CHARACTERIZING THE ROLE OF INNATE AND ADAPTIVE IMMUNE RESPONSES IN THE PATHOGENESIS OF *CHLAMYDIA* GENITAL TRACT INFECTION

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CHARACTERIZING THE ROLE OF INNATE AND ADAPTIVE IMMUNE RESPONSES IN THE PATHOGENESIS OF *CHLAMYDIA* GENITAL TRACT INFECTION

Lauren C. Frazer, PhD

University of Pittsburgh, 2013

Sexually transmitted infections with *Chlamydia trachomatis* are highly prevalent and can lead to irreversible tissue damage if infection ascends from the cervix to the upper reproductive tract. The goal of these studies was to determine key immune responses involved in either the development of oviduct pathology or protection from infection. Using the mouse model of *Chlamydia* genital tract infection, we determined that increased neutrophil recruitment and delayed apoptosis were associated with enhanced oviduct damage. Despite the important role of neutrophils in *Chlamydia*-induced tissue damage, we were unable to detect a central role for IL-17, IL-22, or IL-23 in neutrophil recruitment or the development of pathology. We found that IL-17 did promote Th1 immunity to infection but was not required for normal resolution of infection. Finally, we determined that expression of the adaptor molecule MyD88 by CD4+ T cells was required for efficient resolution of *Chlamydia* from the genital tract due to its role in enhancing the survival of these cells. These studies revealed that increased influx, survival, or activation of innate immune cells in the genital tract was associated with enhanced disease while prolonged survival of CD4+ T cells was associated with more efficient clearance of bacteria from the genital tract.

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ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
CDC	Centers for Disease Control and Prevention
CM3.1	Plasmid-cured strain of *Chlamydia muridarum*
CTD153	Plasmid-cured strain of *Chlamydia trachomatis* serovar D
D/UW-3/Cx	Wild-type strain of *Chlamydia trachomatis* serovar D
EB	Elementary body
FTOC	Fallopian Tube Organ Culture
hsp60	Heat shock protein 60
IFU	Inclusion forming units
i.p.	Intraperitoneal
i.v.	Intravenous
MMP	Matrix metalloproteinase
MOMP	Major Outer Membrane Protein
Nigg	Wild-type strain of *C. muridarum*
NOS	Nitric oxide synthase
ompA	outer membrane protein A, gene for MOMP
PBMC	Peripheral blood mononuclear cell
PFU	Plaque forming units

PI	Propidium Iodide
PID	Pelvic Inflammatory Disease
RB	Reticulate body
SFU	Spot forming units detected by ELISPOT
SNP	Single nucleotide polymorphism
TFI	Tubal Factor Infertility
TLR	Toll-like Receptor
UGT	Upper genital tract
WHO	World Health Organization

1.0 INTRODUCTION

1.1 *CHLAMYDIA TRACHOMATIS*

1.1.1 Lifecycle

Chlamydia trachomatis is a gram-negative obligate intracellular bacterium that infects columnar epithelial cells lining mucosal surfaces. Replication occurs within a membrane bound vacuole known as an inclusion, and the lifecycle of *Chlamydia* consists of two forms: the infectious elementary body (EB) and the replicative reticulate body (RB). EBs infect host cells and differentiate into RBs, which after multiplying on a scale of several hundred fold, convert back into EBs. Infectious EBs are released after approximately 72 hours via either membrane lysis or controlled release of the inclusion from the cell via a process known as extrusion (1). Both EBs and RBs are transcriptionally active, and in a cell free culture system EBs were found to transcribe 655 genes and RBs to transcribe 893 genes (2). In order to sustain metabolic activity, EBs required an external source of glucose-6-phosphate, and RBs required adenosine triphosphate (ATP) (2). Thus, the intracellular niche of *Chlamydia* permits the acquisition of required resources from the host cell and also acts as a protective barrier against host defense mechanisms. This point was reinforced by the discovery that *Chlamydia trachomatis* lacks enzymes required for synthesis of the essential amino acid tryptophan (3-5). Serovars of

Chlamydia that infect the urogenital tract express a functional version of the last enzyme in this pathway, tryptophan synthase β. This enzyme synthesizes tryptophan from indole and serine, which are provided by commensal bacteria found in the genital tract. This reaction permits evasion of a key mechanism of host defense, IFNγ-induced tryptophan limitation, and serovars that lack tryptophan synthase β do not infect the urogenital tract (5, 6).

1.1.2 Serovar Classifications

Chlamydia trachomatis is classified into serovars based on the sequence of the ompA gene, which encodes the major outer membrane protein (MOMP). *Chlamydia trachomatis* serovars A-C infect the ocular epithelium, serovars D-K infect the urogenital epithelium, and serovars L1-L3 exhibit more invasive characteristics by spreading into the lymph nodes draining infected sites. MOMP represents 61% of the outer membrane protein content of EBs (7), but this classification strategy has been widely criticized due to its failure to represent the genetic relatedness of the genome beyond ompA (8, 9). Indeed, recombination of ompA genes is not an infrequent occurrence, which results in the phylogenetic clustering of strains that are otherwise genetically dissimilar (8).

1.1.3 Epidemiology

1.1.3.1 Sexually transmitted infections

Chlamydia trachomatis was the most common reportable infection in the United States in 2011, with over 1.4 million cases reported to The Centers for Disease Control (CDC) (10). Surveillance data indicate that the rates of infection are highest in women between the ages of 15

and 24, with approximately 3500 cases per 100,000 individuals (10). The prevalence of infection in the United States between 1998 and 2008 was 6.8% of sexually active females between the ages of 14-19 years of age (10). *Chlamydia trachomatis* urogenital infections are also highly prevalent globally. Estimates from the World Health Organization (WHO) indicate that in 2005, 98 million individuals were infected at any one time, which represented 3.53% of females and 2.22% males in the world (11).

1.1.3.2 Trachoma

Ocular infections with *Chlamydia trachomatis* are a devastating problem in the developing world. Infections are common in areas where individuals do not have access to clean water or proper sanitation, since infection is spread by contact with infected ocular or nasal secretions. Estimates from the WHO indicate that 71.2% of individuals that live in areas of endemic infection reside on the African continent (12). Disease resulting from *C. trachomatis* ocular infection progresses through several stages (13). Active trachoma is initiated by infection of the ocular epithelium with *C. trachomatis* and is characterized by the presence of conjunctival inflammation. Repeated infections can eventually result in scarring of the conjunctiva, inversion of the eyelids, and scratching of the cornea by direct contact with the inverted eyelashes (i.e. trachomatous trichiasis). Progression to blindness occurs when the cornea becomes opacified due to the combination of trachomatous trichiasis, opportunistic ocular infections, and exposure to environmental insults. According to the WHO, there are currently 21 million individuals with active trachoma and 7.3 million cases of trachomatis trichiasis worldwide (12). Studies indicate that 2.2 million people are visually impaired due to trachoma, and this number includes 1.3 million people that are irreversibly blind (14).

1.1.4 Pelvic inflammatory disease and reproductive tract sequelae

The CDC defines pelvic inflammatory disease (PID) as the presence of lower abdominal pain that cannot be attributed to another cause in association with cervical motion and adnexal tenderness in the presence of one of the following additional criteria: detection of *C. trachomatis* (Ct) or *Neisseria gonorrhoeae* (GC), sexual contact with someone with Ct or GC, temperature > 38°C, >10,000 WBC/mm^3, or evidence of inflammation or infection in the pelvis or peritoneum (15).

PID occurs when infection ascends from the lower to the upper genital tract (UGT) and in some cases into the peritoneal cavity. Ascension of bacteria to the Fallopian tubes results in irreversible tissue damage. We determined that mice experiencing several abbreviated infections with *C. muridarum* developed lower levels of genital tract pathology than those sustaining one infection that was not treated with antibiotics (16). We have also determined that primary infection with a plasmid-deficient strain of *Chlamydia* that does not cause disease can prevent upper tract damage upon challenge infection with a fully virulent strain of *Chlamydia* by inducing a memory response that reduces infection dramatically (16, 17). These data indicate that if the duration or the magnitude of infection in the upper genital tract is reduced, tissue damage can be avoided.

A prospective trial named the prevention of pelvic inflammation (POPI) trial was recently conducted in the England with the goal of determining if testing and treatment for *Chlamydia* could prevent the development of PID over a period of 12 months (18). Women were randomized to receive immediate testing and treatment for *C. trachomatis* or storage of their sample for testing after one year. The incidence of PID over the course of a year was 9.5% (7/74) in women that were positive at enrollment but not treated and 1.6% (1/63) in women that

were tested and treated immediately. Interestingly, 79% (30/38) of cases of PID occurred over in women that were negative at baseline, and 62% (16/26) of the these women with available test results were determined to have *C. trachomatis* when they presented with PID (18). These data indicate that delayed treatment is associated with an increased risk of PID and that annual screening for *C. trachomatis* may not be frequent enough to prevent the majority of individuals from developing PID. These conclusions were supported by comparison of infertility rates in a cohort of women with clinically suspected PID, which determined that 17% (18/101) of women who delayed seeking treatment for *Chlamydia* PID for 3 or more days were infertile or experienced an ectopic pregnancy compared to none (0/13) of the women who were treated immediately (19).

Repeated infections also represent a risk factor for the development of sequelae from *Chlamydia* genital tract infection. In a study examining a cohort of commercial sex workers from Nairobi, Kenya, it was determined that repeated *C. trachomatis* infections was an independent risk factor for the development PID (20). In a retrospective study of 11,000 women from Wisconsin with *Chlamydia*, it was determined that the risk of ectopic pregnancy and PID increased with the number of *Chlamydia* infections (21).

The presence of the clinical signs of PID is not necessarily predictive of whether an individual will go on to be infertile, since the diagnostic criteria are relatively non-specific. UGT damage was directly examined by laparoscopy in a study of 2500 women with clinical symptoms of PID (22). Cases were defined as women with clinically diagnosed PID and abnormal findings on laparoscopy and controls were women with clinically diagnosed PID with normal findings on laparoscopy. The authors determined that the presence of salpingitis on laparoscopy was associated with an increased risk of ectopic pregnancy in the first pregnancy after laparoscopy,

and ectopic pregnancy was diagnosed in 9.1% (100/1309) of cases and 1.4% (6/451) of controls. In addition, 16% (209/1309) of cases and 2.7% (12/451) of controls were infertile, and 141 of 209 (67%) infertile patients from the case group were diagnosed with tubal factor infertility (TFI) compared to none of the infertile individuals in the control group. This study also found that the severity of PID upon initial laparoscopy and the number of times an individual was diagnosed with PID were associated with an increased risk of TFI. The control group in this study consisted of women with symptoms of PID but normal findings on laparoscopy, and 87% of these women were determined to have a lower genital tract infection (22).

A separate study determined that the presence of UGT infection in women with clinically suspected PID was directly correlated with the development of salpingitis (23). In this study, all of the 16 patients with *C. trachomatis* infection of the Fallopian tubes and/or the endometrium exhibited signs of salpingitis by laparoscopy (23). None of the 24 patients that were negative for infection at the cervix exhibited salpingitis (23). However, 5 of 11 patients that had CT or GC in the cervix but not the UGT also exhibited signs of endometritis and had detectable salpingitis on laparoscopy.

Not all women with upper genital tract infection with *Chlamydia* exhibit symptoms of acute PID. Subclinical PID is defined as the presence of endometritis in women that do not meet the CDC's diagnostic criteria for acute PID, and subclinical PID is associated with the presence of UGT infection with *C. trachomatis* (24). *C. trachomatis* infection was detected in the endometrium of 20% of women with acute PID, 10% of women with subclinical PID, and 2% of women without endometritis (25). Of women with *C. trachomatis* infection at the cervix, *C. trachomatis* was detected in the endometrium of 27% of women with subclinical PID and 41% of women with acute PID (25). Subclinical PID is a significant risk factor for infertility. In a

separate study, it was determined that women who were diagnosed with subclinical PID on enrollment had a 40% reduced rate of pregnancy compared to controls (26). In addition, women with sexually transmitted infections without subclinical PID did not have a decreased rate of fertility (26).

These studies indicate that women may be at risk for upper reproductive tract damage even in the absence of symptoms of acute PID. Ascension of bacteria to the UGT can occur in the presence of non-specific abdominal symptoms that both the patient and the physician are unable to recognize. Studies demonstrating that annual testing is not frequent enough to prevent the development of PID from *Chlamydia* (18) and that a delay in treatment for longer than 3 days can increase the risk of infertility (19) indicate that only a vaccine will be able to prevent the tissue damage caused by this pathogen. Unfortunately, the immune response is both the primary mediator of protection and the cause of genital tract damage. Years of research have focused on differentiating the damaging from the beneficial immune response.

1.2 IMMUNE MEDIATORS OF DISEASE AND PROTECTION

1.2.1 Neutrophils

1.2.1.1 Role of neutrophils in pathology

Examination of leukocytes in the endometrial biopsies of women with clinically suspected PID revealed that UGT infection and salpingitis could be diagnosed with a 92% sensitivity and 87% specificity by the presence of ≥1 plasma cell in the endometrial stroma per x 120 field and ≥ 5 neutrophils in the endometrial epithelium per x 400 field (23). This study led to our current

diagnostic criteria for endometritis (23). A separate study of women at risk for PID found that increased vaginal levels of neutrophil alpha defensins, a marker of neutrophil activation, could be detected in women with endometritis from *Chlamydia* (27). In addition, significantly higher numbers of neutrophils were detected in the endocervical brush samples from women with *C. trachomatis* infection before treatment relative to after treatment (28).

These reports indicate a potential role for neutrophils in the development of *Chlamydia*-induced immunopathology. Studies in the mouse model have supported this correlation by revealing that enhanced and/or prolonged neutrophil influx into the oviducts is associated with the development of hydrosalpinx (29, 30). In addition, mice deficient in CXCR2, the receptor for neutrophil chemokines including CXCL2, develop reduced levels of acute inflammation in the genital tract and lower rates of hydrosalpinx (31). More severe disease is observed in strains of mice with elevated levels of CXCL2 (32). Intravaginal infection of mice with a plasmid deficient strain of *C. muridarum*, CM3.1, results in significantly reduced production of CXCL2 and decreased neutrophil influx into the oviducts, which is associated with decreased oviduct pathology compared to infection with wild-type *C. muridarum* (17, 33). In addition, TLR2 deficient mice exhibit significantly lower levels of CXCL2 in their lower genital tract secretions and significantly decreased oviduct pathology following *C. muridarum* infection (34). Finally, a direct correlation between neutrophils and the development of pathology was made in the guinea pig model, where administration of a polyclonal anti-neutrophil antibody successfully depleted neutrophils during ocular infection with *Chlamydia caviae* and resulted in significantly decreased pathology (35).

Neutrophils likely contribute to pathology by releasing cytokines, reactive oxygen species, and proteases, which can directly damage vulnerable tissues. For example, neutrophil

release of the proteolytic enzyme matrix metalloproteinase-9 (MMP9; gelatinase B) has been implicated in the development of scarring and fibrosis of the murine oviduct after chlamydial infection (36-38). A strain of mice with an increased susceptibility to oviduct pathology exhibited enhanced MMP9 activity in the upper genital tract early during acute infection (36). In addition, mice treated with MMP inhibitors or mice deficient in MMP9 developed less severe oviduct pathology (37, 38). Finally, mice with an impaired ability to generate reactive oxygen species due to a deficiency in NADPH oxidase (*p47phox*$^{-/-}$) sustain lower rates of *Chlamydia*-induced hydrosalpinx (39).

The correlation between neutrophils and disease has been examined extensively in populations where trachoma is endemic. Analysis of gene expression in conjunctival swabs from individuals in Tanzania revealed an association between trachomatous scarring and significantly increased expression of MMP 7, 9, and 12 (40). Increased expression of the neutrophil chemokine CXCL5 as well as the proinflammatory cytokines TNFα and IL-1β was also detected in these patients relative to individuals without trachomatous scarring (40). Similar results were observed in a study using microarray to compare conjunctival gene expression in individuals from Ethiopia with and without trachomatous trichiasis (41). In a study of individuals from The Gambia, a SNP in the promoter region of the gene for the neutrophil chemokine IL-8 (IL-8 251TT) was associated with a significantly decreased risk of developing scarring trachoma (42). The protection provided by this SNP was further enhanced by the presence of a protective MMP9 allele (MMP9 Q279R), which resulted in a 70% increase in protection from developing scarring trachoma (42). This SNP resulted in a non-synonymous substitution of an arginine for a glutamine in the MMP9 active site. Levels of MMP9 expression have been directly correlated with the level of conjunctival inflammation and the presence of ocular *Chlamydia* infection in

individuals from The Gambia (43). In addition, increased MMP9 activity has been observed in the conjunctival biopsies of children with active trachoma (44). Immunohistochemistry conducted on these specimens revealed expression of MMP9 by neutrophils and CD68+ macrophages infiltrating the conjunctival epithelium and stroma (44).

1.2.1.2 Role of neutrophils in host defense

Studies in animal models have argued against a significant contribution of neutrophils in control of chlamydial infection. Antibody-mediated depletion of neutrophils during ocular infection of guinea pigs did impact resolution of infection (35). Mice deficient in MHC class II exhibit a robust innate inflammatory response, but infection remains at peak levels indefinitely (45). In addition, CXCR2 knockout mice resolve infection normally despite a reduced neutrophil influx (31). These in vivo findings are likely due to the intracellular lifecycle of *Chlamydia*. In contrast, neutrophils are highly effective at killing *Chlamydia* in vitro. Ingested EBs are rapidly internalized and degraded in the phagolysosomes of neutrophils (46). Incubation of *Chlamydia* with neutrophils at a 1:1 ratio results in the uptake of about 60% of bacteria within 15 minutes (47). After 10 hours of incubation, infectivity is reduced by more than 95% (47). Enzymes present in the phagolysosomes of neutrophils eliminate the bacteria. Lysozyme purified from human neutrophils has been shown to induce a 76% reduction in inclusion formation after incubation with EBs for one hour (48). Generation of reactive oxygen species is not crucial for neutrophil-mediated killing of *Chlamydia*, since neutrophils from patients with myeloperoxidase deficiency or phagocyte NADPH oxidase deficiency effectively kill internalized microorganism in vitro (49), and resolution of infection is normal in NADPH oxidase deficient mice (39).

1.2.2 CD8+ T cells

1.2.2.1 Role of CD8+ T cells in host defense in mice

Similar to neutrophils, CD8+ T cells have been shown to possess anti-chlamydial activity, but the importance of these cells in host defense remains controversial. In the mouse model, a deficiency in CD8+ T cells does not impair infection control. Mice deficient in expression of MHC class I molecules ($\beta_2$microglobulin$^{-/-}$ mice; TAP1$^{-/-}$ mice) exhibit normal resolution of both primary and challenge infection with *C. muridarum* (45, 50). Mice deficient in CD8 or treated with anti-CD8 antibody also resolve infection normally (50), as do C57BL/6 and antibody deficient mice (μMT$^{-/-}$ mice) depleted of CD8+ T cells upon challenge infection (51). In addition, mice deficient in pathways used by CD8+ T cells to lyse target cells including perforin (50, 52), Fas (52), Fas ligand (FasL), (52), and both perforin and FasL (52) resolve infection normally without exhibiting a compensatory cytokine response. Lastly, adoptive transfer of CD8+ T cells isolated from the spleens of mice that had resolved both primary and secondary genital tract infection with *C. muridarum* conferred no protection when transferred into immunologically normal mice prior to infection (53).

CD8+ T cells can provide a limited degree of protection in specific adoptive transfer models. CD8+ T cell clones isolated from the spleens of mice after intravenous (i.v.) injection of *C. muridarum* have been demonstrated to promote resolution of infection when transferred into intravaginally infected nude mice and to produce both IFNγ and TNFα in a *Chlamydia*-specific manner (54). In two separate studies, CD8+ T cells isolated from the spleen of mice infected either intraperitoneally (i.p.) or intravenously (i.v.) with *C. trachomatis* serovar L2 were protective upon subsequent i.v. infection with the same strain of *Chlamydia*, and this protection

was IFNγ dependent (55, 56). Detailed characterization of a CD8+ T cell line isolated after i.p. injection of L2 revealed that upon adoptive transfer, it only resulted in a 3-fold reduction in L2 in the spleen after i.v. infection (57). However, immunization of mice with a vaccinia virus expressing CrpA, the antigen recognized by these cells, resulted in a 18-fold reduction in splenic bacterial burden upon i.v. challenge with L2, which correlated with a robust antigen-specific IFNγ response (57). An additional study demonstrated that the degree of protection provided by CD8+ T cells was antigen specific. Although CD8+ T cells specific for a protein named class 1 accessible protein-1 (Cap1) could lyse *Chlamydia*-infected cells in vitro, immunization with a Cap1 expressing vaccinia virus conferred minimal protection upon subsequent i.v. challenge with L2 (58). These murine studies must be interpreted with caution because L2 is a human strain, which exhibits an increased susceptibility to IFNγ in a murine host (59). In addition, CD8+ T cells examined in these studies were elicited after i.v. or i.p. infection, which may not reflect the immune response that results from an intravaginal infection.

1.2.2.2 Role of CD8+ T cells in pathology in mice

Studies in the mouse model also indicate that CD8+ T cells contribute to the development of *Chlamydia*-induced immunopathology. Mice deficient in CD8+ T cells or perforin develop reduced levels of oviduct pathology upon intravaginal infection with *C. muridarum* (50, 52, 60). Adoptive transfer studies revealed that CD8+ T cell production of TNFα was a significant mediator of oviduct pathology and did not significantly contribute to host defense (50).

1.2.2.3 CD8+ T cell responses detected in humans

CD8+ T cells expand in response to *Chlamydia* genital tract infection in humans. *Chlamydia*-specific CD8+ T cells have been isolated from the peripheral blood of individuals with positive serology for *Chlamydia* or a reported history of *Chlamydia* infection (61-63). Further analysis of the MHC restriction of these CD8+ T cells revealed a combination of MHC class Ia restricted and non-restricted responses. The authors of one study determined that MHC class Ia non-restricted CD8+ T cells were able to lyse *Chlamydia*-infected cells, but the MHC class Ia restricted CD8+ T cells were only able to do so if infected cells were also pulsed with their target antigen, OmcB (63). However, lysis of the target cell was not necessary for this clone to inhibit chlamydial growth in fibroblasts (62). A second study determined that both HLA-A2 restricted and non-restricted CD8+ T cell clones were able to produce IFNγ and lyse *Chlamydia*-infected cells (63). The authors also noted that the level of IFNγ production and the rapidity of target cell lysis were increased in the MHC non-restricted clones relative to the restricted clones (63).

CD8+ T cells specific for MOMP peptides have been isolated from the peripheral blood of both males and females with genital tract infection with *C. trachomatis* who expressed the MHC alleles HLA-A2 and HLA-B51 (64). These cells were found to lyse cervical epithelial cells expressing the appropriate MHC that were either pulsed with peptide or infected with *C. trachomatis* (64). Interestingly, all of the infected individuals expressing HLA-A2 (N=12) recognized at least one of the MOMP peptides examined. It is likely that the high response rate in this study was related to the 17 day pre-stimulation protocol utilized before analysis of CD8+ T cell responses (64). A follow-up study showed that these patients did indeed have MOMP peptide specific CD8+ T cells in the peripheral blood using direct ex-vivo staining with tetramers, and sorting of tetramer stained cells revealed *Chlamydia*-specific lytic activity (65).

Examination of CD8+ T cells in the genital tract of women infected with *Chlamydia* revealed an increased number of CD8+ T cells relative to uninfected women (66). Comparison of the phenotype of CD8+ T cells in the blood and endocervix of these women showed an increased frequency of effector memory CD8+ T cells (CD45RA-CCR7-) in the endocervix but decreased perforin expression by these CD8+ T cells relative to those in the blood (66). The antigen specificity of these cells was not determined, and the ability of CD8+ T cells to recognize *Chlamydia*-infected cells in the genital tract may be hampered by immune evasion mechanisms. In vitro, infection of a human endocervical cell line with *C. trachomatis* serovar D was shown to downregulate MHC class I expression in both infected cells and non-infected cells in the same well (67). Although incubation with IFNγ did result in increased expression of MHC class I in the presence of infection, this upregulation was less so than that observed for cells that were mock-infected, and application of supernatants from *Chlamydia*-infected cells to uninfected cells resulted in class I downregulation (67). However, MHC class I expression by conjunctival epithelial cells was previously detected by immunohistochemical staining of biopsies isolated from children with active trachoma (68).

1.2.2.4 Association of CD8+ T cell responses in humans with chlamydial disease

The association between CD8+ T cell responses and disease resulting from *Chlamydia* genital tract infection has not been extensively examined in humans. In a study of commercial sex workers in Nairobi, Kenya, the MHC class I allele HLA-A31 was associated with the development of *Chlamydia* PID in women with documented *Chlamydia* infection (20). The correlation of this allele with CD8+ T cell function was not determined.

Examination of the correlation between immune responses and disease is more feasible in regions where ocular infection with *Chlamydia* is endemic. Almost all of the individuals in the

population are exposed to *Chlamydia*, but only some individuals develop disease. For example, in a study of individuals from The Gambia with and without trachomatous scarring, approximately 90% of individuals in both the case and control groups had detectable antibody to *C. trachomatis* (69). In this study, it was determined that individuals with the MHC Class I allele HLA-A*6802 were 3 times more likely to develop scarring than individuals without this allele. Interestingly, in a follow-up study, *Chlamydia*-specific CD8+ T cell responses were not detected in the peripheral blood of individuals with and without trichiasis or trachomatous scarring that possessed the HLA-A*6802 allele. This is likely because only responses to predicted epitopes in the proteins MOMP, macrophage infectivity potentiator (MIP), and heat shock protein 70 (hsp70) were examined, but responses to infected cells were not examined (70). In another study of peripheral blood responses of individuals from The Gambia with HLA-B8 and HLA-B35 alleles, cytolytic responses were detected in response to predicted MOMP and heat shock protein 60 (hsp60) peptides in 6 of 26 people examined, and responses were only detected for CD8+ T cells isolated from children that were in the process of resolving infection or adults without scarring. No responses were detected for the PBMCs of adults with trachomatous scarring (71). A later study using tetramers to examine HLA-A2 specific responses to MOMP peptides in the peripheral blood of children from The Gambia, revealed an association between positive tetramer staining and the presence of active infection or repeated episodes of infection (72). No association was found between tetramer binding and the presence of clinical symptoms (72).

1.2.3 CD4+ T cells

1.2.3.1 Role of CD4+ T cells in host defense in mice

CD4+ T cells have been repeatedly demonstrated as essential for control of *Chlamydia* in the mouse model of genital tract infection. It was shown in some of the first studies characterizing the mouse model of *Chlamydia muridarum* infection that athymic nude mice could not resolve infection at all (73). Another early study showed that SCID mice exhibited minimal clearance of infection from the genital tract, and disseminated infection was common (74). These findings were recapitulated by a study showing that TCRβ chain deficient mice could not resolve infection in contrast to TCRδ chain deficient mice, which controlled infection normally (75). The specific role of CD4+ T cells was demonstrated in a study where MHC Class $II^{-/-}$ and $CD4^{-/-}$ mice exhibited significantly delayed resolution of infection (45). During secondary genital tract infection, depletion of CD4+ T cells from C57BL/6 or B cell ($\mu MT^{-/-}$) deficient mice was shown to significantly delay resolution of infection (51).

Adoptive transfer studies have supported the central role for CD4+ T cells in resolution of infection in the mouse model. Adoptive transfer of *Chlamydia*-specific T cell lines that were enriched for CD4+ T cells promoted faster resolution of infection than a line of mixed CD4+/CD8+ T cells (76), and transfer of CD4+ T cells from mice that had resolved either primary or secondary intravaginal infection with *C. muridarum* conferred significant protection when transferred into naïve syngenic recipients (53). An additional study showed that adoptive transfer of a *C. muridarum* specific CD4+ T cell line that produced IL-2, IFNγ, and TNFα elicited clearance of infection in nude recipient mice (77).

1.2.3.2 Role of CD4+ T cells in pathology in mice

Studies in mice indicate that the magnitude of the CD4+ T cell IFNγ response is inversely correlated with the development of oviduct pathology. MHC Class $II^{-/-}$ mice (45) and $IFN\gamma^{-/-}$ mice (78) develop severe oviduct pathology, which is likely the result of the significantly increased bacterial burden in the genital tract of these mice. C57BL/6 mice exhibit a more robust CD4+ T cell IFNγ response and decreased IL-10 production relative to C3H/HeN or Balb/c mice, and this is associated with faster resolution of *Chlamydia* genital tract infection and lower levels of oviduct pathology (30, 79). $IL\text{-}10^{-/-}$ mice rapidly prime a *Chlamydia* specific CD4+ T cell IFNγ response and show faster resolution of primary infection, resistance to challenge infection, and dramatically reduced oviduct pathology (80). Transfer of CD4+ T cells specific for chlamydial protease-like activating factor (CPAF) that were capable of producing IFNγ resulted in faster resolution of infection and prevention of oviduct pathology (81). Upon challenge infection, a decreased frequency of neutrophils and an increased frequency of IFNγ producing T cells in the genital tract have been associated with reduced levels of ascending infection and decreased pathology (16). A renewed susceptibility to genital tract infection after resolution of primary infection coincides with the departure of *Chlamydia* specific T cells from the genital tract (82).

1.2.3.3 CD4+ T cell responses detected in humans

The role of CD4+ T cell responses to *Chlamydia* is not as clear in humans. IFNγ production by PBMCs in response to the *Chlamydia* hsp60 is correlated with protection. A study examining the PBMC responses of commercial sex workers in Nairobi, Kenya determined that production of IFNγ in response to hsp60 was associated with reduced risk of incident *Chlamydia* genital

tract infection (AHR: 0.2, 95% CI, 0.02-1.0) (83). Indeed, none of the 29 women in the study with a detectable IFNγ response to hsp60 developed an incident infection over the 20-month follow-up period, although there was an annual rate of infection of 24% in the study population as a whole (83). A subsequent study revealed that CD4+ T cells (PBMCs depleted of CD8+ T cells) produced IFNγ at a significantly higher frequency than CD8+ T cells (PBMCS depleted of CD4+ T cells) in response to EBs and hsp60-1 antigen (84a). The authors also detected a direct correlation between the number of IFNγ spot forming cells (SFCs) in the peripheral blood and endometrium (84). The protective role of T cell responses to *Chlamydia* hsp60 was supported by a study in Australia. Women presenting with their first *C. trachomatis* infection and uninfected women exhibited similar levels of IFNγ production by PBMCs in response to hsp60, but levels were significantly reduced in women with repeated infections or with *Chlamydia* PID (85).

Genital tract infection with *C. trachomatis* promotes trafficking of lymphocytes to the endocervix. Significantly increased numbers of CD3+ T cells were detected in the cervix of women with *C. trachomatis* before treatment compared to after treatment, but there was no alteration in the frequency of CD4+ and CD8+ T cells relative to those detected in the peripheral blood (28). The frequency of CD3+ T cells expressing CD45RO, CCR5, and CD103 (αEβ7) was significantly higher than in the peripheral blood. Interestingly, there was a highly significant correlation between the number of neutrophils and the number of CD3+ T cells in the endocervix ($r = 0.82$, $P < 0.0001$) (28). This study did not examine the antigen specificity of this response.

Peripheral blood responses to chlamydial antigens have also been detected in individuals from trachoma endemic areas. A study of individuals from The Gambia with and without trachomatous scarring determined that stimulation of PBMCs with MOMP or EBs resulted in increased levels of IFNγ production relative to unstimulated samples in both groups, but only

individuals without scarring had detectable levels of IFNγ in response to hsp60 (86). In addition, only the individuals with scarring trachoma had a detectable IL-4 response to hsp60 (86). In this study, significant associations were detected between specific MHC class II alleles and immune responses. They were as follows: HLA-DRB1*11: increased IFNγ production in response to EBs, HLA-DRBI*1304: increased proliferation in response to hsp60, HLA-DQB1*06: reduced proliferation in response to EBs, HLA-DRB1*06: increased frequency of IL-4 producing PBMC in response to hsp60 (86). An additional study examining the peripheral blood responses of children in The Gambia detected increased proliferation of PBMCs in response to EBs, MOMP, and hsp60 in children that had recently resolved their trachomatous inflammation relative to those with continued inflammation; however, no differences were found in antigen-specific IFNγ production between the groups (87).

1.2.3.4 CD4+ T cell responses and disease in humans

A protective role for CD4+ T cells was demonstrated in a study of commercial sex workers from Nairobi, Kenya (20). For women with HIV and *Chlamydia* genital tract infection, the risk of developing PID was inversely correlated with the number of CD4+ T cells in the peripheral blood (20). A CD4+ T cell count of less than 400 was associated with a 21.7 fold increased risk of developing *Chlamydia* PID, but there was no association between the CD8+ T cell count and risk of PID (20). In an additional study from Nairobi, Kenya of women that were seeking treatment for infertility, the presence of specific MHC class II alleles was associated with infertility and *C. trachomatis* seropositivity (88). HLA-DQA*0101 (OR: 4.9) and HLA-DQB*0501 (OR: 6.8) were associated with an increased risk of *C. trachomatis* associated infertility and HLA-DQA*0102 (OR: 0.2) was associated with a decreased risk (88). The

interaction between these alleles and CD4+ T cell responses to *Chlamydia* was not examined in this study.

1.2.3.5 Mechanism of CD4+ T cell mediated control of infection

The phenotype of mononuclear cells infiltrating the genital tract of mice infected with *C. muridarum* has been examined in detail (89). The peak in *Chlamydia*-specific IFNγ production in the iliac lymph nodes (ILN) and genital tract was detected at 7 days post infection, while the number of *Chlamydia*-specific mononuclear cells in the genital tract peaked at 3 weeks post infection. Th1 cells dominated the antigen specific response to infection in this model, and very little IL-4 was detected at any point during infection (89).

One protective mechanism that CD4+ T cells use to control *Chlamydia* genital tract infection is the induction of nitric oxide production by IFNγ. Direct contact between CD4+ T cells and epithelial cells via LFA-1/ICAM-1 interactions enhances epithelial cell nitric oxide production and clearance of infection (90, 91). In addition, the protective capacity of a clonal population of CD4+ T cells transferred into nude mice was reversed by systemic administration a nitric oxide synthase (nos) inhibitor (92). Mice treated with an anti-IL-12p40 antibody but not those treated with an IL-4 neutralizing antibody exhibit prolonged infection, and neutralization of IL-12 was associated with significantly reduced production of IFNγ upon restimulation of splenocytes (75). Although, *Ifnγ*$^{-/-}$ mice clear 99.9% of *C. muridarum* infection from the genital tract with near normal kinetics (74, 75), and *inos*$^{-/-}$ mice appear to clear infection normally (93), significant deficits in host defense were observed in this mice. Mice deficient in IFNγ developed signs of systemic illness and increased rates bacterial dissemination (74, 75). They also exhibited a chronic low level of infection and prolonged neutrophil infiltration (74). Mice

deficient in inos developed a persistent infection that was reactivated when they were treated with cyclophosphamide at 120 days post-infection (39). In vitro experiments demonstrated that IFNγ was not able to completely eradicate bacteria in the absence of inos activity (39).

In vitro studies indicated that *Chlamydia*-specific CD4+ T cell clones could promote resolution of infection in vitro by inos-dependent mechanisms as well as inos-independent degranulation-dependent mechanisms (94). This degranulation-dependent mechanism related to expression of a protein known as placenta-specifc 8 (Plac8) (95). Although Plac8 knockout mice resolved infection normally during the first three weeks, complete elimination of bacteria from the genital tract was delayed, and treatment with an inos inhibitor lead to reactivation of infection. In addition, inhibition of inos in Plac8 knockout at the time of infection resulted in almost a complete impairment in elimination of the last three logs of bacteria from the genital tract, which was associated with the development of severe genital tract pathology and disseminated infection (95).

MHC class II expression by the infected mucosal epithelium is likely required for CD4+ T cell mediated resolution of infection. MHC Class II expression by epithelial cells was detected in the conjunctival biopsy specimens from children with active trachoma (68). Mechanistic experiments using a panel of *C. muridarum* specific T cell clones revealed that pre-treatment of murine oviduct epithelial cells with IFNγ induced the upregulation of MHC class II expression and improved clearance of *Chlamydia* from infected monolayers in vitro (96). If IFNγ was added at the time of infection or if IFNβ was also added to the epithelium, upregulation of MHC class II was reduced, and recognition of the infected epithelium by the clones was impaired (96).

1.2.4 Cytokines

1.2.4.1 IL-10

The role of IL-10 in *Chlamydia* pathogenesis is complex. Studies in a human Fallopian tube organ culture (FTOC) model showed that IL-10 was protective against tissue damage induced by infection with *Chlamydia trachomatis* (97). A protective role for IL-10 was supported by epidemiologic studies, which demonstrated an increased risk for moderate to severe tubal damage in women with TFI that possessed IL-10 polymorphisms associated with decreased IL-10 expression (98). In contrast, a study of commercial sex workers from Kenya determined that IL-10 production in response to *Chlamydia* hsp60 was associated with an increased risk of incident infection. However, upon multivariate analysis, the 5.3-fold increased risk of incident infection associated with an IL-10 response was no longer statistically significant (83). The contradictory conclusions made by the authors of these studies show that the role of IL-10 needs to be further examined.

In the mouse model, it is clear that IL-10 plays a pathologic role. Strains of mice with increased IL-10 production in response to intravaginal *Chlamydia* infection exhibit delayed resolution and increased disease (79). IL-$10^{-/-}$ clear infection rapidly, and ascension of bacteria to the oviduct is reduced (80). This improved infection control is associated with a 5 to 50 fold increase in the number of *Chlamydia*-specific IFNγ producing T cells in the genital tract between days 15 and 85 post infection (80). IL-$10^{-/-}$ antigen presenting cells (APCs) are more efficient at priming and activating a Th1 response to *Chlamydia* (99). These APCs rapidly express cytokines and activation markers in response to EBs (99). Transfer of IL-$10^{-/-}$ dendritic cells pulsed with EBs results in significant protection against subsequent vaginal challenge (80).

1.2.4.2 IL-1

In vitro experiments and studies in the mouse model point to a pathologic role of IL-1 receptor (IL-1R) signaling during *Chlamydia* infection. A direct role for IL-1 signaling and oviduct damage was observed in a human Fallopian tube organ culture (FTOC) model in vitro (97). Inhibition of the IL-1 response to *Chlamydia trachomatis* infection by addition of IL-1 receptor antagonist (IL-1RA) led to decreased tissue damage. This study also demonstrated that addition of IL-1α to the FTOC could induce tissue damage even in the absence of *Chlamydia*, which was in line with the observation that the level of tissue damage observed during *Chlamydia* infection was out of proportion with the number of cells that were actually infected. In addition, IL-1 was shown to induce production of the neutrophil chemokine IL-8, which could further promote tissue damage in vivo by promoting the influx of neutrophils (97)

The pathogenic role for IL-1 signaling was demonstrated in the mouse model where IL-1β$^{-/-}$ mice (100) as well as IL-1R$^{-/-}$ mice (101) exhibited decreased levels of oviduct pathology despite moderate increases in the bacterial burden. In addition, mice deficient in IL-1RA developed more severe oviduct pathology although the bacterial burden in the genital tract was reduced (101). The primary cell types responsible for IL-1β production in the genital tract were determined to be neutrophils and macrophages (100), and IL-1β production by these cells is likely an important contributor to tissue damage.

The role of IL-1R signaling has not been extensively studied in humans. One study examining SNPs in IL-1β and IL-1RA detected no association with the development of tubal pathology in women with positive *Chlamydia* serology (102). This study was not exhaustive as the authors only analyzed two SNPs in IL-1β as well as the number of tandem repeats in the second intron of IL-1RA (102). There is not enough data to draw conclusions about the role of

IL-1 in human disease. Based on in vitro data and findings in the mouse model, IL-1R signaling is likely to contribute to disease.

1.2.4.3 TNFα

Studies in the mouse model indicate that TNFα is not crucial for host defense against *Chlamydia muridarum* and promotes tissue damage in the genital tract. TNFα$^{-/-}$ mice resolve infection normally, and oviduct pathology is significantly reduced (50). TLR2$^{-/-}$ mice or mice infected with a plasmid-cured strain of *C. muridarum*, CM3.1, develop less severe oviduct pathology despite normal resolution of infection, and this reduced pathology was associated with significantly decreased TNFα in the genital tract (17, 34). Mechanistic studies determined that TNFα production by CD8+ T cells contributes to pathology in this model (50).

