The role of integrins and human IDO in the immune response to Chlamydia trachomatis

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The role of integrins and human IDO in the immune response to

*Chlamydia trachomatis*

A dissertation presented

by

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The role of integrins and human IDO in the immune response to

*Chlamydia trachomatis*

Abstract

*Chlamydia trachomatis* is the most common sexually transmitted bacterial infection in the United States. Genital *C. trachomatis* infections cause serious morbidity, including pelvic inflammatory disease and infertility. Multiple arms of the immune response are activated during genital *C. trachomatis* infection, yet this pathogen survives in its preferred human host. The lack of a vaccine and natural immunity to *C. trachomatis* in humans necessitate a better understanding of why the immune response fails to clear the infection.

Immune protection from *C. trachomatis* is dependent on interferon-γ (IFN-γ) derived from CD4⁺ Th1 cells. Prior to our study, the integrin receptor required for CD4⁺ Th1 cell trafficking to the uterus during *C. trachomatis* infection had not been identified. We found that *C. trachomatis* infection resulted in the recruitment of *C. trachomatis*-specific CD4⁺ T cells expressing the integrin α4β1. Genetic ablation or antibody blockade of α4β1, but not α4β7, function led to defective CD4⁺ T cell trafficking to the uterus and high bacterial load. These data show that integrin α4β1 is necessary for CD4⁺ T cell-mediated protection against *C. trachomatis* infection in the genital mucosa.
IFN-γ induces cell-autonomous antimicrobial responses, such as the expression of indoleamine 2,3-dioxygenase (IDO) in human cells. IDO converts tryptophan to kynurenine, which has been shown in human cell culture to restrict C. trachomatis, a tryptophan auxotroph. The *in vivo* function of IDO on C. *trachomatis* pathogenesis has been less characterized because IDO is not strongly expressed in mice following *Chlamydia* infections or IFN-γ stimulation. The absence of an animal that modeled the human cell-autonomous response prompted us to generate a transgenic mouse with tamoxifen-inducible human IDO (hIDO) expression. We found that hIDO-expressing cells from these transgenic mice restricted *C. trachomatis* growth in a tryptophan-dependent manner and suppressed CD4⁺ Th1 cells. *In vivo*, we observed that uterine hIDO expression initially limited *C. trachomatis* growth but ultimately exacerbated bacterial burden. These findings demonstrate the hIDO expression in this model is not sufficient to clear genital *C. trachomatis* infection. Our results imply that humans lack natural immunity to genital *C. trachomatis* because the pathogen evades the IDO response that is generated by IFN-γ.
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"Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled."

-Sir William Ramsay
CHAPTER 1: General introduction
THE HUMAN PATHOGEN: *CHLAMYDIA TRACHOMATIS*

**Major public health concern**

*C. trachomatis* is a Gram-negative bacterium causative of serious reproductive and ocular disease. Despite its sensitivity to antibiotic treatment and effective diagnostic tests to detect the infection, *C. trachomatis* continues to be the most prevalent bacterial sexually transmitted infection. In the United States alone, over 1.4 million *Chlamydia* cases were reported to the Centers for Disease Control and Prevention (CDC) in 2014, far outpacing the number of *Neisseria gonorrhoea* cases (about 350,000) that same year (CDC, 2015). The increase in yearly reported cases of *C. trachomatis* (2.8% increase since 2013) is greater than the United States population growth of 0.7% demonstrating that the rise in absolute case number is not simply due to population growth. It is challenging to determine the exact cause for the higher rate of *C. trachomatis* infections since such trend could be driven by behavioral (increase in sexual transmission) or clinical (better screening or failed treatment) factors. Globally, the prevalence of *C. trachomatis* is estimated to be 4.2%, which corresponds to an alarming 132 million predicted new case per year (Newman et al., 2015). The economic burden of genital *C. trachomatis* infection is estimated to be over USD $500 million per year (Owusu-Edusei et al., 2013).

In regards to ocular disease, trachoma results from *C. trachomatis* infection of the eye and is the leading infectious cause of blindness worldwide.
with an estimated 1.3 million cases of blindness. A further 8.2 million people have trichiasis, an inversion of the infected eyelid that scars the cornea leading to blindness if left untreated. Ocular C. trachomatis infection is a continuing problem in endemic regions of the world including Africa, South America, and Asia. Although it is difficult to quantify the societal burden of a lifetime of blindness, one study argues that the aggregate loss of economic activity arising from visual disability due to blinding trachoma could be up to USD $8 billion (Burton and Mabey, 2009).

**Disease**

In industrialized countries, the most significant health and economic burden of C. trachomatis arises from complications in the reproductive organs of infected women. C. trachomatis can ascend to the upper genital tract where it causes pelvic inflammatory disease (PID), ectopic pregnancy, and ultimately infertility. C. trachomatis is often subclinical with as many as 85 to 90% of female cases being asymptomatic. These asymptomatic cases may persist for months until the infection causes pain and discomfort or is spontaneously cleared in some patients (Peipert, 2003). In men, up to 50% of cases are asymptomatic (Redgrove and McLaughlin, 2014). The lack of clinical symptoms suggests that C. trachomatis infections are underreported and that an infected person may inadvertently transmit the pathogen for a prolonged duration. About 15% of untreated cases of sexually transmitted C. trachomatis result in PID
(Brunham et al., 2015). During pregnancy, Chlamydia-infected mothers are more prone to postpartum endometritis, preterm labor, and low birth weight (Andrews et al., 2000). During delivery there is a 23-70% probability of C. trachomatis transmission from infected mother to child. In most cases, infants will develop conjunctivitis; however, pulmonary infections from exposure to C. trachomatis during birth have been reported (Tiller, 2002).

C. trachomatis infections are diagnosed by nucleic acid amplification test or by culture. Screening is suggested for at risk populations including young, sexually active adults as well as symptomatic patients with pelvic pain. Patients who are Chlamydia-positive or who have been diagnosed with PID receive broad antimicrobial treatment due to the high frequency of co-infection with other sexually transmitted pathogens (CDC, 2010). Failure to comply with the entire course of antibiotic treatment is a contributing factor to relapsing or persistence C. trachomatis infection (Somani et al., 2000).

To date the cause(s) of the divergent clinical outcomes of C. trachomatis infections in humans are not yet fully understood. A segment of C. trachomatis-infected people will spontaneously clear the infection without antibiotic treatment while others will develop complications to different degrees (Geisler, 2010). In a study of previously identified C. trachomatis-positive patients who did not receive antibiotics, 32% became culture negative in 45-day follow up examination (Parks et al., 1997). However, this study did not assess whether these culture-negative patients were still C. trachomatis free after 45 days or if
they developed PID. Interestingly, a study found that two leukocyte antigen variants were associated with recurrent *C. trachomatis* infection. Furthermore, a G-C-C haplotype variation in *il10* (encodes for the cytokine interleukin-10) was associated with a lower incidence of recurrent infection (Wang et al., 2005). It is probable that the collective contribution of these factors as well as other genetic (host and pathogen) and behavioral drivers ultimately influence the disease outcome in *C. trachomatis*-infected humans.

Given the high prevalence of *C. trachomatis* infections, a safe and effective vaccine would greatly reduce the global burden caused by this pathogen. Other measures such as the expansion of sexual education to promote barrier use and readily available, affordable diagnostics would reduce the rate of transmission and increase awareness. Nonetheless, a safe vaccine that provides long-lasting protection to the recipient and those he/she is in intimate contact with would be the most cost-effective measure to control and eventually eradicate *C. trachomatis* (Owusu-Edusei et al., 2015). In order to develop and test widely efficacious vaccines, researchers and clinicians must first understand the underlying biology of the pathogen and host species to exploit weaknesses in the prior and strengths in the latter. Given that clinical trials have a very limited space for exploratory experimentation due to ethical concerns, establishing animal models that recapitulate the human infection is a necessary process for discovery and translational medicine. Humanized animal models provide useful insights into *C. trachomatis* and host biology. The next
sections present a summary of relevant findings in the fields of *C. trachomatis* and the host response to infection.

**Phylogeny**

All members of the family Chlamydiaceae are Gram-negative, obligate intracellular bacteria adapted to infect specific host species, ranging from humans to animals. The genus *Chlamydia* encompasses nine species and includes the human pathogen *C. trachomatis* (Nunes and Gomes, 2014). Moreover, there are three *C. trachomatis* biovars each with multiple serovars. The ocular biovar includes the serovars A-C, which have a tropism for epithelial cells in the eyelid and are causative of blinding trachoma in endemic regions (Hu et al., 2013). The genital biovar, consisting of serovars D-K, infect endocervical squamous columnar epithelial cells and ascend to the upper genital epithelium causing inflammatory disease in reproductive organs and infertility (Buckner et al., 2013). Lastly, the lymphogranuloma venereum (LGV) biovar includes serovars L1-L3. The LGV biovar, clinically the most invasive of the three biovars, infects the urogenital and anorectal regions with the potential to become a systemic infection by spreading to lymphatic tissue if left untreated (McLean et al., 2007). LGV *C. trachomatis* is able to survive longer than trachoma strains in mononuclear phagocytes potentially providing a mechanism for migration to lymph nodes (Yong et al., 1987).
Life cycle

The biphasic developmental cycle of *C. trachomatis* is truly interesting in that it alternates from an infectious, extracellular form termed elementary body (EB) to an intracellular, non-infectious, replicative state called reticulate body (RB) (Figure 1-1). Once inside the host cell, *C. trachomatis* dwells inside a parasitophorous vacuole known as an inclusion and replicates by binary fission (Moulder, 1991). Prior to replication, the immotile EB must first make contact with the mucosal epithelial cell and successfully induce its uptake. *Chlamydia* outer member protein 2 (OmcB) binds reversibly with heparin sulfate proteoglycans (HSPGs) present on the surface of epithelial cells (Rosmarin et al., 2012; Su et al., 1996). This binding affinity between OmcB and HSPGs is different between biovars and may provide an explanation for the selective tissue tropism (Moelleken and Hegemann, 2008). A previous study demonstrated that the major outer membrane protein (MOMP) of *C. trachomatis* is an adhesin that promotes electrostatic interactions with host cells (Su et al., 1990).
Figure 1-1. Developmental cycle of *Chlamydia*.
Extracellular EBs bind to mucosal epithelial cells in the genital tract inducing their uptake. Shortly after, EBs differentiate to RBs within the relative safety of the inclusion that hides the full repertoire of bacterial antigens from host cell recognition. Secreted virulent factors promote host lipid transport to the inclusion membrane. In addition, Chlamydial effector proteins alter the cell cycle of the host cell in order to delay or promote apoptosis depending on the differentiation state of the pathogen. RBs will replicate and then transition asynchronously to EBs approximately a day after the start of infection. EBs are released into the environment by host cell lysis or extrusion. This process enables the next round of infections in the mucosal epithelium causing continual inflammation and damage to the reproductive tissue. Figure from (Bastidas et al., 2013) *Copyright 2013 Cold Spring Harbor Laboratory Press.*
The *C. trachomatis* genome is relatively small and compact, composed of a single chromosome of 1.04 million base pairs and a 7-kilobase pair plasmid. The L2 serovar chromosome encodes 889 coding sequences while the plasmid has a total of 8 coding sequences (Stephens et al., 1998; Thomson et al., 2008). *C. trachomatis* has three different types of secretion systems (T2SS, T3SS, and T5SS) with the T3SS gene content taking up approximately 10% of the D serovar genome (Betts-Hampikian and Fields, 2010). EBs inject pre-packaged effector proteins inside the host cell through the T3SS to facilitate invasion (Saka et al., 2011). The secreted effector translocated actin-recruiting phosphoprotein (Tarp) rapidly remolds the host cytoskeleton by nucleating actin and recruiting the actin-related protein 2/3 (ARP2/3) complex to enable EB intake (Jiwani et al., 2013).

The successful development of the inclusion and bacterial replication depends on the divergence of host lipid-rich endosomes and multivesicular bodies from the Golgi apparatus to the inclusion. Inclusion membrane proteins (Incs) recruit RAB GTPases and SNARE proteins to the inclusion, thereby hijacking the host cell’s master regulators of membrane fusion (Damiani et al., 2014; Südhof and Rothman, 2009). This process is particularly important for the acquisition of eukaryotic lipids that *C. trachomatis* is unable to synthesize endogenously such as cholesterol, sphingomyelin and phosphatidylinositol. The pathogen requires these lipids for a range of biological processes including inclusion formation and stability, growth, and RB to EB conversion (Elwell and
Engel, 2012). Consequently, disruption of host vesicle transport to the inclusion, either genetically or chemically, severely impairs *C. trachomatis* growth (Reiling et al., 2013).

In addition to lipid redirecting, *C. trachomatis* must appropriate amino acids from the host cell in order to translate proteins needed for growth and differentiation. Genital *C. trachomatis* is a natural auxotroph for tryptophan, hence its survival and infectious state depend on its ability to acquire this amino acid or produce it from indole using tryptophan synthase B (TrpB) (Kari et al., 2011). The relationship between the host cell, *C. trachomatis*, and tryptophan will be discussed at length in further sections of my dissertation. Experimentally treating infected cells with an inhibitor of eukaryotic protein synthesis (cycloheximide) promotes *C. trachomatis* growth, suggesting that the pathogen diverts free host amino acids in the cytoplasm for its own protein synthesis (Ripa and Mårdh, 1977). *C. trachomatis* can also manipulate lysosomes in the proximity of the inclusion to acquire amino acids (Ouellette et al., 2011).

*C. trachomatis* secretes a multitude of effector proteins that manipulate the host cell cycle and control host cell survival and death for the completion of its developmental cycle. *C. trachomatis* induces pro-survival signals by binding to the EphrinA2 receptor tyrosine kinase thereby robustly activating the phosphoinositide 3-kinase (PI3K) pro-survival pathway (Subbarayal et al., 2015). Similarly, *C. trachomatis* activates the mitogen-activated protein/extracellular signal–regulated kinases (MEK/ERK) pathway independently of canonical Ras-
Raf-MEK signaling to prolong survival and delay apoptosis (Gurumurthy et al., 2010). A proteomic screen observed that activator protein 1 (AP-1), a critical transcription factor in cell proliferation and stress, was stabilized upon C. trachomatis infection to promote pathogen growth (Olive et al., 2014). On the other hand, C. trachomatis can block apoptosis by downregulating the central tumor suppressor p53 through the phosphorylation of the ubiquitin ligase Murine Double Minute 2 (MDM2) (González et al., 2014; Siegl et al., 2014). The activation of these pro-survival, potentially pro-carcinogenic pathways during C. trachomatis infection may provide an explanation for the positive correlation in women with squamous cervical cancer that are co-infected with human papillomavirus (HPV) and C. trachomatis (Smith et al., 2002).

In addition to the EB and RB forms, C. trachomatis can differentiate to a reversible persistent state during stressful conditions within the host cell (Morrison, 2003). For example, antibiotic treatment with azithromycin induces the differentiation of C. trachomatis to a culture-negative, viable, metabolically active state. In vitro azithromycin treatment of C. trachomatis-infected epithelial cells results in large, atypical, aberrant inclusions with altered bacterial antigen levels (Dreses-Werringloer et al., 2001). Treatment with suboptimal concentrations of the cytokine interferon-gamma (IFN-γ) transforms C. trachomatis inclusions to a similar persistent state (Beatty et al., 1993). The removal of IFN-γ from cell cultures allows aberrant C. trachomatis inclusion to differentiate back to a replicative or infectious state and resume the
developmental life cycle (Beatty et al., 1995). Key studies demonstrated that intracellular tryptophan depletion causes the persistent state in cell culture models. Intracellular depletion of this essential amino acid is induced by IFN-γ signaling in human cell culture through the enzyme indoleamine 2,3-dioxygenase (IDO) (Beatty et al., 1994a; Beatty et al., 1994b). The persistent C. trachomatis state has been seen in infected human and animal tissues as well, confirming that this state is not a cell culture artifact (Hogan et al., 2004).

The final event of the developmental cycle is the release of new EBs that will infect epithelial cells in the vicinity. EBs are released into the mucosal environment by either extrusion or host cell lysis two to three days after the start of the infection, depending on the Chlamydia species. Not surprisingly, the host cell machinery is needed for extrusion of the inclusion including actin, myosin, and Rho GTPases. The infected cell survives after inclusion extrusion but under certain conditions may require plasma membrane repair (Beatty, 2007). During lysis, Chlamydia consecutively permeabilizes the membranes of the inclusion, nucleus, and plasma membrane to release EBs and kill the host cell (Hybiske and Stephens, 2007). The pathogen’s developmental cycle is then repeated once again in surrounding cells.

The Chlamydia literature is rich with studies identifying roles for effector proteins in modulating the machinery and processes of the host cell. Thus far, I have introduced a handful of essential host pathways that are modulated by C. trachomatis to enable the completion of its developmental cycle. For the sake of
brevity the exhaustive list of effectors will not be discussed here. However, I must mention the secreted serine protease Chlamydial-like activity factor (CPAF), an effector protein that became a contentious topic in the field during my doctoral studies. Recent revelations questioned the physiological functions of CPAF in the processing of bacterial and host cellular proteins. Initial studies probing the role of CPAF determined that this effector degraded RFX-5 and USF-1, which resulted in the downregulation of major histocompatibility complex (MHC) class I and II (Zhong et al., 2000). In total, CPAF was proposed to cleave or degrade 16 proteins affecting numerous cellular pathways (Chen et al., 2012). These studies concluded that CPAF-mediated processing was happening intracellularly during *C. trachomatis* infection. The claims of these findings were later questioned once researchers came to realize that the cleaving activity of CPAF was not fully inactivated with the standard cell lysis treatment. Only a harsher experimental treatment (8M urea) proved to effectively eliminate the proteolytic activity of CPAF. This realization raised concerns because it was no longer clear which proteins were real targets of CPAF as opposed to artifacts of postlysis degradation (Tan and Sütterlin, 2014).

A reverse genetic study conclusively determined that CPAF was important for the cleavage of host cell vimentin filaments and the nuclear envelope protein lamin-associated protein-1 (LAP1), but was not as promiscuous as previously thought. The CPAF-deficient *C. trachomatis* strain produced fewer EBs after each round of *in vitro* infection, indicating significant
biological importance (Snavely et al., 2014). The next section will discuss how recent innovations in experimental *Chlamydia* genetics are changing the field for the better as shown with CPAF.

**Developments in genetic manipulation**

For decades, *Chlamydia* species were considered genetically intractable organisms due to the difficulty of experimental genetic manipulation. The need to propagate *C. trachomatis* in mammalian cell cultures and the bacteria’s biphasic, obligate intracellular lifestyle posed significant obstacles. Initial experimental introduction of foreign DNA was carried out by electroporation of *Chlamydia* with substantial quantities of DNA. Although successful, this method yielded a low frequency of transformants (Binet and Maurelli, 2009). Evidence of horizontal gene transfer from eukaryotes demonstrated that *Chlamydia* species were naturally capable of picking and incorporating foreign DNA from the environment (Stephens et al., 1998). Additionally, lateral gene transfer between *Chlamydia* species was witnessed in serovar D, which contains large regions of high homology to the serovars E and F (Jeffrey et al., 2010). These examples of natural introduction of foreign DNA prompted researchers to device novel efficient ways of genetically manipulating *C. trachomatis* to more accurately study gene function.

The development of a relatively simple system for the stable transformation of *C. trachomatis* with recombinant DNA has been one of the
most important advancements in the field. In short, *C. trachomatis* EBs are incubated with calcium chloride and a shuttle plasmid, heat-shocked, and selected for antibiotic resistance during passaging. During antibiotic selection, non-transformed inclusions are large and aberrant while successful transformants show normal inclusion morphology (Wang et al., 2011). Transformation with recombinant DNA has recently allowed researchers to study gain-of-function *C. trachomatis* mutants. For example, this protocol was used to generate *C. trachomatis* strains that express fluorescent proteins in order to better study its biology using microscopy and flow cytometry (Vromman et al., 2014). As with other genetically tractable microbes, the trans complementation of *C. trachomatis* mutants with transformation can now be used to rescue loss-of-function phenotypes to confirm gene function.

Endogenous *C. trachomatis* gene expression can be experimentally inhibited by chemical mutagenesis or targeted transposon insertion for forward or reverse screens. A recent study generated and mapped 934 unique chemically mutagenized *C. trachomatis* strains using whole-genome sequencing (Kokes et al., 2015). With the advent of *C. trachomatis* transformation, the TargeTron system (based on retrotransposition) provides a platform to specifically target insertions in the genome for gene inactivation. The TargeTron system was successfully used to eliminate IncA in *C. trachomatis* (Johnson and Fisher, 2013). Although useful, the low efficiency of
transformation is a drawback for plasmid based mutagenesis techniques to be used on a genome wide scale.
INNATE AND ADAPTIVE IMMUNITY

The species-specific relationship with *Chlamydia* and the host is complex and fascinating. Elucidating *C. trachomatis* pathogenesis invariably delves into the biology of the host, as the pathogen’s life cycle is fundamentally linked to its intracellular niche. In higher organisms like humans, *C. trachomatis* must evade the innate and adaptive immune responses to complete its developmental cycle. Host defenses range from anatomical impediments to antigen-specific lymphocytes that trigger antimicrobial signaling cascades. *C. trachomatis* stimulates diverse arms of the immune system, yet infections only elicit a partial immune response that do not clear the pathogen or prevent re-infection (Batteiger et al., 2010). Comprehensive investigations of host responses to *C. trachomatis* permit the assessment of protective pathways from inadequate ones. This information can then be used to develop better animal models to test vaccine candidates. I have summarized the innate, adaptive, and cell-autonomous host defenses that beleaguer *C. trachomatis* during infection.

**Extracellular barriers**

The first defense in the reproductive system is the mucosal lining. The antimicrobial peptides human defensin 5 (HD5) and human neutrophil alpha defensin 1-3 (HNP1-3) are produced in higher amounts by epithelial cells and neutrophils during *C. trachomatis* infection (Porter et al., 2005). Aside from their
direct bacterial inhibition, defensins are also chemoattractants for immune cells and trigger the production of pro-inflammatory interleukin-1β (IL-1β) (Chertov et al., 1996; Perregaux et al., 2002). C. trachomatis must survive the usual biology of the reproductive tract such as cell shedding, vaginal flora competition, and the mucus layer to establish an infection (Eggert-Kruse et al., 2000).

Detection

Pattern recognition receptors (PRRs) are innate molecules that recognize pathogen associated molecular patterns (PAMPs), conserved microbial regions that trigger immune activation (Kawai and Akira, 2010). Epithelial cells, though not professional immune cells, can recognize C. trachomatis and secrete antimicrobial factors. C. trachomatis-infected epithelial cells produce IL-8, a chemokine that recruits neutrophils to areas of inflammation (Baggiolini and Clark-Lewis, 1992; Buchholz and Stephens, 2006). The adaptor protein myeloid differentiation primary response (MYD88) and toll-like receptor 2 (TLR2) are needed for IL-8 secretion in epithelial cells during C. trachomatis infection (O'Connell et al., 2006). The cytoplasmic receptor nucleotide-binding oligomerization domain-containing 1 (NOD1) recognizes C. trachomatis peptidoglycan and induces IL-8 through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Buchholz and Stephens, 2008; Welter-Stahl et al., 2006). In addition, stimulation of NOD1
results in IL-1β secretion in human trophoblasts (Kavathas et al., 2013). Interestingly, certain functional polymorphisms in NOD1 correlated with higher risk of tubal factor infertility in *C. trachomatis*-infected women (Branković et al., 2015).

DNA sensor cyclic GMP-AMP synthase (cGAS) and signaling adaptor stimulator of IFN genes (STING) are required for the generation and recognition of cyclic GMP-AMP and production of IFN-β during *C. trachomatis* infection. *C. trachomatis* directly synthesizes cyclic di-AMP with its own diadenylate cyclase. Cyclic di-AMP is a ligand for STING that induces IFN-β production through the phosphorylation of interferon regulatory factor 3 (IRF3) (Barker et al., 2013). Interestingly, it has been shown that high levels of IFN-β expression results in exacerbated pathology in the murine genital tract (Zhang et al., 2014).

Moreover, inflammasome-mediated activation of caspase 1 promotes the growth of *C. trachomatis* inside the host cell (Abdul-Sater et al., 2009). The signaling cascade triggered by PRRs is needed for microbial restriction but also results in immunopathology that can permanently damage tissue (Darville and Hiltke, 2010).

