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Evaluation Of Peripheral And Mucosal T Cell Phenotypes Of Chlamydia-Infected Women

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EVALUATION OF PERIPHERAL AND MUCOSAL T CELL PHENOTYPES OF CHLAMYDIA-INFECTED WOMEN

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ABSTRACT

Chlamydia trachomatis (CT) infection is the most prevalent bacterial sexually transmitted infection worldwide and women are disproportionately affected due to its reproductive complications. T cell phenotypes present during CT infection have been well characterized in the murine model, but remain to be elucidated in humans. Therefore, studies aimed at increasing our understanding of T cell phenotypes in CT-infected humans were the focus of this dissertation. The first chapter of this dissertation reviews various aspects of chlamydial infection while in the second chapter aims to address the influence of CT infection on T cell phenotypes. We first compared differences in peripheral blood T cell phenotypes from CT-infected women vs. CT-seronegative controls and found that CT-infected women had higher expression of $CD4^+$ and $CD8^+$ T cell activation markers (CD38 and HLA-DR), $CD4^+$ T helper 1 (Th1)- and Th2-associated effector phenotypes (CXCR3 CCR5 and CCR4), and homing markers (CXCR3 and CCR7, but not CCR5). We then evaluated changes in T cell phenotypes after CT treatment and their association with CT reinfection, comparing peripheral blood T cell phenotypes in CT-infected women at an initial visit (prior to treatment) and follow-up visits (at 3 and 6 months). We found $CD4^+$ and $CD8^+$ T cells had higher expression of activation markers, homing, and Th-2 associated chemokine receptors in their initial visit with active infection versus at the 3-month follow-

up visit when infection had cleared, suggesting T cell phenotypes return to a basal state in the absence of infection. We also saw a decrease in same T cells phenotypes in women who became CT reinfected at their first follow-up visit, possibly because of a lower CT load at this visit. Our study also demonstrated that certain T cell phenotypes differ in women with vs. without CT reinfection at follow-up visits, suggesting some phenotypes may be associated with protective immunity. In the third dissertation chapter, we evaluated differences in circulating (peripheral) vs. mucosal T cell phenotypes in CT-infected women. We found a higher proportion of genital mucosal T cells were activated and expressed CCR5 and Th1-associated CKRs compared to peripheral T cells, but a lower proportion of mucosal T cells expressed homing CKR CCR7, Th-2 associated CKR CCR4, and $CXCR3^{+}CCR4^{+}$ for both T cell subsets. T cell phenotypes differed in the peripheral vs. genital mucosa compartments in CT-infected women. Since CT infects mucosal epithelial cells, finding a higher frequency of activated T cells and Th-1 phenotypes in the mucosa likely reflects an adaptive immune response to infection. Our findings could be useful in understanding adaptive immune mechanisms in human CT infections and aid vaccine development.

Keywords: *Chlamydia trachomatis*, T cell phenotype, reinfection, characterization, female genital tract, peripheral blood, mucosa

DEDICATION

To my God and my dear family

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PERIPHERAL BLOOD T CELL PHENOTYPES IN WOMEN WITH *CHLAMYDIA TRACHOMATIS* INFECTION

DISTINCT PERIPHERAL VERSUS MUCOSAL T CELL PHENOTYPES IN CHLAMYDIA-INFECTED WOMEN

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
CMI	Cell Mediated Immunity
CD	Cluster of Differentiation
CKR	Chemokine Receptors
CT	*Chlamydia trachomatis*
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immuno-SPOT Assay
EB	Elementary Body
IFN-γ	Interferon-gamma
IL	Interleukin
NAAT	Nucleic Acid Amplification Test
PBMC	Peripheral Blood Mononuclear Cells
MHC	Major Histocompatibility Complex
MMC	Mucosal Mononuclear Cells
MOMP	Major Outer Membrane Protein
PID	Pelvic Inflammatory Disease
RB	Reticulate Body
STI	Sexually Transmitted Infection
TFI	Tubal Factor Infertility
TNF-α	Tumor Necrosis Factor-alpha

CHAPTER 1

INTRODUCTION

Chlamydia trachomatis (CT) is an obligate intracellular bacterium that causes the most prevalent bacterial sexually transmitted infection (STI) worldwide, accounting for over 131 million new infections annually (1, 2). Many CT infections are asymptomatic (~70-90% of endocervical infections) and go unrecognized; as a result, many infected patients do not receive treatment and have infections persisting for months to years (3). The test recommended by the Centers for Disease Control and Prevention (CDC) for CT detection is a nucleic acid amplification tests (NAAT) (4). The management of CT infection includes: CT treatment using a CDC-recommended antimicrobial for both the infected patient and their partner (in order to prevent subsequent CT reinfection), repeat CT testing in about 3 months to identify reinfection, and in pregnant women, a test of cure (TOC) 3-4 weeks after treatment (5). Early CT detection and treatment is necessary in order to limit persisting CT infection that can lead to complications, especially in women (e.g., pelvic inflammatory disease [PID], ectopic pregnancy, or tubal factor infertility [TFI]) (3, 6). Additionally, the natural history of CT infection also includes the following outcomes: resolution of infection, persisting infection, and reinfection after repeat CT exposure (7, 8). About 10-20% of CT-infected patients are re-infected within several months after treatment, suggesting some humans do not develop protective immunity (9).

The CT immune paradigm as established in murine models is that local induction of T helper type (Th) 1 dependent cell-mediated immunity (CMI) usually involves interferon gamma (IFN-γ) (10, 11). Additionally, resistance to or protection against subsequent reinfection by CT in experimental animals is associated with the presence of major histocompatibility complex (MHC) Class II antigen presenting cells (APC) that activate and recruit specific T cells in the genital tract (10, 12-14). Recruitment and trafficking is facilitated by chemotactic factors knows as chemokines that bind to chemokine receptors (CKRs), which help localize T cells to the site of infection. Our understanding of trafficking CKRs in humans during CT infection is limited. This first chapter reviews the current knowledge of the immune responses to CT based on *in vitro* and *in vivo* (animal and human) studies and discusses how immune responses are related to epidemiology and clinical manifestations of CT infection. This introduction also describes T cell characterization, the specific receptors used in our studies, and chlamydia-specific literature on T cell phenotypes.

Chlamydia in vitro

Brief History

CT infections have been ailing humans for a very long time, with evidence of early trachoma cases found in ancient societies like Egypt, Rome, Greece, Arabia, and China (15). The first cytoplasmic inclusions were described from scrapings taken from conjunctival orangutans in 1907. It wasn't until 1911 that Lindner described cytoplasmic inclusions in cervical swab specimens from mothers and in the inflamed eyes of newborns

(16). As the years passed, CT knowledge increased, CT detection methods were developed, and finally in 1959, CT was first isolated from the genital tract of a mother with a child with conjunctivitis (17). The development of *in vitro* chlamydia culture techniques opened the way for new technologies to be employed for CT research. In the mid-1960s, it was established that *Chlamydia* organisms are obligate intracellular pathogenic bacteria rather than parasites (18, 19). In the 1970s and 1980s, through the development of serological and serotyping techniques, the first CT seroprevalence and serotype data became available. The development of the polymerase chain amplification (PCR) (20) technique changed the face of chlamydia diagnostic research resulting in the first successful CT nucleic acid amplification test (NAAT by PCR in 1989) (21). Initially the screening guidelines included CT culture, however the CT NAAT first became commercially available in 1993 and later became a part of the established CT screening guidelines (22).

Life Cycle

Chlamydiae are obligate intracellular anaerobic bacteria with a biphasic life cycle (developmental cycle) (23). Outside host cells, chlamydiae generally exist in an infectious metabolically inactive state known as an elementary body (EB) that is compact and contains adenosine tri-phosphate (ATP) and ATPase stores (23, 24). EBs are coated by a protein that spans the cell membrane bilayer called the major outer membrane protein (MOMP; also, known as OmpA) (25, 26). Chlamydiae EBs enter a host cell by pinocytosis, endocytosis, or receptor-mediated endocytosis (27). Once chlamydiae have entered the host cell, the EB reorganizes into a new form known as a reticulate body (RB) and is contained within inclusions by activating ATPases and reduction of MOMP disulfide

bonds (27-29). The RB is the replicative non-infectious form of CT that replicates via binary fission (23, 29). Once the replicative process is complete, RBs transform back into EBs which exit the host cell by two means: 1) lysis (likely protease-mediated) via an ordered series of membrane ruptures, beginning with the inclusion membrane then the nuclear membrane and lastly the plasma membrane; 2) extrusions where the inclusion protrudes through the plasma membrane and tethers to the exterior over time (30). The chlamydiae developmental cycle in cell culture takes anywhere from 48 to 72 hours to be completed (29, 30).

CT consists of 15 major serovariants that are classified based on serotyping of MOMP. Serovars A-C cause trachoma, a disease that primarily affects the mucosal linings in the eyes and is transmitted through poor hand and facial hygiene in developing countries (31, 32). Serovars D-K are sexually transmitted and can infect the urogenital tract, which may result in urethritis in men and women and cervicitis in women, and the anorectum, which may result in proctitis (31, 32). Lastly, serovar L, which is further subdivided into L1 to L3, is sexually transmitted and can lead to an invasive ulcerative disease of the genital or anorectal sites known as lymphogranuloma venereum (LGV) (1, 31, 32). LGV strains are more invasive than serovars A-K strains, with more rapid replication, more tissue inflammation, involvement of lymph nodes that can lead to large, swollen lymph nodes (in genital LGV, the enlarged nodes are known as “buboes”), and ability to cause systemic infection (32).

In cell culture, there exist a persistent state of chlamydiae which is marked by an aberrant enlarged RB form (33). This state is induced by exposing CT-infected cells to IFN-γ, (33, 34) tryptophan depletion, (34) penicillin treatment, (35, 36) iron deprivation,

(37) and HSV co-infection or nutrient starvation (33, 34, 38, 39). In this state, chlamydiae are believed to have arrested all metabolic functions.(33) Upon removal of any of the aforementioned factors, the metabolic functions resume and the normal life cycle is reestablished (33, 34).

Immune responses

In vitro studies have demonstrated that IFN-γ has several modes of action that seem to limit CT replication and can lead to clearance of infection. First, IFN-γ can upregulate indoeamine-2,3-dioxygenase (IDO), a host cell enzyme that degrades tryptophan stores (40). Second, IFN-γ can upregulate the expression of inducible nitric oxide synthase (iNOS), an enzyme that catalyzes nitric oxide NO and has been shown to be an important antibacterial molecule (41-43). Third, IFN-γ upregulates transferrin receptor expression on infected cells causing iron deficiency, which in turn limits CT replication (44). However, CT has devised at least one means to escape IFN-γ induced killing by using a tryptophan synthase to convert indole into tryptophan. This survival mechanism has been demonstrated for CT serovars D-K (trachoma serovars A-C do not have the tryptophan synthase) (45).

It has been previously shown that infection of epithelial and dendritic cells with CT led to the production of IL-18 and IL-12 in these cells and synergistically (IL-12 upregulates IL-18R production) induced IFN-γ production by natural killer (NK) cells (46). This was an interesting finding that demonstrated the contribution of IFN-γ by an innate immune cell and the potential role NK cells may play in controlling CT infection. In 2010, a study conducted on human fallopian tube cells revealed that hypoxia could nullify the

antichlamydial properties of IFN-γ and allowing CT to grow (47). A later study evaluated host immune responses under reactivated CT growth in hypoxic conditions and confirmed there was diminished IFN-γ, IL-6 and IL-8 production under hypoxic conditions (48). In 2015, an *in vitro* study revealed that the combination of IL-22 and TNF-α, both Th22 cytokines, may have a protective role against CT infection. This study demonstrated that murine oviduct epithelial cells produced Th22 cytokines that were synergistically able to inhibit CT growth while maintaining epithelial survival (49). Overall, *in vitro* studies have provided great insight on IFN-γ mechanisms that control CT infection and have also revealed mechanisms of CT persistence, reactivation, and immune evasion.

Chlamydia in vivo (animal)

Murine model immunology

Highlighted in this section are select studies in chronological order that brought us to our current knowledge of chlamydia immunity based on the murine model, which has established the paradigm for immune responses, and was initially developed by Barron et al in 1981 (50). Since then, studies have investigated the major immune responses responsible for protective and pathologic responses, infecting mice with either CT or *C. muridarium* (a murine specific species of chlamydia).It was established that mice naturally clear genital chlamydial infections in roughly 4 weeks and develop protective adaptive immunity (31). A study in gene-knock out mice by Morrison et al. in 1995 found that knocking out MHC class II and CD4 led to failure or delayed clearance of CT infection (51). Another study around the same time revealed that local Th1-like responses are

induced by genital infection in mice, as evidenced by $CD4^+$ T cells secreting IFN-γ and IL-2 (11).

Subsequently, studies focusing on T cell dynamics during active CT infection began to emerge. In 1998, a mouse model with ascending CT infection after intravaginal inoculation demonstrated the recruitment of MHC class II antigen presenting cells (APCs) into genital tissue during early infection and sensitizing the already implicated Th1 $CD4^+$ T cells in order to prevent ascending infection (13). Incidentally, later that year Igietseme et al. demonstrated that the route of infection (intranasal, intravaginal, oral, and subcutaneous) determined the robustness of CMI responses, with intravaginal and intranasal routes able to induce IFN-γ secreting T lymphocytes and intranasal inducing higher antibody responses (10). Not long after, in another study where the IFN-γ gene had been knocked out, it was demonstrated that IFN-γ was required for chlamydia clearance. Upon adoptive transfer of cytotoxic T lymphocytes (CTL) into mice with or without the defective IFN-γ gene, it was determined that CTL alone were not sufficient to overcome the defect, but were required for the protective effect seen in the mice without the defect (52). Subsequently in the following year, a murine model study determined that resolution of CT infection in the murine genital tract did not require $CD4^+$ T cell Fas-mediated apoptosis or $CD8^+$ T cell perforin-mediated cytotoxicity (53). In 2000, a study revealed a differential regulation of $CD4^+$ T cell recruitment between the upper and lower genital tract that revealed that these cells played different roles at these sites and could be linked in protective immune responses and pathologic inflammatory responses (tubal pathology) (14).

By the turn of the century, numerous studies had been conducted regarding immune responses to CT infections in mice. In 2002, a comprehensive review by Morrison et al. informed that common to murine CT genital tract infections were: 1) the development of protective immunity; 2) the development of histopathology in the upper genital tract; 3) the importance of the Th1 $CD4^+$ T cells in clearance and protective immunity; 4) the induction of CT-reactive CD8+ T cells following infection that were not required for clearance; and 5) an unclear role of B cells and antibody in protective immunity (31). A 2005 study that assessed the induction and development of mature dendritic cells (DCs) using *C. trachomatis* EBs that were either live or UV irradiated proved that the use of live EBs allowed for the development of phenotypically distinct DCs and more effective for strongly promoting protective immunity (54). The following year, a study was conducted evaluating antigen-specific $CD8^+$ T cell responses at the genital mucosa of retrogenic mice and found these T cells proliferated in the draining lymph node and then migrated to the genital mucosa and produced IFN-γ. The study also found that the activation of naïve $CD4^+$ T cells occurred outside the genital tract (55).

Murine studies in subsequent years pushed for improved understanding of the immunogenicity of CT proteins in order to aid in vaccine development. One such study evaluated $CD8^+$ T cell epitopes using MHC class I tetramers against CT and found consensus epitopes that could be used in vaccine studies (56). Another study assessed CT MOMP along with vaccine adjuvants and demonstrated that a high frequency of dual positive T cell populations ($CD4^+$IFN-γ^+TNF-α^+ and $CD4^+$IFN-γ^+IL-17^+) helped confer protection against infection (57). These studies solidified MOMP as a strongly immunogenic CT antigen and a great vaccine candidate.

More recent studies evaluated the role of different cytokines that may play a role in the immune response in chlamydial infections. In particular, one study in 2013 demonstrated that IL-23 induced IL-17 and IL-22 production during *C. muridarium* genital infection, however the absence of these cytokines had little to no influence on disease pathogenesis (58). Another study in early 2015, demonstrated that an excess production of proinflammatory cytokines IFN-γ and IL-12 impaired memory $CD8^+$ T cell development by differentiating T lymphocytes into short lived effector cells (SLEC) rather than the desired memory precursor effector cells (MPEC) (59).

Up to this point, a great deal had been established in the murine model regarding T cell immune functional responses. However, it wasn't until 2010 in a study that evaluated mRNA levels of chemokine receptor genes upregulated during active infection that a chemokine receptor double knockout mouse was developed (60). This knockout established that CXCR3 and CCR5 together were essential for T cell trafficking to the murine female genital tract (60). A later study in 2015 by Noguiera et al. that used the CXCR3 and CCR5 double knock out mouse model demonstrated that protective immunity against CT could be induce both CD4 and CD8 T cells by priming intranasally and challenging intravaginally. This study strengthened the notion of cross mucosal protective responses that would be important in later studies (61). Later in 2015, a mucosal vaccine candidate against CT that generated two waves of protective memory T cells was developed by Stary et al. using UV-light-inactivated CT (CT-Uv) conjugated to charge-switching adjuvant nanoparticles (cSAPs) (62). Upon immunizing the mice with various conjugates, they characterized the mucosal immune responses to uterine CT re-challenge, demonstrating that previously developed circulating effector T cells and other effector T

cells that trafficked to the genital mucosa and establish tissue resident memory (T_{RM}) cells were either re-localized or reactivated (respectively) in response to active infection and were essential for CT clearance and long-lived protection (62).

More recent studies evaluating CT immunity in the murine models explore aspects of innate and partial immunity. One evaluated the role of NK cells in regulating the balance between T regulatory (T_{Reg}) cells and either Th1 or Th17 cells in chlamydial lung infection (63). Another revealed that induction of partial immunity by a MOMP vaccine in both partners (male and female) sufficiently protected females against sexual transmission of CT through a synergistic effect that allowed for the development of sterilizing immunity (64).

Other animal models

While our understanding of chlamydia immunity is largely shaped by studies in the murine model, other animal models, including the guinea pig, pig, and macaque have also contributed knowledge in this area. The murine model is primarily used because it is low cost and provides a means to tease out various mechanisms, but the duration of infection greatly differs from that of humans (65). Guinea pigs are naturally infected by *C. caviae*. Guinea pigs are more relevant models for humans than mice because they have similar estrous cycles and $CD8^+$ T cell infiltration patterns to humans, however cost and upkeep of guinea pigs is more demanding than for mice (65, 66). Another animal model is the pig, which is naturally infected by *C. abortus* and *C. suis*, but can also be infected by CT. Pigs are excellent models for studying genital CT infections because they have very similar

reproductive tracts to humans, but natural pig infection does not lead to tubal infertility (65).

The final model that can be used in studying CT is the pigtailed macaque, which is naturally infected with CT. Unlike in mice where the predominant protective T cell is $CD4^+$, in macaques $CD8^+$ T cells predominate and loss of these cells impairs protection (67, 68). Macaques are able to develop CT-related scarring and fibrosis in their fallopian tubes, making them excellent models from that perspective. However, they are very costly to maintain (65, 69). Ultimately, animal models have provided much insight into understanding immunity to chlamydiae, however differences in these models (e.g., infectious species, estrous cycle, T cell distribution frequencies, etc.) make translating findings to humans difficult. Therefore, studies focused on elucidating the function and phenotype of immune cells in CT infection is humans are warranted.

Chlamydia in vivo (human)

Disease epidemiology

There are over 131 million new CT infections annually worldwide and CT is the most prevalent bacterial sexually transmitted infection (STI) worldwide (2). In the U.S., >1.5 million CT cases are reported annually, (70) however it is estimated that up to 3 million cases actually occur annually (71). Centers for Disease Control and Prevention (CDC) surveillance data has shown that African Americans are disproportionately affected by CT infection, having a 6-fold higher rate than Caucasians (70). Women are also disproportionately affected by CT infection because of their increased risk for upper genital

tract complications such as pelvic inflammatory disease (PID), which can lead to chronic pelvic pain, ectopic pregnancy, and infertility; CT is a leading preventable cause of infertility worldwide. CT infection in pregnant women can lead to complications during child birth, including miscarriage, stillbirth, and premature labor, and neonatal complications of conjunctivitis and respiratory disease. Additionally, CT infection increases the risk for HIV transmission, likely in part by localizing $CD4^+$ T cells that express CXCR4 and CCR5 (well known CKRs that are involved in HIV viral entry) to the endocervix (72). Therefore, it is important to study and understand CT infection in humans. Highlighted below are studies focused on the natural history, clinical pathology, and limited protective immunity in humans developed CT infection.

Natural history

The natural history and progression of CT infection in humans is highly variable. Several studies have evaluated clearance of CT infection in humans over time. Untreated CT infection in humans can persist for months to years, as evidenced by a study in 2005 that retrospectively tested urogenital specimens collected from women every 6 months for several years (73). They demonstrated that among 82 women with CT infection at baseline, 54% naturally cleared infection after one year of follow-up and 94% had cleared after 4 years (73). Two other retrospective studies revealed that CT resolution was predicted by older age, (74, 75) and another demonstrated that race and days since sexual activity played a role in CT resolution (76). Several prospective studies did not find associations of resolution with age, testing interval, or prior CT infection (77-80). However, one prospective study revealed a trend toward higher resolution with longer testing intervals,

male sex, and prior CT infections (8). These studies highlighted natural clearance of untreated CT infection in which some women had cleared infection between their initial screening visit and a visit when they returned for treatment of the positive screening test, a phenomenon termed spontaneous resolution of infection of CT infection.

