THE ROLE OF INNATE-LIKE B1 CELLS IN THE REGULATION OF SEX-DEPENDENT IMMUNE RESPONSES TO *CHLAMYDIA* INFECTION

by

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For my Mom.

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ABSTRACT

Chlamydia infections predominantly impact women's health and the biological basis underlying the sex-dependent disparity is unclear. I demonstrate that female mice are significantly more susceptible to respiratory *Chlamydia* infection than their male counterparts, indicated by significantly greater body weight loss and higher bacterial loads. Furthermore, female mice produce more *Chlamydia*-specific antibodies, whereas male mice generate robust IFN-ɤ-mediated cellular immunity, but less IL-10- and IL-13-mediated responses. Remarkably, female mice have inherently more innate B1 cells, which migrate to the lungs and draining lymph nodes upon *Chlamydia* infection *in vivo*. Finally, I demonstrate that B1 cells, particularly the ones from female mice, produce a large quantity of IL-10 upon *Chlamydia* stimulation and potently induce inhibitory regulatory CD4 T cells differentiation *in vitro*. Together, I show that male and female mice are inherently different in B1 cells, which play a key role in host susceptibility to *Chlamydia* infection by regulating T cell responses.

LIST OF ABBREVIATIONS USED

°C	degrees Celsius
μg	microgram
μl	microliter
μm	micrometer
ACK	ammonium-chloride-potassium
AMI	acute myocardial infarction
ANOVA	analysis of variance
APC	antigen-presenting cell
BAFF	B cell activating factor
BALT	bronchus-associated lymphoid tissue
Bcl6	B cell lymphoma protein 6
BCR	B cell receptor
BLIMP-1	B lymphocyte-induced maturation protein-1
BMDC	bone marrow derived dendritic cells
BS	bovine serum
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
CCHD	chronic coronary heart disease
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
Cm	*Chlamydia muridarum*
Ct	*Chlamydia trachomatis*
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
DC	dendritic cells
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EB	elementary body
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FAS	fatty acid synthase
FASL	FAS ligand
FBS	fetal bovine serum
fDC	follicular dendritic cell
FOB	follicular B
FOXP3	forkhead box P3
FVU	first void urine
GATA	GATA-binding protein
GC	germinal center
GFP	green fluorescent protein
GM	growth medium
GMCSF	granulocyte/monocyte colony-stimulating factor
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
hrs	hours
HSP	heat-shock protein
i.p.	intraperitoneal
i.v.	intravenous
ICOSL	inducible T cell costimulator ligand
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
IFU	inclusion forming unit
Ig	immunoglobulin
IL	interleukin
IL-10 GFP	interleukin-ten ires gfp-enhanced reporter
ILB	innate-like B
kg	kilogram

LGV	lymphogranuloma
LPS	lipopolysaccharide
MEM	minimal essential medium
mg	milligram
MHC	major histocompatibility complex
mins	minutes
ml	milliliters
MLN	mediastinal lymph node
mm	millimeters
mM	millimolar
MOI	multiplicity of infection
MOMP	major outer membrane protein
MSM	men who have sex with men
MZB	marginal zone B
NETs	neutrophil extracellular trap
nIgM	natural IgM
OVA	ovalbumin
PAMPs	pattern associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	plasma cell
PCR	polymerase chain reaction
PD1	programmed cell death protein 1
PDL1	programmed cell death protein 1 ligand 1
PDL2	programmed cell death protein 1 ligand 2
pg	picogram
pH	potential of hydrogen
PID	pelvic inflammatory disease
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
PTX	pertussis toxin

qPCR	quantitative polymerase chain reaction
RAG	recombination-activating genes
RB	reticulate body
RNA	ribonucleic acid
ROR	retinoid-related orphan receptor
RPMI	Roswell Park Memorial Institute medium
s	seconds
SD	standard deviation
SEM	standard error of mean
SLAM	signaling lymphocytic activation molecule
SLE	systemic lupus erythematosus
SLO	secondary lymphoid organ
SPG	sucrose-phosphate-glutamic buffer
STAT	signaling transducer and activator of transcription
STI	sexually transmitted infection
T-bet	T-box transcription factor
TCR	T cell receptor
TFH	T follicular helper
TGF	transforming growth factor
Th	T helper
TLR	toll like receptor
TMB	tetramethylbenzidine substrate solution
TNF	tumour necrosis factor
Tr1	type 1 regulatory T cell
Treg	regulatory T cell
VLA-4	very late antigen 4
xid	x-linked immunodeficiency
XLA	x-linked agammaglobulinemia
XPB-1	xeroderma pigmentosum type B

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CHAPTER 1 INTRODUCTION

1.1 *Chlamydia* infection and characteristics

Chlamydia are a group of obligate intracellular bacteria that infect the epithelium of mucosal surfaces (1). There are currently three known species of *Chlamydia* that infect humans: *Chlamydophila pneumoniae, Chlamydophila psittaci,* and *Chlamydia trachomatis*.

1.1.1 Overview of *Chlamydia* associated diseases

Both *Chlamydophila pneumoniae* and *Chlamydophila psittaci* predominantly cause respiratory infections in humans. *C. pneumoniae* has no animal reservoir and is currently only found in humans (2). It is estimated to be the cause of 10% of community-acquired pneumonias and 5% of bronchitis and sinusitis infections (2). Though it only makes up a small percentage of community-acquired infections, serological studies show seroconversion in up to 50% of adults in the United States (2). Infections with *C. pneumoniae* are generally mild but there is the possibility of severe complications including encephalitis, myocarditis, and chronic infections (3).

Past infection with *C. pneumoniae* has been linked to numerous diseases including heart disease, Alzheimer's disease, and asthma. In 1988, the first evidence linking heart disease and *C. pneumoniae* titers was demonstrated in a cohort of men with acute myocardial infarction (AMI) and chronic coronary heart disease (CCHD) (4). The study demonstrated a significantly higher percentage of seroconversion among men suffering from AMI or CCHD compared to matched controls (4). Though this evidence was correlational in nature, Gaydos *et al.* found more direct evidence for the role of *C.*

pneumoniae in coronary heart disease when they demonstrated the ability of *C. pneumoniae* to infect key components of the atherosclerotic lesion including macrophages and aortic smooth muscle cells (5).

A study from 1998 demonstrated a possible link between *C. pneumoniae* and Alzheimer's disease. In this study, Balin *et al.* demonstrated that the affected area of the brain from Alzheimer's patients contained *C. pneumoniae* while no control patients were found to have evidence of *C. pneumoniae* (6). The mechanism in which *C. pneumoniae* interacts with or causes Alzheimer's disease has not been elucidated.

Finally, there are numerous studies linking *C. pneumoniae* and asthma (7). These studies have almost exclusively compared control and test groups by looking at the percentage of patients who had seroconverted (7). In 1998, more direct evidence was found in a small case study in which three asthma patients on steroid treatment were found to have serological evidence of *C. pneumoniae* infection (8). These patients were treated with clarithromycin or azithromycin successfully and were able to stop oral-steroids and return to inhaled asthma therapies (8).

The second species of *Chlamydia* known to cause respiratory infections in humans is *C. psittaci*. Unlike *C. pneumoniae*, *C. psittaci* is a zoonotic pathogen (9). Primarily infecting birds, *C. psittaci* can be transmitted to humans from the fluids of infected birds and more rarely from handling birds or from the environment of infected birds (9). Though transmission to humans is relatively rare, infection with *C. psittaci* has a high mortality rate (9).

The last species of *Chlamydia* known to infect humans is *Chlamydia trachomatis* (*Ct*). *Ct* can be broken down into serovars A-C, L1-L3, and D-K. Serovars A-C are the

causative agents of trachoma (9), an irreversible blindness caused by chronic inflammation followed by scarring of the conjunctiva (11). Trachoma is currently the leading cause of preventable blindness (11).

Serovars L1-L3 cause the sexually transmitted infection lymphogranuloma veneruem (LGV) (10). LGV is most commonly found in the men who have sex with men (MSM) population and HIV patients (12). LGV differs from other genital *Chlamydia* infections by travelling to lymphatics where it can go on to cause severe systemic disease (12).

The final serovars, D-K, infect columnar epithelial cells and cause conjunctivitis, infant pneumonitis, and urogenital infections (10). Conjunctivitis from *Ct* is usually related to a genital infection in which the patient auto-inoculates the eye with the bacterium (13). It has also been demonstrated that the common house fly, *Musca domestica*, can transmit *Ct* and lead to trachoma (14). In the case of infants, the infection arises almost exclusively after vaginal birth from an infected mother (15). When born to an infected mother, the infant may also acquire neonatal pneumonitis (16). The incidence of infants acquiring conjunctivitis or pneumonia from infected mothers has been reported to be as high as 44% and 17%, respectively, and therefore is a significant health burden (17).

Ct is most recognized for being the causative agent of urogenital infections. According to the Center for Disease Control Sexually Transmitted Disease Surveillance Report in 2016, *Chlamydia* was the most common bacterial sexually transmitted disease in the United States with almost 1.6 million reported infections (18). Unfortunately, *Ct* infections are overwhelmingly asymptomatic with 70% of women and 50% of men

showing no clinical symptoms, so the incidence is likely even higher than reported (10, 18).

Initial *Ct* infection can present as urethritis, cervicitis, salpingitis, epididymo-orchitis, or proctitis (10). Due to the asymptomatic nature of the infection, untreated infections can lead to secondary morbidities, especially in women. One such morbidity is pelvic inflammatory disease (PID) which occurs in up to one third of untreated women (19). PID is characterized by chronic inflammation of the upper genital tract of females and has been shown to lead to problems with fertility later in life including ectopic pregnancies and tubal infertility (10, 20).

Infection with *Ct* poses other risks for both males and females. There has long been an association between sexually transmitted infections (STI) and increased human immunodeficiency virus (HIV) risk (21). Specifically, *C. trachomatis* has been shown in studies with pig-tailed macaques to increase HIV infectivity through prolonged inflammation (22). In another study, which was done in human cell lines, it was demonstrated that *Ct* infection prior to HIV exposure allows the virus to cross the mucous membrane more efficiently (23).

1.1.2 Sex-associated epidemiology and consequences of *Chlamydia* infection

Interestingly both *Ct* and *C. pneumoniae* have been shown to have sex-specific epidemiology and sequelae following infection.

Since *Ct* became a reportable disease in the United States in 2000, females have always had a higher incidence compared to males (18). This is not the case for all STIs in the report, as both gonorrhea and syphilis are reported at similar rates between the sexes

(in the case of syphilis, the trend is skewed towards males if the MSM group is included but is similar among heterosexual males and females) (18). In women, *Ct* has been linked to increased risks when infections occur concomitantly with other STIs. For example, infection with *Ct* has been correlated with increased risk of future cervical cancer from human papilloma virus (HPV) (24). It has also been observed that while women can develop severe reproductive issues following *Ct* infection, a clear link between *Ct* and infertility has not been established in men (25).

Ocular *Ct* infections also have higher incidence in female compared to males, with trachoma being diagnosed 2-4 times as often in females (26). Furthermore, following ocular infection with *Ct*, females are more likely to have complications including trichiasis, corneal opacity, and scarring (26).As described above, the rate of vertical transmission for ocular *Ct* is high but it is not known if there is a difference in the rates of vertical transmission between the sexes (17).

Sex differences have been found following infection with *C. pneumoniae*. While the same sex-specific epidemiology is not observed, there are sex-specific consequences following *C. pneumoniae* infection. In one study, the seroprevalence of *C. pneumoniae* has been shown to be higher in males (3). It is important to note that differences observed in *C. pneumoniae* are not as striking as those observed in *Ct* and seroprevalence does not allow for a yearly incidence to be calculated (3). The sex-dependent incidence of *C. pneumonia* would have to be studied more thoroughly in order to draw any conclusions. Sex-specific consequences of *C. pneumoniae* infection have been identified in women. In a study completed in 2010, antibody against *C. pneumoniae* was measured in men and women over approximately 27 years (27). It was determined that women who tested

positive for anti-*C. pneumoniae* antibodies at the beginning and end of the study were more likely to have decline in lung function but it did not correlate with lung function in men (27).

While sex-specific differences have been characterized in *Chlamydia* infections, the underlying cause that leads to these differences has not yet been identified.

1.1.3 Life cycle of *Chlamydia*

Chlamydia has a unique biphasic life cycle (Figure 1.1). In the beginning of the life cycle, *Chlamydia* is an infectious but metabolically inactive elementary body (EB) (28). EBs are small, approximately 0.3 µm, and appear as dense round objects when viewed by electron microscopy (28). The EB has been shown to enter host cells through a variety of mechanism including phagocytosis (29), pinocytosis (30), and clathrin-mediated endocytosis (31). EB entry occurs 0-2 hrs post-infection and is followed by bacteria-driven changes in the phagocytic compartment that lead to the formation of an inclusion (32). EBs differentiate into the non-infectious metabolically active reticulate bodies (RB) 2-12 hrs post-infection (32). RBs are approximately 1 µm and appear granular when viewed by electron microscopy (28). The differentiation into RBs quickly leads to active protein synthesis; as early as 15 mins post-differentiation new proteins can be detected (33). The RBs divide rapidly by binary fission and create an inclusion body within the host cell (32). These RBs will re-differentiate into EBs in response to a currently unidentified signal and exit the host cell either by host cell lysis or extrusion (28, 34).

In some cases, *Chlamydia* will not immediately progress from the division of RBs

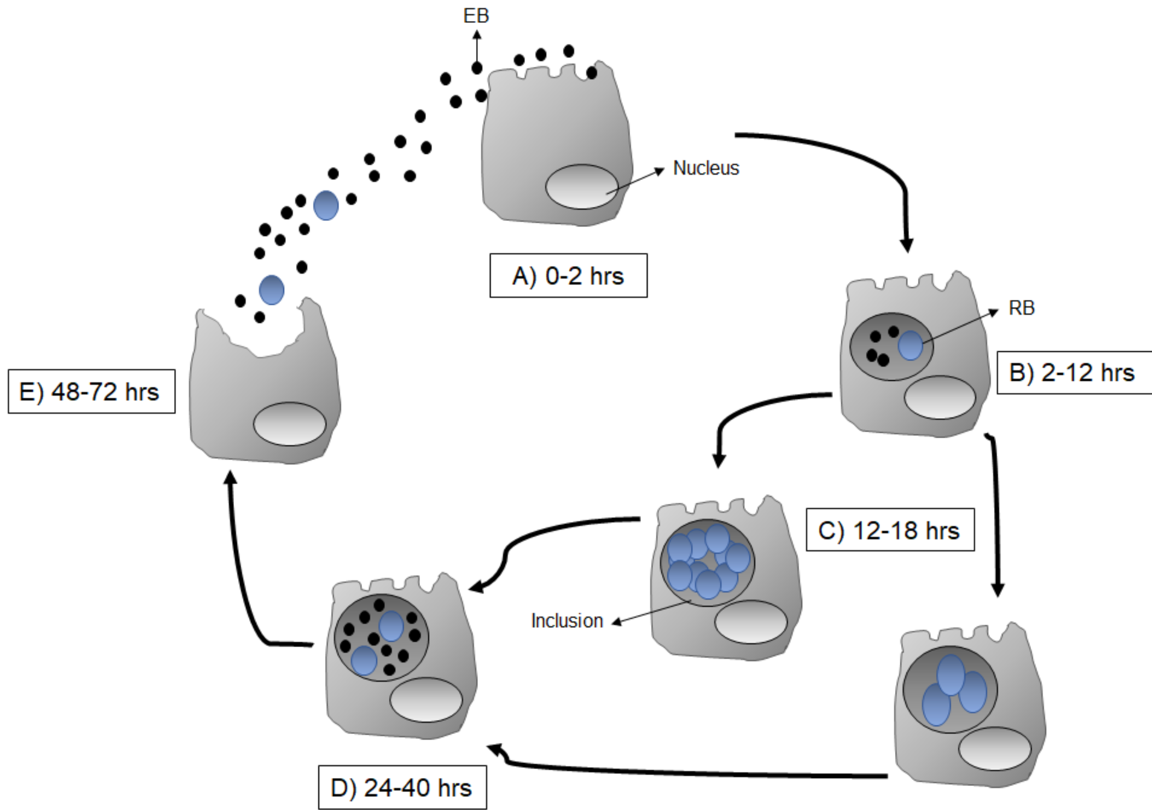


Figure 1.1: The life cycle and replication of *Chlamydia*. (A) *Chlamydia* elementary bodies (EBs) attach and enter the host cell. (B) 2-12hrs post-infection, the EBs differentiate into reticulate bodies (RBs) in an inclusion. (C) The RBs differentiate rapidly by binary fission in the inclusion (left arrow) or go into a persistent state (right arrow). (D) Once replication is complete, approximately 24-40 hrs after initial infection, or after persistence, the RBs will re-differentiate into EBs. (E) 48-72 hrs after the infection, EBs will be released from the host cell by lysis or extrusion and go on to infect neighboring cells to restart the life cycle. (28, 32)

to the re-differentiation into EBs but will instead go into a state of persistence (28). *In vitro*, persistence has been demonstrated in response to specific stimuli. The first observation of persistence was made in the 1970s, where penicillin was added to a culture of L cells containing *C. psittaci* (35). Using electron microscopy, it was observed that in the presence of penicillin, replication was stalled at the RB stage and lead to large abnormal RBs compared to controls (35). When the treated cells were placed in penicillin-free medium, the RBs returned to a normal shape and size and the replication cycle continued (35). More recently, *C. psittaci* has been shown to exhibit a persistent phenotype in response to phage infection (36).

Persistence has also been observed in *C. pneumoniae* models. Originally, it was observed in response to a continuous infection model (37) and shortly thereafter, was observed in response to IFNγ treatment (38).

In 1992, a similar phenomenon was first identified in *C. trachomatis* in response to stresses, including heat shock (39), followed by observations in response to amino acid starvation (40) and iron starvation (41). *C. trachomatis* persistence has also been demonstrated as the natural progression of infection in human monocytes (42), suggesting that this is not only a phenomenon seen *in vitro* but may play a role during *in vivo* infections.

Compared to *in vitro*, there is less evidence for *in vivo* persistence of *Chlamydia* infection. In 1995, *Ct* RBs that resembled persistent forms described *in vitro* were observed in synovial membranes of reactive arthritis and Reiter's syndrome patients (43). Similarly, *C. pneumoniae* was observed in patients with degenerative aortic valve stenosis (44). While this may suggest *in vivo* persistence, the inability to test the viability

of the *Chlamydia* remains a downfall of *in vivo* studies (45); therefore, concrete evidence for *in vivo* persistence has not yet been identified.

1.1.4 Mouse models of *Chlamydia* infection

To study *Chlamydia* infection *in vivo*, models have been established in a variety of animals with the most commonly used being guinea pigs and mice (46). In mice, both lung and genital models have long been established using the mouse pneumonitis strain of what is now known as *Chlamydia muridarum* (*Cm*) (47–49).

Originally isolated in 1942 by Nigg and Eaton, *Cm* was thought to be a virus (50). Eventually, the intracellular bacterium was characterized as a strain of *Ct* (51). In 1999, the 16S and 23S ribosomal RNA sequences of *Chlamydia* strains were analyzed and it was determined that the mouse pneumonitis strain belonged to a different species and was renamed *Cm* (49). *Cm* is now widely used to study *Chlamydia* infection *in vivo*. Because certain aspects of host responses to *Cm* infection differ in the respiratory and genital tract models, both infection models have been utilized in the field in order to obtain a comprehensive understanding of *Chlamydia* pathogenesis.

1.2 Host responses to *Chlamydia* infection

1.2.1 Innate immune responses to *Chlamydia* infection

During *in vivo Chlamydia* infections, the immune system of the host will initially respond through the activation of the innate immune system. This initial non-specific immune response is key for the control of early infection.

Defense against *Chlamydia* infection in the lungs and genital tract begins with the presence of antimicrobial peptides in the mucosa of the lungs, vagina, and cervix (52–54). Numerous antimicrobial peptides have been demonstrated to have activity against *Chlamydia* (55) but the bacterium has demonstrated the ability to downregulate these innate defenses through the serine protease chlamydial-proteasome/protease-like activity factor (CPAF) (56). While antimicrobial peptides are an important primary response, they are not sufficient to inhibit infection and can be subverted by *Chlamydia*.

Once *Chlamydia* enters epithelial cells and protein synthesis begins, the epithelial cells begin to secrete numerous inflammatory cytokines and chemokines including interleukin (IL) 8, granulocyte-monocyte colony stimulating factor (GMCSF), IL-6, IL-1α, and C-X-C motif chemokine ligand 1 (CXCL1) (57). Together, these cytokines and others work to create inflammatory conditions and recruit innate immune cells to the site of infection (58, 59).

Many of the chemokines produced in response to *Chlamydia* function as neutrophil chemoattractants (59). Neutrophils are the most abundant immune cell in human blood and have important functions in host defense and tissue repair (60). Neutrophils develop in the bone marrow and are released into the blood where they circulate and patrol for signs of infection or tissue damage (60).

Once at the site of infection, neutrophils have a multitude of ways to directly and indirectly assist in bacterial clearance. Neutrophils are professional phagocytes and contribute to bacterial clearance principally through phagocytosis (61). Neutrophils are activated to phagocytose bacteria in response to the crosslinking of receptors on the cell surface which leads to changes in the cytoskeleton of the neutrophil, allowing for

phagocytosis of the recognized pathogen (60, 61). Once internalized, the phagosome will fuse with granules containing antimicrobial agents leading to killing of the internalized pathogen (60).

Neutrophils have a unique secondary mechanism to kill pathogens, known as neutrophil extracellular traps (NETs). First described in 2004, NETs are chromatin webs released from neutrophils that contain granule contents (62). Pathogens are trapped by the chromatin "netting" and exposed to granule contents at a high concentration, allowing for direct killing of pathogens (62). Evidence for NET-mediated killing has not yet been described in *Chlamydia* infection.

While neutrophils may contribute to bacterial clearance, they have also been linked to pathological outcomes. In ocular *Chlamydia* infection models, neutropenic mice have significantly less pathology (63). Similarly, *Cm*-induced hydrosalpinx is decreased in neutropenic mice following genital tract infection, suggesting a role for neutrophils in the promotion of infertility following *Chlamydia* infection (64). A similar association has also been identified in humans. In 2002, a study examined pathological outcomes following *Neisseria gonorrhea*, *Trichomonas vaginalis*, and *Chlamydia trachomatis* in women (65). It was determined that elevated neutrophil-derived defensins were associated with endometriosis, a cause of infertility in women (65, 66).

Though the role of neutrophils during *Chlamydia* infection is controversial, neutrophils do have an important role in bridging innate and adaptive immunity by recruiting antigen presenting cells (APCs) (67, 68).

APCs are key components of the innate immune system. Professional APCs, such as dendritic cells (DCs), B cells, and macrophages, share key characteristics which

include the expression of pattern recognition receptors (PRRs), the ability to phagocytose, the expression of major histocompatibility complex (MHC) II, and the expression of co-stimulatory molecules upon activation (69).

APCs recognize non-self through the use of PRRs. A specific subset of PRR, known as Toll-like receptors (TLRs), have been shown to play a role in pattern recognition during *Chlamydia* infection (70).

TLRs are transmembrane proteins that recognize a variety of pathogen associated molecular patterns (PAMPs) (71). TLRs 1-9 are found in humans and mice, TLR10 is found only in humans, while TLRs 11-13 are only found in mice (72). TLRs can be separated based both on location and what PAMPs they recognize (71). TLRs 1, 2, 4-6, and 10 are expressed on the cell surface while TLRs 3, and 7-13 are expressed in intracellular compartments (72–76).

It has been demonstrated that various TLRs have a role in *Chlamydia* infection. In a mouse model of pneumonia using *Cm*, it was demonstrated that TLR2 is necessary to clear *Cm* infection *in vivo* while TLR4 aids in the clearance of infection but is not necessary (77). Interestingly, although *Chlamydia* is Gram negative, TLR4 was not found to be the most important TLR for recognition of *Chlamydia* (72, 77). A more important role for TLR2 over TLR4 for recognition of *Cm* has also been demonstrated in a murine model of genital tract infection (78).

Once APCs are activated, the cells upregulate MHCII and co-stimulatory molecules on the cell surface (79, 80). This upregulation is much stronger in DCs compared to macrophages and B cells (81). Internalized proteins are processed by APCs

in endolysosomes and broken down into peptides to be presented on MHCII to T cells (79).

During presentation to T cells, APCs act as a bridge between innate immunity and the activation of the adaptive immune response. When APCs present to T cells there are three signals required for the T cells to be properly activated (82). The first signal comes from MHCII on APCs presenting peptides to the T cell receptor (TCR) (82). The second signal required are co-stimulatory molecules (82). The third signal necessary for T cell activation is cytokine production by the APC (82). Together, these signals allow APCs to activate and influence the adaptive immune system.

1.2.2 Adaptive immune responses to *Chlamydia* infection

While innate immune responses are rapid and non-specific, adaptive immune responses are antigen-specific and take longer to develop. Adaptive immunity can be broken into two main arms; cell mediated responses that are primarily regulated by T cells, and humoral responses that are primarily regulated by B cells. Both arms of the adaptive immune system are key in the response to *Chlamydia* infection.

1.2.2.1 T cells and cell mediated responses

T cells are the predominant drivers of adaptive cell mediated immunity and can be broken down into two major subsets: cluster of differentiation (CD)4$^+$ T helper cells and CD8$^+$ cytotoxic T cells. T helper cells can further be subdivided into 5 major categories that include T helper (Th) 1, Th2, Th17, regulatory T cells (Treg), and T follicular helper cells (TFH).

Th1 cells develop from naïve $CD4^+$ T cells when stimulated with IL-12 and under the regulation of the master transcription factor T-bet and signal transducer and activator of transcription (STAT) 4 (83). Once differentiated, Th1 cells produce interferon (IFN)γ and tumour necrosis factor (TNF)α (83). The Th1 subset is important for immunity to intracellular bacteria such as *Mycobacterium tuberculosis*, viruses including respiratory syncytial virus, and certain fungi (83, 84).

Th1 responses have been shown to be protective following *Chlamydia* infection (85). In mouse models of respiratory and genital tract infection using *Cm*, protective Th1 responses are significantly upregulated compared to Th2 responses following infection and both IL-12 and IFNγ are believed to be important components of the Th1 response (86, 87). IFNγ is believed to contribute to *Chlamydia* clearance through the induction of indoleamine 2,3-dioxygenase (IDO) (88). The production of IDO in response to IFNγ causes the degradation of tryptophan, which in turn inhibits chlamydial growth (88). IFNγ from Th1 cells also contributes to *Chlamydia* clearance through the induction of IFNγ-dependent inducible nitric oxide synthase, possibly from epithelial cells (42, 89). Th1 cells are also important in *Chlamydia* because Th1 responses inhibit the development of Th2 cells (90)

Th2 cells develop when naïve $CD4^+$ T cells are stimulated with IL-4 under the regulation of the master transcription factor GATA-binding protein (GATA)3 and STAT6 (83). Th2 cells are characterized by the production of IL-4, IL-5, and IL-13 and are important for the clearance of helminths (83).

Th2 responses are not able to clear a primary *Chlamydia* infection in mice, but interestingly, are strongly activated in human female genital tracts following infection,

where Th2 cells and responses are thought to inhibit immunopathology (91, 92). There may be a balance of Th2 cells required to inhibit pathology but not so many that the cells would inhibit more protective responses.

Th17 cells are another subset of Th cells and develop when naïve $CD4^+$ T cells are stimulated with IL-6 and transforming growth factor (TGF) β (83). Under the control of the transcription factors retinoid-related orphan receptor (ROR)α and RORγt and signaling via STAT3, Th17 cells produce IL-17A, IL-17F, and IL-22 (83, 93). Th17 responses are important for clearance of extracellular bacteria and fungi (83).

The role of Th17 cells during *Chlamydia* infection can be both protective and non-protective. In humans, women with genital *Ct* infection have upregulated IL-17 secretion in the genital mucosa, suggesting an involvement of Th17 cells in human infection (94). In mouse models of genital infection, Th17 responses have been shown to cause tissue damage, while in murine respiratory models, IL-17 signaling recruits neutrophils and leads to *Chlamydia* susceptibility (95, 96). Conversely, it has also been shown in both respiratory and genital tract mouse models that Th17 cell-mediated responses promote protective Th1 cell generation (97, 98). Together these results demonstrate both pathogenic and protective roles for Th17 cells during *Chlamydia* infection and suggests a need for a balanced response to infection.

Tregs are important mediators of immune regulation (83). Tregs arise when naive $CD4^+$ T cells are stimulated with TGFβ under the control of the master transcription factor forkhead box (FOX)P3 and STAT5 (83). Once differentiated, these $FOXP3^+$ Tregs produce TGFβ, IL-10, and IL-35 (99). It is, however, important to note that $FOXP3^+$ Tregs are only one subtype of Tregs. Tr1 cells are a type of Treg known to be activate by

and produce large amounts of IL-10 but do not have a defined master transcription factor (100, 101).

The role of Tregs during *Chlamydia* infection varies. Tregs are necessary to inhibit asthma responses following *C. pneumoniae* infection, suggesting a protective role for these cells (102). Conversely, Tregs have also been shown to convert into Th17 cells following genital *Cm* infection, leading to neutrophil infiltration and increased pathology (103). The role of Tregs may vary depending on infection model.

The final subset of $CD4^+$ T cells are TFH cells, which play a key role in shaping and generating humoral responses (104). Pre-TFH cells develop from naïve $CD4^+$ T cells under control of the master transcription factor B cell lymphoma protein (Bcl)6 in response to IL-6 and IL-21 and will develop into full TFH cells following interactions with cognate B cells (105–107). TFH cells express high levels of CXC-chemokine receptor (CXCR)5, allowing them to migrate to the germinal centres (GCs) of lymph nodes and secondary lymphoid organs (SLOs) where they promote humoral B cell responses (105, 106). The role of TFH cells in *Chlamydia* infection has not specifically been studied but is related to the role of antibody responses during infection which will be discussed in 1.2.2.2.

The other major subset of T cells is $CD8^+$ T cells, or cytotoxic T cells. $CD8^+$ T cells produce large amounts of IFNγ, TNFα, and IL-2 after activation and directly kill target cells through fatty acid synthase (FAS)-FAS ligand (FASL) interactions as well as perforin and granzyme excretion (83, 108).

During respiratory *Cm* infection, the depletion of $CD8^+$ T cells results in a significantly higher bacterial burden in the lungs of mice which correlates with a trend for

increased mortality in the mice, suggesting that $CD8^+$ T cells are important for the clearance of bacteria during *Cm* infection (109). Interestingly, perforin, granzyme, and FAS-mediated apoptosis are not necessary to clear genital *Cm* infection, suggesting that $CD8^+$ T cells contribute to bacterial clearance by another mechanism (110). It was suggested that TNFα production could contribute to infected cell apoptosis but TNFα knock out mice clear bacteria efficiently (110, 111). These studies suggest that while $CD8^+$ T cells may aid in bacterial clearance, they are not necessary. Furthermore, studies of genital tract *Cm* infection in CD8 knock out mice demonstrated that there is less pathology in the absence of $CD8^+$ T cells, so overall $CD8^+$ T cells are not protective or necessary for a successful immune response to *Chlamydia* (111).

1.2.2.2 Humoral responses

Humoral responses are primarily mediated by B cells but require a complex network of events in order to occur. This process begins in the T cell zone of lymphoid tissues where APCs activate naïve T cells to differentiate into pre-TFH cells (107). Pre-TFH cells express CXCR5 which allows them to traffic to B cell follicles rich in CXCL13 (112). In the periphery of the B cell follicle, pre-TFH cells will find cognate B cells and form stable conjugates that will move into the germinal center (GC) (107). Follicular dendritic cells (fDCs) are important in maintaining GC reactions through long-term presentation and retention of antigen (113). Follicular B cells (FOB) with high affinity receptors will differentiate into PCs or memory B cells following cognate interactions with TFH cells (114).

In 1983, the first study linking antibody responses and *Chlamydia* infection was done in humans (115). Endocervical swabs and serum samples were taken from 95 women with active *Ct* infections to test bacterial burden, serum anti-*Ct* antibody levels, and local anti-*Ct* antibody levels (115). It was found that secretory immunoglobulin (Ig)A levels were higher in females with lower bacterial burdens, suggesting that antibody responses aid in the control of chlamydial shedding (115).

Following this study, many different groups undertook studies creating monoclonal antibodies against *Chlamydia* proteins to study bacterial neutralization *in vitro* to better understand antibody responses. Monoclonal IgG3 and IgG antibodies against major outer membrane protein (MOMP) were found to neutralize *Chlamydia in vitro* through a mechanism that followed internalization (116, 117). In another study, a monovalent Fab fragment against *Chlamydia* MOMP was able to inhibit the attachment of *Chlamydia* to cells, and therefore prevent entry of the bacteria into host cells (118). Finally, a study using monoclonal IgA was found to decrease infectivity and vaginal shedding when *Chlamydia* was pre-incubated with the antibody before infecting mice, supporting the original human study (119). Together these studies suggested that multiple immunoglobulin subtypes had the ability to effect *Chlamydia* infection and may play a role in the immune response to the bacterium. It is, however, important to note that demonstrating antibody neutralization *in vitro* does not prove that this occurs *in vivo*. More studies need to be undertaken to elucidate the *in vivo* neutralization capabilities of these antibodies.