**Innate immune cells**

Innate immune cells are promptly recruited to the site of infection to control bacterial growth. The first arrivers, neutrophils, kill *C. trachomatis in vitro*
and may significantly contribute to the tissue pathology by releasing highly inflammatory factors in the endometrium (Register et al., 1986). The earliest IFN-γ response derives from natural killer (NK) cells, which are recruited as early as 12 hours after infection (Tseng and Rank, 1998). Epithelial cell derived IL-18 and dendritic cell (DC) produced IL-12 attracts and promotes IFN-γ secretion in NK cells during C. trachomatis infection (Hook et al., 2005). IFN-γ-mediated antimicrobial pathways in epithelial cells will be discussed at length in the next section. Although NK cells are more commonly known for their cancer cell killing activity, the antimicrobial activity of this innate immune cell type is becoming more evident (Adib-Conquy et al., 2014; Zamai et al., 2007). The role of NK T cells during genital C. trachomatis infection has not been explored extensively.

Professional phagocytic cells, such as macrophages and DCs, in the genital tract are both antimicrobial agents as well as liaisons with adaptive immune cells. In vitro infected macrophages restrict C. trachomatis growth by targeting the pathogen for lysosomal destruction. Unlike epithelial cells, the Golgi apparatus is not disrupted in macrophages during infection suggesting that C. trachomatis is less adapted to survive in this cell type. (Sun et al., 2012). Infected human epithelial cells release granulocyte-macrophage colony stimulating factor (GM-CSF) to trigger the recruitment and maturation of monocytes in the genital tract (Porcella et al., 2015). Clinically, it is speculated that reactive arthritis takes place when infected monocytes/macrophages
migrate to the synovial fluid in joints causing localized inflammation (Villareal et al., 2002).

The innate immune system is instrumental in the initial control of pathogen growth. Ultimately, successful innate immunity recognizes the pathogen and slows its growth sufficiently for the host organism to mount a timely, robust adaptive immune response. The clearance of *C. trachomatis* depends on this precise relay between the innate and adaptive arms of immunity.

**Antigen presentation**

Professional antigen presenting cells (APCs) play a crucial role in initiating the adaptive immune response. *C. trachomatis* antigen presentation on MHC class I or class II is the first step in the activation and differentiation of pathogen-specific CD8⁺ T cells or CD4⁺ T cells, respectively. DCs activate lymphocytes by binding to the cognate T cell receptor, co-stimulating the surface receptor CD28, and releasing differentiation-inducing cytokines (Friedl et al., 2005; Harris and Ronchese, 1999; Kapsenberg, 2003). In the murine uterus, there are three resident populations of MHC II⁺ APCs: CD11b⁺ CD11c⁻ CD103⁻ macrophages, CD103⁺ CD11b⁻ DCs, and CD103⁻ CD11b⁺ DCs. Interestingly, the two DC subsets activate different populations of CD4⁺ T cells. These macrophages do not induce a *C. trachomatis*-specific T lymphocyte response using *in vitro* cultures (Stary et al., 2015).
The role of MHC is to present peptide fragments on the cell surface for recognition by the appropriate T cell. MHC class I molecules present peptides that are found in the cytoplasmic compartment of cells. All nucleated cells are able to express MHC class I on their surface. MHC class I consists of three \( \alpha \) subunits and one \( \beta_2 \)-microglobulin that initially are partially folded and bound to calnexin. This complex then binds to a series of chaperone proteins and to the transporter associated with antigen processing (TAP) on the endoplasmic reticulum (ER). TAP transports cytosolic peptides to the ER that bind to MHC class I to finalize the folding of the complex. The antigen-loaded MHC class I molecule is transported from the ER to the cell surface via the Golgi apparatus for extracellular signaling (Vyas et al., 2008). The co-receptor CD8 enables the interaction of \( \text{CD}8^+ \) T cells with MHC class I molecules on APCs. Matching T cell receptors (TCRs) recognize the antigen presented on the groove of the MHC class I molecule. This recognition between TCR and antigen-loaded MHC class I activates \( \text{CD}8^+ \) T cells (Artyomov et al., 2010).

MHC class II presents peptides that are derived from proteins in the extracellular environment. Protein antigens are processed inside endosomes, where they are cleaved by proteases upon acidification of the vesicle. The MHC class II molecule is located in the ER, where it is bound to the invariant chain that prevents premature peptide loading. The invariant chain is processed into a shorter fragment called class II-associated invariant-chain peptide (CLIP) that remains bound to the groove of MHC class II (Bikoff et al., 1993). In the
endosome, HLA-DM binds to the MHC class II molecule to displace CLIP and allow peptide antigens to bind to the groove. The antigen-loaded MHC class II molecule is then directed to the cell surface for presentation (Sanderson et al., 1994). The co-receptor CD4 binds to MHC class II to permit activation of CD4⁺ T cells with the correct TCR.

IFN-γ signaling has many effects on T cells during infection. One of those functions is in the control of antigen processing and presentation. The importance of IFN-γ for MHC class I regulation is evident by its role in the induction of several components that are crucial in this antigen presentation pathway. For example, IFN-γ upregulates proteasome subunits (LMP-2, LMP-7, and MECL-1) and activators (PA28α and PA28β) that increase the efficiency of MHC class I presentation (Boes et al., 1994; Groettrup et al., 1996). Additionally, the TAP proteins, MHC class I heavy chain, and β2-microglobulin are expressed more robustly in the presence of IFN-γ (Epperson et al., 1992; Shirayoshi et al., 1988; Wallach et al., 1982).

IFN-γ regulates CD4⁺ T cell immunity through MHC class II. B cells, macrophages, and DCs are professional APCs that constitutively express MHC class II genes. However, IFN-γ further enhances the expression of MHC class II genes in these professional APCs. Other cell types, such as mucosal epithelial cells, do not constitutively present MHC class II on their surface, yet are able to express MHC class II when exposed to IFN-γ. Mechanistically, this cytokine is key to the induction of the MHC class II transactivator (CIITA), a master positive
activator protein that controls the transcription of MHC class II genes (Steimle et al., 1994). Moreover, IFN-γ upregulates other constituents of MHC class II such as the α and β chains, the invariant chains, HLA-DM, and lysosomal proteases (cathepsins). Therefore, IFN-γ allows CD4⁺ T cells to recognize antigen on cell types that would not normally express MHC class II in order to exert their effector activities (Schroder et al., 2004). For example, epithelial cells in the intestinal mucosa express MHC class II upon IFN-γ stimulation, which directly affects CD4⁺ T cell immunity since CD4⁺ T cells recognize antigen loaded on MHC class II. Furthermore, IFN-γ produced by CD4⁺ T cells is required for MHC class II expression in intestinal epithelial cells demonstrating that IFN-γ expression skews towards a Th1 cell response (Thelemann et al., 2014).

The relationship between IFN-γ-producing CD4⁺ T cells and MHC class II expression is of significance in other mucosal tissues as well. Findings from the Starnbach lab suggest that IFN-γ signaling is needed for MHC class II presentation on C. trachomatis-infected genital epithelial cells and for CD4⁺ T cell-mediated protection in the uterus (unpublished data from Dr. Catarina Nogueira). Yet it is still not entirely clear whether other immune cells that produce IFN-γ drive MHC class II expression on genital epithelial cells to the same degree that CD4⁺ T cells do, and whether CD4⁺ T cells need to directly interact with the infected epithelial cell to effect pathogen clearance. Further studies are needed to elucidate how IFN-γ affects CD4⁺ T cells through antigen presentation on epithelial cells in the genital tract during C. trachomatis infection.
**B cell response**

The importance of the adaptive immune response to *Chlamydia* is evident in severe combined immunodeficiency (SCID) mice where pathogen burden is significantly increased (Magee et al., 1993; Pal et al., 2009). SCID mice are characterized by a deficiency in humoral and cell-mediated immunity due to the lack of functional B and T cells (Bosma and Carroll, 1991). However, the relative contribution of B cells and antibodies in providing immunity during *C. trachomatis* infection is not completely clear.

A study showed that during primary infection, B cell-deficient mice µMT mice had similar bacterial load to wildtype controls. Infected wildtype mice did produce high-titers of anti-*Chlamydia* immunoglobulin IgG2a, IgG2b, and IgA while µMT mice did not have any detectable antibodies. When re-challenged µMT mice did have significantly more burden than control groups indicating a possible role of B cells and antibodies with effective memory development (Su et al., 1997). Other studies noted that *Chlamydia*-specific CD4⁺ T cell priming was enhanced by B cells to limit bacterial spread (Li and McSorley, 2013) and that MOMP antibodies blocked entry into host cells (Peeling et al., 1984).

Contrary, a recent study found that mature B cells were not necessary to confer protection during a primary or secondary *C. trachomatis* infection. Similarly, passive transfer of serum from immunized mice did not provide protection to recipient mice (Nogueira et al., 2015). Collectively, these finding indicate that B cells are not the crucial cell type needed for clearance, given that
**C. trachomatis** is an intracellular pathogen. In some models, B cells may still play a minor role in the development of T cell memory to **C. trachomatis**.

**CD8⁺ T cell immunity**

Robust cell-mediated immunity is needed to eliminate intracellular pathogens. Effector CD8⁺ T cells restrict intracellular pathogens by granzyme and receptor mediated killing of the infected cell or by inducing antimicrobial pathways via cytokine secretion (Harty et al., 2000). A small fraction of activated CD8⁺ T cells then differentiate to a memory phenotype that quickly responds to re-infection (Kaech et al., 2002).

Theoretically, CD8⁺ T cells should readily target **C. trachomatis**-infected epithelial cells and clear the infection. *Chlamydia* antigens become available in the host cell cytosol following the translocation of bacterial effectors, thus providing peptides for MHC class I loading (Starnbach et al., 2003). Although activated CD8⁺ T cells are present in **C. trachomatis**-infected mice, this endogenous CD8⁺ T cell population does not confer protection (Loomis and Starnbach, 2006).

Two studies dissected this shortcoming in protective CD8⁺ T cell immunity and discovered two different mechanisms of defective memory development. One of these studies demonstrated that excessive amounts of inflammatory cytokines had an adverse effect on CD8⁺ T cell immunity. High levels of IFN-γ and IL-12 during genital **C. trachomatis** infection inhibited
protective memory development by increasing the proportion of short-lived effector CD8⁺ T cells. Transiently decreasing IFN-γ and IL-12 levels increased the protective *C. trachomatis*-specific memory CD8⁺ T cell phenotype resulting in lower burden in mice (Zhang and Starnbach, 2015).

The other study demonstrated that the inhibitory receptor programmed death-1 (PD-1) was upregulated on *C. trachomatis*-specific CD8⁺ T cells following genital infection. The elevated expression of PD-1 on CD8⁺ T cells skewed memory differentiation to favor a less protective subset. Genetic ablation or antibody blockade of PD-1 and programmed death ligand-1 (PD-L1) restored the protective memory CD8⁺ T cell phenotype during secondary infection (Fankhauser and Starnbach, 2014). These findings suggest that the defective memory recall during *C. trachomatis* infection is similar to the impaired CD8⁺ T cell phenotype observed in other chronic infections (Wherry et al., 2004).

Studies in human cell culture have characterized CD8⁺ T cells *ex vivo*, yet it is still unclear whether CD8⁺ T cells provide significant protection in the human genital tract. Infected human DCs in culture secrete IL-12 and activate *C. trachomatis*-reactive CD8⁺ T cells (Matyszak and Gaston, 2004; Matyszak et al., 2002). The pro-inflammatory cytokine IL-12 is key in mounting a T helper (Th) 1 response that generates IFN-γ-mediated immunity (Trinchieri, 2003). Moreover, the *Chlamydia* protein OmcB was identified as a human leukocyte antigen (HLA) A0101-restricted antigen that was highly stimulatory of human CD8⁺ T
cells isolated from the peripheral blood of donors. These clones drastically inhibited *C. trachomatis* growth *in vitro* independently of lytic action (Gervassi et al., 2004). CD8<sup>+</sup> effector memory T cells from the human endocervix have low perforin content, suggesting that *in vivo* this cell population is less conducive to localized immune damage (Ibana et al., 2012). In non-human primates, CD8<sup>+</sup> T cells do provide protection against ocular *C. trachomatis* infection (Olivares-Zavaleta et al., 2014).

**CD4<sup>+</sup> T cell immunity**

CD4<sup>+</sup> T cells play a very important role in conferring protection during *C. trachomatis* infection. CD4 or MHC class II knockout mouse lines have greater burden in the genital tract (Morrison et al., 1995). Similarly, transient antibody-mediated depletion of CD4<sup>+</sup> T cells results in higher *C. trachomatis* levels. Adoptive transfer of *C. trachomatis*-specific, transgenic CD4<sup>+</sup> T cell clones confers protection to previously naïve mice after genital infection (Gondek et al., 2012).

CD4<sup>+</sup> T cells are activated and recruited from the iliac lymph nodes to the genital tract after *C. trachomatis* infection (Roan et al., 2006). Naïve CD4<sup>+</sup> T cells are first activated by DCs through cognate antigen presented on MHC class II. (Bousso, 2008). Chlamydial MOMP antigens are preferentially loaded on MHC class II (Karunakaran et al., 2015). Stimulated CD4<sup>+</sup> T cells then
differentiate to distinct helper subsets contingent on additional information provided by cytokines and other signaling molecules (Zhu et al., 2010). CD4\(^+\) Th2 cells, characterized by secretion of IL-4, do not confer protection to infected mice despite promoting high levels of anti-Chlamydia antibody titers (Hawkins et al., 2002). Adoptive transfer of pre-skewed C. trachomatis-specific CD4\(^+\) Th2 cells exacerbate infection in the genital tract (Gondek et al., 2009). Similarly, the generation of regulatory T cells (T regs) is detrimental for pathogen clearance as well (Stary et al., 2015). T helper 17 (Th17) cells release the neutrophil recruiting cytokine IL-17 that may contribute to oviduct pathology during Chlamydia infection (O'Meara et al., 2014). On the other hand, CD4\(^+\) Th1 cells are necessary and sufficient for genital C. trachomatis restriction (Gondek et al., 2009). In humans, C. trachomatis elicits a CD4\(^+\) Th1 cell response as well (Meoni et al., 2009).

CD4\(^+\) T cells must correctly migrate from the draining lymph nodes to the site of the infection in order to exert their effector activity. The expression of chemokine and adhesion receptors on leukocytes is required for proper trafficking (Sallusto and Baggiolini, 2008). In addition, cells in the mucosa must express the correct addressins for leukocytes to recognize the area of inflammation (Rosen, 2004). During genital C. trachomatis infection, the chemokine receptors CXCR3 and CCR5 are upregulated on the surface of pathogen-specific CD4\(^+\) T cells. Both chemokine receptors are needed for proper homing to the genital tract as genetic deletion or transient antibody
blockade suppressed protective CD4⁺ T cell homing to the uterus resulting in higher *C. trachomatis* burden (Olive et al., 2011).

Integrins are adhesion receptors critical for T cell homing. Integrins are attached to the cytoskeleton and bind to ligands on other cells or the extracellular matrix. These receptors are heterodimers composed of one α and one β unit that form unique combinations with specific affinity for certain ligands. Lymphocyte function-associated antigen 1 (LFA-1) (also known as integrin αLβ2) is generally involved in the slowing and stopping of lymphocytes in circulation and facilitating T cell-APC interactions via binding to cells expressing intercellular adhesion molecule 1 (ICAM-1) (Hogg et al., 2011). Tissue-specific homing requires the induction of different sets of integrin heterodimers. T cell homing to the gut is dependent on integrin α4β7 binding to the ligand mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) (Berlin et al., 1993). The expression of α4β7 on T cells is driven by retinoic acid and DC-mediated imprinting in the Peyer’s pouches lining the gut (Iwata et al., 2004). The integrin α4β1 is another tissue-specific adhesion receptor that mediates homing to non-gastrointestinal tissues. Integrin α4β1 is important for T cell homing to the central nervous system and is a major target for the treatment of autoimmune diseases such as experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (Yednock et al., 1992). The integrin receptor important for *C. trachomatis*-specific CD4⁺ T cell homing to the upper genital
tract had not yet been identified when I began my doctoral studies. Chapter 2 presents the characterization of integrins on CD4$^+$ T cells during genital *C. trachomatis* infection.

Upon reaching the genital tract, CD4$^+$ T cells recognize *Chlamydia*-infected epithelial cells via antigen loaded MHC class II on the cell surface (Jayarapu et al., 2009). Following contraction, a memory CD4$^+$ T cell population develops and protects previously challenged mice from *C. trachomatis* reinfection (Gondek et al., 2012). The high levels of IFN-γ secreted by CD4$^+$ Th1 cells elicit antimicrobial, cell-autonomous pathways in infected epithelial cells that ultimately reduce *C. trachomatis* load (MacMicking, 2012). The various arms of innate and adaptive immunity and their interactions with *C. trachomatis* during infection are summarized in Figure 1-2.
Figure 1-2. Innate and adaptive immunity restricts *C. trachomatis* growth.  
(A) EBs in the extracellular environment can be uptaken and neutralized by APCs. *C. trachomatis*-specific antibodies bind to EBs to prevent entry to the epithelial cell and trigger antibody receptor-mediated destruction.  
(B) Inflammatory cytokines produced by NK cells, CD4+ T cells, and CD8+ T cells recruit and activate additional immune cells, while stimulating downstream antimicrobial pathways in the infected cell.  
(C) CD8+ T cells may directly kill the infected cell via MHC class I recognition.  
Figure from (Roan and Starnbach, 2008) Copyright 2007 Blackwell Publishing Ltd.
CELL-AUTONOMOUS IMMUNITY

The host cell employs diverse effectors for protection against intracellular pathogens (Mascie-Taylor and Karim, 2003). Signaling molecules expressed by immune cells, such as cytokines, induce these effector pathways in infected cells (Bezbradica and Medzhitov, 2009). These pathways protect the host by completely eliminating the pathogen or by limiting its growth when eradication is not possible. This section will present important effectors that are induced during C. trachomatis infection, with a focus on IFN-γ-inducible pathways in mice and humans.

Oxidative and nitrosative stress

Chemically reactive oxygen and nitrogen radicals kill pathogens inside the host cell (Slauch, 2011). The production and location of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly regulated to prevent damage to the host cell (Ray et al., 2012). Bacterial infection activates the inducible nitric oxide synthase (iNOS), an enzyme that produces nitric oxide. In murine cells, priming with IFN-γ and a secondary signal—tumor necrosis factor α (TNF-α), lipopolysaccharide (LPS), or IL-1α—leads to the production of high levels of nitric oxide during C. trachomatis infection. In cell culture, chemically blocking nitric oxide production exacerbates C. trachomatis levels
(Mayer et al., 1993). Interestingly, iNOS-deficient mice resolve primary *C. trachomatis* infection to a similar degree as wildtype animals, although with a modest increase in dissemination (Igietseme et al., 1998). These mice deficient in iNOS have higher rates of genital tract pathology, namely hydrosalpinx (Ramsey et al., 2001).

**Interferon-inducible GTPases**

IFNs bind to their cognate receptors and stimulate intracellular Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) signaling that induces the expression of IFN-stimulated genes. IFNs induce four major families of GTPases involved in cell-autonomous, antimicrobial immunity. These four families include guanylate-binding proteins (GBPs), immunity-related GTPases (IRGs), very large IFN-inducible GTPases (VLIGs), and myxovirus resistance (MX) proteins (Pilla-Moffett et al., 2016). The function of VLIGs has not yet been characterized while MX proteins provide mostly viral resistance. On the other hand, GBPs and IRGs play a critical part in conferring resistance to *Chlamydia* infections. GBPs and IRGs are induced robustly by type I IFN-α/β, type II IFN-γ, and type III IFN-λ (Bekpen et al., 2005). Additionally, TNF-α, IL-1β, and TLR agonists can also activate the expression of GBPs, demonstrating that these GTPases are broadly inducible (Degrandi and Konermann, 2007).
*Chlamydia* species are well adapted to survive the immune response of their preferred host species, often times causing chronic infections. Because of this very narrow host tropism, *Chlamydia* species are less adapted to thrive in their non-preferred host species, resulting in more effective immunity. The murine proteins IRGm3, IRGa6, and IRGb10 co-localize to inclusions containing human *C. trachomatis* but not those of the murine strain *Chlamydia muridarum*. The tagging of the *C. trachomatis* inclusion by IRGs redirects the pathogen to autophagosomes for lysosomal fusion and degradation (Al-Zeer et al., 2009). IRGs also disrupt the transportation of sphingomyelin and other host lipids to the inclusion thereby limiting pathogen growth (Nelson et al., 2005). The mouse adapted species *C. muridarum* counteracts IRGb10 to promote its survival within the host cell (Coers et al., 2008). Unlike the human strain, *C. muridarum* encodes a *Yersinia* virulence factor YopT that cleaves GTPase targets, which may contribute to its survival in murine cells (Nelson et al., 2005). *C. trachomatis* is adapted to survive in human cells and is consequently cleared in C57BL/6 wildtype mice, its non-preferred host. Genetic deletion of IRGm1 and IRGm3 permits higher *C. trachomatis* growth in murine cell cultures and mice (Bernstein-Hanley et al., 2006a; Coers et al., 2011).

A loss-of-function study identified several IFN-γ-inducible GBPs that promote the activation of phagocyte oxidase, antimicrobial peptides, and autophagy effectors to restrict bacterial infections (Kim et al., 2011). Human GBP1 and 2 co-localize with *C. trachomatis* inclusions to slow bacterial growth
(Tietzel et al., 2009). These two GBPs are needed for the restriction of *C. trachomatis* through autophagy in human macrophages (Al-Zeer et al., 2013; Haldar et al., 2014). Another study found that a cluster of murine GBPs on chromosome 3 is required for cell-autonomous immunity to *C. trachomatis* in murine cells (Haldar et al., 2014). The elucidation of inducible GTPase function during immunity continues to be an area of active investigation. Appendix C presents preliminary findings regarding the function of murine GBPs during genital *C. trachomatis* infection.

**IDO**

For simplicity, the biological functions of IDO can be categorized into two general areas of study: antimicrobial cell-autonomous activity and immune cell regulation. These two properties of IDO caught my interest when I first began my doctoral studies. Our work characterizing the functions of human IDO during genital *C. trachomatis* infection is summarized in chapter 3 and appendix B. IDO is an enzyme that catalyzes the cleavage of tryptophan to produce kynurenine. There are two different forms of IDO in mammalian cells—IDO1 and IDO2—with similar biochemical function. Most studies, including my own, have focused on IDO1 due to the greater availability of tools and larger body of literature relative to IDO2. It has recently been shown that IDO2 signaling in B cells contributes to autoimmunity, yet its role during *C. trachomatis* remains unknown (Merlo et al., 2016).
IDO1 (here onto referred to as ‘IDO’) is robustly induced in both hematopoietic (APCs) and non-hematopoietic (endothelial and epithelial cells) lineages following inflammatory cues. The expression of IDO is strongly induced in human epithelial cells following IFN-γ treatment while murine cells only modestly upregulate IDO (Roshick et al., 2006). The cell-autonomous function of IDO starves invading tryptophan auxotrophs by reducing the intracellular pool of tryptophan available for pathogen growth. Genital C. trachomatis is a natural tryptophan auxotroph and reversibly transitions to a persistent state to survive amino acid starvation (Leonhardt et al., 2007). It is hypothesized that C. trachomatis evolved to survive IDO-mediated tryptophan starvation by scavenging indole, a precursor of tryptophan. This natural adaptation of genital C. trachomatis to tryptophan starvation in human cells motivated us to develop a mouse model that better represented the human IDO response.

A number of studies have shown that IDO expression suppresses inflammatory cells of the immune system (Munn and Mellor, 2013). Kynurenine metabolites produced by IDO’s enzymatic activity bind to the aryl hydrocarbon receptor (AHR) resulting in effector T cell suppression, anergy, or death (Opitz et al., 2011). Additionally, amino acid deficiency and uncharged tryptophan-tRNAs activate general control nonderepressible 2 (GCN2) kinase and mammalian target of rapamycin (mTOR) in T cells causing cell cycle arrest (Cobbold et al., 2009). While these signaling pathways are suppressive to
inflammatory effector T cells they are stimulatory to T regs. Plasmacytoid DCs activate mature T regs through IDO signaling. T regs dampen the inflammatory immune response by suppressing Th1 cells, thus weakening host immunity to pathogens and tumor cells. It is thought that this function of IDO preserves host tissue integrity by dampening inflammation (Baban et al., 2009).
FOCUS OF DISSERTATION

Multiple immune pathways—innate, adaptive, and cell-autonomous—become activated during *C. trachomatis* infection, yet frequently this pathogen is not eliminated in its preferred human host. The unaddressed clinical need for an effective vaccine and incomplete understanding of why humans lack natural and acquired immunity to *C. trachomatis* prompted me to investigate the immune response that is generated during infection. As I began my doctoral studies, I was most intrigued by CD4⁺ T cell immunity. I sought to learn more about the CD4⁺ T cell response to genital *C. trachomatis* infection. This interest guided me to investigate how IFN-γ driven, cell-autonomous pathways conferred (or failed to provide) resistance to *C. trachomatis*. In practice, I chose to use new genetically modified mouse lines to test hypotheses examining the role of integrins and human IDO on CD4⁺ T cell function and *C. trachomatis*. These mouse lines would ideally answer my original research questions or would, at minimum, improve future animals models. This experimental approach proved to be most fruitful in testing important hypotheses in the field that were only partially answered with cell culture assays.