Polymorphisms in the TNFα promoter have been correlated with disease in humans. In a case-control study of individuals from The Gambia with and without scarring trachoma or trachomatous trichiasis, possession of the TNF-308A allele resulted in increased TNFα production in response to EBs and a significantly increased risk of trichiasis (103). This allele was also associated with an increased risk of severe Fallopian tube damage in women with TFI associated with *C. trachomatis* infection (98), although no correlation was found between this allele and the level of TNFα produced by PBMCs in response to EBs (104).

1.2.4.4 IL-17

IL-17 is critical for host defense against *C. muridarum* pulmonary infection. Resolution of infection, prevention of bacterial dissemination, and the development of Th1 immunity were compromised in the absence of IL-17-mediated signaling in this model (105, 106). This

protective role was due to the ability of IL-17 to induce IL-12 production by dendritic cells (105). In addition, IL-17 and IFNγ have been demonstrated to synergistically induce nitric oxide production during infection of murine lung fibroblasts and macrophages, thus leading to increased killing of *Chlamydia* in vitro. However, these studies were conducted in the absence of T cells, so the physiologic relevance is questionable (107). In contrast, elevated levels of IL-17 have been associated with increased disease and enhanced neutrophil recruitment in the same model (108, 109).

Studies examining the role of IL-17 in protective vaccination strategies in the mouse model have had conflicting results. One study found that intranasal infection with live EBs was significantly more protective against subsequent intravaginal infection with *C. muridarum* than intranasal inoculation with UV-inactivated EBs + CpG or any of the intramuscular vaccination strategies tested. The authors determined that increased protection was associated with decreased *Chlamydia*-specific IL-17 production but increased IFNγ by splenocytes restimulated before challenge (110). These findings were in contrast to another study that demonstrated that after subcutaneous immunization with chlamydial peptides and a variety of adjuvants, the vaccination strategy that induced the highest frequency of IFNγ/IL-17 and IFNγ/TNFα double positive cells was the most protective. In this study, this protective vaccination strategy also induced the highest level of IL-17, TNFα, and IFNγ production upon restimulation of splenocytes in vitro, which makes drawing conclusions about the protective role of any one cytokine difficult (111).

The role of IL-17 in humans has not been extensively studied. Expression of IL-17A and the related genes S100A7 and CXCL5 was associated with the presence of trachomatous conjunctival inflammation in children in Tanzania (112). However, there was no association

between these genes and the presence of a current *Chlamydia* infection (112). A study of women infected with *C. trachomatis* detected IL-22 and IL-17 production by CD4+ T cells isolated from the cervical washes (113), but no correlation was made between the presence of these cells and disease.

1.3 SUMMARY

In the following chapters, we will describe our studies of immune responses implicated in both disease and protection in the mouse model of *Chlamydia* genital tract infection. We begin by comparing neutrophil influx and apoptosis during infection with strains of *Chlamydia* that are either pathogenic or non-pathogenic. We go on to examine the role of IL-17 in promoting both neutrophil influx and the Th1 response. We then extend these studies to the describe role of IL-17, IL-22, and IL-23 in this model. Finally, we turn our attention to the protective immune response by examining the role of the adaptor molecule MyD88 in promoting CD4+ T cell mediated resolution of infection from the genital tract.

2.0 ENHANCED NEUTROPHIL LONGEVITY AND RECRUITMENT CONTRIBUTE TO THE SEVERITY OF OVIDUCT PATHOLOGY DURING *C. MURIDARUM* INFECTION

2.1 ABSTRACT

Our previous studies showed that intravaginal infection of mice with a plasmid-deficient strain of *C. muridarum*, CM3.1, did not induce the development of oviduct pathology. In the studies described in this chapter, we determined that infection with CM3.1 resulted in a significantly reduced frequency and absolute number of neutrophils in the oviducts during acute infection. This reduction in neutrophils was associated with significantly lower levels of neutrophil chemokines in the oviducts and decreased production of neutrophil chemokines by oviduct epithelial cells infected with CM3.1 in vitro. Infection with CM3.1 also resulted in an increased frequency of late apoptotic/dead neutrophils in the oviduct. Examination of the ability of *C. trachomatis* to prevent neutrophil apoptosis in vitro revealed that *C. trachomatis* D/UW-3/Cx exhibited an enhanced ability to prevent neutrophil apoptosis when compared to plasmid-deficient CTD153, and this effect was dependent on the presence of $CD14^{high}$ monocytes. The presence of monocytes also resulted in enhanced neutrophil cytokine production and increased production of tissue damaging molecules in response to D/UW-3/Cx relative to CTD153. Attempts to use antibody-mediated depletion to discern the specific role of neutrophils in infection control and pathology in vivo revealed that although $Ly6G^{high}$ neutrophils were

eliminated from the blood and oviducts with this treatment, immature neutrophils and high levels of tissue damaging molecules were still detectable in the upper genital tract. These data support the role of neutrophils in *Chlamydia*-induced pathology and reveal that novel methods of depletion must be developed before their role can be specifically determined in vivo.

2.2 INTRODUCTION

Studies in the mouse model have repeatedly found an association between enhanced and/or prolonged neutrophil influx into the oviducts with the development of hydrosalpinx (29, 30). In addition, mice deficient in the chemokine receptor CXCR2 display reduced acute inflammation and lower rates of hydrosalpinx, and strains of mice with elevated CXCL2 production exhibit worse disease (31, 32). Neutrophils likely contribute to pathology by releasing mediators that directly damage reproductive tract tissues, and neutrophil release of the proteolytic enzyme matrix metalloproteinase-9 (MMP9) has been implicated in the development of scarring and fibrosis of the murine oviduct after chlamydial infection (36, 37). In addition, mice deficient in NADPH oxidase (*p47phox*$^{-/-}$) sustain lower rates of *Chlamydia*-induced hydrosalpinx (39).

These studies indicate that factors resulting in increased numbers of neutrophils and increased neutrophil activation in reproductive tract tissues may promote the development of *Chlamydia*-induced pathology. Our data in the mouse model indicate that immunologically normal mice infected with a plasmid-deficient strain of *C. muridarum*, CM3.1, develop minimal chronic oviduct pathology despite sustaining an infection of normal intensity and duration (17). This reduced pathology is associated with reduced numbers of neutrophils in the oviducts on late days of infection (17). In the studies outlined in this chapter, we determined that the neutrophil

response was significantly increased in the oviducts during acute infection with wild-type *C. muridarum* Nigg compared to CM3.1 and established potential mechanisms whereby this enhanced neutrophil response leads to increased pathology.

2.3 MATERIALS AND METHODS

2.3.1 Strains, cell lines, and culture conditions

The *C. muridarum* strains Nigg (provided by Roger Rank) and CM3.1 as well as the *C. trachomatis* strains D/UW-3/Cx and CTD153 used in this study were previously described (17, 114). All chlamydial strains were propagated in L929 cells (115). Bacteria were titrated by plaque assay (114) or as inclusion forming units (IFU) using fluorescently tagged anti-chlamydial lipopolysaccharde monoclonal antibody (Bio-Rad, Hercules, CA). Bacterial titers were confirmed using real-time PCR for chlamydial 16S rRNA (115). Live chlamydiae were used for all experiments. In vitro experiments comparing responses with D/UW-3/Cx and CTD153 were repeated with bacteria from at least two different preparations in order to account for variability in chlamydial stimulation resulting from isolation or titration techniques.

2.3.2 Animals

Six to eight week old female C3H/HeOuJ and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were between 8-12 weeks of age at the time of infection. Mice were given food and water ad libitum in an environmentally controlled room

with a cycle of 12 hours of light and 12 hours of darkness. All animal experiments were preapproved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.3.3 Murine infection and monitoring

Seven days prior to infection mice were subcutaneously injected with 2.5 mg of depot-medroxyprogesterone acetate (Depo-Provera®; Upjohn, Kalamazoo, MI) to synchronize mice in a state of anestrous and to facilitate successful intravaginal infection (116). Mice were infected with $1x10^5$ inclusion forming units (IFU) of *C. muridarum* Nigg or CM3.1 intravaginally. Mice were monitored for cervicovaginal shedding (117), and IFUs were calculated as previously described (30). Bacterial burden was measured in the oviducts by serially diluting a 100 μl aliquot of the homogenized oviducts in 1xPBS for titration using a plaque assay (114).

2.3.4 Processing of oviducts for flow cytometry

Oviducts were harvested into 1 ml of media (RPMI+1%FBS) and minced with scissors. For measurement of cytokines and bacterial burden, 100 μl was removed and stored at -80°C until analysis. The tissue was repeatedly passed through a 70 μm filter to yield a single cell suspension. For surface staining, single cell suspensions were resuspended in cold FACS buffer (PBS pH 7.2, 0.5% BSA, and 2mM EDTA) at $4x10^7$ cells per/ml. To block non-specific antibody binding, 25 μl ($1x10^6$ cells) of cell suspension and 25 μl of diluted Fc Block (1μl Fc in 24 μl FACS buffer; BD Pharmingen; Clone: 2.4G2) were combined in a 96 well V-bottom plate and incubated on ice for 20 minutes. Staining antibodies were then added in 50 μl of FACS buffer for a final dilution of 1.25 μl antibody per 100 μl total volume. After incubating for 20

minutes on ice, stained cells were washed, resuspended in Live/Dead® Fixable Stain (Invitrogen, Carlsbad, CA), and incubated for 20 minutes on ice in the dark. After washing, cells were incubated in 2% paraformaldehyde at 4ºC until analysis. Flow cytometric data were acquired using an LSR II Analyzer (BD Biosciences) and analyzed via FlowJo software (Tree Star, Ashland, OR).

The antibodies used for examination of the oviducts during infection with Nigg and CM3.1 were as follows: PerCP-Cy5.5 anti-mouse CD45 (BD Biosciences, Clone: 30-F11), PE-Cy7 anti-mouse CD11c (BD Biosciences, Clone: HL3), APC anti-mouse F4/80 (eBioscience, Clone: BM8), and PE anti-mouse Ly6G/C (eBioscience, Clone: RB6-8C5). Viability was determined using Live/Dead® violet (Invitrogen, Carlsbad, CA). Flow cytometric analysis was conducted for groups of C3H/HeOuJ (N=5) mice infected with Nigg or CM3.1 on days 7, 9, 11, 13, 15, and 21 in a single experiment. Similar analysis was conducted for groups of C57BL/6 (N=3) in two independent experiments. The absolute numbers of cells were calculated by multiplying the cell count obtained by the flow cytometer by the following dilution factor: # of cells counted by hemocytometer/ total # of cells analyzed by the flow cytometer.

The antibodies used to examine the leukocyte populations in the oviducts of mice on day 11 of the neutrophil depletion experiment were as follows: PerCP-Cy5.5 anti-mouse CD45 (BD Biosciences, Clone: 30-F11), APC anti-mouse F4/80 (eBioscience, Clone: BM8), FITC anti-mouse CD11b (BD Biosciences, Clone: M1/70), PE anti-mouse Ly6G (eBioscience, Clone: 1A8), and V450 anti-mouse Ly6G/C (BD Biosciences, Clone: RB6-8C5).

2.3.5 In vivo analysis of neutrophil apoptosis

Groups of 3 C57BL/6 mice were infected with Nigg or CM3.1 and euthanized on days 9, 13, and 15 of infection in a single experiment. Oviducts were harvested and treated as described for flow cytometry except that volumes were scaled down for staining of $2x10^5$ cells, Annexin V binding buffer (BD Biosciences) was used instead of FACS buffer and 7-amino-actinomycin D (7-AAD, BD-Biosciences) was used instead of Live/Dead® Stain. The antibody cocktail included: V450 anti-Annexin V (BD Biosciences), PE anti-mouse Ly6G (eBioscience, Clone: 1A8), and FITC anti-mouse Ly6G/C (eBioscience, Clone: RB6-8C5).

2.3.6 In vivo administration of anti-Ly6G antibody

Groups of C3H/HeOuJ mice were treated intraperitoneally with rat anti-mouse Ly6G mAb (Clone 1A8, BioXCell, West Lebanon, NH) (300 μg in 100 μl PBS) every other day or every third day from D-1 to D20 post infection. A control group of C3H/HeOuJ mice was treated similarly with rat IgG2a (Clone 2A3; BioXCell, West Lebanon, NH). Both groups were infected with $1x10^5$ IFU of *C. muridarum* Nigg as indicated above. Mice were euthanized either on day 11 of infection for analysis of the acute response or on day 42 for examination of pathology. For mice euthanized on day 11, one oviduct was harvested and analyzed for cytokines, cellular influx and bacterial burden. The remainder of the genital tract was either fixed in 10% formalin for H&E staining or embedded in O.C.T.® Compound (Tissue-Tek®, Sakura Finetek, Torrance, CA) and frozen for immunohistochemical analysis. For mice euthanized on day 42, doxycyline (3 mg/ml) was administered (100 μl i.p.) every day from day 21 to day 26. On day 42, genital tracts were fixed in formalin for histologic analysis. None of the mice exhibited signs of serum

sickness over the course of antibody administration. A total of 5 mice per group were administered 300 μg of anti-Ly6G or IgG every other day and were euthanized on day 11 or day 42 post-infection in a single experiment. In two independent experiments, groups of mice were treated with 300 μg of anti-Ly6G or IgG every third day, with a total of 6 mice per group euthanized on day 11 post-infection, and a total of 9 mice per group euthanized on day 42. Flow cytometry revealed similar depletion of $Ly6G^{high}$ cells in the oviducts and peripheral blood with both treatment regimens.

2.3.7 Analysis of peripheral blood neutrophils

Mice treated with anti-Ly6G or IgG were bled at the time of euthanize on day 11 for determination of peripheral blood neutrophil counts. For flow cytometric analysis, non-specific antibody binding was blocked by incubation of 100 μl of peripheral blood with 50 μl of diluted Fc Block™ (2μl Fc in 48 μl FACS buffer; BD Pharmingen, Clone: 2.4G2) for 20 min on ice. Antibody staining was then conducted for 20 min on ice using 1.25 μl of each antibody and enough FACS buffer to bring the volume of antibody cocktail to 50μl. The following antibodies were used: PerCP-Cy5.5 anti-mouse CD45 (BD Biosciences, Clone: 30-F11), APC anti-mouse F4/80 (eBioscience, Clone: BM8), FITC anti-mouse CD11b (BD Biosciences, Clone: M1/70), PE anti-mouse Ly6G (eBioscience, Clone: 1A8), and V450 anti-mouse Ly6G/C (BD Biosciences, Clone: RB6-8C5). Red blood cell lysis was then conducted using VitaLyse® (BioE, St. Paul, MN) according to the manufacturer's instructions.

2.3.8 Microscopic histopathological assessment

Mice were euthanized on specified days following intravaginal infection; the genital tracts were removed en bloc, fixed in 10% buffered formalin and embedded in paraffin. Longitudinal 4 μm sections were cut and stained with hematoxylin and eosin. Each anatomic site (ectocervix, endocervix, uterine horn, oviduct) was assessed independently for the presence of neutrophilic inflammation, lymphocytic/monocytic inflammation, plasma cells, dilatation, and fibrosis and assessed using a four-tiered semi-quantitative scoring system to evaluate the extent of inflammation and pathology as previously described (30, 32).

For immunohistochemical analysis of neutrophils in genital tract tissues, tissues frozen in O.C.T.® Compound (Tissue-Tek®, Sakura Finetek, Torrance, CA) were cut into 5 μm sections, fixed in acetone, and endogenous peroxidase activity was blocked with 0.3% H_2O_2. Sections were incubated for one hour with rat anti-mouse Ly6G (BD Biosciences, Clone: 1A8) at a dilution of 1:2000, and the immunohistochemical procedure was performed according to the manufacturer's instructions using the anti-rat Ig HRP detection kit (BD Biosciences). The reaction was visualized with diaminobenzidine chromogen, and sections were mounted and coverslipped with the Tissue-Tek® SCA™ coverslipper (Sakura Finetek USA, Torrance, CA).

2.3.9 Analysis of oviduct epithelial cell chemokine and cytokine expression.

The oviduct epithelial cell line BM1.11 has been previously described (118). Total RNA was isolated from *Chlamydia*-infected BM1.11 cells 6 hours post infection with Nigg or CM3.1 (MOI: 1) using an RNeasy RNA isolation kit (Qiagen, Valencia, CA), and RNA was examined by quantitative real-time PCR using our previously published protocol (115). Primer pairs for

amplification of the genes analyzed in this study were purchased from SABiosciences (Frederick, MD). Transcription of the mouse gene *cacybp* served as an endogenous reference, and data were analyzed by the $2^{-\Delta\Delta CT}$ method (119) using BioRad proprietary software. Each sample was assayed in triplicate, and three independent transcriptional experiments were performed. mRNA data were correlated with protein expression for two of the three experiments using multiparametric bead array (Millipore, Billerica, MA).

2.3.10 Isolation of human neutrophils.

Peripheral venous blood was collected from healthy adult volunteers after obtaining informed consent. Blood was collected into 10 ml BD Vacutainer® tubes containing sodium heparin and was used within 1 hour. All procedures were conducted at room temperature. Blood was diluted with an equal volume of PBS (w/o calcium and magnesium, pH=7.4; Invitrogen, Carlsbad, CA), and 20 ml of diluted blood was layered over 25 ml of Mono-Poly Resolving Media (density 1.114; MP Biomedicals, Solon, OH). The gradient was spun (300 x g) at 25°C for 40 minutes in a swinging bucket rotor with no brake. The granulocyte band was removed and cells were washed twice with media (RPMI 1640/10% FBS). Cells were plated at a density of $5x10^6$ cells/ml with 200 µl per well in round bottom, polypropylene 96 well plates (Costar, Corning, NY). Purity was confirmed at >90% neutrophils using morphological analysis of Wright-Giemsa stained (Ricca Chemical Co., Batesville, IN) cytospin preparations and flow cytometry for $CD11b^{+}CD14^{low/neg}$ cells. Neutrophils were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For add-back experiments, the upper band of monocytes was isolated from the density gradient. Cells from this band were added to neutrophils from the same gradient. In experiments using highly purified neutrophil preparations, $CD14^{high}$ cells were removed from

gradient purified neutrophils by negative selection with magnetic beads (Miltenyi Biotec, Auburn, CA).

2.3.11 Detection of cytokines in vitro and in vivo.

Highly purified or gradient purified neutrophils (5×10^6/ml) were incubated with media, $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (100ng/ml; Axxora LLC, San Diego, CA), or an MOI of 5 of D/UW-3/Cx or CTD153. Cell free supernatants were collected and stored at -80°C until cytokine analysis. Cytokines were quantified using a multiparametric bead array (Millipore; Billerica, MA) for IL-8, IL-6, TNFα, IL-1β, and G-CSF. Total MMP9 was assayed using a multianalyte profiling assay (Fluorokine® MAP; R&D systems, Minneapolis, MN). These analyses were conducted on duplicate samples in three independent experiments for highly purified neutrophils and for at least two independent experiments for gradient purified neutrophils. Levels of proinflammatory molecules were also measured in the homogenized oviducts of C3H/HeOuJ or C57BL/6 mice infected with Nigg and CM3.1. Levels of CXCL1, CXCL2, GM-CSF, IL-1β, IL-6, and TNFα were measured via multiparametric bead array (Millipore, Billerica, MA). Total MMP9 was assayed using a multianalyte profiling assay (Fluorokine® MAP; R&D systems, Minneapolis, MN). Levels of cytokines and chemokines were determined in the oviducts in 2 independent experiments using C3H/HeOuJ mice with 3 or 5 mice euthanized per group per day as well as in a single experiment using C57BL/6 mice with 3 mice euthanized per group per day.

2.3.12 Analysis of human neutrophil apoptosis.

Human neutrophils (5×10^6/ml) were purified by density gradient centrifugation and were either depleted of $CD14^{high}$ cells or not further purified. Neutrophils that were depleted of $CD14^{high}$ cells are referred to as highly purified while populations that were isolated solely by gradient centrifugation are referred to as partially purified. Cells were stimulated with Pam_3Cys (100 ng/ml) or infected at an MOI of 5 with D/UW-3/Cx or CTD153 for 20 hrs. Apoptosis of partially purified neutrophils was determined via both methods below, and apoptosis of highly purified neutrophils was determined via TUNEL staining alone. *TUNEL:* TUNEL staining was conducted according to the manufacturer's instructions (*In situ* cell death detection kit; Roche, Indianapolis, IN) to detect DNA strand breaks characteristic of apoptotic cells. Staining was analyzed via flow cytometry. Relative levels of apoptosis were quantified by calculating the ratio of the percentage of TUNEL-positive cells in a stimulated group to the percentage of TUNEL-positive cells in the media control. *Annexin V binding and propidium iodide staining:* Annexin V binds to exposed phosphotidylserine in a calcium-dependent manner, and is an indicator of early apoptosis. Propidium iodide (PI) stains cells with disrupted plasma membranes, and is indicative of late apoptosis or necrosis. Neutrophils were stained with Annexin V and PI according to the manufacturer's instructions (Annexin V-FITC Kit; Miltenyi Biotec, Auburn, CA). Labeled cells were analyzed by flow cytometry. Staining indicated the stage of cell death as follows: Alive: Annexin V^- PI^-; Early apoptotic: Annexin V^+PI^-; Late apoptotic/ necrotic: Annexin V^+PI^+. Apoptosis of gradient purified neutrophils was analyzed via TUNEL in duplicate wells in 6 independent experiments and via Annexin V/PI staining in 3 independent experiments. Apoptosis of highly purified neutrophils was analyzed via TUNEL in duplicate wells in 3 independent experiments.

2.3.13 Intracellular flow cytometry.

For analysis of cell specific cytokine production, GolgiPlug™ (1:1000 final dilution; BD Biosciences) was added to preparations of partially purified neutrophils or neutrophils with additional monocytes added after 3 hrs of incubation with media, D/UW-3/Cx (MOI: 5) or Pam_3Cys (100ng/ml). After a total of 6 hrs in culture, cells were stained with Pacific Blue anti-human CD14 (BD Biosciences, Clone: M5E2) and APC-Cy7 anti-human CD11b (BD Biosciences, Clone: ICRF44). After surface staining, cells were fixed and permeabilized according to manufacturers instructions (Cytofix/Cytoperm™Kit, BD Biosciences). Intracellular staining was conducted using PE anti-human IL-6 (eBioscience, Clone: MQ2-13A5) and APC anti-human TNFα (eBioscience; Clone: MAb11). Intracellular cytokine staining was conducted on duplicate wells in two independent experiments.

2.3.14 Statistics.

Statistical comparison of oviduct leukocyte influx, oviduct cytokine/chemokine levels, and the course of lower genital tract infection over time were analyzed via two-way ANOVA with Bonferroni post-test. Mann-Whitney U test was used to determine significant differences in the pathological data between groups. Differences in levels of gene transcription, apoptosis, neutrophil cytokine production, and cellular infiltrate into the oviduct on a single day were determined via Student's *t*-test, or by ANOVA as appropriate. Prism software was utilized for all statistical analysis. Values of $P < 0.05$ were considered significant.

2.4 RESULTS

2.4.1 Lower numbers of neutrophils were detected in the oviducts in response to infection with plasmid-deficient CM3.1 compared to wild-type Nigg.

Our previous studies in the mouse model indicated that intravaginal infection with CM3.1, a plasmid-deficient strain of *C. muridarum*, resulted in an infection of equivalent magnitude and duration to wild-type Nigg, but in contrast to Nigg, CM3.1 did not induce the development of oviduct pathology (17). Histological scoring on day 42 revealed that the reduced pathology observed upon infection with CM3.1 was associated with significantly lower numbers of neutrophils in the oviducts relative to infection with Nigg (17). However, in those studies the kinetics of leukocyte influx into the oviducts was not examined during active oviduct infection.

In order to describe the kinetics of the immune response in detail, groups of C3H/HeOuJ mice were intravaginally infected with wild-type *C. muridarum* Nigg or plasmid-deficient CM3.1, and the oviducts were harvested. Flow cytometric analysis revealed that CM3.1 resulted in significantly lower numbers of $CD45^+$ inflammatory cells in the oviducts relative to Nigg (Figure 1A). Further characterization of $CD45^+$ cells revealed a significantly decreased number (Figure 1B) and percentage of neutrophils (Figure 1C) in the oviducts in response to infection with CM3.1. We also determined that the number of macrophages and dendritic cells were reduced in the oviducts during infection with CM3.1 compared to Nigg (data not shown). The number of neutrophils in the oviducts on day 13 correlated directly with the oviduct dilatation scores determined on day 42 for mice similarly infected ($r=0.65$, $P < 0.05$ by Spearman nonparametric correlation). Histological analysis supported our flow cytometric findings revealing that on days 14, 21, and 28 post infection, CM3.1 resulted in significantly reduced

neutrophilic infiltrates in the oviducts (Figure 1E-I). The presence of reduced acute inflammation in the oviducts of mice infected with CM3.1 was not due to reduced bacterial burden since titration of live bacteria from the oviducts confirmed our previous findings that bacterial burden did not differ between the strains (data not shown)(17). In addition, a repeat experiment performed in C57BL/6 mice revealed lower numbers of neutrophils acutely and reduced chronic oviduct pathology in response to infection with CM3.1 (data not shown) indicating that the differences observed were not mouse strain dependent.

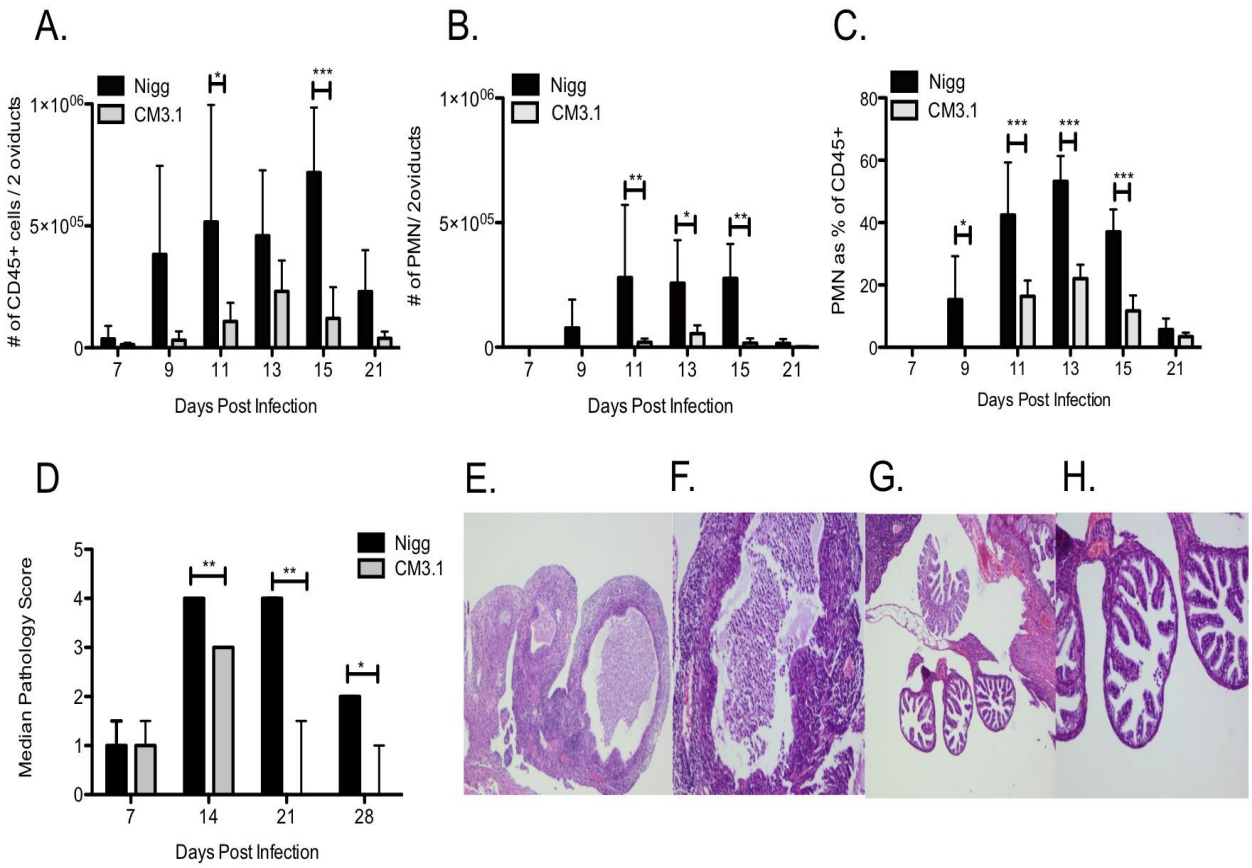

Figure 1: Mice infected with CM3.1 exhibited a reduced neutrophil response in the oviducts during acute infection. Groups of C3H/HeOuJ mice were infected with *C. muridarum* Nigg (black bars) or CM3.1 (grey bars) and euthanized on the indicated days. (A-C) Flow cytometric analysis was used to determine (A) the number of live $CD45^+$ cells, (B) the number of neutrophils ($CD45^+Gr\text{-}1^{high}Cd11c^-F4/80^-$), and (C) the frequency of neutrophils in the oviducts of individual mice. Bars represent the mean ± SD of values from 5 mice per group at each time point in one representative experiment of three. $P < 0.0001$ by two-way ANOVA for CM3.1 vs. Nigg for A-C. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for individual days by two-way ANOVA with Bonferroni post test analysis. (D) In a separate experiment, histological analysis of oviducts revealed significantly reduced neutrophillic inflammation on days 14, 21, and 28 post infection with CM3.1 compared to Nigg. Bars are representative of the median and interquartile range of scores of 5 C3H/HeOUJ mice per day. * $P < 0.05$, ** $P < 0.01$ by Mann-Whitney U test. Photomicrographs are representative of the oviducts of (E, 40x; F, 100x) Nigg and (G, 40x; H, 100x) CM3.1-infected animals on day 21 post-infection.

2.4.2 Decreased neutrophil chemokine production was detected in vivo and in vitro in response to infection with CM3.1.

Our previous studies revealed that lower numbers of neutrophils in the oviducts of CM3.1 infected mice were associated with decreased levels of the neutrophil chemokine CXCL2 (MIP-2) in lower genital tract secretions (17). However, lower genital tract secretions do not reflect the inflammatory milieu in the oviduct, which is the site most vulnerable to *Chlamydia*-induced pathology. Measurement of neutrophil chemokines in homogenized oviduct tissues from Nigg or CM3.1 infected C3H/HeOuJ mice revealed that levels of CXCL1 (Figure A), CXCL2 (Figure 2B), and GM-CSF (Figure 2C) proteins were significantly decreased during infection with plasmid-deficient CM3.1 relative to infection with Nigg.

Chlamydia primarily infects and replicate within the columnar epithelial cells of the oviduct, and infection of the oviduct epithelial cell line Bm1.11 induces a robust cytokine and chemokine response within hours of infection (120). In order to determine if oviduct epithelial cell chemokine production contributed to the differences we observed in the oviducts of Nigg or CM3.1 infected mice, Bm1.11 cells were incubated with media or infected with an MOI of 1 of Nigg or CM3.1. Analysis of gene transcription after 6 hours revealed significantly augmented transcription of CXCL1, CXCL2, and GM-CSF upon infection with Nigg compared to CM3.1 (Figure 2D). These transcriptional differences led to differences in chemokine production as revealed by measurement of protein levels in supernatants collected after 24 hours of infection (data not shown). These findings indicate that an impaired ability to induce oviduct epithelial cell chemokine production likely contributes to the reduced neutrophil response observed in the oviducts of CM3.1 infected mice.

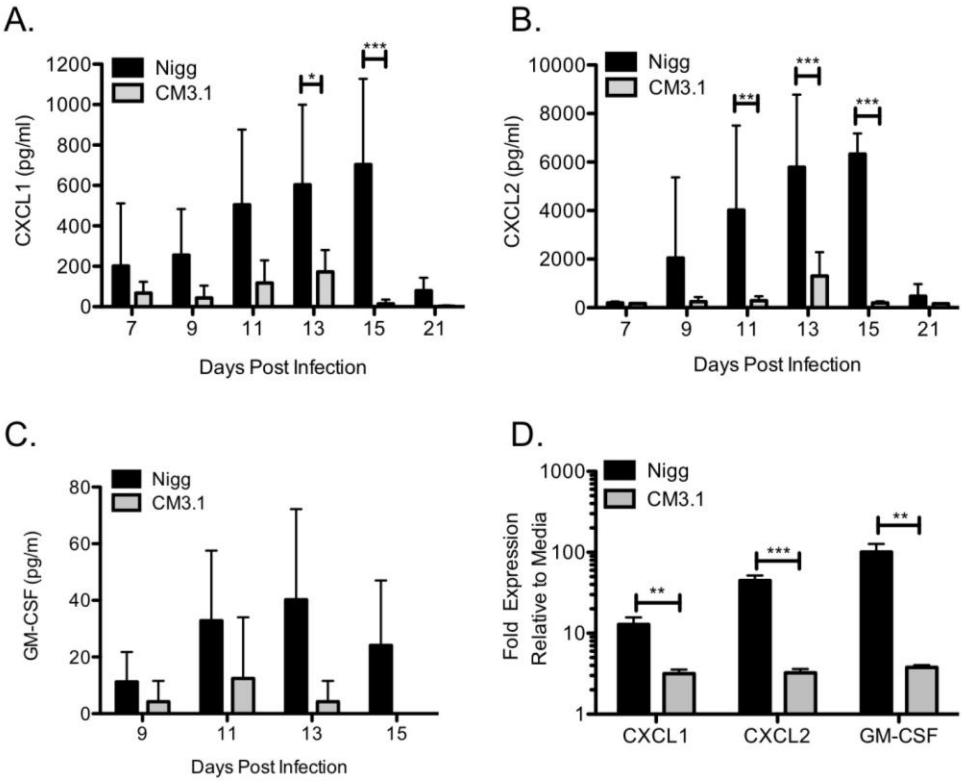

Figure 2: Infection with CM3.1 elicited lower levels of neutrophil chemokine production in vivo and in vitro. Significantly reduced levels of (A) CXCL1, (B) CXCL2, and (C) GM-CSF were detected in the oviduct homogenates of CM3.1 infected mice (grey bars) compared to mice infected with Nigg (black bars). Bars represent the mean ± SD of values from 5 mice per group of a single representative experiment of three. $P < 0.0001$ by two-way ANOVA for CM3.1 vs. Nigg for CXCL1, CXCL2, and GM-CSF over the interval measured. *$P < 0.05$, ***$P < 0.001$ for individual days by two-way ANOVA with Bonferroni post test analysis. (C) Analysis of RNA from Bm1.11 cells infected with Nigg or CM3.1 (MOI: 1) for 6 hrs revealed significantly reduced induction of CXCL1, CXCL2, and GM-CSF when expression was normalized to the housekeeping gene *cacybp* and compared to media. Bars represent the mean ± SD of triplicate wells of a single experiment that was performed three times. **$P < 0.01$, *** $P < 0.001$ for Nigg vs. CM3.1 by Student's *t*-test.

2.4.3 Infection with wild-type *Chlamydia* delayed neutrophil apoptosis significantly more than infection with plasmid-deficient strains both in vivo and in vitro.

Neutrophils are a terminally differentiated population of cells that undergo spontaneous apoptosis in vitro and in vivo. Although analysis of apoptosis in vivo is difficult due to the rapid clearance of apoptotic cells by phagocytes, we sought to determine if neutrophil longevity was enhanced in the oviducts during infection with Nigg relative to CM3.1, which could potentially be influenced by the ability of Nigg, but not CM3.1, to activate TLR2 (17). Groups of C57BL/6 mice were intravaginally infected with Nigg or CM3.1, and oviducts were harvested on days 9, 13, and 15 post infection. Single cell suspensions generated from the oviducts were stained with the neutrophil specific marker Ly6G (Clone: 1A8) as well as with Annexin V and 7-amino-actinomycin D (7-AAD) to differentiate viable ($AnnexinV^{-}7\text{-}AAD^{-}$), early apoptotic ($AnnexinV^{+}7\text{-}AAD^{-}$), and late apoptotic/dead ($7\text{-}AAD^{+}$) neutrophils. Infection with Nigg resulted in a significantly increased frequency of viable neutrophils on days 9 and 13 post-infection (Figure 3A, E). Although the frequency of early apoptotic neutrophils did not differ between the strains (Figure 3B, E), the degree of phosphatidylserine exposure by early apoptotic neutrophils, as evidenced by the mean fluorescence intensity (MFI) of Annexin V staining, was significantly lower on days 9 and 13 of infection in mice infected with Nigg (Figure 3D, E). Phosphotidylserine is externalized as cells undergo apoptosis (121), and this increased expression may indicate that cells in the early apoptotic gate of mice infected with CM3.1 may be more advanced in their progression towards late apoptosis. Correspondingly, Nigg infected oviducts contained a significantly reduced frequency of late apoptotic/dead neutrophils (Figure 3C, E). This reduced frequency of late apoptotic/dead neutrophils may be due to a combination of factors including ongoing influx of viable neutrophils from the blood, which would be

influenced by the magnitude of the chemokine gradient induced in the oviducts by infection (Figure 2A-C). It is also possible that direct stimulation of neutrophils by wild-type Nigg delays neutrophil apoptosis to a greater extent than plasmid-deficient CM3.1.

To compare the ability of wild-type and plasmid-deficient *Chlamydia* to delay apoptosis in vitro, it would be necessary to isolate large numbers of unstimulated neutrophils from a single mouse. Isolating adequate numbers of neutrophils from murine peripheral blood is not possible due to the low blood volume of mice, and cells isolated from murine bone marrow are generally immature and overly manipulated, rendering them non-responsive to chlamydial stimulation. Thus, human peripheral blood neutrophils were used for these studies because large numbers of cells can be isolated from the blood of a single donor, and the isolation techniques do not induce premature neutrophil activation. To analyze the response of human neutrophils to *Chlamydia*, we used a human plasmid-deficient strain named CTD153, which was derived from serovar D (D/UW-3/Cx) using novobiocin treatment (115). Similar to CM3.1, this strain does not stimulate TLR2 or accumulate glycogen and exhibits a similar set of plasmid-responsive chromosomal loci (115).

Analysis of neutrophil apoptosis in vitro was first conducted on populations of human neutrophils that were isolated by density gradient centrifugation and then further depleted of $CD14^{high}$ monocytes using magnetic beads. These highly purified human neutrophils were incubated with D/UW-3/Cx, CTD153, or media, and apoptosis was quantified using TUNEL staining. TUNEL detects DNA fragmentation, which is characteristic of cells that are undergoing or have completed apoptosis. This technique revealed that incubation of highly purified neutrophils with D/UW-3/Cx or CTD153 (MOI: 5) delayed neutrophil apoptosis at 20 hrs when compared to incubation with media alone (data not shown, $P < 0.05$, Student's *t*-test).

However, no difference was detected when the ability of DUW-3/Cx or CTD153 to delay apoptosis was compared (data not shown, $P = 0.134$, Student's *t*-test). In addition, stimulation with the TLR2 agonist Pam_3Cys (100 ng/ml) did not delay the apoptosis of these highly purified neutrophils (data not shown, P = 0.67, Student's *t*-test).

Previous studies with TLR agonists supported our findings that TLR2 stimulation does not delay the apoptosis of highly purified neutrophils, but these studies also demonstrated that the presence of a small percentage of monocytes could facilitate a TLR-mediated delay in apoptosis (122, 123). Thus, we explored the ability of D/UW-3/Cx and CTD153 to differentially influence the apoptosis of neutrophils that were isolated by density gradient centrifugation but were not completely depleted of $CD14^{high}$ monocytes. In these partially purified preparations, neutrophils consistently represented greater than 90% of cells and $CD14^{high}$ monocytes represented approximately 1% of cells. In contrast to our findings with highly purified neutrophils, these partially purified neutrophils exhibited significantly reduced apoptosis upon incubation with D/UW-3/Cx when compared to neutrophils incubated with CTD153 or the TLR2 agonist, Pam_3Cys (Figure 3F). Titration of inclusion forming units at 1 hr of incubation revealed no difference in bacterial load between neutrophils incubated with D/UW-3/Cx or CTD153, and at 20 hrs, when apoptosis was measured, no viable *Chlamydia* were recovered (data not shown).

