between Days 9–21, and by Day 28 there was the appearance of the beginning of another chlamydial shedding peak when compared to the WT mice course of infection. The course of infection with the group of *Chlamydia* stimulated ASC−/− BMDCs transferred into WT mice resembled that observed by the transferred ASC−/− BMDCs into WT mice group. Chlamydial shedding from the *Chlamydia* stimulated ASC−/− BMDCs group began with an overall increase from Days 3–12, followed by a steady decline through Day 28, and ending with an increase in the number of IFUs observed when compared to the unstimulated and *Chlamydia* stimulated groups. Our adoptive transfer data suggest that the loss of ASC increases chlamydial
shedding, while at the same time causes a decrease in the host immune response required for infection clearance.

![Adoptive Transfer Chlamydia Infectivity](image)

**Figure 13:** Cotransferred *Chlamydia* primed ASC−/− DCs. Bone marrow derived ASC−/− DCs transferred into WT mice yielded a decrease of chlamydial shedding during the course of infection similar to WT.

6.9 ASC deficiency inhibits BMDC CARD12 expression

The expression of ASC in unstimulated and *Chlamydia* stimulated WT and ASC−/− BMDCs was assessed by immunoblotting (Figure 14A) and showed that ASC expression was completely abolished in both groups of ASC−/− BMDCs. Additionally, in this study
study we show that *Chlamydia* stimulated ASC<sup>−/−</sup> BMDCs have increased expression of caspase-11, pro–caspase–1 and IL-18 when compared to WT BMDCs (Figure 14B). We also checked the expression of components of ASC-dependent inflammasomes; NLRP3, CARD12, AIM2, along with caspase-8. The results indicate no significant changes in expression of NLRP3, AIM2, or caspase-8 between WT BMDCs and ASC<sup>−/−</sup> BMDCs. Interestingly CARD12 was not expressed in ASC<sup>−/−</sup> BMDCs.
Figure 14: Western blot analysis of Chlamydia stimulated WT and ASC−/− BMDCs protein expression. A. ASC deficiency resulted in the loss of CARD12 expression, however, no significant changes were observed with the other ASC–dependent inflammasome components NLRP3 or AIM2. B. Casp–8 expression remained similar, while the absence of ASC increased pro–Casp–1, Casp–11, and, IL–18.

6.10 Chlamydia stimulated ASC−/− BMDCs exhibit mitochondrial damage

Numerous studies have indicated that the mitochondria functions as a regulator of NLRP3 inflammasome activation (31, 32), yet little is known about the role of the inflammasome on mitochondrial dynamics. Therefore, we investigated the impact of ASC on mitochondrial morphology. The morphology of mitochondria in WT and ASC−/− BMDCs in the presence or absence of Chlamydia was evaluated by electron transmission microscopy (TEM) (Figure 15). The results showed that the ultrastructure of nearly all tubular mitochondria was well preserved in unstimulated and stimulated WT DCs and
unstimulated ASC<sup>−/−</sup> DCs. However, in stimulated ASC<sup>−/−</sup> BMDCs we observed some discernible changes in mitochondrial morphology compared to the normal narrow pleomorphic cristae in unstimulated and stimulated WTBM DCs and unstimulated ASC<sup>−/−</sup> BMDCs, mitochondria of stimulated ASC<sup>−/−</sup> DCs were observed to have undergone a gradual disruption of the cristae. In some of the abnormal mitochondria, few remnants of the cristae were observed.

It should be noted that we also analyzed several apoptotic protein expressions within the BMDCs of both WT and ASC<sup>−/−</sup>. The expression levels of Bcl–2, Bax, ANT1/2/3/4, and Cytochrome C were not detectable from either Chlamydia stimulated BMDC group. These findings indicate that ASC deficiency preserves the mitochondrial outer member following Chlamydia stimulation and does not result in an apoptotic BMDC.
Figure 15: TEM images of WT and ASC -/- BMDCs pre- and post- *Chlamydia* stimulation. Deletion of ASC leads to morphology changes only within the BMDC mitochondria stimulated with *Chlamydia*. Magnification is 30,000X; scale bar indicates 400nm.

