Understanding Acquired Immunity to *Chlamydia* in the Female Reproductive Tract

By

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Table of Contents

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List of Figures

Abstract

Sexually transmitted infections (STIs) affect over 68 million people in the U.S. annually and are highly prevalent among young people. Antibiotic and antiviral drugs have widely been used to treat STIs in patients and this treatment strategy has led to the development of incurable multidrug resistant pathogens. Thus, there is an urgent need for the development of effective vaccines to control the transmission of STIs in the population.

While vaccines have been developed to protect the public against some STIs such as Human Papilloma Virus, many other sexually transmitted pathogens such as *Gonorrhea*, Herpes Simplex Virus (HSV) and the intracellular bacterium *Chlamydia Trachomatis*, are uncontrolled in the population. Women are particularly vulnerable to such infections as these pathogens often wreak havoc on the female reproductive system. Indeed, *Chlamydia trachomatis* can cause pelvic inflammatory disease (PID), ectopic pregnancy, and even infertility in some women. Despite the striking threat of STIs to women's health, our understanding of how immunity develops in the female reproductive tract (FRT) is incomplete.

Much of our knowledge of immunity in the FRT derives from studies using mouse models of genital HSV and LCMV infections. These studies have highlighted an important role for memory T cells, especially those resident in the tissue (T_{RM}) as well as local antibody secretion in the FRT for clearing viral infections. In addition, clusters of memory T cells and antigen presenting cells (APCs) termed memory lymphocyte clusters (MLCs) were shown to be vital for mounting secondary immune responses and clearance of HSV

infection. Although there have been quite a few studies of viral FRT infections, very little is known about how immunity develops in the FRT after bacterial infections.

Chlamydia muridarum (*Cm*) infects the upper female reproductive tract and is a great model to study intracellular bacterial infection and development of FRT pathology in mice. This pathogen replicates inside host epithelial cells and requires CD4 T cells for clearance of infection, complementing studies of human *Chlamydia trachomatis* infections in which resistance to infection correlates with interferon gamma production. Using *Cm* infection in mice, we sought to determine the immune requirements for secondary protection in the FRT.

We first hypothesized that antigen-independent immunity could lead to local protection against FRT *Chlamydia* infection. To test this theory, we developed a pet shop mouse co-housing model at UC Davis based on previous work that established this method as a useful way to generate non-specific immunity against certain pathogens. This model consisted of co-housing inbred laboratory mice with “dirty” pet shop mice, which transmitted endemic pathogens to the lab mice. The goal of this model was to better recapitulate an experienced human immune system in laboratory mice. After the co-housing period, the laboratory mice were challenged with *Cm* and the severity of infection was measured. Using the pet shop co-housing model, we did not detect any advantage of non-specific immunity on resistance to *Chlamydia* infection. Therefore, to achieve effective immunity against genital *Chlamydia* infection, antigen-specific immunity was likely required.

The development of T_{RM} and MLCs in the female reproductive tract has been observed after genital *Cm* infection much like genital HSV infection. Hence, we

hypothesized that these immune factors were required for antigen-specific immunity to *Chlamydia* infection in the FRT. We tested the requirement of resident immunity for secondary protection using parabiosis surgery and found that resident immune memory was completely dispensable for local FRT protection against *Chlamydia*. Furthermore, resident immunity was irrelevant to secondary protection whether this immunity was generated through local intravaginal immunization or distal intranasal immunization. Experiments examining protection after intranasal immunization demonstrated that circulating immunity was completely protective against bacterial burden and the development of pathology after intravaginal rechallenge infection. CD4 T cells were required to control vaginal infection in intranasal immune mice, demonstrating that these lymphocytes were likely the main mediators of circulating immunity. The findings in this study have greatly clarified the required immune components for secondary immunity to *Chlamydia* infection of the female genital tract.

In summary, this work advances our understanding of how immunity to a sexually transmitted bacterial pathogen develops in the female reproductive tract, a previously unaddressed gap in knowledge in the field.

Chapter 1

Introduction: Overview of Chlamydia Immunology

Chlamydia Background

Chlamydia are obligate intracellular, aerobic Gram-negative bacteria that primarily infect host epithelial cells. The genus *Chlamydia* consists of 13 species (1), one of which, *Chlamydia trachomatis* (*Ct*), is a major threat to human health, and another of which, *Chlamydia muridarum* (*Cm*) is a mouse-adapted species used to model *Chlamydia* infection in mice. Serovars A-C of *Ct* cause ocular trachoma, which leads to significant scarring of the eye tissue and eventual blindness (2). Serovars D-K of *Ct* cause genital tract infections that can result in cervicitis, salpingitis, ectopic pregnancy, pelvic inflammatory disease (PID) and infertility in women, and urethritis, proctitis, and epididymitis in men (2). *Chlamydia* contain a plasmid that is non-conjugative and nonintegrative, and much of the pathogenicity of *Chlamydia* spp. is attributable to the plasmid (3). *Ct* plasmids have high sequence identity (3), indicating little genetic variation, which is likely the reason that antibiotic resistance is not observed in clinical *Ct* isolates (4).

Chlamydia have a biphasic life cycle that consists of an infectious elementary body (EB) akin to a spore, and an intracellular replicating reticulate body (RB) (5). EBs initially enter the host cell and differentiate into RBs which replicate inside a host cell vacuole termed an inclusion (5). The cycle concludes when RBs differentiate back into EBs which

exit the host cell through lysis or extrusion (5). *Chlamydia*'s intracellular lifecycle presents many challenges to studying this pathogen. The genome of *Chlamydia* is largely intractable due to the difficulty of delivering plasmids through physical barriers. The EB has a rigid cross-linked cell wall, low metabolic activity, and highly condensed genetic material, whereas the RB, while metabolically active, is shielded by 4 different membranes (5). Furthermore, gene expression in *Chlamydia* varies over time, so if a gene is targeted at one phase of the replication cycle, the desired phenotype may not be apparent at other points in the cycle (5). Surprisingly, despite the intractability of the genome, *Chlamydia* do seem to have a natural ability to exchange genetic information (5). Scientists have recently managed to make alterations to *Chlamydia* bacteria using shuttle vectors (5-8) which will be extremely useful for future studies of *Chlamydia* virulence factors and identifying numerous unknown proteins (5).

Clinical *Chlamydia trachomatis* infections

There were over 1.7 million reported cases of genital *Chlamydia trachomatis* infections in the United States in 2018 , most commonly reported in women and in young people ages 16-24. Genital *Ct* infections are asymptomatic in 70-90% of women, making them difficult to diagnose. Physicians have therefore employed a "screen and treat" approach to broadly screen the most at-risk populations and rapidly treat them with antibiotics. Typically azithromycin is used to treat *Ct* infections and works by blocking chlamydial protein synthesis within infected cells, thereby limiting its growth (9). The urgency to treat *Ct* infections is due to the ability of these infections to ascend to the upper female

reproductive tract and cause PID which can lead to ectopic pregnancy and infertility. Although this approach can lead to a lower incidence of PID in patients (10), it is hypothesized that frequent use of antibiotics has led to the observed increase in *Ct* infection rates. This is presumably because antibiotics can "arrest" the development of immunity in some patients. Therefore, clinicians and scientists greatly support the generation of a *Chlamydia* vaccine to protect the population from this devastating pathogen.

FRT Pathology after genital *Chlamydia* infection

An effective *Chlamydia* vaccine should not only control bacterial loads in the reproductive tract, but also should limit the development of pathology. As mentioned previously, women can develop severe reproductive sequalae including PID, salpingitis, and tubal factor infertility. However, it is still not clear how quickly *Ct* infections progress to PID. Studies with 2-week follow-up visits after initial testing have reported PID rates as high as 3% among women while studies with later follow-ups have projected lower estimates (11). Answering this question would be useful for our understanding of how efficient of an immune response is necessary to prevent these negative sequalae.

Little is known about the mechanisms driving reproductive tract pathology in women, however, organ culture and mouse models have been informative in elucidating possible mechanisms of pathology. Epithelial cells, the targets of *Chlamydia* infection, can themselves be a source of inflammatory mediators of pathology. Infected epithelial cells produce IL-1α upon infection *in vitro* (12), and fallopian tube cultures in which IL-1

and IL-8 signaling was blocked were protected from *Chlamydia*-induced pathology (13). Furthermore, women infected with *Ct* had significantly elevated levels of IL-8 in their cervico-vaginal lavage fluid compared to uninfected controls (14). Interestingly, TLR2 and live replicating bacteria are required for IL-8 signaling in human cell lines *in vitro* (15).

Neutrophils may also play a large role in pathology as they are found in high abundance in women with Ct-related PID (16). They are likely recruited to the female reproductive tract by epithelial cell production of IL-8, a natural neutrophil chemoattractant (17), and this is supported by mouse studies demonstrating high levels of KC (IL-8 mouse equivalent) and MIP-2α in genital tract tissues of infected mice that accompanies influx of neutrophils into the tissue (18). Neutrophil influx into the FRT results in a pathological condition called pyosalpinx, which is characterized by large amounts of pus in the oviduct (19). This accumulation of polymorphonuclear cells in the oviducts seems to begin after the first week of infection in mice, and then peaks after the second week of infection, correlating with the induction of pathology (18). Following pyosalpinx and the receding of neutrophils, is the induction of hydrosalpinx, or dilation and filling of the oviduct with serous fluid accompanying a dramatic increase in leukocytes including neutrophils, lymphocytes, and plasma cells (19, 20). FRT pathology in mice is also associated with neutrophil and myeloid cell products such as phagocytic oxidase and matrix metalloproteinases (21, 22) indicating that secretion/production of these factors may be required for tissue damage to occur.

Adaptive immune responses may also contribute to protection against or development of pathology in women. For instance, women who displayed low IFN-γ and high IL-10 responses after PBMC stimulation with Chsp60 had an increased risk of

reinfection and development of PID (23). Additionally, among HIV-infected women, low CD4 T cells counts were a risk factor for the development Ct-induced PID (24), indicating that CD4 Th1 cell responses may be protective against reproductive tract pathology in women. T cells skewed away from Th1 type cells may be detrimental to the preservation of reproductive health in Ct-infected women (25). These human studies, along with *in vivo* studies of TLR2-deficient mice demonstrating these mice are protected against pathology (26) and re-infection with *Chlamydia* (27), indicate that pathological and bactericidal immune responses may not be inextricably linked. This dichotomy between pathology and protection is optimistic for the design of an effect vaccine that can control *Chlamydia* infections without damaging the reproductive tract tissue.

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Primary and secondary immune response to *Chlamydia* infection

Innate Immune Response-Cell Intrinsic Immunity

To initiate an immune response to a pathogen, the host must first recognize the pathogen's molecular patterns (PAMPs) through Patter Recognitions Receptors (PRRs). The mechanism of this recognition of *Chlamydia* PAMPs is still being uncovered, however, it is clear that certain Toll-like receptors (TLRs) are required to initiate an innate immune response to *Chlamydia*. TLR2 is required for the secretion of TNF-α, IL-6, and CXCL-2 in the genital tract after *Cm* infection (26). While few TLR2 ligands are currently known, scientists have speculated that glycogen produced by *Chlamydia* may be the ligand for TLR2, as enzymatically synthesized glycogen activates TLR2 signaling (28, 29). TLR3 is required for most of the production of IFN-β in response to *Chlamydia*

infection (30), and the known ligands for this receptor are dsRNA (31) and dsDNA (32). Therefore, there are multiple TLRs that are capable of sensing *Chlamydia* infection of the FRT mucosa and initiating an immune response.

IFN-β secretion can also be triggered by sensing of *Chlamydia* through stimulator of interferon genes (STING) (33) by direct recognition of bacteria-derived cyclic dinucleotides or indirect recognition of dsDNA (34)(35). Chlamydial ligands such as PG may activate other receptors such as nucleotide-binding oligomerization domain-containing protein (NOD)-1 and -2 to initiate an inflammatory response (36) (37). Alternatively, NOD1/2 signaling may be triggered by *Chlamydia* infection-induced ER stress as shown in human cell lines and mice (38)(39). *Chlamydia* can also be sensed through cytosolic pathways including canonical inflammasome activation that results in IL-1β and IL-18 secretion, non-canonical caspase-11-mediated inflammasome activation, and an absentin melanoma (AIM)-2-dependent dsDNA-sensing pathway (40-43).

Intracellular nutritional immunity is also extremely effective at limiting *Chlamydia* replication, as *Chlamydia* are highly dependent on nutrients from host cells (28). Human cells can up-regulate the enzyme indoleamine-2,3-dioxygenase (IDO) in the presence of IFN-γ which depletes tryptophan within the cell, thereby starving *Chlamydia* (44-46). *Ct* then transforms into a persistent form by upregulating its *trp* operon which allows it to convert indole produced by the vaginal microbiota into tryptophan for food (47, 48). In this state *Ct* can survive in the presence of IFN-γ for a while, but is unable to replicate (44). This dynamic between *Ct* and human epithelial cells does not occur during *Cm* infection of murine cells. Murine genital epithelial cells do not up-regulate IDO and therefore *Cm* has not adapted to synthesize its own tryptophan (49-51). Instead, mouse cells use

immunity-related GTPases (IRGs) to eradicate *Cm* (51, 52). These IRGs guide the process of inclusion rupture via ubiquitin pathways (53)(28).

Like myeloid cells, epithelial cells are capable of producing nitric oxide (NO) in the presence of intracellular pathogens. Murine epithelial cells primed with IFN-γ are capable of limiting *Cm* growth in a NO-dependent manner (54). Human cells however, only produce a limited amount of NO during *Ct* infection, possibly due to an ability of *Ct* to block NOS2 expression through polyamine synthesis (50, 55, 56). Therefore NO production may not be a prominent mechanism of *Ct* killing during human infection.

Innate Immune Response - Cellular Immunity

Neutrophils, monocytes and natural killer (NK) cells are the first cells recruited to the reproductive tract after genital *Chlamydia* infection (57)(58). CD11b+ cells and Ly6G+ cells (markers of monocytes and polymorphonuclear cells, respectively) are detected in the vaginal tissues of infected mice as early as 3-days after infection (59). This timing of recruitment to the FRT correlates well with a loss of protection observed early during the course of infection in mice depleted of neutrophils, demonstrating a requirement for neutrophils in early control of *Chlamydia* infection (60). Neutrophils are also found in large quantity in human reproductive tract tissue of *Ct* positive women (16) and human neutrophils can effectively neutralize *Ct* with or without the presence of antibody or complement (61)(62). This microbicidal effect of neutrophils was dependent on peroxidase (62).