Annexin V staining in conjunction with propidium iodide (PI) allows for discrimination of cells that are viable, apoptotic, and necrotic. Annexin V/ PI staining revealed that incubation of partially purified neutrophils with D/UW-3/Cx resulted in a significantly lower percentage of apoptotic neutrophils (20 ± 3.4% apoptotic)(data not shown) when compared to incubation with CTD153 (36.5 ± 1.4% apoptotic; $P < 0.05$, One way ANOVA)(data not shown) or media alone

(44.7 ± 5.7% apoptotic; $P < 0.05$, One way ANOVA) (data not shown). There was no significant difference in the percentage of cells that were late apoptotic/ necrotic between any of the groups.

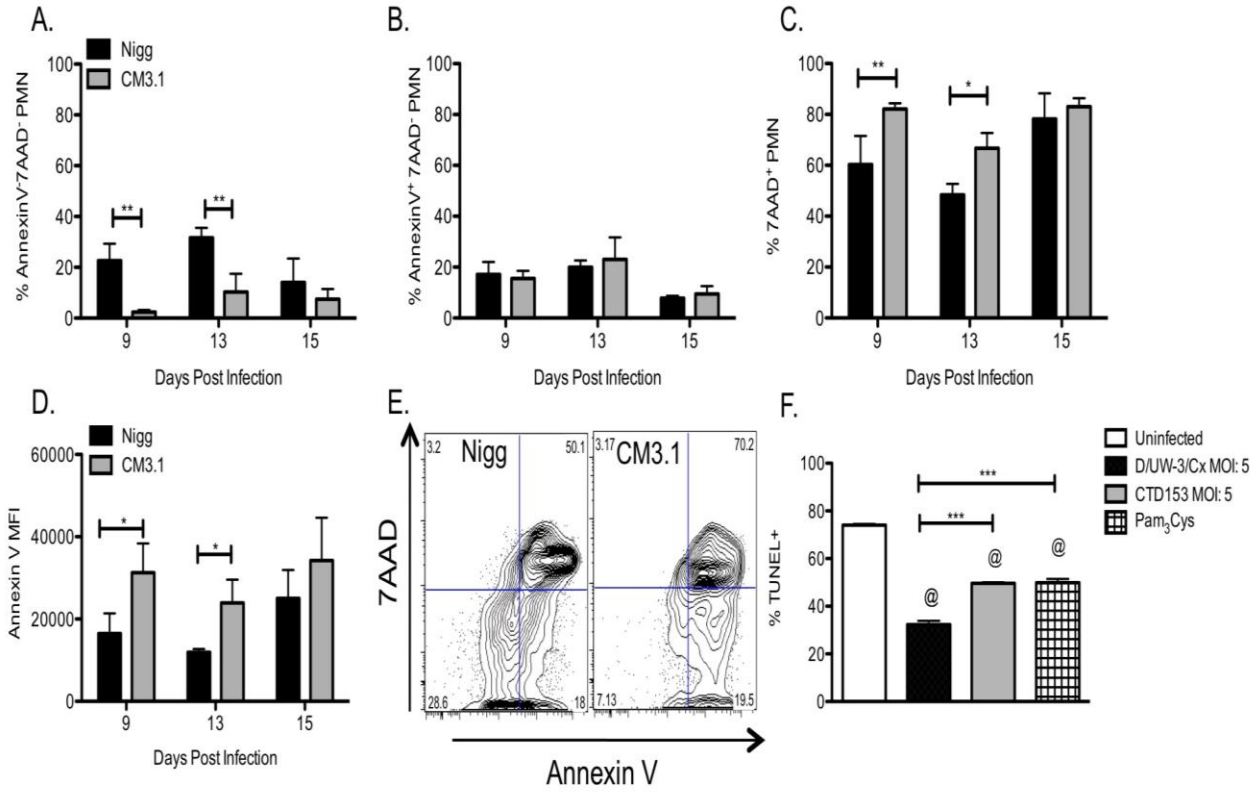

Figure 3: Plasmid-deficient *Chlamydia* exhibited an impaired ability to delay neutrophil apoptosis in vivo and in vitro.

Flow cytometry was used to determine the frequency (A) viable neutrophils (Annexin V^- $7AAD^-$), (B) early apoptotic neutrophils ($AnnexinV^+7AAD^-$), and (C) late apoptotic/dead neutrophils ($7AAD^+$) in the oviducts of mice infected with CM3.1 (grey bars) or Nigg (black bars). Bars represent the mean ± SD of 3 mice per day from a single experiment. * $P < 0.05$, ** $P < 0.01$ by two-way ANOVA with Bonferroni post test analysis. (D) Significantly higher levels of Annexin V expression were detected on early apoptotic neutrophils ($Annexin^+7AAD^-$) on days 9 and 11 during infection with CM3.1. Bars represent the mean fluorescence intensity (MFI) ± SD of 3 mice per day from a single experiment. * $P < 0.05$ by Student's *t*-test. (E) A representative flow diagram of oviduct cells gated on $Ly6G^{high}$ (Clone: 1A8) neutrophils. (F) TUNEL of partially purified human neutrophils after 20 hrs of incubation with media (white bar), Pam_3Cys (100ng/ml) (checkered bar), or an MOI of 5 of D/UW-3/Cx (black bar) or CTD153 (grey bar). Bars represent the mean ± SD of the percentage of apoptotic neutrophils in duplicate wells from one experiment of 6. *** $P < 0.001$ for comparison between treatments; @ $P < 0.05$ vs. uninfected, one-way ANOVA with Tukey's multiple comparison test.

2.4.4 Infection with CM3.1 resulted in a significantly lower level of proinflammatory and tissue damaging molecules in the oviducts than infection with Nigg.

The reduced inflammatory cell influx observed in the oviducts of CM3.1 infected mice (Figure 1) is likely explained by a combination of decreased chemokine production (Figure 2) and increased apoptosis (Figure 3). However, the true correlate of tissue damage in Nigg vs. CM3.1 infected mice is likely to be the release of proinflammatory cytokines and pathogenic mediators. Our previous studies revealed that IL-1β is associated with the development of oviduct pathology in response to *C. muridarum* infection (100), and IL-6 and TNFα are well-characterized mediators of inflammation and fibrosis. In addition, mice deficient in MMP9 exhibit reduced levels of hydrosalpinx compared to wild-type mice (37). To determine if reductions in levels of these inflammatory mediators and tissue damaging molecules could be associated with the absence of pathology observed during infection with CM3.1, we intravaginally infected groups of C3H/HeOuJ mice with Nigg or CM3.1. Oviducts were harvested over the course of acute infection and quantification of protein levels revealed that infection with CM3.1 resulted in significantly reduced levels of IL-1β (Figure 4A), IL-6 (Figure 4B), TNFα (Figure 4C), and MMP9 (Figure 4D). Parallel findings were determined for oviducts of C57BL/6 mice infected with Nigg vs. CM3.1 (data not shown).

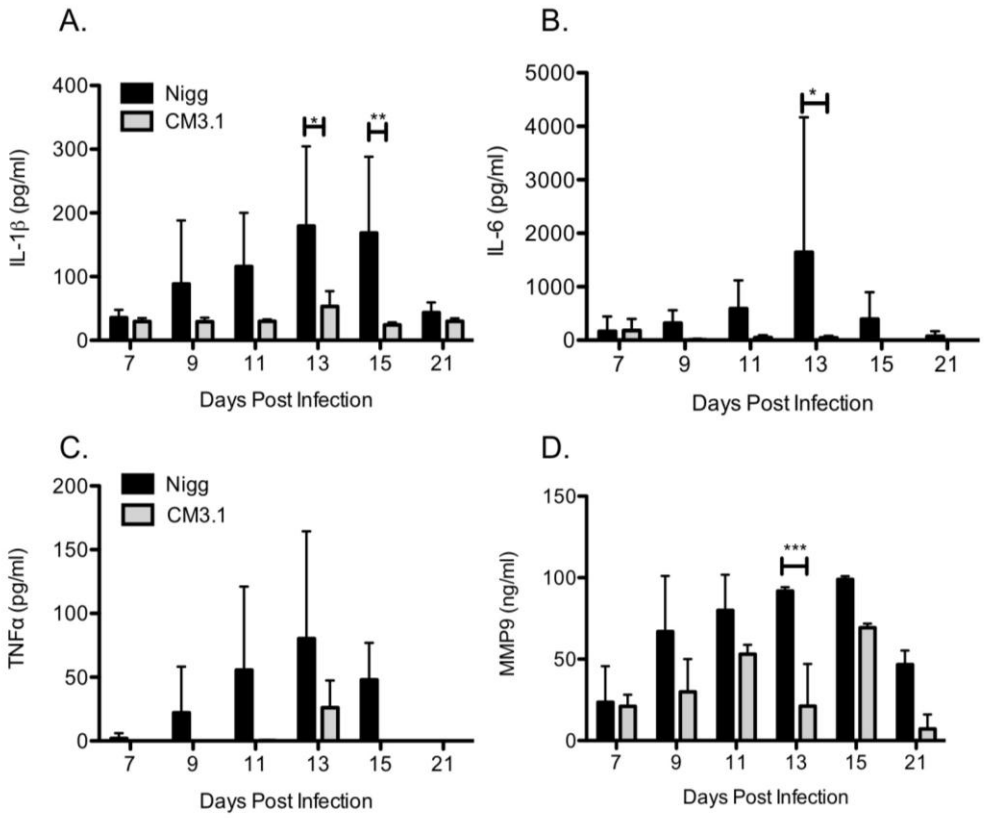

Figure 4: Infection with CM3.1 resulted in significantly reduced levels of proinflammatory and tissue damaging molecules in the oviducts.

Analysis of the homogenized oviducts of mice infected with Nigg (black bars) or CM3.1 (grey bars) revealed significantly lower levels of (A) IL-1β, (B) IL-6, (C) TNFα, and (D) MMP9. Bars represent the mean ± SD of values from 5 C3H/HeOuJ mice per group of a single independent experiment of two. $P < 0.0001$ for IL-1β and MMP9, $P < 0.01$ for TNFα, and $P < 0.05$ for IL-6 by two-way ANOVA for CM3.1 vs. Nigg over the interval measured. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for individual days by two-way ANOVA with Bonferroni post test analysis.

2.4.5 Highly purified neutrophils exhibited low levels of cytokine production, but incubation with D/UW-3/Cx led to enhanced production of cytokines compared to CTD153 when monocytes were present.

Our finding that monocytes were involved in the differential apoptosis observed for D/UW-3/Cx and CTD153 (Figure 3E) prompted investigation into the effect of monocytes on neutrophil cytokine production. Highly purified neutrophils were generated by depleting $CD14^{high}$ cells from gradient purified neutrophils using magnetic beads. These highly purified neutrophils were incubated with D/UW-3/Cx, CTD153, or Pam_3Cys for 20 hours. Measurement of cytokines in the supernatants revealed extremely low levels of TNFα (Figure 5A), IL-6 (data not shown), and IL-1β (data not shown) with all cytokines detected at levels less than 65 pg/ml. Although IL-8 production was more robust, with amounts up to 6000 pg/ml detected upon incubation with *Chlamydia*, no difference in cytokine production after incubation with D/UW-3/Cx and CTD153 was observed (data not shown).

Cytokine production was then analyzed in preparations of partially purified neutrophils, which were isolated by density gradient centrifugation but not depleted of $CD14^{high}$ monocytes. Flow cytometric analysis revealed that even when the population of $CD14^{high}$ monocytes represented less than 1% of cells, the frequency of $CD14^{high}$ cells producing TNFα in response to D/UW-3/Cx was ~7 fold higher, and the frequency producing IL-6 was ~ 700 fold higher than neutrophils in the same preparation (Fig. 5Figure 5B). In addition, the relative amount of cytokines produced by the neutrophil or the $CD14^{high}$ monocyte population was documented by analysis of the mean fluorescence intensity (MFI) of cytokine producing cells. In response to incubation with D/UW-3/Cx, the MFI of $TNF\alpha^{+}$ $CD14^{high}$ cells (468 ± 117) was 13 fold higher

than that of neutrophils (37 ± 1) (Figure 5B). Similarly, the MFI of IL-6 producing $CD14^{high}$ cells (335 ± 6) was 8 fold higher than neutrophils (42 ± 5) (Figure 5B). These results prompted our investigation of the ability of monocytes to respond to *Chlamydia* and influence neutrophil cytokine production.

When monocytes were added to populations of partially purified neutrophils, an increase in the percentage of neutrophils producing TNFα (Figure 5C) and/or IL-6 (data not shown) during incubation with D/UW-3/Cx or Pam_3Cys was observed. Although we did not see a difference in cytokine production upon stimulation with D/UW-3/Cx and CTD153 in preparations of highly purified neutrophils, we did see significantly enhanced levels of TNFα (Figure 5D), IL-6 (Figure 5D), IL-1β (Figure 5D), and G-CSF (data not shown) in the supernatants of partially purified populations of neutrophils after 3 hours of incubation with D/UW-3/Cx relative to CTD153 (Figure 5D). However, these differences were likely attributable to a small percentage of monocytes, since flow cytometry revealed both low MFIs and frequencies of neutrophils producing cytokines in these preparations relative to monocytes (Figure 5B). In addition, measurement of cytokines in the supernatants of highly purified neutrophils after 3 hrs revealed that neutrophil cytokine production was undetectable at this time point (data not shown). In contrast to the other proinflammatory parameters measured, we determined that MMP9 was robustly produced by highly purified neutrophils but not monocytes after 3 hours of incubation in vitro (data not shown). Thus, the augmented MMP9 production observed in response to D/UW-3/Cx in gradient purified populations of neutrophils (Figure 5D), although likely influenced by the cytokine milieu induced by chlamydial stimulation of monocytes, can be attributed to the neutrophils.

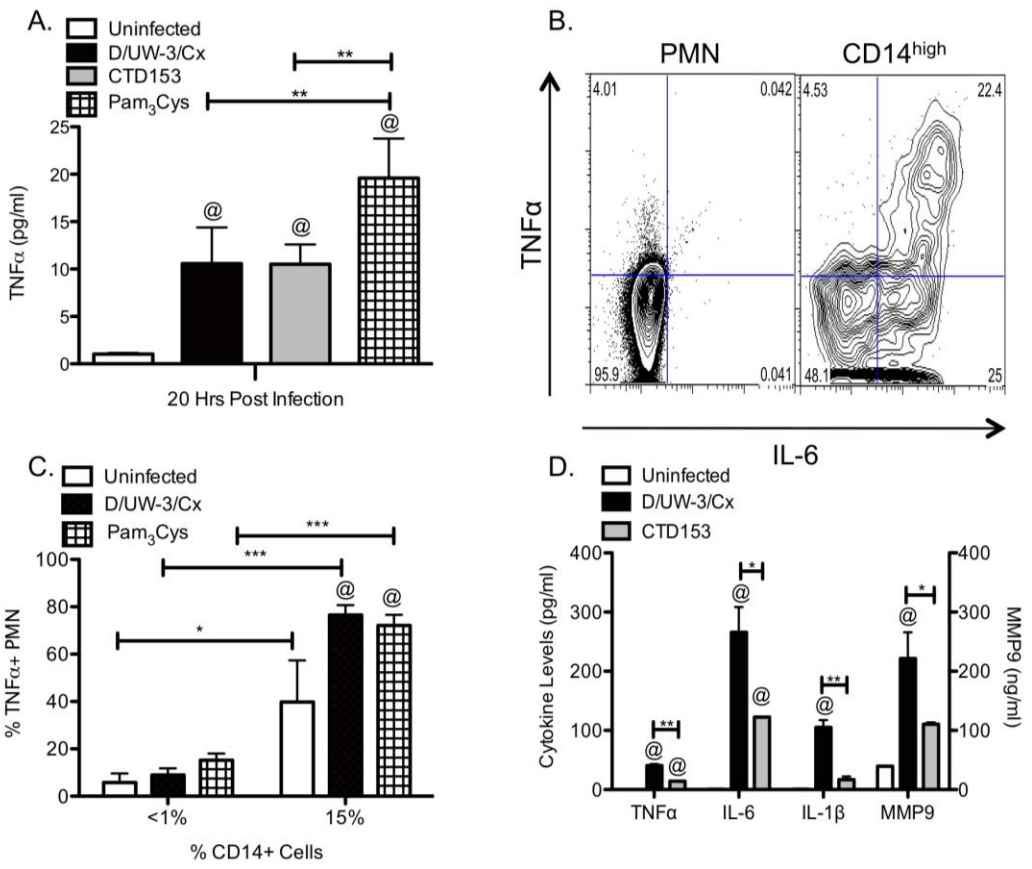

Figure 5: Highly purified human neutrophils produced low levels of cytokines in response to *Chlamydia*, but in the presence of monocytes, D/UW-3/Cx induced significantly higher levels of proinflammatory molecules than CTD153.

(A) Low levels of TNFα were detected in the supernatants of highly purified neutrophils after 20 hrs of incubation with Pam_3Cys (100ng/ml)(checkered bar), or an MOI of 5 of D/UW-3/Cx (black bar), or CTD153 (grey bar). Bars represent the mean ± SD of data pooled from 2 of 3 independent experiments. (B) Plot is gated on neutrophils (PMN, $CD14^{low/neg}FSC/SSC^{high}$) or $CD14^{high}$ monocytes (≤1% of cells) in the same well after stimulation with D/UW-3/Cx (MOI: 5) for 6 hours. (C) Increasing the frequency of $CD14^{high}$ monocytes from ≤1% to 15% significantly increased the percentage of neutrophils producing TNFα after 6 hrs of stimulation with D/UW-3/Cx or Pam_3Cys. Bars represent the mean ± SD of duplicate wells from one representative experiment of two. (D) Incubation of preparations of partially purified neutrophils containing ≤1% monocytes with D/UW-3/Cx (MOI: 5) for 3 hrs resulted in increased release of TNFα, IL-6, IL-1β, and MMP9 relative to incubation with CTD153. Bars represent the mean ± SD of duplicate wells from a representative experiment, which was conducted at least twice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between treatments, @ $P < 0.05$ vs. uninfected by one way ANOVA with Tukey's multiple comparison test.

2.4.6 Depletion of neutrophils from the oviduct using anti-Ly6G antibody cannot be accomplished during *C. muridarum* infection.

We determined that decreased numbers of neutrophils and reduced levels of proinflammatory molecules were present in the oviducts of mice infected with CM3.1. In order to prove that neutrophils contribute to the development of chronic oviduct pathology after chlamydial infection, we attempted to deplete these cells from the genital tract using anti-Ly6G (clone 1A8), an antibody specific for murine neutrophils. We treated mice with 300 μg (i.p.) of anti-Ly6G (Clone: 1A8) or control IgG every other day or every third day from day -1 to day 21 post infection with *C. muridarum* Nigg. Antibody treatment was discontinued on day 21 to avoid the development of serum sickness induced by a host response to foreign antibody. This time point was chosen based on our data indicating that the neutrophil response in the oviducts normally resolves by day 21 (Figure 1B). Since neutrophil depletion might lead to prolonged infection, which would lead to a continued influx of neutrophils after antibody administration was stopped, mice were administered doxycycline on days 22-26 to insure the infection resolved promptly after antibody treatment was stopped.

Flow cytometric analysis of the peripheral blood on day 11 revealed that $Ly6G^{high}$ cells were largely absent upon administration of anti-Ly6G antibody (Figure 6A). However, the reduced numbers of $Ly6G^{high}$ neutrophils in the peripheral blood did not lead to compromise in control of infection in the lower genital tract (Figure 6B) or a reduction in chronic pathology in either the oviducts (Figure 6C) or uterine horns (Figure 6D).

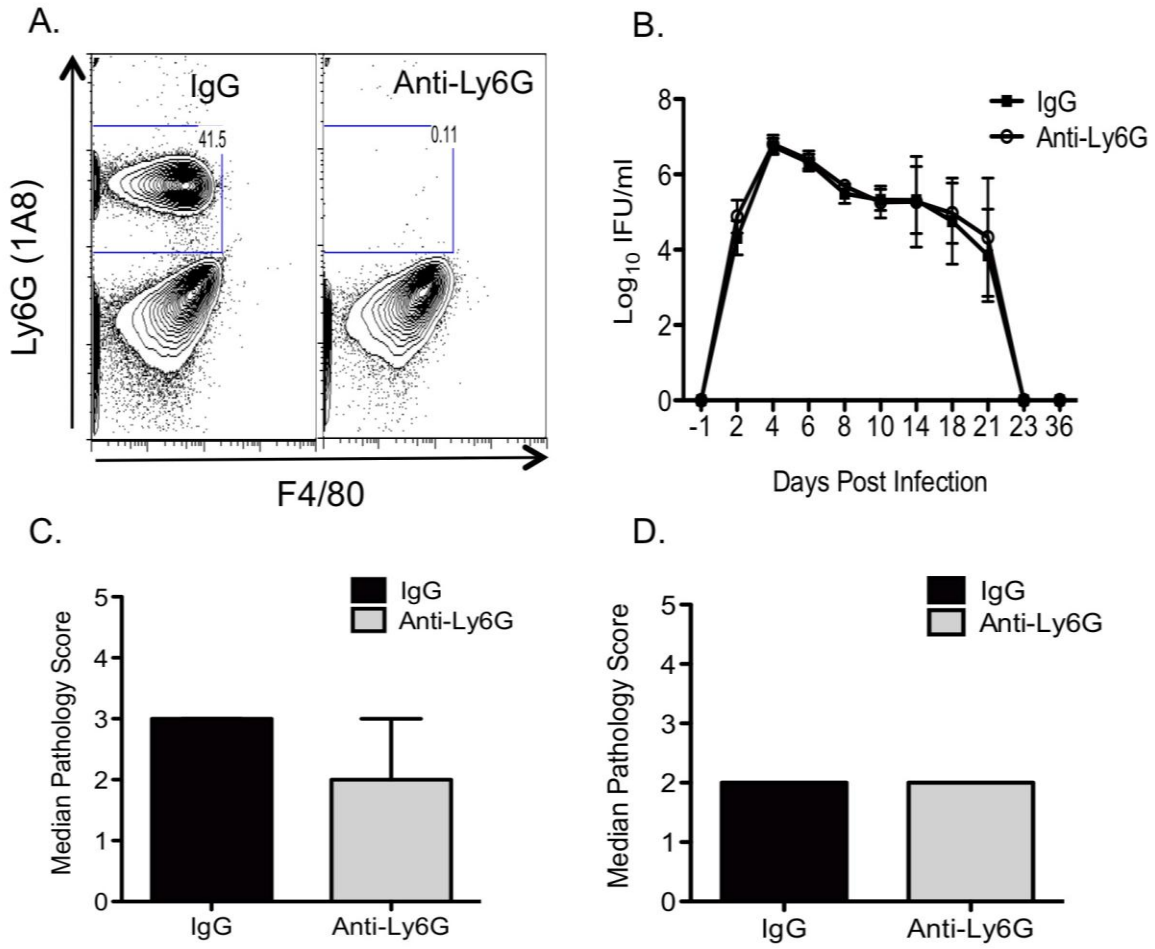

Figure 6: Anti-Ly6G antibody treatment effectively depletes Ly6G^{high} neutrophils from the peripheral blood during *C. muridarum* infection but does not alter the course of infection or the development of pathology.

(A) Flow cytometric analysis of peripheral blood on day 11 post-infection revealed that Ly6G^{high} cells were no longer detectable in the blood of mice treated with anti-Ly6G antibody. Plots are gated on CD45^{+} cells. (B) Titration of bacteria from lower genital tract swabs revealed no difference in the course of infection between mice administered IgG (black squares) or anti-Ly6G (clear circles) and infection quickly resolved with doxycycline treatment. Data points represent the mean ± SD of 5 mice per group from one representative experiment of three. $P > 0.05$ via two-way RM ANOVA. Histologic examination of (C) oviduct dilatation and (D) uterine horn distention on day 42 post infection revealed no difference between IgG (black bars) and anti-Ly6G (grey bars) treated mice. Bars represent the median and interquartile range of 9 mice per group from two independent experiments. $P > 0.05$ by Mann-Whitney U test.

Results presented in this chapter as well in previous studies point to neutrophils as key players in the development *Chlamydia*-induced pathology (30, 32, 36, 37). Thus, we were

surprised to find no reduction in chronic pathology upon depletion of $Ly6G^{high}$ high neutrophils from the peripheral blood (Figure 6A). In order to explain these results, depletion experiments were repeated with groups of mice euthanized on day 11 of infection for analysis of leukocyte infiltrates into the tissue. Flow cytometry revealed that the number of $CD45^{+}$ cells was not significantly reduced upon administration of anti-Ly6G antibody (data not shown), but these animals did exhibit a significantly increased frequency of $CD11b^{+}F4/80^{+}$ macrophages (Figure 7A) and a decreased frequency of $CD11b^{+}F4/80^{-}$ myeloid cells (Figure 7B) compared to mice treated with IgG. Further analysis of the phenotype of $CD11b^{+}F4/80^{-}$ cells revealed that in the oviducts of mice treated with IgG, these cells were 89.6% ± 6.9% $Ly6G^{high}$ neutrophils, whereas upon administration of anti-Ly6G, these cells were only 1.9 ± 2.0% $Ly6G^{high}$ neutrophils (Figure 7B). However, in the oviducts of mice treated with anti-Ly6G antibody, the frequency of $CD11b^{+}F4/80^{-}$ cells exhibiting intermediate staining with Ly6G/C (Clone: RB6-8C5) was robust (66.4 ± 9.5%) (Figure 7B). These findings indicated that a less differentiated population of granulocytes, characterized by low levels of Ly6G/C expression (124), had migrated into the tissue in the absence of $Ly6G^{high}$ neutrophils. In order to confirm the identity of these cells as immature neutrophils, we conducted histologic analysis on day 11 post-infection. Scoring of H&E stained oviducts revealed large numbers of cells exhibiting neutrophil or band morphology in the tissues of both anti-Ly6G and IgG treated animals (Figure 7C, E), with both groups having a median score of 4, which is the highest score in our semi-quantitative scoring system (30, 32). In addition, immunohistochemical staining revealed that this acute inflammation did indeed express Ly6G (Figure 7D, F). Thus, despite repeated efforts to deplete neutrophils with high levels of antibody and frequent administration, we were unable to prevent the influx of immature neutrophils that expressed low levels of Ly6G on their surface. These immature neutrophils are

likely capable of mediating the pathology we observed despite anti-Ly6G treatment, as levels of MMP9 in homogenized oviduct tissues, despite being reduced by 3 fold (IgG: 27±15.3 ng/ml; anti-Ly6G: 96 ± 3 ng/ml)(data not shown), were not absent, as would be expected if neutrophils had been completely depleted from the tissues. In addition, levels of the proinflammatory molecules TNFα, IL-6, and IL-1β measured in the homogenized oviducts on day 11 were not reduced upon administration of anti-Ly6G antibody (data not shown).

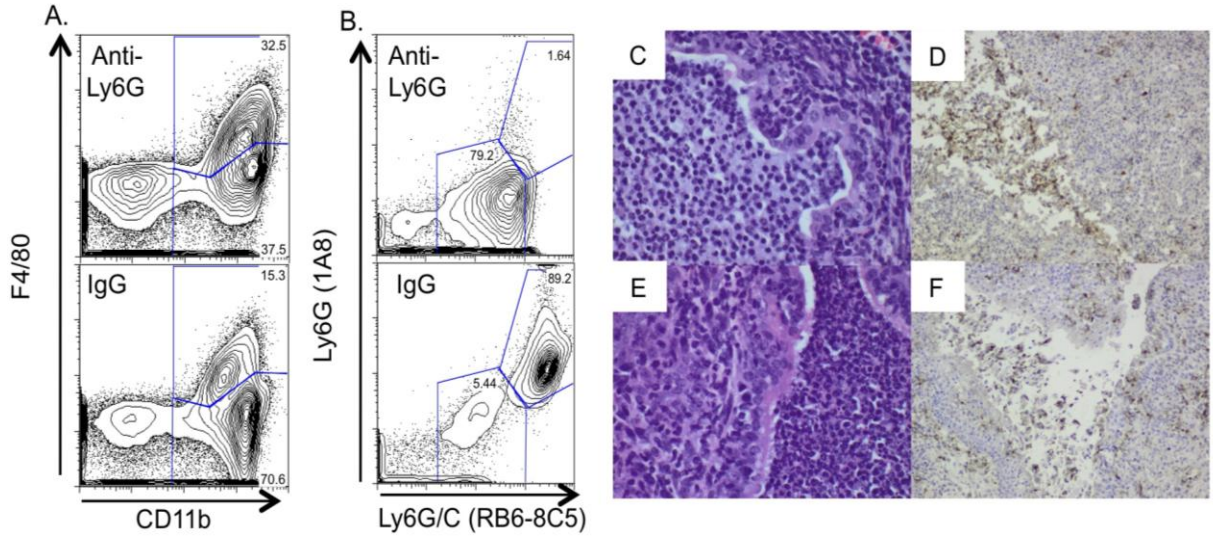

Figure 7: Treatment with anti-Ly6G antibody results in the elimination of $Ly6G^{high}$ neutrophils in the oviducts and a compensatory increase in macrophages and $Ly6G/C^{low}$ immature neutrophils.

(A) Flow cytometric analysis of the oviducts on day 11 post infection revealed an increased frequency of $CD45^+$ cells that were macrophages ($CD11b^+F4/80^+$) and a substantial remaining population of cells with surface marker expression characteristic of neutrophils ($CD11b^+F4/80^-$) upon treatment with anti-Ly6G compared to IgG. (B) Further analysis of $CD11b^+F4/80^-$ cells in the oviducts revealed the absence of $Ly6G^{high}$ neutrophils but a high frequency of $Ly6G/C^{low}$ myeloid cells in mice treated with anti-Ly6G. Plot is gated on $CD45^+CD11b^+F4/80^-$ cells. (C-F) Histologic examination of H&E stained sections (400x magnification) on day 11 post infection revealed cells exhibiting neutrophilic morphology in the oviduct upon treatment with (C) anti-Ly6G or (E) IgG. Immunohistochemical staining of upper genital tract tissues with anti-Ly6G antibody confirmed that cells expressing this antigen were present in the tissues of mice treated with (D) anti-Ly6G or (F) IgG.

2.5 DISCUSSION

Our previous study revealed that intravaginal infection of mice with a plasmid deficient strain of *C. muridarum*, CM3.1, results in the development of significantly lower levels of oviduct pathology compared to infection with wild-type *C. muridarum* Nigg (17). In addition, primary infection with CM3.1 prevents the development of oviduct pathology upon challenge with Nigg (17). Although CM3.1 leads to an infection of normal magnitude and duration, this strain stimulates an immune response that is able to induce resistance to reinfection with Nigg. Furthermore, the immune response effectively resolves infection without inducing collateral tissue damage. The reduced pathology is associated with decreased levels of the neutrophil chemokine CXCL2 in lower genital tract secretions and decreased neutrophil infiltrates in the oviducts on day 42 post-infection (17). Given previous data associating neutrophils with the development of oviduct pathology resulting from chlamydial infection (29, 30), it is not surprising that in the current study we detected a dramatically decreased neutrophil response in the oviducts of mice infected with CM3.1 during acute infection. In this chapter, we explored the mechanisms leading to this reduced neutrophil response as well as downstream effects that might contribute to protection from oviduct tissue damage during infection with plasmid-deficient chlamydiae.

We determined that both decreased recruitment and increased apoptosis contributed to the blunted neutrophil response observed in the oviducts of mice infected with CM3.1. Kinetic experiments revealed that levels of the neutrophil chemokines CXCL1, CXCL2, and GM-CSF were reduced in the oviducts upon infection with CM3.1. We determined that oviduct epithelial cell production of these chemokines was reduced in vitro upon infection with CM3.1 relative to Nigg, which is an important finding given the fact that the primary location of chlamydial

infection in the oviducts is the epithelium. These data correspond with our findings that CM3.1 exhibits a reduced ability to induce cytokine production by cervical epithelial cells (17). In addition, a previous study revealed that mice deficient in CXCR2, the receptor for CXCL1 and CXCL2, exhibit significant reductions in acute inflammation in the oviducts during *C. muridarum* infection, indicating that these chemokines play a role in migration of neutrophils to the genital tract (31).

In this study we also explored the ability of plasmid-deficient *Chlamydia* to prevent neutrophil apoptosis in vivo and in vitro. Neutrophils are terminally differentiated and rapidly undergo spontaneous apoptosis after release from the bone marrow (125). Bacterial products as well as cytokines have been demonstrated to delay neutrophil apoptosis (126-129), and a differential ability to prevent neutrophil apoptosis could contribute to the differences in numbers of neutrophils we detected in the oviducts upon infection with Nigg or CM3.1. Flow cytometric analysis of apoptosis revealed a decreased frequency of viable neutrophils and an increased frequency of late apoptotic/dead neutrophils in the oviducts during acute infection with CM3.1. Although no difference was detectable in the frequency of early apoptotic neutrophils, we detected increased phosphotidlyserine exposure by early apoptotic neutrophils isolated from mice infected with CM3.1. Phosphotidlyserine is externalized as cells undergo apoptosis (121), and this increased expression may indicate that cells in the early apoptotic gate of mice infected with CM3.1 may be more advanced in their progression towards late apoptosis. However, it is also possible that there are differences in the rate of phagocytsis of apoptotic neutrophils by macrophages, which could be due to an increased number of macrophages in the oviducts of mice infected with Nigg as well as potential differences in macrophage activation between the strains.

In order to more directly elucidate the ability of wild-type and plasmid-deficient *Chlamydia* to delay neutrophil apoptosis, we undertook analyses in vitro. Neutrophils are notoriously difficult to experimentally manipulate, and isolation of adequate quantities of non-activated highly purified neutrophils from mice is exceedingly difficult. Thus, we utilized a plasmid-deficient strain of *C. trachomatis*, named CTD153, which was derived from the human strain D/UW-3/Cx (115). This strain exhibits a similar phenotype to CM3.1, in that it does not accumulate glycogen or stimulate TLR2 (115). Although we determined that D/UW-3/Cx and CTD153 were equally efficient at delaying the apoptosis of highly purified neutrophils after 20 hrs of incubation, we determined that partially purified preparations of neutrophils containing a population of ~1% monocytes exhibited delayed apoptosis upon incubation with D/UW-3/Cx relative to CTD153. Thus, it is possible that differences in monocyte production of cytokines such as IL-1β, TNFα, and G-CSF were responsible for the differential ability of D/UW-3/Cx and CTD153 to delay neutrophil apoptosis (130-133). Differences in apoptosis were not due to discrepancies in bacterial burden since we verified equivalent bacterial loading by titration of *Chlamydia* after 1 and 3 hrs of incubation. To our knowledge, this is the first study to demonstrate that direct stimulation with *C. trachomatis* can delay neutrophil apoptosis.

Analysis of cells contributing to this cytokine milieu revealed that highly purified neutrophils produced miniscule levels of cytokines relative to monocytes. These findings agree with those from a study examining cytokine production by neutrophils and monocytes during stimulation with TLR agonists or *Mycobacterium bovis* BCG, which showed that neutrophils are ineffective producers of proinflammatory cytokines relative to monocytes (134). The reduced ability of neutrophils to produce cytokines relative to monocytes can likely be attributed to the enhanced expression of TLR2, TLR4, and CD14 by monocytes relative to neutrophils (122,

135). Monocyte cytokine production is likely responsible for our observation that increasing the percentage of $CD14^{high}$ cells from 1% to 15% enhanced neutrophil IL-6 and TNFα production. In contrast to proinflammatory cytokines, highly purified neutrophils and not monocytes produced large amounts of MMP9. However, neutrophil release of MMP9 was influenced by monocyte cytokine production since preparations of neutrophils that contained monocytes produced more MMP9 in response to D/UW-3/Cx than CTD153, but preparations of neutrophils depleted of $CD14^{high}$ cells did not exhibit differences between the strains. Our data indicating that neutrophils are the primary producers of MMP9 in response to *Chlamydia* are supported by in vivo studies revealing neutrophils as the main producers of MMP9 during *C. muridarum* genital tract infection (36). Thus, although neutrophils weakly contribute to proinflammatory cytokine production relative to monocytes in vitro, the presence of an inflammatory milieu during acute infection likely augments the contribution of neutrophils to this response. For example, when neutrophils were flow sorted from the cervices of mice infected with *C. muridarum*, they were determined to produce IL-1β at levels equivalent to macrophages (100). In addition, proinflammatory cytokines have been demonstrated to augment neutrophil expression of TLRs and to promote neutrophil activation (122, 133, 135, 136).

Previous studies reveal that proinflammatory cytokines contribute to the development of *Chlamydia*-induced genital tract pathology. Mice deficient in TNFα or IL-1β exhibit significantly reduced levels of hydrosalpinx (50, 100). In addition, oviduct pathology is significantly reduced upon infection of mice deficient in MMP9 (37). The combined reduction of all of these inflammatory mediators may explain the absence of oviduct pathology in mice infected with CM3.1.

A previous study in mice using anti-Ly6G/C (Clone: RB6-8C5), which binds not only neutrophils but also subsets of monocytes, dendritic cells, and CD8+ T cells, detected no alteration in the course of infection but did reveal reduced rates of hydrosalpinx resulting from *C. muridarum* genital tract infection (137-140). Although the authors detected a significant decrease in MMP9 levels, no reduction in acute inflammation was observed in the oviducts suggesting a lack of specificity for the outcome (137). Recently, anti-Ly6G (Clone: 1A8) antibody has been determined to specifically deplete neutrophils from mice (141, 142). Thus, in order to directly determine the role of neutrophils during *C. muridarum* genital tract infection, we treated mice with anti-Ly6G antibody (Clone: 1A8) from day -1 to day 21 of infection. Flow cytometric analysis revealed successful depletion of $Ly6G^{high}$ neutrophils from the peripheral blood and oviducts. Neutrophils are eliminated from the circulation within minutes of injection of anti-Ly6G/C (Clone: RB6-8C5) antibody (143) indicating that the inability to detect $Ly6G^{high}$ neutrophils in the circulation upon administration of anti-Ly6G (Clone: 1A8) is unlikely due to interference of depletion antibody bound to the surface of peripheral blood neutrophils with the binding of flow cytometric antibodies specific for the same epitope. However, it does appear that the decrease in neutrophils was compensated for by an increase in macrophages and immature $Ly6G^{low/int}$ neutrophils in the oviducts. The presence of these immature $Ly6G^{+}$ neutrophils in the upper genital tract was verified by morphologic assessment of H&E stained tissue sections and immunohistochemistry for Ly6G. With the influx of these inflammatory cells, it is not surprising that no difference in the course of infection or the development of pathology were detected upon administration of anti-Ly6G antibody. In our experimental setup, mice were treated with doxycycline on days 22 thru 26 post infection. Although tetracycline antibiotics can inhibit matrix metalloproteinases (MMPs), the MMP response that occurs after

day 21 is negligible (36, 38). Thus, administration of doxycycline likely did not contribute to our inability to detect a difference in pathology between mice treated with anti-Ly6G or control antibody.

Dissemination of bacteria from the genital tract to other organs such as the lungs, liver, and kidney occurs during acute infection of immunologically normal mice with *C. muridarum* (74). Localized and systemic infection with this organism drives the production of colony stimulating factors, which enhance release of neutrophils and immature band forms from the bone marrow. These neutrophils are then rapidly recruited to the site of infection. Although antibody treatment can rapidly eliminate the mature neutrophils with high levels of Ly6G expression, the immature bands, with lower levels of Ly6G expression, are not neutralized and survive to infiltrate the tissue. Indeed, a previous study revealed an increased frequency of bands in the peripheral blood upon treatment with anti-Ly6G/C antibody during *C. muridarum* infection (137). We hypothesize that this is the reason for failure of anti-Ly6G antibody to successfully deplete neutrophils from the genital tract tissues. These findings bring into question the ability of anti-Ly6G antibody to successfully eliminate neutrophils in any model that induces robust production of colony stimulating factors and requires that histological examination of tissues be performed to confirm the absence of cells with neutrophil morphology. In a local conjunctival infection with *Chlamydia caviae*, administration of anti-neutrophil antibody resulted in successful depletion of neutrophils in the tissue and significantlyejkri9 reduced levels of pathology (35). These findings support the role of neutrophils in the development of *Chlamydia*-induced pathology and the use of antibodies to deplete neutrophils during localized infection. A potential key factor in the success of neutrophil depletion in the *C. caviae* study was the use of a polyclonal antibody that was specific for an array of proteins expressed by

neutrophils, which prevented reduced expression of a single protein from hindering successful depletion, as occurred in our studies (35).

