THE ROLE OF MAST CELLS AND TOLL-LIKE RECEPTOR 2 (TLR2) IN HOST DEFENCE AND PATHOLOGY FOLLOWING *CHLAMYDIA* INFECTION

by

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ABSTRACT

Chlamydia trachomatis (*Ct*) is the most common bacterial sexually transmitted infection and the leading cause of infectious blindness worldwide. A comprehensive understanding of immune responses to *Ct*, and how they lead to effective host defence or chronic pathology, is essential to designing an efficacious vaccine. This study investigated how mast cells and the pathogen recognition receptor toll-like receptor 2 (TLR2) contribute to host immune responses during *Ct* infection of the female genital tract. Primary human cord blood-derived mast cells (CBMCs) stimulated with *C. trachomatis* (*Ct*) produced a number of pro-inflammatory cytokines and chemokines, such as TNF, IL-1β, IL-6, GM-CSF, IL-23, CCL3, CCL5 and CXCL8. Transmission electron microscopy revealed that *Ct* is rarely taken up by CBMCs *in vitro*. Furthermore, *Ct* was unable to replicate efficiently within these cells. These data suggest that human mast cells primarily detect and respond to extracellular *Ct*. Similarly, murine bone marrow-derived mast cells also produced a variety of mediators in response to *C. muridarum* (*Cm*), including CXCL1, CXCL2, CCL2, CCL3, IL-6, and IL-12p70, although these responses did not reach significance due to variability across cultures. A number of these responses were diminished in TLR2$^{-/-}$ BMMCs, indicating that they were TLR2-dependent. To investigate the pathophysiological role of mast cells *in vivo*, mast cell-deficient (Wsh) mice and wildtype (WT) C57BL/6 mice were infected intravaginally with *Cm* for 50 days. Over the course of infection, bacterial shedding was equivalent. However, at day 50, Wsh mice had significantly reduced oviduct pathology compared to WT. When splenocytes from infected mice were restimulated with heat-inactivated *Cm*, cells from Wsh mice had a marked reduction of IFN-γ, IL-13, and IL-17A, compared to WT, indicating that they had broadly suppressed memory responses. This was attributed to a decreased infiltration of classical dendritic cells (cDCs), particularly CD103^{+} DCs, into the draining lymph node of Wsh mice at three days post-infection. Given the TLR2-dependent nature of BMMC responses to *Cm,* we also infected TLR2$^{-/-}$ and WT mice, on C57BL/6 and BALB/c backgrounds, intravaginally with *Cm* for 50 days. In the C57BL/6 background, TLR2$^{-/-}$ mice had increased bacterial loads at days 14 and 21 post-infection, but this was resolved by day 28. In the BALB/c background, TLR2$^{-/-}$ mice had decreased bacterial shedding compared to WT at day 3, but displayed comparable bacterial burden over the remaining course of infection. At day 50, both C57BL/6 and BALB/c TLR2$^{-/-}$ mice exhibited a trend toward reduced oviduct pathology, but significant differences were not observed. In both strains, TLR2-deficiency was associated with diminished IFN-γ, IL-13 and IL-17A responses when splenocytes were restimulated with heat-inactivated *Cm*. Taken together, these findings indicate that both mast cells and TLR2 contribute to acute inflammation and the development of chronic pathology following *Chlamydia* genital tract infection. To our knowledge, these are the first studies examining mast cells in the context of *Chlamydia* genital tract infection, and the first studies employing two strains of TLR2$^{-/-}$ mice. These findings have important implications for the current understanding of *Chlamydia* pathogenesis, and may assist in future efforts for novel immunotherapies and vaccine design.

LIST OF ABBREVIATIONS USED

ACK	ammonium potassium chloride
ANOVA	analysis of variance
APC	antigen presenting cell
AP-1	activator protein 1
BALF	bronchoalveolar lavage fluid
BMMC	bone marrow-derived mast cell
BMDC	bone marrow-derived dendritic cell
BS	bovine serum
BSA	bovine serum albumin
CAP	community-acquired pneumonia
CBMC	cord blood-derived mast cell
CCL	CC-chemokine ligand
CD	cluster of differentiation
cDC	classical dendritic cell
CNS	central nervous system
Cm	*Chlamydia muridarum*
Cp	*Chlamydia psittaci*
CPAF	chlamydia protease-like activity factor
Cpn	*Chlamydia pneuomoniae*
Ct	*Chlamydia trachomatis*
CXCL	CXC-chemokine ligand
DAMP	damage-associated molecular pattern
DC	dendritic cell
DLN	draining lymph node
EB	elementary body
EM	electron microscopy
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
gDNA	genomic DNA
GM-CSF	granulocyte-macrophage colony stimulating factor
GT	genital tract
HIV	human immunodeficiency virus
hpi	hours post-infection
HPV	human papilloma virus
HRP	horseradish peroxidase
hSCF	human stem cell factor
HSP	heat shock protein
HTB	HEPES-Tyrode's buffer
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IFU	inclusion forming units
Ig	immunoglobulin
IKK	Iκβ kinase

IL	interleukin
ILN	iliac lymph node
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
KC	keratinocyte chemoattractant
LGV	lymphogranuloma venereum
LRR	leucine-rich repeat
LTA	lipoteichoic acid
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MKK6	mitogen-activated protein kinase kinase 6
MMP	matrix metalloproteinases
MOI	multiplicity of infection
MOMP	major outer membrane protein
MSM	men who have sex with men
MyD88	myeloid differentiation primary response gene 88
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κB
NO	nitrix oxide
NTC	no template control
OD	optical density
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
PGN	peptidoglycan
p.i.	post-infection
PID	pelvic inflammatory disease
PRR	pathogen recognition receptor
P/S	penicillin/streptomycin
qPCR	quantitative PCR
RB	reticulate body
RPMI	Roswell Park Memorial Institute (medium)
rRNA	ribosomal RNA
SAP	streptavidin alkaline phosphatase
SD	standard deviation
SE	standard error of the mean
SPG	sucrose-phosphate-glutamate
STI	sexually transmitted infection or soybean trypsin inhibitor
TAB2	TAK-1 binding protein 2
TAK1	TGF-β-activated kinase 1

TEM	transmission electron microscopy
TGF	transforming growth factor
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TIR	toll-interleukin receptor
TIRAP	toll-interleukin 1 receptor domain containing adaptor protein
TNF	tumour necrosis factor
TNFR1	tumour necrosis factor receptor 1
TLR	toll-like receptor
TRAF6	TNF-receptor associated factor 6
Treg	T regulatory cell
TSLP	thymic stromal lymphopoietin
WT	wildtype

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CHAPTER 1 INTRODUCTION

Chlamydiae are obligate intracellular bacteria that infect the mucosal epithelium of the genital, respiratory and ocular tracts [1]. *Chlamydia trachomatis* (*Ct*) is a global public health concern, as it is the most commonly reported bacterial sexually transmitted infection (STI) and the prevailing cause of preventable blindness worldwide [2]. Upon primary infection, invading *Ct* organisms elicit a variety of orchestrated immune responses from the host. The interplay between the immune cells and the pathogen determines the balance between host resistance and persistent infection, ultimately leading to disease outcomes. A more thorough understanding of the immune response to *Ct* infection is critical to the development of novel immunotherapies, predictors of pathology and a safe and effective vaccine.

1.1 *Chlamydia* infection and associated diseases

1.1.1 Sexually transmitted *Chlamydia* infections

Chlamydia trachomatis is the most common bacterial STI worldwide, with over 130 million new infections occurring each year [3]. *Chlamydia* continues to be a public health concern in Canada, as both the incidence and prevalence of *Ct* infections has been rising since the 1990s [4]. Among reported cases, young women are disproportionately represented, with the highest rate of *Ct* infections in females aged 20-24 [4]. Notably, this demographic experiences infections at a frequency of more than seven times the national rate in Canada, positioning young women as a particularly high risk group for acquiring *Ct* infections [4].

Three biovars of *Ct* (serovars D-K) are able to cause sexually transmitted genital infection in males and females [5]. Common symptoms of *Ct* infection in males include dysuria, urethral discharge and testicular pain, whereas females may present with dysuria and abnormal vaginal discharge or bleeding [2]. An estimated 50% of infected men and 70% of infected women may experience asymptomatic infections, during which no clinical signs are present [5]. If left undetected and/or untreated, a fraction of *Ct* infections will resolve themselves. However, untreated *Ct* infections, particularly in women, can lead to a spectrum of reproductive sequelae, including pelvic inflammatory disease (PID), ectopic pregnancy, and infertility [6]. A recent study estimated that as many as 16% of women develop PID following an untreated *Ct* infection [7]. These conditions are thought to arise from the ascension of *Ct* microorganisms from the lower genital tract to the upper genital tract, thereby promoting a chronic inflammatory response which leads to occlusion and scarring of the fallopian tubes [6]. In addition to genital tract pathologies, *Ct* infections are associated with an increased risk of acquiring HIV-1 [8] and high-risk HPV strains leading to cervical cancer [9].

The L-serovars (L1, L2, L3) of *Ct* are able to cause particularly aggressive genital infections that lead to a condition called lymphogranuloma venereum (LGV) [10]. LGV strains of *Ct* are tissue-invasive, and can spread to the local lymph nodes [11]. LGV infection leads to a genital ulcer-adenopathy syndrome, which typically presents as a painful swelling of the inguinal lymph nodes [11]. For decades, LGV has primarily affected the southern hemispheres and was rarely seen in developed nations. However, recently, clusters of cases have emerged in Western European populations of men who

have sex with men (MSM), reigniting an interest in understanding and preventing LGV infections [12].

Despite many efforts, no effective vaccine for *Chlamydia* currently exists. Due to the significant economic burden incurred by health care systems treating women with *Ct* sequelae, many countries have developed screening and treatment programs. *Ct* infections can be effectively detected in urine or genital swab samples by nucleic acid amplification tests, as recommended by the World Health Organization [2] and the Centre for Disease Control [13]. Genital *Ct* infections are usually treatable with antibiotics, however, reinfections are common, and persistent infection and treatment failure are also causes for concern [14]. Although antibiotic resistant strains of *Ct* have been rarely detected, accumulating evidence suggests that emergence should be closely monitored [15].

1.1.2 Other *Chlamydia* infections

The leading cause of infectious blindness worldwide is trachoma resulting from ocular infections with *Ct* [16]. According to the World Health Organization, trachoma is a significant health threat in 42 countries, with almost 200 million people living in endemic areas at risk [16]. Currently, an estimated 1.9 million people suffer from visual impairment or blindness caused by *Ct* infection [16]. Most of these infections are attributable to *Ct* serovars A-C, which can transmitted by hand-to-eye contact with infected surfaces, fomites, or flies [17]. In addition, genital serovars can cause conjunctivitis in neonates born to infected mothers [18]. Given the prevalence of asymptomatic genital tract infections in women, this is a significant concern. Infection of

the ocular tract with *Ct* triggers an inflammatory reaction of the conjunctiva that manifests as irritation, redness and discomfort [19]. If left untreated, this inflammation can become chronic, leading to scarring trachoma and blindness [20]. Similar to genital tract infections with *Ct*, ocular infections are also commonly asymptomatic, thereby facilitating transmission of the disease [19].

Chlamydia pneumoniae (*Cpn*) is a respiratory pathogen and an important cause of community-acquired pneumonia (CAP). *Cpn* infection is quite common, with serological evidence of a previous infection found in up to 50% of young adults and 75% of elderly individuals [21]. *Cpn* is primarily transmitted by aerosols, but genital *Ct* serovars can also cause pneumonia in neonates born to infected mothers [22]. Classical *Cpn* respiratory infections present with mild, flu-like symptoms that may evolve into a persistent cough accompanied by pharyngitis, bronchitis or pneumonia [23]. Mortality rates for *Cpn* infection are generally low, and infections are readily treatable with antibiotics [21]. However, *Cpn* infections have been implicated in the development of other diseases, such as cardiovascular disease and atherosclerosis [24] as well as reactive arthritis [25] and lung cancer [26, 27].

Chlamydia psittaci (*Cp*) is a zoonotic pathogen that primarily infects birds, but can be transmitted to humans via inhalation of aerosols [28]. Acute infection, referred to as psittacosis, typically manifests as flu-like symptoms and/or pneumonia [28]. However, *Cp* can also spread through the bloodstream to other organs such as the liver, spleen, meninges, and CNS, resulting in complications such as hepatitis, arthritis, myocarditis, and encephalitis [28]. The prevalence of *Cp* is quite low in the general population, with

most exposures occurring in pet owners handling birds [29]. *Cp* infections are treatable with antibiotics and instances of mortality have become rare [28].

1.1.3 *Chlamydia* biology and replication cycle

Chlamydiae are Gram-negative, obligate intracellular bacteria. The best studied family within this phylum is the *Chlamydiaceae* family, which is comprised of 11 pathogenic species including *C. trachomatis* [30]. *Chlamydia* strains are highly genetically conserved, sharing roughly two-thirds of their encoded proteins [30].

All *Chlamydia* species share a unique biphasic life cycle, which consists of two distinct forms (illustrated in Figure 1.1). The elementary body (EB) is ~0.3 μm in size, and is the infectious form of the bacterium adapted for the harsh conditions of extracellular life. The larger reticulate body (RB), measuring ~0.8 μm, is the intracellular form of the bacterium involved in growth and replication [31]. EBs attach to the host cell and enter via endocytosis in a membrane-bound compartment, deemed the chlamydial inclusion [32]. The inclusion body redirects itself from the classical endolysosomal pathway, and bacterial protein synthesis begins [33]. During this process, a number of effectors are secreted from the inclusion that act to modulate the host cell [34]. The EB differentiates into the reticulate body, which replicates exponentially by binary fission. At 20-40 hours post-infection, RBs differentiate back into EBs [17]. The newly synthesized EBs exit the host cell by cytolysis or extrusion and go on to infect neighbouring cells [35].

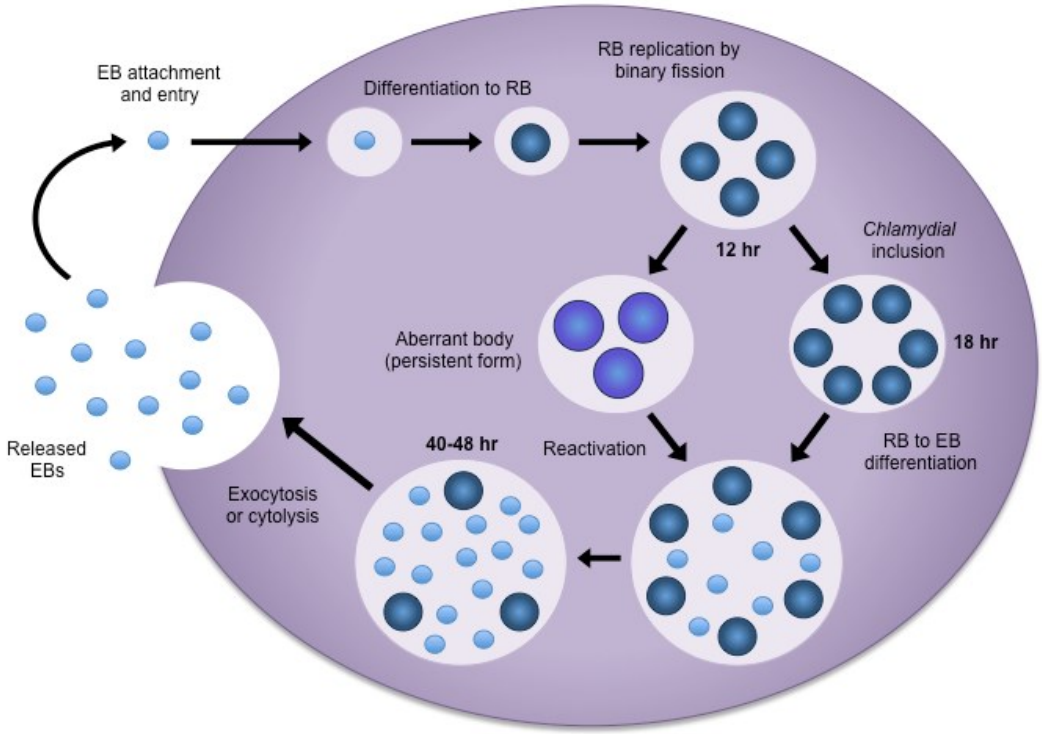


Figure 1.1 Schematic of the *Chlamydia* life cycle. Chlamydia EBs attach to the cell and enter via endocytosis. Within the membrane-bound chlamydial inclusion, EBs differentiate into RBs. RBs then replicate by binary fission. Under conditions of stress, RBs may transform into the persistent form of the bacteria, called the aberrant body. When cellular stresses are removed, aberrant bodies can be reactivated and resume the life cycle. Under normal conditions, RBs transition back into EBs, which are released from the host cell by exocytosis or cytolysis. Released EBs infect neighbouring cells, thereby perpetuating the Chlamydia life cycle. (Modified from [17] and [31].)

1.1.4 Persistent and chronic *Chlamydia* infections

Chlamydial persistence has been studied extensively *in vitro*, and is thought to contribute to the clinical manifestations of chronic pathology and recurrent infections. Chlamydial persistence has been defined as a long-lasting association with the host cell, in which the bacterium enters a viable but non-replicative growth stage that effectively evades immune responses and antibiotics [36, 37]. Persistent infections are associated with the morphological transition of reticulate bodies to enlarged, aberrant bodies under conditions of stress [31]. The formation of aberrant bodies *in vitro* has been described following the addition of antibiotics [38-40] or the removal of nutrients such as amino acids [36]. The most well-studied induction of persistence is the effect of IFN-γ [41]. High levels of IFN-γ are able to effectively restrict chlamydial growth, but moderate to low levels result in the development of aberrant bodies [42]. The most important mechanism in host cells that underlies this phenomenon is the induction of the enzyme indoleamine 2,3-dioxygenase (IDO), which starves the chlamydial inclusion of the essential amino acid tryptophan [43]. After the removal of IFN-γ from culture or the addition of tryptophan, aberrant bodies are able to reactivate and resume a normal life cycle [44]. In addition to IFN-γ-dependent persistence, *Chlamydia* has been shown to enter a spontaneous persistent state in monocytes [45] and aberrant bodies have been observed in the presence of bacteriophages [46]. Ultrastructural analyses of diseased tissues from human patients [47] and animal models [48] have suggested persistent infection can occur *in vivo*, but additional evidence is needed to confirm this.

Based on this phenomenon of persistence, discussion has been generated as to where *Chlamydia* fits on the spectrum of pathogen versus commensal bacteria. Some

argue that persistent infection more closely resembles commensalism [37]. This idea is supported by findings that many veterinary species of *Chlamydia*, closely related to *Ct*, are able to colonize the gastrointestinal tract of their respective animal hosts without initiating significant inflammatory responses [49]. Hyporesponsiveness, or failure to respond to recurrent exposure to microorganisms, may be referred to as immune tolerance [50]. Immune tolerance has been extensively studied in the gastrointestinal tract, which is another mucosal surface that is frequently exposed to foreign antigens. In this system, the pathogen recognition receptor (PRR) toll-like receptor 2 (TLR2) has emerged as a regulator of tolerance [51-54]. For example, the commensal bacteria *Bacteroides fragilis* has evolved mechanisms to activate TLR2 [51]. This activation initiates immunosuppressive responses, thereby facilitating subsequent colonization in the gut [51]. Currently, it is unclear whether *Ct* may utilize similar mechanisms to exploit host immune responses and colonize the genital tract. Nonetheless, it is conceivable that some aspects of immune tolerance may contribute to persistent or recurrent infection *in vivo*.

1.2 Animal models of *Chlamydia* infection

In order to understand the mechanisms of *Chlamydia* infection and identify host factors that limit pathogenesis, a number of animal models have been employed, including non-human primates, pigs, guinea pigs and sheep [55]. The most commonly used animal model of *Chlamydia* genital tract infection is the mouse. Due to its small size, ease of handling, and low procurement and maintenance costs, the mouse is widely considered to be the most practical and cost-effective of the available models.

Importantly, intravaginal inoculation of mice with *Chlamydia* causes acute disease that closely resembles infection in humans. Following a single infectious dose, bacteria are able to ascend the genital tract, resulting in the formation of hydrosalpinges that mimic tubal factor infertility in women [55].

In order to synchronize their estrous cycles and enhance susceptibility, mice must be administered progesterone prior to infection [56]. Most laboratory strains of mice are able to clear the infection without antibiotics in approximately four weeks, but disease outcomes such as the formation of genital tract pathology can range considerably [57]. Strains of mice that are designated to be "resistant" to *Chlamydia*, such as A/J mice, are able to clear genital tract infections rapidly, and do not develop oviduct pathology characterized by the presence of hydrosalpinges [57]. Mice that are considered to be "susceptible" to *Chlamydia* develop ascending infections, and this results in frequent and severe hydrosalpinx formation [57]. Examples of susceptible strains include C57BL/6 and BALB/c mice [57]. Previous reports have shown inoculating dose [58], age [59] and hormonal fluctuation [60] to be important factors influencing the course of infection and development of pathology.

There are two models of murine genital tract infections with *Chlamydia*, utilizing either the human pathogen *Ct* or the mouse pneumotitis biovar *C. muridarum* (*Cm*) [55]. Intravaginal inoculation of mice with *Ct* results in mild genital tract disease that is rapidly resolved. In order to study upper genital tract pathology in this model, *Ct* must be inoculated in the uterine horn [61]. Although the *Ct* model uses the human pathogen of interest, high infectious doses must be used to cause clinical disease, and some argue the validity of this model because it bypasses the natural route of infection [55]. *Cm* is a

murine pathogen that was first cultured from the lungs of mice [62]. Intravaginal inoculation with *Cm* results in an acute infection that is able to ascend to the upper genital tract, mimicking many factors of *Cm*-induced tubal infertility in humans. Although *Ct* and *Cm* are closely related, there are important genetic variations between the two strains. For example, *Cm* has been shown to be sensitive to IFN-γ-mediated inhibition of chlamydial growth, while *Ct* is not [63]. This has been attributed to *Ct* encoding a functional tryptophan synthase to escape host-mediated tryptophan starvation, which is the main host defence induced by IFN-γ [64]. Thus, although both models of genital tract infection may be useful, differential host-pathogen interactions must be considered, especially when considering potential translations to human medicine

1.3 Innate host responses to *Chlamydia*

Mechanisms of host defence elicited by *Chlamydia* infection can be classified into two distinct, but interdependent arms of the immune system: innate and adaptive immunity. Innate immunity is the first line of defence against pathogens, and is characterized by physiological barriers, such as epithelial cells and mucus production; the release of inflammatory mediators, such as cytokines and chemokines; and the actions of early immune cells to detect and kill invading microbes. Innate immunity to *Chlamydia* involves a myriad of players in the immune system, and of those, this introduction will focus on constituents that are most relevant to the presented studies. A special focus will be placed on mast cells and toll-like receptor 2 (TLR2) due to their principal significance in this research.

1.3.1 Epithelial cells

Initiation of *Ct* infection begins as EBs invade the epithelial cells of the cervix [17]. Although epithelial cells are not considered to be immune cells, their primary infection has a pivotal role in initiating and sustaining the immune response that follows. Epithelial cells possess a number of pathogen recognition receptors on their surface and within the cell, such as TLRs [17]. These receptors function as innate immune sensors that are able to detect conserved chlamydial pathogen-associated molecular patterns (PAMPs) and initiate an inflammatory response [65]. Infected epithelial cells produce a number of pro-inflammatory cytokines including granulocyte-monocyte colony stimulating factor (GM-CSF), IL-1α, IL-6 and tumor necrosis factor (TNF) [66]. In addition, they produce a number of chemokines such as CXC-chemokine ligand 1 (CXCL1), CXCL8 (IL-8), CXCL10, CXCL16 and CC-chemokine ligand 5 (CCL5) that function to recruit effector immune cells to the site [66, 67]. Release of IL-12p70, IFN-α and IFN-β from epithelial cells helps to polarize the environment toward a protective T helper 1 (Th1) response, leading to IFN-γ production [68]. Epithelial cells are also able to release antimicrobial peptides such as the cathelicidin LL37, although it has been demonstrated that the chlamydial factor CPAF (chlamydia protease-like activity factor) can effectively degrade these proteins *in vitro* [69]. Overall, epithelial cells have an important role in beginning to coordinate the anti-chlamydial immune response in the genital tract. However, *Ct* has evolved mechanisms to bypass this line of defence, and it has been proposed that some of these early inflammatory mediators may cause collateral tissue damage leading to pathology [1].

1.3.2 Toll-like receptor 2 (TLR2)

Toll-like receptors are a group of highly conserved transmembrane pathogen recognition receptors that were first identified in *Drosophila* [70]. TLRs are important innate immune sensors that detect pathogen associated molecular patterns (PAMPs) from a variety of microbes, as well as damage associated molecular patterns (DAMPs) from damaged cells. TLRs signal through specific adaptor molecules and subsequent downstream pathways that induce the production of mediators that initiate inflammatory responses [71]. The human genome encodes ten TLRs that each have defined roles in host defence [72].

Toll-like receptor 2 was first described in humans in the late 1990s, along with TLRs 1, 3, 4 and 5 [73]. All TLRs share a unique structure composed of an extracellular leucine-rich repeat (LRR) domain responsible for ligand recognition, a transmembrane domain, and an intracellular domain that transmits signals [74]. For ligand recognition and subsequent signalling, TLR2 requires dimerization with TLR1, TLR6 or TLR10 [74]. TLR2 is expressed on a wide variety of cells, including immune cells [75] and epithelial cells [74]. A number of microbial and synthetic products are able to activate TLR2, including lipoproteins, and cell wall components such as lipotechoic acid (LTA) [76], β-glucan [77] and zymosan [78].

1.3.2.1 Overview of TLR2 signalling

TLR2 can be functionally expressed on the extracellular surface of cells, as well as within intracellular endosomes [79]. While both pathways are dependent on the adapter molecule MyD88, and involve the formation of heterodimers with either TLR1 or TLR6, their downstream signalling cascades are distinct (reviewed in [79]).

Engagement of extracellular TLR2 can activate two pathways, which culminate in the transcription of genes mediated by the transcription factors NF-κB (nuclear factor κB) and/or AP-1 (activator protein 1). Upon recognition of an extracellular ligand, TLR2 dimerizes with either TLR1 or TLR6 (illustrated in Figure 1.2A). The intracellular toll-interleukin receptor (TIR) domain of the activated TLR2 molecule binds the TIR domain of TIRAP (toll-interleukin 1 receptor domain containing adaptor protein). TIRAP then recruits the adaptor protein MyD88 (myeloid differentiation primary response gene 88), which is central to multiple TLR signalling pathways. This complex is joined by the IRAKs (interleukin-1 receptor-associated kinases). IRAK4 phosphorylates IRAK1 and IRAK2. IRAK1 and IRAK2 are subsequently able to dissociate from the complex to activate TRAF6 (TNF receptor-associated factor 6). The ubiquitination of TRAF6 results in the sequential association and activation of TAB2 (TAK1-binding protein 2) and TAK1 (TGF-β activated kinase 1). TAK1 can then activate two different pathways: the IKK-NF-κB pathway, and the MAPK pathway. The IKK-NF-κB pathway involves the IKK (IκB kinase) enzyme complex; this consists of the catalytic subunits IKK-α, IKK-β and the regulatory unit NEMO (NF-κB essential modulator). TAK1 phosphorylates IKK-β, allowing the IKK complex to phosphorylate and ubiquitinate IκB. This leads to IκB degradation, ands allows NF-κB to translocate to the nucleus where it promotes

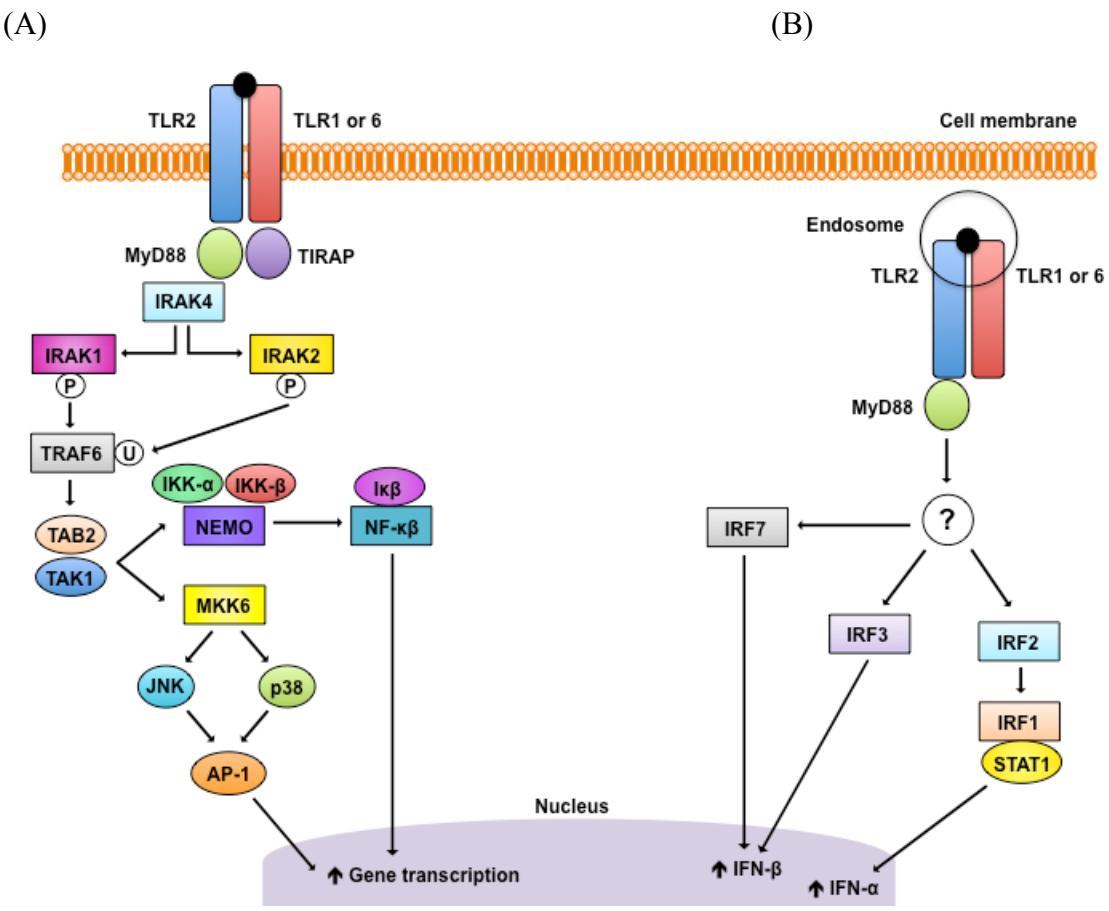


Figure 1.2 Overview of TLR2 signaling. (A) TLR2 expressed on the extracellular surface dimerizes with TLR1 or TLR6 upon ligand binding. The activated TLR2 molecule binds TIRAP, which then recruits MyD88 and subsequently, IRAK4 to the complex. IRAK4 phsophorylates IRAK1 and IRAK2, which then ubiquitinate TRAF6. This results in the recruitment and activation of TAB2 and TAK1. TAK1 can activate two pathways; firstly, TAK1 can assemble the IKK complex consisting of IKK-α, IKK-β and NEMO. The IKK complex can then phosphorylate Iκβ, thereby allowing the transcription factor NF-κB to enter the nucleus to stimulate gene transcription. Conversely, TAK1 can activate MKK6, leading to JNK/p38 activation and gene transcription by AP-1. (B) TLR2 expressed on the intracellular endosome also requires dimerization with TLR1 or TLR6 upon ligand binding. Via a MyD88-dependent, but currently unknown pathway, this results in the activation of IRF7, IRF3 and IRF2. IRF7 and IRF3 promote transcription of IFN-β. IRF2 signals through IRF1 and STAT1 to induce the transcription of IFN-α. (Modified from [79].)

transcription of downstream genes. Conversely, TAK1 can activate MKK6 (mitogen-activated protein kinase kinase 6), leading to p38 and JNK (c-Jun N-terminal kinase) activation. This triggers gene transcription activity by AP-1.