NK cells have been detected in the FRT of infected mice as early as 24 hrs. post infection (63). NK cells are an early source of IFN-γ in the reproductive tract and contribute

to the skewing of T helper cell responses towards the Th1 subset, seemingly through modulation of dendritic cell (DC) function (63, 64). Human epithelial cell and DC cytokines (IL-18 and IL-12, respectively) are responsible for NK cell production of IFN-γ, indicating there may be a feedback loop of directing cell function between DCs and NK cells (65).

Macrophages (m$\emptyset$) likely first encounter *Chlamydia* in the reproductive tract tissue, where tissue-resident macrophages are situated (56, 66). M$\emptyset s$ may take up *Chlamydia* through mannose receptors and complement receptor 3 through recognition of mannose-glycosylated major outer membrane protein (MOMP) and iC3b opsonization, respectively (67-70). The time-frame in which *Chlamydia* can exist within macrophages/monocytes is controversial, but it is clear that *Chlamydia* cannot replicate normally within these cells the way they do in epithelial cells (66). *Chlamydia* enter a state of persistence in monocytes within 24 hrs. and are incapable of forming a mature inclusion within these cells (70). However, they are able to retain their infection and growth capability (71, 72). M1 skewed m$\emptyset s$ are less permissive to *Chlamydia* bacteria than M2 macrophages, but induce the persistent state of *Chlamydia* (73). It is likely that this intracellular persistence in mØs is how *Chlamydia* are capable of infecting distal tissues such as the spleen and the gut (74)

There are multiple intracellular mechanisms used by mononuclear phagocytes to prevent chlamydial inclusion formation. Lysosomal degradation of intracellular bacteria is an important pathway for eradicating *Chlamydia* from macrophages. *Chlamydia* are incapable of preventing phagosome-lysosome fusion in these cells and the bacteria are rapidly acidified as a result (75). In contrast, Rab GTPases in epithelial cells direct *Chlamydia*-containing vesicles to non-degradative vesicular compartments (75)(66).

Chlamydia can also induce autophagy in møs, but not in epithelial cells (75). The formation of an autophagolysosome in møs leads to *Chlamydia* degradation, and autophagy pathways can be enhanced in the presence of IFN-γ (76)(77). Perforin-2 is a protein with antimicrobial capability that is constitutively expressed in macrophages and is up-regulated further during *Chlamydia* infection (78). This protein is required to prevent *Chlamydia* inclusion maturation in macrophages and its proposed bactericidal function is to sensitize bacteria to lysosome-mediated lysis through puncturing the outer cell wall (78, 79). Finally, møs can kill *Chlamydia* through the production of reactive oxygen (ROS) and reactive nitrogen species (RNS). Inducible nitric oxide synthase is produced during *Chlamydia* infection as a result of down-stream signaling through TLR2 and results in ROS acidification of the bacteria (56)(80). In møs deficient in the ROS-generating enzyme NADPH-oxidase, *Chlamydia* are able to form inclusions in which they can replicate and survive (81).

Although a requirement for møs in T cell priming during *Chlamydia* infection has not been demonstrated, there is evidence that møs may be capable of priming antigen-specific T cells (66). Upon *Chlamydia* infection, monocytes up-regulate costimulatory receptors such as CD40, CD80, and CD86 (56)(82). They also may support Th1 differentiation through the production of IL-12 and IFN-γ (56)(83).

Dendritic cells are antigen presenting cells that have been shown to stimulate *Chlamydia*-specific T cell responses (84-86). *In vitro*, dendritic cells take up live *Chlamydia* and upregulate the costimulatory molecules CD80, CD86, and CD40, as well as MHC class II expression (87). *Chlamydia muridarum*-infected DCs also secrete IL-12, TNF-α, and stimulate IFN-γ production by Chlamydia-specific T cells (87). DCs pulsed

with UV-inactivated elementary bodies (UV-EBs) do not up-regulate costimulatory markers to the same degree and are less potent at secreting Th1-forming cytokines (87). DCs pulsed with heat-killed EBs (HKEBs) stimulate a weaker IFN-γ response in T cells and a greater IL-10 response (88). Interestingly, DCs pulsed with either live or dead *Chlamydia* are protective against intravaginal *Chlamydia* challenge, but HKEBs or UV-EBs injected directly into mice are not protective (87-89). This discrepancy may be due to the inability of DCs to be primed as strongly *in vivo* or tolerogenic anti-inflammatory subsets may be the primary DC subsets taking up *Chlamydia in vivo* as described previously during *Ct* vaccination in mice (90). In summary, dendritic cells are likely critical for initiating adaptive immune responses during *Chlamydia* infection *in vivo*.

Adaptive Immune Response

The adaptive immune response can be mediated by a multitude of cells including CD4 T cells, CD8 T cells, and B cells, as well as antibody. During a primary *Chlamydia* infection, MHC class-II$^{-/-}$ and nude mice are both incapable of resolving infection of the reproductive tract, indicating a likely role for CD4 T cells in bacterial clearance (91). MHC class-I on the other hand does not appear to be required for bacterial clearance, implying a minimal role for CD8 T cell responses (91). The importance of CD4 T cells and the dispensability of CD8 T cells for primary clearance is supported by experiments using antibody depletion of the lymphocyte populations (92).

No requirement for B cells has been detected for clearing *Chlamydia* from the FRT, however, μMT mice lacking B cells and antibody have worsened disseminating infection in the peritoneal cavity and spleen (93). We recently demonstrated that B cell antibody

secretion, but not MHC class-II presentation, was necessary to control disseminating infection in mice (94). T-independent IFN-γ production is also required to control disseminating infection, possibly to enhance bacterial killing in innate cells or skew the antibody response to the appropriate isotype (95). Very little is known about the germinal center response and the process of antibody formation during *Chlamydia* infection and the field would benefit from further research into this area.

The CD4 T helper cell response to *Chlamydia* is diverse with Th1, Th17, Tregs all previously detected during infection (96). An in-depth summary of all the studies assessing the requirements of T helper cell responses can be found in chapter two of this dissertation (97). However, the status of this area of research is that we still do not know which T helper subsets are required for clearing *Chlamydia* from the FRT. Ongoing research in our lab will resolve this issue. Previous studies suggest that CD4 T cells may not only use cytokines to clear bacteria from the FRT, they may also use cytotoxic molecules to kill infected cells. Indeed, “killer-like” CD4 T cell clones have been detected in FRT of infected mice and are a very attractive explanation for how CD4 T cells may clear an obligate intracellular infection from host epithelial cells (98, 99).

After a primary *C. muridarum* infection is resolved, mice develop robust immunity to secondary *Chlamydia* infection. It has been previously demonstrated that antibody and CD4 T cells both play a role in this secondary immunity (100, 101). The Fc portion of the IgG2c antibody isotype appears to be critical for antibody-mediated control of *C muridarum*, however, the exact mechanism of antibody-mediated protection has not been described (102). Functions of CD4 T cells needed for secondary *Chlamydia* clearance are also unknown.

Tissue resident T cells (T_{RM}) are non-recirculating and stably reside in tissues for long periods of time (103). Upon re-activation, they can recruit effector cells into tissues to mediate pathogen clearance (104, 105). T_{RM} may also exist in memory lymphocyte clusters (MLCs) or anatomical clusters of memory lymphocytes and antigen presenting cells that are distinct from tertiary lymphoid organs (106). MLCs lack organized germinal centers and HEVs but are able to initiate secondary immune responses locally (106). The studies contained in this dissertation aimed to determine the necessity of T_{RM} and/or MLCs to secondary Chlamydial immunity as well as the sufficiency of circulating immune CD4 T cells and antibody.

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Chapter 2

Diversity in the T cell response to Chlamydia-sum are better than one

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Abstract

Chlamydia trachomatis is responsible for an increasing number of sexually transmitted infections in the United States and is a common cause of serious pathology in the female reproductive tract (FRT). Given the impact and incidence of these infections, the production of an effective *Chlamydia* vaccine is a public health priority. Mouse models of *Chlamydia* infection have been utilized to develop a detailed and mechanistic understanding of protective immunity in the FRT. These studies reveal that MHC class-II restricted *Chlamydia*-specific CD4 T cells are critical for primary bacterial clearance and provide effective protection against secondary infection in the FRT. Despite the clear importance of IFN-γ produced by CD4 Th1 cells, there are also suggestions of wider functional heterogeneity in the CD4 T cell response to *Chlamydia* infection. Understanding the role of this diversity in the CD4 T helper cell response in the FRT should allow a more nuanced view of CD4 T cell biology in the context of *Chlamydia* infection and may be critical for vaccine development. Here, we summarize our current understanding of CD4 T helper subsets in the clearance of *Chlamydia* and discuss some areas where knowledge needs to be further extended by additional experimentation.

Introduction

The *Chlamydiaceae* family consists of 11 different species of *Chlamydia*. *Chlamydia trachomatis* (*Ct*) and *muridarum* (*Cm*) infect human and mouse reproductive tracts, respectively, and will be highlighted in this review (1). *Chlamydiae* are gram-negative, obligate intracellular bacteria (1). Their typical life cycle is bi-phasic, consisting of elementary (EBs) and reticulate bodies (RBs). The spore-like elementary bodies are built to withstand the noxious extracellular environment, while reticulate bodies acquire nutrients and replicate inside a host cell vacuole known as an inclusion (2). After replication, bacteria are released from the host cell by one of two mechanisms: lysis or extrusion. During lysis, permeabilization of the inclusion, and nuclear and plasma membranes all lead to rupture of the host cell and release of EBs (3). Extrusion occurs when EBs exit the cell by budding off from the plasma membrane, leaving the host cell uncompromised (3). After exiting the initial target cell, *Chlamydia* initiate the replication cycle again in a neighboring host cell.

The incidence of *Chlamydia* infection is over 100 million worldwide cases (4), and a study of women in the UK estimates that 5% of 16–24-year-old women are infected (5). Furthermore, *Ct* infections are responsible for 35% of incidents of pelvic inflammatory disease (PID) in 16–24 year old patients and 29% of tubal factor infertility cases (TFI), making this pathogen a substantial threat to the reproductive health of young women (5, 6). Due to the asymptomatic nature of this infection, patients run the risk of developing severe complications prior to seeking medical attention. Efforts to regularly screen patients and treat them with antibiotics have been implemented to address this problem

(7). While employment of this strategy has coincided with reduced incidence of PID, the incidence of *Ct* infections is still rising (7, 8). Indeed, antibiotic use may be limiting acquired immunity to infection and thus contributing to the rising incidence of infection (9, 10). Therefore, an effective vaccine would be the preferred method of diminishing the frequency of *Ct* infections and associated pathology in the population. Clinical reports of *Ct* infections suggest that primary infection can be resolved naturally in some women, as evidenced by swab collections at clinical follow-up visits that are Ct negative (11, 12). Mouse studies support a model in which adaptive immunity, particularly CD4 T cells, are required to clear primary *Chlamydia* infection from the female reproductive tract (FRT). These data suggest that a vaccine targeting adaptive CD4 T cells will be most promising in protecting patients from *Ct* infection.

Protective immunity in clinical infection

Precisely defining the factors contributing to *Chlamydia* immunity in humans is a daunting task for researchers evaluating clinical studies. Indeed, many studies investigating the duration of the infection and the host factors that influence the resolution of infection are confounding (13). However, these studies point to some important characteristics about natural human *Chlamydia* infection, including the simple fact that some women can naturally resolve the infection. A 5-year study of a cohort of Colombian women showed that approximately 50% of women cleared *Ct* without any reported treatment after 1 year, and 94% were able to clear infection after 4 years (14). These clinical observations indicate that many women naturally generate adequate protective responses to

Chlamydia, although the long delay in this immune development may predispose some of these individuals to severe complications associated with prolonged infection (15). Exactly how a subset of women is able to spontaneously clear or resist primary *Chlamydia* infection is poorly understood.

There are several genetic and environmental factors linked to resistance or susceptibility to *C. trachomatis* (*Ct*) infection in women. The HLA class II variant DQB1*06 is reported to be associated with *Chlamydia* infection in North American adolescents (16), pointing to a major role for CD4 T cells in *Chlamydia* immunity. Interestingly, HIV-infected women that lack healthy CD4 T cells have an increased risk of developing Chlamydial PID (17), suggesting that CD4 T cells are required for clearing infection and/or regulating pathology. Peripheral blood mononuclear cell (PBMC) secretion of IFN-γ or IL-13, cytokines that are produced by T helper cells, has been associated with resistance to Ct infection in a cohort of female sex workers in Kenya (18). Detection of these cytokines may indicate that CD4 T cell differentiation is heterogeneous in the FRT. Women lacking an IL-10 variant produced higher levels of this cytokine after infection, and this correlated with increased susceptibility to re-infection (19). Thus, the development of an appropriate effector CD4 T cell response appears to be required for resolution of *Ct* infection and counter regulation of these responses may allow reinfection.

CD4 T cell subsets in *Chlamydia* immunity

Despite a clear requirement for CD4 T cells in protective immunity to *Chlamydia*, the precise role of CD4 T helper cell subsets remains incompletely defined. Our laboratory

previously developed multiple MHC class-II tetramers that allow *ex vivo* detection of expanded *Chlamydia*-specific CD4 T cells during infection (33). This technical approach allows identification of CD4 T cell responses without predicting effector capacity. Using tetramers, we detected expansion of Th1, Th17 and Tregs, but virtually no $GATA3^{+}$ Th2 cells, in the draining lymph nodes and reproductive tract of mice infected with intravaginal Cm (33). This finding is somewhat at odds with reports in mice and humans regarding the presence of IL-13 or IL-13-producing CD4 T cells (17, 35). There are multiple possible explanations for this discrepancy. Perhaps the IL-13-producing Th cells identified in mice are producing this cytokine in a GATA3-independent manner (36). Indeed, other transcription factors such as E4BP4 have been shown to regulate IL-13 production in T helper cells (37). Another possibility for the presence of IL-13-producing CD4 T cells in the mouse study by Johnson et al. is the technique used to identify these cells (35). The authors cultured these cells *in vitro* for many passages in order to create cell lines (35). It is well established that cell lines do not often reflect the *in vivo* phenotype of the cells originally responding to the infection (38). In the next sections, we will present our view of what is known regarding the heterogeneity of T helper cell subsets responding to *Chlamydia* infection.

Th1 cells

T helper type 1 cells characteristically facilitate the clearance of intracellular bacterial pathogens (39), and are essential for the resolution of Salmonella, Mycobacteria, and Leishmania infections in mice (40). Th1 cells secrete the key cytokine IFN-γ, which allows

infected macrophages to eliminate intra-phagosomal bacteria by inducing production of toxic radicals, including nitric oxide, which can directly destroy the pathogen (41).