Spontaneous resolution of CT infection is particularly interesting because despite disease clearance in some instances, these individuals may still become reinfected and develop complications (74, 75). One prior study revealed that spontaneous resolution of genital CT infection occurred in about 20% women who returned 1 to 12 months after treatment for repeat testing. The study also determined that spontaneous resolution was associated with decreased reinfection risk, with about a four-fold lower reinfection rate in those with spontaneous resolution of CT infection vs. persisting infection at the time of presenting for treatment of a positive CT screening test (81). Nonetheless, despite there being a group of individuals who can resolve CT, most have persisting infection that could lead to the development of adverse outcomes.

Clinical pathology

CT infection is a major cause of preventable infertility. Over one million women in the U.S. suffer from PID annually (82). Approximately 10-15% of CT-infected women develop symptomatic PID and approximately 18% of women who develop PID progress on to infertility (83-86). There are varying estimates of CT-associated tubal factor infertility (TFI; blocked fallopian tubes) that exist. One statistical modeling study reported ~45% of TFI cases are attributed to CT infection (87).

Various studies have been conducted assessing CT immune responses associated with TFI. Over the years, studies have been conducted linking various CT antigens to TFI while also seeking to determine immune correlates of pathology. A 1998 study revealed that 23 women with TFI had positive ELISA tests for CT HSP60, associating this CT protein with fallopian tube damage (88). Two years later, another study supported the finding that HSP60 is an important antigen in studying chlamydial pathology in patients with PID and TFI patients, while also confirming that in humans Th1 cells primarily produce IFN-γ against CT infection (89). A follow-up study by the same group in 2003 revealed that along with IFN-γ, HSP60 also induced IL-10 production (90). In subsequent years studies continued to look at tubal pathology associated with CT HSP60 (91). One particular study demonstrated that IL-1, produced by epithelial cells, induced IL-8, a chemoattractant for neutrophils. This study proposed that IL-1 is the initial proinflammatory cytokine activated by active CT infection and blocking of this cytokine eliminated pathology (92). Two studies were conducted to determine CT antigens in women with acute infection and in women with and without TFI. Both studies confirmed the HSP60 was important for the development of TFI along with 3 other CT antigens (e.g., CT443) (93, 94). Recently, a study in Nigeria evaluated differences in 150 women divided into 3 groups as follows: 50 CT positive infertile women, 50 CT positive fertile women, and 50 CT negative women serving as controls. This study found higher IL-10 but lower IFN-γ levels in infertile vs. fertile CT positive women, a cytokine profile which may contribute to the development of TFI (ref). Overall these studies improved our understanding of CT-related immunopathology and demonstrate the need for improved control measures in order to prevent CT-related TFI.

Immune protection

Our understanding of cell mediated immunity that may be associated with protection and pathology caused by CT infection in humans is rather limited. Some humans (~15-20%) continue to have repeat CT infections within months after treatment, (95, 96) possibly indicative of little or no protective immunity. Yet, others do not become reinfected suggesting in some cases they may have some degree of protective immunity. Limited studies have evaluated mucosal and peripheral T-cell immune responses in CT- infected humans (9, 97-99). However, these studies have not comprehensively investigated T-cell immune responses interrelated to clinical correlates of protection. There are scant studies that affirm murine model findings by implicating IFN-γ as contributing to protective immunity to CT infection in humans. In particular, two human studies reported a peripheral CT-specific IFN-γ response correlated with protection against initial and repeat CT infection, but they did not look at reproductive complications of CT or mucosal immune responses (99, 100). Barral et al. stimulated PBMCs with various antigens including MOMP and then using enzyme-linked immuno-spot assay (ELISPOT) assays demonstrated that IFN-γ not IL-17 was associated with protection against CT reinfection in 42 sexually active adolescent females (99). Cohen et al. showed that IFN-γ production by PBMCs stimulated with CT heat-shock protein (HSP) 60 correlated with protection against incident infection in commercial sex workers, many who likely had prior infection (100).

Recent studies have elucidated various aspects of CT-specific immune responses in the peripheral and mucosal compartments for both T cell subsets in humans. A study by Agrawal et al. using CT HSP60 evaluated both mucosal and peripheral immune responses

in women with primary and recurrent CT infection and determined that mucosal immune responses were marked by acute inflammation, unlike those in PBMCs where there were no observable differences in inflammatory markers. Interestingly, this study showed that IFN-γ was the only cytokine found to be involved in modulating CT infection directly by causing acute inflammation and indirectly by modulating heat shock protein expression (101). A follow-up study by Ondondo et al. compared differences in IFN-γ production in systemic and mucosal mononuclear cells taken from commercial sex workers and stimulated with EB and 3 genetically variant HSP60 proteins. They found that at both sites, IFN-γ responses correlated with preferential targeting of $CD4^+$ T cells that largely mediated Th1 and to some degree Th2 responses (98). Subsequently, Ibana et al. evaluated the cytotoxic function of $CD8^+$ T cells in CT-infected women, assessing perforin production, and found that CT-infected women had low perforin content in their $CD8^+$ effector memory cells, which likely reflects the unique microenvironment involved in immune modulation of protective responses and tolerance (102). Together these studies recapitulated various aspects of CT-specific immune T cell functions and laid the foundation for more targeted studies.

Most recently, there have been studies focused on understanding the role of other cytokines such as IL-17 that may that may be protective as well as antigens that may be linked to clearance. A study evaluating the efficacy of antigens for developing a CT vaccine stimulated PBMCs with inactivated CT EBs and measured IFN-γ and IL-17 by ELISPOT. It determined that the presence of circulating CD4 IFN-γ memory T cells was greatly reduced 2 months after CT infection diagnosis, suggesting that an effective vaccine would require a stronger response than that elicited by natural infection (103). In the past year,

one study sought to determine the role of both $CD4^+$ and $CD8^+$ T cells in CT infection resolution after natural exposure by screening using a CT proteomic library. This study identified specific T cell responses associated with CT infection resolution and also identified antigens specific for spontaneous resolution.

Introduction Summary. There are many cases of untreated CT infections, largely because most infected women are asymptomatic and infection may be missed without screening. Thus, the CDC recommends annual CT screening in all sexually active women age <25 years and older women with risk factors (104). Prevention and control efforts (education, testing, treatment, etc.) have not reduced the high CT infection prevalence (105-107), supporting the need for a CT vaccine. However, efforts to develop CT vaccines have been hindered in part by an incomplete understanding of immunity mediating pathologic and protective responses to CT in humans.

Ultimately, CT infection control efforts have been ineffective in reducing the high Ct infection prevalence; a vaccine is needed. Animal studies suggest IFN-γ-producing Th1-associated cellular immune responses play the primary role in protective immunity. Protective immune responses to Ct in humans remain to be fully elucidated. A CT vaccine could significantly help combat CT infection, thus preventing adverse outcomes associated with infection such as infertility. The next step in expediting vaccine development is increasing our understanding of CT immune responses and characterizing T cell phenotypes (CKR expression) as highlighted in this dissertation. Highlighted in the section below are specifics on how T cells are characterized as wells as CT specific T cell phenotypes in murine models and humans.

Phenotypic chemokine receptor characterization

T cell phenotypes background

Before discussing CT-specific T cell phenotypes, it is important to first delve into some specifics about T cell subset characterization and T cell phenotyping based on CKR expression, cytokine production, and master transcription factors. Additionally, below are brief descriptions about the receptors used in this dissertation in order to provide a foundation for further discussion in the subsequent sections and chapters. Moreover, this section also discusses T cell phenotypes specific to chlamydia by establishing the known paradigm in murine models while revealing knowledge gaps in human studies.

T cell subset characterization. To date, various subtypes of $CD4^+$ T cells have been described including but not limited to: Naïve $CD4^+$ T cells (T_N), Th1, Th2, Th9, Th17, Th22, T_{Reg}, and follicular helper T cells (T_{FH}). The three primary approaches used to characterize these subtypes of T cells include: phenotype (surface marker expression), cytokine (polarizing and effector) production, and master transcription factors. The first two approaches to characterizing T cells are primarily observed by staining with antibodies and have a designated cluster of differentiation (CD) for specific proteins (e.g., chemokine receptors or cytokines). Phenotype is usually defined by surface marker expression of chemokine receptors and other CD receptors on the cell surface. Polarizing cytokines induce the proliferation of naïve T cells into specific T cell subsets, which then produce specific effector cytokines. The final characteristic is one that either the mRNA or the actual protein can be stained for, but there are limitations involved with looking at mRNA alone due to post-translational modifications. Therefore, looking at specific protein

expression using flow cytometry techniques (cell surface or intracellular cytokine staining) or quantitative enzyme-linked immunosorbent assay ELISA along with qRT-PCR may be the best means to comprehensively characterize T cells.

Chemokine receptors. Chemokine receptors are membrane bound proteins with 7 transmembrane domains coupled to trimeric G proteins that play a role in signal transduction and function as potent chemoattractant and activators of leukocytes. The N-terminus is extracellular, while the C-terminus is intracellular and contains some amino acid residues that can be phosphorylated by G-coupled receptor kinases (108). Chemokine receptors play an essential role in inflammatory migration of T-cell subtypes and because CKRs expression varies with microenvironments, tissue localization, or even with disease states, they can also play a key role in cell phenotyping (109). $CD4^+$ T_N are marked by the expression of CKR CCR7 (110). CXCR3 and CCR5 are CKRs that are expressed on T cell subsets and may denote immune cells that traffic in response to inflammation (e.g., Th1 responses) (109, 111-113). Comparably, CCR4 is a predominant phenotypic CKR associated with Th2 responses, with contributions from CXCR3 and CRTH2 (109, 111-114). However, there are no chemokine receptors that have been associated with Th9 (115). The CKRs CCR4, CCR5, and CCR6 are co-expressed on Th17 cells (116). Similarly, CCR4 and CCR6 along with CCR10 are the phenotypic CKRs for Th22 cells (117). T_{Reg} ($CD4^+CD25^+FoxP3^+CD127^{+/-}$) exist in various forms, including naïve (n) T_{Reg}, T_{Reg} effector ($T_{Reg\text{-}Eff}$), and effector memory ($T_{Reg\text{-}EM}$). These various T_{Reg} cell forms have variable CCR7 CKR expression with $T_{Reg\text{-}Eff}$ being generally negative, nT_{Reg} being positive, and $T_{Reg\text{-}EM}$ being either positive or negative (118). On the other hand, T_{FH} cells are known

for their role in antigen specific B-cell immunity and predominantly express CKR CXCR5 as a phenotypic marker (4). There exists some debate about the use of chemokine receptors to phenotype cells, particularly regarding Th1 and Th2 subtypes where polarizability between the two exists (113, 114, 119).

Cytokines. Another aspect to consider when characterizing T-cell subtypes is cytokine production. Cytokines are effector small proteins secreted by immune cells. Cytokines that aid in T-cell characterization can be classified as either polarizing (activating) or effector (produced) cytokines. $CD4^+$ T_N cells are polarized or activated by APC to produce IL-2. Specific activation of T_N cells can result in various subtypes depending on the activating cytokine. Stimulation of $CD4^+$ T_N with IL-12 + IFN-γ polarizes T_N cells towards Th1 T cells producing IFN-γ and TNF-α (113, 114, 119). Similarly, stimulating $CD4^+$ T_N cells with IL-4 polarizes T_N cells into Th2 T cells that produce IL-4, IL-5 and IL-13 (109, 112-114, 118, 120). Likewise, stimulating $CD4^+$ T_N with IL-4 and TGF-β polarizes T_N cells into T_H9 cells that produce IL-9, (115) and stimulating with IL-1, IL-6, IL-23 and TGF-β polarizes T_N cells towards Th17 cells that produce IL-17, IL-21, IL-22, IL-23, IL-25, and IL-26 (116). The use of IL-6 and TNF-α facilitates the formation of TH22 cells that produce IL-22, TNF-α, and IL-2+TGF-β, resulting in T_{Reg} cells producing IL-10 and TGF-β (110, 115, 117, 118, 120). The formation of a T_{FH} cell requires IL-6 plus IL-21, resulting in T_{Reg} cells that produce IL-21 (4, 110, 118, 120). The combined use of CKRs and cytokines to characterize T cells provides us with a more specific picture of the specific T-cell subsets. However, because there are various subtypes that produce the same cytokines and chemokines, sometimes another step is required in order to fully characterize specific T-cell subtypes.

Master transcription factors. The third and final means to characterize T cells involves looking at the expression of the master transcription factors that facilitate T subset formation. Transcription factors are proteins that help regulate gene expression, allowing for DNA to be transcribed into RNA and ultimately protein. Master transcription factors associated with T-cell subtypes can be assessed at either the mRNA or protein levels. The transcription factors associated with various T-cell subsets are as follows: T-Bet → Th1; GATA3 → Th2; IRF4 → Th9; ROR-γt → Th17; AHR → Th22; Fox-P3 → nT_{Reg} and T_{Reg-EM}; Bcl-6 → T_{FH} (4, 109, 110, 115, 117, 118, 120). Uniquely, Fox-P3 lineage $T_{Reg-Eff}$ has plasticity that allows for further polarization using (Tbet/GATA3/ROR-yt) into their respective T-cell subtypes (118). The combined use of CKRs, cytokine production, and master transcription factor expression provides the best means to fully characterize T-cell subtypes.

Applications. Perhaps debate on CKRs used for characterizing T cell phenotypes exists because the migration of CKRs associated with a specific phenotype vary based on disease states or are tissue-specific (121, 122). There is evidence for disease-specific phenotypes, including a review where HIV, HSV-2, and CT were compared for chemokine receptor expression in the female genital tract (123, 124). Nanki et al. reported that there was a lack of correlation between CKRs in Th1/Th2 cytokine expression, but they focused primarily on mRNA expression and therefore did not account for posttranslational modifications (125). This raises an interesting question because CXCR3 in T cells has been demonstrated to have various functions, in particular the most predominant is expression and trafficking

of Th1 cells, establishing CXCR3's vital role in this subset (126). Additionally, cytokine production is an excellent approach to characterize T cell subtypes, however, if used alone this approach can also be confounded by the variability in cytokine levels based on disease or tissue. Transcription factors are outstanding because one is looking directly at gene expression, but there still exists some debate because looking only at mRNA does not account for post-transcriptional and post-translational modifications. Most papers employ phenotypic and cytokine production characterization techniques (109, 117). In particular, the heterogeneity of memory T cells in peripheral blood and tissues demonstrates the importance of using CKRs in order to further understand these subsets (127). Ultimately, the best and most comprehensive means for T-cell characterization would be to employ at least two or three of the aforementioned characterization approaches. However, if chemokine roles are well-established within an animal model for a specific disease, it may be worthwhile to assess the expression of those chemokine receptors on human T cells.

Table 1

T cell subset characterization

T-Cell Subtype	Phenotype (CKRs)	Polarizing Cytokines	Effector Cytokines	Master Transcription Factor	T Cell Receptors and CDs	Primary Function
TN	**CCR7+**	APCs	IL-2		CD3+CD4+ CD45RA+	Proliferation and differentiation
Th1	**CXCR3+** CCR5 +CCR4-	IL-12, IFN-γ	IFN-y, TNF-α	**T-Bet**	CD3+CD4+	Production of pro-inflammatory cytokines
Th2	**CCR4+** &CRTH2+	IL-4	IL-4, IL-5, IL-13	**GATA3**	CD3+CD4+	Production of anti-inflammatory cytokines; promote allergic response; evoke strong antibody response
Th9	CCR9	IL-4, TGF-β	IL-9	**IRF4**	CD3+CD4+	Humoral immunity through B cell interactions; functions on many cell types including mast cells
Th17	CCR4+CCR5+ CCR6+	IL-1, IL6, IL-23, TGF-β	IL-17, IL-21, IL-22, IL-25,IL-26	**ROR-yt**	CD3+CD4+	Proliferation and differentiation
TH22	CCR4+CCR6+ CCR10+	IL-6, TNF-α	IL-22, TNF-α	**AHR**	CD3+CD4+	Mucosal immune response; inflammatory diseases; barrier defense
nTReg	CCR7+	TGF-β, IL-2	IL-10, TGF-β	**Foxp3**	CD3+CD4+ CD25+CD45RA+ CD45RO- CTL4- CD127-	Maintenance of self-tolerance
TReg-Eff	CCR7-	TGF-β, IL-2	IL-10, TGF-β	**Foxp3** +(Tbet/GATA3/RORyt)	CD3+CD4+CD25+CD45RA-CD45RO+ CTL4+CD127-	Maintenance of self-tolerance
TReg-EM	CCR7+/-	TGF-β, IL-2	IL-10, TGF-β	**Foxp3**	CD3+CD4+ CD25+CD45RA-CD45RAO- CTL4+CD127+	Maintenance of self-tolerance
TFH	CXCR5	IL-6, IL-21	IL-21	**Bcl-6**	CD3+CD4+	Antigen specific B cell immunity

Chemokine (C-X-C motif) receptor 3 (CXCR3). CXCR3 is known to bind 3 chemokines (126). CXC-Ligand(L)9, also known as monokine induced by gamma-interferon (MIG); CXCL10, also known as interferon induced protein (IP-10); and CXCL11, also known as interferon inducible T cell alpha chemoattractant (I-TAC) (128). Once any of the three ligands bind CXCR3, they are able to induce migration of activated T cells to mucosal sites. CXCR3 plays a major role in the localization of effector and regulatory T cells and plays a role in the generation of memory T cells (126, 128). Most notably, CXCR3 has

been shown to be preferentially expressed along with CCR5 on Th1 CD4+ IFN producing T cells in response to certain inflammatory reactions at mucosal sites (12, 122, 126, 128, 129).

Chemokine (C-C motif) receptor 4 (CCR4). CCR4 is a chemokine receptor that binds the following five chemokines: chemokine ligand (CCL) 2 or monocyte-chemoattractant protein 1 (MCP 1); CCL4 or macrophage inflammatory protein 1 (MIP 1); CCL5 or regulation on activation, normal T cell expressed and secreted (RANTES); CCL17 or thymus- and activated regulated chemokine (TARC); and CCL22 or macrophage derived chemokine (MDC) (12, 129-132). CCR4 is primarily expressed on Th2 CD4 IL-4 secreting T cells and is associated with homing to the skin (12, 122, 129, 130, 133). Interestingly, CCR4 has been shown to be upregulated on CD4 T cells in the peripheral blood of patients with Crohns disease and was significantly correlated with disease activity (134).

Chemokine (C-C motif) receptor 5 (CCR5). The CCR5 receptor binds CCL3 or MIP1-alpha, CCL4 or MIP1-beta, and CCL3L1 as well as CCL5, also known as RANTES (129, 130, 135-137). CCR5 can be a HIV co-receptor along with CXCR4, that serves to facilitate viral entry (138). Functional CCR5 expression on T cell has been shown to be beneficial in preventing adverse pathology associated with CT, yet interestingly the delta-32 mutation on CCR5 which renders it non-functional is beneficial in preventing HIV entry (139, 140). However, CCR5 is also a chemokine receptor that is expressed on T cells along with CXCR3 on Th1 CD4+ IFN producing T cells in response to inflammation (12, 128-130).

Chemokine (C-C motif) receptor 7 (CCR7). CCR7 is a secondary lymphoid homing receptor the binds the following chemokines: CCL19 (or MIP 3-beta) and CCL2121, primarily produced by lymph nodes, stromal cells in the spleen T zone, and Peyer's patches (141). CCR7 enables T cells to migrate to lymphoid organs and receive antigens by APCs (141-143). In humans CCR7 helps in segregating two different types of memory T cells: central and effector memory (144, 145). Although antigen experienced, central memory cells express CCR7 and lack immediate T cell effector function, while effector memory cells downregulate CCR7 expression and readily produce cytokine upon antigen re-sensitization (142-145).

Cluster of differentiation 38 (CD38). CD38 is a lymphocyte ectoenzyme that can catalyze beta-NAD+ into nicotinamide via its extracellular domain (146). CD38 has a short intracellular tail and only known natural binding protein ligand is tumor necrosis factor ligand (TNF-L) (146). CD38 exhibits signal transduction properties such as being able to regulate intracellular calcium levels. CD38 serves as an activation marker and is most notably used to assess HIV T cell activation status and as a prognostic marker in leukemia (146-149).

Human Leukocyte Antigen-D Related (HLA-DR). HLA-DR is a major histocompatibility (MHC) class II cell surface protein which along with its +9-mer ligand peptide form a T cell receptor (TCR) ligand (150). Generally, HLA-DR molecules are upregulated during signaling and therefore allow it to serve as an excellent activation maker (151). HLA-DR

along with CD38 have been used dual activation markers for studies correlating viral load in HIV and HSV-2 studies with degree of inflammation (149, 150, 152-154).

Table 2

Phenotypic receptors, their ligands and function

Receptor	Ligands	Function
CXCR3	CXCL9 (MIG); CXCL10 (IP-10); CXCL11 (I-TAC)	Homing of Th1 T cells to mucosal sites
CCR4	CCL2 (MCP 1); CCL4 (MIP 1b); CCL5 (RANTES); CCL17 (TARC); CCL22 (MPC)	Homing of Th2 T cells to the skin and lungs
CCR5	CCL3 (MIP 1a); CCL4 (MIP 1b); CCL3L1; CCL5 (RANTES)	Homing to inflammatory sites (with CXCR3) is Th1 associated
CCR7	CCL19 (MIP 3b); CCL22 (MPC)	Homing to secondary lymphoid organs and memory segregation
CD38	TNF-L	Activation maker
HLA-DR	≥9-mer peptide	Activation maker

T cell phenotypes in CT infection

Leukocyte migration is essential for immune surveillance of various sites in the body as modulated by chemotactic cytokines (chemokines) (128). Chemokines serve as factors that regulate the induction, recruitment, and persistence of immune effector cells in the genital mucosa during chlamydial infections (155). Understanding the trafficking and activation of CT-specific T cells after infection is critical in developing therapeutic interventions that facilitate protection. Early studies that sought to improve our understating of homing receptors that mediate the recruitment of T cells in the murine models demonstrated that chemokines along with integrins played a major role in $CD4^+$ T cell localization to the murine genital tract during CT infection (14, 155).

