THE ROLE OF REGULATORY T AND B CELLS IN HOST RESPONSES TO CHLAMYDIA GENITAL TRACT INFECTION

by

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For my son Jack,

may you discover the joy in learning (and hard work!)

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ABSTRACT

The existence of regulatory T and B cells as important cellular entities in immune responses to infection has been established. The focus of this study was to dissect the role of regulatory T and B cells in regulating the pathogenesis of *Chlamydia* infection, a common cause of immunopathological disease sequelae. The functional role of $CD4^+Foxp3^+CD25^+$ regulatory T cells (Tregs) in host responses to *Chlamydia* was investigated in an *in vitro* co-culturing system and a murine model of *Chlamydia* genital tract (GT) infection. Remarkably, *Chlamydia* infection subverted the immune suppressive role of $CD4^+Foxp3^+$ Tregs; instead of hampering immune responses, Tregs promoted pathological T helper (Th17) responses to *Chlamydia* in both *in vitro* and *in vivo* settings. Anti-CD25 monoclonal antibody treatment depleted Tregs prior to *Chlamydia* GT infection and markedly reduced the frequency and the total number of Th17 but not T helper 1 (Th1) cells at both immune induction and memory phases. Importantly, Treg-depleted mice displayed significantly attenuated neutrophilic inflammation and reduced severity of oviduct pathology. To our knowledge, this is the first demonstration that the level of endogenous host Tregs has a major impact on the development *Chlamydia*-associated diseases. In addition to Tregs, we investigated the role of B cells as a source of counter-regulatory IL-10, an immunosuppressive anti-Th1 cytokine, in host responses to *Chlamydia*. B cells were major IL-10 producers in response to *Chlamydia in vitro*. In particular, an $IL\text{-}10^+$ $CD43^-$ B cell subset strongly suppressed $CD4^+$ T cell proliferation and IFN-γ production *in vitro*. Notably, dendritic cells were required for the generation of $CD43^-$ $IL\text{-}10^+$ B cells in response to *Chlamydia in vitro*. Importantly $CD43^-IL\text{-}10^+$ B cells were potently expanded in the local draining lymph node by day 5 following *Chlamydia* GT infection and selective deficiency of IL-10 in B cells markedly enhanced Th1 responses and decreased bacterial burden, reducing the severity of oviduct pathology. To our knowledge, this is the first report demonstrating a regulatory role for $IL\text{-}10^+$ B cells in *Chlamydia* infection. Collectively, these results demonstrate that regulatory T and B cells have critical and distinct roles in modulating protective and pathological immune responses to *Chlamydia*.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Ab	antibody
AID	activation-induced cytidine deaminase
AP-1	activator protein-1
APC	antigen presenting cell
APRIL	a proliferation inducing ligand
ATP	adenosine triphosphate
B10pro	B10 progenitor
BAFF	B cell activating factor
BCR	B cell receptor
BMDC	bone marrow-derived dendritic cell
Breg	regulatory B cell
BS	bovine serum
BSA	bovine serum albumin
CD	cluster of differentiation
CIA	collagen-induced arthritis
Cm	*Chlamydia muridarum*
CMI	cell-mediated immunity
COPD	chronic obstructive pulmonary disease
CSR	class switch recombination
Ct	*Chlamydia trachomatis*
CTLA4	cytotoxic T lymphocyte antigen 4
DAMP	Damage-associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity

DTR	diphtheria toxin receptor
EAE	experimental autoimmune encephalomyelitis
EB	elementary bodies
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FasL	Fas ligand
FBS	fetal bovine serum
FDC	follicular DC
FO	follicular
Foxp3	forkhead box P3
GATA-3	GATA-binding protein 3
GFP	green fluorescent protein
GFPKI	GFP knock-in
GITR	glucocorticoid-induced TNFR family related gene
GM-CSF	granulocyte/monocyte colony stimulating factor
GT	genital tract
HBSS	Hanks balanced salt solution
Hsp60	heat-shock protein 60
i.p.	intraperitoneal
i.v.	intravenous
i.vag.	intravaginal
IBD	inflammatory bowel disease
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IFU	inclusion forming unit
Ig	immunoglobulin

IKK	IκB kinase
IL	interleukin
ILB	innate-like B cell
ILN	iliac lymph node
iNOS	inducible nitric oxide synthase
IPEX	immune disregulation, polyendocrinopathy, enteropathy, X-linked
IRAK	IL-1 receptor-associated kinase
IRES	internal ribosome entry site
IRF4	interferon regulatory transcription factor 4
iTreg	inducible regulatory T cell
IκB	inhibitor of κB
KO	knockout
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MD-2	myeloid differentiation factor 2
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MyD88	myeloid differentiation primary response protein 88
MZ	marginal zone
NALT	nasopharynx-associated lymphoid tissue
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κB
NK	natural killer

NLR	NOD-like receptor
NO	nitric oxide
NOD	nuclear oligomerization domain
nTreg	natural regulatory T cell
p.i.	post-infection
P/S	penicillin/streptomycin
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerC	peritoneal cavity
PGE_2	prostaglandin E_2
PID	pelvic inflammatory disease
PLO	peripheral lymphoid organs
PMA	phorbol myristate acetate
PRR	pattern recognition receptor
qPCR	quantitative PCR
RA	retinoic acid
rad	radiation absorbed dose
RAG	recombination activating gene
RB	reticulate bodies
RORγt	retinoic acid-receptor related orphan receptor γt
s.c.	subcutaneous
SCID	severe combined immunodeficiency
SHM	somatic hypermutation
SPG	sucrose phosphate glutamic acid

STAT	signal transducer and activator of transcription
STI	sexually-transmitted infection
T2-MZP	transitional 2 marginal zone precursor
TAK	TGF-β-activated kinase
T-bet	T-box expressed in T cells
Tconv	conventional T cell
TCR	T cell receptor
TF	transcription factor
TFI	tubal factor infertility
TGF-β	transforming growth factor β
Th	T helper cell
TIR	Toll/IL-1 receptor
TIRAP	TIR-domain containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
Tr1	type 1 regulatory T cell
TRAM	TRIF-related adaptor molecule
Treg	regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSDR	Treg-specific demethylation region
UL	uterine lavage
WT	wild-type

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CHAPTER 1 INTRODUCTION

Infectious diseases elicit potent host immune effector mechanisms designed to eliminate pathogenic organisms and infected cells. Counter-regulatory mechanisms keep immune effector cells under control during homeostasis and responses to infection in order to minimize collateral tissue damage caused by inappropriate or excessive immune activation. However, immunosuppressive mechanisms may also provide an opportunity for pathogens to evade host immunity and a lack of effective immunity is a contributing factor in chronic infectious diseases.

Chlamydiae are a group of highly successful intracellular bacterial pathogens that infect mucosal epithelial cells of the ocular, respiratory and genital tracts, causing a spectrum of pathologies in humans. Chronicity is a hallmark of *Chlamydia*-caused infections, which commonly result in immune-mediated tissue damage and fibrosis. The underlying immune mechanisms that render the host susceptible to persistent infection and mediate immunopathological responses are not fully understood.

1.1 *Chlamydia* infection and associated diseases

The genus *Chlamydia* comprises a group of obligate intracellular bacteria responsible for a wide range of diseases in both humans and animals (1). *Chlamydia* preferentially target host epithelial cells and establish infections at the single layer of mucosal epithelium lining the ocular, respiratory and genital tracts. *Chlamydia* species share a unique biphasic life cycle consisting of two distinct forms of the bacterium: the small infectious but metabolically inert elementary body (EB) and the larger metabolically active reticulate body (RB). The infection cycle takes up to 48 hours to complete and is initiated when *Chlamydia* EB are endocytosed by the host cell (Figure 1.1). Once inside the cell, EB reside within host endosomes where they transition into RB, which then divide by binary fission. As the number of RB progeny increases, the endosome membrane expands to accommodate them, generating a macroscopically visible ‘inclusion’ within the cell (2). RB then revert back into infectious EB and are released to infect neighboring cells (2). *Chlamydia* utilizes various strategies to evade

immune-mediated elimination, including the production of proteins that modulate host cellular functions to block endosome-lysosome fusion, inhibit apoptosis and interfere with immune-recognition (2-4). Moreover, certain stimuli such as host cytokines, antibiotics or nutrient deprivation can cause *Chlamydia* RB to enter a persistent state inside host cells in which they are viable but dormant until the stimulus is removed (1). Indeed, a large body of evidence indicates that *Chlamydia* characteristically establish persistent infections in humans leading to chronic inflammatory pathologies (5;6). Infections caused by *Chlamydia* can result in severe morbidities and represent a significant public health problem worldwide.

1.1.1 *Chlamydia* genital tract (GT) infection

The trachomatis biovar of *Chlamydia trachomatis* (serovars D to K) is responsible for more sexually-transmitted infections (STI) than any other bacterial pathogen (6;7). In the female genital tract (GT), *C. trachomatis* establishes infections in columnar epithelial cells lining the endocervix causing inflammation and mucopurulent cervicitis (8). The female GT is comprised of two distinct compartments: the lower GT (vagina, ectocervix) and the upper GT (endocervix, uterus, oviducts). Initial mucosal *Chlamydia* infections of the GT are essentially local and elicit acute inflammatory responses resulting in redness, edema, and mucopurulent discharge at the infection site. However, over time the infection can ascend upward into the uterus, oviduct and ovaries causing inflammation, tissue damage and fibrosis (i.e. scar formation) (6), which can obstruct the oviduct causing the lumen becoming fluid-filled and dilated, described as 'hydrosalpinx' (9). These pathological sequelae profoundly disrupt reproductive functions of the oviduct (9;10).

While GT infections are treatable with antibiotics, a prominent feature of *C. trachomatis* epidemiology is that a high percentage (up to 75%) of infections in women are subclinical or asymptomatic and often go untreated (11). Up to 30% of untreated *C. trachomatis* infections progress into pelvic inflammatory disease (PID), which can lead to serious long-term sequelae such as infertility and ectopic pregnancy in 10-20% of

patients due to oviduct fibrosis (11;12). It is estimated that 30-40% of cases of female infertility are caused by post-infection oviduct damage, i.e. tubal factor infertility (TFI) (13). *C. trachomatis* infections are also associated with adverse pregnancy outcomes including miscarriage, preterm labor and preeclampsia (11;14). In total, *Chlamydia* GT infections and associated pathologies represent a significant socioeconomic and public health burden for Canadians (15).

1.1.2 Other *Chlamydia* infections

While *C. trachomatis* GT infection is the most high profile chlamydial disease in industrialized countries, *Chlamydia* species have numerous tissue tropisms and cause a spectrum of diseases. The *C. trachomatis* lymphogranuloma venereum (LGV) biovar (serovars L1-L3) can also be transmitted sexually but is invasive, causing systemic lymphatic infections. Although rare in industrialized countries, this biovar is currently endemic in developing countries among homosexual males (16). The trachoma biovar of *C. trachomatis* (serovars A to C) infects the human conjunctiva causing corneal scarring (i.e. trachoma) and remains the leading cause of preventable blindness worldwide, particularly in areas of poverty and poor sanitation conditions (17;18). Genital tract serovars D to K can also cause infections of the conjunctiva and respiratory tract of infants born to infected mothers. The rate of vertical transmission of *C. trachomatis* from mothers to their infants is reportedly as high as 50-70% and frequently results in acute conjunctivitis and neonatal pneumonia (11;19).

Like *C. trachomatis*, *C. pneumoniae* is a common pathogen of humans. *C. pneumoniae* causes approximately 10-15% of cases of community-acquired pneumonia and is also an agent of acute respiratory infections such as sinusitis, pharyngitis, laryngitis and bronchitis (20). Serological surveys indicate that *C. pneumoniae* is ubiquitous, with ~50% of healthy young adults and 75% of elderly persons exhibiting evidence of prior infection (5;21). Respiratory infections with *C. pneumoniae* in early life may play a role in the etiology of nonallergic asthma: a recent study in a mouse model indicates that neonatal respiratory *Chlamydia* infection induces changes in lung

structure causing respiratory dysfunction that persists into adulthood (22). Respiratory chlamydial infections acquired in infancy may persist for months or years in the absence of specific treatment—both *C. pneumonia* and *C. trachomatis* have been found in lung washings from children with chronic respiratory disease (23). Chronic lung infections by *Chlamydia* throughout life may not only exacerbate asthmatic symptoms but also play a role in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD) (24). Chronic pulmonary *C. pneumoniae* infection is also associated with significantly increased risk of developing lung cancer (25).

In addition to causing local infection and tissue damage, both *C. trachomatis* and *C. pneumoniae* can disseminate from their sites of primary infection via circulating monocytes and establish long-term residence in distant anatomic sites such as synovial tissue and arterial vasculature (26). Within monocytes/macrophages, *Chlamydia* can exist in a developmentally-arrested state of persistence that elicits and propagates inflammation provoking various chronic inflammatory diseases (26;27). Indeed, *C. trachomatis* is among the most common infectious causes of reactive arthritis, with approximately 4-15% of women developing arthritis following primary *C. trachomatis* GT infection (28). The presence of *C. pneumoniae* in vascular tissue is closely associated with atherosclerosis (5) and, more recently, *C. pneumoniae* has been implicated in chronic diseases of the central nervous system including Alzheimer's disease and multiple sclerosis (29). Collectively, *Chlamydia*-caused infections impose significant morbidities afflicting people of all ages worldwide.

1.2 Animal models of *Chlamydi*a infection

Humans are the exclusive natural host for *C. trachomatis* but, clearly, studying the natural history of untreated *Chlamydia* infections in humans is both ethically and practically problematic. In order to study host responses to *Chlamydia* infection, several animal models of *Chlamydia* have been developed using natural host-pathogen combinations in rodents, guinea pigs, pigs, sheep and non-human primates. By far, the most commonly used model of experimental infection is the mouse (30).

While human serovars of *C. trachomatis* can establish limited infections in mice, the species *C. muridarum*—formerly known as the mouse pneumonitis biovar of *C. trachomatis*—is a natural pathogen of mice and is used extensively in both respiratory and GT infection models (30). In the mouse model of *C. muridarum* GT infection, female mice are pretreated with progesterone to synchronize their estrous cycles to the luteal phase, which is most permissive to infection (31). A single intravaginal dose of *Chlamydia* establishes robust infection that ascends into the upper GT and persists for approximately 30 days (9;30). The gross anatomy of the mouse female upper GT is distinct from that of humans (Figure 1.2) but the microscopic anatomy and physiology are comparable. Indeed, *C. muridarum* infections in mice result in severe upper GT pathologies that closely resemble pathological sequelae observed in the genital tracts of *C. trachomatis*-infected women, including damaged epithelia, increased oviduct dilatation and occlusion, and hydrosalpinx formation (9).

Of particular interest to the study of *Chlamydia*-caused disease is the naturally-occurring variation in susceptibility to *C. muridarum*-induced infection and inflammatory pathology among mouse strains: C57BL/6 mice are the most resistant whereas BALB/c and C3H/HeN mice are susceptible strains, displaying greater and more prolonged bacterial burden and more severe tissue pathology following *Chlamydia* infection in both respiratory and GT infection models (9;32). These inherent strain-dependent differences offer potential insight into host genetic factors involved in pathological responses to *Chlamydia*. Furthermore, all strains resolve chlamydial GT infections with sterilizing clearance and are strongly protected from secondary challenge infections (30). Given these features, the mouse model is well-suited for studying immune mechanisms that mediate protective immunity to *Chlamydia* as well as identifying some of the immunopathological processes responsible for *Chlamydia*-induced disease.

1.3 Host responses to *Chlamydia* infection

The mammalian immune system is able to defend against infectious agents through the actions of distinct but interdependent innate and adaptive arms of immunity. The

innate immune system is a powerful first line of defense that involves physical/physiological barriers (e.g. epithelial cells, low pH), secretion of inflammatory mediators (e.g. cytokines, chemokines, complement, acute-phase proteins), and mobilization of immune cells that eliminate invading microbes and infected host cells (e.g. neutrophils, macrophages, natural killer (NK) cells) (33). Cells of the innate immune system are rapidly activated upon infection via the triggering of various pattern-recognition receptors (PRRs) that detect invariant features of microbes/microbial products termed pathogen-associated molecular patterns (PAMPs) (34). Innate immune responses are designed to contain pathogens through non-specific mechanisms of inflammation but effective host defense may also require the induction of a highly specific adaptive immune response mediated by T and B lymphocytes (33). During primary infection, the adaptive immune response takes several days to develop but provides long-lasting immunity against secondary infection through the formation of pathogen-specific memory responses. Upon subsequent encounters with the same pathogen, immune memory responses are rapidly and robustly activated, forming the basis of protective immunity.

Complex signals from the innate immune system are responsible for initiating and instructing adaptive immune responses. Antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) engulf pathogens by phagocytosis and degrade their protein constituents into antigenic peptides to be presented on the cell surface within the cleft of major histocompatibility complex (MHC) molecules. Upon recognizing PAMPs, APCs undergo a maturation process and present antigen to naïve $CD4^+$ T cells, leading to their activation and clonal expansion. Based on the type of pathogen encountered, APCs produce distinct cytokines and chemokines that instruct the differentiation of $CD4^+$ T cells into discrete subsets with specialized effector functions (35). Activated $CD4^+$ T subsets provide help to B cells resulting in the production of high affinity antigen-specific antibodies. Together, adaptive T and B cell responses direct effector functions of innate immune cells in an antigen-specific manner, greatly enhancing their ability to target and destroy the offending microorganism (36).

The success of host immune defense relies heavily on the generation of appropriate $CD4^+$ T cell responses. Because different effector $CD4^+$ T cell subsets are specialized to protect against distinct types of infection, the induction of non-protective lineages can compromise host defenses and allow pathogens to persist. Failure to resolve infection can sustain chronic inflammatory responses that drive tissue damage and fibrosis. Fibrosis is commonly associated with repeated injury and occurs when normal parenchymal tissue is replaced by excessive deposition of extracellular matrix components (ECM) due to abnormal activation and proliferation of fibroblasts and myofibroblasts (37). While fibrogenesis is a complex, progressive process that is incompletely understood, it is clear that profibrotic processes are initiated by inflammatory reactions involving components of both innate and adaptive arms of the immune system (37). Clinical and experimental studies support the notion that deleterious host inflammatory responses drive the pathogenesis of *Chlamydia*-caused disease; however, the immune mechanisms involved in this process remain unclear (38).

1.3.1 Innate responses

1.3.1.1 Pattern recognition receptors (PRRs)

In order to initiate an immune response, cells of the innate immune system must first be able to distinguish non-infectious self from infectious non-self (36). This innate immune recognition is facilitated by pattern recognition receptors (PRRs), which are germ-line encoded and have broad specificities for molecular structures unique to micro-organisms termed pathogen-associated molecular patterns (PAMPs). PAMPs can include bacterial cell wall components, viral and bacterial nucleic acids, lipids, and lipoproteins that are generally shared among large groups of pathogens (39). In addition to PAMPs, PRRs also recognize endogenous stress-induced self molecules termed damage-associated molecular patterns (DAMPs) (40). The sensing of PAMPs and/or DAMPs by PRRs triggers a signalling cascade within the host cell leading to the activation of various transcription factors that induce production of antimicrobial peptides, cytokines,

chemokines and type I interferons (IFN). Together, these molecules orchestrate early host responses to infection and provide essential signals for the activation of adaptive immune responses.

Toll-like receptors (TLRs) are an important group of PRRs expressed on the cell membrane and/or endosomal membrane components of both immune and non-immune cells. In mammals, 12 members of the TLR family have been identified, with TLR1-9 being conserved in both mice and humans. TLRs are comprised of an ectodomain that facilitates ligand binding, a transmembrane domain and an intracellular signal transduction domain known as a Toll/IL-1 receptor (TIR) domain. TLRs can be broadly divided into two subgroups based on their cellular localization and the type of PAMPs they recognize. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface and mainly bind PAMPs associated with microbial membrane components whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular vesicles such as endosomes, lysosomes and endoplasmic reticulum (ER) and sense microbial nucleic acids (39).

TLRs transduce signals within the host cell to activate nuclear factor-κB (NFκB) and mitogen-activated protein kinase (MAPK) pathways, which initiate inflammatory responses (39;41). NF-κB activation results in the production of proinflammatory cytokines, including TNF-α, IL-6, pro-IL-1β and pro-IL-18 (41). Although some redundancy exists among the signaling pathways induced by different TLRs, individual TLRs are able to mediate distinct biological responses by recruiting different cytoplasmic adaptor molecules such as MyD88 and TRIF to their intracellular TIR domain (Figure 1.3). Accordingly, TLR signaling can be classified as either MyD88-dependent or TRIF-dependent (39). All TLRs except TLR3 recruit MyD88 and signal through MyD88-dependent pathways to activate NFκB and MAPK pathways. Conversely, TLR3 and a subset of TLR4 signalling pathways recruit TRIF and initiate a TRIF-dependent pathway that activates IRF3 as well as NFκB and MAPK pathways, resulting in the production of both type I IFNs and proinflammatory cytokines. Because most TLRs signal through

MyD88-dependent pathways, MyD88-deficient mice are frequently used to model impaired TLR function (42).

A single pathogen expresses numerous PAMPs and can potentially interact with multiple TLRs. To date, the TLRs most studied in the context of *Chlamydia* infection are TLR3, TLR4 and, especially, TLR2. *C. muridarum* induces production of type I interferon-β (IFN-β) by oviduct epithelial cell lines via TLR3- and TRIF-dependent pathways (43;44) but the role of TLR3 signaling in host defense against *Chlamydia in vivo* has not been investigated. TLR4 recognizes Gram-negative bacteria via lipopolysaccharide (LPS), a major outer membrane constituent of all Gram-negative bacteria with the aid of two accessory proteins myeloid differentiation factor-2 (MD-2) and CD14 (45). While chlamydial LPS can activate TLR4 signaling (albeit with 100-fold less potency than LPS from *Escherichia coli*), studies using TLR4-deficient mice indicate that TLR4 is dispensable for bacterial clearance in both the GT and respiratory *Chlamydia* infection models and does not mediate *Chlamydia*-induced immunopathology in the GT (46;47). Rather, TLR2 seems to be the dominant PRR for triggering host inflammatory cytokine production in response to *Chlamydia* (46;48-50). TLR2 forms heterodimers with TLR1 or TLR6 to bind triaclyated lipopeptides from Gram-negative bacteria or diacylated lipoprotetins from Gram-positive bacteria, respectively, and also interacts with non-TLR molecules to recognize additional PAMPs (39;51). TLR2 has been observed to traffic to and co-localize with chlamydial inclusions within epithelia *in vitro* (52), suggesting an active role for TLR2 signaling in the intracellular detection of *Chlamydia*. Indeed, TLR2 mediates the production of early proinflammatory mediators such as TNF-α, IL-6 and CXCL2 by fibroblasts and macrophages as well as IL-6 and GM-CSF production by oviduct epithelia in response to *C. muridarum* infection *in vitro* (46;53). *In vivo*, the role of TLR2 in host responses to *C. muridarum* infection is strikingly disparate between lung and GT infection sites. In the lung infection model, TLR2-deficient mice develop prolonged infection and more severe inflammation than their wild-type counterparts indicating that TLR2 is required for effective bacterial clearance (47). Conversely, TLR2-deficient mice in the GT infection model exhibit bacterial clearance comparable to wild-type mice but develop markedly less *Chlamydia*-

induced oviduct pathology, implicating TLR2 as a mediator of pathological inflammatory responses but not bacterial clearance in the GT (46). Nonetheless, TLRs appear to be important in the development of protective immune responses to *Chlamydia* GT infection; MyD88-deficient mice exhibit reduced production of IFN-γ by NK cells and enhanced non-protective T helper 2 (Th2)-polarized adaptive immune responses, which is associated with increased bacterial burden in the upper GT and more severe oviduct pathology relative to wild-type controls (54;55).

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are a more recently described family of PRRs that reside in the cytosol and sense intracellular bacterial PAMPs as well as host-derived DAMPs produced upon cellular stress or injury (56). NOD1 and NOD2 detect bacterial cell wall peptidoglycan (PGN) components introduced into host cytoplasm by bacterial secretion systems and activate NFκB and MAPK pathways (56). There is evidence that *Chlamydia* infection activates NOD proteins. Upon *in vitro* infection of host cells, *C. pneumoniae* induces NOD1- and NOD2-mediated NFκB activation and, similarly, NOD1 is involved in NFκB activation in response to *C. trachomatis* and *C. muridarum* (57-59). However, NOD1-deficiency does not impact the production of proinflammatory cytokines or alter the course of infection and oviduct pathology during *Chlamydia* GT infection *in vivo* (58).

Whereas NOD1 and NOD2 stimulation activate NFκB and MAPK pathways, other NLR family members are involved in caspase activation. Certain NLR proteins (e.g. NLRC4 or NLRP3) are important components of multi-protein complexes termed inflammasomes that generate active caspase-1, which in turn mediates the maturation of IL-1β and IL-18 from immature pro-IL-1β and pro-IL-18 precursors (60). IL-1β in particular is a potent proinflammatory mediator implicated in regulating inflammation and fibrosis during *Chlamydia* infections. Notably, pro-IL-1β may be the key effector molecule induced downstream of TLR2 in response to *Chlamydia* infection (61;62). While caspase-1 deficiency or administration of capase-1 inhibitors in *Chlamydia*-infected mice confers significant protection from oviduct pathology (63), the role of

inflammasome activation in *Chlamydia*-induced IL-1β production is not definitive. On one hand, the NLRP3 inflammasome has been shown to mediate IL-1β production by cervical epithelial cells and monocytes in response to *Chlamydia in vitro* and is required for IL-1β production during *C. pneumoniae* respiratory infection (61;64;65). However, a recent study determined that NLRP3 inflammasome activation is not required for either IL-1β secretion or the development of oviduct pathology during *Chlamydia* GT infection (62).

1.3.1.2 Mucosal epithelial cells

The lower and upper compartments of the female GT are distinct microenvironments. The lower GT is non-sterile, i.e. colonized by commensal microflora, but resistant to pathogens due largely to stratified squamous (type II) mucosal epithelia (>25 layers) and the acidic environment maintained by commensals such as lactobacilli, which together provide a substantial physical/physiological barrier to invading microbes. In contrast to the barriers present in the lower GT, the upper GT is sterile and lined with a single layer of columnar (type I) mucosal epithelia (66). These columnar epithelial cells of the upper GT are the primary cellular hosts of *Chlamydia* and provide the initial host-derived signals that direct the influx of inflammatory cells to the infection site (32;67-69). Upon encountering *Chlamydia*, epithelial cells produce a wide array of proinflammatory cytokines and chemokines including IL-α/β, IL-6, CXCL2 (IL-8 in humans), TNF-α, IFN-β, and GM-CSF (32;67-69). Notably, whereas other bacteria that invade epithelia typically induce a rapid but transient cytokine response, the production of inflammatory cytokines by *Chlamydia*-infected epithelial cells is delayed for ~24h post-infection, requires bacterial protein synthesis and is sustained throughout the bacteria's growth cycle of 2 to 4 days (67). The release of IL-1α by *Chlamydia*-infected epithelial cells upon cell lysis is believed to further amplify inflammatory responses by stimulating cytokine production from uninfected neighbouring cells (70).

1.3.1.3 Neutrophils and macrophages

In general, the female GT contains few leukocytes and must recruit immune cells during host responses to infection (71). Chemotactic factors released by *Chlamydia*-infected and damaged epithelia cause innate immune cells to be rapidly mobilized from circulation into the genital infection site. Neutrophils are the first and most abundant cell population recruited during the acute phase of *Chlamydia* infection (68;72). Neutrophils are characterized as professional phagocytic cells and aid host defense via the removal of microbial products and cellular debris and by engulfing pathogens. Following phagocytosis, neutrophils kill pathogens via the production of superoxides, reactive nitrogen species, proteolytic enzymes and antimicrobial peptides (73). Although neutrophils can phagocytose extracellular EBs and transiently reduce the number of infectious EBs during the first few days of infection, these cells are ultimately dispensable for bacterial clearance (74;75). Rather, the duration and intensity of neutrophilic inflammation is strongly correlated with the development of *Chlamydia*-induced pathology (9;76;77). Mice depleted of neutrophils or deficient in CXCR2, which mediates neutrophil recruitment, develop significantly less severe *Chlamydia*-induced oviduct pathology and reduced rates of infertility compared to wild-type counterparts (74;78). Oviduct pathology is also enhanced in mouse strains with high levels of CXCL2 expression, which is associated with more vigorous neutrophil infiltration (79).

Destruction of host epithelium is a central feature of the pathophysiology of *Chlamydia* infection and it is increasingly apparent this epithelial damage results largely from the activity of neutrophils (80). The granules of neutrophils contain proteolytic enzymes such as matrix metalloproteinase 9 (MMP-9), which degrades type IV collagen—a major component of the basal lamina to which epithelial cells are anchored. This degradation causes large sheets of epithelia to detach and slough off into the lumen of the uterus/oviduct (80). It has been suggested that epithelial sloughing may contribute to the dissemination of *Chlamydia* infection from the lower to upper GT (80). MMPs also promote ECM remodeling, which is instrumental to fibrosis, and can have additional immunomodulatory effects by amplifying chemokine and cytokine activity, potentially providing a positive feedback loop for epithelial cell-derived inflammatory responses

(81;82). Indeed, both animal model and human studies implicate MMP-9 as a significant molecular factor in host susceptibility to *Chlamydia*-induced disease sequelae (83-85).

The initial wave of neutrophil infiltration is followed by the recruitment of monocytes/macrophages (86). In the presence of TLR ligands and IFN-γ/TNF-α in the local environment, macrophages can be polarized toward a classically activated/inflammatory M1 phenotype, which mediate pathogen killing via the production of reactive oxygen/nitrogen species and can also function as APCs (87). The activation of phagocyte oxidase in neutrophils and macrophages triggers the release of tissue-damaging superoxide molecules; mice deficient in phagocyte oxidase have lower rates of *Chlamydia*-induced oviduct pathology (88). Neutrophils and macrophages also produce cytokines including TNF-α, IL-6 and IL-1β that are linked to the development of infection-induced fibrosis in multiple models (37). Macrophages are a major source of IL-1β induced by *Chlamydia* and, as previously discussed, IL-1β is implicated in the development of oviduct pathology and infertility during *Chlamydia* infection in both mice and humans (62;70;89;90).

1.3.1.4 Antigen presenting cells (APCs)

APCs are responsible for initiating adaptive immune responses to infection. Dendritic cells (DCs) are important APCs that act as sentinels in peripheral tissue, sensing the presence of pathogens using various PRRs. Upon encountering chlamydial products and/or inflammatory cytokines, DCs undergo a maturation process characterized by the upregulation of MHC class II and costimulatory molecule expression and secretion of specific cytokines (91-93). The cytokine profile of DCs is influenced by the type of microorganism recognised and local factors produced by various tissue-resident and immune cells such as epithelial cells, NK cells, and macrophages (94). Mature DCs migrate to the lymph node and present antigen to naïve T cells. Unlike PAMPs, antigens do not possess intrinsic features to indicate the nature of their origin. In this regard, the cytokine milieu generated by PRR-activated DCs provides an essential biological context for antigens and instructs the polarization of the ensuing adaptive immune response. DCs

are a highly heterogeneous cell type; the best-studied subsets are classical myeloid or conventional DCs (cDCs) and plasmacytoid DCs (pDCs). $CD11b^+$ cDCs are the major DC population involved in the induction of T cell responses to *Chlamydia* GT infection (95) and form a critical bridge between innate and adaptive immunity to *Chlamydia* by instructing the generation of appropriate $CD4^+$ T cell responses.

1.3.2 Adaptive responses

1.3.2.1 $CD4^+$ T helper cell responses

$CD4^+$ T helper (Th) cells play a central role coordinating complex effector immune responses during microbial infection. Previous studies have demonstrated that $CD4^+$ T cells are an essential component of host defense against *Chlamydia* infection: mice deficient in T cells or possessing genetic deletions in TCR$\alpha\beta$, CD4, or MHC class II molecules have profoundly impaired abilities to resolve GT infection (96-98). Upon activation, $CD4^+$ T cell clones proliferate and polarize into distinct effector cell lineages that produce characteristic profiles of cytokines and chemokines that mobilize specific immune cells in order to eradicate different types of pathogens. It is important to note that the GT lacks mucosa-associated lymphoid tissue (MALT), which distinguishes it from other mucosal sites in the body such as the gastrointestinal and nasopharynx/bronchial tracts (71). As such, primary responses to genital pathogens are induced exclusively at distal sites such as the spleen and local draining iliac lymph nodes (ILN)(71).

T helper (Th) differentiation is initiated upon T cell receptor (TCR) binding with its cognate antigen in the context of self-MHC class II molecules (referred to as signal 1) and costimulatory molecules expressed by mature APCs (signal 2). Th lineage decisions are ultimately determined by the local cytokine environment (signal 3) generated by innate immune responses to the invading pathogen (99) (Figure 1.4). These cytokine signals activate different specific signal transducer and activator of transcription (STAT)

proteins, which can then induce the expression of lineage-specific 'master regulator' transcription factors (TFs) that form a complex network of lineage-specific gene expression while suppressing gene transcription of opposing Th lineages (100) (Figure 1.5). The transcriptional programs of fully-differentiated Th subsets are stabilized by epigenetic mechanisms such as DNA methylation and chromatin remodeling (101).

Th1 polarization is initiated by STAT1 activation via IFN-γ or IL-27 signaling, which leads to the induction of T-bet, master regulator of Th1 differentiation (99). T-bet induces IFN-γ production and upregulates IL-12Rβ2 expression on the T cell surface, rendering the T cell responsive to IL-12 from APCs (99). IL-12 stimulates STAT4-dependent signaling to maintain T-bet expression and cytokine production, which further reinforces Th1 lineage commitment (Figure 1.5). T-bet also antagonizes Th2 and Th17 differentiation programs by inhibiting the function of competing lineage transcription factors GATA3 and RORγt, respectively (99;102). Th1 cells primarily produce IFN-γ and co-ordinate cell-mediated immunity (CMI), which is essential for host defense against numerous viral and intracellular bacterial pathogens (103).

For Th2 cells, polarization is initiated by IL-4-mediated activation of STAT6, which activates transcription of GATA3, the master regulator of Th2 lineage commitment (99) (Figure 1.5). Th2 cells produce IL-4, IL-5, and IL-13 which are essential for optimal antibody production and coordinate the elimination of helminths and other extracellular parasites (103).

In mice, the initiation of Th17 differentiation requires STAT3 activation by proinflammatory cytokine IL-6 in the context of transforming growth factor β (TGF-β) (99;104) (Figure 1.5). TGF-β signalling inhibits negative regulators of STAT3 signalling, such as SOCS3, as well as competing lineage-specific TFs such as T-bet and STAT6, thereby disabling negative feedback from Th1/Th2 lineage cytokines. STAT3 induces the expression of IL-21, which provides an autocrine feedback loop via STAT3-dependent signals. These include the expression IL-23R to make differentiating cells responsive to IL-23 signalling, a critical growth and stabilization factor for Th17 cells,

and the induction of master TF retinoic acid receptor-related orphan receptor-γt (RORγt), which coordinates the expression of Th17 lineage cytokines IL-17A, IL-17F, and IL-22 (105). The TF Runx1 promotes IL-17A production through co-operative binding with RORγt but can also inhibit IL-17A by associating with Foxp3 (106). In addition to Runx1 and RORγt, TFs such as RORα, aryl-hydrocarbon receptor (AhR) and interferon regulatory factor 4 (IRF4), which is induced by IL-1β signaling, participate in Th17 development (107-110).

Th17 cells produce two IL-17 family cytokines, IL-17A and IL-17F, which signal as homo- or heterodimers through a complex of IL-17 receptor (IL-17R) subunits IL-17RA and IL-17RC (111). IL-17RA is expressed on a wide range of cell types and at high levels on immune cells; in contrast IL-17RC is expressed at low levels on hematopoietic cells and high levels on non-immune cells (105). While both IL-17RA and IL-17RC are required for IL-17A/F signaling in mice, IL-17A has a higher affinity to IL-17RA whereas IL-17F has a higher affinity to IL-17RC (112). The differential expression of IL-17RA and IL-17RC may therefore account for some of the distinct functions of IL-17A and IL-17F. However, IL-17A and IL-17F have many overlapping functions: both exhibit potent proinflammatory activity by inducing the production of proinflammatory cytokines TNF, IL-1β, IL-6 and granulopoeitic factors (GM-CSF, G-CSF) as well as chemokines (CXCL1, CXCL2) promoting the generation, recruitment and activation of neutrophils (105;113). IL-17A and IL-22 also induce the production of antimicrobial peptides such as β-defensins and lipocalin-2 by mucosal epithelial cells (114;115). Strong evidence indicates that Th17 responses play an important role in host defense against mucosal infection by extracellular bacterial and fungal pathogens (105;113), including *Streptococcus pneumonia* (116), *Neisseria gonorrhoea* (117) and *Candida albicans* (118). Indeed, humans with genetic deficiencies in IL-17RA and IL-17F are highly prone to mucocutaneous disease caused by *C. albicans* and *Streptococcus aureus* (119;120). Likewise, humans deficient in STAT3, an important TF in Th17 differentiation, suffer from *S. auerus* infection and mucocutaneous candidiasis, which is linked to defective Th17 responses (120;121). However, while providing a protective

role during certain infections, Th17 cytokines can also cause persistent or recurrent neutrophil infiltration that substantially augments tissue damage in others (122;123).

1.3.2.2 Th1/Th2 paradigm in *Chlamydia* infection

As previously discussed, Th1 and Th2 subsets mediate different types of immune responses suited for distinct classes of pathogens. Furthermore, Th1 and Th2 responses antagonize the differentiation of the opposing lineage, causing immune responses to be polarized (103). For many years, the balance of Th1 and Th2 responses has provided a useful paradigm for understanding pathological and protective immunity in many diseases, including *Chlamydia* infection.

Th1 responses and CMI are required for host defense against *Chlamydia*. Mice deficient in IFN-γ or IFN-γ receptor (IFN-γR), or treated with anti-IFN-γ antibodies exhibit significantly impaired ability to control the infection (96;124-126). Adoptive transfer of Th1, but not Th2, cells can confer protection against infection (127). IFN-γ limits *Chlamydia* growth in epithelial cells directly by activating indoleamine 2,3-dioxygenase (IDO), inducing tryptophan catabolism and starving *Chlamydia* of host-derived tryptophan. IFN-γ also activates inducible nitric oxide synthase (iNOS) which catalyzes the production of antimicrobial reactive nitrogen intermediates such as nitric oxide (NO) (128-130). IFN-γ can impede *Chlamydia* indirectly by augmenting host immune responses via promoting M1 polarization, antigen presentation and chemokine expression. The quality of Th1 responses to *Chlamydia* is understood to be a critical determinant in pathogenesis—inadequate or suboptimal Th1 responses delay the clearance and promote latent or persistent infection, which fuels chronic inflammatory responses and causes tissue damage (72).

In stark contrast, Th2 responses are linked to pathological host responses to *Chlamydia* infection. The deleterious role of Th2 responses is largely attributed to the antagonistic effects of Th2-polarizing TFs and cytokines on the differentiation of protective Th1 responses. Studies in humans show that gene expression levels for Th2

cytokines are significantly higher in women with *Chlamydia*-induced infertility whereas as *Chlamydia*-seropositive women without fertility disorders produce higher levels of Th1-associated cytokines IFN-γ and IL-12 (131). These observations suggest that Th1-biased responses protect from chronic infection sequelae. Th2 cytokines, particularly IL-13, promote tissue remodelling and fibrosis (132) and may therefore contribute directly to GT tissue scarring during *Chlamydia* infection. Thus, traditional conceptualizations of *Chlamydia* immunity have been framed within the Th1/Th2 paradigm, with Th1 responses being protective and Th2 responses a non-protective and malignant subversion of Th1 development.

1.3.2.3 Potential role of Th17 responses in *Chlamydia* infection

The discovery of Th17 cells has revised the Th1/Th2 paradigm in a wide range of inflammatory disease models. Defining the role of IL-17A and Th17 cells in host responses to *Chlamydia* may likewise enrich our understanding of how protective and pathological responses are regulated. IL-17A and Th17 responses are induced by *Chlamydia* in both the respiratory and GT infection models (133-135). Recent evidence indicates that early IL-17A production, likely from innate sources, benefits host defense against *Chlamydia* by promoting DC maturation and IL-12 production to support the downstream induction of Th1 responses (136). The role of IL-17A production by Th17 cells in *Chlamydia* infection is much less understood. Relatively high Th17 responses have been reported in mice with *Chlamydia*-susceptible mouse strains in the respiratory model and are associated with increased neutrophil infiltration and disease susceptibility (134;137). IL-17A may also contribute to fibrosis directly by promoting epithelial-mesenchymal transition and increased collagen production (138) but the direct role of IL-17A in fibrosis is controversial (37). Collectively, while Th17 responses appear non-protective in *Chlamydia* infection, it is unclear whether Th17 responses are directly pathological since they tend to coincide with reduced protective Th1 responses and increased bacterial burdens. IFN-γ can inhibit Th17 differentiation, thus lower Th1 responses may result in higher Th17 responses due to reduced counter-regulation (139;140). Indeed, in addition to having prolonged infections, *Chlamydia*-infected IFN-

γ-deficient mice display enhanced Th17 responses and neutrophilic inflammation in the GT (133). Similarly, *Chlamydia*-susceptible BALB/c mice exhibit lowered Th1 responses and delayed clearance concomitant with high levels of IL-17A production in the later phase of respiratory infection compared to resistant C57BL/6 mice (137). However, *Chlamydia*-susceptible C3H/HeN exhibit Th1 responses comparable to C57BL/6 but significantly greater IL-17A/Th17 responses during respiratory infection (134), indicating that additional factors are involved in the balance of Th1/Th17 responses. Due to these complex interactions, the precise contribution of IL-17A and Th17 responses to host defense against *Chlamydia* infection requires further characterization.