TLR2 activation can also lead to the induction of type I interferons (IFNs) (Figure 1.2B). It has been proposed that this occurs through TLR2 signaling within the endosome, which leads to the activation of IRF (interferon regulatory factor) family members. Similar to the extracellular pathway, ligand binding induces TLR2 to form a heterodimer with TLR1 or TLR6 and this leads to MyD88 activation. Although subsequent players in the signaling cascade remain to be elucidated, this leads to the ultimate activation of IRF7, IRF3 and IRF2. IRF7 and IRF3 are known to induce production of IFN-β, while IRF2 functions with STAT1 to stimulate IFN-α transcription.

1.3.2.2 Role in host defence against bacterial pathogens

TLR2 has been implicated as a major component of host defence against many bacterial pathogens. Traditionally, TLR2 is the major PRR for Gram-positive bacteria. These bacteria have cell walls composed of peptidoglycan (PGN), lipoteichoic acids and other extracellular proteins that have been identified as classical TLR2 ligands [79]. Alternatively, TLR2 has also been shown to play a role in the detection of Gram-negative bacteria, which typically have a thin PGN layer surrounded by lipopolysaccharides (LPS) [79]. Lipoproteins are produced by all bacteria, and are known to stimulate TLR2 [79].

Activation of TLR2 typically results in the transcription of inflammatory genes, including cytokines and chemokines such as TNF, IL-6, and CXCL8 (IL-8) [79]. These mediators function to initiate subsequent inflammatory responses and recruit immune

effector cells to the site of infection. However, animal models have revealed that TLR2-mediated responses may be protective during some bacterial infections, but may exacerbate others. For example, TLR2$^{-/-}$ mice are more susceptible to systemic infection with *Listeria monocytogenes* [80] and *Staphylococcus aureus* [81], exhibiting increased bacterial burdens and mortality rates compared to wildtype mice. In both of these studies, MyD88$^{-/-}$ mice were even more susceptible to disease, suggesting that there is some functional redundancy among TLRs [80, 81]. Conversely, in a model of respiratory infection with the Gram-negative bacterium *Burkholderia pseudomallei*, TLR2$^{-/-}$ mice displayed decreased bacterial burdens over the course of disease, as well as reduced lung and organ pathology [82]. Interestingly, although Gram-negative bacteria are generally thought to signal through TLR4, TLR4$^{-/-}$ mice displayed a clinical course of disease indistinguishable from wildtype in response to this pathogen [82]. According to these data, TLR2 is implicated in a range of bacterial infections. Whether TLR2-mediated immune responses are protective or pathogenic may depend on a myriad of circumstances, such as the mode of infection, the tissue site, and the bacterium itself.

1.3.2.3 Evidence for TLR2 in host defence against Chlamydia

TLR2 is widely regarded to play an important role during *Ct* genital tract infection. In this model, TLR2 has been shown to be critical for the immune responses of several cell types. For example, following *Cm* stimulation *in vitro*, macrophage activation, production of cytokines, and killing capacity all required TLR2 signalling [83, 84]. In human endocervical epithelial cells, cytokine production in response to the major outer membrane protein (MOMP), an important chlamydial antigen, was shown to be

dependent on TLR1/TLR2 heterodimers [85]. Multiple studies have described the recruitment of TLR2 and MyD88 to the chlamydial inclusion, suggesting that TLR2-dependent stimulation might occur intracellularly [86, 87]. Other suggested ligands for TLR2 detection of *Ct* include chlamydial LPS [88], and bacterial lipoproteins such as macrophage inhibitory potentiator (mip) [89]. A recent report confirmed the long-speculated presence of peptidoglycan in the *Ct* cell wall [90], which may function as an additional TLR2 ligand.

In the respiratory model of *Cm* infection, TLR2 appears to be protective, and possibly exerts anti-inflammatory effects. $TLR2^{-/-}$ mice subjected to high dose, but not sublethal dose, respiratory infections lost more body weight, although bacterial burden was comparable to wildtype [84]. This was accompanied by an increase in cytokines such as TNF, IFN-γ, and IL-17 in the lung of $TLR2^{-/-}$ mice [84]. In neonatal mice lacking TLR2, respiratory infection was prolonged compared to wildtype mice, and this was associated with persistent neutrophils, decreased IFN-γ levels and increased IL-17 in the lung [91]. Whether TLR2 is protective or pathogenic during *Chlamydia* genital tract infection is unclear. A study by Darville *et. al.* reported that TLR2 knockout (KO) mice had a similar course of infection to wildtype mice, but had reduced cytokine secretion from the genital tract and developed significantly less oviduct pathology [92]. Furthermore, plasmid-deficient strains of *Cm*, that do not stimulate TLR2-dependent cytokine production, do not induce genital tract pathology in mice [93]. A more recent study found that mice deficient in TLR2 developed hydrosalpinx as severe as those from wildtype mice, but mice deficient in TNF receptor 1 (TNFR1) had significantly reduced pathology [94].

Studies in humans have examined genetic variation in TLR2 genes and its effect on the clinical course, severity and outcomes of *Ct* infection. In a large cohort of Dutch women with or without *Ct* infection, there were no significant differences in TLR2 genotypes between the two groups [95]. However, within the group of women infected with *Ct,* one TLR2 haplotype was associated with protection against tubal pathology, and also negatively correlated with the severity of clinical disease [95]. A study comparing genotypes of women with and without tubal factor infertility did not find an association with polymorphisms in TLR2, although one TLR4 genotype was associated with an increased incidence of genital tract infections [96]. Similarly, single nucleotide polymorphisms (SNPs) in TLR2 were not associated with *Ct* infections among women with PID, while variants in TLR1 and TLR4 were [97]. Few of these studies have been reported, and they are often limited to sampling from a specific geographic region or ethnic group. Therefore, the impact of TLR2 on the manifestations of clinical *Ct* disease in humans requires further investigation.

1.3.3 Neutrophils and macrophages

The inflammatory reaction initiated by the mucosal epithelium coordinates an influx of effector cells to the submucosa, termed the immune inductive site. During early infection, the immune inductive site is populated abundantly by neutrophils and macrophages [17].

Neutrophils are short-lived phagocytic cells that are among the first to arrive to the site of infection [98]. In primary *Chlamydia* infection, neutrophils have been demonstrated to be important in gaining initial control of bacterial spread and

dissemination. Early studies revealed that human neutrophils are able to inactivate *Ct in vitro* [99, 100], and neutrophil depletion using an anti-Ly6G (Gr-1) monoclonal antibody *in vivo* leads to an increased bacterial burden during the initial phase of infection [101]. However, neutrophil infiltration that is sustained, or ascends to the upper genital tract, has been implicated in the development of oviduct pathology in mice [102-104]. It is thought that this occurs through the continued production of inflammatory mediators, such as cytokines and matrix metalloproteinases (MMP) that result in tissue damage and fibrosis [103]. This is further supported by evidence that suggests *Ct* may have an evolved mechanism to delay neutrophil apoptosis *in vitro* [102]. In humans, elevated levels of neutrophil defensins in the genital tract were strongly correlated with the development of endometritis following *Ct* infection [105]. Taken together, this evidence suggests that while neutrophils are important in facilitating early bacterial clearance, persistent neutrophils at the site of infection may contribute to chronic genital tract pathology.

During early *Ct* infection, macrophages also migrate to the genital tract submucosa [106]. At the site of infection, macrophages phagocytose bacteria [107] and are stimulated to produce pro-inflammatory cytokines, such as TNF and IL-6, through the activation of their TLRs [89]. Macrophages can be infected by *Ct*, but intracellular bacterial replication is limited [108]. Suppression of chlamydial growth inside macrophages occurs through the trafficking of bacteria to lysosomes for destruction, and the induction of host cell autophagy [108, 109]. During late stage infection of macrophages *in vitro*, *Ct* has been detected residing in double-membraned autophagosomes and co-localizing with the autophagy marker LC3 [108]. Another important role of macrophages in host defence against *Chlamydia* is to facilitate a

protective T helper response via their capacity to present antigen [110]. However, macrophages infected with *Ct* may actually facilitate T cell apoptosis, which is a potential explanation underlying the phenomenon of persistence [111]. Furthermore, a recent report described the phagocytosis of chlamydial extrusions by macrophages [112]. Within these extrusions, *Ct* was able to evade macrophage-mediated killing and eventually replicate and release [112]. Thus, although macrophages contribute to bacterial elimination and the generation of a protective immune response, *Ct* has evolved mechanisms that can render macrophages as "Trojan horses" that may contribute to persistent infection.

1.3.4 Dendritic cells

Dendritic cells (DCs) are critical antigen presenting cells (APCs) during *Ct* infection. DCs are recruited to the cervical mucosa, where they phagocytose and process antigens. DCs then migrate to the regional lymph node, where they present antigens to T cells to initiate T cell activation [98]. It is well established that DCs activate T cells during *Ct* infection by both MHC class I and class II receptors, and are also an important source of Th1 cytokines. For example, stimulation of DCs with *Cm* induces production of CXCL10 and IL-12, which function to recruit T cells and polarize them to a Th1 response, respectively [113].

DCs can be divided into several subsets that are distinguished based on their ontogeny, localization and functionality [114]. Plasmacytoid dendritic cells (pDCs) are a small subset of DCs that accumulate in the blood and lymphoid tissues. Unlike most DCs, pDCs express low levels of MHC class II and co-stimulatory molecules. When activated,

pDCs produce large amounts of type I IFN [115]. In mice, pDCs can be distinguished by the expression of markers such as B220, CD317 (also known as BST2 or PDCA-1) and Siglec-H, which are not expressed by other DC subsets [114].

The other major subset of DCs is the classical dendritic cells (cDCs). cDCs have a pivotal role in recognizing tissue injury or infection, and have a superior ability to process and present antigens to T cells [116]. cDCs are resident in both lymphoid and non-lymphoid tissues, and are differentiated primarily by their expression of CD11b, CD8, and CD103 in mice [116]. $CD11b^+$ cDCs, or "myeloid" DCs, exhibit high levels of MHC class II, and appear to have a prominent role in the induction of $CD4^+$ T cell-mediated immunity [117]. Conversely, $CD8^+$ cDCs are specialized in their ability to cross-present exogenous antigens on MHC class I molecules to prime $CD8^+$ cytotoxic T cells [118]. $CD103^+$ cDCs are considered largely analogous to $CD8^+$ DCs in their origin and function. However, $CD103^+$ cDCs tend to populate connective tissues, and are particularly enriched in the Peyer's patches of the intestine [114].

Currently, the kinetics of dendritic cell subsets and their relative contributions to *Ct* infection are not well understood. During *Ct* infection in women, both myeloid and plasmacytoid DCs have been shown to migrate to the cervical mucosa [119]. The presence of pDCs was associated with mucopurulent cervicitis and heightened inflammation in the lower genital tract [119]. In a mouse model of intravaginal *Cm* infection, cDCs (defined as $CD11c^+CD3^-CD19^-CD11b^+$) dominated the genital tract over the course of infection while both pDCs (defined as $CD11c^+CD3^-CD19^-B220^+$) and cDCs were seen to influx to the iliac lymph node [120]. Consistent with clinical data in humans, pDCs have also been implicated in genital tract pathology in mice, possibly

through the modulation of non-protective T cell responses such as regulatory T cells and $CD8^+$ T cells [121]. In the lower genital tract, $CD11b^+$ cDCs have been reported to inhibit Th1 immunity by the production of anti-inflammatory IL-10 [122].

Due to their capacity to influence the T helper response, DCs have generated much interest in the context of designing a *Chlamydia* vaccine. For example, the adoptive transfer of DCs pulsed with live *Cm* EBs has been shown to confer protection against subsequent infection [123]. Transfection of DCs with the chlamydial MOMP [124] and adoptive transfer of DCs pulsed with CPAF [125] have shown similar protection, although these approaches did not completely mitigate oviduct pathology following *Cm* challenge. Overall, DCs are essential to coordinating a protective T helper response during *Ct* genital infection. However, the specific roles of DC subsets during infection remain to be elucidated.

1.3.5 Mast cells

Mast cells are innate immune cells that populate tissues that interact with the external environment, such as the skin, the gastrointestinal tract, and the lung [126]. Mast cells arise from bone marrow progenitors, and circulate in the blood in an immature form before migrating to the tissues where they undergo maturation [126]. This maturation process critically requires the mediators stem cell factor (SCF, also known as kit-ligand) and IL-3 [126]. Mast cells tend to associate in close proximity to the vasculature, as well as the nervous system [126]. Once they have become resident in the tissue, mast cells are long-lived, and can persist for months to years [127]. Mast cell populations are primarily

replenished by immature progenitors from the blood, but in some instances have been shown to proliferate locally [128].

A distinctive feature of mast cells is their secretory granules, which can store a variety of preformed mediators [129]. These stored constituents may include histamine, cytokines, such as TNF, and proteases, such as cathepsins, chymases, and tryptases [129]. These granules can be released into the extracellular environment in response to a variety of stimuli, such as immunoglobulin E (IgE) receptor ligation or complement components, in a process known as degranulation [129]. In addition, mast cells can also produce a number of soluble mediators that are not released through degranulation. These include an array of cytokines, chemokines, and newly synthesized lipid mediators [126]. Recent reports have also described type I IFN production from mast cells, which exhibit expression patterns that are unique from tissue structural cells [130, 131].

Depending on the tissue where they reside, mast cells exhibit phenotypic and functional differences. For example, in mice, distinct mucosal and connective tissue-type mast cell populations have been referred to in the literature, based on their granule contents, abilities to degranulate in response to certain peptides, and profile of lipid mediators [126]. In humans, mast cells have been distinguished based on the protease content of their granules; they may contain tryptase only, or tryptase and chymase [126]. It has been proposed that instead of distinct subsets, there exists a spectrum of mast cell types, and these are dynamic in different contexts, such as tissue types or inflammatory conditions [126].

1.3.5.1 Allergic responses

Mast cells are perhaps best known for their traditional role in allergy. Allergic diseases are characterized by a pattern of inflammation that involves IgE activation [132]. Examples of allergic inflammation include anaphylaxis, allergic asthma, and hypersensitivity reactions of the skin [133]. Allergic inflammation develops as a result of interactions between many immune cells, and their production of inflammatory mediators [133]. Mast cell activation has been centrally implicated in the initiation of many of these processes [132].

Systemic anaphylaxis is the most severe case of allergic reaction, which is driven primarily by IgE-mediated activation of mast cells [127]. In the classic model of anaphylaxis, exposure to an allergen results in the binding of the antigen to IgE immunoglobulins. This antigen-IgE complex then binds to FcεRI receptors expressed on the surface of mast cells [127]. Cross-linking of the receptor induces mast cell activation and subsequent degranulation, leading to a substantial release of histamine, prostaglandins, cytokines, and other vasoactive substances [133]. This is followed by an influx of additional immune cells, such as $CD4^+$ T cells and eosinophils [132]. This process can manifest in patients as mild clinical signs, such as changes in the skin, or can result in a systemic, life-threatening reaction characterized by reduced blood pressure and bronchoconstriction [134].

Mast cells also play a role in allergic inflammation through a number of IgE-independent mechanisms. In response to stimuli, mast cells are able to selectively produce a number of cytokines and chemokines that help to shape the tissue microenvironment [127]. Of particular interest in allergic diseases are the cytokines IL-4,

IL-5 and IL-13, which are associated with a Th2-polarized response [132]. These mediators are able to increase vascular permeability, modulate adhesion molecules on endothelial cells, and recruit additional immune cells to the site [127]. More specifically, this cytokine milieu stimulates the proliferation and differentiation of cells such as eosinophils and B cells, thereby driving antibody-mediated responses [127]. Other cytokines that are released by mast cells, such as TNF and IL-1β, may also have a role in allergic disease by amplifying inflammatory responses [132]. Mast cell-derived leukotrienes, such as LTB_4 and LTC_4, can have powerful effects on vasodilation, bronchoconstriction, and the recruitment of immune cells such as eosinophils and neutrophils [126]. Moreover, mast cell proteases that are important in tissue remodeling processes may also contribute to epithelial tissue damage and fibrosis [132]. Overall, mast cells are key players in the initiation of allergic responses through multiple mechanisms.

1.3.5.2 Responses to bacterial pathogens

Mast cells are located in close proximity to both the external environment and the vasculature. This places them at an ideal site to detect pathogens, and expedite the recruitment of effector cells. In recent years, accumulating evidence has demonstrated that mast cells have a pivotal role in host defence against a wide variety of pathogens, including bacteria.

Mast cells can be activated during bacterial infection through several mechanisms. Firstly, mast cells can be activated by direct interactions with a pathogen, via PRRs such as TLRs [126]. Mast cells express a variety of TLRs, including TLRs 1-4

and 6-9 on murine mast cells, and all human TLRs with the exception of TLR8 [135]. Notably, some studies have been unable to detect TLR4 expression in human mast cells [136]. Depending on the bacterial ligand, mast cells are highly selective in their responses. For example, murine mast cells activated with LPS have been reported to produce TNF, IL-1β, IL-6, and IL-13 via TLR4 signaling [137]. Conversely, murine mast cells stimulated with PGN produce TNF, IL-4, IL-5, and IL-13 in a TLR2-dependent manner [137]. In one study, murine mast cells were found to degranulate in response to PGN but not LPS [137]. However, several other studies have failed to replicate this effect [138, 139]. Mast cells can also be activated indirectly by bacterial pathogens through the Fcγ receptor [126]. This can occur through the binding of bacterial antigens to pathogen-specific antibodies, or through B cell superantigens, which are pathogen-derived immunoglobulin-binding proteins [126]. This type of mast cell activation generally leads to degranulation, and the release of cytokines and lipid mediators [126]. Lastly, mast cells can be activated through receptors for various complement products, leading to cytokine production, and in some cases, degranulation [126]. When discussing mast cell activation by bacterial pathogens, it is also important to consider how mast cell responses may be shaped by the cytokine milieu in the microenvironment. For example, human mast cells pre-treated with IL-5 secreted significantly higher amounts of cytokines such as TNF, CCL3, and GM-CSF following IgE activation [140]. Therefore, "priming" with certain mediators may predispose mast cells toward enhanced or suppressed responses.

Mast cell-derived mediators are important in facilitating the recruitment of effector cells to the site of bacterial infection. One of the best examples of this is mast cell-mediated neutrophil migration. In this process, mast cells produce cytokines, such as

IL-1 and TNF, which induce vasodilation and enhance adhesion molecule expression on endothelial cells [126]. At the same time, the production of chemotactic signals, such as CXCL8 or mast cell proteases, enhances neutrophil migration from the vasculature [126]. Lastly, the production of growth factors, such as GM-CSF, enhances the survival of these effector cells in the tissues [126]. This has been illustrated in multiple animal models of bacterial infection. One example comes from a study by Huang *et. al.*, where the inoculation of human mast cell tryptase βI into the murine lung resulted in a 100-fold increase in the number of neutrophils [141]. Furthermore, when mast-cell deficient *W/Wv* mice were given βI prior to *Klebsiella pneumoniae* infection, they were less susceptible to respiratory disease [141]. In human mast cells, *Pseudomonas aeruginosa* was seen to induce IL-1α and IL-1β production, and this was required for neutrophil transendothelial migration *in vitro* [142]. It has also been suggested that mast cell mediators can directly influence neutrophil function. For example, mice with IL-6 expression ablated only in mast cells exhibited impaired bacterial clearance and increased mortality following *Kleibsiella pneumoniae* infection, and this was associated with bacterial persistence within infiltrating neutrophils [143]. These studies demonstrate the importance of both mast cell-derived factors, and subsequent neutrophil activity, in controlling bacterial infection.

Given their close proximity to the epithelium at mucosal surfaces, mast cells are also regarded to be important in the regulation of epithelial barrier function. For example, a co-culture model of the mast cell line HMC-1 with human epithelial cells revealed that, following *Pseudomonas aeruginosa* infection, mast cell-derived factors enhanced epithelial integrity and prevented apoptosis [144]. This was reflected *in vivo*, as mast cell-

deficient Wsh mice infected with *P. aeruginosa* exhibited increased epithelial permeability and bacterial dissemination in the lung [144]. In a report by Wu *et. al.*, a monolayer of T84 intestinal epithelial cells were found to transport bacterial PGN to HMC-1 mast cells *in vitro* [145]. Subsequent mast cell activation, via TLR2 and NOD2, promoted increased permeability of the epithelial monolayer, thereby compromising barrier function [145]. These findings have demonstrated an important relationship between mast cells and epithelial cells during bacterial infection. However, specific mediators that affect the mast cell-epithelial cell axis remain to be elucidated.

Mast cells can also have profound effects on adaptive immunity. One of the best-studied mechanisms of this is the ability of mast cell-derived products to influence the maturation, migration and function of DCs [146, 147]. In response to *P. aeruginosa*, mast cells produce CCL20, which may function as a chemotactic ligand for CCR6 receptors on immature DCs, thereby recruiting them to the site [148]. At the site of infection, it has recently been reported that mast cells and DCs can directly communicate through the formation of synapses [149]. It is suggested that these synapses facilitate the transfer of antigen from mast cells to DCs, which can then be presented to T cells [149]. The indirect effect of mast cell mediators in enhancing dendritic cell maturation and migration has been better established. Using Mcpt5-CreTNF mice, in which TNF is specifically deleted in mast cells, it was shown that mast cell-derived TNF provides a critical signal for the maturation and migration of $CD8^+$ DCs to the draining lymph node [150]. TNF production from mast cells also had a direct effect on the T helper response, by boosting the $CD8^+$ T cell priming abilities of $CD8^+$ DCs [150]. In the context of bacterial infection, similar effects have been seen. In a skin infection model using *Staphylococcus aureus*,

mast cell-deficient Wsh mice had impaired mobilization of plasmacytoid and $CD11b^+$ DCs into the draining lymph node [146]. This was dependent on the histamine H2 receptor, and was restored by local mast cell reconstitution [146].

Due to the actions outlined above, mast cell responses are frequently protective *in vivo*. For example, studies using *W/Wv* mice have shown that mast cell-deficient animals are unable to control bacterial dissemination during *Citrobacter rodentium* infection [151], and are not protected from *Helicobacter pylori* following vaccination [152]. In both of these instances, mast cell reconstitution rescued the disease phenotype [151, 152]. Additional studies in the improved $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (Wsh) model found that mast cell-deficient mice infected with *Mycoplasma pulmonis* had more severe bacterial burdens and clinical signs of pneumonia; however, this phenotype was not completely restored to wildtype by reconstitution [153]. Despite their protective functions, in some models of bacterial infection, mast cells have been shown to exacerbate disease and pathology. For example, during *Escherichia coli* infection of the bladder, local mast cells produced immunosuppressive IL-10, leading to tolerance and bacterial persistence [154]. In another study, mast cell-derived TNF was found to enhance bacterial growth and mortality following intraperitoneal inoculation with *Salmonella typhimurium* [155].

Overall, it is clear that mast cells have an important role during bacterial infections *in vivo*. However, due to their highly selective responses following activation, mast cell contributions to the immune response are dependent on circumstances such as the type of stimulus, the cytokine milieu in the microenvironment, and their location at tissue site.

1.3.5.3 Evidence for mast cells in host defence against Chlamydia

Currently, mast cells have only been investigated in the context of *Chlamydia pneumoniae*. *In vitro*, primary human mast cells were shown to upregulate TNF, CXCL8 (IL-8) and CCL2 (MCP-2) in response to *Cpn*, at both the mRNA and protein levels [156]. Responses to TNF and CXCL8, but not CCL2, were reduced when the bacterium was UV-inactivated [156]. IL-10 and TGF-β were also measured following activation with *Cpn*, but were not detected [156]. An additional study reported that, following incubation with *Cpn*, a portion of bone marrow-derived mast cells showed positive immunofluorescent staining for an anti-*Cpn* LPS antibody, indicating that they could be infected [157]. Infected mast cells were also reported to have altered morphology, including prominent protrusions [157]. When *Cpn*-infected mast cells were co-cultured with pancreatic beta cells, beta cells populations were reduced and had abrogated ATP and insulin production [157]. Taken together, these studies indicate that mast cells can be activated and possibly infected by *Cpn*, and their subsequent responses can impact nearby cells.

A recent study by Chiba *et. al.* found that mast cells have a detrimental effect in mice following lung infection with a human strain of *Cpn* [158]. Following infection, Wsh mice had improved survival, fewer neutrophils and macrophages in bronchoalveolar lavage fluid (BALF), and more rapid clearance of bacteria, despite cytokine and chemokine levels being comparable to WT [158]. These neutrophils and macrophages from the lungs of Wsh mice had decreased bacterial burdens compared to those from WT mice [158]. Importantly, Wsh mice had abrogated immune cell infiltration in the lung, exhibiting patches of neutrophils and lymphocytes rather than the diffuse pattern

observed in WT mice [158]. Mice that were treated with cromolyn, a mast cell stabilizer, exhibited similar characteristics to Wsh mice, while mice that were administered a mast cell degranulation inducer had exacerbated disease [158]. Reconstitution of Wsh mice with mast cells restored the WT phenotype [158]. Taken together, these observations suggested that during *Cpn* infection, mast cell degranulation recruits immune cells to the lung [158]. However, this infiltration is detrimental to the host, as *Cpn* is able to survive and replicate within these cells, thereby leading to more severe pathology [158].

Overall, these studies provide evidence that mast cells respond to *Cpn in vitro*, and suggest that they play a detrimental role during respiratory infection *in vivo*. However, further study is warranted to investigate the effect of mast cells during infection with other *Chlamydia* species at additional mucosal surfaces.

1.4 Adaptive host responses to *Chlamydia*

Innate immune responses to pathogens successively activate the adaptive arm of immunity. Adaptive immune responses are specific, and function in parallel with innate immune responses to eliminate pathogens and prevent subsequent infections. Adaptive immunity consists of two major components: cell-mediated responses and antibody responses. In the context of *Chlamydia* genital tract infection, these responses will be discussed as they pertain to the development of protective versus pathological outcomes.

1.4.1 $CD4^+$ T cell responses

$CD4^+$ T cell responses are central to protective immunity during *Chlamydia* infections. It has been well characterized that gene knockout mice lacking $CD4^+$ T cells are unable to control infection in the lower genital tract [159]. After inoculation with *Ct* in the upper genital tract, $CD4^+$ T cell responses were required for clearing primary infection in mice and were also protective against reinfection [160]. Furthermore, the adoptive transfer of $CD4^+$ T cells, but not $CD8^+$ T cells, from previously infected donors conferred resistance to *Cm* genital tract infection in naïve mice [161].