A key murine study demonstrated that concomitant expression of both CXCR3 and CCR5 are required for T-cell mediated immunity against CT in the genital tract (60). CXCR3 directs T-cell migration inducing cellular responses that drive chemotactic migration. CXCR3 along with CCR5, are both classified as inflammatory homing receptors, that are preferentially expressed on Th1 T cells. Similarly, CXCR3 and CCR5 help in propagating inflammatory dynamics and immune activation in the genital tract of CT-infected mice, facilitating clearance of infection (60, 128, 155). These studies currently set the paradigm for CKRs responsible for T cell trafficking in the murine models. Chemokine mediated immune responses in murine genital tract revealed that following infection, Th1 $CD4^+$ T cells become activated, actively proliferate and are recruited to the genital mucosa, where they serve as the major producers of IFNγ (11, 31, 60).

Initial studies have demonstrated that integrin α4β1 was also required for $CD4^+$ T cell trafficking. One particular murine study revealed that CD4+ T cells robustly produce α4β1, but not α4β7, and that knocking out α4β1 resulted in defective trafficking to the mucosal site as well as a high CT load (156). In mice, a CD8+ T cell response has only a minimal role on primary CT clearance despite subsequent expansion (61). In mice T cells, $CD8^+$ T cells are able to control bacterial replication if elicited by immunization before CT infection. Ultimately, in murine CT infection, CD4 T cells along with the chemokine receptors CCR5, CXCR3, and integrin α4β1, have been shown to be essential for T cell recruitment into the genital mucosa, however the majority of trafficking mechanisms in remain to be elucidated (157).

In humans, much of chemokine receptor dynamics remains to be understood. One study revealed that both in mice and humans, the dysregulation of CCR5 receptor function

during chlamydial infection likely resulted in adverse pathology such as TFI (139). Another study showed that functional CCR5 protects against CT-related arthritis in patients with high synovial bacterial burden (140).

A key human study evaluating limited T cell phenotypes showed that HLA-DR levels are higher at the mucosal site compared to blood. T cell HLA-DR levels remain the same in the blood at time of infection and at a first follow-up visit (median of 35 days) after treatment (158). They also found CCR5 was elevated in both infected and treated cervical T cell populations compared to blood (138, 158). This study also found that the homing marker CCR7 was expressed at lower levels in CT-infected women compared with controls (158), suggesting that T cells had already homed to secondary lymphoid organs from the peripheral compartment (144). Thus, CCR7, a chemokine receptor that controls homing to secondary lymphoid organs and divides memory T cells into two distinct subsets, was found to be significantly higher in the human peripheral blood vs. mucosal endocervical cells (145, 158). This study also investigated two integrins, alpha E beta 7 ($\alpha_E\beta1$) and $\alpha4\beta7$, known be involved in tethering and rolling, and found that overall there was a higher expression of $\alpha_E\beta1$ on CD4 and CD8 T cells at the endocervix than the peripheral blood but a lower expression of $\alpha4\beta7$ at the endocervix than the periphery (158).

Primed cells are said to be in an active state and markers that help determine this state are known as activation makers. For example, the activation status in HIV and HSV2 immune studies, CD38 and HLA-DR have been frequently used as markers of cell activation and directly correlated to viral load (149, 153, 154). There are sparse chlamydia studies that use both CD38 and HLA-DR in conjunction as markers of activation and possibly correlate them to CT load. Limited attention has been given to CCR4, a chemokine

receptor that serves to regulate leukocyte trafficking and has been implicated in T helper type 2 (Th2) responses. The balance between Th1 and Th2 is crucial in determining the outcome of physiological and pathological responses that can be associated with CT infection (129, 133). Although limited, activation marker HLA-DR, chemokine receptors CCR5 and CCR7, and integrins $\alpha_E\beta1$ and $\alpha4\beta7$ have been shown to play some role in CT-infection in humans. Nonetheless, in humans there is still much to be understood about the role of Th-associated and homing chemokine receptors and activation markers on both CD4 and CD8 T cells during CT infection.

Aims of the dissertation

There is limited knowledge on immunological responses to CT infection in humans. Immune mechanisms contributing to CT clearance and immunity in humans have not been fully elucidated. It is unclear how much knowledge of immune mechanisms to CT gained through animal studies is applicable to human CT infection. The CT epidemic continues despite prevention and control measures; a CT vaccine is needed. The gained understanding from this study should increase our overall knowledge about CT trafficking and CKRs in humans in order to expedite vaccine development. The knowledge gained form this study could be used in developing an efficacious vaccine that involves the co-administration of chemotactic factors and vaccine adjuvants, which may boost APCs sensitization and thus propagate a more robust immune response while bolstering efficient recruitment of effectors to clear the infections (155).

Differences in chemokine receptor expression

In order to improve our understanding of T cell phenotype distribution we devised three aims for this study. The first aim of this study is to compare the T cell phenotypic distribution between individuals that have never been infected with chlamydia (CT seronegative controls) and women with active chlamydial infection in order to ascertain differences in expression patterns. The overall hypothesis is that women with CT-infection likely have higher expression of CKRs and activation markers than seronegative controls. This is likely because CT infection elicit T cell priming or activation and CKR expression in those actively infected in order to prepare them for homing to the site of infection.

The second aim of this study is to evaluate differences in peripheral blood T cell phenotypes in paired blood specimens from women with active CT infection at a pre-treatment visit versus phenotypes in these same women at 3- and or 6-month follow-up visits where these same women may or may not have CT reinfection. The hypothesis is that CT infection leads to changes in T cell phenotypes that are further influenced by clearance or presence of reinfection after treatment. Therefore, at the subsequent visit it may not be necessary to localize as many cells and we would therefore see decreased CKR expression over time. In reinfected women with poorly established immune responses, homing CKR expression may be severely dysregulated and therefore effector T cells may not reach the site of infection.

Site-specific differences during chlamydial infection

The third aim is evaluating site specific differences in T cell phenotype distribution between the peripheral and mucosal compartments of CT-infected women. The hypothesis is that because the mucosal compartment is the site of active infection, we expect to see a

higher activation status as well as a downregulation of T cell phenotypes (homing and T-associated) as these cells arrive at the genital mucosa.

Through the use of 10 color flow cytometry, this study will phenotypically characterize mononuclear cells taken from CT-seronegative controls and women with or without CT infection at various time points by looking at several chemokine receptors (CCR4, CCR5, CCR7 and CXCR3) and activation markers (HLA-DR and CD38) expressed on T cell subsets (CD4 and CD8) in the peripheral and genital mucosal compartments.

PERIPHERAL BLOOD T CELL PHENOTYPES IN WOMEN WITH *CHLAMYDIA TRACHOMATIS* INFECTION

by

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ABSTRACT

T cell phenotypes involved in the immune response to *Chlamydia* have been well characterized in animal models, but not fully elucidated in humans. To address the influence of *Chlamydia trachomatis* (CT) infection on T cell phenotypes in humans, we compared differences in peripheral blood T cell phenotypes from CT-infected women vs. CT-seronegative controls and found that CT-infected women had higher expression of $CD4^+$ and $CD8^+$ T cell activation markers (CD38 and HLA-DR), $CD4^+$ T helper type 1 (Th1)- and Th2-associated effector phenotypes ($CXCR3^+CCR5^+$ and $CCR4^+$), and $CD4^+$ and $CD8^+$ homing markers (CXCR3 and CCR7, but not CCR5). To evaluate changes in T cell phenotypes after CT treatment and their association with CT reinfection, we compared T cell phenotypes in paired peripheral blood specimens from CT-infected women at an initial visit (prior to treatment) and follow-up visits (3- and 6-months). We found $CD4^+$ and $CD8^+$ T cells had higher expression of activation markers and homing and Th-2 associated chemokine receptors prior to treatment versus at follow-up after infection had cleared, suggesting T cell phenotypes return to a basal state in the absence of CT infection. We also saw a decrease in the same phenotypes in women who became CT reinfected at their first follow-up visit, perhaps because of a lower CT load at this visit. Our study demonstrated T cell phenotypes differ in women with vs. without CT infection, and phenotypes change in CT-infected women after treatment and may be further influenced by presence of CT reinfection. Findings could be useful in understanding adaptive immune mechanisms in human CT infections.

INTRODUCTION

Chlamydia trachomatis (CT) is an intracellular pathogen that can infect columnar epithelial cells in the genital tract (GT) (1) and sometimes lead to significant reproductive morbidity in women, including tubal factor infertility and ectopic pregnancy. CT infection is the most prevalent bacterial sexually transmitted infection (STI) worldwide, with over 131 million new infections annually (2). In the majority of CT-infected individuals, the infection is asymptomatic (3). Therefore, control efforts rely mostly on CT screening, which is recommended in women age<25 years and older women as well as males in populations with a high CT infection prevalence or who have risk factors, and then providing timely treatment for infected individuals (4). Limited data suggest that ~50% of CT-infected individuals naturally clear infection in about one year after detection (5, 6), suggesting effective immune-mediated clearance can occur. Yet, up to 20% of CT-infected patients become reinfected within months after treatment, implying some individuals may not develop protective immunity (7).

Chlamydia-specific cellular immune responses and T cell phenotypes have been well characterized in murine *Chlamydia* infection models. It has been established that T helper type1 (Th1) responses, primarily mediated by interferon gamma (IFN-γ), are essential for protective immunity (8-10). Certain chemokine receptors (CKRs), especially CXCR3 (chemokine [C-X-C motif]) and CCR5 (chemokine [C-C motif]), have been shown to be essential for preferential localization and migration of *Chlamydia*-specific T cells to the GT (11-14). T cells in mice that lack CXCR3 and CCR5 do not migrate to the

GT upon chlamydial infection (11, 12), suggesting these T cell receptors play an important role in the immune response to *Chlamydia*. Since *Chlamydia* is a mucosal pathogen, understanding how immune cells traffic from the peripheral blood to the mucosal surface via CKRs is critical for studying protective immune responses.

Although murine models of *Chlamydia* have provided some key immunological findings associated with protection, there is insufficient data on immune responses to CT in human CTs. Only sparse studies have investigated CT-specific cellular immune responses and T cell phenotypes in humans (15-18). In one such study, Ficarra et al. reported a higher expression of HLA-DR and CCR5 on endocervical $CD3^+$ T cells vs. peripheral blood CD3+T cells from CT-infected women (16) However, this study did not further segregate $CD3^+$ T cells into $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell phenotypes and only investigated a small number of CKRs (16). The few studies that have evaluated the association of cytokine production by CT-specific T cells to protection against CT infection incidence or reinfection in women have yielded contradictory results, with one study implicating the Th2 cytokine IL-4 in CT immunity (17), another study suggesting a protective role for the Th1 cytokine IFN-γ (18), and a third study identifying both Th1 and Th2 cytokines (IFN-γ and IL-13, respectively) in protective immunity (15). However, none of these studies evaluated T-cell phenotypes and the expression of CKRs in CT infection. Furthermore, no single study in CT-infected humans has comprehensively and categorically (distribution, activation, homing and Th-associated T cell phenotypes) investigated $CD4^+$ and $CD8^+$ T cell phenotypes in circulating peripheral blood T cells in CT-infected women, nor have they compared those T cell phenotypes with those of CT-negative controls.

Our study had two aims. The first aim was to investigate the key phenotypic differences between T cells obtained from CT-infected individuals vs. CT-seronegative controls, with respect to T cell distribution, expression of CKRs associated with homing and cellular migration (CXCR3, CCR5, and CCR7), cell surface activation markers (HLA-DR and CD38), and expression of CKRs indicative of Th-associated phenotypes (CCR4 [Th2-associated], $CXCR3^{+}CCR5^{+}$ [Th1-associated] and $CXCR3^{+}CCR4^{+}$), in order to determine the influence of CT infection on T cell phenotypes. Our second aim was to analyze how T cell phenotypes changed after CT treatment at follow-up and whether they were influenced by presence of CT reinfection at follow-up.

MATERIALS AND METHODS

Study Population

This study investigated T cell phenotypes in females ≥16 years of age presenting to the Jefferson County Department of Health (JCDH) STD Clinic in Birmingham, Alabama, USA for treatment of a positive screening CT nucleic acid amplification test (NAAT). The cohort has been previously described (19). Briefly, these women were returning for treatment of a positive CT screening NAAT and had not been treated for CT at the time of screening because they had no signs of CT infection or known exposure to a CT-infected partner. Exclusion criteria were pregnancy, having known concomitant infection with HIV, syphilis, and/or gonorrhea, having a prior hysterectomy, or receiving an antibiotic with anti-CT activity in the prior 30 days. At enrollment, participants were interviewed regarding their demographics, symptoms, sexual history, hormonal contraception use,

antibiotic use, and reported partner treatment. A pelvic examination was performed, in which an endocervical swab was collected for repeat NAAT to confirm CT infection and examination findings were noted. Blood was collected and stored until processed for peripheral blood mononuclear cells (PBMCs). All participants then received directly observed CT therapy with azithromycin 1g, given as a single oral dose. In this study, we evaluated T cell phenotypes in PBMCs collected from 90 study participants who completed the study and stored PBMCs from 6 healthy, low-risk CT-seronegative controls (based on testing their sera with a CT elementary body ELISA (20)). Informed consent was obtained from all of the participants in the study, which was approved by the UAB IRB and JCDH.

PBMC isolation and cryopreservation

As previously described (19), PBMCs were isolated and then frozen (in 90% FBS+10% DMSO) in liquid nitrogen at the UAB Center for Clinical and Translational Sciences (CCTS) Specimen Processing and Analytical Nexus (SPAN) (21, 22), until later thawed for immune studies.

Flow cytometry

Methods have been previously reported (23). Briefly, cryopreserved PBMCs were thawed and labeled with LIVE/DEAD fluorescent reactive dye (Invitrogen, Carlsbad, CA) and stained with surface antibodies against CD3-APCefluor780 (UCHT1; eBiosciences, San Diego, CA); CD4-Qdot 655 (S3.5) and CD8-Qdot 605 (3B5) (both from Life Technologies, Eugene, OR); CXCR3-APC-CD183 (1C6), CCR4 BV421-CD194 (1G1), CCR5-PE-Cy7-CD195 (2D7), CCR7-Alexa Fluor700-CD197 (150503), CD38-FITC (HB7) and HLA-

DR-PE (L243) (all from BD Biosciences, San Jose, CA). Cells were then incubated for 30 minutes and centrifuged at 1200 rpm for 10 minutes. PBMCs were fixed with 2% formalin before analysis. Stained cells were acquired using an LSR II flow cytometer (BD Immunocytometry Systems, San Jose, CA). Using 10-color flow cytometric analysis, we investigated T cell phenotypes classified by the markers listed above. Data were analyzed using FlowJo software (v9.8.5, TreeStar, Ashland, OR). Lymphocytes were analyzed based on forward and side scatter profiles after exclusion of dead cells. Gates were set based on Fluorescence Minus One (FMO) control gates and applied to all samples from each participant for each time point.

Statistical analysis

Analyses were performed using Statistical Analysis System (SAS) version 9.2 (SAS Institute, Cary, NC). Comparisons of peripheral T cell phenotypes (the percentage of T cells expressing a cell surface marker) in CT-infected women vs. CT-negative controls were performed using the Wilcoxon rank-sum test. T cell phenotype comparisons between paired PBMC samples from initial and subsequent visits from CT-infected women were performed using the Wilcoxon signed-rank test. Associations of T cell phenotypes with demographical, clinical, and behavioral characteristics were evaluated using the Wilcoxon rank-sum test. A P value of <0.05 was considered statistically significant.

RESULTS

Study population

In the 90 CT-infected women, the median age was 21.5 years (range 16-32 years), the majority were African American (96.7%), and all were non-Hispanic ethnicity (**Table 1**). Prior CT infection was reported and/or documented in the medical record in 53.3% and the majority (54.4%) were asymptomatic.

The influence of CT infection on T cell activation and CKR

We sought to understand the effect of CT infection on T cell activation and CKR expression by evaluating differences in T cell phenotypes on PBMCs from CT-infected individuals (prior to treatment) and CT-negative controls. **Fig. 1** depicts the gating strategy used to analyze CD4 and CD8 T cell subsets for specific surface marker expression. We first analyzed the distribution of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets and found a significantly higher percentage of $CD3^+CD8^+$ T cells in CT-infected women vs. CT-negative controls (P=0.0281) (**Fig. 2a**).

To ascertain the influence of CT infection on activation of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, we evaluated the activation status by measuring CD38 and HLA-DR expression on these T cell subsets. Our investigations revealed a higher proportion of activated $CD38^+CD4^+$, $CD38^+CD8^+$, and $HLA\text{-}DR^+CD8^+$ T cells in CT-infected women compared with CT-negative controls (P<0.0001; P<0.0001; P=0.0002, respectively). Our findings also revealed a higher proportion of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells that co-expressed CD38 and HLA-DR (both P<0.0001) in CT-infected women (**Fig. 2b-c**).

We next evaluated the expression of CKRs ($CCR5^+$, $CCR7^+$, and $CXCR3^+$) involved in T cell trafficking during CT infection. CCR5, an inflammatory homing CKR, was found to have a lower expression on $CD4^+$ T cells in CT-infected women ($P<0.0112$). In contrast, CCR7, an indicator of active migration of T cells into the lymphatics, was found to be significantly up-regulated in $CD4^+$ and $CD8^+$ T cells from CT-infected women compared to CT-negative controls ($P<0.0001$ for both). Our study also revealed a significant increase in $CXCR3^+$ expression on $CD8^+$ but not $CD4^+$ T cells of CT-infected women compared to CT-negative controls ($P<0.0001$) (**Fig. 2d-e**).

As Th1 responses have been associated with protection against CT (8, 9, 24, 25) and the CT-induced cytokine milieu (24) could alter Th polarization, we analyzed differences in Th2-associated ($CCR4^+$) and Th1-associated ($CXCR3^+CCR5^+$) CKRs in CT-infected women vs. CT-negative controls (**Fig. 2f-g**). CT-infected women had significantly higher expression of Th1-associated and Th2-associated CKRs ($P=0.0048$; $P<0.0001$, respectively) (**Fig. 2f**). On $CD8^+$ T cells, both $CCR4^+$ and $CXCR3^+CCR5^+$ expression was significantly higher in CT-infected women vs. CT-negative controls ($P=0.0006$; $P<0.0001$, respectively) (**Fig. 2g**). There were also higher proportions of $CXCR3^+CCR4^+$ $CD4^+$ and $CD8^+$ T cells in CT-infected women (both $P<0.0001$) (**Fig. 2f-g**).

Changes in T cell phenotypes after CT-infected women are treated and clear infection

An enhanced activation status and increased expression of homing markers on peripheral T cells after CT infection is acquired suggests a potential for migration of CT-specific T cells to the GT. We wanted to understand how expression of these markers changed in women after receiving CT treatment and having cleared their infection. We therefore

evaluated T cell phenotypes at the first follow-up visit (3-month) in a subset of the CT infected women who were CT-negative at the first follow-up visit (n=33) and compared them to their initial visit prior to treatment. We found a higher proportion of $CD8^+$ T cells (P=0.0016) and a trend towards a higher proportion of $CD3^+CD4^+$ T cells (P=0.0687) at the initial visit (**Figure 3a**). We also found a higher expression of the activation markers CD38, HLA-DR, and dual $CD38^+HLA\text{-}DR^+$ at the initial visit (CD4: P<0.0001; P=0.0005; P<0.0001, respectively; CD8: all P<0.0001) (**Figure 3b-c**). There was no difference in the expression of homing CKR CCR5 on $CD4^+$ T cells, however we saw a decreased expression of CCR5 on $CD8^+$ T cells at the first follow-up visit (P=0.0027). We also found decreased expression of homing CKRs CXCR3 and CCR7 at the first follow-up visit (all P<0.0001) for both T cell subsets (**Figure 3d-e**).

Our analysis of Th-associated CKRs revealed a high frequency of the Th2-associated phenotype at the initial visit, based on expression of CCR4 on $CD4^+$ T cells, that decreased at the first follow-up visit, (P<0.0001). We saw a decrease in the dual expression of Th1-associated CKRs CXCR3 and CCR5 between the initial and first follow-up (3-mo) visits (P=0.0046). We also saw a decrease in the dual expression of $CXCR3^+CCR4^+$ on $CD4^+$ T cells at the first follow-up visit (P<0.0001) (**Fig. 3f**). We noted similar reductions at the first follow-up visit for CCR4 and $CXCR3^+CCR4^+$ expression on $CD8^+$ T cells (both P<0.0001) (**Fig. 3g**).

To assess whether the T cell phenotypes further changed with longer follow-up, we evaluated differences in T cell phenotypes from the initial visit to the first follow-up visit (3-month) then to a second follow-up visit (6-month) in paired PBMCs from a subset of women with initial CT infection who did not have reinfection at follow-up (n= 12). We

only saw further significant decreases in receptor expression for a select group of receptors between the first and second follow-up visits (**Fig. 4a-g**). However, the general trend was a continued decrease of these markers.