IFN-γ is also important for the resolution of *Chlamydia* infection. IFN-γ-mediated destruction of *Chlamydia* can occur in multiple cells *in vitro* including macrophages and epithelial cells (42). Additionally, IFN-γ up-regulates IDO in host cells, resulting in tryptophan degradation, which is known to starve *Chlamydia* and severely inhibit its growth (43). Consistent with this *in vitro* activity, Th1 responses can facilitate protection against *Chlamydia* infection *in vivo* (27, 44, 45). Th1 cells are initially polarized by IL-12, secreted by dendritic cells, while IFN-γ production by natural killer, CD8, or Th1 cells maintains this type I polarization. Mice lacking IL-12 display prolonged periods of vaginal *Chlamydia* shedding, but can eventually resolve infection (22). In marked contrast, mice lacking IFN-γ display lower bacterial burdens in the FRT but can develop systemic disease (27, 45). These observations indicate distinct roles for Th1 cells in the local reproductive tract versus systemic organs. The phenotype of IL-12-deficient mice resembles CD4-depleted mice in that they shed high numbers of *Cm* bacteria at the FRT mucosa (27, 45), suggesting they are deficient in Th1 responses at the tissue site. In contrast IFN-γ-deficient mice shed low levels of bacteria from the FRT, while B cell-deficient mice clear Cm from the FRT completely (22, 33). Both mice, however, develop severe concurrent systemic infection, indicating that IFN-γ and humoral responses may be necessary for containing disseminating infection (33, 46, 47). CD4 T cells are likely the main source of IFN-γ in these scenarios since NK or CD8 T cell depletion during primary or secondary Cm infection causes only mildly increased bacterial burdens (24, 29, 48). This might suggest that CD4 T cell production of IFN-γ to direct antibody class-

switching is critical to preventing disseminated primary infection, and this notion is supported by a recent study (47). The source of this protective IFN-γ is likely to be Tfh cells in the draining lymph node, a CD4 subset that has not been carefully studied in the context of *Chlamydia* infection. Indeed, in a study utilizing a *Salmonella* vaccine, repeated antigen exposure generated robust germinal center formation that was dependent on IFN-γ, the primary source of which was Tfh cells (49). The antibody response generated from these germinal centers enhanced pathogen clearance. It is possible that the humoral immune response to *Chlamydia* functions through a similar mechanism, where IFN-γ-producing T follicular helper cells direct the generation of protective antibody.

The development of *Chlamydia*-specific tools to track T cell responses has allowed more precise measurement of T helper cell subset development in response to *Chlamydia*l antigens. Two independent TCR transgenic lines, one responding to *C. trachomatis* (NR1) (50) and the other specific for *C. trachomatis* and *C. muridarum* (21) have been generated. When used as part of an adoptive transfer system, both of these TCR transgenic populations develop into Th1 effector cells in response to challenge with their respective Chlamydial species. The latter TCR transgenic line was protective against *C. muridarum* and exhibited polyfunctionality, secreting both TNF-α and IL-2 in conjunction with IFN-γ, which lends support to previous evidence documenting polyfunctional CD4 T cell responses to *Chlamydia* (18, 33, 51). An extensive and elegant vaccine study using the NR1 mouse showed that NR1 cells differentiate into Th1 cells upon challenge with UV-*Chlamydia trachomatis* (UV-Ct) conjugated to an adjuvant linked to a charge-switching particle (UV-Ct-cSAP). These *Chlamydia*-specific Th1 cells were protective against *Ct* challenge in immunized mice. Interestingly, these protective Th1

cells were primed mainly by $CD103^+$ DCs in the uterus or iliac lymph node as opposed to non-protective UV-Ct specific T cell responses, which were primed by tolerogenic $CD103^+$ DCs. Th1 cells in UV-Ct-cSAP immunized mice reduced *Chlamydia* bacterial burdens in the reproductive tract by 10-fold compared to UV-Ct immunized mice. However, this protection was equivalent to that conferred by live *Ct* infection, characterized by an initial bacterial load of 100,000 IFU (52). This level of protection may be sub-optimal, as it allows a significant amount of bacteria to enter the tissue, greatly contrasting the robust protection observed after *C. muridarum* infection that consists of approximately 10 IFU of bacteria in the FRT upon secondary challenge (24).

Although it is clear that Th1 cells make a significant contribution to *Chlamydia* immunity, the phenotypes of mice lacking Th1 immunity are not synonymous with the those of mice possessing a deficiency in MHC class-II, which display high levels of bacterial shedding and non-resolving infections (23). It seems possible therefore that other cytokines produced by polyfunctional Th1 cells, such as TNF-α and IL-2 (51), serve to restrain bacterial infection in the absence of IFN-γ. Alternatively, there may well be contributions from additional CD4 T helper cell subsets within the female reproductive tract that control *Chlamydia* infection, but remain poorly characterized.

Th2 cells

The prominent functions of Th2 cells are in assisting mast cells during allergy and hypersensitivity reactions as well as in immunity to helminth infections (39). They can also coordinate tissue repair responses (53, 54). A study of human *Chlamydia* infection

revealed there are significantly more IL-4 producing antigen-specific CD4 T cells in PBMCs from *Chlamydia*-infected female patients than non-infected control subjects (55). Robust cellular production of this cytokine was detected at enrollment, and at 1- and 4-month follow-up visits. The author's suggest that this finding "implies that Type 2 immunity was evolutionarily selected to control genital *C. trachomatis* infection" (55). While Th2 cells may be detected during active *Chlamydia* infection, it is less clear that Th2 cells are actually protective. Because women were treated with antibiotics prior to follow-up visits, associations of Th2 responses with the ability to control infection, or reduced pathology could not be measured in the study (55).

The potential role of Th2 cells during *Chlamydia* infection has not been extensively characterized. As noted above, very few $GATA3^+$ CD4 T cells were detected during active infection in a study using MHC Class-II tetramers to identify endogenous polyclonal responses (33). Furthermore, genetic deletion of IL-4 or IL-4Rα did not affect *Chlamydia* bacterial burdens relative to wildtype mice (33, 56). Additionally, the protective capacity of an *in vitro* generated Th2 clone specific to *Chlamydia* muridarum has been examined. When transferred into nude mice, this Th2 clone provided no protection against *Cm* compared to mice lacking T cells. Using the same approach, transfer of Th1 clone provided robust protection against *Cm*, and infection was cleared after 30 days (57). Likewise, adoptive transfer of NR1 TCR transgenic T cells that had previously been skewed toward a Th2 phenotype resulted in higher bacterial burdens compared to mice receiving Th1 cells. Intriguingly, transferred Th2 cells responding to infection eventually skewed towards a Th1 phenotype (44). Collectively, these results suggest that few Th2 cells are generated in the mouse model of infection and that artificially generated Th2

cells lack the capacity to clear *Chlamydia* infection *in vivo*. Thus, if further data confirms that Th2 cells are involved in human *Chlamydia* immunity, alternative animal models may be required to study Th2-mediated protection.

Although few classical Th2 cells are elicited during murine infection, eosinophils are recruited to the FRT during *C. trachomatis* infection in mice (56). A reduction in eosinophil frequency in IL-4-deficient mice correlated with increased severity of upper reproductive tract pathology (56). Recent data suggest that multifunctional CD4 T cells producing both IFN-γ and IL-13 may be protective against reproductive tract pathology (35, 58). Since few Th2 cells are detected in the FRT during *C. muridarum* infection, it is possible that IL-13 production from this unusual population orchestrates the recruitment and/or retention of reparative eosinophils (59, 60). While these type 2 immune responses may play an important role in tissue protection and repair following damage of the FRT, it seems unlikely that these responses effectively reduce the bacterial burden of *Chlamydia* (56). Future clinical studies should clarify which cells produce IL-13 following stimulation of human PBMCs (18).

Th17 cells

Th17 cells typically orchestrate the clearance of extracellular bacteria through the recruitment of neutrophils (39). Additionally, they contribute to the maintenance of barrier function at mucosal surfaces and Th17 responses have been implicated in pathology associated with autoimmune conditions (61, 62). Recent studies suggest that Th17 cells play a role in the development of pathology during *Chlamydia* infection. For example,

antibody neutralization of IFN-γ causes CD4 T helper cells to divert towards a Th17 phenotype that corresponds to worsened reproductive tract pathology, and only slightly increased bacterial burden (63). The exacerbated pathology may be due to the greater influx of neutrophils and monocytes into the tissue mediated by enhanced Th17 cell production (63). BALB/c mice deficient in IL-17, a canonical Th17 cytokine, exhibit significantly reduced FRT pathology after *Chlamydia* infection when compared to wildtype controls (64). IL-17-deficient mice also displayed a corresponding reduction in neutrophil and monocyte infiltration, which could explain the differences in pathology. Interestingly, bacterial shedding was decreased in IL-17-deficient mice compared to WT controls (64). It is unclear whether CD4 T cells were directed toward a more robust Th1 phenotype in *Chlamydia*-infected IL17-deficient mice, but this could explain the observed reduction in bacterial loads. The effect of IL-17-deficiency in mice on a C57BL/6 background differed markedly from the findings in BALB/c mice. C57BL/6 IL-17-deficient mice displayed diminished Th1 responses and neutrophil recruitment. This effect of IL-17 deficiency on Th1 development is supported by studies using other bacterial infection models (62). Interestingly there were no significant changes in bacterial clearance or FRT pathology in C57BL/6 IL-17-deficient mice compared to WT mice (65). The opposing phenotypes observed in BALB/c and C57BL/6 backgrounds are likely due to strain-specific differences in CD4 T cell differentiation. However, a pathological role for Th17 cells has been observed in many other studies, supporting a similar function for these cells in *Chlamydia* infection (66-68).

Tregs

Induced regulatory T cells dampen pro-inflammatory T cell responses through direct cell-cell contact mechanisms as well as through the secretion of immune-suppressive cytokines (69). However, during *Chlamydia* infection Treg-induced immunosuppression has deleterious outcomes for the host in controlling infection. In a clinical trial of inactivated *Ct* immunization, a subset of vaccinated subjects experienced worse symptoms of ocular trachoma than control subjects (70). This deleterious clinical outcome was also observed in mice immunized with UV treated *Ct*, since they experienced higher bacterial burdens than unimmunized controls after infection (52). When this same UV-Ct was physically linked to an adjuvant, CD4 T cell responses were directed away from Treg development and toward a protective Th1 response. This lineage choice was influenced by the DC subset priming the naïve T cells during the primary response to vaccination. Some reports indicate that Treg formation is dependent on $CD11b^{-}$ plasmacytoid DCs while other studies pinpointed $CD103^{+}$ $CD11b^{-}$ cDCs in the priming of Treg responses (52, 71). In contrast, protective Th1 responses appear to be primed by $CD103^{-}$ $CD11b^{+}$ DCs (52). In addition to Treg differentiation driving the Chlamydial T cell responses away from a protective Th1 lineage, the induction of Tregs may assist the development of other pro-inflammatory responses that encourage pathology. For instance, co-culturing of Tregs with conventional T cells (Tconv) resulted in increased production of IL-17A in the supernatant (72). Additionally, Treg-depleted mice had diminished Th17 responses, which correlated with reduced oviduct pathology, but not inhibited bacterial clearance

(72). The available data therefore point to T regulatory cells having an overall harmful influence on the host during *Chlamydia* infection.

Conclusion

The *Chlamydia* community has repeatedly demonstrated in animal models the importance of Th1 immunity in fighting infection. Clearly, canonical Th1 cytokines IL-12 and IFN-γ are required for optimal anti-Chlamydial immune responses. However, an area that has remained relatively unexplored is the examination of transcription factors in the regulation of *Chlamydia*-specific CD4 T cell lineages. In preliminary studies, our laboratory has observed that T-bet, the transcription factor that classically defines Th1 cells, is not required for *Chlamydia* clearance from the reproductive tract. This finding actually fits well with previous observations showing a marked discrepancy between mice lacking MHC class-II and those lacking IFN-γ (23, 73). These observations highlight a need to understand cytokines relevant to protection against *Chlamydia* and how they are transcriptionally controlled, especially if through non-canonical pathways. Together, these studies suggest a more nuanced understanding of how CD4 T cells regulate bacterial growth in the reproductive tract.

Other investigators have suggested that tissue resident memory development may be a more comprehensive model to understand protection than Th1 development. However, while vaccine-induced tissue resident memory cells (T_{RM}) can protect against *Chlamydia*, the role of this population has not been clearly defined in *C. muridarum* infection. Another MHC class II-restricted immune response that is often overlooked is

the development of T follicular helper cell response to *Chlamydia*. The importance of B cells and antibody during *Chlamydia* infection suggests a requirement for this subset that will demand greater investigation. While Th2, Th17, and Treg responses appear to play roles in regulating pathology, there is less evidence that they contribute to bacterial clearance. Thus, there remains the potential that a non-classical CD4 effector response plays a major role in bacterial clearance from the FRT during *Chlamydia* infection. Future studies are certainly required to examine this possibility more carefully. In summary, a heterogeneous CD4 T cell response is induced during *Chlamydia* infection and multiple populations likely contribute to protection and pathology. The development of an effective *Chlamydia* vaccine will depend on the ability of immunologists to define these responses in more detail and devise immunization approaches that induce similar responses in naive individuals.

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Chapter 3

Chlamydial Immunity in a "Dirty" Mouse Co-housing Model

Jasmine C. Labuda and Stephen J. McSorley

Introduction

The immunological landscape of nonlymphoid tissues (NLT) from mice housed in specific pathogen free (SPF) conditions resembles that of a neonate human (1). These naïve tissues are devoid of most lymphocytes, including memory T cells (1). In contrast, NLT samples from pet shop mice, previously infected SPF mice, and adult humans, are replete with memory lymphocytes generated from immunological events (1-3). These resident lymphocyte populations are capable of providing protection against pathogens in a non-antigen-specific manner (1). Previously, a mouse model was generated to study this non-specific protection: genetically identical SPF mice were co-housed with pet shop mice to generate an "experienced" immune system in the laboratory mice (1, 4). Co-housing with these "dirty" mice increased non-specific lymphocytes in laboratory mice, resulting in enhanced control of a range of systemic pathogens (1, 4). However, this "dirty" mouse model has only been used in models of systemic infection rather than a typical local mucosal infection.

Chlamydia trachomatis (*Ct*) is an obligate intracellular Gram-negative bacterium that causes a local infection of the upper reproductive tract in humans and mice (5). The adult human female reproductive tract (FRT) is replete with lymphocytes and lymphoid structures that are likely to influence the outcome of genital tract infections such as *Ct* (1,

6, 7). In the current study, we sought to determine whether increasing previous immunological experience in the relatively lymphopenic SPF laboratory mice could create a mouse model more relevant to human *Chlamydia* infection. To answer this question, we used the mouse adapted species *Chlamydia muridarum* that is commonly used to model this genital tract infection in mice. Previous work has demonstrated that enrichment of non-specific resident memory T cells in the FRTs of CCR7-deficient SPF mice enhances resistance to *Chlamydia muridarum* infection (8). Therefore, we hypothesized that increasing non-specific lymphocytes in SPF mice, both in the blood and FRT, would influence the clearance of *Chlamydia muridarum*. In this study, we found that co-housing "dirty mice" with SPF laboratory mice increased the frequency of effector memory T cells (T_{EM}) in the blood of the lab mice, and was protective against systemic *Listeria* infection, as previously described (1). However, we did not identify any effect of non-specific immunological exposure on the course of genital *Chlamydia* infection.