Considering the potential role of antibody in the response to *Chlamydia*, experiments were carried out in mice to better understand the role of B cells and

antibodies following infection. To this end, mice were treated with anti-IgM from birth in order to inhibit the development of B cells and then challenged intravaginally with *Cm* (120). Surprisingly, B cell deficient mice cleared *Cm* infection as well as control mice and upon secondary challenge, B cell deficient mice were equally as resistant to reinfection as the controls (120). Overall, this study suggested that B cell responses were unnecessary for the control of primary or secondary *Chlamydia* infection.

In a subsequent study, μMT/ μMT antibody-deficient mice were infected with *Cm* in order to study antibody depletion more specifically (121). While results from primary infection were similar to those seen in B cell-deficient mice, the μMT/ μMT mice were more susceptible to secondary infection, suggesting that B cell responses are important for the control of subsequent *Chlamydia* infections but are not necessary to clear primary infection (121).

While the role of antibody production in secondary *Chlamydia* infections is protective, antibody responses have also been linked to immunopathology. A correlation between anti-*Chlamydia* heat shock protein (HSP)60 antibody and PID was identified in cohort of women in 1997 and confirmed in a model using southern pig-tailed macaques (122, 123). Anti-*Chlamydia* HSP60 antibody has also been correlated with tubal infertility and an increased risk for the development of cervical cancer (124, 125).

A more recent study identified that antibody to certain *Cm* proteins were correlated with pathology in the genital tracts of mice (126). Specifically, they identified that mice who developed hydrosalpinx following infection had antibodies against the *Cm* proteins TC0582 and TC0912c, while mice without hydrosalpinx had antibodies against a

variety of other proteins (126). These results suggest that certain antibody responses may result in pathology following *Cm* infection, while others may not have the same effect.

Overall, the role of antibody responses to *Chlamydia* infection are controversial. While antibody helps protect against secondary infection, it may also lead to severe pathology and is unnecessary for protection against primary infection.

1.3 Innate-like B cells

The study of B cell responses to *Chlamydia* infection has so far focused on the role of FOB cells and adaptive antibody production. In our study, we also considered the role of innate-like B cells, a subset of B cells that is characterized by the ability to respond to infection independent of Th cells (127).

1.3.1 Identified subsets and characteristics

Innate-like B cells consist of two major subsets: marginal zone B (MZB) cells and B1 cells (128). In contrast to antibody produced by FOB cells, the antibody made by innate-like B cells is low affinity and often polyreactive (129). MZB and B1 cells share many of the same functions so we will focus on B1 cells herein because they are a key aspect of this research.

The term marginal zone (MZ) was originally coined in 1929 for the area in the spleen between the red and white pulp (130). The cells of the MZ include reticular cells, macrophages, and marginal zone B (MZB) cells (130). The spleen is a key organ in the defense against pathogens and due to slow blood flow through the MZ, it is a key site of antigen surveillance for the circulatory system (131, 132). MZB cells reside exclusively

in the spleens of mice (130, 133). In mice, MZB cells are currently characterized as $B220^{hi}CD21^{hi}CD23^{-}$ cells (134). Human MZB were proposed in 2004 and are characterized as $IgM^{+}IgD^{+}CD27^{+}$ (135). In humans, MZB cells have also been identified in the subcapsular sinus of lymph nodes, the tonsils, and the subepithelium of Peyer's patches (136–139).

B1 cells were first identified in 1983 but were originally called Ly-1 B cells (140). Though these cells were originally observed in the spleen, it was later shown that a large population resides in the peritoneal and pleural cavities, where they are the main B cell population (140, 141). It has been shown that peritoneal-derived B1 cells differ significantly from the originally identified splenic B1 cells (142). Specifically, the surface IgM levels, the response to phorbol 12-myristate 13-acetate (PMA), and the expression of numerous genes differ significantly between the two principle B1 cell populations (142). Currently it is understood that B1 cells also reside in bone marrow, blood, omentum, secondary lymphoid tissues, and mucosal sites (143, 144). Whether these B1 cells more closely resemble the peritoneal or splenic B1 cell populations has not yet been described.

In 1992, the original $CD5^{+}$ Ly-1 B cells were shown to be distinct from FOB cells and $CD5^{-}$ Ly-1 B "sister" cells were discovered and renamed B1a cells and B1b cells, respectively (145). The term B1 cell arose from the discovery that B1 cell ontogeny began earlier in development compared to MZB cells and FOB cells (collectively called B2 cells) (146). Currently, B1 cells in mice are identified as $CD19^{+}CD43^{+}IgM^{hi}B220^{lo}CD5^{+}$ B1a cells and $CD19^{+}CD43^{+}IgM^{hi}B220^{lo}CD5^{-}$ B1b cells.

A human counterpart to murine B cells has been identified but the identification was controversial. In 2011, B cells with the phenotype $CD20^+CD27^+CD43^+CD70^-$ were proposed as human B1 cell equivalents (147). To identify B1 cells, the researchers sorted B cell populations from adult peripheral blood and umbilical cords and tested the ability of the B cell subsets to conform to typical murine B1 cell characteristics including the secretion of natural IgM (nIgM) without antigen exposure, the ability to activate T cells, and tonic intracellular signaling (147). Using these parameters, the aforementioned phenotype was identified.

Soon after the original identification of human B1 cells, two independent studies observed that the identified population may have been T cell doublets, due to expression of CD3 on the putative B1 cells (148, 149). The original researchers did not identify $CD3^+$ events and so it was unlikely that the putative B1 cells were T cell doublets (150). Following this, a separate research group characterized the putative B1 cells as pre-plasmablasts (151). In this study, human "B1" cells were differentiated *in vitro* into $CD20^-$ cells and gene expression profiling supported a plasmablast-like phenotype (151).

In 2016, human B1 cells were finally defined separately from all other antibody producing circulating B cells (152). Based on the ability to secrete nIgM and through gene expression analysis, four circulating B cell subsets were identified: $CD19^+CD20^+CD27^+CD38^{lo/int}CD43^+$ B1 cells, $CD19^+CD20^-CD27^{hi}CD38^{hi}$ conventional plasmablasts, $CD19^+CD20^+CD27^{hi}CD38^{hi}$ "20^+38^{hi}" B cells, and $CD19^+CD20^+CD27^+CD43^-$ memory B cells (152). In order to demonstrate that human B1 cells are separate from pre-plasmablasts, the populations were cultured with IL-2, IL-6, and IL-15 for 3 days to promote plasmablast differentiation (152). It was demonstrated

that B1 cells did not expand into plasmablasts following attempted activation but, instead, the "$20^{+}38^{hi}$" B cells had significant expansion, suggesting that these cells are the true pre-plasmablast subset in peripheral blood (152). From this study, a clear phenotype for human B1 cells was identified.

1.3.2 Ontogeny of B1 cells

Early in the history of B1 cells, when they were originally identified as Ly-1 B cells, it was discovered that these B cells did not share the same progenitors as FOB cells (141). In the first study on Ly-1 B cell ontogeny, peritoneal cells were transferred to irradiated recipient mice intravenously (i.v.) and were able to reconstitute themselves in the cavity, demonstrating for the first time that Ly-1 B cells are self-renewing (141). When bone marrow was transferred from adult mice to irradiated mice in the same study, only FOB cells were reconstituted in the recipient mice (141). Finally, when cells from the spleen, liver, or bone marrow from young animals was used to reconstitute mice, both FOB and Ly-1 B cells were reconstituted in the irradiated mice (141). Together, this study demonstrated for the first time that B1 cells are self-renewing and have distinct progenitors from FOB cells. Subsequent studies also demonstrated that Ly-1 B cell progenitors could arise from the fetal omentum (153).

After the identification of Ly-1 "sister" cells and the nomenclature change to B1 cells, more studies were conducted on B1 cell ontogeny. It was found that B1b cells could be reconstituted from the fetal liver like B1a cells but they could also be reconstituted from adult bone marrow (145). Following these early studies, there was controversy in the field regarding whether the lineage differentiation between B1 cells

and B2 cells occurred completely separately or if B1 cells arose from a developmental step following initial B2 cell initiation (134).

In 2011, this was finally clarified by the discovery of B1 cell progenitors in the fetus at embryonic day 9-9.5 in the yolk sac and intra-embryonic para-aortic splanchnopleura (154). At day 11, these precursors can be identified in the fetal liver (154, 155). More recently, precursors for B1 cells were identified in the spleen of adult mice (134). Splenic cells were isolated and injected i.v. into $RAG^{-/-}$ mice where lin^{-} $CD19^{+}B220^{lo/-}CD43^{-}$ cells gave rise to B1 cells but not FOB cells (134).

Importantly, a key molecule necessary for B1 cell development has been identified. Mice lacking Bruton's tyrosine kinase (BTK) are known as x-linked immune deficiency (Xid) mice (156). Xid mice lack B1 cells and precursor B1 cells but also have lower levels of conventional B cells (156, 157). Specifically, there is still one third to one half of normal conventional B cell numbers but there is almost a complete abrogation of B1 cell development (158). Xid mice allow for the study of immune responses in the absence of B1 cells but because there is also a slight deficiency in B2 cells in this model, the results have to be confirmed using other methods.

Xid mirrors the human disease x-linked agammaglobulinemia (XLA) where BTK deficiency also results in B cell development arrest but to a more severe degree (156). In humans with XLA, there is a severe block in all B cell development and extremely low production of immunoglobulin (156, 159). The almost complete lack of immunoglobulin suggests B1 cells are also deficient in XLA but considering that B1 cells were just recently confirmed in humans, there would need to be specific studies conducted to identify a B1 cell deficiency in XLA.

Together, these studies demonstrate conclusively that B1 cells arise separately from B2 cells and are, in fact, individual B cell lineages.

1.3.3 Role of B1 cells in autoimmunity

B1 cells produce large amounts of natural antibody (143). In B1 cells, the principal natural immunoglobulin is IgM but IgA and IgG can also be produced (129, 160). While this antibody has an important role in the immune response to pathogens (discussed in 1.3.4), it has also been related to autoimmune responses because it is often polyreactive (129). Since the original identification of B1 cells, there has been a large amount of research into the role of these cells in autoimmune diseases.

Ly-1 B cells were originally identified in New Zealand Black (NZB) and the F1 progeny of NZB and New Zealand White mice (BWF_1), which are typically used as mouse models of autoimmune disease (140). Since the identification of B1 cells, they have been associated with various autoimmune diseases including systemic lupus erythematosus (SLE) (140). Various roles for B1 cells have been identified in SLE and so it will be used here as an example to demonstrate how B1 cells can contribute to autoimmune responses.

The BWF_1 mice that were used to first characterize Ly-1 B cells are used as models of the autoimmune disease SLE (161). SLE is an autoimmune disorder that is predominantly found in females and has symptoms that greatly vary between patient but can include rashes, arthritis, oral ulcers, kidney complications, and anemia (162, 163). The strongest evidence for a role for B1 cells in SLE came from a B1 cell depletion study (164). In this study, distilled water was injected into mice i.p. to lyse the cells that reside

in the peritoneal cavity (164). When B1 cells were eliminated from the mice, the pathogenic IgG, thought to be responsible for kidney pathology, was greatly reduced (164). The renal pathology of these mice was greatly reduced compared to controls who did not receive water injections, suggesting that B1 cells are a predominant contributor of pathogenic antibody in the mouse model of SLE (164).

Subsequent studies on the role of B1 cells in SLE mice have identified antibody-independent roles of these cells in disease progression which instead focus on the ability of these cells to shape the adaptive immune system through antigen presentation and cytokine production (162).

There has been evidence that B1a cells contribute to pathology by acting as antigen presenting cells in a model of SLE using New Zealand Mixed (NZM) mice (an inbred strain from BWF_1). It was found that there was an accumulation of B1a cells with high expression of the co-stimulatory molecules CD80 and CD86 in NZM mice (165). When compared to B2 cells from the NZM mice *in vitro*, B1a cells had stronger antigen presenting capabilities as demonstrated by an increase in T cell proliferation in response to B1a cells as APCs and increased production of IFNγ (165). These findings were elaborated on in 2007, when Ishikawa & Matsushima demonstrated that B1 cell homing to the peritoneal cavity in NZM mice was impaired due to enhanced CXCL13 levels in diseased organs (such as the liver or lungs) (166). *In vitro*, they demonstrated that the B1 cells at the disease sites were capable of interacting with TFH cells to produce autoreactive antibody and were capable of activating T cells through antigen presentation (166). Together, these studies demonstrated a clear role for B1 cells in SLE by shaping the adaptive immune response through antigen presentation.

In 1994, a study was performed in BWF_1 mice where researchers injected anti-IL-10 into the mice and found that the onset of autoimmune disease was slower than in control mice (167). Knowing that IL-10 administration has been shown to specifically deplete B1 cells, this suggests that depletion of B1 cells can lower the severity of SLE (168). B1 cells were identified to be key producers of IL-10 in 1992 and may, therefore, support their own expansion during lupus by producing large amounts of IL-10 (169). Together these experiments further demonstrate the detrimental role that B1 cells play during SLE and also suggest a role for B1 cell cytokine production in SLE progression.

It is evident from studies in SLE that B1 cells are more than antibody producing factories in the context of autoimmunity. They are also able to affect adaptive immune responses through antigen presentation and cytokine production.

1.3.4 Role of B1 cells in infection

B1 cells are also important mediators of immunity to infection. During infection, the role of B1 cells differs greatly from the role of FOB cells and this begins with how the cells are activated. *In vitro*, B1 cells have been shown to upregulate the PC transcription factors B lymphocyte-induced maturation protein-1 (BLIMP-1) and xeroderma pigmentosum type B (XPB-1) in response to stimulation with agonists of TLR 1/2, 2/6, 4, 7, and 9, while TLR agonists alone are not enough to induce the differentiation of FOB cells (170). Interestingly, *in vivo*, cross-linking of the B cell receptor (BCR) has been shown to enhance the response of B1 cells to innate antigen when it is activated in concert with TLR signaling (171). The BCRs of B1 cells are less

diverse than those of FOB cells and are primarily reactive to common pathogen antigens and self-antigens (172).

Following activation, B1 cells migrate from the pleural and peritoneal cavities to the site of infection (172). The exact mechanism that allows for migration of these cells is controversial and may be dependent on the model. One generally understood principle of B1 cell migration is the involvement of the omentum but the signals and surface receptors involved in emigration are not fully understood (173).

The chemokine CXCL13 is involved in the recruitment of B cells to lymph node and spleen follicles (174). Knowing this, the role of CXCL13 in B1 cell homing has been investigated and it is now understood that CXCL13 is necessary for the proper homing of B1 cells to the peritoneal cavity (144). Because CXCL13 is known to be important in B1 cell homing, its potential role in B1 cell redistribution following antigenic challenge has been studied in various models. During influenza infection, B1 cells upregulate the CXCL13 receptor CXCR5, but interestingly, CXCL13 did not improve the migration of these cells, suggesting that it is not necessary for B1 cell migration following influenza challenge (173). In another study, the role of all G protein coupled receptors was examined by injecting pertussis toxin (PTX)-treated B1 cells into recombination-activating gene 2 $(RAG2)^{-/-}$ mice and challenging with lipid A from lipopolysaccharide (LPS) (175). In mice that received PTX-treated B1 cells, there was a severe downregulation of B1 cell egress from the peritoneal cavity (175). When the specific receptors involved were investigated, it was determined that CXCL13 from the omentum was necessary for B1 cell migration in response to lipid A (175). Together, these data do

not provide a definitive role for CXCL13 but suggest that, in some cases, it may be necessary for B1 cell egress to sites of infection.

Various integrins have also been studied to examine a possible role in B1 cell migration. Integrins are heterodimeric trans-membrane proteins made of individual α and β subunits and have been long understood to play a role in cell migration (176, 177). In a skin model using complete Freund's adjuvant to induce inflammation, B1 cell migration to inflamed skin was found to be dependent on the integrin α4β1 (also called very late antigen 4 [VLA-4]) (178). VLA-4 is constitutively expressed on B1 cells and when it is blocked by an antibody, B1 cells no longer migrate to sites of inflammation (178). It is important to note that as an immediate response to infection, VLA-4 is downregulated, potentially allowing for release from the peritoneal cavity before being re-activated allowing migration to the site of inflammation (173). This hypothesis is supported by work that demonstrated the downregulation of multiple integrin subunits, including α4 and β1, in the hours following LPS challenge (175).

In influenza infection, the integrin CD11b has been associated with the migration of B1 cells to the mediastinal lymph node (MLN) (173). In this model, type 1 IFNs produced at the site of infection activate CD11b on the surface of B1a cells allowing for the egress of B1 cells from the pleural cavity to the MLN (173). Similar results are found using systemic LPS challenge in which B1a cells that migrate from the peritoneal cavity to the spleen during infection are a $CD11b^+$ subset (179). Together, these studies suggest an important role for CD11b expression in the migration of B1 cells from both the pleural and peritoneal cavities.

Following migration, B1 cells produce natural antibody, secrete cytokines in response to infection, and can act as APCs.

B1 cells are often characterized by their ability to produce natural antibody, and this function of B1 cells has been well studied. B1a and B1b cells are able to produce large amounts of nIgM and IgA at the site of infection or the draining lymph node for the site (143, 180–183). This rapid antibody secretion is important in many different infections and provides an important source of antibody before the adaptive immune system is activated (143).

Following migration to the MLN in response to influenza infection, B1a cells produce large amounts of polyreactive IgM, while B1b cells have not been shown to migrate and therefore do not contribute to the antibody response (143, 180). The IgM produced by B1a cells is non-specific and as little as 10% of the IgM produced actually binds the virus; nevertheless this IgM is important for initial control of the infection (143, 180).

In response to systemic LPS challenge, B1a cells are also the primary responders and will migrate to the spleen, as described above, where one subset of B1a cells immediately differentiates into antibody producing PCs, while another subset will undergo one round of division before they differentiate (179). Together, these B1a cells will allow for rapid IgM secretion and the divided cells will allow for the maintenance of B1a cells in the spleen (179). Similarly, in response to i.p. injection of *Francisella tularensis* -derived LPS in a vaccine model, B1a cells will differentiate into PCs to produce IgM in the spleen and peritoneal cavity following secondary *F. tularensis* challenge (181). In this model, antigen-specific B1a cells produced IgM following

secondary challenge, suggesting that B1a cell activation can result in long-term protective responses (181).

In another model of systemic infection, using i.v. injection of *Borrelia hermsii*, B1b cells were the primary responders (182). B1b cell-derived IgM responses to *B. hermsii* were demonstrated to be antigen specific and long lasting, similar to B1a cell responses to *F. tularensis*. (181, 182).

While FOB cells are the predominant B cell subset involved in adaptive humoral responses, B1 cells have been shown to have a role in adaptive antibody production through nIgM production. In 1998, Boes *et al.* determined that mice with impaired nIgM secretion had inefficient IgG responses to T cell-dependent antigens, suggesting that nIgM secretion played a role in the promotion of proper antigen-specific humoral responses (184). Similar results were reported in an influenza model that demonstrated that both antigen-specific IgM and nIgM had to be present during infection in order to mount proper antigen-specific IgG responses (185). The mechanisms that led to these responses was not characterized but suggests that B1 cells have a role in shaping the adaptive immune response to infections.

It is important to note that while IgM is the most common antibody produced in response to B1 cell activation, IgA production has been demonstrated in other models. Specifically, B1 cell-derived IgA is important for the response to commensal bacteria in the gut (183).

Together, these results demonstrate that antibody production by B1 cells is rapid following antigen challenge and the resulting antibody can have long lasting immune functions.

B1 cells also influence immune responses to infection through cytokine secretion. While much of the focus of B1 cell-derived cytokine research has been on IL-10 production, there is also evidence that B1 cells produce GMCSF (169, 186–190).

In the peritoneal cavity, the majority of IL-10 is produced by B1 cells (169). These cells are known to have regulatory functions through the modulation of Th1 responses (187, 188). IL-10 production can indirectly downregulate $IFN\gamma^{+}$ $CD4^{+}$ T cell responses through the modulation of dendritic cell function (188).

B1 cell-derived GMCSF comes from a specific B1a subset called innate response activator B (IRA) cells (189). IRA cells produce GMCSF in response to systemic or localized infections, such as in the lungs (189, 190). IRA cells support bacterial clearance through the production of IgM induced by autocrine signaling from IRA cells (189, 190).

Together, B1 cell-derived cytokines promote adaptive immunity through the regulation of T cell responses and innate immunity through the production of natural antibody.

B1 cells from the spleen and peritoneal cavity are also able to act as APCs to induce naïve T cell differentiation. Antigen presenting functions of B1 cells have been demonstrated both *in vivo* and *in vitro* (165, 191–194). These studies are further supported by observations that B1 cells are able to phagocytose both *Escherichia coli* and *Staphylococcus aureus* and by observations that B1 cells are able to migrate to draining lymph nodes in response to infection (180, 195). Together, these studies support an important role for B1 cells as a bridge between the innate and adaptive immune response through antigen presentation.

The role of B1 cells in *Chlamydia* infection has not been thoroughly studied but our lab has previously looked at the role of splenic B1 cells in a genital tract infection model of *Chlamydia* using *Cm* (196). In this study, splenic B1 cells were shown to produce IL-10, IL-6, and GMCSF in response to *Cm* infection and acted as APCs to naïve T cells (196). While this study only examined splenic and not peritoneal B1 cells, the data demonstrates that B1 cells can respond to *Cm* infection and have a role in shaping adaptive immune responses to infection.

1.4 The role of sex in immune responses to infection

In this study, we sought to determine if sex-specific immune responses could explain the sex-specific epidemiology and consequences of *Chlamydia* infection in humans. Sex-specific differences in innate and adaptive immunity have been identified and will be discussed herein.

1.4.1 Sex differences in the immune system

Sex differences have been identified in both the innate and adaptive immune system at rest and in response to certain infections.

The most obvious control of sex differences in immune responses is hormonal influence. In the innate immune system, sex-specific hormones contribute to sex differences in a variety of cell types including plasmacytoid DCs, macrophages, and invariant natural killer T cells (197–199). In adaptive immunity, the role of sex-hormones is less clear but female hormones are thought to contribute to Th1 responses (200).

The X chromosome also plays a role in sex-dependent immune responses. The X chromosome in humans contains more immune related genes than any other chromosome but for the most part females do not receive double copies of these genes due to X inactivation (200, 201). In some cases, such as TLR7, genes may escape silencing, resulting in two copies of the gene and possibly contributing to sex differences (202).

While some sex-dependent immune differences have clear genetic and hormonal causes, other sex differences have been observed in which the cause has not been fully elucidated.

It has long been known that antibody responses in males and females differ but the mechanism leading to this difference has not been identified. In 1967, blood antibody titers of males and females of varying ages were collected and compared to one another (203). It was discovered that females have significantly higher IgM levels from age 4.5 years until adulthood (203). In a later study using patients with additional X chromosomes, it was demonstrated that the number of X chromosomes correlated with IgM titers (204); for example patients with XXXY or XXX genotypes had similar IgM levels in which were higher than patients with XX or XXY genotypes (204). While this study suggests a role for the X chromosome in heightened IgM responses, a direct mechanism has not been identified.

A multitude of other sex-dependent differences can be found at baseline in the immune system but overall these differences are primarily mediated by sex hormones, the X chromosome, and unknown mechanisms.

1.4.2 Sex differences in infections

Sex-dependent differences have also been shown in immune responses following viral, parasitic, and bacterial infections (205). The role that sex plays in the immune response to *Chlamydia* infection has not been examined but other infections have been shown to have sex-specific susceptibility and immune responses.

In murine studies using *Listeria monocytogenes*, a Gram positive intracellular bacteria, female C57BL/6J, BALB/c, C3H/HeN, and CBA/J mice were all more susceptible to infection compared to males of the same strain (206). Female mice of all four strains had higher bacterial burden and mortality compared to male counterparts (206). In each strain, IL-10 was upregulated in the serum of female mice and so IL-10 knock out mice were infected to see if this difference could contribute to sex-dependent susceptibilities (206). In the IL-10 knock out mice, male and females had similar bacterial burden and susceptibilities, demonstrating that sex-specific differences in IL-10 production following infection are responsible for the sex differences observed in *L. monocytogenes* infection (206). This study demonstrates that male and female mice have different susceptibilities and immune responses to other intracellular infections.

Pseudomonas aeruginosa susceptibility also differs between male and female mice (207). Following lung infection, female mice had higher bacterial burdens, lost more weight, and had higher levels of inflammatory mediators in the lungs compared to male mice (207). The mechanism behind the difference in susceptibility was not fully defined but this study demonstrates that male and female mice have different susceptibilities and immune responses to other lung infections.

Females have been shown to be more resistant to *Treponema pallidum*, the causative agent of syphilis (208). To determine if this resistance is immune mediated, blood samples were collected from male and female patients with active syphilis infections and analyzed by flow cytometry (208). In females, there was an upregulation of both $CD4^+$ and $CD8^+$ T cells compared to males following *T. pallidum* infection that was not seen among other cell types (208). A direct mechanism for how these cell differences relate to differences in susceptibility to infection was not determined but the results from this study demonstrate that sex-dependent susceptibilities to genital tract infections are correlated with sex-specific immune responses.

Together these studies suggest that in certain intracellular, lung, and genital tract infections, sex-dependent susceptibilities are correlated or directly related to sex-specific immune responses.

1.5 Rationale, Objective, and Hypothesis

1.5.1 Rationale

The reason(s) for the sex-specific epidemiology and outcomes of *Chlamydia* infection has never been fully researched. Considering that male and female immune responses differ significantly in multiple aspects, it is possible that sex-specific immune responses contribute to the sex-specific differences in *Chlamydia* infection. Successful clearance of primary *Chlamydia* infections requires strong Th1 responses while humoral responses are unnecessary and may even be pathogenic (85, 121, 122). It has long been understood that females have strong humoral responses compared to males but the

potential role of this difference has not been examined in the context of *Chlamydia* infection (203).

In this study we first wanted to establish that murine models of infection would induce the same sex-specific susceptibility that is observed in human infection. We also wanted to determine if any observed responses were caused by sex-specific immune responses. To do this, we examined conventional humoral responses by analyzing TFH and FOB cell populations but we also decided to examine the role of B1 cells during *Chlamydia* infection.

Our lab has previously determined that splenic $CD19^{+}CD43^{+}$ cells can contribute to the immune response to *Chlamydia* infection but peritoneal B1 cells have not been examined in this context (196). B1 cells are heightened in SLE, which has a strong female-dominant epidemiology, suggesting that B1 cells may have sex-dependent functions (140, 209). Considering that these cells are involved in sex-specific diseases and can respond to *Chlamydia* infection, we included them in this study.

Sex-specific epidemiology and consequences have been described in multiple *Chlamydia* infections, including ocular, respiratory and genital tract. Given that male and female genital tracts differ greatly in gross pathology which may influence susceptibility to infection, we decided to pursue a lung model of *Chlamydia* infection in order to conduct equitable studies to examine the impact of sex on immune responses to *Chlamydia* infection.

1.5.2 Objective and Hypothesis

The objective of this study was to determine if sex-specific immune responses occurred following *Chlamydia* infection and to characterize any differences that were observed. Based on the current literature I hypothesis that female mice are more susceptible to *Chlamydia* infection than their male counterparts. I further hypothesize that B1 cells play an important role in regulating sex-dependent immune responses during *Chlamydia* infection.

CHAPTER 2 MATERIALS AND METHODS

2.1 *Chlamydia*

2.1.1 *Chlamydia muridarum* (*Cm*) propagation

McCoy cells (American Type Culture Collection, Manassas, VA, USA) were used to propagate *Cm*. McCoy cells were grown and passaged in T-175 tissue culture flasks in McCoy medium, which consists of Minimum Essential Medium (MEM) (Invitrogen, Oakville, ON, CA), 2.2 g sodium bicarbonate (Bioshop, Burlington, ON, CA), 5% fetal bovine serum (FBS) (Sigma Aldrich, Oakville, Ontario), 10 μg/ml gentamicin (Invitrogen) and 2 μg/ml fungizone (Invitrogen). Prior to propagating *Cm*, McCoy cells from one T-175 flask were split into ten 150 mm tissue culture dishes and incubated at 37ºC for 3-5 days or until confluent.

Once confluent, McCoy medium was removed from the plates and 3 ml *Cm*-containing culture supernatant was added to infect McCoy cells for 10 mins. Following this, 12 ml of growth medium (GM), consisting of MEM, 5% FBS, 10.06% Glucose (BioShop), 7.5% sodium bicarbonate, 10 mM HEPES (Wisent Bio Products, Saint-Jean-Baptiste, Québec), 200 mM L-Glutamine (Wisent Bio Products), 1X vitamins (Invitrogen), 50 mg/ml gentamicin and 10 μg/ml cyclohexamide (Sigma Aldrich), was added to the cultures and the plates were returned to 37ºC for 2 days. Supernatant and monolayer derived *Cm* was purified from culture supernatants and cell pellets, respectively, and stored in small aliquots at -80ºC.

To purify supernatant-derived *Cm*, the culture supernatant was collected and centrifuged (335×g) for 10 mins at 4ºC to remove cellular debris. Following this low spin, supernatant was collected into pre-chilled centrifuge tubes and ultra-centrifuged in a Beckman J2-21 centrifuge (22,500×g, 1 hr, 4ºC). The supernatant from ultra-spin was

discarded and the pellets were washed with 10 ml sucrose phosphate glutamic acid (SPG) buffer (220 mM sucrose/4 mM potassium phosphate monobasic/7 mM potassium phosphate dibasic/5 mM monosodium glutamate, pH=7.2). Pellets were combined into 2 centrifuge tubes and re-centrifuged (22,500×g, 1 hr, 4ºC). The final pellets were resuspended in 1 ml SPG and frozen in small aliquots at -80ºC.

To purify monolayer-derived *Cm*, 5 ml of pre-chilled SPG was added to the monolayer of each culture plate and the cells were collected using a cell scraper. The cells were sonicated 3 times on ice for 15 s and then centrifuged (335×g, 10 mins, 4ºC). The supernatant was collected and spun in a Beckman J2-21 centrifuge (22,500×g, 1hr, 4ºC). Subsequently, the pellets were resuspended in 10 ml SPG buffer and loaded onto a two-layer cushion, with 30% Isovue-370 (Bracco Diagnostics, Princeton, NJ, USA) in 30 mM Tris-HCl layered over 50% sucrose (Sigma Aldrich) in 30 mM Tris-HCl. The gradients were centrifuged (22,500×g, 1 hr, 4ºC, slow acceleration, no deceleration) and the supernatant was discarded. The final interface pellets were collected and resuspended in 4 ml SPG buffer and frozen in small aliquots at -80ºC.

2.1.2 *Cm* quantification

2.1.2.1 Quantification by quantitative polymerase chain reaction (qPCR)

To quantify *Cm* by qPCR, genomic DNA was first extracted from 100 µl of sample. To lyse samples, 500 µl DNAzol (Invitrogen) was added to each sample. The samples were mixed by inversion and left at room temperature for 3 mins. Next, genomic DNA was precipitated by adding 250 µl 100% ethanol and mixed by inversion. The samples were centrifuged (10,400×g, 15 mins, 4ºC) and the supernatant was discarded.

The genomic DNA was washed twice by adding 750 µl 70% ethanol, mixing by inversion, and centrifuging (10,400×g, 5 mins, 4ºC). After both washes, the pellet was dried by wiping excess liquid from the tube with a kim wipe. The DNA was solubilized by adding 100 µl of 8 mM NaOH and gently pipetting to dissolve the pellet. To bring the pH to approximately 7.0, 3.2 µl of HEPES was added to each sample. The samples were stored at -20ºC for future quantification.

To quantify the extracted DNA, 15 µl of reaction mixture was mixed with 5 µl of genomic DNA at 1:10 with DNase/RNase free water in a qPCR plate. The reaction mixture consisted of primers specific to the gene encoding the 16s ribosomal RNA of *Cm* (forward: 5'-CGC-CTG-AGG-AGT-ACA- CTC-GC -3', reverse: 5'-CCA-ACA-CCT-CAC-GGC-ACG-AG-3'), 10 µl 2x SYBR green (Promega, Madison, WI, USA), and 4 µl distilled DNase and RNase free water per sample. The plate was covered and centrifuged (335×g, 5 mins, 4ºC) before being run in a 7900HT Fast Real-Time PCR System on the following cycle: 50°C for 2 mins, then 95°C for 10 mins followed by 40 repeats of 95°C for 15 s followed by 60°C for 1 min, then 95 °C for 15 s, 60°C for 15 s, and 95°C for 15 s. The results were quantified by comparing to a known standard.

2.1.2.2 Quantification by inclusion forming unit (IFU) assay

McCoy cells were plated at a density of 7×10^4 cells per well in a 96 well plate and incubated overnight at 37ºC. *Cm* needing quantification was diluted 1:500 in GM and then further serial diluted 1:2 in a v-bottom plate. The serial diluted 100 µl samples were added to the 96 well plate of McCoy cells in duplicates with 11 total dilutions. An

additional two wells received GM alone as a control. The culture plate was centrifuged (1300×g, 1 hr, 36ºC) to facilitate the infection and then incubated at 37ºC for 40 hrs.

At 40 hrs post infection, the supernatant was discarded and 200 µl of methanol was added to each well and allowed to incubate for 20 mins. After incubation, the methanol was discarded and the plate was left to air dry. Once dry, the plate was either stained immediately or wrapped in parafilm and stored at 4ºC for up to 1 week.

Giménez staining was used to visualize *Cm* within McCoy cells. To prepare the dye, 2 ml Giménez stock was diluted in 5 ml phosphate buffer and filtered through a 0.2 µm filter. Dye was added to each well (60 µl) and incubated at room temperature for 30 s. After incubation, the stain was discarded and the plate was rinsed with water. As a counter stain, 0.8% Malachite green was added to each well (60 µl) and incubated for 90 s. Following the incubation, the stain was discarded and the plate was rinsed. Malachite green staining was performed a second time with a 30 s incubation and the plate was rinsed.