The effect of CT reinfection at the first follow-up visit on the distribution of T cell phenotypes

The significant steady decline of activation marker, homing, and Th-associated CKR expression across all three visits in women who did not have CT reinfection at follow-up suggested their T cell phenotype distribution had been influenced by presence of CT infection. It was however unclear whether CT reinfection would lead to similar T cell phenotype distributions as seen at the initial visit when women had active CT infection or whether adaptive immune responses may influence T cell distributions with subsequent infection. Thus, we compared T cell phenotypes between the baseline and first follow-up visit in a subset of women who had CT reinfection at follow-up (n = 18). We found a reduced expression of activation makers CD38 and HLA-DR and their co-expression for both T cell subsets at the first follow-up visit despite the presence of CT reinfection (All $P<0.0001$) for both $CD4^+$ and $CD8^+$ T cells (**Fig. 5b-c**). Except for CCR5, there was lower expression of the other homing CKRs, CCR7 and CXCR3, for both $CD4^+$ and $CD8^+$ T cells at the first follow-up visit when compared to the initial visit prior to treatment (all $P<0.0001$) (**Fig. 5d-e**). Similarly, we also found higher expression of Th2-associated CKR CCR4 ($P<0.0001$), dual expression of Th1-associated CKRs CXCR3 and CCR5 ($P=0.0005$), and the dual expression of CXCR3 CCR4 for $CD4^+$ T cells ($P<0.0001$) at the initial visit (**Fig. 5f**). Changes in T cell phenotypes on $CD8^+$ T cells were similar to $CD4^+$

cells, marked by a decline in CCR4, $CXCR3^+CCR5^+$ and $CXCR3^+CCR4^+$ expression between the initial and first follow-up visits (All $P<0.0001$) (**Fig. 5g**). Additionally, despite finding no notable differences in CKR expression on $CD4^+$ T cells when comparing women with vs. without CT reinfection at the first follow-up (3-mo) visit but, we found that the dual expression of CXCR3 and CCR5 or CCR4 on $CD8^+$ T cells was higher in women without CT reinfection ($P<0.0001$; $P=0.0008$, respectively) (**Sup. Fig. 1a-b**).

DISCUSSION

Our study is the first to provide in-depth profiling of $CD4^+$ and $CD8^+$ T cell phenotypes involved in CT infection in humans. One of the aims of the study was to investigate differences in peripheral T cell phenotypes in CT-infected women compared with CT-negative controls in order to improve our understanding of how T cell phenotypes change in response to CT infection. Evaluation of the major T cell subsets revealed that CT-infected women had a higher proportion of $CD3^+CD8^+$ T cells and a trend towards a higher proportion of $CD3^+CD4^+$ T cells than CT-negative controls. We also observed an overall higher activation status in both CD4 and CD8 T cells in CT-infected women, suggesting that CT primed circulating T cells in preparation for homing to the site of infection at the mucosa. Our study also revealed an increased expression of CKR homing markers $CCR7^+$ on $CD4^+$ and both $CCR7^+$ and $CXCR3^+$ on $CD8^+$ T cells in CT-infected women, consistent with ongoing activation and migration of T cells after CT infection acquisition.(11, 16) Interestingly, we also observed a higher expression of CCR5 on $CD4^+$ T cells in CT-seronegative controls, however this difference was marginal and did not

appear on $CD8^+$ T cells. These findings together seem to indicate that there is an expansion of T cells in response to CT infection, notably with an increase in the magnitude of T cells' activation, CKR expression, and homing ability.

Similarly, our evaluation of Th-associated phenotypes revealed a predominance of Th2-associated CCR4 CKR expression in CT infected women, along with some contributions from Th1-associated $CXCR3^+CCR5^+$ CKR expression. These findings suggest that CT-primed $CD4^+$ cells have the potential to produce cytokines capable of polarizing T cells towards either a predominant Th2-associated phenotype, which has been associated with CT persistence (26-28), or a Th1-associated phenotype, which has been associated with protective immune responses against *Chlamydia* (8, 9, 24, 25). High CCR4 expression has not been reported in CT infection, however, in Crohn's disease and ulcerative colitis it has been demonstrated that CCR4 expression was upregulated in the peripheral blood along with the suppression of CXCR3 and CCR5 (29). This finding is further supported by another study that found that CT infected women elicited robust Th2 responses and much lower Th1 responses (17). $CD8^+$ T cells revealed similar findings, with an increase in expression both $CXCR3^+CCR5^+$ and $CCR4^+$ CKRs in CT-infected women, suggesting a complementary role with $CD4^+$ Th-associated T cells responsible for CT control and clearance (12, 30, 31).

The second aim of our study focused on evaluating how T cell phenotypes changed after CT treatment at follow-up and whether CT reinfection influenced CKR and activation marker expression. The finding of a higher proportion of $CD4^+$ T cells at the initial visit prior to treatment vs. follow-up is consistent with their role in adaptive immune responses to ongoing CT infection. Our finding of a higher proportion of $CD8^+$ T cells at the initial

visit also suggest involvement of these cells in the immune responses to active CT infection. In women without active CT infection at the first (3-month) and second (6-month) follow-up visits, the proportion of activated T cells was significantly lower than seen at the initial visit prior to treatment of the infection. Similarly, we found a lower expression of CCR7 (a secondary lymphoid organ and memory marker) (32, 33), and CXCR3 (an inflammatory homing marker) (34) on both T cell subsets at follow-up in women without active CT infection, suggesting distinct trafficking potential for these T cells in CT infection. In the Ficarra et al. study, they found no difference in the expression of HLA-DR on $CD3^+$ T cells at the time of infection vs. a follow-up visit, (median of 35 days between visits); however, similar to our findings, they found that CCR7 expression decreased on $CD3^+$ T cells on follow-up (16). They found an increased expression of $CD3^+CCR5^+$ at the first follow-up visit yet, while we found no change in $CD4^+CCR5^+$ and a decrease in expression of $CD8^+$ $CCR5^+$ T cells at follow-up. Differences in our study findings from the Ficarra et al. study may reflect that their study did not segregate $CD3^+$ T cells into $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell phenotypes, and therefore $CD3^+$ T cells other than $CD4^+$ and $CD8^+$ may have contributed to the differences. Overall, our findings in women without active infection at subsequent visits suggest that CCR7, CXCR3, and perhaps CCR5, play a role in the localization of T cells during active CT infection and in the absence of CT infection, these CKRs go back to their basal expression level.

The declining expression of CKRs on T cells over time across visits in the peripheral compartment suggests that once these cells are activated upon CT infection, they eventually migrate to the site of infection, the GT. Our investigation into receptors that are associated with different Th phenotypes revealed Th1- and Th2-associated markers

steadily declined at follow-up, irrespective of presence CT reinfection at follow-up. Our analysis of CD8 T cells revealed similar findings along with the emergence of dual CXCR3 and CCR5 expression being higher in non-reinfected women. Overall, these finding suggest a complementary role of $CD8^+$ with CD4+ T cells in controlling CT infection, as evidenced by similar CKR expression in both T cell subsets. Our findings on changes in the Th-associated phenotype markers following CT infection may be useful in future studies on the T cell functions that contribute to CT infection clearance and protection from reinfection.

In a small subset of women who became reinfected at the first follow-up visit, we also observed a decrease in the expression of makers/receptors leading to differences in T cell phenotypes, when compared to their initial visit (prior to treatment). One possible reason for this finding may be that the CT load at subsequent visits is usually lower (35), and therefore, the reduced antigen load may not lead to the same degree of up-regulation of CKR expression as is seen at the initial CT infection with a higher CT load. Another explanation for this finding may be that the timing of treatment may have an effect on expression of CKRs particularly in women who become reinfected with CT.

One of our most notable findings was that there was a higher proportion of both $CD4^+$ and $CD8^+$ T cells expressing $CXCR3^+CCR4^+$ T cells in CT-infected women at their initial visit prior to treatment compared to follow-up visits. Furthermore, at the first follow-up (3-mo) visit, our finding that the dual expression of CXCR3 and CCR4 on CD8+ T cells was higher in women non-reinfected vs. reinfected women substantiates the importance of these CKRs. A study evaluating the migration of T cells based on CXCR3 and CCR4 expression determined that T cell migration was partially dependent on CXCR3, but

independent of CCR4 expression (36). Rather, CCR4 seemed to play a role in T cell retention at inflamed sites. This study also reported that the absence of both markers resulted reduced the development of idiopathic arthritis. These T cells have also been implicated as regulatory T cells in inflamed human liver tissue as well as in T cells from the synovial fluid of patients with juvenile idiopathic arthritis (34, 37, 38). Together our findings seem to suggest that this unique phenotype seems to play an immunomodulatory role in women actively infected with CT and this could contribute to lower the risk for reinfection, although further studies are warranted to establish their function and their role in CT reinfection.

Our study had some limitations. One may consider whether the T cell profiles are CT-specific, as concomitant genital infections or use of hormonal therapy could influence T cell phenotypes (39). We did not find any association of concomitant bacterial vaginosis or trichomoniasis or use of hormonal contraception with any of the T cell phenotypes (**data not shown**); patients with gonorrhea or HIV were excluded and the number of participants with trichomoniasis were too small for meaningful analyses; thus, the T cell phenotype distributions were likely CT-specific. A future direction to confirm the CT-specificity of the changes in the T cell phenotypes will be to explore using CT antigen specific-multimer (tetramer/pentamer) staining, which we believe will confirm our findings. Additionally, generalizability of our findings could be limited due to the fact that our cohort was mostly comprised of African American women.

In summary, our comprehensive investigation of peripheral blood T cell phenotypes in CT-infected women vs. CT-negative controls revealed that CT infection leads to overall higher activation and up-regulation of various CKRs, as well as markedly

increased proportion of $CCR4^+$ and $CXCR3^+CCR5^+$ $CD4^+$ and $CD8^+$ T cells. Our analysis of differences in T cell phenotypes between paired peripheral blood specimens from an initial visit before CT-infected women were treated and their follow-up visits revealed a higher activation status and expression of homing and Th2-associated CKRs on T cells at the initial visit, with a steady decline over time over subsequent follow-up visits. The down regulation of CKR expression may indicate that some of these T cells could be going back to basal CKR expression in the absence of infection. Our study also points to role for T cells co-expressing CXCR3 with CCR5 or CCR4 in modulating CT-infection for both T cell subsets in CT-infected women prior to treatment and a role perhaps a for CD8 T cells expressing these CKRs in preventing reinfection. Our future studies will evaluate regulatory and memory T cell phenotypes, as well as address the functional role of these T cells in CT-infected women. We will also look at the relationship between T cell phenotypes and function as it relates to risk for CT reinfection, with the goal of identifying immune correlates of protection that could be useful for CT vaccine development efforts.

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DISCLOSURE

The authors declare no disclosures.

AUTHOR CONTRIBUTIONS

BMOO, RKB, KG, SJ, SS and WMG designed the study. CP and WMG conducted clinical procedures. BMOO, RKB, KG, SJ, SS, LB, and RK conducted laboratory experiments. JYL and WMG performed statistical analyses. BMOO, RKB, KG, SJ, SS, and WMG interpreted the data and wrote the manuscript.

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Table 1Participant Characteristics among Chlamydia-Infected Women (n=90)

Subject Characteristics	***n* (%)**
Age	
Median (range)	21.5 (16-32)
Race	
Black	87 (96.7)
White	3 (3.3)
Ethnicity	
Non-Hispanic	90 (100)
Hormonal contraception*	29 (32.0)
Median prior sex partners	
Last 3 mo. (range)	1 (1-5)
Prior chlamydia	48 (53.3)
Asymptomatic	49 (54.4)
Cervicitis*	19 (21.1)
Co-Infections	
Candidiasis	12 (13.3)
Bacterial vaginosis	26 (28.9)
Trichomoniasis	4 (4.4)

*missing for 1 participant

Fig. 1. Gating Strategy on Mononuclear Cells. Representative flow cytometry plots showing the gating strategy used to measure chemokine receptor expression, activation markers, and T helper associated phenotypes on T cells. PBMCs were first gated on lymphocytes based on FSC and SSC profile, then multiplets were excluded before gating on $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cells and well as dual and single gates demonstrating CKR expression with gates determined by negative samples or FMO.

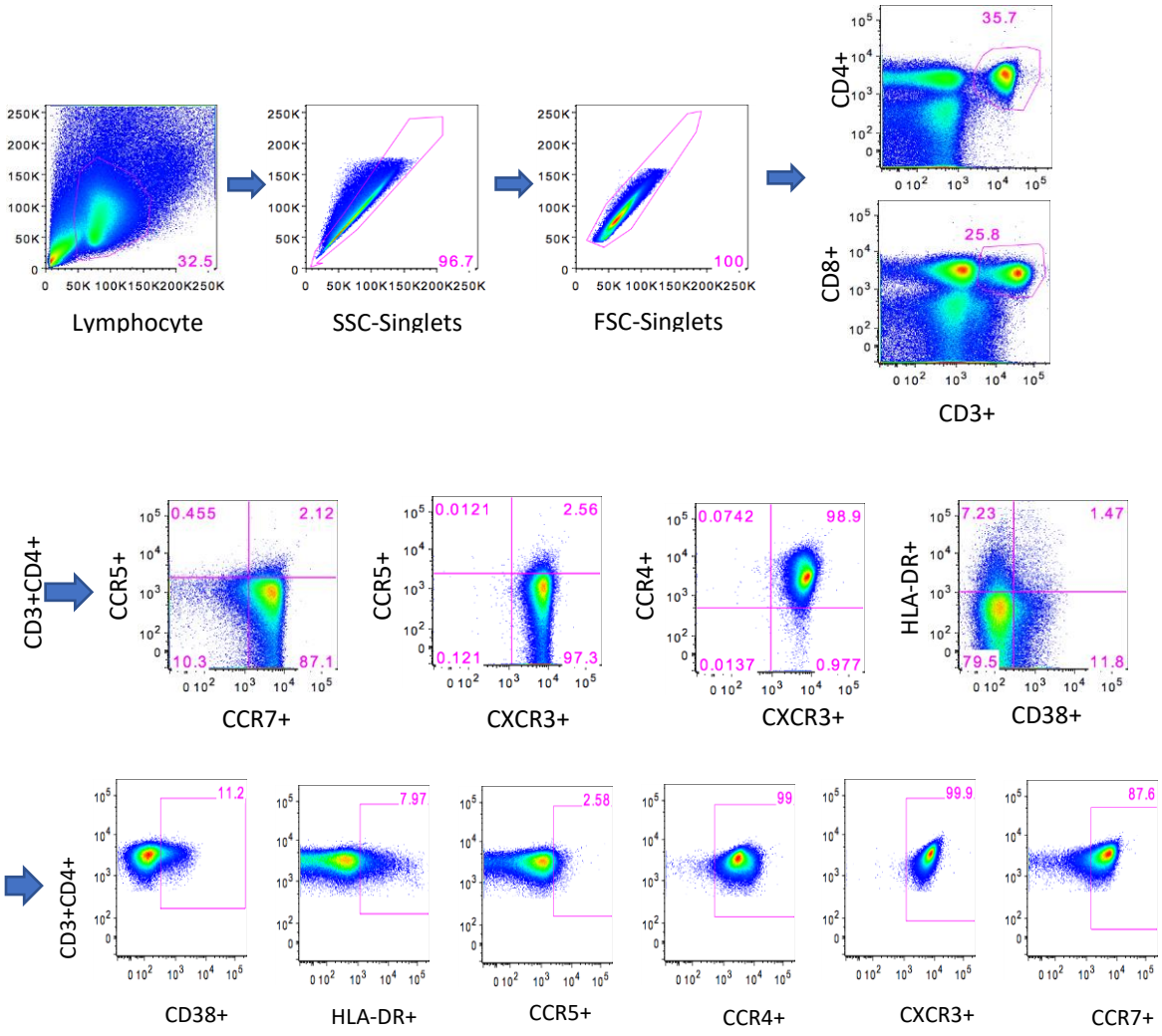
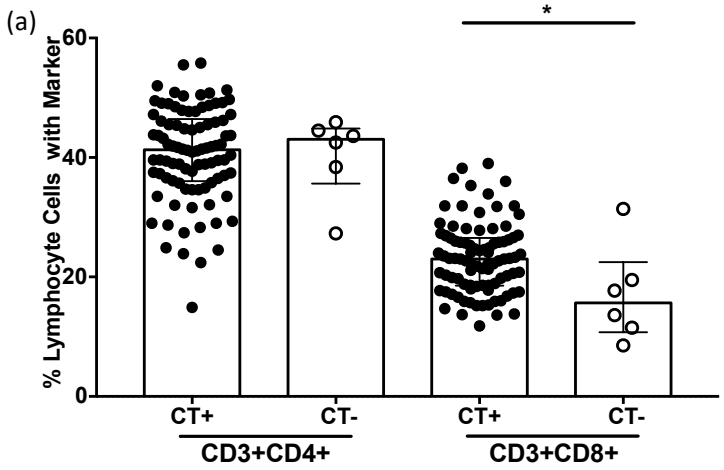
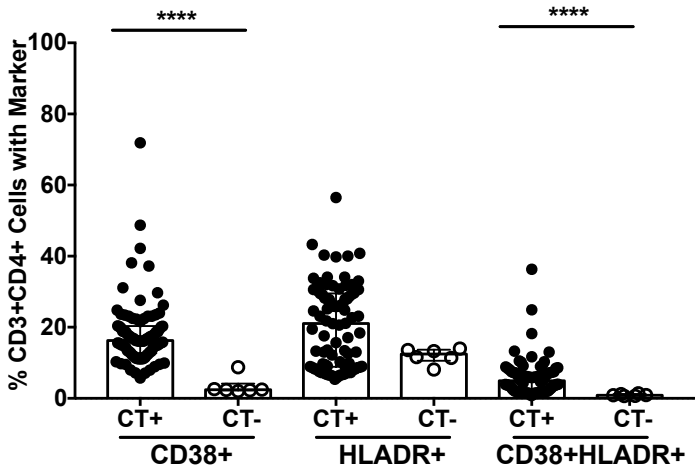


Figure. 2. Differences in T cell distribution and homing CKR expression between chlamydia-infected (CT+) women vs. chlamydia-seronegative (CT-) controls. (**a**) Scatter bar plot comparison of T cell distribution PBMCs from subjects with CT infection (CT+ [closed circles] n=90) at the initial visit prior to treatment vs. CT-seronegative controls (CT- [open circles] n=6) across T cell subsets ($CD4^+$, $CD8^+$). (**b-c**) Scatter bar plots depicting activation makers (CD38, HLA-DR and $CD38^+HLADR^+$) on PBMCs from CT+ women vs. CT- controls for both (**b**) $CD3^+CD4^+$ and (**c**) $CD4^+CD8^+$ T cell subsets. (**d-e**) Scatter bar plots depicting the expression of homing CKRs ($CXCR3^+$, $CCR5^+$, and $CCR7^+$) on PBMCs from CT-infected women (CT+ [closed circles] n=90) vs. CT-negative controls (CT- [open circles] n=6) in both (**d**) $CD3^+CD4^+$ and (**e**) $CD4^+CD8^+$ T cell subsets. (**f-g**) Scatter bar plots comparing the expression of chemokine receptors $CCR4^+$, $CXCR3^+CCR5^+$, and $CXCR3^+CCR4^+$ on PBMCs of CT+ women (n=90) and CT- controls (n=6) for both (**f**) $CD3^+CD4^+$ and (**g**) $CD3^+CD8^+$ T cell subsets. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or NS (not significant) with data represented as median and interquartile range (IQR).