Methods

Mice

C57BL/6 mice 6 weeks old were purchased from the Jackson Laboratory and housed in SPF (conventional) conditions. "Dirty" mice were purchased from Kasch's Kritters (Citrus Heights, CA), Petco (Davis, CA), Pet Supplies Plus (Woodland, CA), or wild-caught in traps with the help of the Bales Lab at UC Davis. Lab mice were conventionally housed with "dirty" mice at a 3 to 1 ratio for approximately 2 months before analysis. All animal

procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Bacterial Strains and Infections

Salmonella enterica serovar Typhimurium strain BRD509 (aroA aroD mutant) was injected intravenously to induce systemic bacterial infection in mice. *S*Tm was grown in LB broth at 37°C overnight and 5 x 10^5 CFU injected at an OD_{600} of 0.4-0.6 in a 200µl volume of PBS.

Listeria Monocytogenes strain LM10403s was injected i.v. to induce systemic bacterial infection in mice. *LM* was grown in Brain-Heart Infusion media at 37°C overnight to an OD_{600} of 0.8 and 4.5 x 10^4 CFU was injected in 200µl of PBS.

Chlamydia muridarum from the ATCC was propagated in HeLa cells as previously described (9). *Chlamydia* infections were delivered at a dose of 10^5 IFU in 5µl of SPG buffer intravaginally to induce an upper reproductive tract infection.

Bacterial Enumeration

Spleens and livers from *S*Tm-infected mice were homogenized in PBS and serial dilutions were plated on MacConkey agar plates and incubated overnight at 37°C. The next day bacteria were enumerated on plates and the total CFU/organ calculated. For *Listeria*-infected mice, the bacteria were enumerated the same way except BHI plates were used and only livers were measured. For *Chlamydia* infection experiments, mice were swabbed at regular intervals every 3-4 days with calcium alginate swabs from Puritan. Swabs were agitated in 500µl of SPG buffer with 2 glass beads. Supernatants were frozen at -80°C

until end of experiment. For analysis, swabs were thawed, serially diluted, and plated on a HeLa cell monolayer, centrifuged for 1hr at 37^{o}C and incubated for approximately 16-20 hrs in media + cycloheximide. After incubation, cells were washed and fixed with 100% methanol for 15 min. After fixation, cells were stained with mouse *Chlamydia* immune serum for 45-60 min, washed and then stained with secondary FITC-conjugated goat anti-mouse IgG antibody and washed again. *Chlamydia* inclusions were enumerated under a fluorescent microscope and total IFU/swab were calculated.

Flow Cytometry

Blood was collected by cheek bleeds, ACK lysed, and washed with FACS buffer. Spleens were harvested and homogenized through a filter into a single cell suspension, ACK lysed, and washed with FACs buffer. Cells were incubated in Fc block for 10-30 min and then washed. Cells were stained in antibodies resuspended in Fc block for 30 min at room temperature. Antibodies used in the experiments included: CD11b PE, CD11c PE, B220 PE, F4/80 PE, CD8 PerCP eFluor 710, CD4 FITC, CD44 PE-Cy7, CD4 APC, CD62L FITC. Samples were run on a BD Fortessa.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version 8.

Results

Higher frequency of memory CD4 T cells in blood and spleen of "dirty" mice

To determine the frequency of the immune memory cells in the pet shop mice that would be co-housed with SPF mice, we analyzed the blood and spleens of pet shop mice for memory CD4 and CD8 T cell frequencies. The frequency of CD4 T cells in the blood of pet shop mice was around 10%, 2x higher than that of the SPF lab mice, and 30% in the spleen, more than 3x higher than the SPF mice (Figure 1). There were no significant differences found in the CD8 T cell frequencies in the blood and spleen of pet shop mice compared to SPF mice (Figure 1).

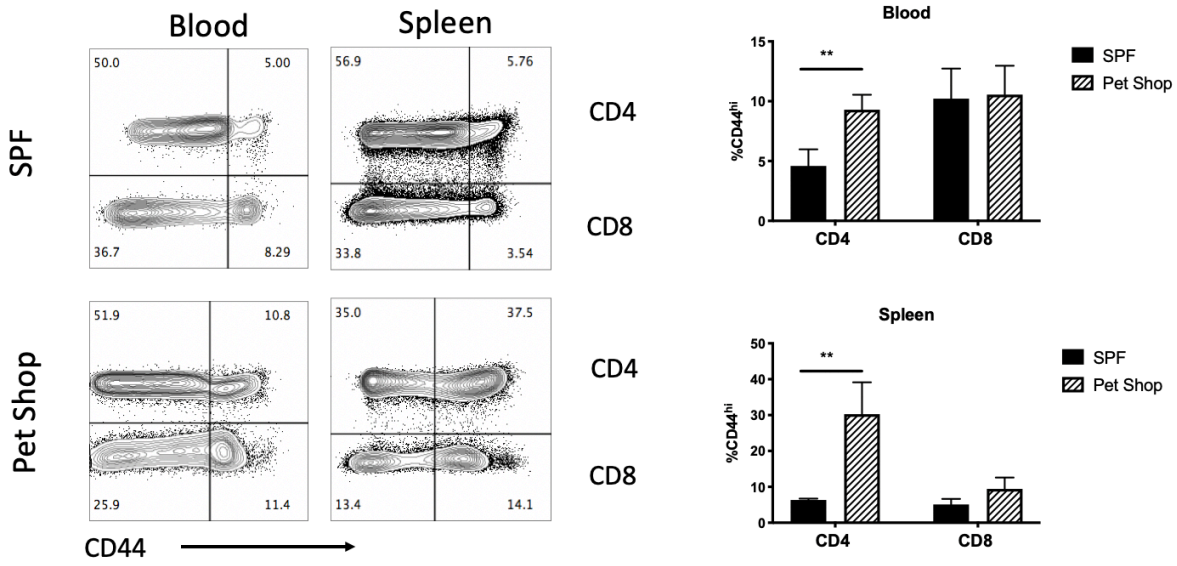

Figure 1: Analysis of memory T cells in blood and spleens of SPF and pet shop mice. Memory CD4 and CD8 T cell populations were analyzed by flow cytometry in the blood and spleens of SPF laboratory mice and pet shop mice. $CD44^{hi}$ CD8 and CD4 T cells were measured, quantified, and compared using T-tests. N=4 mice per group, p=*p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Co-housing with "dirty" mice induces increased frequency of peripheral blood memory T cells in SPF mice

To determine whether exposing SPF mice to pet shop mice increases immunological memory in SPF mice, we co-housed female SPF mice with female pet shop mice in cages at a three to one ratio for two months. Blood was sampled after one or two months and analyzed by flow cytometry for effector memory (T_{EM}) CD4 and CD8 T cell ($CD44^{hi}$ $CD62L^{-}$) frequencies (Figure 2). After one or two months a substantial increases in T_{EM} was observed in the blood of co-housed SPF mice (Figure 2, right panel), comparable to that of pet shop mice (Figure 2, left panel). Thus, co-housing with locally sourced pet shop mice increases the percentage of T_{EM} in the blood of laboratory mice, similar to laboratory mice co-housed with pet shop mice in Minnesota (1).

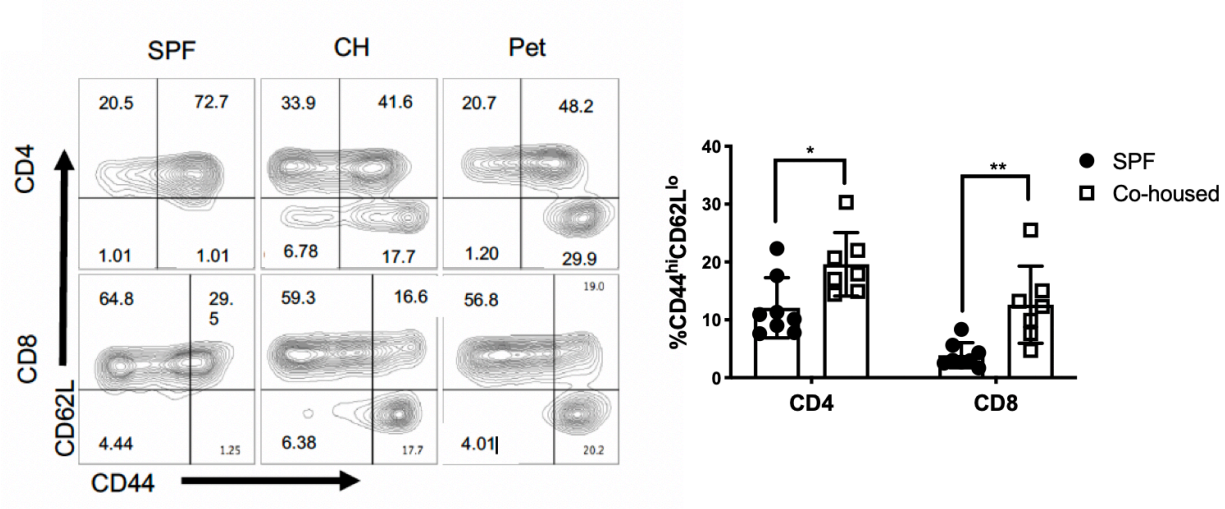

Figure 2: Increase in effector memory T cells in blood after co-housing with pet shop mice. Female laboratory mice were co-housed with female pet shop mice for two months. $CD62L^{-}$ $CD44^{hi}$ T_{EM} in peripheral blood were analyzed by flow cytometry in SPF, co-housed, and pet shop mice after one month (left panel) and two months (right panel).

Mean frequencies were compared using t-tests. N=7 mice per group, p=*p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Co-housing with "dirty" mice protects against systemic *Listeria* infection

In order to determine whether the modest increase in T_{EM} in co-housed mice had any appreciable effect on disease outcome, we challenged these mice with pathogens previously shown to be affected by co-housing and others that had not been previously studied in this model. It was previously shown that co-housing with pet shop mice was protective against systemic *Listeria monocytogenes* infection and *Plasmodium berghei* infection in laboratory mice, two infections that mainly require CD8 T cells for clearance from the host (10-12). Co-housing was likely protective through activity of non-specific memory CD8 T cells as these cells have previously been reported to protect against *Listeria* infection (13, 14). To confirm that our co-housing model was also protective against pathogens that require CD8 T cells for their elimination, we challenged co-housed laboratory mice with 4.5 x 10^4 CFU of *Lm* intravenously and euthanized the mice after three days. Livers from infected SPF and co-housed mice were assessed for *Listeria* bacterial loads. Co-housing with pet store mice significantly lowered the burden of *Listeria* in the livers of infected mice (Figure 3A), consistent with the previous study (1).

To determine whether pet shop mouse co-housing has any effect on systemic infection models that require CD4 T cells for protection, we infected co-housed mice with 5 x 10^5 CFU of *Salmonella* Typhimurium(15, 16) intravenously and euthanized mice at one-week intervals for four weeks. The spleens and livers of infected SPF and co-housed mice were analyzed for *S*Tm bacterial loads at each time point since these are the primary

sites of systemic *Salmonella* infection (17). No differences in bacterial load were found in the spleens or livers of co-housed and SPF mice at any time-point (Figure 3B). Therefore, induction of CD4 T_{EM} through co-housing with "dirty" mice did not affect the outcome of *Salmonella* Typhimurium infection in laboratory mice.

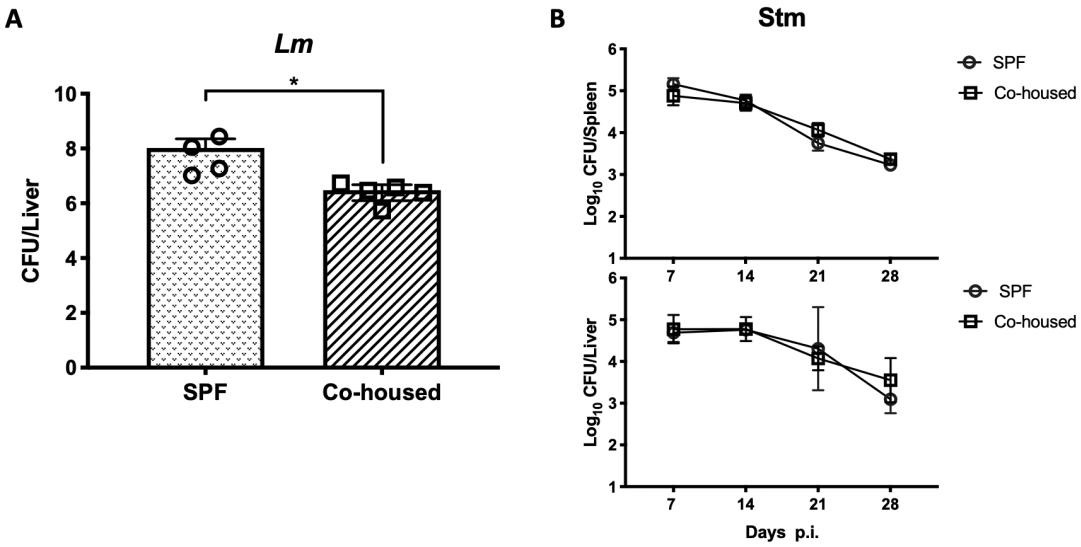

Figure 3: Co-housing with "dirty" mice protects against systemic *Listeria monocytogenes* infection, but not *Salmonella* Typhimurium infection. Female laboratory mice were co-housed for two months with female "dirty" mice and then SPF and co-housed mice were infected intravenously with 4.5 x 10^4 CFU of *L.m.* (A) or 5 x 10^5 CFU of *S*tm (B). Mice were euthanized after three days and *L.m.* bacterial loads were measured in the livers (A). Alternatively, mice were euthanized 7, 14, 21, and 28 days after *S*tm infection and bacterial loads were measured in spleens and livers (B). N=3-5 mice per group. Significance was determined with a Mann-Whitney test (A) or an ANOVA (B). p=*p $<$ 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Exposure to "dirty" mice has no effect on a localized reproductive tract infection

Although co-housing with "dirty" mice did not limit the systemic CD4-dependent pathogen *Salmonella* Typhimurium, we wondered whether localized infection with a CD4 T cell-sensitive bacterium (18, 19) could be better controlled in our pet shop co-housing model. To test this theory, we co-housed lab mice with feral mice or pet shop mice as described in Figure 2. Co-housed mice were infected intravaginally with 10^5 IFU of *Chlamydia muridarum* (*Cm*). SPF, pet shop co-housed, or feral co-housed laboratory mice were vaginally swabbed every 3-4 days over a month-long period. No significant differences in bacterial shedding were detected among any of the groups (Figure 4).