### 6.11 Effect of ASC on DC immunometabolism

The damage exhibited by the mitochondria from the *Chlamydia* stimulated ASC -/- BMDC prompted us to examine the glycolytic pathway. We hypothesized that ASC deficiency might cause changes in the DC metabolic pathway. To determine the effect of ASC deficiency on immunometabolism in *Chlamydia* stimulated DCs, we evaluated the pyruvate production and glycolytic enzyme expressions (Figure 16). Results showed that
pyruvate production in ASC−/− BMDCs was markedly reduced compared to WT BMDCs in response to *Chlamydia* stimulation (Figure 16A). HEK II, ENO-1, and TIM protein levels were not affected by the absence of ASC pre- or post-*Chlamydia* stimulation (Figure 16B). Interestingly, PDHA1 expression was gradually reduced in stimulated ASC−/− BMDCs. PDHA1 is encoded by the PDHA1 gene and provides the primary link between glycolysis and the tricarboxylic acid (TCA) cycle by catalyzing the irreversible conversion of pyruvate into acetyl-CoA, which is utilized for protein acetylation and fatty acid synthesis. Those results suggest that ASC deficiency inhibited a needed alternation in metabolism of DCs and down–regulated the conversion of pyruvate into acetyl-CoA. Both of which are required to meet the increased bioenergetics and biosynthetic demands of an activated DC, specifically by funneling metabolites into pathway for lipid and protein synthesis. This downregulation in the conversion of pyruvate into acetyl-CoA coincides with the damaged mitochondrial cristae seen in infected ASC−/− DCs (Figure 15).
Figure 16: ASC−/− results in a decrease of pyruvate production in Chlamydia stimulated BMDCs. A. Pyruvate concentration post Chlamydia stimulation were significantly reduced within the ASC−/− BMDCs (p<0.05). B. Glycolytic enzymes, HEK II, ENO-1, and TIM protein levels were not affected by the absence of ASC pre- or post-Chlamydia stimulation. PDHA1 expression was gradually abolished in pulsed ASC−/− BMDCs.
CHAPTER VII
DISCUSSION

*C. trachomatis* infections are the most common bacterial STI within the US, affecting the mucosal tissues of humans, mammals, and, birds. A persistent infection within the host exerts a cytotoxic effect on the reproductive system, contributing to tubal pathology and ending with TFI (29). Initiation of adaptive immunity is required for *Chlamydia* clearance; however, the recruitment of inflammasomes, activation of inflammatory caspases, and, the ensuing inflammatory response is also known to be a contributing factor to Chlamydia induced pathology (10). It has been suggested that host inflammatory and anti – microbial immune responses to infection involving cytokines are the key determinants of *Chlamydia*-induced tubal pathology (30). Therefore, there needs to be a way to regulate the delicate balance between a protective anti – microbial and a pathologic immune response during *Chlamydia* infection (27).

IL–10 is a multifunctional anti–inflammatory cytokine with diverse effects on most hemopoietic cell types, including regulation of DC and Th cell growth and differentiation (33, 34). As expected, an increase in the production of IL–10 was reported following *C. trachomatis* infection; however, it should also be noted that a shorter course of infection was observed in the absence of the cytokine (35, 36). Our
group decided to further investigate the connection of *Chlamydia* and IL–10 in hopes of discovering unknown links between IL–10 and *Chlamydia* induced pathologies.

To investigate the role of IL-10 in the protective and immunopathogenic responses during chlamydial infection, we tested the hypothesis that IL-10 regulates inflammasome activation in DCs exposed to *Chlamydia*, thereby influencing the APC function and the induction of immunity that controls inflammatory tubal pathology and infertility. Results of this study suggest that IL-10 deficiency limits tubal damage and infertility in *Chlamydia* infected mice, and it enhances the antigen handling function of DCs while suppressing caspase-induced DC apoptosis. The capacity to assemble NLRP3 inflammasome was also diminished in *Chlamydia* stimulated IL-10−/− DCs. This study describes the regulatory role of IL-10 in DC inflammasome assembly, which might be part of the mechanism that forms the basis for the rapid *Chlamydia* clearance, retention of normal tissue architecture and essentially normal fertility rates in *Chlamydia* infected IL-10−/− mice (27).