To quantify *Cm*, the plate was visualized under a microscope and purple inclusion bodies were counted. The number of IFU of the duplicates were counted and the titer of *Cm* was determined by multiplying by the dilution factor.

2.2 Mice

Congenic C57BL/6 CD45.1 mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and bred in-house at the Isaak Walton Killam Health Centre (IWK) animal facility. IL-10-IRES-eGFP knock-in (IL-10GFP) or Tiger (IL-10 ires gfp-enhanced reporter) mice were obtained from Dr. Richard Flavell (Yale University, CT,

USA) and bred in house at the IWK animal facility. OTII TCR transgenic mice used for co-culture were obtained from Dr. Jean Marshall and housed at the Carleton Animal Care Facility at Dalhousie University.

All mice were housed under pathogen free conditions and all animal procedures were approved by the Ethics Committee according to the Canadian Council for Animal Care guidelines.

2.3 *In vivo* studies

2.3.1 Respiratory infection

Mice were anesthetized using ketamine (37.5 mg/kg) (Bioniche, Lavaltrie, Québec)/Rompun (xylazine) (7.5 mg/kg) (Bayer Healthcare, Mississauga, Ontario) through i.p. injection. Once asleep, approximately 4×10^3 IFU supernatant-derived *Cm* diluted in 25 µl of SPG buffer was delivered with a P100 micropipette into the nose of the mouse. Body weight was taken at the same time daily for the duration of the experiment. A body weight loss of 20% was used as the humane endpoint.

2.3.2 Tissue processing following respiratory infection

During sacrifice, the spleen, MLN, and lungs of infected mice were placed in Roswell Park Memorial Institute 1640 medium (RPMI)/5% bovine serum (BS) on ice. For splenocyte isolation, spleens were mashed with glass slides into 10 ml RPMI/5%BS into a Petri dish. The glass slides and Petri dish were washed with 10 ml RPMI/5%BS and the suspension was centrifuged (525×g, 10 mins, 4ºC). The supernatant was discarded following centrifugation and red blood cells were lysed with ACK buffer. Next,

the cells were filtered through a 40 µm filter and counted using 0.4% Trypan blue exclusion and a hemocytometer

Lymph node samples were mashed with glass slides into a 60 mm dish in 1 ml of RPMI/5%BS. The dish and glass slides were rinsed with 1 ml RPMI/5%BS and pipetted into a 40 µm filter cap tube. The cells were counted as above.

Before lungs were removed from the mice, bronchial alveolar lavage fluid was collected by inserting a needle into the trachea of the mice, washing with 1ml phosphate buffered-saline (PBS), and then aspirating the liquid from the lungs. Following this, the large lobe of the lungs was placed in 500 µl SPG to use for bacterial burden quantification, and the rest of the lungs were used for cell isolation. In some experiments, lung cells were not isolated but instead, the remaining lung lobes were fixed in 1 ml formalin.

For lung cell isolation, samples were rinsed in Hank's balanced salt solution (HBSS) (Wisent) and placed in 1 ml of HBSS. The tissue was cut into small pieces using scissors. Lung samples were incubated in a 37ºC water bath with collagenase II (Sigma Aldrich) (80 µg/ml) for 20 mins followed by 10 mins with ethylenediaminetetraacetic acid (EDTA) (10 mM). Samples were filtered through a 40 µm strainer and the red blood cells were lysed using ACK. The cells were counted as above.

To process lungs for bacterial burden, the lung lobes were homogenized in SPG with a tissue homogenizer at the highest setting until tissue was disrupted. The samples were centrifuged (1000×g, 10 mins, 4ºC) and the supernatant was collected for future DNA extraction and bacterial quantification.

Blood was collected from mice by cheek bleed. Using a 22-gauge needle, mice were cheek bled into a 1.5 ml tube. The blood was left to coagulate at room temperature and then centrifuged (21,100×g, 30 mins, 4ºC). The serum was collected and stored at -80ºC until antibody detection.

2.3.3 Cell tracking during respiratory infection

In some experiments, peritoneal cells were tracked using *in situ* staining. In these experiments, carboxyfluorescein succinimidyl ester (CFSE) (stock 20 mM in dimethyl sulfoxide [DMSO]) was diluted in PBS to a final concentration of 50 µg/ml and injected i.p. 6 hrs before intranasal infection. Control mice received DMSO vehicle control diluted in PBS. The dye or vehicle was injected slowly over 20 s with a 23-guage needle and the belly of the mouse was gently tapped. Six hrs post-CFSE injection, mice were infected intranasally, as described above.

2.4 *In vitro* studies

2.4.1 *In vitro* cell stimulation

Peritoneal cells were collected by injecting 4.5 ml of peritoneal wash buffer containing PBS, 0.5% bovine serum albumin (BSA), and 5 mM EDTA into the peritoneal cavity of the mouse following sacrifice. The peritoneum of the mouse was massaged for 20 s and then the buffer was aspirated from the cavity. The cells were centrifuged (525×g, 10 mins, 4ºC) and then counted. Splenocytes were collected according to 2.3.2.

Once isolated, the cells were plated in a 96 well plate at a density of 2×10^6 cells per well in 200 µl of complete RPMI (cRMPI) containing RPMI medium, 10% FBS, 1%

L-glutamine, 1% penicillin-streptomycin (p/s) and 1% HEPES. The cells were stimulated with 50 µl of SPG buffer containing *Cm* at multiplicity of infection (MOI)=1 or 50 µl of SPG buffer alone. After 24 hrs the supernatant was collected and frozen at -20ºC for future analysis by enzyme-linked immunosorbent assay (ELISA) (2.6.1). The cell pellets were collected and stained for flow cytometry staining and analysis (2.7).

2.4.2 Co-culture

To obtain bone marrow derived dendritic cells (BMDCs) for co-culture *in vitro*, bone marrow was isolated by removing the femurs and tibias of mice and placing in RPMI on ice. The end of the femur and tibia were cut off and the bone marrow was flushed from the bone using a 23-gauge needle and syringe with RPMI containing 5% BS. The marrow was cut using scissors and centrifuged (525×g, 10 mins, 4ºC). Red blood cells were lysed by adding 5 ml ACK buffer (0.1 mM EDTA, 0.15 M NH_4Cl, 1 mM $KHCO_3$) to each sample. To stop the reaction, 10 ml RPMI/5%BS was added after 5 mins. The samples were filtered through a 40 µm filter and washed twice with RPMI/5%BS, and counted as above. The cells were plated at a density of 3×10^6 cells in 10 ml R10 (cRPMI containing 50 µM β-mercaptoethanol) with 10% GMCSF-containing culture supernatant to induce BMDC differentiation. On day 3, an additional 10 ml R10/10% GMCSF was added to the culture. On day 6, the non-adherent dendritic cells were collected by aspirating the culture medium and lightly rinsing the culture plate. The cells were counted as above.

To obtain different B1a, B1b, and B2 cells for co-culture, peritoneal cells (2.4.1) and splenocytes (2.3.2) were isolated and resuspended in sorting buffer, which contains

PBS and 0.5% BS and 2 mM EDTA, at 1×10^8 cells/ml. The samples were incubated with the appropriate fluorochrome-conjugated anti-mouse monoclonal antibodies (Table 1) for 20 mins at 4ºC. After incubation, the samples were diluted with a large volume of sorting buffer and centrifuged (525×g, 10 mins, 4ºC) to wash away unbound antibody. The samples were resuspended in sorting buffer at a final concentration of 4×10^7 cells/ml or a minimum of 2 ml for sorting. B cell subsets were sorted by a fluorescent-activated cell sorting (FACS)Aria machine (BD Biosciences, San Jose, CA, USA) and collected into tubes that were pre-coated with PBS/5%BS. In most experiments, to obtain B1a and B1b cells, peritoneal cells were stained with anti-CD19, anti-CD43, anti-B220, and anti-CD5 fluorochrome conjugated antibodies to isolate $CD19^+CD43^+B220^{lo}CD5^+$ (B1a) or $CD19^+CD43^+B220^{lo}CD5^-$ (B1b) populations. In most experiments, B2 cells were isolated by staining splenocytes with anti-CD19, anti-CD43, anti-CD21, and anti-CD23 fluorochrome conjugated antibodies to isolate $CD19^+CD43^-CD21^{lo}CD23^+$ cells. In one set of experiments, splenocytes or peritoneal cells were stained with anti-CD19, anti-CD43, anti-CD5, and anti-programmed cell death protein 1 ligand (PDL)2 fluorochrome conjugated antibodies to isolate $CD19^+CD43^+CD5^+PDL2^+$ (B1a-like), $CD19^+CD43^+CD5^-PDL2^+$ (B1b-like), $CD19^+CD43^-CD5^+PDL2^+$ (B2-like), and $CD19^+CD43^-CD5^-PDL2^-$ (B2-like) populations. Single stains were setup for machine compensation. To do this, 10 µl of sample was diluted in 90 µl sorting buffer containing the antibody and stained in a 1.5 ml tube.

After sorting, B cell subsets or non-adherent BMDCs were cultured with naïve OVAII or CD45.1 T cells at various ratios of T cells to antigen presenting cells, including

Table 2. 1: List of fluorochrome-conjugated primary antibodies used in flow cytometry experiments.

Specificity	Host	Isotype	Clone	Manufacturer
B220-PeCy7	Rat	IgG2aκ	RA3-6B2	eBioscience
CD4-FITC	Rat	IgG2aκ	RM4-5	eBioscience
CD19-FITC	Mouse	IgAκ	MB19	eBioscience
CD19-PeCy7	Rat	IgG2aκ	eBio1D3	eBioscience
CD43-APC	Rat	IgG2aκ	S7	BD Biosicences
CD43-PE	Rat	IgM	eBioR2/60	eBioscience
CD62L-FITC	Rat	IgG2aκ	MEL-14	eBioscience
CXCR5-PE	Rat	IgG2aκ	SPRCL5	eBioscience
Fixable viability dye eFluor 506	n/a	n/a	n/a	eBioscience
ICOS-PerCP-eFluor710	Golden Syrian hamster	IgG	15F9	eBioscience
PD1-APC	Rat	IgG2bκ	RMP1-30	eBioscience
PDL2-FITC	Rat	IgG2aκ	122	eBioscience
PDL2- PerCP-eFluor710	Rat	IgG2aκ	122	eBioscience
TCRβ-APC-eFluor70	Armenian hamster	IgG	H57-597	eBioscience

1:8, 1:4, 1:2, and 1:1. The cells were either stimulated with OVAII alone (100 µg/ml) or OVAII/heat-killed *Cm* (inactivated for 30 mins at 65ºC) (1:2,500 dilution). When CD45.1 mice were used to isolate T cells, anti-CD3 (10 µg/ml) and anti-CD28 (5 µg/ml) antibodies were used for stimulation. After 72 hrs the supernatant was collected and frozen at -20ºC for future analysis by ELISA.

2.5 *Ex vivo* antigen recall assay

Splenocytes isolated during sacrifice were plated at a density of 1×10^6 cells/well (100 µl) in a 96 well plate with triplicate wells for each mouse in each stimulation. The cells were stimulated with heat-killed *Cm* (100 µl) at final dilutions of 1:100, 1:200, or 1:400 or with medium alone. The plate was incubated at 37ºC for 72 hrs. Following incubation, the supernatant was collected and frozen at -20ºC for future use.

2.6 ELISAs

2.6.1 Measuring cytokine levels using ELISA

Cytokine levels in culture supernatant were assessed using specific antibody pairs for mouse B cell activating factor (BAFF), GMCSF, IFN-γ, IL-4, IL-6, IL-10, IL-13, IL-17A, IL-21, and IL-33 (eBioscience, San Diego, CA, USA).

ELISA plates (Costar Maxisorp 96-well flat bottom) were coated with 50 µl of capture antibody diluted in coating buffer according to the manufacturer's protocol (eBioscience) and left at 4ºC overnight.

The following day, the plates were washed 3 times. Washing was done by adding 200 µl PBS/0.1% Tween-20 (Sigma Aldrich) to each well, dumping the wash buffer, and

then patting the plates on paper towel. Once washed, 200 µl of assay diluent (eBioscience) was added to each well and incubated at room temperature for 2 hrs. During incubation, standards (eBioscience) were diluted to 2,000 pg/ml (4,000 pg/ml in the case of IL-10) in assay diluent and then serially diluted 1:2 to create a standard with 8 total concentrations (12 in the case of IL-10). At the same time, samples were diluted in assay diluent if the concentration was expected to exceed the standard curve based on previous experiments. Following incubation, the plates were washed 3 times and 50 µl of the samples and standards were added to the appropriate wells. In each plate, 50 µl of assay diluent was added to 4 wells as a control. The plates were returned to 4ºC overnight.

The following day, the plates were washed 5 times. Detection antibody was diluted according to the manufacturer's protocol and 50 µl was added to each well. The plates were incubated at room temperature for 2 hrs. After the incubation, the plates were washed 5 times and 50 µl streptavidin/horse-radish peroxidase (HRP) (eBioscience) diluted in assay diluent was added to each well. The plates were incubated in the dark at room temperature for 20 mins. Following incubation, the plates were washed 7 times and 50 µl of 1X tetramethylbenzidine substrate solution (TMB) (eBioscience) was added to each well. The plates were developed in the dark until the 7th well of the standard was visibly blue or the blank wells started to become blue. The reaction was stopped using 50 µl 0.2 M H_2SO_4. Within 30 mins the plates were read at 450 nm using a BioTek Synergy HT plate reader (BioTek, Winooski, VT, USA) and analyzed using Gen 5 (BioTek).

2.6.2 Measuring antigen-specific antibody responses using ELISA

To measure antibody against *Cm*-specific antigens in mouse serum, ELISA plates were coated with 50 µl *Cm* derived proteins (5 µg) or heat-killed *Cm* (500 IFU) diluted in 0.05 M $NaHCO_3$ and incubated at 4ºC overnight.

The following day, the plates were washed 3 times (as in 2.6.1) and 200 µl of PBS/0.1% Tween-20/2% BSA was added to each well. The plates were incubated for 1 hr at room temperature. During incubation, serum was serially diluted from 1:100 to 1:6400 with PBS/0.1% Tween-20/2% BSA. The best dilution from preliminary experiments was used for future experiments. Once the incubation was complete, the plates were washed 3 times and 100 µl of sample was added to the appropriate wells.

On the final day, the pates were washed 5 times and 100 µl goat anti-mouse IgG with HRP (Invitrogen) (5 µg/ml) diluted in PBS/0.1% Tween-20/2% BSA was added to each well. The plates were incubated at room temperature for 20 mins and the washed 7 times. After washing, 50 µL TMB was added to each well and the plates were developed in the dark. The reaction was stopped with 50 µl 0.2 M H_2SO_4. Within 30 mins the plates were read at 450 nm using a BioTek Synergy HT plate reader (BioTek, Winooski, VT, USA) and analyzed using Gen 5 (BioTek).

2.7 Flow cytometry

All antibodies used for flow cytometry are listed in Table 1.

Samples to be stained were moved into mini tubes for staining and centrifuged (525×g, 10 mins, 4ºC). The samples were washed with FACS buffer (0.5% BS/PBS) by adding 200 µl to each sample, centrifuging (755×g, 3 mins, 4ºC), and discarding the

supernatant. Next, 50 µl of 10% inactivated rat serum/FACS buffer was added to each well and incubated at 4ºC for 15 mins. During incubation, the appropriate fluorochrome-conjugated anti-mouse monoclonal antibodies for that experiment were prepared in the dark by diluting the antibodies in FACS buffer. After incubation, 50 µl of the appropriate antibody cocktail was added to each well and the samples were incubated in the dark for 20 mins at 4ºC.

Following incubation, the samples were washed twice with 100 µl FACS buffer. Once washed, the samples were fixed by adding 200 µl 1% Formalin/PBS and stored at 4ºC for up to 2 days. All samples were read on the FACSFortessa (BD Biosciences). Flow cytometry data were analyzed using FCS Express 4 Flow Cytometry software (De Nova Software, Los Angeles, CA, USA).

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 Software (GraphPad, San Diego, CA, USA). The D'Agostino and Pearson omnibus normality test was used to determine if data were parametric or non-parametric. To compare between two groups with parametric data, a Student's t-test was used while a Mann-Whitney test was used for non-parametric data. To compare 3 or more groups with parametric data, a 1-way ANOVA with Bonferroni post-test was used. To compare 3 or more groups with non-parametric data, a Kruskal Wallis test with Dunn's post-test was used. To compare differences while considering two independent variables, a 2-way ANOVA was used. All data are shown as mean±standard error of mean (SEM) if $n \geq 3$, or as mean if $n < 3$. *P* values < 0.05 were considered significant.

CHAPTER 3 RESULTS

3.1 Characterization of responses to *Cm in vivo*

3.1.1 Female mice lose significantly more weight than male mice following intranasal *Cm* infection

The epidemiology and responses to *Chlamydia* infection in humans have been shown to differ between males and female. To study the cause of this dichotomy in animal models, we first had to investigate if male and female mice showed different clinical responses to intranasal *Cm* infection which would mirror the human data.

Over the course of intranasal infections, body weight was recorded every day as a clinical score of disease progression in the mice (Figure 3.1). We observed that females lost significantly more weight compared to male mice. From days 0-9 post-infection, male and female mice had similar body weight loss trends in two independent experiments (Figure 3.1). At approximately days 7-9, a difference in body weight loss began to become apparent between the sexes (Figure 3.1). During the 12-day infection, female body weight loss became significantly higher than males from days 10-12 (Figure 3.1B). In both experiments, male and female mice started to regain weight near the end of infection (Figure 3.1). Although all mice survived infection, the body weight data shows that male and female mice have statistically divergent responses to intranasal *Cm* and suggests that they may serve as a useful animal model to study the sex differences observed in humans following *Chlamydia* infection.

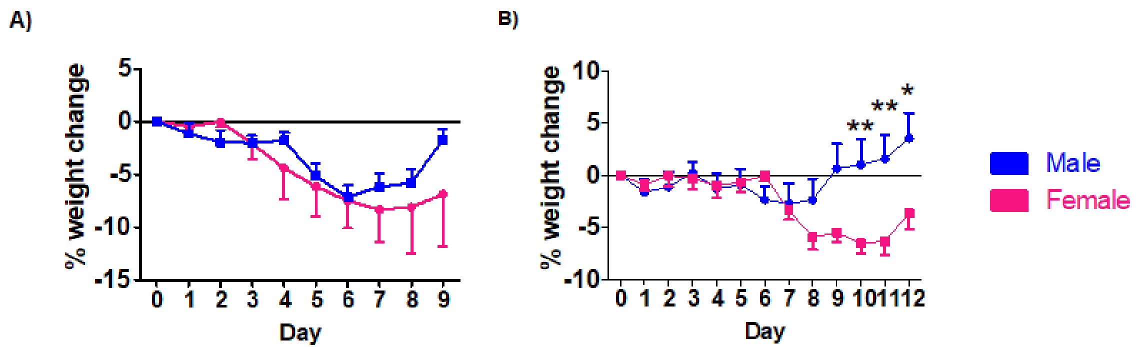


Figure 3. 1: Body weight loss following intranasal *Cm* infection differs significantly between male and female mice. Mice were infected intranasally with *Cm* at Day 0 and weighed daily over the course of the experiment. A) Day 9 experiment B) Day 12 experiment. Data are shown as mean±SEM, male n=6/time point and female n=5/time point. *p<0.05, **p<0.001 using 2-way repeated measures ANOVA.

3.1.2 Female mice have a significantly higher bacterial burden in the lungs compared to male mice following intranasal *Cm* infection

Next, we sought to determine if the observed body weight changes between male and female mice could be dependent on bacterial burden. At days 6, 9, and 12 post-infection, bacterial burden from the lungs was quantified by qPCR (Figure 3.2). Bacterial burden was significantly higher in females at day 9 post-infection which is approximately the same time that male and female body weight begins to differ (Figure 3.2B). Before this, bacterial burden did not differ between the sexes (Figure 3.2B) and by day 12, the bacterial burden between males and females was no longer significantly different (Figure 3.2C). The change in burden over time suggests that throughout the infection male mice had a steady control of bacterial replication while the bacteria were better able to replicate in the female lungs. Importantly, in the end, both male and female mice could control the infection. The difference in body weight change coincides with the time point at which bacterial burden between male and female mice begins to diverge. It is possible that body weight loss reflects the less controlled infection in female mice. Together, these data demonstrate that the bacterial burden between male and female mice differs significantly and that it may be associated with increased weight loss in female mice.

3.1.3 Female mice produce more antigen-specific antibody following infection compared to male mice

Given that we observed a significant difference in bacterial burden at day 9, we wanted to see if this would be reflected in the antibody response. To this end, we

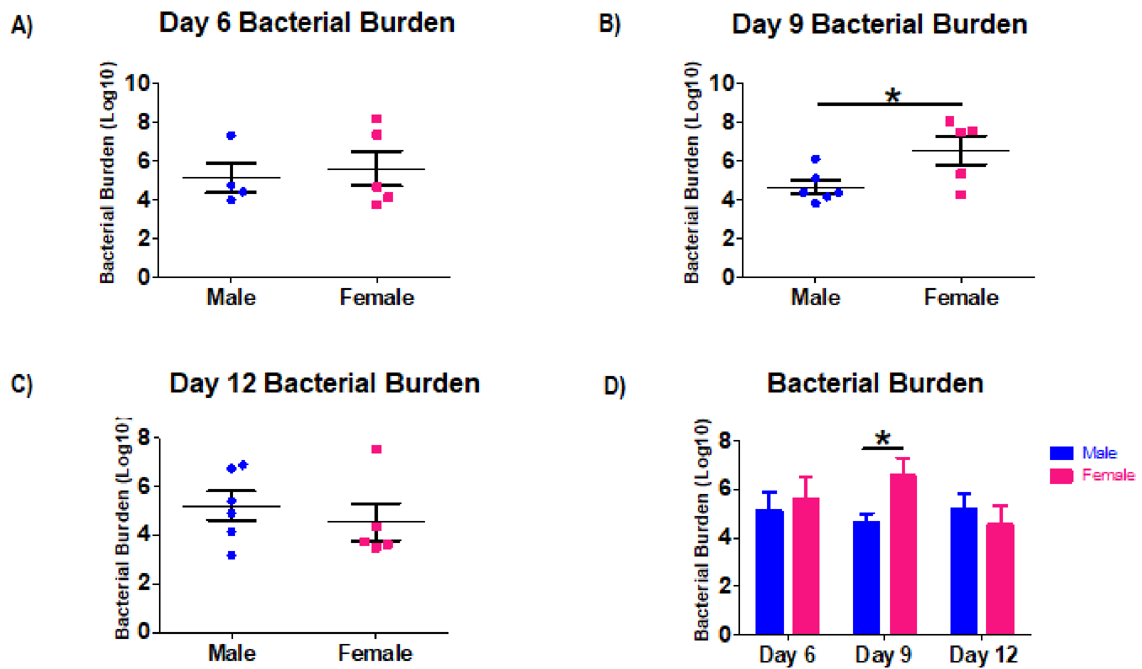


Figure 3. 2: Bacterial burden is significantly higher in female mice at day 9 post-infection. At various time points following infection, lungs were collected and homogenized. Genomic DNA was isolated from the homogenate and bacterial burden was measured by qPCR. A) Bacterial burden in the lungs taken on day 6 post-infection. B) Bacterial burden in the lungs taken on day 9 post infection. C) Bacterial burden in the lungs taken on day 12 post-infection. D) Bacterial burden shown over time from day 6-12 post infection. Each dot represents an individual mouse. All data are shown as mean±SEM. *p<0.05 using Mann Whitney test.

collected serum from naïve mice as well as infected mice at day 6, 9, and 12 post-infection and measured total IgG responses using direct ELISA (Figure 3.3).

To measure the IgG response to *Cm*, heat-killed *Cm* was used. We found that females had a higher amount of total IgG at day 9 post-infection (Figure 3.3A). To better understand the antibody differences between male and female mice, we also tested the total IgG against pathogenic (Figure 3.3B) and non-pathogenic (Figure 3.3C) proteins isolated from *Cm* (126). We noted that females had a significantly higher IgG response to the pathogenic protein TC0582 at day 9 post-infection but this was not mirrored in the second pathogenic protein tested (TC0912C) (Figure 3.3B). In response to the non-pathogenic proteins TC0047 and TC0326, females had a higher response at day 9, although this was not significant (Figure 3.3C). The differences in total IgG response between males and females *in vivo* suggests that females have strong humoral responses which may be detrimental, considering the controversial role of humoral responses during *Chlamydia* infection.

Given that male and female mice had differences in both bacterial burden and total IgG responses at day 9 post-infection, we wanted to determine if there was a relationship between these two observations. To this end, we performed correlational analysis between bacterial burden and antibody levels at day 9 post-infection (Figure 3.4). We found that bacterial burden in the lungs correlated significantly with the IgG response measured at day 9 post-infection to *Cm* (Figure 3.4A), pathogenic proteins (Figure 3.4B), and non-pathogenic proteins (Figure 3.4C).

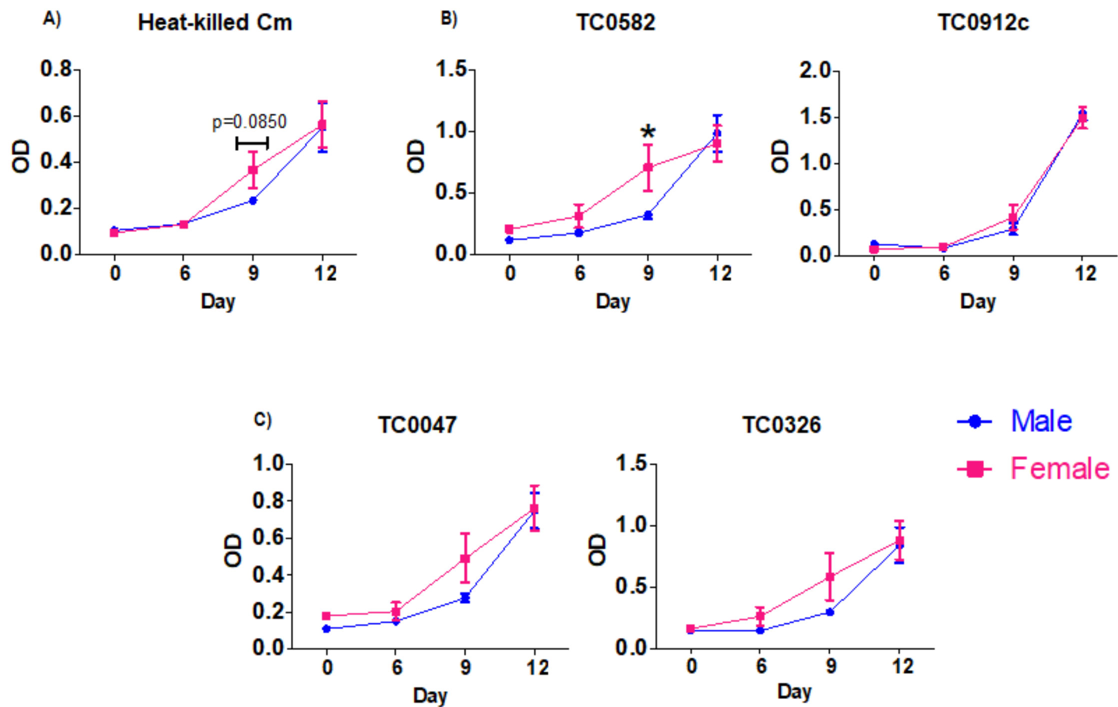


Figure 3. 3: Females produce more IgG in response to *Cm* infection. Blood samples taken by cheek bleed from naïve mice and at days 6, 9, and 12 post-infection. The samples were coagulated and serum was collected. Total IgG levels were tested against various antigens by direct ELISA. A) The response to heat-killed *Cm* over time. B) The response to the pathogenic proteins TC0582 and TC0912c over time. C) The response to the non-pathogenic proteins TC0047 and TC0326 over time. For day 0 n=2/sec, for day 6 male n=4 and female n=5, for day 9 and 12 male n=6 and female n=5. *p<0.05 using Mann Whitney test at each time point.

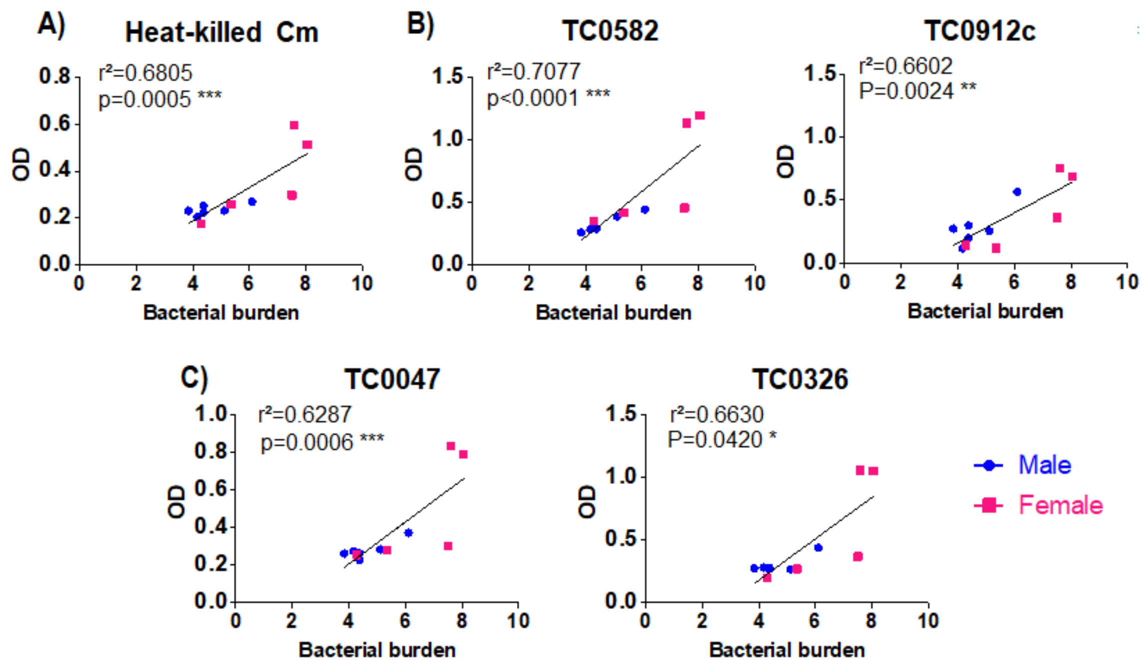


Figure 3. 4: Bacterial burden correlates significantly with IgG levels at day 9 post-infection. Following intranasal infection with *Cm*, lungs were collected for bacterial burden and blood was collected for serum. Bacterial burden was measured by qPCR while serum was tested by direct ELISA. The correlation between bacterial burden and the total IgG response to heat-killed *Cm* (A), the pathogenic proteins TC0582 and TC0912c (B), and the non-pathogenic proteins TC0047 and TC0326 (C) at day 9 post infection. Male n=6, female n=5. Linear regressions were used for r^2. $^*p<0.05$, $^{**}p<0.001$, $^{***}p<0.001$ using Pearson (TC0912c) or Spearman correlation.

Together, this demonstrates that there are differences in the total IgG response to *Cm* infection between males and females *in vivo* and that these differences are somehow related to bacterial burden.

3.1.4 Male mice have significantly higher Th1 and Th17 responses following infection while female mice have strong Th2 and Treg responses

Considering the differences in male and female mice following infection, we wanted to characterize how splenocytes from infected mice would respond to secondary challenge to understand the overall responses to *Cm* infection in both sexes. In this view, we stimulated splenocytes from infected mice with heat-killed *Cm ex vivo* for 72 hrs. Following incubation, the culture supernatant was collected and analyzed by ELISA. Specifically, we measured cytokines that represent various $CD4^+$ T cell responses: IFNγ for Th1, IL-13 for Th2, IL-17A for Th17, IL-10 for Tregs, and IL-21 for TFH cells. In order to confirm that the cytokines are coming from T cells further experiments would need to be done but the cytokines represent the overall responses.