Consideration of our data together with multiple prior reports correlating increased neutrophils with enhanced oviduct disease after chlamydial infection supports an important role for these cells and their products in oviduct tissue damage (29, 30, 36, 37). Our findings of decreased neutrophil chemokines, shortened neutrophil lifespan, and decreased levels of proinflammatory molecules produced by neutrophils in the oviducts of mice infected with CM3.1, where chronic oviduct pathology is notably absent, is further suggestive of a key role for these acute inflammatory cells in causing reproductive tract sequelae. Unfortunately, the inability to deplete neutrophils from the local site of infection in this model prevents us from directly proving the deleterious role of these cells. However, these data indicate a need to limit acute inflammation in women with chlamydial infection, potentially through frequent screening, early treatment, or the use of drugs that specifically inhibit acute inflammatory mediators (144, 145).

2.6 ACKNOWLEDGEMENTS

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3.0 IL-17 CONTRIBUTES TO GENERATION OF TH1 IMMUNITY AND NEUTROPHIL RECRUITMENT DURING *CHLAMYDIA MURIDARUM* GENITAL TRACT INFECTION BUT IS NOT REQUIRED FOR MACROPHAGE INFLUX OR NORMAL RESOLUTION OF INFECTION

3.1 ABSTRACT

IL-17 contributes to development of Th1 immunity and neutrophil influx during *Chlamydia muridarum* pulmonary infection, but its role during *C. muridarum* genital tract infection has not been described. We detected similar numbers of *Chlamydia*-specific Th17 and Th1 cells in iliac nodes of wild-type mice early during genital *C. muridarum* infection, while Th1 cells predominated later. *Il17ra*$^{-/-}$ mice exhibited a reduced *Chlamydia*-specific Th1 response in draining iliac nodes and decreased local IFNγ production. Neutrophil influx into the genital tract was also decreased. However, *il17ra*$^{-/-}$ mice resolved infection normally, and no difference in pathology was observed when compared to wild-type. Macrophage influx and TNFα production were increased in *il17ra*$^{-/-}$ mice providing a compensatory mechanism to effectively control chlamydial genital tract infection despite a reduced Th1 response. In *ifnγ*$^{-/-}$ mice, a marked increase in cellular infiltrates and chronic pathology was associated with an increased Th17 response. Although neutralization of IL-17 in *ifnγ*$^{-/-}$ mice decreased neutrophil influx, macrophage infiltration remained intact and bacterial burden was not increased. Collectively,

these results indicate that IL-17 contributes to generation of Th1 immunity and neutrophil recruitment but is not required for macrophage influx or normal resolution of *C. muridarum* genital infection. These data highlight the redundant immune mechanisms operative at this mucosal site and the importance of examining site-specific responses to mucosal pathogens.

3.2 INTRODUCTION

Expansion of the Th1/Th2 paradigm to include the Th17 subset has provided important insight into the complexities of the mucosal immune response. Initially, IL-17 was identified as a key factor in the development of autoimmune inflammation and pathology (146-148); however, subsequent data have demonstrated both protective and pathologic roles for IL-17 depending upon the pathogen and target tissue (105, 106, 109, 149-155). In addition, Th17 cells link innate and adaptive immunity (156) and play a role in augmenting memory responses (147, 157), providing an attractive target for promoting vaccine-induced immunity.

Resolution of chlamydial infection and protection from pathology is associated with a strong Th1 response and IFNγ production (45, 51, 74, 75, 89). Recent work has demonstrated that IL-17 is critical for host defense against *C. muridarum* pulmonary infection, as bacterial clearance and development of Th1 immunity were compromised in the absence of IL-17 (105). In this mouse model, IL-17 was necessary for dendritic cell production of IL-12p70 and downstream development of a protective Th1 response (105). However, elevated levels of IL-17 have been associated with increased disease susceptibility during *C. muridarum* respiratory tract infection via recruitment of neutrophils (108, 109). Enhanced neutrophil influx and neutrophil release of matrix metalloproteases has been directly linked to tissue pathology in mouse models

of *C. muridarum* genital tract infection (29, 36). In addition, human studies have suggested that increased neutrophil activation is associated with the presence of *C. trachomatis* genital tract infection as well as with endometritis (27). Due to the pleiotropic effects of IL-17 on Th1 immunity and neutrophil induction and the significance of these responses in host defense to chlamydiae, it is important to directly examine the role of IL-17 in protection versus pathogenesis in the genital tract model of chlamydial infection.

We determined the numbers of chlamydial antigen-specific Th17 and Th1 cells in the draining iliac nodes of wild-type C57BL/6 mice after genital infection with *C. muridarum.* Although similar numbers of Th17 and Th1 cells were observed on day 7 of infection, by day 20, Th1 cells predominated. We then infected IL-17 receptor deficient (*il17ra*$^{-/-}$) mice on the C57BL/6 background. Decreased IFNγ production was observed in both NK cells in the cervical tissues and *Chlamydia*-specific CD4^{+} T cells in the iliac nodes of *il17ra*$^{-/-}$ mice. In addition, neutrophil influx was decreased in the *il17ra*$^{-/-}$ mice. Despite diminished Th1 and neutrophil responses, infection resolved normally and pathology was similar to wild-type mice. Enhanced macrophage influx and macrophage production of TNFα provided a potentially compensatory host defense mechanism in the *il17ra*$^{-/-}$ mice.

To further determine whether IL-17 plays a protective role during *C. muridarum* genital infection, we evaluated the role of IL-17 in the absence of a protective IFNγ response, removing any inhibition of IL-17 that might be imposed by IFNγ. *Ifnγ* $^{-/-}$ mice clear the majority of chlamydiae from their genital tract, indicating a role for alternative mechanisms of bacterial control. We observed a significantly increased Th17 response and markedly elevated neutrophil infiltrates in *ifnγ*$^{-/-}$ mice. Although neutralization of IL-17 in *ifnγ*$^{-/-}$ mice resulted in a significant decrease in neutrophil numbers in the oviducts, control of infection was not further compromised

and pathology was not improved. Alternate immune mechanisms continue to control infection, demonstrating the marked redundancy of host defense mechanisms operative at this mucosal site. Macrophage influx was sustained during IL-17 depletion, indicating a potential role for these cells as important mediators of host defense in the genital tract.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Mice homozygous for the *Ifnγ*^{tm1Ts} targeted mutation (*ifnγ*$^{-/-}$) and wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17 receptor deficient (*il17ra*$^{-/-}$) mice on the C57BL/6 background have been previously described (150). All mice were infected between 8-12 weeks of age. Mice were infected in groups of five except where otherwise indicated. Mice were given food and water ad libitum in an environmentally controlled room with a cycle of 12 hours of light and 12 hours of darkness. All animal experiments were approved by the University Institutional Animal Care and Use Committee.

3.3.2 Reagents and bacteria

Chlamydia muridarum (Nigg 1942), was cultured in mycoplasma-free McCoy or HeLa229 cells and chlamydial elementary bodies (EBs) were harvested from infected cells as previously described (158). Where specified, gradient purified *C. muridarum* Nigg EBs inactivated under ultraviolet light were used as antigen at a concentration of 5 μg/ml (159). Immunostimulatory

peptides (RplF$_{51-59}$) GNEVFVSPAAHIIDRPG, (PmpG$_{1303-311}$) SPIYVDPAAAGGQPPA, and (PmpE/F$_{2351-359}$) AFHLFASPAANYIHTG were synthesized and purified by Synthetic Biomolecules (San Diego, CA). These chlamydial peptides correspond to MHC class II epitopes that were discovered by Karunakaran *et al.* using immunoproteomics (160). Pooled peptides were solubilized in DMSO (4 mg/ml) and used at a concentration of 2 µg/ml each in media as stimulatory antigen for ELISPOT assays.

3.3.3 Murine infection and monitoring

Seven days prior to infection mice were subcutaneously injected with 2.5 mg of progesterone (Depo-Provera® Upjohn, Kalamazoo, MI) to synchronize all mice in a state of anestrous and to facilitate successful intravaginal infection (116). Mice were infected with $3x10^5$ inclusion forming units (IFU) of *C. muridarum* Nigg intravaginally. Mice were monitored for cervicovaginal shedding as described (117) and IFUs were calculated as previously described (30).

3.3.4 Cytokine and chemokine analysis of genital tract secretions and iliac node supernatants

Genital tract secretions were collected from mice on multiple days throughout the course of infection as previously described (32, 79). At specified intervals prior to and during infection, an aseptic surgical sponge (2 X 5 mm) (DeRoyal, Powell, TN.) was inserted into the vagina of an anesthetized animal and retrieved 30 minutes later. The sponges were stored at -70°C until cytokine assay. Each sponge was placed in a Spin-X microcentrifuge tube (Fisher Scientific,

Pittsburgh, PA) containing a 0.2 mM cellulose acetate filter and incubated in 300 ml of sterile phosphate-buffered saline (PBS) plus 0.5% bovine serum albumin (BSA) and 0.05 Tween 20 for 1 h on ice and then centrifuged for 5 minutes. Spin-X filters were first pre-blocked with 0.5 ml of sterile PBS plus 2% BSA and 0.05% Tween 20 for 30 min at 25°C, centrifuged, and washed twice with 0.05 ml of sterile PBS. Samples were kept on ice and promptly loaded into an ELISA plate prepared for a specific cytokine assay. Genital tract sponge eluates were assayed for cytokines and chemokines using Quantikine ELISA kits (R&D Systems, Minneapolis, MN) for IL-17, IL-6, TNFα, KC (CXCL1), and MIP-2 (CXCL2). IL-17, IFNγ, and IL-12p70 levels in iliac node mononuclear cell supernatants were similarly analyzed. TGF-β was quantified using a luciferase bioassay as previously described (161). Cytokines and chemokines in oviduct homogenates were quantified using the Multiplex Cytometric Bead Array (Millipore, Billerica, MA).

3.3.5 Quantification of antigen-specific IL-17 and IFNγ producing $CD4^+$ T cells by ELISPOT

C. muridarum antigen-specific IFNγ-and IL-17-producing $CD4^+$ T cells from infected iliac nodes were quantified using a previously described ELISPOT assay (153). Iliac nodes were harvested from nine C57BL/6 mice on days 7 and 20 post-infection. Cells from the nodes of 3 mice were pooled and processed to single cell suspensions. $CD4^+$ T cells were enriched by Magnetic Activated Cell Sorting (MACS; Miltenyi Biotec, Auburn, CA), and were routinely >90% pure. Cell culture plates (Multiscreen-HA; Millipore, Billerica, MA) were coated overnight at 4°C with monoclonal purified anti-mouse IFNγ (clone R4-6A2; eBioscience, San Diego, CA) or monoclonal purified anti-mouse IL-17 (clone 50101.111; R&D Systems, Minneapolis, MN) in

PBS. The plates were then incubated with blocking solution (DMEM containing 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FBS; all additives from Sigma-Aldrich, St. Louis, MO). Iliac node cells were plated at an initial concentration of $1x10^5$ cells/well in antibody-coated plates before being serially diluted to a concentration of ~3125 cells/well. Irradiated splenocytes from uninfected mice were used as antigen presenting cells at a concentration of $1x10^6$ cells/well. Cells were cultured in the presence of IL-2 alone (10 U/ml final concentration), *C. muridarum* UV-EBs (5 µg/well) + IL-2, or pooled *C. muridarum* peptides (1 µg/ml/peptide-see above) + IL-2. Spots were visualized using streptavidin-alkaline phosphatase (DakoCytomation, Ft. Collins, CO) and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium (Sigma-Aldrich, St. Louis, MO) as substrate. Spots were quantified using CTL-ImmunoSpot® S5 UV Analyzer and CTL ImmunoSpot® Professional Software Version 5.0 reader. Data are expressed as mean number of spot-forming-cells per million iliac node cells ± SD calculated from triplicate determinations.

3.3.6 In vivo neutralization of IL-17 in *ifnγ*$^{-/-}$ mice

Five *ifnγ*$^{-/-}$ mice were treated intraperitoneally with rat anti-mouse IL-17 MAb (Clone 50104**;** R&D Systems, Minneapolis, MN) (100 µg in 100 µl PBS) every other day from D-1 to D19 post-infection. A control group of five *ifnγ*$^{-/-}$ mice were treated similarly with Rat IgG2a (Clone 2A3; BioXCell, West Lebanon, NH). Both groups were infected with $3x10^5$ IFU of *C. muridarum* Nigg as indicated above. Mice were sacrificed on day 21 of infection. One oviduct was harvested and analyzed for bacterial burden, cytokines, and cellular influx (see section Processing of cervical and oviduct homogenates for flow cytometry). The second oviduct was

fixed in formalin and used for histopathological assessment. None of the mice exhibited signs of serum sickness over the course of antibody administration.

3.3.7 Assessment of lymphocyte proliferation and in vitro chlamydial antigen-specific cytokine responses

Iliac nodes of *il17ra*$^{-/-}$ mice and C57BL/6 mice infected intravaginally with *C. muridarum* were harvested on days 7, 21, and 35. Nodes of infected *ifnγ*$^{-/-}$ and C57BL/6 mice were harvested on days 7, 21 and 28. Nodes were processed to a single cell suspension and placed in culture with media alone, ConcanavalinA (ConA; 5µg/well), or UV-EBs (5µg/well) with or without anti-CD4 (1 µg/well, Clone RM4-5; BD Biosciences, San Diego, CA), which sterically blocks antigen-specific T cell proliferation due to CD4-TCR co-clustering. T cell proliferation was measured by incorporation of tritiated thymidine (1µCi/ well) after 96 hours of culture, and expressed as counts per minute (cpm) as measured by scintillation counter. Supernatants were collected for quantification of cytokines and chemokines as described above.

3.3.8 Processing of cervical and oviduct homogenates for flow cytometry

For analysis of the cervical immune response, three C57BL/6 and three *il17ra*$^{-/-}$ mice were sacrificed on day 4, 8, 9, or 14 post-infection. Cervical tissues were harvested in 90ul of media (RPMI+1%FBS). Tissues were minced, and the volume was expanded to 500ul with media. For analysis of cervical cytokines, 100ul was removed and stored at -80ºC. Collagenase I (1mg/ml; Sigma-Aldrich, St. Louis, MO) was added to bring the volume to 1 ml, and the suspension was incubated at 37°C for 20 minutes with shaking to disperse the cells. After incubation, 4 µl of

0.1M EDTA and 500 µl of media were added, and the homogenate was repeatedly passed through a 70µm filter to yield a single cell suspension.

Single cell suspensions of at least $5x10^5$ cells were stimulated with PMA (50ng/ml final concentration; Sigma-Aldrich, St. Louis, MO) and ionomycin (500ng/ml final concentration; Sigma-Aldrich) for 4 hrs at 37°C in the presence of GolgiPlug™ (1:1000 final dilution; BD Biosciences, San Diego, CA) prior to analysis for cytokine production via flow cytometry. After incubation, cells were washed and prepared as described below.

For surface staining, single cell suspensions of stimulated or unstimulated cells were resuspended in cold FACS buffer (PBS pH 7.2, 0.5% BSA, and 2mM EDTA) at $4x10^7$ cells per/ml. To block non-specific antibody binding, 25 µl ($1x10^6$ cells) of cell suspension and 25 µl of Fc Block™(CD16/CD32, BD Pharmingen, San Diego, CA) were combined in a 96 well V-bottom plate and incubated on ice for 20 minutes. The following antibodies were utilized for cell surface staining in our experiments: CD45 PerCP-Cy5.5(Clone 30-F11), Ly6G/C FITC, PE, and eFluor 450 (Clone RB6-8C5), F4/80 APC and PerCP (Clone BM8), Ly6G PE-Cy7 (Clone 1A8), NK1.1 PE-Cy7(Clone PK136), CD3 Alexa Fluor 700 (Clone 17A2), CD4 eFlour 450 (Clone RM4-5), CD8α FITC (Clone 53-6.7), all from eBioscience, San Diego, CA. Antibodies for CD11b FITC (Clone M1/70) and CD11c PE and PE-Cy7 (Clone HL3) were obtained from BD Pharmingen, San Diego, CA. Staining antibodies were added in 50ul of FACS buffer for a final dilution of 1.25ul antibody per 100ul total volume. After incubating for 20 minutes on ice, stained cells were washed, resuspended in Live/Dead® Fixable Stain (Invitrogen, Carlsbad, CA), and incubated for 20 minutes on ice in the dark. After washing, cells were fixed in 2% paraformaldehyde.

If cells were stimulated for intracellular cytokine staining, suspensions were treated as described above for surface and Live/Dead staining. They were then fixed and permeabilized according to manufacturer's instructions (BD Biosciences Cytofix/Cytoperm™ Kit, San Diego, CA). Intracellular cytokine staining was conducted as described above for surface staining. Stained cells were washed and incubated in 2% paraformaldehyde until analysis. The following antibodies were utilized for intracellular cytokine staining: IFNγ APC (Clone XMG1.2), IL-17A PE (Clone TC11-18H10), TNFα FITC (Clone MP6-XT22), all from BD Pharmingen, San Diego, CA.

For analysis of the oviducts, five C57BL/6 and five *ifnγ*$^{-/-}$ mice were sacrificed on days 7, 14, or 21 following intravaginal infection. Oviducts were homogenized as for cervical tissues but did not require collagenase treatment to yield single cell suspensions. Aliquots were removed as described above for cervical samples except that the total volume of oviduct sample plus media was brought to 1 ml prior to removal of 100ul for titration of bacterial burden or cytokine levels. Staining was conducted as described above. Flow Cytometric data were analyzed via FACSDiva or FlowJo Software.

3.3.9 Quantitative PCR analysis to determine *C. muridarum* burden in the oviducts

Aliquots (5µl) were removed from 100µl of frozen oviduct homogenates or genital tract swab eluate, and diluted into 195 µl of Epicentre DNA Extraction Solution, before being processed according to the manufacturer's instructions. Real time PCR reactions were carried out using iQ Sybergreen supermix (BioRad, Hercules, CA) in a BioRad iCycler using primers directed against chlamydial 16S rRNA (Sense: 5' CGTTAATACCCGCTGGATTTGAG 3'; Antisense: 5'

GCCCCGATCTTTGACAACTAAC 3') in a two step reaction, 95°C, 10 sec.; 55°C 30 sec for a total of 40 cycles. Melt curve analysis showed that the accumulation of SYBR green-bound DNA was gene specific and not due to primer dimers. The samples were assayed in duplicate. The chlamydial load in the oviducts of each mouse, expressed as genome equivalents, was extrapolated from a standard curve generated using 16s rRNA amplified PCR product of known concentration and adjusted for the presence of two copies of this gene in *C. muridarum*.

3.3.10 Quantification of infectious *C. muridarum* in oviduct homogenates

In the experiment using *ifnγ*$^{-/-}$ mice and C57BL/6 mice that were not treated with antibody, a 100 µl aliquot of the homogenized oviducts was serially diluted in 1xPBS for titration of bacteria using a plaque assay (114). On day 7 and day 14, the limit of detection of the plaque assay was ≤ 99 organisms and on day 21, the limit of detection was ≤ 19 organisms. Samples with a negative titer were assigned a value of zero for statistical analysis.

3.3.11 Microscopic histopathological assessment

Mice were sacrificed on specified days following intravaginal infection; the genital tracts were removed en bloc, fixed in 10% buffered formalin and embedded in paraffin. Longitudinal 4-µm sections were cut and stained with hematoxylin and eosin. Each anatomic site (ectocervix, endocervix, uterine horn, oviduct) was assessed independently for the presence of neutrophilic inflammation, lymphocytic/monocytic inflammation, plasma cells and fibrosis and assessed using a four-tiered semi-quantitative scoring system to evaluate the extent of inflammation and fibrosis as previously described (30, 32).

3.3.12 Statistics

Statistical comparisons between the murine strains for level of infection and cytokine production over the course of infection were made by a two-factor (days and murine strain) analysis of variance with post hoc Tukey test as a multiple comparison procedure. The Wilcoxon rank sum test was used to compare the duration of infection in the respective strains over time. Mann-Whitney U test was used to determine significant differences in the pathological data between groups. Differences in lymphocyte proliferation, in vitro cytokine production, ELISPOT cytokine data, and oviduct homogenate cytokine and flow cytometric data were analyzed by the Student's *t*-test, or by ANOVA as appropriate. SigmaStat software was utilized for all statistical analysis (SPSS). Values of $P < 0.05$ were considered significant.

3.4 RESULTS

3.4.1 *C. muridarum* antigen-specific $CD4^+$ Th1 and Th17 cells were present in similar numbers in the iliac nodes of C57BL/6 mice on day 7 post-infection, but Th1 cells predominated by day 20.

To compare the Th1 and Th17 responses during genital tract infection in wild-type mice, ELISPOTs were performed using purified $CD4^+$ T cells harvested from iliac nodes on day 7 and day 20 post-infection (Figure 8). The primary objective of this experiment was to define that Th17 cells are detectable in the draining lymph nodes following *C. muridarum* genital tract infection in wild-type mice, and examine the kinetics of this response in relation to development

of the Th1 response. Similar numbers of *Chlamydia*-specific Th1 and Th17 cells were detected on day 7 (Figure 8A). In contrast, by day 20, Th1 cells were significantly increased with respect to Th17 cells (Figure 8B) indicating that as infection resolves in wild-type mice, the Th1 response predominates. The reduction in T cell numbers detected at day 20 when compared to day 7 is likely due to reduced bacterial burden and reduced antigen stimulation related to in vivo clearance of infection. It is possible that a percentage of cells produced both IFNγ and IL-17 (111). Experiments were then conducted to examine the role of the IL-17/Th17 response during genital tract chlamydial infection.

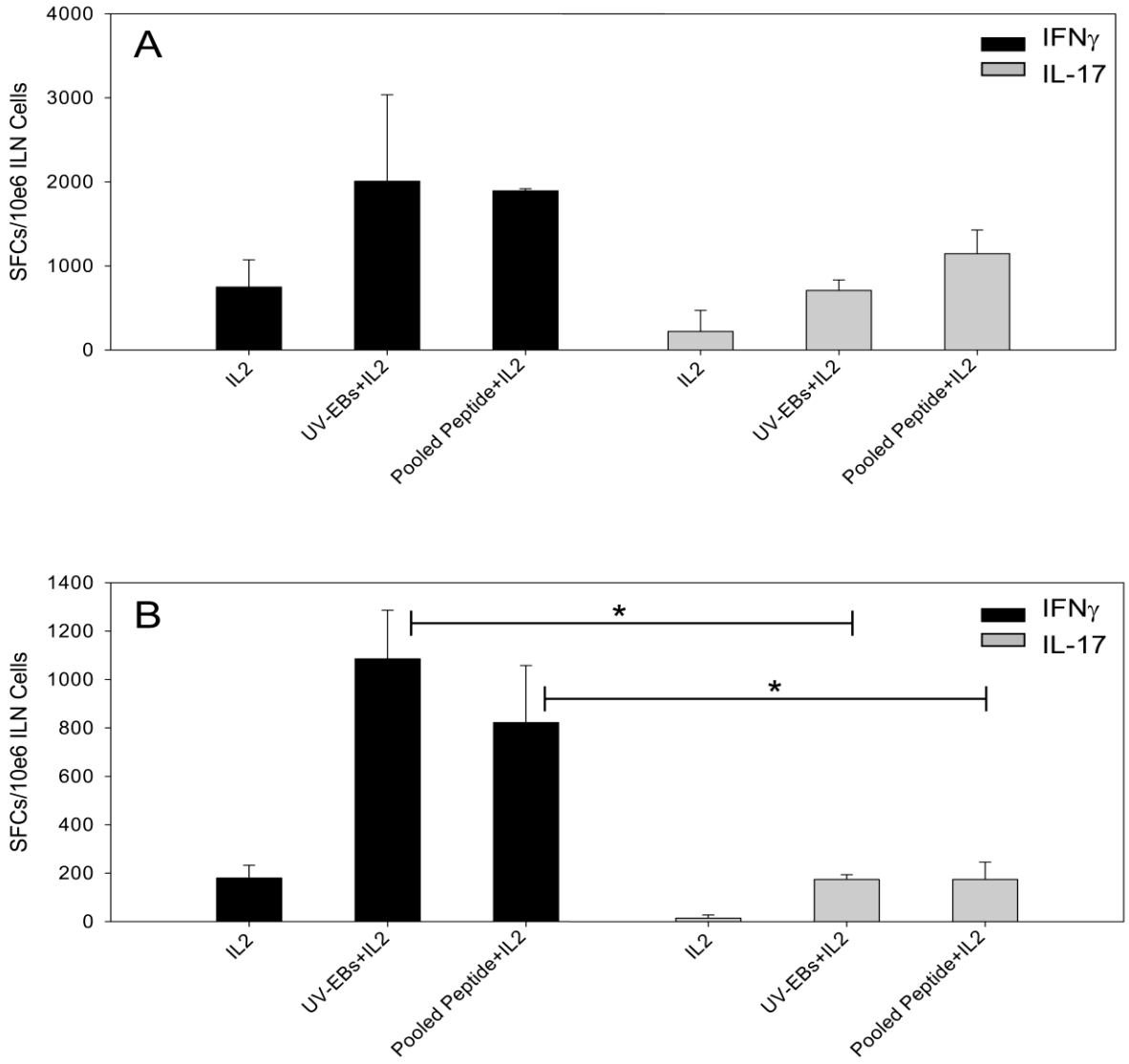

Figure 8: *C. muridarum* antigen-specific Th1 and Th17 cells were detected in similar numbers in the iliac nodes of C57BL/6 mice on day 7 post-infection, but Th1 cells predominated on day 20.

Numbers of antigen-specific IFNγ (black bars) and IL-17 (gray bars) producing CD4+ T cells in iliac nodes of C57BL/6 mice were quantified by ELISPOT on (A) day 7 and (B) day 20 post-infection. Significantly increased numbers of IFNγ-producing CD4+ T cells and IL-17-producing CD4+ T cells were noted on both (A) day 7 and (B) day 20 post-infection after stimulation with UV-EBs or C. muridarum pooled peptide when compared to stimulation with IL-2 alone ($P < 0.05$ by one-way ANOVA). On day 7 post-infection, numbers of UV-EB- and pooled peptide-specific Th1 (IFNγ-producing) (black bars) and Th17 (IL-17-producing) (gray bars) cells were not significantly different. By day 20, numbers of UV-EB- and pooled peptide-specific Th1 (IFNγ-producing) cells were significantly greater than Th17 (IL-17-producing cells) (* P <0.05 by one-way ANOVA). The data are representative of two individual experiments in which the cells from three groups of three mice each were pooled and analyzed.

3.4.2 The Th1 response was reduced in the iliac nodes of *il17ra*$^{-/-}$ mice.

Significantly lower production of IFNγ by *Chlamydia*-specific CD4+ T cells from the iliac nodes of *il17ra*$^{-/-}$ mice was detected on days 7, 21, and 35 post-infection when compared to C57BL/6 mice (Figure 9A). Although we noted a contraction of the T cell response in the wild-type mice at day 20 in the previous experiment (Figure 8B), it is not possible to directly compare the ELISPOT data, which measures the antigen-specific cytokine producing cells, with the protein levels detected by ELISA in this experiment due to differences in methodology and experimental read-out. Since IL-12p70 is essential for induction of Th1 immunity, we assayed IL-12p70 in supernatants of *Chlamydia*-stimulated iliac node cells harvested from infected mice. Levels were significantly reduced in *il17ra*$^{-/-}$ mice on days 7 and 21 post-infection when compared to wild-type mice (Figure 9). Despite reduced IFNγ and IL-12p70, IL-4 was not increased indicating that a shift towards a Th2-like response did not occur (Figure 9C).

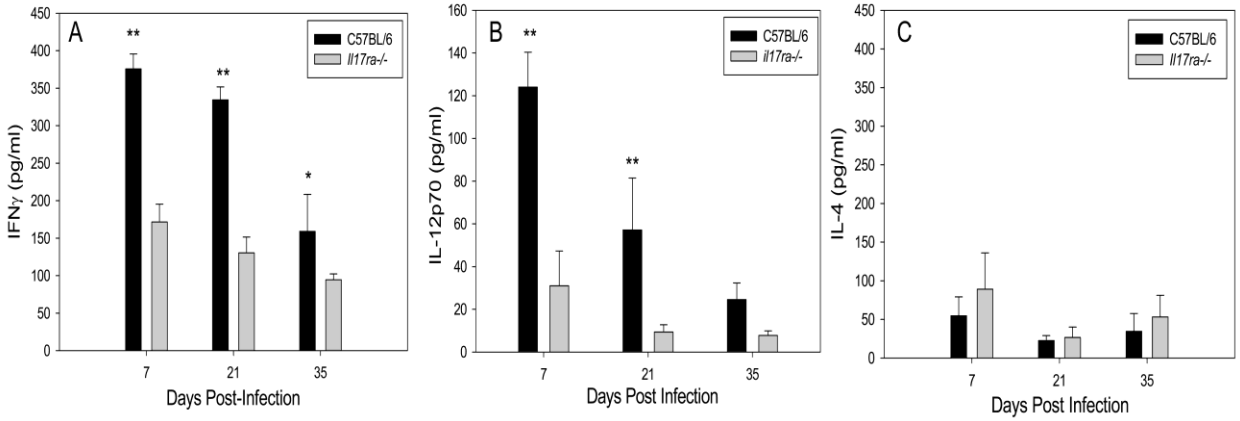

Figure 9: *Il17ra*$^{-/-}$ mice displayed reduced IFNγ and IL-12p70 production in the iliac nodes.
Iliac node mononuclear cells from individual *il17ra*$^{-/-}$ mice or C57BL/6 mice intravaginally infected with *C. muridarum* were stimulated in vitro with UV-inactivated EBs for 96 hours. Supernatants were analyzed for (A) IFNγ, (B) IL-12p70, and (C) IL-4. Levels of IFNγ and IL-12p70 were significantly reduced in *il17ra*$^{-/-}$ mice. Addition of a blocking anti-CD4 antibody resulted in >95% inhibition of the IFNγ response, and levels were undetectable in cells incubated with media alone (data not shown). Data points represent the mean ± SD of values from 5 mice in a single independent experiment. * $P < 0.050$; ** $P < 0.001$, Student's *t*-test.

3.4.3 IL-17RA deficiency results in decreased cervical NK cell IFNγ production.

Significantly lower levels of IFNγ were detected in genital secretions of the infected *il17ra*$^{-/-}$ mice on days 2 and 4 post-infection (Figure 10A) and in cervical homogenates on day 4 and day 8 (data not shown). Flow cytometric analysis of cervical tissues revealed decreased numbers of IFNγ-producing NK1.1^{+} cells (Figure 10B, C) on day 4 and day 8 in *il17ra*$^{-/-}$ mice compared to wild-type. Cytometric analysis of NK1.1 and CD3 markers revealed that both NK and NKT cell populations were significantly decreased in the *il17ra*$^{-/-}$ mice, but NK cells (NK1.1^{+}CD3^{-}) were the predominant producers of IFNγ (>80% of NK1.1^{+} cells) in the cervix (data not shown).

The decreased Th1-IFNγ response observed in the absence of IL-17 signaling parallels findings in the pulmonary model of *C. muridarum* infection during anti-IL-17 administration

(105). The marked reduction in NK cell IFNγ is similar to results obtained during infection of *il17ra*$^{-/-}$ with the intracellular pathogen *Francisella tularensis* (154). In both of these pulmonary infection models, increased IL-4 production was noted. We did not detect increased levels of IL-4 during chlamydial genital tract infection indicating that alternative cell types might be activated in the genital tract to prevent this shift.

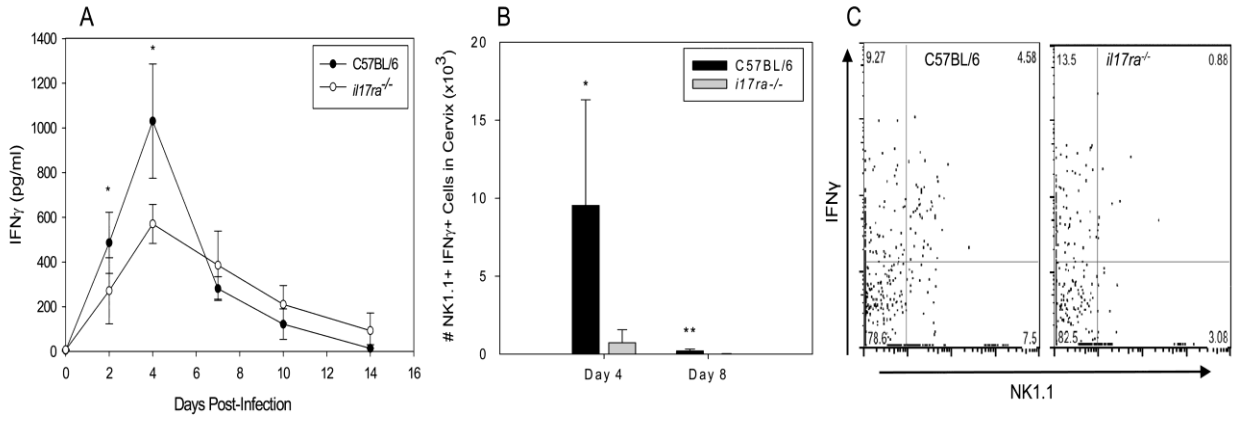

Figure 10: *Il17ra*$^{-/-}$ mice displayed reduced IFNγ production and fewer IFNγ producing NK cells in the lower genital tract.

(A) IFNγ levels were significantly lower in genital secretions of *il17ra*$^{-/-}$ mice (open circles) on day 2 and 4, but levels were similar to wild-type (black circles) from days 6-14. Data points represent the mean ± SD of 5 mice per group of a single individual experiment. * $P < 0.050$ by two-way RM ANOVA with multiple comparisons procedure. (B, C) Cell specific IFNγ production in infected *il17ra*$^{-/-}$ and C57BL/6 mice was measured via flow cytometric analysis of intracellular cytokine staining and revealed significantly decreased numbers of IFNγ-producing NK cells on day 4 and day 8 in the cervix of *il17ra*$^{-/-}$ mice. (B) Bars represent the mean number ± SD of live NK1.1^{+}IFNγ^{+} cells in the cervical tissues from 3 mice of a single individual experiment. (C) Flow cytometric plot is gated on live cells. * $P < 0.050$, ** $P < 0.010$ Student's *t*-test.

3.4.4 Neutrophil recruitment was decreased in the lower genital tract of *il17ra*$^{-/-}$ mice during *C. muridarum* infection.

In addition to promoting Th1 immunity, IL-17 has been shown to contribute to neutrophil recruitment in chlamydial pulmonary infection models (108, 109). Therefore, we measured

neutrophil chemokines in vaginal secretions of infected C57BL/6 and *il17ra*$^{-/-}$ mice. Lower levels of the neutrophil chemoattractants CXCL1 (KC) (Figure 11A) and G-CSF (data not shown) were observed in the secretions of *il17ra*$^{-/-}$ mice. However, levels of CXCL2 (MIP-2) were not compromised in the *il17ra*$^{-/-}$ mice (Figure 11B). The percentage and absolute number (data not shown) of neutrophils in the cervix were similar on day 4 but were decreased in *il17ra*$^{-/-}$ mice on days 8 and 14 of infection (Figure 11C, D). We suspect that the decrease in CXCL1 expression detected early on in the genital secretions of the *il17ra*$^{-/-}$ mice is maintained in the tissue throughout infection, driving lower influx of neutrophils over time.

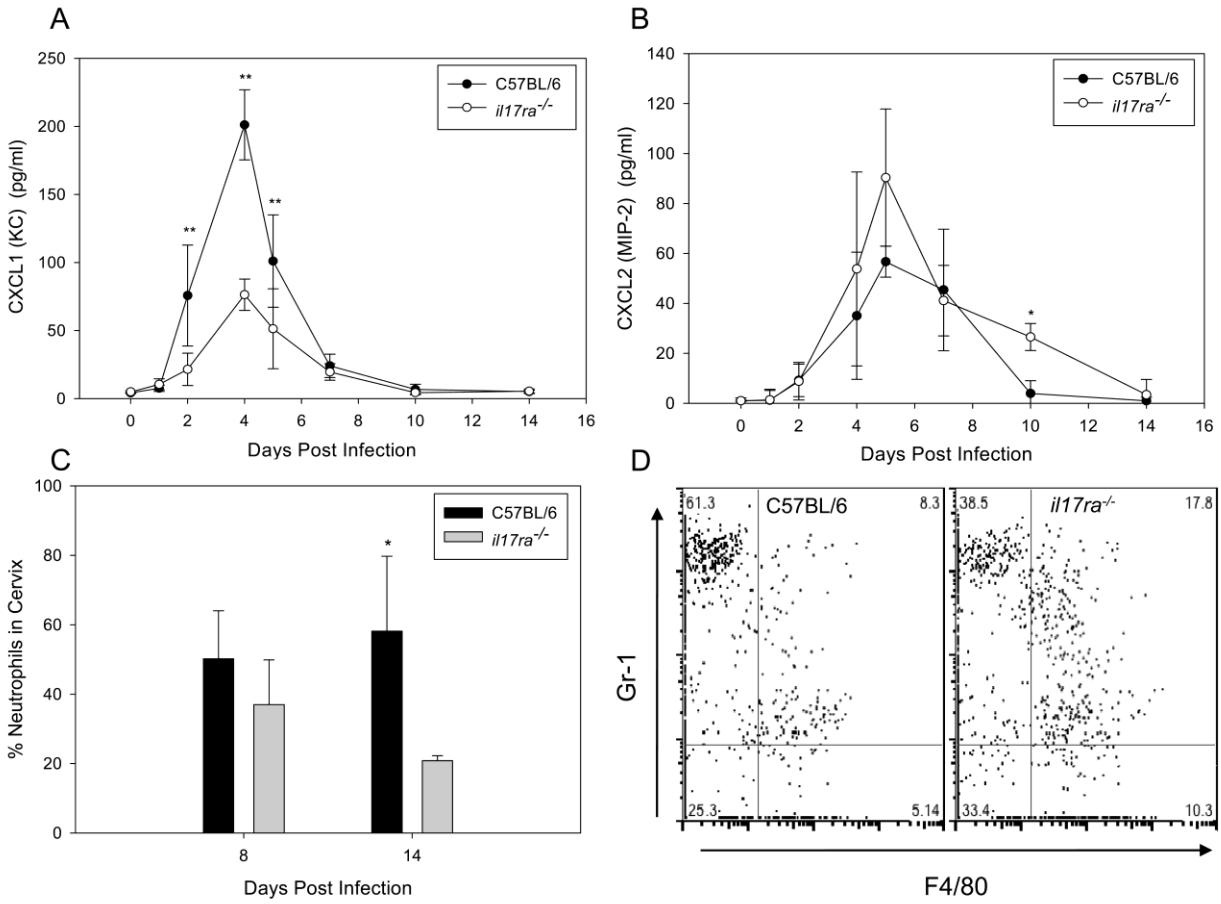

Figure 11: *Il17ra*$^{-/-}$ mice exhibited reduced chemokine levels and fewer neutrophils in the lower genital tract during early *C. muridarum* infection.

Il17ra$^{-/-}$ mice (open circles) and C57BL/6 mice (black circles) were intravaginally infected with *C. muridarum*. Measurement of neutrophil promoting chemokines in vaginal secretions revealed significantly decreased (A) CXCL1 (KC) ($P < 0.001$, two-way RM ANOVA), but secretion of (B) CXCL2 (MIP-2) remained intact ($P < 0.050$, two-way RM ANOVA; * $P < 0.01$, ** $P < 0.001$ for individual days). Data points represent the mean ± SD of five mice analyzed in a single individual experiment. (C, D) The influx of neutrophils into the cervix of *i17ra*$^{-/-}$ mice was decreased on day 8 and day 14 (* $P < 0.02$, Student's *t*-test). (C) Bars represent the percentage of CD45+ cells that were neutrophils ($CD45^{+}Ly6G/C^{high}F4/80^{-}$) ± SD for 3 mice of a single individual experiment. (D) Flow plot is gated on live CD45+ cells. Neutrophils are Ly6/C (Gr-1) ^{high} $F4/80^{-}$. Macrophages are Ly6G/C $(Gr\text{-}1)^{+}$ $F4/80^{+}$

3.4.5 *Il17ra*$^{-/-}$ mice exhibited a normal course of infection and no difference in chronic pathology compared to wild-type mice.