The adaptive immune response is critical for *C. trachomatis* clearance. Specifically, protection from *Chlamydia* infection in the genital mucosa is dependent on IFN-γ-producing CD4⁺ Th1 cells. I first wanted to answer how these protective CD4⁺ T cells were trafficking to the upper genital tract to exert
their effector function and decrease *C. trachomatis* burden. Although other adhesion receptors expressed by CD4\(^+\) T cells had been characterized, the integrin receptor required for *C. trachomatis*-specific CD4\(^+\) T cell-mediated protection in the upper genital tract had not been identified. In chapter 2, I describe our effort to identify and characterize integrin receptors necessary for the recruitment of protective CD4\(^+\) T cells to the upper genital tract following *C. trachomatis* infection.

A long-standing question in the field is, why *C. trachomatis* infections fail to induce protective immunity in humans but are cleared in mice? The species-specific differences in the IFN-γ-driven, cell-autonomous response provided a clue to address this question. In contrast to humans, mice do not restrict *C. trachomatis* growth by IDO-mediated tryptophan depletion. Studies in human cell culture have shown a role for IDO in cell-autonomous host defense and immune cell regulation, but limitations in current animal models precluded studies that characterized the human-like IDO response on *C. trachomatis* pathogenesis *in vivo*. In chapter 3 I present a humanized mouse model with inducible expression of human IDO. I report the effects that ectopic hIDO expression had on 1) *C. trachomatis* growth in murine cell cultures, 2) *C. trachomatis* in the uteri of mice, and 3) CD4\(^+\) Th1 cells. In Appendix B, I present preliminary results of a new animal model for persistent *C. trachomatis* infection. This model may become a platform to research the host and pathogen interactions that lead to pathology and absence of protective
immunity during chronic infections. In appendix C, I present findings concerning the in vivo role of GBP proteins during genital C. trachomatis infection.

Understanding why host immunity fails to provide resistance to C. trachomatis infection is both interesting and important to the field. In this dissertation, I answered two main questions: how do CD4⁺ T cells home to the uterus? And what is the effect of the principal human IFN-γ effector pathway (IDO) on CD4⁺ T cells and C. trachomatis? A greater understanding of these host defense mechanisms will better inform future research efforts and the design of vaccines that elicit long-lasting, protective immunity.
CHAPTER 2: Integrin $\alpha 4\beta 1$ is necessary for CD4$^+$ T cell-mediated protection against genital *Chlamydia trachomatis* infection
ATTRIBUTIONS

I designed, performed, and analyzed the experiments presented in chapter 2 in collaboration with Dr. Andrew Olive. Dr. Michael Starnbach helped with experimental design and data interpretation.

I wrote the text of this chapter. Portions of this chapter were published in the Journal of Immunology (Davila et al., 2014).
ABSTRACT

_C. trachomatis_ infection is the most common sexually transmitted bacterial infection in the United States and a significant health burden worldwide. Protection from _Chlamydia_ infection in the genital mucosa is dependent on IFN-γ derived from CD4⁺ Th1 cells. These CD4⁺ T cells must home successfully to the genital tract to exert their effector function and reduce pathogen load. Although some adhesion receptors expressed by CD4⁺ T cells in the genital tract have been characterized, the integrin receptor required for _C. trachomatis_-specific CD4⁺ T cell-mediated protection had not been explored previously. Here, we found that _C. trachomatis_ infection of the upper genital tract led to the recruitment of _C. trachomatis_-specific CD4⁺ T cells robustly expressing the integrin α4β1. Interfering with α4β1, but not α4β7, function resulted in defective CD4⁺ T cell trafficking to the uterus and high bacterial load. We conclude that integrin α4β1 is necessary for CD4⁺ T cell-mediated protection against _C. trachomatis_ infection in the upper genital mucosa. By identifying homing molecules required for successful CD4⁺ T cell trafficking to _C. trachomatis_-infected tissues, future research efforts will be better equipped to design vaccines that elicit protective immunity in the genital mucosa.
C. trachomatis is the most common cause of bacterial sexually transmitted infection in the United States and the leading cause of preventable blindness worldwide (Brunham and Rey-Ladino, 2005). C. trachomatis is an obligate intracellular pathogen that infects conjunctival and genital tract epithelial cells. In the upper genital tract, complications from C. trachomatis infection include pelvic inflammatory disease, ectopic pregnancy and infertility (Cohen and Brunham, 1999; Roan and Starnbach, 2008). The high frequency of infection, low incidence of acquired immunity, and lack of an effective vaccine make C. trachomatis a continuing public health concern.

Protection of the genital mucosa from C. trachomatis is dependent on the production of IFN-γ (Perry et al., 1999). IFN-γ protects through the upregulation of IDO, NOS and IRGs that interfere with various aspects of the pathogen’s developmental cycle and reduce growth (Coers et al., 2008; Mayer et al., 1993; Nelson et al., 2005; Thomas et al., 1993). Mice that are deficient in IFN-γ production have delayed resolution of infection in the genital mucosa (Gondek et al., 2009). CD4⁺ T cells must produce IFN-γ in order to mediate protection, as transfer of Chlamydia-specific CD4⁺ T cells only protect naïve mice against challenge when IFN-γ is produced by those T cells. It is also critical that antigen-specific, IFN-γ-secreting CD4⁺ T cells efficiently traffic to the genital
mucosa in response to *Chlamydia* infection in order to drive protective immunity (Gondek et al., 2012; Johansson and Lycke, 2001).

Homing receptors mediate the migration of immune cells towards specific signals in order to exit the circulation and enter target tissues (Springer, 1994). Integrins are a family of adhesion receptors consisting of α and β heteroduplexes that direct signaling from both outside and inside of the cell membrane (Richard, 2002). The role of certain integrin members on leukocytes has been studied extensively. For example, LFA-1 has been shown to play a crucial function in the arrest of leukocytes in the blood vessels at the site of inflammation (Makgoba et al., 1988; Springer et al., 1987). Other integrin heterodimers, namely α4β1 and α4β7, provide tissue-specificity to T cells when homing to different areas of the body. Descriptions of how lymphocytes traffic to the gastrointestinal tract and central nervous system (CNS) have been reported. Lymphocyte recruitment to the gastrointestinal tract is largely mediated by the chemokine receptor CCR9 and the integrin receptor α4β7 (Mora et al., 2003). On the other hand, integrin α4β1 regulates trafficking to the CNS. In these models, interfering with α4β1 and α4β7 profoundly impaired immune cell recruitment to the respective tissues (Bauer et al., 2009; Yednock et al., 1992). Integrin-specific antibodies are used clinically to block immune cell infiltration and provide relief from autoimmune diseases such as ulcerative colitis and multiple sclerosis (Feagan et al., 2005; Steinman, 2005). Unfortunately, our understanding of how CD4⁺ T cells traffic to the genital mucosa has been
limited, including what combination of adhesion receptors is required for successful migration.

In this study, we interrogated the importance of α4β1 and α4β7 integrin heterodimers in promoting *C. trachomatis*-specific CD4+ T cell recruitment to the genital mucosa and protecting mice from *C. trachomatis* infection. We found that integrin α4β1 was dramatically increased on the surface of both polyclonal and *C. trachomatis*-specific CD4+ T cells in the uterus following infection. Blocking or deleting integrin α4β1, but not α4β7, on pathogen-specific CD4+ T cells resulted in the impairment of trafficking to the uterus and a decrease in the protective capacity of CD4+ T cells. We conclude that integrin α4β1 is necessary for CD4+ T cell-mediated protection against *C. trachomatis*. Identifying the receptors required for CD4+ T cell trafficking to the genital tract in response to *C. trachomatis* is important for the further characterization of protective immune cell populations.
RESULTS

*C. trachomatis* infection leads to robust integrin α4β1 surface expression on bulk CD4⁺ T cells in the uterus

Previous reports differ regarding the levels of α4β1 and α4β7 expression on T cells in the uterus during *Chlamydia* infection (Hawkins et al., 2000; Kelly et al., 2009; Perry et al., 1998). As a first step to resolve these discrepancies, we examined the surface expression of α4, β1, and β7 on CD4⁺ T cells responding to *C. trachomatis* infection in the genital mucosa. To test differences in surface integrin expression, mice were infected transcervically with *C. trachomatis*. Seven days later, the uterus and draining (iliac) lymph nodes were isolated and examined for the surface expression of integrins on endogenous CD4⁺ T cells by flow cytometry using fluorescent antibodies. We found that α4 and β1 were dramatically upregulated on the surface of CD4⁺ T cells in the uterus relative to those present in the draining lymph nodes of infected mice (Figure 2-1A). In contrast, the surface expression of β7 was only modestly increased on CD4⁺ T cells in the genital mucosa compared to α4 and β1. We next quantified the absolute number of α4⁺β1⁺- and α4⁺β7⁺-expressing CD4⁺ T cells in the genital tract (Figure 2-1B). Very few CD4⁺ T cells were found in the uterus during steady state in naïve mice. These low cell numbers precluded conclusive interpretations about integrin staining differences in naïve mice. Following infection, the absolute number of α4⁺β1⁺ CD4⁺ T cells...
Figure 2-1. *C. trachomatis* infection leads to robust integrin α4β1 surface expression on polyclonal CD4+ T cells in the genital tract. C57BL/6 mice were transcervically infected with *C. trachomatis*. Seven days following infection the indicated tissues were harvested and prepared for flow cytometry. After gating on live cells that were CD3+CD4+ the surface expression of α4, β1 and β7 was quantified. 

(A) The integrin surface expression was analyzed by comparing the geometric mean fluorescence intensity (gMFI) ratio of CD4+ T cells localized in the genital mucosa to those in the draining lymph node. 

(B) The absolute numbers of α4+β1+ and α4+β7+ CD4+ T cells were quantified in the genital tract of naïve or mice infected with *C. trachomatis* for seven days. 

(C) The absolute numbers of activated α4+β1+ and α4+β7+ CD4+ T cells were quantified in the genital tract of naïve or infected mice; activation was determined by CD44+ staining. Data show mean ± standard error of the mean (SEM) of at least five mice per group from one of two independent experiments. * p < 0.05, ** p < 0.01, and **** p < 0.0001 (unpaired t-test).
in the upper genital tract significantly increased whereas the number of α4+β7+ cells did not. We next compared the number of activated α4+β1+ or α4+β7+ CD4+ T cells responding to the genital mucosa by gating for populations expressing high levels of CD44. Interestingly, both activated α4+β1+ and α4+β7+ CD4+ T cell numbers were significantly increased in infected animals relative to naïve controls (Figure 2-1C). Nonetheless, there was a more robust recruitment of activated α4+β1+ CD4+ T cells to infected uteri compared to α4+β7+ CD4+ T cells. These results show that while both α4+β1+ and α4+β7+ CD4+ T cells are found in the infected genital mucosa, β1 is more highly expressed. These observations on endogenous T cells suggest that α4β1 is the primary integrin driving CD4+ T cell recruitment to the genital mucosa in response to C. trachomatis infection.

**Infection leads to increased α4β1 surface expression on C. trachomatis-specific CD4+ T cells in the uterus**

During C. trachomatis infection, antigen-specific T cells are primed and recruited specifically to the genital tract. However, inflammatory cytokines can also activate bystander T cells at the site of infection independently of antigen specificity (Di Genova et al., 2010). Previous work characterizing integrin receptors required for T cell recruitment to the genital mucosa focused exclusively on memory CD4+ T cell lines (Hawkins et al., 2000), polyclonal CD4+ T cells (Kelly et al., 2009), or bulk CD4+ T cells (Perry et al., 1998) but never examined naïve C. trachomatis-specific CD4+ T cells. However, only by
using naïve antigen-specific T cells is it possible to properly model primary infection. To directly interrogate the integrin profile of *C. trachomatis*-specific CD4⁺ T cells, we took advantage of *C. trachomatis*-specific TCR transgenic CD4⁺ T cells that are locked into specificity for the *Chlamydia* antigen Cta1 (referred to subsequently as NR1 cells). Based on the results described in the previous section, we hypothesized that NR1 cells would display significantly increased surface β1, rather than β7, upon trafficking to infected uteri. To test this prediction, we transferred 10⁶ NR1 cells intravenously into congenic naïve animals that were then infected transcervically the following day with *C. trachomatis*. In agreement with our observations with polyclonal CD4⁺ T cells (Figure 2-1), 8 days after infection there was over a 10-fold increase in the surface expression of integrin β1 on NR1 cells in the uterus compared to NR1 cells in the draining lymph nodes (Figure 2-2A). To better understand the dynamics of integrin expression, we monitored the surface expression of integrins on NR1 cells in the genital tract and draining lymph nodes at three time points following *C. trachomatis* infection. Three, 8, and 13 days after infection were chosen to coincide with T cell activation, peak of infiltration to the site of infection, and contraction, respectively (Figure 2-2B and 2-2C). There was a surge of both α4⁺β1⁺ and α4⁺β7⁺ NR1 cells in the genital tract 8 days after infection compared to what was observed with these populations 3 days after infection (Figure 2-2B). Even though both NR1 populations increased
Figure 2-2. *C. trachomatis* infection leads to robust α4β1 surface expression on *C. trachomatis*-specific CD4+ T cells responding to the genital tract.

CD90.1+ NR1 cells were transferred into CD90.2+ mice. The next day mice were infected with *C. trachomatis* and NR1s analyzed at indicated time point.

(A) Integrin gMFI ratio of NR1 cells in uterus / lymph nodes at day 8.

(B) Absolute numbers of α4β1+ and α4β7+ NR1 cells.

(C) Quantification of the trafficking kinetics of α4β1+ and α4β7+ NR1 cells.

Data show mean ± SEM of at least eight mice per group from one of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001 (unpaired t-test).
during infection, the magnitude of infiltration of the $\alpha_4\beta_1$+ NR1 cells to the uterus was far more robust ($**p<0.001$) than the $\alpha_4\beta_7$+ population ($p<0.05$) (Figure 2-2B, left). Surface integrin profiles of NR1 cells in the draining lymph nodes were statistically indistinguishable between these two time points (Figure 2-2B, right). Thirteen days after infection the decline of NR1 cells (Figure 2-2C) corresponded to the resolution of \textit{C. trachomatis} infection in the genital mucosa. Even at this late time point, $\alpha_4\beta_1$+ NR1 cells were found significantly more frequently than $\alpha_4\beta_7$+ NR1 cells in the genital tract. These observations suggest that $\alpha_4\beta_1$+ NR1 cells are either recruited or retained in the uterus for a longer period of time than other T cell populations.

Given that several findings in this work relied on the use of integrin antibodies, we wanted to validate the efficiency of $\alpha_4\beta_7$ and $\beta_7$ integrin staining to accurately interpret the data presented. We were worried that the limited $\beta_7$ staining observed could be due to poor antibody function. To test antibody staining, we activated \textit{C. trachomatis}-specific CD4$^+$ T cells (NR1 cells) \textit{in vitro} in the presence of retinoic acid (RA) or dimethyl sulfoxide (DMSO) vehicle control and examined integrin surface staining by flow cytometry 5 days later (Figure 2-3). It has been previously shown that RA induces integrin $\alpha_4\beta_7$ and is important for T cell homing to the gastrointestinal tract (Iwata et al., 2004). Consequently, we used RA treatment as a positive control to induce high levels of surface integrin $\alpha_4\beta_7$ expression on NR1 cells to test the staining effectiveness of the $\beta_7$ and $\alpha_4\beta_7$ antibodies used in this study. As shown in Figure 2-3, activation of
Figure 2-3. The limited β7 and α4β7 antibody staining observed following T cell activation is not an effect of poor antibody function.

*C. trachomatis*-specific CD4+ T cells were activated *in vitro* in the presence of RA (dashed black line) or DMSO vehicle control (solid black line). The surface staining function of β7 (left panel) and α4β7 (right panel) antibodies was tested by flow cytometry 5 days post culture and compared to isotype antibody background staining (gray filled). Data is representative of one of three independent experiments.
NR1 cells in the presence of RA led to a strong upregulation of surface β7 expression which was detected using either the β7 antibody (Figure 2-3, left panel) or α4β7 antibody (Figure 2-3, right panel). In agreement with previous findings, activation of NR1 cells in the absence of RA led to a minimal increase in β7 on the surface of T cells suggesting that β7 upregulation on C. trachomatis-specific CD4+ T cells was not robust under RA-free activation conditions. Taken together, we were now confident that the results presented here describing the low levels of β7 expression on activated C. trachomatis-specific CD4+ T cells were due to underlying biology and not an effect of poor antibody function. These findings demonstrate that both polyclonal and C. trachomatis-specific CD4+ T cells, preferentially express α4β1 on their surface in the murine genital mucosa in response to C. trachomatis infection.
Blocking integrin α4 but not α4β7 increases *C. trachomatis* burden

Since we identified a strong upregulation of α4β1 on the surface of CD4+ T cells responding to *C. trachomatis* infection, we next assessed the functional role of β1 and β7 in promoting protective immunity. Our group has previously shown that transfer of *in vitro*-activated Th1-skewed NR1 cells into naïve mice confers significant protection from *C. trachomatis* infection compared to mice that receive no T cells (Gondek et al., 2012). We hypothesized that if specific integrins were required for recruitment to the genital mucosa, blocking these integrins on activated NR1 cells would alter their protective capacity. To test this and determine the relative contributions of α4β1 and α4β7 to CD4+ T cell-mediated protection, we selectively blocked integrin receptors using antibodies (Figure 2-4). NR1 cells from naïve mice were harvested and polarized *in vitro* to a Th1 phenotype. IFN-γ production by these NR1 cells was assayed to confirm their Th1 phenotype by flow cytometry prior to transfer. One million NR1 Th1 cells were pre-treated with antibody that blocked α4 (blocked both α4β1 and α4β7), α4β7, or an isotype control and then transferred intravenously into host mice that were then transcervically infected with *C. trachomatis* the following day. To ensure robust blockade of integrins, each group of mice was also treated with antibody 1 and 3 days following infection. Five days after infection, *C. trachomatis* burden in the uterus was measured using quantitative PCR (qPCR). Mice that were treated with blocking antibody against α4 had a higher *C. trachomatis* burden in the genital tract than isotype control treated animals,
Figure 2-4. Antibody blockade of α4 but not α4β7 exacerbates C. trachomatis burden in the genital mucosa.

NR1 cells were skewed *in vitro* to a Th1 phenotype for 5 days. NR1 cells were pretreated with the indicated antibodies and then transferred intravenously into naïve recipients. The following day, mice were infected transcervically with *C. trachomatis*. Groups were injected with the respective integrin or isotype control antibody 1 and 3 days following infection. Five days after infection, the genital tract was isolated and genomic DNA was purified. The levels of *Chlamydia* 16S DNA relative to the levels of host GADPH were quantified using qPCR. Data show mean ± SEM of at least five mice per group from one of two independent experiments. * p < 0.05 and ** p < 0.01 (unpaired t-test).
similar to bacterial levels found in mice that received no NR1 cells at all. On the other hand, mice treated with α4β7 blocking antibody had significantly lower C. trachomatis levels than the no transfer group and similar bacterial burden to the isotype antibody treated mice. Despite the lack of an antibody that specifically blocked α4β1, the importance of α4β1 can be inferred indirectly. Because blocking α4 with antibody prevented both α4β1 and α4β7 signaling, the differences observed in protection between α4 and α4β7 antibody treatment groups pointed towards a function of α4β1. Since the integrin α4 chain dimerizes with either β1 or β7 (von Andrian and Mackay, 2000), the results shown in Figure 2-4 indirectly confirm that only α4β1 is required for CD4+ T cell-mediated protection against C. trachomatis in the genital mucosa.

**A reduction in CD4+ T cells is responsible for the higher C. trachomatis burden in anti-α4 antibody-treated mice**

Although α4 blockade was sufficient to prevent protection in the genital mucosa, we had not yet determined the mechanism responsible for higher burden. We predicted that the loss of protection seen in mice treated with α4-blocking antibody was due to diminished recruitment of NR1 cells to the infected genital mucosa. To test this possibility, we monitored the trafficking of NR1 cells to the infected genital tract after integrin blockade. NR1 cells were skewed to the Th1 phenotype *in vitro*, and then transferred into congenically
mismatched host mice. The next day mice were infected transcervically with *C. trachomatis*. Prior to transfer, NR1 cells were pretreated with individual integrin blocking antibodies or an isotype control. Mice were also treated with the same blocking antibody or isotype control 1 and 3 days after infection. Five days after infection, we examined the number of NR1 cells present in the genital tract and draining lymph nodes by flow cytometry. We noted that the number of NR1 cells in the genital mucosa was significantly diminished following treatment with the α4 blocking antibody relative to the isotype treated control mice (Figure 2-5A and 2-5B). In contrast, α4β7 antibody treatment did not impact NR1 cell recruitment to the uterus, as absolute numbers were similar between isotype- and α4β7-treated groups. The absolute numbers of NR1 cells present in the draining lymph nodes were not significantly different between the groups which suggested no general defect in trafficking of NR1 cells following antibody treatment. We also calculated a migration index (the ratio of live NR1 cells in uteri to draining lymph nodes within the same animal) to normalize for mouse-to-mouse variability (Figure 2-5C). We found that the migration index was profoundly decreased in mice treated with α4 blocking antibody compared to isotype-treated mice. These results demonstrated that blocking α4 prevented efficient CD4+ T cell trafficking from the draining lymph nodes to the uteri following *C. trachomatis* infection. The migration index of mice treated with α4β7 antibody was not statistically different from isotype control treated mice demonstrating that α4β7 played a limited role.
Figure 2-5. Blockade of α4 but not α4β7 impairs *C. trachomatis*-specific CD4+ T cell trafficking to the genital tract following infection.

(A) Representative flow cytometry plots with NR1 population frequencies.

(B) Absolute number of NR1 cells after antibody treatment.

(C) Migration index comparing the NR1 cells in the uterus to those in lymph nodes (lower migration index ratio indicates decreased uterine NR1 cell recruitment).

(D) CD44+CD62Llow NR1 cell numbers in the genital mucosa.

Data show mean ± SEM of at least five mice per group from one of two independent experiments. * p < 0.05, ** p < 0.01, and *** p < 0.001. (unpaired t-test).
in NR1 cell trafficking to the genital mucosa in response to *C. trachomatis* infection.

We next examined if antibody treatment altered the recruitment of effector CD4\(^+\) T cell populations to the genital mucosa. NR1 cells were stained for CD44 and CD62L in order to evaluate the presence of activated CD4\(^+\) T cells at the site of the infection. The absolute number of CD44\(^+\)CD62L\(^{low}\) NR1 cells in the uterus was significantly decreased only in the α4 antibody-treated group compared to isotype-treated mice (Figure 2-5D). Moreover, anti-α4β7 antibody treatment did not significantly decrease the absolute number of effector NR1 cells in the genital tract relative to the isotype-treated group. These results suggested that disrupting the integrin α4β1 but not α4β7 on NR1 cells was sufficient to eliminate CD4\(^+\) T cell-mediated protection following *C. trachomatis* infection in the genital tract.