(b)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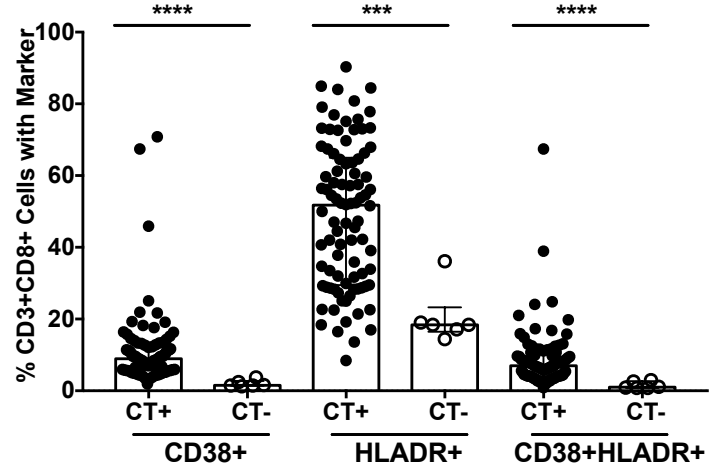
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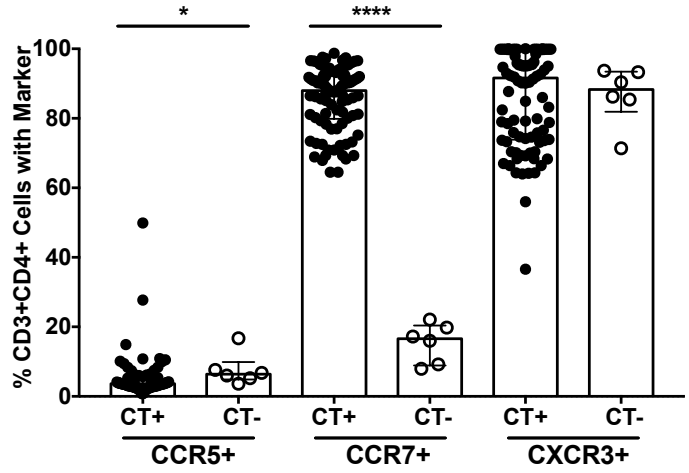
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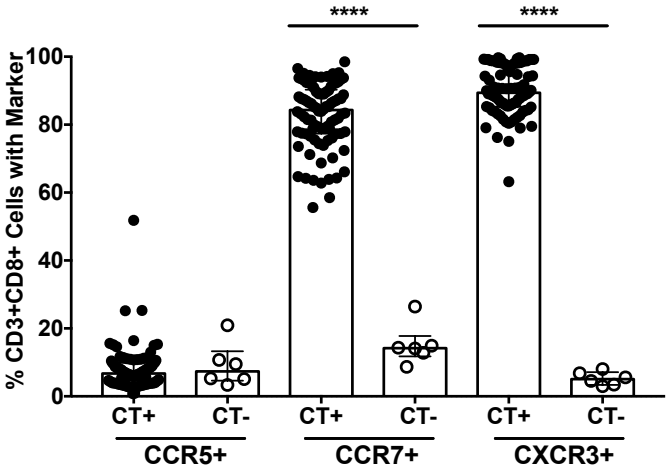
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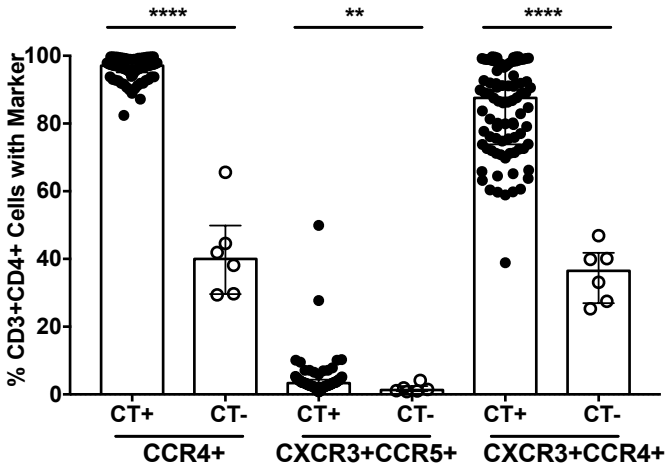
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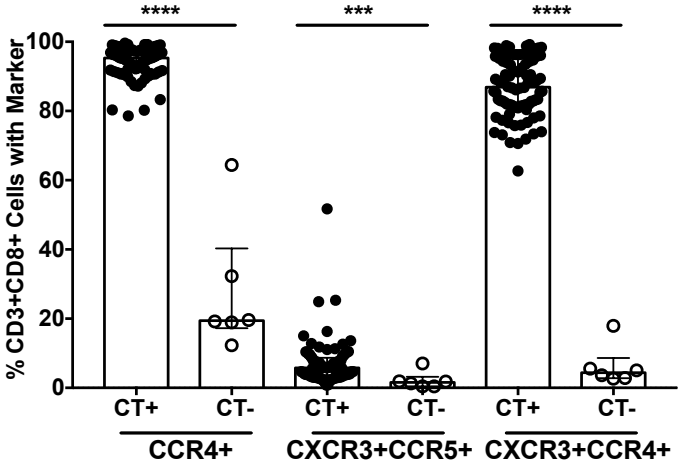
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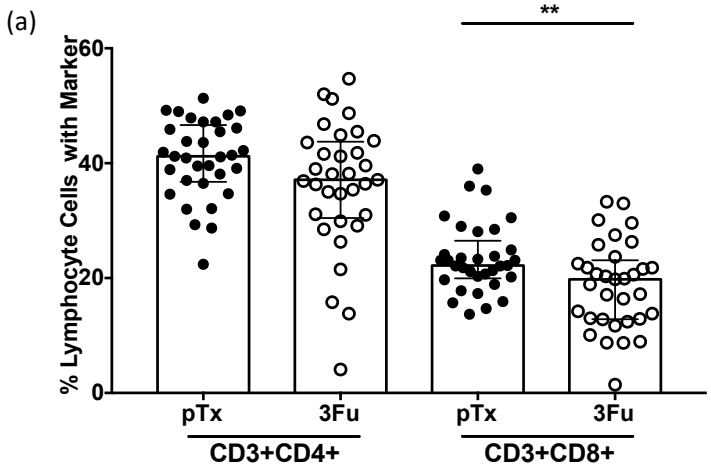
Fig. 3. Changes in T cell phenotypes at the first follow-up visit after treatment has cleared the CT infection. (**a**) Scatter bar plot comparison of T cell distribution of paired PBMCs from women with CT infection at their initial visit prior to treatment (pTx [closed circles] n=33) vs. CT-negative at their first (3-month) follow-up visit (3Fu [open circles] n=33) across T cell subsets ($CD4^+$, $CD8^+$). (**b-c**) Scatter bar plots depicting activation makers (CD38, HLA-DR and $CD38^+HLADR^+$) on paired PBMCs from CT+ pTx vs. CT- 3Fu women for both (**b**) $CD3^+CD4^+$ and (**c**) $CD4^+CD8^+$ T cell subsets. (**d-e**) Scatter bar plots depicting the expression of homing CKRs ($CXCR3^+$, $CCR5^+$, and $CCR7^+$) on paired PBMCs from CT+ (pTx [closed circles] n=33) vs. CT- (3Fu [open circles] n=33) women in both (**d**) $CD3^+CD4^+$ and (**e**) $CD4^+CD8^+$ T cell subsets. (**f-g**) Scatter bar plots comparing the expression of chemokine receptors $CCR4^+$, $CXCR3^+CCR5^+$, and $CXCR3^+CCR4^+$ on paired PBMCs of CT+ pTx (n=33) vs CT- 3Fu women (n=33) for both (**f**) $CD3^+CD4^+$ and (**g**) $CD3^+CD8^+$ T cell subsets. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$, or NS (not significant) with data represented as median and interquartile range (IQR).



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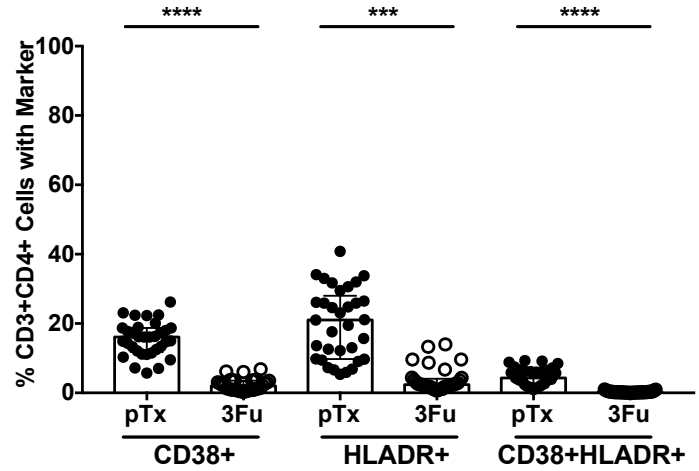
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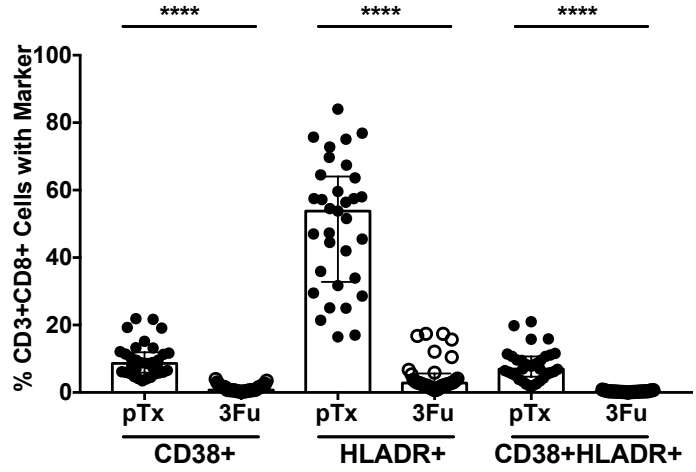
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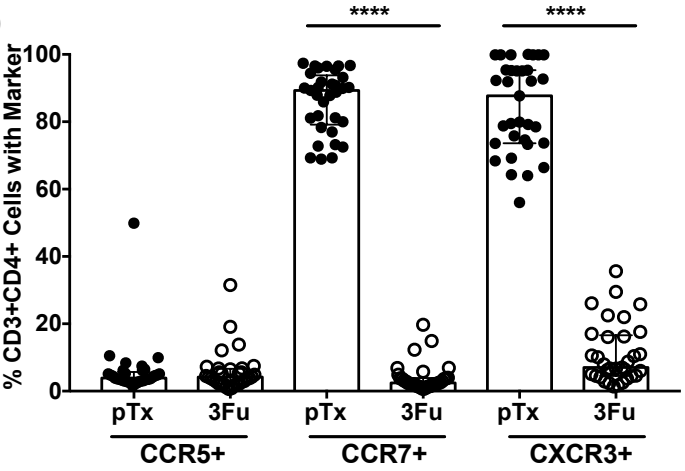
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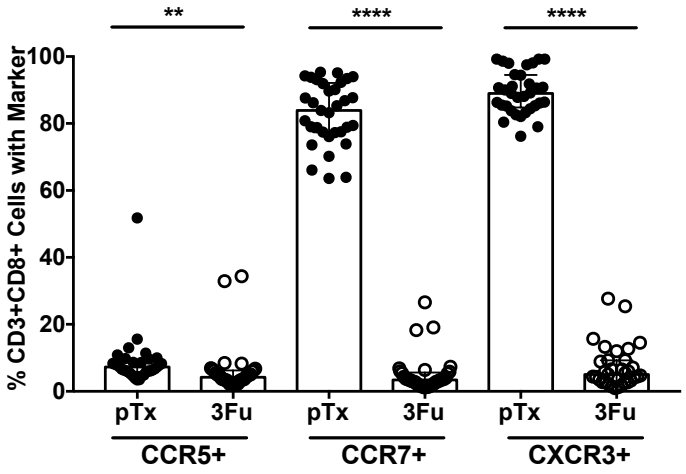
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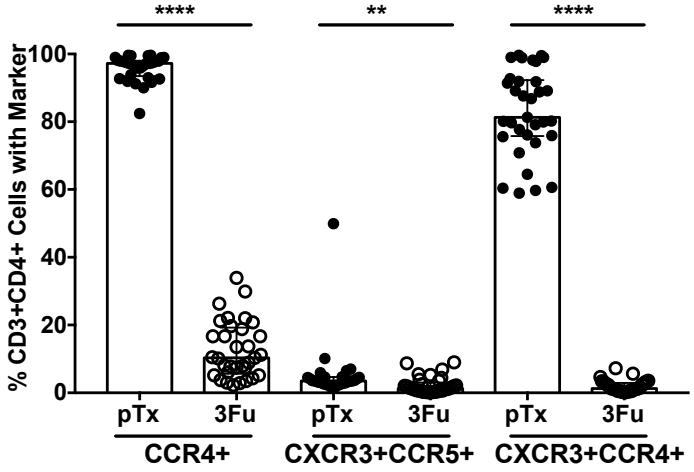
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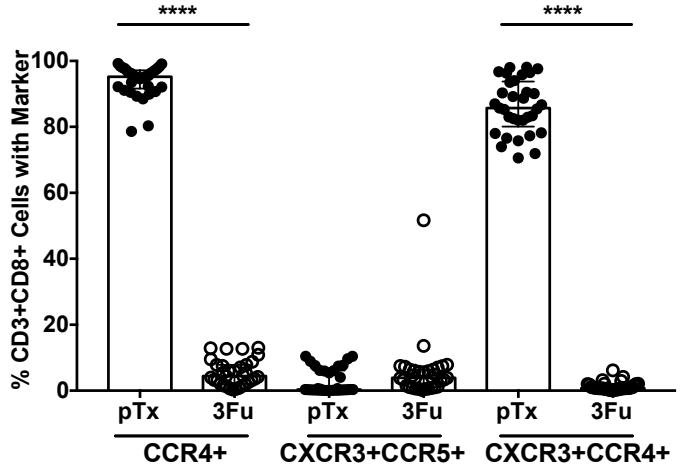
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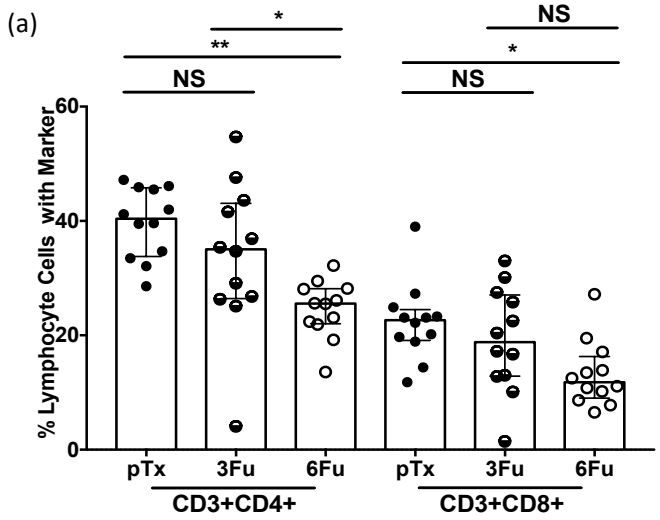
Fig. 4. Evaluation of T cell phenotypes between an initial visit prior to CT treatment and two follow-up visits in which CT infection is absent. (**a**) Scatter bar plot comparison of T cell distribution of paired PBMCs from women with CT infection at their initial visit prior to treatment (pTx [closed circles] n=12) vs. those CT-negative at their first (3-month) follow-up (3Fu [open circles] n=12) and second (6-month) follow-up (6Fu [open circles] n=12) across T cell subsets ($CD4^+$, $CD8^+$). (**b-c**) Scatter bar plots depicting activation makers (CD38, HLA-DR and $CD38^+HLADR^+$) on paired PBMCs from CT+ pTx vs. CT- 3Fu vs. CT- 6Fu women for both (**b**) $CD3^+CD4^+$ and (**c**) $CD4^+CD8^+$ T cell subsets. (**d-e**) Scatter bar plots depicting the expression of homing CKRs ($CXCR3^+$, $CCR5^+$, and $CCR7^+$) on paired PBMCs from CT+ (pTx [closed circles] n=12) vs. CT- (3Fu [open circles] n=12) vs. CT- (6Fu [open circles] n=12) women in both (**d**) $CD3^+CD4^+$ and (**e**) $CD4^+CD8^+$ T cell subsets. (**f-g**) Scatter bar plots comparing the expression of chemokine receptors $CCR4^+$, $CXCR3^+CCR5^+$, and $CXCR3^+CCR4^+$ on paired PBMCs of CT+ pTx (n=12) vs. CT- 3Fu (n=12) vs. CT- 6Fu (n=12) women for both (**f**) $CD3^+CD4^+$ and (**g**) $CD3^+CD8^+$ T cell subsets. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$, or NS (not significant) with data represented as median and interquartile range (IQR).



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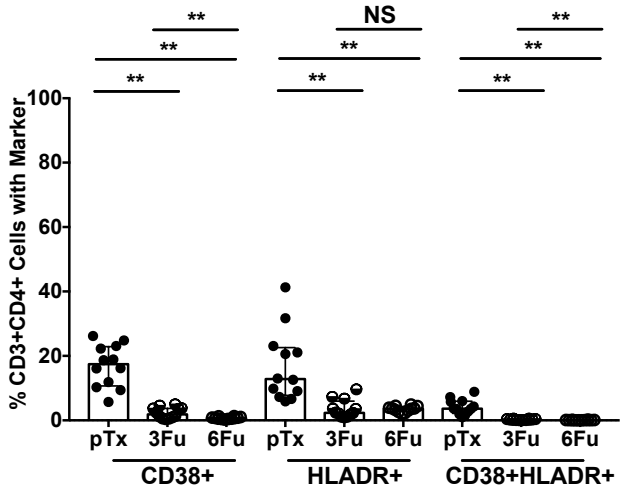
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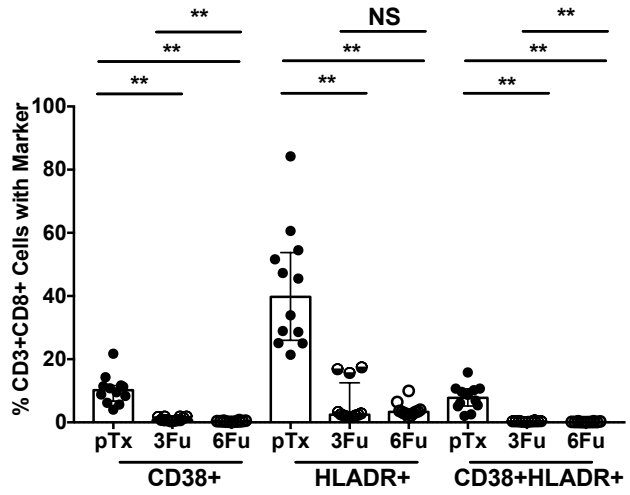
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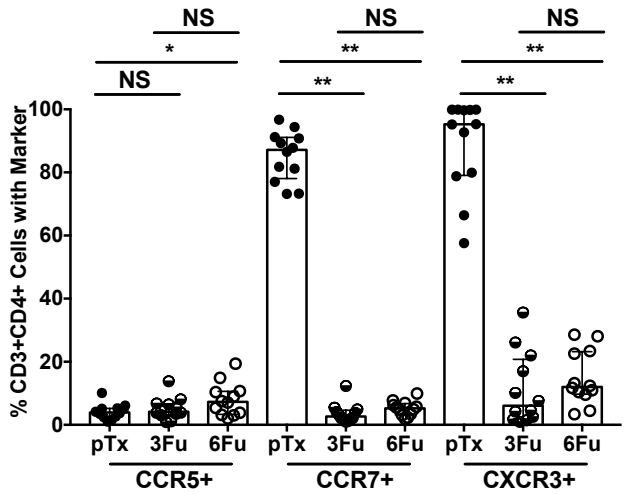
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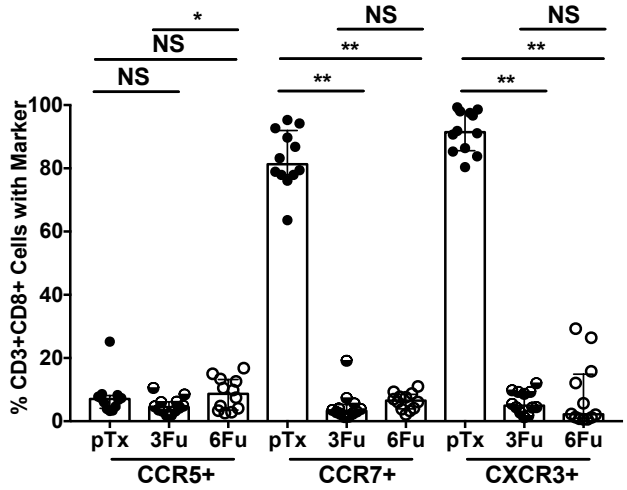
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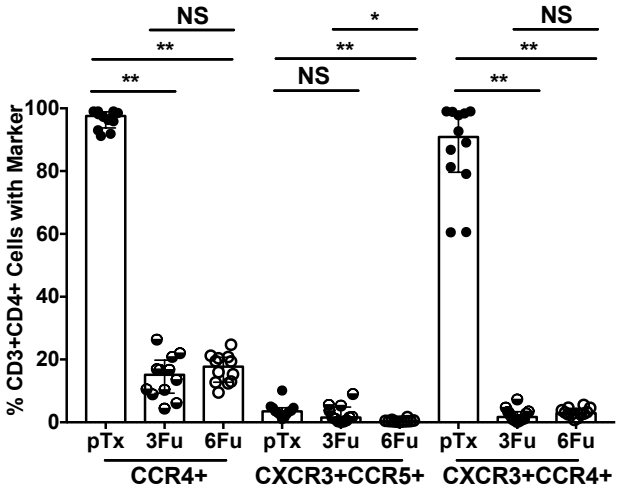
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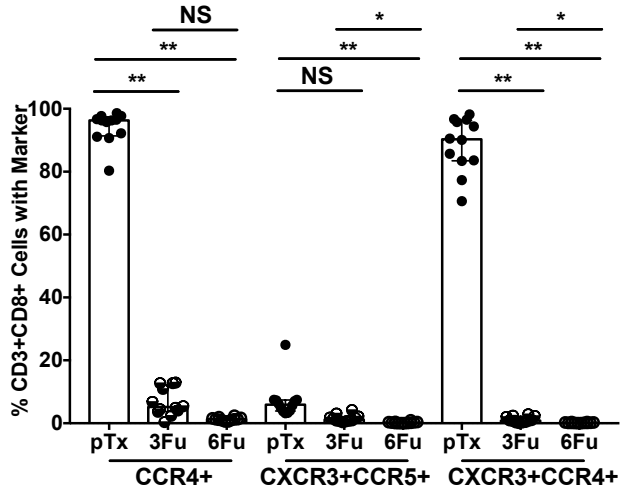
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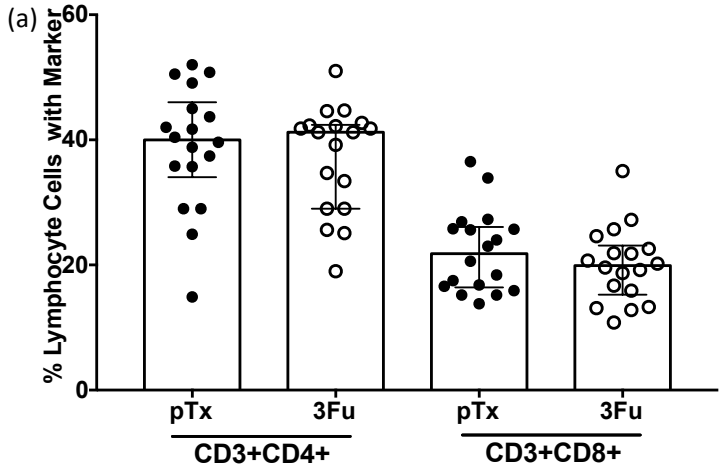
Fig. 5. The effect of CT reinfection at the first follow-up visit on T cell phenotypes. (**a**) Scatter bar plot comparison of T cell distribution of paired PBMCs from women with CT infection at their initial visit prior to treatment (pTx [closed circles] n=18) vs. CT reinfection at their first (3-month) follow-up visit (3Fu [open circles] n=18) across T cell subsets ($CD4^+$, $CD8^+$). (**b-c**) Scatter bar plots depicting activation makers (CD38, HLA-DR and $CD38^+HLADR^+$) on paired PBMCs from CT+ pTx vs. CT+ 3Fu women for both (**b**) $CD3^+CD4^+$ and (**c**) $CD4^+CD8^+$ T cell subsets. (**d-e**) Scatter bar plots depicting the expression of homing CKRs ($CXCR3^+$, $CCR5^+$, and $CCR7^+$) on paired PBMCs from CT+ (pTx [closed circles] n=18) vs. CT+ (3Fu [open circles] n=18) women in both (**d**) $CD3^+CD4^+$ and (**e**) $CD4^+CD8^+$ T cell subsets. (**f-g**) Scatter bar plots comparing the expression of chemokine receptors $CCR4^+$, $CXCR3^+CCR5^+$, and $CXCR3^+CCR4^+$ on paired PBMCs of CT+ pTx (n=18) vs CT+ 3Fu women (n=18) for both (**f**) $CD3^+CD4^+$ and (**g**) $CD3^+CD8^+$ T cell subsets. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$, or NS (not significant) with data represented as median and interquartile range (IQR).