In the murine model of *Cm* infection, $CD4^+$ T cells migrate to the vaginal mucosa by day three post-infection, where they are the predominant T cell population [106]. This recruitment has been shown to depend on expression of the chemokine receptors CXCR3 and CCR5, as well as production of CCL3, CCL5, and CXCL10 from the local inflammatory site [162]. As the infection progresses, these $CD4^+$ T cells form clusters throughout the uterine tissues, where they can persist for months [106].

$CD4^+$ or T helper (Th) cell responses can be further categorized into several distinct subsets [163]. T helper 1 (Th1) responses are characterized by production of IFN-γ, and have particular importance during intracellular infections. T helper 2 (Th2) responses involve production of IL-4, IL-5, and IL-13, and occur during parasitic infections. T helper 17 (Th17) cells are associated with extracellular pathogens, and are distinguished by their production of the IL-17 family cytokines including IL-17A and IL-17F. Regulatory T cells (Treg) are generally thought to be immunosuppressive, and are associated with the production of inhibitory cytokines such as IL-10 and TGF-β [164].

In mouse models of *Cm* infection, IFN-γ-producing Th1 cells have been established to be an important component of bacterial clearance [17]. This notion is supported by a number of studies, as mice deficient in either CD4 [159, 161], MHC class II [159], IL-12 [165], IFN-γ [166] or the IFN-γ receptor [167] are all unable to control *Cm* infection. Studies have suggested that Th1 cells exert their protective effects primarily through the induction of IDO in infected epithelial cells, which is dependent on IFN-γ [165]. This results in starvation of the chlamydial inclusion within the host cell, and subsequent suppression of bacterial replication [165]. Alternatively, these $CD4^+$ T cells have been shown to induce nitric oxide (NO) in epithelial cells and exhibit some cytolytic capacity *in vitro* [168].

Genital tract infection in women elicits robust Th2 responses. In endometrial samples from 12 women infected with *Ct*, genome-wide microarray analysis revealed a number of Th2-associated factors were upregulated, including expression of matrix metalloproteinases and $CD4^+$ T cell expression of the Th2 regulator GATA-3 [169]. It has been suggested that these responses may have evolved as a means to eliminate bacterial colonization while minimizing immunopathological damage [170]. However, in murine models of genital tract infection, Th2 immunity appears to be notably absent [171]. This Th1/Th2 paradigm poses important challenges for translating experimental findings from murine to human systems.

Recently, Th17 responses have been implicated in the pathogenesis of *Ct* genital tract infection. Cervical lavages from women infected with *Ct* or gonorrhea were found to have higher levels of IL-17 than women with viral STIs, or uninfected women [172]. In murine models of *Cm* respiratory [173] and genital tract infection [174], IL-17 promotes

the recruitment of neutrophils to the site of infection. In the genital tract model specifically, IL-17 also contributes to the generation of robust Th1 immunity. This is illustrated by intravaginal *Cm* infection of IL-17 receptor A (IL-17RA) knockout mice, which were found to have decreased local IFN-γ production and Th1 responses in the draining lymph node compared to WT [174]. In the absence of IFN-γ, Th17 responses are enhanced, leading to tissue damage [171]. It has also been described that, rather than exerting an immunoregulatory response, Tregs promote Th17 responses during *Cm* genital tract infection [175]. Furthermore, Tregs were seen to convert to Th17 cells during infection, resulting in increased oviduct pathology [175]. Overall, the contribution of Th17 responses remains controversial in *Chlamydia* infection. However, many current studies support the notion that achieving a balance between T helper responses is essential to generating protective immunity while avoiding immunopathology.

1.4.2 $CD8^+$ T cell responses

Compared to the well-established protective role of $CD4^+$ T cells, the role of $CD8^+$ T cells in *Chlamydia* immunity remains contentious. During *Cm* infection, $CD8^+$ T cells are elicited and similar migration patterns are observed following infection as for $CD4^+$ T cells, although in lesser numbers [106]. $CD8^+$ T cells exert a number of effector functions that are important for eliminating intracellular pathogens, including release of antimicrobial peptides, production of cytokines and granule exocytosis leading to cytolysis [110]. *In vitro*, both human and mouse $CD8^+$ T cells are able to recognize and lyse *Ct* infected cells [176]. However, depletion of $CD8^+$ T cells does not significantly alter the course of *Cm* infection in mice [161, 177]. In fact, $CD8^{-/-}$ mice develop reduced

hydrosalpinx, which has been attributed to a lack of $CD8^+$ T cell-derived TNF production [177]. Therefore, although $CD8^+$ T cells are traditionally helpful during intracellular infections, they do not appear to provide significant protection during *Ct* genital tract infections.

1.4.3 B cell and antibody responses

A role for B cells in *Ct* infection was first proposed when levels of bacteria recovered from the endocervix of infected women were found to correlate with IgM and IgG levels in the sera [178]. In particular, IgA correlated inversely with bacterial quantification, suggesting that humoral responses might impact chlamydial shedding [178]. Early murine studies of B cells in *Cm* infection relied on mice rendered B cell-deficient through the administration of anti-IgM from birth [179]. When these mice were inoculated intravaginally with *Cm*, there were no significant differences in the course of infection compared to wildtype mice [179]. In addition, these mice remained resistant to secondary infection [179]. An alternative model of B cell-deficiency using gene knockout mice ($muMT^{-/-}$ mice) confirmed that primary infection resembles that of wildtype mice [180]. However, these mice did exhibit enhanced susceptibility to secondary infection [180]. It has since been demonstrated that resistance to reinfection may be mediated by the synergistic actions of B cells and $CD4^+$ T cells. For example, B cell-deficient $muMT^{-/-}$ mice that were also depleted of CD4 were unable to resolve secondary infection [181]. In another study, $muMT^{-/-}$ mice had significantly less local *Chlamydia*-specific $CD4^+$ T cell priming accompanied by a disseminating bacterial infection [182]. This provides a potential mechanism through which B cells contribute to protective immunity.

The precise role of chlamydial-specific antibodies in secondary infection remains unclear. A number of studies have demonstrated the ability of antibodies to bind to and neutralize *Ct in vitro* [183-185]. Indeed, the adoptive transfer of monoclonal antibodies against antigens such as the MOMP has been shown to be protective against genital tract infection [186, 187]. However, the presence of antibodies against other antigens, such as chlamydial heat shock protein 60 (HSP60), has been correlated with tubal pathology, infertility, and the development of cervical cancer in women [188, 189]. One study examined antisera from infected mice to see which of 257 *Cm* antigens were recognized [190]. Mice that developed hydrosalpinx preferentially recognized two antigens, while mice that did not develop pathology preferentially recognized ten separate antigens [190]. Taken together, these data suggest that antibody responses against specific immunodominant antigens may be protective, while others may contribute to tissue damage. This has important implications for repeated *Chlamydia* infections as well as vaccine design.

1.5 Rationale

Our understanding of *Chlamydia* infection and pathogenesis has advanced significantly in recent years, however, some aspects of the immune response remain to be elucidated. Outcomes of *Chlamydia* infection, including pathology, are the end result of a complex interplay between the pathogen, effector immune cells, and inflammatory mediators. In models of bacterial infection, mast cells have been shown to profoundly influence this dynamic through their role as sentinel cells. Importantly, mast cell-derived mediators have been shown to have direct effects on the recruitment of neutrophils, the

mobilization and function of dendritic cells, and subsequent alterations in adaptive immunity. At this time, mast cells have not been studied within the context of *Chlamydia* genital tract infections. This includes *Ct* infection in humans, as well as *Cm* infection in the relevant murine model. The presence of mast cells in the female genital tract has been established in several species, including humans and mice [191-193]. This places mast cells in an ideal location for the recognition of sexually transmitted pathogens. Previous studies on the closely related *Cpn* strain have indicated that mast cells can respond by producing cytokines *in vitro* [156, 157]. During *Cpn* respiratory infection *in vivo*, mast cells enhanced pathology by recruiting immune effector cells to the lung [158]. These studies indicate that mast cells are likely to respond to *Cm* and *Ct*, and may play an important role in pathology following genital tract infection.

TLR2 is an important pathogen recognition receptor that is central to host defence against bacterial pathogens, and the generation of immune tolerance *in vivo*. TLR2 has been investigated in the context of *Chlamydia* genital tract infections, but conflicting reports [92, 94] have contributed to a poor understanding of its functions and downstream effects. Some studies have implicated TLR2 as an important mediator of oviduct pathology during *Cm* genital tract infection in mice [92], while others have found TLR2 does not significantly alter the course or outcome of infection [94]. Thus, the role of TLR2 in *Chlamydia* genital tract infection of humans and mice remains unresolved. In many immune cells, TLR2 has been shown to be important for the release of inflammatory mediators in response to *Chlamydia* [83, 89, 92]. Since TLR2 has been shown to mediate mast cell recognition of other bacterial products [136, 194], it is plausible that TLR2 may be an important component of mast cell responses to *Chlamydia*

as well.

1.5 Objective and hypothesis

The objective of the study was to examine how mast cells and TLR2 contribute to the immune response during *Chlamydia* infection *in vitro* and *in vivo*. Based on the current literature, I hypothesize that mast cells and TLR2 have important roles in host defence and the development of pathology during *Chlamydia* infection.

CHAPTER 2 MATERIALS AND METHODS

2.1 Mice

Female mice were used between 7-16 weeks of age. C57BL/6, B6.Cg-$^{KitW\text{-}sh}$/HNihrJaeBsmGlliJ (Wsh) and B6.129-*Tlr2*^{tm1Kir}/J (TLR2$^{-/-}$) mice were bred from stocks obtained from Jackson Laboratories (Bar Harbor, ME, USA). BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA, USA). BALB/c TLR2$^{-/-}$ mice were bred "in house".

Mice were housed at the Carleton Animal Care Facility at Dalhousie University under pathogen-free conditions. For some experiments, mice were transported to the IWK Health Centre Animal Facility and housed in the quarantine facility for the duration of the study. All procedures were approved by the Dalhousie University Committee on Laboratory Animals according to the Canadian Council for Animal Care guidelines.

2.2 *Chlamydia* purification

McCoy cells (ATCC, Manassas, VA, USA) were seeded into 10-20 100 mm culture plates and allowed to grow until confluent. Cell monolayers were infected with 3 ml crude *Cm* or *Ct* supernatant to a total of 20 ml growth medium (MEM (Invitrogen, Oakville, ON), 5% FBS (Sigma-Aldrich, Oakville, ON), 10.06% glucose (BioShop, Burlington, ON), 7.5% sodium bicarbonate (BioShop), 10 mM HEPES (Wisent Bio Products, Saint-Jean-Baptiste, QC), 200 mM L-glutamine (Wisent Bio Products), 1X vitamins (Invitrogen), 50 mg/ml gentamicin (Invitrogen), 10 μg/ml cyclohexamide (Sigma Aldrich)) and incubated at 37°C for 72 hours.

Culture supernatants were collected and centrifuged in a Beckman Allegra X-12R centrifuge (1200 rpm, 10 min, 4°C) to remove debris. Supernatants were collected and centrifuged again in a Beckman J2-21 centrifuge (22 500 g, 1 hour, 4°C). Pellets were washed with sucrose phosphate glutamic acid (SPG) buffer (220 mM sucrose, 4 mM potassium phosphate monobasic (KH_2PO_4), 7 mM potassium phosphate dibasic (K_2HPO_4), 5 mM monosodium glutamate (L-glutamic acid monosodium salt hydrate), pH=7.2) and combined to produce more concentrated stocks. Pellets were resuspended in SPG buffer and aliquoted into small volumes for storage at -80°C. These batches were designated supernatant-derived *Cm* or *Ct* and utilized consistently for *in vitro* experiments.

For monolayer-derived *Cm* or *Ct* purification, 10 ml SPG buffer was added to each dish and cell monolayers were harvested using a cell scraper. Dishes were rinsed with an additional 10 ml SPG buffer. Cell suspensions were sonicated three times on ice, then centrifuged in a Beckman Allegra X-12R to remove debris (1200 rpm, 10 min, 4°C). Supernatants were collected and centrifuged in a Beckman J2-21 centrifuge (22 500 g, 1 hour, 4°C). To purify EBs, pellets were resuspended in SPG buffer and passed through a discontinuous density gradient consisting of 30% Isovue-370 (Bracco Diagnostics, Princeton, NJ, USA) in 30 mM Tris-HCl over 50% sucrose (Sigma Aldrich) in 30 mM Tris-HCl. Samples were loaded onto the two-layer cushion and centrifuged (22 500 g, 1 hour, 4°C) with slow acceleration and no brake. For *Cm* purification, pellets containing EBs were collected at the bottom of the cushion. For *Ct* purification, the purified EB layer was collected from between the Isovue solution and sucrose cushion. Pellets were washed once with SPG, centrifuged (22,500 g, 1 hour, 4°C), and resuspended in SPG

buffer. Stocks were aliquoted into small volumes for storage at -80°C. Monolayer-derived *Cm* was used consistently for *in vivo* experiments. Bacterial titres of each preparation were determined by the IFU assay and qPCR as described below.

2.3 Bacterial quantification

2.3.1 Intravaginal swab collection and processing

To assess bacterial shedding, intravaginal swabs were obtained at days 3, 7, 14, 21, 28, 35 and 42 post-inoculation with *Cm*. Immediately after collection, calcium alginate swabs (Thermo Fisher Scientific, Waltham, MA, USA) were placed in a microtube containing 300 µl SPG buffer and kept on ice. Swabs were spun with sterile glass beads at 4°C for one hour. Swabs were removed from tubes prior to freezing samples at -80°C.

2.3.2 Real-time quantitative polymerase chain reaction (RT-qPCR)

Genomic DNA was extracted from 30 µl bacterial stock, 100 µl intravaginal swab samples or 250 µl *in vitro* cultures by the addition of 500 µl DNAzol (Invitrogen or Molecular Research Center, Inc., Cincinnati, OH, USA). DNA was precipitated with 250 µl 100% ethanol. Samples were washed twice with 70% ethanol and resuspended in 100 µl 3 mM sodium chloride (NaCl). Genomic DNA was stored at -20°C until use.

The bacterial copy number in each sample was determined using *Chlamydia*-specific 16S ribosomal RNA (rRNA) primers (forward primer sequence: 5'-CGCCTGAGGAGTACACTCGC-3', reverse primer sequence: 5'-

CCAACACCTCACGGCACGAG-3') (Integrated Technologies, Inc., Montreal, QC). SYBR green master mix (Qiagen, Mississauga, ON) was combined with primers and nuclease-free H_2O according to manufacturer's instructions. Genomic DNA samples were diluted 1:10 with nuclease-free H_2O. 5 µl gDNA dilutions were added to 15 µl master mix for 20 µl reactions. No template controls (NTC) contained nuclease-free H_2O in place of gDNA template. Samples were run on a 7900H fast real-time PCR machine.

Bacterial copy numbers were calculated based on a DNA standard generated from purified *Cm* with a known titre. Copy numbers are expressed as a $\log_{10}$ value.

2.3.3 Inclusion forming unit (IFU) assay

McCoy cells were seeded into a 96-well flat bottom tissue culture plates and grown overnight until confluent. Bacteria preparations were serially diluted across cell monolayers on the plate. The plate was centrifuged (1300 g, 1 hour, 37°C) and incubated at 37°C for 40 hours. Supernatants were discarded and cells were fixed with by adding 200 µl 100% methanol to each well. Giménez staining solution was prepared by mixing 2 ml Giminéz stock (Sigma Aldrich) with 5 ml phosphate buffer (Sigma Aldrich). Fixed cells were stained with Giminéz (20 sec) and 0.8% Malachite green (Sigma Aldrich) (90 sec), rinsing the plate with tap water between stains. The resulting cytological stain allowed for the visualization of purple *Chlamydial* inclusion bodies within cells stained green. Inclusion forming units (IFU) were counted under a microscope to calculate the bacterial stock titre in IFU/ml.

2.4 *In vitro* primary cultures

2.4.1 Bone marrow-derived mast cells (BMMCs)

Bone marrow was flushed from the femur and tibia of mice using 5 ml RPMI (GE Healthcare Life Sciences, Logan, UT, USA) with 10% FBS (Sigma Aldrich) in a 25 5/8 G needle. Cell suspension was then passed through a 100 μm strainer. Cells were cultured in RPMI supplemented with 10% FBS, 1% P/S (Thermo Fisher Scientific) 50 μM 2-Mercaptoethanol (Sigma Aldrich), 200 μM PGE_2 (Sigma Aldrich) and 20% WEHI 3B cell culture supernatant (produced "in house"; cells obtained from ATCC) as a source of mouse IL-3. After 3-4 weeks, cytospins of BMMC cultures were stained with Alcian blue to determine mast cell purity. BMMCs were used when purity was determined to be >95%.

2.4.2 Cord blood-derived mast cells (CBMCs)

Cord blood was obtained from umbilical cords from consenting mothers at the IWK Health Centre, following elective cesarian section procedures. Leukocytes were isolated from cord blood using Ficoll-Pacque Plus and cultured for 5 weeks in serum-free StemSpan medium (Stem Cell Technologies) supplemented with 100 ng/ml human stem cell factor (hSCF) (Peprotech, Rocky Hill, NJ, USA), 10 ng/ml human IL-6 (hIL-6) (eBioscience, San Diego, CA, USA) and 10 ng/ml human IL-3 (hIL-3) (BioLegend, San Diego, CA, USA). After 5 weeks, cells were cultured in RPMI supplemented with 10% FBS, 1% P/S, 15 mM HEPES, 50 μM 2-Mercaptoethanol, 10 ng/ml hSCF and 10 ng/ml hIL-6. CBMC purity was analyzed by flow cytometry using anti-human CD117 antibody

(refer to Table 2.1). CBMCs were used when purity determined to be >95%.

2.5 *In vitro* activations

2.5.1 BMMC activations

Prior to activation, BMMCs were cultured overnight in the absence of PGE_2, in RPMI supplemented with 10% FBS, 10% WEHI 3B culture supernatant, 1% P/S and 50 μM 2-Mercaptoethanol. These BMMCs were washed with RPMI and resuspended in RPMI supplemented with 10% FBS, 20 mM HEPES, 100 μg/ml soybean trypsin inhibitor (Sigma Aldrich), 20 μg/ml leupeptin (Sigma Aldrich) and 3 ng/ml mouse IL-3. BMMCs were plated on a 96-well plate for a final concentration of 2 x 10^6 cells/ml. BMMCs were incubated for 24-48 hours with *Cm* (MOI=0.5, 1, 5), 50 μg/ml peptidoglycan (Sigma Aldrich), 10 μg/ml Pam_3CSK_4 (EMC Microcollections, Tubingen, Germany) or medium alone. At 24 or 48 hour time points, 5 μl P/S was added to each well and the plate was centrifuged (10 min, 300 g, 4°C). Supernatants were harvested for cytokine detection by ELISA or Luminex array.

2.5.2 CBMC activations

Prior to activation, CBMCs were cultured overnight without hIL-6, in RPMI supplemented with 10% FBS, 1% P/S, 15 mM HEPES, 50 μM 2-Mercaptoethanol and 10 ng/ml hSCF. Rested CBMCs were washed with RPMI and resuspended in RPMI supplemented with 10% FBS (Sigma Aldrich), 15 mM HEPES, 10 ng/ml hSCF and 100 μg/ml STI. CBMCs were plated on a 96-well plate for a final concentration of 1 x 10^6

cells/ml and incubated with *Ct* (MOI=0.5, 1 or 5), 50 µg/ml peptidoglycan, 10 µg/ml Pam_3CSK_4 or medium alone for 6-48 hours. At time points, 5 µl P/S was added to each well and the plate was centrifuged (10 min, 300 g, 4°C). Supernatants were harvested for cytokine detection by ELISA or Luminex array. In some experiments, cell pellets were harvested in DNAzol for genomic DNA isolation and qPCR analysis.

2.5.3 β-hexosaminidase assay

Prior to activation, CBMCs were cultured overnight without hIL-6, as described above. Rested CBMCs were washed twice with HEPES Tyrodes buffer (HTB) (137 mM Na, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM HaH_2PO_4, 1 mM HEPES, 0.1% BSA, pH=7.3) and kept strictly at 4°C until the time of activation. *Cm* (MOI=0.5, 1, 5) and A23187 Ca^{2+} ionophore (10^{-6} M) were prepared in HTB buffer pre-warmed to 37°C. CBMCs at a concentration of 0.25 x 10^6/ml were incubated with activators or medium alone for 15 minutes or 1 hour at 37°C. At time points, cells were immediately placed on ice for 5 minutes to stop degranulation. 5 µl P/S was added to each tube before pelleting cells (10 min, 300 g, 4°C). Supernatants were collected into new tubes, and both supernatant and pellet samples were stored at -80°C until the time of assay.

Cell pellets were disrupted by repeated freeze-thaw cycles, by transferring samples between a 37°C water bath and liquid N_2 at least six times. Pellets were centrifuged (30 sec, 20,000 g, 4°C). 50 µl pellet and supernatant samples were added to a 96-well plate. 50 µl HTB was added to blank wells. 50 µl 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NAG) was added to each sample and incubated for 1 hour at 37°C.

The reaction was stopped by adding 200 µl 0.1 M carbonate buffer. Absorbance (OD value) was measured at 405 nm.

Values are expressed as % degranulation, and were calculated using the following method: $(OD_{sup} - OD_{HTB}) \div [(OD_{sup} - OD_{HTB}) + (OD_{pellet} - OD_{HTB})] \times 100\%$.

2.5.4 *Ex vivo* antigen recall stimulation assay

Splenocytes from infected mice were processed as described below (refer to section 2.7) and adjusted to a concentration of 5×10^6 cells/ml. Cell suspensions were plated at 150 µl/well on a 96-well plate. Aliquots of purified *Cm* stocks were heat-inactivated at 65°C for 40 min. Heat-inactivated *Cm* stocks were diluted 1:50, 1:100 and 1:200 in RPMI 10% FBS. 50 µl bacterial dilutions or medium were added to splenocytes in triplicate wells. The plate was incubated at 37°C for 72 hours. Supernatants were collected for cytokine detection by ELISA.

2.6 *In vivo* genital tract infection model

At ten and three days prior to infection, mice were administered 2.5 mg Depo-Provera (Pfizer, Kirkland, QC) in 1X PBS via subcutaneous injection in the scruff of the neck. To prepare for genital tract infection, mice were anesthetized by intraperitoneal injection of 37.5 mg/kg ketamine (Bioniche, Lavaltrie, QC) and 7.5 mg/kg Rompun (xylazine) (Bayer Healthcare, Mississauga, ON). 5×10^5 IFU of *Cm* in SPG buffer to a total volume of 10 µl were delivered intravaginally using a P20 micropipette.

2.6.1 1X vs 5X dose model

Genital tract sections were obtained from previous experiments by Melanie Tillman for mast cell staining. At ten and three days prior to infection, mice were administered Depo-Provera as described above. Mice were anesthetized using isoflurane for genital tract infection. The 1X dose group was inoculated on day 0 with 3 x 10^5 IFU of *Cm*. The 5X dose group was inoculated on days 0, 2, 4, 6 and 8 with 6 x 10^4 IFU of *Cm* for a total of 3 x 10^5 IFU per mouse. Mice were sacrificed and organs were collected on day 30.

2.6.2 Immunization model

Genital tract sections were obtained from previous experiments by Tyler Brown for mast cell staining [195]. Four weeks prior to infection, mice were immunized intranasally with a recombinant adenovirus vector expressing CPAF. At three and ten days prior to infection, mice were administered Depo-Provera as described above. Mice were challenged intravaginally with 5 x 10^4 IFU of *Cm*.

2.7 Tissue sample processing

Following sacrifice, mouse tissue samples were placed in RPMI supplemented with 5% bovine serum (Sigma Alfrich) (5% BS/RPMI) and placed on ice until processing. For spleen cell isolation, whole spleens were mashed with frosted glass slides in a 100 mm petri dish containing 10 ml 5% BS/RPMI. Slides and dishes were each rinsed with an additional 10 ml of media. Samples were centrifuged (1200 rpm, 10 min, 4°C). Red blood cells were lysed by adding 4 ml ammonium chloride potassium (ACK)

buffer for 5 minutes exactly. The reaction was inactivated by the addition of 20 ml 5% BS/RPMI. Samples were centrifuged (1200 rpm, 10 min, 4°C) and resuspended in 20 ml RPMI supplemented with 10% FBS, 1% L-glutamine, 1% P/S and 1% HEPES (complete RPMI). Samples were pipetted through 40 μm cell strainers to remove remaining tissue debris and obtain single cell suspensions. Cell concentrations were determined by diluting samples in 0.4% Trypan blue and counting using a hemacytometer. Once cell numbers were determined, cells were resuspended to a concentration of 5 x 10^6 cells/ml.

For lymph node processing, lymph nodes were mashed with frosted glass slides in a 60 mm petri dish containing 1 ml 5% BS/RPMI. Slides and dishes were each rinsed with an additional 1 ml of media. Samples were centrifuged (1200 rpm, 10 min, 4°C) and resuspended in complete RPMI. Samples were passed through 40 μm cell strainers to obtain single cell suspensions. Cell counts were determined using 0.4% Trypan blue and a hemacytometer. Experiments where the cell yield was determined to be less than 50% of the average cell yield from the iliac lymph node were excluded from analysis.

2.8 Cytokine quantification

2.8.1 Cytokine enzyme-linked immunosorbent assay (ELISA)

Cytokine and chemokine concentrations were determined using ELISA kits (eBioscience, R&D, Peprotech) for IL-1β, IL-6, IL-10, IL-13, IL-17A, IFN-γ, MIP-1α/CCL3 and TNF. Optimal primary and secondary antibody concentrations were recommended by the manufacturer. Primary antibodies in bicarbonate ($NaHCO_3$) buffer (pH 8.3-8.5) were coated onto 96-well plates at 50 μl/well and incubated at 4°C overnight. Plates were washed three times by adding 250 μl wash buffer (PBS with 0.1%

Tween20 (BioShop)) per well and patted dry on paper towel. Wells were blocked by adding 100 µl 2% BSA (bovine serum albumin) (Sigma Aldrich) in PBS or 200 µl manufacturer's dilution buffer per well for 2 hours. Plates were washed three times, and samples or standards were added at the appropriate dilution and incubated at 4°C overnight. The following day, plates were washed three times. Secondary antibodies in 0.2% BSA or manufacturer's dilution buffer were added at 50 µl/well for 2 hours. Plates were washed three times, and 50 µl/well of either streptavidin-HRP (streptavidin-horse radish peroxidase) (eBioscience) or SAP (streptavidin-alkaline phosphatase) (Invitrogen) was added for 30 min. Plates processed using streptavidin-HRP were washed and 50 µl/well 1X TMB substrate (eBioscience) solution was added. When colour change was observed, 50 µl/well of 2 N H_2SO_4 was added to stop the reaction. Plates were read at 450 nm. Plates processed using SAP were washed and 50 µl/well substrate solution (Invitrogen) was added for 30 min, then 50 µl/well amplification solution (Invitrogen) was added. 50 µl/well of 0.2 M H_2SO_4 was added to stop the reaction when colour change was observed. Plates were read at 490 nm using Gen5 software (BioTek, Winooski, VT, USA).

2.8.2 Luminex array

Magnetic Luminex Screening Assay kits were purchased for human and mouse from R&D Systems and run according to manufacturer's instructions using provided reagents. ProcartaPlex Multiplex Immunoassays for mouse were purchased from eBioscience and run according to manufacturer's instructions using provided reagents. Plates were read on a Bio-Plex 200 system (Bio Rad, Montreal, QC).

2.9 Electron microscopy

To prepare cell samples for electron microscopy, CBMCs were washed with RPMI and resuspended in RPMI supplemented with 10% FBS, 50 mM HEPES, 10 ng/ml hSCF and 100 µg/ml STI. CBMCs were plated on a 24-well plate for a total of 4 x 10^6 cells/well. CBMCs were incubated with *C. trachomatis* (MOI=20) for 6 or 40 hours at 37°C. In parallel, McCoy cells were plated on a 6-well plate for a total of 2 x 10^6 cells/ml. McCoy cells were incubated with *Ct* (MOI=0.5) for 6 or 40 hours at 37°C. At 6 and 40 hour time points, CBMC wells were collected for individual samples while duplicate wells of McCoy cells were pooled to obtain equivalent cell numbers. Non-adherent CBMCs were collected and McCoy cell monolayers were detached with 0.5 ml TryplE (Thermo Fisher, Boston, MA, USA). Cells were centrifuged (10 min, 300 g, 4°C) to remove medium. Cell pellets were resuspended in 1 ml 2.5% glutaraldehyde in 0.1 M sodium cacodylate and stored at 4°C. Samples were further processed by technicians at the Dalhousie Faculty of Medicine Electron Microscopy Facility. Samples were visualized on a 120 kV JEOL 1230 Transmission Electron Microscope.