Day 9 post-infection, the splenocyte cultures of male mice had significantly higher production of IFNγ compared to females, suggesting a stronger protective Th1 response (Figure 3.5A). IL-17A levels were also higher in male cultures, representative of a Th17 response (Figure 3.5A). The cytokine levels of IL-10, IL-13, and IL-21 did not differ significantly between the sexes at day 9 post-infection (Figure 3.5A). At day 12 post-infection, female mice had significantly higher levels of both IL-13 and IL-10, representative of Th2 and Treg responses respectively (Figure 3.5B). The sex differences

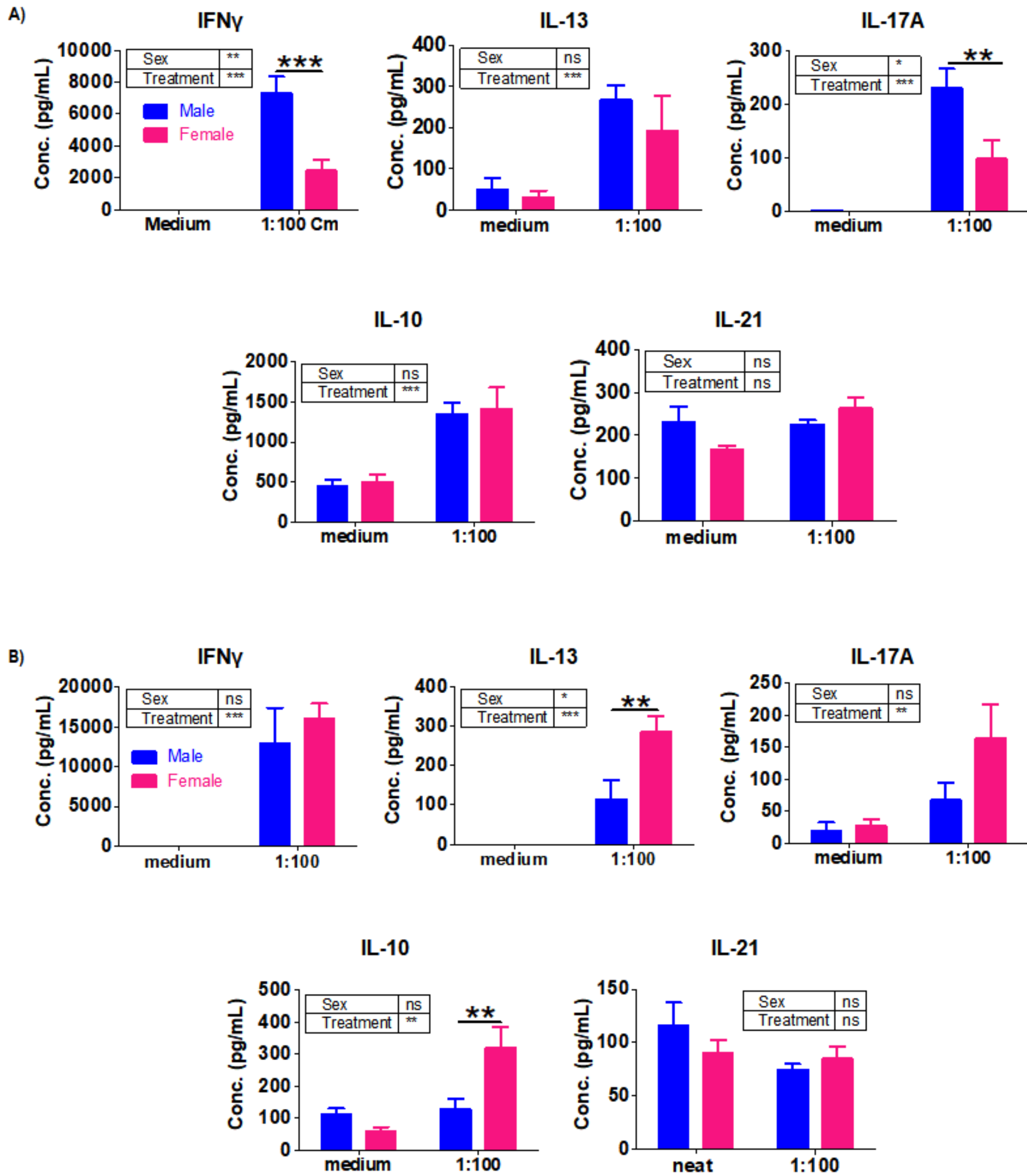


Figure 3. 5: Cytokine responses following day 9 and 12 *ex vivo* antigen recall assay differ between male and female mice. Splenocytes isolated from mice infected intranasally with *Cm* 9 (A) and 12 (B) days post-infection were cultured with heat-killed *Cm*. After 72 hrs, culture supernatants were collected and the cytokines IFNγ, IL-13, IL-17A, IL-10, and IL-21, were measured by ELISA. 1:100 indicates the dilution of *Cm*. Male n=6, female n=5. Data are shown as mean±SEM. *p<0.05, **p<0.01, ***<0.001 using 2-way ANOVA.

observed at day 9 post-infection in IFNγ and IL-17A were not replicated and the level of IL-21 did not differ between the sexes (Figure 3.5B).

Together, the recall assay demonstrates that the adaptive immune responses of male and female mice differ significantly. Importantly, while male mice had an upregulation of protective Th1 responses, female mice had higher non-protective Th2 and Treg responses. These data may provide a basis for the observed differences in both body weight loss and control of bacterial burden between the sexes.

3.1.5 Female mice have significantly more B1, B2, and TFH cells in the lungs following intranasal *Cm* infection

Given that male and female mice differed in both clinical responses and overall immune responses to infection, we wanted to further investigate how these differences were mediated at a cellular level. To this end, we examined various T and B cell populations in the lungs and MLN following intranasal *Cm* infection.

Day 9 post-infection, the B2 cell compartment was significantly expanded in the lungs of female mice compare to males (Figure 3.6A). This upregulation of B2 cells coincides with significantly higher antibody responses in females, suggesting that this expanded B2 compartment may be responsible for this difference. By day 12, this difference begins to dissipate (Figure 3.6B). We also observed no difference in antibody responses at day 12 post-infection, further supporting that the expanded B2 cell population in females plays a role in the observed dichotomy in humoral immunity.

Surprisingly, we did not observe the same sex-specific differences in the MLN following infection (Figure 3.7). Instead, we saw no difference in B2 cells between male

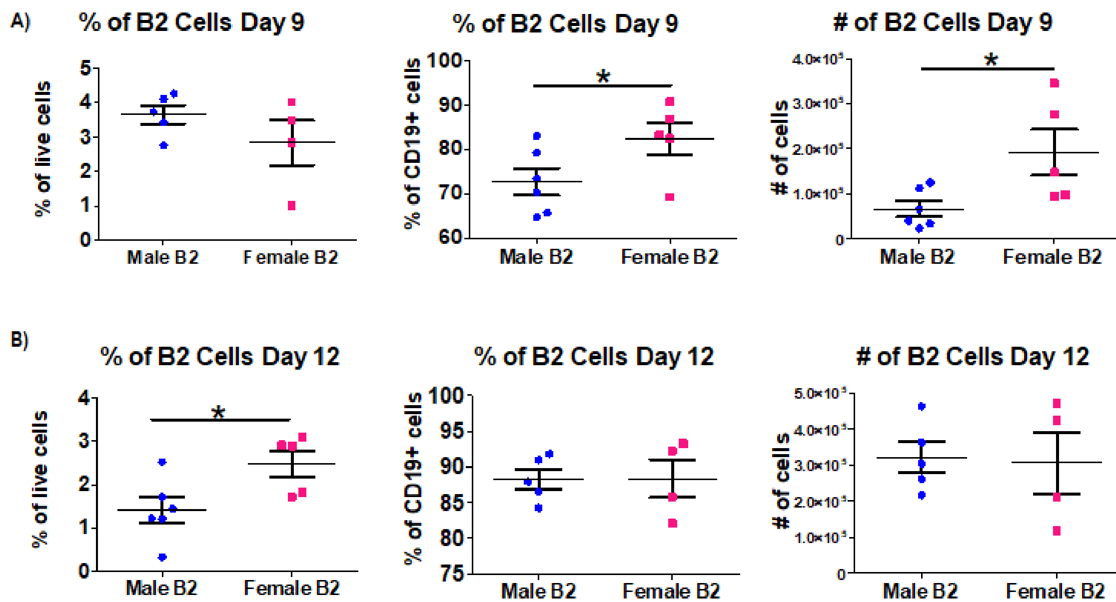


Figure 3. 6: Females have more B2 cells in the lungs at day 9 post-infection. Mice were infected intranasally with *Cm*. Cells from the lungs were isolated at various time points and analyzed by flow cytometry. A) B2 cell proportions out of live, total B cells, and total numbers of B2 cells at day 9 post-infection. B) B2 cell proportions out of live, total B cells, and total numbers of B2 cells at day 12 post-infection. Each dot represents an individual mouse. Data are graphed as mean±SEM. *p<0.05 using Mann Whitney test.

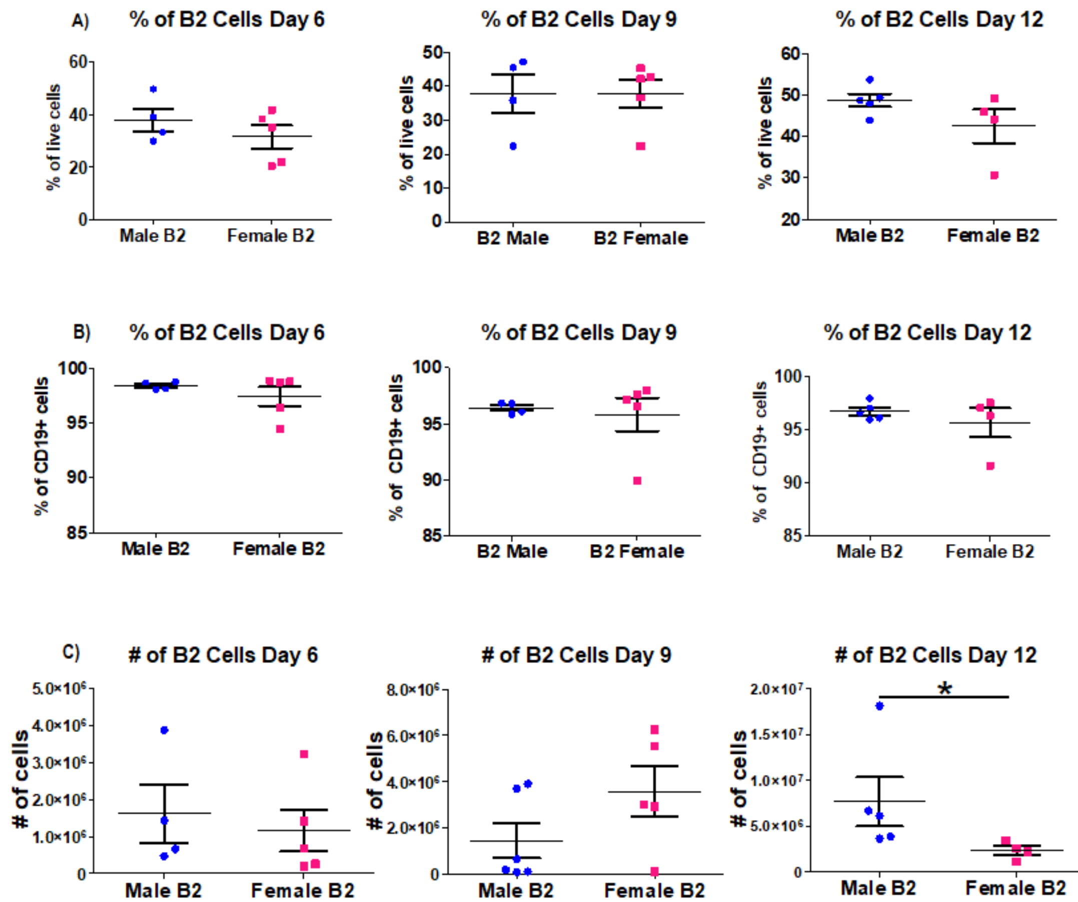


Figure 3. 7: Male mice have significantly more B2 cells in the MLN 12 days post-infection. Mice were infected intranasally with *Cm*. Cells from the MLN were isolated at various time points and analyzed by flow cytometry. The frequency of B1 cell subsets out of live (A), out of total B cells (B), and the total cell number (C) are shown at days 6, 9, and 12. Data are shown as mean±SEM where each dot represents an individual mouse. *p<0.05 using Mann Whitney test.

and female mice at day 6 or 9 (Figure 3.7A, B). We did, however, observe a significantly higher number of B2 cells in the male MLN following infection at Day 12 (Figure 3.7C).

It is likely that these cells are related to the upregulation of male antibody production at this time point considering that the male response is significantly lower at day 9 but successfully rebounds by day 12 post-infection.

It is important to also consider B1 cells in the response to *Cm* infection as they have been shown to be involved in the immune response to other intranasal infection such as influenza. Given that B1 cells have been shown to migrate to the lungs quickly in response to influenza infection, we sought to demonstrate a similar response in our model (210). To this end, we labelled peritoneal cells *in situ* with CFSE. Three days post-infection, we observed clear migration of $CFSE^{+}CD19^{+}$ cells from the peritoneal cavity to the lungs and to a lesser extent the MLNs (Figure 3.8A). This migration demonstrates the ability of peritoneal B cells to respond to *Cm* infection *in vivo*. Of these B cells, a significant percentage were $CD43^{+}$ and there was close to an even split of B1a ($CD5^{+}$) and B1b ($CD5^{-}$) cells that migrated to the lungs (Figure 3.8B). This migration demonstrates the ability of B1 cells to respond to *Cm* infection *in vivo*.

To fully characterize the B1 cell response in the lungs, we analyzed the frequency of B1 cells out of live, out of total B cells, and the total cell number of B1 cells present following infection (Figure 3.8C). Interestingly, there was a significant upregulation of both subsets of B1 cells in female mice compared to males (Figure 3.8C). Given that a population of these cells are $CFSE^{+}$, this upregulation in female mice is likely due to a combination of cell migration and expansion. We also examined B1 cells in the MLNs at

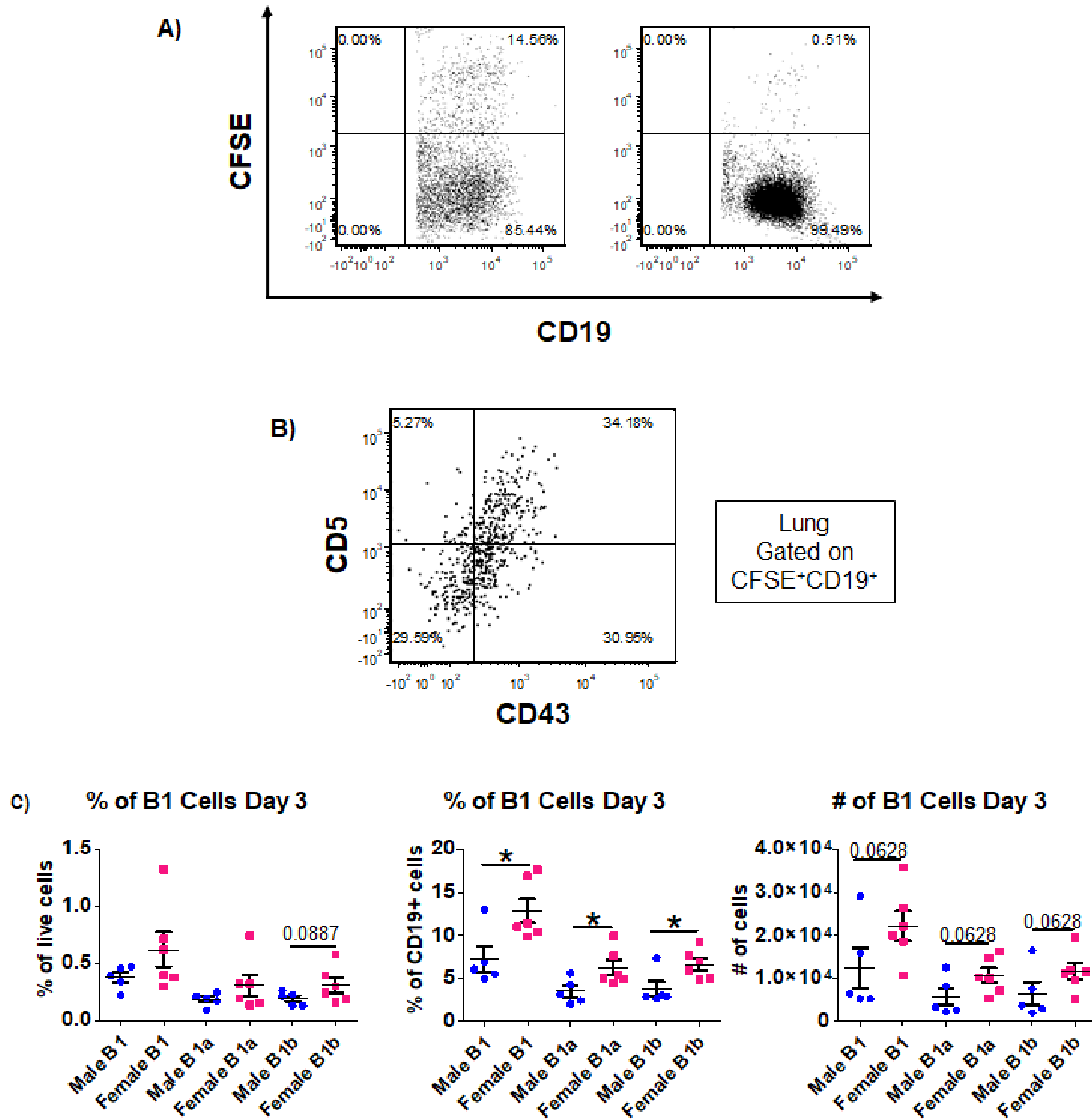


Figure 3. 8: B1 cells migrate from the peritoneal cavity to the lungs following intranasal infection with *Cm* and differ between male and female mice. Mice were injected intra-peritoneally with CFSE 6 hrs before intranasal infection with *Cm*. After 3 days, mice were sacrificed and cells from the lungs and MLNs were isolated and analyzed by flow cytometry. A) Representative FACS plots showing CFSE^{+}CD19^{+} cells in the lungs and MLN. B) Representative FACS plot of CD43 and CD5 expression in B1 cells that migrated to the lungs. C) Frequency of B1 cell subsets out of live, out of total B cells, and the total cell number in the lungs. *p<0.05 using Mann Whitney test.

days 6, 9, and 12 post-infection and did not observe the same sex-specific differences in the number of B1 cells (Figure 3.9).

Together these data demonstrate that in the lungs specifically, both B2 and B1 cells are significantly upregulated in females following intranasal *Cm* infection. This difference in B cell populations likely accounts for the increased antibody responses observed in female mice.

Knowing that TFH cells are key in the creation of antigen-specific antibody responses, we wanted to characterize this population in both the lungs and MLNs of male and female mice following intranasal *Cm* infection.

At day 9 post-infection, females had significantly more TFH cells in their lungs compared to males, which coincides with increases in antibody production and B cell numbers in the lungs of female mice (Figure 3.10A). By day 12 post-infection, there was no longer a significant difference in TFH cells between the sexes which mirrors the pattern observed in bacterial burden where by day 12 post-infection males and females had similar responses (Figure 3.10B). Considering this, it seems that following *Cm* infection *in vivo*, female mice have increased humoral responses initially but by day 12 post-infection when the mice begin to recover, the humoral response of males and females even out.

The differences in TFH numbers observed in the lungs were not mirrored in the MLN (Figure 3.11). Instead, at day 12 post-infection, there was a significant upregulation of TFH cells in the male MLN (Figure 3.11C). This coincides with an increase of B2 cells in the MLN of male mice and therefore further supports that an increased humoral

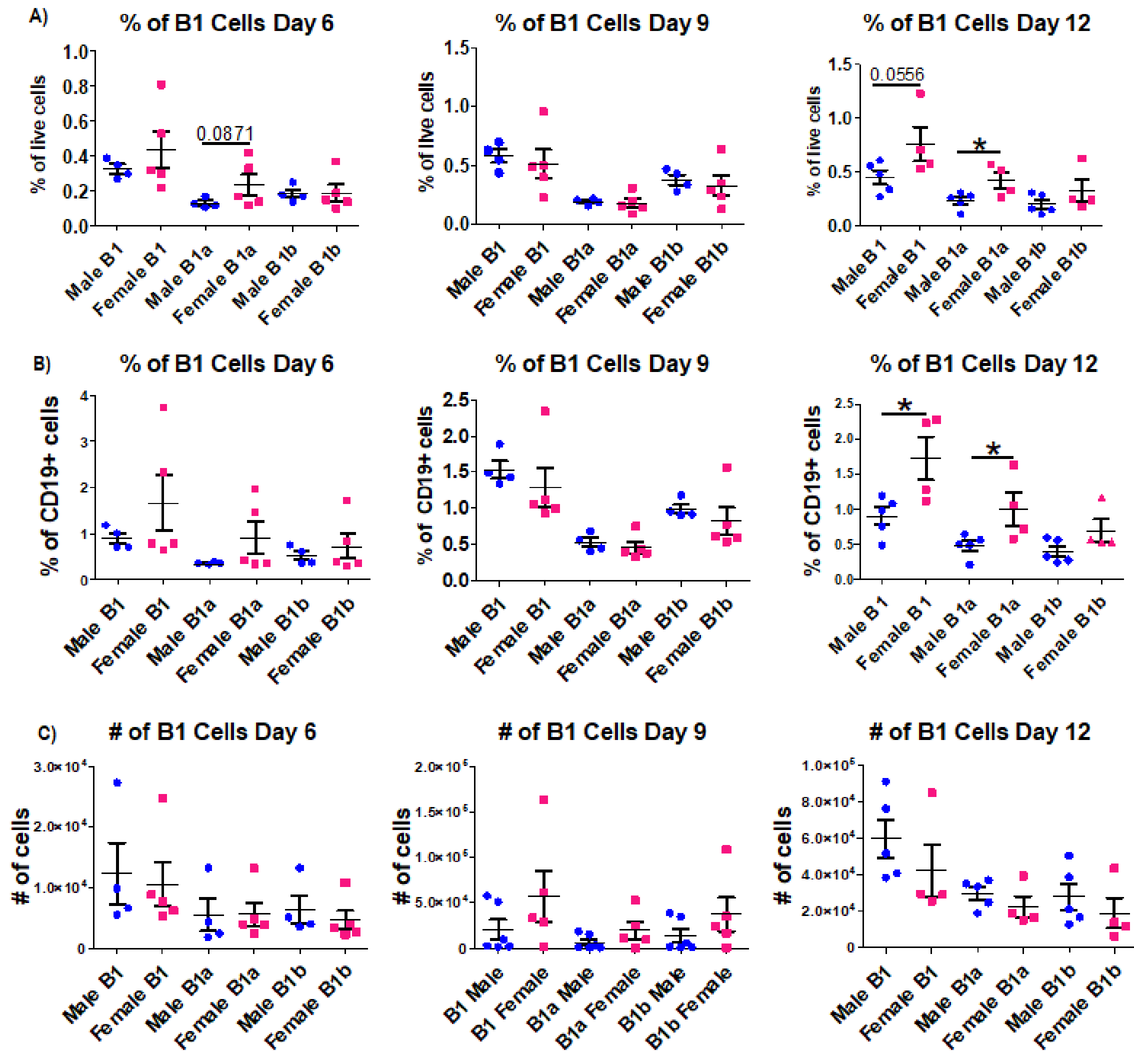


Figure 3. 9: B1 cells do not differ between males and females at day 12 post infection in the MLN. Mice were infected intranasally with *Cm*. Cells from the MLNs were isolated at various time points and analyzed by flow cytometry. The frequency of B1 cell subsets out of live (A), out of total B cells (B), and the total cell number (C) are shown at days 6, 9, and 12 post-infection. Data are shown as mean±SEM where each dot represents an individual mouse. *$p<0.05$ using Mann Whitney test.

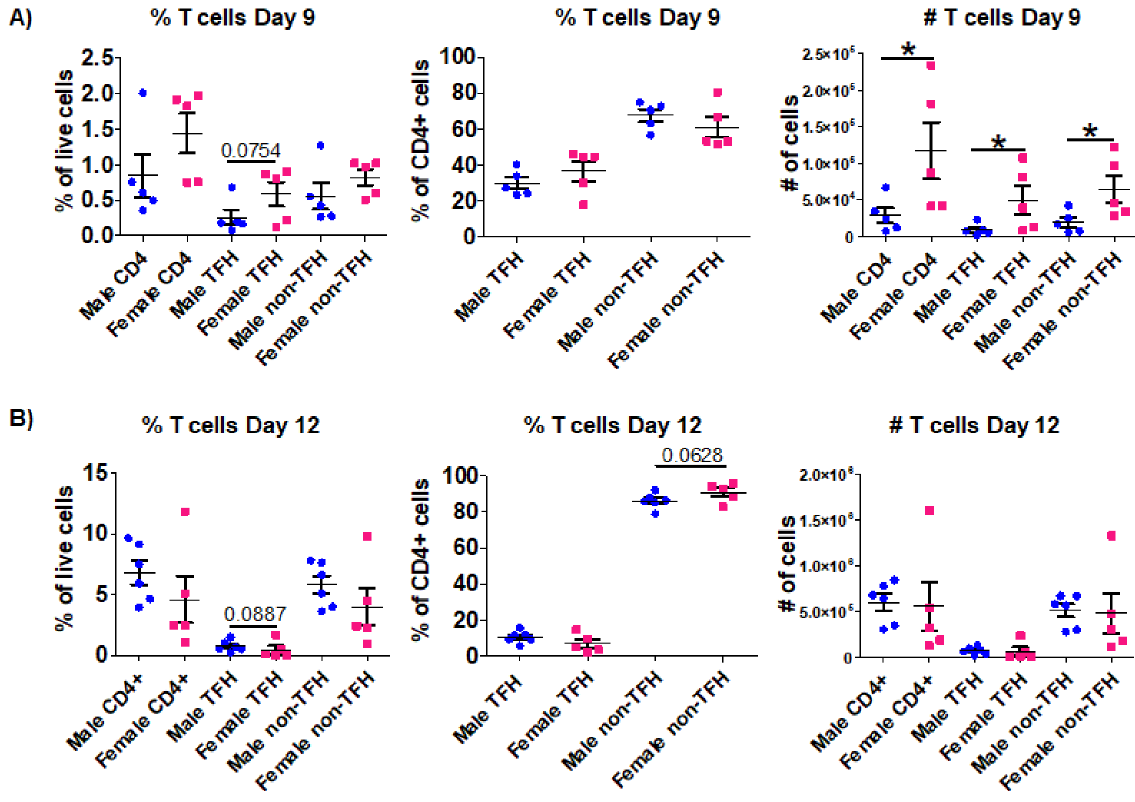


Figure 3. 10: Females have more TFH cells in the lungs at day 9 post-infection. Mice were infected intranasally with *Cm*. Cells from the lungs were isolated at various time points and analyzed by flow cytometry. A) T cell proportions out of live, total T cells, and total numbers of T cells at day 9 post-infection. B) T cell proportions out of live, total T cells, and total numbers of T cells at day 12 post-infection. Each dot represents an individual mouse. Data are graphed as mean±SEM. *p<0.05 using Mann Whitney test.

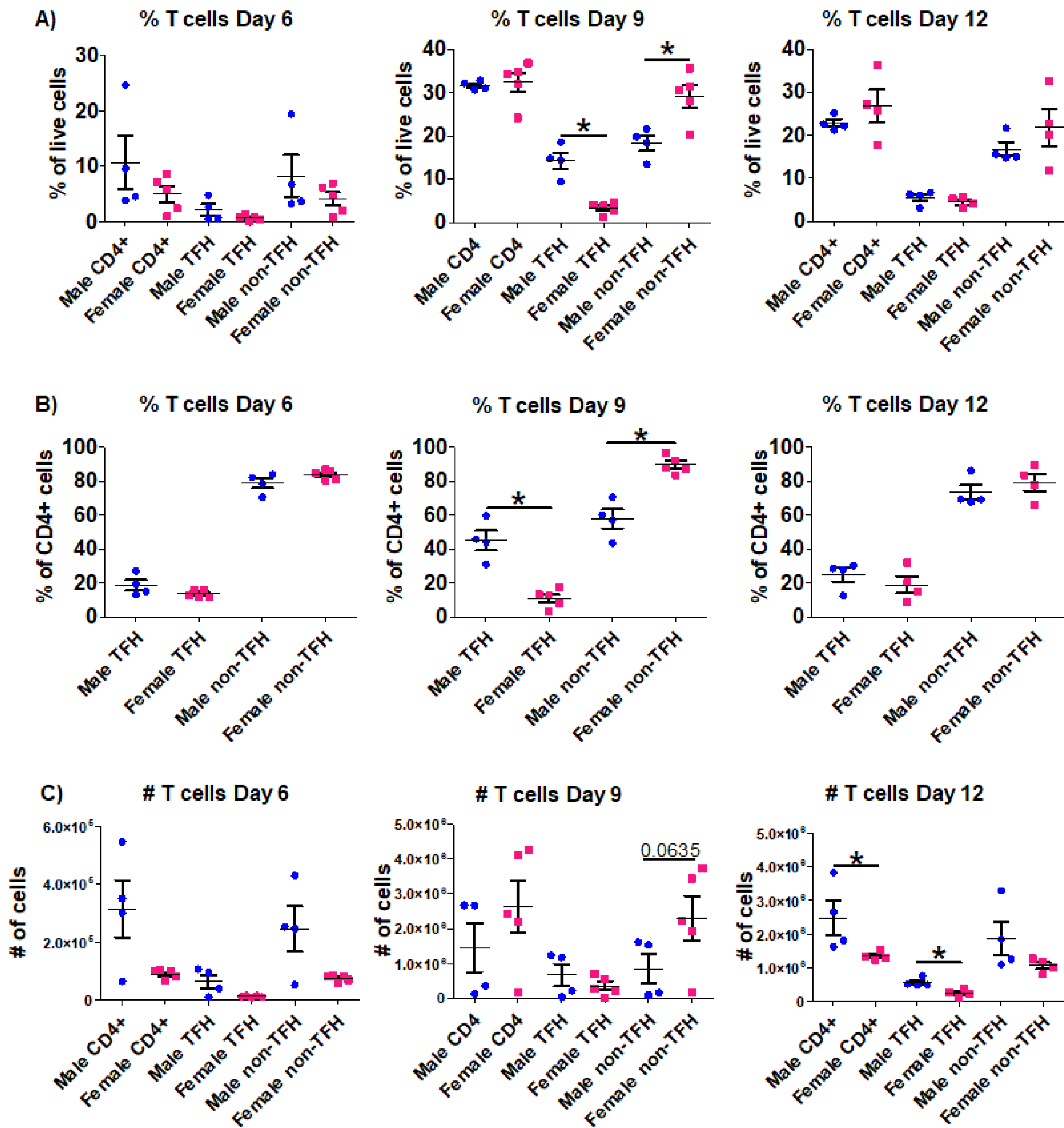


Figure 3. 11: Males have significantly more TFH cells in the MLN 9 days post-infection. Mice were infected intranasally with *Cm*. At day 6, 9, and 12 post-infection, MLNs were isolated and analyzed by flow cytometry. A) The proportion of T cells out of total cells throughout infection. B) The proportion of T cell subsets out of total T cells. C) The number of T cells. Each dot represents an individual mouse and data are shown as mean±SEM. *$p<0.05$ using Mann Whitney test.

response in the MLN of male mice at day 12 may contribute to the recovery of male humoral responses.

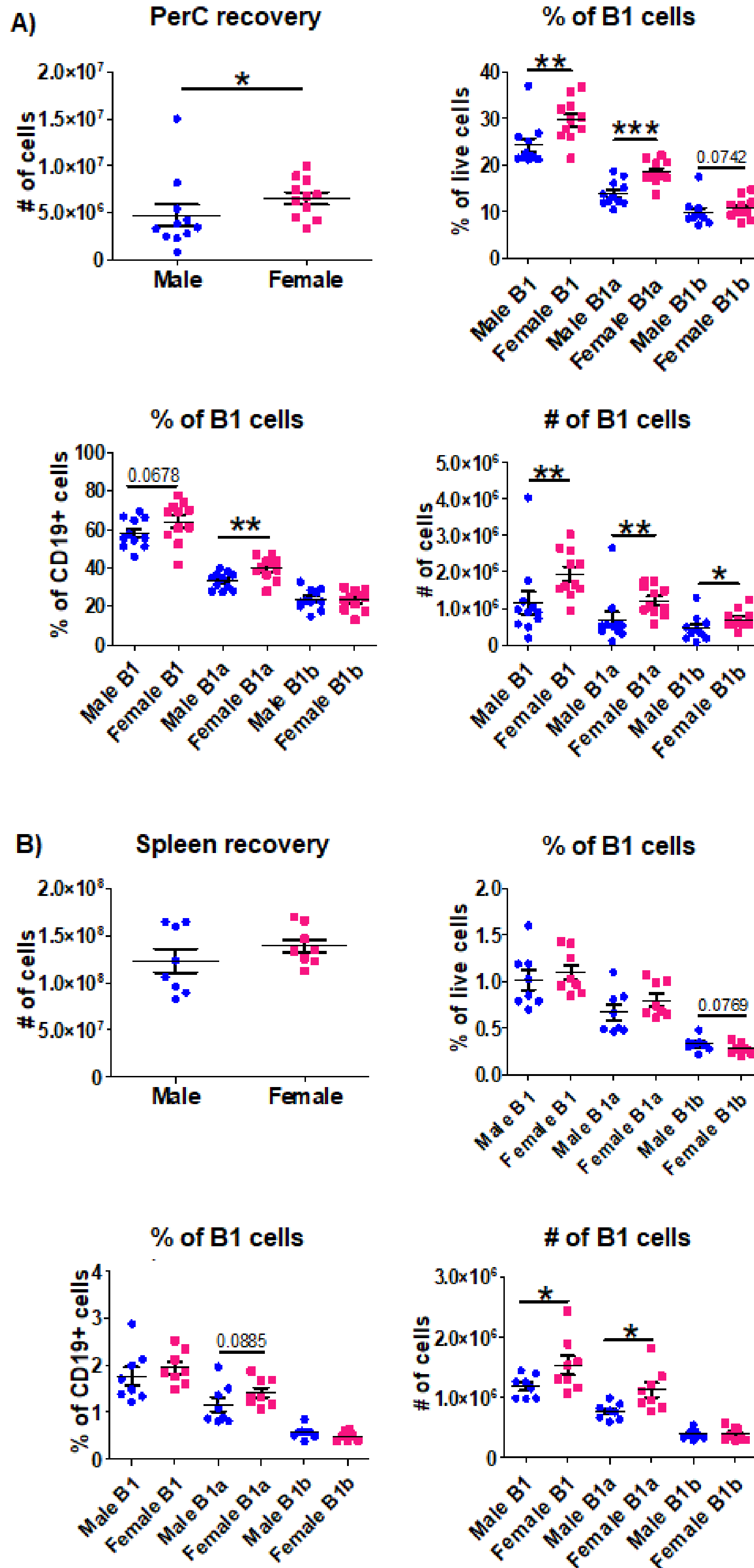
3.1.6 Female mice have significantly more B1 cells in the peritoneal cavity and spleen at rest

Given that B1 cells have not been studied in *Cm* infection and considering that the sex-specific differences in B1 cells observed in the lungs following infection have not been described before, we wanted to investigate these cells further to better understand how they differ between male and female mice. One study identified that females had higher total B cells in the peritoneal cavity at rest but since this study only examined total $CD19^+$ cells, we wanted to determine if there was a difference specifically in B1 cells at rest (211).