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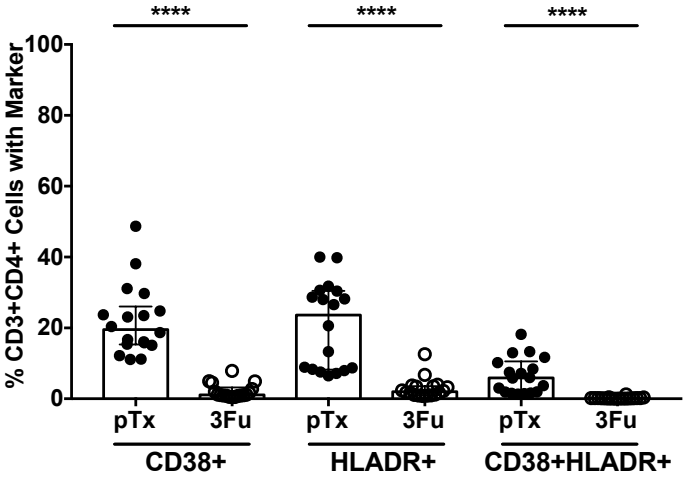
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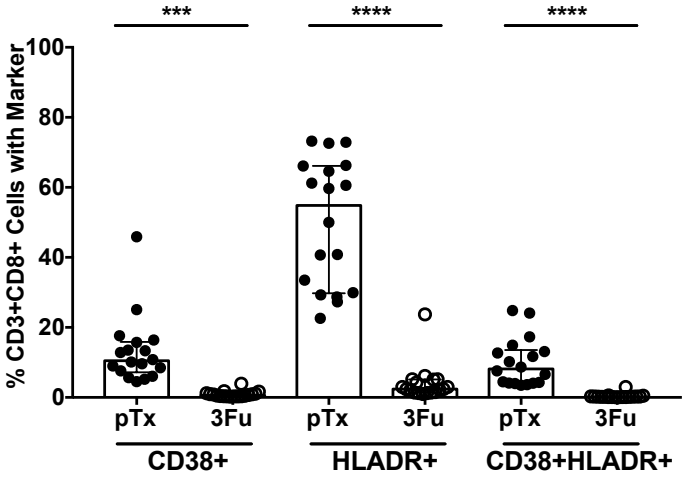
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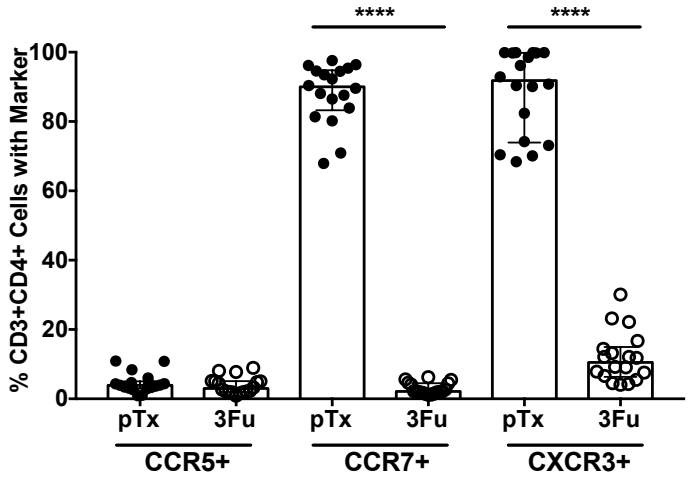
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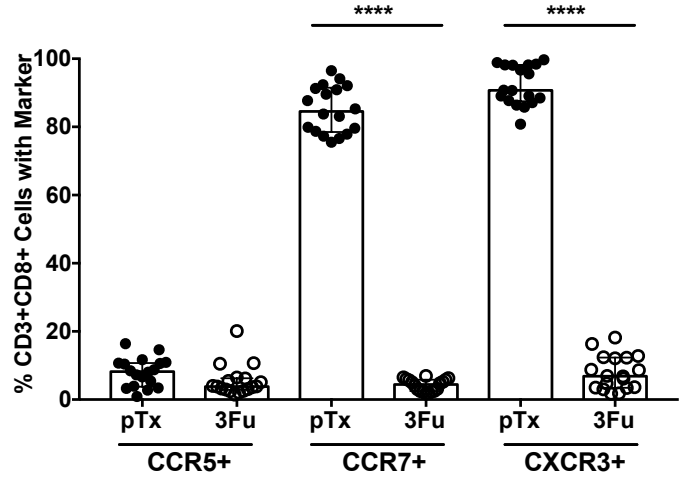
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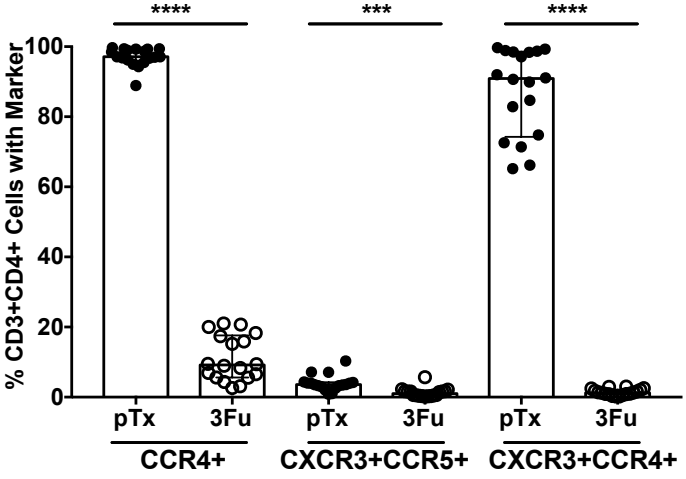
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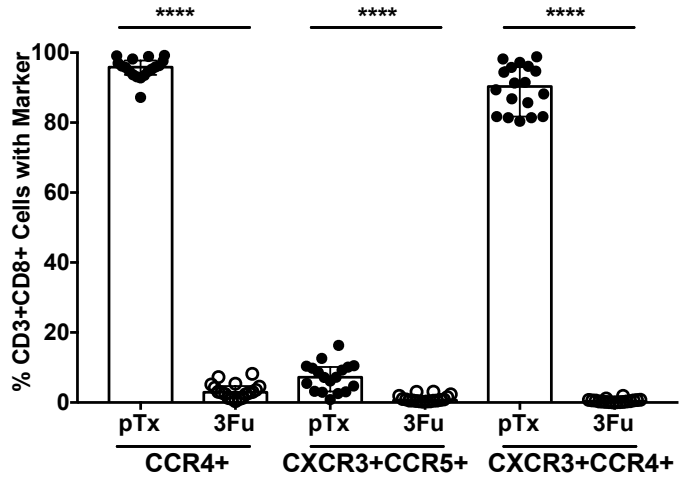
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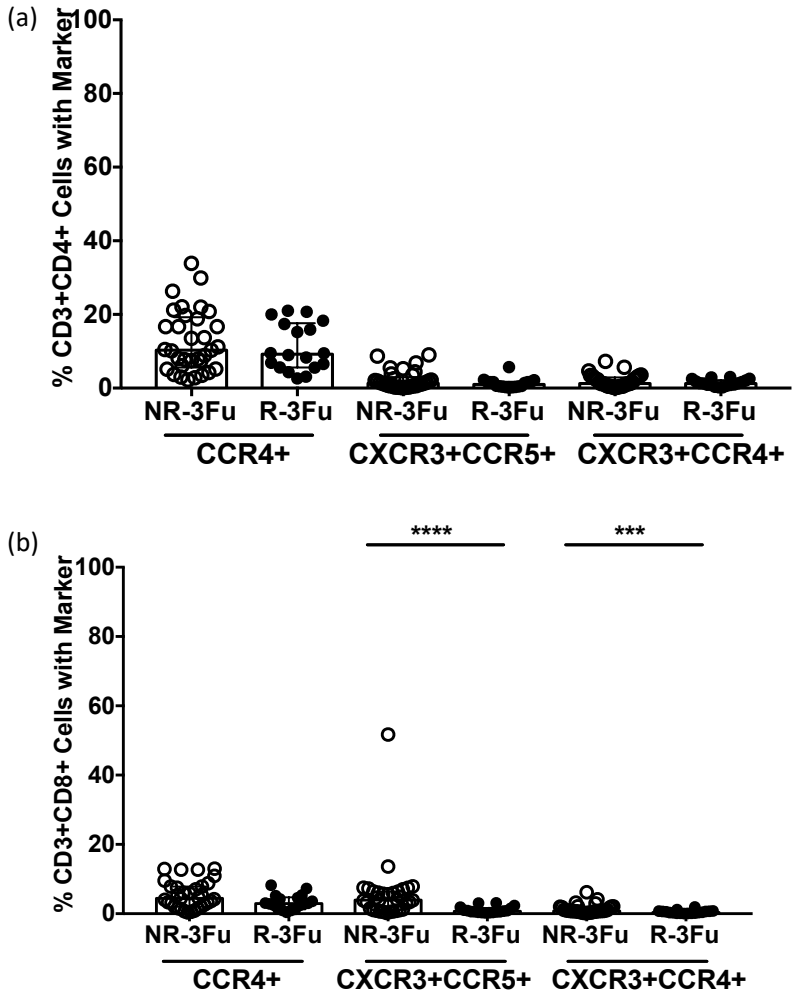
Supplemental Fig. 1. CT reinfection at 3 months between women with vs. without infection. (**a-b**) Scatter bar plots comparing the expression of chemokine receptors $CCR4^+$, $CXCR3^+CCR5^+$, and $CXCR3^+CCR4^+$ on paired PBMCs of CT- non-reinfected (NR)-3Fu (n=33) vs CT+ reinfected (R)-3Fu women (n=18) for both (**a**) $CD3^+CD4^+$ and (**b**) $CD3^+CD8^+$ T cell subsets. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$, or NS (not significant) with data represented as median and interquartile range (IQR).



DISTINCT PERIPHERAL VERSUS MUCOSAL T CELL PHENOTYPES IN CHLAMYDIA-INFECTED WOMEN

by

BRIAN M. O. OGENDI, RAKESH K. BAKSHI, STEFFANIE SABBAJ, JEANNETTE Y. LEE AND WILLIAM M. GEISLER

Submitted to *American Journal of Reproductive Immunology*

Format adapter for dissertation

ABSTRACT

Problem: Differences in circulating (peripheral) and mucosal T cell phenotypes in chlamydia-infected women remain largely unknown.

Method of study: Thirteen paired mononuclear cell specimens from blood and cervicovaginal lavages from chlamydia-infected women were stained and analyzed using ten-color cell surface flow cytometry for T cell distribution, activation status, homing and T helper (Th)-associated chemokine receptors (CKRs).

Results: A higher proportion of genital mucosal T cells were activated ($CD38^{+}HLA\text{-}DR^{+}$) and expressed CCR5 and Th1-associated CKR $CXCR3^{+}CCR5^{+}$ compared to peripheral T cells, but a lower proportion of mucosal T cells expressed homing CKR CCR7, Th-2 associated CKR CCR4, and $CXCR3^{+}CCR4^{+}$ for both T cell subsets.

Conclusion: T cell phenotypes differed in the peripheral vs. genital mucosa compartments in chlamydia-infected women. Since chlamydia infects mucosal epithelial cells, the finding of a higher frequency of activated T cells and Th-1 phenotypes in the mucosa likely reflects an adaptive immune response to infection.

KEYWORDS

mucosal, peripheral, *Chlamydia trachomatis*, T cell, phenotype, chemokine, receptor,

BACKGROUND

Chlamydia trachomatis (CT) infection is highly prevalent and associated with adverse reproductive outcomes. Our limited understanding of the T cell phenotypes involved in CT infection comes primarily from murine models. In particular, the concomitant expression of $CXCR3^+$ and $CCR5^+$ on $CD4^+$ T cells has been shown in murine models to play a critical role in localizing protective effector T cells to the genital mucosa, and loss of either receptor impairs protective immunity [1]. A single human study by Ficarra et al. evaluated $CD3^+$ T cell phenotypes in the peripheral blood and genital mucosa of women with uncomplicated CT infection, but did not differentiate $CD3^+$ T cells into $CD4^+$ or $CD8^+$ T cells and only evaluated a limited number of T cell phenotypes [2]. No study in CT-infected humans has comprehensively investigated $CD4^+$ and $CD8^+$ T cell phenotypes in the peripheral blood (the compartment where circulating lymphocytes reside) and genital mucosa (the site of active CT infection), which was the aim of our study.

METHODS

Ethics statement

This study was approved by the UAB IRB and the Jefferson County Department of Health. All subjects provided written consent prior to enrollment in the study.

Study participants

Women presenting to a STD clinic in Birmingham, AL, USA for treatment of a positive urogenital screening CT nucleic acid amplification test were enrolled in a CT immune

response study, in which they had blood collected for peripheral blood mononuclear cells (PBMCS) and a cervicovaginal lavage (CVL) for mucosal mononuclear cells (MMCs) and were treated with azithromycin 1g. The majority of the enrolled women were non-Hispanic African American, with an average age of 22.6 years. Only 3 (23.1%) did not have a history of prior CT infection and 8 (61.5%) reported use of hormonal contraception. Four (30.8%) women were diagnosed with cervicitis and one (7.7%) with pelvic inflammatory disease.

Mononuclear cell isolation

PBMCs and MMCs were isolated in accordance with the UAB Center for Clinical and Translational Sciences (CCTS) Specimen Processing and Analytical Nexus (SPAN) protocol of centrifugation through lymphocyte separation medium (Mediatech, Inc, Manassas, VA). PBMCs were then frozen (in 90% FBS+10% DMSO) in liquid nitrogen until later thawed for immune studies. Immune studies were performed on fresh viable MMCs, not frozen, due to the low recovery rate upon thawing frozen MMCs.

Flow cytometry

PBMCs and MMCs were stained with fluorescent antibodies against cell surface markers and analyzed using 10-color flow cytometry by previously reported methodology [3]. Briefly, cryopreserved PBMCs and freshly isolated MMCs were labeled with LIVE/DEAD fluorescent reactive dye (Invitrogen, Carlsbad, CA) and stained with surface antibodies against CD3-APCefluor780 (UCHT1; eBiosciences, San Diego, CA); CD4-Qdot 655 (S3.5) and CD8-Qdot 605 (3B5) (both from Life Technologies, Eugene, OR); CXCR3-APC-CD183 (1C6), CCR4 BV421-CD194 (1G1), CCR5-PE-Cy7-CD195 (2D7), CCR7-Alexa Fluor700-CD197 (150503), CD38-FITC (HB7) and HLA-DR-PE (L243) (all antibodies were purchased from BD Biosciences, San Jose, CA). Cells were then incubated

for 30 minutes and centrifuged at 1200 rpm for 10 minutes. Both PBMCs and MMCs were fixed with 2% formalin before analysis. Using an LSR II flow cytometer (BD Immunocytometry Systems, San Jose, CA), $CD3^+$ events were acquired from each sample. Data were analyzed using FlowJo software (v9.8.5, TreeStar, Ashland, OR). Lymphocytes analysis was based on forward and side scatter profiles after exclusion of dead cells. Gates were either set using negative sample or Fluorescence Minus One (FMO) control and were then applied to all samples from each participant.

Statistical analysis

Statistical analyses were performed using Statistical Analysis System (SAS) version 9.2 (SAS Institute, Cary, NC). Comparisons of T cell phenotypes between paired PBMCs and MMCs from 13 CT-infected women were done using the Wilcoxon signed-rank test.

RESULTS AND DISCUSSION

We first investigated differences in the proportion of T cell subsets ($CD4^+$ and $CD8^+$) in the peripheral blood vs. genital mucosa. We found a higher proportion of $CD4^+$ and $CD8^+$ T cells in the peripheral blood, although the difference was only significant for the former ($CD4^+$: P=0.0105; $CD8^+$: NS) (Figure 1A). It is unclear whether this is a normal difference in these T cell subsets between peripheral blood and the genital mucosa or if this is a change due to the presence of chlamydia.

We next evaluated activation status of T cells with the hypothesis that mucosal T cells would be more activated than peripheral blood T cells, as CT is a mucosal pathogen. We found a higher proportion of T cells expressing the activation markers CD38 and HLA-

DR as well their co-expression ($CD38^+HLA\text{-}DR^+$) at the genital mucosa for both $CD4^+$ and $CD8^+$ T cells ($CD4^+$: P=0.0266, P=0.0134, P=0.0007; $CD8^+$: P=0.0005, NS, P=0.0012) (Figure 1B). Our HLA-DR finding is consistent with Ficarra et al. finding higher HLA-DR expression in mucosal $CD3^+$ T cells; they did not evaluate CD38 [2].

We then evaluated expression of homing chemokine receptors (CKRs) at the peripheral blood vs. mucosal site anticipating a lower proportion of these receptors at the mucosa due to down-regulation of these receptors upon arrival at the site of infection. We found lower expression of CCR7, an important T lymphocyte homing and memory marker [4], in the genital mucosa for both $CD4^+$ and $CD8^+$ cells (both P=0.0002) (Figure 1C). We found no significant difference in the expression of CXCR3, a marker of effector and memory cells that is also important in T cell trafficking, on $CD4^+$ cells between compartments; however, CXCR3 expression on $CD8^+$ cells trended towards being higher in the peripheral blood (P=0.0771) (Figure 1C). We found expression of CCR5, an inflammatory homing marker that influences trafficking, to be higher in the genital mucosa for both $CD4^+$ and $CD8^+$ T cells (P=0.0266 and P=0.0105) [2]. Our finding of lower CCR7 expression and higher CCR5 expression in the genital mucosa for both CD4+ and CD8+ T cells is consistent with the Ficarra et al. findings showing the same differences in $CD3^+$ T cells; they did not evaluate CXCR3 expression [2]. Overall, our evaluation of homing markers suggests CCR5 but not CXCR3 in humans may be the key marker for homing of effector T cells to the genital tract for an adaptive immune response against CT. While CCR7 is an important homing and memory marker [4], our finding of a low frequency of CCR7 expression at the genital mucosa is consistent with the idea that CCR7 is down-regulated once cells exit the lymphatic system and enter mucosal tissues, perhaps due to a

shift towards these cells becoming resident memory T cells.

Finally, we investigated T helper (Th)-associated phenotypes, not studied by Ficarra et al. [2], to assess whether T cells preferentially polarized towards Th1 or Th2 phenotypes in peripheral blood and the genital mucosa, anticipating a higher expression of Th1-associated CKRs in both compartments, given that murine studies have shown $CD4^+$ Th1 responses are essential for immunity to chlamydia [1]. Our data, on the contrary, demonstrates a higher expression of Th2-associated CKR $CCR4^+$ in the peripheral blood ($CD4^+$: P=0.0002) (Figure 1D). However, in support of this idea, we found higher expression of Th1-associated $CXCR3^+CCR5^+$ in the genital mucosa ($CD4^+$: P=0.0046), suggesting $CD4^+$ T cells may polarize from Th2 to Th1 phenotypes once they arrive at the mucosa, the site of active infection. We observed a similar trend in expression of these markers in $CD8^+$ T cells ($CCR4^+$: P=0.0002; $CXCR3^+CCR5^+$: P=0.0171) (Figure 1D). The comparable expression of CCR4 and CXCR3 CCR5 for both $CD4^+$ and $CD8^+$ T cells suggests that both of these T cell subsets are involved in response to CT infection. We also found a higher expression of $CXCR3^+CCR4^+$ T cells in the peripheral blood ($CD4^+$ and $CD8^+$ both P=0.0002) (Figure 1D); this T cell phenotype has been previously reported to be expressed on regulatory T cells in inflamed human liver tissue [5] and in synovial fluid of patients with juvenile idiopathic arthritis [6], suggesting it may regulate Th cell polarization and/or could contribute to immunopathology in CT infection.