2.10 Flow cytometry

Cells for flow cytometry were taken from *in vitro* cultures or isolated from mouse tissues as described. 1-2 x 10^6 cells per sample were transferred to microcentrifuge tubes or a 96-well plate for staining. Samples were blocked with 20 µl 3% FBS or 50 µl 10% rat serum in PBS supplemented 0.01 M sodium azide (FACS buffer) for 20 minutes. Fluorochrome-conjugated antibodies (refer to table 2.1) were diluted in 100 µl FACS buffer or 90 µl BD Horizon Brilliant Stain buffer (BD, Mississauga, ON) when two or

more Brilliant Violet fluorochromes were used. Antibodies were added to samples for 30 minutes. In cases when fixable viability dye was used, samples were washed once with FACS buffer before eFluor506 (eBioscience) was added separately to samples for 20 minutes. Samples were washed once with 900 µl FACS buffer and an additional time with 900 µl cold 1X PBS. Samples were fixed in 1% PFA (paraformaldehyde) for at least 1 hour, then resuspended in 1X PBS for reading. Single colour controls were prepared similarly using UltraComp eBeads (eBioscience). One half-drop of UltraComp beads per sample was incubated with 100 µl fluorochrome-conjugated antibodies for 15-30 minutes. Beads were fixed in 1% PFA and resuspended in 1X PBS, in a manner identical cell samples. Fluorescence minus one controls were used for gating on positive populations. All samples were run on a BDR LSR Fortessa. Flow cytometry data was analyzed using FCS Express 4 software (De Novo Software, Glendale, CA, USA).

2.11 Histology

Genital tracts were excised and fixed in 10% buffered formalin. Histology samples were embedded in paraffin, cut into 5 µm longitudinal sections and stained with hematoxylin and eosin (H&E) by staff in the pathology department at the IWK Health Centre. Slides were examined using a Leica DFC 490 microscope and Leica Application Suite software (Leica Microsystems, Wetzlar, Germany). To measure oviduct dilation, the cross-sectional distance of the oviduct was determined in µm. The largest oviduct dilation measurement per mouse was reported.

For toluidine blue staining, 5 µm longitudinal sections of genital tracts embedded in paraffin were dewaxed by three washes in xylene (5 min), followed by one wash for 5

minutes and one wash for 2 minutes in each of 100% ethanol, 95% ethanol and 70% ethanol. Slides were briefly rinsed in water and 0.033 N HCl. Staining was performed overnight (18-20 hours) in 0.5% toluidine blue solution. Slides were rinsed again in 0.033 N HCl and water, then dried overnight. Slides were examined using an Olympus BX40CY microscope. Mast cells were distinguished based on the dark purple staining of their granules. A manual counting strategy was developed where a total of 50 fields were counted per tissue section using 100X oil immersion magnification. Fields were counted five across, five down, five across, five up, and so on, using the endometrial edge as a horizontal guide. Fields of view were excluded where stained tissue occupied less than 90% of the area. The total mast cell count per 50 fields was converted to the mast cell count per mm^2 tissue.

2.12 Statistical analyses

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad, La Jolla, CA, USA). Data sets were tested for normality using a Shapiro-Wilk test. To compare two groups, a Student's *t* test was used. In cases where the data set was not normally distributed, a Mann-Whitney test was used. For paired nonparametric analyses, a Wilcoxon signed rank test was used. To compare more than two groups, one-way analysis of variance (ANOVA) was used. In instances that multiple comparisons of the means were made, the Bonferroni method was used. All data are shown as the mean ± standard error of the mean (SEM) or standard deviation (SD). *P* values <0.05 were considered statistically significant.

Table 2.1 List of fluorochrome-conjugated antibodies used for flow cytometry.

Specificity	Clone	Manufacturer
Anti-mouse CD3 BV421	145-2C11	BD Biosciences
Anti-mouse CD3 BV711	145-2C11	BD Biosciences
Anti-mouse CD4 BV510	RM4-5	BD Biosciences
Anti-mouse CD8α BV650	53-6.7	BD Biosciences
Anti-mouse CD11b FITC	M1/70	eBioscience
Anti-mouse CD11c APC	N418	eBioscience
Anti-mouse CD19 PE-Cy7	6D5	BioLegend
Anti-mouse CD40 PE	3/23	BD Biosciences
Anti-mouse CD45R/B220 BV786	RA3-6B2	BD Biosciences
Anti-mouse CD80 FITC	16-10A1	eBioscience
Anti-mouse CD86 BV650	GL-1	BioLegend
Anti-mouse CD103 PE	2E7	eBioscience
Anti-human CD117 APC	104D2	BioLegend
Anti-human CD117 PE	104D2	StemCell Technologies
Anti-mouse CD199 (CCR9) BV421	CW-1.2	BD Biosciences
Anti-mouse CD317 (BST2/PDCA-1) PE-eFluor610	eBio927	eBioscience
Anti-mouse MHC class II AlexaFluor700	M5/114.15.2	BioLegend
Anti-mouse NK 1.1 PerCPN-Cy5.5	PK136	eBioscience
Anti-mouse Siglec-H eFluor710	eBio440c	eBioscience
Anti-mouse TCR-β APC-eFluor780	H57-597	eBioscience
Fixable viability dye eFluor506	N/A	eBioscience

CHAPTER 3 MAST CELL RESPONSES TO *CHLAMYDIA* INFECTION

3.1 Results

3.1.1 Human mast cells selectively produce pro-inflammatory cytokines and chemokines in response to *C. trachomatis.*

While previous reports suggested that human mast cells produce cytokines in response to *C. pneumoniae* [156], human mast cell responses to *C. trachomatis* (*Ct*) have not been reported. To address this, human cord blood-derived mast cells (CBMCs) were stimulated with *Ct*, the human strain of *Chlamydia* that causes sexually transmitted infections and ocular trachoma. In order to gain a full understanding of mast cell cytokine and chemokine responses, a total of 29 soluble mediators were measured in culture supernatants. Following incubation with *Ct* for 24 hours, a number of cytokines were produced by CBMCs including TNF, IL-1β, IL-6, GM-CSF, and IL-23 (Figure 3.1). Several chemokines were also produced, namely CCL3 (MIP-1α), CCL5 (RANTES), and CXCL8 (IL-8). IL-1RA, a receptor antagonist that binds to the IL-1β receptor but does not transmit a signal [196], was also significantly elevated following stimulation with *Ct.* CCL2 (MCP-1), IL-2, IL-22, and CCL11 (eotaxin) were all detected in culture supernatants, but concentrations were comparable between CBMCs activated with *Ct* and medium controls (data not shown). IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-17C, IL-17E, IL-17F, IL-18, IL-27, IL-33, IL-36 (IL-1F8), CXCL10 (IP-10), and VEGF-A were detected in a low range (<6 pg/ml) or not detected in culture supernatants (data not shown). These results indicate that human mast cells respond to *Ct* by selectively producing a number of cytokines and chemokines.

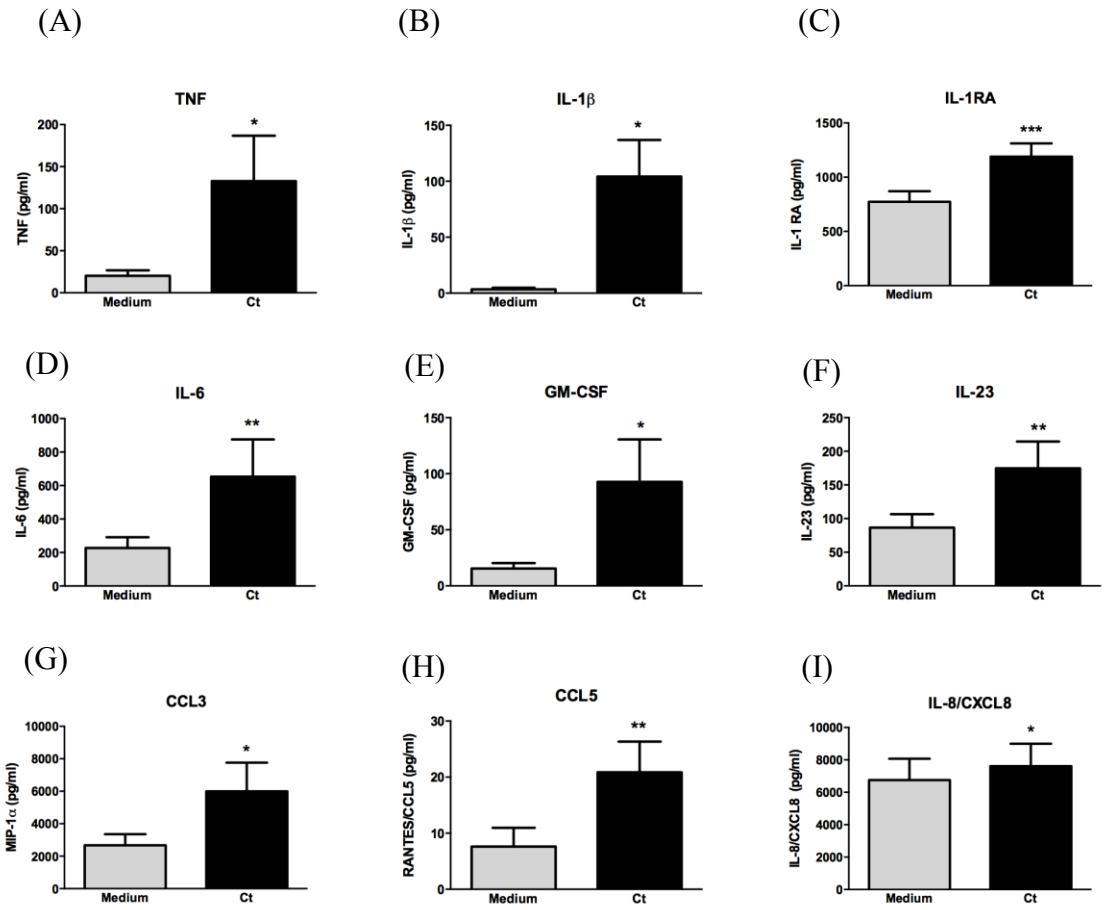


Figure 3.1 Human mast cells produce a variety of pro-inflammatory cytokines and chemokines in response to *C. trachomatis*. Human CBMCs were stimulated with *Ct* (MOI=1) or incubated with medium alone for 24 hours. Supernatants were collected and analyzed for the presence of 29 cytokines and chemokines by Luminex array. Graphs show all of the mediators that were significantly different between groups, including TNF (A), IL-1β (B), IL-1RA (C), IL-6 (D), GM-CSF (E), IL-23 (F), CCL3 (G), CCL5 (H) and IL-8/CXCL8 (I). Data represent six CBMC cultures from four independent experiments. Data are graphed as the mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined by a Student's *t* test.

Previous reports have suggested a detrimental role for type I interferon (IFN) production during *Ct* genital tract infection [197]. Because mast cells can be a good source of type I IFNs in response to viral infection [130], we investigated whether human mast cells produced type I IFNs in response to *Ct.* Following incubation for 24 hours with *Ct,* IFN-β was not elevated above baseline conditions (Figure 3.2A). Furthermore, IFN-α (B) and IFN-ω (C) were not detected in CBMC supernatants. Therefore, *Ct* stimulation does not induce type I IFN production from human mast cells.

3.1.2 Murine mast cells produce select pro-inflammatory mediators in response to *C. muridarum*.

As the mouse model of *Chlamydia* genital tract infection is widely used, we sought to compare cytokine and chemokine profiles produced by human and murine mast cells. Bone marrow-derived mast cells (BMMCs) from female mice were activated with *C. muridarum* (*Cm*), the murine strain of *Chlamydia*. Pam_3CSK_4, a TLR2 agonist, and peptidoglycan (PGN), a component of bacterial cell walls, were used as positive controls as these compounds are known to induce cytokine responses from human and murine mast cells [194, 198-200]. Culture supernatants were analyzed for production of IL-6 and CCL3, both of which were strongly produced by human CBMCs in response to *Ct*. Indeed, IL-6 and CCL3 were both detected in supernatants from BMMCs following incubation with *Cm* at all three doses for 24 hours (Figure 3.3 A, B). Both cytokines were also detected at 48 hours at similar levels (C, D). However, only CCL3 responses were statistically significant in cultures activated with *Cm* versus medium controls. These data indicate that murine mast cells also produce inflammatory mediators in response to *Cm*,

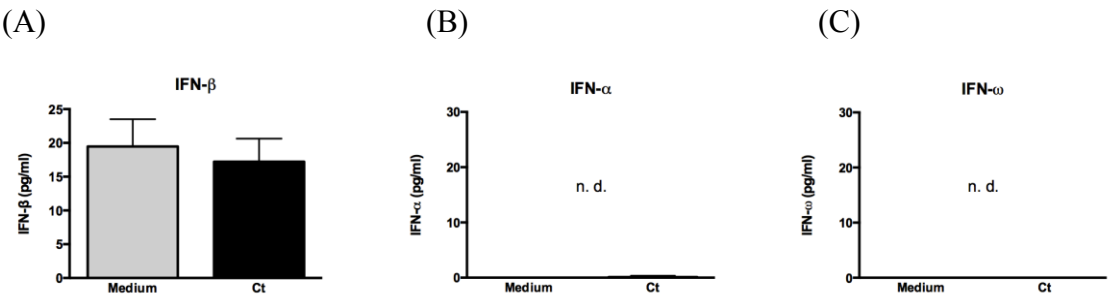


Figure 3.2 Human mast cells do not produce type I IFNs in response to *C. trachomatis.* Human CBMCs were stimulated with *Ct* (MOI=1) or incubated with medium alone for 24 hours. Supernatants were collected and analyzed for production of IFN-β (A), IFN-α (B) and IFN-ω (C) by Luminex array. Data represent seven separate CBMC cultures examined in three independent experiments. Data are graphed as the mean ± SEM (n.d.= not detected). Statistical significance was determined using a student's *t* test.

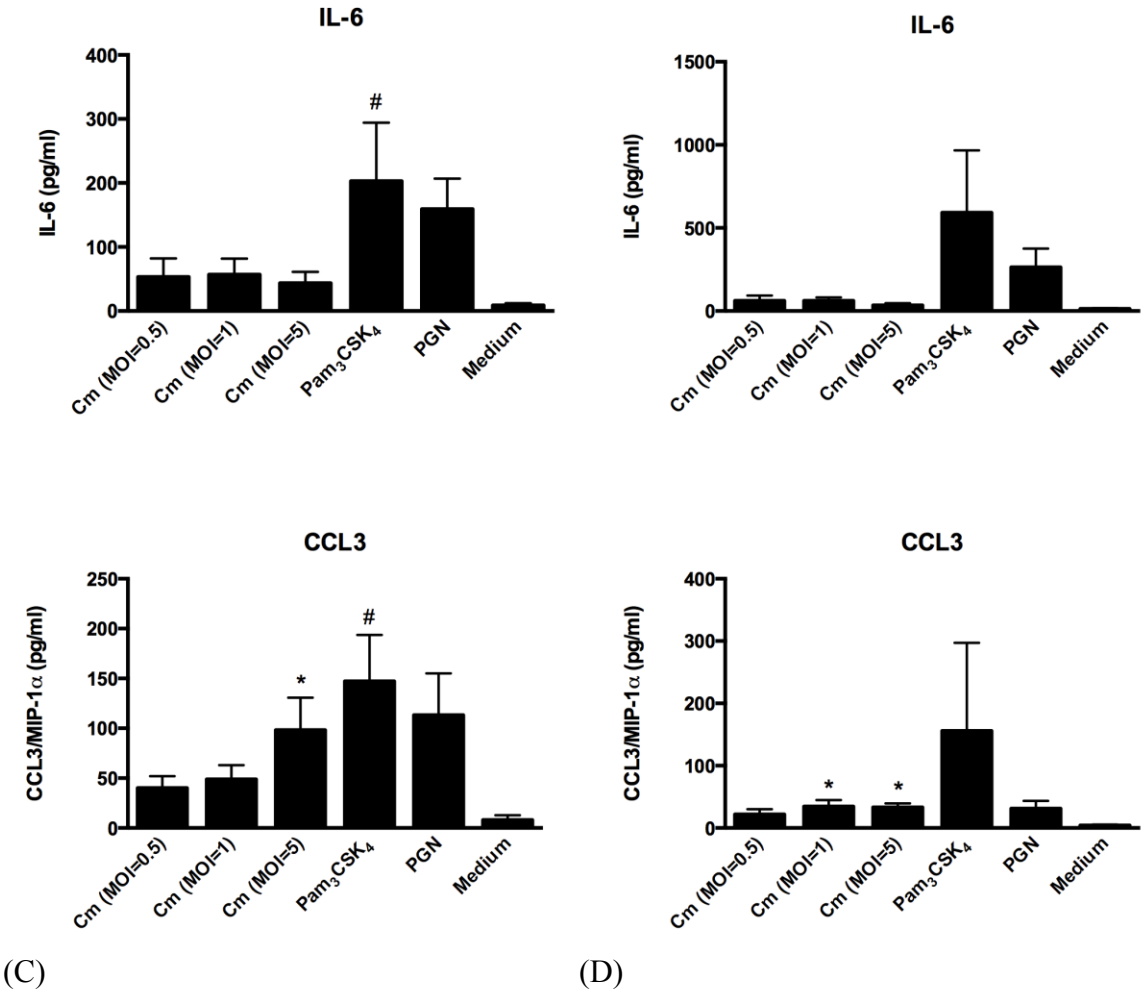


Figure 3.3 Murine mast cells produce CCL3 in response to *C. muridarum*. BMMCs from female mice were incubated with *Cm* (MOI=0.5, 1, 5), Pam_3CSK_4 (10 μg/ml), peptidoglycan (PGN) (50 μg/ml) or medium alone. Production of IL-6 is shown at 24 hours (A) and 48 hours (B). Data represent eight separate BMMC cultures at 24 hours and seven separate cultures at 48 hours. Production of CCL3 was measured at 24 hours (C) and 48 hours (D). Data represent five BMMC cultures at 24 hours and four cultures at 48 hours. Data are graphed as the mean ± SEM. Significant differences among all groups relative to medium controls were tested using one-way ANOVA (#$p<0.05$). A second post-hoc one-way ANOVA was performed excluding positive controls (Pam_3CSK_4, PGN) to determine significance of *Cm* responses relative to medium controls (*$p<0.05$).

namely CCL3. Moreover, these cytokine profiles share some common characteristics with those from human mast cells.

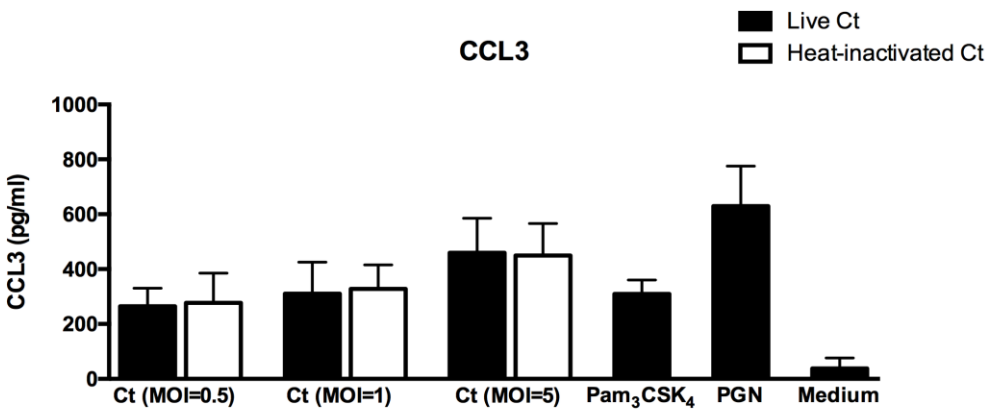
3.1.3 Live and heat-inactivated *Chlamydia* induce divergent responses in human and murine mast cells.

To determine whether *Chlamydia* EBs must be viable to induce cytokine responses from mast cells, heat-inactivated bacteria were used to stimulate BMMCs and CBMCs. Heat-inactivated and live bacteria were prepared from the same *Cm* or *Ct* stocks at an equivalent MOI (0.5, 1 or 5). CCL3 levels were measured as an indicator of cytokine production, as it was produced by both CBMCs and BMMCs in response to *Ct* or *Cm*, respectively. At 24 hours post-stimulation, supernatants from CBMCs incubated with heat-inactivated *Ct* contained similar cytokine levels to those incubated with live *Ct* (Figure 3.4A). This was consistent at all three doses of *Ct.* Conversely, BMMCs incubated with heat-inactivated *Cm* had decreased CCL3 production compared to live *Cm*. This effect was consistent across all three doses of *Cm*, and statistically significant at MOI=1 and 5. These results highlight important differences between human interactions with *Ct* versus murine interactions with *Cm*.

3.1.4 *C. trachomatis* does not induce human mast cell degranulation.

A unique feature of mast cells is their ability to store soluble mediators in granules, which can be triggered for release by a number of stimuli in a process called degranulation. β-hexosaminidase is an enzyme stored in large quantities in mast cell granules, so its release is commonly used as an indicator of mast cell degranulation [201].

(A)



(B)

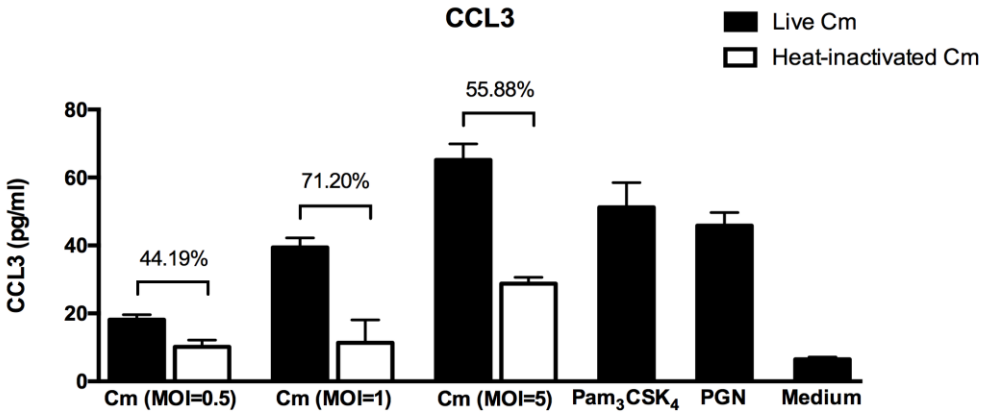


Figure 3.4 Live and heat-inactivated *C. trachomatis* induce similar cytokine responses from human mast cells, while heat-inactivated *C. muridarum* elicits decreased cytokine responses from murine mast cells. Human CBMCs were stimulated with live or heat-inactivated *Ct* (MOI=0.5, 1, 5), Pam_3CSK_4 (10 μg/ml), peptidoglycan (PGN) (50 μg/ml) or medium alone for 24 hours. Murine BMMCs were stimulated with live or heat-inactivated *Cm* (MOI=0.5, 1, 5), Pam_3CSK_4 (10 μg/ml), peptidoglycan (PGN) (50 μg/ml) or medium alone for 24 hours. Supernatants were collected and analyzed for cytokine production by ELISA. Data represent three separate CBMC cultures from two independent experiments and two BMMC cultures from one independent experiment. Data are graphed as the mean ± SEM. Numbers above bars represent the % decrease in cytokine production using heat-inactivated bacteria relative to live.

To determine whether human mast cells undergo degranulation in response to *Ct*, β-hexosaminidase release was measured following incubation of CBMCs with varying doses of *Ct*. Calcium ionophore (A23187) was used as a positive control, as it is known to induce mast cell degranulation [202]. At 15 minutes and 60 minutes post-stimulation with *Ct*, the calculated percentage degranulation values were equivalent to or below medium controls (Figure 3.5). Therefore, human mast cells do not degranulate in response to *Ct*.

3.1.5 *C. trachomatis* may be taken up, but does not replicate effectively within human mast cells.

As intracellular bacteria, *Chlamydiae* require a permissive host cell to complete their replicative life cycle. *Ct* is able to productively infect many cell types including immune cells [111, 203]; however, it is currently unknown whether *Ct* is able to infect mast cells. To address this, we first investigated the extent to which *Ct* is able to replicate within human mast cells. CBMCs were incubated with *Ct* at varying doses for 3-48 hours, which is the approximate time of the *Chlamydia* life cycle [31]. Total genomic DNA from CBMCs was analyzed to determine the bacterial load at each time point. At three hours, *Chlamydia*-specific 16S rRNA was detectable at all three doses of *Ct* (Figure 3.6). At 48 hours, bacterial copy numbers were either comparable or lower than the earlier time point. In some cases, copy numbers were not detectable above medium controls. These results were consistent among several CBMC donors. Overall, this indicates that *Ct* is not able to replicate effectively within human mast cells.

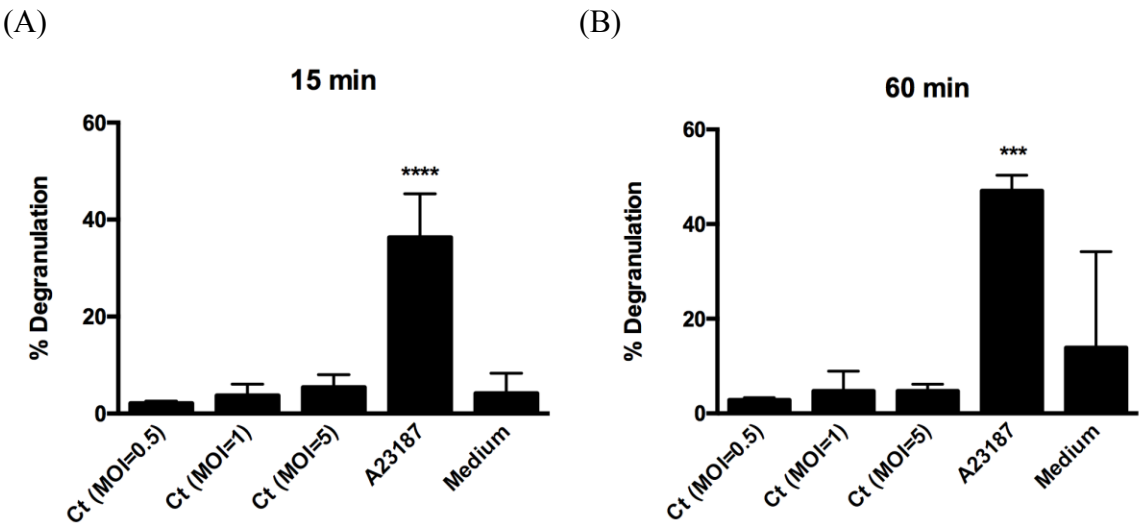


Figure 3.5 Human mast cells do not undergo degranulation following activation with *C. trachomatis*. CBMCs were incubated with *Ct* (MOI=0.5, 1 or 5), A23187 (calcium ionophore) or medium alone for 15 (A) or 60 minutes (B). At time points, culture supernatants and cell pellets were collected. β-hexosaminidase released into culture supernatants was measured relative to β-hexosaminidase retained in cell pellets. Data represent four CBMC cultures in two independent experiments. Data is presented as the mean % degranulation ± SEM. Statistical significance was determined relative to medium controls by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

(A) (B)

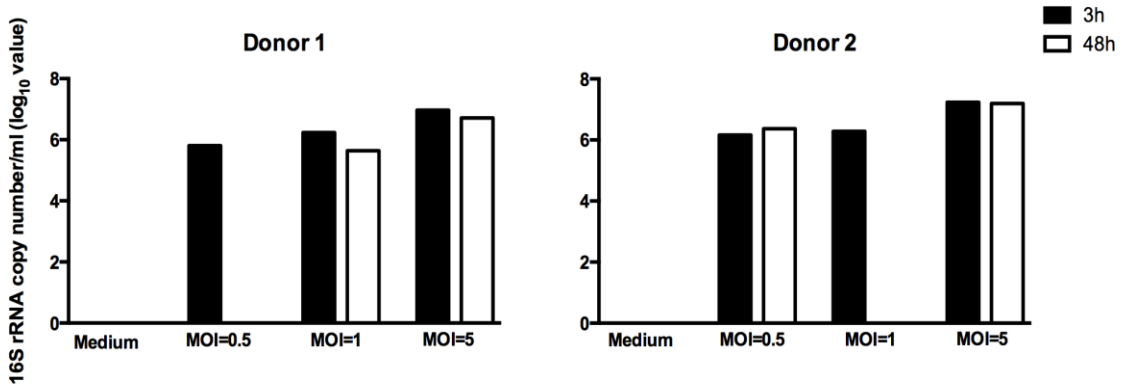


Figure 3.6 *C. trachomatis* does not replicate effectively within human mast cells. Human CBMCs were incubated with *Ct* (MOI=0.5, 1, 5) or medium alone for 3 or 48 hours. At these time points, supernatants were removed and cells were washed twice with PBS. Cell pellets from triplicate wells were disrupted by the addition of DNAzol and pooled. Genomic DNA was isolated and *Chlamydia*-specific 16S rRNA was quantified by qPCR relative to a known DNA standard. Data shown are representative of four separate CBMC cultures from two independent experiments. Each graph represents a single CBMC culture, due to variation between donors. Data are graphed as the mean 16S rRNA copy number/ml on a $\log_{10}$ scale.