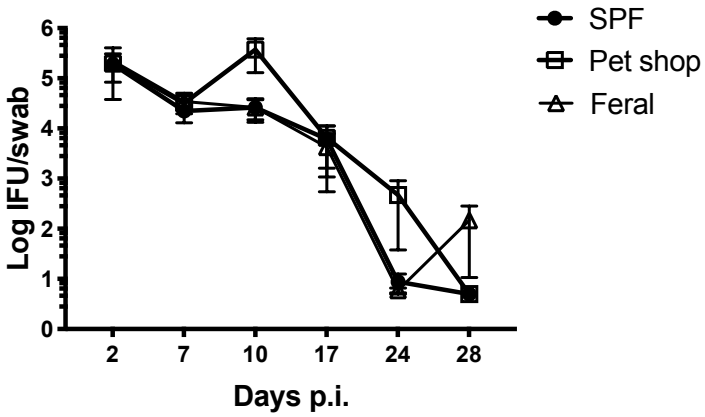

Figure 4: Co-housing with feral or pet shop mice does not influence *Chlamydia muridarum* infection in laboratory mice. After two months of co-housing with female pet shop or feral mice, co-housed female mice or SPF mice were synchronized s.c. with 2.5mg of Depo Provera (medroxyprogesterone) and challenged intravaginally with 10^5 IFU of *Chlamydia muridarum*. Bacterial shedding was measured through vaginal swabs which were later analyzed for *Cm* bacteria. N=4-8 mice per group. Significance was determined using a Two-way Repeated Measures ANOVA.

We considered that sexual intercourse may be required for sufficient exposure of the FRT to infectious agents to induce a strong enough protective memory T cell response at this tissue site. To this end, we co-housed female laboratory mice with either male pet shop mice or female pet shop mice for two months. Mice were rested for an additional month to ensure all litters were born and none of the animals were pregnant. Laboratory mice co-housed with either male or female pet shop mice were synchronized and infected as described in Figure 4. Bacterial shedding from the vagina was measured by swabs and analyzed over a month. Our results demonstrate that there were no significant differences between the groups over the course of the entire month of the infection, indicating that co-housing with male mice did not alter *Chlamydia* infection in female laboratory mice (Figure 5). In conclusion, co-housing of laboratory mice with pet shop mice does not have a meaningful effect on *Chlamydia* infection of the female reproductive tract.

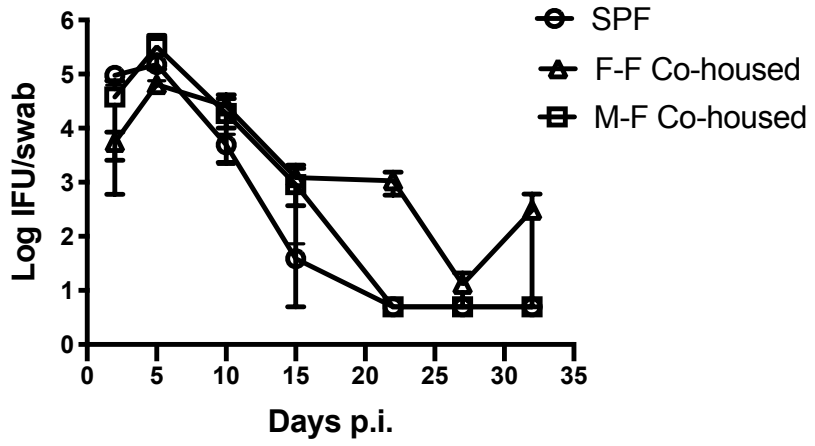

Figure 5: Sexual exposure to pathogens does not influence outcome of *Chlamydia muridaurm* infection. Female laboratory mice were co-housed for two months with either male or female pet shop. Mice were then separated and rested for another month to ensure none of the mice were pregnant. SPF and co-housed mice were synchronized s.c.

with 2.5mg of Depo Provera (medroxyprogesterone) and challenged intravaginally with 10^5 IFU of *Chlamydia muridarum*. N=4-5 mice per group. Significance was determined using a Two-way Repeated Measures ANOVA.

Discussion

Specific pathogen free housing conditions were developed to control animal exposure to infectious agents that may confound infectious diseases studies, as such studies usually aim to attribute observed immunological responses to a particular pathogen of interest (2). However, a major caveat of the SPF housing model that has been overlooked until recently is that animals housed in such conditions do not develop an immune system comparable to adult humans or wild mice (1, 2). This is evident by a lack of memory T cell populations and ectopic lymphoid structures in blood and non-lymphoid tissues of laboratory mice compared to wild mice and humans (1, 6-8, 20, 21). Diminished lymphocyte frequencies in these laboratory mice is likely due to a lack of exposure to common pathogens that a mouse would normally incur living in the wild (1). However, a new animal model was recently developed to overcome this abnormality, but permits maintenance of genetically controlled experiments (1, 4, 22). This model involves co-housing genetically controlled laboratory mice with feral mice or mice from pet stores that carry common murine pathogens, termed “dirty” mice (1, 23). Co-housing with “dirty” mice exposes laboratory mice to new pathogens and promotes the development of a more antigen-experienced immune system (1, 23). This exposure also increased the overall

frequency of lymphocytes in mice and conferred non-specific protection against systemic pathogens (1, 4, 22).

The female reproductive tract is a mucosal tissue that is exposed to the outside environment and frequently comes into contact with various pathogens. Indeed sexually transmitted infections such as the upper genital tract pathogen *Chlamydia trachomatis*, are common among young women (24), yet there is limited understanding of why some women are able to control this infection better than others (25). Human studies of *Chlamydia* infections in women indicate existence of memory lymphocyte clusters in the genital tract that may play a role in fighting *Chlamydia* infections (21). This hypothesis is supported by a study demonstrating that CCR7 knockout mice have increased non-specific memory T cells in the reproductive tract and better control *Chlamydia* infections compared to wildtype mice (8). Given this observation and the fact that CD4 T cells are required for primary clearance of *Chlamydia* infection (18, 19), we hypothesized that boosting memory CD4 T cell frequencies in laboratory mice via pet shop co-housing may more accurately model a human immune response to *Chlamydia* and possibly enhance protection against infection.

To test this hypothesis, we set up a pet shop co-housing model at UC Davis that utilized mice from various pet store retailers and feral mice for co-housing in cages with laboratory mice. Our co-housing set up was successful at inducing an enhanced circulating CD4 and CD8 T cell immune response in laboratory mice marked by an increase in effector memory T cells within these populations (Figure 2). However, our co-housing model could not achieve the same induction of blood T_{EM} as that observed in a previous study (1). This could be due to location and environmental differences between

Minneapolis and Davis/Sacramento as well as cleanliness standards or housing practices within the specific animal facilities. Like the previous study, our co-housing model resulted in enhanced control of *Listeria monocytogenes* infection in laboratory mice, albeit the protection was more modest (Figure 3) (1). Thus, our study confirms an effect of co-housing on pathogens that require CD8 T cells for their control.

We wondered whether pet shop co-housing would induce protective T cell responses against an infection that primarily requires CD4 T cells for clearance. However, there was no effect of co-housing on systemic *Salmonella Typhimurium* infection (Figure 3), or localized *Chlamydia muridarum* infection (Figures 4 and 5). The effect on *Chlamydia* infection was negligible regardless of whether the female laboratory mice were housed with female or male pet shop mice. Hence, in the current study, we did not detect any influence of co-housing on CD4 T cell-dependent pathogens, however, we cannot currently distinguish whether the influence of co-housing is specific to cell type or certain pathogens.

Overall, the pet-shop co-housing model appears to be useful for modeling human disease under specific conditions such as infections with CD8-dependent pathogens. Additionally, this model would be ideal in infection models where protection from non-antigen-specific lymphocytes has been previously demonstrated. It should be noted that this model is not broadly influential in all immune contexts, and during our study, we observed that many of the co-housed mice become chronically ill based on observations of lack of grooming and sluggishness. The previous study observed such illness but claimed the mice fully recovered after six weeks (1, 23), differing from our model where many of the mice did not recover. Therefore, co-housing with pet shop mice may not

model a healthy adult human with previous pathogen exposure, but rather a human that is chronically ill and whose immune system is constantly being stimulated. However, co-housing with feral mice may be a more realistic model of previous exposure to infections in humans, as these mice are generally healthy and can still induce an increase, although modest, in circulating lymphocytes in laboratory mice. The better health status of feral mice compared to pet store mice likely has to do with the housing conditions of pet shop mice which are usually cramped and less hygienic. Therefore, we propose the use of feral mice in future co-housing models that aim to replicate an adult human immune system.

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Chapter 4

Circulating Immunity Protects the Female Reproductive Tract from *Chlamydia* Infection

Jasmine C. Labuda, Oanh H. Pham, Claire E. Depew, Kevin D. Fong, Bokyung S. Lee, Jordan A. Rixon, and Stephen J. McSorley

Abstract

Anatomical positioning of memory lymphocytes within barrier tissues accelerates secondary immune responses and is thought to be essential for protection at mucosal surfaces. However, it remains unclear whether resident memory in the female reproductive tract (FRT) is required for Chlamydial immunity. Here, we describe efficient generation of tissue-resident memory CD4 T cells (CD4 T_{RM}) and memory lymphocyte clusters (MLCs) within the FRT after vaginal infection with *Chlamydia*. Despite robust establishment of localized memory lymphocytes within the FRT, naïve mice surgically joined to immune mice, or mice with only circulating immunity following intranasal immunization, were fully capable of resisting *Chlamydia* infection via the vaginal route. Blocking the rapid mobilization of circulating memory CD4 T cells to the FRT inhibited this protective response. These data demonstrate that secondary protection in the FRT can occur in the complete absence of tissue resident immune cells. The ability to confer robust

protection to barrier tissues via circulating immune memory provides an unexpected opportunity for vaccine development against infections of the female reproductive tract.

Introduction

Naïve CD4 T cells circulate through lymphoid tissues and blood until an infection initiates lymphocyte clonal expansion and the acquisition of specific effector function (1, 2). Expanded effector CD4 T cells access inflamed or infected tissues where they regulate pathogen control and coordinate tissue repair (3, 4). In the aftermath of this host response, an elevated frequency of memory CD4 T cells returns to circulation and a discrete population of non-circulating memory lymphocytes is retained within the tissues (5, 6). For many barrier tissues, the non-circulating tissue-resident memory T cells (T_{RM}) are critical for the rapid deployment of secondary immune responses upon pathogen re-exposure (6-11). The female reproductive tract (FRT) is a barrier tissue that is thought to depend heavily upon T_{RM} since it regularly encounters pathogens, but lacks organized lymphoid tissues (12).

The lack of organized lymphoid structures during steady-state and the immunologically restrictive nature of the FRT, makes establishing immunity in this mucosal tissue a complex process. In certain contexts, lymphocytes as well as circulating antibody cannot easily enter the FRT mucosa (9, 10), suggesting that protective immune memory is contained within the tissue itself. Indeed, previous reports document the establishment of resident memory lymphocytes and mucosal antibody secretion as two important local protective mechanisms (7, 9, 13). Memory lymphocyte clusters (MLCs) are lymphoid structures that form at the interface of the FRT epithelium and lamina propria

and consist of memory T cells and antigen presenting cells (13). Upon secondary infection, these MLCs can efficiently recruit effector cells in a CD4 T cell-dependent manner to clear FRT infections (9, 13). Thus, the generation of FRT MLCs is thought to be an important prerequisite for the development of new vaccines against important reproductive tract pathogens.

A confusing aspect of this model is the observation that distal mucosal immunization in the lung can often induce local immunity in the FRT. It is not yet clear whether such distal immunization induces seeding the FRT with T_{RM}, formation of MLCs, and/or secretion of local antibody (14, 15). An alternative hypothesis is that distal immunization generates circulating memory responses that are recruited to the infected FRT and mediate pathogen clearance in the absence of T_{RM} or MLCs (15, 16).

In this study, we examined whether local or distal immunization with the bacterial pathogen *Chlamydia muridarum* (*Cm*) is protective against intravaginal challenge with *Chlamydia*. Our data reveal that CD4 T_{RM} efficiently populate the FRT and that MLCs are generated after local, but not distal, immunization. Using parabiosis, we demonstrate that the establishment of local resident memory lymphocytes in the FRT is dispensable for protective immunity. Indeed, circulating immunity induced locally or distally was completely sufficient for protection against local infection and FRT pathology. The depletion of lymphocytes revealed that local control of protection was mediated by circulating memory CD4 T cells.

Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory at 8-16 weeks of age and housed under specific pathogen free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee at UC Davis.

Bacterial infection

Mice were synchronized by subcutaneous injection of 2.5mg Depo provera, 7 days prior to intravaginal infection. *Chlamydia muridarum* was purchased from ATCC and propagated using established culture conditions (5, 6). For intravaginal infections, 10^5 inclusion forming units (IFU) of *Cm* was suspended in 5µl of SPG buffer and pipetted into the vagina. For intranasal infection, 10^5 IFU of *Cm* (in 20µl of SPG buffer) was pipetted into the nasal cavity of anesthetized mice.

Enumeration of bacteria

Chlamydia bacterial burden was measured by cervico-vaginal swabbing and culture. Vaginal swabs were placed in 500µl of SPG buffer with glass beads and disrupted for 5 min. Serial dilutions were generated from swab supernatants and subsequently plated on HeLa229 monolayers. Infected cells were fixed with methanol and stained using a primary mouse anti-*Chlamydia* MOMP antibody that was a kind gift from the Caldwell laboratory. Antibody-stained HeLa cells were further incubated with a goat, anti-mouse, FITC-conjugated IgG secondary antibody and inclusion forming units (IFU) enumerated using a Keyence microscope.

ELISPOT Assays

ELISPOT plates were prepared using the Mouse Interferon Gamma ELISPOT Kit from BD, following the manufacturer's protocol. Single cell suspensions were prepared from tissues and labeled for negative selection with Miltenyi Biotec CD4 selection kits. Cells from the FRT underwent a second round of positive CD4 selection to remove epithelial cell contamination. CD4 T cells were routinely isolated to 80-90% purity using this approach. After column enrichment, 10^5 splenocyte cells or all the recovered FRT cells (10^5 or less) were plated into precoated ELISPOT plates and incubated overnight with medium, heat-killed elementary bodies (HKEBs), heat-killed *Salmonella Typhimurium* (HKST), or anti-CD3/CD28 (5µg/ml and 1µg/ml). Plates were subsequently washed with 0.5% PBS Tween and stained with detection antibody and an HRP-conjugated antibody. After the final wash, AEC substrate was added to wells and spots counted using an AID ELISPOT Reader (Autoimmune Diagnostika).