1.3.2.4 Role of $CD8^+$ T cell responses

$CD8^+$ T cells are important effectors of adaptive immunity during certain intracellular infections due their ability to kill host cells harbouring pathogens and produce type I cytokines IFN-γ and TNF-α (141). However, although $CD8^+$ T cells can recognize and kill *Chlamydia*-infected cells *in vitro* (142), $CD8^+$ T cell responses are vastly inferior to $CD4^+$ T cells in their protective effects *in vivo* and are dispensable in host defense against *Chlamydia* genital tract infection: mice that are $CD8^+$ T cell-depleted or deficient in perforin and/or Fas ligand (FasL), or β2-microglobulin resolve infection normally (97;143-145). In addition to providing little to no protection, $CD8^+$ T cells appear to contribute to *Chlamydia*-induced genital tract pathology via TNF-α production (146).

1.3.2.5 Role of antibody responses

B cells are central to the adaptive immune system and are responsible for generating high affinity antigen-specific immunoglobulin (Ig) molecules that neutralize invading pathogenic microbes and target them for destruction by innate effector cells (147). During infection, B cells that recognize their cognate protein antigen via the B cell

receptor (BCR) internalize and efficiently process it in order to present peptides to $CD4^+$ T cells specific for the same pathogen, which have been previously primed by activated DCs. Engagement of CD40 on B cells by CD40L on $CD4^+$ T cells initiates a signaling pathway leading to the activation of NFκB (148). Within the B cell, NFκB mediates the transcription of immunoglobulin (Ig) genes and the enzyme activation-induced cytidine deaminase (AID) that is required for the diversification of Ig genes through the processes of class-switch recombination (CSR) and somatic hypermutation (SHM) (149). Together these processes generate B cells that produce high affinity pathogen-specific antibodies with the ability to interact with certain effector molecules in order to facilitate efficient pathogen elimination. In addition to the well-known role of B cells in producing antibody, B cells can modulate T cell activation via antigen presentation (147). DCs are generally considered the most efficient APC for naïve $CD4^+$ T cell priming but B cells can also function as APCs in the initiation of T cell responses, particularly when antigen is limiting. Antigen presentation by B cells is also implicated in optimal Th expansion and memory formation (150). B cells also produce cytokines that influence T cells directly or indirectly by modulating APC maturation/activity. Thus, B cells are multifaceted immune effectors and can serve distinct functions in the immune response.

In contrast to other mucosal sites that primarily produce IgA and IgM, the female GT predominantly contains IgG and the majority of antibodies present in the GT enter via transudation from systemic circulation rather than local production (151). While it is well-documented that *Chlamydia* infection elicits strong humoral responses in the serum and local cervical secretions of mice as well as humans during active infection (7) and that specific antibodies can neutralize *Chlamydia* infectivity *in vitro* (152;153), B cells and humoral immunity are widely considered to be of secondary importance in antichlamydial defense (124;154;155). This notion is derived in part from the fact that the intracellular lifestyle of *Chlamydia* makes it largely inaccessible to neutralizing antibodies and, in general, immunity to intracellular pathogens is regarded to be independent of the humoral arm of adaptive immunity (156). Furthermore, experimental vaccines that elicit high titres of *Chlamydia*-specific antibody are ineffective (157) and B cell-deficient mice are reported to resolve GT infection as efficiently as their wild-type

counterparts (154;158), supporting that B cells are dispensable. Indeed, B cell-deficient mice are only slightly less resistant to reinfection than B cell-competent mice, illustrating the dominant role for $CD4^+$ T cell responses in protecting against reinfection by *Chlamydia* (154;155;159).

There is, however, evidence that B cells and antibody can compensate for lack of $CD4^+$ T cells in protecting against reinfection (143;160). In mice rendered susceptible to infection via $CD4^+$ T cell-depletion, immunity to secondary infection can be restored by passive transfer of immune serum (160). Immunity to secondary infection is also significantly compromised in B cell-deficient mice depleted of $CD4^+$ T cells prior to reinfection compared to $CD4^+$ T cell-depleted B cell-competent mice (143). Mechanisms through which antibody might protect from reinfection include impeding *Chlamydia* attachment and early colonization of the mucosa, as well as promoting FcR-mediated and antibody-dependent cellular cytotoxicity (161;162).

It should be noted that many of the initial early studies examining the role of B cells in *Chlamydia* infection utilized B cell-deficient mice (157). Congenitally B cell-deficient mice have more recently been shown to possess significant immune abnormalities—including decreased T cell numbers and TCR repertoires, altered splenic architecture and impaired FDC development—that may alter immune responses independent of B cell-deficiency itself (163-166). Therefore, direct comparisons between such mice and their B cell-competent counterparts must be interpreted cautiously.

1.3.3 Immune regulation during infection

The immune system is endowed with powerful inflammatory responses that are capable of eliminating a wide range of pathogenic microbes but also have the potential to cause damage to host tissue. Immune regulatory mechanisms serve to limit the activation of immune responses in order to protect the host from the aberrant or excessive inflammation (167). During immune cell development in primary lymphoid organs, complex pathways including clonal deletion of self-reactive T and B cells help establish a

lack of responsiveness to self-antigens, resulting in central tolerance (168). In addition to these central processes, the immune system is equipped with mechanisms to ensure tolerance in the periphery in order to prevent inappropriate immune activation against self-antigens as well as against innocuous foreign antigens from commensal microorganisms or the environment. Cell-intrinsic signals can control the activation of lymphocytes by inducing programmed death or anergy and these intrinsic forms of tolerance also help limit excessive immune responses to foreign antigens (167). In addition, there are cell-extrinsic mechanisms that actively suppress inappropriate or excessive immune responses and this form of tolerance is mediated by specialized subsets of T and B lymphocytes, as well as some regulatory subsets of DCs and macrophages (167;169). Regulatory T and B cells also control the magnitude and quality of immune responses to infection, which can elicit excessive inflammation that is potentially more damaging to the host than the virulence of the pathogen that elicited them. It is now apparent that all adaptive immune responses involve the concomitant activation of regulatory cells (167). While these regulatory elements are crucial for controlling the extent and duration of host immune responses, excessive regulation may hinder effective clearance, promoting persistent infection and inflammation (170). An appropriate balance between effector and regulatory immune networks is essential for resolving infection with minimal damage to host tissue.

1.3.3.1 Regulatory T cells (Tregs)

Regulatory T cells (Tregs) play a critical role in the maintenance of immune tolerance and suppression of autoimmune disease. Tregs constitute approximately 5-10% of peripheral $CD4^+$ T cells in mice and humans and express the lineage-specific transcription factor forkhead box 3 (Foxp3). Null mutations of the Foxp3 gene cause severe multi-organ autoimmune diseases in humans termed immune disregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and an analogous fatal lymphoproliferative disorder in mice (Scurfy mice) characterized by overproduction of proinflammatory cytokines and $CD4^+$ T cell hyperactivation (171;172). Because the loss of Foxp3 so devastatingly impacts Treg activity, Foxp3 is regarded as the master

regulator of Treg function. In support of this role, ectopic expression of Foxp3 in conventional $CD4^+$ T cells has been shown to upregulate Treg-associated markers CD25 glucocorticoid-induced TNFR-related gene (GITR) and cytotoxic T lymphocyte-associated 4 (CTLA-4) and confer suppressive function *in vitro* and *in vivo* (173;174). However, more recent work indicates that Foxp3 controls only a portion of the transcriptional program associated with $Foxp3^+$ Tregs and Foxp3 alone is not sufficient to establish full Treg phenotype and function (175-177). Rather, Foxp3 acts to amplify and stabilize epigenetic modifications within Treg precursor cells that are established by TCR and IL-2R signaling (178). Nonetheless, Foxp3 remains a highly specific and reliable marker for identifying Tregs in mice.

$Foxp3^+$ Tregs can be divided into two major subsets based on their origin: 1) naturally occurring Tregs (nTregs) that develop directly from the thymus, and 2) Tregs that differentiate from naïve conventional $CD4^+$ T cells in the periphery. Both subsets are characterized by Foxp3 expression and the inability to produce effector Th lineage cytokines such as IL-2, IFN-γ or IL-4.

1.3.3.1.1 Regulatory T cell subsets

Natural Tregs (nTregs) have a characteristic $CD4^+CD25^+Foxp3^+$ phenotype and are produced by the thymus as a distinct $CD4^+$ T cell subpopulation. Natural Tregs possess T cell receptors (TCRs) that recognize self-antigen (179;180), which has provoked much investigation into how nTregs develop during the thymic selection process designed to delete self-reactive T cell clones. A large body of research indicates that TCR recognition of self-peptide/MHC complexes and CD28-mediated co-stimulation are important factors for thymic Treg development (181). Importantly, thymic Treg development appears to depend on a specific threshold of TCR affinity for self-antigen well below the threshold of self-reactivity required to elicit conventional $CD4^+$ T cell responses in the periphery (181;182). TCR and CD28 signaling cascades result in the development of Foxp3- Treg precursors that express CD25 (IL-2Rα) and can utilize IL-2 (and to a lesser extent IL-7 and IL-15) to induce Foxp3 expression (183;184). Foxp3

actively suppresses T cell-intrinsic IL-2 production so nTregs are reliant on exogenous IL-2 secreted by activated conventional T cells for survival in the periphery (185). Constitutive high expression of CD25 is a key feature of nTregs that allows these cells to efficiently absorb IL-2 in their vicinity (186;187).

In contrast to $Foxp3^+$ Tregs derived directly from the thymus, so-called 'inducible' $Foxp3^+$ Tregs (iTregs) can be generated from mature conventional $CD4^+$ T cells in the periphery (Figure 1.4). The differentiation of iTregs requires TCR activation in the context of TGF-β and IL-2: TGF-β induces the activation of Smads that bind conserved non-coding regions in the Foxp3 locus and co-operate with TCR and IL-2-mediated signals to induce Foxp3 expression (188-190) (Figure 1.5). Additional signals may also contribute to efficient iTreg generation, particular in *in vivo* settings (191). For instance, iTreg development is promoted under conditions of suboptimal co-stimulation and low doses of high affinity TCR ligands (192-194) as well as by the presence of retinoic acid (RA), a vitamin A metabolite produced by certain APC populations such as $CD103^+$ DCs (195;196). Being derived from conventional $CD4^+$ T cells, iTregs differ from nTregs in their repertoire of TCR specificity and have been found to recognize foreign antigens including allergens, commensal microbiota, tumor antigens, and alloantigens in addition to self antigens (197). iTreg generation at mucosal tissues has been well-documented and these cells are thought to play an important role maintaining peripheral tolerance to microbial flora and innocuous environmental antigens (191;198). Although inflammation-associated cytokines such as IL-6, IL-12, IL-27 and IFN-γ can inhibit the development of iTregs, iTreg generation has been observed in states of chronic inflammation such as asthma, colitis and chronic infection (191;198).

In addition to iTregs, other extrathymically-derived Treg subsets including type 1 regulatory (Tr1) cells and Th3 cells have been identified (Figure 1.4). Tr1 cells differentiate from conventional $CD4^+$ T cells in the periphery in the presence of IL-10 and IL-27 (199) and lack Foxp3 expression, but can be characterized by their production of IL-10 in the absence of IL-2 or IL-4 (200). Th3 cells are TGF-β-producing cells with

variable expression of Foxp3 that are involved in oral tolerance. Some Th3 clones that express low levels of TGF-β also produce IL-4 and IL-10 (201).

1.3.3.1.2 Mechanisms of suppression

Tregs must be activated via TCR ligation in order to exert suppression but, once activated, Tregs suppress bystander cell responses in an antigen-nonspecific manner (202). nTregs are functionally mature and 'antigen-primed' upon exiting the thymus and therefore a much lower (~100-fold) threshold concentration of peptide is required to activate nTregs compared to naïve T cells (187). This high antigen sensitivity of nTregs allows them to become activated during the initial stages of an immune reaction and may also facilitate their ability to quickly form aggregates around APCs, outcompeting naïve T cells (203). Both nTregs and iTregs have been shown to effectively suppress T cell responses *in vivo* and likely share similar mechanisms of suppression (204;205). Many of these mechanisms require cell-to-cell contact and target responding T cells directly and/or indirectly by modifying APC function and include: 1) limiting the bioavailability of IL-2, 2) production of granzyme B or perforin, 3) expression of inhibitory surface receptors such as CTLA-4 that inhibit DC maturation and function, and 4) expression of ectonucleotidases that degrade proinflammatory ATP and generate anti-inflammatory adenosine (202). In addition, Tregs secrete inhibitory cytokines such as IL-10, TGF-β, and IL-35. It is likely that soluble factors are most effective when Tregs are in close proximity to target cells, which could contribute to contact-dependent suppressive function (206). Notably, suppression mediated by Tr1 cells is contact-independent and IL-10-dependent (207).

1.3.3.1.3 Modulation of Treg function by TLRs

The dominant suppressive function of nTregs under steady state conditions would appear to present a significant obstacle to the induction of effective immune responses to infection, particularly in the context of an acute infection. In answer to this puzzle, recent

studies have ascertained that nTregs themselves are subject to regulation and can be transiently over-ridden by certain molecular signals, most notably TLR agonists. On one level, TLRs control nTreg activity by eliciting production of proinflammatory cytokines such as IL-6 by APCs that render effector $CD4^+$ T cells refractory to Treg-mediated suppression (208). On another level, it has been suggested that Tregs can sense the presence of pathogens directly via TLRs and there is evidence that TLR engagement by human Tregs differentially modulates their function. Stimulation of TLR5 by flagellin enhances suppressive activity and Foxp3 expression (209) whereas stimulation of TLR8 on Tregs reverses suppression (210).

TLR2 has garnered significant attention as a potent modulator of nTreg function, both at the steady state and during infection. In mice, TLR2 appears to play an essential role in nTreg expansion *in vitro* and *in vivo*; indeed, TLR2-deficient mice possess ~50% fewer nTregs cells than their wild-type counterparts (211). Independent studies reported that Foxp3 expression and suppressive activity of murine Tregs are transiently abrogated by synthetic TLR2 ligand Pam3Cys in the context of IL-2 and TCR triggering (211;212) but an additional study did not confirm this effect (213). However, both these studies found that Pam3Cys induced Treg proliferation and enhanced survival. Notably, a recent study in mice using highly purified nTregs suggests that TLR agonists do not directly activate Tregs and that nTregs at steady-state only express TLR1 and very low levels of TLR2 (214). In humans, high concentrations of Pam3Cys are reported to attenuate Treg function and induce production of IL-17A and IL-17AF, although the impact on Foxp3 expression is variable (215;216). In contrast, TLR2 ligation by endogenous heat shock protein 60 (Hsp60) co-activates human Tregs and enhances their suppressive activity (217). These disparate results could be due in part to the distinct heterodimerization of TLR2 with TLR1 or TLR6 to sense Hsp60 or Pam3Cys, respectively. Collectively, it is uncertain whether the impact of TLR2 signaling on Treg function is indirect (e.g. via the activation of accessory cells) or direct and varies depending on the nature of the TLR2 agonist.

1.3.3.1.4 Relationships between Treg lineages and Th17 responses

Th17 cells share a close developmental relationship with iTregs. Activated $CD4^+$ T cells stimulated with TGF-β co-express Foxp3 and RORγt and possess a dual potential to become iTreg or Th17 cells. In the presence of TGF-β alone, particularly at high concentrations, Foxp3 inhibits IL-17A expression by competing with Runx1 for interaction with RORγt, thereby antagonizing RORγt function (106;218). IL-6 acts as potent regulator of iTreg versus Th17 differentiation by suppressing Foxp3-mediated inhibition of RORγt, allowing RORγt-dependent transcription and Th17 differentiation to occur (104) (Figure 1.5).

In contrast to the reciprocal relationship between Th17 and iTregs, nTregs have been found to positively correlate with Th17 levels in various models of inflammation (219-222). TGF-β production has been previously implicated as the molecular mechanism behind the Th17-promoting activity of nTregs (223). T cell-specific deficiency in TGF-β severely inhibits Th17 development *in vivo* (224) and $CD4^+CD25^+$ Tregs in the presence of IL-6 are sufficient to support Th17 differentiation in the absence of exogenous TGF-β *in vitro* (223). However, more recent work indicates that Tregs are dispensable as a source of TGF-β for Th17 differentiation; instead, developing Th17 cells themselves provide the relevant TGF-β in an autocrine manner (225). Consistent with this finding, two recent independent studies have shown that $CD25^+$ Tregs promote Th17 responses by sequestering IL-2 (220;226). IL-2-activated STAT5 inhibits IL-17A transcription and binds the same sites across the IL-17A locus as IL-6 (and/or IL-21, IL-23) -activated STAT3 (227;228). Due to the opposing effects of STAT5 and STAT3 (Figure 1.5), relative levels of IL-2 versus IL-6 signaling can directly affect Th17 differentiation (228).

It is generally accepted that Tregs are a stable lineage committed to suppressive function under steady-state conditions (229;230). However, recent studies indicate that a portion of $Foxp3^+$ Tregs can be reprogrammed into Th17 cells in the presence of inflammatory cytokines (231;232). *In vitro* studies have shown that $Foxp3^+$ Tregs

stimulated in the presence of IL-6 or IL-1β may lose Foxp3 expression and up-regulate IL-17A production (233-235). Evidence for Treg to Th17 conversion has also been observed *in vivo* wherein adoptively-transferred $CD4^+Foxp3^+$ Tregs covert into $Foxp3^-IL\text{-}17A^+$ effector cells in response to experimental autoimmune encephalomyelitis (EAE), intestinal inflammation, and cutaneous *Leishmania major* infection (236;237). The stability of Foxp3 expression is linked to epigenetic modifications of the Foxp3 locus, particularly DNA methylation at a conserved noncoding sequence termed the Treg-specific demethylation region (TSDR) (238). nTregs possess a fully demethylated TSDR and exhibit stable Foxp3 expression upon TCR stimulation (239) whereas TGF-β–generated iTregs have a highly methylated TSDR and lose Foxp3 expression when restimulated in the absence of exogenous TGF-β (240). However, IL-6 can destabilize epigenetic modifications at the TSDR in nTregs, downregulating Foxp3 expression (241). As previously discussed, the relative levels of STAT5 and STAT3 activation within $CD4^+$ T cells can directly dictate IL-17A production (227;228). IL-2 is vital for the induction and maintenance of Foxp3 expression in both nTreg and iTregs (242;243) and IL-2 derived from conventional $CD4^+$ T cells *in vivo* may be a critical factor in stabilizing Treg phenotype/ function and resisting Th17 conversion in inflammatory settings (244-247).

The notion of Treg plasticity and/or instability remains controversial. An important factor to consider is that $Foxp3^+$ Tregs are a heterogeneous population and some subpopulations may be more stable than others. Recent work suggests that observed $Foxp3^+$ to $Foxp3^-$ cell conversions result from the instability of peripherally-induced adaptive $Foxp3^+$ iTregs that are not fully committed to the Treg lineage, not the reprogramming of bona fide fully-committed nTregs (248;249). Altogether, $Foxp3^+$ Treg cells are considered predominantly stable, with only a minor fraction become unstable under specialized inflammatory or lymphopenic circumstances (230).

1.3.3.1.5 Current understanding of Tregs in infection

Foxp3$^+$ Tregs are indispensible for controlling the differentiation and expansion of Th subsets as part of their role in maintaining immune homeostasis. Tregs have also been found to modulate antimicrobial immune responses in a broad range of viral, fungal, bacterial and parasitic infection models (250;251). In general, experimental evidence supports a role for Tregs in dampening pathogen-directed immune responses, which can protect the host from immune-mediated pathology but may also favor persistent infection (170;250).

In murine models of mycobacterial infection, Foxp3$^+$ Tregs have been observed to proliferate and accumulate in the lung and draining lymph nodes (252-254). Independent investigations into the role of Tregs have yielded controversial conclusions depending on the model used and the extent to which Tregs are manipulated. Adoptive transfer of a high (1:2) ratio of CD4$^+$CD25$^+$ Tregs to CD4$^+$CD25$^-$ T cells into RAG$^{-/-}$ mice significantly inhibits clearance of *M tuberculosis* infection compared to recipients of CD4+CD25- T cells alone (252). Similarly, conditional depletion of Tregs in a mixed bone marrow chimera model of *M. tuberculosis* resulted in more efficient control of bacterial growth than Treg-replete mice. However, the rigorous Treg depletion in this model caused robust, non-specific immune activation so it is not certain whether Tregs specifically regulate anti-mycobacterial responses (253). Further investigation suggested that *M. tuberculosis* infection expands pathogen-specific Tregs and, upon adoptive transfer, these Tregs potently suppressed the recruitment of effector CD4$^+$ and CD8$^+$ T cells to the lung (254). Yet, other studies suggest that Tregs have a limited impact on the outcome of mycobacterial infection. Mice partially depleted of Tregs via anti-CD25 antibody prior to *M. tuberculosis* infection had improved bacterial clearance during only the early stage of infection and SCID mice reconstituted with CD4$^+$CD25$^+$ T cells with conventional CD4$^+$ T cells at physiologically relevant ratio (1:10) exhibited survival kinetics and clearance similar to recipients of conventional CD4$^+$ T cells alone (255). Consistent with this picture, anti-CD25-antibody-mediated depletion of Tregs prior to *M. bovis BCG* infection or immunization slightly enhanced mycobacteria-specific effector Th1 responses but this was not sufficient to improve bacterial clearance or improve

resistance to *M. bovis* or *M. tuberculosis* challenge (256). However, in a separate study in which Tregs were depleted both before and after immunization, Treg-depleted mice were slightly more protected against *M. tuberculosis* challenge (257). Overall, there is a general trend that Tregs suppress immune activation in response to *M. tuberculosis* and aid the establishment of persistent infection.

In addition to facilitating persistence, Tregs can also limit immunopathology induced by infection. *Helicobacter pylori* can persistently colonize the human stomach for life and, although it can cause chronic gastritis in some individuals, the majority of those infected remain asymptomatic. In mouse models, *H. pylori* infection induces development of adaptive Tregs that protect from immunopathology (258). Experimental depletion or ablation of Tregs in *H. pylori*-infected mice dramatically reduces bacterial burden but generates severe immune-mediated tissue damage (259;260). Human *H. pylori* infection is also associated with increased gastric $Foxp3^+$ Tregs (259) and protection from gastritis is similarly correlated with Treg abundance (261).

While Tregs are generally held to dampen host resistance and limit immune pathology during chronic infections, the role of Tregs in acute infection is less-studied and more controversial (250). A recent study in an oral *Candida albicans* infection model found that Tregs were required for the optimum induction of Th17 responses, which reduced fungal burden and enhanced resistance (226). Tregs have also been associated with host protection during acute *Escherichia coli* infection; Treg depletion resulted increased bacterial burden and high systemic levels of inflammatory cytokines (262), suggesting that lack of Tregs exacerbates inflammatory responses to the bacteria. Similarly, in a model of sepsis induced by cecal ligation and perforation, adoptive transfer of *ex vivo*-activated $CD4^+CD25^+$ Tregs enhanced bacterial clearance and host resistance, presumably by controlling overwhelming inflammation (263). However, some models of acute infection suggest that Tregs can compromise host defense by inhibiting adequate induction of effector immune responses. For instance, Tregs impede the priming of protective $CD8^+$ T cell responses to *Listeria monocytogenes* infection (264). Similarly, decreased resistance to *L. monocytogenes* during pregnancy is

associated with the expansion of maternal Tregs and can be reversed upon the ablation of Tregs (251;265).

1.3.3.2 Regulatory B cells (Bregs)

In addition to Treg subsets, some B cell populations have also been shown to regulate immune responses. The first evidence that B cells may negatively regulate immune responses came in the 1970s from guinea pig studies in which the transfer of total but not B cell-depleted splenocytes inhibited delayed-type hypersensitivity (DTH) reactions (266;267). Subsequently, studies in autoimmune disease models, especially EAE and collagen-induced arthritis (CIA), have demonstrated that IL-10-producing B cells are required for restraining pathogenic T cell responses and suppressing inflammation (268;269). Negative regulation of immune responses by so-called regulatory B (Breg) cells have since been described in multiple models of inflammation, autoimmunity and infection (270). The most widely recognized and extensively studied Breg subsets in mice are marginal zone (MZ) B cells (and their direct precursors) and regulatory B10 cells (270;271).

1.3.3.2.1 Origins of regulatory B cells

In contrast to Treg lineages, Bregs are not observed under steady state conditions *in vivo* and appear to arise out of inflammatory responses (272). Furthermore, there is currently no known conclusive surface marker or master transcription factor that distinguishes Bregs from conventional B cells. Thus, Bregs are defined by their production of IL-10 and ability to suppress immune reactions *in vitro* and/or *in vivo* (270;273). A variety of Breg phenotypes have been described, all of which share markers with previously defined conventional B cell subsets (Table 1.1). B cells have traditionally been categorized into B-1 and B-2 lineages based on their ontogeny: B-1 cells develop early in life from fetal liver and are maintained by a process of self-renewal—although B-1 cells can be reintroduced into irradiated mice from adult bone

marrow (274;275)—whereas B-2 cells are continuously produced by adult bone marrow and include follicular (FO) and marginal zone (MZ) B cell subsets (275). However, the increasing appreciation for the diversity of B cell functions has led to an alternative system for classifying B cells as belonging to the innate or adaptive arm of the immune system (276).

Adaptive B cell immunity is mediated by FO B cells, the most abundant B cell subset in the periphery. FO B cells possess a somatically rearranged, antigen-specific BCR and populate follicles within the spleen and lymph nodes where they interact with $CD4^+$ T cells to generate high affinity antibodies, as previously discussed. Innate-like B (ILB) cells include B-1 and MZ B cells. FO and MZ B cells arise from shared bone marrow precursors that differentiate via transitional B cell stages in the spleen: T1, T2 and T2-MZ precursor (T2-MZ P) B cells (277;278). In the spleen, FO and MZ B cells occupy distinct microenvironments: FO B cells home to follicles in response to a CXCL13 gradient generated by follicular DCs while MZ B cells are retained in the marginal zone of the spleen where they interact with circulating blood-borne antigens exposed on MZ macrophages, DC or neutrophils (278). B-1 cells are the major B cell population in the peritoneal and pleural cavities and a minor B cell population in the spleen (~5%), but are rare (<1% of $CD19^+$ cells) among B cells in the lymph nodes (275). B-1 cells can be divided into two subpopulations based on CD5 expression—$CD5^+$ B-1a and $CD5^-$ B-1b cells—that produce mainly IgM and IgA antibodies, respectively(275).

Whereas the antigen receptors of FO B cells are randomly generated and recognize a highly diverse range of antigens, ILB cells possess germline-encoded polyreactive BCRs that are skewed toward recognizing antigens with certain specificities, such as microbial or self-ligands (275;278;279). Under steady state conditions, the spontaneous release of polyreactive 'natural' IgM by ILB cells is important for neutralizing pathogens, enhancing antibody-dependent complement activation, and promoting adaptive B cell responses by concentrating antigen within B cell follicles (275). Along with BCRs that recognize microbial molecular patterns such as polysaccharides, ILB cells express high levels of TLRs and are poised for rapid activation

in response to microbial products (275;278;279). Upon TLR and/or BCR ligation, B-1 and MZ B cells produce high levels of low affinity polyspecific antibodies as well as cytokines (e.g. IL-6 and/or IL-10) independently of T cells within 1-3 days of encountering pathogens (278). ILB cells provide a frontline of defence against infection during the temporal gap required for the development of adaptive B cell responses.

The origin and development of Bregs from conventional B cell subsets is currently controversial (271;280). While reports of multiple Breg phenotypes may reflect the existence of several distinct Breg subsets, the substantial overlap among the phenotypes reported suggests that Bregs are developmentally related and potentially derived from a common precursor (271;280;281). Based on cumulative data, the major activating signals for Bregs include TLR ligands, CD40-CD40L interactions and BCR stimulation (270). It has been proposed that Bregs can be subdivided into 'innate-type' and 'adaptive-type' Bregs according to their mode of activation: innate-type Bregs are activated by TLR signaling alone whereas adaptive-type Bregs require additional signals via the CD40-CD40L pathway and/or BCR stimulation (272;273).

1.3.3.2.2 Innate-type regulatory B cells

Innate-type Bregs are essentially TLR-activated ILB cells that exert suppressive function (273). ILB cells are uniquely poised to produce large amounts of IL-10 in response to microbial stimulation. Whereas TLR2 or TLR4 ligation promote FO B cells to produce IFN-γ and IL-6, TLR2 or TLR4 stimulation triggers MZ B cells to produce high levels of IL-10 and some IL-6 (282). Furthermore, peritoneal B-1 cells are known to be a major source of IL-10 produced in response to TLR4 agonist LPS *in vivo* (283). LPS-activated B cells from male mice efficiently induce tolerance to male skin transplants in female mice (284). In addition, CpG-stimulated B cells have been shown to ameliorate late-phase experimental allergic conjunctivitis (285) and can inhibit Th1 responses in neonatal mice (286).

1.3.3.2.3 Adaptive-type regulatory B cells

In addition to TLR stimulation, the generation and function of Bregs in some settings has been shown to require signals from the adaptive immune system (272;273). The best evidence that TLR stimulation alone does not empower Bregs with suppressive function comes from experiments using chimeric mice within the EAE model (287). EAE is induced by immunization of myelin oligodendrocyte glycoprotein peptide (MOG35-55) together with killed *M. tuberculosis*, which serves as an agonist for TLR2 and TLR4. Mice with B cell-restricted deficiency in TLR2, TLR4 or MyD88 have impaired B cell-derived IL-10 production, develop exacerbated Th1 and Th17 responses and fail to resolve disease (287;288). However, even with B cell-intrinsic TLR signaling intact, mice that have B cell-restricted CD40-deficiency or that express an irrelevant BCR also develop severe chronic EAE (287).

CD40 engagement can increase production of IL-10 by TLR-activated B cells (282;288). CD40L signals may be provided by interactions with cognate $CD4^+$ T cells and the intensity of these signals are likely correlated with the number of antigen-reactive T cells available (270). This suggest that optimal IL-10 production requires interactions with CD40L expressed on antigen-activated $CD4^+$ T cells that, along with BCR stimulation, typically occurs in an antigen-specific manner during the course of an adaptive immune response. Notably, CD40 signals can also play a role in T cell-independent B cell activation. Anti-CD40 agonist mAbs alone are sufficient to induce IL-10 production from immature T2-MZP B cells (289) and T2-MZP B cells from lupus-prone (MRL/*lpr*) mice secrete high levels of IL-10 in response to *ex vivo* CD40 stimulation, independently of BCR signaling (290). Moreover, regulatory DCs can induce B1, MZ, and MZ precursor B cells to differentiate into IL-10-producing Bregs via production of IFN-β and CD40-CD40L (291).

Several lines of evidence support that BCR signaling also plays an important role in the generation of Bregs. BCR and CD40 signals induce B cell proliferation and survival through several common molecules so there may be some redundancy or synergy between the pathways (292;293). In a model of CIA, splenic B cells from

arthritic mice were shown to produce IL-10 upon stimulation *ex vivo* with both antigen (i.e. collagen II) and anti-CD40 mAbs (294). Upon adoptive transfer, these cells prevent as well as ameliorate CIA by inhibiting Th1 responses via IL-10-dependent mechanisms (294). Regulation of inflammation and autoimmunity by B10 Bregs appears to require activation by specific antigen and B10 cells can produce polyreactive and self-reactive antibodies, suggesting that self-antigens might trigger early B10 cell development (295-298). Consistent with this notion, B10 numbers are ~90% reduced in transgenic mice that have fixed BCR specificity (299) and are almost doubled in mice that overexpress CD19, a B cell co-receptor that enhances signaling (296). Furthermore, mice with defective BCR signaling develop exacerbated EAE corresponding with a profound loss of IL-10 production by B cells (300). The expansion and regulatory function of B10 cells *in vivo* is inhibited by lack of B cell-expressed IL-21R, MHC class II and CD40 (298;301), indicating that B10 cells require cognate interactions with $CD4^+$ T cells (302). Indeed, agonistic CD40 treatment of B cells *in vitro* promotes B10 maturation.

Agonists of TLR4 and TLR9 have been shown to promote IL-10 production by B10 cells (295). However, because MyD88-deficient mice are reported to develop normal B10 cell numbers, TLR agonists are proposed to act as polyclonal mitogens following the initial antigen encounter (298;299). It is likely that both TLR and BCR signaling are required for optimum B10 cell activation. Interestingly, apoptotic cell products can induce IL-10 production from B cells and may represent a stimulus for B10 development through combined self-reactive BCR and TLR signals (295;303).

In light of the more complex signaling requirements for adaptive Bregs, the generation of B10 and MZ-like Breg subsets has been proposed to occur in distinct two-phase models (Figure 1.6). Tedder *et al.* propose that B10 development begins with B10 progenitor (B10pro) cells that exist within phenotypically defined B-1, MZ and T2-MZP subsets. B10pro cells are then selected on the basis of affinity for self-antigen (Figure 1.6a) and, upon exposure to TLR ligands and/or CD40 stimulation, these B10pro cells mature into IL-10-producing B10 cells, which are enriched in $CD1d^{hi}CD5^+$ B cell population (271;295). Alternatively, Mauri *et al.* assert that T2-MZP B cells are the

most likely common precursor for Bregs (270). In the first phase of their model (Figure 1.6b), initial activation of naïve T2-MZP B cells via TLRs releases a first wave of IL-10 production. Subsequently, these B cells receive signals via BCR and/or CD40 for complete activation, which promotes proliferation and survival as well as stabilizes IL-10 production (270). Once in the periphery, Mauri *et al.* suggest the Bregs can express a wide range of phenotypes depending on the type, kinetics and/or magnitude of their regulatory function (270). While considerable progress has been made in our understanding of Breg biology in recent years, the origins and development of Bregs remains an area of ongoing debate.

1.3.3.2.4 Mechanisms of suppression

The predominant mechanism through which Bregs regulate immune responses is via production of IL-10 (270;295). IL-10 produced by B cells has been shown to suppress Th1 and Th17 responses and also inhibit TNF-α production from monocytes (270). B cell-derived IL-10 can indirectly promote suppression by converting conventional $CD4^+$ T cells into IL-10-producing Tr1 cells (282;290) or by serving as a source of IL-10 required to maintain endogenous pools of Tregs (304).

Notably, several IL-10-independent mechanisms of Bregs-mediated suppression have also been described. For instance, LPS-activated B cells have been shown to suppress Th1 immunity and prevent diabetes in non-obese diabetic mice via TGF-β production as well as expression of express Fas ligand (FasL), which can induce $CD4^+$ T cell apoptosis (305). In a recent study, B cells were described to suppress antitumor immunity by maintaining nTreg homeostasis via GITR ligand expression, independent of IL-10 production (306). Similarly, B cells activated via TLR2 can induce Tr1 cell differentiation via IL-10-independent, contact-dependent mechanisms (307).

1.3.3.2.5 Current understanding of Bregs in infection

Like Tregs, Bregs have been demonstrated to play an important role in controlling immune-mediated tissue damage in mouse models of autoimmune disease such as inflammatory bowel disease (IBD), EAE and CIA. A role for Bregs in controlling effector responses to infectious disease has also been observed in multiplemodels. In many cases, the discovery of counter-regulatory B cell function stems from the observation that resistance to a particular pathogen is enhanced in B cell-deficient mice.

B cells can suppress effector immune responses during acute systemic bacterial infection. MZ B cells are one of the first cells in contact with bloodborne pathogens and are well-equipped to mount the first line of host defenses. MZ B cells produce large amounts of IL-10 via TLR2 and TLR4 ligation in response to *Listeria monocytogenes in vitro* (308) and this MZ B cell-derived IL-10 plays a detrimental role in *L. monocytogenes* infection *in vivo* by suppressing protective host TNF-α and IFN-γ production, increasing host susceptibility (308). More recently, B10 were also found to regulate innate responses to *L. monocytogenes* infection. B10 cells preferentially expand upon infection and dampen macrophage activation via IL-10 production, inhibiting bacterial clearance (309). *Salmonella typhimurium* can also directly stimulate high levels of IL-10 production by B cells via TLR2 and TLR4 *in vitro*. Systemic *S. typhimurium* infection potently induces a population of IL-10-producing $CD138^{+}$ plasma-like B cells within 1 day post-infection (310). These B cells exert IL-10-dependent suppression of neutrophil, NK cell and Th1 responses. The rapidness of Breg responses suggests they are derived from innate-like B cells and the authors of this study speculate that splenic B-1a or MZ B cells may be the precursors of IL-10-expressing Bregs induced by *S. typhimurium* (311). B cells from C57BL/6 mice produce IL-10 in response to *Brucella abortus*, which dampens pro-inflammatory Th1 responses responsible for clearing the infection (312). IL-10 produced by B-1 cells is also implicated in dampening IFN-γ-producing NK/NKT cell responses to *Francisella tularensis* infection, impairing resistance (313). Collectively, IL-10 produced by B cells during acute responses to bacterial infection has a generally deleterious role by inhibiting protective CMI responses.

Bregs are well-known to limit immunopathology in models of autoimmune disease and there is some evidence that they also perform this function during chronic infection. During chronic infection by *H. felis*, a close relative of *H. pylori*, B cells restrain severe gastric pathology by suppressing excessive Th1-mediated responses (307). The regulatory function of B cells in this model requires B cell-intrinsic TLR2 and MyD88 signaling. However, while these signals induced B cells to produce IL-10 *in vitro*, IL-10^+ B cells did not appear in the gastric mucosa *in vivo* and B cell-derived IL-10 was dispensable in suppressing Th1-mediated immunopathology. Rather, B cells regulated excessive Th1 responses indirectly by efficiently inducing IL-10-producing Tr-1 cells via contact-dependent mechanisms (307).

1.3.3.3 Evidence for immune regulation during *Chlamydia* infection

The Th1/Th2 paradigm has long been proposed as a regulatory mechanism underlying different susceptibilities among mouse strains. However, while our understanding of regulatory immune cells and their role in infectious diseases increases, the role of these cells in host responses to *Chlamydia* remains undefined. Genetic studies in humans have linked susceptibility to trachoma and *Chlamydia*-induced tubal factor infertility with polymorphisms that generate high IL-10-producing phenotypes (314). IL-10 has a well-documented immunosuppressive role in experimental infections models (315) and it has been suggested that IL-10-mediated suppression of Th1 responses contributes to bacterial persistence and chronic *Chlamydia* infection (316). However, a more recent study has also linked low IL-10-producing alleles to enhanced risk of *Chlamydia*-induced TFI (317), indicating that an appropriate balance of IL-10 is required to protect from infection-induced immunopathology. Thus, IL-10 may also play an important role in resolving inflammation in the infection site. Although IL-10 was initially described as a Th2 cytokine, it is now known to be produced by other T cell subsets including Tregs, as well as macrophages, DCs, NK cells and B cells (318). The relevant source of counter-regulatory IL-10 produced by the host in response to *Chlamydia* infection has not been clearly identified.

Chlamydia infection has been reported to induce Foxp3$^+$ Treg expansion in both mice and humans. Foxp3$^+$ cells accumulate in the conjunctiva of patients with ocular *C. trachomatis* infection and increased levels of Foxp3 transcripts correspond with the highest phase of clinical infection (319). More recently, *in vitro* stimulation of PBMCs from patients with trachoma and healthy controls with chlamydial EB induced Foxp3$^+$ Treg expansion, although Treg expansion was not significantly associated with disease (320). To date, the role of Tregs in human GT infections has not been examined. However, mouse models of *Chlamydia* GT infection indicate that Foxp3$^+$ cells expand in the genital mucosa and draining lymph nodes in response to infection (321-323). *Chlamydia*-infected mice deficient in ICOS (inducible costimulatory molecule) display reduced Treg numbers that correspond with enhanced Th1 responses and accelerated bacterial clearance, but also more severe oviduct immunopathology (321). This correlation suggests that Foxp3$^+$ Tregs may dampen protective immune responses while preventing damaging inflammation.

There is some evidence for the involvement B cells with regulatory functions in models of infection by *Chlamydia* species related to *C. trachomatis*. Regulatory B cell subsets including CD19$^+$CD43$^-$CD1d^{hi} B cells have been described as being present in the spleens of *C. pneumonia*-infected mice but their role is uncharacterized (324). B cells have been found to play an essential role in moderating inflammation during intraperitoneal infection with *C. abortus*, potentially via TGF-β production (325;326). As previously discussed, B cells are considered to play a minor role in primary responses to *Chlamydia* GT infection and potential regulatory roles for B cells have not been investigated. However, a splenic ILB cell population consistent with MZ-like B cells has been shown to potently produce IL-10 in response to a clinical *C. trachomatis* GT serovar *in vitro* (327) but the role of these cells during *in vivo* responses is unclear.

1.4 Hypotheses, Objectives and Rationale

The immune response to *Chlamydia* infection has the potential to be both protective and severely damaging to host tissue. Regulatory mechanisms can play a

crucial role in determining the strength of the immune response to infection, either to the benefit or detriment of the host and their precise role is highly dependent on the nature of the pathogen and infection. The overarching goal of this project is to understand the role of immune regulatory mechanisms in shaping protective versus pathological immune responses during *Chlamydia* GT infection using a mouse model. Specifically, we have chosen to examine the roles of two prominent regulatory lymphocyte populations—regulatory T cells (Tregs) and regulatory B cells (Bregs)—that have been shown to play instrumental roles in other models of bacterial infection but whose role in *Chlamydia* infection is unclear.

1.4.1 Hypothesis 1

Endogenous Treg populations are a critical modulator of host responses to *Chlamydia* GT infection through divergent effects on $CD4^+$ T cell lineage differentiation—specifically by inhibiting protective Th1 responses while promoting pathological Th17 responses.

1.4.1.1 Objective

Our first objective is to determine the role of Tregs with respect to Th1/Th17 lineage differentiation in response to *C. muridarum in vitro* and *in vivo* and examine the impact of Treg depletion on bacterial clearance and oviduct pathology during *C. muridarum* GT infection.

1.4.1.2 Rationale

Previous work has determined that nTregs are sufficient to induce the *de novo* differentiation of Th17 cells and also convert into IL-17A-producing cells themselves in the presence of IL-6 *in vitro*. This pathway has yet to be studied in the context of *Chlamydia*-stimulated antigen-presenting cells (APCs) so I will first establish an *in vitro*

system to examine the direct relationship between various levels of nTregs and Th1/Th17 differentiation. We will next determine whether endogenous nTreg levels within the host modulate Th1 and Th17 responses and, furthermore, whether relative Th1/Th17 responses correlate with susceptibility to *Chlamydia* infection and associated GT pathology. In this regard, nTreg activity represents a potential mechanism for establishing persistent infection and augmenting inflammatory pathology *in vivo*.