CONCLUSIONS

In summary, our investigation of differences in T cell phenotypes between peripheral blood and the genital mucosa of CT-infected women revealed a greater proportion of activated T cells at the genital mucosa, a lower expression of the homing and memory marker CCR7 and higher expression of homing marker CCR5 in the mucosa, and more frequent polarization towards a Th2-associated phenotype in the blood vs. Th1-associated phenotype in the genital mucosa. Thus, T cell phenotypes are distinct at the peripheral blood vs. genital mucosa sites in women with CT infection. This highlights the importance of studying both peripheral and mucosal compartments in CT infection and our findings provide knowledge that will be important in future studies aimed at evaluating CT-specific function of these specific T cell phenotypes.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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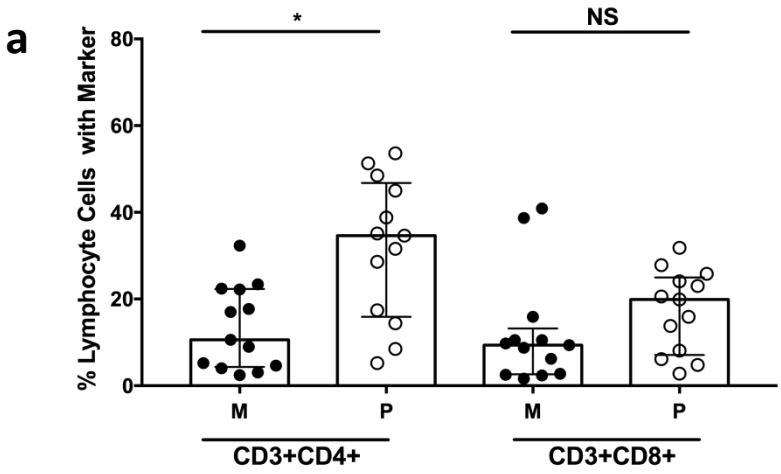
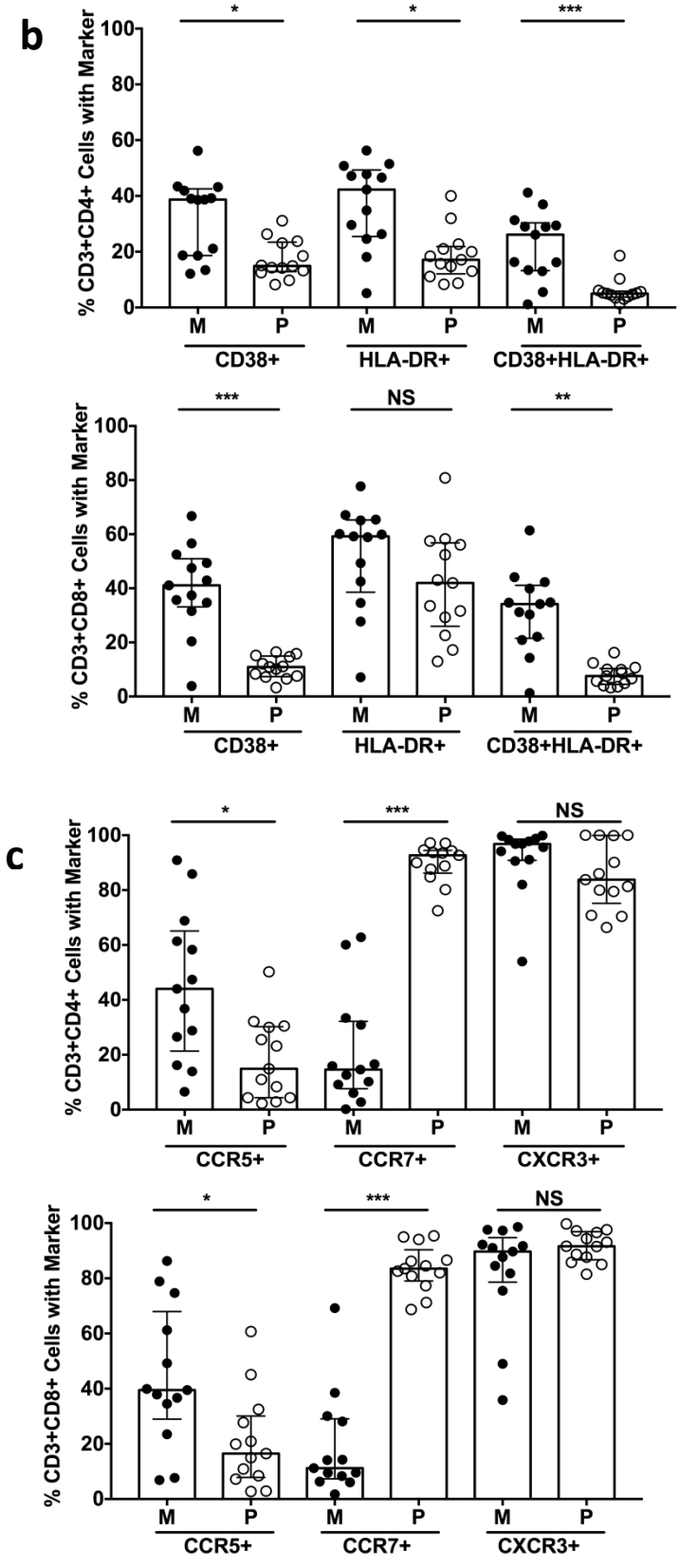
Fig. 1 Differences in T cell phenotypes from the peripheral blood vs. genital tract mucosal in chlamydia-infected women. a-d Scatter-bar plots comparing mucosal (M, n=13 [closed circles]) vs. peripheral (P, n=13 [open circles]). **a** T cell distribution of both T cell subsets; **b** Activation markers CD38+HLA-DR+; **c** Homing CKRs CCR5+, CCR7+, CXCR3+; **d** T helper associated CKRs CCR4+ (Th2-associated), CXCR3+CCR5+ (Th1-associated), CXCR3+CCR4+ expressed on both CD3+CD4+ (left) and CD3+CD8+ (right) T cell subsets. Findings show that at the genital tract mucosa in chlamydia-infected women, there is enhanced T cell activation along with a higher expression of CCR5, a lower expression of CCR7, and association with a Th1 phenotype; in the peripheral blood, Th cells expressed predominantly a Th2-associated phenotype. * $P \leq 0.05$, ** $P < 0.001$, *** $P < 0.0001$, or NS (not significant) by the Wilcoxon signed-rank test. Data are represented as median and interquartile range (IQR).

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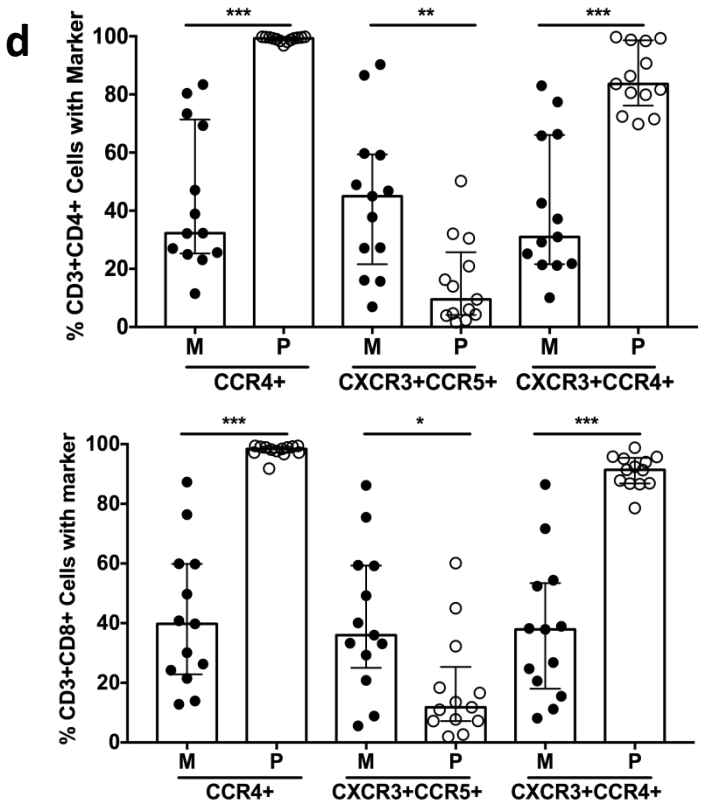
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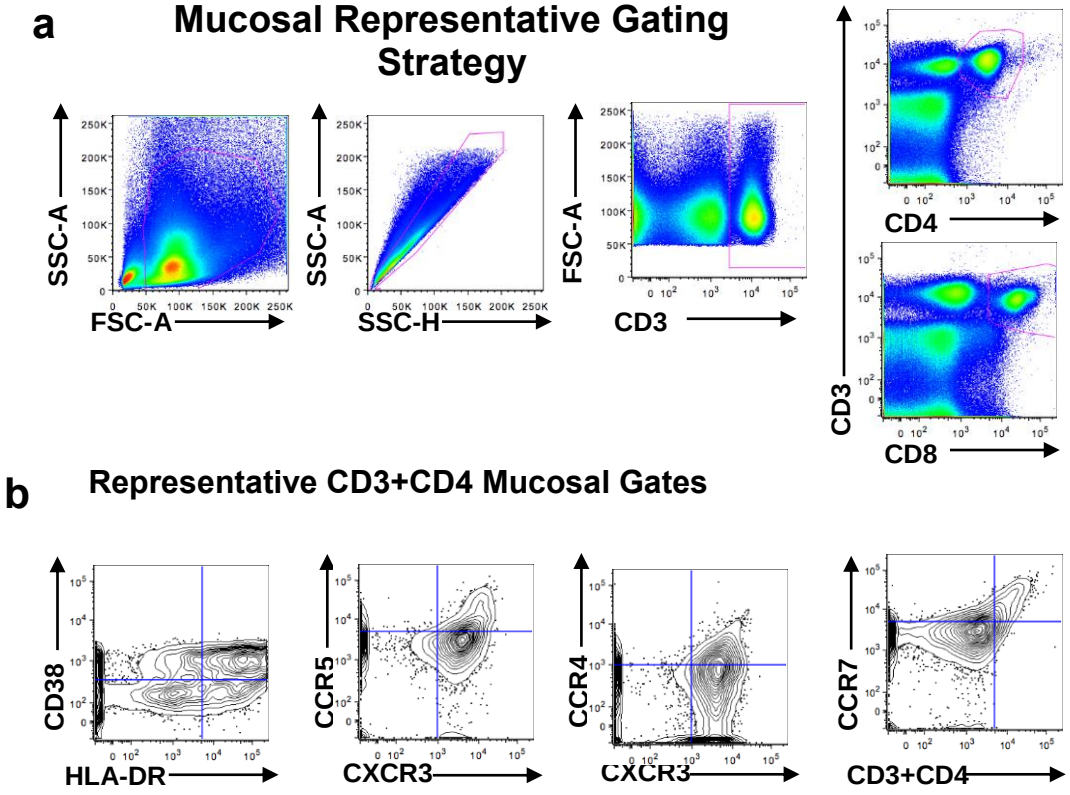
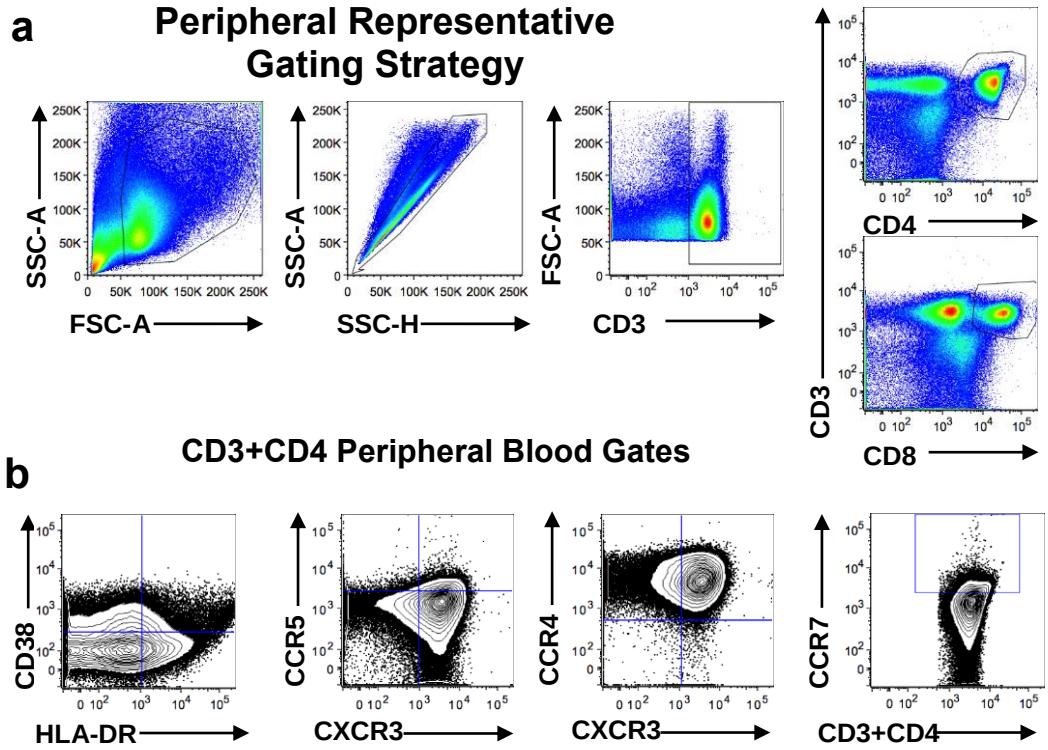
Supplemental Fig. 1 Mucosal mononuclear cell gating strategy. a Gating strategy for segregation of mononuclear cells from the genital mucosa of chlamydia-infected women into CD3 T lymphocytes and then into CD4 and CD8 T cells. **b** Flow plots of gates depicting CD3+CD4+ T cells further gated for the expression of activation markers and chemokine receptors (CKRs).



Supplemental Fig. 2 Peripheral blood mononuclear cell gating strategy. a Gating strategy for separation of peripheral blood mononuclear cells from chlamydia-infected women into CD3 T lymphocytes and then into CD4 and CD8 T cells. **b** Flow plots of dual and single gates depicting CD3+CD4+ T cells further gated for the expression of activation markers and chemokine receptors (CKRs).



SUMMARY DISCUSSION

The rates of CT infection have not decreased despite screening and control efforts (105-107). Studies in humans evaluating the natural progression of CT infection are limited by challenges stemming from ethical constraints, human variation (e.g., sexual behaviors, genetics and etc.), and availability of well-characterized cohorts. The few studies that have been conducted in humans evaluating immune responses revealed that IFN-γ correlated with protection against initial and repeat CT infection (99, 100), however approximately 15-20% of individuals continue to have repeat CT infections within months after treatment (95, 96), possibly indicative of little or no protective immunity. These scant studies that affirm established murine model paradigm implicate Th1 responses but fail to provide insight into T cell phenotypes and homing dynamics.

A recent murine vaccine study demonstrated that protective immunity against CT could induce both $CD4^+$ and $CD8^+$ T cells by priming intranasally and challenging intravaginally (61). A subsequent study evaluated a noel mucosal vaccine candidate against CT that generated two waves of protective memory T cells (62). The vaccine was developed by utilizing a unique CT-adjuvant conjugate nanoparticle to inoculate and then re-challenged the immunized mice. This study demonstrated that previously developed circulating effector T cells and effector T cells that trafficked to the genital mucosa and establish T_{RM} cells were either re-localized or reactivated (respectively) in response to active infection and proved crucial for bacterial clearance and long-lived protection (62). Interestingly, in a recent review looking at the effects of ovarian steroid hormones and CT

APC presentation and immune responses reveal that hormone levels play a significant role in determining T cell prevalence and in infection dynamics. The finding in this review were important because female contraceptives and the menstrual cycle could affect vaccine efficacy. Ultimately, findings in this review were difficult to collectively interpret, perhaps due to the use of various cell lines and models as well as varying levels and combinations of hormones (159). Overall, animal models have offered a great deal of understanding into chlamydial immunity, however differences in infectious species, estrous cycle, T cell distribution frequencies, duration of infection and immune responses make translating findings into humans difficult. One prominent human study evaluated limited chemokine receptor expression (CCR5 and CCR7), one activation maker (HLA-DR) and two integrins (($\alpha_E\beta1$ and $\alpha4\beta7$), comparing expression of these markers on endocervical cells vs. peripheral blood mononuclear cells from CT-infected women (158). The study revealed that there was a higher expression of HLA-DR and CCR5 at the mucosa (158). However, this study looked at only a limited number of CKRs and did not further delineate $CD3^+$ T lymphocytes into $CD4^+$ and $CD8^+$ T cells; therefore, no real conclusions could be made about Th-associated and homing phenotypes in CT infection. Therefore, more in-depth studies focusing on evaluating immune T cell CKR dynamics in human CT infection are warranted. As such, this dissertation discusses two studies (second and third chapters) aimed and increasing our understanding of T cell phenotypes during CT infection in humans.

Dissertation findings

Peripheral blood T cell phenotypes

The second chapter of the dissertation study was to evaluate differences in the peripheral blood T cell phenotypes of women with versus without active genital CT infection as well as CT-seronegative controls. As such, our study is the first to comprehensively evaluate peripheral blood $CD4^+$ and $CD8^+$ T cell phenotypes involved in CT infection in humans by evaluating differences in the T cell distribution, CKR homing, activation status, and T helper-associated phenotypes. The first aim in this study was to evaluate these phenotypes in CT-infected versus CT-seronegative controls. Evaluation of the major T cell subsets revealed that CT-infected women had a higher proportion of $CD3^+CD8^+$ T cells and a trend towards a higher proportion of $CD3^+CD4^+$ T cells than CT seronegative controls. We also observed an overall higher activation status in both T cell subsets of CT-infected women versus seronegative controls, suggesting that CT led to activation of circulating T cells in preparation for homing to the site of infection to mount a local immune response. Our study also revealed an increased expression of CKR homing markers $CCR7^+$ on $CD4^+$ T cells and both $CCR7^+$ and $CXCR3^+$ on $CD8^+$ T cells in CT-infected women, also consistent with ongoing activation and migration of T cells after CT infection acquisition (60, 158). These findings together may indicate that the CT driven expansion of T cells may influence the magnitude of T cells' activation, CKR expression, and homing ability during infection.

We also evaluated Th-associated phenotypes and found a predominance of Th2-associated (CCR4) CKR expression in CT-infected women at the initial visit (just prior to treatment), along with some contributions from Th1-associated ($CXCR3^+CCR5^{+)}$ CKR

expression. These findings suggest that CT-primed $CD4^+$ cells have the potential to produce cytokines capable of polarizing T cells towards either a predominant Th2-associated phenotype, which has been linked to CT persistence and pathology (160-162), or a Th1-associated phenotype, which has been associated with protective immune responses against *Chlamydia* (10, 11, 155, 163). The analysis of $CD8^+$ T cell phenotypes revealed similar findings, with an increase in expression both $CXCR3^+CCR5^+$ and $CCR4^+$ CKRs in CT-infected women, suggesting a complementary role with $CD4^+$ Th-associated T cells responsible for protection or pathology in CT infection (61, 164, 165). Additionally, T regulatory cells play a role in driving cell fates towards either Th1 or Th2 phenotypes, however this was not evaluated in this study but this phenotype is of interest in future studies.

The second aim of this study focused on evaluating how T cell phenotypes changed at follow-up (after CT treatment) and whether CT reinfection at follow-up influenced T cell phenotype expression dynamics. We found that the proportion of activated T cells steadily declined in women without active infection at the first (3-month) and second (6-month) follow-up visits. Likewise, CCR7 (a secondary lymphoid organ and memory maker (147, 166)), and CXCR3 (an inflammatory homing maker (128)) was lower for both T cell subsets at the follow-up visits in women without active CT infection, suggesting a distinct fate for these T cells in CT infection. Unlike at the initial visit prior to treatment where we saw enhanced expression of homing, T-associated and activation T cell phenotypes, at follow-up visits there was lower expression of these T cell phenotypes, suggesting they were returning to basal expression. Ficarra et al. reported similar findings regarding CCR7 expression decreasing at the first follow-up visit (with a median time of 35 days between

visits and a range of 14-113) (158). Along with minimal expression of CCR5 on $CD8^+$ T cells, our findings suggest that CCR7 and CXCR3 CKRs play a role in the localization of T cells during active CT infection and in the absence of CT infection, these CKRs go back to their basal expression level. The continued decrease in CKR expression over a 6-month time period (corresponding to follow-up visits out to 6 months) in the peripheral compartment further substantiates that these cells were initially primed or activated in preparation for migration at the time of their initial infection but in the absence of reinfection at follow-up, they did not remain primed for infection and returned to a basal state. Intriguingly, in a small subset of women who become reinfected at the first follow-up visit (3-month), we also observed a decrease in T cell phenotypes when compared to their initial visit (prior to treatment). A reason for this finding may be that these women had a lower CT load at reinfection (vs. their initial infection) and therefore may not have had the same degree of up-regulation of CKR expression compared with their initial infection (167). An alternative explanation may be that the timing of treatment in the course of the infection (i.e., earlier vs. later stages of infection) may have an effect on the expression of CKRs, particularly in women who become reinfected with CT who we may be very early in their infection before the onset of CKR expression. Finally, there may be varying degrees of adaptive immunity that develop after the initial infection that could influence T cell phenotypes. One of the limitations of our study, as often seen in studies of human CT infection, is that we don't know the precise duration of infection.

Our investigation into receptors that are associated with different Th phenotypes revealed a higher proportion of Th2-associated $CCR4^+CD4^+$ T cells at the initial visit prior to treatment which also steadily declined across both follow-up visits, irrespective of the

occurrence of CT reinfection. We also saw a decrease in the expression of Th1-associated $CXCR3^+CCR5^+CD4^+$ T cells in women with or without CT reinfection at both follow-up visits. CCR5 and CXCR3 has been shown to be important in genital tract homing in CT-infected mice (60) and in the peripheral blood T cells of patients with Grave's disease (168). Interestingly, on $CD8^+$ T cells we found that the expression of CXCR3 and CCR5 was higher in women without CT reinfection at the first follow-up visit, further substantiating the importance of this phenotype. Alternatively, high CCR4 expression has not been demonstrated in humans. A study that evaluated CKR expression in Crohn's disease and ulcerative colitis, reported similar findings that CCR4 expression was upregulated in the peripheral compartment along with the suppression of CXCR3 and CCR5 (134). This suggests that in infections with inflammation localized to a mucosal site, the peripheral T cells seem to first express high levels of CCR4 but as they traffic to the site of inflammation, there is a shift in this expression. This finding is further supported by another study that found that CT-infected women elicited robust Th2 responses during active infection and had much lower Th1 responses ,suggesting that Th1 responses develop more slowly (133). Our analysis of $CD8^+$ T cells revealed similar findings, again suggesting a complementary role of $CD8^+$ with $CD4^+$ T cells in controlling CT infection, as evidenced by similar CKR expression in both T cell subsets.