ELISAs

Heat-killed *Chlamydia muridarum* (5 x 10^5 IFU) was coated onto a 96- well ELISA plate (Corning Costar) with PBS and incubated overnight at 4^{o}C. Serum was diluted 1:10,000 for the IgG ELISA, and 1:10 for IgA ELISA. Vaginal lavage (VL) samples were diluted 1:10. All samples were serially diluted 1:2. Plates were washed 3x with 0.05% PBS-Tween20 (PBST) and then 0.5% BSA was incubated on the plate for 1hr at RT to block non-specific binding. Diluted serum or VL samples were added to plates after blocking and incubated for 2.5 hrs at RT. Plates were then washed 4x with PBST and anti-mouse IgA-biotin (eBioscience) or anti-mouse IgG-biotin (eBioscience) diluted 1:500 was added to plates and incubated for 1hr at RT. Plates were washed 4x with PBST and then

incubated with avidin-HRP (eBioscience Ready-Set-Go) for 30 min at RT. Plates were washed again and then TMB substrate was added for 15 min and stopped with sulfuric acid.

Serum Transfers

Cm-immune or naïve mice were bled and serum was collected. For passive immune serum transfer, mice were injected i.p. with 200ul of serum diluted up to 500ul in PBS every 3 days. For intravaginal serum transfers, mice were given 20ul of immune serum or naïve serum pipetted directly into the vaginal vault.

Flow Cytometry

Intravascular lymphocyte staining was performed by injecting 3µg/ul of antibody specific for CD45 or CD90 into the tail vein of mice, 3 min prior to euthanasia. Single cell suspensions were blocked using 24G2 antibody, 10-30 min prior to antibody staining. Antibodies used in this study include: APC: Cd11b, CD11c, F4/80, B220 CD44, CD4. FITC: CD62L, CD11b, CD11c, F4/80, B220, CD69, CD4. AF700: CD4, CD44. PEcy7: CD44. PerCP EF710: CD90.2, CD8. BV650: CD45.1, CD45.2. EF450: CD69, CD3. PE Texas Red CD62L. PE: P2RX7, CD11b, CD11c, f4/80, B220. APC EF780: CD11b, CD11c, F4/80, B220. Samples were analyzed on a BD LSR Fortessa or FacsSymphony and results were analyzed using FlowJo software.

Lymphocyte isolation

Spleens were processed as previously described (6). FRTs were initially diced into small tissue pieces before being digested in 25ml flasks containing collagenase IV in 5% RPMI medium. FRTs were disrupted for an hour and then strained carefully to remove any larger tissue clumps. FRT lymphocytes were subsequently isolated from these suspensions using 44% and 67% Percoll gradients.

Parabiosis

Mouse parabiosis was performed, as previously described (6). Briefly, CD45.1 mice were infected with *Cm* intravaginally and allowed to completely resolve the primary infection and develop memory responses for approximately 2 months. These CD45.1 immune mice were then surgically joined to CD45.2 naïve mice and monitored carefully to ensure successful surgery and wound healing. Similarly, naïve mice were surgically joined to other naïve mice and referred to as “surgery controls”. The blood of all co-joined mice was assessed after 12-14 days of parabiosis to confirm that blood chimerism had occurred. Following successful parabiosis, surgery was reversed and separated mice were rested for two weeks before further experimentation. Parabionts (both naïve and memory) were infected intravaginally with *Chlamydia* and bacterial shedding was examined, as described above. In all protection experiments, groups of naïve and immune mice that had not undergone surgery were also infected as controls.

Histology

The entire reproductive tract was harvested from female mice and frozen in OCT on dry ice. Tissue blocks were sectioned on a Leica CM3050S cryostat at 10μm thickness and

sampled at 50μm intervals throughout the tissue. Slides were fixed in ice cold acetone and frozen at –80^{o}C until stained. For staining, slides were thawed and rehydrated in PBS before 5% BSA was used to block for 30 min. Slides were subsequently stained with anti-mouse CD4 PE/Dazzle (clone RM4-5, Biolegend) for 2 hours before being washed in PBS and counterstained with DAPI. Slides were imaged using a Leica SP8 STED 3x microscope using stitching of 1248μm x 1248um images to achieve an image of the entire reproductive tract section. To enumerate clusters in reproductive tract tissues, ImageJ software was first used to mask and calculate the area of DAPI staining for each tissue section. Another investigator, blinded to treatment groups, counted the number of CD4+ clusters with a minimum radius of 75μm in each tissue section. The area of DAPI for each tissue section was extracted and the cluster number per tissue was divided by the DAPI area of each tissue. This method was used to account for variation in the area of tissue sections.

Histopathological Analysis

Naïve or infected mice were euthanized at 30-days post-secondary infection by carbon dioxide asphyxiation and cardiac exsanguination. The female reproductive tract (ovary, oviduct, uterus, cervix and vagina) was immersion fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin, sectioned 5 μm thick, and stained with hematoxylin and eosin. Histopathologic evaluation was performed by a board-certified veterinary anatomic pathologist after masking and randomization of samples. Samples were evaluated for the presence and severity of acute inflammation, chronic inflammation, erosion, dilation and fibrosis. Acute inflammation was defined by neutrophilic infiltration

and edema. Chronic inflammation was defined by lymphohistiocytic infiltration. Erosion was defined by the loss of mucosal epithelial cells, with or without breach of the basement membrane. Dilation was defined by distention of the lumen. Fibrosis was defined by either an increase in fibroblasts or an increase in collagenous connective tissue. Parameters were evaluated using an ordinal scoring system based on lesion severity and distribution on a 0–4 point scale.

Lymphocyte Depletion

Anti-CD4 clone GK1.5 (300µg) and Isotype control antibody clone LTF2 antibodies (purchased from Bio X Cell) were administered to mice i.p. every 3-4 days beginning 4 days prior to secondary infection and ending 18 days post infection.

Statistics

Statistical analysis was performed by using an unpaired *t* test for normally distributed continuous-variable comparisons and a Mann-Whitney U test for nonparametric comparisons (Prism; GraphPad Software, Inc.).

Results

Chlamydia-specific CD4 T_{RM} populate the FRT after local infection with *Chlamydia*

To determine whether CD4 T_{RM} are generated after intravaginal exposure to *C. muridarum*, C57BL/6 mice were infected with 10^5 IFU *Cm* and reproductive tract tissues examined 65 days later. There was an obvious increase in the percentage of antigen-experienced effector CD4 T cells ($CD44^{hi}$ $CD62L^{-}$) in the reproductive tract and most of these cells expressed phenotypic markers of tissue-resident memory ($CD69^{+}$ $P2RX7^{+}$) (Fig 1A and B). The absolute number of CD4 T cells in the FRT increased markedly after *Chlamydia* infection and much of this increase was due to an increase in resident memory cells (Fig. 1B). IFN-γ, ELISPOT analysis confirmed that CD4 T cells in the FRT and spleen were reactive to *Chlamydia* heat-killed elementary bodies (HKEBs) (Fig 1C and 1D). Thus, *Chlamydia*-specific CD4 T_{RM} populate the FRT of C57BL/6 mice after the resolution of intravaginal *Cm* infection.

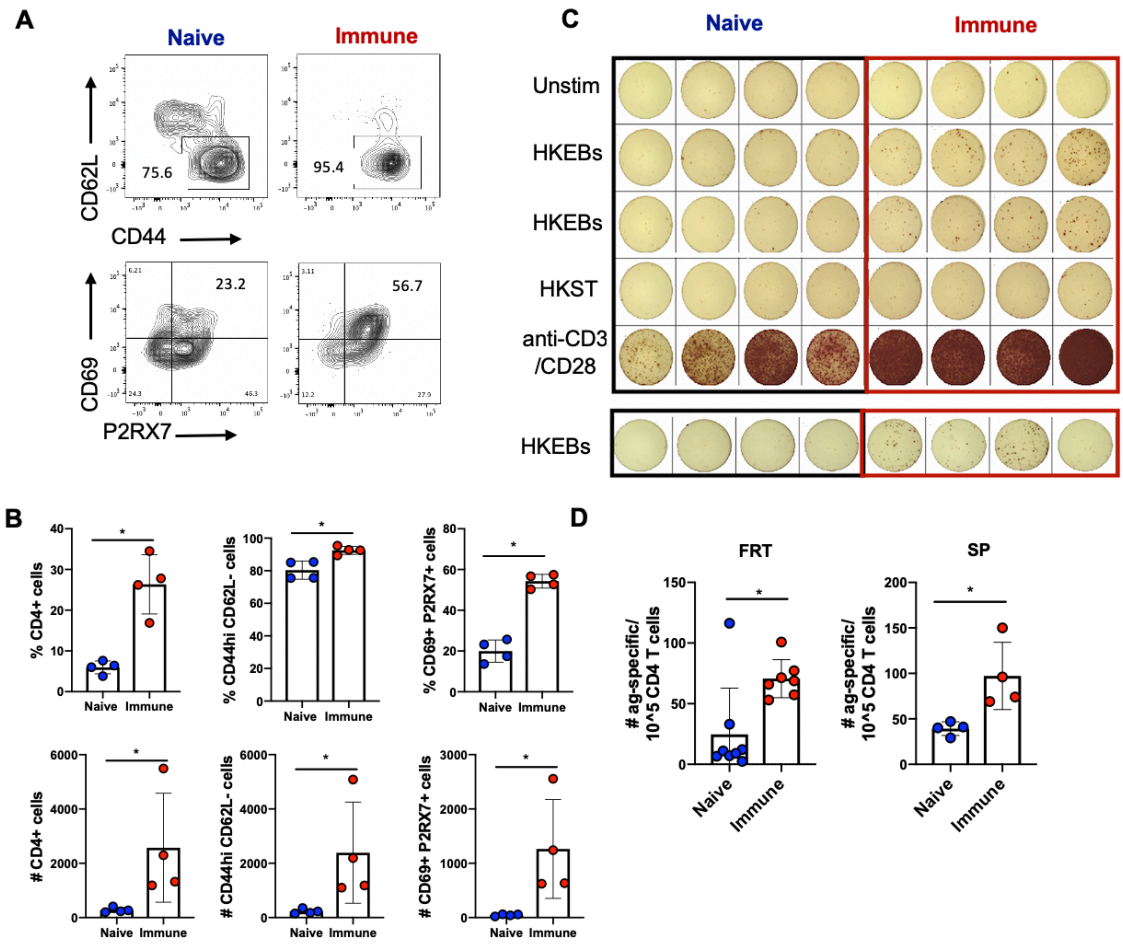

Figure 1: Chlamydia-specific CD4 T_{RM} form in the FRT after intravaginal *Chlamydia* infection. Mice were infected I.Vag with 10^5 IFU of *C. muridarum* and euthanized after 50-65 days, weeks after the infection has cleared. A) Flow plots of $CD44^{hi}$ $CD62L^{lo}$ CD4+ memory T cells and CD69+ P2RX7+ CD4+ T_{RM} in the FRT B) Quantification of percentage and number of CD4 T cells, CD4 memory T cells, and CD4 T_{RM}. C) ELISPOT analysis of Cm-specific CD4 T cells in the spleen (top panels and FRT (bottom panels) and D) quantification. Significant differences were calculated using a Mann-Whitney t-test. Data are means ± STD, *$p < 0.5$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. N= 3-4 mice per group, data are representative of two experiments.

Circulating immunity is sufficient for secondary protection against *Chlamydia* infection

Next, we used parabiosis surgery to examine whether these non-circulating memory cells were essential for immunity to secondary *Chlamydia* infection. *Cm*-immune (CD45.1) mice were surgically joined to congenic (CD45.2) naïve mice and subsequently surgically separated after a period of shared circulation (Fig. 2A). Successful parabiosis was confirmed by monitoring CD45.1 and CD45.2-expressing CD4 T cells in peripheral blood (Fig. 2C, left panel). Intravenous (IV) injection of CD90.2 antibody prior to tissue harvest confirmed that very few memory cells within the FRT are exposed to blood circulation in Cm-immune mice (Fig 2B). As expected, the FRT of paired surgery control mice contained a 50:50 mix of IV-negative host and donor CD4 T cells expressing CD69 (Fig. 2D). Similarly, naïve parabionts had a mix of host and donor CD4 T cells (Fig. 2D), indicating efficient movement of donor immune memory cells into the FRT of naïve partners. In marked contrast, immune parabionts displayed a higher proportion of host CD4 T_{RM} within the FRT (Fig. 2C and D). Thus, efficient chimerism occurs in the blood, but not the FRT, of parabiont immune mice, consistent with previous reports (7). As expected (Fig. 1), CD4 T_{RM} in the FRT of immune mice displayed high surface levels of CD44 and CD69 (Fig. 2C, right panels, and D). Intriguingly, circulating cells from the immune parabiont that entered the FRT of the naïve parabiont also expressed high levels of CD69 (Fig 2C and D).

This parabiosis approach allowed the generation of mice with equivalent circulating memory, but disparate FRT memory populations. After vaginal *Cm* challenge, all naïve

and surgery control mice displayed high levels of bacterial shedding from the reproductive tract over 14 days (Fig 2E). In marked contrast, immune parabionts successfully resisted secondary infection, demonstrating the presence robust protective memory within the FRT (Fig. 2E-red triangles). Surprisingly, naïve parabionts also displayed a robust protective response to secondary infection of the FRT, with almost no bacterial shedding (Fig 2E-blue triangle). Thus, circulating memory transferred to naïve mice during parabiosis was fully sufficient to protect mice from *Chlamydia* infection of the FRT.