1.4.2 Hypothesis 2

Bregs are an important source of IL-10 responsible for negatively regulating protective Th1 responses during *Chlamydia* infection.

1.4.2.1 Objective

Our second objective is to determine whether B cells are a relevant source of counter-regulatory IL-10 produced in response to *Chlamydia* and, if so, determine the impact of B cell-derived IL-10 on conventional $CD4^+$ T cell responses *in vitro* and *in vivo* and examine the role of IL-10-producing B cells in bacterial clearance and oviduct pathology during *Chlamydia* GT infection.

1.4.2.2 Rationale

IL-10 clearly has a counter-regulatory role during *Chlamydia* infection but the relevant cellular sources of endogenous IL-10 within *Chlamydia*-infected hosts remain to be elucidated. An increasing body of evidence indicates that IL-10-producing B cells critically regulate host responses to a range of pathogens, including intracellular bacteria. Given that murine B cells have been shown to produce IL-10 in response to *C. trachomatis* stimulation *in vitro,* we anticipate that B cells may be an unappreciated regulator of host Th1 responses to *Chlamydia* GT infection. Breg-mediated suppression of effector immune responses represents a possible mechanism for establishing persistent

infection and chronic inflammation; however, IL-10-producing Bregs could potentially limit the extent of immunopathology in the mucosal infection site.

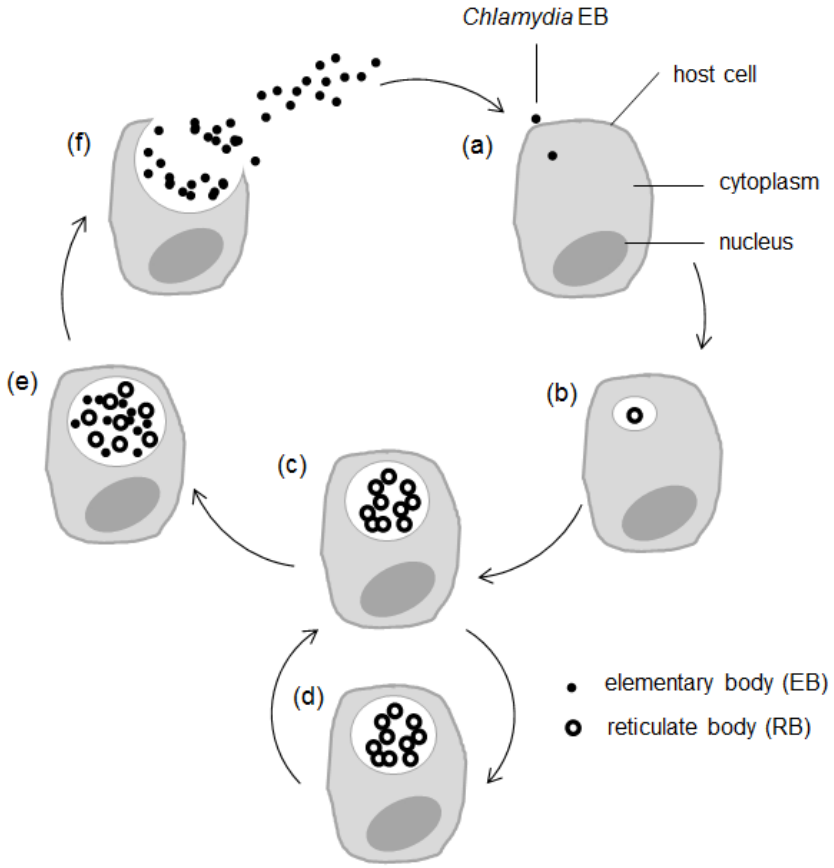


Figure 1.1 Schematic of *Chlamydia* life cycle within host epithelial cells.

Chlamydia species have a biphasic life cycle that consists of two morphologically distinct forms of the bacterium—the small (~0.2-0.3μm) infectious elementary body (EB) and the larger (~0.8μm) metabolically-active reticulate body (RB)—and occurs as follows: (a) *Chlamydia* elementary bodies (EBs) attach to epithelial cells and enter via receptor-mediated endocytosis; (b) within ~2 h post-infection, EBs transform into metabolically-active reticulate bodies (RBs) within host-derived membrane inclusion; (c) RBs divide by binary fission; (d) in the presence of certain stimuli (e.g. IFN-γ, penicillin, amino acid or iron deprivation), *Chlamydia* can enter a persistent state in which RB division halts until the stimulus is removed; (e) RBs revert back into EBs and (f) infectious EBs are released from the cell by spontaneous lysis or apoptosis of infected cell or extrusion of inclusions (1;6).

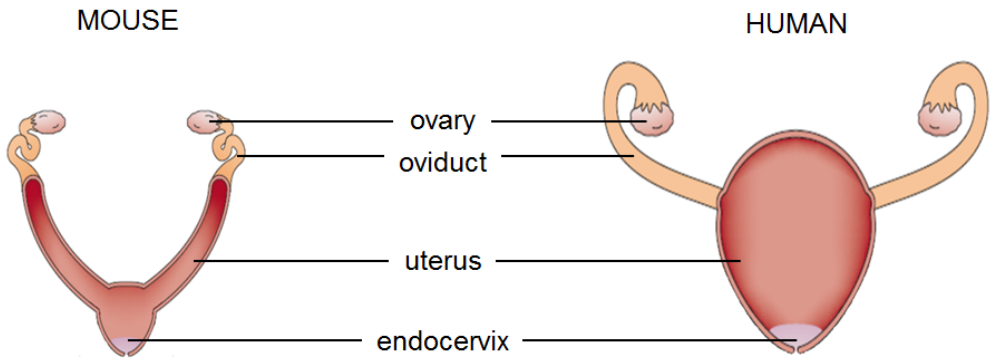
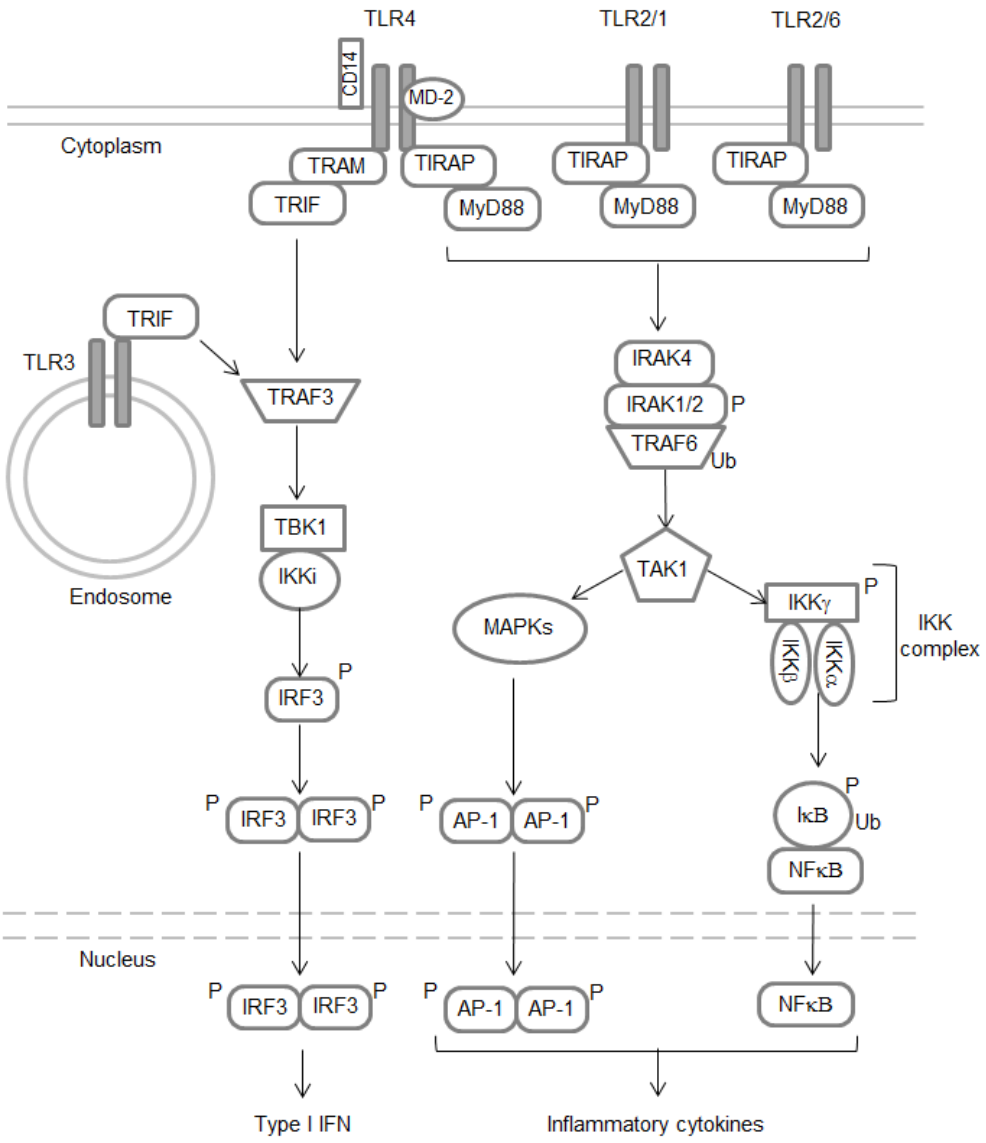


Figure 1.2 Comparative gross anatomy of female upper genital tract from mouse and human.

In the female mouse (left), the uterus consists of two lateral horns that join into a single body (corpus) whereas human females (right) have an inverted pear-shaped uterus. In the mouse, the oviduct is a long narrow coiled tube that directly connects the ovary and uterine horn; in humans the oviduct is relatively uncoiled and is open to the intra-abdominal cavity near the ovary (9;328;329). Modified from (329).

Figure 1.3 MyD88-dependent and TRIF-dependent Toll-like receptor (TLR) signaling pathways.

Upon ligand binding, TLRs form homo- or heterodimers and recruit one or more Toll/IL-1 receptor (TIR)-domain-containing adaptor proteins—MyD88, TIRAP, TRIF, or TRAM—to the cytoplasmic domains of the receptors. TIRAP acts as a bridge to recruit MyD88 to TLR2 and TLR4 signaling, whereas TRIF is used in TLR3 signaling and, in association with TRAM, in TLR4 signaling. In the MyD88-dependent pathway, MyD88 associates with IRAK family members IRAK4, IRAK1 and/or IRAK2. IRAK4 phosphorylates IRAK1 and/or IRAK2 promoting their association with TRAF6, which serves as a platform to recruit and activate TAK1. Activated TAK1 then activates the IKK complex (IKKα, IKKβ, and IKKγ), which in turn catalyzes the phosphorylation and subsequent degradation of inhibitor of κB (IκB). IκB degradation allows NF-κB to translocate to the nucleus. At the same time, TAK1 also activates the MAPK pathway, which results in the phosphorylation and activation of AP-1. NF-κB and AP-1 control inflammatory responses by inducing the expression of numerous inflammatory cytokine genes. In the TRIF-dependent pathway, TRIF recruits TRAF3 to activate TBK1 and IKKi, which mediate the phosphorylation and dimerization of IRF3. Activated IRF3 then translocates to the nucleus to induce the transcription of type I IFN, particularly IFN-β (39;330). Modified from (331).


TLR4
TLR2/1
TLR2/6
CD14
MD-2
Cytoplasm
TRAM
TIRAP
TIRAP
TIRAP
TRIF
MyD88
MyD88
MyD88
TRIF
TLR3
TRAF3
IRAK4
IRAK1/2
P
TRAF6
Ub
TBK1
IKKi
TAK1
MAPKs
IKKγ
P
IKK
complex
IKKβ
IKKα
Endosome
P
IRF3
P
IRF3
IRF3
P
P
AP-1
AP-1
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IκB
Ub
NFκB
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NFκB
Type I IFN
Inflammatory cytokines

Figure 1.3

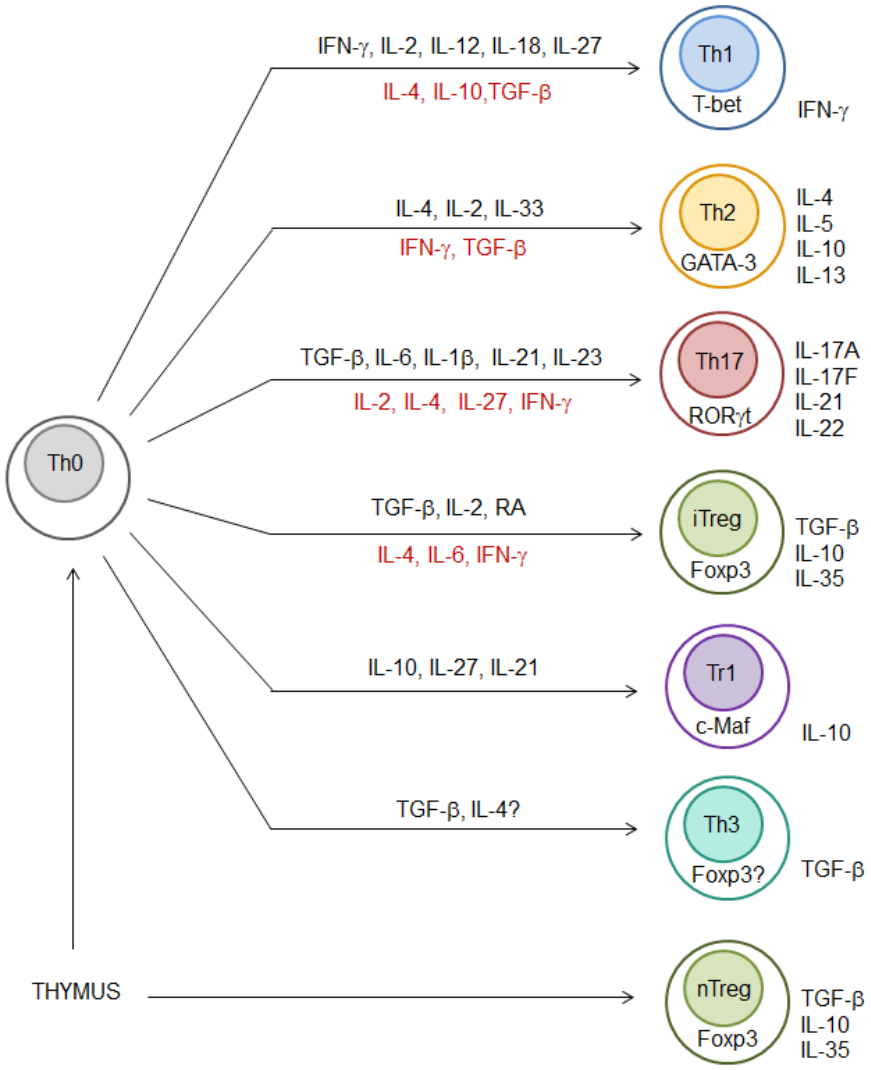

Figure 1.4 Cytokine signals regulate the differentiation of Th1, Th2 and Th17 and regulatory T cell lineages from conventional CD4$^+$ T cells.

Following maturation in the thymus, conventional CD4$^+$ T cells and natural CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (nTregs) exit and enter into the periphery. Upon activation, conventional CD4$^+$ T cells (Th0) are polarized into distinct effector T helper (Th)—Th1, Th2, Th17—or adaptive regulatory T cell lineages—iTreg, Tr1, Th3—by cytokines in the local environment (99;332-334). Cytokines that positively regulate the differentiation of a particular lineage are shown in black (on top of arrow) whereas cytokines that suppress differentiation are shown in red (underneath arrow). Fully differentiated lineages express a characteristic transcription factor and cytokine repertoire.

Figure 1.5 Role of cytokines in regulating transcription factor activity during Th1, Th2, Th17 and iTreg differentiation.

Signalling through TCR/CD28 triggers the nuclear accumulation of shared, activation-dependent transcription factors (e.g. NFAT, NFκB and AP-1), IL-2Rα (CD25) expression and autocrine IL-2 signaling (102). $CD4^+$ T cell lineage fate is coordinated by cytokine receptor signals that mediate gene activating and silencing events at both the epigenetic and transcription factor (TF) level. Th1 polarization is initiated by IFN-γ-mediated STAT1 activation leading to expression of T-bet and cofactors HLX and RUNX3, which activate IFN-γ transcription, and IL-12Rβ2. IL-12 activates STAT4, which further promotes IL-12Rβ2 and T-bet expression (99;102). Th2 polarization is initiated by IL-4-mediated STAT6 activation that induces GATA-3. STAT6 and GATA-3 bind the Th2 cytokine locus containing genes for IL-4, IL-5 and IL-13 (99;102). Th17 polarization is initiated by IL-6 in the context of TGF-β. IL-6-activated STAT3 induces autocrine IL-21 signaling resulting in further STAT3 activation and expression of RORγt, leading to IL-17A transcription (105). Binding of Runx1 and RORγt further enhances IL-17A gene expression (106). The differentiation of iTregs requires TGF-β and IL-2 signaling. TGF-β activates Smad2/3 which, together with IL-2-activated STAT5, promotes Foxp3 expression (188;190). IL-2-activated STAT5 also promotes expression of IFN-γ or IL-4 in Th1 and Th2 cells, respectively, but inhibits IL-17A production in Th17 cells(100).

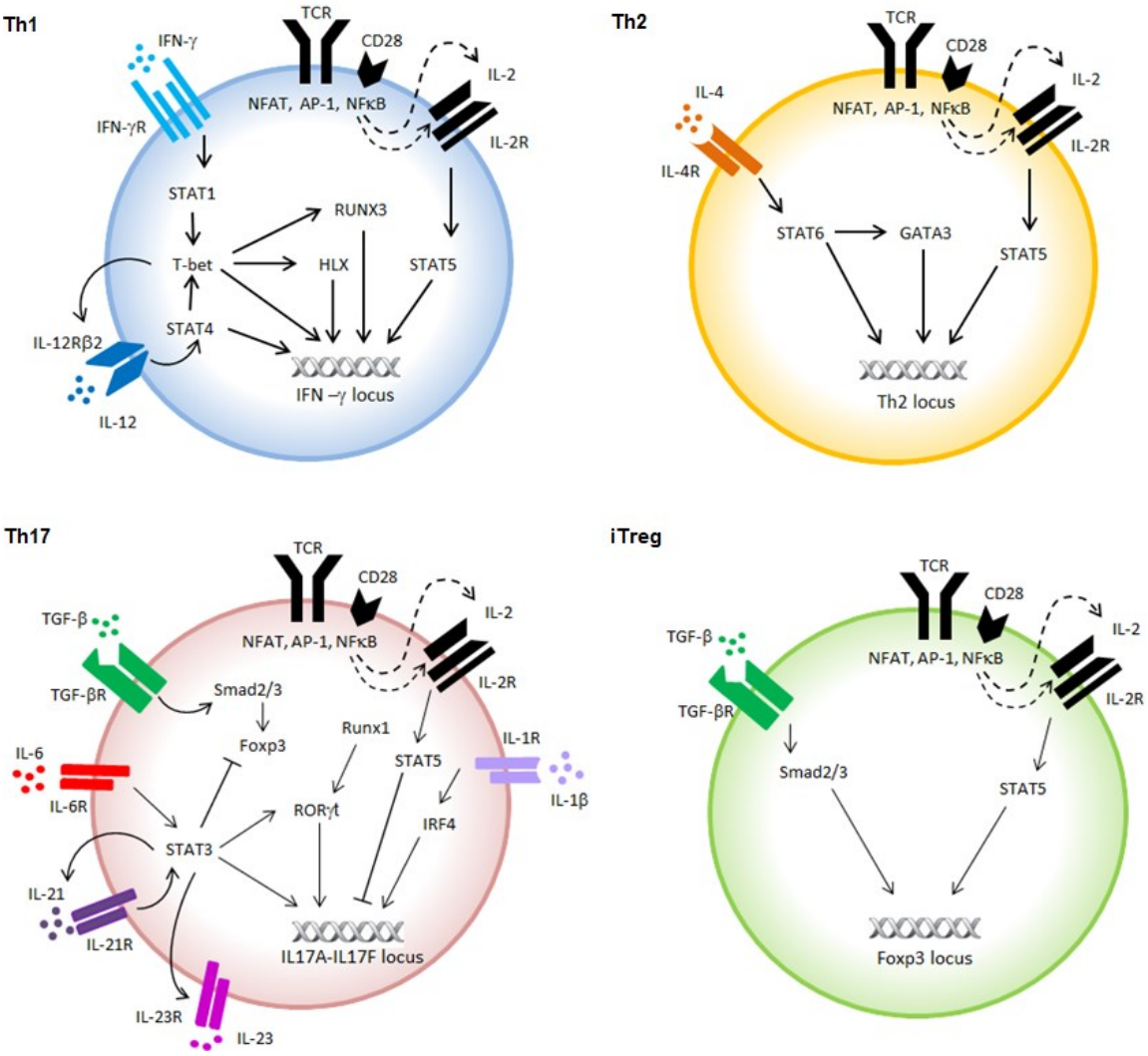
Th1
TCR
CD28
IFN-γ
IL-2
NFAT, AP-1, NFκB
IFN-γR
IL-2R
STAT1
RUNX3
T-bet
HLX
STAT5
STAT4
IL-12Rβ2
IFN –γ locus
IL-12
Th2
TCR
CD28
IL-2
IL-4
NFAT, AP-1, NFκB
IL-2R
IL-4R
STAT6
GATA3
STAT5
Th2 locus
Th17
TCR
CD28
TGF-β
IL-2
NFAT, AP-1, NFκB
IL-2R
TGF-βR
Smad2/3
Foxp3
Runx1
IL-1R
IL-6
STAT5
IL-6R
RORγt
IL-1β
IRF4
STAT3
IL-21
IL-21R
IL17A-IL17F locus
IL-23R
IL-23
iTreg
TCR
CD28
TGF-β
IL-2
NFAT, AP-1, NFκB
IL-2R
TGF-βR
Smad2/3
STAT5
Foxp3 locus

Figure 1.5

Table 1.1 Surface phenotype of conventional B cells subsets in mice

B cell subset		Surface phenotype
B-1	PerC B1a	$IgM^{hi}IgD^{lo}CD43^{+}CD23^{-}CD21^{-}CD1d^{mid}CD11b^{+}CD5^{+}$
	PerC B1b	$IgM^{hi}IgD^{lo}CD43^{+}CD23^{-}CD21^{-}CD1d^{mid}CD11b^{+}CD5^{-}$
	Splenic B1a	$IgM^{hi}IgD^{lo}CD43^{+}CD23^{-}CD21^{-}CD1d^{mid}CD11b^{-}CD5^{+}$
	Splenic B-1b	$IgM^{hi}IgD^{lo}CD43^{+}CD23^{-}CD21^{-}CD1d^{mid}CD11b^{-}CD5^{-}$
B-2	T1	$IgM^{hi}IgD^{lo}CD43^{-}CD23^{-}CD21^{lo}CD1d^{lo}CD11b^{-}$
	T2	$IgM^{hi}IgD^{hi}CD43^{-}CD23^{+}CD21^{mid}CD1d^{lo}CD11b^{-}$
	T2-MZ P	$IgM^{hi}IgD^{hi}CD43^{-}CD23^{+}CD21^{hi}CD1d^{lhi}CD11b^{-}$
	Marginal zone (MZ)	$IgM^{hi}IgD^{lo}CD43^{-}CD23^{-}CD21^{hi}CD1d^{hi}CD11b^{-}$
	Follicular (FO)	$IgM^{lo}IgD^{hi}CD43^{-}CD23^{+}CD21^{mid}CD1d^{mid}CD11b^{-}$

Modified from (275;335).

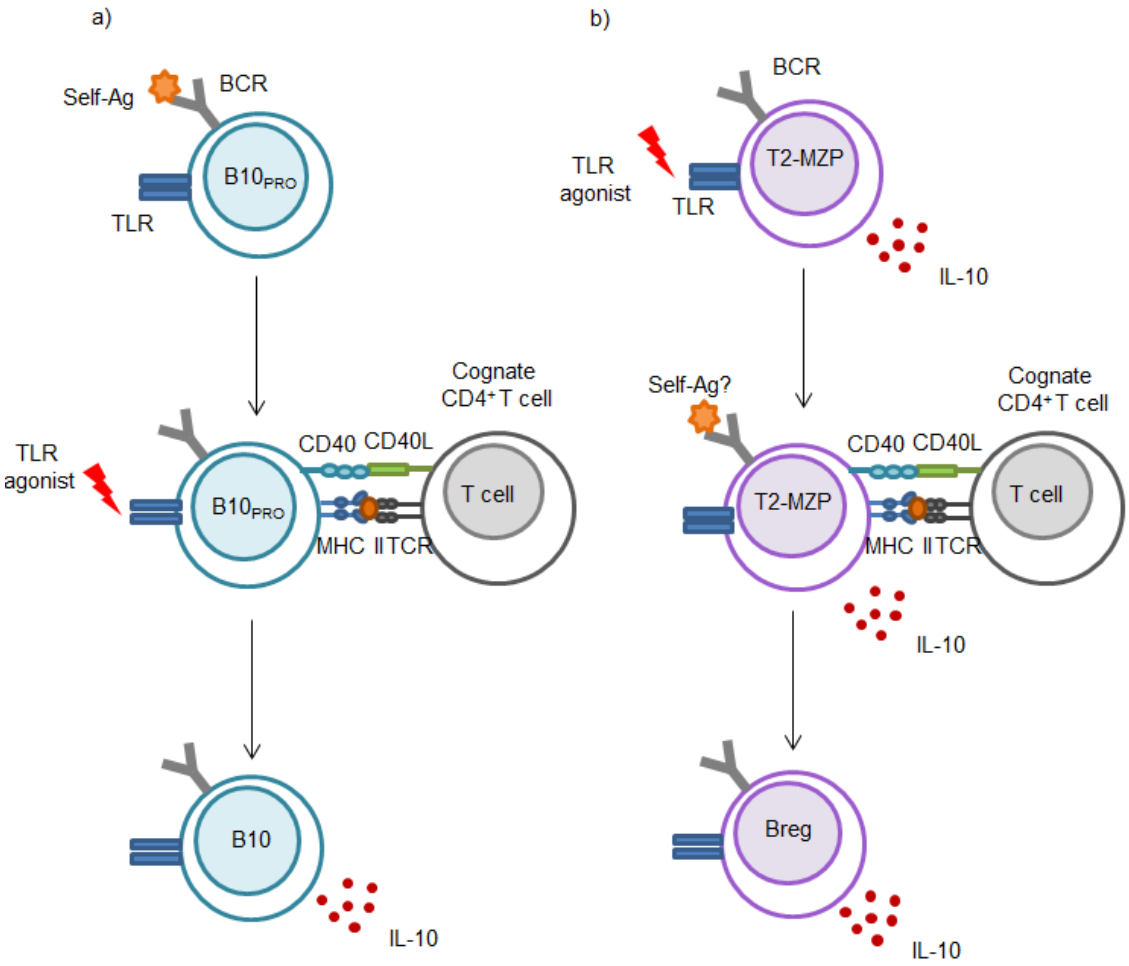

Figure 1.6 Two-step models of regulatory B cell generation.

The generation of B10 and T2-MZP Breg subsets has been proposed to occur in distinct two-phase models. (a) In the model proposed by Tedder *et al.* B10 progenitor (B10pro) cells are first selected on the basis of affinity for self-antigen (271). Upon exposure to TLR ligands and/or CD40 stimulation, B10pro cells mature into IL-10-producing B10 cells enriched within a $CD1d^{hi}CD5^{+}$ B cell population (271;295). (b) In the model proposed by Mauri *et al.*, T2-MZP B cells are a likely common precursor for adaptive Bregs (270). Initial activation of naïve B cells via TLRs releases a first wave of IL-10 production. In a second phase, B cells receive signals via BCR and/or CD40 for complete activation, which promotes proliferation and survival as well as stabilizes IL-10 production (270). Modified from (270;271;295).

CHAPTER 2 MATERIALS AND METHODS

2.1 Mice

Mice were normally used between 6 to 10 weeks of age. C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Congenic C57BL/6 CD45.1 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). MyD88-deficient mice were obtained from Dr. Jean Marshall (Dalhousie University, Nova Scotia) with the permission from Dr. Shizuo Akira (Osaka University, Japan). OTII TCR transgenic mice were also obtained from Dr. Jean Marshall. IL-10-deficient mice were obtained from Dr. Andrew Stadnyk (Dalhousie University, Nova Scotia). B cell-deficient (μMT) mice and IL-6-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

FoxP3-IRES-eGFP knock-in (Foxp3-GFP) mice expressing a functional chimeric GFP protein in cells with active Foxp3 transcription (104) were kindly provided by Dr. Mohamed Oukka (Harvard Medical School, MA, USA). To confirm the genotype of Foxp3-GFP mice used in our studies, tissue samples (ear punches) were collected and processed for genotyping by polymerase chain reaction (PCR) using a "REDExtract-N-Amp™ Tissue PCR Kit" (Sigma, Oakville, Ontario) as per manufacturer's instructions. All primers were synthesized by Integrated DNA Technologies, Inc. (Montreal, Quebec). The following primers were used: knock-in forward primer 5'-ACG CCC CAA CAA GTG CTC CAA-3', knock-in reverse primer 5'-GTG TGA GTC AGT AGG ACT GCA-3', and internal ribosome entry site primer (IRES) 5'-ACC CCT AGG AAT GCT CGT CAA G-3'. PCR products were analyzed by electrophoresis (APPENDIX A1a). Additionally, Foxp3-GFP genotype was confirmed by phenotypic analysis of Foxp3-GFP-expressing $CD4^+$ T cells among peripheral blood mononuclear cells (PBMCs) by flow cytometry (APPENDIX A1b).

IL-10-IRES-eGFP knock-in (IL-10GFP or *tiger* (interleukin-ten ires gfp-enhanced reporter) (336)) mice were obtained from Dr. Richard Flavell (Yale University, CT,

USA). In these mice, an IRES-eGFP construct follows the genomic coding sequence of the IL-10 gene, resulting in cytoplasmic GFP expression during IL-10 gene transcription (336). To confirm the genotype IL-10GFP mice used in our studies, tissue samples were collected and prepared as described in the previous section. For IL-10GFP genotyping, the following primers were used: forward primer 5'-GTG TGT ATG AGT CTG CTG GAC-3', reverse primer 1 5'-GTG TGG CCA GCC TTA GAA TAG-3', and reverse primer 2 5'-GGTTGCCTGACCATCGATG-3'. PCR products were analyzed by electrophoresis (APPENDIX A2).

Mice were housed at the Izaak Walton Killam Health Centre (IWK) animal facility under pathogen-free conditions. All animal procedures were approved by the Ethics Committee according to the Canadian Council for Animal Care guidelines.

2.2 Tissue sample processing

Mouse tissues collected during sacrifice were normally placed in sterile Hanks balanced salt solution (HBSS) (Invitrogen, Oakville, Ontario) or 1X phosphate-buffered saline (PBS) and kept on ice until processing. Cell isolation were performed in RPMI-1640 media (Invitrogen, Oakville, Ontario) supplemented with 5% heat-inactivated bovine serum (BS) (Fisher Scientific, Ottawa, Ontario), i.e. 5% BS/RPMI. For spleen cell isolation, spleens were placed in a 100-mm Petri dish containing 5 ml of 5% BS/RPMI and mechanically disrupted using sterile frosted-end glass slides. An additional 10 ml of media was used to rinse slides and samples were centrifuged at 1200 rpm for 10 min at 4^{o}C. The cell pellet was resuspended and red blood cells were lysed by incubation in 4 ml of ammonium chloride potassium (ACK) buffer (0.1 mM EDTA, 0.15 M NH_4Cl, 1 mM $KHCO_3$ prepared from chemicals purchased from BioShop (Burlington, Ontario)) for 5 min at room temperature followed by addition of 5-10 ml 5% BS/RPMI. Samples were centrifuged for 10 min and resuspended in 10 ml 5% BS/RPMI and gently pipetting through 40-µm cell strainers (BD Falcon™, BD Biosciences, Mississauga, Ontario) to remove tissue debris. The concentration of cells in single-cell suspension was determined by diluting sample in 0.4% Trypan Blue Solution (Life Technologies,

Burlington, Ontario) and counting by trypan blue-dye exclusion method using a hematocytometer. Once cell number was determined, cells were finally resuspended at a fixed concentration in RPMI-1640 (Invitrogen, Oakville, Ontario) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (P/S) (all purchased from Fisher Scientific, Ottawa, Ontario), i.e. 10% FBS, 1% L-glutamine, 1% P/S RPMI medium. For lymph node cell isolation, lymph nodes were placed in a 60-mm Petri dish containing 1 ml of 5% BS/RPMI and mechanically disrupted using sterile frosted-end glass slides. An additional 2 ml of media was used to rinse slides and then samples were gently pipetted through 40-μm strainers to remove tissue debris, after which cell number was determined by trypan blue-dye exclusion method using a hematocytometer. Samples were then centrifuged at 1200 rpm for 10 min at 4^{o}C and pellet was resuspended at a fixed concentration in 10% FBS, 1% L-glutamine, 1% P/S, RPMI medium. To process GT tissue, the uterus and upper portion of the cervix was excised and then minced with sterile surgical scissors in 500 μl HBSS containing 2 mg/ml collagenase II (Life Technologies, Burlington, Ontario) and incubated at 37^{o}C for 20 min. The suspension was then passed through a 40-μm cell strainer using the end of a sterile 3 ml syringe plunger in a 100-mm Petri dish containing 5 ml 5% BS/RPMI. Samples were centrifuged at 1200 rpm for 10 min at 4^{o}C. The pellet was resuspended and red blood cells were lysed by incubation in 4 ml ACK buffer for 5 min at room temperature followed by addition of 5-10 ml 5% BS/RPMI. Samples were centrifuged for 10 min and resuspended in 5% BS/RPMI, after which cell number was determined by trypan blue-dye exclusion method using a hematocytometer. Samples were then centrifuged at 1200 rpm for 10 min at 4^{o}C and pellet was resuspended at a fixed concentration in 10% FBS, 1% L-glutamine, 1% P/S, RPMI medium. To perform uterine lavage (UL), the genital tract was removed *en bloc* and the uterine horns were cut at the level of the uterine corpus. The ovary and oviducts were removed, and each uterine horn was flushed with 100 μl of sterile 1XPBS. Samples were briefly centrifuged to pellet cells and the lavage fluid was collected and frozen at -80^{o}C until assayed. Pellets were resuspended in 500 μl 10%FBS, 1%L-glutamine, 1% P/S RPMI medium and subsequently stained for flow cytometry analysis.

2.3 *In vitro* cultures

2.3.1 Generation of bone marrow-derived dendritic cells (BMDCs)

To collect bone marrow, the femurs and tibia of naïve mice were flushed with 5% BS/RPMI into Petri dishes containing 5-10 ml of 5% BS/RPMI. Samples were centrifuged at 1200 rpm for 10 min at 4^{o}C. The pellet was suspended and red blood cells were lysed by incubation in 1xACK buffer for 5 min at room temperature followed by addition of 5-10 ml 5% BS/RPMI. Samples were centrifuged for 10 min and resuspended in 10 ml R10 medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (P/S), and 50 μM β-mercaptoethanol (all purchased from Fisher Scientific, Ottawa, Ontario) and 20 ng/ml (200 U/ml) recombinant mouse granulocyte–macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Burlington, Ontario). The concentration of cells in single-cell suspension was determined by trypan blue-dye exclusion method using a hematocytometer. At day 0, 3x10^{6} cells were seeded per 100-mm tissue culture dish in 10 ml R10 medium and incubated at 37^{o}C, 5% CO_2. Cells were fed by adding an additional 10 ml R10 medium at day 3. On day 6, loosely adherent cells were collected by gently pipetting off the suspension and used as the source of dendritic cells.

2.3.2 *In vitro* antigen recall stimulation

Aliquots of purified *C. muridarum* were heat-inactivated by 30 min incubation at 65^{o}C and used as heat-killed crude *C. muridarum* antigen for *in vitro* stimulation. For *in vitro* recall experiments, 200 μl of single-cell suspensions prepared from spleen and ILN of *C. muridarum*-infected mice were seeded in 96-well plates at a concentration of 1x10^{6} cells per well in10% FBS, 1% L-glutamine, 1% P/S, RPMI medium. 5 μl of 1:50 dilution of heat-killed *C. muridarum* (approximately equivalent to a multiplicity of infection (MOI) of 0.01) was added to each well. Wells were mixed by pipetting and cultures were incubated for 72 h at 37^{o}C. The culture supernatants were used for detecting cytokine content by ELISA.

2.3.3 *In vitro* stimulation of spleen cells

Spleens were removed from naive IL-10GFP or wild-type mice and single-cell suspensions were prepared as described above. Cells were seeded in 96-well plates at a concentration of $1x10^6$ cells per well in 200 µl 10% FBS, 1% L-glutamine, 1% P/S, RPMI medium and stimulated with $1x10^5$ inclusion forming units (IFU) of live *C. muridarum* (MOI 0.1) at 37°C, 5% CO_2 for indicated time periods. In some experiments, stimulation conditions also included $1x10^6$ IFU heat-killed *C. muridarum* (MOI 1.0), 100 or 200 ng/ml of lipopolysaccharide (LPS) (Sigma, Oakville, Ontario), or 1 µg/ml of CpG oligonucleotides (InvivoGen). At indicated timepoints, cells were transferred to fresh 96-well V-bottom plates for staining to analyze IL-10-expressing immune cells by flow cytometry. In order to examine the proliferation of IL-10GFP cells in some experiments, cells were labeled with 5 µM "Cell Proliferation Dye eFluor®670" (eBioscience, San Diego, CA, USA) as per manufacturer's protocol prior to culture. To assess cellular division by flow cytometry, the gate for "undivided" cells was determined by the fluorescent intensity of "Cell Proliferation Dye eFluor®670" in unstimulated cells from control wells.

In experiments using DC-depleted splenocytes, $CD11c^+$ DCs were depleted from splenocytes prior to culture using direct-conjugate "CD11c Microbeads" (Miltenyi Biotech, Auburn, CA, USA) as per the manufacturer's protocol. CD11c-depleted splenocytes and whole splenocytes were then seeded in 24-well plates at a concentration of $2x10^6$ cells per well in 1 ml 10% FBS, 1% L-glutamine, 1% P/S, RPMI medium and stimulated with $2x10^5$ IFU of live *C. muridarum* (MOI 0.1) for 48 h. In some experiments, splenic B cells were purified from naïve splenocytes by labeling cells with a 1:200 dilution of phycoerythrin (PE)-conjugated anti-mouse CD19 monoclonal antibody and then performing positive selection by magnetic activated cell sorting (MACS) using "Anti-PE Microbeads" (Miltenyi Biotech, Auburn, CA, USA) as per the manufacturer's protocol. Purified B cells were then seeded in 96-well plates at a concentration of $1x10^6$ cells per well in 200 µl 10% FBS, 1% L-glutamine, 1% P/S RPMI medium and stimulated with $1x10^5$ IFU of live *C. muridarum* (MOI 0.1) for 24 to 72 h. For experiments using cells from wild-type or MyD88-deficient mice, ~100 µl of culture

supernatants were transferred into a fresh 96 well plate at indicated timepoints and stored at -20^{o}C until assayed for cytokine levels by enzyme-linked immunosorbent assay (ELISA).

2.3.4 Treg and Tconv co-culture

Spleens from removed from naïve donor mice and single-cell suspensions were prepared. $CD4^{+}CD25^{+}$ regulatory T cells (Tregs) and conventional $CD4^{+}CD25^{-}$ T cells (Tconv) were purified by MACS using the "$CD4^{+}CD25^{+}$ Regulatory T cell isolation kit" Miltenyi Biotech, Auburn, CA, USA) as per the manufacturer's protocol. Purity for both populations was assessed by flow cytometry to be >90% based on CD25 expression. Alternatively, spleens from Foxp3-GFP mice were used as a source of donor cells and labeled with anti-mouse CD4 were sorted into $CD4^{+}Foxp3^{+}$ Treg and $CD4^{+}Foxp3^{-}$ populations by flow cytometry using a FACSAria cell sorter (Becton Dickinson, San Jose, CA, USA). Purity was assessed by flow cytometry to be >95% for Tregs and >98% for Tconv based on Foxp3 expression. For experiments examining cellular proliferation, cells were labeled with 5 μM "Cell Proliferation Dye eFluor®670" as per manufacturer's protocol.

Treg and Tconv co-cultures were performed in 96-well tissue culture plates in a total volume of 200 μl per well 10% FBS, 1% L-glutamine, 1% P/S, RPMI medium. The day prior to Treg and Tconv purification (day-1), 50 μl containing $2x10^{4}$ bone marrow-derived dendritic cells (BMDCs) were seeded into each well and stimulated with media alone, live *C. muridarum* (MOI 1.0) or 100 ng/ml LPS for 24 h at 37^{o}C, 5% CO_2. On day 0, 100 μl of $1x10^{5}$ naïve Tconv cells alone or with titrated ratios of Treg cells were added directly to appropriate wells containing 50 μl of $2x10^{4}$ BMDCs. 50 μl of soluble anti-CD3 (0.5-1 μg/ml) (eBioscience, San Diego, CA, USA) was added to appropriate 'stimulated' and 50 μl of fresh media was added to unstimulated well and incubated for 48-72 h at 37^{o}C, 5% CO_2. For experiments in which purified Tregs were cultured in the absence of Tconv, 10 ng/ml of recombinant mouse IL-2 (R&D Systems, Burlington, Ontario) was added at day 0. At the endpoint of the experiment, ~100 μl of culture

supernatant was transferred into a fresh 96 well plate and stored at -20^{o}C until assayed. Cells were transferred to fresh 96-well V-bottom plates for staining to analyze by flow cytometry. To assess cellular division by flow cytometry, the gate for "undivided" $CD4^{+}$ T cells was determined by the fluorescent intensity of "Cell Proliferation Dye eFluor®670" in unstimulated cells from control wells.