We next wanted to attest that the high *C. trachomatis* burden and limited CD4\(^+\) T cell recruitment observed in Figures 2-4 and 2-5 were not due to poor α4β7 antibody blockade. In Figure 2-6 we pre-treated *C. trachomatis*-specific CD4\(^+\) T cells *in vitro* with the same unlabeled α4β7 or isotype antibody used in Figures 2-4 and 2-5 and stained for integrin α4β7 surface expression. *C. trachomatis*-specific CD4\(^+\) T cells were activated *in vitro* for 5 days in the absence or presence of RA. Following activation, *C. trachomatis*-specific CD4\(^+\) T cells were pre-treated with unlabeled isotype control (black line) or unlabeled α4β7 (gray line) antibody for 30 minutes. NR1 cells were then washed and
Figure 2-6. Pretreatment of *C. trachomatis*-specific CD4⁺ T cells with unlabeled α4β7 antibody leads to efficient blockade of α4β7. *C. trachomatis*-specific CD4⁺ T cells were activated *in vitro* in the absence (left panel) or presence (right panel) of retinoic acid. Following activation, *C. trachomatis*-specific CD4⁺ T cells were pre-treated with unlabeled isotype (black line) control or unlabeled α4β7 antibody (gray line) for 30 minutes. Cells were then washed and stained with fluorescent-labeled α4β7 antibody and analyzed using flow cytometry. Data is representative of one of three independent experiments.
stained with fluorescent-labeled α4β7 antibody to assay α4β7 surface levels. Activation in the presence of RA led to more robust surface expression of α4β7 on NR1 cells that were pre-treated with isotype control antibody relative to the α4β7 blocking treatment. *C. trachomatis*-specific CD4⁺ T cells pre-treated with unlabeled α4β7 antibody did not stain positive regardless of RA treatment. Therefore, treatment of activated *C. trachomatis*-specific CD4⁺ T cells with the unlabeled α4β7 antibody used in these experiments resulted in efficient blockage of α4β7 on T cells, which validated the antibody blockade experiments.

**Integrin β1-deficient *C. trachomatis*-specific CD4⁺ T cells are unable to protect the uterus**

To complement the antibody-blocking experiments that showed that α4β1 is required for *C. trachomatis*-specific CD4⁺ T cells to home to and protect the genital mucosa, we generated TCR transgenic mice in which the T cells were deficient in either integrin β1 or β7. Because complete loss of β1 results in embryonic lethality (Fässler and Meyer, 1995), we used a CRE-Flox system to generate NR1 cells conditionally deficient in integrin β1. NR1 transgenic mice were crossed to *Lck-CRE* and *Itgb1*<sup>flox/flox</sup> animals such that only the lymphocytes were deficient in β1. We also crossed NR1 transgenic mice with *Itgb7<sup>−/−</sup>* mice to generate *C. trachomatis*-specific CD4⁺ T cells deficient in integrin β7. We first confirmed that integrin surface expression was significantly
Figure 2-7. CD4⁺ T cells from *C. trachomatis*-specific integrin deficient mice show altered integrin surface expression. 
(A) Integrin sufficient or deficient NR1 cells were skewed *in vitro* to a Th1 phenotype for 5 days. After stimulation, live NR1 cell were gated to show the percentage of β1⁺β7⁻ and β1⁻β7⁺ populations within each group. 
(B) The quantification of α4⁺β1⁺β7⁻ or α4⁺β1⁻β7⁺ NR1 cells is shown as percentage of total NR1 cells. Data show mean ± SEM of at least three mice per group from one of three independent experiments. * p < 0.05 and *** p < 0.001 (unpaired t-test).
altered for each knockout T cell genotype (Figure 2-7A and 2-7B). Interestingly, loss of \( \beta_1 \) led to a concomitant increase of surface \( \beta_7 \) on NR1 cells similar to a previous report showing that \( \alpha_4\beta_7 \) heterodimers form more readily in the absence of the integrin \( \beta_1 \) chain (DeNucci et al., 2010). The loss of integrin \( \beta_7 \) also led to an increase of the percentage of integrin \( \beta_1^+ \) NR1 cells after \textit{in vitro} activation.

We next confirmed that NR1 cells deficient in individual integrins proliferated normally. Integrin deficient or sufficient NR1 cells were harvested from mice and polarized \textit{in vitro} for 5 days to a Th1 phenotype. We found no significant differences between groups in the total number of recovered NR1 cells 5 days following activation, demonstrating that all the genotypes were viable (Figure 2-8A). We next examined activation and cytokine production for each group. For all genotypes examined, NR1 cells were robustly activated as determined by staining for the activation markers CD25 and CD44 (Figure 2-8B). When we assayed cytokine profiles using intracellular cytokine staining (ICCS), we found similar levels of IFN-\( \gamma \) and TNF-\( \alpha \) in all groups, demonstrating that loss of integrin \( \beta \) chains did not negatively impact Th1 differentiation (Figure 2-8C). Given these observations, the absence of either integrin \( \beta_1 \) or \( \beta_7 \) did not interfere with expansion, activation, and Th1 cytokine production of NR1 cells \textit{in vitro}.

Upon showing that the various genotypes of NR1 cells had normal effector phenotypes, we then tested whether deficiency in either the integrin \( \beta_1 \)
Figure 2-8. Integrin-deficient C. trachomatis-specific CD4⁺ T cells have normal expansion, activation, and Th1 cytokine production.  

(A) The indicated NR1 genotypes were skewed in vitro to a Th1 phenotype for 5 days. The expansion of NR1 cells was compared for the three genotypes. 

(B) The activation of live NR1 cells was assessed by gating for CD25⁺CD44⁺ cells. 

(C) TNF-α and IFN-γ production was examined using ICCS. 

Data show mean ± SEM of at least three mice per group from one of three independent experiments (unpaired t-test).
or β7 chain would adversely affect the protective capacity of *C. trachomatis*-specific CD4⁺ T cells *in vivo*. Based on our previous results from antibody blocking experiments, we hypothesized that integrin β1-deficient NR1 cells would be unable to protect the genital mucosa from *C. trachomatis* infection. We transferred 10⁵ wildtype, β1⁻/⁻ or β7⁻/⁻ Th1-skewed NR1 cells into naïve mice 1 day prior to transcervical infection with *C. trachomatis* (Figure 2-9). Five days after infection, we harvested the upper genital tract and quantified the levels of *C. trachomatis* using qPCR. As expected, mice that received wildtype NR1 cells had significantly lower *C. trachomatis* burden compared to mice that received no transfer. Mice that received integrin β1⁻/⁻ NR1 cells were not protected against *C. trachomatis* infection as indicated by bacterial burdens similar to mice that received no NR1 cells. In line with previous findings using antibody blockade, we found that mice receiving integrin β7⁻/⁻ NR1 cells were significantly protected against *C. trachomatis* infection relative to mice that received no transfer, and trended toward being even more protective than wildtype NR1 cells. These findings showed that integrin β7 on NR1 cells was dispensable for protecting the uterus from *C. trachomatis* infection. In summary, integrin β1, but not β7, was necessary for *C. trachomatis*-specific CD4⁺ T cells to protect against infection in the genital mucosa.
Figure 2-9. *C. trachomatis*-specific CD4⁺ T cells deficient of integrin β1 are unable to protect mice from infection.
After 5 days of *in vitro* stimulation, 10⁵ NR1 cells were transferred intravenously into naïve hosts. The following day, mice were transcervically infected with *C. trachomatis*. Five days after infection, the genital tract was harvested and genomic DNA was purified. The levels of *Chlamydia* 16S DNA relative to the levels of host GADPH were quantified using qPCR. Data show mean ± SEM of at least five mice per group from one of three independent experiments.* p < 0.05 and ** p < 0.01 (unpaired t-test).
Integrin β1 deficiency impairs *C. trachomatis*-specific CD4+ T cell homing to the uterus

We next sought to understand the mechanisms responsible for the loss of protective capacity in integrin β1−/− *C. trachomatis*-specific CD4+ T cells. Our previous data using antibody blockade showed that CD4+ T cell trafficking to the genital mucosa was inhibited and therefore provided limited protection. Here, we used a competitive homing assay to test the trafficking potential of integrin-deficient *C. trachomatis*-specific CD4+ T cells. We directly compared the migration of integrin-sufficient and deficient NR1 cells under identical conditions within the same infected host. We transferred an equal number of CD45.2+/CD90.2+ β1−/−, β7−/− or wildtype NR1 cells and CD45.2+/CD90.1+ wildtype NR1 cells into congenically mismatched CD45.1+ recipients (Figure 2-10). The next day, we infected mice transcervically with *C. trachomatis*. Seven days after infection, we isolated and processed tissues to quantify the numbers of both NR1 populations using flow cytometry. We found that integrin β1−/− NR1 cells were far less efficient than wildtype NR1 cells in trafficking to the genital mucosa, while integrin β7−/− NR1 cells outcompeted their wildtype counterparts (Figure 2-11A). None of the experimental groups showed defects in their migration to the draining lymph node. Interestingly, we observed a higher percentage of integrin β1−/− NR1 cells in the lymph nodes compared to the wildtype NR1 cells, inverse of what was seen in the genital mucosa. These results were likely due to a decreased ability of integrin β1−/− NR1 cells to leave
Figure 2-10. Schematic diagram of competitive homing assay with *C. trachomatis*-specific CD4$^+$ T cells. An equivalent ratio of CD45.2$^+$/CD90.1$^+$ integrin wildtype NR1 cells and CD45.2$^+$/CD90.2$^+$ wildtype, $\beta_1^{-/-}$, or $\beta_7^{-/-}$ NR1 cells were transferred intravenously into CD45.1$^+$/CD90.2$^+$ host mice. The next day, mice were infected transcervically with *C. trachomatis*. Figure courtesy of Dr. Xuqing Zhang.
β1-deficient C. trachomatis-specific CD4+ T cells are unable to traffic efficiently to the genital tract following infection. An equivalent number of CD45.2+/CD90.1+ integrin wildtype NR1 cells and CD45.2+/CD90.2+ wildtype, β1+/−, or β7+/− NR1 cells were transferred intravenously into CD45.1+/CD90.2+ host mice. The next day, mice were infected transcervically and organs harvested 7 days later.

(A) Representative flow plots of integrin sufficient and deficient NR1 cells.

(B) The migration index within each group was calculated by comparing the percentage of CD90.2+ to CD90.1+ NR1 cells in the uterus to the percent of CD90.2+ to CD90.1+ NR1 cells in lymph nodes (left) or spleen (right). A lower migration index indicates lower integrin-deficient NR1 cell trafficking to the uterus. Mean ± SEM of at least 9 mice per group from one of three independent experiments. ** p < 0.01, *** p < 0.001, and **** p < 0.0001 (unpaired t-test).
the circulation to enter the infected genital mucosa. To normalize for mouse-to-mouse variation in absolute NR1 cell numbers, we calculated a migration index for each mouse by comparing the ratio of the two transferred NR1 cell populations in the uterus (integrin deficient CD90.2⁺% : integrin sufficient CD90.1⁺%) to the ratio of the transferred populations in the draining lymph nodes or spleen within the same animal (Figure 2-11B). A smaller migration index indicated less efficient trafficking of integrin-deficient NR1 cells specifically to the uterus relative to the circulation. We found a dramatically lower migration index for integrin β1⁻/⁻ NR1 cells demonstrating that trafficking to the uterus during infection was significantly impaired relative to wildtype NR1 cells. Intriguingly, the migration index for integrin β7⁻/⁻ NR1 cells was significantly higher than wildtype, which suggested enhanced homing of C. trachomatis-specific CD4⁺ T cells to the uterus in the absence of integrin β7. These results showed that integrin β7 was not only dispensable, but that deficiency of integrin β7 enhanced antigen-specific CD4⁺ T cell migration to the genital tract during C. trachomatis infection. These findings collectively reveal that integrin β1 plays a crucial in trafficking of CD4⁺ T cells to the genital mucosa and that absence of β1 negatively affects the protective capacity of C. trachomatis-specific CD4⁺ T cells.
DISCUSSION

The orchestration of events required for a successful T cell response determines whether an intracellular pathogen will be eliminated from the host. In the case of *C. trachomatis* infection, a robust CD4⁺ Th1 cell population that homes to the genital tract provides the vigorous IFN-γ response necessary for bacterial clearance (Gondek et al., 2012). The integrin receptors important for *C. trachomatis*-specific CD4⁺ T cell-mediated protection in the upper genital tract had not been characterized previous to this study. Using *C. trachomatis*-specific CD4⁺ T cells, we demonstrated that perturbing integrin α4β1 but not α4β7, through antibody blockade or genetic deletion, resulted in impaired T cell trafficking to the genital tract and a loss of protective capacity following *C. trachomatis* infection. Together these observations show that integrin α4β1 is necessary for CD4⁺ T cell-mediated protection against *C. trachomatis* infection in the murine genital tract.

It was previously shown that the chemokine receptors CXCR3 and CCR5 are required for *C. trachomatis*-specific CD4⁺ T cells to home to and protect the genital mucosa following infection with *C. trachomatis* (Olive et al., 2011). Here, we extended these studies to identify integrin receptors important for CD4⁺ T cell-mediated protection. When we examined the surface expression of integrins β1 and β7 on both bulk and antigen-specific CD4⁺ T cells, we found that relative integrin β1 expression was dramatically increased on the majority
of CD4\(^+\) T cells found in the uterus following \textit{C. trachomatis} infection (Figures 2-1A and 2-2A). These findings were in line with Perry et al. who demonstrated that surface integrin \(\beta 1\) was upregulated on bulk lymphocytes localized in the genital tract but not in the intestinal mucosa following infection with the mouse-adapted species \textit{C. muridarum} (Perry et al., 1998). Although most CD4\(^+\) T cells were expressing high levels of surface integrin \(\beta 1\), this report also noted a minor percentage of integrin \(\beta 7^+\) CD4\(^+\) T cells in the genital tract (Perry et al., 1998). Separate reports concluded that integrin \(\alpha 4\beta 7\) was the dominant integrin receptor expressed on CD4\(^+\) T cells in the genital tract. However, these studies had significant experimental constraints that limited their interpretations. For example, Kelly et al. did not interrogate the integrin profile during the primary response or differentiate between antigen-specific and bystander CD4\(^+\) T cells in the genital tract (Kelly and Rank, 1997). Another report examined the surface integrin receptors on a memory CD4\(^+\) T cell line in culture following stimulation but did not assess \textit{in vivo} integrin dynamics (Hawkins et al., 2000). Memory CD4\(^+\) T cells can exert effector activities with greater ease than primary T cells (Croft et al., 1994), therefore examining memory cells is not indicative of the trafficking properties of T cells in a primary response. Because these studies did not examine naïve, antigen-specific CD4\(^+\) T cells they were unable to recapitulate the initial activation events during primary \textit{C. trachomatis} infection. In addition, these reports did not examine how perturbation of distinct integrin
complexes altered the protective capacity of CD4\(^+\) T cells responding to genital *C. trachomatis* infection.

We found that integrin \(\alpha_4\beta_1\) drove *C. trachomatis*-specific CD4\(^+\) T cells to the infected genital mucosa (Figures 2-5 and 2-11) and was required to mediate protective immunity following *C. trachomatis* infection (Figures 2-4 and 2-9). While integrin \(\alpha_4\beta_1\) was the dominant integrin in the genital mucosa, we did identify a second integrin \(\beta_7^+\) population that when perturbed did not alter immunity to *C. trachomatis* infection. The existence of two NR1 cell populations with distinct integrin profiles and identical TCR specificity demonstrated that integrin levels were not hardwired but rather imprinted during T cell activation as has been suggested previously (Iwata et al., 2004). Our results also imply that only CD4\(^+\) T cells with the correct integrin profile extravasated into the infected genital mucosa. We speculate that while integrin \(\alpha_4\beta_1\)-expressing CD4\(^+\) T cells entered the infected tissue efficiently, the \(\alpha_4\beta_7^+\) CD4\(^+\) T cells remained in the circulation and failed to provide protection in the genital mucosa.

Because the entire uterus, including its associated vasculature, was harvested in all experiments it was not possible to distinguish between those T cells present in blood vessels and those that had completed transendothelial migration. It is feasible that the \(\alpha_4\beta_7^+\) CD4\(^+\) T cell population still contributes to immunity elsewhere in the mouse, but this remains to be tested.

Interestingly, one report uncovered a mechanism that controls distinct integrin expression on T cells in the lungs of mice. Ruane et al. found that a
subset of residing DCs imprinted lung T cells to express α4β7. T cells that expressed α4β7 did not mediate protection in the lungs but rather provided gut mucosal immunity (Ruane et al., 2013). Given that a subset of lung DCs was able to imprint a population of T cells to express α4β7, we speculate that a similar process also occurs in the genital tract. While the majority of CD4+ T cells were primed to express integrin α4β1, which mediated immunity in the genital mucosa during C. trachomatis infection, a subset of uterine DCs also imprinted α4β7 on a fraction of the CD4+ T cell population for systemic mucosal immunity. This hypothesis will need to be examined in future experiments.

It is well established that integrin α4β1 binds to the addressin vascular cell adhesion molecule 1 (VCAM-1) and the extracellular matrix protein fibronectin (Alon et al., 1995; Mariano et al., 1990). It has been shown that surface VCAM-1 increases on endothelial cells lining microvessels following inflammation (von Andrian and Mackay, 2000). For example, patients with CNS autoimmune disorders are often treated with natalizumab, an anti-α4 antibody that blocks α4β1 and α4β7 interaction with their ligands. It is thought that natalizumab treatment decreases undesirable inflammation in the CNS by interfering with integrin α4β1-mediated immune cell recruitment to this sensitized area in the body (von Andrian and Engelhardt, 2003). Previous studies showed that surface VCAM-1 became abundant on murine and human genital cells following Chlamydia infection. In contrast, the expression of MAdCAM-1, the binding partner for α4β7, was reported to be expressed
robustly in the gut but only modestly in the genital tract (Johansson et al., 1999; Perry et al., 1998). The noticeable increase of surface VCAM-1 in the genital mucosa after *Chlamydia* infection corresponded to the upregulation of surface α4β1 on *C. trachomatis*-specific CD4+ T cells in the uterus observed in our study.

Several other signals stimulate the rapid increase of VCAM-1 on vaginal epithelial cells including IFN-γ and herpes simplex virus infection (Parr and Parr, 2000). Given that VCAM-1 can be selectively upregulated on both endothelial and non-endothelial cells, responding CD4+ T cells may require integrin α4β1 signaling for multiple steps in order to provide protection. Previous studies have characterized the importance of integrin α4β1 to slow/arrest lymphocytes in the blood vessel, but integrin α4β1 may also mediate the interactions between effector CD4+ T cells and infected epithelial cells as has been suggested to occur during *Chlamydia* infection (Jayarapu et al., 2010). It remains unknown whether *C. trachomatis*-specific CD4+ T cells directly interact with infected epithelial cells in the genital mucosa or if their antimicrobial effects occur by altering the cytokine milieu at the site of infection. In this study we observed that integrin β1 and β7 were dispensable for proliferation and differentiation to a Th1 phenotype (Figure 2-8). Consequently, we conclude that the function of integrin β1 in mediating protection is to allow successful *C. trachomatis*-specific CD4+ T cell trafficking to the uterus rather than playing a role in activation or production of IFN-γ. Future studies should explore the interaction between IFN-γ-producing
CD4\(^+\) T cells and the infected epithelium to determine whether integrin β1 is required for cellular interactions \textit{in vivo} within the genital mucosa. Additional studies will need to determine if T cell recruitment and effector activity, mediated by integrin α4β1, contributes to genital tract pathology following \textit{C. trachomatis} infection. Although integrin β1 is found primarily in complex with integrin α4 on T cells, our findings did not entirely eliminate the possibility that integrin β1 can form additional heterodimers on CD4\(^+\) T cell and that these heterodimers may play a role in the retention of T cells in the genital tract following infection.

The results obtained in this study further elucidate the essential homing receptors required for an effective CD4\(^+\) T cell defense in the genital mucosa. It remains to be determined whether CD4\(^+\) T cells responding to other pathogens in the genital tract also require the same homing receptors (CXCR3, CCR5, and α4β1) for successful trafficking during \textit{C. trachomatis} infection. Generating a robust and long-lived protective T cell response is crucial to clear intracellular pathogens and avoid recurrent cycles of inflammation and associated pathology. Vaccine efforts against intracellular pathogens should examine whether the appropriate T cell population, with the necessary homing molecules, is being generated to ensure protection and minimize pathology. In addition to the current treatments for autoimmune diseases in the gut and the CNS, integrin and chemokine receptor targeted therapies could be used to selectively shape the recruitment of desired T cells to other mucosal tissues.
MATERIALS AND METHODS

Mice

C57BL/6, B6.PL-Thy1a (CD90.1 congenic), C57BL/6, B6.SJL-Ptprc
Pep3/BoyJ (CD45.1 congenic), B6.Cg-Tg548Jxm/J (Lck-CRE), C57BL/6-
Itgb7tm1Cgn/J (Itgb7\textsuperscript{−/−}), and B6;129-Itgb1\textsuperscript{tm1Efu}/J (Itgb1\textsuperscript{flox/flox}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and NR1 mice were previously described (Roan et al., 2006). B6;129-Itgb1\textsuperscript{tm1Efu}/J (Itgb1\textsuperscript{flox/flox}) mice were crossed with C57BL/6 for greater than 10 generations. C. trachomatis-specific integrin-deficient mice were generated by breeding NR1 mice to either Lck-CRE and Itgb1\textsuperscript{flox/flox} or Itgb7\textsuperscript{−/−} mice. All mice were maintained in facilities managed by the Harvard Medical School Center for Animal Resources and Comparative Medicine. To normalize for the murine estrous cycle, mice were treated subcutaneously with 2.5 mg of medroxyprogesterone 7 days prior to infection. Harvard’s institutional animal care and use committee approved all the experiments described.

Growth, isolation and detection of C. trachomatis

C. trachomatis serovar L2 (434/Bu) was propagated using McCoy cell monolayers grown in Eagle’s minimum essential media (MEM) (Invitrogen, Grand Island, NY) plus 10% fetal bovine serum (FBS), 1.5 g/l sodium bicarbonate, 0.1 M nonessential amino acids, and 1 mM sodium pyruvate.
Infected McCoy cells were detached from plates using sterile glass beads and then sonicated to disrupt *C. trachomatis* inclusions. Density gradient centrifugation was used to purify elementary bodies. Aliquots were stored at -80°C in a medium containing 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid (SPG).

**Transfer of NR1 cells, infection of mice, and tissue preparation**

*C. trachomatis*-specific CD4+ T cells were isolated from the lymph nodes and spleens of naïve donor NR1 mice. Recipient mice received 10⁶ NR1 cells and were infected the following day with 10⁶ *C. trachomatis* inclusion forming units (IFU) in 10 μl of SPG media. We used the NSET device (ParaTechs) to bypass the cervix and directly infect the uterine horns. The uterus was harvested and disaggregated by digestion with 1 mg/ml of type XI collagenase (Sigma, St. Louis, MO) and 50 Kunitz/ml of DNase (Sigma) for 30 min at 37°C. Single cell suspensions from tissues were obtained by mechanical disaggregation prior to staining. Suspensions of splenocytes were treated with a hypotonic buffer to lyse red blood cells prior to use.

**Flow cytometry**

Single cell suspensions were stained immediately for activation markers or stimulated for 5 hours with 100 ng/ml phorbol myristate acetate (PMA) (Alexis Biochemical), 1 mg/ml ionomycin (Calbiochem), and 1:1000 brefeldin A (BD
Biosciences) in RPMI 1640 media for ICCS. Cells were treated with anti-FCγR (BioXCell) before staining with combinations of the following antibodies: anti-β1 Pacific Blue, anti-β7 FITC, anti-TCRβ2 allophycocyanin, anti-CD90.1 peridinin chlorophyll protein, anti-CD45.2 phycoerythrin (PE), anti-CD90.2 FITC, anti-IFN-γ PE, anti-TNF-α PE-cy7, anti-CD25 PE, anti-CD44 PE or Pacific Blue, anti-CD62L FITC (Biolegend), anti-CD3ε allophycocyanin, anti-α4 PE (BD Biosciences), anti-CD4 Qdot605 and a LIVE/DEAD dead cell stain kit (Invitrogen). The efficacy of all antibodies used in this study was confirmed extensively. For cytokine staining, cells were permeabilized using a Cytofix/Cytoperm Plus Kit following manufacturer’s instructions (BD Biosciences). Cell number was determined with AccuCheck Counting Beads (Invitrogen). Flow cytometry data were collected on a modified FACSCalibur (Cytek Development) or an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star).