In agreement with the previous study, we found that female mice had significantly more total cells in the peritoneal cavity compared to male mice (Figure 3.12A) (211). When we examined B1 cells specifically, we found that females had significantly more B1 cells, B1a cells, and B1b cells compare to males. Together these results demonstrate that there is an innate difference in peritoneal B1 cells in male and female mice.

Considering that there is a population of B1 cells in the spleen, we also examined splenic B1 cells in male and female mice at rest (Figure 3.12B) (142). In contrast to the peritoneal cavity, the total cell recovery from the spleen did not differ between male and female mice (Figure 3.12B). However, female mice did have a higher number of B1 cells, specifically B1a cells, in the spleen compared to males (Figure 3.12B). Together these

Figure 3. 12: B1 cells in the peritoneal cavity and spleen differ between males and females. The peritoneal cells and splenocytes of naive male and female mice were isolated and stained for flow cytometry. The cell recovery, frequency of B1 cells out of live cells, out of $CD19^{+}$ cells, and the number of B1 cells are shown for peritoneal cells (PerC) (A) and splenocytes (B). Peritoneal cell graphs are pooled from 3 individual experiments and splenocyte graphs are pooled from 2 individual experiments. Each dot represents an individual mouse. Data are graphed as mean±SEM. *$p<0.05$, **$p<0.001$, ***$p<0.001$ using Student's t test (A: panel 2 B1a and panel 3. C: all panels.) or Mann Whitney test.


A)
PerC recovery
# of cells
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
*
Male
Female
% of B1 cells
% of live cells
40
30
20
10
0
**
***
0.0742
Male B1
Female B1
Male B1a
Female B1a
Male B1b
Female B1b
% of B1 cells
% of CD19+ cells
100
80
60
40
20
0
0.0678
**
# of B1 cells
# of cells
5.0×10⁶
4.0×10⁶
3.0×10⁶
2.0×10⁶
1.0×10⁶
**
*
B)
Spleen recovery
2.0×10⁸
1.5×10⁸
1.0×10⁸
5.0×10⁷
0.0769
0.0885
3.0×10⁶
2.0×10⁶
1.0×10⁶

data show that there are significant differences between male and female B1 cell populations in the areas where B1 cells predominantly reside at rest.

Because B1 cells differ in both the spleen and peritoneal cavity at rest, it suggests that there is a fundamental difference is the development of these cells between male and female mice. Considering this, we decided to examine the B1 cell population in the lungs and MLNs at rest because we saw differences in these organs following infection and wanted to determine if this was an innate difference or if it was induced by *Cm* infection.

In the lungs we observed no significant differences in the total cell recovery or among the B1 cell populations (Figure 3.13A). Conversely, in the MLN the cell recovery was significantly higher in females compared to males and females had significantly more B1a cells at rest (Figure 3.13B). At day 6 post-infection we did not observe a difference in B1 cell number between male and female mice in the MLN, suggesting a dynamic change in the B1 cell population of the MLN following *Cm* infection.

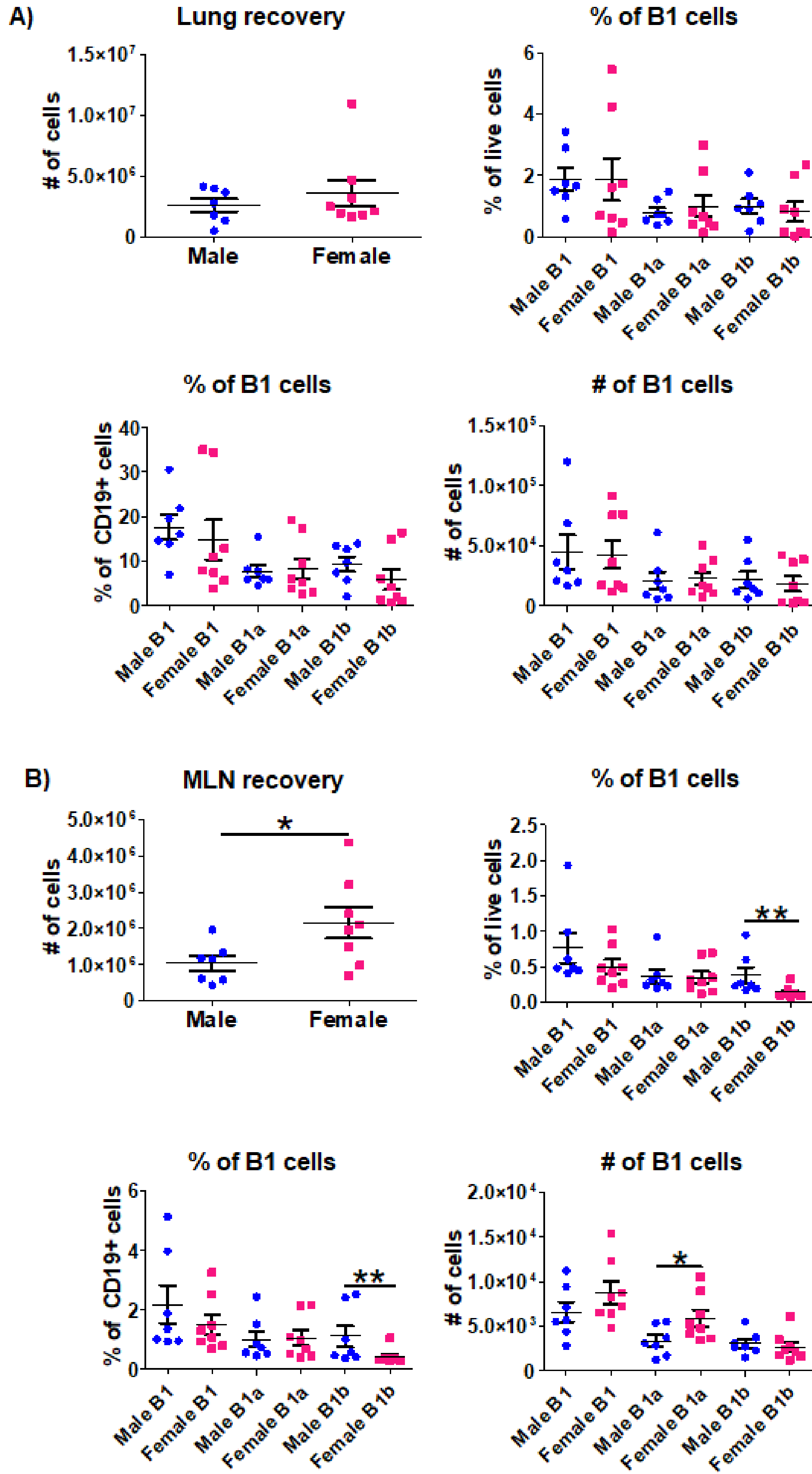
Together, the characterization of B1 cells at rest demonstrates that there are sex-specific differences in B1 cell populations at rest but these do not account for all the differences observed following infection, such as in the lungs. Our data also demonstrate that it is important to consider the sex of the mouse when investigating the roles of B1 cells as we have identified multiple differences at rest.

3.2 Characterization of sex differences in response to *Cm* infection *in vitro*

3.2.1 Female IL-10^{+} B1 cells expand significantly following *Cm* infection *in vitro*

Knowing that there are significant differences in B1 cells in male and female mice following *in vivo* infection, we wanted to better characterize the specific roles of B1 cells

Figure 3. 13: Females have more B1a cells in the MLN compared to males at rest. Cells from the lungs and MLNs of naive male and female mice were isolated and stained for flow cytometry. The cell recovery, frequency of B1 cells out of live cells, out of $CD19^+$ cells, and the number of B1 cells are shown for lungs (A) and MLNs (B). Graphs are pooled from 2 individual experiments. Each dot represents an individual mouse. Data are graphed as mean±SEM. *p<0.05, **p<0.001, ***p<0.001 using Mann Whitney test.

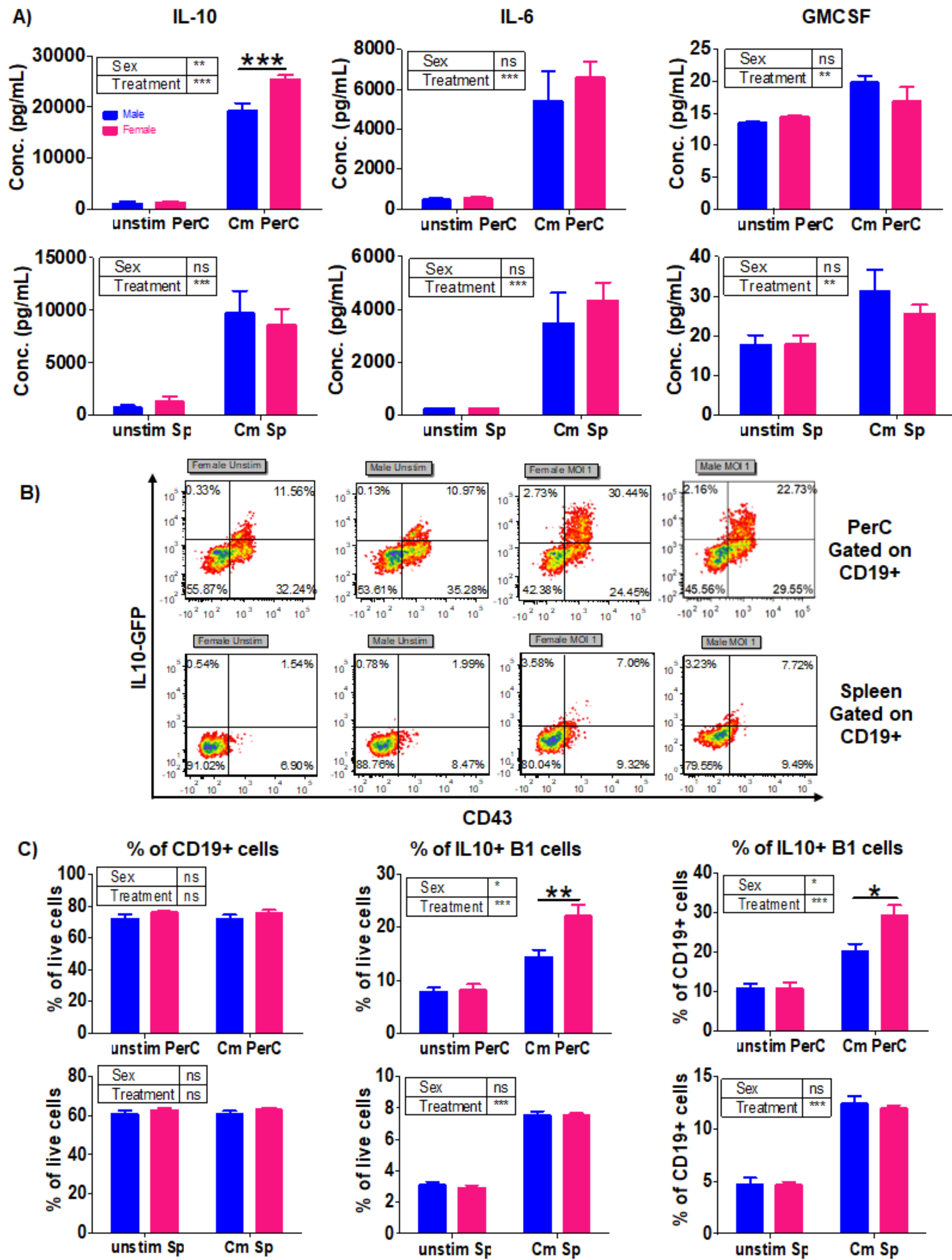

A)
Lung recovery
# of cells
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
Male
Female
% of B1 cells
% of live cells
6
4
2
0
Male B1
Female B1
Male B1a
Female B1a
Male B1b
Female B1b
% of B1 cells
% of CD19+ cells
40
30
20
10
0
Male B1
Female B1
Male B1a
Female B1a
Male B1b
Female B1b
# of B1 cells
# of cells
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
Male B1
Female B1
Male B1a
Female B1a
Male B1b
Female B1b
B)
MLN recovery
# of cells
5.0×10⁶
4.0×10⁶
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
*
Male
Female
% of B1 cells
% of live cells
2.5
2.0
1.5
1.0
0.5
0.0
**
Male B1
Female B1
Male B1a
Female B1a
Male B1b
Female B1b
% of B1 cells
% of CD19+ cells
6
4
2
0
**
Male B1
Female B1
Male B1a
Female B1a
Male B1b
Female B1b
# of B1 cells
# of cells
2.0×10⁴
1.5×10⁴
1.0×10⁴
5.0×10³
0
*
Male B1
Female B1
Male B1a
Female B1a
Male B1b
Female B1b

in vitro to see how they might affect the overall immune response in female mice.

We initially began our *in vitro* studies by analyzing responses in whole peritoneal exudate and splenocytes, knowing that there are B1 cells in both populations (Figure 3.14). Obvious differences in the responses of male and female whole peritoneal exudate cells were apparent 24 hrs post-infection (Figure 3.14). When stimulated with *Cm*, female peritoneal cells produced significantly higher amounts of IL-10 compared to males (Figure 3.14A). The upregulation of IL-10 in females is particularly interesting considering that they had lower Th1 responses *in vivo* and IL-10 is a potent regulatory cytokine. Both IL-6 and GMCSF production did not differ between the sexes (Figure 3.14A). When the same experiment was done in splenocytes, there was no apparent sex difference in IL-10 production (Figure 3.14A). Much like in peritoneal cells, IL-6 and GMCSF production in splenocyte cultures did not differ between the sexes (Figure 3.14A).

When we analyzed B1 cells in these cultures by flow cytometry, we found that following stimulation with *Cm*, female peritoneal cell cultures had a significant upregulation of IL-10^+ B1 cells (Figure 3.14B and C) but we did not see the same trend in splenocytes. This suggests that following *Cm* infection, B1 cells in the peritoneal cavity are activated and begin to upregulate IL-10. Given that we know peritoneal B1 cells migrate to the lungs following infection, the IL-10 produced by migrated B1 cells may explain the suppressed Th1 responses in female mice.

Figure 3. 14: Female peritoneal cells stimulated with *Cm* produce more IL-10 and contain more $IL10^+$ B1 cells than male cells. Peritoneal cells and splenocytes were isolated from mice and stimulated *in vitro* with *Cm* at MOI=1 for 24 hrs. The culture supernatant was collected for ELISA and the cells were analyzed by flow cytometry. A) Supernatant from the culture was tested for IL-10, IL-6, and GMCSF by ELISA. B) Flow plots showing $CD19^+CD43^+IL10^+$ cells 24 hrs post stimulation. C) The frequency of B cells and the proportion of $IL10^+$ B1 cells out of live or out of total B cells are shown for peritoneal cells and splenocytes. Data shown are representative of two experiments and graphed as mean±SEM. For peritoneal cells n=4 for males, n=5 for females, for splenocytes n=5 mice per sex. *p<0.05, **p<0.01, **P<0.001 using 2-way ANOVA with Bonferroni post-test.


A)
IL-10
IL-6
GMCSF
Conc. (pg/mL)
Sex
Treatment
Male
Female
unstim PerC
Cm PerC
unstim Sp
Cm Sp
B)
Female Unstim
Male Unstim
Female MOI 1
Male MOI 1
PerC Gated on CD19+
Spleen Gated on CD19+
IL10-GFP
CD43
C)
% of CD19+ cells
% of IL10+ B1 cells
% of live cells
% of CD19+ cells

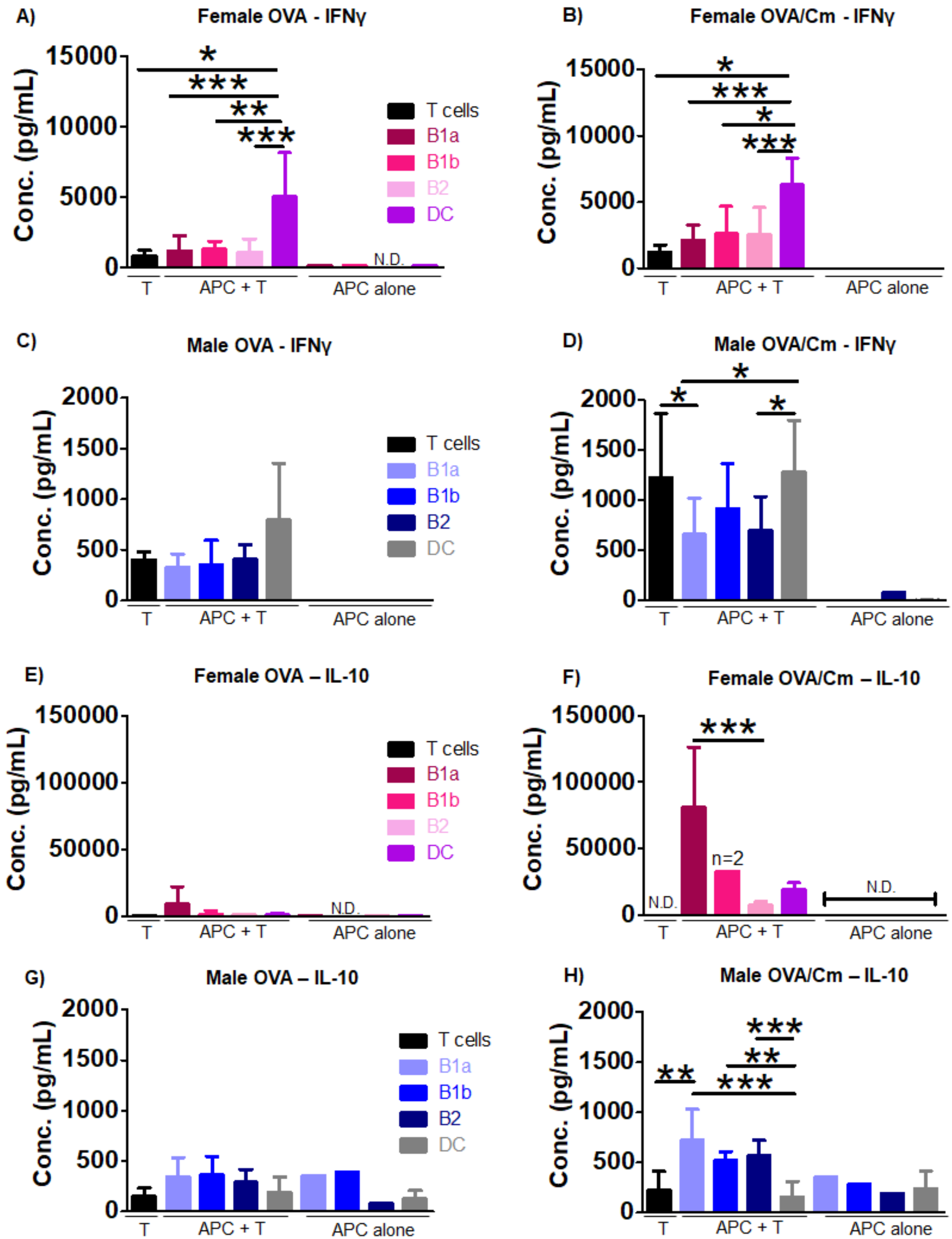
3.2.2 Dendritic cells and B cells function as antigen presenting cells in a subset and sex-dependent manner in response to *Cm*

Given that there are multiple subsets of B cells present in the peritoneal cavity and we observed that B1 cells are involved in responses to *Cm*, we wanted to examine how using B2, B1a, B1b cells or DCs as APCs would affect cytokine responses *in vitro*. To this end, we isolated APCs from male and female mice and co-cultured them with naïve OVA-specific T cells with or without heat-killed *Cm* and measured cytokine responses. In cells isolated from female mice, we found that using DCs as APCs resulted in the strongest IFNγ response, while B1a, B1b, and B2 cells resulted in significantly less IFNγ production (Figure 3.15A and B). In the cultures using cells from male mice, we found a significantly higher response when DCs were used as APCs compared to B1a and B2 cells but it was comparable to the response induced by B1b and T cells (Figure 3.15C and D). When male B1a cells were used as APCs, the resulting IFNγ response was lower than the T cell control, suggesting that these APCs suppressed IFNγ production (Figure 3.15C and D).

When measuring IL-10 responses, using B1a cells as APCs elicited the strongest response in females (Figure 3.15E and F). In males, any cells as APCs resulted in similar IL-10 responses in the presence of OVA alone or with heat-killed *Cm* (Figure 3.15G and H). Together these data demonstrate that different B cell subsets in both male and female can act as APCs in a subset-specific manner.

Knowing that IL-10 production differed in the cultures in Figure 3.14A, we wanted to examine if the strength of the co-culture responses differed between the sexes (Figure 3.16). We found that the amount of cytokines produced in co-culture differed

Figure 3. 15: B cell subsets in the peritoneum can act as APCs and have subset-dependent abilities to induce cytokine production *in vitro*. B cell subsets were isolated by FACS sorting and co-cultured with $CD62L^+$ OVA-specific T cells in the presence of OVA or a combination of OVA and heat-killed *Cm* for 72 hrs. The supernatant was collected and the levels of IFNγ and IL-10 from female (A-D) and male (E-H) sorts were measured by ELISA. T cell alone bars contain n=3-12 while APC + T bars contain n=5-16 unless otherwise indicated. N.D. represents no data available. *$p<0.05$, **$p<0.001$, ***$p<0.001$ using 1-way ANOVA with Bonferroni post-test (D) or Kruskal Wallis test with Dunn's post-test (A-C, E-H).



A)
Female OVA - IFNγ
Conc. (pg/mL)
15000
10000
5000
0
*
***
**
***
T cells
B1a
B1b
B2
DC
N.D.
T
APC + T
APC alone
B)
Female OVA/Cm - IFNγ
Conc. (pg/mL)
15000
10000
5000
0
*
***
*
***
T
APC + T
APC alone
C)
Male OVA - IFNγ
Conc. (pg/mL)
2000
1500
1000
500
0
T cells
B1a
B1b
B2
DC
T
APC + T
APC alone
D)
Male OVA/Cm - IFNγ
Conc. (pg/mL)
2000
1500
1000
500
0
*
*
*
T
APC + T
APC alone
E)
Female OVA – IL-10
Conc. (pg/mL)
150000
100000
50000
0
T cells
B1a
B1b
B2
DC
N.D.
T
APC + T
APC alone
F)
Female OVA/Cm – IL-10
Conc. (pg/mL)
150000
100000
50000
0
***
n=2
N.D.
N.D.
T
APC + T
APC alone
G)
Male OVA – IL-10
Conc. (pg/mL)
2000
1500
1000
500
0
T cells
B1a
B1b
B2
DC
T
APC + T
APC alone
H)
Male OVA/Cm – IL-10
Conc. (pg/mL)
2000
1500
1000
500
0
***
**
**
***
T
APC + T
APC alone

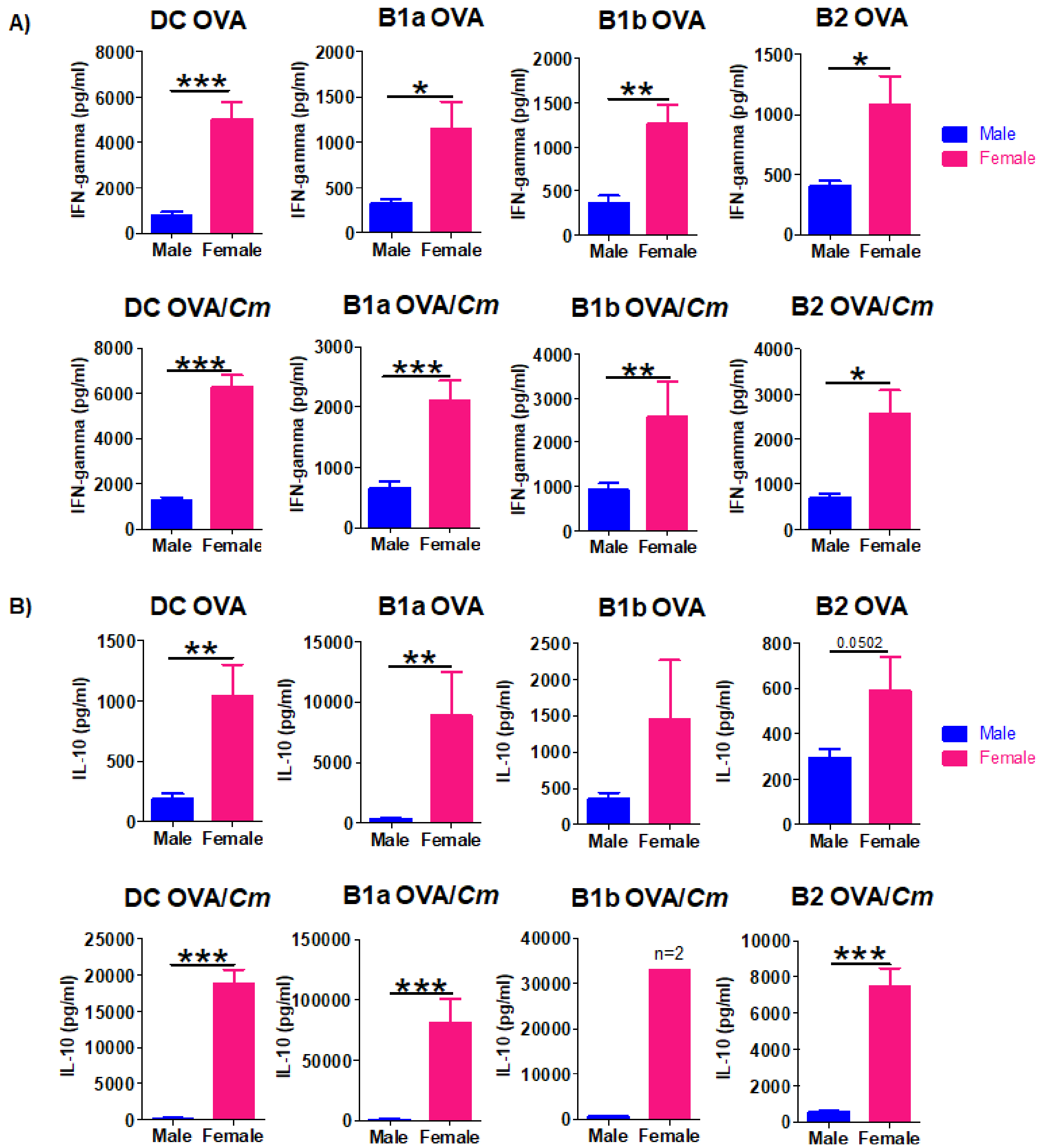


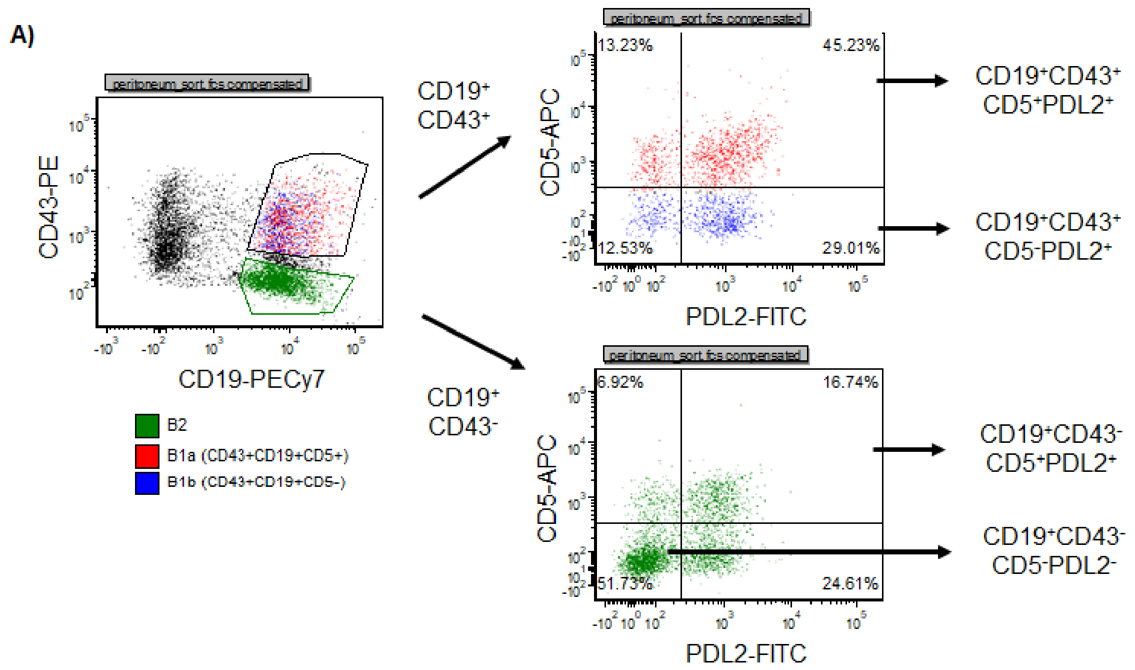
Figure 3. 16: B cell subsets have sex-dependent abilities to induce cytokine production *in vitro*. B cell subsets were isolated by FACS sorting and then cultured with CD62L^{+} OVA-specific T cells in the presence of OVA or a combination of OVA and heat-killed *Cm* for 72 hours. The supernatant was collected and the levels of IFNγ (A) and IL-10 (B) were measured by ELISA each bar containing n=5-16 unless otherwise indicated .. *p<0.05, **p<0.001, ***p<0.001 using Student's t test or Mann Whitney test within stimulations.

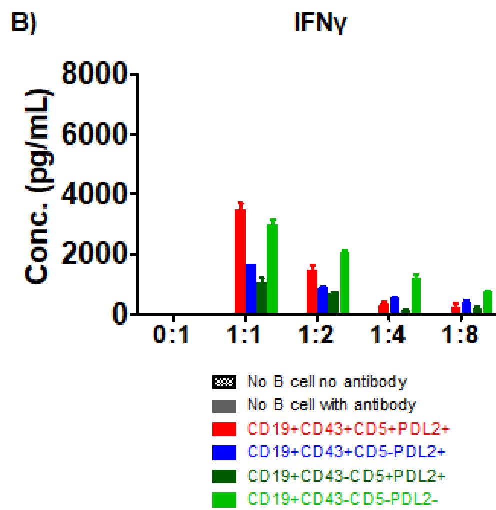
significantly between males and females (Figure 3.16).

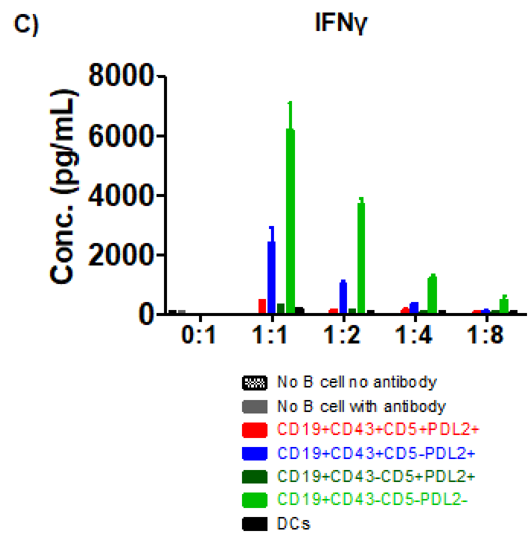
In the case of IFNγ, each cell subset resulted in significantly higher cytokine production if it was isolated from female mice (Figure 3.16A). APCs isolated from female mice were also able to induce significantly more IL-10 production compared to cells from male mice (Figure 3.16B). Together these data suggest that female APCs are better able to activate T cells to produce cytokines compared to male APCs.

Typically, B1a cells are defined as $CD19^{+}CD43^{+}CD5^{+}$ and B1b cells are defined as $CD19^{+}CD43^{+}CD5^{-}$ but we noted in our experiments that some populations of B1 cells also expressed PDL2. To examine the role of these populations further, we used these subsets as APCs in co-culture (Figure 3.17A, FACS plots are colour coded according to conventional subset markers). We noted two interesting trends from these experiments. Firstly, when $PDL2^{+}$ B1a cells are used as APCs in co-culture, the resulting IFNγ levels are higher than co-cultures with $PDL2^{+}$ B1b cells and two subsets of B2 cells (Figure 3.17B). These data suggest that PDL2 expression can alter the role of APC subsets, as B1a cells were not strong inducers of IFNγ in Figure 3.15. Secondly, when we repeated these co-cultures using B cell subsets from the spleen, the resulting cytokine levels differed greatly (Figure 3.17C). In the spleen co- cultures, when $PDL2^{+}$ B1a cells were used as APCs, the resulting levels of IFNγ were lower than cultures with other subsets as APCs (Figure 3.17C). Instead, $PDL2^{+}$ B1b cells induced the strongest IFNγ response (Figure 3.17C). These results demonstrate that B cells in the peritoneal cavity and spleen have divergent functions as APCs.