2.3.5 B cell suppression assays

At day -1, spleens from removed from naïve wild-type or IL-10-deficient mice mice and single-cell suspensions were prepared. $30x10^{6}$ cells were seeded in 100-mm tissue culture dishes in 10 ml of 10% FBS, 1% L-glutamine, 1% P/S RPMI medium and stimulated with $30x10^{6}$IFU (MOI 1.0) of heat-killed *C. muridarum* or media as an 'unstimulated' control for 24 h at 37^{o}C, 5% CO_2. On day 0, B cells were purified from cultures by labeling cells with a 1:200 dilution of PE-conjugated anti-mouse CD19 monoclonal antibody and then performing positive selection by MACS using "Anti-PE Microbeads" as per the manufacturer's protocol. Purity was assessed by flow cytometry to be >90%. For assays utilizing $CD43^{-}$ and $CD43^{+}$ B cell populations as suppressors, *in vitro* cultures pre-stimulated with heat-killed *C. muridarum* as described above were labeled with anti-mouse CD19 and anti-mouse CD43 monoclonal antibodies and sorted into $CD19^{+}CD43^{-}$ and $CD19^{+}CD43^{+}$ populations by flow cytometry using a FACSAria cell sorter. Purity was assessed by flow cytometry to be >99% for $CD19^{+}CD43^{-}$ cells. Purified B cells were then irradiated with 1000 radiation absorbed dose (rad) of gamma radiation from a cesium source to halt B cell proliferation. To confirm that *C. muridarum*-stimulated B cells continued to produce IL-10 following irradiation, purified B culture supernatants were assessed by ELISA at 24, 48, and 72 h following irradiation (APPENDIX A3). For responder cells, spleens were removed from OTII mice and single-cell suspensions were prepared. Cells were labeled with 5 μM "Cell Proliferation Dye eFluor®670" as per manufacturer's protocol. 100 μl of $1x10^{5}$ naïve responder cells alone were added to titrated ratios (1:1, 1:2, 1:5, or 1:10) of B cells or media alone (0:1) to make 200 μl total well volume. Cultures were then stimulated with 50 μl/well of soluble anti-CD3 (5 μg/ml) or were left unstimulated as a control by adding 50 μl/well of

fresh media and incubated for 48-72 h at 37°C, 5% CO_2. At the endpoint of the experiment, ~100 µl of culture supernatant was transferred into a fresh 96-well plate and stored at -20°C until assayed. Cells were transferred to fresh 96-well V-bottom plates for staining to analyze by flow cytometry. To assess cellular division by flow cytometry, the gate for "undivided" $CD4^+$ T cells was determined by the fluorescent intensity of "Cell Proliferation Dye eFluor®670" in unstimulated cells from control wells.

2.4 *In vivo* models

2.4.1 Genital tract infection model

Ten and three days prior to infection, mice were injected subcutaneously (*s.c.*) with 2.5 mg of progesterone (Depo-Provera®, Pfizer Canada, Kirkland, Quebec) in 100 µl of sterile 1XPBS to synchronize estrus cycles. To establish genital infection, mice were immobilized with an intraperitoneal (*i.p.*) injection of 75 mg/kg Ketamine (Sandoz Canada Inc.) and 15 mg/kg Xylazine (Rompun®, Bayer) anesthesia, placed in a supine position and inoculated intravaginally with $1x10^6$ inclusion forming units (IFU) of *C. muridarum* in 10 µl sucrose-phosphate-glutamate (SPG) buffer using a P20 micropipette. For experiments involving chimeric mice with a day 30 endpoint, an inoculating dose of $5x10^5$ IFU was used.

2.4.2 Generation of mixed bone marrow chimeras

To prepare bone marrow, donor mice were euthanized and their fur was soaked in 70% ethanol. The femurs and tibia of both legs were excised and connective tissue was carefully removed. Bone marrow was extracted by inserting a syringe with 30 G needle into the bone marrow cavity and flushing with 3-5 ml of RPMI 1640 containing 5% bovine serum (BS) into Petri dishes containing 5 ml of 5% BS/RPMI. Clusters within the extracted bone marrow were dissociated by pipetting. Samples were centrifuged (1200 rpm for 10 min at 4°C). The cell pellet was suspended and red blood cells were lysed by

incubation in ACK buffer for 3 min at room temperature followed by addition of 5-10 ml 5% BS/RPMI. The suspension was centrifuged (1200 rpm for 10 min at 4^{o}C), resuspended in 5% BS/RPMI and counted by trypan blue-dye exclusion method using a hematocytometer. Donor bone marrow preparations were depleted of T cells by labeling cells with PE-conjugated antibodies against CD4, CD8α and Thy1.2 and performing negative selective by MACS using "Anti-PE Microbeads" as per manufacturer's protocol. T cell depleted bone marrow cells were combined into aliquots of ~10x10^{6} cells comprised of 80% μMT and 20% IL-10$^{-/-}$ cells or 80% μMT and 20% wild-type cells to generate IL-10$^{-/-}$ B and wild-type B chimeras, respectively. Female recipient mice (5 weeks old) were administered antibiotic-laced drinking water (0.2% w/v neomycin sulfate, BioShop, Burlington, Ontario) *ad libitum* for 72 h prior to irradiation. Recipient mice were exposed to consecutive doses of 750 rad and 450 rad of gamma irradiation from a cesium source delivered approximately 18 h apart. Within 1 h following second dose of irradiation, mice were placed in restraint device to administer tail vein injections and approximately 10x10^{6} bone-marrow cells in 100 μl of sterile PBS were delivered intravenously. Mice were monitored daily for bodyweight changes and administered 0.2% antibiotic-laced drinking water *ad libitum* for two weeks. Recipients were then left an additional 4 weeks for engraftment, at which point peripheral blood was analyzed for the presence of donor-derived cells. CD45.1 and CD45.2 expression showed that recipient mice were >96% reconstituted with donor cells.

2.4.3 *In vivo* antibody-mediated Treg depletion

Anti-CD25 monoclonal antibody (mAb) (rat IgG1, clone PC61) was purified by sequential ammonium sulphate precipitation (50% then 33%) from supernatants of the TIB222 hybridoma culture (ATCC, Manassas, VA, USA). After precipitation, the pellet was dissolved in LPS-free PBS and extensively dialysed against LPS-free PBS solution. The concentration of antibody was determined by bicinchoninic acid assay (Thermo Scientific) using purified rat IgG (Sigma, Oakville, Ontario) as a standard. The LPS content was determined by *Limulus Amebocyte Lysate* (LAL) test (Associates of Cape Cod, East Falmouth, MA, USA). The antibody preparation that had the LPS content less

than 1 EU/μg was used for *in vivo* study. The functional activity of in-house produced PC61 antibody was confirmed by comparing to commercial product from eBioscience and the dose of PC61 was determined to be 300 μg per mouse based on 50% *in vivo* Treg depletion rate.

To deplete Tregs *in vivo*, mice were injected subcutaneously with 300 μg of PC61 anti-CD25 monoclonal antibody in 300 μl sterile PBS 1 day prior to intravaginal *C. muridarum* infection. Purified rat IgG (Sigma, Oakville, Ontario) was used as an isotype control in some experiments. To confirm the efficacy of $CD4^+Foxp3^+$ Treg depletion, peripheral blood samples were collected from the tail vein of PC61-treated and control Foxp3-GFP mice using heparinized capillary tubes at different time points following injection. Following red blood cell lysis, PBMCs were stained for CD4-expression and the frequency of Foxp3-GFP-expressing cells among $CD4^+$ PBMCs was analyzed by flow cytometry.

2.4.4 B cell adoptive transfer

Female wild-type and IL-10-deficient donor mice were sacrificed by anesthetic (isoflurane) overdose and their abdomens were soaked in 70% ethanol. Spleens were excised and placed in ~500 μl of sterile collection media (HBSS or PBS) and kept on ice until processed into single-cell suspensions. To purify B cells, single-cell suspensions were labeled with a 1:200 dilution of PE-conjugated anti-mouse CD19 monoclonal antibody and positive selection by MACS was performed using "Anti-PE Microbeads" as per the manufacturer's protocol. The number of cells recovered in CD19-positive fractions was determined by trypan blue-dye exclusion method using a hematocytometer. Aliquots of 17.5 x 10^6 CD19-positive donor cells were prepared in 100 μl sterile PBS. For adoptive transfer, progesterone-pretreated female μMT recipient mice were placed in restraint device to administer tail vein injections and donor cells were delivered intravenously. At 24 h following transfer (day 0) recipient mice were infected intravaginally with 1x10^6 IFU of *C. muridarum*.

2.5 Preparation of *Chlamydia muridarum*

Chlamydia muridarum (*C. muridarum*) was originally obtained from Xi Yang (University of Manitoba, Winnipeg, Manitoba) and propagated in McCoy cells (ATCC, Manassas, VA, USA) according to procedures described by Li *et al.* (337) and Yang *et al*. (338). Briefly, McCoy cells monolayers were infected for 48 to 72 h and harvested with sterile glass beads. The cell suspensions were subjected to brief sonication on ice, and cellular debris was removed by centrifugation at 500 x g for 10 min. The supernatants were collected and spun at 30,000 x g for 30 min to pellet the bacteria. The infectious elementary bodies (EBs) were purified by discontinuous density gradient centrifugation using 30% Isovue-370 (Bracco Diagnostics, Princeton, NJ, USA) and 50% sucrose (Sigma, Oakville, Ontario). The titre of purified EB preparations was determined using the inclusion forming unit (IFU) assay. *C. muridarum* EB stocks were stored in sucrose-phosphate-glutamic acid (SPG) buffer in small aliquots and frozen at -80°C until needed.

2.6 Bacterial quantification

2.6.1 Genital swab collection and processing

To assess *C. muridarum* shedding from the lower genital tract, cervicovaginal material was collected by swabbing mice vaginally with a calcium alginate swab (Fisher Scientific, Ottawa, Ontario). Swabs were immediately placed in a collection tube containing 500 µl of SPG buffer (or 300 µl in later experiments), briefly vortexed with sterile glass beads (4 mm diameter) and rotated for 1 h at 4^{o}C. The swabs were then removed and discarded and remaining fluid was frozen at -80^{o}C for bacterial load quantification by quantitative PCR or inclusion forming unit (IFU) assay.

2.6.2 Real-time quantitative polymerase chain reaction (qPCR)

To quantify the bacterial burden recovered from vaginal swabs of *C. muridarum*-infected mice, total nucleic acid from 100 µl of each sample of swab fluid was extracted

using DNAzol (Invitrogen, Oakville, Ontario) as per the manufacturer's instructions. The number of bacteria in each sample was determined by quantitative PCR with *Chlamydia*-specific primers for 16S ribosomal RNA (rRNA) using SYBR green supermix (Qiagen, USA) in a 7900H fast real-time PCR machine (Applied Bioscience, Foster City, CA, USA). The forward primer was 5'-CGCCTGAGGAGTACACTCGC-3' and the reverse primer was 5'-CCAACACCTCACGGCACGAG-3'. All primers were synthesized by Integrated DNA Technologies, Inc. (Montreal, Quebec, Canada). Bacterial copy number was calculated using a standard based on known copy numbers of *C. muridarum* DNA extracted from purified *C. muridarum* preparations using the same procedure and is expressed as log10 value of copies of 16S rRNA per ml.

2.6.3 Inclusion forming unit (IFU) assay

To determine the bacterial burden collected by genital swabs in *Chlamydia*-challenged mice, McCoy cells were grown in Minimum Essential Medium (MEM) (Invitrogen, Oakville, Ontario) plus 5% FBS, 2 μg/mL fungizone (Life Technologies, Burlington, Ontario) and 25 μg/mL of gentamicin (Life Technologies, Burlington, Ontario) and seeded in 96-well plates at a concentration of 4.5 $x10^4$ cells in 200 μl/well and incubated at 37°C and 5% CO_2 overnight to generate a monolayer. The following morning, McCoy cell culture media was carefully removed to avoid disturbing monolayer and replaced with 200 μl/well of Growth Media (MEM plus 10% FBS (Fisher Scientific, Ottawa, Ontario), 0.5% glucose (BioShop, Burlington, Ontario), 0.225% sodium bicarbonate (Bioshop, Burlington, Ontario), 20 mM HEPES (Invitrogen, Oakville, Ontario), 2% L-glutamine (Fisher Scientific, Ottawa, Ontario), 2% 100 X MEM vitamin solution (Life Technologies, Burlington, Ontario), 10 μg/mL gentamicin (Life Technologies, Burlington, Ontario) and 1 μg/ml cyclohexamide (Invitrogen, Oakville, Ontario) and McCoy cells were then inoculated with 10-20 μl of genital swab samples (neat or diluted in SPG buffer) and serial dilutions of purified-*Chlamydia* with known titers as standards. Plates were incubated for 1 h at 37°C and 5% CO_2 and then centrifuged at 1300 x g for 1 h at 36°C. Following centrifugation, plates were incubated for 40- 42 h at 37°C and 5% CO_2. Following incubation, the cell monolayer was fixed by

carefully removing culture media and adding 200 µl of 100% methanol to each well and incubating at room temperature for 10 min. Methanol was then removed and plates were left uncovered to air dry for 2-3 min before proceeding with *Chlamydia*-specific staining.

To stain for *Chlamydia*, plates were washed three times with 0.01% Tween-20 (BioShop, Burlington, Ontario))/PBS buffer. To wash plates, ~200 µl of 0.01% Tween-20/PBS buffer was added to each well then plate was immediately dumped out and patted dry using paper towel. To block plates, 50 µl/well of 10% bovine serum (BS) was added to each well and plates were incubated for 1 h at room temperature. Plates were washed once with 0.01% Tween-20/PBS buffer. 50 µl of genus-specific biotin-conjugated rabbit anti- *Chlamydia* polyclonal primary antibody (Biodesign International, Saco, ME, USA) diluted 1:376 in dilution buffer (1:1 10% BS and 0.01% Tween-20/PBS) was added to each well and plates were incubated overnight at 4^{o}C. The following day, plates were washed seven times with 0.01% Tween-20/PBS buffer and 50 µl of streptavidin-conjugated Texas red (Sigma, Oakville, Ontario) secondary antibody diluted 1:376 in dilution buffer was added to each well. Plates were incubated for 1 h in the dark at room temperature and then washed three times with 0.01% Tween-20/PBS buffer. 200 µl of PBS was added to each well and plates were wrapped in aluminum foil and stored at 4^{o}C until analysis by fluorescent microscopy within 24 h. The fluorescent inclusions were counted (16 fields per well) using an automatic stage Olympus IX71 microscope (Olympus, Tokyo, Japan) and the number of inclusion forming units was calculated based on the known titres of *Chlamydia* used as standards.

2.7 Immunofluorescence staining and flow cytometry

The primary antibodies used in these experiments are listed in Table 2.1. FACS buffer (1% BS/PBS) was used for all surface-staining procedures. Cells were first blocked with diluted anti-mouse CD16/CD32 antibody (eBioscience, San Diego, CA, USA) or 2% rat serum in PBS for 15-20 min at 4°C. Staining was performed in 96-well V-bottom plates with up to 1-2x10^{6} cells per well. To wash cells, 100 µl of FACS buffer was added to each well and the plate was centrifuged at 1200 rpm for 3 min at 4^{o}C. The

buffer was discarded and the cell pellet was resuspended by briefly vortexing. For surface marker labeling, cells were incubated with 50 µl of fluorochrome-conjugated anti-mouse monoclonal antibody/antibodies for 15-20 min at 4°C. For experiments in which primary biotinylated anti-mouse antibodies were used, cells were washed once and then incubated with 50 µl of secondary streptavidin conjugated antibodies purchased from eBioscience (San Diego, CA, USA) diluted in FACS buffer for 15-20 min at 4°C. Cells were washed 2 times after staining and fixed in 200 µl of 1% formalin/PBS and stored in the dark at 4°C until analysis by flow cytometry within 72 h.

For determining the cell type and the frequency of IFN-γ-, IL-17A-, or IL-10-producing cells, single-cell suspensions were stimulated in culture with heat-killed *C. muridarum* for 72 h followed by amplification with 50 ng/ml phorbol myristate acetate (PMA) (Sigma, Oakville, Ontario) plus 2 µg/ml ionomycin (Sigma, Oakville, Ontario) in the presence of 10 µg/ml Brefeldin A (eBioscience, San Diego, CA, USA) and 2 µM monensin (eBioscience, San Diego, CA, USA) for the last 4-6 h of culture. In some experiments, cells were directly stimulated with heat-killed *C. muridarum*, PMA plus ionomycin, Brefeldin A and monensin for 4-6 h total. Cells were harvested and surface staining was performed according to the procedure described above, up to the final washing step. To perform intracellular cytokine staining (ICCS), 100 µl of "IC Fixation Buffer (eBioscience, San Diego, CA, USA) was added to each well and the plate was incubated for 10-15 min in the dark at room temperature. 100 µl of "1X Permeabilization Buffer" (eBioscience, San Diego, CA, USA) was added to each well and plate was centrifuged at 1200 rpm for 5 min at 4°C. Supernatant was discarded and cells were washed once more with 200 µl "1X Permeabilization Buffer". Cells were resuspended and labeled with anti-mouse IFN-γ, IL-17A, or IL-10 monoclonal antibodies or isotype-matched control antibody diluted in 100 µl of "1X Permeabilization Buffer" and incubated in the dark at room temperature for 30-45 min. After staining, cells were washed once more and fixed in 200 µl of 1% formalin/PBS and stored in the dark at 4°C until analysis by flow cytometry within 24 h.

A total of 100,000 to 500,000 events were collected on a FACScaliber (BD Biosciences, Mississauga, ON, Canada), and the data were analyzed using FCS Express 4 Flow Cytometry software (De Nova Software, Los Angeles, CA, USA).

2.8 Cytokine ELISA

The concentrations of cytokines and chemokines in culture supernatants and mouse tissue were determined by ELISA using antibody pairs specific for mouse IFN-γ, IL-17A, IL-1β, TNF-α, IL-4, IL-6, IL-10, IL-12p70, IL-27, IL-23, and CXCL2 (eBioscience, San Diego, CA, USA). The optimal concentration of each antibody was specified by the manufacturer. 96-well flat-bottom Nunc Maxisorp™ plates were coated with 50 µl/well of capture antibody directed against cytokine of interest diluted in 0.01M $NaHCO_3$ buffer. The plate was incubated for ~24 h at 4°C and washed once. To wash plates, ~200 µl of 0.01% Tween-20/PBS buffer was added to each well then plate was immediately dumped out and patted dry using paper towel. 100 µl of blocking buffer (1% bovine serum albumin fraction V (BSA) (Bioshop, Burlington, Ontario) in 1xPBS) was added to each well and the plate was incubated at room temperature for 1-2 h. The plate was then washed 3 times. Samples and standards were diluted in 1%BSA/PBS and added to appropriate wells in 50 µl volumes. Serial two-fold dilutions of standard were performed beginning with the concentration recommended by manufacturer and at least 8 dilutions were performed to generate a standard curve. The plate was incubated overnight at 4°C and then washed 3 times. 50 µl/well of biotinylated detection antibody diluted in 1% BSA/PBS was added to each well and the plate was incubated at room temperature for 1-2 h. The plate was washed 3 times. 50 µl/well of streptavidin-horse radish peroxidase (eBioscience, San Diego, CA, USA) diluted in 1% BSA/PBS was added to each well and the plate was incubated for 20 min at room temperature in the dark. The plate was then washed 5 times. 50 µl/well of "1xTMB ELISA Substrate Solution" (eBioscience, San Diego, CA, USA) was added directly to each well and the plate was monitored for color change and the reaction stopped using 50 µl/well of 0.2 M H_2SO_4. The plate was

immediately read at 450 nm using an ELISA plate reader and data were collected and analyzed using Gen5 software.

2.9 Histology

Mice were sacrificed at day 10 and 46 post-infection; the genital tracts were removed *en bloc* and fixed in 10% buffered formalin. Samples were then embedded in paraffin and longitudinal 5 μm sections were cut and stained with hematoxylin and eosin (H&E) by staff at the histology lab in the IWK Health Centre. The oviduct region was examined at ×50 magnification and images were acquired using a Leica DM2700M microscope (Leica Microsystems). To assess oviduct dilation, the cross-sectional diameter of each oviduct in the left and right uterine horns within each group was measured in μm using ImageJ software (National Institutes of Health). When multiple oviduct loops were present, the one with the greatest diameter was reported. Measurements for individual oviducts are presented as a box whiskers plot: the line within the box represents the median, box limites the 25^{th} and 75^{th} percentiles, and the whiskers extend to the minimum and maximum values. A blinded analysis on histological assessment was conducted using randomly selected samples.

For immunohistochemical Ly6G staining, slides containing 5 μm thick paraffin-embedded GT tissue sections were deparaffinized by two washes of Xylene (3 min each) followed by two washes of 100% ethanol (30 seconds (s) each), one wash of 95% ethanol (30 s), one wash with 70% ethanol (30 s) and two washes with distilled water. Slides were incubated in 10 mM sodium citrate buffer at 95^{o}C for 10-30 min, cooled for 20 min and washed twice with 0.5% Tween-20/PBS buffer, which was used for all subsequent washes. Slides were incubated in 3% hydrogen peroxide at room temperature for 10 min and washed twice. For primary antibody staining, slides were first incubated with blocking buffer (2% goat serum, 1% BSA, 0.1% Triton X-100, 0.05% Tween 20, 0.05% sodium azide, PBS) for 30 min at room temperature and then incubated with rat anti-mouse Ly6G (Gr-1) antibody (BD Pharmingen) diluted 1:4000 in primary antibody buffer (1% BSA, 0.5% Triton X-100, 0.05% sodium azide, PBS) overnight at 4°C. After

washing three times, the tissue sections were then incubated with biotinylated secondary goat anti-rat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500 in 0.5% Tween-20/PBS buffer for 60 min, washed three times and then incubated with a peroxidase conjugate (ABC, Vector Laboratories) for 30 min. Slides were washed three times with 0.5% Tween-20/PBS buffer and staining was developed using diaminobenzidine. To better visualize staining, slides were counterstained with haematoxylin by staff at the histology lab in the IWK Health Centre. Ly6G-positive cells were counted in the oviduct region in 5 different high power fields (×400) per section and averaged for each individual. The results are expressed as the number of cells per high power field and presented as mean ± SEM of individuals within each group.

2.10 Statistical analyses

Tests for statistical significance were performed using GraphPad Prism 4 (GraphPad) software. The one- or two-tailed unpaired Student's *t* test and the Mann-Whitney *U* test were used to determine the significance of the differences between two groups. For comparison of multiple groups, the analysis of variance (ANOVA) test followed by multiple comparison of means was applied. All data are shown as mean ± standard error of the mean (SEM) and *P* values ≤ 0.05 were considered statistically significant.

Table 2.1. List of fluorochrome-conjugated primary antibodies used in FACS staining

Specificity	Clone	Manufacturer
Anti-human/mouse B220 (CD45R)	RA3-6B2	eBioscience
Anti-mouse CD11b	M1/70	eBioscience
Anti-mouse CD11c	N418	eBioscience
Anti-mouse CD19	1D3	eBioscience
Anti-mouse CD1d	1B1	eBioscience
Anti-mouse CD3	145-2C11	eBioscience
Anti-mouse CD4	GK1.5	eBioscience
Anti-mouse CD43	S7	BD Pharmingen
Anti-mouse CD45.1	A20	eBioscience
Anti-mouse CD45.2	104	eBioscience
Anti-mouse CD5	53-7.3	eBioscience
Anti-mouse CD8α	53-6.7	eBioscience
Anti-mouse Gr-1 (Ly-6G)	RB6-8C5	eBioscience
Anti-mouse IFN-γ	XMG1.2	eBioscience
Anti-mouse IgD	11-26	eBioscience
Anti-mouse IgM	eB121-15F9	eBioscience
Anti-mouse IL-10	JES5-16E3	eBioscience
Anti-mouse IL-17A	eBio17B7	eBioscience
Anti-mouse Thy1.2	30-H12	eBioscience
Rat IgG1 κ isotype control	eBRG1	eBioscience
Rat IgG2a κ isotype control	eBR2a	eBioscience
Rat IgG2b κ isotype control	eB149/10H5	eBioscience

CHAPTER 3 $CD4^+FOXP3^+$ REGULATORY T CELLS PROMOTE TH17 RESPONSES AND OVIDUCT PATHOLOGY UPON INTRACELLULAR *CHLAMYDIA MURIDARUM* GENITAL TRACT INFECTION

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3.1 Introduction

Chlamydia trachomatis (*C. trachomatis*) is an intracellular bacterial pathogen that infects mammalian hosts by targeting mucosal epithelial cells lining the ocular, respiratory and genital tract (GT). At these various mucosal sites, *Chlamydia*-caused infections commonly result in chronic inflammation, tissue damage and fibrosis due to repeated and/or persistent *Chlamydia* infection (7;339). In the case of *C. trachomatis* GT infection, the most common bacterial sexually transmitted infection, serious sequelae such as pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility are the primary clinical complications identified in a portion of *Chlamydia*-infected individuals (6;340). Mouse models of GT infection with *C. muridarum* closely mimic both acute and chronic phases of *C. trachomatis* infection in women and have been extensively used in research. A reliable surrogate marker for fibrotic oviduct occlusion and infertility in mice is the development of hydrosalpinx, which is characterized by accumulation of inflammatory exudates and oviduct dilation (341). Histological or cytological examinations of samples collected from mice and human subjects have revealed a profound chronic accumulation of neutrophils associated with *Chlamydia* infections (74;77;341;342). Experimental blockade or attenuation of neutrophil infiltration during *C. muridarum* infection results in significantly reduced tissue damage and oviduct pathology without delaying bacterial clearance (74;77), indicating that prolonged neutrophilic inflammation is an important pathological host response to *Chlamydia* infection. In addition, inappropriate $CD8^+$ T cell responses (particularly TNF-α-producing $CD8^+$ T cells) and production of proinflammatory cytokines downstream of

Toll-like receptor 2 (TLR2), such as IL-1β, have also been demonstrated to play important roles in oviduct pathology in mice (46;62;146;343). However, a comprehensive understanding about the vital mechanisms controlling *Chlamydia*-caused pathological sequelae remains lacking.

T cell-mediated immunity is essential in host defense against *Chlamydia* infection and has a role in mediating *Chlamydia*-induced inflammation (6;7;146;343). Among the different $CD4^+$ T helper (Th) responses including Th1, Th2 and Th17, IFN-γ-producing $CD4^+$ Th1 cells are the primary response induced in mouse models of *Chlamydia* infection and have a well-established protective role. Mice deficient in IFN-γ or IFN-γ receptors are unable to clear genital *C. muridarum* infection and display massive inflammatory responses in the genital tract (124;344). Recent work has demonstrated that $CD4^+$ Th17 responses are also induced by *Chlamydia* infection (133;136). Th17 cells are differentiated from conventional αβ $CD4^+$ T cells in local draining lymph nodes under the influence of a cytokine milieu containing the immunosuppressive cytokine TGF-β and proinflammatory cytokines IL-1β, IL-6 and IL-23 (105). IL-17A and IL-17F, the major effector cytokines of Th17 cells, is a proinflammatory cytokines with potent biological activity in neutrophilic responses via stimulating the production of cytokines and chemokines involved in neutrophil generation, maturation and mobilization to sites of inflammation (105;345;346). IL-17A also promotes the optimum priming of Th1 responses to *C. muridarum* by upregulating dendritic cell (DC) expression of co-stimulatory molecules, class II MHC molecules and IL-12p70 (136;347). The role of IL-17A/Th17 responses in *Chlamydia* infection is complex. Delivery of neutralizing anti-IL-17A antibody before or shortly after respiratory *C. muridarum* infection markedly attenuates bacterial clearance (135;136;347), indicating that IL-17A production is critically required for host defense against *Chlamydia*. However, *Chlamydia*-susceptible mouse strains BALB/c and C3H/HeN are shown to have higher levels of IL-17A/IL-17R responses than *Chlamydia*-resistant C57BL/6 mice (134;137). Collectively, these studies suggest that IL-17A-mediated responses to *C. muridarum* represent a double-edged sword that can promote bacterial clearance but also potentially damage host tissue when improperly controlled. Elucidating immune mechanisms that effectively regulate IL-

17A/Th17 responses during *Chlamydia* infection may shed the light on the pathogenesis of *Chlamydia*-associated diseases.

$CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells represent approximately 5-10% of peripheral $CD4^+$ T cells in normal hosts and are well-documented to prevent autoimmunity by suppressing the activation of auto-reactive lymphocytes (348). In addition to their critical immunosuppressive role in self-tolerance and immune homeostasis, accumulating evidence indicates that Tregs also modulate host-pathogen interactions (250). Tregs are classically known to inhibit a wide range of immune responses including proliferation and cytokine production by $CD4^+$ and $CD8^+$ T effectors and this suppressive activity has been demonstrated to protect hosts from immunopathology and tissue damage associated with vigorous pathogen-directed immune responses (250;251). However, Treg-mediated suppression can also facilitate persistent infections by dampening the induction of effective antimicrobial immunity (250;251). Tregs have been ascribed both beneficial or detrimental roles during infection depending on the nature of the infectious agent (e.g. parasitic, viral, fungal or bacterial) and/or whether the infection is acute or chronic (250;251). Emerging evidence suggests that the presence of TLR ligands, inflammatory mediators and abundant survival cytokines during the acute infection may subvert Treg suppressive function (223;235;246;349). In contrast to having a suppressive role, Tregs have been shown to promote *de novo* generation of Th17 cells in the presence of TLR ligands *in vitro* and *in vivo* (226;246). More recently, Tregs were demonstrated to enhance host resistance to mucosal fungal infection via the promotion of Th17 responses (226). Although several murine and human studies report that *Chlamydia* infection induces expansion of $Foxp3^+$ Tregs (319-323), our understanding about the role of Tregs during *Chlamydia* infection remains rudimentary.

Given the importance of $CD4^+$ T helper responses in *Chlamydia* infection and the known ability of Tregs to modulate effector responses, we investigated the role of Tregs in regulating host responses to *C. muridarum* infection within an *in vitro* co-culture system and *in vivo* model of genital tract infection. Here, we have explicitly

demonstrated that Tregs have a divergent role in controlling the induction and differentiation of $CD4^+$ T helper responses upon *C. muridarum* infection. While Tregs had no obvious role in regulating protective Th1 responses, they served as a prominent inducer of and direct contributor to IL-17A/Th17 responses during *C. muridarum* infection, which, in turn, facilitated the development of *C. muridarum*-induced immunopathology *in vivo*.

3.2 Results

3.2.1 Tregs have a divergent role in regulation of Th1, Th2 and Th17 responses *in vitro* upon *C. muridarum* infection

To understand how Tregs modulate effector $CD4^+$ T cell responses to *C. muridarum*, we first utilized the conventional *in vitro* Treg suppression assay and assessed the impact of *C. muridarum* infected-DCs versus non-infected DCs on effector $CD4^+$ T cell proliferation and T helper cytokine production in the absence or presence of various amounts of Tregs. Without *C. muridarum* infection, Tregs suppressed conventional $CD4^+$ T cell (Tconv) proliferation in a classic dose-dependent manner, with approximately 50% reduced proliferation at 1:1 ratio of Treg:Tconv comparing to Tconv alone (Figure 3.1a). While *C. muridarum* infection markedly enhanced the overall magnitude of $CD4^+$ T cell proliferation (Figure 3.1a), Tregs still displayed a comparable pattern of dose-dependent suppression in the presence of infection (Figure 3.1a). However, substantial differences were observed in $CD4^+$ T helper cytokine profiles in this co-culture system. Tregs were able to inhibit IFN-γ production in a dose-dependent manner under non-infected conditions but this inhibitory activity was abolished upon *C. muridarum* infection (Figure 3.1b). In comparison, IL-4 production in the culture supernatants were significantly suppressed by increased densities of Tregs, and *C. muridarum* infection only altered their overall magnitudes of production (Figure 3.1c). In sharp contrast to Th1/Th2 cytokine profiles, IL-17A production was promoted by increasing densities of Tregs and was further amplified by *C. muridarum* infection (Figure 3.1d). Using Tregs and Tconv cells from CD45.2 and CD45.1 congenic

C57BL/6 mice, respectively, intracellular IL-17A staining clearly revealed IL-17A-positive staining in Tconv ($CD4^+CD45.2^-$) cells. The Th17 frequency was increased from 0.1% of Tconv cells in the co-culture with non-infected DCs to 1.2% with *C. muridarum*-infected DCs, and further increased substantially to 6.6% in the presence of Tregs (Figure 3.1e). These results clearly demonstrate divergent roles of Tregs in modulating $CD4^+$ T helper responses to *C. muridarum* infection *in vitro*: upon *C. muridarum* infection, Tregs lost their regulatory capacity over Th1 responses while serve as a suppressor of Th2 responses and a promoter for Th17 responses.

3.2.2 MyD88-dependent signals in both DCs and Tregs themselves are required for Tregs to convert into Th17 cells during *C. muridarum* infection *in vitro*

We noticed a clear IL-17A-positive population of Tregs ($CD4^+CD45.2^+$) in our co-culture system (Figure 3.1e). To further verify that IL-17A was not produced by any contaminating Tconv cells in the Treg preparation, we sorted Tregs from Foxp3-GFP mice and directly stimulated these Tregs with anti-CD3 in the presence of non-infected DCs, *C. muridarum*-infected DCs or LPS-treated DCs. As shown in Figure 3.2a, while approximately 4% Tregs displayed a IL-$17A^+$Foxp3-GFP^+ phenotype upon LPS stimulation, *C. muridarum*-infected DCs stimulated robust IL-17A production with approximately 33% of Tregs becoming IL-$17A^+$Foxp3-GFP^+. Since Tregs express a spectrum of TLRs and the engagement of TLRs has been reported to directly modulate Treg function (223;350;351), we wondered whether IL-17A-production by $Foxp3^+$ Tregs is a direct or indirect effect of TLR signaling in Tregs. To this end, we utilized Tregs and DCs derived from C57BL/6 mice or MyD88-deficient mice and set up a combinatorial co-culture system. We found that the absence of MyD88-dependent signaling in either Tregs or DCs completely abolished IL-17A production upon *C. muridarum* infection or LPS stimulation (Figure 3.2b). Accordingly, we detected little to no proinflammatory cytokines such as IL-6, IL-1β and TNF-α in MyD88-deficient DC cultures upon *C. muridarum* infection (Figure 3.2c). Taken together, our *in vitro* results suggest that Tregs are not only a potent inducer of Th17 differentiation but are themselves a significant contributor to Th17 populations responding to *C. muridarum* infection. Moreover, this

process of Treg to Th17 conversion requires activation of MyD88-dependent signaling pathways in both DCs and Tregs.

3.2.3 *C. muridarum* infection induces expansion of Tregs that precedes Th17 differentiation *in vivo*

Given the potent role of Tregs in promoting Th17 differentiation from both Tconv cells and themselves upon *C. muridarum* infection *in vitro*, we next examined the kinetics of Foxp3$^+$ cells and Th17 responses at immune induction sites (i.e. the draining iliac lymph nodes (ILN) and spleen) as well as the genital infection site during *C. muridarum* genital tract infection in Foxp3-GFP mice. We observed a rapid expansion of Foxp3$^+$CD4$^+$ cells in the ILN as early as day 3 post-infection, which continued to increase at day 5 and returned back to naïve levels by day 10 post- infection (Figure 3.3a). Consistent with our *in vitro* data, in addition to the conventional CD4$^+$ Th17 cells, a portion of IL-17A-producing CD4$^+$ T cells were also Foxp3-GFP$^+$ (Figure 3.3b/c). Unlike the early expansion of Foxp3-GFP Tregs, however, both Foxp3-GFP$^+$ and Foxp3-GFP$^-$ Th17 populations were not significantly increased relative to naïve mice until day 5 post-infection (Figure 3.3b). Thus, it appears that the expansion of Tregs precedes the induction of Th17 response *in vivo* and, in conjunction with our *in vitro* observation, suggests that a certain threshold for the number or relative density of Tregs over Tconv cells may be required to effectively induce Th17 responses to *C. muridarum* infection. The kinetics of Tregs and Th17 responses in the spleen appeared to concurrently peak at day 5 post-infection (Figure 3.3c). Contrary to the lymphoid organs, the number of total CD4$^+$ T cells in the GT was very low in naïve mice and gradually increased, peaking by day 10 post-infection. Along with increased numbers of effector CD4$^+$ T cells, both Tregs and Th17 cells accumulated in the infected GT with comparable frequencies throughout infection. In comparison, the FoxP-GFP$^+$IL-17A$^+$ double positive Th17 cells were present in the GT at a very low level (Figure 3.3c).

3.2.4 PC61 treatment prior to *C. muridarum* genital tract infection significantly attenuates Th17 responses without affecting Th1 responses *in vivo*

The rapid expansion of $CD4^+Foxp3^+CD25^+$ Tregs in the ILN upon *C. muridarum* genital tract infection led us to hypothesize that the majority of these expanded Tregs originate from pre-existing endogenous Tregs and that their frequency may directly affect the magnitude of Th17 responses to *C. muridarum* infection. To test this hypothesis, we used anti-CD25 mAb (clone PC61) to deplete pre-existing Tregs in naïve mice 24 h prior to *C. muridarum* genital tract infection. We found that PC61, but not control rat IgG, reduced the frequency of pre-existing $Foxp3^+$ cells in peripheral blood by ~40% and >50% in ILN 24 hours post-injection and Treg levels remained low in PC61-treated mice until day 13 post-injection (Figure 3.4a). Although PC61 treatment has been previously reported to deplete recently-activated $CD4^+$ Tconv cells (352), the total number of $CD4^+Foxp3^-$ Tconv cells was markedly induced in the ILNs of both PC61-treated mice and control mice at day 5 post-infection compared to their naïve counterparts and the level of $CD4^+Foxp3^-$ Tconv induction was perhaps even greater in PC61-treated group (Figure 3.4b/c). Conversely, the total number of $CD4^+Foxp3^+$ Tregs in PC61-treated mice was approximately 50% of that observed in control mice, both naïve and at 5 days post-infection (Figure 3.4b/c). PC61 treatment in our model therefore resulted in prominent depletion of $CD4^+Foxp3^+$ Tregs with no apparent effect on $CD4^+Foxp3^-$ Tconv cells. Paralleling the reduced frequency of Tregs in PC61-treated mice, we observed a significantly reduced percentage and total number of Th17 cells at day 5 post-infection in the ILN of PC61-treated mice comparing to control (Figure 3.4d/e). In contrast to the Th17 profile, the frequency and total number of Th1 cells were not altered by PC61 treatment at the same time point. While no difference in Th1 or Th17 profiles was observed between groups at day 10 post-infection, PC61-treatment did result in significantly lower levels of Th17 memory responses in the spleen at day 46 post-infection (Figure 3.4f). Taken together, our *in vitro* and *in vivo* data collectively demonstrate a prominent role for Tregs in the regulation of Th17, but not Th1, responses to *C. muridarum* infection.

3.2.5 PC61 treatment prior to *C. muridarum* genital infection markedly reduces oviduct pathology without altering the level of bacterial shedding in the genital tract

Having characterized the role of Tregs in controlling different $CD4^+$ T helper responses, we further examined the impact of PC61-treatment in bacterial clearance and inflammatory responses during *C. muridarum* infection. Remarkably, PC61-treated mice displayed less acute inflammatory infiltration in the oviduct (pyosalpinx) by day 10 post-infection, and significantly reduced oviduct dilation (hydrosalpinx) by day 46 post-infection (Figure 3.5a/b). Furthermore, the incidence of grossly apparent hydrosalpinx in PC61-treated versus control mice was ~16% and ~57%, respectively (n=6-7 mice per group) at day 46 post-infection. In sharp contrast to apparent amelioration of acute phase inflammation and chronic phase oviduct pathology in PC61-treated mice, the level of bacterial shedding was comparable between groups throughout infection (Figure 3.5c), indicating that Treg-mediated immune mechanisms involved in the development of oviduct pathology have no obvious role in controlling genital bacterial shedding.

To further understand the impact of PC61-mediated Treg depletion on acute phase inflammation in the genital tract, we performed uterine lavage (UL) to characterize cells infiltrating the uterus. PC61-treated mice had significantly fewer overall cells recovered by UL compared to control mice at days 4 and 7 post-infection (Figure 3.5d). The majority (~90%) of UL cells from control mice at day 4 post-infection had a $CD11b^+Ly6G^+$ neutrophil phenotype whereas only ~66% of UL cells from PC61-treated mice were $CD11b^+Ly6G^+$--with the remaining portion having a mixed $CD11c^+$ or $F4/80^+$ phenotype consistent with monocytes (data not shown)—which translated into ~5-fold reduction in the absolute numbers of neutrophils in UL samples from PC61-treated mice (Figure 3.5e). Immunohistochemical anti-Ly6G staining also revealed a significantly reduced number of neutrophils in PC61-treated mice compared to controls at day 10 post-infection (Figure 3.5f). Consistent with a well-established role of IL-17A/Th17 responses in stimulating production of neutrophil chemoattractants such as CXCL2 to mobilize neutrophils, IL-17A and CXCL2 levels in genital tract homogenates were positively correlated at day 5 post-infection (Figure 3.5g) and soluble IL-17A levels from

PC61-treated mice were significantly lower compared to control mice (Figure 3.5h). Collectively, these data suggest that modulation of IL-17A is a potential mechanism through which PC61-mediated Treg depletion attenuated neutrophilic inflammation during *C. muridarum* infection.

3.3 Discussion

In this study, we have demonstrated that Tregs have a divergent role in controlling $CD4^+$ T helper responses during intracellular *C. muridarum* genital tract infection. In contrast to their inability to control Th1 responses to *C. muridarum in vitro* and *in vivo*, Tregs displayed a novel innate-like property by potently promoting Th17 differentiation from conventional $CD4^+$ T cells and by themselves converting into IL-17A-producers. Correspondingly, *in vivo* depletion of pre-existing Tregs by PC61 anti-CD25 mAb resulted in diminished Th17 responses to *C. muridarum* genital tract infection. Consistent with reduced IL-17A responses, PC61-treated mice displayed striking amelioration of neutrophil infiltration and oviduct pathology in genital tract tissue relative to control mice, despite comparable bacterial shedding throughout the acute and chronic phases of infection.