**Th1 polarization and protection against C. trachomatis**

CD4⁺ T cells were harvested from the lymph nodes and spleens of naïve NR1 mice and enriched with a mouse CD4 negative isolation kit (Invitrogen) following the manufacturer’s protocol. CD4⁺ T cells were cultured in media consisting of RPMI 1640 (Invitrogen), 10% FBS, L-glutamine, HEPES, 50 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 mg/ml streptomycin. NR1 cells were activated by co-culture with irradiated or mitomycin-treated splenocytes
pulsed with 5 mM of Cta1_{133-152} peptide at a stimulator:T cell ratio of 4:1. Th1 polarization was achieved by supplying cultures with 10 ng/ml of IL-12 (Peprotech, Rocky Hill, NJ) and 10 mg/ml of anti-IL-4 antibody (Biolegend). After 5 days of stimulation, NR1 Th1 cells were transferred intravenously into naïve recipient mice. In integrin antibody blocking experiments, NR1 cells were treated with 100 ug of anti-α4, anti-α4β7 or isotype antibody (BioXCell) for 1 hour at room temperature prior to transfer into recipients. The next day, mice were challenged with 5x10^6 C. trachomatis IFUs and the upper genital tract was analyzed for burden 5 days after infection. Mice were treated intraperitoneal with 200 ug of the respective antibody at 1 and 3 days post-infection for integrin blocking experiments.

**Competitive homing**

For this assay an equivalent number of integrin wildtype NR1 cells (CD45.2+/CD90.1+) and congenic wildtype, Lck-CRE/Itgb1^{floxed}/, or Itgb7^{−/−} NR1 cells (CD45.2+/CD90.2+) were combined and transferred into congenically mismatched CD45.1^{+} hosts. The following day, mice were transcervically infected with 5x10^6 C. trachomatis IFUs. Tissues were harvested and analyzed 7 days after infection.
**DNA extraction and quantitative PCR**

Bacterial burden was evaluated by quantifying *C. trachomatis* 16S DNA relative to mouse GADPH DNA (Roan et al., 2006). The uterus was homogenized and DNA was extracted using the DNeasy blood and tissue kit (Qiagen). DNA was analyzed using *C. trachomatis*- and mouse-specific primer pairs and dual-labeled probes. Threshold values were detected by an ABI Prism 7000 sequence system. The ratio of *C. trachomatis* to host DNA was obtained using a standard curve.

**RNA extraction and qRT-PCR**

To isolate RNA, uterine tissue homogenates were first treated with TRIzol reagent (Ambion). Samples were then processed using a RNA extraction kit (Qiagen) per the manufacturers protocol. Each qRT-PCR reaction contained purified RNA (25 ng), SYBR Green RT-PCR Master Mix, reverse transcriptase, (Qiagen) and the primers: integrin β1 forward
(5'-GGCAGAGAGCTCCGGCCAG-3'), integrin β1 reverse
(5'-CAATAGGGGTATCAGCCTCCCT-3'), integrin β2 forward
(5'-CAACGTCAGAAGCTGGGCG-3'), integrin β2 reverse
(5'-CATGGTGCTCGTCCGCTCACTGCAAGCTGGC-3'), integrin β7 forward
(5'-GGACGCTCAAGCCTTCGAGG-3'), integrin β7 reverse
(5'-GTGAACACTAGACGAGGAG-3'), integrin α1 forward
(5'-GTCACCAATCCGAAGGGAG-3'), integrin α1 reverse
(5'-GTAGGGCTGACATCAGAAC-3'), integrin α4 forward
(5'-CTGAGGAAATTCCACCACTCC-3'), integrin α4 reverse
(5'-GCAGAAACTTGGGAGACAGC-3'), integrin αE forward
(5'-GTGCTTCAGGCTTGGCCT-3'), integrin αE reverse
(5'-CTACAGTAGGCGGAGTCC-3'), integrin αM forward
(5'-GAATATGTTGGCGCGTCTC-3'), integrin αM reverse
(5'-CTCAGAGGGTTGGGAGCCG-3'), integrin αL forward
(5'-GACGCTGGCAACAGATGC-3'), integrin αL reverse
(5'-CATAGGTCCTCCAGACTCTGA-3'), L-selectin forward
(5'-GGCGGGGAAGGATCCTGG-3'), and L-selectin reverse
(5'-GCATTTTCCCAGGTCATGGG-3'). Expression for all genes of interest was normalized to GAPDH expression in the same sample. Data was gathered by an ABI Prism 7000 sequence system and fold changes were calculated relative to baseline expression of uninfected control mice.

**Statistical analysis**

Statistical significance between groups was determined using an unpaired two-tailed t-test and depicted within figures as * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.
CHAPTER 3: Human IDO inhibits CD4$^+$ Th1 cells and does not clear

*Chlamydia trachomatis* in the murine upper genital tract
ATTRIBUTIONS

I designed, performed, and analyzed the experiments presented in chapter 3 in collaboration with several scientists. Drs. Michael Starnbach and Jörn Coers provided guidance with experimental design and data analysis. Drs. Zhe Shi and Jörn Coers engineered the transgenic hIDO mouse lines. Dr. Sara Martin ran the HPLC samples that I provided to her. Noah Harrison helped with molecular assays and mouse experiments. Christian Berrios provided the SV40 plasmid and protocol used to make MEF lines. Vikram Juneja gave advice on in vivo tamoxifen induction. Madeleine Haff helped with mouse colony maintenance.

I wrote the text of this chapter.
ABSTRACT

IFN-γ induces species-specific cell-autonomous responses, such as the robust expression of IDO in human cells and IRGs in mice. In human cell culture, IDO converts tryptophan (trp) to kynurenine (kyn) to restrict C. trachomatis, a trp auxotroph. In contrast to humans, mice do not restrict C. trachomatis growth by IDO-mediated trp depletion. Although studies in human cells have demonstrated a role for IDO in cell-autonomous host defense and immune cell regulation, limitations in current animal models have precluded an examination of the impact that a human-like IDO response has on the pathogenesis of C. trachomatis infections in vivo. To address this issue, we generated a humanized mouse model with tamoxifen-inducible expression of human IDO (hIDO). hIDO expression in murine cells restricted C. trachomatis growth in a trp-dependent manner and suppressed CD4⁺ Th1 cells. In the murine upper genital tract, hIDO initially limited C. trachomatis growth at an early time point (day 2 post infection) but exacerbated bacterial burden at a later point of the infection (day 7). These results show that hIDO profoundly inhibits CD4⁺ Th1 cells and that its expression is not sufficient to clear genital C. trachomatis infection. Our findings suggest that insufficient IDO-mediated antimicrobial restriction and suppression of protective CD4⁺ T cells contributes to the failed human immune response to C. trachomatis.
INTRODUCTION

Ascending infection of genital *C. trachomatis* can cause pelvic inflammatory disease that results in ectopic pregnancy and infertility (Haggerty et al., 2010). The limited natural and acquired human immunity to *C. trachomatis* infection demands a better understanding of defects in immune mechanisms that would otherwise provide protection to the host. Due to the significant morbidity caused by this pathogen, developing better animal models to understand host-pathogen interactions is of great importance to the development of an effective vaccine.

*Chlamydia* species are Gram-negative, obligate intracellular bacteria with a biphasic life cycle (Abdelrahman and Belland, 2005). The infectious EB form of *C. trachomatis* invades epithelial cells and establishes an intracellular replicative niche, a vacuolar compartment known as the inclusion. Within inclusions, EBs convert into morphologically distinct RBs that replicate through binary fission. Intracellular bacterial growth is restricted by the lymphocyte-derived cytokine IFN-γ (Lampe et al., 1998). In order to endure the host immune response *Chlamydia* species evolved to resist IFN-γ-dependent host defense pathways. Because IFN-γ-inducible, cell-autonomous host defenses can vary substantially between distinct vertebrate species, *Chlamydia* species evolved counter-immune strategies uniquely adapted to their preferred hosts. Illustrating this principle, IFN-γ induces a pronounced increase in the expression of IDO in
human but not mouse genital epithelial cells as part of a cell-autonomous defense program against intracellular pathogens (Beatty et al., 1994a; Taylor and Feng, 1991). IDO is an enzyme that catalyzes the first and rate-limiting step of trp degradation via oxidative cleavage of the aromatic indole ring (Yamamoto and Hayaishi, 1967). Depletion of intracellular trp starves the natural trp auxotroph *C. trachomatis* of an essential host-derived nutrient thereby halting bacterial replication. In response to trp starvation *C. trachomatis* converts into an inactive yet viable persistent state. Once intracellular trp stores are replenished, the persistent non-replicative form of *C. trachomatis* can revert back to the replicative form (Shemer et al., 1987).

The presence of trp biosynthesis genes varies in different *Chlamydia* species. While the rodent-adapted species *C. muridarum* lack a *trp* operon, genital strains of the human-adapted species *C. trachomatis* harbor an incomplete *trp* operon (Xie et al., 2002). Although *C. trachomatis* cannot generate trp *de novo*, the partial *trp* operon encodes a functional trp synthase which can convert exogenously supplied indole to trp (McClarty et al., 2007). Indole-producing microbes in the genital tract could potentially provide *C. trachomatis* with the indole substrate needed to produce limiting amounts of trp (Aiyar et al., 2014). It is tempting to speculate that by consuming indole *C. trachomatis* avoids the detrimental effects of complete trp starvation and survives IFN-γ-driven nutritional immunity of its preferred human host.

Contrary to human cells, murine epithelial cells fail to induce robust IDO
expression in response to IFN-γ priming and accordingly fail to restrict *C. trachomatis* growth by IDO-mediated trp depletion (Roshick et al., 2006).

Consistent with these *in vitro* studies, it was shown that IDO-deficient mice restrict *C. trachomatis* infections as efficiently as wildtype animals (Nelson et al., 2005). Instead of using an IDO-dependent cell-autonomous defense pathway, murine cells upregulate a set of mouse-specific, IFN-γ-inducible IRG proteins. IRGs effectively restrict *C. trachomatis* growth both *in vitro* and *in vivo* (Bernstein-Hanley et al., 2006b; Coers et al., 2008). To do so, IRGs bind to and rupture *C. trachomatis* inclusions, thereby destroying the intracellular niche essential for *C. trachomatis* growth and survival (Haldar et al., 2015; Ling et al., 2006; Martens et al., 2005). As a pathogen not adapted to rodents, *C. trachomatis* lacks mechanisms to evade mouse-specific IRG-driven host defenses and is quickly cleared by the murine host. These cell-autonomous differences in the antimicrobial response between humans and mice have limited our ability to model human *C. trachomatis* infection (Coers et al., 2008; Coers et al., 2009), highlighting the need for humanized mouse models to study *C. trachomatis* pathogenesis. Other cell-autonomous, IFN-γ-inducible genes such as the GBP5s are important for *C. trachomatis* restriction in cell cultures yet their *in vivo* role remain unexplored (Haldar et al., 2014).

*C. trachomatis* stimulates various arms of the adaptive immune response with cell-mediated immunity playing a key role in pathogen restriction. Although CD8⁺ T cells are important for protection against ocular *C. trachomatis* in
macaques, the murine endogenous CD8\(^+\) T cell response to \textit{C. trachomatis} is curtailed and does not provide protection (Fankhauser and Starnbach, 2014; Olivares-Zavaleta et al., 2014; Zhang and Starnbach, 2015). Contrary to endogenous CD8\(^+\) T cells, CD4\(^+\) T cells are needed to provide immunity in mice. The expression of CD4 and MHC class II is necessary for \textit{C. trachomatis} restriction (Morrison et al., 1995). Moreover, \textit{C. trachomatis}-specific CD4\(^+\) T cells that present a Th1 phenotype are sufficient to provide protection in the murine uterus (Gondek et al., 2012).

In addition to antimicrobial trp sequestration, studies have found two distinct mechanisms of IDO-mediated immunomodulation. First, the enzymatic activity of IDO cleaves trp thereby generating kyn, a metabolite that can be toxic to certain inflammatory cell types but stimulatory of others (Fallarino et al., 2002; Mellor and Munn, 2004). Second, a report discovered a non-enzymatic signaling role for IDO in the induction of long-term tolerance in DCs (Pallotta et al., 2011). IDO-mediated tolerance fulfills important physiological functions, for example, preventing fetal allograft rejection (Munn et al., 1998). However, its activity has also been linked to various diseases: IDO expression and kyn abundance correlates with increased tumor size, a weaker inflammatory T cell response, and higher infectious disease burden in HIV patients (Favre et al., 2010; Soliman et al., 2010). To date our understanding of IDO has been limited by the lack of \textit{in vivo} models for a human-like IDO response. Mouse models lacking IDO expression or overexpressing IDO in APCs fail to mimic the IDO
inducibility that is a unique feature of IFN-γ-primed human epithelial cells.

Longitudinal human studies have shown that C. trachomatis re-infections are prevalent, indicating an absence of long-lasting, protective immunity (Oh et al., 1996; Walker et al., 2012). It is unclear what microbial and host factors prevent the development of an effective immune cell response to C. trachomatis in humans. We hypothesize that the robust human-specific IDO response does not provide sterilizing immunity to C. trachomatis.

To overcome the limitations of current mouse models and test our hypothesis, we developed a humanized mouse model that expressed hIDO upon stimulation with an experimentally supplied inducer. Using this newly developed mouse line, we assessed the interplay of hIDO-mediated cell-autonomous restriction and T cell modulation during genital C. trachomatis infection. We found that the induction of hIDO expression in murine cells restricted C. trachomatis growth in a trp-dependent manner. In the murine genital tract, hIDO expression limited C. trachomatis initially but ultimately exacerbated burden. Localized hIDO expression in the uterus resulted in a severe reduction of activated C. trachomatis-specific CD4+ Th1 cells. In vitro, hIDO-expressing cells and kyn directly inhibited effector CD4+ Th1 cells. These findings demonstrate that hIDO is not sufficient to clear genital C. trachomatis infections and that it suppresses CD4+ Th1 cells. The design of effective therapeutics and vaccines will be better informed by a clear understanding of the interplay of hIDO cell-autonomous activity and immune regulation.
RESULTS

Robust induction of hIDO expression and kynurenine production in murine cells

To observe the effects of hIDO expression, we generated hIDO transgenic mice (hIDO\textsuperscript{stopFL}) by targeting the ROSA26 locus using a construct encoding the human \textit{IDO1} cDNA, preceded by a \textit{loxP} flanked STOP cassette. The construct was marked by a signaling deficient truncated version of human \textit{CD2} under the control of an internal ribosomal entry site (IRES) downstream of the inserted \textit{IDO1} cDNA (Figure 3-1A). We confirmed the presence of the hIDO\textsuperscript{stopFL} knockin construct via Southern blot (Figure 3-1B) and PCR (Figure 3-1C). In order to spatially and temporally control expression of hIDO, hIDO\textsuperscript{stopFL} mice were bred with Cg-Tg(CAG-cre/Esr1) mice that express the Cre-ER(T) fusion protein that is activated by tamoxifen (tam), but not by estradiol.

To begin characterizing this new inducible hIDO system, we harvested and immortalized mouse embryonic fibroblasts (MEFs) from embryos containing both hIDO\textsuperscript{stopFL} and Cg-Tg(CAG-cre/Esr1) alleles (henceforth referred to as ‘ER-hIDO’) or lack of for control ‘wildtype’ cells. MEFs were treated with tam for 24 hours and then selected for the expression of CD2 using an enrichment column. To quantify hIDO transcript induction, we extracted RNA from MEFs 24 hours after plating and measured hIDO mRNA in relation to glyceraldehyde 3-phosphate dehydrogenase (\textit{GAPDH}) using qRT-PCR.
Figure 3-1. Robust induction of hIDO expression and kynurenine production in murine cells.
(A) Schematic diagram of hIDO\textsuperscript{stopFL} knockin cassette.
(B) Southern blot of wildtype and hIDO\textsuperscript{stopFL} knockin bands.
(C) Agarose gel of wildtype and hIDO\textsuperscript{stopFL} knockin PCR products.
(D) hIDO mRNA expression was normalized to GAPDH and fold changes determined relative to wildtype MEFs using qRT-PCR.
(E) Frequency of hIDO\textsuperscript{+} CD2\textsuperscript{+} MEFs assessed by flow cytometry.
(F) HPLC was used to measure kyn in MEF supernatants ± DL-MT.

Data show mean ± SEM of triplicates from one of three independent experiments. *** p < 0.001 (unpaired t-test).
Induced ER-hIDO MEFs had significantly higher levels of hIDO expression (~100 fold higher) relative to the control group confirming successful transcription after tam treatment (Figure 3-1D). Previous findings have shown that IFN-γ-treated cultured human epithelial cells upregulate IDO to a similar degree (Roshick et al., 2006).

To assess the total population of hIDO+ CD2+ MEFs and confirm hIDO protein expression, we stained with an antibody that only recognized the human form of IDO. Using flow cytometry, we observed that the majority (96.9%) of ER-hIDO MEFs were positive for both surface CD2 and intracellular hIDO, thus demonstrating that the hIDO protein was successfully expressed (Figure 3-1E). To confirm hIDO enzymatic activity, we used high-performance liquid chromatography (HPLC) to quantify the presence of kyn in cell supernatants as determined by a standard curve. Only ER-hIDO MEFs produced kyn, with a highest concentration of ~10 uM (Figure 3-1F). Furthermore, the levels of kyn decreased drastically when ER-hIDO MEFs were treated with an IDO competitive inhibitor, 1-methyl-DL-tryptophan (DL-MT). Wildtype MEFs had little to no detectable kyn in the supernatants. Collectively, these results confirmed that this novel engineered hIDO system was inducible and robustly expressed at the transcript and protein level. Equally important, the presence of kyn demonstrated that the expressed hIDO protein was enzymatically active and that these murine cells secreted kyn into the supernatant at comparable levels to human cells treated with IFN-γ (De Ravin et al., 2010).
We also compared induced to uninduced ER-hIDO MEFs and observed leaky expression of hIDO transcript in the uninduced group (Figure 3-2A). Previous studies have commented on the potential leakiness of Cg-Tg(CAG-cre/Esr1) mice corroborating our observations here (Jaisser, 2000). Furthermore, we observed that hIDO expression leakiness in the infected uninduced ER-hIDO MEFs resulted in an intermediate C. trachomatis restriction between wildtype and induced ER-hIDO MEF levels (Figure 3-2B). Consequently, we compared induced ER-hIDO MEFs to wildtype cells from here on to overcome the confounding effect of leakiness.
Figure 3-2. Leaky hIDO expression in uninduced ER-hIDO MEFs results in intermediate *C. trachomatis* restriction phenotype. 

(A) RNA was harvested from MEFs 24 hours after seeding for qRT-PCR analysis. *hIDO* mRNA expression was normalized to *GAPDH*. Fold changes were determined relative to control wildtype MEFs. 

(B) MEFs were plated and infected with *C. trachomatis*. DNA was harvested at 24 (left) and 42 (right) hours post infection to measure *C. trachomatis* load using qPCR. 

Data show mean ± SEM of triplicates from one of three independent experiments. * p < 0.05, ** p < 0.01, and **** p < 0.0001 (unpaired t-test).
hIDO-expressing murine cells limit *C. trachomatis* growth in a tryptophan-dependent manner

We next explored how the expression of hIDO would affect *C. trachomatis* restriction in murine cells. As with *C. trachomatis* infection of human cells, we predicted that hIDO expression in murine cells would decrease *C. trachomatis* load in a trp-dependent manner. To test this hypothesis, we plated wildtype or ER-hIDO MEFs and infected cells with *C. trachomatis* (Figure 3-3A). DNA from infected cells was harvested at 24 and 42 hours post infection to measure *C. trachomatis* load using qPCR. At both time points, *C. trachomatis* burden was significantly reduced in ER-hIDO relative to wildtype MEFs. Adding trp to ER-hIDO MEFs rescued *C. trachomatis* growth similar to wildtype MEF levels demonstrating that hIDO restriction was mediated through trp deprivation.

To substantiate that hIDO activity mediated the restriction of *C. trachomatis* growth, MEFs were infected with *C. trachomatis* in the presence or absence of the IDO inhibitor DL-MT (Figure 3-3B). DNA was harvested from MEFs at 24 and 42 hours post infection to measure *C. trachomatis* load using qPCR. Treatment of ER-hIDO MEFs with DL-MT fully rescued *C. trachomatis* growth similar to wildtype levels demonstrating that restriction was indeed mediated by hIDO activity. Similar results were observed with the use of the L-MT inhibitor (Figure 3-3C). We selected DL-MT rather than other IDO inhibitors because of its higher purity and racemic mixture. These findings show that
murine cells expressing hIDO restricted *C. trachomatis* in a trp and hIDO-dependent manner.
Figure 3-3. hIDO-expressing murine cells limit *C. trachomatis* growth in a tryptophan-dependent manner.

(A) MEFs were infected with *C. trachomatis* ± exogenous trp. DNA was harvested to measure *C. trachomatis* levels using qPCR.

(B) MEFs were infected with *C. trachomatis* ± DL-MT.

(C) MEFs were infected with *C. trachomatis* ± L-MT. DNA was extracted from samples at 24 (left) and 42 (right) hours post infection to measure *C. trachomatis* load using qPCR. Data show mean ± SEM of triplicates from one of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001 (unpaired t-test).
IFN-γ signaling restricts *C. trachomatis* growth independent of hIDO

IDO in humans and mice is only one of many hundreds of IFN-γ-stimulated genes (de Veer et al., 2001). Consequently, we next explored the degree of hIDO-mediated *C. trachomatis* restriction relative to the entire antimicrobial effect of IFN-γ signaling. MEFs were pretreated with recombinant murine IFN-γ or vehicle control for 2 hours and subsequently infected with *C. trachomatis* (Figure 3-4A). DNA was harvested at 24 and 42 hours post infection to measure *C. trachomatis* load using qPCR. At 24 hours post infection, *C. trachomatis* restriction was comparable between untreated ER-hIDO MEFs and IFN-γ-treated wildtypes demonstrating that initially hIDO was sufficiently capable of preventing bacterial growth to a similar degree as IFN-γ treatment. However, at 42 hours post infection IFN-γ treatment resulted in a more robust *C. trachomatis* restriction than hIDO alone. These observations show that although hIDO expression reduced *C. trachomatis* growth independently of IFN-γ, it was not sufficient to restrict bacterial growth as effectively as IFN-γ treatment. Furthermore, this data suggested that *in vivo* IFN-γ signaling would mask any hIDO phenotype during *C. trachomatis* infection.

We initially interrogated the *in vivo* phenotype of hIDO using a different mouse line with *hIDO* cDNA knocked in immediately downstream of an IFN-γ-inducible promoter, IRGm2 (henceforth referred to as m2-hIDO). Wildtype or m2-hIDO mice were infected transcervically with *C. trachomatis* (Figure 3-4B). Uteri were then harvested at 3 and 7 days post infection. We isolated DNA from
Figure 3-4. IFN-γ signaling restricts *C. trachomatis* growth independent of hIDO.

(A) MEFs were pretreated with recombinant murine IFN-γ (white bars) or vehicle control (black bars) for 2 hours and subsequently infected with *C. trachomatis*. DNA was harvested to measure *C. trachomatis* load using qPCR.

(B) Wildtype or m2-hIDO mice were infected transcervically with *C. trachomatis*. Uteri were harvested for *C. trachomatis* burden analysis using qPCR.

(C) Wildtype mice were infected transcervically with *C. trachomatis* or left uninfected for control groups. At 7 days post infection, uterine RNA was harvested for gene expression analysis using qRT-PCR. Genes were normalized to GAPDH mRNA levels and fold changes assessed in infected mice relative to naïve groups. Data show mean ± SEM from one of at least two independent experiments. * p < 0.05, ** p < 0.01, and *** p < 0.001 (unpaired t-test).
uterine homogenates to assess *C. trachomatis* burden using qPCR. We observed no significant differences in burden at either time point, which suggested that IFN-γ signaling sufficient mice restricted *C. trachomatis* irrespective of hIDO expression.

To assess if IFN-γ-inducible, antimicrobial GTPase genes in the uterus were upregulated robustly in response to *C. trachomatis*, wildtype mice were infected transcervically or left uninfected for control groups (Figure 3-4C). At 7 days post infection, uterine RNA was then harvested for gene expression analysis of a subset of IFN-γ-inducible, cell-autonomous defenses using qRT-PCR. Genes of interest were normalized to GAPDH levels in each sample. Fold changes in infected mice were obtained relative to the uninfected control group. *IRGm1, IRGm3*, and *Gbps 2,5, and 7* were significantly upregulated in the uterus following *C. trachomatis* infection. Collectively, these results confirmed that potent IFN-γ-inducible, antibacterial genes restricted *C. trachomatis* in the uterus independent of hIDO expression. Sufficiency of IFN-γ elicited immunity in the genital tract precluded the interrogation of specific hIDO-mediated restriction of *C. trachomatis in vivo*. 
Despite initial *C. trachomatis* restriction, mice expressing hIDO develop higher *C. trachomatis* burden in the genital tract

Given the findings in the previous section, we decided to cross ER-hIDO mice to IFN-γ receptor 1 knockout (*ifngr<sup>-/-</sup]*) animals to eliminate mouse-specific IFN-γ signaling pathways and define the specific contribution of hIDO during genital *C. trachomatis* infection. We developed a protocol that selectively induced hIDO expression in the upper genital tract through the administration of localized 4-hydroxytamoxifen (htam). We noticed that uninduced ER-hIDO mice had leaky hIDO expression (Figure 3-5). As with *in vitro* experiments, we opted to compare induced ER-hIDO mice to controls without the hIDO cassette to avoid the confounding effects of expression leakiness.