Despite dampened Th1 and neutrophil responses in the absence of IL-17 signaling, no delay in bacterial clearance from the lower genital tract was observed in *il17ra*$^{-/-}$ mice (Figure 12). The degree of chronic oviduct dilatation and uterine horn distension observed after resolution of infection was similar between wild-type and *il17ra*$^{-/-}$ mice. (Figure 13A, B) on days 35 and 56 post-infection. In addition, no differences in histopathology between wild-type and *il17ra*$^{-/-}$ mice were detected in the oviducts (Figure 13C, D), uterine horns (Figure 13E, F) or cervix (data not shown) on days 35 and 56 post-infection.

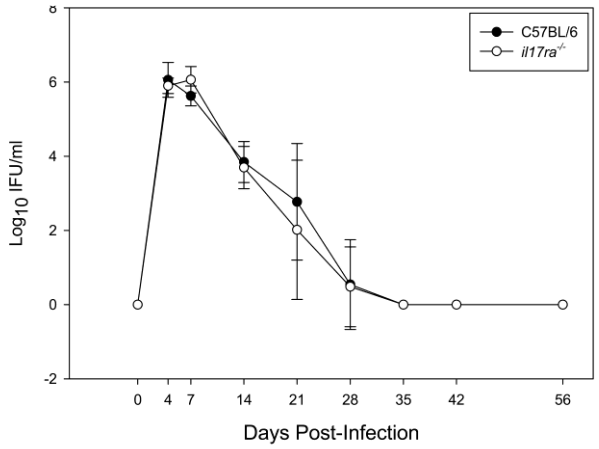

Figure 12: C57BL/6 and *il17ra*$^{-/-}$ mice exhibit no difference in the course of *C. muridarum* infection.
Groups of five C57BL/6 (black circles) and five *il17ra*$^{-/-}$ mice (open circles) were intravaginally infected with *C. muridarum,* and the course of infection was monitored via endocervical swabs. Titration of live bacteria revealed that the bacterial burden in the lower genital tract was similar between strains. Data points represent the mean ± SD of IFU values from both culture positive and negative mice at each time point of a single experiment that is representative of 2 independent experiments.

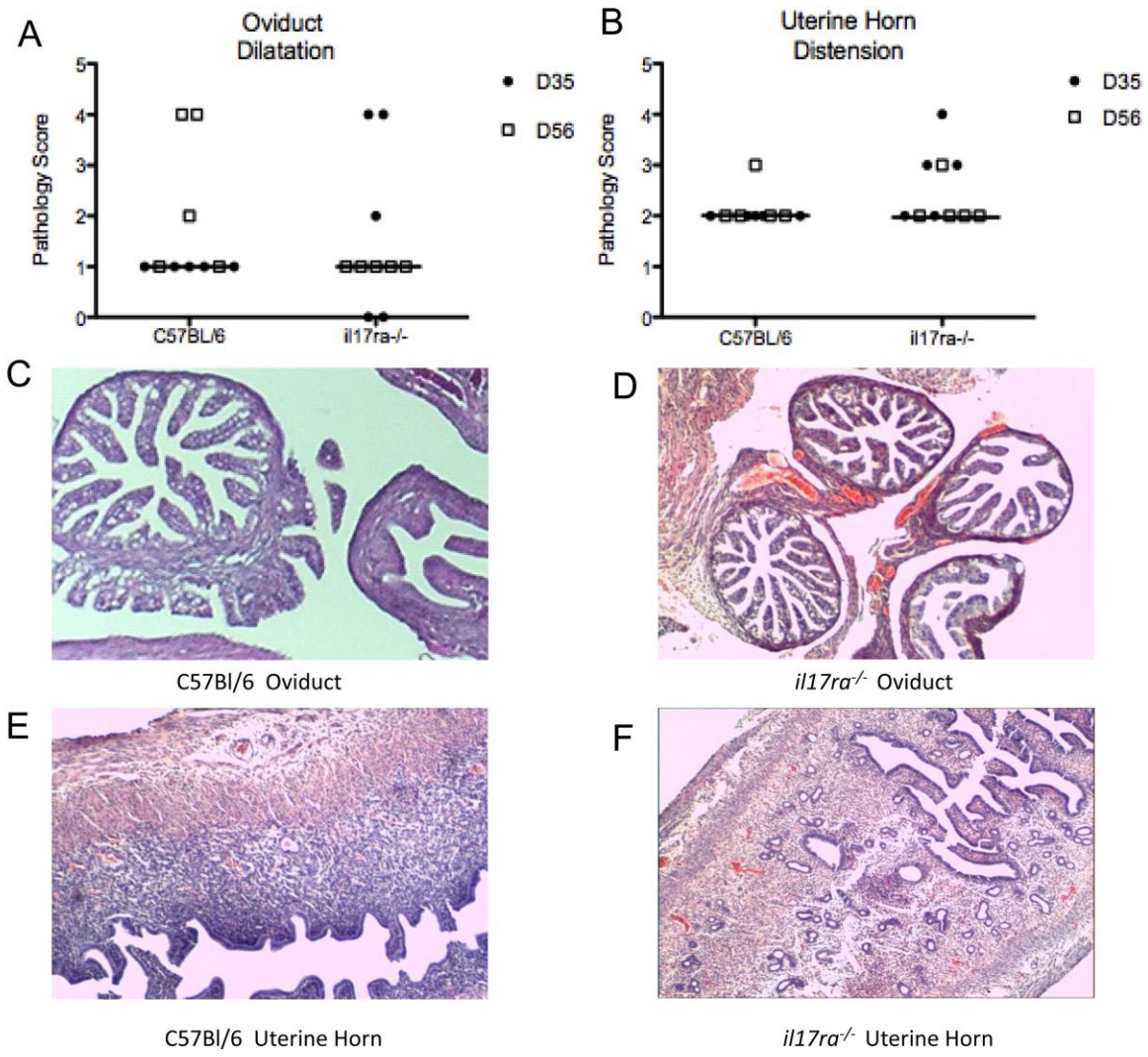

Figure 13: *Il17ra*$^{-/-}$ mice did not exhibit enhanced genital tract pathology during *C. muridarum* genital infection. Genital tract tissues were removed en bloc from *C. muridarum*-infected mice at 35 and 56 days post infection (n=5 mice per strain, per time point). Tissues were scored for (A) oviduct dilatation and (B) uterine horn distension. Scores for individual mice on day 35 are indicated by closed circles and scores for day 56 are indicated by open squares. The median score is indicated by a dark line. No differences in median pathology scores were noted between groups on either day examined (Mann-Whitney U test). Tissues were also examined grossly and histologically. (C) Oviducts from C57BL/6 and (D) *il17ra*$^{-/-}$ mice displayed similar inflammatory scores and oviduct dilatation. Uterine horns from (E) C57BL/6, and (F) *il17ra*$^{-/-}$ mice exhibited no significant differences in inflammation or fibrosis. Tissues are representative samples of the (C, D 40x) oviducts and (E, F 40x) uterine horns from C57BL/6 mice and *il17ra-/-* mice on day 56 post-infection.

3.4.6 Macrophage influx and cytokine production were increased in *il17ra*$^{-/-}$ mice.

The effective control of chlamydial genital tract infection in *il17ra*$^{-/-}$ mice contrasted sharply with the outcome of respiratory tract infection in IL-17 depleted mice, where the lack of a measureable Th1 response resulted in uncontrolled infection (105). In the lung model, IL-17 depletion also resulted in enhanced neutrophil and macrophage inflammation, which was attributed to the increased bacterial burden observed in the absence of IL-17 (105). Flow cytometric analysis of cervical tissues revealed that the percentages of macrophages were increased in *il17ra*$^{-/-}$ mice on days 9 and 14 (Figure 14A; Figure 11D). In addition, the absolute number of macrophages in the cervix was significantly elevated on day 9 (Figure 14B). Remarkably, a significantly higher frequency of TNFα+ macrophages was found in cervical tissues of *il17ra*$^{-/-}$ mice using intracellular cytokine staining (Figure 14C). In addition, increased levels of TNFα were observed in the secretions of *il17ra*$^{-/-}$ mice during the early days of infection (Figure 14D). Significantly increased levels of IL-6 were detected in the secretions of the *il17ra*$^{-/-}$ mice (P = 0.048, Two-way RM ANOVA; data not shown), providing corroborative evidence of enhanced macrophage activation in these mice. These findings suggest a previously undescribed role for IL-17 in diminishing monocyte/macrophage influx and activation in an infected tissue.

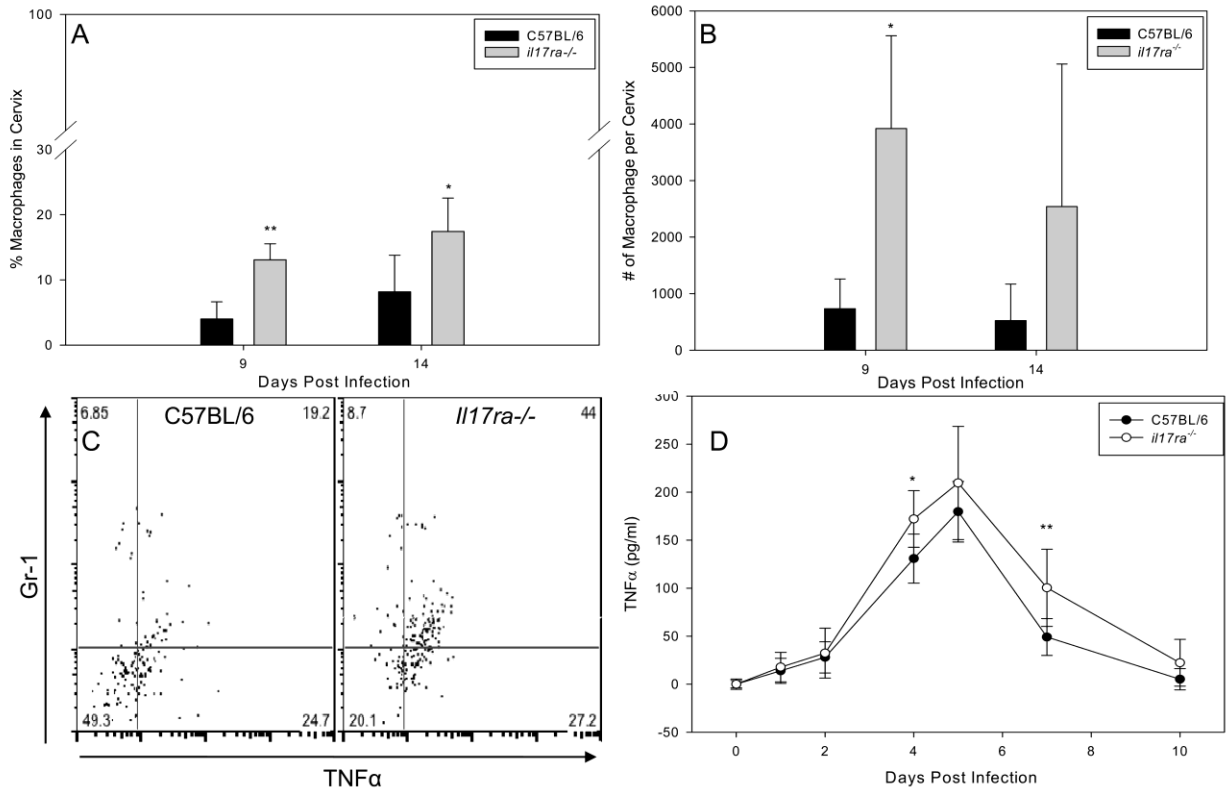

Figure 14: Macrophage influx and activation were increased in the cervix of *il17ra-/-* mice.

(A) Flow cytometric analysis of cervical tissues on day 9 and day 14 post infection revealed that the percentage of $CD45^+$ cells that were macrophages ($CD45^+Gr\text{-}1^+F480^+$) was significantly higher in the cervical tissue of *il17ra-/-* mice (** $P < 0.005$; * $P < 0.01$; two-way ANOVA with multiple comparisons). (B) The absolute number of macrophages was also increased in the cervical tissue of in *il17ra-/-* mice (* $P< 0.050$ for day 9). Bars represent mean ± SD of 3 mice analyzed in a single independent experiment. (C) The percentage of macrophages that were TNFα+ was significantly higher in the cervical tissues of *il17ra-/-* mice on day 9 ($P = 0.007$; Student's t-test). Plot is gated on live $CD45^+CD3^-F480^+$ cells. (D) TNFα levels were significantly higher in the secretions of *il17ra-/-* mice ($P <0.001$ via two-way RM ANOVA with multiple comparisons procedure. * $P < 0.02$ ** $P < 0.005$ on days 4 and 7 respectively). Bars represent mean ± SD of 5 mice from a single independent experiment.

3.4.7 *Ifnγ*$^{-/-}$ mice exhibited chronic low-level shedding of *C. muridarum* and increased neutrophil infiltrates throughout the genital tract in response to chlamydial infection.

Because IFNγ has been shown to negatively regulate the Th17 response (162), we hypothesized that the severe pathology previously described in the absence of IFNγ during chlamydial genital tract infection of *ifnγ*$^{-/-}$ mice (74, 75)(6,32) may be due to enhanced induction of Th17 cells and IL-17-mediated increases in neutrophil recruitment. After intravaginal infection, similar levels of bacterial shedding were observed in wild-type and *ifnγ*$^{-/-}$ mice over the first three weeks, with both groups eliminating 99% of the chlamydial organisms from their genital mucosa (74, 75). Thereafter, the wild-type mice became culture negative, while *ifnγ*$^{-/-}$ mice alternated between culture negative and culture positive states (Figure 15), suggesting that they remained infected at extremely low levels.

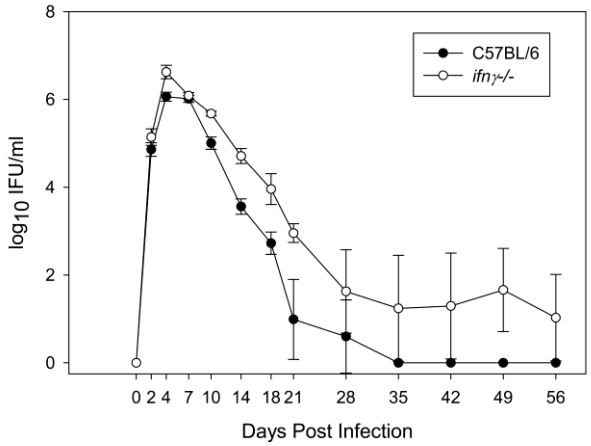

Figure 15: *Ifnγ*$^{-/-}$ mice exhibited chronic low-level shedding of *C. muridarum* from the genital tract.
Consistent with previously published reports (6,32) *ifnγ*$^{-/-}$ mice (open circles) and C57BL/6 mice (filled black circles) showed similar clearance over the first 3 weeks of infection. However, wild-type C57BL/6 mice became culture negative, whereas *ifnγ*$^{-/-}$ mice continued to exhibit low-level shedding of chlamydiae often alternating between culture negative and positive states. Data points represent the mean ± SD of five mice and include both culture positive and negative mice at each time point.

Gross examination of peritoneal contents of *ifnγ*$^{-/-}$ mice sacrificed 35 days post-infection revealed ascites (10 of 10 mice), fibrinous peritonitis (8 of 10 mice), adhesions between the mesosalpingeal tissues and the small intestine (8 of 10 mice), and frank purulence in the uterine horns (5 of 10 mice). These gross pathologic findings are similar to the findings described in humans with Fitz-Hugh-Curtis Syndrome. When the genital tracts harvested 35 days post-infection were analyzed histologically, the oviducts, uterine horns, and mesosalpingeal tissues from *ifnγ*$^{-/-}$ mice demonstrated a marked increase in neutrophilic and lymphocytic/monocytic inflammation (Figure 16A, D, G) when compared to wild-type mice ($P < 0.002$ by Mann-Whitney U test).

An increase in neutrophilic inflammation with destruction of mucosal epithelial cells, loss of luminal plicae, and a marked inflammatory exudate consisting of fibrin and neutrophils was observed in oviducts from *ifnγ*$^{-/-}$ mice (Figure 16C) when compared with oviducts from wild-type mice (Figure 16B). Similarly, increased neutrophilic inflammation, mucosal erosions and overlying exudate were apparent in the uterine horns of *ifnγ*$^{-/-}$ mice (Figure 16E, F). Mesosalpingeal tissues of the *ifnγ*$^{-/-}$ mice (Figure 16I) displayed increased inflammation as well as fat necrosis when compared to mesosalpingeal tissues of wild-type mice (Figure 16H).

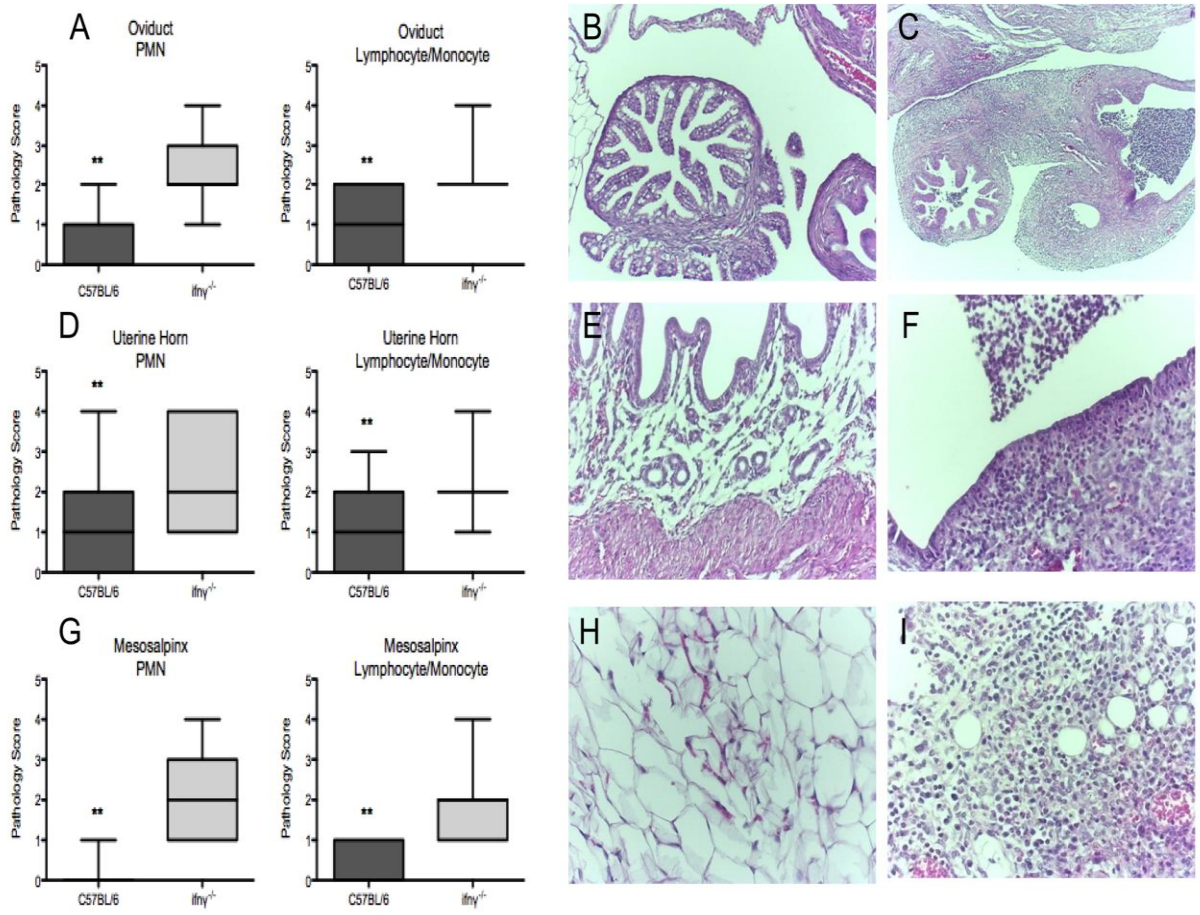

Figure 16: *Ifnγ*$^{-/-}$ mice exhibited enhanced genital tract pathology and an amplified neutrophil response to *C. muridarum* genital infection.

Genital tract tissues were removed en bloc from *C. muridarum*-infected mice 35 days post infection (C57BL/6 N=21; *ifnγ*$^{-/-}$ N=15). Tissues were scored for neutrophilic (PMNs) and lymphocytic and monocytic inflammation. Histopathologic analysis of the (A-C) oviducts, (D-F) uterine horns, and (G-I) mesosalpingeal tissues (G-I) of (B, E, H) C57BL/6, and (C, F, I) *ifnγ*$^{-/-}$ mice shows significantly increased neutrophil and lymphocyte/monocyte infiltration in tissues from *ifnγ*$^{-/-}$ mice (** $P < 0.002$ by Mann-Whitney U test). Boxes extend from the 25th to 75th percentiles and bars from the 5th to 95th percentiles. Photomicrographs of (C, 4x) oviducts, (F, 20x) uterine horns, and (I, 20x) mesosalpingeal tissues from *ifnγ*$^{-/-}$ mice demonstrate a marked increase in neutrophilic infiltrates, edema and loss of tissue structure compared to similar tissues from C57BL/6 mice [(B, 4x) oviducts, (E, 20x) uterine horns, and (H, 20x)] mesosalpinx.

3.4.8 Increased levels of Th17-related cytokines and chemokines were detected in genital tract secretions and oviducts from *ifnγ*$^{-/-}$ mice compared to wild-type mice infected with *C. muridarum.*

Th17 cells have been shown to promote neutrophil influx through induction of neutrophil chemokines. We examined the cytokine and chemokine milieu in the lower genital tract and oviducts of *ifnγ*$^{-/-}$ and C57BL/6 mice following *C. muridarum* genital tract infection. Analysis of genital tract secretions from infected wild-type mice revealed an increase in IL-17 during the first 5 days of infection, after which IL-17 levels fell to baseline by day 10 (Figure 17A). The high levels of IL-17 detected in secretions of the wild-type mice on days 1-5 may reflect production from NK cells induced early after infection (Figure 10B, C) (39).

Although moderate increases in IL-17 were detected in *ifnγ*$^{-/-}$ mice during the first 5 days of infection, these levels were significantly lower than those of infected wild-type mice. However, by day 10, high IL-17 levels were detected in the secretions of *ifnγ*$^{-/-}$ mice, and remained elevated through day 28 (Figure 17A). In addition, high levels of the Th17 cytokine IL-22 were found throughout the course of infection in *ifnγ*$^{-/-}$ mice (data not shown).

Th17 responses are initiated by the combination of IL-6 and TGF-β and supported by DC IL-23 production. Th17 cells induce the production of neutrophil chemoattractants such as GM-CSF, IL-6 and CXC chemokines including CXCL1 (KC), and CXCL2 (MIP-2) (4,15,26,42,43). Significantly increased levels of the Th17-promoting cytokines TGF-β (data not shown) and IL-6 (Figure 17B) were observed in the genital tract secretions of *ifnγ*$^{-/-}$ mice when compared to wild-type mice. TNFα was also significantly increased in genital tract secretions from *ifnγ*$^{-/-}$ mice when compared to wild-type mice (data not shown). Thus, the cytokine milieu in the lower

genital tract of *ifnγ*$^{-/-}$ mice following *C. muridarum* infection is conducive to induction of a Th17 response. The neutrophil chemokine CXCL1 (KC) (Figure 17C) and GM-CSF (data not shown) ($P < 0.001$ by two-way RM ANOVA) were significantly increased in genital tract secretions of infected *ifnγ*$^{-/-}$ mice when compared to wild-type.

Oviducts were harvested from *ifnγ*$^{-/-}$ and wild-type mice on days 7, 14, and 21 representing early, mid- and late- infection in immunologically normal mice. Similar levels of cytokines were noted in oviducts from wild-type and *ifnγ*$^{-/-}$ mice on day 7. However, on days 14 and 21, the levels of IL-17 (Figure 17D), IL-6 (Figure 17E), and TNFα (data not shown) were increased in oviduct homogenates from *ifnγ*$^{-/-}$ mice, as were the chemokines CXCL1 (KC) (Figure 17F) and CXCL2 (MIP-2) (data not shown). Thus, conditions in the upper genital tract of *ifnγ*$^{-/-}$ mice also reflect a Th17-favorable environment.

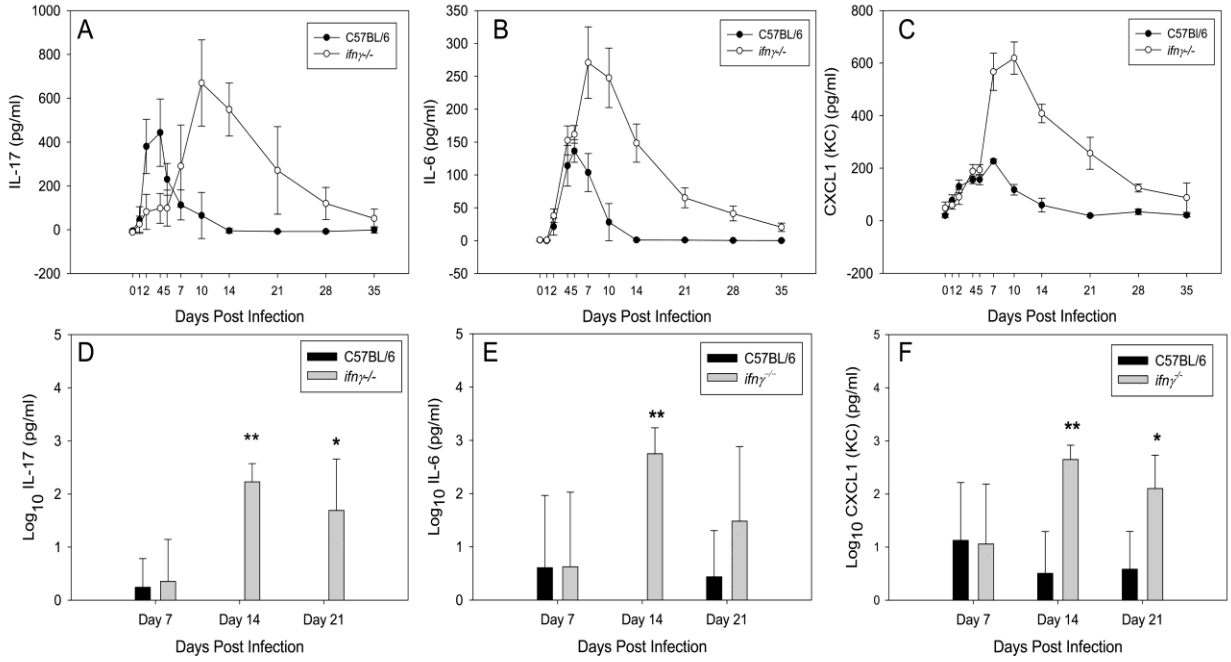

Figure 17: Genital tract secretions and oviduct homogenates from *ifnγ*$^{-/-}$ mice demonstrated increased levels of Th17-related cytokines and chemokines following *C. muridarum* genital tract infection.

C57BL/6 mice (filled dark circles) infected with *C. muridarum* exhibit early (A) IL-17 responses in genital secretions that are significantly higher than those of *ifnγ*$^{-/-}$ mice (open circles) on days 2-5 post-infection. IL-17 levels in secretions from infected *ifnγ*$^{-/-}$ mice (open circles) during days 7-35 post-infection were significantly higher than those from wild-type mice. (B) IL-6 and (C) CXCL1 (KC) were significantly increased in the *ifnγ*$^{-/-}$ mice from day 7-35 of infection. Each data point represents the mean ± SD of values from five mice in each group at each time point of a single individual experiment. $P < 0.05$ by two-way RM ANOVA for A, B, and C. (D-F) Oviducts from *C. muridarum* infected C57BL/6 mice and *ifnγ*$^{-/-}$ mice were harvested on day 7, 14, and 21 post-infection. On day 7 post-infection, cytokine levels were similar in oviducts from *ifnγ*$^{-/-}$ and C57BL/6 mice. On day 14, levels of (D) IL-17, (E) IL-6, and (F) CXCL1 (KC) were significantly elevated in oviducts from *ifnγ*$^{-/-}$ mice when compared to wild-type. On day 21, (D) IL-17 and (F) CXCL1 (KC) levels were significantly elevated in oviducts from *ifnγ*$^{-/-}$ mice. (** $P \leq 0.001$; * $P < 0.02$ by two-way ANOVA). Each bar represents the mean ± SD of values from homogenates of five mice at each time point in a single individual experiment.

3.4.9 Oviducts from *ifnγ*$^{-/-}$ mice exhibited increased bacterial burden and enhanced neutrophil and Th17 infiltrates.

To determine the role of pathogen-driven inflammatory cell recruitment to the oviduct, chlamydial burden was quantified by plaque assay and via real-time PCR for the *Chlamydia* 16S rRNA gene. Significantly higher levels of viable bacteria were detected in the oviducts of *ifnγ*$^{-/-}$ mice on day 21 (Figure 18A). Consistent with these observations, we detected significantly increased amounts of chlamydial genomic DNA in the oviducts of *ifnγ*$^{-/-}$ mice compared to wild-type on days 14 and 21 ($P < 0.050$, Student's *t*-test) (data not shown).

Flow cytometric analysis revealed increased numbers of neutrophils (Figure 18B) and CD4^{+} T cells (data not shown) in the oviducts of *ifnγ*$^{-/-}$ mice on days 14 and 21 when compared to wild-type. IL-17 producing CD4+ T cells were detected in the oviducts of *ifnγ*$^{-/-}$ mice on day 21 (Figure 18C) by intracellular cytokine staining; whereas, these cells were absent in the oviducts of wild-type mice on this day (data not shown).

CD4+ T cells in the iliac nodes of *ifnγ*$^{-/-}$ mice produced significantly greater amounts of IL-17 when stimulated with UV-EBs when compared to wild-type on days 7 (data not shown), 21 (Figure 18D), and 28 (data not shown). Sustained infection in the *ifnγ*$^{-/-}$ mice indicates that the enhanced Th17 and neutrophil responses were not effective compensatory bacterial control mechanisms.

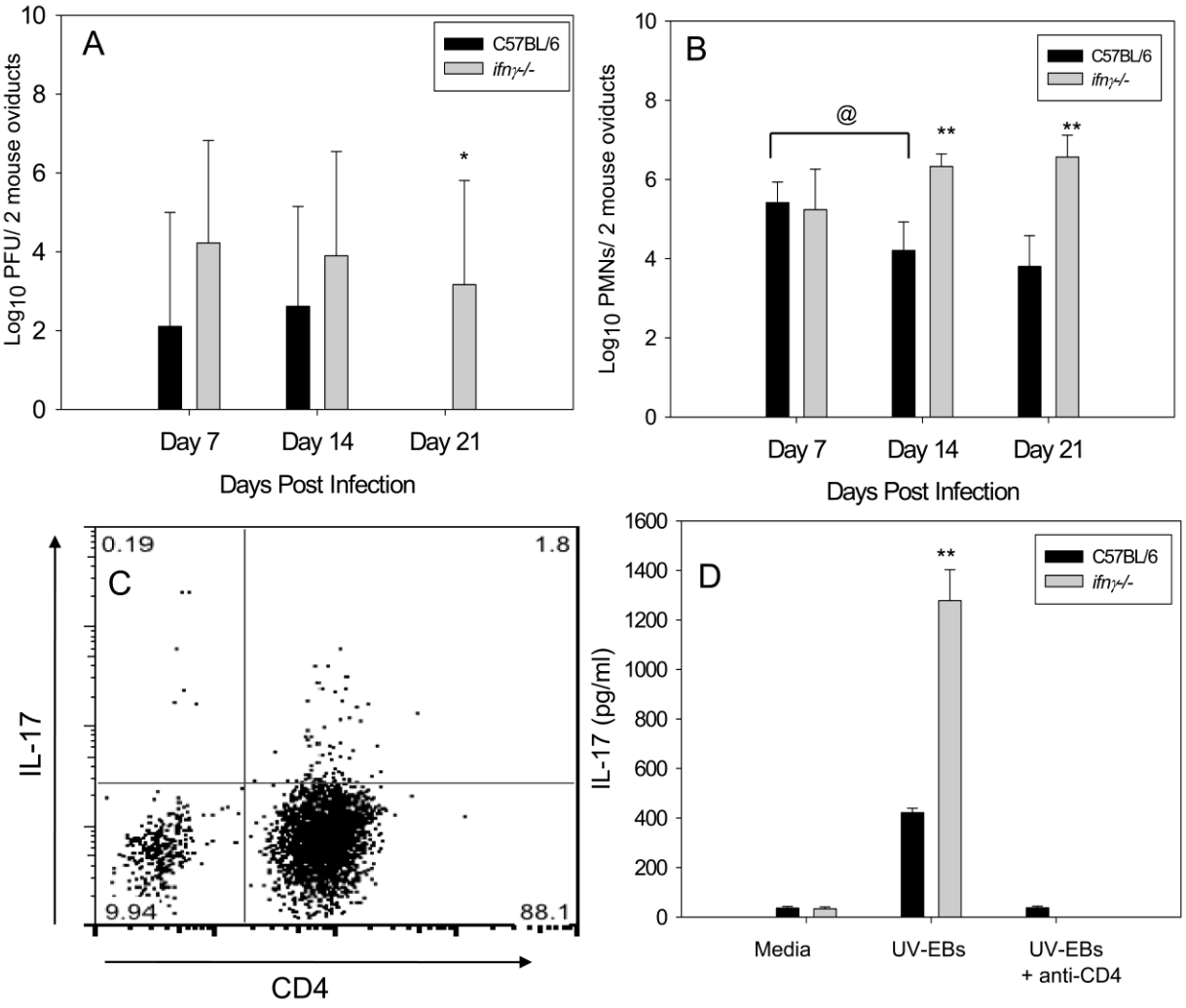

Figure 18: Increased bacterial burden in the oviducts of *ifnγ*$^{-/-}$ mice was associated with increased infiltration of neutrophils and Th17 cells.

(A) Bacterial burden in the oviducts of 5 *ifnγ*$^{-/-}$ and 5 wild-type mice sacrificed on days 7, 14, and 21 post-infection in a single independent experiment were analyzed by plaque assay and a significant increase in viable *Chlamydia* in the oviduct was noted on day 21; * $P < 0.02$ two-way ANOVA. (B) Single cell suspensions generated from two pooled oviducts of each mouse were analyzed by flow cytometry for live neutrophils (Gr-1^{high}Cd11c-F4/80^{-}). Data points represent the mean ± SD of values from 5 mice per group at each time point in a single independent experiment; ** $P \leq 0.001$ by two-way ANOVA for wild-type versus *ifnγ*$^{-/-}$; $^{@}$ $P < 0.02$ by two-way ANOVA for day 7 vs. day 14 in wild-type mice. (C) Th17 cells (CD3+CD4+ IL-17+) were detected in the oviducts of *ifnγ*$^{-/-}$ mice on day 21 post-infection. Plot is gated on CD3+ cells. (D) Iliac node mononuclear cells from individual mice sacrificed on day 21 were stimulated in vitro with UV-inactivated EBs with and without anti-CD4 for 96 hours and supernatants were analyzed for IL-17. Data points represent the mean ± SD of values from 5 mice per group at each time point in a single independent experiment; ** $P < 0.001$, Student's *t*-test.

3.4.10 In vivo neutralization of IL-17 in *ifnγ*$^{-/-}$ mice resulted in a significant decrease in G-CSF and neutrophil influx but no increase in bacterial burden.

Since oviduct epithelial cells secrete chemokines in response to chlamydial infection (18), it was possible that the enhanced neutrophil response was simply due to increased bacterial burden in the absence of IFNγ. Therefore, we examined the contributions of IL-17 to enhanced neutrophil recruitment and to the outcome of infection in *ifnγ*$^{-/-}$ mice by administration of anti-IL-17 antibody. G-CSF was absent in the oviducts of the anti-IL-17-treated *ifnγ*$^{-/-}$ mice on day 21 post-infection (Figure 19A), indicating that IL-17 was effectively neutralized and that an immune response had not been mounted against the administered antibody. Flow cytometric analysis of leukocytes in the oviducts of anti-IL-17 and IgG2a-treated mice revealed a 65% reduction in frequency of neutrophils in the anti-IL-17 treated group (Figure 19B). Frequencies of monocytes, CD4^{+}, and CD8^{+} T cells were not significantly altered by anti-IL-17 treatment (Figure 19B). Despite the significant reduction in neutrophils observed on day 21, no increase in bacterial DNA was found in the oviducts of anti-IL-17 treated *ifnγ*$^{-/-}$ mice when compared with the IgG2a-treated *ifnγ*$^{-/-}$ group (data not shown). The reduction in neutrophil influx observed in anti-IL-17-treated *ifnγ*$^{-/-}$ mice also did not alter oviduct pathology. Histological examination revealed severe inflammation and oviduct dilatation in both groups (Figure 19C, D). Thus, in the absence of IFNγ, IL-17 plays a direct role in driving neutrophil influx into the oviducts, but IL-17 and the downstream increase in neutrophils does not contribute to the control of infection. Sustained infection and leukocyte infiltration resulted in severe pathology in *ifnγ*$^{-/-}$ mice, even in the absence of IL-17-mediated neutrophil influx.

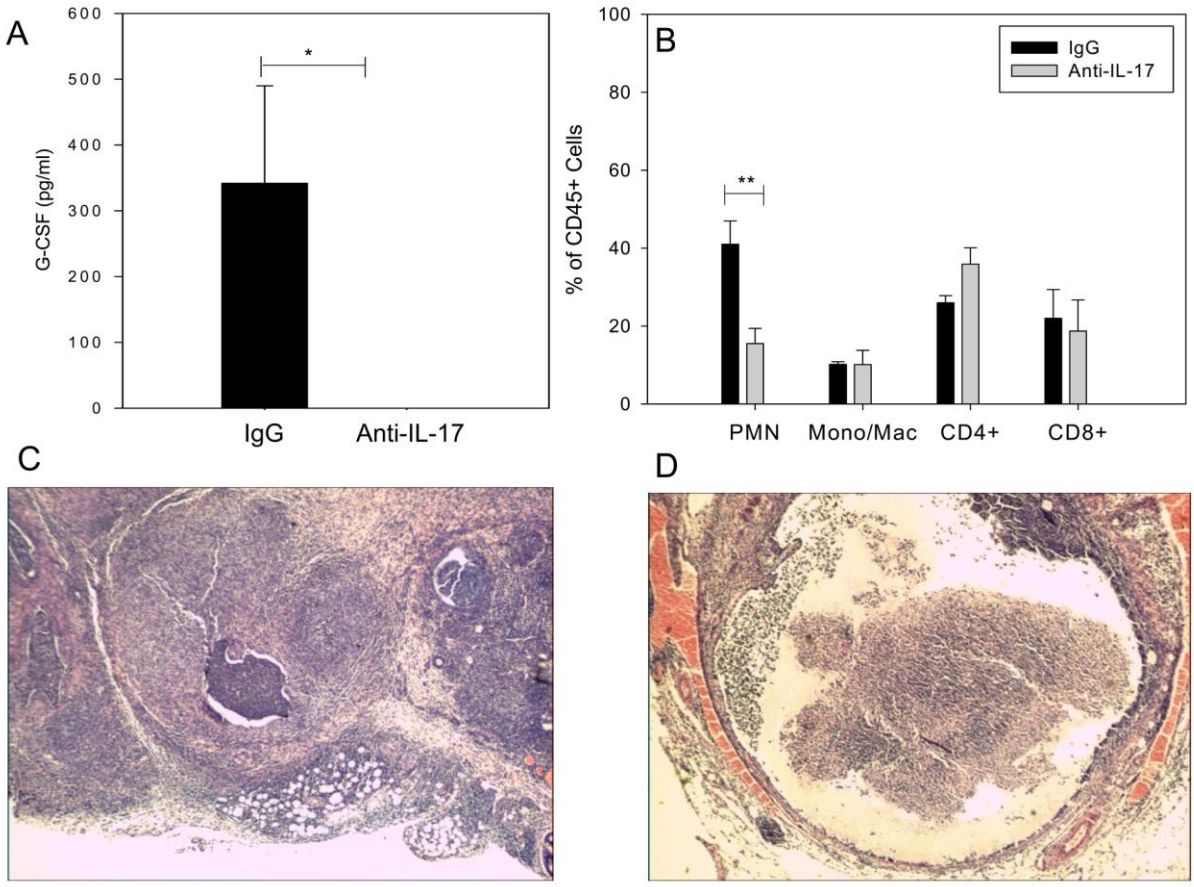

Figure 19: Administration of anti-IL-17 to *ifnγ*$^{-/-}$ mice led to significantly reduced G-CSF and decreased numbers of neutrophils in the oviducts but did not improve pathology.