Tregs have previously been correlated with increased Th17 levels in various models of inflammation but the relevance of Tregs in the induction of Th17 responses to intracellular bacterial infection is unclear (219-222). In this study, we have demonstrated a prominent role for Tregs in promoting Th17 responses during *C. muridarum* infection *in vitro* and *in vivo*. Mechanistically, Tregs may simply serve as a cellular source of TGF-β, a key Th17 promoting cytokine, to promote conventional Th17 differentiation under the influence of a proinflammatory cytokine-enriched environment upon infection (223). Several Th17 promoting proinflammatory cytokines such as IL-6, IL-1β and IL-23 were readily detected in *C. muridarum*-infected DC in a dose-dependent manner (APPENDIX B1). Accordingly, IL-17A production by Tregs and Tconv was markedly reduced in *in vitro* cocultures using IL-6-deficient DCs compared to the wild-type control DCs upon *Chlamydia* or LPS stimulation (APPENDIX B2), highlighting a critical role

for proinflammatory IL-6 in promoting Th17 responses in the presence of Tregs. However, recent work indicates that Tregs are dispensable as a source of TGF-β for Th17 differentiation *in vitro* and *in vivo* (220;225). Alternatively, Tregs may promote Th17 development by virtue of constitutive high CD25 (IL-2Rα) expression to actively consume IL-2 (220). Tregs form stable, long-lasting associations with lymph node DCs *in vivo* (353) and the high density of Tregs residing in proximity of Tconv cells can deplete IL-2 availability to activated Tconv, thereby facilitating Th17 development since IL-2-activated STAT5 inhibits IL-17A production (220;227). In agreement with our observation, a recent study also demonstrated that reducing Treg numbers *in vivo* via PC61-mediated Treg depletion led to significantly decreased Th17 responses to *C. albicans* infection and reduced neutrophil influx into the infected mucosa (349). Notably, although reduced Th17 responses and attenuated neutrophil mobilization ameliorated immunopathology during *C. muridarum* infection, these cellular changes severely impaired host resistance to *C. albicans* infection. This indicates that the Th17-promoting activity of Tregs can lead to completely disparate biological outcomes depending on the specific pathogen.

Of importance, a significant portion of Th17 cells induced by *C. muridarum* infection were Foxp3$^+$IL-17A$^+$ and indeed originated from Tregs themselves. Consistent with this observation, Foxp3$^+$IL-17A$^+$ cells have also been observed *in vivo* during *C. albicans* and *Leishmania major* infections (226;246). The process of Treg to Th17 conversion requires activation of signaling pathways in both DCs and Tregs that are mediated by MyD88, a common adaptor protein shared by TLR and IL-1 signaling pathways (354). It is conceivable that the MyD88-TLR pathway in DCs is required to create proinflammatory cytokine environment to initiate the conversion process; however, whether the MyD88-TLR pathway and/or MyD88-IL-1 pathway in Tregs themselves are involved in Treg to Th17 conversion is unclear. On one hand, TLR2 ligation has been shown to directly promote Treg proliferation and Th17 conversion by human Foxp3$^+$ Tregs (211;215). On the other hand, MyD88-IL-1 pathway has also been shown to potently promote Th17 conversion from Tregs due to high level of IL-1R expression by Tregs (235). Regardless of how Tregs convert into Th17 cells, the

Foxp3$^+$IL-17A$^+$ population observed in *C muridarum*-infected mice was most prominent in the primary immune induction site, i.e. the ILN, but not peripheral genital infection site, which supports the idea that these double-positive cells represent a transitional stage of Foxp3$^+$ Tregs converting to Th17 (231). As such, it remains to be determined whether and how Treg-derived Th17 cells contribute to Th17-mediated inflammation during *Chlamydia* infection *in vivo.*

Tregs are generally viewed as a suppressor of Th1 immune responses. Nonetheless, Treg-depletion prior to infection in our model did not significantly alter Th1 responses to *C. muridarum* infection. Th1 polarizing and promoting cytokines IL-12p70 and IL-27 were readily detected in supernatants from *C. muridarum*-infected DCs (APPENDIX B1), suggesting that *C. muridarum*-infected DCs might subvert the immunosuppressive functions of Tregs by providing a cytokine environment that favors the induction of Th1 responses. Consistent with our observation, Treg-depletion in other intracellular bacterial infection or immunization models using *Mycobacterium bovis* BCG has also been shown to have very limited impact on Th1 responses (256;257). However, in an experiment in which we adoptively transferred additional Tregs into the host 24 hours prior *C. muridarum* infection, we indeed observed a significant reduction of Th1 responses in these mice in ILN at day 5 post-infection compared to control mice. Accordingly, the effect of Tregs in promoting Th17 was delayed to day 18 post-infection (APPENDIX B3). Therefore, the level of Tregs present in the host at the moment of *C. muridarum* infection dictates the overall kinetics of Th1 and Th17 responses. The observed inability of Tregs to regulate Th1 responses in our setting and some other studies may be due to a relatively low level of endogenous Tregs in C57BL/6 mice (355). Overall, it is conceivable that Tregs have divergent roles as a suppressor of Th1 and promoter of Th17 responses.

In this study, we have employed the widely-used strategy of PC61/anti-CD25 mAb treatment to deplete CD4$^+$Foxp3$^+$ Tregs prior to infection *in vivo*. PC61 has been shown to cause a significant reduction in Foxp3$^+$ Tregs by targeting these cells for elimination by phagocytes via antibody-dependent cellular cytotoxicity (356). While

PC61 does not result in complete depletion of Foxp3$^+$ Tregs, the partial elimination achieved by this method has been found to adequately alter the immunological balance in order to examine the role of Tregs (356). The ability of PC61 to deplete activated effector T cells has been the major concern for using this Ab. However, recent studies have demonstrated that the impact of PC61 on non-Tregs varies from model to model depending on the nature of immune responses (357). In our model, we did not see any impact of PC61 treatment on the number of Tconv cells; rather, depletion was restricted to the Foxp3$^+$ Treg compartment. Interestingly, anti-CD25 mAb has actually been speculated to enhance Th17 differentiation by binding IL-2R on the surface of activated Tconv and blocking IL-2 signaling. However, we and others indeed observe PC61 to have the opposite effect on Th17 responses (226;358). Thus, we conclude that PC61 did not significantly target CD25 on Tconv cells in our study and, instead, exerted its effect mainly by depleting Foxp3$^+$ Tregs. With this approach, our data for the first time suggest that a higher level of pre-existing CD4$^+$Foxp3$^+$ Tregs in *Chlamydia*-infected hosts may predispose the development of *Chlamydia*-associated diseases. This notion may help conciliate some observations in the field. For instance, *Chlamydia*-resistant C57BL/6 mice indeed carry significantly fewer endogenous Tregs relative to *Chlamydia*-susceptible BALB/c mice (355). Furthermore, TLR2-deficient mice—which have ~50% fewer endogenous Tregs than their wild-type C57BL/6 counterparts(211) —also develop significantly less oviduct pathology during *C. muridarum* infection compared to wild-type mice, despite having comparable courses of infection (46). A recent follow-up study indicated that IL-1β—a Th17-promoting cytokine—is the key molecule downstream of TLR2 responsible for this phenomenon (359). In our view, therefore, the magnitude of Treg responses dictates the intensity of Th17 responses as well as the extent of acute and chronic inflammation. While high levels of IL-17A are strongly implicated in tissue-damage, IL-17A can also serve essential protective functions provided that the kinetics and magnitude of its induction are appropriately regulated. Therefore, the relative abundance and/or activity of Foxp3$^+$CD25$^+$Tregs play a critical role in modulating the development of protective immunity versus immunopathology during *Chlamydia* infection.

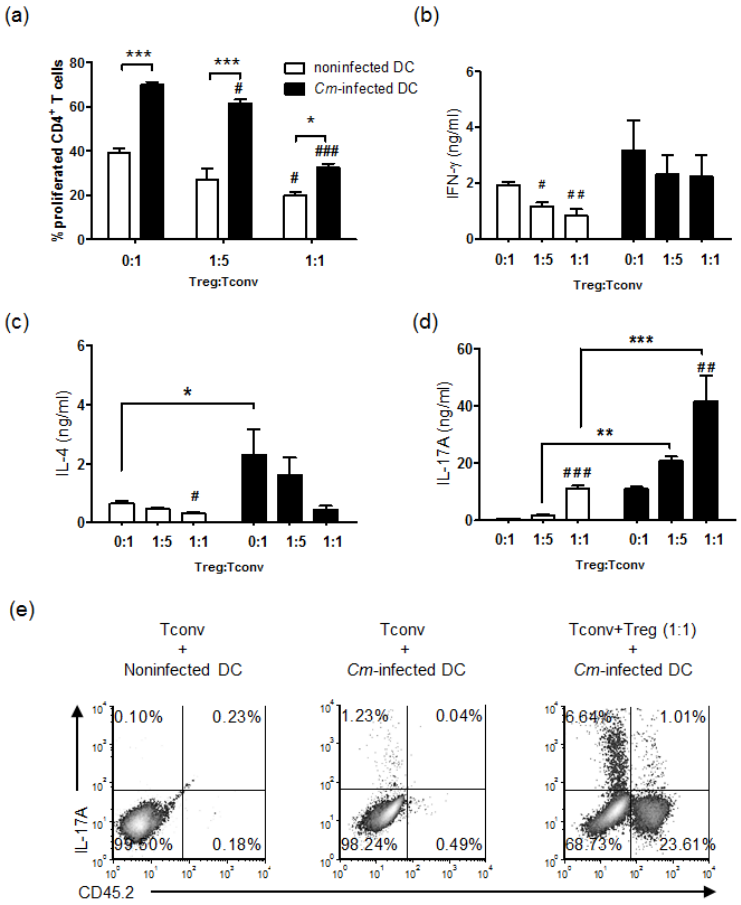

Figure 3.1 Tregs have a divergent role in regulation of Th1, Th2 and Th17 responses *in vitro* upon *C. muridarum* infection.

Purified Tconv (CD45.1) cells were labeled with eFluro670 dye, co-cultured with Treg (CD45.2) cells at different ratios, and stimulated with or without anti-CD3 in the presence of noninfected or *C. muridarum* (*Cm*)-infected BMDCs for 72 h. The percentages of Tconv proliferation were determined by flow cytometry based on intracellular eFluor670 dilution relative to non-stimulated condition (a), and the levels of IFN-γ (b), IL-4 (c) and IL-17A (d) in culture supernatants at 48 h were measured by ELISA. The IL-17A production by Tconv (CD45.1) cells and Treg (CD45.2) cells under different conditions were determined by ICCS (e). Data are presented as mean ± SEM of three to six replicates and representative of at least two independent experiments. **P*≤0.05, ***P*≤0.01, ****P*≤0.001 using two-way ANOVA test; #*P*≤0.05, ##*P*≤0.01, ###*P*≤0.001 vs. Tconv cells alone using one-way ANOVA test.

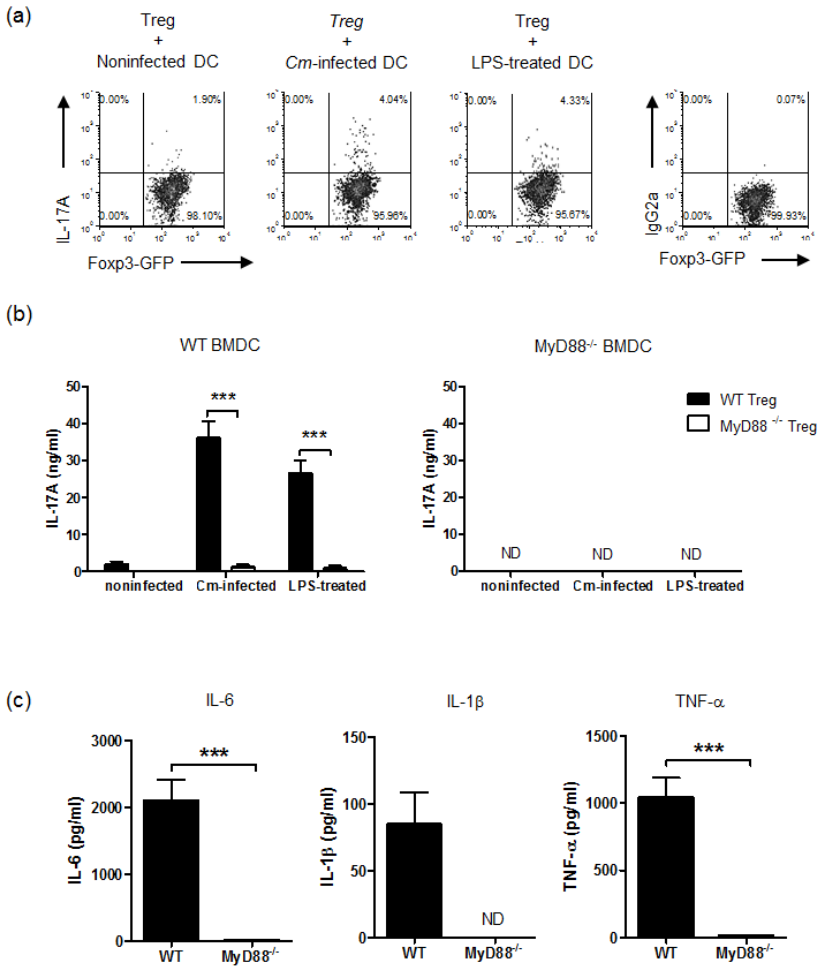

Figure 3.2 *C. muridarum* induces IL-17A production by Tregs via MyD88-dependent mechanisms *in vitro.*

Purified Foxp3-GFP Treg cells were stimulated with anti-CD3 in the presence of noninfected or *C. muridarum* (*Cm*)-infected or LPS-treated BMDCs for 48 h and IL-17A expression was determined by ICCS (a). DCs and Treg cells were prepared from wild-type (WT) or MyD88$^{-/-}$ mice and cultured in various combinations under different conditions for 48 h. IL-17A levels in supernatants derived from Treg-containing cultures (b), and IL-1β, IL-6 and TNF-α levels (c) in supernatants from non-infected, *Cm*-infected or LPS-treated DC cultures were measured by ELISA. Data are presented as mean ± SEM of three to six replicates and are representative of at least two independent experiments. ***$P \leq 0.001$ using two-way ANOVA test in (b), or Student's *t*-test in (c). ND, Not detectable.

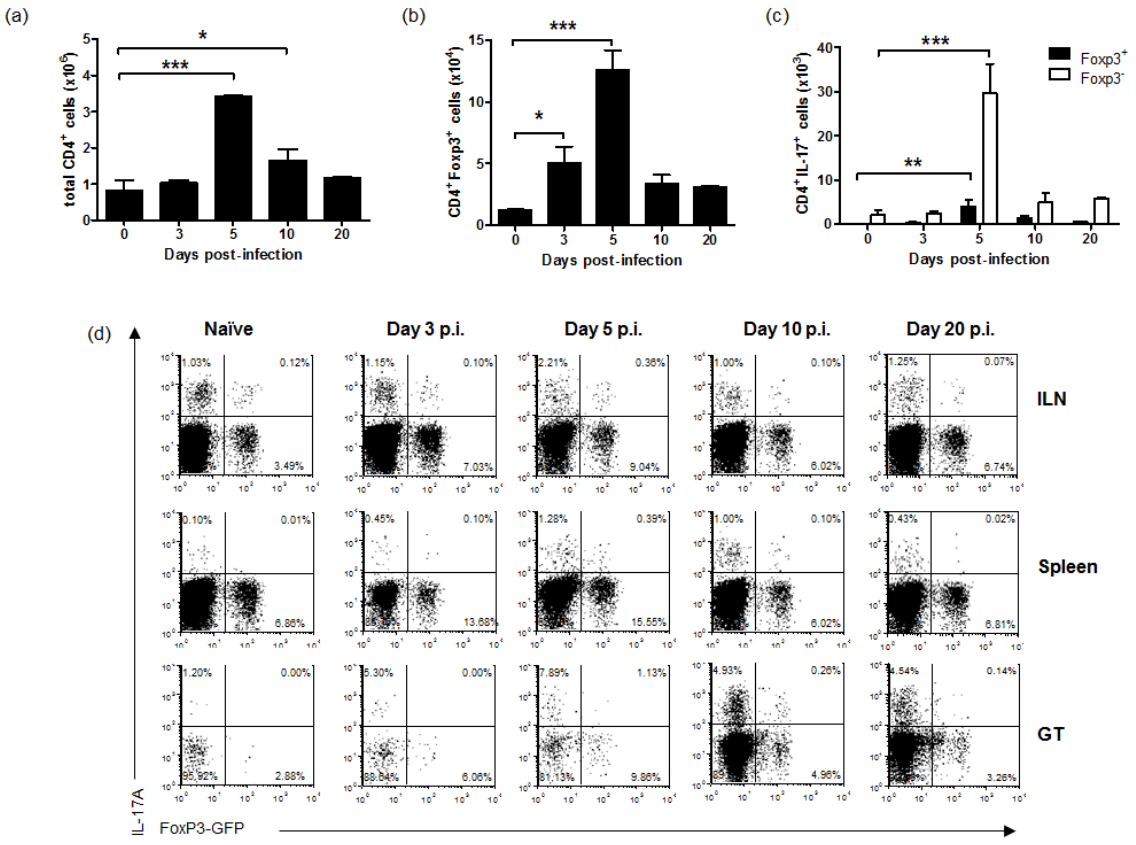

Figure 3.3 Foxp3+ Treg expansion precedes Th17 differentiation in the draining lymph nodes upon *C. muridarum* genital tract infection

Foxp3-GFP mice were intravaginally infected with *C. muridarum*. The absolute number of $CD4^+$ T cells and $CD4^+Foxp3^+$ Treg cells (n=4-10 mice/timepoint) (a, b) and the absolute numbers of $Foxp3^+$ and $Foxp3^-$ Th17 cells (n=3-5 mice/timepoint) (c) in the iliac lymph nodes (ILN) at days 0, 3, 5 and 10 post-infection were determined by intracellular IL-17A staining Data are presented as mean ± SEM and are representative of at least two independent experiments. (d) Representative dot plots of Foxp3-GFP expression and IL-17A staining on CD4-gated cells in the ILN, spleen and genital tract (GT). *$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ vs. day 0 using one-way ANOVA test. p.i., post-infection.

Figure 3.4 PC61-mediated Treg depletion attenuates Th17 responses upon *C. muridarum* genital tract infection

FoxP3-GFP mice were treated with PC61 or IgG antibody or PBS control at day -1 and infected intravaginally with *Cm* at day 0. (a) The frequencies of $Foxp3^+$ cells out of $CD4^+$ T cells in ILN of all treatment groups were determined by FACS analysis on days 0, 1, 4, 11 and 13 post-depletion. The frequencies (b) and the absolute numbers (c) of $Foxp3^+CD4^+$ cells and $Foxp3^-CD4^+$ cells in ILN of control and PC61-treated mice at day 1 post- depletion (but no infection) and day 5 post-infection were determined (n=3 mice/group). (d) Representative FACS plots of intracellular IFN-γ and IL-17A staining on CD4-gated T cells on day 5 post-infection. (e) The percentage and absolute number of Th1 and Th17 cells in the ILN of Treg-depleted and non-depleted mice at days 5 and 10 post-infection (n=5-6 mice/group/ timepoint). (f) IL-17A and IFN-γ production from *in vitro* heat-killed *C. muridarum* Ag-restimulated splenocytes at day 46 post-infection was measured by ELISA, (n=6 mice/ group). Data are presented as mean ± SEM. $*P \leq 0.05$,** $P \leq 0.01$,*** $P \leq 0.001$ using one-way ANOVA test in (a, *top panel*), or two-way ANOVA test in (a, *bottom panel*), (c), and (e), or Student's *t*-test in (f).

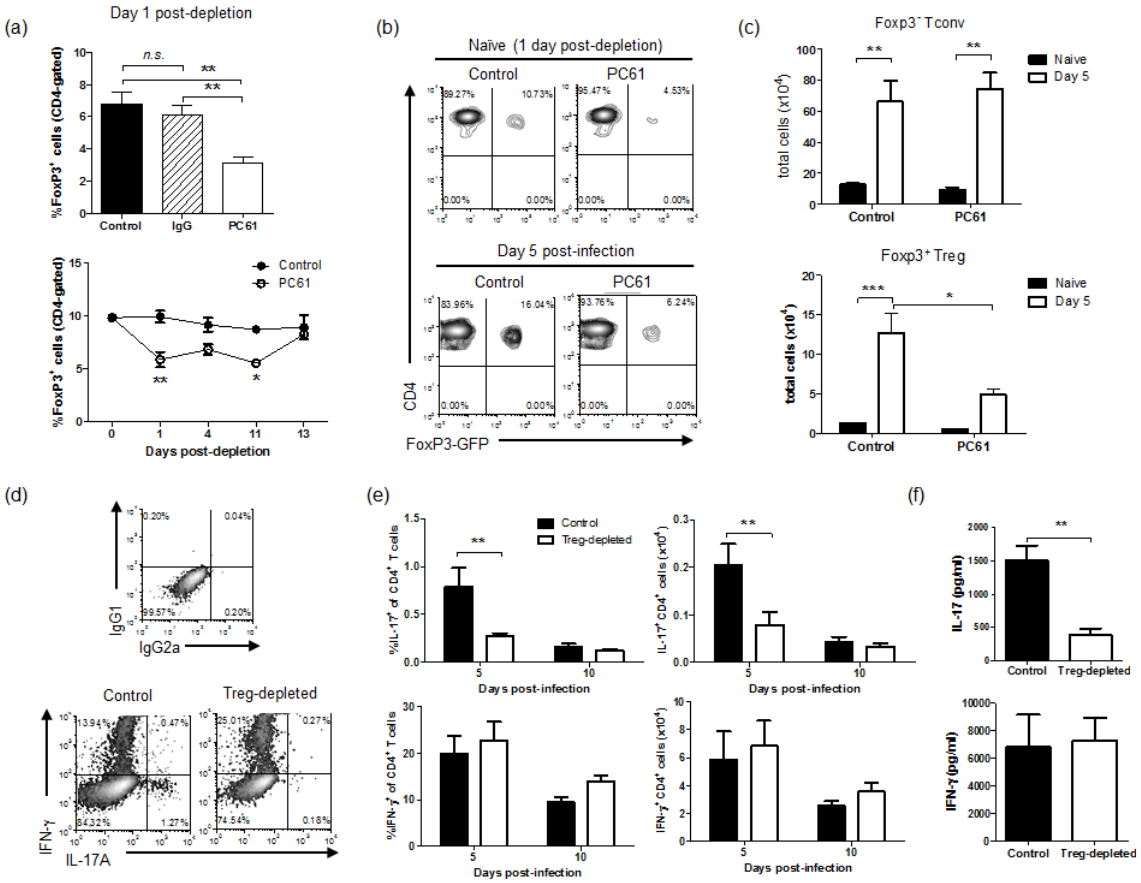
(a)
Day 1 post-depletion
%FoxP3+ cells (CD4-gated)
n.s.
**
**
Control
IgG
PC61
Control
PC61
Days post-depletion
(b)
Naïve (1 day post-depletion)
Control
PC61
Day 5 post-infection
CD4
FoxP3-GFP
(c)
Foxp3+ Tconv
total cells (x10^4)
Naive
Day 5
Foxp3+ Treg
***
*
(d)
IgG1
IgG2a
Control
Treg-depleted
IFN-γ
IL-17A
(e)
Control
Treg-depleted
%IL-17+ of CD4+ T cells
IL-17+ CD4+ cells (x10^4)
%IFN-γ+ of CD4+ T cells
IFN-γ+ CD4+ cells (x10^4)
Days post-infection
(f)
IL-17 (pg/ml)
IFN-γ (pg/ml)
Control
Treg-depleted

Figure 3.4

Figure 3.5 PC61-mediatedTreg depletion attenuates neutrophil infiltration and oviduct pathology despite unaltered *C. muridarum* genital shedding

Mice were treated with or without PC61 at day -1 and infected intravaginally with *C. muridarum* at day 0. (a) Oviducts (indicated by black arrows) from control and Treg-depleted mice at days 0, 10, and 46 post-infection were stained with H&E (n=3-4 mice/group/time point). (b) The oviduct dilation at day 46 post-infection. Data are presented as the median with upper and lower ranges. (c) *C. muridarum* shedding from the GT was measured by quantitative PCR of genital swab samples taken throughout infection. Dotted line on y-axis represents assay detection limit (n=4-19 mice/group/time point). (d) The absolute number of cells recovered in UL samples from control and Treg-depleted mice at day 0, 4 and 7 post-infection (n=3-5 mice/group/time point). (e) The frequency and total number of $CD11b^{+}Gr\text{-}1^{+}$ cells in live-gated uterine lavage samples (n=5 mice/group). (f) Representative anti-Gr-1 mAb immunohistochemical staining of oviduct tissue sections from control and Treg-depleted mice at day 10 post-infection,. Bar graph represents absolute number of $Gr\text{-}1^{+}$ cells per high power field (h.p.f.) at 400X magnification (n=3 to 4 mice/group). Data are presented as mean ± SEM. (g) Correlation analysis of IL-17A and CXCL2 levels in GT homogenates at day 5 post-infection measured by ELISA, n=11. (h) IL-17A levels in UL fluid at day 5 post-infection measured by ELISA (n=6 mice/group). Data are presented as mean ± SEM. $*P \leq 0.05$ and $**P \leq 0.01$ using Student's *t*-test in (e), (f), and (h), or Mann-Whitney *U* test in (b) or correlation analysis in (g). p.i., post-infection.

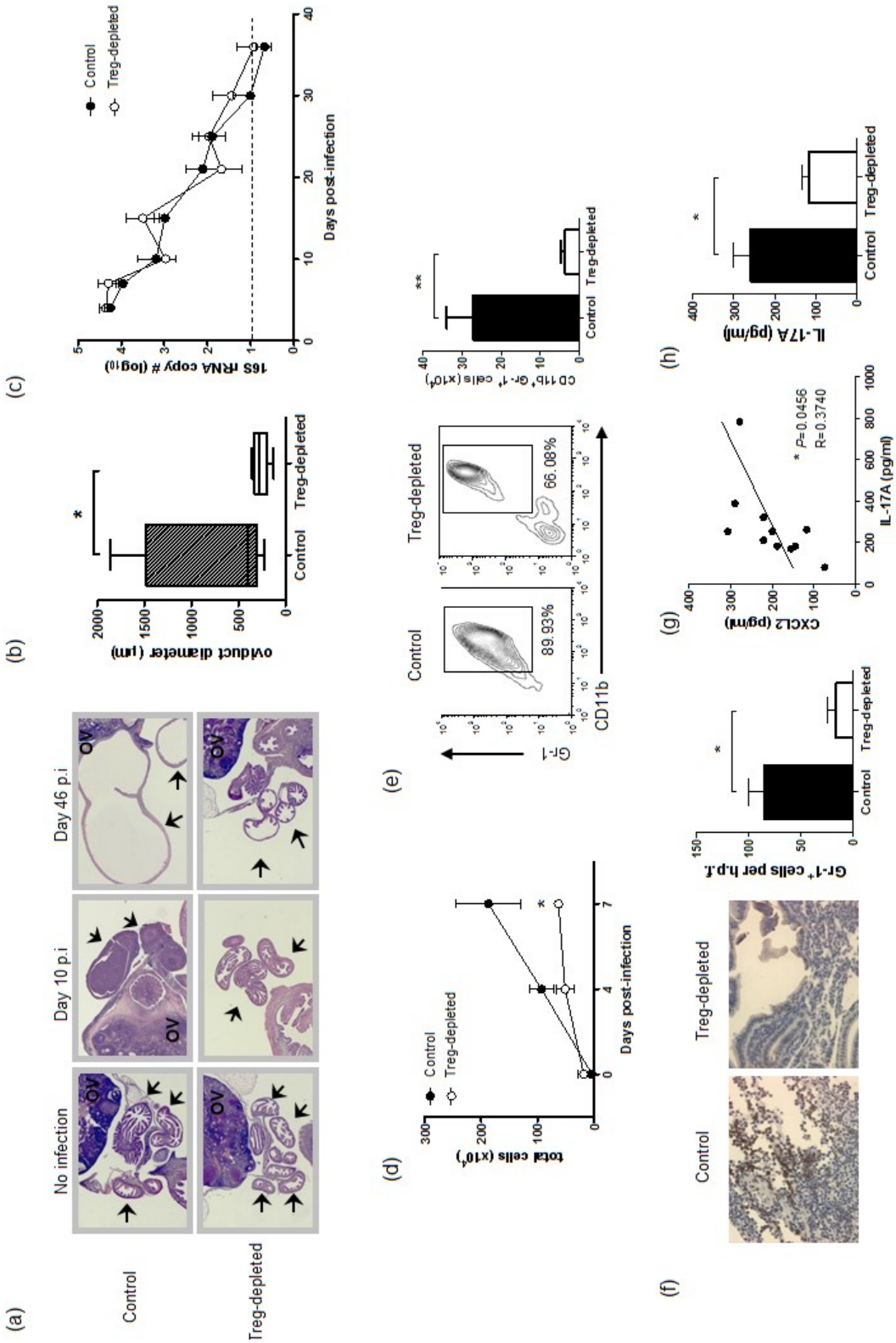
(a)
No infection
Day 10 p.i
Day 46 p.i
Control
Treg-depleted
ov
(b)
ovidact diameter (µm)
Control
Treg-depleted
(c)
16S rRNA copy # (log10)
Days post-infection
Control
Treg-depleted
(d)
total cells (x10⁴)
Days post-infection
Control
Treg-depleted
(e)
Control
Treg-depleted
89.93%
66.08%
Gr-1
CD11b
CD 11b⁺Gr-1⁺ cells (x10⁴)
(f)
Control
Treg-depleted
Gr-1⁺ cells per h.p.f.
(g)
CXCL2 (pg/ml)
IL-17A (pg/ml)
P=0.0456
R=0.3740
(h)
IL-17A (pg/ml)
Control
Treg-depleted

Figure 3.5

CHAPTER 4 CD43$^-$, BUT NOT CD43$^+$, CD1d^{hi}CD5$^+$ IL-10-PRODUCING B CELLS SUPPRESS T HELPER 1 (T_H1) RESPONSES DURING *CHLAMYDIA MURIDARUM* GENITAL TRACT INFECTION

4.1 Introduction

Chlamydia species are obligate intracellular bacterial pathogens of mucosal epithelium lining the surfaces of ocular, respiratory, and genital tracts (GT). *Chlamydia trachomatis* (*Ct*) is the most common sexually transmitted bacterial pathogen worldwide (360). While a large portion of GT infections are initially subclinical, such infections often become persistent, leading to chronic inflammation with serious long-term complications including pelvic inflammatory disease and infertility (6;9;360). Intravaginal inoculation of *Chlamydia muridarum* (*Cm*) in mice leads to tubal pathology similar to that observed in women (9).

Due to the intracellular lifestyle of *Chlamydia*, cell-mediated immune responses are important in controlling the infection. In particular, CD4$^+$ T helper 1 (Th1) cells are critical for resolving primary infection and preventing bacterial ascension into the upper GT via interferon-γ (IFN-γ) production (6;360). Mice with genetic deletions in CD4, IFN-γ or IFN-γ receptor all have profoundly impaired abilities to resolve *Chlamydia* infection and display massive inflammatory responses in the GT (96;124;361). In both mice and humans, CD4$^+$ Th1 responses are highly regulated and weakened Th1 responses commonly lead to reduced host resistance and prolonged *Chlamydia* infection (6;360;362).

B lymphocytes are essential to humoral immunity and are also important modulators of cellular responses via their interactions with dendritic cells (DCs) and T lymphocytes within lymphoid organs. In mice, three mature B subsets with different functional activities, phenotypes and/or topographic locations are identified: B1 cells (including CD5$^+$ B1a and CD5$^-$ B1b subsets), follicular B (FO) B cells and marginal zone B (MZ) B cells (273;276). Although FO and MZ B cells belong to the 'B2' lineage that

develops after B1 cells during ontogeny, these subsets have distinct functional roles. FO B cells, also called adaptive B cells, are responsible for classic T-cell-dependent B cell responses and the production of high-affinity antigen-specific antibodies. In contrast, MZ B cells, as well as B1 cells, produce low affinity IgM antibody upon microbial stimulation independently of T-cell help and are therefore termed innate-like B (ILB) cells (273;276;363-365). Whereas FO B cells comprise the vast majority of B cells in peripheral lymphoid organs (PLO), B1 cells are relatively rare in PLO but highly enriched in the coelomic cavities (275). Initial rapid T-cell independent IgM responses from ILB cells are the first line of host defense against blood-borne and mucosal pathogens (364;365). However, B cells also possess important antibody-independent functions including antigen-presentation and cytokine production, particularly production of immune suppressive cytokine IL-10 (272;273;276). Hence, B cells can positively or negatively regulate immune responses depending on the engagement of specific functions of different B cell subsets. Previous attempts to characterize the role of B cells in anti-*Chlamydia* immunity using B cell-deficient mice in models of respiratory, GT and peritoneal infection have yielded conflicting results (154;155;326;366). Although *Chlamydia* infection elicits substantial antibody responses, *Chlamydia*-specific antibodies are generally considered to provide minimal protection during primary genital infection (6;7;154;160). However, passive immunization with immune serum containing anti-*Chlamydia* antibodies is able to enhance Th1 responses and confer protection against secondary *Chlamydia* genital challenge (160;161). Despite numerous investigations examining the contribution of antibody in *Chlamydia* infection, however, the role of other functional activities of B cells in host responses to *Chlamydia* remains elusive.

Recently, regulatory B (Breg) cells have become recognized as discrete B cell entities that modulate immune responses in numerous inflammatory disease models, including systemic bacterial infection, mainly via production of IL-10 (270;272;273;275;278;309;310). Two Breg populations—$CD1d^{hi}CD21^{+}CD23^{+}$ transitional 2 (T2)-MZ precursor (289) and $CD1d^{hi}CD5^{+}$ B10 cells (296)—have been described in mice, but the relationship of these populations to conventional innate-like and adaptive B subsets is unclear. Alternatively, IL-10-producing Bregs can be classified

as 'innate type' or 'adaptive type' based upon signal(s) required for their activation/induction (272;273). Major signalling pathways involved in Breg generation include Toll-like-receptor (TLR) ligation and signals involved in adaptive immunity such as B-cell receptor (BCR) stimulation and multiple co-stimulatory signals, particularly the CD40-CD40L pathway (270;272;273). Bregs induced by TLR stimulation alone are proposed to be innate-type whereas Bregs requiring BCR and/or CD40-CD40L signals in the presence or absence of TLR stimulation are proposed to be adaptive-type (272;273). However, no definitive marker currently exists to differentiate innate- from adaptive-type and the specific role of innate versus adaptive Bregs in modulating immune responses is ambiguous.

In this study, we examined the phenotype and function of *Chlamydia*-induced IL-10-producing B cells *in vitro* and *in vivo*. We demonstrated that CD43 is a novel indicator of innate and adaptive-type IL-10-producing B cell populations activated by TLR signals alone or TLR plus DC-derived signals, respectively. Unlike $CD43^+$IL-10-producing B cells, $CD43^-$IL-10-producing B cells displayed bona fide Breg activity and potently modulate host responses to *Chlamydia* infection *in vivo*. Our data for the first time demonstrate a distinct role for $CD43^-$ adaptive Bregs over $CD43^+$ innate counterparts in controlling mucosal responses against intracellular bacterial infection.

4.2 Results

4.2.1 *C. muridarum* induces robust IL-10 production from multiple ILB cell subsets *in vitro*

To identify immune cells that produce IL-10 in response to *Chlamydia* (*Cm*), *Cm*-stimulated splenocytes from IL-10GFP mice were analyzed by FACS. Remarkably, *Cm* induced a distinct $IL\text{-}10GFP^+$ population that was comprised of >94% of $CD19^+B220^+$ B cells and ~5% of $CD11c^+$ DCs (Figure 4.1a). IL-10 production from purified B ($B220^+$) cells was significantly greater than non-B ($B220^-$) fraction at 48 h post-infection, confirming that B cells are a major source of IL-10 induced by *Cm in vitro* (Figure 4.1b).

Notably, *Cm* elicited large quantities of IL-6 in addition to IL-10 but appeared to suppress low levels of endogenous IFN-γ production from purified B cells. No detectable amounts of IL-4, IL-17A or IL-12p70 were measured (APPENDIX C1). Phenotypical analyses were conducted by comparing IL-10GFP$^+$ with IL-10GFP$^-$ B cell populations. While IL-10GFP$^-$ B cells displayed a classic IgM^{lo}IgD^{hi} phenotype of conventional FO B cells (273;276;363), IL-10GFP$^+$ B cells exhibited a striking IgM^{hi}IgD^{lo} phenotype—a characteristic feature of ILB cells, including B1 and MZ B cells (273;363). In addition, IL-10GFP$^+$ B cells displayed higher fluorescence intensities of CD1d, CD5, and CD43 relative to IL-10GFP$^-$ B cells (Figure 4.1c). While CD1d was only expressed by a small fraction (~6%) of IL-10GFP$^-$ B cells, it was generally up-regulated by all IL-10GFP$^+$ B cells (Figure 4.1d). Approximately 28% of IL-10GFP$^+$ B cells concurrently carried CD1d^{hi}CD5$^+$ B10 (Figure 4.1d) and CD43$^+$CD5$^+$ B1a phenotypes (Figure 4.1e). Thus, approximately 50% of *Cm*-induced IL-10GFP$^+$ B cells expressed CD43 and half of these CD43$^+$IL-10GFP$^+$ cells were CD43$^+$CD5$^+$CD1d^{hi} (Figure 4.1d/e). Other *Cm*-induced IL-10GFP$^+$ B populations were CD43$^-$CD1d$^{hi/lo}$ CD5$^{+/-}$ (~50%) and CD43$^+$CD1d$^{hi/lo}$ CD5$^-$ (~25%) (Figure 4.1f), indicating that *Chlamydia* induces robust IL-10 production from a heterogeneous population of ILB cells *in vitro*. Heat-killed (HK)-*Cm*, LPS and CpG also stimulated IL-10-production from B cells although a reduced potency was evident (APPENDIX C2).

4.2.2 CD43$^+$ and CD43$^-$ ILB cells have different signal requirements for IL-10 production

The concept of innate and adaptive Bregs has been proposed (272;273) so we wondered whether such distinct populations were present among *Cm*-induced IL-10-producing ILB cells. Since *Cm* induced both CD43$^+$ and CD43$^-$ IL-10-GFP$^+$ B cells (Figure 4.1e/f) and CD43 is normally expressed by B1 cells or transiently upregulated by B2 subsets during plasma cell differentiation (275;367), we analyzed IL-10GFP levels in conjunction with kinetics of B-cell proliferation and CD43 expression following *Cm* infection. A clear IL-10GFP signal was evident at 24 h post-infection but IL-10GFP$^+$ B cell proliferation was not evident until 48 h post-infection (Figure 4.2a) indicating that

while IL-10 production is independent of B cell proliferation, IL-10-producing ILB cells are nonetheless able to proliferate. We noticed that $CD43^+$ and $CD43^-$ IL-10-producing ILB cell frequencies were comparable at both 48 h (2.2% vs 3.4%) and 72 h (6.6% vs 6.6%), implying that the two populations may be generated concomitantly from distinct origins. Of interest, the proportions of $CD43^-$ IL-10-producing ILB and total IL-10-producing B cells were markedly reduced in cultures of purified B cells compared to the whole splenocyte cultures (Figure 4.2b). This indicates a requirement for other immune cells to induce IL-10 production by $CD43^-$ ILB cells. In addition, *Cm*-induced IL-$10GFP^+$ B cells appeared to proliferate more within whole splenocyte cultures than in purified B-cell cultures (Figure 4.2b), suggesting that $CD43^-$IL-$10GFP^+$ B cells may proliferate better than $CD43^+$ counterparts. Because TLR engagement is well-known to elicit IL-10-production by ILB cells, we next examined the role of TLR signalling in *Cm*-induced IL-10 B cell responses using $MyD88^{-/-}$ mice. Unsurprisingly, $MyD88^{-/-}$ B cells produced little to no IL-10 following *Cm* stimulation. Similar results were observed using whole splenocytes (Figure 4.2c), confirming that TLR signalling is essential for IL-10 production from all subsets of B cells upon *Cm* infection. DCs are known to provide important signals to B cells including CD40/CD40L interaction, which promotes B cell proliferation and survival (368). Therefore, we questioned whether the generation of $CD43^-$IL-10^+ ILB cells in whole splenocyte cultures was dependent and/or promoted by signals from DCs. To test this hypothesis, we utilized a simple DC-depletion method. Remarkably, DC-depletion markedly reduced the frequencies of IL-$10GFP^+$ B cells by ~45% (8.4% vs 4.7%) and ~80% (5.1% vs 0.9%) in *Cm*-treated and LPS-treated cultures, respectively. Reduced frequencies of IL-$10GFP^+$ B cells under both conditions were due almost entirely to elimination of the $CD43^-$IL-$10GFP^+$ B cell population (Figure 4.2d). Together, these data show that $CD43^+$ and $CD43^-$ IL-10^+ ILB cells have different signalling requirements—TLR alone versus TLR- plus DC-mediated signals—which classify these B subsets as innate and adaptive Breg populations (272;273), respectively. Regulatory B10 cells are reported to contain both $CD43^+$ and $CD43^-$ fractions (271) so we analyzed CD43 levels on IL-$10GFP^+CD1d^{hi}CD5^+$ cells in *Cm*-stimulated culture. Consistently, DC-depletion markedly attenuated generation of $CD43^-$ B10 cells, which accounted for ~58% of total $CD1d^{hi}CD5^+$ B10 cells in *Cm*-stimulated whole splenocyte

cultures (Figure 4.2d). Collectively, our data demonstrate that CD43 is a novel indicator distinguishing innate and adaptive IL-10-producing ILB populations.