Mice were treated transcervically with htam and then infected with *C. trachomatis* the following day. To measure gene induction, RNA was extracted from uterine lysates at 2 and 7 days post infection and analyzed using qRT-PCR (Figure 3-6A). Only uterine tissues from *ifngr<sup>-/-</sup>* ER-hIDO mice expressed robust levels of hIDO transcript, which demonstrated that hIDO could be experimentally induced *in vivo*. In addition, we also monitored the changes in expression levels of *IRGm1* and *IRGm3* in the genital tract of *C. trachomatis*-infected animals. We wanted to verify that *ifngr<sup>-/-</sup>* and *ifngr<sup>-/-</sup>* ER-hIDO mice were comparably lacking this mouse-specific IRG resistance system previously shown to clear *C. trachomatis in vivo* (Bernstein-Hanley et al, Coers et al).
Figure 3-5. Detection of leaky hIDO expression in uninduced ER-hIDO mice.
Mice were treated transcervically with htam (induced ER-hIDO) or DMSO vehicle (uninduced ER-hIDO). Uteri were harvested at 5 days post treatment. RNA was isolated from uterine homogenates to measure hIDO expression normalized to GAPDH using qRT-PCR. hIDO fold changes in uteri were determined relative to control wildtype groups. Data show mean ± SEM (unpaired t-test).
Despite initial *C. trachomatis* restriction, mice expressing hIDO develop higher burden in the genital tract. Mice were induced transcervically and the following day infected with *C. trachomatis* in the upper genital tract. Uteri were harvested at 2 and 7 days post infection. (A) hIDO, IRGm1, and IRGm3 levels were normalized to GAPDH and fold changes compared to wildtype groups using qRT-PCR. (B) Kyn was measured by HPLC. (C) *C. trachomatis* levels were analyzed by qPCR. Data show mean ± SEM of at least three mice per group from one of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001 (unpaired t-test). ND, not detected.
IRGs were not induced in both IFN-γ signaling deficient groups relative to wildtype mice.

We then quantified kyn levels in the genital tract by HPLC to confirm proper enzymatic activity in vivo following htam induction (Figure 3-6B). We detected significantly higher kyn levels in the uteri of infected ER-hIDO mice relative to control groups at both time points. The higher concentration of kyn at day 7 suggested that the metabolite accumulated in the genital tract following induction. These results further confirmed that the hIDO system was inducible and robustly expressed in the uterus.

Given the in vitro phenotype in ER-hIDO MEFs, we expected hIDO-expressing mice to have lower C. trachomatis burden than control groups. To quantify C. trachomatis levels, we extracted DNA from infected uteri lysates and analyzed samples using qPCR (Figure 3-6C). At 2 days post infection, ifngr−/− ER-hIDO mice had a lower burden than ifngr−/− controls although higher than wildtype mice, showing that hIDO expression conferred limited protection during the innate response to C. trachomatis. Surprisingly, ifngr−/− ER-hIDO mice developed significantly higher C. trachomatis burden relative to both control groups at 7 days post infection. Therefore, these results demonstrated that in vivo hIDO expression alone was not sufficient to clear genital C. trachomatis infection.
hIDO suppresses *C. trachomatis*-specific CD4\(^+\) Th1 cells in the genital tract

The higher *C. trachomatis* burden in ER-hIDO mice during the adaptive phase of immunity prompted us to consider the immunomodulatory properties of IDO. We focused on characterizing the effect of hIDO expression on *C. trachomatis*-specific CD4\(^+\) T cells as they have been shown to be both necessary and sufficient for pathogen restriction in the upper genital tract (Gondek et al., 2012). We hypothesized that hIDO expression would negatively impact CD4\(^+\) T cells in the genital tract. To test this prediction, we harvested *C. trachomatis*-specific CD4\(^+\) T cells from a mouse line whose TCRs were locked in specificity to a *C. trachomatis* antigen Cta1 (NR1 cells) (Roan et al., 2006). To analyze *C. trachomatis*-specific CD4\(^+\) T cells in vivo, CD90.2\(^+\) recipient mice were treated transcervically with htam and then adoptively given 9x10\(^5\) CD90.1\(^+\) congenically mismatched NR1 cells intravenously. Recipients were then infected transcervically the following day. Uteri and draining lymph nodes were collected at 4 days post infection to assess gene expression, *C. trachomatis* burden, and NR1 cell abundance. In the upper genital tract, we confirmed that hIDO transcript was robustly expressed while IRGm1 and IRGm3 were similarly not induced in IFN-y receptor deficient groups (Figure 3-7A). At 4 days post infection, bacterial burden was similar between *ifngr\(^{-/}\) and *ifngr\(^{-/-}\) ER-hIDO mice, and significantly higher than wildtype mice (Figure 3-7B). The similar burden in
Figure 3-7. hIDO suppresses C. trachomatis-specific CD4\(^+\) Th1 cells in the murine upper genital tract.

CD90.2\(^+\) recipient mice were induced transcervically and given 9x10\(^5\) CD90.1\(^+\) C. trachomatis-specific CD4\(^+\) T cells (NR1 cells). Recipient mice were infected transcervically with C. trachomatis the following day. Uteri and draining lymph nodes were harvested at 4 days post infection.

(A) Gene expression in uteri normalized to wildtype mice using qRT-PCR.

(B) C. trachomatis burden in uteri measured by qPCR.

(C) Representative plots of adoptively transferred live NR1 cells.

(D) Quantification of live NR1 cells.

(E) Live CD25\(^+\) CD44\(^+\) NR1 cell numbers.

(F) Live IFN-\(\gamma\) TNF-\(\alpha\) NR1 Th1 cells were assessed by flow cytometry.

Data show mean ± SEM of at least three mice per group from one of two independent experiments. * \(p < 0.05\), ** \(p < 0.01\), and *** \(p < 0.001\) (unpaired t-test).
*ifngr*⁻/⁻ and *ifngr*⁻/⁻ ER-hIDO mice gave us confidence that any differences seen in the NR1 cell response would not be due to variations in *C. trachomatis* antigen levels.

To determine NR1 cell abundance, uteri and draining (iliac) lymph nodes were processed into cell suspensions and stained with antibodies for CD90.1, CD4, and the *C. trachomatis*-specific TCRvα2 for flow cytometry analysis. Upon gating, we observed an NR1 cell frequency decrease in *ifngr*⁻/⁻ ER-hIDO uteri relative to *ifngr*⁻/⁻ controls despite their similar bacterial burden (Figure 3-7C). This difference in NR1 cell presence in the uterus was not seen in the draining lymph nodes. Upon quantification of absolute NR1 cell numbers using cell-counting beads, we noted that *ifngr*⁻/⁻ but not *ifngr*⁻/⁻ ER-hIDO mice had significantly more NR1 cells in the uterus compared to wildtypes (Figure 3-7D). The fewer NR1 cells observed in wildtype uteri was due to lower *C. trachomatis* burden in this group relative to *ifngr*⁻/⁻ mice. We attributed the fewer NR1 cells observed in *ifngr*⁻/⁻ ER-hIDO uteri to hIDO expression. Unlike uterine samples, the presence of NR1 cells in the draining lymph nodes was not statistically different.

We then wanted to know if activation of *C. trachomatis*-specific CD4⁺ T cells in the uterus was affected by hIDO expression. Live activated NR1 cells were quantified by gating for high expression of the activation markers CD25 and CD44 by flow cytometry (Figure 3-7E). The absolute number of live CD25⁺ CD44⁺ NR1 cells present in the uterus was significantly lower in *ifngr*⁻/⁻ ER-
hIDO mice relative to ifngr<sup>−/−</sup> controls. Contrary, activated NR1 cell numbers in the draining lymph nodes were not statistically different. These results demonstrate that the fewer activated NR1 cells observed in uteri were due to hIDO expression.

We also investigated whether hIDO would affect the effector population of C. trachomatis-specific CD4<sup>+</sup> Th1 cells. Th1 cells were of greatest interest given their ability to provide protection to C. trachomatis-infected mice. Live NR1 Th1 cells were quantified by gating for high expression of the Th1 hallmark cytokines IFN-γ and TNF-α using ICCS and flow cytometry (Figure 3-7F). The IFN-γ<sup>+</sup> TNF-α<sup>+</sup> NR1 cell population in the uterus was significantly decreased in ifngr<sup>−/−</sup> ER-hIDO mice relative to ifngr<sup>−/−</sup> controls. NR1 Th1 cell abundance in the draining lymph nodes was not statistically different. Jointly, these findings indicate that in vivo hIDO expression had a profound negative impact on activated C. trachomatis-specific CD4<sup>+</sup> Th1 cells in the upper genital tract.

**hIDO expression directly inhibits CD4<sup>+</sup> Th1 cells**

We hypothesized that during infection, C. trachomatis-specific CD4<sup>+</sup> T cells were recruited to the uterus and became suppressed by hIDO-expressing genital epithelial cells. Therefore to assess whether hIDO-expressing cells were directly inhibiting CD4<sup>+</sup> T cells, we designed a co-culture system with wildtype or ER-hIDO MEFs plus activated CD4<sup>+</sup> T cells. Briefly, CD4<sup>+</sup> T cells were
isolated from naïve wildtype mice and labeled with carboxyfluorescein succinimidyl ester (CFSE). CFSE-labeled cells dilute CFSE with each cell division. T cells were then activated via anti-CD3ε and anti-CD28 antibodies in wells previously seeded with MEFs. Cells were co-cultured in the absence or presence of DL-MT. At 4 days post co-culture setup, T cells were stained and analyzed using flow cytometry. We first examined live CD4+ T cells that became activated (CD44+ ) and had proliferated substantially (CFSE low ) (Figure 3-8A). hIDO-expressing MEFs significantly reduced the CD44+ CFSE low CD4+ T cell population relative to wildtype MEF co-cultures. Blocking hIDO activity with DL-MT rescued CD4+ T cell activation and proliferation in the ER-hIDO MEF co-cultures, which demonstrated that T cell inhibition was caused directly by hIDO expression.

We then assessed the impact of ER-hIDO MEFs on CD4+ Th1 cells using the same co-culture system described above. We identified and quantified live IFN-γ+ CD4+ T cells using ICCS and flow cytometry (Figure 3-8B). Co-culture with ER-hIDO MEFs significantly restricted the CD4+ Th1 cell population relative to wildtype MEF co-cultures. Interestingly, blocking hIDO activity with DL-MT dramatically increased the CD4+ Th1 cell population in co-cultures with either MEF genotype. These findings suggest that DL-MT triggered pro-Th1 signals in these CD4+ T cells. Taken together, these data show that ER-hIDO MEFs directly inhibited CD4+ Th1 cells, as seen with NR1 cells in the upper genital tract of hIDO-expressing mice.
Figure 3-8. hIDO and kynurenine directly inhibit CD4⁺ Th1 cells.
(A) Quantification of live CD44⁺ CFSE<sub>low</sub> CD4⁺ T cells ± DL-MT 4 days post coculture.
(B) Live IFN-γ⁺ CD4⁺ T cells in co-cultures were assessed by ICCS.
(C) Live CD44⁺ CFSE<sub>low</sub> CD4⁺ T cells at day 6 after seeding. White bars show samples treated at day 0 with 4, 20, 100, or 500 µM (from left to right) of a kyn cocktail. Red bars represent groups treated at day 4.
(D) Live IFN-γ⁺ CD4⁺ T cells were assessed by ICCS using the same experimental setup and color representation as in (C).

Data show mean ± SEM of triplicates from one of three independent experiments. ** p < 0.01, *** p < 0.001, and **** p < 0.0001 (unpaired t-test in (A) and (B) and one-way ANOVA with Dunnett’s post-test in (C) and (D) comparing to vehicle treated).
Kynurenine treatment directly and severely restricts effector CD4⁺ Th1 cells

We next wanted to test if direct kyn treatment would phenocopy the hIDO-mediated suppression of CD4⁺ Th1 cells discussed in the prior sections. Although previous studies have shown that kyn treatment affects naïve T cells (Terness et al., 2002), we set out to characterize the consequence of kyn exposure on two CD4⁺ T cell differentiation states, naïve and effector. A naïve CD4⁺ T cell may be exposed to IDO-expressing DCs during activation in the lymph nodes (day 0 of infection) or as an effector cell in the genital tract via contact with IDO-expressing epithelial cells (starting at approximately day 4 post infection) (Moffett and Namboodiri, 2003; Shayda et al., 2009). In order to test both situations in a reductionist setting, experimental T cell groups were treated with kyn at day 0 or day 4 after seeding. A naïve T cell reaches its full effector state after 4 days of in vitro activation (Jelley-Gibbs et al., 2000). CD4⁺ T cells were isolated from naïve wildtype mice, plated, and activated via anti-CD3ε and anti-CD28 antibodies. Experimental groups were treated at day 0 or day 4 after seeding with various concentrations of an equimolar cocktail of kyn and its downstream metabolites (L-kynurenine, 3’-hydroxy-DL-kynurenine, 3’-hydroxyanthranilic acid, anthranilic acid, and quinolinic acid) following a previously described protocol (Desvignes and Ernst, 2009).

CD4⁺ T cell activation and proliferation was assessed by gating for positive CD44 and low CFSE staining at day 6 after the start of the culture
(Figure 3-8C). When treated at day 0, the two highest concentrations of kyn significantly decreased the percent of activated, proliferated CD4\(^+\) T cells relative to the untreated control group. When treated at day 4 only the highest concentration of kyn had a statistically significant negative effect. These findings demonstrated that treatment at day 0 had a more profound negative effect on activation and proliferation because T cell expansion was most robust during the first three days of activation.

We then examined the effect of direct kyn exposure on IFN-γ\(^+\) CD4\(^+\) Th1 cells using the same experimental setup (Figure 3-8D). With increasing concentration of kyn there was a more pronounced decrease of live IFN-γ\(^+\) CD4\(^+\) T cells. Interestingly, kyn treatment had a more severe suppression when given at day 4 rather than at the start of the culture. Even the lowest concentration of kyn significantly restricted the IFN-γ\(^+\) CD4\(^+\) T cell population when given at day 4 but not at day 0. These results demonstrate that kyn treatment had a greater negative impact when given to effector CD4\(^+\) T cells compared to when given at the naïve T cell stage. Furthermore, these data confirm that kyn treatment acted directly on CD4\(^+\) T cells independent of APCs and trp levels.
DISCUSSION

In this study we engineered a new inducible hIDO mouse model and present its characterization in the context of *C. trachomatis* infection. To our knowledge, this was the first study to examine *C. trachomatis* pathogenesis in a humanized IDO mouse. The cell-autonomous, antimicrobial activity of hIDO indeed decreased pathogen levels in a reductionist *in vitro* system; however, hIDO activity did not completely eradicate *C. trachomatis* in infected MEFs since trp or IDO inhibitor addition fully rescued pathogen growth (Figure 3-3). In cell culture, hIDO restriction was initially on par with the protection mediated by comprehensive IFN-γ signaling (Figure 3-4A, 24 hours post infection). However, at a later time point (Figure 3-4A, 42 hours post infection) it became clear that hIDO alone was not sufficient to restrict *C. trachomatis* to the same degree as IFN-γ treatment. Similar trends were observed *in vivo* where IFN-γ signaling sufficient mice limited *C. trachomatis* growth in the genital tract independent of hIDO expression (Figure 3-4B). We then validated the induction of numerous antimicrobial, IFN-γ-inducible genes in the genital tract following transcervical *C. trachomatis* infection (Figure 3-4C), as a mechanism for pathogen control. These robust IFN-γ responses masked the phenotype of hIDO *in vivo*.

Only by eliminating IFN-γ signaling pathways in mice were we able to interrogate the direct role of hIDO during *C. trachomatis* infection *in vivo*. During the innate phase of immunity (Figure 3-6C, 2 days post infection) hIDO
expression moderately reduced *C. trachomatis* levels, showing only partial protection. Timing-wise, the *in vivo* 2-day post infection time point corresponded most closely with the 42-hour post infection time point in MEFs. The results from these two experiments proved that hIDO could limit *C. trachomatis* growth to some degree. Remarkably, hIDO-expressing mice developed significantly higher *C. trachomatis* burden relative to controls during the adaptive phase of immunity (Figure 3-6C, 7 days post infection). This sustained burden demonstrated that in this model hIDO expression alone was not sufficient to clear genital *C. trachomatis*.

Aside from its antimicrobial properties, hIDO directly modulated the adaptive immune response. hIDO expression in the uterus limited the total *C. trachomatis*-specific CD4⁺ T cell population in the uterus, but not in the draining lymph nodes (Figure 3-7C and 3-7D). Namely, activated CD4⁺ Th1 cells were negatively affected by hIDO expression in the genital tract (Figure 3-7E and 3-7F). Moreover, we found that non-hematopoietic murine cells expressing hIDO directly inhibited CD4⁺ Th1 cells (Figure 3-8A and 3-8B). Interestingly, the degree of kyn-mediated suppression of CD4⁺ Th1 cells depended on their differentiation state (Figure 3-8C and 3-8D). Effector T cells were more sensitive to the adverse effects of kyn exposure relative to naïve T cells.

One interpretation of these observations is that the dominant cell-autonomous effect of hIDO is to slow rather than clear *C. trachomatis* infection.

As a counter defense to hIDO-mediated trp catalysis, *C. trachomatis*
transitioned to the aberrant, low antigenic form. By modifying its developmental state *C. trachomatis* escaped antigen recognition by APCs early in the infection and was able to later replicate more robustly. Microscopy of *C. trachomatis*-infected uteri from hIDO-expressing mice could be used to detect the presence of aberrant inclusions and confirm these speculations.

Another non-mutually exclusive interpretation is that the kyn byproducts of hIDO activity suppressed protective immune cells, as seen with CD4⁺ Th1 cells, resulting in defective adaptive immunity to *C. trachomatis*. Mechanistically, we cannot explicitly conclude that the inhibition of IFN-γ⁺ CD4⁺ Th1 cells caused the higher burden at 7 days post infection since hIDO-expressing mice also lacked IFN-γ signaling. Nonetheless, CD4⁺ Th1 cells are clearly important in providing resistance to genital *C. trachomatis* infections (Gondek et al., 2012). Therefore, it is likely that in an IFN-γ sufficient background, hIDO-mediated inhibition of IFN-γ producing T cells will directly prevent pathogen clearance. We propose that in humans IDO expression also inhibits protective CD4⁺ Th1 cells, preventing genital *C. trachomatis* clearance. IFN-γ induction of IDO expression in genital epithelial cells could in turn inhibit responding Th1 cells through kyn production. This hypothesis will need to be addressed in future studies to further assess if IDO expression is detrimental for pathogen clearance.

We have yet to determine how hIDO affects other immune cell types during infection. Correlative human studies and animal experiments have
shown that IDO suppresses other inflammatory cell types including CD8\(^+\) T cells, Th17, and NK cells while activating T regs (Baban et al., 2009; Liu et al., 2009; Peng et al., 2014). It is possible that the higher burden seen in ER-hIDO mice at 7 days post infection was due to the stimulation of non-protective T cell helper types. For example, adoptive transfer or stimulation of CD4\(^+\) Th2 cells or T regs exacerbate *C. trachomatis* burden in mice (Gondek et al., 2009; Stary et al., 2015).

Studies that further explore the molecular mechanisms of IDO and kyn signaling are warranted. AHR is an immunomodulatory receptor that upon activation induces IDO expression in DCs. Furthermore, kyn is an endogenous ligand and regulator of AHR (Bessede et al., 2014; Nguyen et al., 2010). Given that trp degradation by IDO generates kyn, it is likely that this IDO-kyn-AHR signaling axis is a positive feedback loop of importance for disease tolerance and therapeutic targeting that is not yet fully understood. The hIDO mouse presented here is an apt platform to test hypotheses with the aforementioned molecules to gain a better understanding of mechanisms important for immune regulation.

The host response to other pathogens is also influenced by IDO. Like *C. trachomatis*, IFN-\(\gamma\)-mediated restriction of *Toxoplasma gondii* in infected human cells is abrogated with the addition of exogenous trp. *T. gondii* scavenges trp from the host cell and lacks a complete trp biosynthetic enzymatic pathway (Blader and Koshy, 2014). Interestingly, IFN-\(\gamma\) induces IDO regardless of
whether an infectious agent is a trp auxotroph. For example, *Mycobacterium tuberculosis* encodes a complete set of trp biosynthetic genes, yet IDO is still induced robustly in the lung during infection (Blumenthal et al., 2012). The number of bacterial and protozoan trp auxotrophs is limited and the acquisition of trp synthesis pathways would allow such pathogens to overcome IDO-mediated immunity (Schmidt and Schultze, 2014). Therefore, IDO-mediated trp depletion has limited potential as a universal host defense pathway. These observations make us question the evolutionary benefit of IDO in humans during *C. trachomatis* infection. Did humans evolve to express robust levels of IDO to restrict trp auxotrophs like *C. trachomatis* or to limit damage in the reproductive system by suppressing inflammatory immune cells (Figure 3-9)? Irrespective, genital *C. trachomatis* strains evolved to survive the human IDO response.

Since the complete removal of IFN-γ signaling has pleiotropic effects, future approaches to humanize IDO mice should selectively eliminate effectors specific to murine immunity to *C. trachomatis*. It would be worthwhile to examine the phenotype of *C. trachomatis* in mice that have hIDO and also lack mouse-specific cell autonomous pathways important for restricting pathogen growth. These new lines will better model human-like *C. trachomatis* infections in the genital tract. GBP and IRG genes are suitable candidates since they provide IFN-γ-mediated protection to *C. trachomatis*-infected murine cells.
Figure 3-9. Proposed model of the dual role of hIDO during C. trachomatis infection.
In a cell-autonomous context, hIDO-mediated depletion of trp temporarily slows C. trachomatis growth (left). The production of kyn by hIDO negatively impacts the CD4+ Th1 cell response (right).
(discussed more in appendices B and C) (Coers et al., 2011; Haldar et al., 2014).

The therapeutic use of IDO inhibitors, currently in clinical trials, is intended to magnify the tumor-reactive cytotoxic T lymphocyte response to increase cancer survival rates (Löb et al., 2009). Certain human cancers express high levels of IDO that correlate with a reduced number of infiltrating, tumor-specific T cells and increased metastasis (Brandacher et al., 2006; Pan et al., 2008). As with the host-pathogen interactions delineated, much remains unknown regarding the functions of IDO in the complex tumor environment. Due to the broadly tolerizing effect of IDO activity, one should consider using IDO inhibitors to “wake up” the immune response to pathogens that cause chronic infections. Pathogens of highest interest are those with no vaccine or cure available that do not elicit sterilizing, natural host immunity. Reduced IDO activity might positively affect the development of primary and memory T cell responses to the pathogen and consequently improve disease outcome.