Another notable finding was a higher proportion of both $CD4^+$ and $CD8^+$ T cells expressing $CXCR3^+CCR4^+$ in CT-infected women at their initial visit (prior to treatment) compared with at follow-up visits. The presence of this unique phenotype suggests an emerging role for this phenotype as evidenced by our findings showing higher expression of these CKRs on $CD8^+$ T cells on women without CT reinfection at the first follow-up

visit. Another study evaluating the migration of T cells based on CXCR3 and CCR4 expression determined that T cell migration was partially dependent on CXCR3, yet independent of CCR4. Instead, this study also reported that CCR4 seemed to play a role in T cell retention at inflamed sites and that the absence of both markers resulted in reduced development of arthritis (122). Overall, our findings suggest that T cells expressing both CCR4 and CXCR3 may have an immune-modulatory role in CT infection. These T cells have also been implicated as regulatory T cells in inflamed human liver tissue as well as in T cells from the synovial fluid of patients with juvenile idiopathic arthritis (128, 169, 170). Together our findings seem to suggest that this unique phenotype seems to play a role in the immunomodulation of CT infection in women, although further studies are warranted to establish their function and their role in CT reinfection.

T cell phenotypes in the genital mucosa versus the peripheral blood

The third chapter of this dissertation was to evaluate site-specific differences in chemokine receptor expression of paired genital mucosal and peripheral blood specimens taken from women with active CT infection for both $CD4^+$ and $CD8^+$ T cells. The proportion of $CD4^+$ T cells was higher in the peripheral compartment than the mucosa, with a similar trend for $CD8^+$ T cells, suggesting that there may be some level of T cell expansion at the periphery in response to CT infection. However, there was a higher activation status of the T cell subsets at the mucosa than the periphery. The enhanced activation status at the mucosa, the site of active CT infection, suggests that APCs are being sensitized to CT antigens and in turn circulating and recruiting T cells. Interestingly, expression of CCR5, an inflammatory homing marker that influences trafficking, was

higher in the mucosa, suggesting that active infection localized to the genital mucosa is dynamically modulating T cell trafficking. This is further supported by the increased expression of homing CKRs CCR7 for both T cell subsets and trending towards higher expression for CXCR3 for $CD8^+$ T cells at the periphery. CCR7 is a secondary lymphoid organ homing and memory marker (147), which in this case is likely being expressed on circulating T cells homing them to the lymphatics and eventually the genital mucosa where CCR7 expression is then downregulated. Similarly, CXCR3, a known mucosal homing marker, has been demonstrated to be an essential CKR for genital mucosa homing during active CT infection (60). CXCR3 expression is also reduced once T cells home to the genital mucosa, suggestive of mucosal residency (i.e., T cells becoming resident memory T cells). These findings suggest that CKRs CCR7 and CXCR3 play a more important role in trafficking T cells to the mucosa and are likely regulated by CCR5 expression.

The evaluation of CKRs that are associated with T helper phenotypes revealed that during active infection, the peripheral compartment predominantly maintains a higher expression of $CCR4^+CD4^+$ T cells, which are associated with a Th2 phenotype. However, the concomitant expression of $CCR5^+CXCR3^+$ on $CD4^+$ T cells was predominantly higher at the genital mucosa, indicative of a shift towards a Th1-associated phenotype at this site. Site-specific differences in Th associated phenotypes suggests that there is a polarization that occurs from Th2 to Th1 when T cells traffic from peripheral circulation to the genital mucosa. This is evidenced by the fact that Th1 responses are essential for CT clearance and establishing immunity in the murine model (60). $CXCR3^+CCR4^+CD4^+$ T cells were found to be more prevalent in the peripheral compartment. This shift may be modulated by a unique subset of cells co-expressing CXCR3 and CCR4 on $CD4^+$ T cells that have not

been reported in CT infection previously, but have been shown to be involved in regulating inflammation at the liver (171) and on synovial fluid during idiopathic arthritis.(172) This may be supported by the fact that we observed a comparable proportion of T cells co-expressing CXCR3 and CCR4 relative to the proportion expressing the predominant Th-associated phenotype at each site. Coincidentally, expression of these CKRs on CD8 T cells was similar, suggesting a complementary role of CD8 T cells in aiding CD4 T cells to control CT infection.

Conclusions

Our comprehensive evaluation of peripheral blood T cell phenotypes in CT-infected women vs. CT-negative controls revealed that CT infection leads to overall higher activation and up-regulation of various CKRs, as well as markedly increased expression of Th-associated CKRs on $CD4^+$ and $CD8^+$ T cells. Our analysis of differences in T cell phenotypes between paired peripheral blood specimens from women initially infected with CT to those with or without active infection at follow-up visits suggests that during active CT infection, T cells acquire the ability to traffic and then after the initial infection has been treated, there is a expression of the same CKRs over time. There was a higher expression of CCR4 in women not reinfected at follow-up, suggesting a role of this CKR during initial CT infection. The down regulation of CKR expression across follow-up visits in those without reinfection may indicate that some of these T cells could be going back to basal CKR expression in the absence of infection. Our study also points to the role for T cells co-expressing CCR4 and CXCR3 in modulating CT-infection in women prior to treatment. Our studies evaluating the mucosal versus peripheral compartment revealed a higher

expression of CCR5, Th1-associated CKRs ($CXCR3^+CCR5^+$), and activation makers (CD38 and HLA-DR) in the mucosal compartment suggesting that these makers play a key role at the site of active CT infection. However, the markedly higher expression of Th2-associated CKR CCR4 in the peripheral compartment implies that these cells are maintained as this phenotype until some T cells that are to be home to the mucosal site become polarized towards Th1 cells. Both Th subtypes polarize from higher Th2 at the periphery to higher Th1 in the mucosa. While both subtypes may play a role in T cell trafficking, they are likely modulated by T cells co-expressing CXCR3 and CCR4 during active infection, which regulates inflammation. Our analysis also highlights the importance of studying T cell phenotypes when seeking to better understand CT immunobiology that may contribute to protection or influence CT infection outcomes.

Future directions

Findings from immune functional studies on PBMCS from the same cohort of women in Dr. Geisler's research program revealed that the predominant Th1 cytokine produced at the time of initial infection was TNF-α (173), rather than IFN-γ, as had been demonstrated in murine models and limited human studies highlighted in the introduction. Coincidentally, in a subsequent immune function study evaluated Th1 responses at follow-up visits after treatment in the same women and found there was a shift towards increased IFN-γ production at the follow-up visits, suggesting that over time there seems to be a development of adaptive immune responses that likely serve to protect against CT reinfection upon re-exposure. This notion is further supported by that same study demonstrating a higher frequency of $CD4^+$ IFN-γ responses at follow-up in women who

did not have reinfection vs. those that did.

Increasing or decreasing levels of specific cytokines may serve to regulate the formation of certain types of memory cells in CT infection. Perhaps what may be occurring in women with or without CT reinfection is the development of short lived effector T cells (SLEC) in reinfected women rather than the desired memory precursor effector cells (MPEC). At the very least, it is possible that there is a skew towards more MPEC cells in non-reinfected women. This is further supported by a study in mice that demonstrated this effect was further enhanced by high expression of IFN-γ and IL-12 together impaired development of memory (59). Therefore, it would be interesting to evaluate in the future the expression of IFN-γ and IL-12 in women initially infected with CT at the pretreatment visit versus women with or without reinfection at follow-up.

Additionally, in the future, an in-depth evaluation of the relationship of other T cell phenotypes (e.g., CCR6 or CCR9) and function with risk for CT reinfection by would be important in order to hopefully identify immune correlates of protection. In particular, experiments that further delineate memory T cell phenotypes (by staining for CCR7 and CD45RA) and the expression of integrin binding receptors on T cells from CT-infected women are warranted. Moreover, future experiments that evaluate the function of T cells for which we have already characterize the phenotype may include: cytotoxicity assays on CD8 T cells to determine their role during CT infection and T regulatory cell suppression assays assessing the function of CXCR3 and CCR4 dual positive cells thought to be immunomodulatory. Since our findings raise the possibility that T cells from the initial CT infection may develop into resident memory T cells, future experiments aimed at evaluating for resident memory T cells may be informative. Ultimately, further examining

these other T cell markers in the setting of CT infection could provide knowledge that will be important in correlating with future studies aimed at evaluating CT-specific function of these specific T cell phenotypes and ultimately could be useful for CT vaccine development efforts that would help counteract increasing rates of CT infection and further contribute to the reduction of CT complications.

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APPENDIX A

IRB APPROVAL FORM

OMB No. 0990-0263
Approved for use through March 31, 2018

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule.

Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [✓] CONTINUATION [ ] EXEMPTION	[✓] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
Mechanisms and Correlates of Immune Protection Against Genital Chlamydia in Humans (CITY Health II - The Center for the Study of Community Health - SIP 14-034 - Prospective Study of Immune Response to Chlamydial Infection to Inform Development of Rational Prevention Strategies)	GEISLER, WILLIAM MICHAEL

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 11/08/2021 IRB Registration No. IRB00000196

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting) _____ or [X] Expedited Review on (date) 6/5/17
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title X110816011
Protocol subject to Annual continuing review.	Mechanisms and Correlates of Immune Protection Against Genital Chlamydia in Humans (CITY Health II - The Center for the Study of Community Health - SIP 14-034 - Prospective Study of Immune Response to Chlamydial Infection to Inform Development of Rational Prevention
IRB Approval Issued: 6/5/17	IRB Approval No Longer Valid On: June 5, 2018

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: irb@uab.edu	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
14. Name of Official Expedited Reviewer	15. Title IRB Member
16. Signature Julius Libin, MD	17. Date 6/5/17

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Investigator's Progress Report

Form version June 25, 2015

☒ Continuing Review (Complete Items 1-12)
—OR—
☐ Final Report—all protocol-related activities are complete, including data analysis (Complete Items 1-11, and Item 13)

—FOR—

☒ Expedited Review
—OR—
☐ Convened (Full) Review

1. Dates

Today's Date	07/06/2015	To help avoid delay, respond to all required items in the format provided, and include requested materials.
Starting Date of Project	03/27/2012	If previous approval expires before approval is officially re-issued by the Office of the IRB, all work on the protocol must cease.
Current IRB Expiration Date	08/15/2015	The IRB recommends applying for continuing review *4-6 weeks* before expiration of current approval. (See schedule.)

2. Principal Investigator (PI)

Name (with degree)	William M. Geisler, MD, MPH	**Blazer ID**	wgeisler
Department	Medicine	**Division**	Infectious Diseases
Office Address	Zeigler Research Building 242	**Office Phone**	205-934-4376
E-mail	wgeisler@uab.edu		
PI Contact who should receive copies of IRB correspondence (Optional)			
Name		**E-mail**	
Phone			

3. UAB IRB Protocol Identification

Protocol Number	X110816011
Protocol Title	Mechanisms and Correlates of Immune Protection Against Genital Chlamydia in Humans
Study Sponsor(s)	NIH
OSP Assigned Number (9 digits)	000410050 (link number)
Note. *If the source or amount of funding for this project has changed or a new OSP # has been assigned to the protocol, include the new or revised funding application and/or provide the new OSP Assigned Number:*	

4. Purpose

In two or three sentences, briefly summarize the purpose of this protocol, and related studies if applicable. Please use non-technical language, and write for adults with general knowledge rather than for specialists.

The purpose of this study is to better understand the cellular immune responses and genetic determinants that protect chlamydia-infected persons from getting chlamydia re-infection after treatment. This knowledge will be important for future chlamydia vaccine studies in humans.

5. Screened, entered, or otherwise accessed by the UAB Investigator(s). Include numbers for individuals, specimens, data records, charts, etc., as applicable to the protocol.

5.a. Number screened for study entry since the start of the project?	515
5.b. Number entered in study since the start of the project? (See Total in 5.e.)	248
5.c. Number entered in study since the last IRB review?	101
5.d. What is the age range for all participants entered in the study since the start of the project (e.g., 18-65)?	16-50

5.e. Complete the table below for cumulative enrollment for each racial and ethnic category. Copy/paste the entire table for additional groups (e.g., controls, sub-studies) if needed.

Racial Categories	Ethnic Categories									Total
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			
	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	
American Indian/ Alaska Native	0			0						
Asian	0			0						
Native Hawaiian or Other Pacific Islander	0			0						
Black or African American	233			0						
White	10			0						
More Than One Race	3			0						
Unknown or Not Reported	0			2						
Total	246			2						

☐ Check the box at the left if demographic information is not available (e.g., not collected for screening; collecting only specimens or data records and did not have access to the information).

6. Conflict of Interest Review Board (CIRB)

Does the Principal Investigator, the institution, or any other person listed on this protocol have a financial conflict of interest, as defined by the UAB CIRB, related to this research? ☐Yes ☒No

If No, continue with Item 7.

If Yes, in the space below, provide the names of the individuals who have a conflict and indicate whether or not a management plan is in place for each person listed.

▶

7. Information Since the Date of Last IRB Review

- Mark at least one checkbox to indicate the type(s) of information received since the Date of Last IRB Review.
- Please summarize each type of information, and provide details and copies as requested.

7.a. You received multi-center trial reports that you have not previously forwarded to the IRB. ☐Yes ☒No Multi-Center Trial Report

Attach a copy and, in the space below, provide the date and source of the report, and summarize the findings and any recommendations:

▶

7.b. You received data and safety or other monitoring reports (e.g., DSMB, sponsor site visit). ☐Yes ☒No Data Safety or Other Monitoring Report

Even if you have already forwarded a copy to the IRB, attach a copy and, in the space below, provide the date and source of the report, and summarize the findings and any recommendations:

▶

7.c. You learned of literature published about this research. Attach the publication or provide its web address, and summarize the published findings here:	☐Yes ☒No Published Literature
▶	
7.d. You learned of other relevant information regarding this research, especially about risks associated with the research. Attach a copy of the source and/or summarize below, and check "Other Information" at right. Also check "Affects Willingness" if this information might affect a participant's willingness to continue in the research, and describe the effects on participants here:	☐Yes ☒No Other Information
	☐Yes ☒No Affects Willingness
▶	
7.e. You have received another type of information. Summarize the information, including details relevant to participants here:	☐Yes ☒No Other Type of Information
▶	

8. Reportable and Non-reportable Problems	
8.a. Have there been any "reportable events" since the IRB's last continuing review of the project? "Reportable events" are those that may constitute unanticipated problems involving risks to participants or others. If yes, attach the UAB Problem Report (even if already reported to the IRB); also attach the UAB Problem Summary Sheet completing Table A; Provide brief narrative summary (2-3 sentences) of any trends or increases in frequency or severity noted for all events over the life of the project, or enter "None noted" here:	☐Yes ☒No Reportable Events since last continuing review (Table A)
▶	
8.b. Have participants experienced harms (expected or unexpected, serious or not serious) that do not meet the UAB IRB criteria for "reportable events" since the IRB's last continuing review of the project? Attach UAB Problem Summary Sheet completing Table B; provide brief narrative summary (2-3 sentences) of any trends or increases in frequency or severity noted for all events over the life of the project, or enter "None noted" here:	☐Yes ☒No Other Events since last continuing review (Table B)
▶	
8.c. Have there been any reportable or non-reportable events over the life of the project? Attach UAB Problem Summary Sheet completing Table A and/or B as appropriate. Note the UAB Problem Summary Sheet is a cumulative report for all events over the life of the project. Provide brief narrative summary (2-3 sentences) of any trends or increases in frequency or severity noted, or enter "None noted" here:	☐Yes ☒No Any reportable or non-reportable events over the life of the project
▶	

9. Events Since the Date of Last IRB Review Mark at least one checkbox to show event(s) that have occurred since the Date of Last IRB Review. Please summarize all events, and provide specific details and/or copies as requested.	
9.a. You have had one or more problems obtaining informed consent. Briefly describe the problem(s) here:	☐Yes ☒No Consent Problems
▶	
9.b. You have received complaints about the research. Briefly describe the number and nature of the complaints:	☐Yes ☒No Complaints

▶	
9.c. One or more participants withdrew, or were withdrawn from, the research. Indicate here the number of withdrawals and the reason for each:	☐Yes ☒No Withdrawals
▶	
9.d. Participants have experienced research-related benefits. For example, "60% of participants in the treatment group appear to have reduced symptoms or reduced severity of symptoms, compared with 10% in the placebo group." Briefly describe the benefits here:	☐Yes ☒No Benefits
▶	
9.e. The risks, potential benefits, or both of this research have changed. Briefly describe the changes here:	☐Yes ☒No Change in Risk or Benefit
▶	
9.f. Does the research involve minors (<18 years of age)? If the study is still open to accrual or the participants are still receiving protocol driven intervention, the PI must either (a) confirm the previously assigned Children's Risk Level (CRL) number or (b) reassign a new CRL and give the reason it has changed in the space provided below:	☒Yes ☐No
▶ The PI (Geisler) confirms the previously assigned CRL of 1 has not changed.	
9.g. Events have occurred that relate to participant safety but do not fit into the categories listed above. Briefly describe the events here:	☐Yes ☒No Other Events
▶	

10. Protocol and/or Informed Consent Modifications **Check the applicable boxes to indicate modifications made since Date of Last IRB Review (Yes to 9.a.) or requested with this renewal (Yes to 9.b. or 9.c.). Please provide the details and materials requested.**	
10.a. Previous Modifications	
Since the last IRB review, have you made modifications to the protocol, consent process, consent document or change in personnel?	☒Yes ☐No
If Yes, have the modifications been approved by the IRB?	
☒Yes—Provide a copy of each amendment form stamped "Approved" by the IRB during this approval period. ☐No—In the space below, justify making the modification without prior IRB approval:	
▶	
10.b. Modifications To Protocol Requested With This Renewal	
Are you requesting IRB review of changes to the protocol (e.g., procedures, personnel, recruitment)? If so, check "Yes" and describe them in the space below. If adding personnel, (1) provide full name and UAB department/division, (2) indicate role in research, (3) and address whether the personnel has a financial conflict of interest. If removing personnel, please provide name(s) of personnel being removed. Indicate beside the name whether you are adding or removing each individual.	☒Yes ☐No Protocol Changes
▶ Adding the following persons to the project: 1. Kanupriya Gupta, PhD, UAB Department of Medicine / Division of Infectious Diseases. 2. Brian Ogendi, B.S., UAB Graduate School.	

2. Brian Ogendi, B.S., UAB Graduate School

Both Kanu Gupta and Brian Ogendi will assist in carrying out laboratory procedures in the project and neither has any conflict of interest. The PI, Dr. William Geisler, will provide the necessary education and research training for Kanu and Brian, and will supervise their research-related duties and function.

10.c. Modifications To Consent Requested With This Renewal	
Are you requesting IRB review of changes to the consent process and/or form(s)? If so, check the applicable "Yes" box and, in the space below, describe the changes.	☐Yes ☒No Consent Process Changes ☒Yes ☐No Consent Document Changes

If the changes affect the consent form(s), indicate the number of consent and/or assent forms used for this protocol, and describe the changes to each form:
(a) describe all changes to IRB-approved forms and the reasons for them;
(b) describe the reasons for the addition of any materials (e.g., addendum consent); and
(c) indicate either (1) how and when you will reconsent enrolled participants or (2) why reconsenting is not necessary.

Also, indicate the number of forms changed or added. For new forms, provide 1 copy. For revised documents, provide 3 copies:
- a copy of the currently approved document (showing the IRB approval stamp, if applicable),
- a revised copy highlighting all proposed changes with "tracked" changes, and
- a revised copy for the IRB approval stamp.

▶ The current consent form is being revised on the last page (AUTHORIZATION FOR USE/DISCLOSURE OF HEALTH INFORMATION FOR RESEARCH page) by removing the word "Public" from Jefferson County Department of Public Health.

11. Gene Therapy, Gene Transfer, Recombinant DNA				
If this study involves	☐Gene therapy	☐Gene transfer	☐Recombinant DNA	☒N/A – go to item 11.
	Complete this item, and include memorandum with original signatures of Gene Therapy Review Panel addressing the risk-benefit ratio, any recommendations, and the CRL if applicable.			

11.a. Has the Panel's assessment of the risk-benefit ratio of this project changed? If yes, please explain below.	☐Yes ☐No Risk-Benefit Change
▶	
11.b. Does the Panel have any recommendations regarding the protocol or the consent form? If yes, please explain below.	☐Yes ☐No Panel Recommendations
▶	

12. Continuing Review—Complete only if you want to renew IRB approval so that protocol-related activities can continue.	
12.a. Accrual Status—Indicate whether the study is "NOT YET OPEN," "OPEN," or "CLOSED" (described below) and provide the details requested for that accrual status.	
NOT YET OPEN: No individuals have been screened or entered.	☐Not Yet Open
OPEN: The study could still enroll more individuals, add more specimens, review more records, etc. • Attach a copy of the most recently approved consent form(s) OR note in the space below that the IRB has waived informed consent and/or use of a consent form (waiver of documentation of informed consent).	☒Open