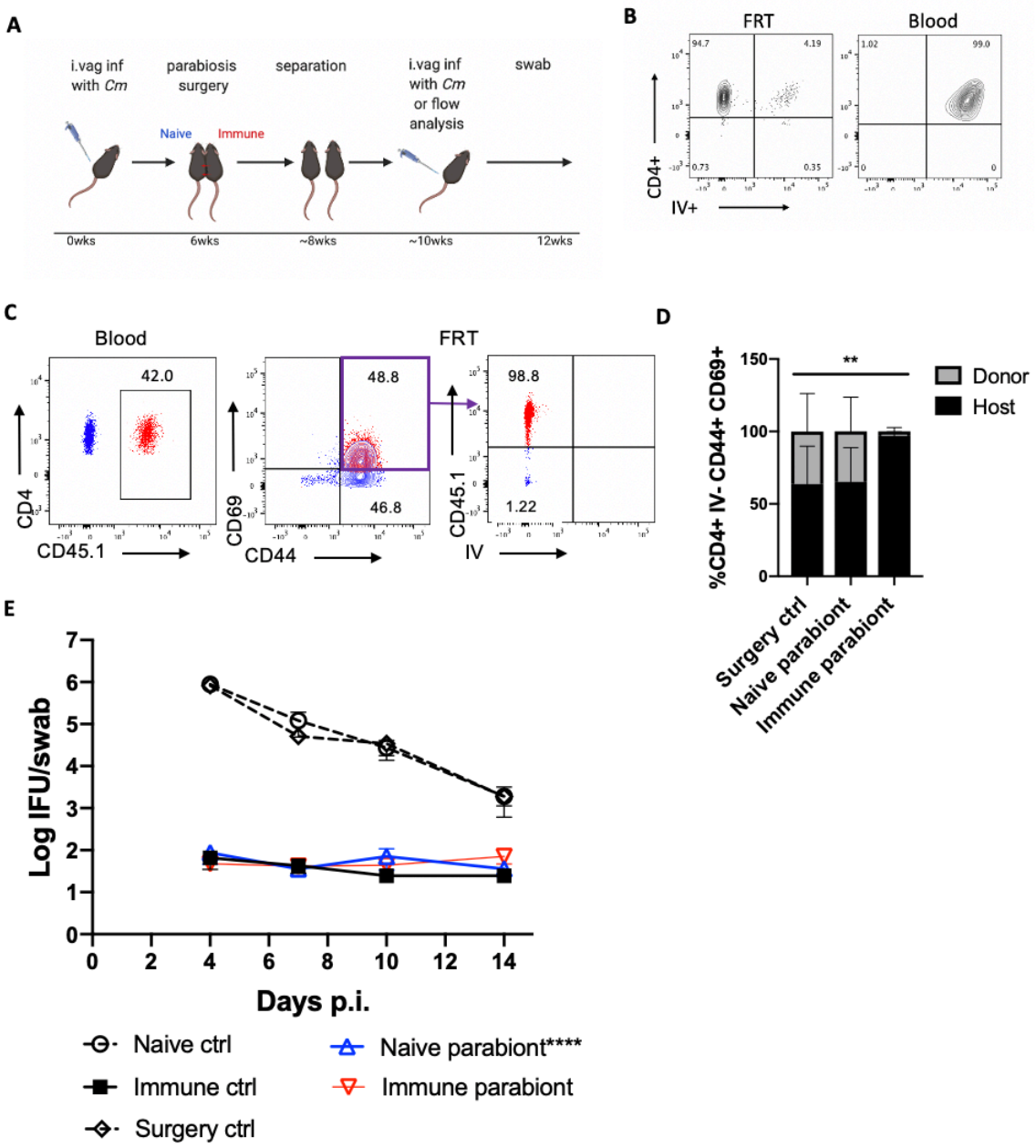

Figure 2: Circulating memory is sufficient for protection against intravaginal *Chlamydia* challenge. Mice were infected I.Vag with 10^5 IFU of *C. muridarum* and joined by parabiosis to naïve congenic, age-matched recipients after 6 weeks. Two weeks later, mice were surgically separated and rested for another two weeks. Mice were re-challenged with *Cm* or euthanized for flow analysis. A) Schematic of experimental set-up.

B) Example of i.v. labeling of lymphocytes in the blood. C) Flow plots showing chimerism in the blood but not FRT of CD45.1+ immune parabionts. Left panels shows CD45.1 positive and negative cells in the blood of parabionts. Right panels show example gating strategy for gating on $CD69^{+}$ $CD44^{HI}$ host and donor cells in parabiont FRTs D) Quantification of host and donor T_{RM} in parabiont FRTs. E) Bacterial shedding of *Cm* from the reproductive tract of parabionts following secondary infection. Significant differences were calculated using a Mann-Whitney t-test (D) and two-way ANOVA (E). Data are means ± STD (D) or SEM (E), *$p < 0.5$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. N= 3-4 mice per group, data are representative of 2 experiments.

Memory Lymphocyte clusters are not required for local protection against *Chlamydia*

Clusters of lymphocytes and APCs, termed memory lymphocyte clusters (MLCs) correlate with protection against herpes simplex virus infection of the lower reproductive tract (13). It has been hypothesized that similar clusters form after *Chlamydia* infection and are essential for secondary protection (12). Thus, MLC formation was measured in immune and naïve parabiotic mice by staining tissue sections for CD4 T cells across the whole FRT (Fig 3A). MLCs (with a minimum radius of 75μm) were enumerated in randomized sections by a blinded investigator (Fig 3B). As expected, numerous MLCs were identified in the FRT of immune parabionts but were absent from the FRT of surgery control mice (Fig. 3C). Similarly, there were no MLCs detected in the FRT of naïve parabiont mice (Fig 3C), despite the fact that these mice resist vaginal infection with

Chlamydia (Fig. 2E). Thus, transfer of immunity via parabiosis does not lead to MLC formation and therefore MLCs are not essential for protection against local *Cm* infection.

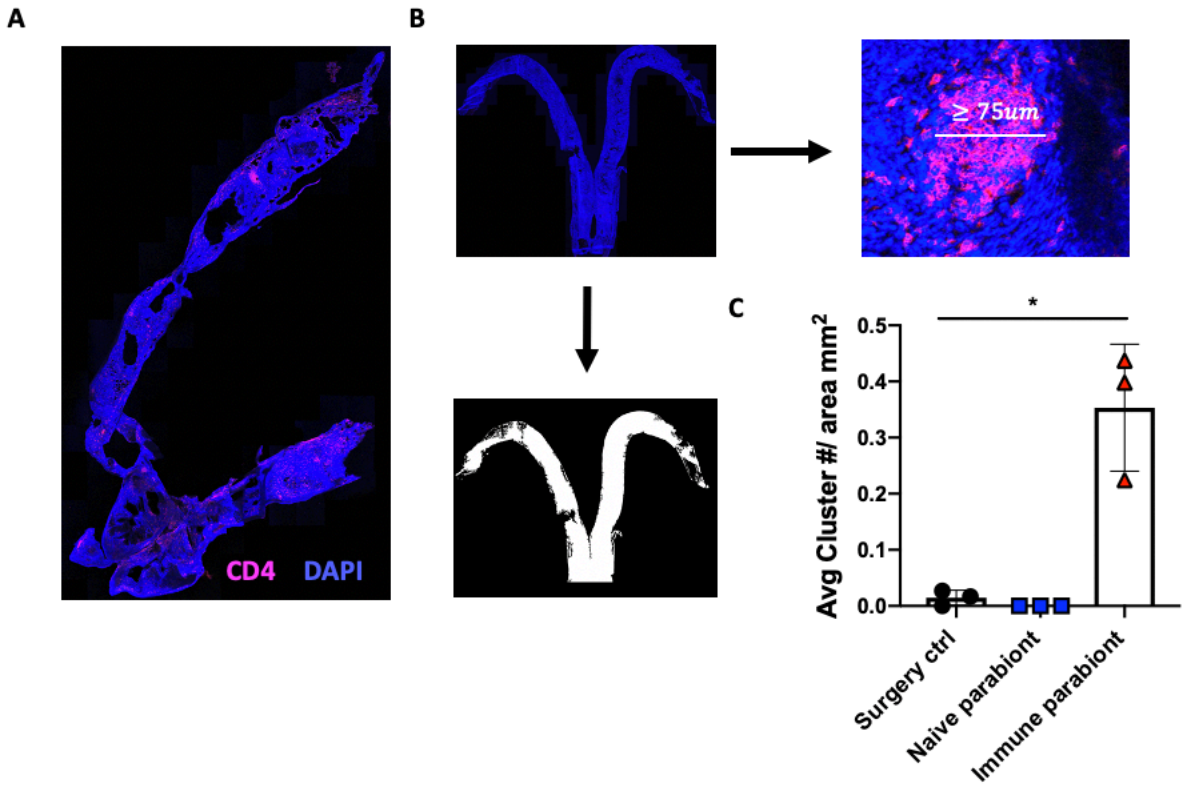

Figure 3: Memory lymphocyte clusters form in the FRT after *Cm* infection but are not required for protection. A) FRTs from parabionts were frozen, sectioned, and stained with DAPI and for CD4+ cells. B) Areas of tissue sections were masked based on DAPI staining and calculated using ImageJ. Clusters of CD4 T cells were counted by eye and divided by the area of DAPI staining. C) Clusters per area averaged per mouse. Significant differences were calculated using a Kruskal-Wallis test (C) and two-way ANOVA (D). Data are means ± STD (C) or SEM (D), *p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001. N= 3 mice per group, data are representative of one experiment.

Distal mucosal immunization with *Cm* protects against infection and pathology in the FRT

Intranasal exposure to live *Cm* protects mice against subsequent vaginal challenge, however, the degree of protection against bacterial load and pathology remains unclear (17). To compare protection in the FRT after local and distal immunization, mice were immunized intranasally or intravaginally with live *Cm* and challenged intravaginally. Distal immunization generated similar robust protection as observed with local immunization (Fig 4A). To determine whether intranasal immunization with live *Cm* was capable of limiting upper reproductive tract (URT) pathology, uninfected, intranasally immunized and intravaginally infected mice, and mice that were only intravaginally infected, were submitted for pathology scoring. All mice were euthanized for pathological analysis 30 days post intravaginal challenge. As expected, uninfected mice did not have high histopathological scores of their reproductive organs (Fig. 4B and C). Mice infected vaginally as a positive control developed severe composite lesion histopathology scores of the uterine horns and oviducts (Fig. 4B and C). In contrast, mice that were immunized intranasally before *Cm* challenge displayed significant protection against URT pathology (Fig. 4B and C). Thus, intranasal immunization is highly protective against local *Chlamydia*-induced infection and pathology of the female reproductive tract.

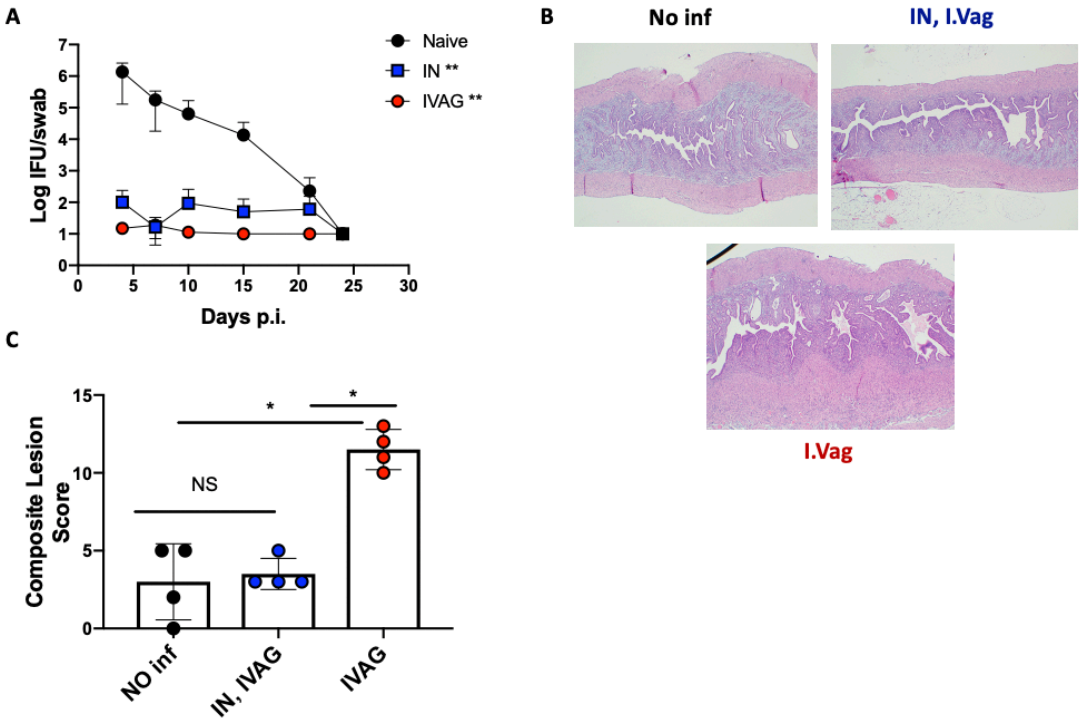

Figure 4: Protection against FRT infection and pathology after I.N. immunization with *Cm.*

Mice were intranasally immunized with 10^5 IFU of *C. muridarum* and then challenged intravaginally 47-days later. A) Bacterial shedding measured by vaginal swabs. Significant differences were calculated using a two-way ANOVA. To determine protection against FRT pathology, mice were immunized intranasally with 10^5 IFU of *Cm* or left unimmunized and challenged 42 days later with the same dose intravaginally. Thirty days after secondary challenge, mice were analyzed for FRT pathology. Uninfected mice were included as controls. B) Example images of H&E staining of mouse uterine tissue at 20x magnification. C) Composite lesion pathology scores of uterine and oviduct tissues. Significance between groups was calculated using a Mann-Whitney test. Data represent one experiment with n=4 mice per group. Data are means ± STD, *$p < 0.5$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Local protection against *Cm* is mediated by circulating memory CD4 T cells

Since parabiosis experiments demonstrated that circulating immunity is sufficient for local protection (Fig 2E), we hypothesized that protection generated by distal immunization is also was mediated by circulating immunity. To examine this possibility, we compared the generation of Cm-specific CD4 T cells in the spleen and FRT after intranasal (IN) or intravaginal (I.Vag) immunization using ELISPOT. While Cm-specific CD4 T cells were detected in the spleen after either IN or I.Vag immunization, only I.Vag immunization generated Cm-specific CD4 T cell seeding of the FRT (Fig 5A). Thus, intranasal immunization provides protection without generating FRT CD4 T_{RM}. To test whether circulating CD4 T cells provide local tissue protection after distal immunization, we depleted CD4+ lymphocytes from intranasally immunized mice (Fig 5B), before intravaginal challenge with *Chlamydia* (Fig 5C). CD4-depleted mice displayed elevated bacterial shedding from the FRT compared to isotype control mice, demonstrating that CD4 T cells provide circulating immunity in intranasally immunized mice (Fig 5C). Increased local and systemic Cm-specific antibody responses were detected after intranasal immunization (Fig S1A), but passive transfer of immune serum transfer failed to affect infection of the FRT (Fig S1C). Transfer of immune serum into the vaginal vault of mice on the same day as vaginal infection completely blocked *Cm* infection (Fig S1B), but this effect was rapidly lost if immune serum transfer was delayed by 1 day (Fig S1B). Thus, immediate encounter with mucosal antibody can prevent *Chlamydia* infection, but has no effect after infection has commenced. These data show that circulating CD4 T cells generated by distal mucosal immunization are required to control *Chlamydia* infection locally in the FRT.