4.2.3 *C. muridarum*-induced CD43$^-$ but not CD43$^+$, IL-10-producing B cells suppress CD4$^+$ T cell and IFN-γ production *in vitro*

Because Bregs are ultimately defined by their ability to suppress immune reactions (270), we next used an *in vitro* suppression assay to determine whether *Cm*-induced IL-10-producing B cells are able to suppress CD4$^+$ T cell responses. As demonstrated in Figure 4.3a, *Cm*-stimulated B cells, but not unstimulated B cells, significantly reduced CD4$^+$ T cell proliferation in a dose dependent manner with approximately 50% suppression at 1:1 ratio of B cells to responders compared to responder cells alone. Thus, *Cm* clearly induces B cells to acquire the ability to suppress CD4$^+$ T cell responses *in vitro*. Remarkably, *Cm*-stimulated B cells, but not unstimulated B cells, markedly suppressed IFN-γ production in the culture supernatants in a dose-dependent manner (Figure 4.3b). In contrast, IL-4 levels were significantly promoted by *Cm*-stimulated B cells but suppressed by unstimulated B cells in a dose-dependent manner, although overall IL-4 level are low (Figure 4.3c). Relative to Th1/Th2 cytokine profiles, B cells had only a minor effect on IL-17A/Th17 response (Figure 4.3d).

Notably, *Cm*-stimulated B cells, but not unstimulated B cells, generally increased IFN-γ levels in co-cultures compared to responder cells alone while also showing dose-dependent suppression (Figure 4.3b). To differentiate IFN-γ production by CD4$^+$ (Th1) and non-CD4$^+$ cells in this assay, we performed intracellular cytokine staining (ICCS) for IFN-γ. While *Cm*-stimulated B cells suppressed the frequency of IFN-γ$^+$ cells among total CD4$^+$ T cell in a dose-dependent manner, these cells also promoted a population of non-CD4 IFN-γ$^+$ cells over responder cells alone (Figure 4.3e). Thus, *Cm*-stimulated B cells exert potent suppressive functions on CD4$^+$ T cell proliferation and IFN-γ production by Th1 cells *in vitro*.

Given the distinct innate versus adaptive nature of $CD43^+$ and $CD43^-$ IL-10-producing ILB cells induced by *Cm in vitro*, we next sought to determine whether these subpopulations differed in function. To this end, $CD43^+$ and $CD43^-$ B subsets were sorted from *Cm*-stimulated splenocytes. For an unknown reason, we were unable to obtain a pure $CD43^+$ B cell population but experienced no difficulty obtaining highly purified $CD43^-$ B cells from the same sample. Nevertheless, we compared the suppressive activity of purified $CD43^-$ B cells to a mixture of $CD43^-$ and $CD43^+$ B cells (51.8% and 47.9%, respectively). Remarkably, $CD43^-$ B cells significantly suppressed $CD4^+$ T cell proliferation in a dose-dependent manner, with ~70% suppression at 1:1 ratio (Figure 4.3f). In contrast, mixed $CD43^{+/-}$ B cells had significantly reduced suppressive function at 1:2 and 1:1 ratios compared to pure $CD43^-$ B cells (Figure 4.3f), indicating that $CD43^-$, but not $CD43^+$, IL-10-producing B cells are a bona fide Breg population induced by *Cm*.

We further examined the role of IL-10 in the suppressive function of $CD43^-$ B cells utilizing cells derived from wild-type versus $IL\text{-}10^{-/-}$ mice. Notably, wild-type $CD43^-$ B cells significantly suppressed CD4+ T cell proliferation starting at a 1:5 ratio to responder cells, with approximately 70% suppression at 1:1 ratio (Figure 4.3g). In contrast, $IL\text{-}10^{-/-}$ $CD43^-$ B cells exhibited significantly reduced capacity to suppress responder cells at all ratios compared to wild-type $CD43^-$ B cells (Figure 4.3g). Consistent with reduced suppressive function of $IL\text{-}10^{-/-}$ $CD43^-$ B cells, IFN-γ levels were significantly greater in co-cultures containing $IL\text{-}10^{-/-}$ B cells at 1:10 and 1:5 ratios compared to wild-type controls (Figure 4.3h). Notably, both wild-type and $IL\text{-}10^{-/-}$ $CD43^-$ B cells suppressed IFN-γ levels at high densities (Figure 4.3h). Together these data show that the suppressive function of *Cm*-stimulated $CD43^-$ Bregs is largely dependent on IL-10, but a potential IL-10-independent mechanism is also evident.

4.2.4 *C. muridarum* genital tract infection induces expansion of $CD43^-$ $CD1d^{hi}CD5^+IL\text{-}10^+$ B cells

Having observed potent IL-10 production by functionally distinct $CD43^+$ and $CD43^-$ B cells in response to *Cm* stimulation *in vitro*, we next examined B cell responses to *Cm in vivo*. *Cm* infection significantly increased the number of total B cells as well as the frequency and absolute number of IL-10-producing B cells in the ILN, which all peaked at day 5 post-infection and returned to basal levels by day 10 (Figure 4.5a/b/c). Notably, IL-10 production was concurrently induced in both B and non-B cells at day 5 post-infection, with the frequency of $IL\text{-}10^+$ non-B cells being significantly greater than IL-10+ B cells (Figure 4.4b/d). Importantly, a large majority of $IL\text{-}10^+$ B cells induced in the ILN by day 3 post-infection were $CD43^-$ and this remained the dominant $IL\text{-}10^+$ B-cell phenotype in the ILN at days 5 and 10 post-infection (Figure 4.4e). Approximately 70% of IL-10-producing B cells at day 5 post-infection carried a phenotype of $CD43^-$ $CD1d^{hi}CD5^+$ consistent with a subset of B10 cells (Figure 4.4e). In contrast to observations in the ILN, total B cell numbers in the spleen remained relatively stable throughout the course of infection (Figure 4.4f). However, the number of $IL\text{-}10^+$ B cells in the spleen significantly decreased at day 5 post-infection, suggesting effluxion of $IL\text{-}10^+$ B cells from the spleen to ILN (Figure 4.4g/h). Although B cells were detected in the GT at both day 5 and day 10 post-infection, these B cells were not IL-10 producers (Figure 4.4j). Together, these data show that *Cm*-induced $CD43^-CD1d^{hi}CD5^+$ Bregs are preferentially expanded and/or recruited to draining ILN in response to *Cm* infection.

4.2.5 IL-10-deficiency in B cells markedly enhances cellular responses and host resistance against *C. muridarum* genital tract infection

Having demonstrated that $CD43^-CD1d^{hi}CD5^+$ Bregs accumulate in the ILN along with other IL-10-producing cells following *Cm* infection, we next generated mixed bone marrow chimeric mice with B cell-intrinsic IL-10 deficiency to examine the specific impact of B cell-derived IL-10 on host responses to *Cm* infection. Following 6 weeks of bone marrow reconstitution, the frequencies of B and $CD4^+$ T cells in the peripheral blood of $IL\text{-}10^{-/-}$ B and wild-type B chimeras were comparable and IL-10 production by

B cells from IL-10$^{-/-}$B chimeras was confirmed to be markedly reduced compared to wild-type B counterparts (Figure 4.5a). At day 10 post-infection, IL-10$^{-/-}$ B chimeras had markedly enhanced cellular responses in the ILN and spleen relative to wild-type B chimeras (Figure 4.5b/c). The increased cellular response in the ILN of IL-10$^{-/-}$ B chimeras was attributed to a marked expansion of B cells and a trend of increase in CD4^{+} T cell numbers (Figure 4.5b/c). Consistent with a role for Bregs in modulating Th1 responses, IL-10$^{-/-}$ B chimeras had significantly increased Th1 cell numbers in the ILN compared to wild-type B chimeras (Figure 4.5d). We noticed that IFN-γ-producing non-CD4 populations were also enhanced in IL-10$^{-/-}$ B chimeras (Figure 4.5d), supporting a role of Bregs in modulating immune cells other than CD4^{+} T cells. In comparison, Th17 responses were not significantly affected by B cell-derived IL-10, although there was a reduced trend of Th17 numbers in IL-10$^{-/-}$ B chimeras relative to wild-type B counterparts (APPENDIX C3). We further verified the critical role of B cell-derived IL-10 on Th1 responses *in vivo* using an adoptive transfer approach in B cell-deficient (μMT) mice receiving IL-10$^{-/-}$ or wild-type donor B cells (Figure 4.5e), and a B cell-depletion method with anti-CD20 monoclonal antibody (mAb) (APPENDIX C4). Collectively, data from these three distinct *in vivo* models clearly demonstrate that IL-10 produced by B cells potently regulate the magnitude of Th1 responses during GT infection.

Consistent with the increased induction of Th1 responses in the ILN, IL-10$^{-/-}$ B cell chimeric mice exhibited significantly reduced bacterial burden by day 20 post-infection compared to wild-type counterparts (Figure 4.5f). Importantly, accelerated bacterial clearance in IL-10$^{-/-}$ B cell chimeras corresponded with reduced severity of hydrosalpinx by day 30 post-infection (Figure 4.5g/h).

4.3 Discussion

The objective of this study was to examine the role of IL-10-producing B cells in host responses to *C. muridarum* infection *in vitro* and *in vivo*. We have identified CD43 as a novel cell surface marker distinguishing innate-type from adaptive-type IL-10-

producing B cells. Remarkably, *C. muridarum*-stimulated $CD43^-$, but not $CD43^+$, B cells possessed potent IL-10-dependent suppressive activity over Th1 immune responses *in vitro*. Furthermore, the majority of IL-10-producing B cells induced by *C. muridarum in vivo* carried a $CD43^-CD1d^{hi}CD5^+$ phenotype. Finally, mixed bone marrow chimeric mice with IL-10-deficiency restricted to the B cell compartment showed significantly increased Th1 responses, enhanced bacterial clearance and reduced pathology during *C. muridarum* infection relative to wild-type controls.

IL-10 is a potent immunosuppressive cytokine strongly implicated in the negative regulation of Th1 responses in *Chlamydia*-infected hosts (318). Genetic analyses on gene polymorphisms in the IL-10 promoter region in *Chlamydia*-infected individuals have established a link between allelic variants in levels of IL-10 production and the risk of developing pathological sequelae (314;369;370). Similarly, $IL\text{-}10^{-/-}$ mice are highly resistant to *Chlamydia* infection, displaying augmented Th1 responses, rapid clearance and less severe GT pathology compared to wild-type counterparts (316). However, cellular sources of IL-10 during *Chlamydia* infection are somewhat ambiguous since IL-10 is produced by a broad range of immune and non-immune cells (318;371). In a DC-based vaccination model, IL-10-deficient DCs stimulated robust protective Th1 immunity against genital *Chlamydia* infection that was superior to that induced by wild-type DCs, suggesting that endogenous IL-10 produced by DCs at the immune induction site potently affects the strength/quality of antichlamydial Th1 responses (316;372). In addition, IL-10-producing DCs were reported to be enriched in the relatively less-inflamed lower GT but not in the heavily inflamed upper GT during *Chlamydia* infection, supporting an anti-inflammatory role for IL-10-producing DCs in controlling cellular responses at the immune effector site (322). In this study, we found that $CD43^-$ $CD1d^{hi}CD5^+$ Bregs were localized to the ILN, indicating that Bregs specifically modulate immune responses in the ILN but not at the immune effector site. Notably, the total number of B cells was markedly expanded in $IL\text{-}10^{-/-}$ B chimeras at day 10 post-infection in the ILN, suggesting that IL-10 may also regulate B cell proliferation in an autocrine manner. However, it remains to be determined whether enhanced B cell proliferation contributes to accelerated bacterial clearance and reduced pathology in these mice.

The concept of innate and adaptive Breg subsets was initially proposed by Mizogouchi (272) and supported by others subsequently (271;273); however, the role of different subsets of Bregs in modulating host responses is largely under-studied. To date, the identification of Breg populations—particularly regulatory B10 cells—in many published studies requires an in vitro stimulation/amplification procedure using LPS and PMA plus ionomycin following agonistic anti-CD40 antibody pre-stimulation (271;309). This protocol is likely to amplify both innate and adaptive Bregs and, indeed, the B10 population is reported to contain both $CD43^+$ and $CD43^-$ fractions (271). Herein, we have established that splenic innate and adaptive Bregs can be identified based on their CD43 expression. We demonstrated a dramatic attenuation of $CD43^-$ IL-10-producing B cells in purified B cell cultures and DC-depleted splenocyte cultures in comparison to whole splenocyte cultures upon *C. muridarum* or LPS stimulation. While MyD88-dependent signals were required for IL-10 production by all B subsets in response to *C. muridarum*, the requirement for DC-derived signals in IL-10 production by $CD43^-$, but not $CD43^+$, B cells would classify these cells as adaptive and innate Bregs, respectively. Given that all *C. muridarum* -induced IL-10-producing B cells *in vitro* carried $IgM^{hi}IgD^{lo}$ phenotype, we infer that *Chlamydia*-induced innate and adaptive $IL\text{-}10^+$ B cells *in vitro* likely originate from B1 and MZ B cells, respectively (270;273;363). A distinct $CD43^+CD1d^{hi}CD5^+$ population accounted for 25-35% of $IL\text{-}10^+$ B cells in whole splenocyte cultures and ~60% of DC-depleted splenocyte cultures. This population phenotypically overlaps regulatory B10 and B1a cells and closely resembles a splenic $IgM^{hi}IgD^{lo}B220^+CD1d^{hi}CD43^+$ population previously described to produce IL-10 in response to a human *C. trachomatis* serovar independently of T cells *in vitro* (327). However, despite being potent IL-10-producers, the results of our in vitro suppression assay did not support a claim that *C. muridarum* -induced $CD43^+IL\text{-}10^+$ B cells are true immunosuppressive Bregs. Furthermore, although the $CD43^+CD1d^{hi}CD5^+$ population is potently induced *in vitro*, they did not appear to be major IL-10 producers during genital *Chlamydia* infection *in vivo*. We believe the limited engagement of $CD43^+IL\text{-}10^+$ B cells *in vivo* could be attributable to the mucosal route of *C. muridarum* infection since B1 cells are extremely scarce or absent from the GT and ILN. Therefore, the *in vivo* role of

$CD43^+CD1d^{hi}CD5^+$ IL-10-producing B cells to *Chlamydia* infection may require different infection models for investigation.

In contrast, $CD43^-CD1d^{hi}CD5^+$ B cells were found to preferentially expand in the ILN upon *C. muridarum* GT infection. Importantly, $CD43^-IL\text{-}10^+$ B cells demonstrated a potent suppressive activity in our *in vitro* suppression assay, supporting that these cells are a bona fide Breg population. *In vitro*, the generation of $CD43^-IL\text{-}10^+$ B cells was fundamentally different from that of $CD43^+IL\text{-}10^+$ B cells in that it required the presence of DCs. The activation/induction of $CD43^-$ Bregs in vivo likely involves TLR-mediated signals, with DCs providing additional stimulation including CD40 ligation. Direct B-DC interaction has been shown to be necessary for B cell proliferation and survival (368). Both membrane bound molecules (e.g. CD40) and soluble molecules such as IFN-β produced by DCs are reported to promote Breg differentiation (291). Activated T cells may supply additional signals, which would be attenuated in our DC-depleted in vitro cultures but available in *in vivo* settings. Cytokines produced by activated T cells and myeloid and/or stromal cells such as IL-21 and BAFF (B cell-activating factor), respectively, can also promote Breg differentiation and expansion (301;373). The *in vivo* kinetics of IL-10-producing B cells in the ILN and spleen during infection suggest that $CD43^-$ Bregs were mobilized from spleen to the ILN in response to *C. muridarum* GT infection. However, the signal(s) required for possible Breg migration from the spleen to ILN is unclear.

Collectively, our data demonstrate for the first time a distinct role for $CD43^-CD1d^{hi}CD5^+$ adaptive Bregs over $CD43^+$ innate-type counterparts in controlling mucosal immune responses. It is also the first report that B cells are a key cellular source of counter-regulatory IL-10 produced during *C. muridarum* infection and potently suppress protective antichlamydial host Th1 responses *in vivo*.

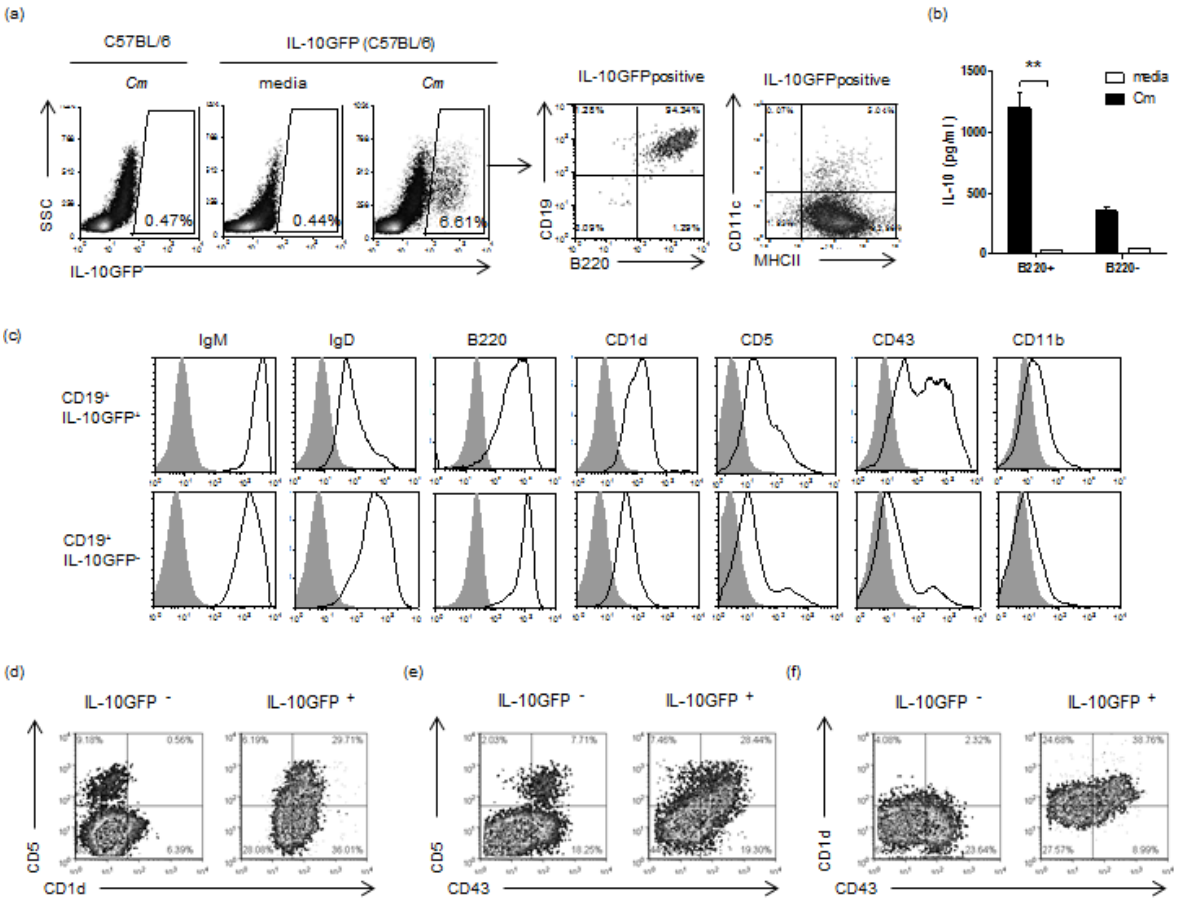

Figure 4.1 *C. muridarum* induces robust IL-10-production from multiple innate-like B (ILB) cell subsets *in vitro*

(a) Splenocytes derived from IL-10-GFP or C57BL/6 mice were stimulated with *C. muridarum* (MOI 0.1) for 48 h *in vitro*. IL-10GFP$^+$ cells were analyzed by flow cytometry. (b) IL-10 levels were measured by ELISA in culture supernatants of B220$^+$ and B220$^-$ fractions of C57BL/6 splenocytes 48 h p.i. Data are presented as mean ± SEM of triplicate wells and are representative of two independent experiments. $^{**}P \leq 0.01$ using two-way ANOVA test. (c-f) Representative FACS plots of surface marker expression of IL-10GFP$^+$ and IL-10GFP$^-$ CD19-gated B cells. Filled histogram represents isotype control. Data are representative of three independent experiments.

Figure 4.2 DC-derived signals are critically required for IL-10 production from CD43$^-$, but not CD43$^+$, ILB cells upon *C. muridarum* or LPS stimulation

eFluor670 dilution-based proliferation rate and CD43 expression by CD19$^+$IL-10GFP$^+$ B cells at 0, 24, 48, and 72 h post-infection (p.i.) with live *C. muridarum* (*Cm*). Values represent percentage out of CD19-gated cells. (b) Frequency and phenotype of IL-10GFP$^+$ B cells in cultures of purified B cells alone or whole splenocytes 72 h p.i. Values in bar graph represent mean ± SEM of triplicate samples. Histogram shows eFluor670 dilution-based proliferation rate of IL-10GFP$^+$CD19$^+$ B cells from purified B cell or whole splenocyte cultures 72 h p.i. (c) IL-10 levels measured by ELISA in culture supernatants of purified B cells or whole splenocytes from wild-type or MyD88$^{-/-}$ mice at 0, 24, 48 and 72 h p.i. Data are presented as mean ± SEM of triplicate wells. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ using two-way ANOVA test. (d) Frequency and phenotype of IL-10GFP$^+$ B cells in whole splenocyte or CD11c$^+$ DC-depleted splenocyte cultures 48 h p.i. or treatments with LPS.

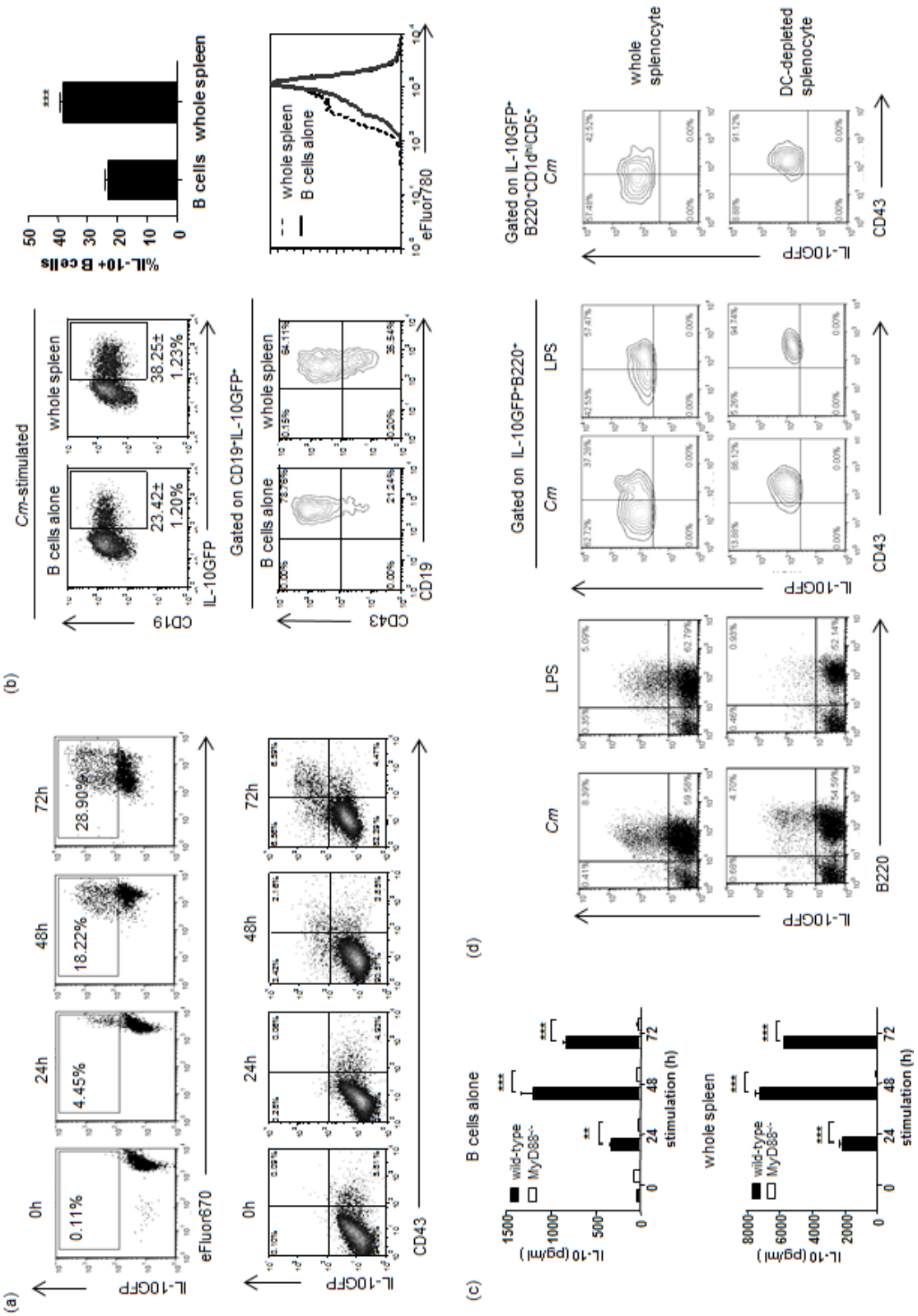
(a)
0h
24h
48h
72h
IL-10GFP
eFluor670
0.11%
4.45%
18.22%
28.90%
CD43
CD19
(b)
Cm-stimulated
B cells alone
whole spleen
23.42± 1.20%
38.25± 1.23%
IL-10GFP
Gated on CD19+IL-10GFP+
%IL-10+ B cells
B cells whole spleen
eFluor780
(c)
B cells alone
whole spleen
wild-type
MyD88-/-
IL-10 (pg/ml)
stimulation (h)
(d)
Gated on IL-10GFP+B220+
Cm
LPS
B220
Gated on IL-10GFP+ B220+CD1dhiCD5+
whole splenocyte
DC-depleted splenocyte

Figure 4.2

Figure 4.3 *C. muridarum*-induced CD43$^-$ B cells suppress CD4$^+$ T cell proliferation via IL-10-dependent and IL-10-independent mechanisms *in vitro*.

B cells from C57BL/6 mice were pre-stimulated with heat-killed *C. muridarum* (*Cm*) for 24 h, irradiated and then co-cultured at 1:1, 1:2, 1:5 or 1:10 ratio with eFluor670-labelled OTII splenocytes with or without anti-CD3 for 72-90 h. (a) % of CD4$^+$ T cell proliferation in the presence of different densities of unstimulated or *Cm*-stimulated B cells. Data are pooled from two individual experiments and presented as mean ± SEM of 5 mice for *Cm*-stimulated B cells. Unstimulated B cells were derived from one mouse. *$P\leq0.05$, *** $P\leq0.01$ versus responder cells alone using one-way ANOVA test. (b) IFN-γ, (c) IL-4 and (d) IL-17A levels measured by ELISA from co-culture supernatants. Data are presented as mean ± SEM of triplicate wells from one representative experiment.* $P\leq0.05$, ** $P\leq0.01$, *** $P\leq0.001$ versus responder cells alone using one-way ANOVA test and, for IFN-γ ELISA data, #$P\leq0.05$ versus 1:10 using one-way ANOVA test. (e) Frequency of IFN-γ producing CD4$^+$ and CD4$^-$ cells in co-cultures determined at 90 h post-stimulation. (f) % of CD4$^+$ T cell proliferation and % of suppression in the presence of different densities of *Cm*-stimulated CD43$^-$ B cells or CD43$^{+/-}$ B cells. Data are presented as mean ± SEM of 3 replicates. * $P\leq0.05$, ** $P\leq0.01$, *** $P\leq0.001$ versus responder cells alone using one-way ANOVA test. *** $P\leq0.001$ using two-way ANOVA test for percent suppression. (g) % of CD4$^+$ T cell proliferation and % of suppression in the presence of *C. muridarum*-stimulated CD43$^-$ B cells from wild-type or IL-10$^{-/-}$ mice. Data are pooled from two independent experiments and presented as mean ± SEM of 6 replicates. * $P\leq0.05$, *** $P\leq0.001$ versus responder cells alone for each group using one-way ANOVA test. * $P\leq0.05$, ** $P\leq0.01$, *** $P\leq0.001$ using two-way ANOVA test for percent suppression. (h) IFN-γ levels measured by ELISA from co-culture supernatants described in (f). Data are presented as mean ± SEM of triplicate wells from one representative experiment.* $P\leq0.05$ using two-way ANOVA test. #$P\leq0.05$, ## $P\leq0.01$ versus 1:10 using one-way ANOVA test.

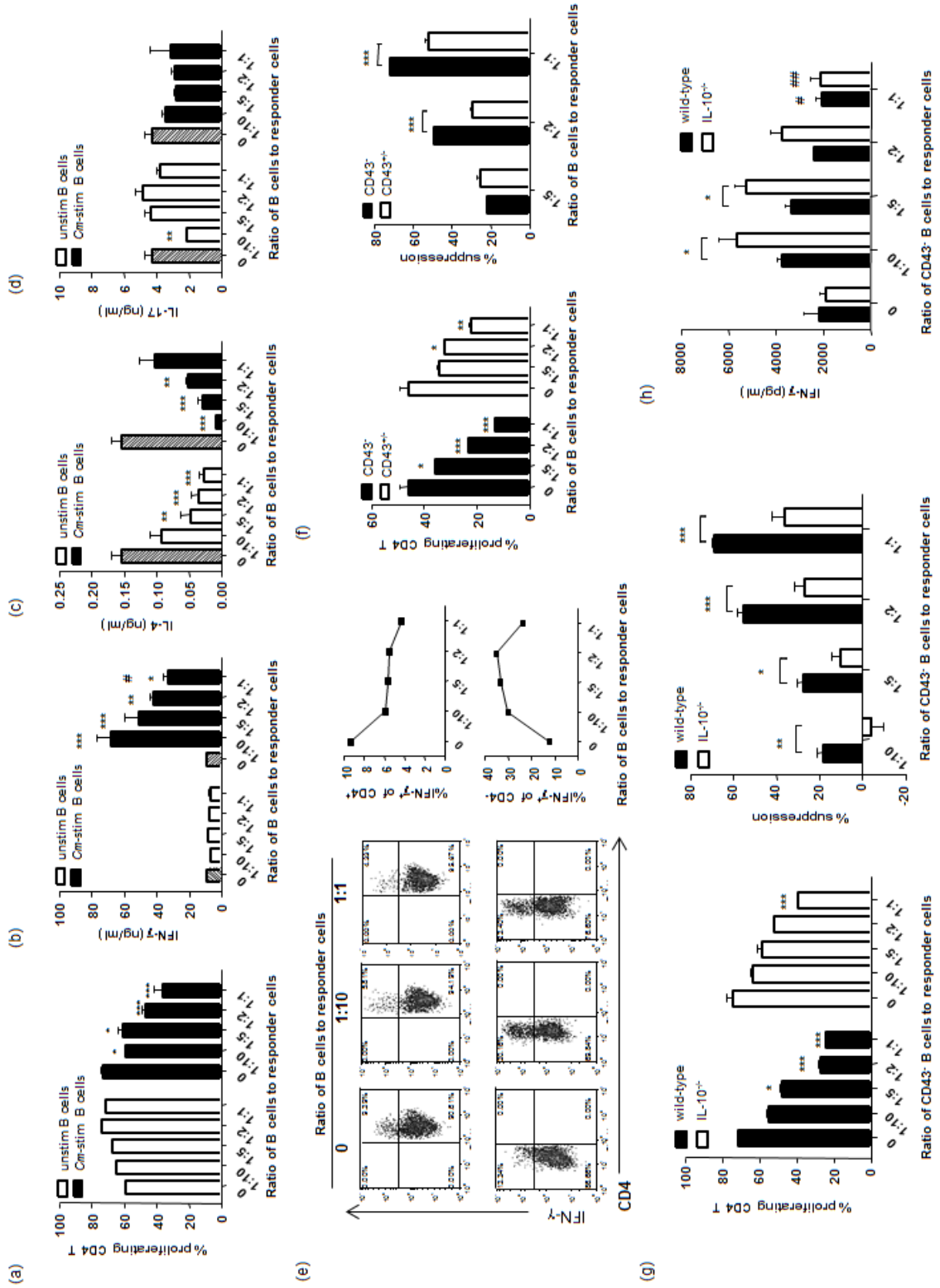
(a)
% proliferating CD4 T
unstim B cells
Cm-stim B cells
Ratio of B cells to responder cells
(b)
IFN-γ (ng/ml)
(c)
IL-4 (ng/ml)
(d)
IL-17 (ng/ml)
(e)
Ratio of B cells to responder cells
0
1:10
1:1
IFN-γ
CD4
%IFN-γ+ of CD4+
%IFN-γ+ of CD4-
(f)
% proliferating CD4 T
CD43-
CD43+/-
% suppression
(g)
wild-type
IL-10-/-
Ratio of CD43- B cells to responder cells
(h)
IFN-γ (pg/ml)

Figure 4.3

Figure 4.4 *C. muridarum* genital tract infection induces expansion of CD43$^-$IL-10$^+$ B cells in the ILN but not in the genital tract.

C57BL/6 mice were intravaginally infected with *C. muridarum* and sacrificed at various time points (n=6 mice per time point). (a, b, d, e) The absolute number of total B cells, the frequency of IL-10$^+$ of total B cells, and the absolute number of IL-10$^+$ B cells and IL-10$^+$ non-B cells, as well as the frequency of CD43$^+$ versus CD43$^-$ IL-10$^+$ B cells in the ILN were determined on days 0, 3, 5 and 10 post-infection (p.i.) (c) Representative dot plots of IL-10 expression in CD19$^+$ B cells and CD19$^-$ non-B cells in the ILN on days 0, 3, 5 and 10 p.i by ICCS. Dot plots in bottom panel show CD19 and CD43 expression by IL-10$^+$CD19$^+$-gated cells. Values in quadrants represent percent of gated cells. (f-g) The absolute number of total B cells and IL-10$^+$ B cells as well as IL-10$^+$ non-B cells in the spleen on days 0, 3, 5 and 10 p.i. (h) Representative dot plots of IL-10 expression in CD19$^+$ B cells and CD19$^-$ non-B cells in the spleen on days 0, 3, 5 and 10 p.i by ICCS. (i) Isotype control for IL-10 staining in spleen and ILN samples. (j) IL-10 expression by CD19$^+$ B cells on days 5 and 10 p.i in the genital tract (GT) cells by ICCS. *$P \leq 0.05$ versus day 0 using one-way ANOVA test in panels a/b; ###$P \leq 0.001$ versus day 0 using one-way ANOVA test in panels d; *$P \leq 0.05$, **$P \leq 0.01$ using two-way ANOVA test in panels d/g.

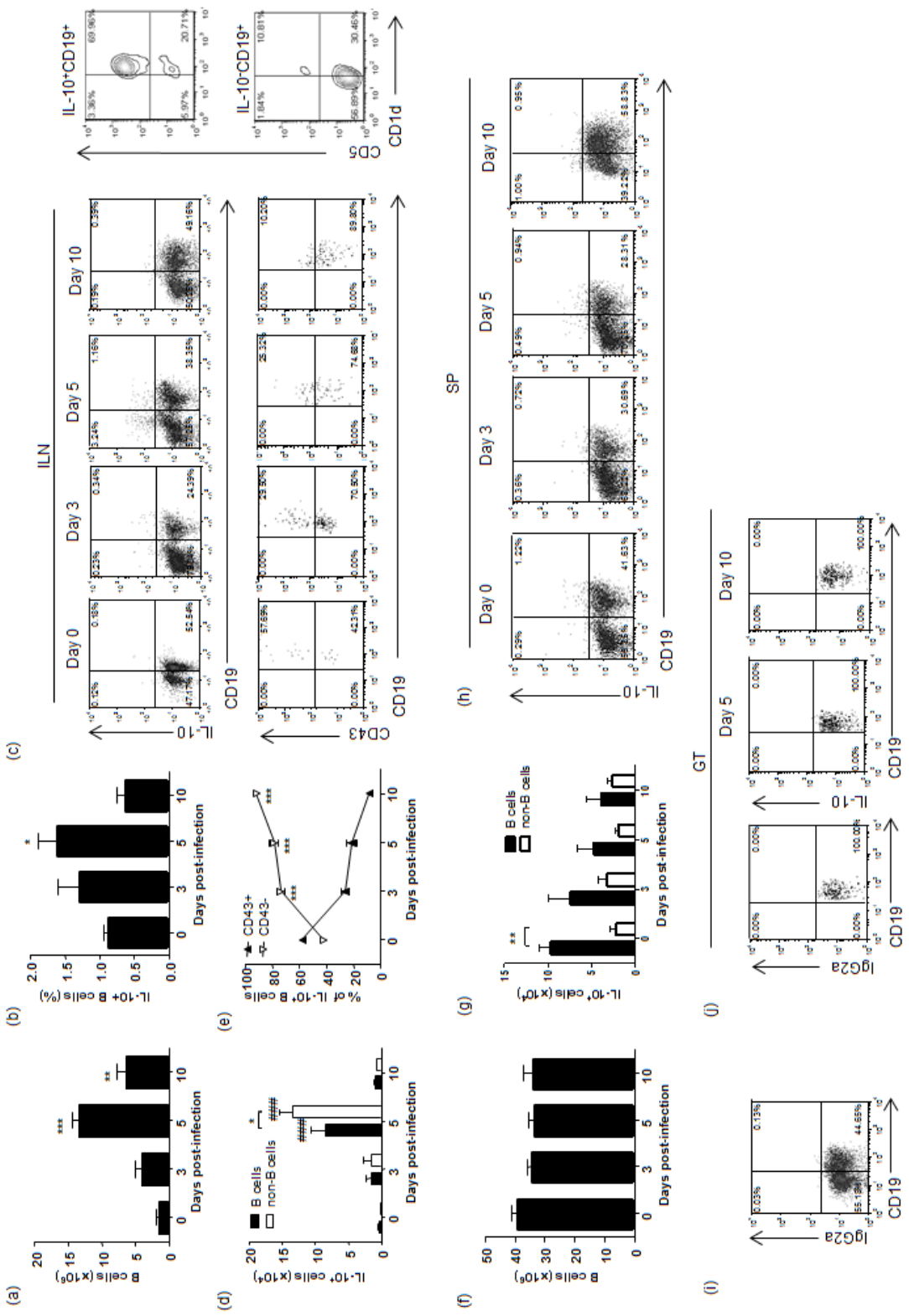
(a)
B cells (x10⁶)
Days post-infection
(b)
IL-10+ B cells (%)
Days post-infection
(c)
ILN
Day 0
Day 3
Day 5
Day 10
IL-10
CD19
CD43
IL-10+CD19+
IL-10-CD19+
CD5
CD1d
(d)
IL-10+ cells (x10⁴)
B cells
non-B cells
Days post-infection
(e)
% of IL-10+ B cells
CD43+
CD43-
Days post-infection
(f)
B cells (x10⁶)
Days post-infection
(g)
IL-10+ cells (x10⁴)
B cells
non-B cells
Days post-infection
(h)
SP
Day 0
Day 3
Day 5
Day 10
IL-10
CD19
GT
(i)
IgG2a
CD19
(j)
Day 5
Day 10
IL-10
IgG2a
CD19

Figure 4.4

Figure 4.5 IL-10-deficiency in B cells markedly enhances cellular responses and host resistance against *C. muridarum* genital tract infection.

(a) Characterization of frequency of $B220^+$ and $CD4^+$ cells in peripheral white blood cells (wbcs) and IL-10-producing B cells in the spleen of wild-type B and IL-$10^{-/-}$ B chimeras at day -1 (n=4-6 per group). (b/c) The absolute numbers of total cells, and the frequency and absolute number of $B220^+$ and $CD4^+$ cells recovered from ILN (b) and spleen (c) of wild-type B and IL-$10^{-/-}$ B chimeras at day 10 post-infection. Data are pooled from two independent experiments and presented as mean±SEM of 6-9 mice per group.* $P \leq 0.05$, *n.s*. = no significance using Student's *t*-test. * $P \leq 0.05$,** $P \leq 0.01$ using two-way ANOVA test. (d) The total number and frequencies of IFN-γ^+CD4^+ and IFN-γ^+CD4^- cells in the ILN of wild-type B versus IL-$10^{-/-}$ B chimeras at day 10 post-infection (n=6-9 mice per group). (e) The absolute numbers of total cells, frequency of IFN-γ^+ among $CD4^+$ T cells, and absolute number of total IFN-γ^+CD4^+ cells recovered the ILN (top panels) and spleen (bottom panels) of B cell-deficient (μMT) recipients of wild-type or IL-$10^{-/-}$ B cells at day 10 post-infection (n=4 mice per group). * $P \leq 0.05$ using one-tailed Student's *t*-test in panels d/e. (f) Levels of *C. muridarum* in genital swab samples from wild-type B and IL-$10^{-/-}$ B chimeras were quantified. (n=4-8 mice per group per timepoint). * $P \leq 0.05$ using two-way ANOVA test. (g-h) Oviducts from wild-type B and IL-$10^{-/-}$ B chimeras at day 30 p.i. were stained with H&E and oviduct dilation was measured (n=10-11 mice per group). Data are presented as box whiskers plot: line within box represents median, box limits 25 and 75 percentiles, whiskers extend to the minimum and maximum values. * $P \leq 0.05$ using Mann-Whitney *U*-test.