Figure 3. 17: PDL2 expression alters B cell-mediated induction of IFNγ. B cell subsets were isolated by FACS and co-cultured with splenic naïve T cells and anti-CD3/CD28. A) Gating strategy for B cell subset sorting. Colours in gating strategy represent conventional B cell subset nomenclature. B) Co-culture with peritoneal B cells. C) Co-cultures with splenic B cells. Ratios on X-axis represent the number of antigen presenting cells: T cells.


A)
peritoneum_sort.fcs compensated
CD43-PE
CD19-PECy7
B2
B1a (CD43+CD19+CD5+)
B1b (CD43+CD19+CD5-)
CD19+
CD43+
CD19+
CD43-
CD5-APC
PDL2-FITC
13.23%
45.23%
12.53%
29.01%
6.92%
16.74%
51.73%
24.61%
CD19+CD43+
CD5+PDL2+
CD19+CD43+
CD5-PDL2+
CD19+CD43-
CD5+PDL2+
CD19+CD43-
CD5-PDL2-


B)
IFNγ
Conc. (pg/mL)
8000
6000
4000
2000
0
0:1
1:1
1:2
1:4
1:8
No B cell no antibody
No B cell with antibody
CD19+CD43+CD5+PDL2+
CD19+CD43+CD5-PDL2+
CD19+CD43-CD5+PDL2+
CD19+CD43-CD5-PDL2-


C)
IFNγ
Conc. (pg/mL)
8000
6000
4000
2000
0
0:1
1:1
1:2
1:4
1:8
No B cell no antibody
No B cell with antibody
CD19+CD43+CD5+PDL2+
CD19+CD43+CD5-PDL2+
CD19+CD43-CD5+PDL2+
CD19+CD43-CD5-PDL2-
DCs

CHAPTER 4 DISCUSSION

4.1 Discussion of major findings

Sex-specific differences in both epidemiology and sequalae of infection have been described in *Chlamydia* but the basis for these differences is not well understood. In this study, we wanted to better understand these differences by determining if sex-specific immune responses arose following *Chlamydia* infection. To this end, our objectives were to determine if there were sex-specific immune differences in the responses to *Cm* infection in mice and to characterize any observed responses. We hypothesized that female mice are more susceptible to *Chlamydia* infection compared to males and that B1 cells play an important role in regulating sex-dependent immune responses during *Chlamydia* infection.

4.1.1 *In vivo* characterization of sex-specific responses to *Cm* infection

4.1.1.1 The immune response to intranasal *Cm* infection differs significantly between the sexes

Male and female mice were infected intranasally with *Cm* to characterize how responses differ between the sexes. A lung model was chosen to avoid the differences in gross anatomy between male and female mice, and to instead focus on solely sex-specific immune responses.

Following intranasal *Cm* infection, body weight loss is used as a clinical score. In our experiments, female mice lost significantly more body weight compared to male mice, suggesting they were sicker following infection. Interestingly, a similar body weight trend has also been observed in *P. aeruginosa* infection (207). Following lung

infection with *P. aeuruginosa*, female mice lost more body weight compared to males and recovered their body weight more slowly (207). While these results are very similar to our own, it is possible that body weight loss and susceptibility are affected by the starting body weight of the mice, as female mice are always initially smaller than males. This possibility has been examined in the context of L. *monocytogenes* infection (206). Following i.v. infection of *L. monocytogenes*, female mice were more likely to succumb to infection compared to males and the authors considered that this difference may be due to the fact that male mice start off slightly larger than females (206). In order to exclude this parameter, the authors decided to dose the bacterial injections by body weight such that the mice received 680 colony forming units per gram of weight (206). When the infections were performed in this manner, the female mice were still more susceptible to infection (206). Together, this suggests that following intranasal *Cm* infection female mice are sicker than male mice.

Next, we measured bacterial burden in the lungs of the mice. We found that initially, male and female mice had similar bacterial burdens but by day 9 the burden in females was significantly higher. At day 12, bacterial burdens were once again similar. Interestingly, the difference in bacterial burden at day 9 coincides with when body weight differences become apparent between the sexes. This suggests that females are more susceptible to infection and become sicker as a result. Over time, the male bacterial burden levels stay relatively stable during the timepoints measured while the females have an increase followed by a decrease at day 12. This suggests that males have a better control of the infection over time while females become sicker initially before controlling

the infection. This is reflected in body weight where females also start to recover by day 12 post-infection.

While this is the first study examining the bacterial burden of male and female mice following intranasal *Cm* infection (to our knowledge), there have been studies in humans comparing bacterial burden in genital tract infections. The sample collection methods vary between papers making it hard to judge the actual burden when comparing males and females. In one study, first void urine (FVU) was used in males and females, in order to try to make the collection methods comparable (212). In this study, they found that males had a higher burden in one genotype of *Chlamydia* but other studies have argued that FVU is not the most accurate way to measure burden in females (212–214). These other studies suggested that self-collected vaginal swabs are more accurate in women, while in men FVU and urethral swabs are equally accurate (213, 214). Endocervical swabs are also considered accurate in women but are not always used because they are invasive (213). In studies using endocervical swabs compared to male urethral swabs, there are higher burdens in females (215, 216). Therefore, it is likely that our observed lung burden mirrors human genital infection burden trends but because of the differences in gross anatomy between the male and female genital tract it is hard to know what studies represent the most accurate burden in humans.

Considering that female mice had higher bacterial burdens, we wanted to examine if females had strong antibody responses. We observed that at day 9 post-infection females had higher total IgG responses to both heat-killed *Chlamydia* and the pathogenic protein TC0582. Together this suggests that females have stronger antibody responses compared to males. Considering the controversy surrounding the role of antibody in the

response to *Chlamydia* infection, these data suggest at least a non-protective response, and at worst a pathological response, in female mice. Considering that this response occurs concurrently with a peak in bacterial burden and the beginning of weight differences, it is possible that female mice are having predominantly antibody-mediated responses allowing for *Chlamydia* to replicate while males are better at controlling the infection.

Strong antibody responses in females compared to males have also been described following influenza infection in mice (217). Following intranasal infection with H1N1 or H3N2 influenza virus, female mice have significantly more neutralizing antibody and anti-influenza IgG in the blood compared to male mice (217). It is therefore possible that we are observing a general difference between males and females and not one that is specific to *Chlamydia* infection.

To further support the hypothesis that increased bacterial burden in females is related to increased antibody production, we performed liner regressions and correlation analyses between the two variables. We found that in both male and female mice there was a significant correlation between bacterial burden and antibody titer, where the higher the bacterial burden, the more antibody there was in the blood. This correlational data does not infer causation but suggests a relationship between the two variables that would need to be studied further. It is also possible that having more bacteria is simply leading to a greater immune response and more antibody so it is hard to draw concrete conclusions from the correlations.

Now that we observed considerable differences in the overall response to *Cm* between male and female mice, we sought to characterize the immune responses during

infection. To this end, we isolated the splenocytes of the mice at day 9 and 12 and performed an *ex vivo* antigen recall assay to characterize the type of response occurring during infection. We found that males had significantly higher IFNγ and IL-17A responses at day 9 post-infection. Considering that IFNγ is key in the response to *Cm* infection, it may be controlling bacterial replication in male mice causing the difference in bacterial burden observed at day 9 post-infection. The concentration of IL-17A observed in the cultures may be related to the increase in IFNγ. In a study from 2009, mice deficient in IL-17A lost more body weight, had higher bacterial burdens, and had more lung pathology compared to control mice following intranasal *Cm* infection (97). In this study, they found that the IL-17A deficient mice had a decrease in Th1 responses and an increase in Th2 responses and identified that IL-17A promotes Th1 responses through a dendritic cell-dependent mechanism (97). It is therefore possible in our study that the IL-17A observed in male mice is working to promote the IFNγ response and, together, efficiently control bacterial burden in male mice. Together, the day 9 recall assay suggests that males have a protective response at day 9 post-infection that is stronger than the response that we observed in females.

At day 12 post-infection, females had a significantly higher concentration of IL-13, representative of a Th2 response, as well as IL-10, suggestive of a regulatory response. Th2 responses are not protective during genital *Cm* infection and therefore will not help the female mice clear infection (218). Similarly, IL-10 production has been shown to diminish protective Th1 responses following *Cm* infection in mice (219). Specifically, IL-10 knock out mice were found to have increased Th1 responses leading to improved bacterial clearance in a lung infection model (219). Together, the responses

at day 9 and 12 suggest that males have an enhanced protective cellular response to *Cm* infection while females have delayed protective immune responses. Instead, female mice initially have an increase in non-protective humoral immune responses. We currently use *ex-vivo* antigen recall cytokine measures to determine T cell responses. To confirm these responses more specifically, the transcription factors from the recall culture cells from could be analyzed by flow cytometry

Knowing that *Cm* infection results in differential overall immune responses in male and female mice, we wanted to identify if these differences were caused by differences in immune cell populations. To this end, we examined B and T cell populations in the lungs and MLN of mice following intranasal *Cm* infection.

We first examined B2 cells following infection, given that females produced significantly more antigen-specific IgG following infection compare to males. We saw that in the lungs, female mice had significantly more B2 cells compared to males 9 days post-infection. Considering that the antibody responses differed significantly at day 9 post-infection, this upregulation in the lungs is likely responsible for upregulated antibody responses in female mice. This could be confirmed by examining antibody *in situ* through immunofluorescent microscopy. By day 12 post-infection, male and female mice had similar B2 cell levels. This also coincides well with antibody levels, as by day 12 post-infection, the antibody differences between male and female mice are no longer observed.

In the MLN, there was no difference between the number of B2 cells day 9 post-infection, but instead, male mice had significantly more B2 cells compared to females at day 12 post-infection. We hypothesize that this upregulation of B2 cells could help

account for the increase in male antibody production at day 12 post-infection. Considering that females had more antibody at day 9, males would have to produce more antibodies than females from day 9-12 to have similar levels by day 12 post-infection.

While B cell increases in the lungs have previously been demonstrated following *Cm* infection, there is no previous work examining differences in B2 cells between male and female mice following infection (220).

We next examined B1 cells in mice given that they are important in respiratory infections, such as influenza, and have yet to be characterized during *Cm* infection (210). In female mice we saw a rapid migration of B1 cells to the MLN 3 days post-infection, that resulted in higher numbers of all B1 cell subsets in the lungs in females compared to males. While B1a cell migration has been observed in response to influenza, this is to our knowledge, the first time B1b cells have been shown to migrate to the lungs in response to infection (180).

Finally, we examined TFH cells in the lungs and MLN, given their importance in mediating the production of antigen-specific antibody production. Interestingly, our observations of TFH cells closely mirrored what we saw when characterizing B2 cell responses. At day 9 post-infection, female mice had significantly higher numbers of TFH cells in the lungs compared to male mice but by day 12 post-infection this difference was no longer present. In the MLN, there were no differences in the number of TFH cells between male and female mice 9 days post-infection but at day 12 post-infection, there was significantly more TFH cells in the MLNs of male mice. This data further supports that while females have strong antibody responses initially, males have a later

upregulation of antibody production which is likely mediated by increases in both B2 and TFH cells in the MLN at day 12 post-infection.

Our data suggest that at least in females, antibody responses are mediated in the lungs rather than in the MLN. A similar phenomenon has previously been described in influenza infection (221). When mice lacking SLOs were challenged with influenza, they were able to produce and maintain neutralizing antibody in induced bronchus-associated lymphoid tissues (BALT), suggesting that an immune response could be initiated at the site of infection (221). Importantly, weight loss was similar in the mice with and without SLOs, suggesting that this response not only occurs, but is sufficient to control influenza infection (221).

The initial increase in antibody production in female coincides with a significant difference in bacterial burden as well as increases in female body weight loss. Considering that *Chlamydia* is intracellular, antibody production would not be sufficient for bacterial clearance. Therefore, the observed upregulation of B2 and TFH cells in female mice may be responsible for the sex-specific differences in bacterial burden and body weight loss.

4.1.1.2 Male and female mice have significantly different B cell populations at rest

Considering the sex-specific difference we observed in B1 cells following infection, we wanted to further characterize the role of these cells in *Cm* infection.

Given that B1 cells have not been characterized extensively and never between the sexes, we wanted to examine B1 cells at rest considering the differences we observed following *Cm* infection *in vivo*. To this end, we isolated B cells from the spleen and

peritoneal cavity, where B1 cells reside at rest, and compared between males and females. We saw a significantly higher number of B1 cells, specifically B1a, in females in both the peritoneal cavity and spleen at rest. While it is possible that these differences are hormone related, it is important to note that the estrous cycles of the female mice were not synchronized in these experiments. Considering that the variation between mice is minimal and that they are likely at different parts of the estrous cycle, the differences are likely not due to sex hormones. The second possibility is a genetic difference between males and females that results in these differences but this has not yet been determined.

Consistent with our results, a study published in 2011 also noted higher total cell recovery from female peritoneal cavities compare to males (211). In this study they examined total $CD19^+$ B cells in the peritoneal cavity and found it to be higher in females but they did not break down B cell populations (211). In this study the percentage of total $CD19^+$ cells did not differ between the sexes but our data demonstrates a clear difference in the percent of B1 cells (211). Therefore, it is important to consider all B cell subsets in the peritoneal cavity individually as there may not be differences in all of the populations between the sexes. To determine if sex hormones contributed to the differences observed in the study, the authors performed ovariectomies on the female mice and compared the cell differences to female mice receiving sham operations (211). The authors found a decrease in $CD19^+$ cells in the peritoneal cavity following ovariectomies but there was also a decrease in the sham B1 levels compared to experiments earlier in the paper (original female B1 numbers were around 3×10^6 while sham mice had approximately 2×10^6 cells) making these numbers hard to compare to male mice (211). While this suggests a role for sex hormones in total $CD19^+$ cell numbers in the peritoneal cavity of

female mice, it is not possible to tell if B cell numbers would still be higher compared to males receiving sham operations as this experiment was not performed. Together, our data build on previously observed sex-specific B cell differences in the peritoneal cavities of mice.

Considering that we observed differences in B1 cells in the lungs following *Cm* infection, we also compared the B1 cell population of male and female mice in the lungs and MLN before infection. In the lungs there were no significant differences between male and female B1 cell populations, while in the MLN there was a higher cell recovery in females and more total B1a cells. While sex differences in B cells at rest have not previously been compared, there are previous studies showing B1 cells in the lungs parenchyma and the MLN. In our study, ~2% of cells in the lungs were B1 cells, while a previous report suggests 0.4-0.6% are B1 cells (180). Importantly, while our studies were performed in C57BL/6 mice, one of the study citing lower percentages was done in BALB/c mice which may account for the difference (180). In the MLN, ~0.5% of cells were B1 cells in our hands, while the study in BALB/c mice reports 0.1-0.3% of cells are B1 cells (180). It is important to note that B1 cells can be found in both strains at rest but that there may be strain specific differences in B cell populations.

4.1.2 *In vitro* characterization of sex-specific responses to *Cm* infection

4.1.2.1 Whole peritoneal exudate responds in a sex-specific manner to *Cm* infection *in vitro*

We began to investigate the specific roles of B1 cells following infection by simply analyzing whole peritoneal exudate and whole splenocytes following *Cm*

infection *in vitro* considering that these are where we observed differences in B1 cells at rest. Following *in vitro* stimulation there was a higher concentration of IL-10 in the culture supernatant of female peritoneal cells while no differences were observed in splenocyte cultures. This first result was interesting for two reasons: first, it showed a sex-specific difference in IL-10 production which has been extensively studied in *Chlamydia* infection, and secondly, it implicated a sex difference that would be in the immune cell population of the peritoneal cavity but not the spleen.

The role of IL-10 in the immune response to *Chlamydia* infection has been studied in both humans and mice. IL-10 production by APCs has been associated with the inhibition of Th1 responses in murine studies (222). Considering that strong Th1 responses are necessary for efficient clearance of primary *Chlamydia* infection, the increase in female IL-10 production in our cultures may lead to a detrimental suppression of Th1 responses in female mice (85). The role of IL-10 in human *Chlamydia* infections has been studied in both males and females. In the semen of men and cervical-vaginal lavage of females, there is a significant up-regulation of IL-10 in *Ct*-infected patients compared to controls (223, 224). In these studies, it is unclear what cells produced IL-10 but it demonstrates that there is an upregulation of IL-10 production following Ct infection which agrees with our culture observations.

The role of IL-10 production from splenic B cells during *Chlamydia* infection has previously been studied in our lab. Specifically, $CD43^{-}CD1d^{hi}CD5^{+}$ IL-10-producing B cells were upregulated following *Chlamydia* infection and could suppress Th1 responses *in vitro* (196). In this study, splenic $CD43^{+}CD19^{+}$ cells were also studied, and it was determined that following *Cm* infection, these cells also produced IL-10 (196)

It is well established that $CD19^+CD43^+$ B1 cells are a major cell population in the peritoneal cavity and differences in splenic and peritoneal B1 cells have previously been described (142). Considering this, we analyzed the IL-10^+ B1 cell population in the peritoneal and splenocyte cultures following *Cm* infection *in vitro* and found that in the peritoneal cell cultures derived from female mice there were significantly more IL-10^+ B1 cells. We also found a higher percentage of IL-10^+ B1 cells in peritoneal cell cultures compared to splenocyte cultures, regardless of sex which has previously been described (225). Specifically, $CD19^+$ cells from the peritoneum and spleen have previously been studied following isolation by magnetic positive selection and stimulation *in vitro* (225). In this study they found that LPS stimulation resulted in an increased percentage of $IL10^+$ B cells, as well as total IL-10 measured by ELISA, in peritoneal-derived cultures compared to splenic cultures (225). While this study focused on total $CD19^+$ cells and not B1 cells, the result is mirrored in our *Cm* stimulations *in vitro* and may have been predominantly driven by B1 cells.

Together our *in vitro* stimulation supports previous work that demonstrated differences in splenic and peritoneal B cells and showed sex-specific immune responses to *Cm* are found in peritoneal exudate cells, and more specifically peritoneal IL-10^+ B1 cells.

Following infection, we have demonstrated that B1 cells in the peritoneal cavity migrate to the lungs and upregulate IL-10 production. Considering that IL-10 is a potent immunosuppressive cytokine, the increase in IL-10^+ B1 cells may be related to the observed decrease in female Th1 responses. Considering that IL-10 is known to be important for the differentiation and function of Tr1 cells, B1-cell derived IL-10 may also

cause the observed increase in regulatory responses in female mice (100, 226). Though this may be one role of B1 cells in the response to *Cm* infection, we wanted to further investigate the antigen presenting capacity of B1 cells and determine if they had multiple roles following *Cm* infection.

4.1.2.2 B cell subsets induce specific immune responses in a sex-dependent manner

To further understand the role of B1 cells as APCs, we followed the *in vitro* whole exudate stimulations with a more specific co-culture model, where peritoneal B cell subsets were used as antigen APCs to naïve T cells. Our co-cultures demonstrated that different subsets of B1 cells elicit different immune responses following infection and that cells from female mice produce significantly stronger cytokine responses in culture compared to male counterparts. In both male and female cultures, DCs were the most capable of inducing strong IFNγ responses, which are known to be important in the immune response to *Chlamydia* (85). For IL-10 production, female B1a cells clearly induced the strongest responses while all of the male peritoneal B1 subsets induced a similar amount of IL-10 (though there is slightly more in the B1a co-culture).

B1a cells have previously been linked to IL-10 in the literature. B1a cells are known to produce IL-10 but in our co-culture model we did not see high IL-10 production in B cell alone wells (227). In order to sort all of the populations during these experiments only a limited number of mice could be used due to time constraints. Because of this, the B cell alone wells do not have a large number of cells and it is possible that IL-10 production was not measurable by ELISA in these wells.

Nevertheless, in co-culture wells, IL-10 was easily measured suggesting that this IL-10 was not coming from the B1a cells.

B1a cell have also previously been shown to induce IL-10 producing cells. In a study from 2015, B1a cells were shown to induce suppressive T cells in culture that are distinct from FOXP3^{+} Tregs or Tr1 cells (228). These novel Tregs (termed Treg-of-B1a cell) produce large amounts of IL-10 and IFNγ though this is not how suppression is mediated (228). It is therefore possible that these cells are contributing to the observed IL-10 levels in our co-culture.

When we compared male and female production of both IFNγ and IL-10 we found that in all APC subsets, female co-cultures resulted in significantly stronger cytokine production. While these experiments were done separately, the difference is consistent across both cytokines and all cell types and in most cases multiple experiments were performed per sex. Cytokine differences between males and females have previously been studied but not in a similar model. Sex difference in IL-10 production have been observed in *L. monocytogenes* infection, where higher IL-10 production by females increases susceptibility to infection (206). In a study using human peripheral blood mononuclear cells (PBMCs), female cells produced more IL-10 in the presence of LPS and the female sex hormone estradiol but in the presence of only LPS, male and female PBMCs produced similar amounts of IL-10 (229). It is important to note that in both of these studies it was not determined what cells were producing IL-10. In another human study, CD4^{+} T cells were sorted from men and women and the percent of IFNγ^{+} cells was measured (230). In this study they found similar IFNγ production by both male and female T cells but it is important to note that this was done without stimulation (230).

Conversely in a mouse model of *Leishmania* infection, IFNγ production in the inguinal lymph nodes was higher in female compared to males but it was not determined what cells produced the IFNγ (231). Together, these studies demonstrate that sex differences have been observed in the same cytokines as our experiment but which sex has higher production appears to be completely dependent on the model used.

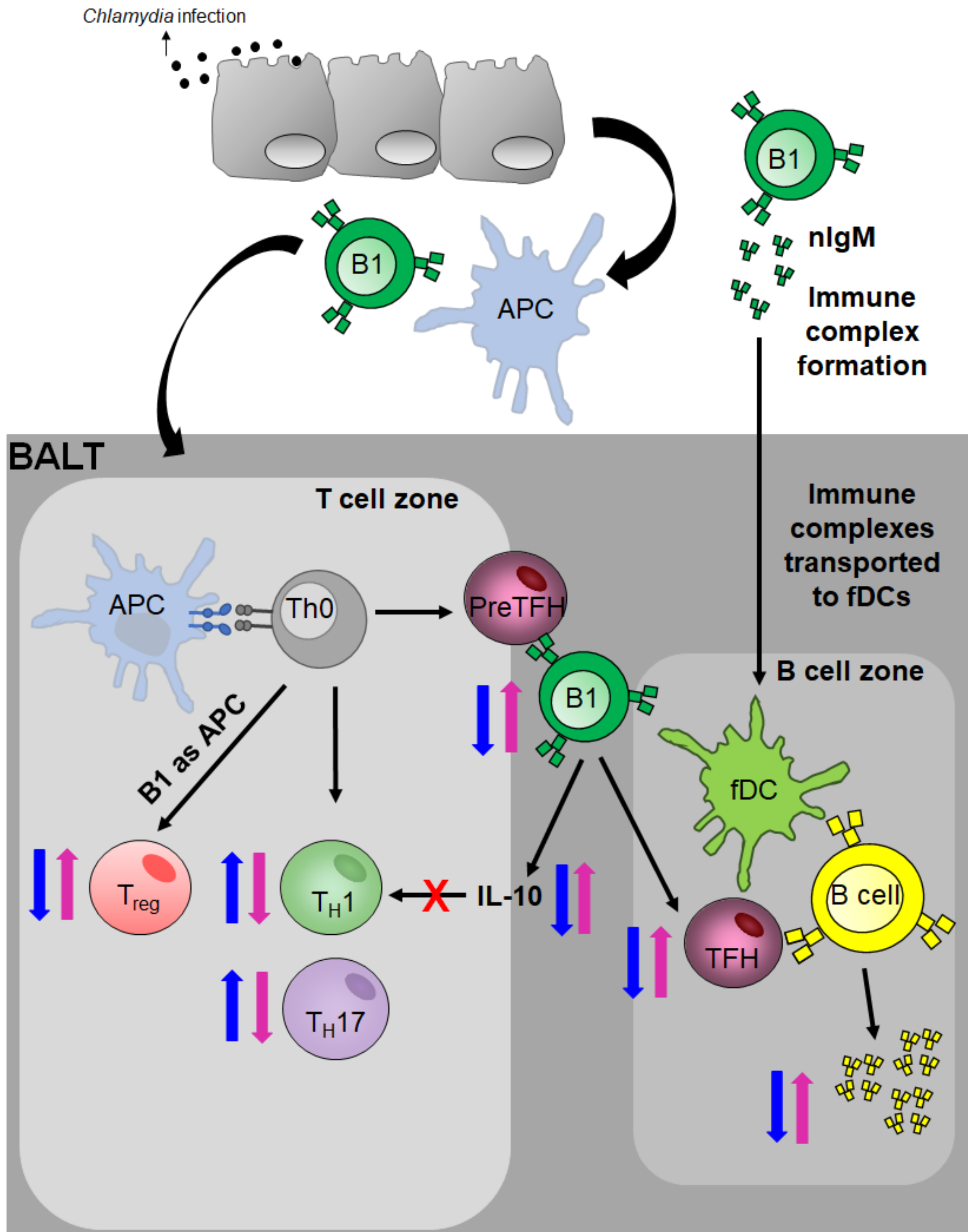
Following this experiment, we repeated co-cultures with PDL2$^+$ B cell subsets from both the peritoneum and spleen. We found that PDL2 expression significantly changed B cell function and these functions differed greatly between the spleen and peritoneal cavity cells. PDL2$^+$ B1a cells have been described in mice and are expanded in the lupus-prone BXBS strain (232). These cells, first described in 2009 and called L2pB1 cells, were found to produce autoreactive antibodies (232). We observed this population in our sorting experiment but we also sorted a previously undescribed PDL2$^+$ B1b population. In the spleen and peritoneal cavity these two populations had opposite cytokine profiles, so whether the ability of the L2pB1 cells differ in the spleen is unknown and the role of PDL2$^+$ B1b cells at both sites is currently unknown.

4.2 Proposed model

The data presented herein clearly demonstrate a significant difference in the responses to intranasal *Cm* infection between male and female mice and has led us to propose a model for how sex-specific immune responses to *Cm* infection are mediated (Figure 4.1).

Following infection with *Cm*, APCs carry peptides to the BALT of the lungs (233). In the T cell zone, APCs will present antigen to naïve T cells where in male mice

Figure 4. 1: Proposed model of sex-specific immune responses in male and female mice following intranasal *Cm* infection. Following *Cm* infection, antigen is brought to the BALT and presented to naïve T cells by both classical APCs but also B1 cells. B1 cell presentation results in the production of IL-10 producing Tregs, which in combination with B1-derived IL-10, work to suppress protective Th1 responses in female mice. In male mice, antigen presentation results in strong Th1 and Th17 responses. Pre-TFH cells will also be induced through antigen presentation, and through interactions with B1 cells and the co-stimulatory molecules ICOSL and CD80, pre-TFH cells will become full-fledged TFH cells. These TFH cells will go on to activate FOB cells and stimulate germinal center (GC) formation, and we propose B1 cells as well, to produce long lived antigen-specific memory B cells and plasma cells. Because of the increase in B1 cells in the lungs of females, there will be a mirrored increase in both TFH and B2 cells, leading to stronger antibody responses in females compared to males. B1 cells may also promote GC formation by secreting nIgM at the site of infection leading to the formation of immune complexes. These complexes will be trafficked to fDCs and further promote GC formation and antibody responses. Overall, the responses noted here will result in strong antibody and regulatory responses in females, resulting in a failure to successfully control bacterial replication and leading to increased morbidity in the form of severe body weight loss. APC, antigen presenting cell; Th0, naïve T cell; Treg, regulatory T cell; Th, T helper cell; TFH, T follicular helper cell; IL-10, interleukin 10; nIgM, natural immunoglobulin M; fDC, follicular dendritic cell; BALT, bronchus-associated lymphoid tissue.



Chlamydia infection
B1
APC
B1
nIgM
Immune complex formation
BALT
T cell zone
APC
Th0
PreTFH
Immune complexes transported to fDCs
B1
B cell zone
B1 as APC
fDC
T_{reg}
T_H1
IL-10
B cell
TFH
T_H17

significant Th1 and Th17 populations are induced. However, in females the IL-10 production from B1 cells dampens induced Th1 responses and results in increases in Treg and Th2 responses in female mice. Furthermore, we observed that in co-culture B1a cells result in significant induction of Treg responses and therefore may also contribute to Treg differentiation by acting as an APC.

In order to induce antibody responses, pre-TFH cells must become full-fledged TFH cells and move to the B cell zone. We observed that TFH numbers are significantly higher in females and believe that these cells go on to induce clonal expansion in FOB cells resulting in the increases we observed in both B2 cell numbers and antibody responses in female mice.

It is unclear why TFH cells are higher in females but we propose a role for B1 cells in this induction. Our lab has demonstrated that B1 cells upregulate a number of co-stimulatory molecules following *Cm* stimulation, including inducible T cell costimulator ligand (ICOSL) (Appendix A1). Importantly, $ICOSL^+$ B cells are necessary to keep pre-TFH cells in the periphery of the B cell zone allowing them to search for antigen-specific B cells which will promote germinal center formation and the production of antigen-specific antibody (107). We also saw an upregulation of CD80 on B1 cells following stimulation (Appendix A1). In the absence of $CD80^+$ B cells, TFH cell numbers are significantly decreased, suggesting an important role for $CD80^+$ B cells in TFH development (234). We hypothesize that it is therefore possible that ICOSL and CD80 provided by B1 cells allows for the successful differentiation of pre-TFH cells to TFH cells by keeping them in the periphery and allowing them to bind to cognate B cells.

We also believe that B1 cells may be able to act as cognate B cells in the B cell zone. B1 cells upregulate important costimulatory molecules including CD40, signaling lymphocytic activation molecule (SLAM), PDL1, and PDL2 following *Cm* stimulation, all of which are important for antigen presentation to TFH cells and the maintenance of TFH cells, germinal centers, and long lived antigen-specific PCs (235, 236). While B1 cells are predominantly characterized in T cell independent interactions, they have also been shown to act as APCs and are able to induce T cell proliferation (193). It is therefore possible that B1 cells can also act as cognate B cells and lead to germinal center formation, leading to the higher TFH cells and antigen-specific antibody that we observed in female mice.

B1 cells may also promote antibody production through nIgM secretion (184, 185). This effect would likely be mediated by complement, where natural IgM production leads to the formation of immune complexes and complement activation (237). These immune complexes are transported by macrophages to the B cell zone where they are passed to naïve B cells and then finally to fDCs that will support germinal center responses (113).

Together, these mechanisms account for the observed outcomes in male and female mice but more work will need to be done to further support the hypotheses presented in this model.

4.3 Implications for human *Chlamydia* infection

Considering that sex-specific epidemiology and sequalae have been described in human *Chlamydia* infections, the findings presented herein may have significant

implications for the understanding of human responses to *Chlamydia* infection (18, 24–27). Our findings support the possibility that sex-specific immune responses are responsible for the significantly higher infection rates and morbidities observed in females compared to males.

Our studies heavily implicate B1 cells as mediators and drivers of detrimental sex-specific responses in females, specifically the formation of regulatory and humoral responses to infection. Unfortunately, since B1 cells have only recently been described in humans, they have not been extensively studied and as of now there are no studies comparing B1 cells between males and females (147). Nevertheless, if we hypothesize that B1 cells in humans are equivalent to murine B1 cells, the control of these cells could be key in inhibiting the consequences of *Chlamydia* infection through preventative vaccination.

The current research into potential *Chlamydia* vaccines primarily focuses on immunity through the promotion of mucosal responses (238). Our data suggests that the key sex-specific differences in immune responses are mediated in the lungs themselves, suggesting that mucosal responses to *Cm* infection will vary greatly between male and female mice. Interestingly, IL-12 and IFNγ have been shown to decrease peritoneal B1 cell numbers (239). Therefore, it is possible that using these cytokines as systemic adjuvants during female mucosal vaccination could impede detrimental responses by lowering B1 cell numbers. In mice, IL-12 was delivered intraperitoneally so further work would need to be done in humans to assess whether systemic adjuvants delivered intravenously would be sufficient in order to avoid the invasiveness of a peritoneal injection (239).

While there are potential implications for human vaccine design from our study, significant research must be done on the role of B1 cells in human *Chlamydia* infection to identify if these results are translatable.

4.4 Future Directions

To better support the model presented herein, further studies to characterize the immune response in mice following respiratory infection would need to be undertaken. Specifically, our model implicates B1 cells as drivers of detrimental responses in female mice, therefore we could consider repeating respiratory infections in B1 cell deficient xid mice, hypothesizing that sex-specific differences would be diminished. Furthermore, we hypothesized that B1 cell derived-nIgM may lead to increased formation of immune complexes in female mice. This could be confirmed by measuring IgM at the site of infection in both males and females. Our current model implicates B1 cells in the T cell zone, the periphery of the B cell zone, and in germinal centers which could be confirmed using immunofluorescent microscopy of lung BALT.

While our studies focused on respiratory infection, significant sex differences are observed in genital *Ct* infection in humans. Unfortunately, while a male model of genital *Cm* infection has been developed in mice, it is not currently used as a standard model (240). Considering this, it may be more appropriate for future studies into sex-specific immune responses to be carried out in humans. Specifically, by examining how immune cell populations respond to *Ct in vitro* and by identifying if there are differences in immune cell populations in males and females who are infected with *Ct*, we can elucidate if responses to *Ct* mirror the responses we observed in respiratory infection.

4.4 Concluding statement

The data from this study have identified key sex-specific differences in the immune response to *Chlamydia* infection. While males are able to control bacterial replication and maintain body weight through the induction of protective Th1 responses, female mice were unable to effectively control bacterial burden, resulting in significant body weight loss, due to the B1 cell-mediated induction of non-protective Treg and humoral responses. Our study demonstrates for the first time that sex-specific differences observed in *Chlamydia* infection are likely mediated by significant differences in immune responses between males and females.