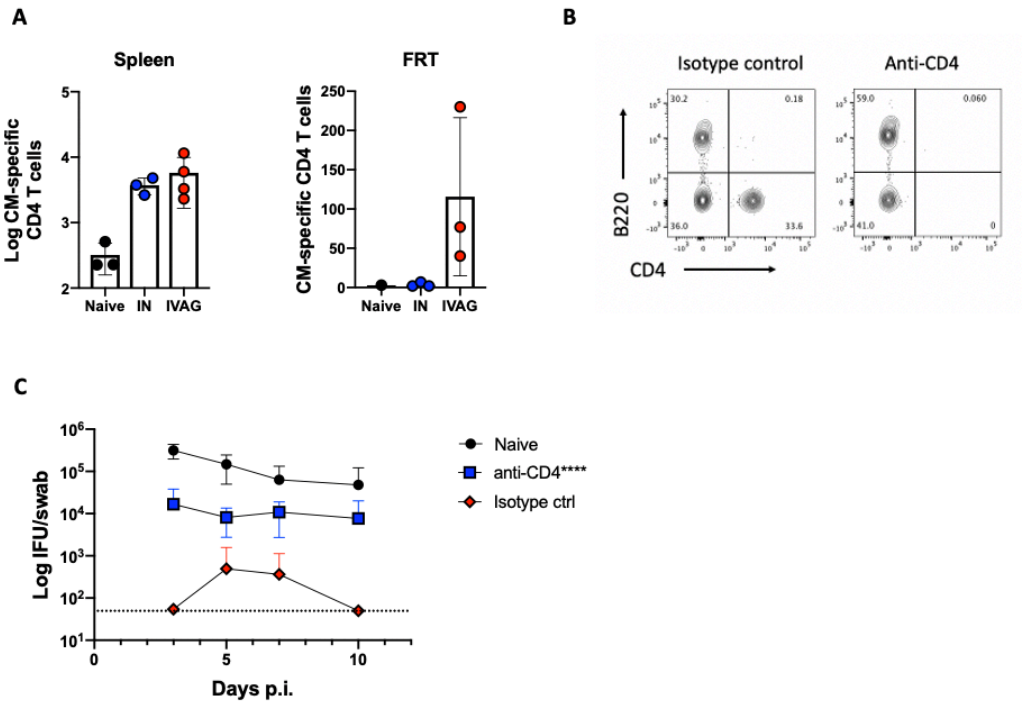

Figure 5: Protection after I.N. immunization is dependent on CD4 T cells.

Mice were intranasally or intravaginally immunized with 10^5 IFU of *C. muridarum* and CD4 T cells from FRTs and spleens were analyzed 56 days later by ELISPOT for IFN-γ secretion after *Chlamydia* stimulation. A) Number of Cm-specific CD4+ T cells in the spleen and FRT of mice from all groups. Significance between groups was calculated using a Mann-Whitney test. To test the requirement of circulating CD4 T cells for controlling secondary infection, mice were immunized intranasally with 10^4 IFU of *Cm* and re-challenged I.Vag with 10^6 IFU 93 days later. Mice were treated with anti-CD4 antibody or isotype control antibody beginning 4 days prior to secondary challenge and through day 18 post infection. B) Example flow plots of B220+ and CD4+ cells in the blood of anti-CD4 and isotype control treated mice 5 days post infection. C) Bacterial shedding curve of secondary infection. Significant differences between groups were calculated using a 2-way ANOVA. Data are means ± STD (C), *p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Discussion

Tissue resident memory cells have been shown to play a vital role in protection in models of genital infection (7, 9, 11, 13, 14), leading to the generalization that T_{RM} protect barrier tissues. In marked contrast, our data demonstrate robust pathogen control within the FRT that is independent of T_{RM}. While efficient generation of T_{RM} and MLCs occurs in mice that resolve primary *Chlamydia* infection, this is not a requirement for protection against local mucosal infection. Using two different experimental models (parabiosis and intranasal immunization) we find solid protection of the FRT in the absence of local T_{RM} or MLCs. Instead, local protection is provided by circulating memory CD4 T cell responses that are induced elsewhere. This finding is important for two reasons. First, it validates the historical and ongoing examination of immune memory within the peripheral blood of patients with current or previous genital tract infection in the search for correlates of protection. Second, it suggests that vaccine development against vaginal infection may not necessarily require induction of local tissue memory responses and traditional parenteral immunization could be sufficient.

Although, our data show that *Chlamydia* infection of the FRT induces strong circulating memory responses, it is also clear that natural *Chlamydia* exposure generates a strong T_{RM} response and the formation of MLCs. While these local memory responses are not essential for protection, it seems likely that they participate in secondary immune responses. Indeed, the presence of local T_{RM} may be more critical in viral infection models due to the faster replication rate of some viruses (HSV, 4-12hrs) (18) compared to *Chlamydia* (48hrs) (19). We suggest that the nature of the pathogen might dictate the

need for this specialized layer of poised immunity at barrier tissues, rather than there being a general requirement for T_{RM} in barrier defense. The longevity of different memory populations is not directly addressed by our study but may be a critical variable. While circulating memory responses can decline over time, our previous study demonstrated a robust elevated population of *Chlamydia*-specific CD4 T cells in SLOs 352 days after primary infection (5). Future studies will be needed to examine whether antigen or bacterial persistence affects the maintenance of this memory pool.

Circulating immunity induced after intranasal immunization consisted of both antigen-specific CD4 T cells and IgG antibodies and recent studies point to an important role for antibody in *Chlamydia* protection (20, 21). Local secretion of Cm-specific IgG was detected in the FRT after intranasal immunization and the injection of serum into the vaginal vault efficiently blocked infection. These data support the idea that antibody is an important component of barrier defense against reproductive tract pathogens. However, depletion of CD4 T cells eliminated almost all of the protective effect of circulating memory, suggesting that circulating T cells are the critical limiting factor in *Chlamydia* immunity. We propose a model in which antibodies secreted luminally may sometimes be sufficient to efficiently block *Chlamydia* attachment or entry into the vaginal epithelium and eliminate infection. However, if this initial barrier defense fails, recruitment of circulating CD4 T cells will be required to control the established infection within the FRT. It is currently unclear whether the antibody secreted in the FRT mucosa after intranasal immunization is derived from serum antibody or from plasma cells within the FRT. This should be a focus of future studies since it may be critical for vaccine development. While our data demonstrate a CD4-dependent mechanism of *Chlamydia* protection, the precise

mechanism of CD4-mediated immunity within the FRT is an enigma. While IFN-γ-mediated induction of cell intrinsic defense mechanisms (22, 23) or cytotoxic killing of infected epithelial (24) seem likely possibilities, this will also require more detailed analysis of CD4 effector function within the FRT.

In summary, our data show a surprising capacity of circulating memory CD4 T cells to efficiently protect against one of the most common human pathogens to infect mucosal surfaces. This capacity to disconnect protection from T_{RM} and MLC formation is encouraging since it suggests a roadmap to successful vaccination without the need for encouraging local tissue specific adaptations that may be more difficult to induce.

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Supplemental Figures

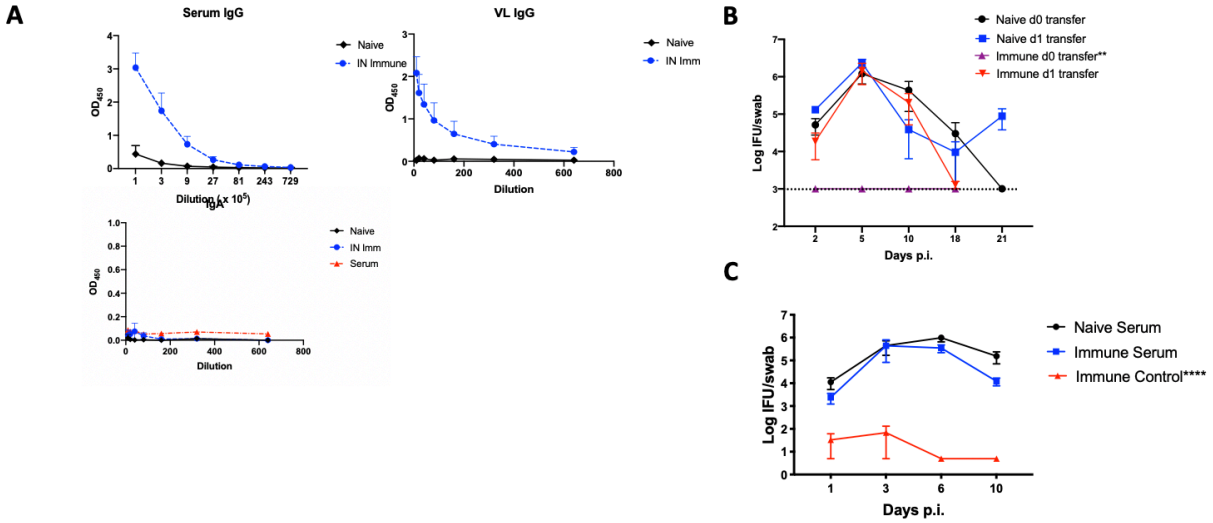

Figure S1: Antibody responses in the FRT after immunization

Mice were immunized intranasally with 10^5 IFU of *C. muridarum* and Cm-specific IgG and IgA levels were measured and compared in the serum and vaginal lavage fluid (VL) of mice after 46 days. A) Relative Cm-specific ELISAs of serum and VL IgA and IgG. To measure the capacity of antibody to protect locally in the FRT, naïve mice were given 20ul of naïve serum or Cm-immune serum I.Vag immediately following I.Vag infection (10^5 IFU) with *Cm* (d0) or one day after infection (d1). B) Bacterial shedding from the FRT over time. In a separate experiment, 200ul of naïve serum or immune serum was injected i.p. into naïve mice every 3 days throughout a primary intravaginal *Cm* infection. A previously infected mouse was included as a control. C) Bacterial shedding from the FRTs of mice from each group. Significant differences between groups were calculated using a 2-way ANOVA. Data are means ± SEM (C), *p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Statement of Author Contribution

Jasmine Labuda designed, performed, and analyzed all experiments and wrote and edited the manuscript. Oanh Pham, Claire Depew, Kevin Fong, Bokyung Lee, and Jordan Rixon helped perform experiments and edit the manuscript.

Chapter 5

Conclusion

The failure of antibiotic treatments to lower the incidence of *Chlamydia trachomatis* (*Ct*) infections in the U.S. highlights an urgent need to develop an effective vaccine against this prolific and devastating pathogen (1). In women, *Ct* can infect the cervical epithelium leading to cervicitis and eventually ascend to the upper reproductive tract where it can cause pelvic inflammatory disease and tubal factor infertility in some cases (2). Women often do not develop immunity after *Ct* infection, possibly due to antigen removal from antibiotic treatment (3). In some untreated cases of *Ct* infection, women are able to resolve infection on their own, indicating that development of protective immunity may be possible (4). Protection against *Chlamydia* re-infection in women correlates with IFN-γ production by CD4 T cells (5). Furthermore, increased *Ct* infections and sequalae have been linked to certain HLA class II variants as well as HIV infection in women (6). Together, these data support a role for CD4 T helper 1 cells in immunity to human *Chlamydia* infections.

In mouse models, MHC class II and CD4 T cells are required for primary clearance of *Chlamydia muridarum* (*Cm*) from the female reproductive tract (FRT) (7, 8). However, Th1 cells appear to be dispensable for clearance of *Cm* from the FRT and are instead important for controlling systemic infection (9). The body of work examining the T helper subsets important for clearing *Chlamydia* from the FRT is extensively reviewed in Chapter 2. The overall findings indicate that more research must be done in this area to complete

our understanding of the T helper cells required for Chlamydial clearance and the mechanism of this clearance.

Another area of *Chlamydia* research that lacks in-depth understanding is the development of immunological memory. Previous work has demonstrated a role for both CD4 T cells and antibody in secondary protection against *Chlamydia* infections (10). However, little is known about how CD4 T cells and antibody work to clear a secondary *Chlamydia* infection from the female reproductive tract. In addition, it is unknown whether tissue resident memory lymphocytes must be established in the FRT mucosa for rapid immune defense. Chapter 4 of this dissertation addresses this issue in detail. Indeed, we demonstrate that contrary to previous studies using viral infection models, immunity to the bacterial pathogen *Chlamydia* can be mediated by circulating memory T cells that are able to rapidly control secondary vaginal infection within days. These circulating memory CD4 T cells can be generated through local intravaginal immunization or distal intranasal immunization (Chapter 4). This observation is vital to informing the design of future vaccines, as it highlights that the generation of localized resident memory may not be necessary. Rather, traditional methods of parenteral vaccination will likely be sufficient for protection against genital *Chlamydia trachomatis* infection.

Intravaginal infection of C57BL/6 mice with *C. muridarum* results in a stable, productive infection that naturally ascends to the upper female genital tract and causes pathology, similar to infection and pathology observed in humans (2). However, a major difference between human infection with *Ct* and murine infection with *Cm* is the duration of the infection. *Ct* establishes a chronic infection and can persist in human cervical epithelial cells whereas *Cm* is acutely cleared from the mouse FRT within 30 days (2)

(11). Furthermore, naïve SPF mice lack immune cells at steady-state in their FRT compared to mice from a pet shop or human FRT tissue (12). This discrepancy could have a large impact on how pathogen-specific immune responses are generated and sustained locally. Therefore, a more accurate mouse model of human *Chlamydia* infection would help advance our understanding of this pathogen and the associated host immune response.

We aimed to address this gap in the field by using a pet shop co-housing mouse model to generate a more "human-like" immune system in laboratory mice (12). Laboratory mice co-housed with pet shop mice exhibited a greater proportion of memory T cells in their blood as previously described (Chapter 3). Despite the importance of memory T cells in clearing *Chlamydia* infection, we did not detect any enhanced protection in the "dirty" laboratory mice (Chapter 3). The reason for this is difficult to interpret due to the complexity of this model, but could be due to a number of factors such as the types of pathogens harbored by the "dirty" mice, how well they were transferred to the lab mice, or a true lack of biological influence of these endemic pathogens on *Chlamydia*-specific immunity. However, improvement upon current mouse models of *Chlamydia* infection should remain an active area of research in the field.

The current study revealed the importance of circulating antigen-specific immune CD4 T cell responses in controlling vaginal *Chlamydia* infection locally. However, there is still little understanding of how these cells clear *Chlamydia* from the FRT. Future studies should use sequencing technology in conjunction with mechanistic experiments to reveal the functions of CD4 T cells that are required to clear *Chlamydia* infection. In addition, our study indicated that antibody likely has an important role in blocking Chlamydial infection

of epithelial cells early in infection. These findings call for further research into the development and function of antibodies in blocking *Chlamydia* infection. The current findings as well as findings from the suggested future studies together would greatly enhance our understanding of protective immunity to genital *Chlamydia* infections.

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