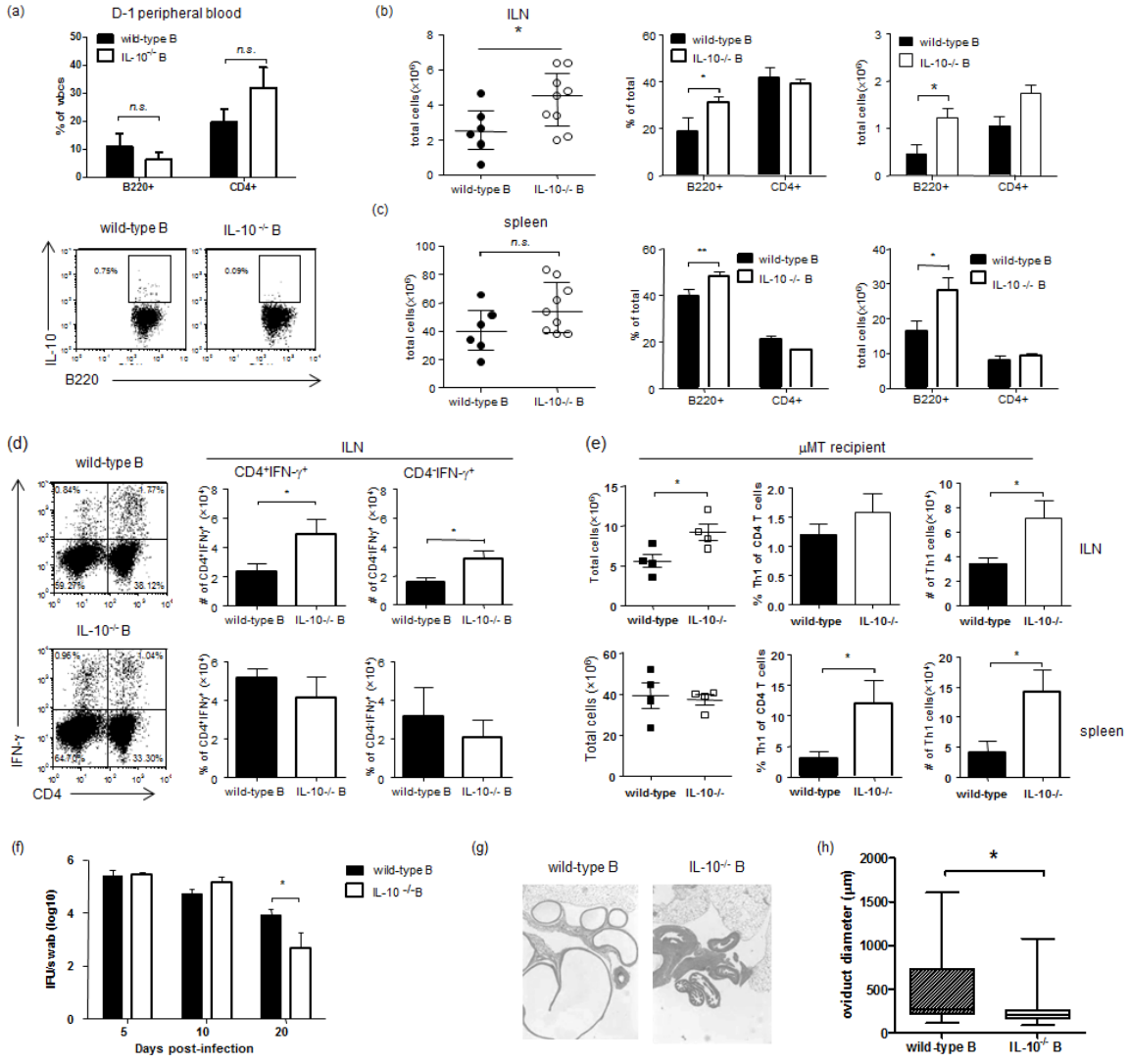
(a)
D-1 peripheral blood
wild-type B
IL-10-/- B
% of vbcs
n.s.
B220+
CD4+
wild-type B
IL-10-/- B
0.75%
0.09%
IL-10
B220
(b)
ILN
*
total cells(x10⁶)
wild-type B
IL-10-/- B
% of total
total cells(x10⁶)
(c)
spleen
n.s.
**
(d)
wild-type B
IL-10-/- B
CD4+IFN-γ+
CD4-IFN-γ+
# of CD4+IFNγ+ (x10⁴)
% of CD4+IFNγ+ (x10⁴)
IFN-γ
CD4
(e)
μMT recipient
Total cells(x10⁶)
% Th1 of CD4 T cells
# of Th1 cells(x10⁴)
wild-type
IL-10-/-
ILN
spleen
(f)
IFU/s wab (log10)
Days post-infection
(g)
(h)
oviduct diameter (μm)

Figure 4.5

CHAPTER 5 DISCUSSION

5.1 Summary of major findings

The current study offers important insight into the immunological mechanisms mediating pathological versus protective responses to *Chlamydia* GT infection. Herein we have demonstrated that the balance of Th17 and Th1 responses is a critical factor in the development of inflammatory pathology during *Chlamydia* infection. Importantly, we have also determined that regulatory T and B cells play distinct roles in regulating Th17 and Th1 responses, respectively.

In contrast to the classic immunosuppressive role of Tregs, we found that Tregs potentiated proinflammatory Th17 responses to *Chlamydia*. Depletion of pre-existing Tregs *in vivo* dramatically reduced Th17 cell numbers and oviduct pathology but did not impact Th1 responses or bacterial clearance during GT infection. We discovered that *Chlamydia* induces IL-10-producing B cells with regulatory function *in vitro* so we next investigated the role of these cells in host responses to infection *in vivo*. Using a bone marrow chimeric mouse model of B cell-intrinsic IL-10-deficiency, we determined that B cell-derived IL-10 is a potent negative regulator of Th1 responses to *Chlamydia*. Mice deficient in B cell-derived IL-10 exhibited enhanced Th1 responses and more rapid bacterial clearance as well as reduced oviduct pathology, although Th17 responses were not significantly affected.

Collectively, these findings constitute a significant advance in our understanding of the impact of regulatory T and B cell activity on Th17 and Th1 responses and, ultimately, the immunological mechanisms governing the outcome of *Chlamydia* genital tract infection. A schematic model is summarized in Figure 5.1.

5.2 Implications and relevance of major findings

5.2.1 Importance of Th1/Th17 balance in *Chlamydia*-induced pathogenesis

This work significantly advances our understanding of the role of Th17 responses in *Chlamydia*-induced immunopathology. Previous attempts to characterize the role of Th17 cells in *Chlamydia* infection using antibody-mediated neutralization of IL-17A or IL-17AR-deficient mice have been confounded by the existence of multiple cellular sources of IL-17A at different stages of infection (138;374). IL-17A production is not restricted to Th17 cells and can occur from numerous innate cell types, including γδ T cells, invariant NKT cells and neutrophils (375). Innate production of IL-17A mediates early neutrophil recruitment and primes Th1 responses in response to *Chlamydia* respiratory infection and is required for adequate bacterial clearance (135;136). Consistent with data from the respiratory infection model, IL-17R-deficient mice exhibit impaired IFN-γ/Th1 responses and reduced neutrophil recruitment during *Chlamydia* GT infection, although these mice display bacterial clearance and oviduct pathology comparable to wild-type controls (133). While this might suggest that IL-17A is dispensable for both protective and pathological responses, it is important to note that IL-17R-deficient mice show an abnormal influx of activated macrophages and TNF-α and IL-6 production in the GT that may be induced to compensate for comprised Th1 responses (133). Because IL-17A appears to bridge innate and adaptive immune responses to *Chlamydia* by supporting Th1 immunity, it has been unclear how Th17 cells are involved in either protective or pathological responses to the infection. Our Treg depletion model provided a unique context for evaluating the role Th17 responses because Th1 responses and bacterial clearance were not significantly affected by depletion. Using this model, we determined that Th17 responses are directly associated with neutrophilic inflammation and pathology in the *Chlamydia*-infected GT.

Chlamydia infections in the GT mucosa cause the release of mediators that promote the influx of inflammatory cells, causing damage to host epithelium (38). It is likely that IL-17A derived from *Chlamydia*-induced Th17 cells promotes successive waves of neutrophil recruitment, extending the intensity and duration of inflammatory

responses (Figure 5.1a). Indeed, the level of acute inflammation induced by *Chlamydia* is frequently judged to be disproportionate to the degree of infectious involvement of local tissues—histological studies report that while relatively few epithelial cells contain chlamydial inclusions, the infection induces a vigorous infiltration of neutrophils that cause large sheets of noninfected epithelia to be sloughed off and/or destroyed (9;80;376). Consistent with the notion that Th17 responses may augment infection-induced inflammation, IL-17A has been shown to synergistically enhance neutrophil recruitment when combined with proinflammatory mediators such as TNF-α (377), which is strongly upregulated in the GT of *Chlamydia*-infected mice (32;69). Intriguingly, it has been proposed that neutrophils may promote *Chlamydia* colonization of the upper GT by causing infected epithelia to slough off into the uterus and subsequently move to upper GT tissues, releasing chlamydial EBs and establishing new infectious foci (80). Neutrophils also appear to play a role in liberating EBs from infected cells during the terminal stages of the chlamydial development cycle (75). However, two independent studies report that neutrophil depletion by antibodies—anti-Ly6G (clone 1A8) or anti-Ly6G/C (clone RB6-8C5)—does not significantly affect the course of *Chlamydia* GT infection (74;77). Nonetheless, these results may be confounded by the inability of 1A8 to deplete immature $Ly6G^{low/int}$ neutrophils from the oviduct (77) and by the collateral depletion of non-neutrophil $Ly6C^{+}$ populations by RB6-8C5 (378). It is currently unclear whether neutrophils intensify chlamydial burden in the upper GT but this activity may represent a parallel mechanism through which IL-17A/Th17 responses promote oviduct inflammation and tissue pathology.

Our observation that B cell IL-10-deficient chimeras exhibited reduced oviduct pathology despite displaying Th17 induction comparable to controls highlights the importance of bacterial burden as the impetus for Th17-elaborated inflammation. Ongoing *Chlamydia* infection may also enhance the pathogenicity of *Chlamydia*-induced Th17 cells by providing continued innate cell-derived IL-23 production, which is crucial for the stability and pathogenic effectiveness of Th17 cells. In addition, *Chlamydia*-infected epithelial cells and monocytes produce prostaglandin E_2 (PGE_2) (379;380), which has been shown to favor IL-23 production by DCs and facilitate IL-23-induced

Th17 expansion (381). Increased Th1 responses such as we observed in IL-10$^{-/-}$ B chimeric mice most likely curbed the impact of Th17-mediated pathological responses by reducing bacterial burden and limiting the course of infection (Figure 5.1a). Our data indicate that, through distinct mechanisms, Treg depletion and B cell IL-10-deficiency both skewed the balance of Th1/Th17 responses to *Chlamydia* toward relatively higher Th1 and reduced oviduct pathology. Together, these observations support a model in which the balance of Th1 and Th17 responses ultimately regulates the outcome of *Chlamydia* GT infection (Figure 5.1b).

5.2.2 Tregs as a host factor in susceptibility to *Chlamydia*-induced pathology

Our data indicate that the magnitude of Th17 responses induced upon *Chlamydia* infection corresponds with the level of pre-existing Tregs within the host, suggesting that endogenous Tregs may be a contributing factor in susceptibility to *Chlamydia*-caused immune pathology. Consistent with this view, *Chlamydia*-susceptible BALB/c mice have an almost two-fold greater number of CD4^{+}CD25^{+} Tregs than C57BL/6 mice (355) and also develop greater Th17 responses to *Chlamydia* respiratory infection than C57BL/6 mice (137). However, there are numerous genetic influences that may contribute to susceptibility among mouse strains and it remains to be determined whether endogenous Treg levels are a dominant factor. This may be a highly relevant consideration for the study of susceptibility to *Chlamydia* within human populations; endogenous Treg levels can vary considerably among women, with frequencies of peripheral CD4^{+}CD25^{+} Tregs in non-pregnant women recently reported to range from 2.3-8.0%, with the median being 4.4% (382).

Because Tregs do not promote Th17 differentiation in the absence of *Chlamydia* (APPENDIX B4), bacterial stimulation appears to be critical to the potential pathological role of Tregs. TLR2 has a recognized pathological role in *Chlamydia* GT infection in mice (46;383) and increasing evidence from human studies indicates that enhanced TLR2 signaling predisposes women to *C. trachomatis*-induced GT pathology. Genetic polymorphisms in both TLR1 and TLR4 correspond with increased risk of *C.*

trachomatis upper GT infection, but only variants in TLR1 (which heterodimerizes with TLR2) were associated with infertility among *Chlamydia*-infected women (384). Furthermore, expression levels of TLR2 in cervical monocytes from women with *Chlamydia*-induced fertility disorders are significantly higher than in women with asymptomatic *Chlamydia* infections or healthy controls, (385). In light of the information presented in our study, the deleterious role of TLR2 in the GT of *Chlamydia*-infected hosts may be associated with Th17 differentiation. TLR2-deficient mice exhibit reduced oviduct pathology during *Chlamydia* GT infection and this is associated with decreased innate cell production of TNF-α, IL-6, and IL-1β (46), which are Th17-promoting cytokines. The critical role of IL-1β in early Th17 differentiation (110) may, in part, account for the prominent deleterious role of IL-1β and caspase activation in oviduct pathology during *Chlamydia* infection (63;89). Mice deficient in TLR2 also have ~50% fewer peripheral $CD4^+CD25^+$ cells, strongly indicating that TLR2 regulates the expansion and maintenance of Treg populations (212). Together these observations suggest that TLR2 signaling could promote Th17 responses to *Chlamydia* by generating a polarizing cytokine environment and by expanding and/or maintaining $CD4^+CD25^+$ Treg populations that support Th17 differentiation.

5.2.3 IL-10^+ Bregs regulate protective Th1 immunity to *Chlamydia* GT infection

In this work we have identified a previously undescribed role for IL-10-producing B cells in regulating Th1 responses to *Chlamydia* GT infection. Previous studies in IL-10-deficient mice have strongly indicated that IL-10 suppresses Th1 responses to *Chlamydia* infection in both the GT and respiratory tract, resulting in delayed clearance and chronic inflammatory pathology (316;322;338). Unfortunately, these models of global IL-10-deficiency offer little insight into whether a single or multiple cell types provide the relevant IL-10 that promotes susceptibility to *Chlamydia*. Because numerous cell types can produce IL-10 in response to microbial infection—including DCs, macrophages, NK cells, B cells and T cells—and IL-10 can impact inflammatory responses at multiple stages, relatively minor differences in the magnitude or source of IL-10 can have a major cumulative effect (315;318). Although lack of IL-10 is

associated with inflammation-driven tissue damage in numerous infectious disease models (318), IL-10-deficient mice exhibit striking absence of *Chlamydia*-induced oviduct pathology and this effect is attributed to the rapid clearance of the bacteria from the GT (316). However, in *Chlamydia*-infected women, genotypes associated with low IL-10 production are at increased risk for severe oviduct pathology, highlighting the importance of IL-10 in preventing tissue damage (314). Indeed, in IL-10-competent hosts, IL-10 may have opposing functions, on one hand dampening Th1 immunity and delaying clearance while, on the other hand, limiting chronic inflammatory tissue damage in the GT (322). The overall levels, kinetics and site of IL-10 production are therefore likely to be important factors in the outcome of *Chlamydia* infection. Our data show that IL-10-producing B cells are induced within the first week of infection and are restricted to the draining LN. At this site, Bregs are important modulators of Th1 responses but do not appear to be involved in local IL-10 production in the genital mucosa. This is an important distinction because mice deficient in B cell-derived IL-10 in our study could have benefited from potential protective effects of IL-10-producing non-B cells on inflammation in the GT.

In our study, the generation of IL-10-producing $CD43^-$ Bregs in response to *Chlamydia* stimulation *in vitro* was strongly promoted by the presence of $CD11c^+$ DCs. Although the precise nature of this DC-B cell interaction in the context of *Chlamydia* infection requires further investigation, there are several recent examples in the literature that may suggest the mechanism(s) through which DCs promote Breg generation. For instance, DCs having a $CD11c^+CD11b^{hi}Ia^{low}$ regulatory phenotype have recently been reported to program splenic ILB cells into $IL\text{-}10^+$ Bregs via IFN-β and CD40L signaling (291). CD40-CD40L signaling is recognized as a major pathway involved in the generation of adaptive type Bregs (270;273) but the role of type I IFNs is less clear. Type I IFNs are known to impact B cell function by promoting production of the B cell survival factors BAFF and a proliferation inducing ligand (APRIL) by DCs and macrophages (386). Type I IFNs have also been shown to directly stimulate maximal IL-10 production from CpG-stimulated B cells, which control acute inflammation in neonates (387). A recent study in humans has characterized an interesting co-operation

between IFN-α-producing pDCs and B cells that promotes the generation and proliferation of IL-10-producing B cells in response to *Staphylococcus aureus* (388). The authors of this study suggested that *S. aureus*-induced TLR signals might exploit pDCs and B cells to establish B cell-mediated down-regulation of host responses (388). In the *Chlamydia* GT infection model, studies using mice deficient in type I IFN (IFN-α and IFN-β) receptor indicate that type I IFNs suppress protective $CD4^+$ T cell responses, promoting bacterial persistence and oviduct pathology (389). Type I IFNs can have a range of immunomodulatory effects that dampen immune responses to infection, including increasing the susceptibility of macrophages and lymphocytes to apoptosis (390-392) but our data showing that DC-B cell interactions promote Breg generation in response to *Chlamydia* might suggest a novel mechanism through which type I IFNs are injurious during *Chlamydia* GT infection.

5.2.4 Implications for human chronic *Chlamydia*-associated diseases

Chlamydia infections in humans are characteristically chronic (5;6). In addition to failing to clear primary *Chlamydia* infections, many individuals are susceptible to reinfection indicating that immunity acquired by natural infection is incomplete (6). Although mouse models cannot fully recapitulate chronic human infections—which can last for years—mouse studies can provide unique insight into how regulatory networks are established. Our data indicate that IL-10-producing Bregs are critical modulators of Th1 immunity to *Chlamydia* and may therefore potential mechanism for inadequate clearance of the bacteria and the failure to develop robust acquired immunity. In addition to generating Bregs that facilitate persistence, the preferential induction of Th17 responses via Treg expansion is likely another contributing factor in *Chlamydia*-associated chronic disease etiology. It is conceivable that *Chlamydia*-specific Th17 cells induced during primary infection may become activated by persistent chlamydial antigens and drive inflammatory disease. Th17 cytokines are implicated in the pathogenesis of numerous chronic inflammatory diseases, including arthritis, asthma, and atherosclerosis (393-395). Interestingly, the ability of *C. pneumoniae* infection to advance atherogenesis in hypercholesterolemic mice has been shown to be partly

dependent on IL-17A (396). Collectively, our data indicate that regulatory networks involving regulatory T and B cells may be the underlying immune mechanisms that render hosts susceptible to persistent *Chlamydia* infections and associated inflammatory pathologies.

5.3 Limitations of experimental systems

5.3.1 Distinguishing nTregs from iTregs

In this study, we did not distinguish between thymically-derived nTreg and peripherally-induced iTreg populations within the Foxp3-expressing Treg populations we examined. The term nTreg has often been applied to pre-existing or endogenous Tregs in the lymphoid organs of naïve mice based on the assumption that they originate from the thymus. However, it is now appreciated that $CD4^+CD25^+Foxp3^+$ cells in the periphery are a mixed population of both thymically-derived nTregs and peripherally-induced iTregs. Phenotypically, both nTregs and iTregs express similar markers including CD25, CTLA-4, GITR, CCR4, and Foxp3. It has been recently suggested that the transcription factor Helios and surface marker neuropilin-1 (CD304) can be used to separate nTregs from iTregs (397-399) but these markers were not well-recognized for this application at the time of our experiments. In mice, Helios expression corresponds closely with Tregs generated in the thymus and ~70% of peripheral $Foxp3^+$ Tregs are $Helios^+$ (398), further supporting the widely-held notion that the majority of Tregs found in peripheral lymphoid organs are thymically-derived nTregs (400). We observed that $Foxp3^+$ Tregs expanded in the draining LN within 3 days of *Chlamydia* GT infection indicating the expansion of pre-existing Tregs rather than *de novo* differentiation, although we cannot definitively exclude iTregs from this population. In support of this assertion, previous studies have found that highly inflammatory environments do not support iTreg differentiation (401). Indeed, in models of acute *Listeria monocytogenes* or *Leishmania major* infection, $Foxp3^+$ expression is restricted to nTregs and the generation of iTregs from conventional $CD4^+$ T cells is not observed (401;402). Based on these observations, it has been previously proposed that nTregs preferentially control highly inflammatory

settings whereas iTregs may be induced to perform suppressive functions downstream of the initial inflammatory response (191;250). Notably, we focused our analysis of Tregs to Foxp3-expressing populations in our study and thus the role of Tr1 and other Foxp3$^-$ Treg subsets have yet to be defined in this model.

5.3.2 *In vivo* Treg depletion

While antibody-mediated methods of Treg depletion have been widely used to study Tregs *in vivo* (356), more recently developed tools for this purpose include Foxp3-diptheria toxin receptor (DTR) mouse strains that allow for Foxp3-targetted conditional Treg ablation. Administration of diphtheria toxin (DT) to these mice results in >90% depletion of Foxp3-expressing cells and Foxp3 cells can be depleted continuously by repeated DT treatment without depleting activated Tconv cells (403). In contrast, the anti-CD25 mAb-mediated depletion used in our model achieved ~50% depletion that was transient. Notably, the magnitude of depletion resulting from this method is approximately representative of the physiological differences in endogenous Treg numbers observed among mouse strains and within the human population (355;382), which may make this model more informative about how such differences can impact host susceptibility to *Chlamydia*. In future studies, however, it would be valuable to determine the impact of more complete Treg depletion on *Chlamydia* infection. It is possible that Tregs provide regulatory role controlling the magnitude of inflammatory responses to *Chlamydia* and that their total ablation may be harmful to the host. In addition, the use of DT-mediated Treg depletion in Foxp3-DTR mice would facilitate the depletion of Foxp3$^+$ Tregs in the later phases of host responses to *Chlamydia* and provide insight into whether Tregs have different roles in late versus early phases of the infection.

5.3.3 Mouse strains

In this study, we utilized cells from MyD88-deficient mice as a model for TLR-deficiency to determine the roles of TLR ligation in DC-mediated Treg conversion as

well as *C. muridarum*-induced IL-10 production by B cells *in vitro*. It is important to note that in addition to its role in TLR pathways, MyD88 is also involved in IL-1R signaling and has been recently implicated in transducing B cell-activating factor (BAFF) signals (404). These functions are meaningful to the interpretation of our *in vitro* data utilizing MyD88-deficient cells. Based on previous reports that IL-1β plays an important role in Treg to Th17 conversion (110;405), it is likely that MyD88-deficient Tregs were unable to produce IL-17A in the presence of *C. muridarum*- or LPS-stimulated DCs due to a lack of responsiveness to IL-1β. Therefore, any potential role for Treg-intrinsic MyD88-mediated TLR signals may have been masked. Future studies using cells deficient in specific TLRs may resolve this issue.

With respect to our *in vitro* B cell stimulation experiments, we assert that the inability of MyD88-deficient B cells to produce IL-10 in response to *Chlamydia* reflects a critical requirement for TLR-mediated signals. Although BAFF has been shown to promote the generation of IL-10-producing B cells (373), B cells do not produce BAFF (406) and therefore purified B cell cultures contained no cellular sources of this cytokine. However, we cannot fully discount a role for BAFF in promoting *C. muridarum*-induced IL-10^+ B cells in the context of cells (e.g. DCs) that could potentially act as a cellular source of BAFF *in vivo* or *in vitro*.

C57BL/6 mice are the background strain for many transgenic GFP reporter mouse strains relevant to this study, including Foxp3-GFP and IL-10GFP mice (104;336). For these practical purposes, the experiments conducted herein were exclusively performed within C57BL/6 mice. Notably, C57BL/6 mice are the least susceptible to *Chlamydia*-induced GT pathology among common inbred laboratory mouse strains including BALB/c and C3H/HeN mice (407;408). Interestingly, BALB/c mice contain significantly more endogenous $CD4^+CD25^+$ cells in their peripheral lymphoid organs (355) and have also been demonstrated to generate more IL-10-producing B cells than C57BL/6 mice in response to *Leishmania major* infection (409). This predisposition to B cell-derived IL-10 production is thought to mediate the high susceptibility of BALB/c mice to chronic *L. major* infection and pathology. Given these observations, it would be

interesting to determine whether our experimental models of Treg depletion and B cell IL-10-deficiency could have a more dramatic effect in reducing the susceptibility of BALB/c mice to *Chlamydia* than observed here in C57BL/6 mice.

5.3.4 Assessment of bacterial burden in the upper versus lower GT

In the mouse model of *Chlamydia* GT infection, measuring chlamydial shedding from cervico-vaginal swabs is a standard parameter for assessing bacterial clearance. However, it has been previously demonstrated that the degree of bacterial burden in distinct regions of the murine GT (i.e. the cervix, uterine horns or oviducts) can differ substantially (410). The bacterial burden data presented in our studies was quantified from *Chlamydia* recovered using cervico-vaginal swabs and thereby reflects the total bacteria shed from the upper and lower GT compartments. Therefore, neither the kinetics of ascending infection nor the magnitudes of oviduct colonization are represented. In terms of IFU per milligram of tissue, the oviduct can carry a higher chlamydial burden than any other region of the GT and this may significantly impact local inflammatory pathology (410;411). It is possible that Treg-depleted mice—which exhibited bacterial shedding comparable to control mice—may have experienced reduced chlamydial burden in upper GT tissues that could have contributed to reducing oviduct pathology. Similarly, while IL-$10^{-/-}$ B chimeras displayed comparable levels of bacterial shedding to control chimeras until day 20 post-infection, it is feasible that the enhanced Th1 responses in the IL-$10^{-/-}$ B cell chimeras limited the ascension of *Chlamydia* infection into the upper GT resulting in reduced burden in the oviduct earlier than indicated by our cervico-vaginal swab measurements.

5.3.5 Use of heat-killed *C. muridarum* for generating IL-10^{+} B cells *in vitro*

In this study, we utilized heat-killed *C. muridarum* to generate IL-10-producing B cells for *in vitro* functional assays in order to avoid potential confounding effects of live bacteria in the co-culture environment. While we noted that heat-killed *C. muridarum*

was a less potent inducer of IL-10$^+$ B cells than live *C. muridarum*, the reason for this difference is not clear. It is possible that conformational B cell epitopes of *C. muridarum* may be thermally denatured by the heat-killing process. However, our data strongly suggest that TLR-mediated, rather than BCR-mediated, signals are responsible for inducing IL-10 production by B cells in response to *C. muridarum*. Nonetheless, heating may also cause the denaturation of proteins and lipids that could serve as PAMPs and thereby alter innate B cells responses to *C. muridarum* (412). In addition to the direct effect on B cell recognition of *C. muridarum*, heat-killing may also alter DC responses to the bacteria. Previous work has indicated that live and dead (UV-irradiated) *C. muridarum* have different effects on DC maturation, with live *C. muridarum* stimulating greater expression of costimulatory molecules and production of IL-12 and TNF-α (413). It is therefore possible that DC-mediated signals required for Breg generation are compromised by the use of heat-killed *C. muridarum*, further adding to the possibility that IL-10$^+$ B cells generated by heat-killed bacteria may not accurately reflect those induced by live *C. muridarum*. Further study will be required to identify the chlamydial PAMP(s) that induce IL-10$^+$ B cells and using purified TLR ligands may represent a better system to evaluate the function of these cells *in vitro*.

5.4 Proposed future directions

5.4.1 Mechanisms of *Chlamydia*-induced Foxp3$^+$ Treg expansion

Further investigation will be required to determine the nature of the signals induced by *Chlamydia* infection that result in Foxp3$^+$ Treg expansion, particularly the specificity of the antigens recognized by these Tregs. Although nTregs are biased toward self-antigen recognition, the ability of nTregs to specifically recognize pathogen-specific antigens and proliferate in response to infected DCs has been convincingly demonstrated in a model of *Leishmania major* infection (402). *In vitro* Treg proliferation has also been reported to occur independently of antigen in the presence of IL-2 and interactions with DCs and some *in vivo* evidence supports that Treg division could be enhanced by molecules that expand or activate DCs such as GM-CSF and TLR ligands (414).

Additionally, the origin of early increased Foxp3$^+$ Treg populations in the ILN during *C. muridarum* infection is not clear. It is generally understood that Tregs traffic from peripheral tissue into draining LNs under steady state conditions and that immune reactions both accelerate this movement and trigger their egress from draining LNs to the site of inflammation (415). With this understanding, there are two potential pathways for *Chlamydia*-induced Foxp3$^+$ Treg expansion in the LN to occur: 1) LN-resident Foxp3$^+$ Tregs are activated by DCs emigrating from the infection site and undergo proliferation and/or, 2) tissue-resident Foxp3$^+$ Tregs activated at the infection site accumulate in the LN. If Tregs are activated in the infection site where they may be exposed to *Chlamydia* products before migrating to the LN, direct TLR ligation may significantly modulate Treg proliferation and function. In particular, it would be interesting to determine whether TLR2 signaling mediates the expansion of Foxp3$^+$ Tregs in response to *C. muridarum* GT infection, as this may provide a mechanism through which TLR2 promotes oviduct pathology during infection.

5.4.2 Cross-regulation of regulatory T and B cell subsets

This work has identified distinct and opposing functions for Tregs and Bregs in shaping conventional CD4$^+$ T cell responses to *C. muridarum*. This finding is novel in the context of recent literature suggesting that Tregs and Bregs collaborate to collectively promote immune suppression; in particular, Bregs are frequently reported to promote Treg differentiation via IL-10 and/or TGF-β production (297;304;416-418). In our model, however, the cellular kinetics in the ILN suggests that Treg expansion is concurrent with, and perhaps even precedes Breg expansion. There is some indirect evidence to suggest that the ability of Tregs to promote Th17 differentiation could enhance Breg generation. Th17 cells produce IL-21, which has been recently demonstrated to play an important role in B10 cell development and IL-10 production (301). IL-17A alone or in combination with BAFF directly enhances B cell survival and proliferation (419;420). Indeed, the few studies investigating the role of Th17 cells in B cell responses have determined that Th17 cells efficiently promote early B cell activation and proliferation (419). Further studies will be required to determine whether Tregs and

Bregs act sequentially during host response to *Chlamydia* and whether the development of one is dependent on the other.

5.4.3 Antichlamydial vaccine design

Vaccination is considered a necessary measure to prevent the continued spread of *Chlamydia* and its associated disease sequelae (8). Altogether, *C. trachomatis* GT infections pose a considerable socioeconomic and public health burden (12;15) and mathematical models project that a fully protective antichlamydial vaccine could potentially eradicate *Chlamydia* within 20 years (421). Early vaccine trials in the 1960s using whole-cell *Chlamydia* preparations provided short-term protection in some individuals but caused severe hyperreactivity in others (422). Since then, antichlamydial vaccine development has utilized a more cautious approach through rational design of subunit vaccines using carefully selected *Chlamydia* antigens (8). The ability of *Chlamydia* to induce both protective and pathological host immune responses poses a significant challenge to current research efforts to design a safe and effective vaccine. As such, a detailed knowledge of the immunological factors that provoke protection versus pathogenesis is required. Our results may provide insight for developing strategies to skew the immune response toward the protective pathway.

5.4.3.1 Route of immunization

Our studies have identified immune regulatory networks activated upon natural *C. muridarum* infection in the GT mucosa but it is highly relevant to vaccine design to understand how regulatory T and B cells influence the induction of immune responses to *Chlamydia* at other mucosal immunization sites. In rodents, there is evidence of a common mucosal immune system that interconnects the nasopharynx/bronchial tract, the gastrointestinal tract and the GT tract and findings also support the existence of a similar interconnecting system in humans (151). The intranasal route of immunization is of particular interest in the design of vaccines against GT pathogens because it efficiently

stimulates cellular immunity at the distant GT effector site (423) and is the best route of immunization for obtaining maximal IgG and IgA antibody responses in vaginocervical secretions (151). Indeed, intranasal immunization with live *Chlamydia* EBs is currently the 'gold-standard' for acquired protection against *Chlamydia* GT infection and oviduct pathology in experimental mouse models (424). Unlike the GT, which does not contain MALT, the nasopharynx/bronchial tract contains nasopharynx-associated lymphoid tissue (NALT) so immune responses are initiated at these inductive sites rather than the draining LN (425). The nature of the mucosal immune system in the nasopharynx/bronchial tract versus GT will likely influence how Treg and/or Breg activity affects antichlamydial immunity at this site.

In addition, our preliminary data in the *C. muridarum* respiratory infection model indicate that $CD43^+$ B-1 cells may enhance the induction of Th1 responses to respiratory *C. muridarum* infection—a finding with potentially important implications for intranasal immunization strategies. The majority of our understanding of B-1 biology has come from rodents; human B-1 cells have only recently been characterized (426). Since the identification of human B-1 cells, a distinct humans $CD11b^+$ B-1 cell subset has been found to possess potent T cell stimulatory activity (427). If human B-1 cells are present in or recruited to sites of *Chlamydia* infection, they could potentially represent an important and previously unappreciated APC that, like DCs, could be targeted by vaccination to elicit potent antichlamydial immunity.

5.4.3.2 Considerations for Tregs

Our data suggests that endogenous Tregs promote Th17 responses to *Chlamydia* GT infection at both inductive and memory phases. In contrast to Th1 responses and specific antibodies, high Th17 responses have been found to have little protective value against secondary *Chlamydia* infection and are instead associated with severe oviduct pathology (428). It would be interesting to determine whether endogenous Tregs hamper the protective efficacy of *Chlamydia* immunization in certain hosts by skewing Th1/Th17 immunity toward higher Th17 responses. In our study, splenocytes from Treg-depleted

mice exhibited normal Th1 but low Th17 responses upon *C. muridarum*-antigen recall and this immune profile has previously been reported to strongly correlate with optimum protective immunity against *Chlamydia* (428). Notably, mice immunized with vaccines that elicit Th1 and a low level of Th17 responses have been shown to be more protected than mice in which only Th1 immunity was induced (429). There is evidence that IL-17A may play a co-operative role with IFN-γ in vaccine-primed protective immunity. In a *M. tuberculosis* vaccination model, antigen-specific Th17 responses were shown to enhance protective immunity by inducing chemokines CXCL9, CXCL10 and CXCL11 to enhance Th1 recruitment to challenge sites (430). A more recent study demonstrated that IL-23-dependent IL-17A production is required for the generation of effective BCG vaccine-induced Th1 immunity to *M. tuberculosis* (431). Altogether, the relative strength of Th1 versus Th17 immune responses is likely an important factor in *Chlamydia* vaccine efficacy.

The concept of depleting endogenous Tregs to enhance the ability of vaccines to induce robust immunity has garnered significant research attention in recent years. In mice, depletion of Tregs using anti-CD25 mAb prior to immunization has been reported to increase protective T cell responses to herpes simplex virus 1 (432) and malaria vaccines (433). Similarly, administration of the denileukin difitox—an IL-2 immunotoxin that targets Tregs for depletion (434)—prior to viral-based vaccination in mice has also been shown to increase antigen-specific T cell responses (435). While there are currently clinical protocols to inhibit or deplete Tregs prior to immunization with tumor antigen vaccines (436;437), such measures may not have practical application in vaccinating large numbers of healthy individuals against *Chlamydia*. Alternatively, understanding the mechanisms that promote the expansion and/or Th17-promoting activity of Tregs may inform the development of vaccines to reduce the impact of Tregs on Th17 polarization. Strategies to overcome or avoid Treg expansion might include the proper choice of adjuvants that support the expansion of effector T cells over that of endogenous Tregs.

5.4.3.3 Considerations for Bregs

In our study, DCs were shown to play a prominent role in the generation of IL-10-producing Bregs in response to *Chlamydia in vitro*. These adaptive-type Bregs, in turn, corresponded with IL-10-producing B cells responsible for dampening protective Th1 responses *in vivo*. DCs are major targets for shaping vaccine-induced immunity through the use of adjuvants (438) and therefore a clear direction for future study will be to elucidate the DC-B cell interactions involved in the generation of Bregs and to determine whether these signals could be discouraged by the use of certain adjuvants. DCs may provide the stimulatory requirements for adaptive Bregs directly, e.g. via IFN-β production and CD40/CD40L interactions (291), BAFF production (373), or by activating antigen-specific $CD4^+$ T cells that subsequently provide help to B cells via cognate interactions (301). In addition to signals provided by DCs and/or $CD4^+$ T cells, *Chlamydia*-induced Bregs also appear to require direct stimulation by MyD88-dependent TLR pathways. Identifying specific chlamydial PAMPs that promote the activation/expansion of Bregs may help engineer adjuvant preparations to reduce the counter-regulatory effects of Bregs on Th1 priming during immunization. Furthermore, in addition to our preliminary data indicating that B-1 cells promote the induction of Th1 responses to *C. muridarum* respiratory infection, we determined that IL-10-deficient B-1 cells stimulated more robust Th1 responses compared to wild-type B-1 cells (APPENDIX D3). Therefore, identifying the chlamydial PAMPs and other signals that activate B-1 cells and/or elicit IL-10 production in response to *C. muridarum* may also inform strategies to optimize Th1 priming during immunization at the respiratory mucosa.

5.5 Concluding remarks

Our understanding of basic immunology has evolved rapidly over the past several years to include regulatory immune mechanisms. The work presented here has established a critical role for both regulatory T and B cells in modulating protective and pathological responses to *Chlamydia* infection. Knowledge gained from these studies contributes substantially to our understanding of host responses to *Chlamydia* and these

findings may also enhance our understanding of other pathogens that cause chronic infections and inflammatory pathology. Diseases caused by *Chlamydia* infection remain a major public health concern in Canada and worldwide. Future work based on the discoveries presented in this study could have significant implications for the design of prophylactic strategies to manipulate regulatory T and B cell functions in order to control *Chlamydia* infections and associated diseases.

Figure 5.1Schematic model summarizing the role of Tregs and Bregs in modulating the balance of Th1 and Th17 responses to *Chlamydia* GT infection and the impact of this regulation on infection outcomes.

(a) (i) *Chlamydia* induces proinflammatory cytokine and chemokine production by epithelial cells and resident macrophages. Neutrophils and some monocytes are rapidly recruited to the infection site. Upon encountering *Chlamydia*, DCs mature and migrate into the draining LN where they initiate $CD4^+$ T cell responses. *Chlamydia*-matured DCs stimulate the differentiation of both Th1 and Th17 cells. (ii) Tregs promote the expansion of Th17 responses, (iii) IL-10-producing B cells dampen the expansion of Th1 responses, (iv) Effector $CD4^+$ T cells migrate to mucosal infection site whereTh1 cells mediate bacterial clearance. Th17 responses do not appear to aid in clearing bacteria but, rather, amplify *Chlamydia*-induced neutrophilic inflammation. (b) Relative to control (i.e. "normal") mice (i), Treg-depleted mice (ii) develop fewer Th17 cells available to potentiate inflammation induced by *Chlamydia*-infected cells. The infection is cleared with normal kinetics and oviduct pathology is reduced. In B cell-IL-10-deficient chimeric mice (iii), heightened Th1 responses clear the bacteria faster and/or limit ascending infection, thereby reducing the *Chlamydia*-induced inflammatory signals for Th17 to amplify. The infection is cleared with accelerated kinetics and oviduct pathology is reduced.