Groups of five *ifnγ*$^{-/-}$ mice infected intravaginally with *C. muridarum* and treated with IgG2a or anti-IL-17 were sacrificed on day 21 post-infection and oviducts were harvested. (A) G-CSF was completely abrogated by IL-17 neutralization (* $P < 0.05$ by Student's *t*-test). Bars represent the mean ± SEM of values from 5 mice per group in a single experiment. (B) Percentages of live neutrophils (Ly6G (1A8)$^{+}$Gr-1^{high}), macrophages (F4/80^{+}), CD4+ T cells (CD3^{+}CD4^{+}CD8^{-}) and CD8+ T cells (CD3^{+}CD4^{-}CD8^{+} cells) were determined by flow cytometry. A 65% reduction in frequency of neutrophils was noted in the anti-IL-17 treated group compared with IgG2a-treated mice on day 21, (** $P < 0.01$ by ANOVA). Data points represent the means ± SD of values from 5 mice per group in a single independent experiment. (C, D) Oviducts were harvested and examined histologically from (C) IgG2a, and the (D) anti-IL-17 treated groups. Oviduct pathology was not improved by anti-IL17 treatment; severe inflammation and oviduct dilatation along with destruction of mucosal epithelial cells, granuloma formation, and a marked inflammatory exudate were noted in both groups. Photomicrographs are representative tissue samples from the oviducts of the (C, 40x) IgG2a-treated and (D, 40x) anti-IL-17-treated groups on day 21 post-infection.

3.5 DISCUSSION

Control of chlamydial infection in the genital tract requires a robust Th1 response (45, 51, 74, 75, 89). Recent investigations indicate that IL-17 potentiates Th1 immunity and consequently plays an important role in the control of intracellular pathogens (105, 154). We investigated the role of IL-17 in the control and pathologic outcome of *C. muridarum* genital tract infection using IL-17 receptor deficient mice and IFNγ deficient mice. Our data demonstrate that IL-17 promotes neutrophil recruitment and augments Th1 immunity but is not required for infection control or *Chlamydia*-induced pathology at this site.

Following *C. muridarum* genital tract infection of C57BL/6 mice, we detected both Th1 and Th17 antigen-specific CD4+ T-cells in the iliac nodes, the draining lymph nodes for the genital tract. We noted a contraction of the T cell response in the iliac node on day 20, when compared with day 7, likely due to resolving infection and reduced antigen burden. Ongoing experiments examining the kinetics of tissue-specific, antigen-specific Th1 and Th17 responses in the genital tract tissues by multi-color flow cytometric analysis, as well as examination of alternative IL-17 and IFNγ-producing cells (i.e. NK, NKT, γδ, and CD4 T cells) operative in the cervix and oviducts of wild-type mice will further define the role of IL-17 during chlamydial infection.

During *C. muridarum* pulmonary infection, IL-17 neutralization had a detrimental impact on disease course and development of *Chlamydia*-specific Th1 responses (105). This parallels findings during pulmonary infection with *Francisella tularensis* LVS, where in the absence of IL-17, Th1 immunity was compromised (154). In contrast, results obtained during pulmonary *M. tuberculosis* infection of *il23p19*$^{-/-}$ mice revealed that infection is eliminated at a normal rate despite the absence of IL-17 (153). We determined that *il17ra*$^{-/-}$ mice did not experience

increased bacterial load or augmented genital tract pathology when inoculated intravaginally with *C. muridarum*. We detected lower levels of IFNγ in the genital tract secretions of *il17ra*$^{-/-}$ mice early during infection and in the iliac node supernatants through day 35. Further examination revealed that the lower levels of IFNγ in the secretions of the *il17ra*$^{-/-}$ mice could be attributed to decreased numbers of IFNγ-producing NK cells in the cervix, which is consistent with the documented role of IL-17 in inducing IFNγ production by NK cells (153). It is possible that this decreased NK cell production of IFNγ contributes to the reduction in the Th1 response we noted in the iliac nodes of *il17ra*$^{-/-}$ mice. Tseng and Rank demonstrated that depletion of NK cells during *C. muridarum* genital tract infection resulted in a shift towards a Th2 response and delayed resolution of infection (39). However, we did not detect increased IL-4 in the secretions of infected *il17ra*$^{-/-}$ mice, indicating that such a shift did not occur. It is possible that alternative pathways are induced in *il17ra*$^{-/-}$ mice that prevent an augmented Th2 response when the NK cell IFNγ response is decreased.

In both the *C. muridarum* and *F. tularensis* pulmonary infection models, Th1 immunity was compromised in anti-IL-17- treated or IL-17- deficient mice, a finding that was associated with significantly decreased dendritic cell production of IL-12p70 (105, 154). We detected reduced levels of both IL-12p70 and IFNγ in the iliac nodes of *il17ra*$^{-/-}$ mice, indicating that priming of the Th1 response was compromised in the absence of IL-17. However, we did not detect the increase in bacterial load or IL-4 secretion that was observed when IL-17 was neutralized during *C. muridarum* pulmonary infection (105). We also observed significantly increased macrophage influx and enhanced macrophage TNFα production in *il17ra*$^{-/-}$ mice. A role for TNFα in contributing to control of *C. muridarum* genital tract infection has been

described (59) suggesting that this inflammatory response may have compensated for deficiencies in the Th1 response resulting in a normal course of infection.

C. muridarum infects both the respiratory tract and genital tract, and this provides a unique opportunity to investigate the potential for site-specific differences in the mucosal inflammatory responses to this pathogen. Although a decreased Th1 response was noted in the absence of IL-17 signaling in both the respiratory and genital tracts, we did not observe increased bacterial burden or a shift towards a Th2 response during genital tract infection of *il17ra*$^{-/-}$ mice. It is possible that these differences may indicate site specificity in the development of the adaptive response to chlamydiae. These site-specific differences could be related to distinct differences in the anatomy of the mucosal immune response in the female genital tract including the lack of inductive mucosal sites analogous to the mucosa associated lymphoid tissue (MALT) of the lungs or related to differences in immunologic priming by genital tract epithelial cells. Alternatively, the differences in responses may reflect variance resulting from the genetic background of the animals used in each study. C57BL/6 mice, the genetic background for both the *il17ra*$^{-/-}$ and *ifnγ*$^{-/-}$ strains used in this study, are less susceptible to pulmonary infection with *C. muridarum* and experience reduced bacterial load, a shortened infection course, and less severe tissue pathology than Balb/c mice (108). Thus, abrogation of the Th17 response may be more detrimental to host defense in the Balb/c strain used for the studies of pulmonary infection (30).

Because IFNγ has been shown to negatively regulate the Th17 response (162, 163), we intravaginally inoculated *ifnγ*$^{-/-}$ mice with *C. muridarum*, to determine the role of an unopposed Th17 response during chlamydial infection. In the absence of CD4^{+} Th1 IFNγ production, we observed a significantly increased CD4^{+} Th17 response and increased neutrophil influx. *Ifnγ*$^{-/-}$

mice displayed significantly worsened pathology and elevated bacterial burden in the lower and upper genital tracts. Administration of an anti-IL17 antibody raised against IL-17A confirmed a direct role for IL-17A in driving neutrophil influx in *ifnγ*$^{-/-}$ mice, but IL-17 neutralization did not lead to enhanced bacterial burden, likely due to maintenance of macrophage influx. We anticipated that if neutrophils were effectors of upper tract pathology, then administration of anti-IL17 antibody might reduce the severity of disease. Unfortunately, the intense infiltration of non-neutrophil leukocytes observed in the absence of IFNγ prevented adequate assessment of the role of neutrophils or IL-17 in pathology. Because the antibody utilized in these experiments targeted IL-17A, it is possible that the continued presence of IL-17F provided sufficient signaling to result in production of neutrophil promoting chemokines and neutrophil influx, albeit at reduced levels, which could explain the absence of an effect on pathology in the antibody treated mice. Further, continued signaling by IL-17F could also prevent enhanced macrophage influx such as that observed in the *il17ra-/-* mice.

Our data have important implications for the prevention and treatment of human *Chlamydia* infections and the strategic design of vaccines and therapeutics to target chronic chlamydial disease. The presence of augmented Th17 responses and enhanced neutrophilic infiltration in the absence of IFNγ indicate that these responses could be used as biomarkers for chronic infection and an inadequate Th1 response in humans. In models of *M. tuberculosis* pulmonary infection, vaccination in the presence of IL-17 inducing adjuvant promotes Th1 immunity and reduced bacterial burden (157). Recent data from Yu *et al* demonstrated that a vaccine consisting of immunodominant chlamydial T-cell antigens afforded protection from infection that was associated with IFNγ/TNFα and IFNγ/IL-17 double positive CD4+ T-cells (111). Indeed, our data reveal that removal of IL-17 blunts Th1 priming during genital tract

infection. Thus, the possibility exists that a chlamydial vaccine that induces a Th17 response may promote more effective Th1 immunity.

In summary, we show for the first time that the Th17 response that occurs in the genital tract due to *Chlamydia* infection promotes but is not essential for induction of neutrophil influx or Th1 immunity. In addition, we show that in the absence of the protective IFNγ response, a heightened Th17 response induces neutrophil influx. The findings that chlamydial genital infection, in contrast to respiratory tract infection, resolves normally in the absence of IL-17 and in the presence of a diminished IFNγ response highlight the importance of examining site-specific responses to infection. Importantly, we determined that removal of IL-17 led to decreased neutrophil influx but enhanced macrophage influx and activation. Thus, it is possible that in an immunologically intact host IL-17 not only drives neutrophil influx and Th1 immunity but also downregulates the monocyte/macrophage response. Future studies will investigate IL-17 mediated mechanisms of monocyte/macrophage inhibition and the role of the macrophage TNFα response in controlling chlamydial infection.

3.6 ACKNOWLEDGEMENTS

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4.0 IL-23 INDUCES IL-22 AND IL-17 PRODUCTION IN RESPONSE TO *CHLAMYDIA MURIDARUM* GENITAL TRACT INFECTION, BUT THE ABSENCE OF THESE CYTOKINES DOES NOT INFLUENCE DISEASE PATHOGENESIS

4.1 ABSTRACT

The cytokine response to *Chlamydia* genital tract infection is crucial for resolution of infection but can also result in irreversible tissue damage. Interleukin 23 (IL-23) induces production of IL-22 and IL-17, and these cytokines are central immune mediators of both host defense and autoimmunity. We determined that the IL-22 receptor was expressed in the genital tract, but infection of mice deficient in IL-22 revealed no difference in the course of lower genital tract infection or the development of oviduct pathology. Infection of IL-23 knockout mice revealed reduced levels of IL-22 and IL-17 in the draining lymph nodes and genital tract but no difference in the kinetics of infection or oviduct damage. IL-23 deficient and wild type mice also exhibited similar susceptibility to infection with low levels of *Chlamydia*. These data demonstrate that a deficiency in IL-23, IL-17, and/or IL-22 does not result in a measureable phenotype and indicate that these cytokines perform either negligible or redundant functions in this model

4.2 INTRODUCTION

Studies in animal models of *Chlamydia* genital tract infection have repeatedly demonstrated a central role for IFNγ production by CD4+ T cells in controlling infection and preventing the development of oviduct disease (53, 74, 75, 78, 164). This protective Th1 response is counterbalanced by the anti-inflammatory cytokine IL-10, which inhibits Th1 activation and delays clearance of *C. muridarum* infection from the genital tract (80).

IL-22 is member of the IL-10 family of cytokines that exhibits complex protective and pathologic effects depending on the disease model examined. Although IL-10 and IL-22 have limited homology, their heterodimeric receptor complexes share a common chain, IL-10 receptor beta, and predominately induce STAT3 activation (165-168). The unique subunit of the IL-22 receptor, IL-22 receptor alpha-1, is expressed exclusively by non-hematopoietic cells including epithelial cells, while the IL-10-specific receptor subunit, IL-10 receptor alpha, is widely expressed by both hematopoietic and non-hematopoietic cells (166, 169). The expression pattern of the IL-22 receptor explains the localization of IL-22-induced responses to environmental interfaces including the skin, lungs, and gastrointestinal tract (149, 169-171). In addition, IL-22 receptor mRNA has been detected in the female reproductive tract including the ovaries, cervix, and placenta (169, 172, 173).

IL-22 promotes mucosal immunity by enhancing epithelial barrier integrity, expression of anti-microbial molecules, and mucin production (149, 170, 174, 175). The importance of IL-22 in mucosal host defense was first documented in models of infection with extracellular bacteria including *Klebsiella pneumoniae* pulmonary infection and *Citrobacter rodentium* intestinal infection, where mice succumbed to infection when IL-22 was inhibited or absent (149, 170). In contrast, IL-22 induces immunopathology in the small intestine in response to peroral infection

with the intracellular parasite *Toxoplasma gondii* (176, 177). The reduced pathology observed upon neutralization of IL-22 in this model was associated with significant decreases in proinflammatory cytokine and chemokine production in the draining lymph nodes and ileum (176). Indeed, IL-22 has been demonstrated to induce production of several neutrophil chemokines (CXCL1, -2, -3, -5, -6, -8) in addition to up regulating expression of matrix metalloproteases (MMP1, -3, -10)(149, 172, 178, 179). Enhanced neutrophil influx and MMP production are clearly associated with oviduct damage in response to *Chlamydia* infection in the mouse model (31, 33, 35, 38, 137). Thus, IL-22 induces responses in other models that are linked with disease development during chlamydial genital infection.

There are a limited number of studies examining the role of IL-22 in the female reproductive tract under both physiologic conditions and in the context of infection. IL-22-producing immature NK cells have been detected in the human uterus, where they have been proposed to play a role in tissue regeneration after cyclic shedding (180). In the context of infectious diseases, mouse models of vaginal infection with *Candida albicans* and *Neisseria gonorrheoae* failed to show a requirement for IL-22 in infection control (181, 182). We previously reported significantly increased levels of IL-22 in genital tract secretions from *C. muridarum*-infected IFNγ-deficient mice, which were associated with a heightened Th17 response, increased neutrophil infiltration, and the development of severe oviduct pathology (78). IL-22 and IL-17 production have also been observed by CD4+ T cells isolated from the cervical washes of women infected with *C. trachomatis* (113). These data indicate that IL-22 may be involved in the pathogenesis of *Chlamydia* genital tract infection.

IL-22 is produced by Th17 cells, Th22 cells, γδ T cells, lymphoid tissue inducer cells, and NK22 cells (171, 175, 183-187). Release of both IL-17 and IL-22 from the aforementioned

cells is enhanced by IL-23 (171) (175, 183, 187-190). IL-22 and IL-17 can cooperatively induce the production of proinflammatory cytokines, neutrophil chemokines, and anti-microbial molecules (149, 175, 179). For example, IL-22 and IL-17 enhance production of S100A8 and S100A9, which form a heterodimeric complex known as calprotectin (175). Calprotectin induces neutrophil chemotaxis, and acts as an alarmin, potently amplifying inflammation (191, 192). The interplay between IL-17 and IL-22 has been shown to dictate whether IL-22 exhibits a tissue-protective or damaging role (193). Herein, we explored the possible cooperative effects of IL-17 and IL-22, by examining the course and outcome of chlamydial infection in mice deficient in IL-23.

The intracellular life cycle of *Chlamydia* dictates that resolution of infection from the genital tract is dependent on the influx of CD4+ T cells. Even in the presence of a robust innate inflammatory cell influx, such as observed in MHC class II deficient mice, infection is sustained at high levels indefinitely (45). Thus, we hypothesized that IL-22-mediated induction of anti-microbial molecules was unlikely to be significantly beneficial in infection control in this model, which would be in accordance with findings in other models of intracellular bacterial infection including *Mycobacterium tuberculosis* and *Listeria monocytogenes* (176, 194, 195). However, given the importance of neutrophil activation and MMP production in development of chlamydial-induced oviduct damage (31, 35, 37, 38, 137), we hypothesized that IL-22-mediated induction of these processes would contribute to pathology, and this cytokine either independently, or in conjunction with IL-17, would play a detrimental rather than protective role during chlamydial genital tract infection. We tested this hypothesis by comparing the course and outcome of *C. muridarum* genital tract infection in mice genetically deficient for IL-22 with

immunologically normal mice. In addition, we utilized mice genetically deficient for IL-23 to determine if reductions in both IL-22 and IL-17 would ameliorate oviduct pathology.

4.3 MATERIALS AND METHODS

4.3.1 Strains, cell lines, and culture conditions

Plaque-purified *C. muridarum* Nigg was used for all experiments and was isolated as previously described (17, 114). All chlamydial strains were propagated in L929 cells (115). Bacteria were titrated by plaque assay (114) or as inclusion forming units (IFU) using fluorescently tagged anti-chlamydial lipopolysaccharde monoclonal antibody (Bio-Rad, Hercules, CA)(30).

4.3.2 Animals

Female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The IL-22 knockout (IL-22 KO) mice were kindly provided by Dr. Wenjung Ouyan and the IL-23p19 knockout (IL-23p19 KO) mice by Dr. Nico Ghilardi, both at Genentech. IL-23p19 heterozygous mice used in these studies were the F1 progeny of a C57BL/6 and IL-23p19 KO mouse. Mice were at least 7 weeks of age at the time of infection. Mice were given food and water ad libitum in an environmentally controlled room with a cycle of 12 hours of light and 12 hours of darkness. All animal experiments were approved by the University Institutional Animal Care and Use Committee.

4.3.3 Immunohistochemical analysis of murine genital tract tissues for the IL-22 receptor

Analysis of IL-22 receptor alpha-1 (IL-22R1) expression was conducted as previously described (196). Genital tract tissues were harvested from C57BL/6 mice, and tissue sections were made as described below (See section Microscopic histopathological assessment**).** Sections were deparaffinized in xylene (3 × 10 minutes) and rehydrated through sequential washings with 100%, 95% and 75% ethanol (2 × 10 minutes). Antigen retrieval was performed by boiling for 10 minutes in 10mM citrate buffer followed by incubation for 30 minutes at room temperature. After peroxidase blocking (3% hydrogen peroxide for 10 minutes), slides were blocked following the Vectastain® ABC blocking protocol for Rat IgG (Vector Laboratories, Burlingame, CA). Receptor expression was visualized using Rat anti-mouse IL-22R1 (R&D Systems, Clone: 496514) at a dilution of 1:50. Additional tissues were incubated with Rat IgG2a (R&D Systems, Clone: 54447) as a control for nonspecific staining.

4.3.4 Murine infection and monitoring

Five to seven days prior to infection mice were subcutaneously injected with 2.5 mg of medroxyprogesterone (Depo-Provera®; Upjohn, Kalamazoo, MI) to induce a state of anestrous (116). Mice were intravaginally inoculated with $1x10^5$ IFU of *C. muridarum* Nigg diluted in 30 µl of sucrose-sodium phosphate-glutamic acid (SPG) buffer unless otherwise indicated. Mice were monitored for cervicovaginal shedding via endocervical swabs (117), and IFU were calculated as previously described (30). Bacterial burden was measured in the oviducts by plaque assay (114). Lower genital tract (LGT) bacterial burden was enumerated for IL-22 KO and C57BL/6 mice in two independent experiments with 5-6 mice per strain per experiment. The

course of infection in the LGT of IL-23p19 KO and C57BL/6 was compared in three independent experiments with 4-5 mice per strain. A group of five IL-23p19 heterozygous mice was added for one of these experiments. Bacteria were titrated from the oviducts of IL-23p19 KO and C57BL/6 mice in a single experiment with 3-4 mice per strain per day of analysis.

To determine the susceptibility of C57BL/6 and IL-23p19 KO mice to low doses of infection, 10-fold serial dilutions of *C. muridarum* Nigg ranging from $5x10^1$ to $5x10^4$ bacteria were resuspended in 30 μl of SPG buffer and intravaginally inoculated into groups of 6-7 mice per dose per strain. On day 6 post-infection, mice were euthanized, and their cervices were immediately processed for detection of infection via IFU (197).

4.3.5 Processing of oviducts for flow cytometry

Oviducts and cervices were processed for flow cytometric analysis as previously described (33, 78). Briefly, tissues were harvested and minced with scissors. For measurement of cytokines and bacterial burden, an aliquot of the minced tissue was stored at -80^{o}C until analysis. Cervices were digested with collagenase I (1mg/ml; Sigma-Aldrich, St. Louis, MO), and then cervices and oviducts were repeatedly passed through a 70 μm filter to yield a single cell suspension. Single cell suspensions were incubated with Fc Block (BD Pharmingen; Clone: 2.4G2), and cell surface proteins were subsequently stained with the indicated antibodies. Stimulation of cells for analysis of intracellular cytokines was conducted as below (See section Detection of *Chlamydia*-specific cytokine production by intracellular flow cytometry). Flow cytometry data were acquired using an LSR II Analyzer (BD Biosciences) and analyzed via FlowJo software (Tree Star, Ashland, OR).

4.3.6 Detection of *Chlamydia*-specific cytokine production by intracellular flow cytometry

For analysis of cytokine production, single cell suspensions generated from the cervix or oviducts of individual mice were incubated overnight in complete medium (DMEM containing 10% FBS, 2 mM glutamine, 100 μM non-essential amino acids, 50 μM β-mercaptoethanol, 100 μg/ml vancomycin and 50 μg/ml gentamicin) with 5 μg/ml of gradient purified UV-inactivated *C. muridarum* elementary bodies (UV-EBs)(159). GolgiPlug™ (1:500 final dilution; BD Biosciences) was added for the last 4 hours of incubation. Cell surface proteins were stained with PerCP-Cy5.5 anti-mouse CD45 (Clone: 30-F11), V450 anti-mouse CD3 (Clone 500A2), and PE anti-mouse CD4 (Clone RM4-5) all from BD Biosciences. After surface staining, cells were fixed, permeabilized, and stained with APC anti-mouse IFNγ (BD Biosciences, clone XMG1.2) according to manufacturers instructions (Cytofix/Cytoperm™Kit, BD Biosciences). Cytokine production by WT and IL-23p19 KO cells was analyzed on days 7, 10, and 14 of infection with 3-4 mice per strain per day in two independent experiments.

4.3.7 Detection of cytokines in lower genital tract secretions and oviduct homogenates

LGT secretions were collected via washing the vaginal vault with 100 μl of phosphate-buffered saline with protease inhibitor (Complete EDTA-free protease inhibitor tablets, Roche Diagnostics) during the first ten days of infection as previously described {Riley, 2012 #10190. IL-17, TNFα, and IFNγ were quantified in these lavages and in the homogenized oviducts of C57BL/6 and IL-23p19 KO mice via multiparametric bead array (Millipore, Billerica, MA). IL-22 was measured by ELISA (R&D Systems, Minneapolis, MN). Cytokines were monitored in

the LGT secretions of C57BL/6 and IL-23p19 KO mice in two independent experiments with 4-5 mice per group, and cytokines were measured in the oviducts of 3-5 mice per group per day.

4.3.8 Assessment of *Chlamydia*-specific cytokine responses in the iliac nodes

Iliac nodes from IL-23p19 KO and C57BL/6 mice infected intravaginally with *C. muridarum* were harvested on days 0, 7, 14, 21, 28, 35, and 56 post infection. Lymph nodes were processed to a single cell suspension and placed in culture with media alone or UV-EBs (5 μg/well). Supernatants were collected after 96 hours in culture for quantification of cytokines as described above. Cytokine production by iliac lymph node mononuclear cells was evaluated using 5 mice per strain per day.

4.3.9 Microscopic histopathological assessment

Genital tracts were removed en bloc, fixed in 10% buffered formalin, and embedded in paraffin. Longitudinal 4-μm sections were cut and stained with hematoxylin and eosin. Oviduct epithelial cell erosion and oviduct dilatation were assessed for tissues harvested on day 42 using a four-tiered semi-quantitative scoring system by a pathologist blinded to the experimental design {Darville, 1997 #10212;Darville, 1997 #10212;Darville, 2001 #10231}. Oviduct pathology for IL-22 KO and C57BL/6 mice was compared on day 42 in two separate experiments with 5-6 mice per group per experiment, and the same comparison was conducted for IL-23p19 KO and C57BL/6 mice.

4.3.10 Statistics

Statistical comparison of flow cytometry data, cytokine levels, or the course of infection was conducted via two-way ANOVA with Bonferroni post-test analysis. A Mann-Whitney U test was used to determine significant differences in pathology scores. A Fisher's exact test was used to determine differences in susceptibility to low dose infection. Prism software (GraphPad Software, LaJolla, CA) was utilized for all statistical analysis. Values of $P < 0.05$ were considered significant.

4.4 RESULTS

4.4.1 Murine genital tract epithelial cells express the IL-22 receptor.

The IL-22 receptor is a dimeric complex of the IL-10 receptor beta chain (IL-10R2), which is ubiquitously expressed, and the IL-22 receptor alpha-1 chain (IL-22R1), which is only expressed by non-hematopoietic cells (166, 168, 169). Although a role for IL-22 receptor signaling has been reported at mucosal sites including the pulmonary and gastrointestinal tracts(149, 170), expression of this protein has not been previously described in the genital tract. Using immunohistochemistry, we detected IL-22R1 expression in the murine ectocervix (Figure 20A), endocervix (Figure 20B), uterine horns (Figure 20C), and oviducts (Figure 20D and E). Receptor expression was localized to the epithelium, and no staining was observed for stromal cells of the genital tract. No staining was observed when sections were incubated with the relevant immunoglobulin isotype (Figure 20F).

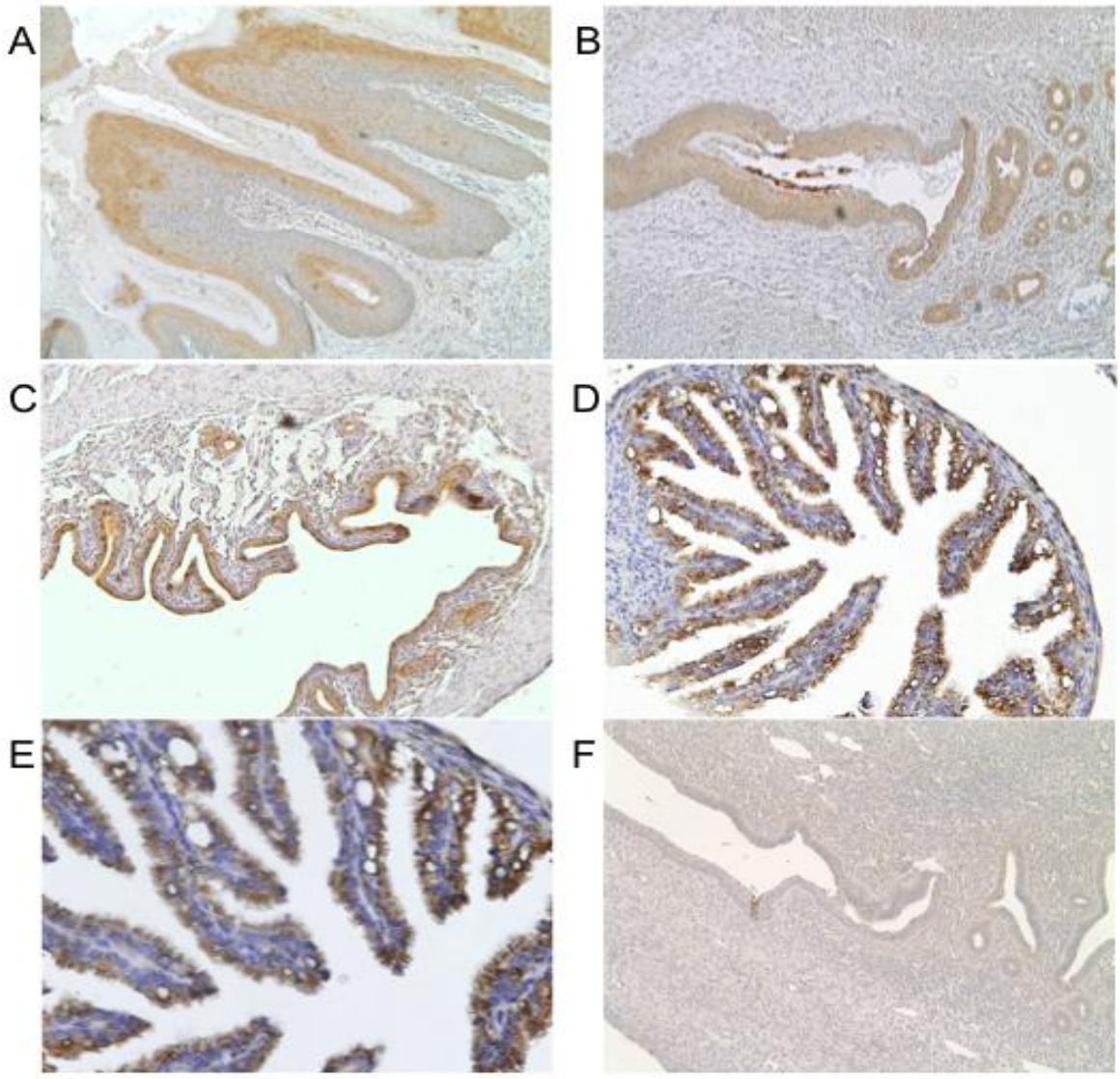

Figure 20: IL-22 receptor expression was detected in the murine genital tract.
Genital tracts from uninfected C57BL/6 mice were stained with anti-IL-22R1. Ectocervix (A; magnification, x100), Squamocolumnar junction (B; magnification, x100), Uterine horn (C; magnification, x100), Oviduct (D; magnification, x200), Oviduct (E; magnification, x400), negative control (Rat IgG2a) uterine horn (F; magnification, x200).

4.4.2 IL-22 deficiency had no effect on bacterial burden or oviduct pathology.

Since we detected expression of IL-22R1, and we previously documented IL-22 in murine genital tract secretions during active *C. muridarum* infection (78), we sought to determine if IL-

22 was involved in resolution of infection from the genital tract. Comparison of the course of lower genital tract infection for C57BL/6 and IL-22 deficient mice revealed that infection resolved with normal kinetics in the absence of IL-22 (Figure 21A). In addition, none of the mice exhibited clinical signs of bacterial dissemination as has been observed in models of infection with extracellular bacteria in the absence of IL-22 (149, 190). We also examined the possibility that IL-22 could influence oviduct pathology in this model. Histological analysis revealed that erosion of the oviduct epithelium was comparable between strains (Figure 21B). In addition, we detected similar degrees of oviduct dilatation in the presence and absence of IL-22, with 5 of 6 mice in both groups developing severe dilatation (Figure 21C).

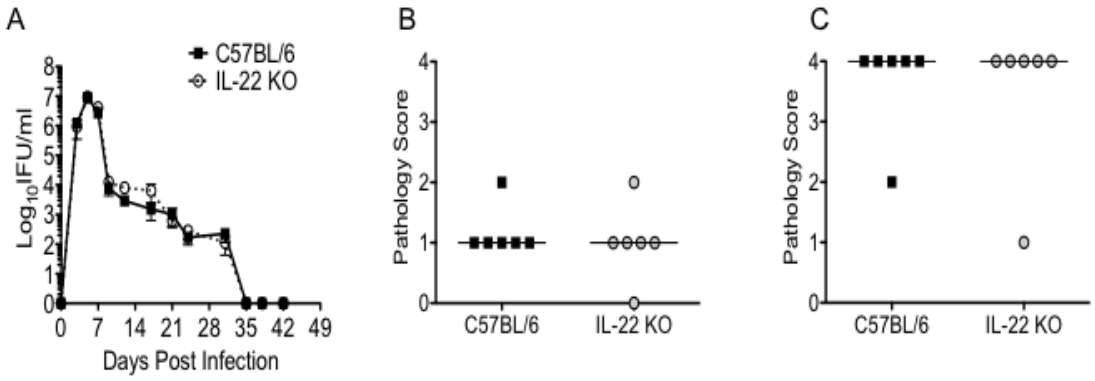

Figure 21: Resolution of *C. muridarum* infection and the development of oviduct pathology were not influenced by the absence of IL-22.

(A) The kinetics of lower genital tract infection for C57BL/6 (black squares) and IL-22 KO mice (open circles, dashed line) does not differ ($P > 0.05$ via two-way repeated measures ANOVA). Data points represent the mean ± SEM of IFU values from 6 mice per strain from a single representative experiment of two. (B,C) Histological analysis of oviduct epithelial cell erosion (B) and oviduct dilatation (C) in genital tracts harvested on day 42 post-infection revealed no difference between the strains. ($P > 0.05$ via Mann-Whitney U-test). Data points represent semi-quantitative scoring of oviduct pathology for individual mice with 6 mice per strain from a single experiment of two. C57BL/6 (black squares) and IL-22 KO mice (open circles). Median indicated by horizontal line.

4.4.3 IL-23 induced IL-17 and IL-22 production in response to *C. muridarum* infection in the genital tract and iliac lymph nodes.

IL-23 is composed of the shared IL-12p40 subunit and the unique IL-23p19 subunit (198). IL-23 enhances the release of IL-17 and IL-22 from both innate and adaptive immune cells (171, 175, 183, 188-190). In order to determine the role of IL-23 in the cytokine response to *C. muridarum* genital tract infection, we intravaginally infected IL-23p19 deficient mice. Lower genital tract secretions were collected for the first 10 days of *C. muridarum* infection, and oviducts were harvested on day 10 post-infection. These time points represent peak days of cytokine production at both sites (33). Examination of cytokine levels in the absence of IL-23 revealed significantly reduced IL-17 (Figure 22A and E) and IL-22 (Figure 22B and F) but no difference in TNFα (Figure 22C and G) or IFNγ (Figure 22D and H) at either of these sites.

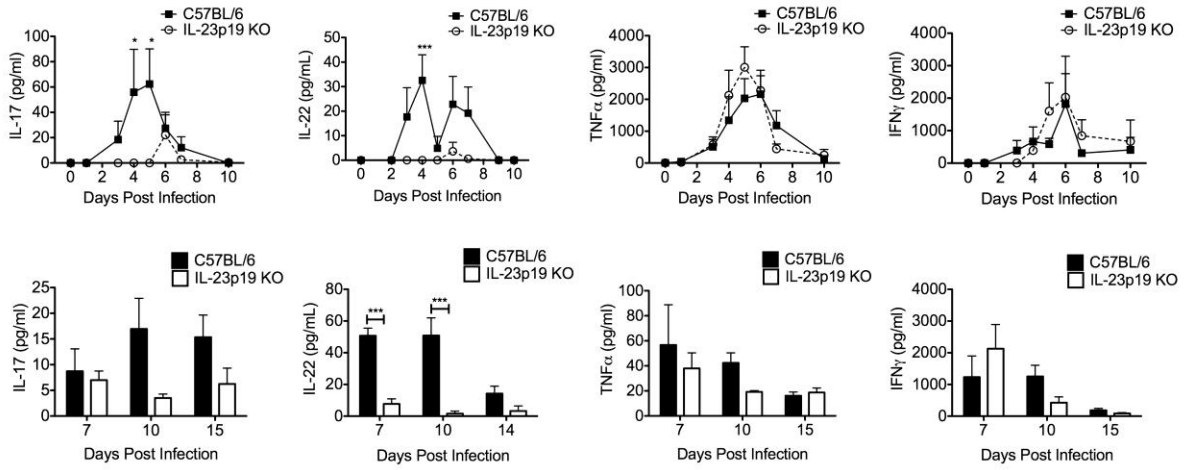

Figure 22: IL-23 induced production of IL-17 and IL-22 but not TNFα or IFNγ in the genital tract during *C. muridarum* infection.

(A-D) Levels of IL-17 (A) and IL-22 (B) in the vaginal lavages of IL-23p19 KO mice (clear circles, dashed line) were significantly reduced compared to C57BL/6 mice (black squares), but no difference was detected for TNFα (C) or IFNγ (D). Data points represent the mean ± SEM for 4-5 mice per strain from one representative experiment of two. $P < 0.05$ for IL-17 and IL-22 (by two-way ANOVA over the interval measured). *, $P < 0.05$; ***, $P < 0.001$. (E-H) Measurement of cytokines in the homogenized oviducts of infected mice revealed significantly reduced levels of IL-17 (E) and IL-22 (F) in the absence of IL-23 but no difference in TNFα (G) and IFNγ (H). Data points represent the mean ± SEM for 3-5 mice strain per day. $P < 0.05$ for IL-17 and IL-22 (by two-way ANOVA over the interval measured). ***, $P < 0.001$.

IL-23 has been previously shown to promote the stability of the Th17 lineage (199). In order to determine the role of IL-23 in the adaptive immune response to *Chlamydia,* we harvested the iliac lymph nodes (ILN) from infected mice and stimulated them with *C. muridarum* elementary bodies. In the absence of IL-23, *Chlamydia*-specific release of both IL-17 and IL-22 was significantly reduced (Figure 23A and B). Similar to our previous findings in IL-17 receptor deficient mice (78), IFNγ production was reduced on day 7 in the ILNs of IL-23p19 deficient mice, but levels were comparable to those detected for wild-type mice by day 14 (Figure 23C). Despite early reductions of IFNγ in the ILN, flow cytometry revealed no

difference in the frequency of *Chlamydia*-specific IFNγ-producing $CD3^+CD4^+$ T cells in either the cervix or oviducts on days 7, 10, or 14 post-infection (data not shown). These findings are in accordance with the detection of normal levels of IFNγ at both of these sites in the absence of IL-23 (Figure 23D and H). These data indicate that IL-23p19 KO mice provide an appropriate model to examine the role of a combined deficiency of IL-17 and IL-22 in chlamydial infection without the confounding effects that would result from reductions in the protective cytokine IFNγ (74, 75, 78).

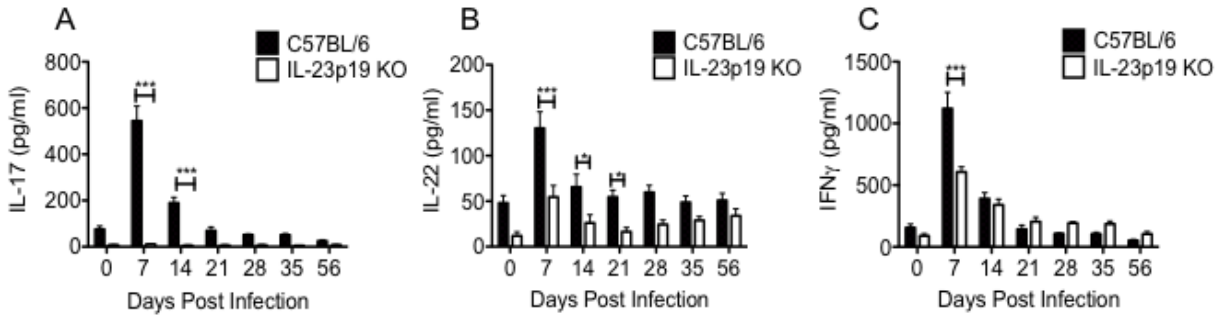

Figure 23: In the absence of IL-23, *Chlamydia*-specific cytokine production was reduced in the iliac lymph nodes. (A-C) Significantly reduced levels of IL-17 (A), IL-22 (B), and IFNγ (C) were measured in the supernatants of Iliac lymph node mononuclear cells from IL-23p19 KO (white bars) mice restimulated in vitro for 96 hours in the presence of UV-EBs relative to those from C57BL/6 mice (black bars). Bars represent the mean ± SEM for 5 mice/strain. $P < 0.0001$ for IL-17 and IL-22 and $P < 0.05$ for IFNγ (by two-way ANOVA over the interval measured). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.4.4 Infection resolved with normal kinetics in the absence of IL-23.