REFERENCES

1. Hafner, L., K. Beagley, and P. Timms. 2008. *Chlamydia trachomatis* infection: Host immune responses and potential vaccines. *Mucosal Immunol.* 1: 116–130.

2. Kuo, C. C., L. A. Jackson, L. A. Campbell, and J. T. Grayston. 1995. *Chlamydia pneumoniae* (TWAR). *Clin. Microbiol. Rev.* 8: 451–461.

3. Grayston, J. T. 1992. Infections caused by *Chlamydia pneumoniae* strain TWAR. *Clin. Infect. Dis.* 15: 757–761.

4. Saikku, P., M. Leinonen, K. Mattila, M. R. Ekman, M. S. Nieminen, P. H. Makela, J. K. Huttunen, and V. Valtonen. 1988. Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* 2: 983–986.

5. Gaydos, C. A., J. T. Summersgill, N. N. Sahney, J. A. Ramirez, and T. C. Quinn. 1996. Replication of *Chlamydia pneumonia in vitro* in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect. Immun.* 64: 1614–1620.

6. Balin, B. J., H. C. Gérard, E. J. Arking, D. M. Appelt, P. J. Branigan, J. T. Abrams, J. A. Whittum-Hudson, and A. P. Hudson. 1998. Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain. *Med. Microbiol. Immunol.* 187: 23–42.

7. Johnston, S. L., and R. J. Martin. 2005. Chlamydophila pneumoniae and Mycoplasma pneumoniae: A role in asthma pathogenesis? *Am. J. Respir. Crit. Care Med.* 172: 1078–1089.

8. Hahn, D. L., D. Bukstein, A. Luskin, and H. Zeitz. 1998. Evidence for *Chlamydia pneumoniae* infection in steroid-dependent asthma. *Ann. Allergy, Asthma Immunol.* 80: 45–49.

9. Harkinezhad, T., T. Geens, and D. Vanrompay. 2009. *Chlamydophila psittaci* infections in birds: A review with emphasis on zoonotic consequences. *Vet. Microbiol.* 135: 68–77.

10. Manavi, K. 2006. A review on infection with *Chlamydia trachomatis*. *Best Pract. Res. Clin. Obstet. Gynaecol.* 20: 941–951.

11. Mariotti, S. P., D. Pascolini, and J. Rose-Nussbaumer. 2009. Trachoma: global magnitude of a preventable cause of blindness. *Br. J. Ophthalmol.* 93: 563–568.

12. Cunningham, S. E., M. D. Johnson, and J. T. Laczek. 2012. Lymphogranuloma venereum proctitis. *Gastrointest. Endosc.* 75: 1269–1270.

13. Postema, E. J., L. Remeijer, and W. I. van der Meijden. 1996. Epidemiology of genital chlamydial infections in patients with chlamydial conjunctivitis; a retrospective study. *Genitourin. Med.* 72: 203–5.

14. Forsey, T., and S. Darougar. 1981. Transmission of chlamydiae by the housefly. *Br. J. Ophthalmol.* 65: 147–150.

15. Schachter, J., M. Grossman, and R. L. Sweet. 1986. Prospective study of perinatal transmission of *Chlamydia trachomatis*. *JAMA* 255: 3374.

16. Beem, M. O., and E. M. Saxon. 1979. Respiratory-tract colonization and a distinctive pneumonia syndrome in infants infected with *Chlamydia trachomatis*. *N. Engl. J. Med.* 296: 306–310.

17. Rosenman, M. B., B. E. Mahon, S. M. Downs, and M. B. Kleiman. 2003. Oral erythromycin prophylaxis vs watchful waiting in caring for newborns exposed to *Chlamydia trachomatis*. *Arch. Pediatr. Adolesc. Med.* 157: 565–71.

18. Center for Disease Control. 2016. *Sexually Transmitted Disease Surveillance 2015*,.

19. Belland, R., D. M. Ojcius, and G. I. Byrne. 2004. Focus: Chlamydia. *Nat. Rev. Microbiol.* 2: 530–531.

20. Weström, L., R. Joesoef, G. Reynolds, A. Hagdu, and S. E. Thompson. 1992. Pelvic inflammatory disease and fertility: A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. *Sex. Transm. Dis.* 19: 185–192.

21. Peterman, T. A., D. R. Newman, L. Maddox, K. Schmitt, and S. Shiver. 2015. Risk for HIV following a diagnosis of syphilis, gonorrhoea or chlamydia: 328,456 women in Florida, 2000–2011. *Int. J. STD AIDS* 26: 113–119.

22. Henning, T. R., K. Butler, D. Hanson, G. Sturdevant, S. Ellis, E. M. Sweeney, J. Mitchell, F. Deyounks, C. Phillips, C. Farshy, Y. Fakile, J. Papp, W. E. Secor, H. Caldwell, D. Patton, J. M. McNicholl, and E. N. Kersh. 2014. Increased susceptibility to vaginal simian/human immunodeficiency virus transmission in pig-tailed macaques coinfected with *Chlamydia trachomatis* and *Trichomonas vaginalis*. *J. Infect. Dis.* 210: 1239–1247.

23. Buckner, L. R., A. M. Amedee, H. L. Albritton, P. A. Kozlowski, N. Lacour, C. L. McGowin, D. J. Schust, and A. J. Quayle. 2016. *Chlamydia trachomatis* infection of endocervical epithelial cells enhances early HIV transmission events. *PLoS One* 11: e0146663.

24. Luostarinen, T., P. B. Namujju, M. Merikukka, J. Dillner, T. Hakulinen, P. Koskela, J. Paavonen, H. M. Surcel, and M. Lehtinen. 2013. Order of HPV/*Chlamydia* infections and cervical high-grade precancer risk: A case-cohort study. *Int. J. Cancer* 133: 1756–1759.

25. Samplaski, M. K. ., T. Domes, and K. A. Jarvi. 2014. Chlamydial infection and its role in male infertility. *Adv. Androl.* 307950: 1–11.

26. Courtright, P., and S. K. West. 2004. Contribution of sex-linked biology and gender roles to disparities with trachoma. *Emerg. Infect. Dis.* 10: 2012–2016.

27. Gislason, T., V. Guethdnason, B. Benediktsdottir, I. Olafsson, T. Aspelund, B. Thjodleifsson, and C. Janson. 2010. *Persistent Chlamydia pneumoniae* serology is related to decline in lung function in women but not in men. Effect of persistent *Chlamydia pneumoniae* infection on lung function. *BMC Pulm. Med.* 10: 44.

28. AbdelRahman, Y. M., and R. J. Belland. 2005. The chlamydial developmental cycle. *FEMS Microbiol. Rev.* 29: 949–959.

29. Byrne, G. I., and J. W. Moulder. 1978. Parasite-specified phagocytosis of *Chlamydia psittaci* and *Chlamydia trachomatis* by L and HeLa cells. *Infect. Immun.* 19: 598–606.

30. Prain, C. J., and J. H. Pearce. 1989. Ultrastructural studies on the intracellular fate of *Chlamydia psittaci* (strain guinea pig inclusion conjunctivitis) and *Chlamydia trachomatis* (strain lymphogranuloma venereum 434): Modulation of intracellular events and relationship with en. *J. Gen. Microbiol.* 135: 2107–2123.

31. Wyrick, P. B., J. Choong, C. H. Davis, S. T. Knight, M. O. Royal, A. S. Maslow, and C. R. Bagnell. 1989. Entry of genital *Chlamydia trachomatis* into polarized human epithelial cells. *Infect. Immun.* 57: 2378–2389.

32. Bastidas, R. J., C. A. Elwell, J. N. Engel, R. H. Valdivia, B. Henriques-Normark, I. Elaine, N. Joanne, M. Kostakioti, M. Hadjifrangiskou, J. Scott, J. Pizarro-Cerdá, A. Kühbacher, P. Cossart, J. Behnsen, and E. Deriu. 2013. Chlamydial intracellular survival strategies. *Cold Spring Habor Perspect. Med.* 3: a010256.

33. Plaunt, M. R., and T. P. Hatch. 1988. Protein synthesis early in the developmental cycle of *Chlamydia psittaci*. *Infect. Immun.* 56: 3021–3025.

34. Hybiske, K., and R. S. Stephens. 2007. Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. *Proc. Natl. Acad. Sci. U.S.A.* 104: 11430–11435.

35. Matsumoto, A, and G. P. Manire. 1970. Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia psittaci*. *J. Bacteriol.* 101: 278–285.

36. Hsia, R. C., H. Ohayon, P. Gounon, A. Dautry-Varsat, and P. M. Bavoil. 2000. Phage infection of the obligate intracellular bacterium, *Chlamydia psittaci* strain Guinea pig inclusion conjunctivitis. *Microbes Infect.* 2: 761–772.

37. Kutlin, A., C. Flegg, D. Stenzel, T. Reznik, P. M. Roblin, S. Mathews, P. Timms, and M. R. Hammerschlag. 2001. Ultrastructural study of *Chlamydia pneumoniae* in a continuous-infection model. *J. Clin. Microbiol.* 39: 3721–3723.

38. Pantoja, L. G., R. D. Miller, J. A. Ramirez, R. E. Molestina, and J. T. Summersgill. 2001. Characterization of *Chlamydia pneumoniae* persistence in HEp-2 cells treated with gamma interferon. *Infect. Immun.* 69: 7927–7932.

39. Kahane, S., and M. G. Friedman. 1992. Reversibility of heat shock in *Chlamydia trachomatis*. *FEMS Microbiol. Lett.* 97: 25–30.

40. Coles, A. M., D. J. Reynolds, A. Harper, A. Devitt, and J. H. Pearce. 1993. Low-nutrient induction of abnormal chlamydial development: a novel component of chlamydial pathogenesis? *FEMS Microbiol. Lett.* 106: 193–200.

41. Raulston, J. E. 1997. Response of *Chlamydia trachomatis* serovar E to iron restriction *in vitro* and evidence for iron-regulated chlamydial proteins. *Infect. Immun.* 65: 4539–4547.

42. Koehler, L., E. Nettelnbreker, A. P. Hudson, N. Ott, H. C. Gérard, P. J. Branigan, H. R. Schumacher, W. Drommer, and H. Zeidler. 1997. Ultrastructural and molecular analyses of the persistence of *Chlamydia trachomatis* (serovar K) in human monocytes. *Microb. Pathog.* 22: 133–142.

43. Nanagara, R., F. Li, A. Beutler, A. Hudson, and H. R. Schumacher. 1995. Alteration of *Chlamydia trachomatis* biologic behaviours in synovial membranes. *Arthritis Rheum.* 38: 1410–1417.

44. Skowasch, D., K. Yeghiazaryan, S. Schrempf, O. Golubnitschaja, U. Welsch, C. J. Preusse, J. A. Likungu, A. Welz, B. Lüderitz, and G. Bauriedel. 2003. Persistence of *Chlamydia pneumoniae* in degenerative aortic valve stenosis indicated by heat shock protein 60 homologues. *J. Heart Valve Dis.* 12: 68–75.

45. Hogan, R. J., S. A. Mathews, S. Mukhopadhyay, J. T. Summersgill, and P. Timms. 2004. Chlamydial persistence: Beyond the biphasic paradigm. *Infect. Immun.* 72: 1843–1855.

46. Yang, X., and R. Brunham. 1998. T lymphocyte immunity in host defence against *Chlamydia trachomatis* and its implication for vaccine development. *Can. J. Infect. Dis.* 9: 99–108.

47. Williams, D. M., J. Schachter, D. J. Drutz, and C. V. Sumaya. 1981. Pneumonia due to *Chlamydia trachomatis* in the immunocompromised (nude) mouse. *J. Infect. Dis.* 143: 238–241.

48. Barron, A. L., H. J. White, R. G. Rank, B. L. Soloff, and E. B. Moses. 1981. A new animal model for the study of *Chlamydia trachomatis* genital infections: Infection of mice with the agent of mouse pneumonitis. *J. Infect. Dis.* 143: 63–66.

49. Everett, K. D., R. M. Bush, and A. A. Andersen. 1999. Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards. *Int. J. Syst. Bacteriol.* 49 Pt 2: 415–440.

50. Nigg, C. 1942. An unidentified virus which produces pneumonia and systemic infection in mice. *Science* 95(2454): 49–50.

51. Williams, and Wilkins. 1984. Genus Chlamydia. In *Bergey's manual of systematic bacteriology* 729–739.

52. Kovach, M. A., M. N. Ballinger, M. W. Newstead, X. Zeng, U. Bhan, F. Yu, B. B. Moore, R. L. Gallo, and T. J. Standiford. 2012. Cathelicidin-related antimicrobial peptide is required for effective lung mucosal immunity in Gram-negative bacterial pneumonia. *J. Immunol.* 189: 304–311.

53. Draper, D. L., D. V. Landers, M. A. Krohn, S. L. Hillier, H. C. Wiesenfeld, and R. P. Heine. 2000. Levels of vaginal secretory leukocyte protease inhibitor are decreased in women with lower reproductive tract infections. *Am. J. Obstet. Gynecol.* 183: 1243–1248.

54. Helmig, R., N. Uldbjerg, and K. Ohlsson. 1995. Secretory leukocyte protease inhibitor in the cervical mucus and in the fetal membranes. *Eur. J. Obstet. Gynecol.* 59: 95–101.

55. Yasin, B., M. Pang, R. I. Lehrer, and E. A. Wagar. 2004. A cumulative experience examining the effect of natural and synthetic antimicrobial peptides vs. *Chlamydia trachomatis*. *J. Pept. Res.* 64: 65–71.

56. Tang, L., J. Chen, Z. Zhou, P. Yu, Z. Yang, and G. Zhong. 2015. *Chlamydia*-secreted protease CPAF degrades host antimicrobial peptides. *Microbes Infect.* 17: 402–408.

57. Rasmussen, S. J., L. Eckmann, A. J. Quayle, L. Shen, Y. Zhang, D. J. Anderson, J. Fierer, R. S. Stephens, and M. F. Kagnoff. 1997. Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial Ppthogenesis. *J. Clin. Invest.* 99: 77–87.

58. Johnson, R. M. 2004. Murine oviduct epithelial cell cytokine responses to *Chlamydia muridarum* infection include interleukin-12-p70 secretion. *Infect. Immun.* 72: 3951–3960.

59. Maxion, H. K., and K. A. Kelly. 2002. Chemokine expression patterns differ within anatomically distinct regions of the genital tract during *Chlamydia trachomatis* infection. *Infect. Immun.* 70: 1538–1546.

60. Kruger, P., M. Saffarzadeh, A. N. R. Weber, N. Rieber, M. Radsak, H. von Bernuth, C. Benarafa, D. Roos, J. Skokowa, and D. Hartl. 2015. Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS Pathog.* 11: e1004651.

61. Lee, W. L., R. E. Harrison, and S. Grinstein. 2003. Phagocytosis by neutrophils. *Microbes Infect.* 5: 1299–1306.

62. Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky. 2004. Neutrophil extracellular traps kill bacteria. *Science* 303(5663): 1532–1535.

63. Lacy, H. M., A. K. Bowlin, L. Hennings, A. M. Scurlock, U. M. Nagarajan, and R. G. Rank. 2011. Essential role for neutrophils in pathogenesis and adaptive immunity in *Chlamydia caviae* ocular infections. *Infect. Immun.* 79: 1889–1897.

64. Lee, H. Y., J. H. Schripsema, I. M. Sigar, C. M. Murray, S. R. Lacy, and K. H. Ramsey. 2010. A link between neutrophils and chronic disease manifestations of *Chlamydia muridarum* urogenital infection of mice. *FEMS Immunol. Med. Microbiol.* 59: 108–116.

65. Wiesenfeld, H. C., R. P. Heine, M. A. Krohn, S. L. Hillier, A. A. Amortegui, M. Nicolazzo, and R. L. Sweet. 2002. Association between elevated neutrophil defensin levels and dndometritis. *J. Infect. Dis.* 186: 792–797.

66. Bulletii, C., M. E. Coccia, S. Battistoni, and A. Borini. 2010. Endometriosis and infertility. *J. Assist. Reprod. Genet.* 27: 441–447.

67. Chertov, O., H. Ueda, L. L. Xu, K. Tani, W. J. Murphy, J. M. Wang, T. J. Sayers, and J. J. Oppenheim. 1997. Identification of human neutrophil derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J. Exp. Med.* 186: 739–747.

68. Bennouna, S., S. K. Bliss, T. J. Curiel, and E. Y. Denkers. 2003. Cross-talk in the innate immune system: Neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J. Immunol.* 171: 6052–6058.

69. Kambayashi, T., and T. M. Laufer. 2014. Atypical MHC class II-expressing antigen-presenting cells: Can anything replace a dendritic cell? *Nat. Rev. Immunol.* 14: 719–730.

70. Shimada, K., T. R. Crother, and M. Arditi. 2012. Innate immune responses to *Chlamydia pneumoniae* infection: Role of TLRs, NLRs, and the inflammasome. *Microbes Infect.* 14: 1301–1307.

71. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783–801.

72. Kawai, T., and S. Akira. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int. Immunol.* 21: 317–337.

73. Hess, N. J., S. Jiang, X. Li, Y. Guan, and R. I. Tapping. 2017. TLR10 Is a B cell intrinsic suppressor of adaptive immune responses. *J. Immunol.* 198: 699–707.

74. Pifer, R., A. Benson, C. R. Sturge, and F. Yarovinsky. 2011. UNC93B1 is essential for TLR11 activation and IL-12-dependent host resistance to *Toxoplasma gondii*. *J. Biol. Chem.* 286: 3307–3314.

75. Koblansky, A. A., D. Jankovic, H. Oh, S. Hieny, W. Sungnak, R. Mathur, M. S. Hayden, S. Akira, A. Sher, and S. Ghosh. 2013. Recognition of profilin by Toll-like receptor 12 is critical for host resistance to *Toxoplasma gondii*. *Immunity* 38: 119–130.

76. Shi, Z., Z. Cai, A. Sanchez, T. Zhang, S. Wen, J. Wang, J. Yang, S. Fu, and D. Zhang. 2011. A novel toll-like receptor that recognizes vesicular stomatitis virus. *J. Biol. Chem.* 286: 4517–4524.

77. Rodriguez, N., N. Wantia, F. Fend, S. Dürr, H. Wagner, and T. Miethke. 2006. Differential involvement of TLR2 and TLR4 in host survival during pulmonary infection with *Chlamydia pneumoniae*. *Eur. J. Immunol.* 36: 1145–1155.

78. Darville, T., J. M. O. Neill, C. W. Andrews, U. M. Nagarajan, L. Stahl, and D. M. Ojcius. 2003. Toll-like receptor-2, but not Toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection. *J. Immunol.* 171: 6187–6197.

79. Trombetta, E. S., and I. Mellman. 2005. Cell biology of antigen processing *in vitro* and *in vivo*. *Annu. Rev. Immunol.* 23: 975–1028.

80. Sallusto, F. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. *J. Exp. Med.* 182: 389–400.

81. Steinman, R. M., M. Pack, and K. Inaba. 1997. Dendritic cells in the T-cell areas of lymphoid organs. *Immunol. Rev.* 156: 25–37.

82. den Haan, J. M. M., R. Arens, and M. C. van Zelm. 2014. The activation of the adaptive immune system: Cross-talk between antigen-presenting cells, T cells and B cells. *Immunol. Lett.* 162: 103–112.

83. Chen, K., and J. K. Kolls. 2013. T cell-mediated host immune defenses in the lung. *Annu. Rev. Immunol.* 31: 605–633.

84. Ottenhoff, T. H. M., F. A. W. Verreck, E. G. R. Lichtenauer-Kaligis, M. A. Hoeve, O. Sanal, and J. T. van Dissel. 2002. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. *Nat. Genet.* 32: 97–105.

85. Brunham, R. C., and J. Rey-Ladino. 2005. Immunology of *Chlamydia* infection: implications for a *Chlamydia trachomatis* vaccine. *Nat. Rev. Immunol.* 5: 149–61.

86. Kelly, K. A., E. A. Robinson, and R. G. Rank. 1996. Initial route of antigen administration alters the T-cell cytokine profile produced in response to the mouse pneumonitis biovar of *Chlamydia trachomatis* following genital infection. *Infect. Immun.* 64: 4976–4983.

87. Caldwell, H. D. 1997. Immunity to *Chlamydia trachomatis* is mediated by T helper 1 cells through IFN-gamma-dependent and-independent pathways. *J. Immunol.* 158: 3344–3352.

88. Taylor, M. W., J. M. Carlin, G. I. Byrne, G. Feng, M. W. Taylor, J. M. Cadin, and G. Byrne. 1993. IFN-gamma-mediated antimicrobial response. Indoleamine 2,3-dioxygenase-deficient mutant host cells no longer inhibit intracellular Chlamydia spp. or Toxoplasma growth. *J. Immunol.* 150: 5529–5534.

89. Igietseme, J., P. Wyrick, D. Goyeau, and R. Rank. 1994. An *in vitro* model for immune control of chlamydial growth in polarized epithelial cells. *Infect. Immun.* 62: 3528–3535.

90. Szabo, S. J., N. G. Jacobson, A. S. Dighe, U. Gubler, and K. M. Murphy. 1995. Developmental commitment to the Th2 lineage by extinction of IL-12 signaling. *Immunity* 2: 665–675.

91. Wang, S., Y. Fan, R. C. Brunham, and X. Yang. 1999. IFN-gamma knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection. *Eur. J. Immunol. J. Immunol.* 29: 3782–3792.

92. Vicetti Miguel, R. D., S. A. K. Harvey, W. A. LaFramboise, S. D. Reighard, D. B. Matthews, and T. L. Cherpes. 2013. Human female genital tract infection by the obligate intracellular bacterium *Chlamydia trachomatis* elicits robust type 2 immunity. *PLoS One* 8: e58565.

93. O'Connor, W., L. A. Zenewicz, and R. A. Flavell. 2010. The dual nature of TH17 cells: Shifting the focus to function. *Nat. Immunol.* 11: 471–476.

94. Masson, L., A. L. Salkinder, A. J. Olivier, L. R. Mckinnon, H. Gamieldien, K. Mlisana, T. J. Scriba, D. A. Lewis, F. Little, H. B. Jaspan, K. Ronacher, L. Denny, S. S. Abdool Karim, and J. A. S. Passmore. 2015. Relationship between female genital tract infections, mucosal interleukin-17 production and local T helper type 17 cells. *Immunology* 146: 557–567.

95. Vicetti Miguel, R. D., N. E. Quispe Calla, S. D. Pavelko, and T. L. Cherpes. 2016. Intravaginal *Chlamydia trachomatis* challenge infection elicits TH1 and TH17 immune responses in mice that promote pathogen clearance and genital tract damage. *PLoS One* 11: e0162445.

96. Zhou, X., Q. Chen, J. Moore, J. K. Kolls, S. Halperin, and J. Wang. 2009. Critical role of the interleukin-17/interleukin-17 receptor axis in regulating host susceptibility to respiratory infection with *Chlamydia* species. *Infect. Immun.* 77: 5059–5070.

97. Bai, H., J. Cheng, X. Gao, A. G. Joyee, Y. Fan, S. Wang, L. Jiao, Z. Yao, and X. Yang. 2009. IL-17/Th17 promotes type 1 T cell immunity against pulmonary intracellular bacterial infection through modulating dendritic cell function. *J. Immunol.* 183: 5886–5895.

98. Scurlock, A. M., L. C. Frazer, C. W. Andrews, C. M. O'Connell, I. P. Foote, S. L. Bailey, K. Chandra-Kuntal, J. K. Kolls, and T. Darville. 2011. Interleukin-17 contributes to generation of Th1 immunity and neutrophil recruitment during *Chlamydia muridarum* genital tract infection but is not required for macrophage influx or normal resolution of infection. *Infect. Immun.* 79: 1349–1362.

99. Tripathi, S. K., and R. Lahesmaa. 2014. Transcriptional and epigenetic regulation of human $CD4^+$ T helper lineage specification. *Immunol. Rev.* 261: 62–83.

100. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. De Vries, and M. G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.

101. Zeng, H., R. Zhang, B. Jin, and L. Chen. 2015. Type 1 regulatory T cells: A new mechanism of peripheral immune tolerance. *Cell. Mol. Immunol.* 12: 566–571.

102. Crother, T. R., N. W. J. Schröder, J. Karlin, S. Chen, K. Shimada, A. Slepenkin, R. Alsabeh, E. Peterson, and M. Arditi. 2011. *Chlamydia pneumoniae* infection induced allergic airway sensitization is controlled by regulatory T-cells and plasmacytoid dendritic cells. *PLoS One* 6: e20784.

103. Moore-Connors, J. M., R. Fraser, S. A. Halperin, and J. Wang. 2013. $CD4^+CD25^+Foxp3^+$ regulatory T cells promote Th17 responses and genital tract inflammation upon intracellular *Chlamydia muridarum* infection. *J. Immunol.* 191: 3430–3439.

104. Pratama, A., and C. G. Vinuesa. 2014. Control of TFH cell numbers: Why and how? *Immunol. Cell Biol.* 92: 40–48.

105. Crotty, S. 2011. Follicular Helper CD4 T Cells (T_{FH}). *Annu. Rev. Immunol.* 29: 621–663.

106. Fazilleau, N., L. Mark, L. J. McHeyzer-Williams, and M. G. McHeyzer-Williams. 2009. Follicular helper T cells: Lineage and location. *Immunity* 30: 324–335.

107. Suh, W.-K. 2015. Life of T follicular helper cells. *Mol. Cells* 38: 195–201.

108. Barry, M., and R. C. Bleackley. 2002. Cytotoxic T lymphocytes: All roads lead to death. *Nat. Rev. Immunol.* 2: 401–409.

109. Magee, M. D., D. M. Williams, J. G. Smith, C. A. Bleicker, B. G. Grubbs, J. Schachter, and R. G. Rank. 1995. Role of CD8 T-Cells in primary *Chlamydia* infection. *Infect. Immun.* 63: 516–521.

110. Perry, L. L., K. Feilzer, S. Hughes, and H. D. Caldwell. 1999. Clearance of *Chlamydia trachomatis* from the murine genital mucosa does not require perforin-mediated cytolysis or Fas-mediated apoptosis. *Infect. Immun.* 67: 1379–1385.

111. Murthy, A. K., W. Li, B. K. R. Chaganty, S. Kamalakaran, M. N. Guentzel, J. Seshu, T. G. Forsthuber, G. Zhong, and B. P. Arulanandam. 2011. Tumor necrosis factor alpha production from $CD8^+$ T cells mediates oviduct pathological sequelae following primary genital *Chlamydia muridarum* infection. *Infect. Immun.* 79: 2928–2935.

112. Breitfeld, D., L. Ohl, E. Kremmer, J. Ellwart, F. Sallusto, M. Lipp, and R. Förster. 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* 192: 1545–52.

113. Heesters, B. A., R. C. Myers, and M. C. Carroll. 2014. Follicular dendritic cells: Dynamic antigen libraries. *Nat. Rev. Immunol.* 14: 495–504.

114. Nutt, S. L., P. D. Hodgkin, D. M. Tarlinton, and L. M. Corcoran. 2015. The generation of antibody-secreting plasma cells. *Nat. Rev. Immunol.* 15: 160–171.

115. Brunham, R. C., C. C. Kuo, L. Cles, and K. K. Holmes. 1983. Correlation of host immune response with quantitative recovery of *Chlamydia trachomatis* from the human endocervix. *Infect. Immun.* 39: 1491–4.

116. Peeling, R., I. W. Maclean, and R. C. Brunham. 1984. *In vitro* neutralization of *Chlamydia trachomatis* with monoclonal antibody to an epitope on the major outer membrane protein. *Infect. Immun.* 46: 484–8.

117. Caldwell, H. D., and L. J. Perry. 1982. Neutralization of *Chlamydia trachomatis* infectivity with antibodies to the major outer protein. *Infect. Immun.* 38: 745–754.

118. Su, H., and H. D. Caldwell. 1991. *In vitro* neutralization of *Chlamydia trachomatis* by monovalent Fab antibody specific to the major outer membrane protein. *Infect. Immun.* 59: 2843–2845.

119. Pal, S., I. Theodor, E. M. Peterson, and L. M. de la Maza. 1997. Monoclonal immunoglobulin A antibody to the major outer membrane protein of the *Chlamydia trachomatis* mouse pneumonitis biovar protects mice against a chlamydial genital challenge. *Vaccine* 15(5): 575–582.

120. Ramsey, K. H., L. S. F. Soderberg, and R. G. Rank. 1988. Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. *Infect. Immun.* 56: 1320–1325.

121. Su, H., K. Feilzer, H. D. Caldwell, and R. P. Morrison. 1997. *Chlamydia trachomatis* genital tract infection of antibody-deficient gene knockout mice. *Infect Immun* 65: 1993–1999.

122. Eckert, L. O., S. E. Hawes, P. Wölner-Hanssen, D. M. Money, R. W. Peeling, R. C. Brunham, C. E. Stevens, D. A. Eschenbach, and W. E. Stamm. 1997. Prevalence and correlates of antibody to chlamydial heat shock protein in women attending sexually transmitted disease clinics and women with confirmed pelvic inflammatory disease. *J. Infect. Dis.* 175: 1453–1458.

123. Peeling, R. W., D. L. Patton, Y. T. Cosgrove Sweeney, M. S. Cheang, A. B. Lichtenwalner, R. C. Brunham, and W. E. Stamm. 1999. Antibody response to the chlamydial heat-shock protein 60 in an experimental model of chronic pelvic inflammatory disease in monkeys (*Macaca nemestrina*). *J. Infect. Dis.* 180: 774–779.

124. Tiitinen, A., H. M. Surcel, M. Halttunen, S. Birkelund, A. Bloigu, G. Christiansen, P. Koskela, S. G. Morrison, R. P. Morrison, and J. Paavonen. 2006. *Chlamydia trachomatis* and chlamydial heat shock protein 60-specific antibody and cell-mediated responses predict tubal factor infertility. *Hum. Reprod.* 21: 1533–1538.

125. Paavonen, J., K. P. Karunakaran, Y. Noguchi, T. Anttila, A. Bloigu, J. Dillner, G. Hallmans, T. Hakulinen, E. Jellum, P. Koskela, M. Lehtinen, S. Thoresen, H. Lam, C. Shen, and R. C. Brunham. 2003. Serum antibody response to the heat shock protein 60 of *Chlamydia trachomatis* in women with developing cervical cancer. *Am. J. Obstet. Gynecol.* 189: 1287–1292.

126. Zeng, H., S. Gong, S. Hou, Q. Zou, and G. Zhong. 2012. Identification of antigen-specific antibody responses associated with upper genital tract pathology in mice infected with *Chlamydia muridarum*. *Infect. Immun.* 80: 1098–1106.

127. Vinuesa, C. G., and P.-P. Chang. 2013. Innate B cell helpers reveal novel types of antibody responses. *Nat. Immunol.* 14: 119–26.

128. Kearney, J. F. 2005. Innate-like B cells. *Springer Semin. Immunopathol.* 26: 377–383.

129. Zhang, X. 2013. Regulatory functions of innate-like B cells. *Cell. Mol. Immunol.* 10: 113–121.

130. Pillai, S., A. Cariappa, and S. T. Moran. 2005. Marginal zone B cells. *Annu. Rev. Immunol.* 23: 161–196.

131. Mebius, R. E., and G. Kraal. 2005. Structure and function of the spleen. *Nat. Rev. Immunol.* 5: 606–616.

132. Cerutti, A., M. Cols, and I. Puga. 2013. Marginal zone B cells: Virtues of innate-like antibody-producing lymphocytes. *Nat. Rev. Immunol.* 13: 118–132.

133. Martin, F., and J. F. Kearney. 2002. Marginal-zone B cells. *Nat. Rev. Immunol.* 2: 323–335.

134. Ghosn, E. E. B., P. Sadate-Ngatchou, Y. Yang, L. A. Herzenberg, and L. A. Herzenberg. 2011. Distinct progenitors for B-1 and B-2 cells are present in adult mouse spleen. *Proc. Natl. Acad. Sci. U.S.A.* 108: 2879–2884.

135. Weller, S., M. C. Braun, B. K. Tan, A. Rosenwald, C. Cordier, M. E. Conley, A. Plebani, D. S. Kumararatne, D. Bonnet, O. Tournilhac, G. Tchernia, B. Steiniger, L. M. Staudt, J.-L. Casanova, C.-A. Reynaud, and J.-C. Weill. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* 104: 3647–3654.

136. Puga, I., M. Cols, C. M. Barra, B. He, L. Cassis, M. Gentile, L. Comerma, A. Chorny, M. Shan, W. Xu, G. Magri, D. M. Knowles, W. Tam, A. Chiu, J. B. Bussel, S. Serrano, J. A. Lorente, B. Bellosillo, J. Lloreta, N. Juanpere, F. Alameda, T. Baró, C. D. de Heredia, N. Torán, A. Català, M. Torrebadell, C. Fortuny, V. Cusí, C. Carreras, G. A. Diaz, J. M. Blander, C.-M. Farber, G. Silvestri, C. Cunningham-Rundles, M. Calvillo, C. Dufour, L. D. Notarangelo, V. Lougaris, A. Plebani, J.-L. Casanova, S. C. Ganal, A. Diefenbach, J. I. Aróstegui, M. Juan, J. Yagüe, N. Mahlaoui, J. Donadieu, K. Chen, and A. Cerutti. 2011. B cell–helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat. Immunol.* 13: 170–180.