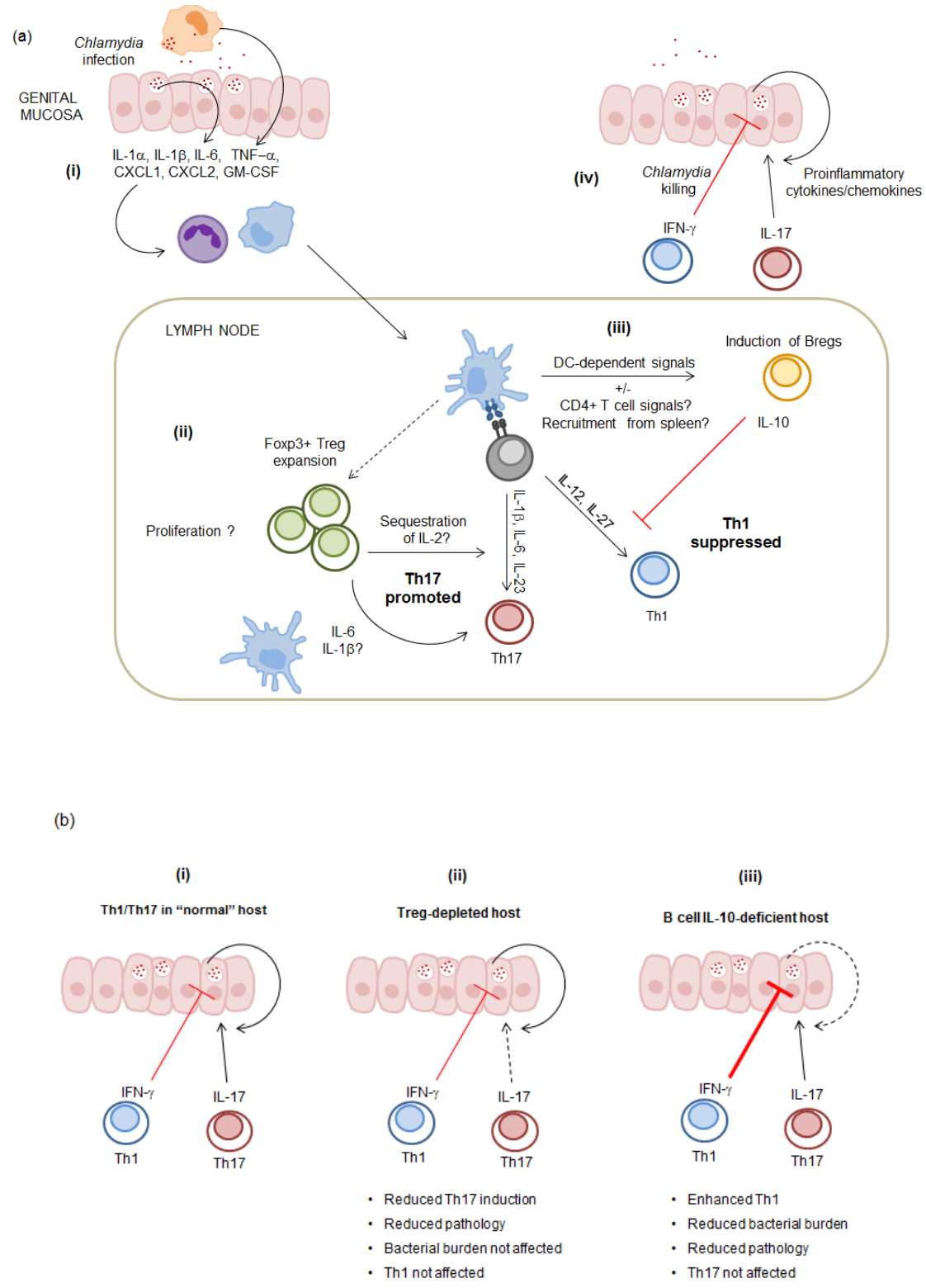
(a)
Chlamydia infection
GENITAL MUCOSA
(i)
IL-1α, IL-1β, IL-6, TNF–α, CXCL1, CXCL2, GM-CSF
(iv)
Chlamydia killing
Proinflammatory cytokines/chemokines
IFN-γ
IL-17
LYMPH NODE
(iii)
DC-dependent signals
+/-
CD4+ T cell signals?
Recruitment from spleen?
Induction of Bregs
IL-10
(ii)
Foxp3+ Treg expansion
Proliferation ?
Sequestration of IL-2?
Th17 promoted
IL-1β, IL-6, IL-23
IL-12, IL-27
Th1 suppressed
Th1
IL-6
IL-1β?
Th17
(b)
(i)
Th1/Th17 in "normal" host
(ii)
Treg-depleted host
(iii)
B cell IL-10-deficient host
IFN-γ
IL-17
Th1
Th17
• Reduced Th17 induction
• Reduced pathology
• Bacterial burden not affected
• Th1 not affected
• Enhanced Th1
• Reduced bacterial burden
• Reduced pathology
• Th17 not affected

Figure 5.1

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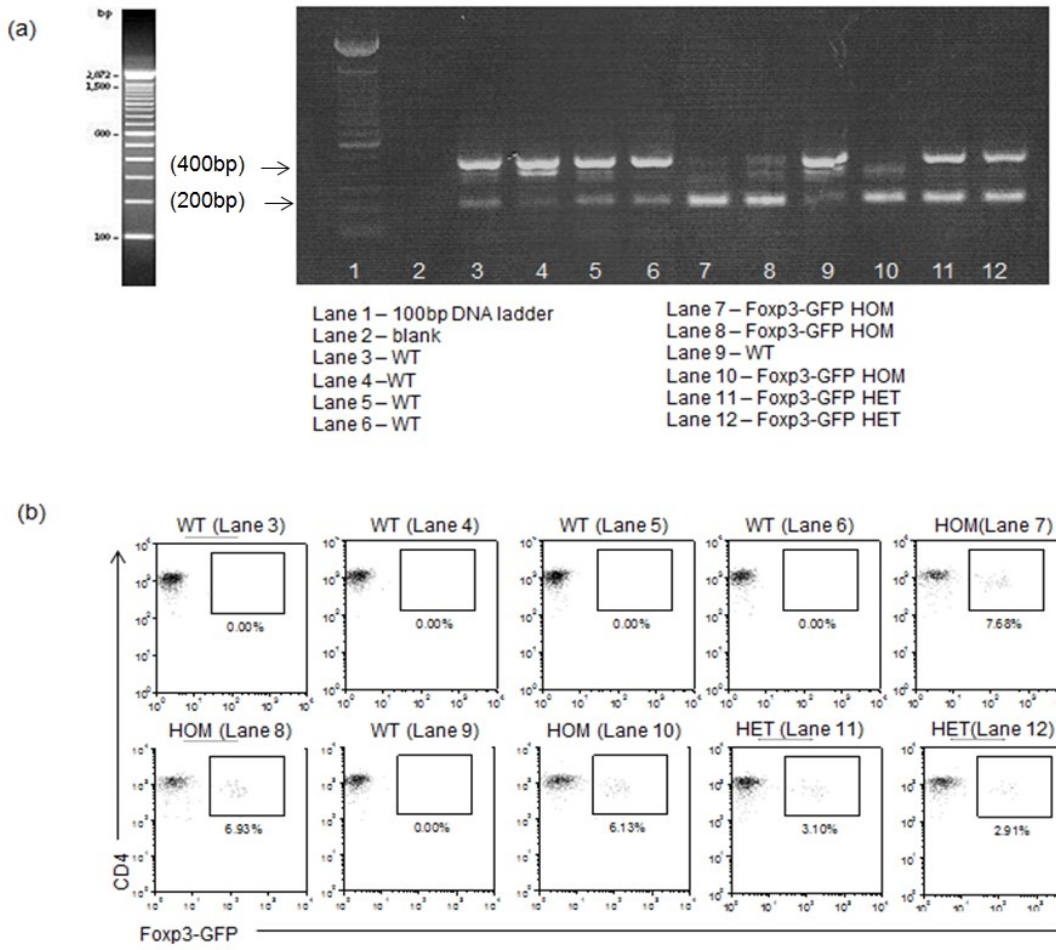
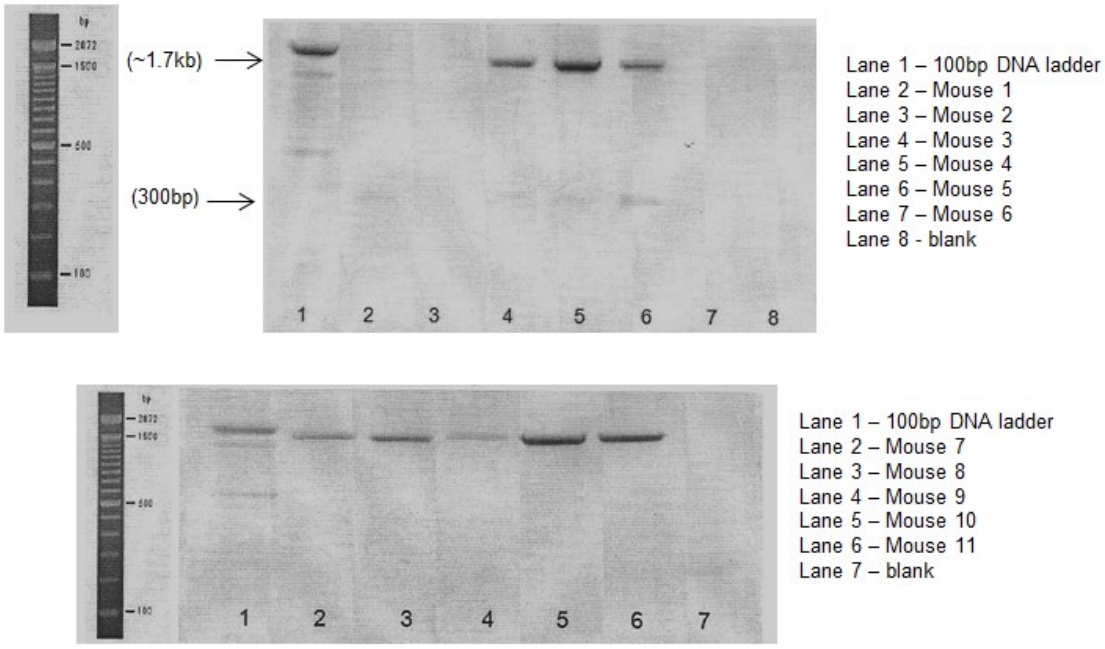
APPENDIX A Supporting material for CHAPTER 2



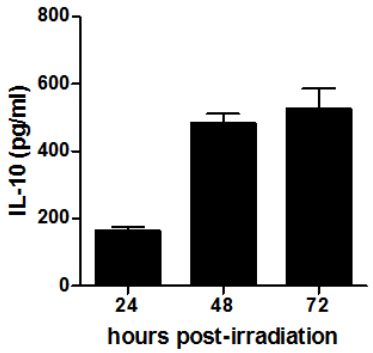
APPENDIX A1 Foxp3-GFP mouse genotyping

(a) Genotyping PCR products were analyzed by electrophoresis on a 1.5% agarose gel alongside a 100bp DNA ladder (Invitrogen, Oakville, Ontario) to determine band sizes. Mice homozygous (HOM) for Foxp3-GFP displayed a single band at 200 base-pairs (bp), wild-type (WT) mice displayed a single band at 400-450bp, and heterozygotes (HET) displayed two bands, one of each size (Dr. Amit Awasthi, personal communication) (b) The phenotype of mice displaying a WT, Foxp3-GFP HET and Foxp3-GFP HOM genotype by PCR in part (a) was analyzed by FACS analysis of CD4-gated PBMCs.



APPENDIX A2 IL-10GFP mouse genotyping

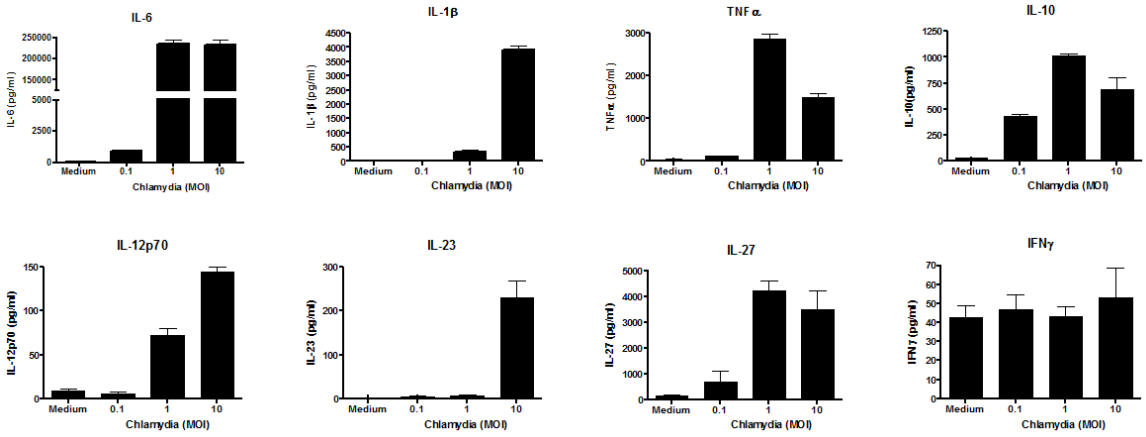
Genotyping PCR products were analyzed by electrophoresis on a 1% agarose gel alongside a 100bp DNA ladder (Invitrogen, Oakville, Ontario) to determine band sizes. The forward and reverse 1 primers bind to non-coding regions of the wild-type IL-10 gene ~350bp apart. The ~1.4kb IRES-eGFP cassette (439) is inserted within this region in the *tiger* IL-10 gene; thus, the presence of a ~1.7kb band indicated the *tiger* IL-10 allele whereas a 350bp band indicated the wild-type IL-10 allele. Heterozygotes displayed two bands, one of each size. Mice homozygous (HOM) for the IL-10GFP (*tiger*) allele displayed a single band at ~1.7kb (mouse 7-11) whereas wild-type (WT) mice displayed a band at 350bp (mouse 1). Heterozygous (HET) individuals displayed one band of each size (mouse 3-5). PCR and electrophoresis shown here were performed by Tyler Brown. No PCR products were detected in samples from mouse 2 and mouse 6.



APPENDIX A3 IL-10 production by irradiated *C. muridarum*-stimulated B cells

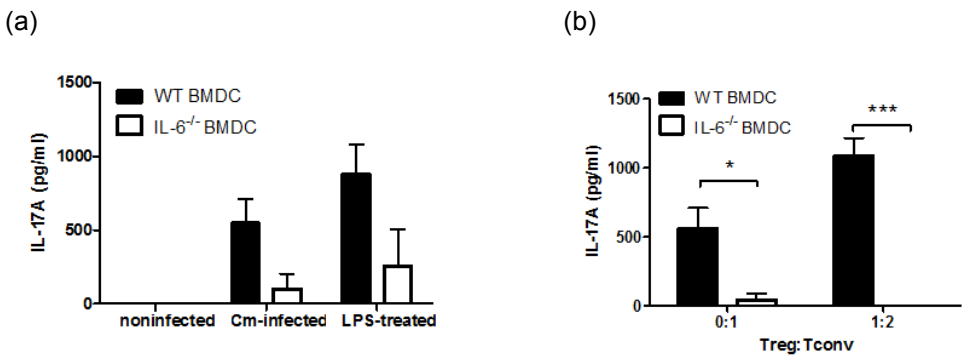
B cells were purified from splenocytes from C57BL/6 mice were stimulated with heat-killed *C. muridarum* (*Cm*) MOI 1.0 for 24 h and irradiated. $1x10^6$ of purified irradiated B cells were then cultured with fresh media in 96-well plates for an additional 72 h. Supernatants were collected at 24, 48 and 72 h and IL-10 levels were detected by ELISA. Data are presented as mean ± SEM of quadruplicate wells/timepoint.

APPENDIX B Supporting material for CHAPTER 3



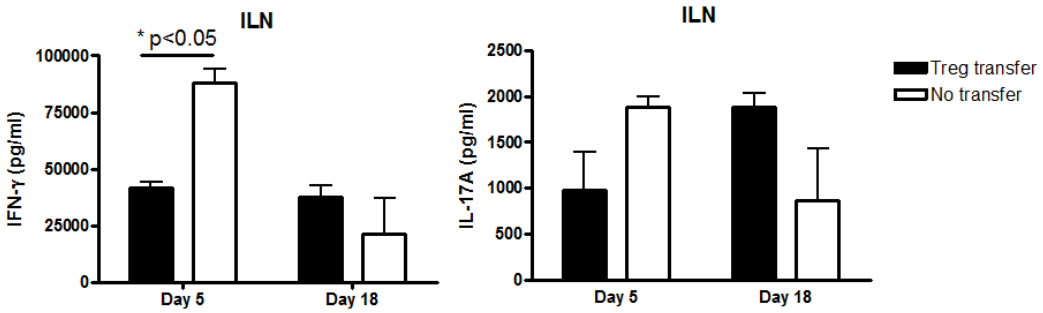
APPENDIX B1 *C. muridarum* induces production of Th1- and Th17-polarizing cytokines by bone marrow-derived DCs *in vitro*.

$1x10^6$ BMDCs were cultured in 24-well plates with live *C. muridarum* at MOI 0.1, 1.0 or 10 or media alone for 72 hours. Concentrations of IL-6, IL-1β, TNF-α, IL-10, IL-12p70, IL-23, IL-27 and IFN-γ were measured in supernatants by ELISA. Data are presented as mean ± SEM of 3-4 replicates.. The experiment shown here was performed by Dr. Elizabeth Acosta-Ramirez.



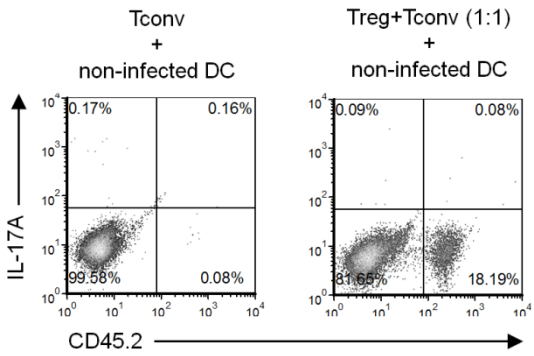
APPENDIX B2 IL-6-deficiency in DCs markedly impairs *Chlamydia*-induced IL-17A production by Tregs themselves and Tconv *in vitro*.

BMDCs were prepared from WT and IL-6-deficient mice and seeded in 96-well plates at 20, 000 cells per well. BMDCs were treated with live *C. muridarum*, LPS or media alone for 24 hr. FACS-sorted $CD4^{+}Foxp3^{-}$ Tconv cells and $CD4^{+}Foxp3^{+}$ Tregs were added to wells as indicated and cultured in the presence of 1μg/ml anti-CD3 for an additional 48 hr. Tregs cultured in the absence of Tconv were treated with 10ng/ml IL-2. IL-17A levels in culture supernatants containing $1x10^{5}$ Tregs (a) or $1x10^{5}$ Tconv and Treg:Tconv (1:2) co-culture supernatants (b) were determined by ELISA. Data are presented as mean± SEM of 3-4 replicates. *P≤0.05, ***P≤ <0.001 using two-way ANOVA test



APPENDIX B3 Adoptive transfer of Tregs prior to infection dampens early Th1 responses but enhances late Th17 responses in the ILN during *C. muridarum* infection.

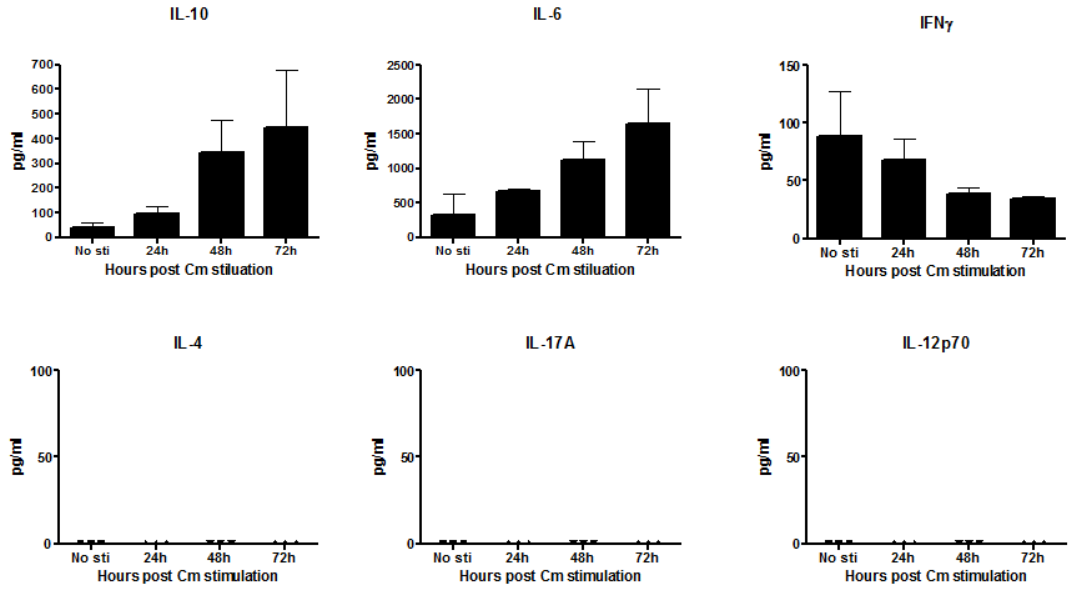
Mice received 1x10^6 CD4$^+$Foxp3$^+$ Tregs sorted by flow cytometry (purity 94.5- 97.5%) from Foxp3-GFP donor mice or 100µl PBS *i.v.*at day -1 and were infected intravaginally with *C. muridarum* at day 0. At day 5 and 18 post-infection, single-cell suspensions were prepared from ILN samples and seeded at a concentration of 1x10^6 cells per well in 96-well plates and restimulated with heat-killed *C. muridarum* for 72 h *in vitro*. IFN-γ and IL-17A production from *in vitro* re-stimulated ILN cells at day 5 and 18 post-infection were measured by ELISA (n=3 mice per group per timepoint). Data are presented as mean ± SEM. *$P \leq 0.05$ using two-way ANOVA test.



APPENDIX B4 Tregs do not promote Th17 responses in the presence of non-infected BMDCs

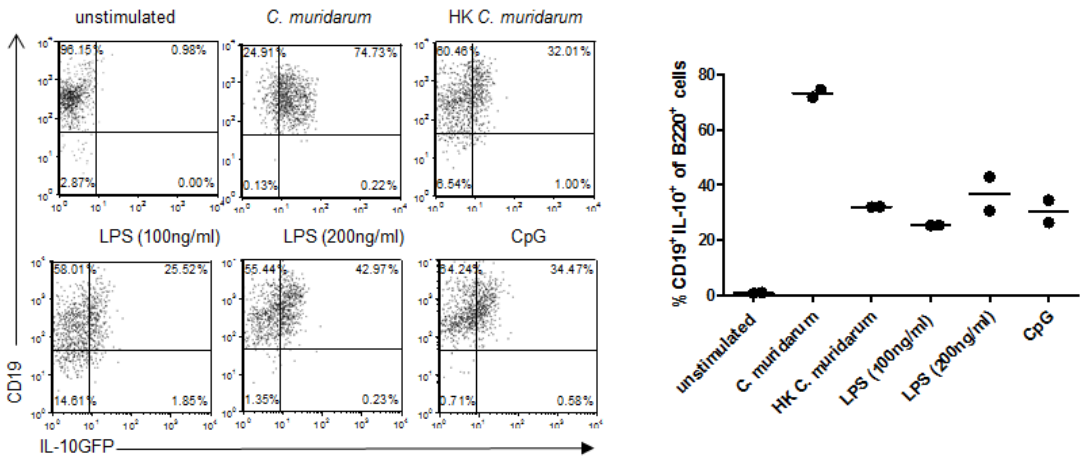
Purified Tconv (CD45.1) cells were co-cultured with or without Treg (CD45.2) cells at 1:1 ratio and stimulated with anti-CD3 in the presence of noninfected BMDCs for 72 h. The IL-17A production by Tconv (CD45.1) cells and Treg (CD45.2) cells under different conditions were determined by ICCS. Data are representative of at least two independent experiments.

APPENDIX C Supporting material for CHAPTER 4



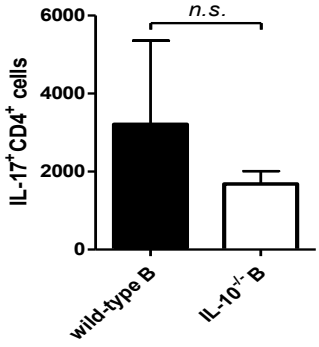
APPENDIX C1 Cytokine profile from purified B cells upon *C. muridarum* stimulation *in vitro*

$CD19^{+}CD220^{+}$ B cells were sorted by flow cytometry from spleens of naïve wild-type mice and cultured in the presence or absence of live *Chlamydia in vitro*. At 24, 48 and 72h post-stimulation, the culture supernatants were collected and the level of IL-10, IL-6, IFN-γ, IL-4, IL-17A and IL-12p70 was determined by ELISA. The data is presented as the mean ± SEM (n=3 mice). This experiment was performed by Chi Yan.



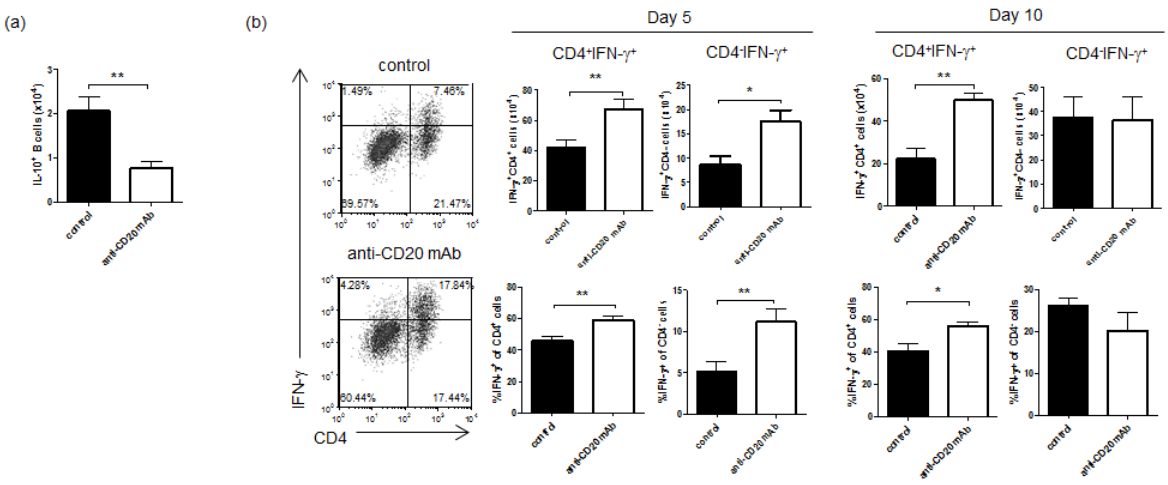
APPENDIX C2 Induction of IL-10 by B cells following stimulation with different TLR ligands or bacterial products.

a) Whole splenocytes from IL-10GFP mice were cultured with media alone (unstimulated) or in the presence of various stimulation conditions: *C. muridarum* (MOI 0.1), heat-killed *C. muridarum* (MOI ~1.0), LPS (100 or 200ng/ml), and CpG oligonucleotides (1µg/ml). Dot plots show IL-10GFP and CD19 expression by B220-gated splenocytes after 96 h. (b) Graph represents percent of CD19+IL-10GFP+ cells out of live B220-gated splenocytes described in (a). Data are presented as two individual replicates for each condition.



APPENDIX C3 IL-10-deficiency in B cells does not significantly alter Th17 responses to *C. muridarum* genital tract infection

The total number of IL-17A^{+}CD4^{+} cells in the ILN of wild-type B versus IL-10$^{-/-}$ B chimeras at day 10 post-infection as determined by ICCS (n=3-4 mice per group). Data are presented as mean ± SEM, n.s. = no significance using Student's *t*-test.



APPENDIX C4 Anti-CD20 antibody delivery markedly enhances Th1 responses to *C. muridarum* genital tract infection.

Mice were infected intravaginally with *C. muridarum* at day 0 and treated with 100µg anti-CD20mAb (rituximab, Rituxan®, Hoffmann-La Roche Ltd., Mississauga, Ontario) in 200µl sterile PBS *i.p.* at day 2 and 4 post-infection. (a) Total number of IL-10^+ B cells in ILN of control and anti-CD20mAb-treated mice at day 5 post-infection as determined by ICCS (n=11-14). (b) The total number of IFN-γ^+ $CD4^+$ and IFN-γ^+ $CD4^-$ cells as well as the frequency of IFN-γ^+ cells among $CD4^+$ and $CD4^-$ cells was determined by ICCS of ILN cells from anti-CD20mAb-treated and control mice at day 5 and 10 post-infection (n=5-9 mice per group). Data are presented as the mean ± SEM. * $P \leq 0.05$, ** $P \leq 0.01$ using Student's *t*-test.

APPENDIX D Investigating the role of B-1 cells in host defense against respiratory *C. muridarum* infection

Summary of findings

In the course of our study, we observed an innate-like subset of splenic $CD43^+$ B cells that produced IL-10 in response to *Chlamydia in vitro* in the absence of DCs (Figure 4.2). The $CD5^+CD43^+$ phenotype of these ILB cells was consistent with B-1a cells (275). Because B-1 cells are a minor population in the spleen, we utilized the peritoneal cavity (PerC) as a source for B cells and found that, indeed, $CD5^+CD43^+$ B-1a cells potently produce IL-10 in response to *C. muridarum in vitro* (APPENDIX D1). Importantly, however, we noted that $CD43^-$ B cells were major IL-10 producers in the ILN during *Chlamydia* GT infection, not $CD43^+$ B cells. We wondered whether the lack of $CD43^+$ B cell involvement in the GT infection model could be due to the scarcity of B-1 cells in peripheral LNs (275). B-1 cells are, however, enriched in the peritoneal and pleural cavities and participate in host responses to certain respiratory pathogens including influenza (440), *M. bovis* BCG (441), and *Paracoccidioides brasiliensis* (442) so we wondered whether B-1 cells may respond to *C. muridarum* respiratory infection. We observed that $CD43^+$ B cells accumulate in the lung and draining mediastinal LN (MedLN) early on following *C. muridarum* respiratory infection APPENDIX D2a). *In situ* labelling of PerC cells with cytoplasmic dye (carboxyfluorescein succinimidyl ester (CFSE)) demonstrated that at least a portion of $CD43^+$ B cells found in the lung and MedLN of *C. muridarum*-infected mice are recruited from the PerC (APPENDIX D2b).

To determine the role of B-1 cells and B-1-cell-derived IL-10 in host responses to infection, we adoptively transferred naïve PerC B cells from wild-type or IL-10-deficient donors into wild-type mice 24h prior to *C. muridarum* lung infection. Unexpectedly, we found that B-1 cells did not suppress but rather promoted Th1 responses and bacterial clearance during *C. muridarum* respiratory infection (APPENDIX D3). These results are consistent with some recent work revealing novel potent T cell-expanding activities of B-1 cells (443). Such studies have illustrated an important contribution of PerC B-1 cells to antigen presentation and $CD4^+$ T cell activation (444;445) and are also in accordance with our *in vitro* observations that *C. muridarum*-stimulated splenic $CD43^+$ B cells do not

suppress $CD4^+$ T cells in co-culture (Figure 4.3). Notably, our experiments showed that Th1 responses to *C. muridarum* were more robustly promoted in mice receiving IL-10-deficient $CD43^+$ B cells (APPENDIX D3), suggesting that while B-1 cells apparently promote Th1 responses, B-1 cell-derived IL-10 also plays a role in regulating Th1 immunity in the respiratory mucosa. A potential explanation for these seemingly contradictory functions may lie in the ability of B-1 cells to promote IL-10-producing $CD4^+$ T cells in addition to Th1 and Th2 lineages, which has been demonstrated to occur *in vitro* (445). Thus, B-1 cell-derived IL-10 may not in itself impede T cell activation but induce IL-10-producing T cells with regulatory functions.

Collectively, our studies and preliminary work illustrate the diversity in B cell functions and the differential involvement of B cell subsets at distinct mucosal sites in response to *Chlamydia*. These findings may help to explain some of the contradictory data about the role of B cells in *Chlamydia* infection. In particular, B cell-deficient mice resolve genital *C. muridarum* infection with similar or faster kinetics than wild-type mice whereas B cell-deficient mice are highly susceptible to *C. muridarum* lung challenge (154;158;366). In these studies, B cell-deficiency is associated with impaired Th1 responses in the lung but not the GT model, which is consistent with our data showing that B-1 cells can promote Th1 responses in the respiratory tract. Additionally, our discovery that distinct ILB cell subsets are involved in antichlamydial responses in the respiratory versus GT mucosa may elucidate the divergent role of other factors in host defense between these sites, such as TLR2 (46;47).

Materials & Methods

Peritoneal cavity (PerC) lavage

To isolate cells from the peritoneal cavity, euthanized mice were soaked in 70% ethanol and the peritoneum was exposed by making a small incision in the abdominal skin and gently pulling skin to each side of the mouse. A 27G needle was carefully inserted hole-side down into the lower abdominal area of the peritoneal cavity (PerC),

taking care not to perforate any organs and aiming for a region where fat was visible. Using a 10ml syringe, 7-10ml of cold sterile PBS was injected forcefully into the intraperitoneal cavity. The needle was then slowly removed, allowing fat to clog the opening at the injection site. Mice were gently shaken to agitate the intraperitoneal lavage fluid. To collect lavage fluid, a 23G needle was inserted hole-side down horizontally into the upper part of the abdomen, taking care not to perforate any organs, and fluid was slowly extracted. The number of cells recovered was determined by diluting sample in 4% Trypan Blue and counting by hematocytometer.

***C. muridarum* respiratory infection model**

Mice were anesthetized by isoflurane and a dose of 4000IFU of *C. muridarum* in 25µl SPG buffer was delivered in intranasally (*i.n.*) with a P20 pipette tip. Mice were monitored daily for bodyweight changes. A 25% loss of original (day 0) bodyweight was used as the humane endpoint.

Lung tissue processing

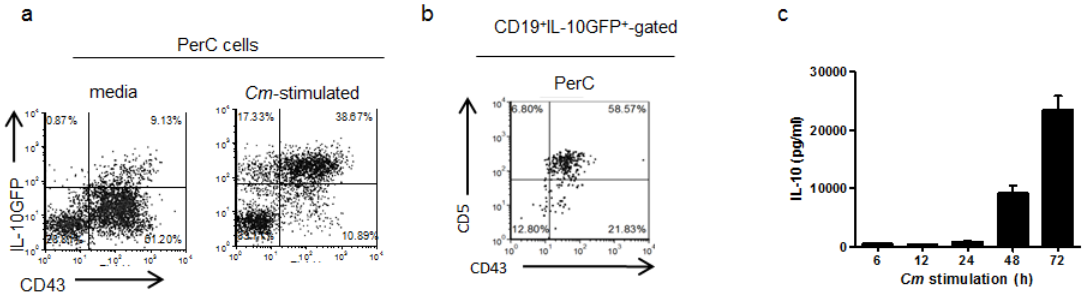
Lungs were excised from euthanized mice was placed in ~500µl of collection media (HBSS or PBS) and kept on ice until processing. Lung tissue was then minced with sterile surgical scissors in 500µl HBSS containing 2mg/ml collagenase II and incubated at 37^{o}C for 20 min. The suspension was then passed through a 40µm cell strainer and washed with 5% FBS/RPMI. The number of cells recovered was determined by diluting sample in 4% Trypan Blue and counting by hematocytometer. In some experiments, bacterial burden in the lung was determined by preparing tissue homogenates from the left lobes of the lung. In these circumstances, the left half of the lung was placed in 500µl SPG and kept on ice until processing. Tissue was homogenized with a homogenizer fitted with a sterile stainless steel probe. Homogenates were then centrifuged at 500xg at 4°C for 15 min, and the supernatants were collected for determination of the bacterial load in lungs by *Chlamydia* IFU assay as described in Chapter 2.

In situ fluorescent labeling of peritoneal cavity cells

To track the migration of B cells from the peritoneal cavity to the lung, spleen and MedLN, we utilized an *in situ* carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling procedure (446). CFSE stock solution (10mM in DMSO) was diluted to 50µg/ml in 1ml sterile PBS and injected *i.p.* into naïve mice. Control mice were injected with 1ml PBS alone. At 6h following *i.p*. administration of CFSE, approximately 70% of PerC lavage cells were CFSE-positive.

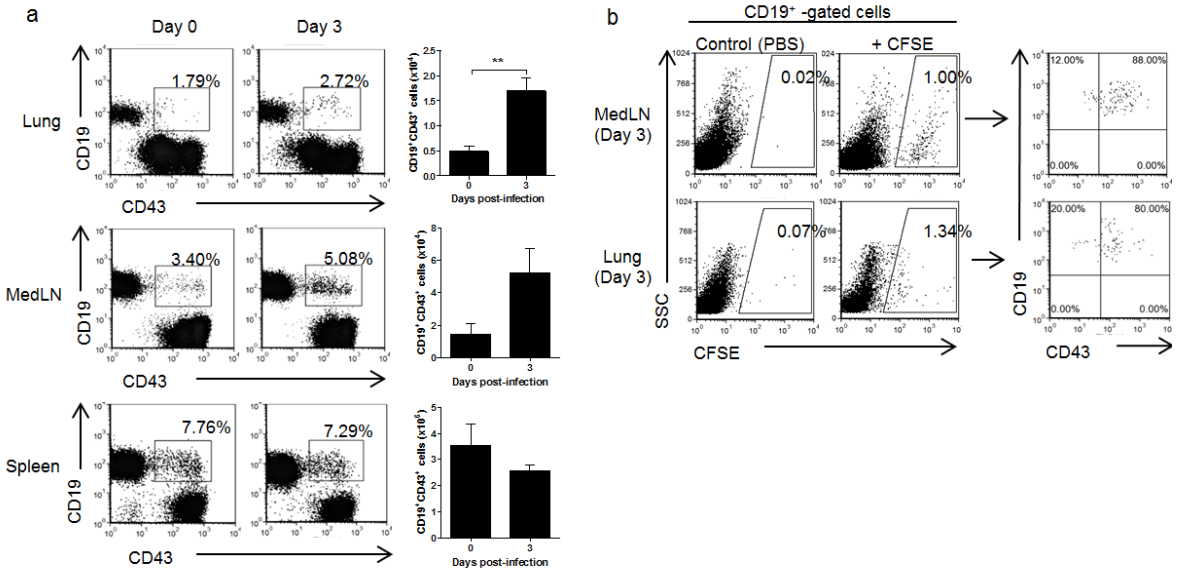
Intratracheal adoptive transfer

Freshly isolated wild-type or IL-10-deficient donor PerC cells were labeled with a 1:200 dilution of PE-conjugated anti-mouse CD19 monoclonal antibody and positive selection by MACS was performed using "Anti-PE Microbeads" as per the manufacturer's protocol. The number of cells recovered in CD19-positive fractions was determined by trypan dye-exclusion method using a hematocytometer. Aliquots of 1 x 10^6 CD19-positive donor cells were prepared in 20µl sterile PBS for intratracheal transfer. To perform intratracheal transfer, recipient mice were anesthetised via *i.p*. injection of ketamine/xylazine anaesthetic. Individual mice were placed in a supine position on a platform and the throat region was soaked in 70% ethanol. The trachea was surgically exposed and 30µl of donor cell preparation was injected into the trachea using a 27G needle. The incision was closed with sterile surgical sutures and the mice were placed in warmed cages and monitored until recovered from anaesthesia. 24 hours following transfer, mice were infected intranasally with 4000 IFU of *C. muridarum*.



APPENDIX D1 *C. muridarum* induces robust IL-10 production by peritoneal CD43+ B-1a cells *in vitro*

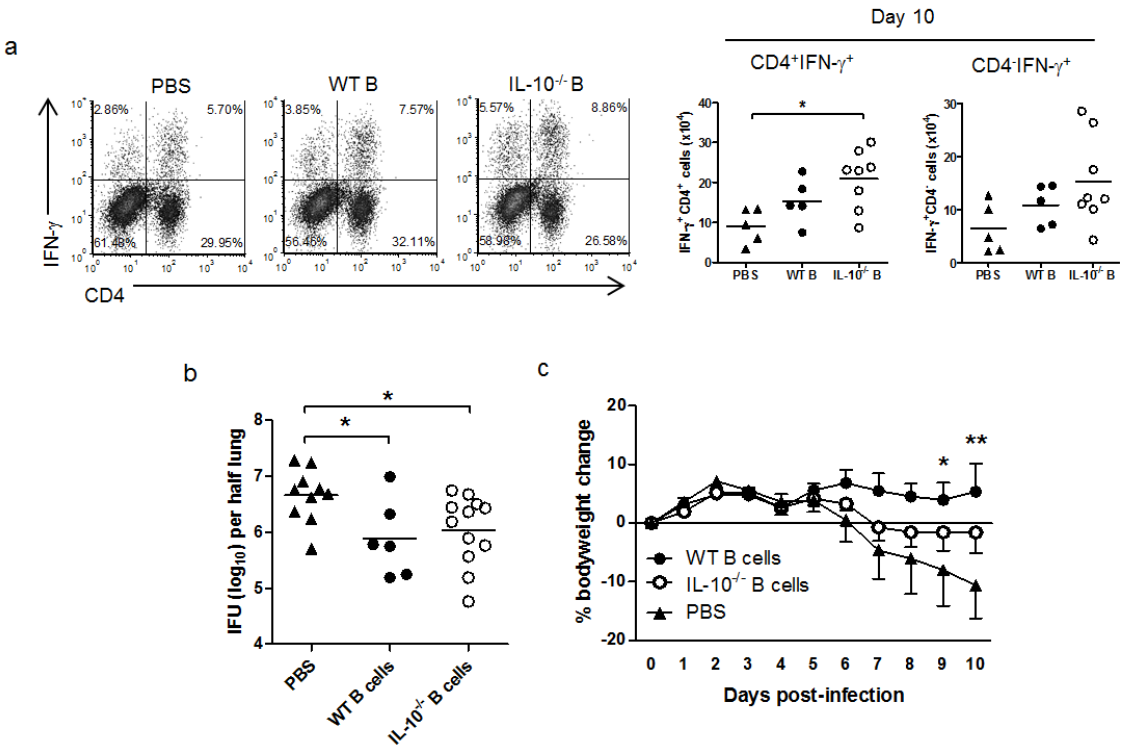
PerC cells from IL-10GFP mice were stimulated with live *C. muridarum* MOI 1.0 for 48-72 h *in vitro*. Cells were then collected and stained with CD19, CD43, and CD5 and analyzed by FACS. (a) FACS analysis of IL-10GFP and CD43 co-expression by CD19-gated peritoneal cavity (PerC) lavage cells at 72h following *C. muridarum* stimulation *in vitro*. (b) CD43 and CD5 co-expression by IL-10GFP^{+}CD19^{+}-gated PerC cells at 48h following *C. muridarum* stimulation *in vitro*. CD19^{+}CD43^{+} PerC B cells sorted from wild-type mice were stimulated with live *C. muridarum* for 72 h and IL-10 levels were measured from culture supernatants at 6, 12, 24, 48, and 72 h post-stimulation (c).



APPENDIX D2 *C. muridarum* respiratory infection induces expansion and recruitment of CD43$^+$ B cells in the lung and MedLN

Mice were infected intranasally with 4000IFU of *C. muridarum* and sacrificed at day 3 post-infection. Single-cell suspensions were prepared from the lung, mediastinal lymph nodes (MedLN) and spleen and stained with CD19 and CD43 and analyzed by FACS.

(a) FACS analysis of CD19$^+$CD43$^+$ cells in the spleen, mediastinal LN (MedLN) and lung at day 0 and 3 post-infection. Value in plot represents percent of live-gated cells. Bar graphs represent absolute numbers, n=4-7 mice/per day, ** $P \leq 0.01$ using Student's *t*-test. In experiments shown in (b), mice were administered a single *i.p.* injection of CFSE (+CFSE) or PBS (PBS) 6 h prior to being infected intranasally with 4000IFU of *C. muridarum*. At day 3 post-infection, mice were sacrificed and single-cell suspensions were prepared from the spleen, MedLN and lung. Cells were stained for CD19 and CD43 and analyzed by flow cytometry. (b) Analysis of CD43 vs CD19 expression by CFSE$^+$CD19$^+$ cells in the MedLN and lung at day 3 post-infection. Data are representative of at least two independent experiments.



APPENDIX D3 Adoptive transfer of PerC B-1 cells enhances host resistance to respiratory *C. muridarum* infection

Mice received PBS or $1x10^6$ of wild-type or IL-$10^{-/-}$ PerC B cells intratracheally 24h prior to intranasal infection. (a) IFN-γ ICCS of cells from the MedLN of mice at day 7 post-infection. Graph represents absolute cell number. n=5-8 mice per group, *$P \leq 0.05$ using one-way ANOVA test (b) Bacterial burden (IFU) from lungs at day 10 post-infection, n=6-12 mice per group, *$P \leq 0.05$ using one-way ANOVA test. (c) Bodyweight change over the course of infection (n=3-5 mice per group), *$P \leq 0.05$, ** $P \leq 0.01$ for WT B cell recipients versus PBS group using two-way ANOVA test. Data are representative of at least two independent experiments.

APPENDIX E Copyright release for published material

September 12, 2013

Jessica Moore-Connors
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Canada
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