Given the role of IL-17 and IL-22 in enhancing mucosal immunity, we sought to determine if the reductions in IL-17 and IL-22 that we observed in the absence of IL-23 impacted the ability of mice to control infection in either the lower or upper genital tract. We followed the course of

lower genital tract infection in C57BL/6, IL-23p19 deficient and IL-23p19 heterozygous mice and found no difference in the kinetics of infection between any of the strains (Figure 24A). We also found no difference in the bacterial burden in the oviducts over the peak days of infection in the absence of IL-23 (Figure 24B).

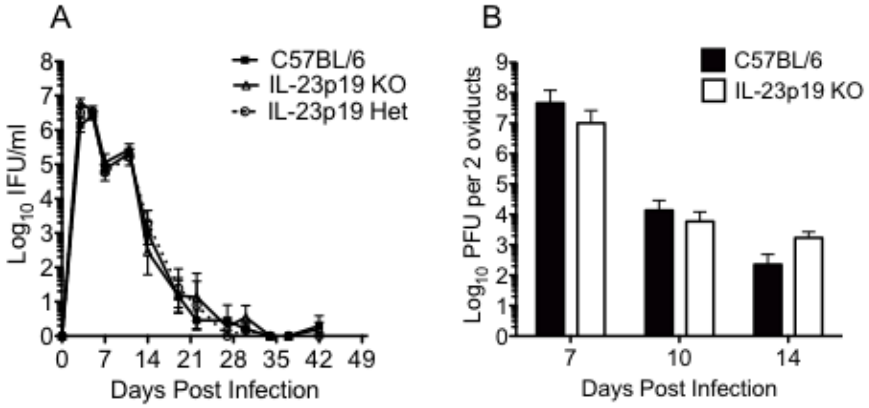

Figure 24: *C. muridarum* infection resolved with normal kinetics in the absence of IL-23.
(A) The kinetics of lower genital tract infection for C57BL/6 (black squares), IL-23p19 KO (open triangles), and IL-23p19 heterozygous mice (open circles) does not differ. Data points represent the mean ± SEM of IFU values from 4-5 mice per strain from a single experiment of three. (B) Analysis of bacterial burden in the oviducts revealed no difference between C57BL/6 (black bars) and IL-23p19 KO mice (white bars). Bars represent the mean ± SEM of PFU for two pooled oviducts of individual mice with 3-4 mice per strain per day.

Although we did not detect a difference in the kinetics of infection when mice were infected with 100,000 bacteria, we recognized that with such a high dose of infection, innate defense mechanisms induced by IL-22 and IL-17 may be overwhelmed. To explore this possibility, we infected C57BL/6 and IL-23p19 deficient mice with doses of *C. muridarum* Nigg ranging from 50 to 50,000 microorganisms. All of the mice from both strains established an active infection upon inoculation with as few as 500 IFU (data not shown). When the innoculum was decreased to 50 IFU, 6 of 7 C57BL/6 mice and 2 of 6 IL-23p19 deficient mice developed an

active infection, but these differences were not statistically significant ($P > 0.05$ Fisher's exact test).

4.4.5 IL-23 was not required for influx of innate immune cells into the oviduct or the development of oviduct pathology.

Innate immune responses are key for the development of chlamydia-induced immunopathology (31, 33-35, 100, 137), and IL-17 and IL-22 have been shown to cooperate in inducing release of neutrophil chemokines and promoting innate inflammation (149, 175, 179). Thus, we examined the influx of innate inflammatory cells into the oviducts of wild-type and IL-23p19 deficient mice on day 10 post-infection. Flow cytometry revealed no differences in the frequency of neutrophils, inflammatory monocytes, or macrophages between the strains at this time point (Figure 25A). In accordance with these data, no improvement in the severity of oviduct dilatation was found in IL-23p19 deficient mice (Figure 25B).

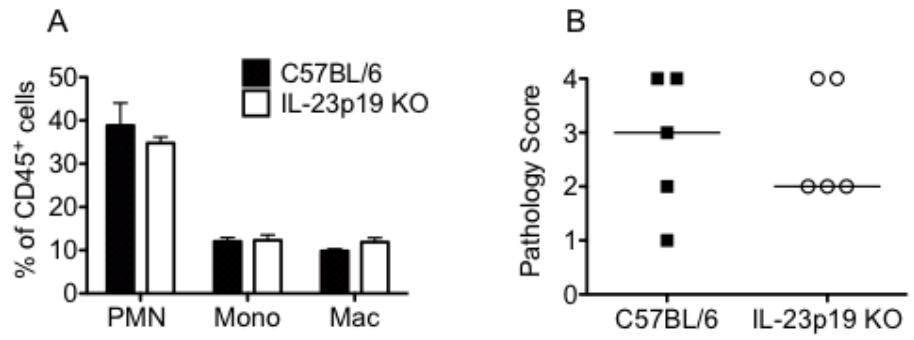

Figure 25: IL-23 was not required for influx of acute inflammatory cells into the oviducts or the development of oviduct pathology.

(A) On day 10 post-infection, flow cytometric analysis revealed no difference in the frequency of innate inflammatory cells in the oviducts of C57BL/6 (black bars) and IL-23p19 KO mice (white bars). Bars represent the mean ± SEM of the frequency of $CD45^+$ cells in the oviducts of 4 mice per strain for one representative experiment of two. PMN: $Ly6G/C^{high}$ $F4/80^{neg}CD11c^{neg}$; Mono (inflammatory monocytes): $Ly6G^{med}$ $F4/80^{neg}CD11c^{neg}$; Mac (macrophages): $F4/80^{pos}$ (B) Histological analysis of oviduct dilatation in genital tracts harvested on day 42 post-infection revealed no difference between the strains ($P > 0.05$ via Mann-Whitney U-test). Data points represent semi-quantitative scoring of oviduct dilatation of individual mice for 5 mice per strain from one representative experiment of two. C57BL/6 (black squares) and IL-23p19 KO mice (open circles). Median indicated by horizontal line.

4.5 DISCUSSION

In the studies outlined in this chapter, we explored the role of Th17 cells and the associated cytokines IL-22, IL-17, and IL-23 in the mouse model of *Chlamydia* genital tract infection. We show for the first time that the epithelial cells of the murine genital tract express the unique subunit of the IL-22 receptor, IL-22R1. We also demonstrate that IL-23 is required for IL-17 and IL-22 production in response to *C. muridarum* infection and is necessary for the maintenance of a chlamydia-specific Th17 response in the draining lymph nodes. However, we were unable to detect a requirement for any of these cytokines in resolution of infection,

susceptibility to low dose infection, or the development of oviduct pathology. The normal resolution of infection observed for IL-23p19 deficient mice could be predicted given these mice developed a Th1 response comparable to wild-type mice. Similarly, no compromise was seen in the ability of innate inflammatory cells to migrate into the oviducts of IL-23p19 deficient mice, and this influx was associated with oviduct pathology similar to that observed for C57BL/6 mice.

The IL-22 receptor is a heterodimer of IL-22R1 and IL-10R2, with expression of IL-22R1 limited to non-hematopoietic cells (169). IL-22R1 expression was previously detected in the human cervix and ovary by microarray (172), but protein expression in vivo has not been previously demonstrated. We detected IL-22R1 expression localized to the epithelium of the ectocervix, endocervix, uterine horns, and oviducts of uninfected C57BL/6 mice. Inflammatory stimuli including LPS, IFNγ, TNFα, and IL-1β may induce higher levels of IL-22R1 expression during acute chlamydial infection (169, 200). Detection of this receptor on the epithelium of the genital tract is in accordance with reports of receptor expression by epithelial cells at environmental interfaces including the skin, lung, and gastrointestinal tract (149, 169-171). Columnar epithelial cells of the genital tract are the site of chlamydial replication and are highly susceptible to infection-induced damage. Thus, the IL-22 receptor is appropriately located to play a role during chlamydial infection. Despite detection of this receptor, we determined that *C. muridarum* infection resolved normally in IL-22 deficient mice. Resolution of infection was also normal for IL-23p19 deficient mice despite nearly undetectable levels of both IL-17 and IL-22 in the lower and upper genital tract. These findings are not unique to *C. muridarum*, as pulmonary infection with the intracellular bacterium *Mycobacterium tuberculosis* resolved normally in the absence of IL-17 and IL-22 (153, 176).

There are several possible explanations for why we observed normal control of chlamydial infection in IL-22 and IL-23p19 deficient mice. It is likely that the intracellular replicative niche of chlamydiae hinders the potential protective capacity of anti-microbial proteins induced by these cytokines, which includes S100A proteins, β-defensins, Reg proteins, and lipocalins (149, 172, 175). Chlamydiae are susceptible to anti-microbial peptides in vitro (201, 202), but innate defense mechanisms have a limited ability to resolve *C. muridarum* infection in vivo independently of the adaptive immune response (45). It is also possible that the ability of chlamydiae to directly stimulate pattern recognition receptors (120) on epithelial cells and tissue resident immune cells obviates the requirement for epithelial-targeting cytokines peripheral to the Th1 response (34, 118, 120). Although IL-17 and IL-22 can induce the production of Th1 chemokines including CXCL9 (149), we observed no deficit in IFNγ production or Th1 migration to the genital tract of IL-23p19 deficient mice. PRR stimulation by *Chlamydia* induces the production of many proinflammatory cytokines that can activate the same pathways as IL-22 and IL-17 (34, 120). These cytokines, in combination with pathways induced directly by PRR stimulation, likely augment production of chemokines necessary for innate and adaptive inflammatory cell influx into the genital tract, thus obviating the requirement for IL-22 and IL-17.

It was previously observed that IL-17 played an important role in inducing the Th1 response to *C. muridarum* infection in the lung and was required for normal resolution of pulmonary infection (105). This contrasts with our findings of only slight reductions in IFNγ and no compromise in resolution of genital tract infection in the absence of IL-17 receptor signaling (78), or IL-23-dependent induction of the Th17-related cytokines, IL-22 and IL-17 (current work). The genital tract is a mucosal site that must maintain a tolerogenic environment for proper

reproductive fitness. Thus, there appears to be a site-specific role for IL-17 in defense against chlamydial infections, and this may hold true for IL-22 as well. Such tissue-specificity is described for *Candida albicans* infection where Th17 cytokines were required for control of oropharyngeal candidiasis (203), but in vulvovaginal candidasis, infection resolved normally in the absence of IL-22, IL-17 and IL-23 (181).

We also determined that *Chlamydia*-induced genital tract pathology was not altered in the absence of either IL-22 or IL-23. It was difficult to predict whether IL-22 would prevent or induce tissue damage in this model given its complex and dual roles in other models. We hypothesized that since IL-17 and IL-22 enhance neutrophil chemokine production and promote MMP release (149, 172, 178, 179), these cytokines would promote damage. We expected to observe decreased epithelial erosion and oviduct hydrosalpinx in IL-22 and IL-23p19 deficient mice. However, we determined that similar degrees of oviduct pathology developed in both the presence and absence of IL-17 (78), IL-22, and IL-23, which indicates that if these cytokines promote pathologic responses, their role is redundant with other cytokines. On the other hand, IL-22 has been demonstrated to enhance epithelial regeneration after an inflammatory insult (149, 174, 193, 195, 196, 204). Despite known regenerative effects of this cytokine, we did not observe any difference in the degree of epithelial erosion in the oviducts of IL-22-deficient and wild-type mice. When fully virulent *C. muridarum* are used for infection, enhanced early control of infection and prevention of ascension of *Chlamydia* to the oviduct may be required to prevent oviduct damage (16, 205). This does not preclude the potential for IL-22 to play a protective and regenerative role in the human genital tract, where *Chlamydia trachomatis* infection is frequently more indolent and chronic in nature (206).

We previously demonstrated that during *C. muridarum* genital infection, IFNγ-deficient mice exhibited significantly increased bacterial burden, enhanced production of Th17-differentiating cytokines, predominant Th17 and neutrophilic responses, an increased IL-22 response, and enhanced genital tract tissue damage (78). *Chlamydia trachomatis* has been shown to induce IL-23 by a combination of toll-like receptor stimulation and endoplasmic reticulum stress signals (207), both of which would be augmented in the presence of a suboptimal Th1 response and increased bacterial burden. $CD4^+$ T cells isolated from the cervix of women actively infected with *C. trachomatis* have been observed to produce IL-17 and IL-22 (113). These cells may play dual roles due to the complex interactions of these cytokines in vivo (149, 193). However, data in the mouse model indicate a primary role for Th1 cells in host defense and resolution of infection, and our data in mice deficient for the IL-17 receptor (78), or for Th17 cells and their downstream cytokines reveal that this pathway is dispensable for inducing a robust Th1 response and for resolution of genital tract infection and does not contribute substantially to protection from tissue damage. Given the fragile nature of the female oviduct, and the documented complex and often tissue injurious roles for IL-17 and IL-22, we propose that chlamydial vaccine strategies should avoid induction of these cytokines and focus on selective enhancement of the IFNγ response to chlamydial antigens.

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5.0 EXPRESSION OF MYD88 BY CD4+ T CELLS IS REQUIRED FOR EFFICIENT RESOLUTION OF *CHLAMYDIA MURIDARUM* GENITAL TRACT INFECTION

5.1 ABSTRACT

MyD88 is required for efficient resolution of *Chlamydia* genital tract infection in the mouse model, but MyD88 mediated signals can also enhance tissue damaging innate immune responses. CD4+ T cells are the primary mediator of clearance of *Chlamydia* from the genital tract. The goal of the studies outlined in this manuscript was to determine if MyD88 expression by CD4+ T cells enhanced resolution of infection. Using the murine model of *Chlamydia* genital tract infection, we found that CD4+ T cell expression of MyD88 was necessary for resolution of infection. This requirement was associated with a reduced ability of MyD88$^{-/-}$ CD4+ T cells to accumulate in the draining lymph nodes and genital tracts when exposed to the same inflammatory milieu as wild-type CD4+ T cells. We also demonstrated that the impaired infection control we observed in the absence of MyD88 could not be recapitulated by deficiencies in TLR or IL-1R signaling. These findings were supported by our in vitro findings of increased apoptosis of MyD88$^{-/-}$ CD4+ T cells in the absence of exogenous ligands for receptors upstream of MyD88. Vaccination strategies should thus avoid activating MyD88 mediated pathways that have the potential to induce tissue damaging immune responses, and

future studies should focus on delineating signaling pathways that directly promote CD4+ T cell survival.

5.2 INTRODUCTION

Due to its obligate intracellular lifecycle, *Chlamydia* is able to evade innate defense mechanisms that are effective against extracellular bacteria, and innate immune responses have been repeatedly correlated with the development of oviduct pathology (31, 33-35, 37). In contrast, studies in the mouse model have revealed that the adaptive immune response is crucial for eradication of both primary (73) and secondary infection (51). In addition, CD4+ Th1 cells are crucial for protection in both mice (45, 51, 74, 75, 77, 89) and women (20, 83, 85). CD4+ T cells directly interact with infected epithelial cells and promote eradication of infection via IFNγ dependent and independent mechanisms (75, 89, 90, 94).

Recognition of pathogens by pattern recognition receptors (PRRs) expressed by innate immune cells is crucial for effective induction of an adaptive immune response (208), but overly robust innate immune activation results in tissue damage. Chlamydiae stimulate several PRRs including Toll-like receptor 2 (TLR2) (34, 209), TLR3 (210), TLR4 (211, 212), and nucleotide-binding oligomerization domain-containing protein 1 (NOD1) (213). Mice deficient in TLR2 develop dramatically reduced levels of oviduct pathology in response to *Chlamydia muridarum* infection, but resolution of infection is not impacted by the absence of this receptor (34). TLR4 and NOD1 do not appear to play a central role in either tissue damage or induction of a protective immune response in the mouse model (34, 213). These findings were corroborated by a study of women with *Chlamydia trachomatis* PID, which revealed that women with specific

polymorphisms in TLR1, a receptor that signals by forming heterodimers with TLR2 (214), exhibited decreased rates of pregnancy, whereas no such association was found with polymorphisms in TLR4 (215). A Dutch study found a nonsignificant association of the TLR4 +896 G allele with tubal factor infertility (216).

MyD88 is an adaptor molecule that is central to signaling via all TLRs except for TLR3 and is required for signaling by the interleukin-1 (IL-1) family of cytokine receptors (217-221). Recognition of ligands by these receptors induces conformational changes that promote homotypic interactions between the Toll/interleukin-1 receptor (TIR) domain of these receptors and those of intracellular adaptor molecules including MyD88 (222-224). Stabilized oligomers of MyD88 then interact via death domains with IL-1 receptor associated kinase (IRAK)1, IRAK2, and IRAK4 to form a Myddosome complex (223, 225-228). This signal transduction cascade leads to NF-κb and AP-1 mediated transcription of pro-inflammatory genes. MyD88 is thus central to promoting innate immune activation and has been implicated in promoting resistance to a multitude of pathogens in the mouse model (Reviewed in (229)). In humans, loss-of-function mutations in MyD88 (230) and IRAK4 (231) have been associated with the development of severe and potentially fatal bacterial infections in children.

MyD88-mediated signals promote cytokine production by innate immune cells in response to *Chlamydia* infection (34, 118, 209, 232). In addition, MyD88$^{-/-}$ mice exhibit significantly impaired control of *Chlamydia muridarum* genital tract infection (197, 232, 233). Prolonged infection was associated with early reductions in natural killer (NK) cell IFNγ production in the cervix and a decreased frequency of CD4+ T cells in the upper genital tract. However, *Chlamydia*-specific CD4+ T cell proliferation and IFNγ production remained largely

intact in the draining lymph nodes, although a small increase in IL-4 production was detected (197).

The importance of MyD88 in promoting adaptive immune responses to pathogens in murine models has been repeatedly attributed to its central role in innate immune activation. However, a requirement for MyD88 expression by adaptive immune cells has also been observed in models of infection and autoimmunity. In a murine model of *Toxoplasma gondii* infection, control of infection was impaired even when MyD88-deficient adaptive immune cells were activated in the presence of normal antigen presenting cells (234). These findings were recapitulated in two independent studies of murine lymphocytic choriomeningitis virus (LCMV) infection, which demonstrated that both CD4+ and CD8+ T cell survival was reduced in the absence of intrinsic expression of MyD88 (235, 236). A requirement for MyD88 expression by CD4+ T cells was also demonstrated in a model of colitis where MyD88-deficient CD4+ T cells exhibited reduced accumulation and cytokine production both in vitro and in vivo (237, 238). Finally, a recent publication demonstrated that CD4+ T cell expression of MyD88 was required for Th17 differentiation and the development of experimental autoimmune encephalitis (EAE) (239). Although the precise mechanism(s) behind this requirement for MyD88 in adaptive immune cells has not been determined, receptors upstream of MyD88 have been implicated in direct co-stimulation of T cells (240-245)

The development of a vaccine against *Chlamydia* requires delineation of immune mechanisms of protection from those that cause pathology. Activation of receptors upstream of MyD88, including TLR2 (34) and IL-1R (101), results in the development of oviduct damage in the mouse model. Although it is clear from these findings that MyD88-mediated signals promote tissue-damaging responses to chlamydial infection, detection of prolonged genital tract

infection in MyD88$^{-/-}$ mice indicates that this molecule also participates in protective immunity. A rapid and robust CD4+ T cell response is key for control of chlamydial infection and protection from disease (16, 45, 246), and MyD88 expression by adaptive immune cells has been implicated in promoting optimum responses in other models (234, 236-238). Using bone marrow chimeric mice and CD4+ T cell adoptive transfer experiments we have determined that intrinsic expression of MyD88 in CD4+ T cells is required for accumulation of CD4+ T cells in infected tissues and efficient resolution of genital tract infection. In vitro and in vivo experiments suggest that the CD4+ T cell specific effects of MyD88 are independent of TLR or IL-1R activation.

5.3 MATERIALS AND METHODS

5.3.1 Strains, cell lines, and culture conditions

C. muridarum Nigg was used for all experiments and was isolated as previously described (17, 114). All chlamydial strains were propagated in L929 cells (115). Bacteria were titrated by plaque assay (114) or as inclusion forming units (IFU) using fluorescently tagged anti-chlamydial lipopolysaccharide monoclonal antibody (Bio-Rad, Hercules, CA) (30).

5.3.2 Animals

C57BL/6 (CD45.2+), B6129SF2/J (C57BL/6;129S), B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J (MyD88$^{-/-}$), B6.129S7-*Rag1*^{tm1Mom}/J (Rag1$^{-/-}$), B6.129S7-*Ifng*^{tm1Ts}/J (IFNγ$^{-/-}$), B6.129S7-*Ifngr1*^{tm1Agt}/J (IFNγR^{-}

$^{/-}$), B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (CD45.1+), B6.PL-*Thy1*^{a}/CyJ (CD90.1+), B6;129S1-*Tlr3*^{tm1Flv}/J (TLR3$^{-/-}$) were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR2$^{-/-}$TLR4$^{-/-}$, TLR7$^{-/-}$ (247), and TLR9$^{-/-}$ (248) mice were kindly provided by Dr. Shizuo Akira. Mice were given food and water ad libitum in an environmentally controlled room with a cycle of 12 hours of light and 12 hours of darkness. The University Institutional Animal Care and Use Committee approved all animal experiments.

5.3.3 Murine infection and monitoring

Female mice of at least 6 weeks of age were subcutaneously injected with 2.5 mg of medroxyprogesterone (Depo-Provera®; Upjohn, Kalamazoo, MI) 5 to 7 days prior to infection to induce a state of anestrous (116). Mice were intravaginally inoculated with 1x10^{5} IFU of *C. muridarum* Nigg diluted in 30 μl of sucrose-sodium phosphate-glutamic acid (SPG) buffer unless otherwise indicated. Mice were monitored for cervicovaginal shedding via endocervical swabs (117), and IFU were calculated as previously described (30). Bacterial burden was measured in the oviducts, lungs, liver, and spleen by plaque assay (114). C57BL/6 mice were used as controls for all strains of knockout mice except TLR3$^{-/-}$ mice, which are on a mixed C57BL/6 and 129S background. Thus, F2 hybrids of C57BL/6 and 129S mice were used as controls for those mice.

5.3.4 Generation of Bone Marrow Chimeras

Mice were injected subcutaneously with 2.5 mg of depot medroxyprogesterone acetate (Depo-Provera®; Upjohn, Kalamazoo, MI) 5 days before irradiation. Recipient mice were prepared for

the immunocompromised state that results from irradiation by replacing their normal diet with antibiotic food (1.2% sulfamethoxazole and 0.2% trimethoprim; Lab Diet, St. Louis, MO) and sterile acidified water (PH 2.5-3) for 10 days prior to the procedure. Mice were irradiated with 2 doses of 500 rads of X-ray irradiation separated by 6 hours. Immediately following the final dose of irradiation, mice were reconstituted by intravenous (i.v.) injection of 7×10^6 bone marrow cells from MyD88$^{-/-}$ (CD45.2+) or WT (CD45.1+) mice. MyD88$^{-/-}$ cells were injected into WT (CD45.1+) recipients and WT (CD45.1+) cells into WT (CD45.2+) recipients. Mice were maintained on acidified water and antibiotic food for 4 weeks following irradiation. Chimerism was verified after 6 weeks using flow cytometry. Mice were re-injected with Depo-Provera® after 6 weeks and infected with 1×10^6 IFU of *C. muridarum* Nigg 1 week later. Data are from one representative experiment of two with 5-6 mice per group.

5.3.5 Generation of Mixed Bone Marrow Chimeras

Recipient mice were fed antibiotic food (1.2% sulfamethoxazole and 0.2% trimethoprim; Lab Diet, St. Louis, MO) and sterile acidified water (PH 2.5-3) for 10 days prior to irradiation. Recipient mice were treated with two doses of 450 rads (900 rads total) of X-ray irradiation separated by 6 hours. Immediately after irradiation, Rag1$^{-/-}$ mice were reconstituted with 2.5×10^6 cells from a Rag1$^{-/-}$ donor and 2.5×10^6 cells from either a MyD88$^{-/-}$, WT (CD45.1+), or IFN$\gamma^{-/-}$ donor. IFNγR$^{-/-}$ mice were injected with 2.5×10^6 cells from a Rag1$^{-/-}$ donor and 2.5×10^6 cells from a WT (CD45.1+) donor. Six weeks after injection, mice were bled to determine the level of engraftment and were injected with Depo-Provera®. Chimeras were infected with 1×10^6 IFU of *C. muridarum* Nigg. Groups consisted of 4-7 mice per donor: recipient combination.

5.3.6 CD4+ T cell Transfer into Rag1$^{-/-}$ mice

CD4+ T cells were isolated from the spleens of naïve C57BL/6 or MyD88$^{-/-}$ mice by negative magnetic selection (CD4+ T cell isolation kit II; Miltenyi Biotech, Auburn, CA). The purity of CD4+ T cells was determined to be > 93% for both strains by flow cytometry prior to transfer. Rag1$^{-/-}$ mice were injected i.v. with 4×10^6 CD4+ T cells from either strain. The frequency of CD4+ T cells in the peripheral blood was determined using flow cytometry at 3 weeks after transfer. Mice were injected with Depo-Provera® 4 weeks after transfer and were infected with *C. muridarum* 5 weeks after transfer. Data are presented from one representative experiment of three with 5-6 mice per group. Rag1$^{-/-}$ mice that did not receive a CD4+ T cell transfer were infected in 3 independent experiments with a total of 15 mice.

5.3.7 CD4+ T cell co-transfer experiment

Ten days prior to receiving the CD4+ T cell transfer, WT recipient mice (CD45.2+ CD90.1+ CD90.2-) were injected with Depo-Provera®. On the day of transfer, CD4+ T cells were isolated from the spleens of naïve WT (CD45.1+CD90.2+) or MyD88$^{-/-}$ (CD45.2+CD90.2+) mice by negative magnetic selection (CD4+ T cell isolation kit II; Miltenyi Biotech, Auburn, CA). Recipient mice were injected i.v. with 4×10^6 cells from both WT and MyD88$^{-/-}$ mice (8×10^6 cells total) and infected intravaginally with 1×10^6 IFU of *C. muridarum* Nigg. Four pools of cells per strain were processed independently from the beginning of the experiment and transferred into groups of 3 mice. The average purity of CD4+ T cells in these preparations was 88% for both strains of mice. Ten days post infection, mice were euthanized, and single cell suspensions were generated from their genital tracts and iliac nodes. The cervix and uterine horns were treated

with collagenase I (1 mg/ml; Sigma-Aldrich, St. Louis, MO) while the oviducts and iliac lymph nodes were mechanically disrupted using the previously described protocol (33, 78). Cells from the cervix, uterine horns, and oviducts were pooled for analysis. Donor-derived cells were enriched using a CD90.2 positive selection kit (Miltenyi Biotech, Auburn, CA) prior to surface staining. The frequency of CD4+ T cells from each strain of mice was determined by flow cytometry. Cells were stained for the following cell surface markers: anti-CD4 PE (Clone: RM4-5), anti-CD3 V450 (Clone: 500A2), anti-CD90.1 FITC (Clone: OX-7), anti-CD90.2 PE-Cy7 (Clone: 53-2.1), anti-CD45.1 PerCP-Cy5.5 (Clone: A20), and anti-CD45.2 APC (Clone: 104). All antibodies were from BD Biosciences (San Jose, CA). Data shown represent the frequencies of donor cells in either the genital tract or iliac lymph nodes from 4 groups of three recipient mice.

5.3.8 In vitro analysis of CD4+ T cell apoptosis

Naïve CD4+ T cells were isolated from the spleens of MyD88$^{-/-}$ (CD45.2) and WT (CD45.1+) mice via negative magnetic selection (Mouse Naïve CD4+ T cell isolation kit; STEMCELL Technologies, Vancouver, BC). Isolated T cells were combined at a 1:1 ratio (1×10^5 cells per strain) in a 96 well plate in complete medium. Complete medium consisted of DMEM (Thermo Fisher Scientific; Pittsburgh, PA), 10% fetal bovine serum (Thermo Scientific Hyclone, Pittsburgh, PA), vancomycin (100 μg/ml; Sigma-Aldrich, St. Louis, MO), gentamicin (50 μg/ml; Life Technologies Gibco, Grand Island, NY), Glutamax (1 mg/ml; Life Technologies Gibco, Grand Island, NY), 2-mercaptoethanol (50 μM; Sigma-Aldrich, St. Louis, MO), and non-essential amino acids (0.5 mg/ml; Life Technologies Gibco, Grand Island, NY). Cells were incubated with the indicated combination of the following reagents: plate-bound anti-CD3 (1

μg/ml, Clone 145-2C11; eBioscience, San Diego, CA), soluble anti-CD28 (1 μg/ml, Clone 37.51; Biolegend, San Diego, CA), IL-2 (5 ng/ml; Peprotech, Rocky Hill, NJ), IL-12p70 (10 ng/ml; Peprotech, Rocky Hill, NJ), and anti-IL-4 (1 μg/ml; Clone: 11B11; eBioscience, San Diego, CA). After three days in culture, cells were either directly stained for flow cytometry or were transferred to a new 96-well plate and incubated with complete medium without any stimulatory reagents for an additional 24 hours prior to antibody staining. Surface staining was conducted using the following antibody combination: anti-TCR β chain V450 (Clone: H57-597), anti-CD4 PE-Cy7 (Clone: RM4-5), anti-CD45.2 APC (Clone: 104), and anti-CD25 PE (Clone: PC61). After surface staining, apoptosis was measured by staining with Annexin V-FITC according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit I). Approximately 10 minutes prior to analysis, 5 μl of 7-Amino-Actinomycin D (7-AAD) was added to the samples. All antibodies and reagents used for staining were from BD Biosciences (San Jose, CA). Data shown represent the mean ± SEM of triplicate wells for each condition from one representative experiment of two.

5.3.9 Statistics

Comparison of the course of infection was conducted via Two-way repeated measures (RM) ANOVA with Bonferroni post-test analysis. A Log-rank (Mantel-Cox) Test was used to compare the duration of infection. Significant differences in the frequency of cells accumulating in the genital tract and lymph nodes in the T cell co-transfer experiment was determined via Mann-Whitney U-test. Apoptosis was compared between strains under different stimulatory conditions in vitro by Two-way ANOVA with Bonferroni post-test analysis. Prism software

(GraphPad Software, LaJolla, CA) was utilized for all statistical analysis. Values of $P < 0.05$ were considered significant.

5.4 RESULTS

5.4.1 MyD88 expression by hematopoietic cells was required for normal resolution of *Chlamydia muridarum* genital tract infection.

Epithelial cells represent the primary niche for *Chlamydia* in the genital tract (249-251), and MyD88 participates in *Chamydia*-induced cytokine production by these cells (118, 209). We first sought to determine if the prolonged infection detected in the absence of MyD88 (197, 232, 233) could be observed for mice with a MyD88 deficient hematopoietic compartment and a wild-type epithelium. Bone marrow chimeras can be utilized for this purpose because hematopoietic cells are more sensitive to irradiation than epithelial or stromal cells. After irradiation, the hematopoietic compartment can be reconstituted by bone marrow from a donor strain of mice while the epithelium retains the genotype of the recipient strain. Wild-type (WT; CD45.2+ or CD45.1+) mice were irradiated, and their bone marrow was reconstituted with either WT (CD45.1+) or MyD88$^{-/-}$ (CD45.2+) cells. Recipient mice with a MyD88 deficient epithelium were not included in this analysis due the potentially confounding effects resulting from the enhanced sensitivity of MyD88 deficient epithelial cells to irradiation (252). The frequency of donor derived CD45+ cells was above 90% for all of the mice (data not shown). In addition, the frequency of CD45+ cells in the peripheral blood that were CD3+CD4+ T cells was similar between the strains (WT donor: 5.4 ± 1.0%; MyD88$^{-/-}$ donor: 6.5 ± 0.87%; $P > 0.05$ by Mann-

Whitney U-test). Indeed, MyD88$^{-/-}$ bone marrow has been previously observed to have no deficiency in its ability to reconstitute irradiated recipient mice (253).

When mice with WT stromal/epithelial cells were reconstituted with MyD88$^{-/-}$ bone marrow (MyD88$^{-/-}$ Donor: WT Recipient), infection was significantly increased ($P < 0.0001$ by Two-Way RM ANOVA; Figure 26A) and prolonged ($P < 0.01$ by Log-rank test; Figure 26B) relative to that observed for recipients of wild-type bone marrow (WT Donor: WT Recipient mice). These data indicate that MyD88 is required in hematopoietic cells for normal resolution of *Chlamydia muridarum* infection even in the presence of wild-type epithelial cells.

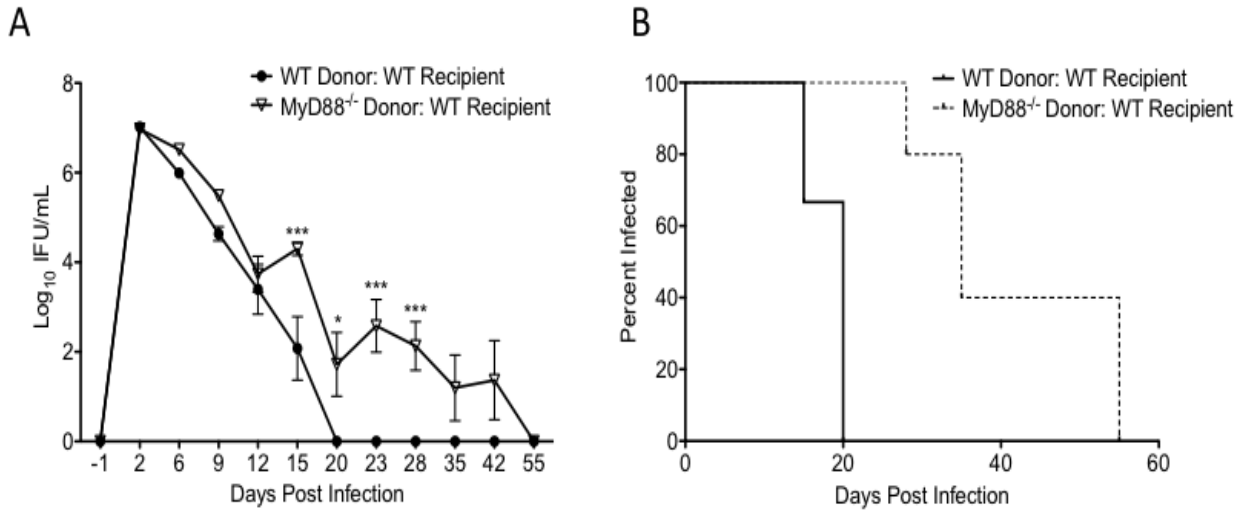


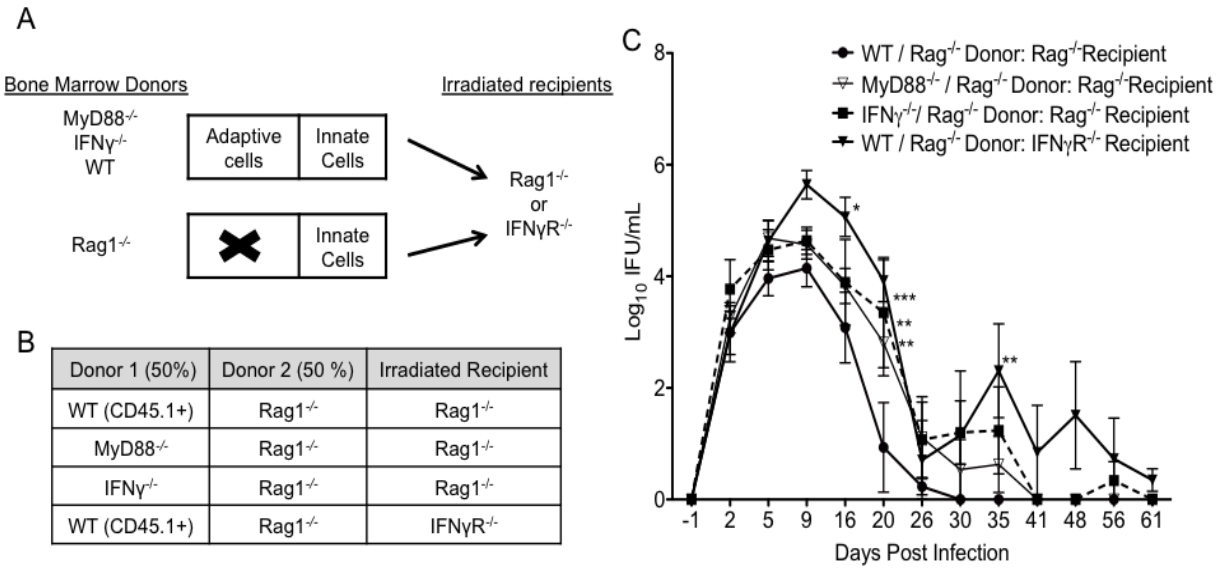
Figure 26: MyD88 was required in hematopoietic cells for normal resolution of *C. muridarum* genital tract infection (A) Bone marrow chimeras were generated with the following strain combinations: WT Donor: WT recipient (black circles) and MyD88$^{-/-}$ Donor: WT Recipient (white triangles). Mice were intravaginally infected with *C. muridarum*, and the course of infection was monitored with lower genital tract swabs. Data points represent the mean ± SEM of 5-6 mice per group from one representative experiment of two. Significance determined via Two-way RM ANOVA with Bonferroni post-test. Comparison of strains on individual days: * $P < 0.05$, *** $P < 0.001$. Comparison of groups over the interval measured: $P < 0.0001$ for WT Donor: WT Recipient vs. MyD88$^{-/-}$ Donor: WT recipient. (B) Infection was significantly prolonged in mice reconstituted with MyD88$^{-/-}$ bone marrow (dashed line) relative to mice with WT bone marrow (solid line). Data points represent the first day of a negative titer in the lower genital tract for the mice described in (A). $P < 0.01$ by Log-rank (Mantel-Cox) Test.

5.4.2 MyD88 expression by adaptive immune cells was required for normal resolution of *C. muridarum* genital tract infection.

MyD88-mediated signals promote activation of innate immune cells in response to *Chlamydia muridarum* (34, 232). In addition, MyD88 expression by adaptive immune cells has been shown to be important in murine models of infection and autoimmunity(234-236, 254). We sought to determine if there was a role for MyD88 in promoting resolution of chlamydial infection in mice with a WT antigen presenting cell (APC) compartment and MyD88 deficiency solely in the adaptive immune cells. To this end, we generated mixed bone marrow chimeras based on the experimental design used by LaRosa *et al* (234). WT, MyD88$^{-/-}$, or IFNγ$^{-/-}$ bone marrow was combined at a 1:1 ratio with Rag1$^{-/-}$ bone marrow and transferred into irradiated Rag1$^{-/-}$ recipients (Figure 27A, B). Rag1$^{-/-}$ mice have normal APCs but no adaptive immune cells. Thus, the bone marrow from WT, MyD88$^{-/-}$, or IFNγ$^{-/-}$ mice served as the only source of adaptive cells, while Rag1$^{-/-}$ bone marrow acted as a source of functional APCs. Irradiation of the Rag1$^{-/-}$ recipients provided a niche for engraftment of the donor-derived bone marrow. Rag1$^{-/-}$ mice were used as recipients to ensure that all T cells were derived from the donor because irradiation cannot eliminate 100% of recipient cells. Irradiated IFNγR$^{-/-}$ mice were reconstituted with mixed WT + Rag1$^{-/-}$ bone marrow as a positive control (Figure 27A, B) due to the central role for IFNγ signaling at the level of the genital tract epithelium (74, 75, 78, 90, 164). Verification of chimerism at six weeks after transfer was performed using disparate markers present on the WT (CD45.1+) and Rag1$^{-/-}$ (CD45.2+) donor cells. Analysis of cells in the peripheral blood revealed as expected, that 100% of CD3+CD4+ and CD3+CD8+ T cells in irradiated Rag1$^{-/-}$ recipients were derived from the WT (CD45.1+) donor while 53 ± 3% of Ly6G/C^{high} innate cells were

derived from the WT donor. In addition, the frequency of CD3+ CD4+ T cells in the peripheral blood did not significantly differ between the groups (data not shown). We were unable to verify the frequencies of MyD88$^{-/-}$ or IFNγ$^{-/-}$ adaptive immune cells since there is no disparate marker between these strains and Rag1$^{-/-}$ mice. However, an identical irradiation protocol, and the same pool of Rag1$^{-/-}$ bone marrow cells were used for all of the groups.