Of particular interest from our study was the observed 3.5 fold downregulation of ASC from IL−10−/− DCs in comparison to WT DCs, which lead to an IL−10 and ASC− dependent inflammasome linkage. Taken in to consideration: 1) The innate immune response to *Chlamydia* infections is driven by the activation of inflammatory caspases, 2) inflammasome assembly is required for inflammatory caspase activation, and, 3) ASC downregulation was associated with decreased chlamydial induced pathology in IL−10−/− mice, we decided to investigate the immunoregulatory effects of ASC on known
*Chlamydia* induced tubal pathologies. It has previously been reported that a single chlamydial infection of the UGT does not result in tubal scarring; however, prolonged exposure to *Chlamydia* due to a chronic persistent infection or frequent reinfection has been associated with chronic inflammation associated with TFI (2). Inflammasome activation produces the essential pro–inflammatory cytokines, which in turn recruit an influx of immune cells necessary to begin the clearance of *C. trachomatis*. Therefore, we examined the association between ASC and *Chlamydia* through an infectivity study following an acute infection. Based on previous data, the expectation was that ASC−/− mice course of infection would be similar to the IL−10 studies. However, the opposite was observed. ASC−/− and WT mice IFU count trend was similar for the first week, but by the second week the course of infection for WT mice began to decline as usual. IFUs reported in the ASC−/− mice began to increase until well into the third week, and then only experienced a slight decrease, ending with an increased number of IFUs in comparison to the WT mice. Our WT mice infectivity data coincides with that of documented primary *Chlamydia* infections (37). The course of infection was also evident within the histological assessments and fertility study, which focused more on the effects of chronic infections.

Successful fertility relies on intact, functioning, and, secreting cells within the UGT. An Immunological Paradigm for Pathogenesis emerges as a consequence of recurrent infections *C. trachomatis*. This induced histopathology results in the influx of required immune cells to the site of infection for clearance, thus damaging ciliated cells
and scarring tissues (2, 8). Hence, the cells can no longer transport the gamete through the UGT (9). We investigated the immunoregulatory effects of ASC on chronic *Chlamydia* induced histology, pathology, and, fertility. As expected from our infectivity study, due to the longer of incidence at higher IFUs, ASC−/− mice displayed more hydrosalpinx, one of the hallmarks of a *Chlamydia* infection, than the WT mice. Routine H&E staining was able to show a deficiency in ASC does not in fact cause unwarranted inflammation prior to infection. This piece of data was extremely important because it allows for a true baseline for all future experiments involving this particular knockout model. Tissue damage within the UGT of ASC−/− mice was more severe in comparison to the UGT of WT mice. A possible explanation of these results involves the difference in chlamydiae bacterial load within the two groups. ASC−/− mice experienced a longer duration at higher IFUs. Such an environment is not only optimal for ascension of the bacteria into the UGT, but also provides enough time for binary fission. As observed in the histological data, fertility analysis confirmed that ASC deficient mice were not predisposed to fertility issues prior to infection, while at the same time proving to be more susceptible to *Chlamydia* induced infertility. These findings also warrant the question if cellular damage was not so widespread, would ASC+/+ show an even greater difference to the WT mice within the infectivity study.

Further analysis into the environment of the infected GT of WT and ASC−/− lead us to more interesting results. Confocal data revealed a decrease in the distribution of immune cell populations within GT ASC−/− mice, which was expected; however, the
exhibited cell profile was not. The population of Macrophages and CD4+ T – cells were significantly reduced, but the infected ASC−/− DC population closely matched that of the infected WT DC population. These findings raised the question if ASC deficiency affects the ability of DCs to function properly, possibly with antigen presentation. An UGT T–cell co–culture revealed that infected ASC−/− DCs lacked the ability to present antigen and overall T–cell proliferation decreased. The co–culture was followed by cytokine and immunoblotting analysis, with both confirming that ASC deficiency causes a loss of the protective immunity seen within WT mice. Because the inflammasome is important in activating the innate immunity and the ensuing adaptive immunity response (9, 38), signaling for these downstream effects relies on the capability of APCs to function properly and in rapid succession for clearance. DCs are important when it comes to antigen presentation; they are the link between the innate and adaptive immune systems post C. trachomatis infection (38). Here we have shown that there exist a disconnect with switching between the immunity systems, and it appears that the DC’s ability to function properly is at the root of the issues.

In an effort to better understand how the absence of ASC affects the ability of DC’s to not only function properly, but also to mature in response to a pathogen, we started to focus on the effects of Chlamydia stimulation on BMDCs. During preliminary Flow Cytometry studies, we began to notice that the expression of ASC deficient BMDC surface markers was in opposition to that of WT BMDC surface marker expressions. During maturation, DCs strongly up-regulate MHC antigens, and costimulatory
molecules such as CD40, CD80 and CD86, which are crucial for effective T-cell activation (38–40). But, in this study we observed a downregulation in both CD40 and MHC II expression, and an upregulation in CD103 expression. Emerging data have begun to identify new DC population subsets, similar to that of T–cells and macrophages. The classical designations of these subsets fall into two categories known either as immunogenic or tolerant (41). Everts and colleagues go on to postulate that the immunogenic DCs display an increased glycolytic and MHC expression, and initiate inflammatory T–cell responses (42). Subsequently, tolerogenic DC subsets are characteristically resistant to maturation, immune modulatory factors, and have low glycolytic levels, which are strongly correlated with an increase in regulatory T–cell responses (43–46). Likewise, our co–culture and adoptive transfer assays resulted in a decrease in the ability of ASC−/− BMDCs to effectively present and activate T–cells.