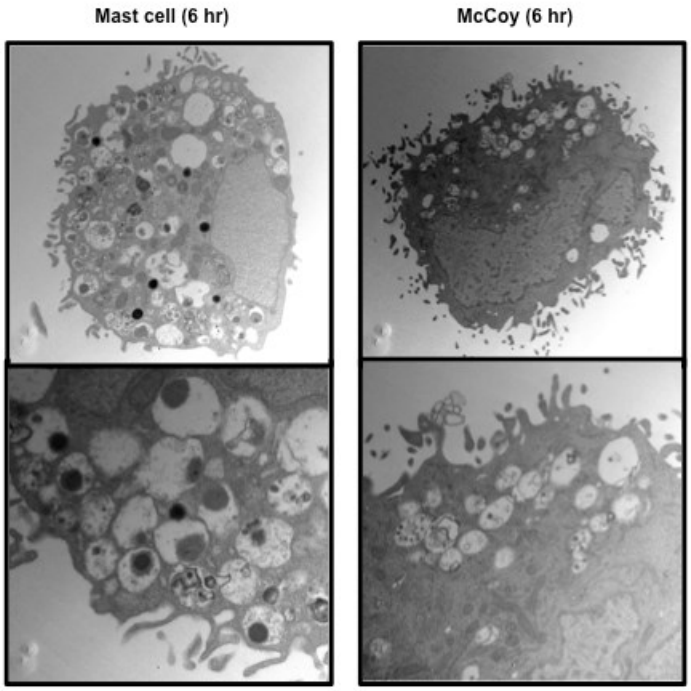
However, in the absence of bacterial replication, it remained unclear as to whether *Ct* was being taken up by mast cells, or whether bacteria perhaps remained bound to the extracellular surface.

To investigate this further, we used a transmission electron microscopy (TEM) approach that would allow us to visualize pathogen-host interactions at the cellular level. To ensure that *Ct* was present in a high enough abundance that these interactions could be adequately captured, CBMCs were incubated with a high dose of *Ct* (MOI=20) for 6 or 40 hours. These time points were chosen in order to capture early entry bacterial entry, as well as the mature inclusion prior to cytolysis if these infective processes were occurring. McCoy cells were used as a positive control to visualize inclusion bodies. These cells are used routinely for *Chlamydia* propagation, and are therefore permissive hosts for the *Ct* life cycle.

Within hours of infection, *Ct* EBs undergo attachment and entry into the host cell via endocytosis [31]. These membrane-bound compartments, deemed early inclusions, contain one or more electron-dense EBs measuring 0.2-0.4 μm in diameter [204]. Structures meeting these criteria were noted in both CBMCs (Figure 3.7A) and McCoy cells (B) at 6 hours post-infection (hpi). However, these features were only detected in <10% of CBMCs compared to >50% of McCoy cells. Furthermore, McCoy cells often presented with multiple early inclusions per cell while this was rarely seen in CBMCs.

At 40 hpi during a typical *Ct* life cycle, the mature inclusion can be distinguished by the following characteristics: (i) it is membrane bound, (ii) may contain both EBs and larger, less electron dense RBs and (iii) may occupy a large portion of the cytoplasm due to the fusion of multiple small inclusions [205]. Inclusions displaying all or some of these

(A) (B)



(C) (D)

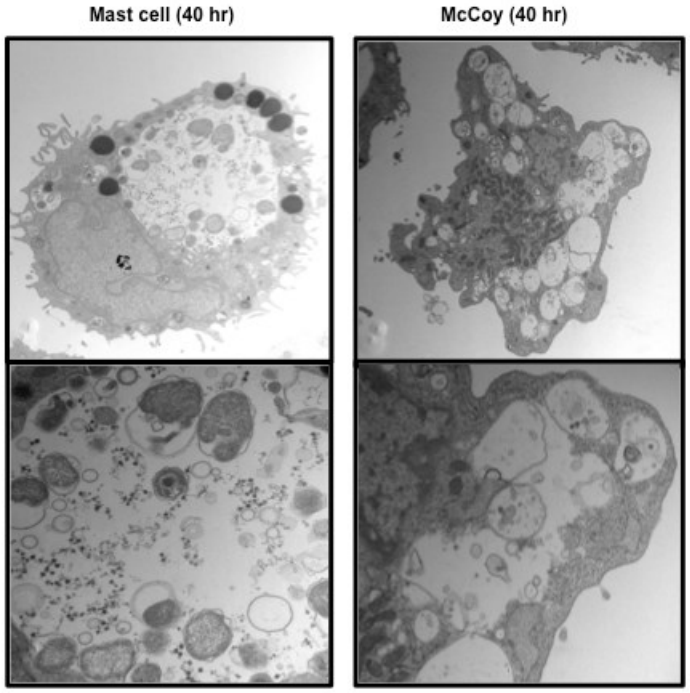


Figure 3.7 ***C. trachomatis*** **can be uptaken by human mast cells.** Human CBMCs were incubated with *Ct* (MOI=20), while McCoy cells (B) were incubated with *Ct* (MOI=0.5) in parallel. At 6 hours (A-B) and 40 hours (C-D), cell pellets were collected and fixed. *Chlamydia* inclusion bodies were visualized by transmission electron microscopy. Images are representative of duplicate samples prepared for each condition.

features were detected in CBMCs, although only <5% of cells examined exhibited these characteristics (Figure 3.7C). Meanwhile, >70% McCoy cells presented with large inclusions and about 5% of the cells examined had already burst, presumably due to host cell lysis (D).

Overall, these data indicate that *Ct* can be taken up by mast cells. However, the high dose of bacteria that was sustained and the low incidence of *Ct* inclusions observed suggest that, compared to epithelial cells, mast cells are more resistant to *Ct* infection.

3.1.6 Mast cells are present in the genital tract, and do not increase in number during *Chlamydia* infection *in vivo*.

Similar to other mucosal surfaces, it is well documented that mast cells populate the uterine and cervical tissues of the female genital tract in mice [193] and humans [206]. However, no reports currently exist describing the kinetics of mast cell populations during intravaginal *Cm* or *Ct* infection. To confirm the presence of mast cells at the site of *Cm* infection, murine genital tract sections were obtained from prior studies in the Wang lab. Tissue sections were stained with toluidine blue, which stains mast cell granules a dark purple colour. At three days post-infection with *Cm*, mast cells were detected in the uterine horns of C3H/HeJ mice (Figure 3.8). Consistent with previous reports [193], mast cells were more abundant in the endometrium, which is the innermost layer of the uterus (Figure 3.8A). Mast cells were also present in the myometrium, but to a lesser extent. Thus, we can conclude that mast cells are present in the murine female genital tract during early *Cm* infection.

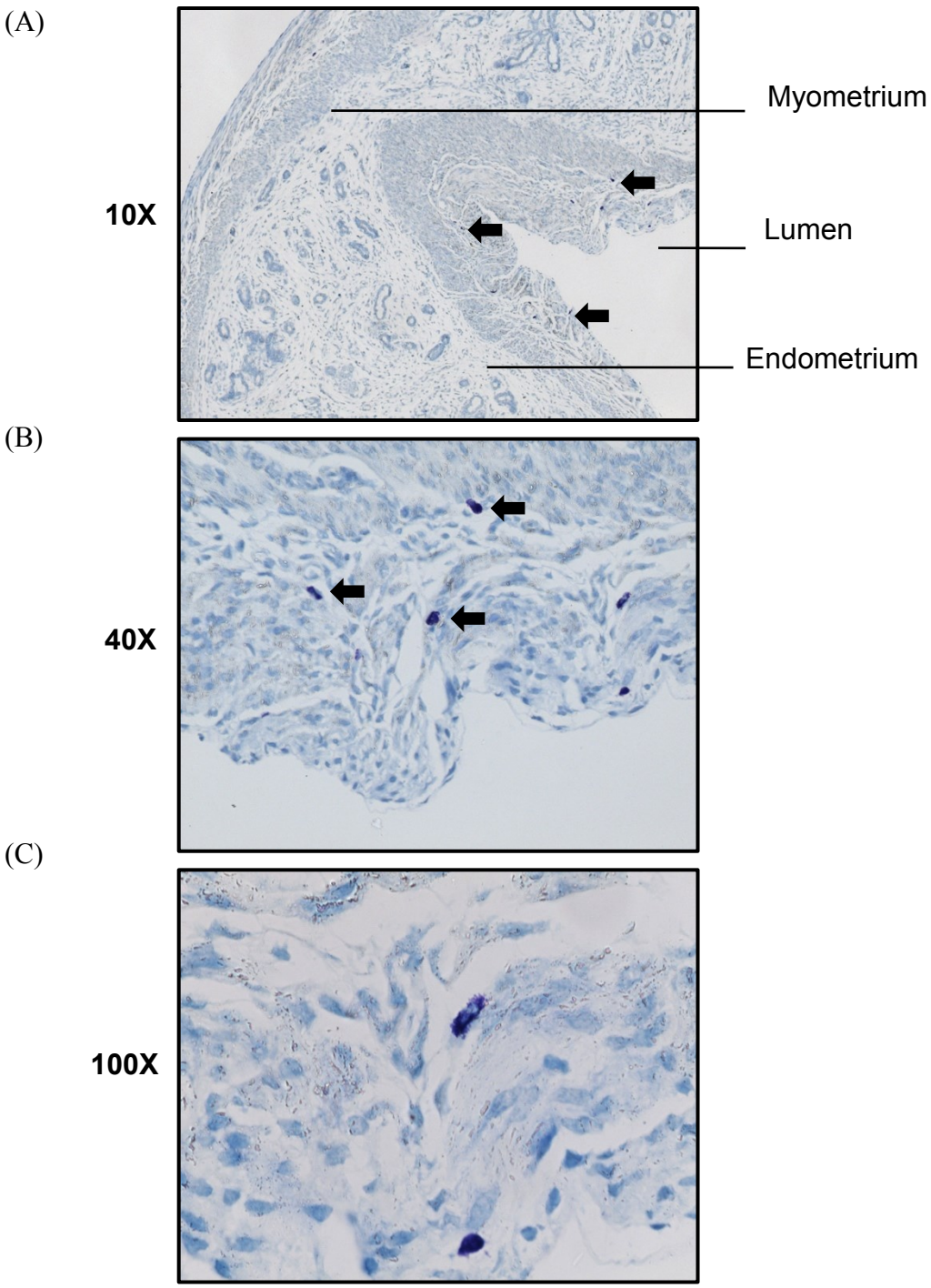


Figure 3.8 Mast cells are present in the murine female genital tract during *C. muridarum* infection. Genital tracts were obtained from female C57BL/6 mice three days post-infection with *C. muridarum*. 5 µm longitudinal sections were stained with 0.5% toluidine blue to detect mast cell granules. Mast cells are stained dark purple. Images show the endometrium and myometrium uterine layers at 10X magnification (A), 40X magnification (B) and 100X magnification (C).

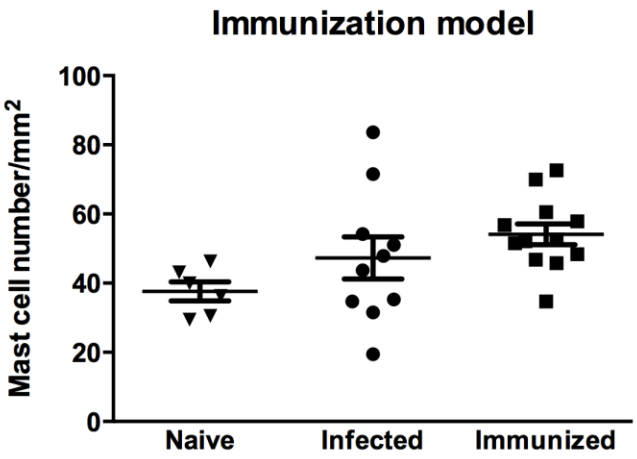
To investigate whether mast cell numbers in the murine female genital tract change during *Cm* infection, we examined genital tract sections from two different infection models. The first was an immunization model of *Cm* infection using female mice on a C3H/HeJ background. Genital tracts were excised from mice that were naïve, *Cm* infected with no prior exposure, or immunized prior to *Cm* challenge (Figure 3.9A). Mast cell numbers were not significantly different across all three groups, although infected and immunized mice did have slightly elevated numbers compared to naïve mice.

The second model we examined was a multiple dose infection model, using female mice on a C57BL/6 background. Mice designated the 1X dose group received a single dose of *Cm* intravaginally. The 5X dose group was inoculated with *Cm* once daily during the first five days of infection, for a total of five doses. Both groups received the same total IFU of *Cm*. Similar to our previous findings in the immunization model, the numbers of mast cells in the genital tracts of both groups were comparable. Taken together, we can conclude that the number of mast cells in the female genital tract does not change significantly among different modes of *Chlamydia* infection, including prior immunization and repeated exposure. Furthermore, the number of mast cells is comparable during infection and in the naive state.

3.1.7 Mast cells are associated with exacerbated oviduct pathology and the development of memory responses *in vivo*.

To investigate the pathophysiological contributions of mast cells during *Cm* genital tract infection *in vivo*, we employed a mast cell-deficient mouse model.

(A)



(B)

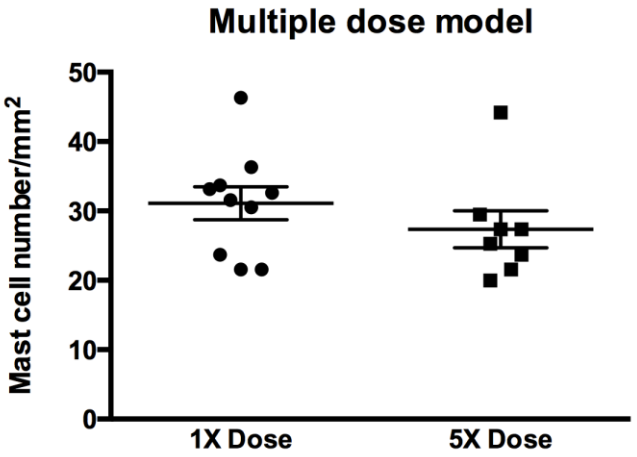
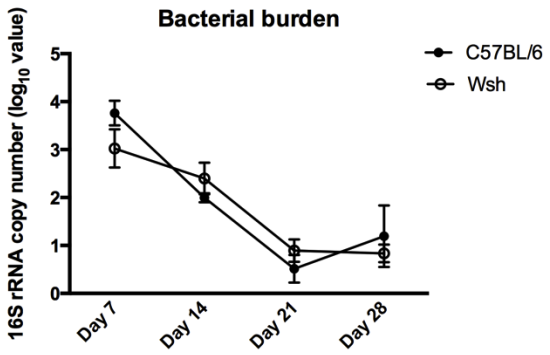


Figure 3.9 Mast cell numbers in the female genital tract do not change following *Cm* infection in immunization or multiple dose infection models. Genital tract sections were obtained from female C3H mice that were infected intravaginally with *Cm*, immunized prior to challenge with *Cm*, or mock infected (naïve) (A). Genital tract sections were obtained from female C57BL/6 mice that were infected intravaginally with *Cm* in either one dose (1X) or five doses (5X) delivered in daily intervals (B). Sections were stained with 0.5% toluidine blue to visualize mast cells. Mast cell numbers were counted per field of view for a total of 50 fields. Mast cell counts are shown as the average number per mm^2 of tissue. Data points represent mast cell counts for each mouse, and are graphed as the mean ± SEM. Statistical significance was tested for using a one-way ANOVA for the three groups in the immunization model and a student's *t* test for the two groups in the multiple dose model ($p<0.05$).

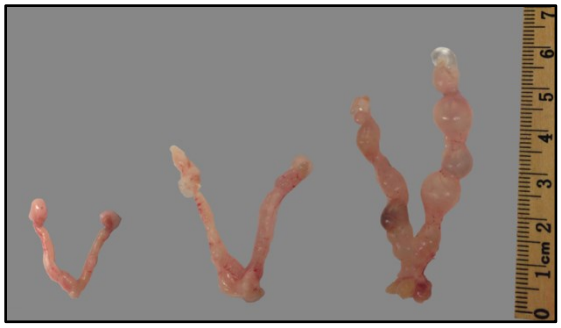
Wsh mice, also known as c-Kit$^{W\text{-}sh}$ or sash mice, have a spontaneous *Kit* mutation that impairs the development of mature mast cells [207]. Female Wsh and wildtype C57BL/6 mice were inoculated intravaginally with *Cm* for 50 days. Over the course of infection, bacterial shedding was comparable, and both groups had mostly cleared the infection by day 28 (Figure 3.10A). Upon sacrifice at day 50, striking changes in gross pathology were observed. Genital tracts from wildtype mice showed severe inflammation and incidence of hydrosalpinx (Figure 3.10B). In contrast, genital tracts from Wsh mice exhibited only occasional hydrosalpinx, and appeared less severely inflamed. At the histological level, Wsh mice had significantly less oviduct dilation compared to wildtype mice (Figure 3.10 C, D). Overall, this indicated that mast cell-deficiency is associated with protection from *Cm* pathology. Thus, the presence of mast cells is associated with exacerbated oviduct pathology.

To compare the memory responses between wildtype and Wsh mice, an antigen recall assay was performed *ex vivo*. Upon restimulation with heat-inactivated *Cm*, splenocytes from Wsh mice produced strikingly lower amounts of IFN-γ, IL-10, IL-17A, and IL-13 compared to wildtype (Figure 3.11). Levels of TNF were comparable between the two groups. These data indicated that Wsh mice had suppressed memory responses, spanning cytokines representative of Th1, Th2, Th17 and immunoregulatory responses. Overall, this demonstrates that mast cells are associated with development of robust memory responses following *Cm* infection *in vivo*.

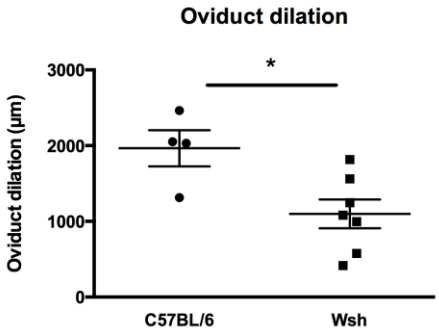
(A)


Bacterial burden
16S rRNA copy number (log10 value)
C57BL/6
Wsh
Day 7
Day 14
Day 21
Day 28

(B)



(C)


Oviduct dilation
Oviduct dilation (µm)
*
C57BL/6
Wsh

(D)

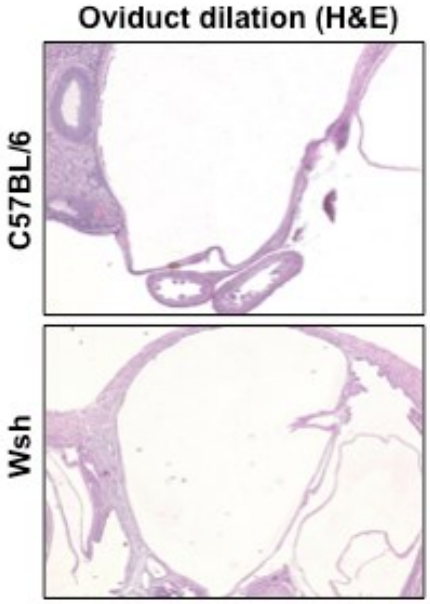

Oviduct dilation (H&E)
C57BL/6
Wsh

Figure 3.10 Mast cells do not contribute to bacterial clearance, but are associated with exacerbated oviduct pathology following *C. muridarum* genital tract infection *in vivo*. Female C57BL/6 and Wsh mice were infected intravaginally with *Cm* for 50 days. Over the course of infection, intravaginal swabs were taken to monitor bacterial shedding by qPCR (A). Data is presented as the average *Chlamydia*-specific 16S rRNA copy number expressed as a $\log_{10}$ value ± SEM. Upon sacrifice, gross genital tract pathology was observed (B). The most severe pathological phenotype per group is shown. Longitudinal genital tract sections were stained with hematoxylin and eosin (H&E) and oviduct dilation was measured for each mouse. The greatest measurement per mouse (μm) is graphed as the mean ± SEM (C). Representative oviducts from the mouse closest to the mean oviduct dilation value for each group are shown (D). Statistical significance was tested for using a student's *t* test. All data represent n=4 C57BL/6 and n=7 Wsh mice.

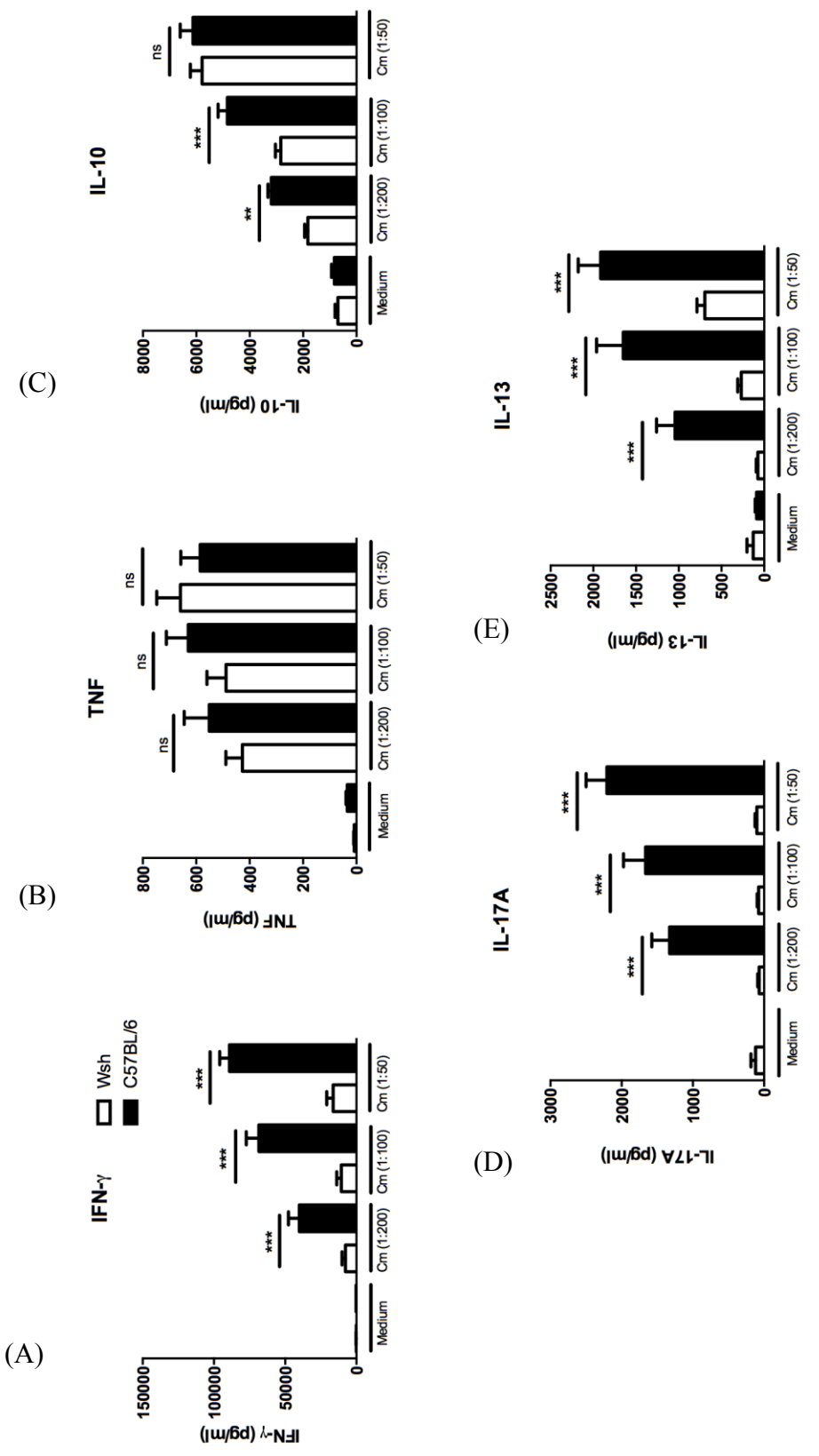

(A)
IFN-γ
Wsh
C57BL/6
IFN-γ (pg/ml)
150000
100000
50000
0
***
***
***
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(B)
TNF
TNF (pg/ml)
800
600
400
200
0
ns
ns
ns
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(C)
IL-10
IL-10 (pg/ml)
8000
6000
4000
2000
0
**
***
ns
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(D)
IL-17A
IL-17A (pg/ml)
3000
2000
1000
0
***
***
***
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(E)
IL-13
IL-13 (pg/ml)
2500
2000
1500
1000
500
0
***
***
***
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)

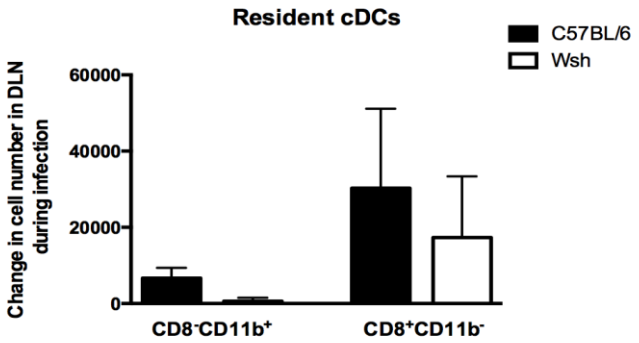
Figure 3.11 Mast cells contribute to the development of memory responses during *Cm* genital tract infection *in vivo*. Female C57BL/6 and Wsh mice were infected intravaginally with *Cm* for 50 days. Upon sacrifice, splenocytes were isolated and restimulated with heat-inactivated *Cm* at dilutions of 1:50, 1:100 or 1:200, or incubated with medium for 72 hours. Supernatants were harvested and analyzed for production of IFN-γ (A), TNF (B), IL-10 (C), IL-17A (D) and IL-13 (E) by ELISA. All data represent n=4 C57BL/6 and n=7 Wsh mice. Data is graphed as the mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined by one-way ANOVA (ns = not significant).

3.1.8 Mast cell products mediate mobilization of dendritic cells to the draining lymph node during *Chlamydia* infection *in vivo*.

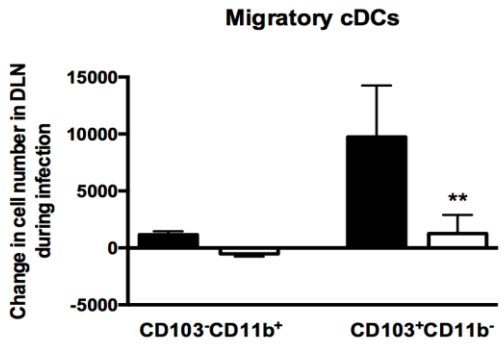
In other models of Gram-negative bacterial infection, mast cells have been shown to release products that result in the mobilization of dendritic cells to the draining lymph node (DLN) [146, 208]. Given the markedly decreased memory responses in Wsh mice, we sought to investigate whether mast cells were having an effect on dendritic cells during early infection. To examine this, female Wsh and wildtype C57BL/6 mice were infected with *Cm* or mock infected with SPG buffer for three days. At this time, the iliac lymph node (ILN) was analyzed for the presence of several dendritic cell subsets. Interestingly, Wsh mice had decreased infiltration of dendritic cells into the draining lymph node compared to wildtype. Though not statistically significant, Wsh mice had less $CD11b^+$ and $CD8^+$ lymphoid tissue resident conventional dendritic cells (cDCs) in the ILN during infection (Figure 3.12A). Moreover, the number of $CD11b^+$ non-lymphoid cDCs in the ILN decreased during infection in Wsh mice (B). The most striking difference was in the migratory $CD103^+$ population, was which was significantly diminished in Wsh mice (Figure 3.12B). Plasmacytoid dendritic cell (pDC) populations were mostly comparable between groups (Figure 3.12C).

These data, taken together, indicate that mast cell-deficiency is associated with a reduced influx of dendritic cells to the iliac lymph node during *Cm* genital tract infection. Therefore, this suggests that mast cells have a pivotal role in mobilizing dendritic cells to the lymph node during early infection.

(A)


Resident cDCs
C57BL/6
Wsh
Change in cell number in DLN during infection
60000
40000
20000
0
CD8-CD11b+
CD8+CD11b-

(B)


Migratory cDCs
Change in cell number in DLN during infection
15000
10000
5000
0
-5000
**
CD103-CD11b+
CD103+CD11b-

(C)

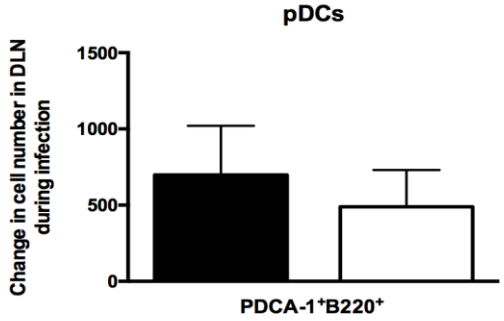

pDCs
Change in cell number in DLN during infection
1500
1000
500
0
PDCA-1+B220+

Figure 3.12 Mast cells mediate mobilization of dendritic cell subsets to the draining lymph node during early *C. muridarum* infection. Female C57BL/6 and Wsh mice were infected intravaginally with *Cm* or mock infected for three days. Upon sacrifice, the iliac lymph nodes were excised and stained for flow cytometric analysis of DC subsets. DCs were defined as live TCR-β^-CD19$^-$CD11c$^+$ cells. Resident cDCs were characterized by expression of CD11b or CD8 (A), while migratory cDCs were characterized by expression of CD11b or CD8 (B). Plasmacytoid DCs were defined as DCs that were PDCA-1$^+$B220$^+$ (C). The number of each cell type present in mock-infected controls was defined as baseline. Mock infected mice were included in each experiment as parallel controls. The change in cell number was calculated by subtracting baseline values from the cell number obtained in each infected mouse. Graphs show the mean change in cell number in the draining lymph node during infection ± SEM. Data represents n=4 infected mice and n=2 mock infected mice for each strain, within two independent experiments. Statistical significance was determined using a Student's *t* test and is shown relative to wildtype. * $p<0.05$, ** $p<0.01$.