Our findings here show that the highly specialized human pathogen C. trachomatis survives the hIDO defense program. hIDO expression in mice resulted in a non-sterilizing, cell-autonomous restriction of C. trachomatis and severe CD4+ Th1 cell suppression. These observations imply that the absence of natural and acquired immunity to C. trachomatis in humans is due to an inadequate cell-autonomous response. Further understanding the balance of IDO-mediated antimicrobial activity and immune suppression will contribute
important insights to the host and pathogen interactions that define clinical outcomes.
MATERIALS AND METHODS

Mouse transgenics, mouse strains used, and colony maintenance

Wildtype (C57BL/6J) and ifngr\(^{-/-}\) (B6.129s7-IFNgr1tm1Ag/J) mice were ordered from The Jackson Laboratory. Transgenic hIDO mice were generated by first introducing hIDO and hCD2 cDNA in the ROSA26 locus. To do so, a previously described targeting vector was altered by replacing GFP with truncated hCD2 (Thai et al., 2007). The open reading frame encoding for hIDO was inserted into the vector using AscI restriction sites. The sequenced construct was transfected into PRX-B6N (Primogenix) embryonic stem (ES) cells derived from C57BL/6N-tac mice. After positive selection with neomycin and negative selection with diphtheria toxin A fragment, ES colonies were PCR screened for insertion into the ROSA26 using primers G1 (5’-TAGGTAGGGGATCGGGACTCT-3’) and G2rev (5’-GCGAAGAGTTTGTCTCAACC-3’) to obtain a 1.3 kb band for the targeted allele or primers G1 (5’-TAGGTAGGGGATCGGGACTCT-3’) and WTrev primer (5’-CTGTAGTAGGATCTAAGCAGGAG-3’) to obtain a 1.6 kb band for the wildtype allele. Individual ES clones were further tested for proper ROSA26 targeting by hybridizing membrane-blotted EcoR1-digested DNA with probeA, resulting in a 15.4 kb wildtype band and 5.1 knockin band. ES cells were injected into blastocyst derived from C57BL/6J mice and chimeras were crossed with C57BL/6J mice. Mice were maintained on a C57BL/6J-C57BL/6N
background and genotyped using forward primer WTSe
(5'-TGCTCTCCAAAGTCGCTC-3') and the reverse primer WTAs (5'-
AATCTGTGGGAAGTCTTGTC-3') to obtain a 320 bp band for the wildtype
allele or the reverse primer hCD2 (5'-CAACCCCTCAGAATCCAGCAAC-3') to
obtain a 720 bp band for the targeted allele. This mouse line was then crossed
with Cg-Tg(CAG-cre/Esr1) mice to obtain ER-hIDO animals. Mice were housed
in facilities managed by the Division of Laboratory Animal Resources at Duke
University Medical Center and by the Harvard Medical School Center for Animal
Resources and Comparative Medicine. Duke’s and Harvard’s Institutional
Animal Care and Use Committee approved all experiments in this study.

MEF cultures and in vitro infections
To isolate MEFs, embryos were harvested at 14 days of gestation. The head
and embryonic sac were removed from the body of the embryo, which was then
minced in Dulbecco’s phosphate buffered saline (DPBS) (Lonza) and treated
with trypsin (Gibco). After washing, MEFs were plated on 10 cm tissue culture
plates (Fisher Scientific) with MEF media composed of Dulbecco’s Modified
Eagle’s Medium (DMEM) (Gibco), 10% FBS (Atlanta Biologics), 50 U/ml
penicillin (Sigma), 50 mg/ml streptomycin (Sigma), 50 mg/ml gentamicin (Irvine
Scientific), and 1.5 g/l sodium bicarbonate (Sigma). MEFs were immortalized
following a previously described method (Zhu et al., 1991). Briefly, HEK 293T
cells with transfected with a lentivirus SV40 large T antigen construct and
packaging vector using Lipofectamine 2000 (Invitrogen). Viral supernatant from HEK 293T plates were mixed with polybrene (Sigma-Aldrich) and then added to MEFs. Blasticidin (Invivogen) was used for selection. Once a line was established, ER-hIDO MEFs were treated with 5 µM tam (Sigma) for 24 hours then stained with a CD2-APC conjugated antibody and enriched using APC-conjugated MACS microbeads and magnetic separation columns following the manufacturer’s protocol (Miltenyl Biotec). For in vitro infections, 2x10^4 MEFs were seeded in 24-well tissue culture plates in antibiotic free (Greiner Bio-One), trp null, 3% FBS media and infected with C. trachomatis at a multiplicity of infection of 3. Plates were then spun at 2000 revolutions per minute for 30 minutes. Groups were treated with 100 mg/L exogenous L-trp, 0.2 mM L-MT, 0.2 mM DL-MT (Sigma-Aldrich), or 0.5 M hydrochloric acid vehicle.

**CD4^+ T cell co-culture and NR1 transfer**

For in vitro co-cultures, CD4^+ T cells were harvested from the spleens and lymph nodes of 6 to 8 week old naïve C57BL/6J mice. Splenic suspensions were treated with a hypotonic buffer of 17 mM Tris-HCl, 150 mM NH₄Cl, pH=7.4 to lyse red blood cells. A mouse CD4 negative isolation kit (Invitrogen) was then used to enrich for CD4^+ T cells as indicated per the manufacturer’s instructions. Tissue culture plates (96-well, flat bottom format) (Greiner Bio-One) were coated with anti-CD3ε antibody overnight at 4° C and washed with DPBS before cell seeding. T cell media consisted of RPMI 1640 (Lonza), 10% FBS, L-
glutamine, HEPES (Calbiochem), 50 mM 2-mercaptoethanol (Sigma), 50 U/ml penicillin, 50 mg/ml gentamicin, and 50 mg/ml streptomycin with anti-CD28 antibody. CFSE (Life Technologies) labeled cells were treated for 8 minutes at 37° C and washed with ice-cold media. 10^5 CD4^+ T cells were added to 10^3 MEF seeded wells. For in vivo adoptive transfer experiments, live NR1 cells were harvested from naïve mice, stained, and quantified by flow cytometry prior to intravenous injections. Synthetic kyn metabolites were purchased from Sigma-Aldrich.

**C. trachomatis strain**

*C. trachomatis* serovar L2 (434/Bu) was propagated in monolayers of McCoy cells in media composed of DMEM, 10% FBS, 1.5 g/l sodium bicarbonate, 0.1 M nonessential amino acids (Hyclone), and 1 mM sodium pyruvate (Hyclone). Infected cells were detached with glass beads and sonicated to disrupt *C. trachomatis* inclusions. *C. trachomatis* EBs were isolated by density gradient centrifugation. EB isolates were titered and stored at -80° C in SPG media.

**In vivo infections and tissue processing**

To synchronize the estrous cycle, mice were injected subcutaneously with 2.5 mg medroxyprogesterone (Pfizer) 7 days before infection. Mice were treated transcervically with 0.16 mg of htam (Sigma) dissolved in DMSO and transcervically infected with 5x10^6 *C. trachomatis* IFUs in 10 µl SPG media the
following day. An NSET device (ParaTechs) was used to bypass the cervix to directly treat and infect uterine horns. Uteri were harvested, minced, and disaggregated via digestion with 1mg/ml collagenase type XI (Sigma-Aldrich) and 50 Kunitz/ml DNase (Sigma) for 30 minute in a 37° C shaker. Draining lymph nodes were mechanically disaggregated with glass slides.

DNA extraction and qPCR

Uteri or MEFs were homogenized in DPBS and DNA extracted with a DNeasy blood and tissue kit (Qiagen). *C. trachomatis* levels were quantified by measuring *C. trachomatis* 16S DNA and host GAPDH DNA using *C. trachomatis* 16S forward (5'-GGAGGCTGCAGTCGAGAATCT-3'), *C. trachomatis* 16S reverse (5'-TTACAACCCCTAGAGCCTTCATCAC-3') and mouse-specific GAPDH (Life Technologies) primers and dual label probes. A standard curve was used to determine the ratio of *C. trachomatis* to mouse DNA. Threshold values were obtained with an ABI Prism 7000 sequence system (Applied Biosystem).

RNA extraction and qRT-PCR

To isolate RNA, uterine tissue homogenates or MEFs were first treated with TRIzol reagent (Ambion). Samples were then processed using a RNA extraction kit (Qiagen) per the manufacturer's protocol. qRT-PCR samples
contained purified RNA (25 ng), SYBR Green RT-PCR master mix, reverse transcriptase (Qiagen), and primers IRGm1 forward (5’-GAGACTGTGGCAACATTG-3’), IRGm1 reverse (5’-CCGATGACTCGAAGTGCATTG-3’), IRGm3 forward (5’- AAAGTTGCCACAAAATATCTGGAAGAC-3’), IRGm3 reverse (5’-GGCTGATGAGGCGCTTGA-3’), hIDO forward (5’-TATATTGGAAATAGCTTCTTGCTT-3’), hIDO reverse (5’-TATAGGTTGCCTTTCCAGCCAGAC-3’), Gbp1 forward (5’-ACCTGGAGACTTCCTGGCT-3’), Gbp1 reverse (5’-TTTATTCCAGCTGGTCCTCTGTATCC-3’), Gbp2 forward (5’-ACCTGGAAACATTCCCTGACC-3’), Gbp2 reverse (5’-ACAGCTCCTCCTCCCGCAGAG-3’), Gbp3 forward (5’-CCAGAAACCAAACACTGGAACCGAA-3’), Gbp3 reverse (5’-TCTCCAGACAAGGCACAGTC-3’), Gbp5 forward (5’-CACTCAGCAACGAGGAGCTGAACT-3’), Gbp5 reverse (5’-TGTTCCTCCTGAGGCAGGACCAGCC-3’), Gbp7 forward (5’-TTGAGGAATGCCAGAGGACCAGT-3’), and Gbp7 reverse (5’-GTCTCCACTATTGATAGCATCCACG-3’). Expressions for all genes of interest were normalized to host GAPDH mRNA expression within the same sample. Data was gathered with an ABI Prism 7000 sequence system and fold changes were calculated relative to baseline expression of control mice or MEFs.
Cell staining and flow cytometry

Cell suspensions were stained for surface and intracellular epitopes using the following antibodies (clones): anti-mouse CD3ε (145-2C11), anti-mouse CD28 (37-51), anti-human CD2 (RPA-2.10), anti-mouse CD44 (IM7), anti-mouse TCR να2 (B20.1), anti-mouse CD90.1 (OX-7), anti-mouse IFN-γ (XMG1.2), anti-mouse CD25 (PC61), anti-mouse TNF-α (MP6-XT22) (Biolegend), anti-human IDO (eyedio) (eBioscience), anti-mouse CD4 (RM4-5) (Life technologies), anti-mouse CD16/32 (2.4G2) (BioXCell), and Live/Dead staining kit (Invitrogen). For ICCS, T cells were stimulated for 3 hours with 100 ng/ml PMA (Alexis Biochemicals), 1 µg/ml ionomycin (Calbiochem), and brefeldin A (BD Biosciences) in RPMI 1640 at 37° C. Cells were permeabilized and fixed with Cytofix/Cytoperm plus kit (BD Biosciences). AccuCheck counting beads (Invitrogen) were added to quantify cell numbers. Samples were assessed by flow cytometry using a modified FACSCalibur (Cytek Development) or an LSRII (BD Biosciences), and data was analyzed by FlowJo software (Tree Star).

Kynurenine quantification using HPLC

All HPLC data were obtained on an Agilent 1100 series HPLC using a Phenomenex C18 reverse-phase column (4.6 x 250 mm, 100Å, 5 µm), equipped with a corresponding guard column. Samples were filtered using 0.2 µm syringe filters prior to injection and were eluted with isocratic 15 mM potassium phosphate, pH=6.4, containing 2.7% acetonitrile at a flow rate of 0.8
ml/min. The area under the curve was quantified, and concentration of kyn was determined relative to a standard curve. The kyn peak was detected at 360 nm at a retention time of 13 minutes (Figure 3-10).

**Statistical analysis**

All statistical analyses were performed using Prism version 5.0d (GraphPad Software) where a p value less than 0.05 was deemed to be a statistically significant difference. In each figure statistically significance differences were represented as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The specific statistical test performed in each experiment was described in figure legends.
Figure 3-10. HPLC analysis of kynurenine abundance.  
(A) Standard curve used to quantify kyn levels in MEF supernatants and uterine lysates.  
(B) Representative HPLC chromatogram of ER-hIDO cell supernatant with kyn peak at a retention time of 13 minutes.
CHAPTER 4: General discussion
SUMMARY OF FINDINGS

The sheer number of people living with *C. trachomatis* and the morbidity caused from these infections continue to be a substantial public health problem. The unmet need for a long-lasting, effective vaccine to prevent *C. trachomatis* infections demands greater attention from researchers, clinicians, and government leaders. Limited protective immunity in many *C. trachomatis*-infected patients leads to recurrent or repeat infections. Understanding the biological reasons for the absence of natural and acquired sterilizing immunity in humans will better inform vaccine efforts. Additionally, further examinations of protective cell-autonomous and adaptive immune responses that do control *C. trachomatis* control are still needed. This dissertation focused on revealing both weaknesses and strengths of the human and murine immune responses to *C. trachomatis*. Specifically, I presented comprehensive findings on protective CD4⁺ T cell homing to the uterus, and the cell-autonomous and regulatory role of human IDO during genital *C. trachomatis* infection. I also included preliminary findings concerning the *in vivo* function of GBP and a nascent model for chronic *C. trachomatis* infection in the murine upper genital tract.

In chapter 2 we interrogated the importance of α4β1 and α4β7 integrin heterodimers in permitting *C. trachomatis*-specific CD4⁺ T cell homing to the upper genital tract and conferring protection to infected mice. We demonstrated that integrin α4β1 was robustly increased on the surface of both polyclonal and
C. trachomatis-specific CD4+ T cells in the uterus after infection. We observed that blocking or deleting integrin α4β1, but not α4β7, on C. trachomatis-specific CD4+ T cells inhibited trafficking to the uterus and reduced the protective capacity of these immune cells. These findings demonstrate that α4β1 is the key integrin receptor for CD4+ T cell-mediated protection against C. trachomatis. Identifying the receptors required for CD4+ T cell trafficking to the genital tract in response to C. trachomatis is important for understanding how protective immune cells get to the site of the infection.

In chapter 3 I presented a new mouse model that inducibly expresses ectopic hIDO. This system was urgently needed because contrary to humans, mice do not restrict C. trachomatis growth by IDO-mediated depletion of the essential amino acid trp. Studies using human cell culture had previously demonstrated a role for IDO in cell-autonomous host defense, but limitations in animal models prevented an assessment of the impact that a human-like IDO response has on genital C. trachomatis infections. We showed that hIDO expression restricted C. trachomatis growth in a trp-dependent manner in murine cells. In the murine genital tract, hIDO expression decreased C. trachomatis levels initially but then resulted in higher bacterial burden. We then questioned the effect of hIDO on the T cell response and observed that C. trachomatis-specific CD4+ Th1 cells were inhibited in the genital tract of hIDO-expressing animals. hIDO-expressing murine cells and kyn treatment directly and robustly inhibited effector CD4+ Th1 cells. Understanding the
balance of hIDO-mediated antimicrobial activity and immune regulation will contribute important insights into the host and pathogen interactions that govern disease.

The appendices include supplemental results to chapters 2 as well as preliminary results from other interesting projects. In appendix B, I present results from a potential animal model for persistent *C. trachomatis* infection. This system may become a platform to research how the host and pathogen biology changes during chronic infections. In appendix C, I show findings concerning the *in vivo* role of GBP proteins during genital *C. trachomatis* infection. The GBP family provides immunity in murine cell culture but their *in vivo* function during genital *C. trachomatis* infection will need additional exploration.
Our findings concern both host cell-autonomous and adaptive immunity to \textit{C. trachomatis} in the murine upper genital tract. I propose the following model of immunity to genital \textit{C. trachomatis} based on interpretations of our results as well as observations from other studies. First, human epithelial cells secrete pro-inflammatory molecules following \textit{C. trachomatis} recognition. These pro-inflammatory signals recruit innate immune cells such as DCs, NK cells, and neutrophils to activate antimicrobial pathways and initiate an adaptive immune response (Buchholz and Stephens, 2006). Importantly, NK cells have the ability to produce IFN-γ, which induces cell-autonomous pathways in genital mucosal epithelial cells (Hook et al., 2005). The IFN-γ-stimulated response differs among host species with human cells robustly inducing IDO and murine cells expressing IRGs (Nelson et al., 2005). In humans, IDO depletes trp slowing \textit{C. trachomatis} growth, likely through the induction of the persistent state.

In addition to the cell-autonomous, antimicrobial effect of IDO, its enzymatic activity produces kyn metabolites that transmit signals to other cells including DCs and T cells (Mellor and Munn, 2004). IDO expression by genital epithelial cells could trigger a tolerogenic response in DCs. These DCs would then induce the differentiation of naïve \textit{C. trachomatis}-specific lymphocytes in the draining lymph nodes to T regs. Conversely, naïve CD4$^+$ T cells that do
differentiate to effector Th1 cells upregulate the surface integrin receptor α4β1 to traffic to the upper genital tract and provide protection against *C. trachomatis* (Davila et al., 2014). These protective Th1 cells secrete robust levels of IFN-γ needed to clear *C. trachomatis* (Gondek et al., 2012).

In the upper genital tract, hIDO-expressing cells suppress *C. trachomatis*-specific CD4+ Th1 cells (Figure 3-7). In humans, a similar IDO-mediated inhibition of protective CD4+ Th1 cells could cause higher burden in the genital tract. Hypothetically, Tregs in the genital tract could be stimulated by IDO expression and suppress effector Th1 cells needed to eliminate *C. trachomatis*. Genetic differences in humans could affect the induction robustness of mentioned signaling molecules. Additionally, haplotype differences could affect the affinity to certain *C. trachomatis* antigens and the ensuing T cell response (Wang et al., 2005). I speculate that IDO-mediated immunity in humans is not sufficient to eliminate genital *C. trachomatis*, as seen in the humanized IDO mouse model.

This working model would be more complete with additional experimentation in mice and isolated human tissues, and correlative studies with *C. trachomatis*-infected patients. The following section will discuss how these proposed experiments would address gaps in the presented findings. Irrespective of my model answering these questions will delve deeper into the biology of *C. trachomatis* and the host.
FUTURE STUDIES

Future investigations should continue examining the effect of IDO on *C. trachomatis* pathogenesis and immune cells. My collaborators and I invested several years conceptualizing, generating, and characterizing the hIDO mice and although we uncovered important findings there is much more potential for discovery and application with these lines. I would prioritize addressing these four key questions that remain unanswered in our studies:

How does hIDO affect the developmental state of *C. trachomatis in vivo*?

Previous studies have demonstrated that *C. trachomatis* inclusions become aberrant and that the organism ceases to replicate under stress, including IDO-mediated trp starvation (Beatty et al., 1994a). We observed that hIDO expression in murine cells (Figure 3-3) and in the genital tract at an early time point (Figure 3-6C, day 2) decreased *C. trachomatis* levels, yet the mechanism of intracellular restriction remains unclear. Does hIDO expression in these cells at this time point halt or slow the growth of *C. trachomatis*? Do most bacteria die under trp starvation (except for a few survivors) or do most bacteria differentiate to a persistent state? Quantifying *C. trachomatis* by DNA extraction and qPCR analysis is a fast and reproducible way of detecting pathogen load, yet it is a technique that does not reveal the biological state of the bacteria. DNA from live or dead organisms is indistinguishable in this assay. On the other hand, microscopy of infected cell cultures and the genital tract would visually
reveal the state of the inclusion—normal or aberrant. Electron or two-photon microscopy images of C. trachomatis-infected hIDO cells would certainly provide an answer to the questions posed above. C. trachomatis viability would then be determined with an infectivity titer to quantify IFUs, as a complementary experiment. I predict that C. trachomatis is not killed in the genital tract of hIDO-expressing mice since higher burden is detected at a later time point (Figure 3-6C, day 7). It is most probable that C. trachomatis growth is initially slowed by hIDO via the induction of the persistent state at 2 days post infection.

Why is C. trachomatis burden higher at 7 days post infection in hIDO-expressing mice? In Figure 3-7 we showed that C. trachomatis-specific CD4⁺ Th1 cells were impaired by hIDO expression in the murine genital tract. It is implicit to think that the inhibition of this immune cell population was sufficient to exacerbate C. trachomatis burden in hIDO-expressing mice, however there could be other important mechanisms happening as well. Furthermore, because experimental hIDO mice were deficient of IFN-γ-signaling, we could not conclude that higher burden was directly caused by hIDO-mediated inhibition of IFN-γ⁺ C. trachomatis-specific Th1 cells. Kyn and hIDO expression could inhibit NK cells or stimulate non-protective helper subtypes such as T regs and Th2 cells, contributing to this burden exacerbation. Moreover, other immune pathways that are protective and independent of IFN-γ signaling could be negatively affected by hIDO activity and contribute to the higher burden seen at 7 days post infection. These unresolved questions could be answered by further
characterizing immune cell populations, and by testing the function of hIDO on mice deficient of specific effectors rather than the entire IFN-γ pathway.

Does hIDO expression improve or worsen upper genital tract pathology following *C. trachomatis* infection? Future studies should examine the histopathology of these mouse lines after *C. trachomatis* infection. Since hIDO expression inhibits inflammatory CD4+ Th1 cells it is possible that these mice will have less severe pathologies. Conversely, Th1 cells help clear *C. trachomatis*, thus suppressing these immune cells could lead to a higher bacterial burden that then causes pathology. These two putative scenarios make predicting the outcome difficult. Although I did not carefully analyze pathology in any of my previous experiments, I did not observe obvious trends in the upper genital tract. Preventing the inflammatory sequela that arises from *C. trachomatis* infections is of utmost clinical importance.

Do human *C. trachomatis* infections recapitulate the phenotype seen in hIDO-expressing mice? The main objective of engineering the hIDO mice was to create small animal models that would be more representative of human *C. trachomatis* infections. It is difficult to determine if we successfully met this goal when there are aspects of the human immune response to *C. trachomatis* that are still unknown. For example, are IDO and kyn expressed at higher levels in the genital tract of *C. trachomatis*-infected humans? Are protective immune cell populations, such as CD4+ Th1 cells, negatively affected by IDO and kyn during human infections? These questions could be addressed by assessing immune
cell populations, IFN-γ levels, *C. trachomatis* load, IDO expression, and kyn abundance in clinical samples (blood or cervical swabs) from infected and control human subjects. I propose that correlative studies with these factors would shed light on the relationship of IDO and kyn with *C. trachomatis* burden, protective T cells, and pathology in humans. Likewise, in mice we showed that integrin α4β1 mediated protective T cell trafficking to the upper genital tract. In humans it is unknown whether the same adhesion receptors are expressed on protective CD4⁺ T cell populations in the genital tract. Gaining insights into these aspects of human biology during *C. trachomatis* infection will better inform basic, translational, and clinical research.
CONCLUDING REMARKS

About 50 years ago, preclinical studies showed that inoculation of non-human primates with ocular *C. trachomatis* resulted in worse inflammation upon exposure compared to the placebo treated group. However, these studies did not assess scarring trachoma, thus it remains inconclusive whether inoculation ultimately prevented blindness in these non-human primates. Clinical trials demonstrated that *C. trachomatis* exposure generated short-lived immunity and decreased scarring trachoma rates in humans. The confounding outcomes between humans and non-human primates arose from incomplete assessments of protection to *C. trachomatis*. Consequently, the medical community became wary of assertively pursuing *C. trachomatis* clinical trials for many years (Mabey et al., 2014). A new generation of nanoparticle based vaccines promises to generate a protective immune response that will prevent *C. trachomatis* diseases in humans (Stary et al., 2015). The results are encouraging in mice, yet it is uncertain whether the vaccine will stimulate the same level of protection in the human mucosa. The failures of past vaccine efforts were due to ill-defined clinical endpoints and an incomplete comprehension of *C. trachomatis*, model organism, and human biology. My hope is that the findings summarized in this dissertation inform future research and vaccine efforts.
APPENDIX A: Supplementary material to chapter 2
C. trachomatis infection alters the gene expression of adhesion receptors in the genital tract

We began the integrin study by surveying the relative expression differences of adhesion receptors in the uterus following infection with C. trachomatis. We selected several members of the α and β integrin chains as well as L-selectin. Wildtype mice were infected transcervically with C. trachomatis or left uninfected for the control group (Figure A-1). Uteri were then harvested and homogenized at 7 days post infection. Uterine RNA was isolated from these samples for qRT-PCR expression analysis. We included the main candidates of our hypothesis—integrins α4, β1, and β7—in this panel to better understand transcriptional changes following C. trachomatis infection. By comparing infected to uninfected samples, we noticed an upregulation of most genes to varying degrees with the exception of integrin αE. This indicated that the murine upper genital tract generally expressed higher levels of these adhesion receptors following C. trachomatis infection. Integrin β2 and L-selection had notably higher levels in the infected groups compared to controls. These adhesion molecules have been previously shown to be important during T cell activation (Bachmann et al., 1997).

Unfortunately, because we assayed the entire uterus, it was impossible to distinguish the relative expression level in specific cell types, such as activated CD4+ T cells responding to C. trachomatis infection. Furthermore, mRNA expression did not necessarily lead to the expression of these integrin
heterodimers on the cell surface as we later observed with flow cytometry analysis. This post-transcriptional information simply could not be obtained using qRT-PCR. Consequently, we proceeded to analyze single cell suspensions of uteri using flow cytometry in chapter 2 to specifically observe the expression of surface integrin receptors on CD4$^+$ T cells. We focused on the integrin heterodimers $\alpha 4\beta 1$ and $\alpha 4\beta 7$ in an effort to identify uterine-specific homing receptors rather than general adhesion molecules upregulated post inflammation.
Figure A-1. *C. trachomatis* infection alters the gene expression of adhesion receptors in the genital tract.