It is becoming increasingly clear that DC activation coincides with cellular metabolism that is tailored towards the bioenergetic and biosynthetic needs of DCs (47). Notable changes include enhanced glycolysis, the accumulation of succinate and the biosynthesis of fatty acids from citrate to support lipid biosynthesis, facilitating the expansion of ER and Golgi apparatus and increasing the biosynthetic capacity that is essential for the maturation and activation of DCs (48–51). Oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) are the main energy sources of DCs in the immature state, and upon LPS stimulation, DCs switch from OXPHOS to anabolic glycolysis (52), in order to rapidly produce ATP for its maturation and activation (53). It
has been established that mitochondrial changes, such as a decrease in mitochondrial transmembrane potential (ΔΨm) along with the release of cytochrome C regulates NLRP3 inflammasome activation (54). Here we have shown that in the absence of ASC, *Chlamydia* stimulated BDMCs exhibit decreased pyruvate production and are unable to undergo the metabolic alterations due to the cristae obliteration. Yet, through our immunoblotting analysis, we have shown that ASC deficiency does not result in the detection of apoptotic proteins, have an effect on the expression of glycolytic proteins or the ASC–dependent inflammasome components NLRP3 or AIM2. There was, however, a decrease in the expression of PDHA1 and the other ASC–dependent inflammasome component CADR12. Interestingly, the NBS domain of NLRC4 is a pocket with specificity for ATP and dATP and a requirement for CARD12 oligomerization, association, and, activation of caspase-1 (55). ASC induced mitochondrial disruption appears to impair the generation of ATP, which may abolish ATP/dATP binding and markedly reduce CARD12 self-association and interaction with pro–caspase–1. Our data also suggest that the mitochondria cristae damage associated with the loss of ASC, may in turn increase reactive oxygen species (ROS) production, and subsequently enhance c-Jun N-terminal kinase (JNK) signaling, which would result in the observed increased expression of caspase-11 and trigger production of both caspase–1 and IL–18 (56).
CHAPTER VIII
CONCLUSION

Infectious agents can damage biological functions of the female reproductive tract with devastating consequences. Among the most common microorganisms involved in STIs and interfering with female fertility is *C. trachomatis*. The impairment from this pathogen can be viewed within the cervix, fallopian tubes and tubal luminal architecture in infected women (2, 8-9, 23). Within this study, we have been able to provide more evidence which supports the claims that ASC is emerging as a key mediator in both protection and inflammation. Additionally, we have suggested a very plausible connection between the role of ASC, GT pathology, infertility, and, *Chlamydia* infections. Our initial data indicates the important immuno–protective role of ASC that may be required for mucosal immunity against chronic *Chlamydia* GT infection and *Chlamydia* induced tubal damage and TFI in the mouse model. However, continued investigations into the mechanisms of chronic *Chlamydia* induced cellular and tissue damage are required in order to develop further our understanding on the pathogenesis of GT disease caused by this specific bacterium, and to direct research into effective ways to control *C. trachomatis* infection, including the possibility of assigning ASC as a potential biomarker for TFI post *Chlamydia* infection. Moreover, this study has allowed us to
propose that ASC is an important molecule in the reprogramming of DC metabolism and in keeping mitochondrial structure intact after stimulation with *Chlamydia* (57–60). In line with that, infected ASC−/− DCs are unable to rapidly activate and effectively present antigen to T–cells, which is crucial for controlling *Chlamydia* clearance and preventing future complications (48, 55, 61, 62). This study was able to establish a baseline for determining the effect of the inflammasome on DC immunometabolism in *Chlamydia* immunity. However, we are only beginning to understand the extent to which ASC–metabolism is interlinked with the functional properties of DCs. There are still many questions which will rely on future investigations. It is of the upmost importance to fully understand the role of ASC in DC immunometabolism, which not only will improve our fundamental understanding of the biology of DCs, but will also be important in the development of metabolism–based approaches to improve the efficacy of DC–based immunotherapies and vaccines against *Chlamydia* infection.
REFERENCES