3.2 Conclusions

Overall, we have demonstrated a novel role for mast cells in *Chlamydia* infection of the genital tract. We have shown that both human and murine mast cells respond to *Chlamydia in vitro* by selectively producing a number of pro-inflammatory cytokines and chemokines. It appears that *C. trachomatis* is able to infect human mast cells, but does not replicate efficiently within them. Studies in a mast cell-deficient mouse model revealed that mast cells are required to mobilize dendritic cells to the draining lymph node during early infection, and this is succeeded by the development of a robust memory response. During late infection, mast cells are associated with the development of oviduct pathology. Thus, in *Chlamydia* genital tract infection, mast cells appear to have a unique role that impacts both acute infection and the development of long-term pathology.

CHAPTER 4 THE ROLE OF TOLL-LIKE RECEPTOR 2 IN HOST DEFENCE AGAINST *CHLAMYDIA* INFECTION

4.1 Results

4.1.1 Murine mast cells produce pro-inflammatory cytokines and chemokines in response to *C. muridarum* in a TLR2-dependent manner.

TLR2 has been shown to be important for host defence in many types of bacterial infection, including respiratory *Chlamydia* infections [84]. To investigate the role of TLR2 in mast cell responses to *Chlamydia*, we generated bone marrow-derived mast cells (BMMCs) from female wildtype (WT) and TLR2$^{-/-}$ mice. BMMCs were incubated with *Cm* or medium alone for 24 hours, and supernatants were analyzed for the presence of 19 cytokines and chemokines through a Luminex assay. In culture supernatants from WT BMMCs stimulated with *Cm*, elevated levels of CXCL1 (KC) (Figure 4.1A), CXCL2 (MIP-2) (B), CCL2 (MCP-1) (C), CCL3 (MIP-1α) (D), IL-6 (E), and TSLP (F) were detected, compared to medium controls. However, these data lacked statistical significance due to inconsistent responses. Notably, two WT cultures produced significant amounts of CXCL1 in response to *Ct*, as we observed a 16-fold and 34-fold increase in production. Similarly, these two cultures had a 10-fold increase of CXCL2 in response to *Ct.* Five out of eight cultures had an increase of 4-fold or greater in levels of CCL3. Conversely, supernatants from TLR2$^{-/-}$ BMMCs stimulated with *Ct* had concentrations of CXCL1, CXCL2, CCL2, CCL3, IL-6, and TSLP that were close to baseline or undetectable. Both WT and TLR2$^{-/-}$ BMMCs produced statistically significant amounts of IL-12p70 following *Cm* stimulation, as well as moderate but not significant

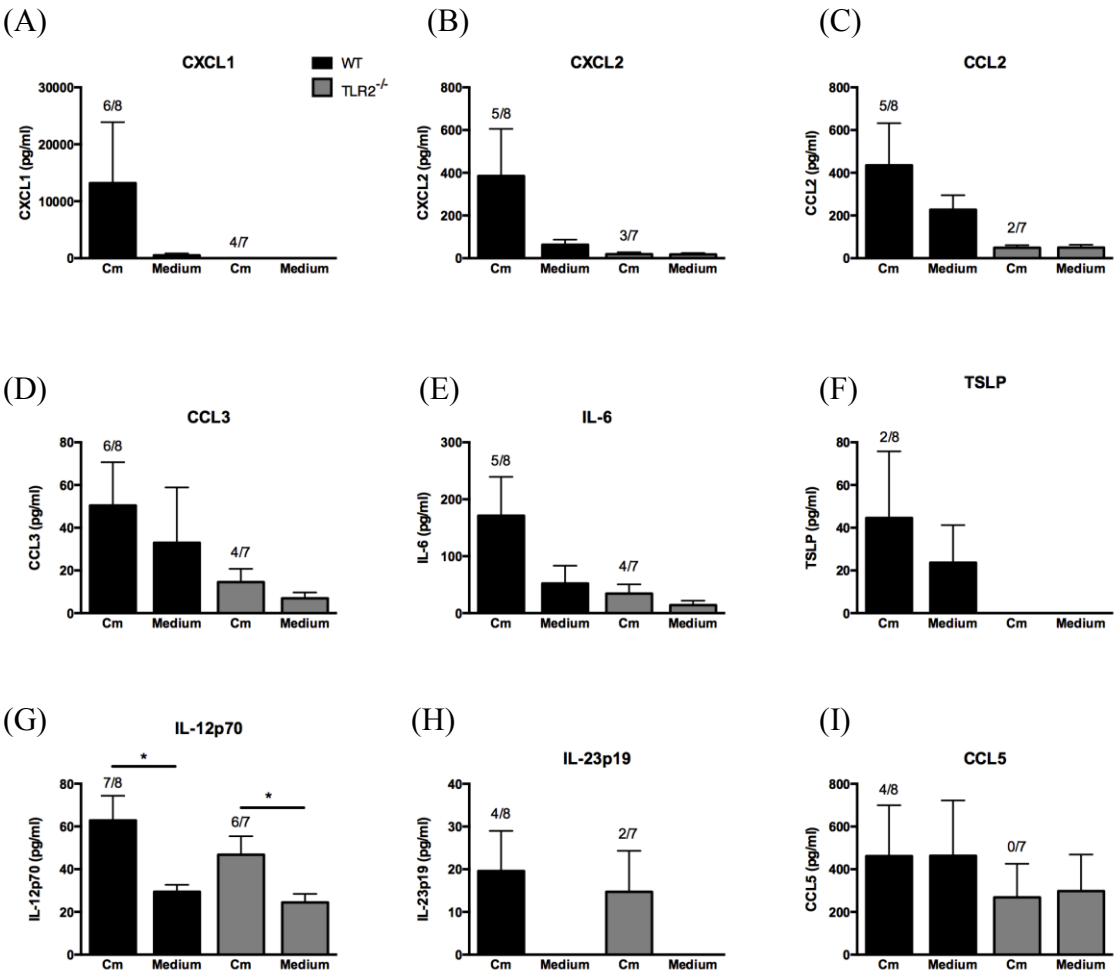


Figure 4.1 Murine mast cells produce cytokines and chemokines in response to *C. muridarum*, and this is partially TLR2-dependent. BMMCs from wildtype and TLR2$^{-/-}$ mice were incubated with *Cm* (MOI=1) or medium alone for 24 hours. Supernatants were harvested and analyzed for 19 cytokines and chemokines by a Luminex multiplex assay. BMMC production of CXCL1 (A), CXCL2 (B), CCL2 (C), CCL3 (D), IL-6 (E), TSLP (F), IL-12p70 (G), IL-23p19 (H) and TNF (I) is shown. Data is graphed as the mean ± SEM and represents n=8 WT BMMC cultures and n=7 TLR2$^{-/-}$ BMMC cultures. Cultures activated with *Cm* were paired with medium controls within independent experiments. Numbers above bars denote the number of cultures that showed >10% increase in *Cm* cultures compared to parallel medium controls. Statistical analyses of *Cm* versus medium controls were performed using a Wilcoxon signed rank test (*$p<0.05$).

levels of IL-23p19. Levels of CCL5 were higher in WT BMMCs, but were not increased by *Cm* stimulation (G). TNF, IL-4, IL-10, and IL-13 were detected in all groups at low levels, but did not show differences between conditions (data not shown). IL-1β, IL-5, IL-15, IL-17A, IL-18, IL-22, IL-25/IL-17E, IL-33, GM-CSF, and CXCL10 (IP-10) were not detected in culture supernatants (data not shown).

In order to illustrate dependence on TLR2, these data were analyzed as the increase in cytokine/chemokine production following stimulation with *Cm* for both WT and TLR2$^{-/-}$ BMMCs (Figure 4.2). In the cases of CXCL1 (A), CXCL2 (B), CCL2 (C), CCL3 (D), IL-6 (E), and TSLP (F), the increase in protein production following stimulation with *Cm* was much higher in WT BMMCs, although this data lacked statistical significance due to variation between cultures. For IL-12p70 and IL-23p19, the increase in protein production following *Cm* stimulation was comparable amongst WT and TLR2$^{-/-}$ cultures.

Taken together, these data indicate that BMMCs selectively produce a number of cytokines and chemokines in response to *Cm*. Furthermore, this process appears to be at least partially dependent on the presence of TLR2.

4.1.2 TLR2 contributes to bacterial clearance, oviduct pathology and the development of memory responses following *Chlamydia* infection *in vivo*

Given that mast cells play a significant role in the development of oviduct pathology as well as memory responses *in vivo*, and many mast cell-derived mediators were seen to be TLR2-dependent, we wanted to further investigate the role of TLR2 in *Cm* infection *in vivo*. To date, TLR2 in the context of *Cm* genital tract infection has only

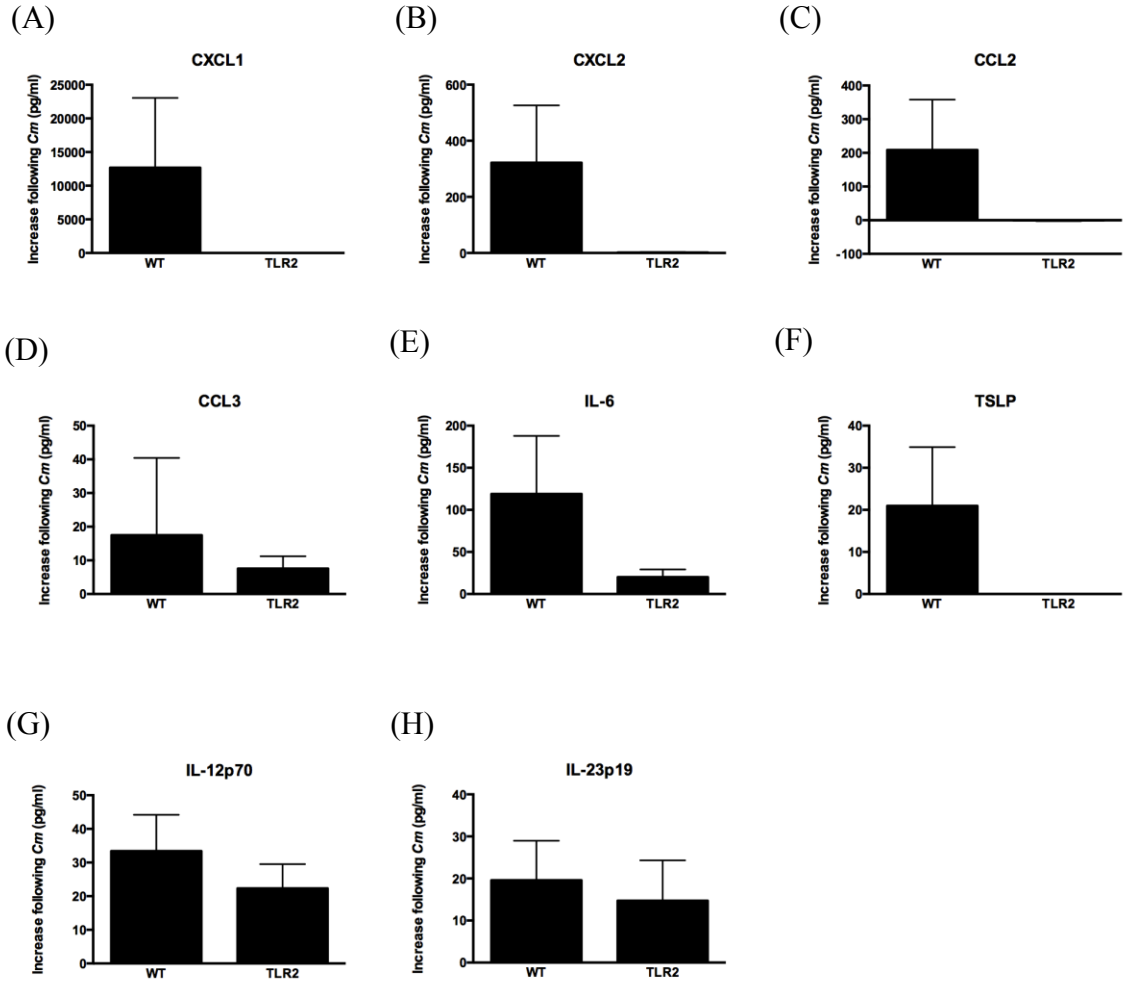


Figure 4.2 Murine mast cell production of cytokines and chemokines in response to *C. muridarum* is partially dependent on TLR2. WT or TLR2$^{-/-}$ BMMCs were incubated with *Cm* (MOI=1) or medium alone for 24 hours. Cytokine and chemokine production was measured by Luminex as in Figure 4.1. Graphs depict the increase in CXCL1 (A), CXCL2 (B), CCL2 (C), CCL3 (D), IL-6 (E), TSLP (F), IL-12p70 (G) and TNF (H) measured in supernatants from cultures activated with *Cm* relative to medium controls. Data represents the mean ± SEM. Statistical analysis was performed using a Mann-Whitney test (A-D, F, H) or a Student's *t* test in cases where data sets passed a Shapiro-Wilk normality test (E, G) (*$p<0.05$).

been investigated in a C57BL/6 background model. To confirm previous reports and extend our findings to an additional mouse strain, we investigated TLR2 deficiency in C57BL/6 and BALB/c mice infected with *Cm* for 50 days.

4.1.2.1 C57BL/6 background model

Female C57BL/6 wildtype and TLR2$^{-/-}$ mice were infected intravaginally with *Cm* for 50 days. Bacterial shedding was similar during early infection, with comparable bacterial burden measurements obtained on days 3 and 7 post-infection (Figure 4.3A). However, during days 14-21, TLR2$^{-/-}$ mice had significantly elevated bacterial loads compared to wildtype. This was resolved by day 28, when most mice in both groups had effectively cleared the infection. Upon sacrifice, it was observed that individuals from both groups had developed hydrosalpinx at a comparable frequency (Figure 4.3B). However, despite an elevated bacterial burden during infection and similar incidence of hydrosalpinx following infection, TLR2$^{-/-}$ mice had remarkably less oviduct dilation than their wildtype counterparts (Figure 4.3 C, D). Taken together, these data suggest that TLR2 may participate in bacterial clearance as well as the development of long-term oviduct pathology.

To characterize the memory response in wildtype and TLR2$^{-/-}$ mice, splenocytes from infected mice were restimulated with heat-inactivated *Cm*. Cells from TLR2$^{-/-}$ mice produced less IFN-γ, IL-13, and IL-17A compared to wildtype (Figure 4.4), although this was not statistically significant. IL-10 production was comparable between both groups. IFN-γ, IL-13, and IL-17A are representative of Th1, Th2, and Th17 responses, respectively. Therefore, these data suggest that TLR2$^{-/-}$ mice develop memory responses

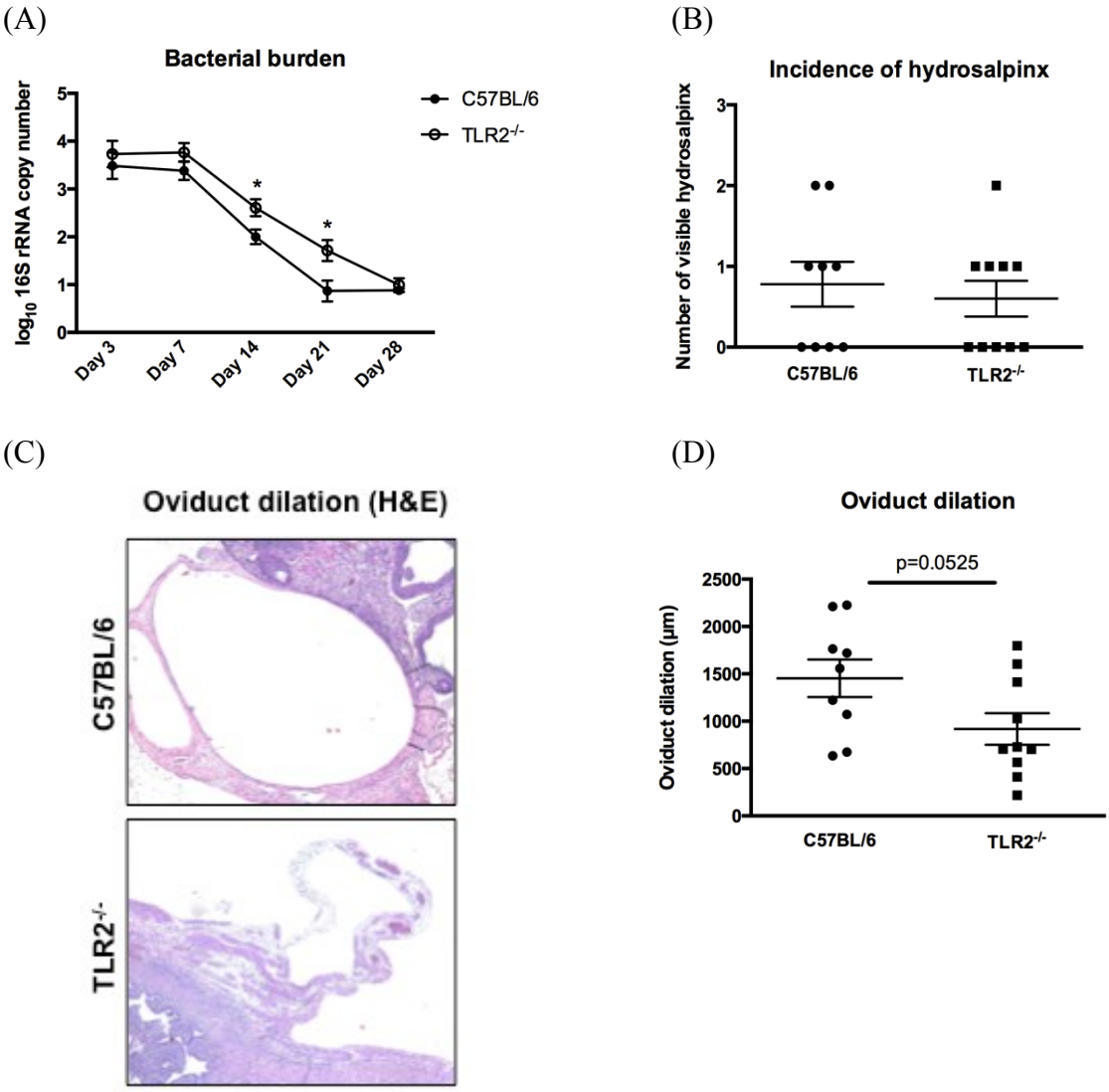


Figure 4.3 TLR2 contributes to bacterial clearance and oviduct pathology during *C. muridarum* infection in a C57BL/6 background genital infection model. Female C57BL/6 wildtype and TLR2$^{-/-}$ mice were infected intravaginally with *Cm* for 50 days. Intravaginal swabs were taken to monitor bacterial shedding by qPCR (A). Data is presented as the average *Chlamydia*-specific 16S rRNA copy number expressed as a $\log_{10}$ value ± SEM. At the time of excision, the incidence of hydrosalpinx was counted (B). Data points indicate the number of visible hydrosalpinx per mouse recorded at the time of sacrifice. Longitudinal genital tract sections were stained with hematoxylin and eosin (H&E) (C). The phenotype closest to the mean for each group is shown. The largest oviduct measurement per mouse (µm) was graphed (D). Data is presented as the mean ± SEM. Statistical significance was tested for using a student's *t* test. All data represent n=9 wildtype and n=10 TLR2$^{-/-}$ mice in two independent experiments.

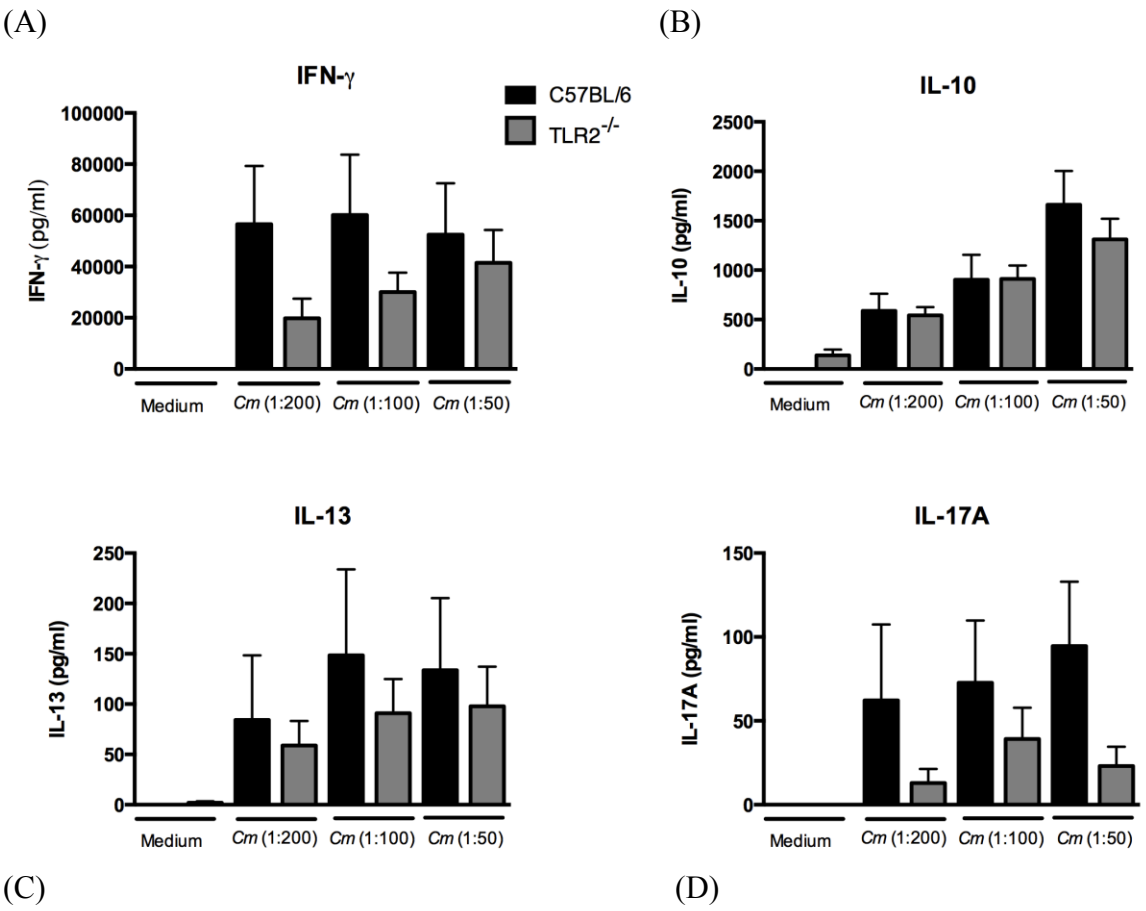


Figure 4.4 TLR2 contributes to development of the memory response during *C. muridarum* genital tract infection *in vivo*. Splenocytes were isolated from C57BL/6 wildtype and TLR2$^{-/-}$ mice that were infected intravaginally with *Cm* for 50 days. Cells were restimulated with heat-inactivated *Cm* at dilutions of 1:50, 1:100 or 1:200, or incubated with medium alone for 72 hours. Supernatants were harvested and analyzed for production of IFN-γ (A), IL-10 (B), IL-13 (C), and IL-17A (D) by ELISA. Data represent n=3 C57BL/6 wildtype and n=7 TLR2$^{-/-}$ mice from one experiment. Data is graphed as the mean ± SEM. Statistical significance was tested using a one-way ANOVA with multiple comparisons ($p<0.05$).

that are overall suppressed compared to wildtype mice.

4.1.2.2 BALB/c background model

In an identical experiment, female BALB/c WT and TLR2$^{-/-}$ mice were also infected with *Cm* for 50 days. In contrast to our observations in the C57BL/6 background, BALB/c TLR2$^{-/-}$ mice had a significantly decreased bacterial load at day 3 post-infection (Figure 4.5A). However, from day 7 onward, bacterial shedding was comparable between the two groups. By day 28, most mice in both groups were able to resolve the infection. At day 50, individuals from both groups had developed hydrosalpinx, although the incidence was less frequent in TLR2$^{-/-}$ mice (Figure 4.5B). Upon examination of the oviducts, wildtype mice had slightly elevated oviduct dilation compared to TLR2$^{-/-}$ mice (Figure 4.5 C, D). Although some divergent trends emerged between BALB/c and C57BL/6 background mice, these data supported the notion that TLR2 may play a role in both bacterial clearance as well as oviduct pathology.

Similar to what was seen in the C57BL/6 model, splenocytes from BALB/c TLR2$^{-/-}$ mice that were restimulated with heat-inactivated *Cm* produced significantly less IFN-γ, IL-13, and IL-17A than those from wildtype mice (Figure 4.6). Strikingly, TNF production from TLR2$^{-/-}$ splenocytes was also significantly impaired. IL-10 levels remained comparable. These results confirmed our previous observations that TLR2 contributes to the development of a robust *Cm*-specific memory response.

To investigate whether dendritic cells were being modulated in the absence of TLR2, we analyzed the expression of activation markers in wildtype and TLR2$^{-/-}$ DCs following *Cm* stimulation (refer to Appendix 1).

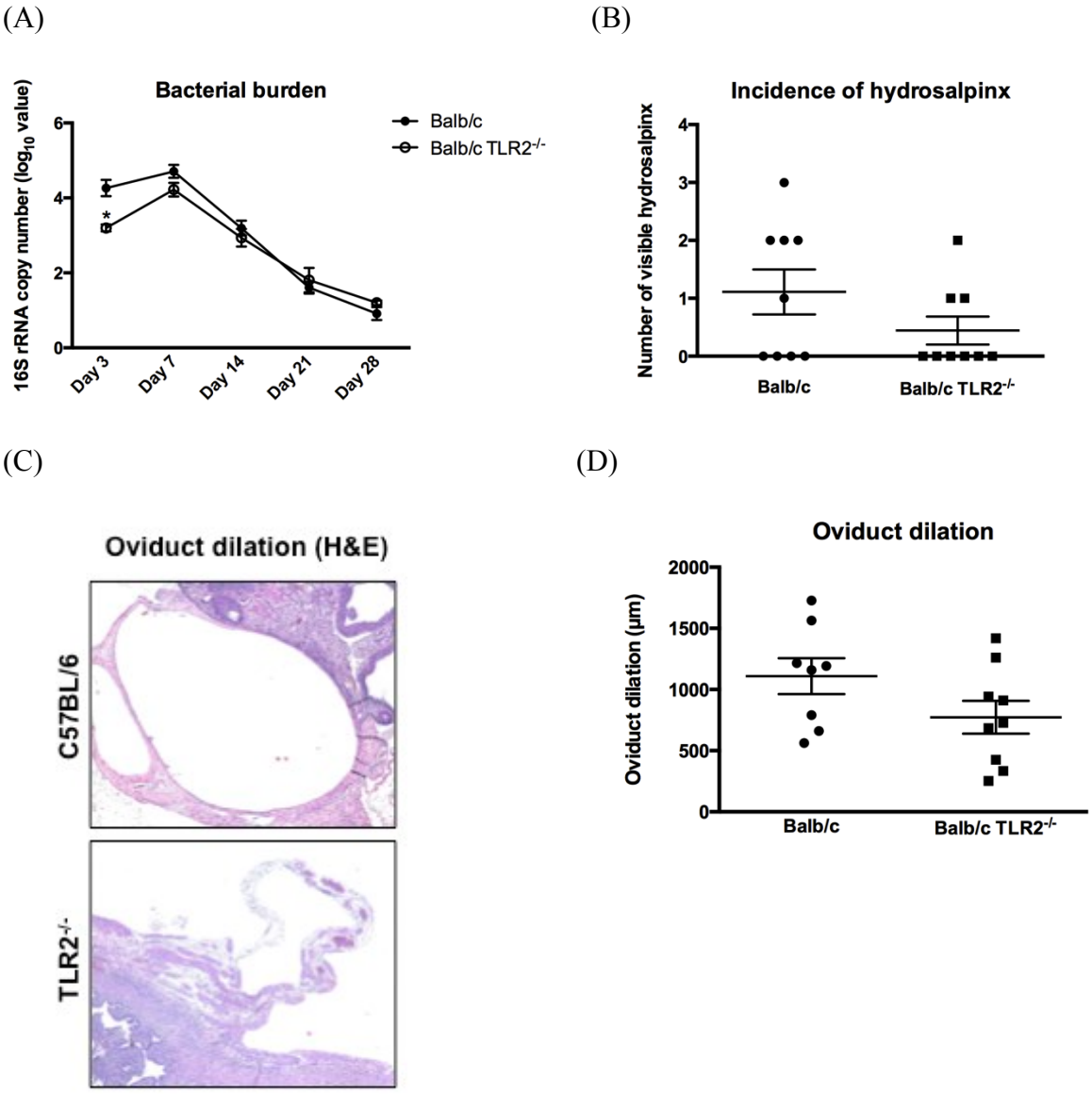


Figure 4.5 TLR2 may contribute to early bacterial clearance, but does not significantly exacerbate pathology in a BALB/c genital tract infection model. Female BALB/c wildtype and TLR2$^{-/-}$ mice were infected intravaginally with *Cm* for 50 days. Intravaginal swabs were taken to monitor bacterial shedding by qPCR (A). Data is presented as the average *Chlamydia*-specific 16S rRNA copy number expressed as a $\log_{10}$ value ± SEM. At the time of excision, the incidence of visible hydrosalpinx was counted (B). Longitudinal genital tract sections were stained with H&E (C). The phenotype closest to the mean for each group is shown. The largest oviduct measurement per mouse (µm) was graphed (D). Data is presented as the mean ± SEM. Statistical significance was tested for using a student's *t* test. All data represent n=9 wildtype and n=9 TLR2$^{-/-}$ mice in one independent experiment.