The chimeras were intravaginally infected with *C. muridarum* 7 weeks after bone marrow transfer. Mice with a MyD88$^{-/-}$ or IFNγ$^{-/-}$ adaptive compartment exhibited a significantly increased infection relative to mice with a WT adaptive immune compartment (Figure 27C). The course of infection did not differ between mice lacking either MyD88 or IFNγ in their adaptive immune cells (Figure 27C). Mice with WT adaptive immune cells but IFNγR$^{-/-}$ stromal/epithelial cells also exhibited a significantly increased infection compared to mice with a WT adaptive immune compartment and IFNγ responsive stromal/epithelial cells (Figure 27B). In addition, comparison of the bacterial burden in the lower genital tract between days 5 and 16 revealed a significantly increased infection only for IFNγR$^{-/-}$ recipient mice ($P < 0.01$ Two-Way RM ANOVA) and not for the Rag1$^{-/-}$ recipients with MyD88$^{-/-}$ or IFNγ$^{-/-}$ adaptive cells ($P > 0.05$ Two-Way RM ANOVA).



Donor 1 (50%)	Donor 2 (50 %)	Irradiated Recipient
WT (CD45.1+)	Rag1$^{-/-}$	Rag1$^{-/-}$
MyD88$^{-/-}$	Rag1$^{-/-}$	Rag1$^{-/-}$
IFNγ$^{-/-}$	Rag1$^{-/-}$	Rag1$^{-/-}$
WT (CD45.1+)	Rag1$^{-/-}$	IFNγR$^{-/-}$

Figure 27: MyD88 expression and IFNγ production by adaptive immune cells as well as IFNγR expression by the stromal compartment was required for normal resolution of *C. muridarum* genital tract infection.

(A, B) Bone marrow chimeras were generated with the following donor: recipient combinations. WT + Rag1$^{-/-}$ Donors: Rag1$^{-/-}$ Recipient (black circles); MyD88$^{-/-}$ + Rag1$^{-/-}$ Donors: Rag1$^{-/-}$ Recipient (white triangles); IFNγ$^{-/-}$ + Rag1$^{-/-}$ Donors: Rag1$^{-/-}$ Recipient (black square, dashed line); WT + Rag1$^{-/-}$ Donors: IFNγR$^{-/-}$ recipient (black triangle). Data points represent the mean ± SEM of 4-7 mice per group. Significance determined via Two-way RM ANOVA with Bonferroni post-test. Comparison of individual days: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ chimeras vs. WT + Rag1$^{-/-}$ Donors: Rag1$^{-/-}$ Recipient group. Comparison of groups over the interval measured: $P < 0.05$ for WT + Rag1$^{-/-}$ Donors: Rag1$^{-/-}$ Recipient group vs. each of the three other groups. $P > 0.05$ for MyD88$^{-/-}$ + Rag1$^{-/-}$ Donors: Rag1$^{-/-}$ Recipient vs. IFNγ$^{-/-}$ + Rag1$^{-/-}$ Donors: Rag1$^{-/-}$ Recipient

5.4.3 MyD88 expression by CD4+ T cells was required for normal resolution of *C. muridarum* genital tract infection.

The mixed bone marrow chimera experiment (Figure 27) essentially permitted observation of the role of MyD88 in CD4+ T cells because neither a deficiency in antibody nor CD8+ T cells influences resolution of primary infection with *C. muridarum* (45, 255). In order to specifically analyze the role of MyD88 in CD4+ T cells, we compared the course of infection in Rag1$^{-/-}$ mice that received CD4+ T cells from either MyD88$^{-/-}$ mice or WT mice. Prior to infection, the

frequency of CD3+CD4+ T cells in the peripheral blood did not significantly differ between the strains (WT: 4.96 ± 0.36%; MyD88$^{-/-}$: 3.70 ± 0.61% of CD45+ cells; $P > 0.05$ by Student's *t*-test). The course of infection in the lower genital tract was both significantly elevated ($P < 0.01$ by Two-way RM ANOVA) and prolonged ($P < 0.001$ by Log-rank Test) upon transfer of MyD88$^{-/-}$ CD4+ T cells relative to transfer of WT CD4+ T cells (Figure 28A, B). The median day of resolution of infection for mice with WT CD4+ T cells was day 23 and with MyD88$^{-/-}$ CD4+ T cells, it was day 47 (Figure 28B). However, Rag1$^{-/-}$ mice that did not receive a T cell transfer shed high levels of bacteria until they began to exhibit symptoms of systemic illness including tachypnea, hunching, lethargy, and death between days 14 and 25 post-infection (Figure 28C). This was observed in a total of 15 mice from three independent experiments. A group of moribund Rag1$^{-/-}$ mice was sacrificed on day 25 post-infection, and *Chlamydia* was detected in the oviducts, lungs, liver and spleen (Figure 28D). Thus, although clearance of infection was impaired in the absence of MyD88 in CD4+ T cells, infection eventually resolved, and dissemination of infection was prevented.

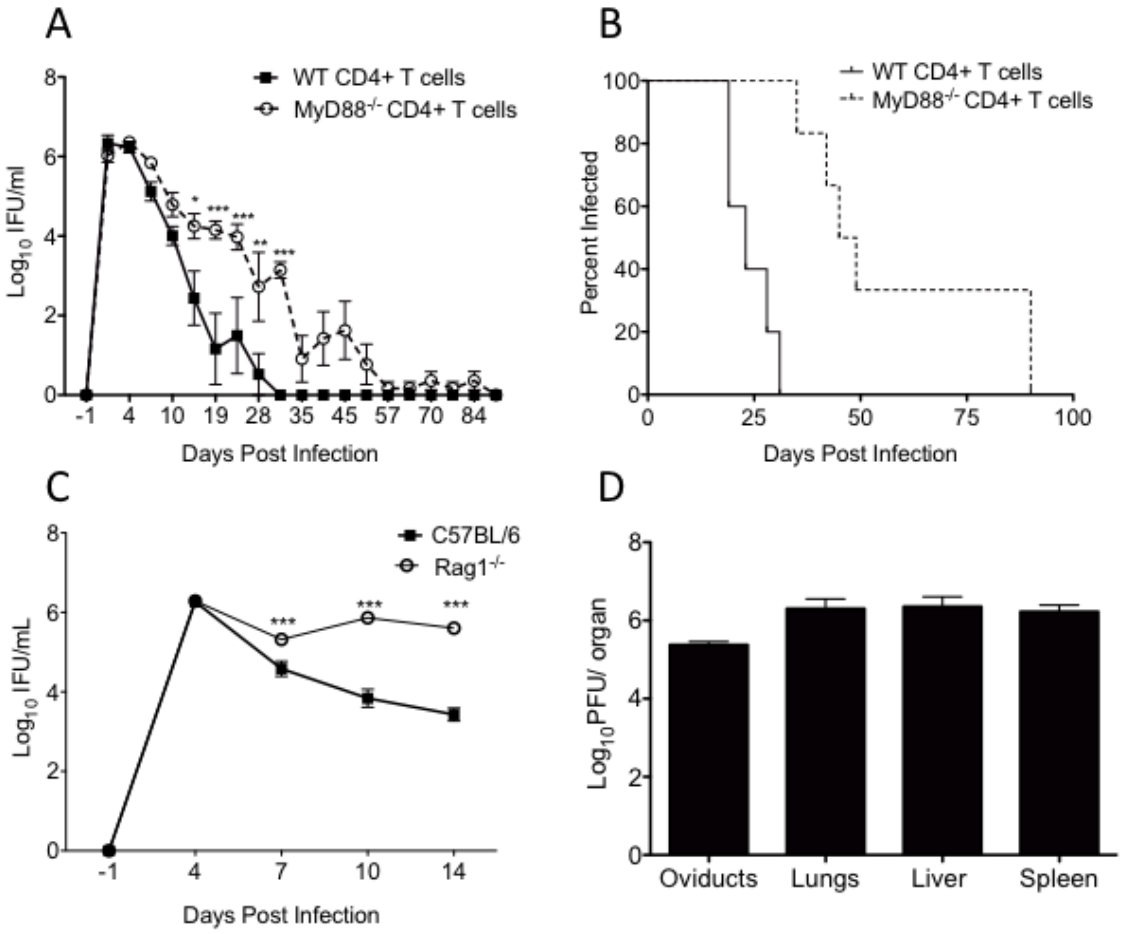


Figure 28: MyD88 was intrinsically required in CD4+ T cells for efficient resolution of *C. muridarum* from the lower genital tract.

(A) Rag1$^{-/-}$ were injected with 4×10^6 CD4+ T cells isolated from the spleens of naïve WT (black squares) or MyD88$^{-/-}$ mice (white circle, dashed line) and intravaginally inoculated with *C. muridarum* 5 weeks later. Data points represent the mean ± SEM of 5-6 mice per group from one representative experiment of three. $P < 0.01$ for WT CD4+ T cell recipients vs. MyD88$^{-/-}$ CD4+ T cell recipients over the interval measured via Two-way RM ANOVA with Bonferroni post-test. Comparison of individual days: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) Infection was significantly prolonged when Rag1$^{-/-}$ mice were reconstituted with MyD88$^{-/-}$ CD4+ T cells (black line) compared to WT CD4+ T cells (dashed line). $P < 0.001$ by Log-rank (Mantel-Cox) Test. (C) Rag1$^{-/-}$ (white circle) infected with *C. muridarum* exhibited a significantly increased infection in the lower genital tract relative to C57BL/6 mice (black squares) starting on day 7. Data points represent the mean ± SEM of 4 mice per group from one representative experiment of three. *** $P < 0.001$ for individual days by Two-Way ANOVA with Bonferroni post-test. (D) Titration of organs from moribund Rag1$^{-/-}$ mice sacrificed on day 25 post infection. Bars represent the mean ± SEM of plaque assay titers from for three mice.

5.4.4 Accumulation of MyD88$^{-/-}$ CD4+ T cells was impaired relative to WT CD4+ T cells.

After demonstrating a role for MyD88 in CD4+ T cells in promoting resolution of *C. muridarum* infection (Figure 28), we sought to define the mechanism responsible for this requirement. MyD88-mediated signals have been implicated in the survival of CD4+ and CD8+ T cells in other models of infection (234-236). To determine if MyD88$^{-/-}$ deficient CD4+ T cells exhibited impaired accumulation in the genital tract and iliac nodes when exposed to the same inflammatory milieu as WT CD4+ T cells, we conducted a co-transfer experiment where a 1:1 ratio of MyD88$^{-/-}$ and WT CD4+ T cells was transferred into immunologically normal mice (Figure 29A). Mice were intravaginally infected with *C. muridarum* at the time of T cell transfer. By day 10 post-infection, there was a significantly decreased frequency of MyD88$^{-/-}$ CD4+ T cells in both the iliac lymph nodes and genital tract (Figure 29B). This difference was particularly striking in the genital tract where an average of 74% of donor cells were from the WT donor (Figure 29B). These data indicate that MyD88 expression by CD4+ T cells is required for accumulation of these cells in the iliac lymph nodes and genital tract.

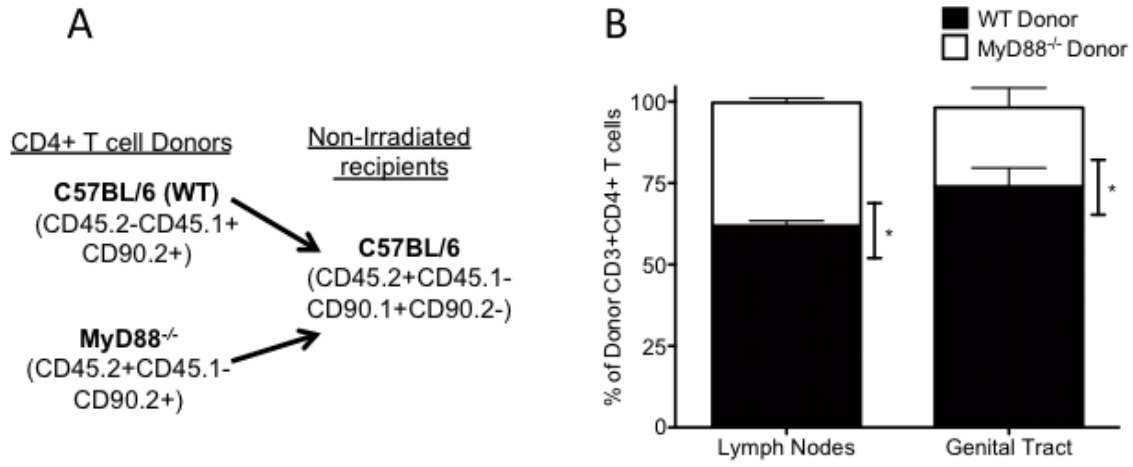


Figure 29: A significantly decreased frequency of MyD88$^{-/-}$ CD4+ T cells relative to WT CD4+ T cells was detected when CD4+ T cells were co-transferred into immunologically normal mice.

(A) Schematic of CD4+ T cell co-transfer experiment. (B) Frequency of donor WT (CD45.1+CD45.2-CD90.2+) and MyD88$^{-/-}$ (CD45.1-CD45.2+CD90.2+) CD4+ T cells isolated from the iliac lymph nodes and genital tract of recipient mice (CD90.2-CD90.1+) on day 10 post infection. Bars represent the mean ± SEM of the frequency of CD3+CD4+ T cells recovered from 4 groups of 3 mice. * $P < 0.05$ by Mann Whitney U-test.

5.4.5 Mice deficient in receptors upstream of MyD88 did not recapitulate the phenotype of MyD88$^{-/-}$ mice.

Detection of decreased accumulation of MyD88$^{-/-}$ CD4+ T cells compared to WT CD4+ T cells (Figure 29B) exposed to the same inflammatory milieu indicated that a MyD88-mediated signal might act to directly co-stimulate T cells during chlamydial infection. Signaling through several Toll-like receptors (TLRs) including TLR2 (240), TLR3 (241), TLR4 (242), TLR5 (243), TLR7 (243), and TLR9 (241) has been observed to directly co-stimulate T cells and promote their survival. We sought to determine if a deficiency in any of these receptors could recapitulate the significantly prolonged infection we observed in the absence of MyD88. We have previously observed that mice deficient in TLR2 or TLR4 resolve infection from the lower genital tract

normally (34). We next infected mice deficient in both TLR2 and TLR4 in order to determine if these receptors served redundant roles; however, infection resolved with normal kinetics in the absence of both of these receptors (Figure 30A). Although TLR3 signaling is not MyD88 dependent, we sought to determine if TLR3-mediated signals could promote resolution of infection since poly(I:C) can promote the survival of CD4+ T cells (241)(Figure 30B). Resolution of infection from mice deficient in TLR3 was normal. We also determined that TLR7 was not required for normal resolution of infection (Figure 30C). *Chlamydia* possesses unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) dinucleotides (256), which represent potential ligands for TLR9 (248). However, $TLR9^{-/-}$ mice also resolved infection normally (Figure 30D). The role of TLR5 was not examined because Chlamydiae are non-motile bacteria that do not express flagellin.

MyD88 is also required for signaling via the IL-1 family of cytokine receptors, which includes receptors for IL-1, IL-18, and IL-33. Mice deficient in the IL-1 receptor exhibit an increased bacterial burden in the lower genital tract, but infection is not significantly prolonged relative to WT mice (101). We have observed that IL-18 deficient mice resolve infection from the genital tract normally (data not shown), which is in agreement with the normal resolution of infection observed in $NLRP3^{-/-}$ mice that have significantly impaired IL-18 production in response to *Chlamydia* (101). Finally, we did not pursue evaluation of IL-33 deficient mice since IL-33 induces Th2 responses and so is not likely to promote resolution of chlamydial infection (221).

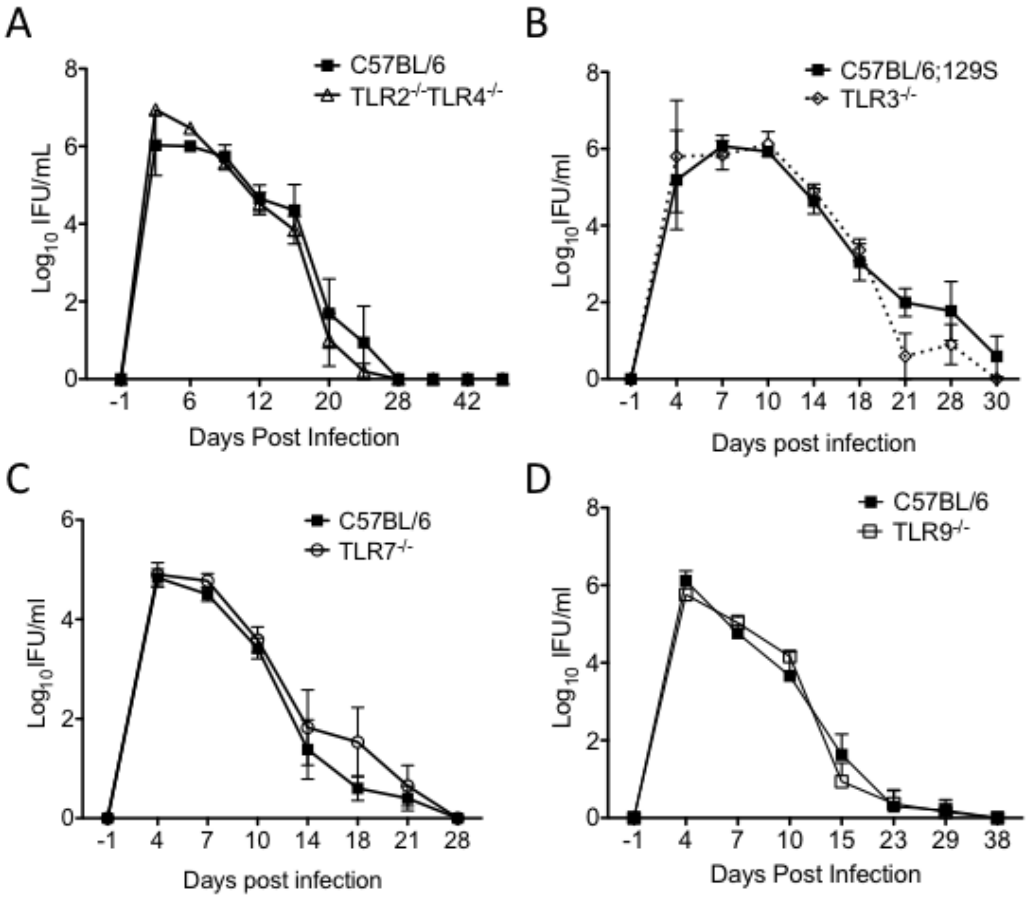


Figure 30: TLR deficiencies did not impair resolution of *C. muridarum*.

Mice deficient in (A) TLR2 and TLR4 (white triangles), (B) TLR3 (white diamonds, dotted line), (C) TLR7 (white circles), or (D) TLR9 (white squares) were intravaginally infected with *C. muridarum*, and the course of infection was compared to the appropriate control strain. C57BL/6 controls were used for all strains except for $TLR3^{-/-}$ mice, where the F2 generation of C57BL/6 and 129S mice was used. No differences were detected between the course of infection in the lower genital tract of any of the knockout strains and their matching control ($P > 0.05$ by Two-way RM ANOVA). Data points represent the mean ± SEM of 5 mice per strain and are from one representative experiment of three for the $TLR2^{-/-}TLR4^{-/-}$ and $TLR9^{-/-}$ mice and from a single experiment with $TLR7^{-/-}$ and $TLR3^{-/-}$ mice.

5.4.6 MyD88 deficient cells exhibited impaired survival upon activation in vitro.

Our in vivo data indicate that deficiencies in receptors upstream of MyD88 do not recapitulate the phenotype of MyD88$^{-/-}$ mice (Figure 30). These findings are similar to what has been described in other murine models (234, 236). We then sought to determine if impaired accumulation of MyD88$^{-/-}$ T cells would occur in vitro in the absence of TLR or IL-1R agonists, supporting the hypothesis that the accumulation defect observed in vivo (Fig. 4B) was independent of receptors upstream of MyD88. Naïve CD4+ T cells (CD25-CD44-) were isolated from the spleens of MyD88$^{-/-}$ (CD45.2) and WT (CD45.1+) mice. These cells were mixed at a 1:1 ratio and stimulated in vitro with different combinations of the following reagents: anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), IL-2 (5 ng/ml), IL-12p70 (10 ng/ml), and anti-IL-4 (1μg/ml) (Fig. 6). A co-culture of MyD88$^{-/-}$ and WT T cells was performed in order to prevent confounding effects that could result from differences in the inflammatory milieu. After 3 days in culture, activated T cells (CD25+) were either examined for their viability based on Annexin V and 7-amino-actinomycin D (7-AAD) staining or were removed from culture and replated for an additional 24 hours with media alone prior to analysis of apoptosis. After 3 days and 3 days + 24 hrs rest, the level of CD25 expression by CD25+CD4+ T cells did not differ between the strains (Figure 31A, B). However, the frequency of apoptotic (AnnexinV+7AAD-) CD4+CD25+ T cells was significantly increased in the absence of MyD88 under all stimulatory conditions tested (Figure 31C). After an additional 24 hrs without stimulation, there was no longer a difference in the frequency of apoptotic cells in the group that had been stimulated with anti-CD3 alone, but the frequency of apoptotic cells was significantly increased in the absence of MyD88 under all other stimulatory conditions (Figure 31D). The frequency of cells from either strain that up-regulated CD25 upon incubation with media alone for three days was negligible, so

that group was not included in analysis of apoptosis. These data indicate that activated MyD88 deficient CD4+ T cells have an increased propensity towards apoptosis even in the absence of exogenous TLR/IL-1R ligands.

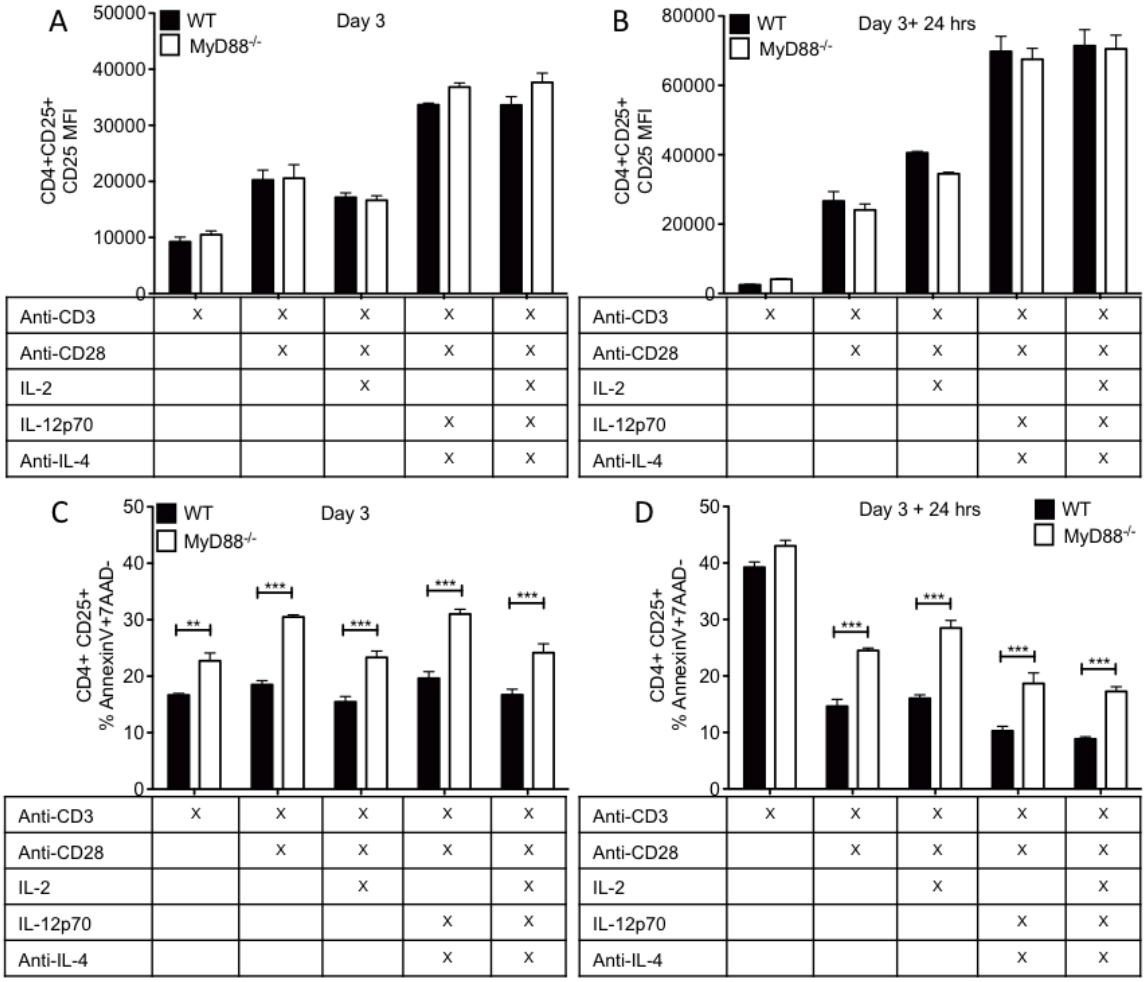


Figure 31: MyD88$^{-/-}$ CD4+ T cells exhibited increased apoptosis when activated in vitro.

Naïve CD4+ T cells from MyD88$^{-/-}$ and WT (CD45.1+) mice were co-cultured in vitro in the presence of the indicated stimulatory reagents for three days. (A, B) The level of CD25+ expression by activated CD4+ CD25+ T cells from each strain was determined after (A) 3 days or (B) after incubation with media alone for an additional 24 hrs. (C, D) The frequency of CD4+ CD25+ cells that were apoptotic (Annexin V+ 7AAD-) was determined after (C) 3 days or (D) 3 days + 24 hrs in media alone. *** $P < 0.001$ by Two-Way ANOVA with Bonferroni post-test. Bars represent the mean ± SEM of triplicate wells from one representative experiment of two.

5.5 DISCUSSION

In the this chapter, we present the results of our studies examining the role of the adaptor molecule MyD88 in the development of an effective adaptive immune response to *Chlamydia* genital tract infection. In doing so we attempt to provide a mechanism for the significantly impaired resolution of infection previously observed in MyD88 deficient mice (197, 232, 233). The increased bacterial burden observed in the absence of MyD88 was associated with the development of severe oviduct pathology (197, 233), which indicated that MyD88-dependent signaling was a double-edged component of the immune response to *Chlamydia* genital tract infection. Mice deficient in receptors upstream of MyD88, including TLR2 knockout mice (34) and IL-1 receptor knockout mice (101), exhibit significantly reduced oviduct pathology. However, signaling via MyD88 is necessary to effectively control infection and prevent the tissue damage that can result from a significantly increased bacterial burden. The goal of the studies outlined in this manuscript was to determine if the role of MyD88 in promoting the adaptive immune response could be separated from its involvement in promoting tissue damaging innate immune responses.

We first explored whether the prolonged infection observed in the absence of MyD88 could at least partially be attributed to a lack of MyD88 in circulating hematopoietic cells. We determined that bone marrow chimeras with a MyD88 deficient hematopoietic compartment and wild-type epithelial/stromal cells did indeed exhibit a significantly prolonged infection. Although the role of MyD88 in enhancing innate and adaptive responses to *Chlamydia* would seem intuitive, it was important to rule out the possibility that a deficiency in MyD88 expression by the epithelium of the genital tract was required to observe impaired resolution of infection. In vitro experiments have shown that genital tract epithelial cells produce significantly lower levels

of cytokines in response to *Chlamydia* in the absence of MyD88 (118). In addition, MyD88 can directly interact with the IFNγ receptor (257), which could be important in IFNγ-mediated clearance of *Chlamydia* from epithelial cells. The significantly prolonged infection we observed for the bone marrow chimeras in the presence of a wild-type epithelium pointed to an important role for MyD88 expression by circulating immune cells, although it did not exclude the possibility that MyD88 signaling at the level of the epithelium participated in controlling infection.

An important technical point regarding the use of bone marrow chimeras in this model is that X-ray irradiation can influence the architecture of the genital tract. We observed that the bacterial burden in the lower genital tract of irradiated mice was 10 to 100 fold lower than that of non-irradiated mice at the peak of infection. In addition, at the time of sacrifice, the uterine horns and cervices of mice that had been irradiated where significantly smaller than those of non-irradiated mice. These effects may preclude the use of this type of experiment in describing immune mediators of genital tract pathology. It also prevented us from analyzing mice with a MyD88-deficient epithelium because these mice exhibit a further increase in susceptibility to radiation-induced damage due to a higher proliferative rate of epithelial cells (252). Despite these caveats, we determined that mice with Myd88 deficient hematopoietic cells exhibited a course of infection that directly paralleled that observed in MyD88$^{-/-}$ mice. These findings indicated that tissue damage resulting from the action of MyD88-mediated signals at the level of the epithelium, as has been described for IL-1 in vitro (97), could potentially be separated from those that were protective.

We next used mixed bone marrow chimeras to more specifically explore the role of MyD88 in promoting clearance mediated by hematopoietic cells, with a focus on MyD88

expression by adaptive immune cells. The mixed bone marrow chimera experimental design was based on a previous manuscript, which showed that MyD88 expression by adaptive immune cells was required for control of *Toxoplasma gondii* infection (234). In these experiments, WT, MyD88$^{-/-}$, or IFNγ$^{-/-}$ bone marrow was mixed with an equal ratio of Rag1$^{-/-}$ bone marrow and transferred into irradiated Rag1$^{-/-}$ recipients. The Rag1$^{-/-}$ bone marrow provided a large pool of normal innate immune cells for priming of the adaptive immune response. In these experiments, mice with MyD88$^{-/-}$ or IFNγ$^{-/-}$ adaptive cells exhibited similar courses of infection, which were significantly increased compared to mice with WT adaptive cells. We also showed that mice with an IFNγR$^{-/-}$ deficient epithelium but normal adaptive immune cells exhibited an early increase in infection that was not observed for the mice with MyD88$^{-/-}$ or IFNγ$^{-/-}$ adaptive cells but an IFNγ responsive epithelium. These findings indicate that IFNγ production by innate immune cells can contribute to early control of infection, but IFNγ provided by adaptive cells is critical for efficient resolution of infection. The mixed-bone marrow chimera experiment also revealed that the prolonged infection observed in the presence of MyD88$^{-/-}$ adaptive immune cells was similar to that observed when adaptive cells could not produce IFNγ. Thus, intrinsic expression of MyD88 may be required for an optimal T cell IFNγ response in the genital tract. However, this is not likely due to a decreased ability of MyD88 deficient CD4+ T cells to differentiate into Th1 cells because that has been repeatedly demonstrated to be unimpaired both in vitro and in the presence of normal APCs in vivo (236, 237, 239).

We used a CD4+ T cell transfer model to confirm our suspicions that MyD88 expression by CD4+ T cells was necessary for eradication of *Chlamydia* from the genital tract, since CD4+ T cells are the only adaptive immune cell required for clearance of primary infection in this model (45). Interestingly, the impaired resolution of infection observed upon transfer of MyD88^{-}

$^{/-}$ CD4+ T cells to Rag1$^{-/-}$ mice was much more pronounced than what we observed in the mixed bone marrow chimera experiments. One potential explanation for this observation is that the length of infection was reduced in the bone marrow chimeras due to effects of irradiation on the architecture of the genital tract. It is also possible that since the mice were provided with a fixed number of CD4+ T cells in the T cell transfer model, and the Rag1$^{-/-}$ recipients do not produce adaptive immune cells, they could not compensate for impairments in infection control with an increased release of adaptive immune cells from the bone marrow compartment. These findings also provided clues that impaired survival of T cells was responsible for the delayed resolution of infection, since release of cells from the bone marrow could replace failing adaptive immune cells in the bone-marrow chimera experiments, but this could not occur upon transfer of a finite number of CD4+ T cells into to the Rag$^{-/-}$ mice. Indeed, impaired survival of MyD88 deficient adaptive immune cells has been previously observed by others characterizing a requirement for MyD88 in CD4+ T cells (235, 237, 238) and CD8+ T cells (236).

Mechanistic experiments revealed that MyD88 deficient CD4+ T cells were impaired in their ability to accumulate in the genital tract and iliac lymph nodes when exposed to the same inflammatory milieu as WT CD4+ T cells. This is similar to what was observed in a CD4+ T cell transfer model of colitis, where naïve MyD88$^{-/-}$ CD4+ T cells exhibited impaired accumulation in a variety of organs when co-transferred with wild-type CD4+ T cells (238). This was also observed in mixed bone marrow chimeras infected with LCMV, where MyD88$^{-/-}$ CD8+ T cells exhibited significantly reduced accumulation in the spleen relative to WT T cells in the same mouse (236). Similar results were obtained upon transfer of LCMV specific MyD88 deficient and WT CD8+ T cells into WT recipients. The detection of a significantly reduced number of MyD88 deficient CD8+ T cells responding to LCMV was associated with an

increased rate of apoptosis and not a defect in proliferation (236). This would explain why we previously observed normal proliferation of *Chlamydia*-specific CD4+ T cells in MyD88$^{-/-}$ mice but a reduced frequency of these cells in the genital tract in the presence of a dramatically increased bacterial burden (197).

We attempted to find a receptor upstream of MyD88 signaling that would explain the deficiencies we observed in its absence. Our current and former studies show that mice with deficiencies in TLR2 (34), TLR2 and TLR4, TLR4 (34), TLR7, TLR9, IL-1R (101), and IL-18R do not exhibit delayed resolution of infection. These negative data could indicate that an untested TIR domain-containing receptor participates in MyD88 mediated control of infection or that a combination of deficiencies can explain this impaired resolution. However, it is also possible that MyD88 plays an unconventional role in promoting T cell survival. Our in vitro apoptosis assays were conducted without the addition of exogenous TLR/IL-1R ligands, and we still observed an increased rate of apoptosis in the absence of MyD88. Although we cannot rule out a role for autologous cytokine production or molecules released from dying cells in co-stimulating these cells, the fact that the level of CD25 expression by MyD88$^{-/-}$ and WT T cells under the stimulatory conditions tested was comparable, indicates that these cells were not exposed to different exogenous activating signals as would occur in the presence of signaling via mediators upstream of MyD88. Rather, these findings indicate that stimulatory signals are able to promote similar levels of activation in MyD88 deficient T cells, but defects arise after the divergence of activating and survival signals. This is similar to what was observed in a model of LCMV (236), where differentiation and activation of MyD88 deficient CD8+ T cells was normal, but accumulation was dramatically impaired. Interestingly, a number of MyD88-mediated signals that are TIR domain independent have been described such as a role for MyD88

in interacting with the IFNγR (257), Phosphatidylinositol 3-kinase (PI-3K) (258, 259), Fas-associated death domain protein (FADD)(260, 261), IFN regulatory factor (IRF)1 (262), IRF5 (262, 263), and IRF7 (264). FADD has been observed to prevent MyD88 mediated proinflammatory signals, so we could speculate that MyD88 could reciprocate by preventing FADD mediated proapoptotic signals (265). Our findings also indicate that the intrinsic requirement for MyD88 in CD4+ T cells for normal resolution of *Chlamydia* genital tract infection is not at all specific to this model. That would explain why similar findings have been observed across several models without an upstream mechanism (234-236).

We were unable to find a receptor upstream of MyD88 that was required for resolution of *Chlamydia* from the genital tract. Thus, direct stimulation of MyD88-dependent receptors on CD4+T cells does not significantly enhance protective immunity during chlamydial infection. In addition, activation of these pathways in CD4+ T cells has been shown to lead to detrimental responses in murine models, including induction of pathologic Th17 responses (239), EAE (239), and IBD (237, 238). These data indicate that activation of TLR and IL-1R receptors on CD4+ T cells should not be incorporated into a vaccination strategy. On the other hand, MyD88 augments the longevity of CD4+ T cells. In the absence of MyD88, small reductions in CD4+ T cell accumulation in the lymph nodes translate into dramatically decreased numbers of CD4+ T cells in the genital tract and impaired resolution of infection. Although the specific mechanisms whereby MyD88 promotes T cell longevity have not been determined, these studies show that MyD88 is clearly a necessary component of an effective adaptive immune response to *Chlamydia.* Determination of signaling pathways that promote CD4+ T cell survival would accelerate vaccine development.

5.6 ACKNOWLEDGEMENTS

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6.0 CONCLUSIONS AND FUTURE DIRECTIONS

These studies show that vaccine strategies must be focused on inducing long-lived Th1 responses to *Chlamydia*. Neutrophils are extremely dangerous for the oviduct, which is vulnerable to direct damage by the immune response and to the ongoing damage that occurs when scarring of the oviduct leads to the accumulation of fluid known as hydrosalpinx. In order for neutrophil-mediated damage to be avoided, the ascension and replication of *Chlamydia* in the UGT must either be prevented or abbreviated. We determined that infection with plasmid-deficient *Chlamydia* resulted in a reduced influx of neutrophils into the oviducts, which was associated with lower levels of neutrophil chemokines and an impaired ability to delay neutrophil apoptosis. Although the mechanism behind the decreased inflammation has yet to be completely elucidated, our previous studies indicated that the plasmid-deficient strain of *C. muridarum* did not signal via TLR2 (17). The ligand(s) that stimulates TLR2 could represent a vaccine target for prevention of the development of oviduct damage. Our studies also show that the Th17 response is likely more harmful than protective in this model. We found that when IFNγ was absent, a markedly enhanced Th17 response in the genital tract was associated with increased neutrophil influx and tissue damage. We did not detect a phenotype in the absence of IL-17, IL-22, or IL-23 in our other studies, but this is likely because these knockout mice were on the Th1-biased C57BL/6 background. Our studies conducted in the absence of IFNγ demonstrate the damage that can occur in the presence of an augmented Th17 response and show that vaccine strategies

that promote a Th17 response to *Chlamydia* either intentionally or inadvertently should be avoided. In addition, we have determined that CD4+ T cell longevity is crucial for efficient resolution of infection from the genital tract. An effective vaccine must prime *Chlamydia-specific* CD4+ T cells in the lymph nodes, and these cells must survive long enough to travel to the genital tract and successfully combat infection.

Future studies will focus on identifying immune responses in the peripheral blood of women that are associated with protection from UGT infection and disease. These studies will be correlated with responses in the endometrium with the hopes of identifying the phenotypes of and antigens recognized by CD4+ T cells that prevent *Chlamydia* from ascending to the vulnerable tissues of the UGT. We will also determine whether sterilizing immunity to *Chlamydia* develops in women in response to genital tract infection and if protective immune responses can de separated from those that are pathologic.

7.0 RELEVANT PUBLICATIONS

1. Scurlock, A. M., L. C. Frazer, C. W. Andrews, Jr., C. M. O'Connell, I. P. Foote, S. L. Bailey, K. Chandra-Kuntal, J. K. Kolls, and T. Darville. 2011. IL-17 contributes to generation of Th1 immunity and neutrophil recruitment during Chlamydia muridarum genital tract infection but is not required for macrophage influx or normal resolution of infection. *Infect Immun* 79: 1349-1362.
2. Frazer, L. C., C. M. O'Connell, C. W. Andrews, Jr., M. A. Zurenski, and T. Darville. 2011. Enhanced neutrophil longevity and recruitment contribute to the severity of oviduct pathology during Chlamydia muridarum infection. *Infect Immun* 79: 4029-4041.
3. Frazer, L.C., A. M. Scurlock, M. M. Riley, M. Mintus, D. A. Pociask, J. E. Sullivan, C. W. Andrews, Jr, T. Darville. 2013. IL-23 induces IL-22 and IL-17 production in response to *Chlamydia muridarum* genital tract infection, but the absence of these cytokines does not influence disease pathogenesis. *Journal of Reproductive Immunology*. Manuscript Submitted.
4. Frazer, L.C., J. E. Sullivan, M. A. Zurenski, M. Mintus, T. E. Tomasask, U. M. Nagarajan, T. Darville. 2013. Expression of MyD88 by CD4+ T cells is required for efficient resolution of *Chlamydia muridarum* genital tract infection. *Journal of Immunology*. Manuscript Submitted.

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