Wildtype mice were transcervically infected with *C. trachomatis*. Seven days following infection, uteri were harvested and RNA isolated for qRT-PCR analysis. Threshold values for the genes of interest were normalized to murine *GAPDH*. The fold change in relative mRNA levels was assessed by comparing infected mice to uninfected control animals. Data show mean ± SEM of at least three mice per group from one of two independent experiments. *p<0.01 (column statistical test).
LFA-1 is induced following activation and is critical for *C. trachomatis*-specific CD4\(^+\) T cell trafficking

The integrin heterodimer \(\alpha L\beta 2\), or more commonly referred to as LFA-1, is instrumental in regulating the function and migration of leukocytes. Previous studies have shown that LFA-1 is an integrin of great importance in T cell recruitment to the site of inflammation during infection (Smith et al., 2007). Therefore due to its essentiality in trafficking, we measured LFA-1 levels in *C. trachomatis*-specific CD4\(^+\) T cells to observe any changes in the absence of either integrin \(\beta 1\) or \(\beta 7\). To address whether LFA-1 was altered in the knockout animals, we measured the surface expression of LFA-1 on NR1 cells from wildtype and integrin knockout mice by flow cytometry (Figure A-2A). NR1 cells from wildtype, \(\beta 1^{-/-}\) and \(\beta 7^{-/-}\) animals were harvested and activated *in vitro* as done previously. LFA-1 expression was assayed five days following activation by flow cytometry. LFA-1 was robustly upregulated on NR1 cells from wildtype, integrin \(\beta 1^{-/-}\), and integrin \(\beta 7^{-/-}\) animals compared to the isotype control. When the histograms were overlaid, we did not see any substantial differences in LFA-1 surface expression between NR1 cells from the three genotypes. These results showed that LFA-1 was robustly upregulated on NR1 cells from the three different genotypes and that there was no deficient or compensatory LFA-1 expression in the integrin knockout NR1 cells following activation.

After confirming robust expression of LFA-1 following *in vitro* activation, we next wanted to test the role of LFA-1 on CD4\(^+\) T cell migration after genital *C. trachomatis* infection.
*trachomatis* infection in mice. We hypothesized that blocking LFA-1 would alter *C. trachomatis*-specific CD4⁺ T cell recruitment to the genital mucosa. We transferred NR1 cells into recipient mice and infected transcervically the following day (Figure A-2B). LFA-1 was blocked systemically using a LFA-1 neutralizing antibody or an isotype antibody for control groups. Spleens and uteri were harvested and processed into single cell suspensions for flow cytometry analysis. We observed that blockade of LFA-1 completely eliminated NR1 cell trafficking to both spleen and uterus. This result demonstrated that LFA-1 was important for general CD4⁺ T cell trafficking as opposed to being a uterine-specific homing receptor. Since the focus of this project was on genital tract homing of CD4⁺ T cells following *C. trachomatis* infection, we decided to not pursue more LFA-1 investigations.
Figure A-2. LFA-1 is induced following activation and is critical for *C. trachomatis*-specific CD4+ T cell trafficking.

(A) NR1 cells from wildtype, β1−/− and β7−/− animals were harvested and activated *in vitro*. LFA-1 expression on NR1 cells was assayed five days following activation using flow cytometry. LFA-1 staining is demonstrated on NR1 cells from wildtype (black line), β1−/− (blue line) and β7−/− (red line) animals compared to the isotype control (gray filled).

(B) NR1 cells were transferred to naïve recipients that were infected with *C. trachomatis* transcervically the following day. Mice were treated systemically with an LFA-1 neutralizing antibody or an isotype control antibody. Spleens and uteri were harvested and processed into single cell suspensions for flow cytometry. Data show mean ± SEM of at least three mice per group from one of two independent experiments.
C. trachomatis-specific CD4\textsuperscript{+} T cells do not express CD103 upon activation

We were curious to know whether CD103 (integrin $\alpha E$) was expressed on C. trachomatis-specific CD4\textsuperscript{+} T cells following activation. Previous studies have shown that CD103 can be expressed on the surface of T cells. The CD103 and $\beta 7$ heterodimer receptor binds to E-cadherin, a ligand expressed on epithelial cells (Cepek et al., 1994). To determine whether CD103 was altered in C. trachomatis-specific CD4\textsuperscript{+} T cells, we measured the surface expression of CD103 on activated NR1 cells by flow cytometry (Figure A-3). NR1 cells from integrin wildtype, $\beta 1^{-/-}$ and $\beta 7^{-/-}$ animals were harvested and activated in vitro. Five days after activation, we examined the surface expression of CD103 on all genotypes. We noted no major differences in CD103 expression between NR1 cells from wildtype, $\beta 1^{-/-}$, and $\beta 7^{-/-}$ animals compared to the isotype control. When the histograms were overlaid, we did not see any substantial upregulation of CD103 surface expression on NR1 cells from these three genotypes. These results revealed that C. trachomatis-specific CD4\textsuperscript{+} T cells did not express CD103 upon in vitro activation.
Figure A-3. *C. trachomatis*-specific CD4$^+$ T cells do not express CD103 upon *in vitro* activation.
NR1 cells from integrin wildtype, $\beta 1^{-/-}$ and $\beta 7^{-/-}$ animals were harvested, activated *in vitro*, and examined for surface expression of CD103 five days later. CD103 surface expression on NR1 cells from wildtype (black line), $\beta 1^{-/-}$ (blue line) or $\beta 7^{-/-}$ (red line) animals compared to the isotype control (gray filled). Data show mean ± SEM of at least three mice per group from one of two independent experiments.
APPENDIX B: Attempt to develop a mouse model for persistent *Chlamydia trachomatis* infection
ATTRIBUTIONS

The experiments presented here were a collaborative effort with Drs. Zhe Shi and Jörn Coers, who engineered the hIDO construct. Drs. Jörn Coers, Michael Starnbach, and I designed and interpreted the experiments.

I wrote the text of this section.
Inadequate elimination of genital *C. trachomatis* from failed antibiotic treatment and non-protective host immunity can result in persistent infections. Unresolved infections and recurrent inflammation in the reproductive organs are major contributors to the morbidity that arises in *C. trachomatis*-infected humans, such as infertility. Developing an animal model for persistent *C. trachomatis* infection will provide a platform to research the host and pathogen interactions that lead to pathology and lack of a long-lived, protective immune response. Here, we generated hIDO-expressing mice deficient of the two major IRG proteins (IRGm1/3) that restrict *C. trachomatis* in the murine genital tract but are not present in human cell-autonomous immunity. At a late time point of infection (day 21), IRGm1/3 \textsuperscript{-/-} ER-hIDO mice had higher levels of *C. trachomatis* in the upper genital tract relative to both wildtype and IRGm1/3 deficient control groups. This new humanized mouse model will further elucidate how *C. trachomatis* survives and establishes persistence in the upper genital tract and be a platform to examine the failed host immune response.
INTRODUCTION

The results presented in chapter 3 attest that mice that express hIDO and are deficient of the IFN-\(\gamma\) response develop sustained \textit{C. trachomatis} burden in the genital tract. Only by entirely eliminating IFN-\(\gamma\) signaling were we able to exclusively interrogate \textit{in vivo} hIDO function on \textit{C. trachomatis} pathogenesis. Although this approach provided clear phenotypes regarding the effect of hIDO expression on \textit{C. trachomatis} load and CD4\(^+\) Th1 cells, the complete removal of IFN-\(\gamma\) signaling has pleiotropic effects. The next generation of humanized mice should include the human-specific cell-autonomous response and also lack mouse-specific cell autonomous pathways important for restricting \textit{C. trachomatis} growth. Consequently, we decided to selectively eliminate two IFN-\(\gamma\) downstream response genes specific to murine immunity to \textit{C. trachomatis} with the objective of generating a more physiologically relevant humanized mouse model for chronic infection.

IRGm1 and IRGm3 (IRGm1/3) provide robust IFN-\(\gamma\)-mediated protection to \textit{C. trachomatis}-infected murine cells and mice during genital infection (Coers et al., 2011). The mechanism of action of IRGs is trp independent and instead compromises the integrity of the pathogen containing vacuole (MacMicking, 2012). Because the mouse is not the preferred host, \textit{C. trachomatis} is very susceptible to an IRG-driven murine epithelial cell defense that is absent in humans. It has been shown that genetic deletions of IRGm1/3 result in mice
having higher *C. trachomatis* burden during the initial phase of infection (day 5 post infection). However, burden in IRGm1/3/−/− animals decreased at time points representing later stages of the adaptive immune response (day 10 and 15 post infection) until *C. trachomatis* load became comparable to wildtype levels. It was then confirmed that an exacerbated CD4⁺ T cell response decreased burden in IRGm1/3/−/− animals and impeded *C. trachomatis* from establishing a chronic infection in mice (Coers et al., 2011).

In chapter 3, we showed that hIDO expression was sufficient to suppress protective *C. trachomatis*-specific CD4⁺ T cells. We hypothesized that hIDO would suppress the exacerbated CD4⁺ T cell response previously seen in IRGm1/3/−/− mice, thus permitting a longer *C. trachomatis* infection in the upper genital tract. Here, we tested whether an IRG deficient mouse with hIDO would develop sustained *C. trachomatis* burden.
RESULTS

We initially crossed ER-hIDO mice (described in chapter 3) to IRGm1/3 $^{−/−}$ animals to obtain a new line with inducible hIDO expression deficient of the two major IRG proteins that restrict C. trachomatis in the murine genital tract. This novel mouse line would be an ideal platform to determine if these three genetic alterations would be sufficient to obtain chronically C. trachomatis-infected mice. To test this, we treated wildtype, IRGm1/3 $^{−/−}$, or IRGm1/3 $^{−/−}$ ER-hIDO mice with htem in the uterus (Figure B-1). Mice were then transcervically infected with C. trachomatis the following day. We harvested uteri at 7 and 21 days post infection and then extracted DNA to measure C. trachomatis burden by qPCR. At 7 days post infection both IRGm1/3 $^{−/−}$ and IRGm1/3 $^{−/−}$ ER-hIDO mice had significantly more C. trachomatis load in the uterus. Mice expressing hIDO had even higher C. trachomatis load than IRGm1/3 $^{−/−}$ groups. This result followed a similar trend to the 7-day post infection ifngr $^{−/−}$ ER-hIDO experiment shown in Figure 3-6 demonstrating that hIDO expression modulates C. trachomatis burden to a similar extent in distinct murine lines. Interestingly, at 21 days post infection mice expressing hIDO still had significantly higher levels of C. trachomatis in the genital tract. Contrary, both wildtype and IRGm1/3 $^{−/−}$ groups had restricted C. trachomatis growth at this late time point suggesting that only mice that express hIDO become chronically infected.
Figure B-1. IRGm1/3-deficient mice that express hIDO are chronically infected with genital *C. trachomatis*.
Mice of indicated genotype were transcervically treated with htam and infected with *C. trachomatis* the following day. Mice were also treated transcervically with htam at 7 and 14 days post infection. Uteri were then harvested at 7 (black bars) and 21 (white bars). DNA was extracted from uterine lysates to measure *C. trachomatis* burden by qPCR. Data show mean ± SEM pooled from three independent experiments. * p < 0.05 and *** p < 0.001 (unpaired t-test).
DISCUSSION

The preliminary results presented here provide a new in vivo platform to test hypotheses that will elucidate the biology of persistent C. trachomatis infections in the genital tract. We showed that mice that express hIDO and are deficient of key IRGs do not clear C. trachomatis. Bacterial burden in the upper genital tract was still present at 21 days post infection, contrary to control mice that restricted C. trachomatis growth. IRGm1/3−/− ER-hIDO mice presented a phenotype of higher burden despite being IFN-γ signaling sufficient. This demonstrates that only three genetic changes (addition of hIDO and removal of IRGm1 and IRGm3) were enough to permit genital C. trachomatis to survive for a longer duration in mice. Future studies should examine later time points to answer if and when C. trachomatis infection in the genital tract is resolved.

It would be very interesting to examine immune cell populations at late stages of infection to assess any functional changes in T cell development. I am particularly interested in seeing how the primary T cell response develops into a memory T cell pool during chronic infection compared to an acute infection. It would be worthwhile to examine both CD4+ T cells and CD8+ T cells as they may each present a different phenotype in this infection model. In models of persistence using lymphocytic choriomeningitis virus (LCMV), inhibitory receptors such as PD-1/PD-L1 become significantly upregulated on T cells during chronic infection relative to T cells responding to an acute LCMV
infection (Barber et al., 2006). A previous study from the Starnbach laboratory showed that *C. trachomatis* infections induced the PD-1/PD-L1 inhibitory pathway. This inhibitory signaling pathway decreased the memory CD8\(^+\) T cell population that was protective against *C. trachomatis* infection. PD-1/PD-L1 genetic deletion or antibody blockade increased the protective memory T cell population, which resulted in a reduction of *C. trachomatis* burden (Fankhauser and Starnbach, 2014). I speculate that chronically infected hIDO mice will have a higher expression of inhibitory receptors and ligands, such as PD-1 and PD-L1, on T cells, APCs, and epithelial cells. The mechanism behind an impaired memory T cell population could be the prolonged *C. trachomatis* antigen stimulation arising from persistent infection and/or direct signaling mediated by hIDO and kyn. Here, we have generated and characterized a suitable *in vivo* system to test these hypotheses and thereby gain a better perspective of the underlying biology of persistent *C. trachomatis* infection.
MATERIALS AND METHODS

Mouse strains and colony maintenance

Wildtype (C57BL/6J) mice were ordered from The Jackson Laboratory. IRGm1/3 \( ^{-/-} \) mice were previously generated and described in (Collazo et al., 2001). Transgenic hIDO mice were crossed with \( Cg-Tg(CAG-cre/Esr1) \) mice to obtain ER-hIDO animals (described in chapter 3). ER-hIDO mice were then crossed with IRGm1/3 \( ^{-/-} \) to obtain experimental groups of IRGm1/3 deficient ER-hIDO mice. Mice were housed in facilities managed by the Harvard Medical School Center for Animal Resources and Comparative Medicine. Harvard’s Institutional Animal Care and Use Committee approved all experiments in this study.

C. trachomatis strain

\( C. trachomatis \) serovar L2 (434/Bu) was propagated in monolayers of McCoy cells in media composed of DMEM, 10% FBS, 1.5 g/l sodium bicarbonate, 0.1 M nonessential amino acids (Hyclone), and 1 mM sodium pyruvate (Hyclone). Infected cells were detached with glass beads and sonicated to disrupt \( C. trachomatis \) inclusions. \( C. trachomatis \) EBs were isolate by density gradient centrifugation. EB isolates were titered and stored at -80° C in SPG media.
**In vivo infections and tissue processing**

To synchronize the estrous cycle, mice were injected subcutaneously with 2.5 mg medroxyprogesterone (Pfizer) 7 days before infection. Mice were transcervically treated with 0.16 mg htam and infected with $5 \times 10^6$ *C. trachomatis* IFUs in 10 µl SPG media the following day. An NSET device (ParaTechs) was used to bypass the cervix to directly treat and infect uterine horns. To maintain the expression of hIDO in the genital tract, mice were treated transcervically with htam at 7 and 14 days post infection.

**DNA extraction and qPCR**

Uteri were homogenized in DPBS and DNA extracted with a DNeasy blood and tissue kit (Qiagen). *C. trachomatis* levels were quantified by measuring *C. trachomatis* 16S DNA and host GAPDH DNA using bacterial and mouse-specific primer pairs and dual label probes. A standard curve was used to determine the ratio of *C. trachomatis* to mouse DNA. Threshold values were obtained with an ABI Prism 7000 sequence system (Applied Biosystem).

**Statistical analysis**

All statistical analyses were performed using Prism version 5.0d (GraphPad Software) where a p value less than 0.05 was deemed to be a statistically significant difference.
APPENDIX C: Examining the role of GBPs in the upper genital tract during *Chlamydia trachomatis* infection
ATTRIBUTIONS

I designed, performed, and analyzed the experiments presented in appendix C in collaboration with Noah J. Harrison. Drs. Jörn Coers and Michael Starnbach were involved in experimental design and data interpretation.

I wrote the text of this section.
ABSTRACT

GBPs play a role in host cell-autonomous immunity to intracellular pathogens. A recent study demonstrated that in murine cell culture GBPs were necessary for IFN-γ-mediated inhibition of *C. trachomatis*. However, the function of GBPs *in vivo* during genital *C. trachomatis* infection was unknown prior to our study. We addressed this question by using mice deficient of GBPs located on chromosome 3 (GBP<sup>chr3-/−</sup>) to test their importance in suppressing *C. trachomatis* growth in the genital tract. We found that *C. trachomatis* burden was similar between wildtype and GBP<sup>chr3-/−</sup> mice at both 3 and 7 days post infection. This result demonstrated that the GBPs on chromosome 3 were not necessary to control *C. trachomatis* growth in the murine genital tract. Gaining a better understanding of the role of the GBP family in immunity will shed light on the IFN-γ response to intracellular pathogens.
GBP proteins belong to a family of IFN-γ-inducible GTPases that are part of the cell autonomous defense against intracellular pathogens (MacMicking, 2012). In mice, 11 functional GBPs have been identified, located on chromosomes 3 and 5. Murine GBPs (mGBPs) 1, 2, 3, 5, and 7 are present on chromosome 3, while in silico analysis recognized mGBPs 4, 6, 8, 9, 10, and 11 on chromosome 5 (Kresse et al., 2008). The human genome also encodes GBPs although in different numbers and locations than in mice. In total, humans have 8 GBPs all located on chromosome 1 (Degrandi et al., 2007; Olszewski et al. 2006). Interestingly, unlike other cell-autonomous genes, such as the IRGs, GBPs are highly conserved in vertebrates (Kresse 2008, Olszewski 2006). Human GBPs (hGBPs) and the mGBPs have a high degree of homology and are upregulated in response to IFN-γ in both species. Given these similarities, the characterization of GBPs in mice could mirror trends present in humans during infection.

Several studies have shown how GBPs restrict the growth and proliferation of a variety of pathogens. For example, hGBP3 controlled influenza activity while hGBP1 suppressed vesicular stomatitis virus and encephalomyocarditis virus in cell cultures (Anderson et al., 1999; Nordmann et al., 2012). GBPs inhibit *Listeria monocytogenes* and *M. tuberculosis* growth providing evidence of their involvement in bacterial restriction (Kim et al., 2012).
*T. gondii* intracellular growth is exacerbated in mice deficient of the 6 GBPs on chromosome 3 (GBP\(^{\text{chr3-/-}}\)) (Yamamoto et al., 2012). A recent study using the same murine cells from Yamaoto et al. demonstrated that IFN-γ-mediated restriction of *C. trachomatis* depended on the same GBPs clustered on chromosome 3. GBP\(^{\text{chr3-/-}}\)-deficient cells had a significantly higher *C. trachomatis* load than wildtype groups following IFN-γ stimulation (Haldar et al., 2014). Previously, no study had characterized the function of GBPs in the genital tract during *C. trachomatis* infection.

GBP signaling also plays a role in inflammation. mGBPs on chromosome 3 activate caspase 11 in response to LPS resulting in pyroptosis (Pilla et al., 2014). mGBP5 is involved in the assembly of the NLRP3 inflammasome in mice (Shenoy et al., 2012). Interestingly, hGBP1 was upregulated in the colonic mucosa of humans with inflammatory bowel disease, indicating that hGBP1 is either an associated or causal factor for this condition (Schnoor et al., 2009). In the genital tract, it is currently unknown whether GBPs contribute to inflammation causative of pathology during *C. trachomatis* infection. Our goal was to gain a better understanding of the role of GBPs in *C. trachomatis* restriction as well as immunopathology in the upper genital tract.
RESULTS

To determine if the previously characterized in vitro C. trachomatis phenotype was replicated in vivo, we infected GBP\textsuperscript{chr3/-} mice with C. trachomatis and analyzed burden in the uterus (Figure C-1). We harvested uteri at 3 and 7 days post infection and isolated DNA for C. trachomatis burden analysis using qPCR. Day 3 represented the innate phase of immunity while day 7 would assess the role of GBPs during adaptive immunity. Surprisingly, C. trachomatis burden was similar between wildtype and GBP\textsuperscript{chr3/-} mice at both 3 and 7 days post infection. These results suggest that the GBPs on chromosome 3 are not necessary to control C. trachomatis growth in the murine upper genital tract as had been shown previously in cell culture.
Figure C-1. GBP<sup>chr3-/-</sup> mice restrict <i>C. trachomatis</i> in the genital tract.
Wildtype or GBP<sup>chr3-/-</sup> mice were infected transcervically with <i>C. trachomatis</i>. Uteri were harvested at 3 (black bars) and 7 (white bars) days post infection. DNA was isolated from uterine homogenates to assess <i>C. trachomatis</i> levels by qPCR. Data show mean ± SEM of at least three mice per group from one of two independent experiments (unpaired t-test).
DISCUSSION

The lack of a statistically significant difference observed here could have several biological implications. Foremost, the simplest explanation is that the specific GBPs tested here do not play an important role in providing immunity to *C. trachomatis* in the genital tract. Given the redundancies in antimicrobial cell-autonomous genes, other proteins with greater ability to restrict *C. trachomatis* may mask the phenotype of GBP$^{\text{chr3}}$-deficient mice. These proteins could be GBPs on chromosome 5, IRGs, or other IFN-γ-inducible genes with antimicrobial activity. If this is true then one could conclude that GBPs on chromosome 3 play a minor to insignificant role in *C. trachomatis* restriction. Generating a mouse that is deficient in all GBPs, those in chromosomes 3 and 5, would truly test the contribution of this family of GTPases in the context of genital *C. trachomatis* infection.

Future experiments in this area of investigation should examine other time points more carefully to better assess the contributions that murine GBPs have *in vivo*. Given that GBPs are cell-autonomous genes, an earlier time point such as day 1 or 2 post infection would better assess any differences in the control of *C. trachomatis* growth that is independent of adaptive immunity. Additionally, GBPs have been implicated in the activation of inflammatory pathways involving caspases. As inflammation often times leads to immunopathology, investigating the role of GBPs in the development of
pathology rather than just *C. trachomatis* restriction would also be interesting. These experiments would better assess the role of GBPs during genital *C. trachomatis* infection.

Pursuing these questions using human cell lines may prove to be a better approach if no interesting phenotype is observed in mice. With the advent of CRISPR-cas genome-editing technology, it would be worthwhile to eliminate all GBPs in human cells to understand their role during infection. Given these new tools, it is truly an exciting time to test the contribution of individual or groups of genes in providing host resistance to pathogens.
MATERIALS AND METHODS

Mouse strains and colony maintenance
Wildtype (C57BL/6J) mice were ordered from The Jackson Laboratory. GBP Chr3-deficient mice were generated and characterized in a previous study (Yamamoto et al., 2012). Mice were housed in facilities managed by the Harvard Medical School Center for Animal Resources and Comparative Medicine. Harvard’s Institutional Animal Care and Use Committee approved all experiments in this study.

C. trachomatis strain
C. trachomatis serovar L2 (434/Bu) was propagated in monolayers of McCoy cells in media composed of DMEM, 10% FBS, 1.5 g/l sodium bicarbonate, 0.1 M nonessential amino acids (Hyclone), and 1 mM sodium pyruvate (Hyclone). Infected cells were detached with glass beads and sonicated to disrupt C. trachomatis inclusions. C. trachomatis EBs were isolate by density gradient centrifugation. EB isolates were titered and stored at -80° C in SPG media.

In vivo infections and tissue processing
To synchronize the estrous cycle, mice were injected subcutaneously with 2.5 mg medroxyprogesterone (Pfizer) 7 days before infection. Mice transcervically infected with 5x10^6 C. trachomatis IFUs in 10 µl SPG media. An NSET device
(ParaTechs) was used to bypass the cervix to directly treat and infect uterine horns.

**DNA extraction and qPCR**

Uteri were homogenized in DPBS and DNA extracted with a DNeasy blood and tissue kit (Qiagen). *C. trachomatis* levels were quantified by measuring *C. trachomatis* 16S DNA and host GAPDH DNA using bacterial and mouse-specific primer pairs and dual label probes. A standard curve was used to determine the ratio of *C. trachomatis* to mouse DNA. Threshold values were obtained with an ABI Prism 7000 sequence system (Applied Biosystem).

**Statistical analysis**

All statistical analyses were performed using Prism version 5.0d (GraphPad Software) where a p value less than 0.05 was deemed to be a statistically significant difference.
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