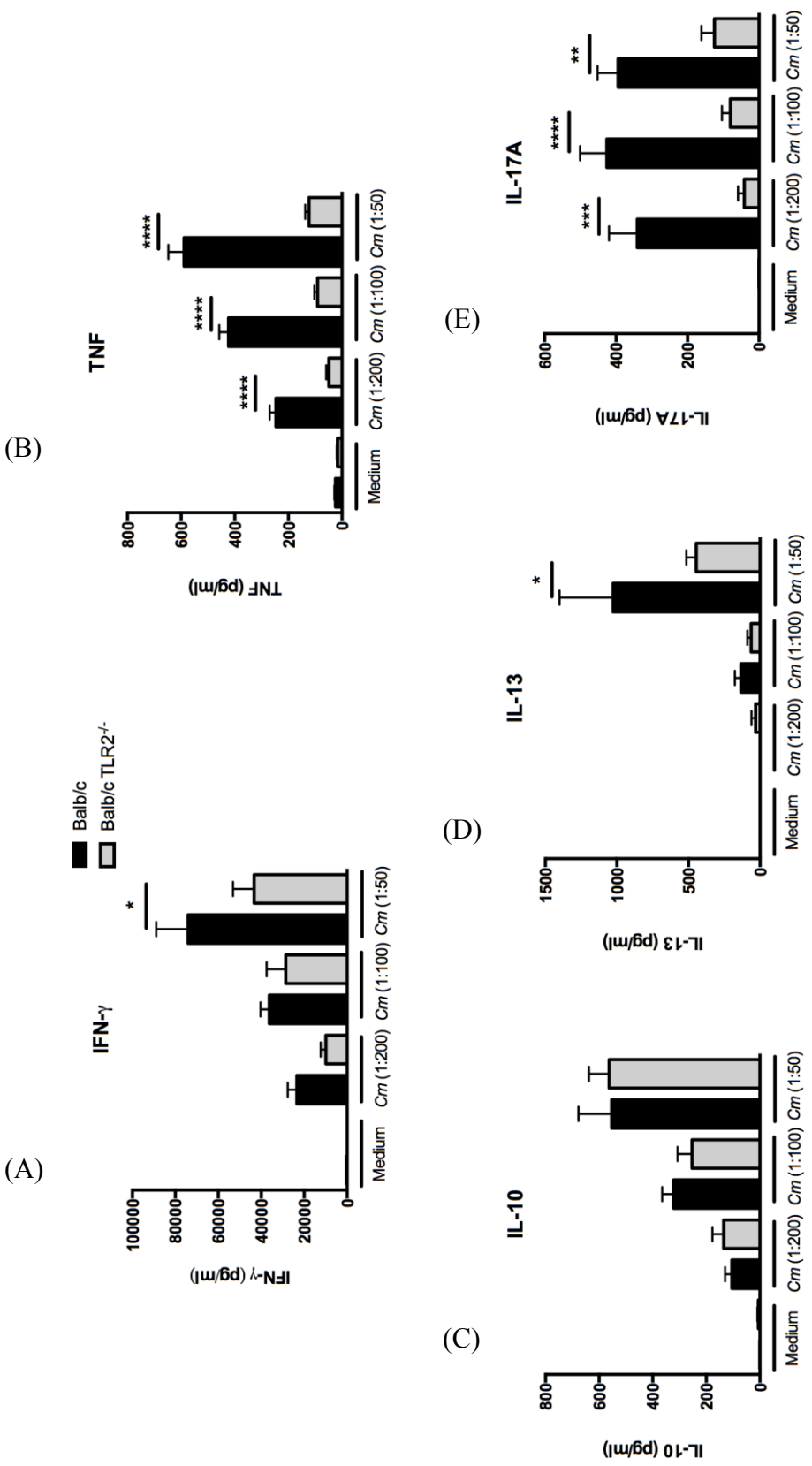

(A)
IFN-γ
Balb/c
Balb/c TLR2-/-
IFN-γ (pg/ml)
100000
80000
60000
40000
20000
0
*
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(B)
TNF
TNF (pg/ml)
800
600
400
200
0
****
****
****
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(C)
IL-10
IL-10 (pg/ml)
800
600
400
200
0
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(D)
IL-13
IL-13 (pg/ml)
1500
1000
500
0
*
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)
(E)
IL-17A
IL-17A (pg/ml)
600
400
200
0
***
****
**
Medium
Cm (1:200)
Cm (1:100)
Cm (1:50)

Figure 4.6 TLR2 contributes to the development of memory responses during *C. muridarum* genital tract infection in a BALB/c model. Splenocytes were isolated from BALB/c wildtype and TLR2$^{-/-}$ mice that were infected intravaginally with *Cm* for 50 days. Cells were restimulated with heat-inactivated *Cm* at dilutions of 1:50, 1:100 or 1:200, or incubated with medium alone for 72 hours. Supernatants were harvested and analyzed for production of IFN-γ (A), TNF (B), IL-10 (C), IL-13 (D) and IL-17A (E) by ELISA. Data represent n=9 BALB/c wildtype and n=9 BALB/c TLR2$^{-/-}$ mice in one independent experiment. Data is graphed as the mean ± SEM. Statistical significance was tested using a one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2 Conclusions

The results outlined above indicated that mast cell cytokine and chemokine responses are partially dependent on the pathogen recognition receptor TLR2. Furthermore, in a mouse model, TLR2 appears to contribute to bacterial clearance as well as the development of oviduct pathology in the context of two genetic backgrounds. The absence of TLR2 also resulted in suppressed memory responses. Overall, these studies have demonstrated the importance of TLR2 in mast cell responses to *Chlamydia*, as well as the physiological implications of TLR2 activation during intravaginal *Cm* infection *in vivo*.

CHAPTER 5 DISCUSSION

5.1 Discussion of major findings

Currently, innate immune mechanisms involved in pathogen recognition and immediate responses to *Chlamydia* infection are not well defined. Understanding these aspects of immunity is important, as early responses can have a profound influence on subsequent effector cells and adaptive immunity. Our studies have identified a novel role for mast cells, and provided additional insight into the role of TLR2 during *Chlamydia* infection of the genital tract. Through a series of studies *in vitro* and *in vivo*, we have demonstrated that mast cells and TLR2 impact acute inflammation following *Chlamydia* infection, and these responses have substantial downstream effects on adaptive immunity.

5.1.1 Characterization of mast cell responses to *Chlamydia*

A primary focus of this study was to characterize how mast cells respond to *Chlamydia*. This was done through the activation of primary murine and human mast cells *in vitro*, with *Cm* or *Ct*, respectively. When BMMCs were incubated with *Cm*, we observed that a number of cytokines and chemokines were present in activated cultures. However, when examined as a group, responses for many mediators were not statistically significant. This effect was due to inconsistent responses across BMMC cultures, as some individual cultures showed notable upregulation of cytokine expression while others did not. This could be attributed to circumstances such as the age of culture (6-14 weeks), length of resting time prior to activation (12-24 hours), or genetic variation among donor mice. Nonetheless, for all mediators shown, with the exception of TSLP, increases >10%

above parallel medium controls were observed in at least half of the activated cultures. Moreover, the increased production of CXCL1 (KC), CXCL2 (MIP-2), CCL2 (MCP-1), CCL3 (MIP-1α), IL-6, and TSLP appeared to be TLR2-dependent, though this was not statistically significant due to the reasons outlined above. Some mediators, such as CCL3 and IL-6, were modestly increased in TLR2$^{-/-}$ cultures activated with *Cm* compared to medium controls, but were more strongly produced by WT cultures. This suggests that TLR2 may function synergistically with other pathogen recognition pathways in the production of certain mediators. Conversely, the induction of IL-12p70 and IL-23p19 was not significantly altered in the absence of TLR2. Overall, of the eight mediators that showed upregulated expression when BMMCs activated with *Cm*, six showed decreased responses in the absence of TLR2. This indicates that TLR2 may contribute to pathogen recognition on mast cells during *Cm* infection in mice. Furthermore, TLR2 likely functions alongside, or synergistically with, other pathogen recognition receptors in the detection of *Cm* and the induction of host defence mechanisms.

In human CBMCs, activation with *Ct* resulted in the statistically significant production of several cytokines and chemokines. The mediator profiles generated from CBMCs activated with *Ct*, and BMMCs activated with *Cm*, shared some commonalities. For example, both human and murine mast cells produced IL-6, CCL3, and IL-23 in response to *Chlamydia*. CBMCs also produced significant amounts of CXCL8 (IL-8), which is considered analogous to CXCL1 and CXCL2 in the murine system [209]. Some mediators, such as IL-1β and IL-1RA, were present in supernatants from CBMCs but not BMMCs. Interestingly, many of the mediators produced by CBMCs are regulated by the transcription factor NF-κB, such as TNF, IL-6, GM-CSF, CCL3 and CXCL8 [210]. The

NF-κB pathway is classically downstream of activated PRRs, such as TLRs [72]. In contrast, the production of IL-1β is indicative of inflammasome activation and caspase-1 mediated cleavage of the pro-IL-1β precusor [211]. An inflammasome-independent pathway for mature IL-1β does exist, but this has been described mostly in neutrophils [212]. Similar to what was seen in the murine system, it appears that multiple inflammatory pathways are activated in CBMCs responding to *Ct*. However, CBMCs also exhibited divergent patterns of mediators from BMMCs. This could be due to host factors in human versus murine cells, such as differential expression of TLRs or other PRRs. This could also be due to variation in *Cm* versus *Ct*, or some combination of these factors.

Mast cell responses to an assortment of bacterial ligands, such as PGN and LPS, have been characterized in both human and mouse (refer to Table 5.1). In comparison with mast cell responses to other bacterial products, mast cells activated with *Chlamydia* exhibit some common features, such as production of IL-6 by BMMCs and IL-1β by CBMCs. In addition, they exhibit some unique features, such as the production of IL-23. Notably, the presence of Th2-associated cytokines such as IL-4 and IL-13, or the immunoregulatory cytokine, IL-10, were not detected in supernatants from BMMCs or CBMCs activated with *Chlamydia*. A previous report from Oksaharju *et. al.* described production of TNF, CCL2 and CXCL8 from CBMCs activated with *Cpn* [156]. In agreement with those findings, CBMCs produced significant amounts of TNF and CXCL8 in response to *Ct*, although CCL2 was not measured. This indicates that there is some degree of similarity among human mast cell responses to different *Chlamydia* species. Overall, it appears that *Chlamydia* stimulation elicits selective production of

cytokines and chemokines from BMMCs and CBMCs. Furthermore, these responses are distinct from the patterns of mediator production described in response to other bacterial ligands.

Many of the mediators produced by mast cells in response to *Chlamydia* are classically pro-inflammatory, or function to recruit effector cells to the site of infection. In the murine system, BMMC production of CXCL1, CXCL2, and CCL2 may be especially biologically relevant as they were present at the highest concentrations in *Cm* supernatants, and were at very low levels or not detected in medium controls. Both CXCL1 and CXCL2 are potent neutrophil chemoattractants in mice, and are important for mobilizing neutrophils from the circulation to the site of inflammation [213]. CCL2 is primarily chemoattractant for $CD4^+$ T cells, monocytes, and macrophages that express the CCR2 receptor [214], but has also been implicated in neutrophil recruitment during bacterial infection [215]. Taken together, these data indicate that mast cells may play an important role in the recruitment of effector cells, particularly neutrophils, during *Cm* infection *in vivo*. Interestingly, this is consistent with observations from Chiba *et. al.* regarding mast cell function during *Cpn* infection of the lung [158].

In humans, CXCL8 is also a powerful neutrophil chemoattractant [209], and was produced at significant levels by CBMCs following *Ct* stimulation. However, the background secretion of CXCL8 by medium controls was quite high. Perhaps more biologically important in the human system is the production of the TNF, IL-1β, and CCL3, which showed significant increases when compared with medium controls, and have important relevance in mouse models of *Chlamydia*. TNF and IL-1β are classical and potent inducers of inflammation, which promote vasodilation, adhesion molecule

expression, and the recruitment of granulocytes, as well as the induction of an acute phase response [211, 216]. Multiple mouse models employing genetic knockouts of TNF [177], TNFR1 [94, 217], TNFR2 [217] and TNFR1/2 [217] have demonstrated that the absence of TNF signaling significantly reduces *Chlamydia*-associated pathology, while the course of infection is unaltered. Similarly, in mouse models of *Cm* infection, IL-1β$^{-/-}$ [218], IL-1R$^{-/-}$ [219] and caspase-1$^{-/-}$ [220] mice all exhibit significantly decreased oviduct pathology. Conversely, CCL3 expression during genital tract infection has been associated with the development of a protective Th1 response [221]. Typically, CCL3 is known for its chemotactic properties that mediate the infiltration of T cells, monocytes, DCs and neutrophils [222]. Although these mediators have pathophysiological importance in murine models of *Cm* infection, it is less clear how early cytokine and chemokine responses influence disease in humans. However, taken together, it seems that CBMC responses to *Ct in vitro* are consistent with a role for mast cells as sentinel cells that detect early infection, initiate inflammatory responses, and recruit effector cells to the site.

Human mast cells have been shown to be important sources of type I IFNs during viral infections [130]. In response to *Ct*, CBMCs did not produce IFN-α, IFN-β or IFN-ω. Typical pathways for type I IFN induction include activation of extracellular TLR4, endosome-associated TLRs, such as TLRs 2, 3, 7, 8 and 9, and other intracellular PRRs [223]. Given that TLR4 is not substantially expressed by CBMCs [136], and most other pathways leading to the induction of type I IFNs require intracellular receptors, this supports the notion that *Ct* is not widely internalized by mast cells. Similar pro-inflammatory responses, devoid of type I IFNs, have been described from mast cells

activated with other Gram-positive and Gram-negative bacteria, such as *Listeria monocytogenes* and *Salmonella typhimurium*, respectively [224]. In these studies, *L. monocytogenes* and *S. typhimurium* engaged TLR activation, but remained bound to the extracellular surface [224]. Moreover, the presence of type I IFNs was found to impair mast cell-mediated host defence mechanisms, such as neutrophil mobilization, *in vivo* [224]. In a mouse model of *Cm* infection, mice deficient in the receptor for type I IFNs ($IFNAR^{-/-}$) exhibited reduced pathology, increased chlamydial-specific T cells in the draining lymph node, and increased $CD4^+$ T cells in the cervical tissues [197]. Thus, type I IFNs appear to be detrimental during *Cm* infection. Taken together, these findings imply that mast cells have evolved highly selective, pathogen-specific responses to *Chlamydia* and other bacterial pathogens.

5.1.2 Infection and replication of *C. trachomatis* within human mast cells

Our TEM studies demonstrated that a proportion of human mast cells are able to take up *Ct*. However, given the high dose of bacteria that was used (1:20 cell to bacterium ratio), and the low percentage of cells that exhibited evidence of chlamydial inclusions, it appears that human mast cells are relatively resistant to *Ct* infection. To further support this, we saw no evidence of bacterial replication within CBMCs. Given that CBMCs express PRRs such as TLR2 on their surface [135], it is plausible that the production of inflammatory mediators may driven, primarily or exclusively, by the detection of extracellular pathogens.

A similar phenomenon has been observed in human blood monocytes infected with *Cpn*. Following infection, there was no increase in bacterial copy numbers between

6 and 48 hours, as detected by the measurement of 16S rRNA by qPCR [225]. However, immunofluorescence for chlamydial LPS and oligonucleotide array both confirmed the presence of *Cpn* in the cytoplasm [225]. In this case, monocytes can be infected, but do not support intracellular replication of *Cpn* [225]. The mechanism that restricts chlamydial replication in monocytes is currently unclear. It has been shown that in human macrophages, *Ct* replication can be suppressed by the trafficking of chlamydial inclusions to lysosomes, and later in infection, by the induction of autophagy [108, 109]. It is possible that similar mechanisms may be employed in human mast cells, however, this was beyond the scope of the current study.

Based on the current findings, it appears that human mast cells respond to *Ct* without substantial uptake or intracellular replication of the bacteria. This further emphasizes the role of mast cells as long-lived sentinel cells during *Chlamydia* infection.

5.1.3 Mast cells as assets and liabilities during *C. muridarum* infection *in vivo*

5.1.3.1 Mast cell-mediated effects on dendritic cells

During early infection with *Cm in vivo*, we observed that mast cell-deficient mice had markedly decreased DC infiltration into the draining lymph node. This effect was particularly significant in the $CD103^+$ population. $CD103^+$ DCs are non-lymphoid tissue resident cells that are typically present in connective tissues [114]. Taken together, this indicates that mast cell-derived mediators have a particular role in mobilizing DCs from the tissue site to the draining lymph node following *Cm* infection. Although not reaching statistical significance, all other cDC subsets that were examined were also substantially decreased in mast cell-deficient mice. However, pDCs, which have poor antigen

presentation capabilities, were not changed. This suggests that mast cells may have a broad influence on antigen-presenting DC populations during *Cm* infection. The reduced infiltration of these critical APCs into the draining lymph node of mast cell-deficient mice provides a reasonable explanation for the universally suppressed *Chlamydia* antigen recall responses observed in Wsh mice. Moreover, this suggests that mast cells might contribute to protection against secondary challenge through impacting the early stages of developing adaptive immunity.

Several other reports have implicated mast cells in DC mobilization during bacterial infection. Studies of Langerhans cells and DC subpopulations in the skin have revealed that mast cell-derived products selectively mediate the mobilization of pDCs and $CD8^+$ DCs to the lymph node in response to PGN from *Staphylococcus aureus* [146, 147]. In our *Cm* genital tract infection model, $CD103^+$ DC infiltration was significantly reduced in the absence of mast cells, but similar effects were seen in all other subsets examined, with the exception of pDCs. This indicates that mast cell-mediated trafficking of specific DC subsets to the lymph node may be pathogen-specific. In addition to mobilization, mast cells have been shown to influence DC maturation through direct crosstalk that enhances the expression of costimulatory molecules [226]. Mast cell "primed" DCs also had a superior ability to induce $CD4^+$ T cell proliferation, and promote Th1 and Th17 responses [226]. Some proposed mechanisms for mast cell-associated effects on DC mobilization and function include mediators such as histamine [146], IL-6 [146], and TNF [150, 227]. Our evidence *in vitro* suggests that murine mast cells do not degranulate, and do not produce significant levels of TNF in response to *Cm*. It is possible that mast cell responses *in vivo* may differ due to the influence of the

microenvironment. Currently, the mechanism for mast cell-mediated DC mobilization in the context of *Cm* genital tract infection is unclear.

5.1.3.2 Mast cell contributions to oviduct pathology

In late stage *Cm* infection, mast cell-deficient mice displayed significantly reduced oviduct pathology, despite a rate of bacterial clearance comparable to wildtype. Thus, it appears that mast cells do not contribute substantially to elimination of the pathogen, although they may contribute to long-term tissue damage following infection. This differs from studies of *Cpn* lung infection, which indicate that mast cells delay bacterial clearance by recruiting immune cells that support chlamydial replication [158]. The pathological effects of mast cells during *Chlamydia* infection may be direct or indirect. For example, it is possible that mast cell-derived mediators that were not investigated, such as proteases, contribute directly to tissue remodeling and fibrosis following infection [228]. Evidence from BMMC responses *in vitro* suggest that mast cells play an indirect role, by initiating and enhancing acute inflammation, and promoting the recruitment of immune effector cells. It has been well characterized in other models of bacterial infection that mast cells have an important role in the recruitment of neutrophils. In our studies, many mediators that were produced by BMMCs in response to *Cm*, such as CXCL1, CXCL2, CCL2, and CCL3, can act as important chemoattractants for neutrophils [209, 213, 215, 229]. In a number of mouse models, excessive or persistent neutrophil infiltration into the genital tract has been associated with exacerbated oviduct pathology [102-104, 175, 230]. Thus, it is plausible to speculate that mast cell-mediated infiltration of neutrophils into the genital tract may be a

contributing factor to the pathology observed in wildtype mice. This is further supported by evidence from Chiba *et. al.*, who observed a similar phenomenon in *Cpn* lung infection [158]; however, future studies are required to confirm this in the genital tract model. Taken together, evidence from *in vitro* and *in vivo* models suggests that mast cells indirectly promote pathology through enhancing the infiltration of immune effector cells.

5.1.3.3 Limitations of the Wsh mouse model

Wsh mice are valuable tools for investigating mast cells *in vivo*. The c-kit/SCF axis, which is exploited by the Wsh model, is critical for the development of mature mast cells. Introducing mutations in the gene *c-kit*, which is the receptor for SCF, results in mice devoid of mature mast cells. The $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (Wsh) model of mast cell deficiency is commonly used [153-155, 158] as this strain is fertile, not anemic, and has normal γδ T cell numbers compared to its predecessors [231]. However, *c-kit* is also expressed at low levels by a number of other cell types [231]. Therefore, *c-kit* mutations in these mice result in pleiotropic effects, besides mast cell-deficiency, that must also be considered while interpreting experimental findings. For example, it has been reported that C57BL/6 background Wsh mice exhibit enlarged and abnormal spleens, featuring increased megakaryocyte and myeloid populations [232]. In the bone marrow of Wsh mice, CD11b and Gr-1 (Ly6G)-expressing populations are also increased, including a marked expansion of circulating neutrophils [232]. This neutrophilia subsequently results in an alteration of the ratio of circulating T cells [232]. Similar findings were reported in a BALB/c background Wsh strain [233]. Moreover, in this model, these expanded $Ly6G^+$ populations were phenotypically similar to myeloid-derived suppressor cells (MDSCs),

and were able to suppress T cell responses *in vitro* [233]. Additionally, it has been observed that Wsh mice have increased proportions of Tregs [234]. These immune alterations pose a challenge for deducing whether the observed effects in our mast cell-deficient model can be attributed to mast cells alone. In order to confirm these findings are principally dependent on the presence of mast cells and mast cell-derived mediators, it would be appropriate to perform a local mast cell reconstitution, or use an additional mast cell-deficient model that is not reliant on *c-kit* [235].

5.1.4 Elucidating the importance of TLR2 during *C. muridarum* infection *in vivo*

5.1.4.1 The relative impact of TLR2 in oviduct pathology

Previous studies investigating the role of TLR2 *in vivo* have afforded inconsistent results. A prominent report by Darville *et. al.* observed that TLR2$^{-/-}$ mice infected with *Cm* had significantly reduced oviduct pathology, although they exhibited a similar course of infection as WT mice [92]. In the field, these findings have contributed to a general perception that TLR2 is a primary mediator of acute inflammation and pathological damage during *Cm* infection [1, 65]. A more recent study by Dong *et. al.,* that employed a notably larger *n*, confirmed that TLR2$^{-/-}$ and WT mice had similar bacterial shedding. In contrast, this study found that TLR2$^{-/-}$ mice did not exhibit reduced pathology [94]. Our studies, in two strains of TLR2$^{-/-}$ mice, indicated that the absence of TLR2 had an effect on bacterial clearance, and a moderate, but not significant, effect on the development of oviduct pathology. In the C57BL/6 background, TLR2$^{-/-}$ mice had increased bacterial shedding compared to WT on days 14 and 21 post-infection. This suggested that TLR2-deficiency delayed bacterial clearance, although this was resolved by day 28. Conversely,

in the BALB/c background, TLR2$^{-/-}$ mice exhibited a decreased bacterial load at day 3, although the successive course of infection was similar to WT. This evidence suggests that, in contrast to previous studies, TLR2 may influence bacterial clearance from the genital tract, although this effect was divergent between the two strains. In both C57BL/6 and BALB/c mice, we observed that oviduct pathology was not significantly attenuated in the absence of TLR2. However, TLR2$^{-/-}$ mice exhibited a clear trend towards reduced incidence of hydrosalpinx and decreased oviduct dilation. Thus, our results do not completely agree nor disagree with previous reports.

A notable aspect of our study was the inclusion of two distinct laboratory mouse strains: C57BL/6 and BALB/c. To our knowledge, this is the first report describing the outcomes of *Cm* infection in BALB/c TLR2$^{-/-}$ mice. This comparison is important in interpreting the impact of TLR2 and other components of immunity, as different mouse strains exhibit immunological variation. For example, C57BL/6 mice are generally Th1-biased in their responses to pathogens, while BALB/c mice are predisposed to Th2 responses [236]. This immunological variation can affect susceptibility, pathological outcomes, and the overall course of infection. In the context of our study, this may explain some discrepancies between strains, such as the kinetics of bacterial clearance or the severity of pathology. However, the results from both strains demonstrate similar trends, which corroborate our results in elucidating the pathophysiological importance of TLR2 during *Cm* infection *in vivo*.

5.1.4.2 The effect of TLR2 on adaptive immune responses

In both C57BL/6 and BALB/c mice, TLR2-deficiency resulted in a markedly decreased recall response. In a pilot experiment, we observed that $TLR2^{-/-}$ BMDCs had impaired maturation following *Cm* activation, and this was true for stimulations with both live and heat-inactivated bacteria. More specifically, $TLR2^{-/-}$ BMDCs did not develop populations expressing high levels of MHC II, CD80 and CD86 that were seen in WT C57BL/6 and Wsh BMDCs. Given these data, we speculate that impaired DC maturation would interfere with the abilities of these cells to present antigens, thereby contributing to the suppressed memory responses that were seen in infected $TLR2^{-/-}$ mice. However, it is difficult to interpret this data, as LPS control stimulations were not optimal. Additional experiments will need to be performed to confirm these results.

5.1.4.3 Limitations of $TLR2^{-/-}$ mouse strains

In contrast to the limitations of the Wsh model, the $TLR2^{-/-}$ mouse model is a specific gene knockout of TLR2. However, since TLR2 has functions outside of pathogen recognition, the absence of this receptor may result in some off-target or developmental effects that could have bearing on an infection model. For example, given that TLR2 has been implicated in the colonization of commensal bacteria in the human gut [51], it is plausible that these mice may have an altered microbiome composition compared to wildtype. A study by Loh *et. al.* found that TLR2/TLR4 double knockout mice had intestinal microbiota similar to wildtype, using denaturing-gel electrophoresis and fluorescence in-situ hybridization [237]. Another study by Albert *et. al.*, performed phylogenetic analysis of 16S rRNA gene clone libraries, and found that although $TLR2^{-/-}$

mice did exhibit colonization by two *Helicobacter* species that were not detected in wildtype mice, the two groups did not differ significantly in their intestinal microbiota [238]. Currently, there are no studies that have investigated the composition of genital tract flora in TLR2$^{-/-}$ mice. Given the literature in the gut microbiome, it is reasonable to postulate that similar results would be found in the genital tract. Nonetheless, commensal bacteria have been shown to be an important factor in modulating host susceptibility to female genital tract infection [238, 239], so these potential discrepancies must be taken in to consideration.

5.3 Implications of major findings

5.3.1 Comparison of mast cell-deficient and TLR2-deficient disease phenotypes

A comparison of the course of disease, oviduct pathology, memory responses, and observations of dendritic cells in Wsh and TLR2$^{-/-}$ mice reveals many similarities between the two strains (refer to Table 5.2). One perspective of this phenomenon is that both mast cells and TLR2 are pivotal innate immune responses that contribute to the initial detection of invading bacteria, and coordinating subsequent effector cells. Consequently, the removal of either of these components from the system has profound downstream effects on the overall immune response, including pathological outcomes and adaptive immunity. Another possibility is that the expression of TLR2 by mast cells, specifically, is particularly important during *Chlamydia* infection. This is supported by the observation that the majority of cytokine and chemokine responses in murine mast cells were TLR2-dependent. This implies that the depletion of mast cells may also represent the deletion of an important component of the TLR2 compartment, and vice

versa. In a tumour model, both mast cells and TLR2 have been shown to be important for recruiting effector cells that reduce tumour growth [240]. At mucosal surfaces, mast cells are one of few resident immune effector cells that express substantial TLR2 [240]. Future experiments to investigate the role of TLR2 on mast cells during *Cm* infection *in vivo* could be performed via the reconstitution of Wsh mice with $TLR2^{-/-}$ mast cells.

5.3.2 Considerations for human disease and vaccine design

Our animal models of *Cm* infection indicate that both mast cells and TLR2 have important roles in genital tract infection (Figure 5.1). *In vivo*, the presence of mast cells and TLR2 was associated with exacerbated oviduct pathology, and TLR2 was further implicated in facilitating bacterial clearance. Mast cells and TLR2 were also essential for the development of robust memory responses, perhaps through modulating the migration and maturation of DCs, respectively. Given the immunological differences in humans versus mice, and the degree of genetic variation between *Cm* and *Ct*, the direct translation of findings from the murine system to humans should be cautioned. That being said, evidence from our studies in human mast cells suggests that they may function similarly in human genital tract infection. For example, human mast cells produced a number of pro-inflammatory mediators, and a number of these were similar, or had analogous functions, to those produced in murine mast cells. Our studies represent the first investigations of mast cells in the context of *Ct* genital tract infections. At this time, mast cells have not been examined in human infection.