137. Tierens, A, J. Delabie, L. Michiels, P. Vandenberghe, and C. De Wolf-Peeters. 1999. Marginal-zone B cells in the human lymph node and spleen show somatic hypermutations and display clonal expansion. *Blood* 93: 226–34.

138. Dono, M., S. Zupo, N. Leanza, G. Melioli, M. Fogli, A. Melagrana, N. Chiorazzi, and M. Ferrarini. 2000. Heterogeneity of tonsillar subepithelial B lymphocytes, the splenic marginal zone equivalents. *J. Immunol.* 164: 5596–5604.

139. Spencer, J., T. Finn, K. A. Pulford, D. Y. Mason, and P. G. Isaacson. 1985. The human gut contains a novel population of B lymphocytes which resemble marginal zone cells. *Clin. Exp. Immunol.* 62: 607–612.

140. Hayakawa, K., R. R. Hardy, D. R. Parks, and L. A. Herzenberg. 1983. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J. Exp. Med.* 157: 202–218.

141. Hayakawa, K., R. R. Hardy, L. A. Herzenberg, and L. A. Herzenberg. 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J. Exp. Med.* 161: 1554–1568.

142. Tumang, J. R., W. D. Hastings, C. Bai, and T. L. Rothstein. 2004. Peritoneal and splenic B-1 cells are separable by phenotypic, functional, and transcriptomic characteristics. *Eur. J. Immunol.* 34: 2158–2167.

143. Baumgarth, N. 2011. The double life of a B-1 cell: Self-reactivity selects for protective effector functions. *Nat. Rev. Immunol.* 11: 34–46.

144. Ansel, K. M., R. B. S. Harris, and J. G. Cyster. 2002. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* 16: 67–76.

145. Kantor, A. B., A. M. Stall, S. Adams, L. A. Herzenberg, and L. A. Herzenberg. 1992. Differential development of progenitor activity for three B-cell lineages. *Proc. Natl. Acad. Sci. U.S.A.* 89: 3320–3324.

146. Kantor, A. B. 1991. The development and repertoire of B-1 cells (CD5 B cells). *Immunol. Today* 12: 389–391.

147. Griffin, D. O., N. E. Holodick, and T. L. Rothstein. 2011. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype $CD20^{+}$ $CD27^{+}$ $CD43^{+}$ $CD70^{-}$. *J. Exp. Med.* 208: 67–80.

148. Perez-Andres, M., C. Grosserichter-Wagener, C. Teodosio, J. J. M. van Dongen, A. Orfao, and M. C. van Zelm. 2011. The nature of circulating $CD27^{+}CD43^{+}$ B cells. *J. Exp. Med.* 208: 2565–2566.

149. Descatoire, M., J.-C. Weill, C. -A. Reynaud, and S. Weller. 2011. A human equivalent of mouse B-1 cells? *J. Exp. Med.* 208: 2563–2564.

150. Griffin, D. O., N. E. Holodick, and T. L. Rothstein. 2011. Human B1 cells are $CD3^{-}$: A reply to “A human equivalent of mouse B-1 cell?” and “The nature of circulating $CD27^{+}CD43^{+}$ B cells.” *J. Exp. Med.* 208: 2566–2569.

151. Covens, K., B. Verbinnen, N. Geukens, I. Meyts, F. Schuit, L. Van Lommel, M. Jacquemin, and X. Bossuyt. 2013. Characterization of proposed human B-1 cells reveals pre-plasmablast phenotype. *Blood* 121: 5176–5183.

152. Quách, T. D., N. Rodríguez-Zhurbenko, T. J. Hopkins, X. Guo, A. M. Hernández, W. Li, and T. L. Rothstein. 2016. Distinctions among circulating antibody-secreting Cell populations, including B-1 cells, in human adult peripheral blood. *J. Immunol.* 196: 1060–9.

153. Solvason, N., X. Chen, F. Shu, and J. F. Kearney. 1992. The fetal omentum in mice and humans: A site enriched for precursors of CD5 B cells early in development. *Ann. N. Y. Acad. Sci.* 651: 10–20.

154. Yoshimoto, M., E. Montecino-Rodriguez, M. J. Ferkowicz, P. Porayette, W. C. Shelley, S. J. Conway, K. Dorshkind, and M. C. Yoder. 2011. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc. Natl. Acad. Sci. U.S.A.* 108: 1468–1473.

155. Montecino-Rodriguez, E., H. Leathers, and K. Dorshkind. 2006. Identification of a B-1 B cell–specified progenitor. *Nat. Immunol.* 7: 293–301.

156. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Müller, A. B. Kantor, L. A. Herzenberg, F. S. Rosen, and P. Sideras. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity* 3: 283–299.

157. Almeida, S. R., L. S. Aroeira, E. Frymuller, M. A. A. Dias, C. S. B. Bogsan, J. D. Lopes, and M. Mariono. 2001. Mouse B-1 cell-derived mononuclear phagocyte, a novel cellular component of acute non-specific inflammatory exudate. *Int. Immununolgy* 13: 1193–1201.

158. Knoops, L., J. Louahed, and J.-C. Renauld. 2004. IL-9-Induced Expansion of B-1b Cells Restores Numbers but Not Function of B-1 Lymphocytes in *xid* Mice. *J. Immunol.* 172: 6101–6106.

159. Bruton, O. C. 1952. Agammaglobulinemia. *Pediatrics* 9: 722–727.

160. Kaveri, S. V., G. J. Silverman, and J. Bayry. 2012. Natural IgM in immune equilibrium and harnessing their therapeutic potential. *J. Immunol.* 188: 939–945.

161. Helyer, B. J., and J. B. Howie. 1963. Renal disease associated with positive lupus erythematosus tests in a crossbred strain of mice. *Nature* 197: 197–197.

162. Berland, R., and H. H. Wortis. 2002. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol.* 20: 253–300.

163. Tan, E., A. Cohen, J. Fried, A. Masi, D. McShane, N. Rothfield, J. Schaller, N. Talal, and R. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 25: 1271–1277.

164. Murakami, M., H. Yoshioka, T. Shirai, T. Tsubata, and T. Honjo. 1995. Prevention of autoimmune symptoms in autoimmune-prone mice by elimination of B-1 cells. *Int. Immunol.* 7: 877–882.

165. Mohan, C., L. Morel, P. Yang, and E. K. Wakeland. 1998. Accumulation of splenic B1a cells with potent antigen-presenting capability in NZM2410 lupus-prone mice. *Arthritis Rheum.* 41: 1652–1662.

166. Ishikawa, S., and K. Matsushima. 2007. Aberrant B1 cell trafficking in lupus. *Front. Biosci.* 1790–1803.

167. Ishida, H., T. Muchamuel, S. Sakaguchi, S. Andrade, S. Menon, and M. Howard. 1994. Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice. *J. Exp. Med.* 179: 305–10.

168. Ishida, H., R. Hastings, J. Kearney, and M. Howard. 1992. Continuous anti-interleukin 10 antibody administration depletes mice of Ly-1 B cells but not conventional B cells. *J. Exp. Med.* 175: 1213–20.

169. O'Garra, A., R. Chang, N. Go, R. Hastings, G. Haughton, and M. Howard. 1992. Ly-1 B (B-1) cells are the main source of B-cell derived interleukin 10. *Eur. J. Immunol.* 22: 711–717.

170. Genestier, L., M. Taillardet, P. Mondiere, H. Gheit, C. Bella, and T. Defrance. 2007. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J. Immunol.* 178: 7779–7786.

171. Alugupalli, K. R., S. Akira, E. Lien, and J. M. Leong. 2007. MyD88- and Bruton's tyrosine kinase-mediated signals are essential for T cell-independent pathogen-specific IgM responses. *J. Immunol.* 178: 3740–3749.

172. Rawlings, D. J., M. A. Schwartz, S. W. Jackson, and A. Meyer-Bahlburg. 2012. Integration of B cell responses through Toll-like receptors and antigen receptors. *Nat. Rev. Immunol.* 12: 282–294.

173. Waffarn, E. E., C. J. Hastey, N. Dixit, Y. Soo Choi, S. Cherry, U. Kalinke, S. I. Simon, and N. Baumgarth. 2015. Infection-induced type I interferons activate CD11b on B-1 cells for subsequent lymph node accumulation. *Nat. Commun.* 6: 8991.

174. Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Förster, J. D. Sedgwick, J. L. Browning, M. Lipp, and J. G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 406: 309–314.

175. Ha, S., M. Tsuji, K. Suzuki, B. Meek, N. Yasuda, T. Kaisho, and S. Fagarasan. 2006. Regulation of B1 cell migration by signals through Toll-like receptors. *J. Exp. Med.* 203: 2541–2550.

176. Huttenlocher, A., and A. R. Horwitz. 2011. Integrins in cell migration. *Cold Spring Harb. Perspect. Biol.* 3: a005074.

177. Hynes, R. O. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell* 110: 673–687.

178. Geherin, S. A., D. Gómez, R. A. Glabman, G. Ruthel, A. Hamann, and G. F. Debes. 2016. IL-10^+ innate-like B cells are part of the skin immune system and require α4β1 integrin to migrate between the peritoneum and inflamed skin. *J. Immunol.* 196: 2514–2525.

179. Yang, Y., J. W. Tung, E. E. B. Ghosn, L.A. Herzenberg, and L.A. Herzenberg. 2007. Division and differentiation of natural antibody-producing cells in mouse spleen. *Proc. Natl. Acad. Sci. U.S.A.* 104: 4542–4546.

180. Choi, Y. S., and N. Baumgarth. 2008. Dual role for B-1a cells in immunity to influenza virus infection. *J. Exp. Med.* 205: 3053–3064.

181. Cole, L. E., Y. Yang, K. L. Elkins, E. T. Fernandez, N. Qureshi, M. J. Shlomchik, L. A. Herzenberg, L.A. Herzenberg, and S. N. Vogel. 2009. Antigen-specific B-1a antibodies induced by *Francisella tularensis* LPS provide long-term protection against F. *tularensis* LVS challenge. *Proc. Natl. Acad. Sci. U.S.A.* 106: 4343–4348.

182. Alugupalli, K. R., J. M. Leong, R. T. Woodland, M. Muramatsu, T. Honjo, and R. M. Gerstein. 2004. B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* 21: 379–390.

183. Macpherson, A. J., D. Gatto, E. Sainsbury, R. Gregory, H. Hengartner, and R. M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288(5474): 2222–2226.

184. Boes, M., C. Esau, M. B. Fischer, T. Schmidt, M. Carroll, and J. Chen. 1998. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J. Immunol.* 160: 4776–4787.

185. Baumgarth, N., O. C. Herman, G. C. Jager, L. E. Brown, L. A. Herzenberg, and J. Chen. 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J. Exp. Med.* 192: 271–280.

186. Shen, P., and S. Fillatreau. 2015. Antibody-independent functions of B cells: A focus on cytokines. *Nat. Rev. Immunol.* 15: 441–451.

187. Maseda, D., K. M. Candando, S. H. Smith, I. Kalampokis, C. T. Weaver, S. E. Plevy, J. C. Poe, and T. F. Tedder. 2013. Peritoneal cavity regulatory B cells (B10 cells) modulate IFN-γ^+CD4$^+$ T cell numbers during colitis development in mice. *J. Immunol.* 191: 2780–95.

188. Sun, C. M., E. Deriaud, C. Leclerc, and R. Lo-Man. 2005. Upon TLR9 signaling, CD5 $^+$ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* 22: 467–477.

189. Rauch, P. J., A. Chudnovskiy, C. S. Robbins, G. F. Weber, M. Etzrodt, I. Hilgendorf, E. Tiglao, J.-L. Figueiredo, Y. Iwamoto, I. Theurl, R. Gorbatov, M. T. Waring, A. T. Chicoine, M. Mouded, M. J. Pittet, M. Nahrendorf, R. Weissleder, and F. K. Swirski. 2012. Innate response activator B Cells protect against microbial sepsis. *Science* 335(6068): 597–601.

190. Weber, G. F., B. G. Chousterman, I. Hilgendorf, C. S. Robbins, I. Theurl, L. M. S. Gerhardt, Y. Iwamoto, T. D. Quach, M. Ali, J. W. Chen, T. L. Rothstein, M. Nahrendorf, R. Weissleder, and F. K. Swirski. 2014. Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis. *J. Exp. Med.* 211: 1243–1256.

191. Margry, B., W. H. Wieland, P. J. van Kooten, W. van Eden, and F. Broere. 2013. Peritoneal cavity B-1a cells promote peripheral CD4$^+$ T-cell activation. *Eur. J. Immunol.* 43: 2317–2326.

192. Vigna, A. F. G., L. C. Godoy, S. Rogerio De Almeida, M. Mariano, and J. D. Lopes. 2002. Characterization of B-1b cells as antigen presenting cells in the immune response to gp43 from *Paracoccidioides brasiliensis in vitro*. *Immunol. Lett.* 83: 61–66.

193. Bamba, H., H. Ishigaki, H. Ishida, K. Kajino, Y. Fujiyama, and K. Ogasawara. 2005. B1-B cells are the main antigen presenting cells in CpG-ODN-stimulated peritoneal exudate cells. *Microbiol. Immunol.* 49: 89–95.

194. Wang, Y., and T. L. Rothstein. 2012. Induction of Th17 cell differentiation by B-1 cells. *Front. Immunol.* 3: 1–7.

195. Gao, J., X. Ma, W. Gu, M. Fu, J. An, Y. Xing, T. Gao, W. Li, and Y. Liu. 2012. Novel functions of murine B1 cells: Active phagocytic and microbicidal abilities. *Eur. J. Immunol.* 42: 982–992.

196. Moore-Connors, J. M., H. S. Kim, J. S. Marshall, A. W. Stadnyk, S. A. Halperin, and J. Wang. 2015. $CD43^-$, but not $CD43^+$, IL-10-producing $CD1d^{hi}CD5^+$ B cells suppress type 1 immune responses during *Chlamydia muridarum* genital tract infection. *Mucosal Immunol.* 8: 94–106.

197. Seillet, C., S. Laffont, F. Trémollières, N. Rouquié, C. Ribot, J. F. Arnal, V. Douin-Echinard, P. Gourdy, and J. C. Guéry. 2012. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol *in vivo* through cell-intrinsic estrogen receptor α signaling. *Blood* 119: 454–464.

198. Calippe, B., V. Douin-Echinard, M. Laffargue, H. Laurell, V. Rana-Poussine, B. Pipy, J.-C. Guery, F. Bayard, J.-F. Arnal, and P. Gourdy. 2008. Chronic estradiol administration *in vivo* promotes the proinflammatory response of macrophages to TLR4 activation: Involvement of the phosphatidylinositol 3-kinase pathway. *J. Immunol.* 180: 7980–7988.

199. Sandberg, J. K., N. Bhardwaj, and D. F. Nixon. 2003. Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Valpha24 NKT cell compartment. *Eur. J. Immunol.* 33: 588–96.

200. Markle, J. G., and E. N. Fish. 2014. SeXX matters in immunity. *Trends Immunol.* 35: 97–104.

201. Bianchi, I., A. Lleo, M. E. Gershwin, and P. Invernizzi. 2012. The X chromosome and immune associated genes. *J. Autoimmun.* 38: J187–J192.

202. Pisitkun, P., J. A. Deane, M. J. Difilippantonio, T. Tarasenko, A. B. Satterthwaite, and S. Bolland. 2006. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* 312(5780): 1669–1672.

203. Butterworth, M., B. McClellan, and M. Allansmith. 1967. Influence of sex on immunoglobulin levels. *Nature* 214: 1224–1225.

204. Rhodes, K., R. L. Markham, P. M. Maxwell, and M. E. Monk-Jones. 1969. Immunoglobulins and the X-chromosome. *Br. Med. J.* 3: 439–441.

205. Fischer, J., N. Jung, N. Robinson, and C. Lehmann. 2015. Sex differences in immune responses to infectious diseases. *Infection* 43: 399–403.

206. Pasche, B., S. Kalaydjiev, T. J. Franz, E. Kremmer, V. Gailus-Durner, H. Fuchs, M. H. de Angelis, A. Lengeling, and D. H. Busch. 2005. Sex-dependent susceptibility to *Listeria monocytogenes* infection is mediated by differential interleukin-10 production. *Infect. Immun.* 73: 5952–5960.

207. Guilbault, C., P. Stotland, C. Lachance, M. F. Tam, A. Keller, L. Thompson-Snipes, E. Cowley, T. A. Hamilton, D. H. Eidelman, M. M. Stevenson, and D. Radzioch. 2002. Influence of gender and interleukin-10 deficiency on the inflammatory response during lung infection with *Pseudomonas aeruginosa* in mice. *Immunology* 107: 297–305.

208. Pope, V., S. A. Larsen, R. J. Rice, S. N. Goforth, C. E. Parham, and M. B. Fears. 1994. Flow cytometric analysis of peripheral blood lymphocyte immunophenotypes in persons infected with *Treponema pallidum*. *Clin. Diagn. Lab. Immunol.* 1: 121–124.

209. Cooper, G. S., and B. C. Stroehla. 2003. The epidemiology of autoimmune diseases. *Autoimmun. Rev.* 2: 119–125.

210. Wang, X., K. Ma, M. Chen, K. H. Ko, B. J. Zheng, and L. Lu. 2016. IL-17A promotes pulmonary B-1a cell differentiation via induction of Blimp-1 expression during influenza virus infection. *PLoS Pathog.* 12: e1005367.

211. Scotland, R. S., M. J. Stables, S. Madalli, P. Watson, and D. W. Gilroy. 2011. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood* 118: 5918–5927.

212. Gomes, J. P., M. J. Borrego, B. Atik, I. Santo, J. Azevedo, A. Brito De Sá, P. Nogueira, and D. Dean. 2006. Correlating *Chlamydia trachomatis* infectious load with urogenital ecological success and disease pathogenesis. *Microbes Infect.* 8: 16–26.

213. Michel, C. E. C., C. Sonnex, C. A. Carne, J. A. White, J. P. V Magbanua, E. C. B. Nadala, and H. H. Lee. 2007. *Chlamydia trachomatis* load at matched anatomic sites: Implications for screening strategies. *J. Clin. Microbiol.* 45: 1395–1402.

214. Wiggins, R., S. Graf, N. Low, and P. J. Horner. 2009. Real-time quantitative PCR to determine chlamydial load in men and women in a community setting. *J. Clin. Microbiol.* 47: 1824–1829.

215. Jalal, H., N. Q. Verlander, N. Kumar, N. Bentley, C. Carne, and C. Sonnex. 2011. Genital chlamydial infection: Association between clinical features, organism genotype and load. *J. Med. Microbiol.* 60: 881–888.

216. Eckert, L. O., R. J. Suchland, S. E. Hawes, and W. E. Stamm. 2000. Quantitative *Chlamydia trachomatis* cultures: Correlation of chlamydial inclusion-forming units with serovar, age, sex, and race. *J. Infect. Dis.* 182: 540–544.

217. Lorenzo, M. E., A. Hodgson, D. P. Robinson, J. B. Kaplan, A. Pekosz, and S. L. Klein. 2011. Antibody responses and cross protection against lethal influenza A viruses differ between the sexes in C57BL/6 mice. *Vaccine* 29: 9246–9255.

218. Hawkins, R. A., R. G. Rank, and K. A. Kelly. 2002. A *Chlamydia trachomatis*-specific Th2 clone does not provide protection against a genital infection and displays reduced trafficking to the infected genital mucosa. *Infect. Immun.* 70: 5132–5139.

219. Yang, X., J. Gartner, L. Zhu, S. Wang, and R. C. Brunham. 1999. IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following *Chlamydia trachomatis* lung infection. *J. Immunol.* 162: 1010–1017.

220. Penttilä, J. M., M. Anttila, M. Puolakkainen, A. Laurila, K. Varkila, M. Sarvas, P. H. Mäkelä, and N. Rautonen. 1998. Local immune responses to *Chlamydia pneumoniae* in the lungs of BALB/c mice during primary infection and reinfection. *Infect. Immun.* 66: 5113–5118.

221. Moyron-Quiroz, J. E., J. Rangel-Moreno, L. Hartson, K. Kusser, M. P. Tighe, K. D. Klonowski, L. Lefrançois, L. S. Cauley, A. G. Harmsen, F. E. Lund, and T. D. Randall. 2006. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. *Immunity* 25: 643–654.

222. Igietseme, J. U., G. A. Ananaba, J. Bolier, S. Bowers, T. Moore, T. Belay, F. O. Eko, D. Lyn, and C. M. Black. 2000. Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: Potential for cellular vaccine development. *J. Immunol.* 164: 4212–4219.

223. Hakimi, H., M. M. Akhondi, M. R. Sadeghi, L. Chamani, M. K. Arababadi, B. N. Ahmadabadi, G. Hassanshahi, and M. S. Fathollahi. 2014. Seminal levels of IL-10, IL-12, and IL-17 in men with asymptomatic *Chlamydia* infection. *Inflammation* 37: 122–126.

224. Spear, G. T., S. R. Kendrick, H. Y. Chen, T. T. Thomas, M. Bahk, R. Balderas, S. Ghosh, A. Weinberg, and A. L. Landay. 2011. Multiplex immunoassay of lower genital tract mucosal fluid from women attending an urban STD clinic shows broadly increased IL1ß and lactoferrin. *PLoS One* 6: e19560.

225. Margry, B., S. C. W. Kersemakers, A. Hoek, G. J. A. Arkesteijn, W. H. Wieland, W. van Eden, and F. Broere. 2014. Activated peritoneal cavity B-1a cells possess regulatory B cell properties. *PLoS One* 9: e88869.

226. Brockmann, L., N. Gagliani, B. Steglich, A. D. Giannou, J. Kempski, P. Pelczar, M. Geffken, B. Mfarrej, F. Huber, J. Herkel, Y. Y. Wan, E. Esplugues, M. Battaglia, C. F. Krebs, R. A. Flavell, and S. Huber. 2017. IL-10 receptor signaling is essential for TR1 cell function *in vivo*. *J. Immunol.* 198: 1130–1141.

227. Duan, B., and L. Morel. 2006. Role of B-1a cells in autoimmunity. *Autoimmun. Rev.* 5: 403–408.

228. Hsu, L.-H., K.-P. Li, K.-H. Chu, and B.-L. Chiang. 2015. A B-1a cell subset induces Foxp3⁻ T cells with regulatory activity through an IL-10-independent pathway. *Cell. Mol. Immunol.* 12: 354–365.

229. Asai, K., N. Hiki, Y. Mimura, T. Ogawa, K. Unou, and M. Kaminishi. 2001. Gender differences in cytokine secretion by human peripheral blood mononuclear cells: Role of estrogen in modulating LPS-induced cytokine secretion in an *ex vivo* septic model. *Shock* 16: 340–343.

230. Bouman, A., M. Schipper, M. J. Heineman, and M. M. Faas. 2004. Gender difference in the non-specific and specific immune response in humans. *Am. J. Reprod. Immunol.* 52: 19–26.

231. Satoskar, A., and J. Alexander. 1995. Sex-determined susceptibility and differential IFN-y and TNF-a mRNA expression in DBA / 2 mice infected with *Leishmania mexicana*. *Immunology* 84: 1–4.

232. Zhong, X., S. Lau, C. Bai, N. Degauque, N. E. Holodick, S. J. Steven, J. Tumang, W. Gao, and T. L. Rothstein. 2009. A novel subpopulation of B-1 cells is enriched with autoreactivity in normal and lupus-prone mice. *Arthritis Rheum.* 60: 3734–3743.

233. Halle, S., H. C. Dujardin, N. Bakocevic, H. Fleige, H. Danzer, S. Willenzon, Y. Suezer, G. Hämmerling, N. Garbi, G. Sutter, T. Worbs, and R. Förster. 2009. Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. *J. Exp. Med.* 206: 2593–2601.

234. Good-Jacobson, K. L., E. Song, S. Anderson, A. H. Sharpe, and M. J. Shlomchik. 2012. CD80 expression on B cells regulates murine T follicular helper development, germinal center B cell survival and plasma cell generation. *J. Immunol.* 188: 4217–4225.

235. Deenick, E. K., and C. S. Ma. 2011. The regulation and role of T follicular helper cells in immunity. *Immunology* 134: 361–367.

236. Good-Jacobson, K. L., C. G. Szumilas, L. Chen, A. H. Sharpe, M. Mary, and M. J. Shlomchik. 2010. PD-1 regulated germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat. Immunol.* 11: 535–542.

237. Sarma, J. V., and P. A. Ward. 2011. The complement system. *Cell Tissue Res.* 343: 227–235.

238. Liang, S., D. Bulir, C. Kaushic, and J. Mahony. 2017. Considerations for the rational design of a *Chlamydia* vaccine. *Hum. Vaccines Immunother.* 13: 831–835.

239. Vogel, L. A., T. L. Lester, V. H. Van Cleave, and D. W. Metzger. 1998. Inhibition of murine B1 lymphocytes by interleukin-12. *Eur. J. Immunol.* 26: 219–223.

240. Pal, S., E. M. Peterson, and L. M. De La Maza. 2004. New murine model for the study of *Chlamydia trachomatis* genitourinary tract infections in males. *Infect. Immun.* 72: 4210–4216.

241. Moore, P. A., O. Belvedere, A. Orr, K. Pieri, D. W. LaFleur, P. Feng, D. Soppet, M. Charters, J. Giri, V. Roschke, B. Nardelli, J. Carrell, S. Sosnovtseva, W. Greenfield, S. M. Ruben, H. S. Olsen, J. Fikes, and D. M. Hilbert. 1999. BLyS: Member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 285(5425): 260–263.

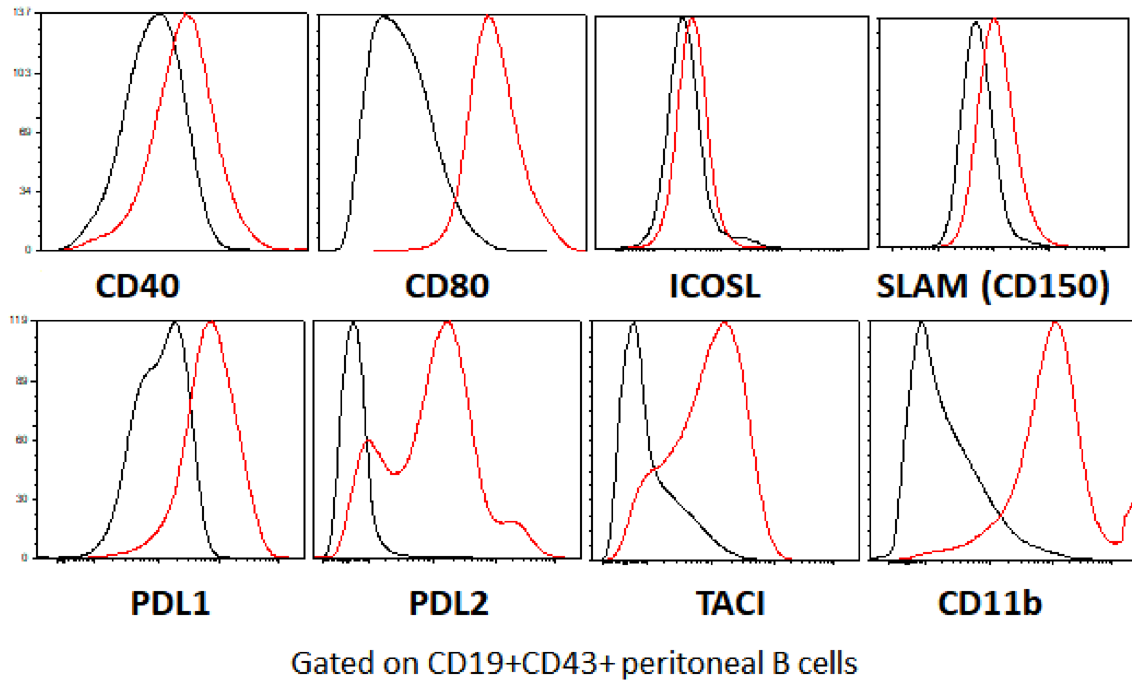
242. Schneider, P., F. MacKay, V. Steiner, K. Hofmann, J.-L. Bodmer, N. Holler, C. Ambrose, P. Lawton, S. Bixler, H. Acha-Orbea, D. Valmori, P. Romero, C. Werner-Favre, R. H. Zubler, J. L. Browning, and J. Tschopp. 1999. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* 189: 1747–1756.

243. Lebien, T. W., and T. F. Tedder. 2008. ASH 50th anniversary review B lymphocytes : how they develop and function. *Am. Soc. Hematol.* 112: 1570–1580.

244. Mackay, F., P. Schneider, P. Rennert, and J. Browning. 2003. BAFF and APRIL: A tutorial on B cell survival. *Annu. Rev. Immunol.* 21: 231–264.

245. Mackay, F., S. A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, and J. L. Browning. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* 190: 1697–710.

APPENDIX A Supporting material for CHAPTER 4



Appendix A1: Peritoneal cells upregulate activation markers following *in vitro Cm* infection. Peritoneal cells were isolated from mice and stimulated *in vitro* with *Cm* (red line) or medium alone (black line) for 16 hrs. Following stimulation, the cell pellets were collected and analyzed by flow cytometry. The experiment shown here was performed by Dr. Jessica Moore-Connors.

APPENDIX B Investigating sex differences in BAFF production

1.1 INTRODUCTION

BAFF belongs to the TNF superfamily of cytokines and acts on B cells (241, 242). The production of BAFF by both DCs and monocytes has been shown to induce proliferation and PC differentiation of B cells and can also act as a survival signal (241–244). Mice lacking the ability to signal via BAFF have reduced mature B cells while mice transgenic for BAFF have significantly expanded mature B cell populations, demonstrating the importance of BAFF in maintaining mature B cell numbers (244, 245). Considering the evidence for increased humoral responses in females, we wanted to investigate if BAFF production differed between the sexes (203).

1.2 MATERIALS AND METHODS

1.2.1 *In vitro* stimulation of male and female BMDCs

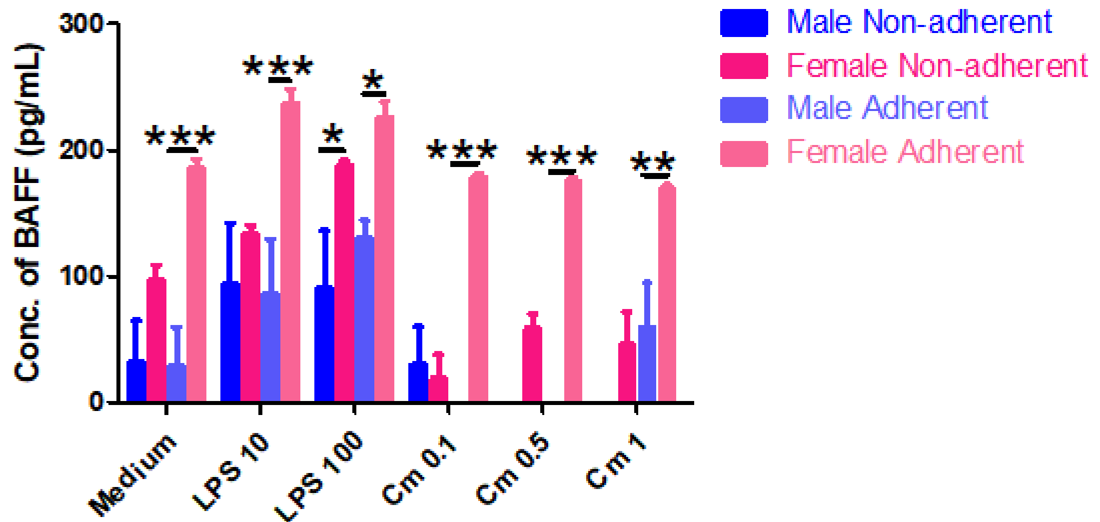
Bone marrow was isolated from male and female mice and differentiated into BMDCs *in vitro* (see 2.4.2). Non-adherent BMDCs were collected from the supernatant by gently washing the plates and aspirating the medium. To isolate adherent BMDCs, the plates were rinsed with PBS and then incubated with 3 ml TrypLE express (Thermo Fisher Scientific, Waltham, MA, USA) for 20 mins at 37° C. Following incubation, the enzyme reaction was stopped by adding cRPMI and the cells were collected. Next, the cells were pelleted by centrifugation (525×g, 10 mins, 4°C). Following the spin, cells were counted and plated in a 96-well plate. The BMDCs were stimulated with medium alone, LPS (Sigma-Aldrich) at concentrations of 10 or 100 ng/ml, or infected with *Cm* at

MOI=0.1, 0.5, or 1. After 24 hrs, the culture supernatant was collected and BAFF levels were measure by ELISA (see 2.6.1).

1.3 RESULTS

1.3.1 Female BMDCs produce more BAFF than male counterparts

Following stimulation with medium alone, LPS, or *Cm*, female adherent BMDCs produced significantly more BAFF compared to male adherent BMDCs. This difference was not as evident in non-adherent BMDCs however female non-adherent BMDCs did still produce significantly more BAFF in response to LPS at 100 ng/ml. Together these results demonstrate that female BMDCs produce more BAFF at rest, as well as in response to LPS and *Cm* stimulation.



Appendix B1: Female bone marrow derived dendritic cells produce more BAFF than male BMDCs. Bone marrow derived dendritic cells were isolated from male and female bone marrow and then stimulated in culture for 24 hours. Cells were plated with medium alone, LPS at 10 ng/ml or 100 ng/ml, or infected with *Cm* at MOI=0.1, 0.5, or 1. The culture supernatant was collected and BAFF production was measured by ELISA. Data are from one experiment with triplicate wells of each stimulation and graphed as mean±SEM. *p<0.05, **p<0.001, ***p<0.001 using 2-way ANOVA.