Traditionally, mast cells have been best known for their role in allergy. However, accumulating evidence has unearthed mast cells as an important component of immunity

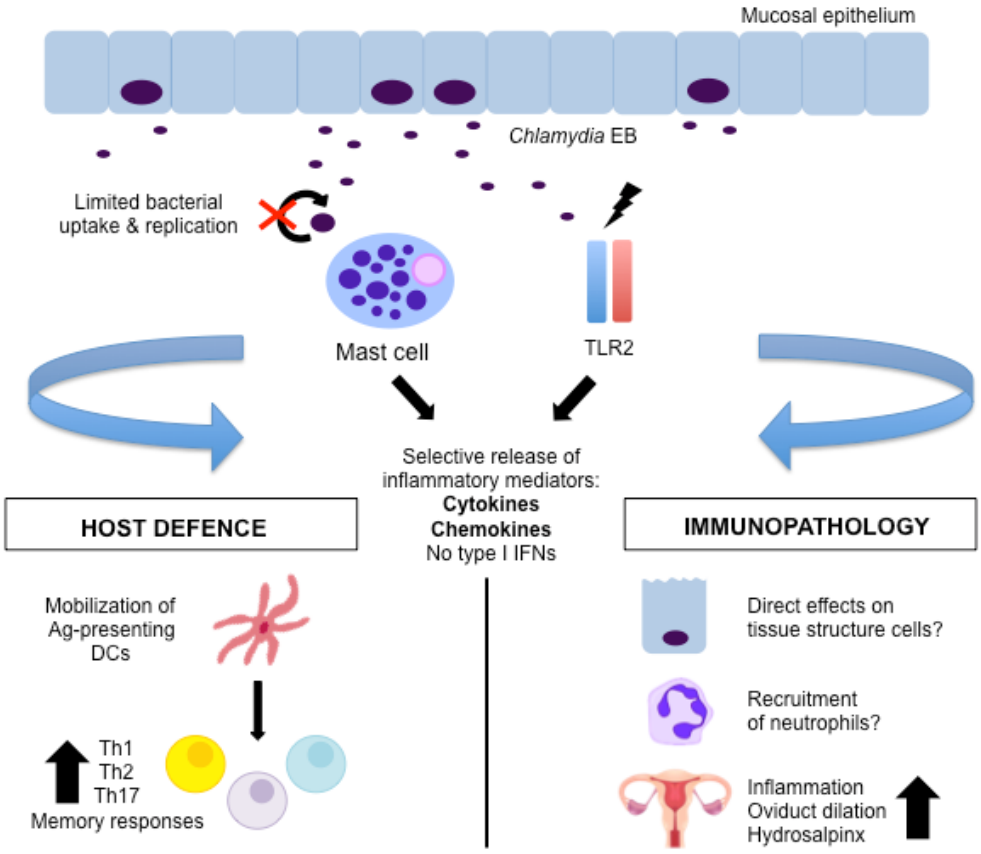


Figure 5.1 Current model of the role of mast cells and TLR2 in host defence and pathology following *Chlamydia* genital tract infection. Invading *Chlamydia* EBs primarily infect the mucosal epithelium of the genital tract. Mast cells are located near the mucosal surface, where they act as sentinel cells to detect invading pathogens. *Chlamydia* EBs are not substantially uptaken by mast cells, and do not replicate robustly within them. Thus, mast cells respond primarily to extracellular bacteria. Following *Chlamydia* stimulation, mast cells selectively release a unique pattern of pro-inflammatory cytokines and chemokines. According to *in vitro* evidence, mast cells do not release type I IFNs and do not undergo degranulation. TLR2 is an important PRR for mast cell responses to *Chlamydia*, and is present on many other cell types. TLR2 activation by *Chlamydia* PAMPs also results in the selective production of cytokines and chemokines. These mast cell-derived and TLR2-dependent mediators contribute to both host defence and immunopathology during infection *in vivo*. Mast cell-derived mediators are important for the mobilization of antigen-presenting DCs to the draining lymph node, where they elicit Th1, Th2 and Th17 responses. TLR2 may also affect DCs and T helper responses through facilitating DC maturation. These processes are important for the development of immunological memory. Conversely, mast cell-derived and TLR2-dependent mediators may cause direct tissue damage to tissue structure cells, or indirect damage through the recruitment of immune effector cells such as neutrophils. Thus, the presence of mast cells and TLR2 is associated with increased inflammation of the genital tract, leading to increased oviduct dilation and hydrosalpinx formation in a mouse model.

during microbial infection. In our studies, human mast cells did not show evidence of substantial *Ct* internalization or replication. However, they did respond by producing a selective profile of pro-inflammatory mediators, which would likely act to promote the recruitment of effector cells to the site. These findings reinforce mast cells as long-lived sentinel cells in mucosal tissues, that function as a first line of defence against pathogens, and participate in both effector cell recruitment and the mobilization of acquired immune responses.

Many efforts to develop a *Chlamydia* vaccine are ongoing, and our studies may be especially pertinent to these endeavors. It is the prevailing opinion that a mucosal vaccination strategy would be the most practical, and elicit the most protective responses against sexually transmitted *Ct* [241]. For a *Chlamydia* vaccine to be optimally efficacious, it should activate both cell-mediated and protective humoural responses without risk for pathology [241]. However, designing an effective mucosal vaccine is challenging, as antigens must cross the epithelial barrier in order to stimulate robust immune responses, and administering antigen alone is rarely effective [242]. As a result, mucosal vaccines often require potent adjuvants [242]. Because mast cells produce mediators that mobilize DCs to the draining lymph node, and contribute to the induction of robust memory responses, mast cells could be appealing adjuvant targets. In theory, immunizing with *Ct* antigens, such as the MOMP or CPAF, in conjunction with a suitable mast cell activator, would enhance T cell and B cell responses, thereby promoting more protective immunity. This approach has been adopted in other vaccination strategies detailed in the literature. For example, one study reported that the intranasal administration of the mast cell activator C48/80, in combination with *Bacillus anthracis*

antigen, resulted in the production of specific neutralizing antibodies in rabbits [243]. However, it should be noted that the administration of this mast cell activator has resulted in adverse effects, such as anaphylaxis, in other animal models [244]. Particular diligence would be required for the appropriation of this vaccine strategy in the context of *Ct*, especially if it were to be administered urogenitally, as mast cell activation was also associated with genital tract pathology *in vivo* [244]. In order to bypass the challenges of activating mast cells directly, a study by St. John *et. al.* described the use of synthetic mast cell granules as adjuvants [245]. These synthetic particles, which contain inflammatory mediators such as the cytokines TNF and IL-12, are constructed to resemble mast cell-derived particles in shape, size and composition [245]. In an influenza model, the use of synthetic mast cell granules as vaccine adjuvants provided protection against lethal challenge, and could be used to harness adaptive immunity towards a Th1-polarized response [245]. An alternative approach would be to elucidate the specific mast cell-derived mediators that influence adaptive immunity, and stimulate their production upon vaccination. As TLR2 was also correlated with enhanced recall responses, it could potentially be manipulated in a similar manner. For example, administering a TLR2 ligand as an adjuvant. However, similar to the caveats of a mast cell-based adjuvant for *Ct,* TLR2 is also associated with the development of genital tract pathology. Moreover, TLR2 activation in certain contexts can exert immunosuppressive responses [51]. Therefore, although these studies provide novel insight into harnessing immune responses for protection from *Ct* disease, further research is warranted.

5.4 Future Directions

5.4.1 Mast cell reconstitutions and additional mast cell-deficient models

As addressed above, Wsh mice exhibit *c-kit*-dependent effects aside from mast cell-deficiency. Although the Wsh model provides a valuable starting point for evaluating the role of mast cells in *Chlamydia* infection *in vivo*, it is necessary to confirm that these findings are not significantly confounded by any associated immune defects. One strategy that is commonly employed in Wsh models is to reconstitute the local tissue site with primary mast cells, such as bone marrow-derived mast cells. One report has described that the mast cell compartment within the uterus can be replenished by local reconstitution of BMMCs injected in the uterine horn [246]. Reconstitution of Wsh mice with wildtype BMMCs prior to *Cm* infection would be important to confirm that the observed effects on pathology and recall responses are attributable to local mast cells. Moreover, given the TLR2-dependence demonstrated by many cytokine and chemokine responses to *Cm*, another interesting approach would be to reconstitute Wsh mice with $TLR2^{-/-}$ BMMCs. This would result in the mast cell-specific deletion of TLR2, therefore providing insight into the importance of this particular receptor on mast cells during *Cm* infection.

5.4.2 Mast cell and TLR2-mediated neutrophil recruitment during early infection

These studies focused primarily on early mast cell responses *in vitro*, and how these contributed to long-term outcomes of infection *in vivo*. Multiple studies in other models of bacterial infection have delineated the importance of mast cell-derived mediators in the recruitment of neutrophils. Paired with the findings that many mediators

produced by BMMCs in response to *Cm* are important chemotactic signals for neutrophils (e.g. CXCL1 (KC), CXCL2 (MIP-2), CCL2 (MCP-1), CCL3 (MIP-1α)), this strongly suggests that mast cells contribute to neutrophil migration during *Cm* infection *in vivo*. Given that neutrophils have been associated with pathology in *Cm* infection, it is possible that the absence of neutrophil chemotactic signals from mast cells may result in reduced neutrophil infiltration, thus contributing to the reduction in oviduct pathology observed in Wsh mice. Since many of these cytokines and chemokines were seen to be TLR2-dependent in BMMCs, decreased neutrophil recruitment may be contributing similarly to the reduction of pathology seen in this model. Some proposed strategies for evaluating neutrophil influx include histological staining or flow cytometric analysis of genital tracts excised at early time points (e.g. 1-5 days post-infection). Alternatively, neutrophil elastase 680 has recently been identified as a useful biomarker for observing *Cm* infection *in vivo* and *ex vivo* using fluorescent optical imaging [247]. Overall, characterizing the influx of neutrophils in Wsh and TLR2$^{-/-}$ mice may provide additional insight into how the course of infection and resultant pathology is attenuated in these models.

5.4.3 Further characterization of dendritic cell responses

In our mast cell-deficient model, we observed abrogated dendritic cell mobilization to the draining lymph node during early infection. Given that Wsh and TLR2$^{-/-}$ mice both exhibited suppressed memory responses, and that the production of many mast cell-derived mediators were dependent on TLR2, it is possible that TLR2$^{-/-}$ mice may also exhibit impaired DC trafficking. For these experiments, it may be valuable

to employ littermates as appropriate wildtype controls to minimize the effects of variables such as age, environment and genetic variation. In a pilot experiment, we observed that DC expression of activation markers was attenuated in TLR2$^{-/-}$, but not Wsh mice. Future experiments to assess the functionality of TLR2$^{-/-}$ DCs may provide further insights. For example, additional experiments could include characterizing cytokine responses from activated TLR2$^{-/-}$ DCs, as well as assessing their ability to present antigen and invoke T cell responses.

5.5 Concluding remarks

The accumulation of studies in the *Chlamydia* field, including *in vitro* observations, animal models of infection, and clinical data from humans, has significantly advanced our understanding of *Chlamydia* infection and pathogenesis. Through the presented work, we have elucidated a novel and significant role for mast cells in *Chlamydia* genital tract infection, and further clarified the impact of TLR2. These studies have expanded our understanding of *Chlamydia* immunity, and may have implications for other sexually transmitted infections or pathogens that mediate chronic inflammation. Currently, *Ct* infection remains a major global public health concern, as it is the most commonly transmitted bacterial STI and the leading cause of infectious blindness worldwide. The development of a *Chlamydia* vaccine has proven to be a significant challenge thus far. Understanding protective and pathological immune responses, such as those discussed in this work, may inspire alternative approaches for the design of a safe and effective vaccination strategy.

Table 5.1 Cytokine and chemokine production by mast cells in response to bacterial products and TLR ligands

Mast cell type	Activator	TLR	Mediators produced
BMMC (murine)	LPS	TLR4	TNF, IL-1β, IL-5, IL-6, IL-10, IL-13, CCL3, CXCL2
	PGN	TLR2	TNF, IL-4, IL-5, IL-6, IL-13
	Streptococcus equi [248]	TLR2	TNF, IL-4, IL-6, IL-12, IL-13, CCL2, CCL7, CXCL2, CCL5
	Chlamydia muridarum	TLR2 and others	IL-6, TSLP, IL-12p70, IL-23p19, CXCL1, CXCL2, CCL2, CCL3
CBMC (human)	LPS [194]	TLR4	TNF*, IL-5, IL-10, IL-13
	PGN	TLR2	IL-1β, GM-CSF
	Zymosan	TLR2	IL-1β, GM-CSF
	Chlamydia pneumoniae [156]	Unknown	TNF, CCL2, CXCL8
	Chlamydia trachomatis	Unknown	TNF, IL-1β, IL-1RA, IL-6, GM-CSF, IL-23, CCL3, CCL5, CXCL8

*Requires pre-treatment with IL-4

Modified from Sandig *et. al.*[135], except where additional references are indicated. Results from the current study are shown in bold.

Table 5.2 Comparison of Wsh and TLR2$^{-/-}$ disease phenotypes following *C. muridarum* infection *in vivo*

		Wsh	C57BL/6 TLR2$^{-/-}$	BALB/c TLR2$^{-/-}$
Bacterial burden		Comparable	↑ (days 14-21)	↓ (day 3)
Oviduct pathology	**Incidence of hydrosalpinx**	-	↓	↓
	Oviduct dilation	↓	↓	↓
Memory responses	**TNF**	Comparable	-	↓↓↓
	IFN-γ	↓↓↓	↓	↓
	IL-13	↓↓↓	↓	↓
	IL-17A	↓↓↓	↓	↓↓↓
	IL-10	Comparable	Comparable	Comparable
DCs	**Migration**	↓	-	-
	Maturation	Comparable	↓	-

Symbols indicate deviations from parallel wildtype controls within each experiment.

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APPENDIX

1.1 INTRODUCTION

Previous data indicated that TLR2$^{-/-}$ mice, on both C57BL/6 and BALB/c backgrounds, had suppressed memory responses following *Cm* infection for 50 days. Moreover, these memory responses exhibited a similar pattern to those of mast cell-deficient Wsh mice that were infected with *Cm* for 50 days. Given these data, we sought to investigate whether dendritic cell maturation was differentially modulated in TLR2$^{-/-}$ and Wsh mice, thereby contributing to the reduction in cytokine production during antigen restimulation. This appendix describes a pilot experiment investigating the maturation of bone marrow-derived dendritic cells (BMDCs) from wildtype (WT), TLR2$^{-/-}$ and Wsh mice following stimulation with *C. muridarum* (*Cm*).

1.2 METHODS

1.2.1 Bone marrow-derived dendritic cells (BMDCs)

Bone marrow was collected from the tibia of female mice using 5 ml RPMI (GE Healthcare Life Sciences, Logan, UT, USA) with 10% FBS (Sigma Aldrich) in a 25 5/8 G needle. Cell suspension was then passed through a 100 µm strainer. Cells were cultured in RPMI supplemented with 10% FBS 1% P/S, 1% L-glutamine (Wisent Bio Products), 1% HEPES (Wisent Bio Products), 5 µM 2-Mercaptoethanol and 10% culture supernatants from X-63 cells expressing GM-CSF. Cells were supplied with fresh GM-

CSF on days 3 and 6. On day 8, non-adherent cells were collected and used for activation.

1.2.2 BMDC activations

Non-adherent cells from BMDC cultures were washed once with RPMI to remove residual GM-CSF. Cells were resuspended in RPMI 10% FBS and transferred to a 24 well plate, with each well containing 2 x 10^6 cells. Cells were incubated for 24 hours at 37°C with either *Cm* (MOI=0.5), heat-inactivated *Cm* (MOI=0.5), LPS (10 ng/ml) (Sigma Aldrich) or medium alone. After 24 hours, replicate wells were pooled and centrifuged (10 min, 300 g, 4°C). Cells were prepared for flow cytometry analysis as described below.

1.2.3 Flow cytometry

BMDC activation was assessed by analyzing expression of CD11c, MHC II, CD80, CD86 and CD40 (refer to Table 2.1). 1 x 10^6 cells per sample were prepared for flow cytometry. Cells were transferred to microcentrifuge tubes and washed twice with PBS supplemented with 0.01 M sodium azide (FACS buffer). Samples were blocked with 20 µl 3% FBS in FACS buffer for 20 minutes. Fluorochrome-conjugated antibodies (refer to table 2.1) were diluted in 100 µl FACS buffer and added to samples for 30 minutes. Samples were washed once with FACS buffer. eFluor506 (eBioscience) fixable viability dye was diluted in FACS buffer and added separately to samples for 20 minutes. Samples were washed once with 900 µl FACS buffer and an additional time with 900 µl cold PBS. Samples were fixed in 1% PFA (paraformaldehyde) for at least 1 hour, then resuspended

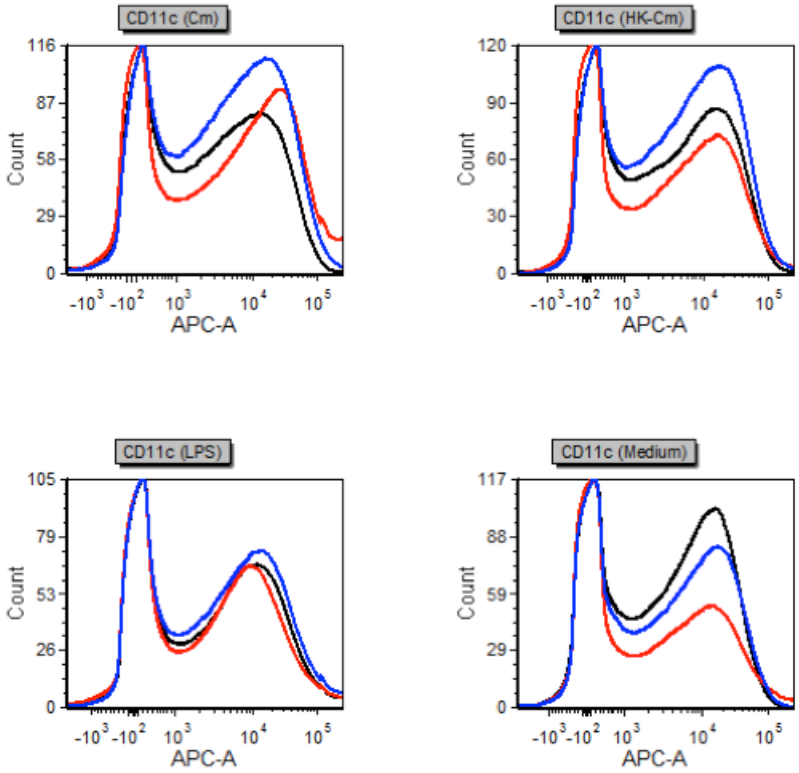
in PBS for reading. Single colour controls and fluorescence minus one (FMO) controls were prepared similarly using pooled cells from all activation conditions. FMO controls were used for gating on positive populations. All samples were run on a BDR LSR Fortessa. Flow cytometry data was analyzed using FCS Express 4 software (De Novo Software, Glendale, CA, USA).

1.3 RESULTS

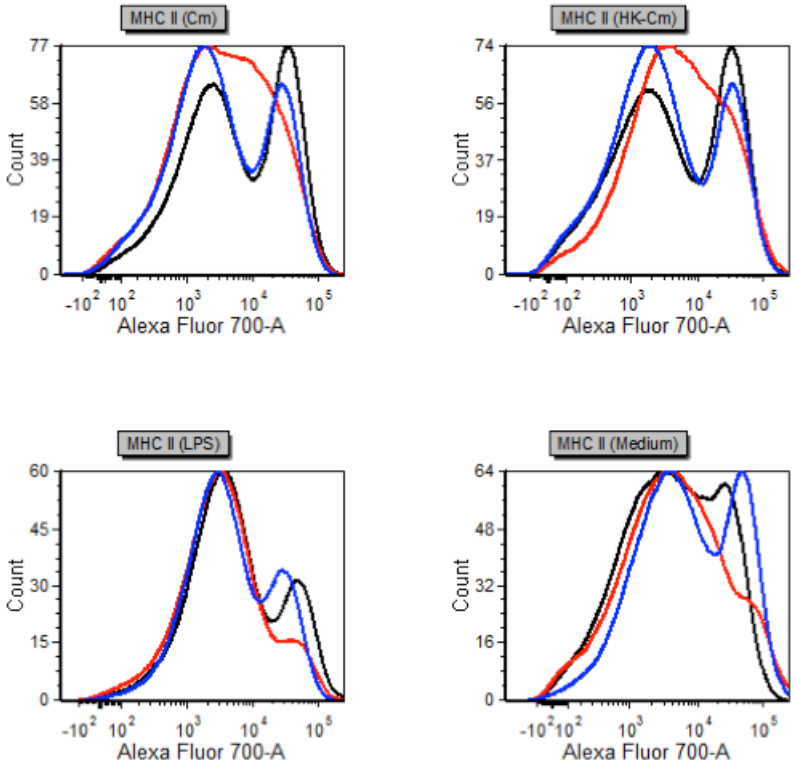
1.3.1 TLR2-deficiency alters the expression of dendritic cell activation markers following Chlamydia stimulation.

Following stimulation with *Cm,* BMDCs from Wsh mice displayed expression of activation markers that was generally similar to WT. Conversely, notable differences were seen in the activation of BMDCs from TLR2$^{-/-}$ mice compared to WT. For example, BMDCs appeared to have impaired development of MHC II^{high} populations compared to wildtype mice. Similar trends were also seen in the expression of CD80 and CD86 following activation with *Cm*. CD40 expression was similar to wildtype under activation conditions. These results may provide some insight into the decreased memory responses of TLR2$^{-/-}$ mice infected with *Cm*, but these experiments must be repeated to gain conclusive evidence.

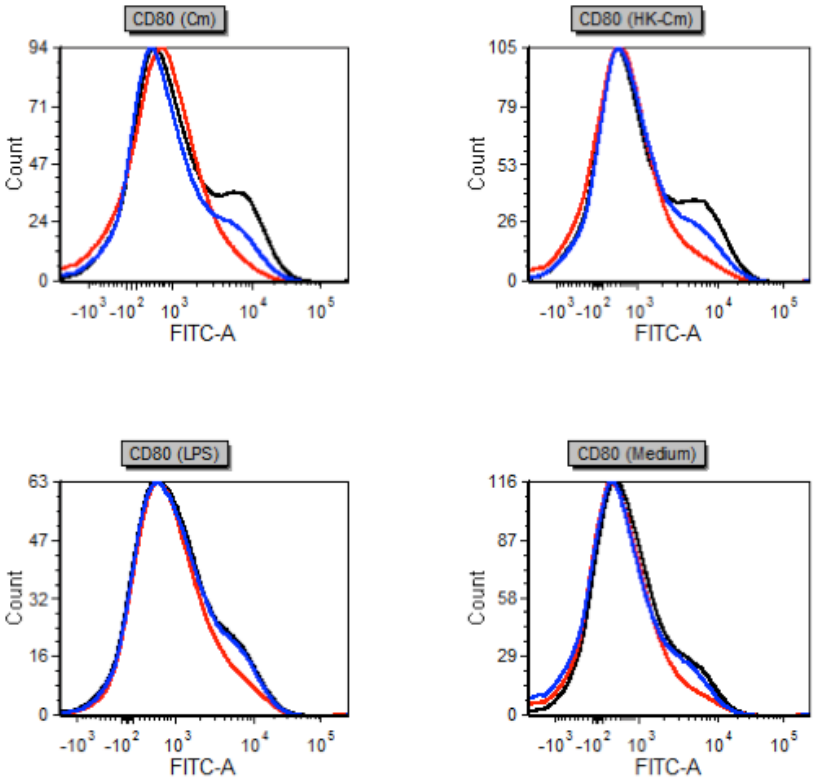
(A)


CD11c (Cm)
CD11c (HK-Cm)
CD11c (LPS)
CD11c (Medium)
Count
APC-A

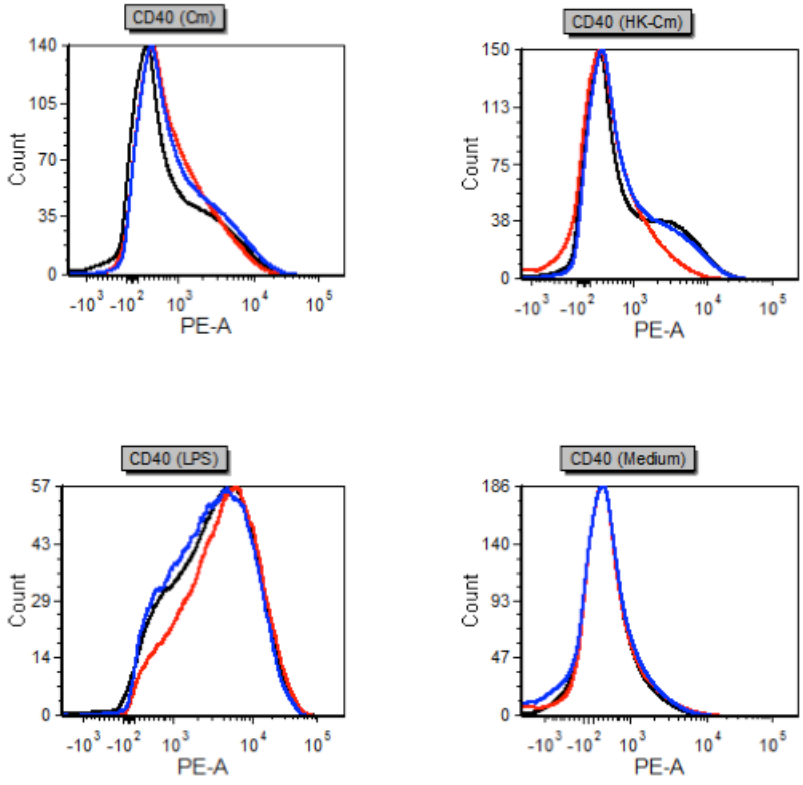
(B)


MHC II (Cm)
MHC II (HK-Cm)
MHC II (LPS)
MHC II (Medium)
Count
Alexa Fluor 700-A

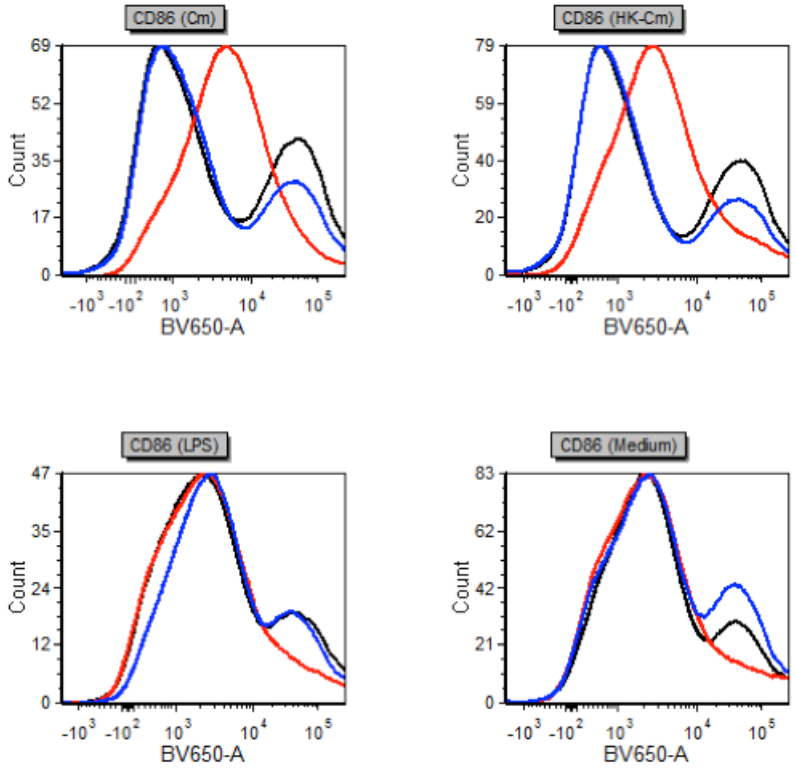
(C)


CD80 (Cm)
CD80 (HK-Cm)
CD80 (LPS)
CD80 (Medium)
Count
FITC-A

(D)


CD40 (Cm)
CD40 (HK-Cm)
CD40 (LPS)
CD40 (Medium)
Count
PE-A

(E)



Appendix Figure 1 Dendritic cells from TLR2$^{-/-}$ mice exhibit altered expression of activation markers following *Cm* stimulation *in vitro*, while dendritic cells from Wsh mice show expression patterns similar to wildtype. Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 (WT), Wsh and TLR2$^{-/-}$ mice. BMDCs were incubated for 24 hours with either *Cm* (MOI=0.5), heat-killed *Cm* (MOI=0.5), LPS (10 ng/ml) or medium alone. At 24 hours, BMDCs were analyzed for expression of CD11c (A), MHC II (B), CD80 (C), CD40 (D) and CD86 (E) under each of the stimulation conditions. Dead cells were excluded from analysis using a fixable viability dye (data not shown). Cells were gated on live for analysis of CD11c expression. Only live CD11c^{+} cells were included for analysis of other markers. For each histogram, black lines represent WT, blue represents Wsh and red represents TLR2$^{-/-}$ BMDCs. Histograms represent three individual wells, pooled and analyzed as N=1 per strain in a single pilot experiment.