Optimization and Use of ELISpot and Proliferation Assays:

Characterizing a CD4+ T-Cell Immune Response to *C. trachomatis* in Adolescent Women

SARAH ANN NELSON

Principal Investigator: Dr. Romina Barral

Abstract   *Chlamydia trachomatis* is the most prevalent STI in the United States, with the highest rates of infection reported among adolescent females[6]. The development of treatment strategies to prevent infection and the associated reproductive pathology requires a better understanding of the CD4+ T-cell immune responses to *Chlamydia* infection. PBMCs isolated from a seropositive donor and a seronegative donor were stimulated with various concentrations of chlamydial antigens (Serovar D EB and CHSP60-1) and underwent 24- and 48-hour incubations. T-cell proliferation and cytokine secretion was quantified by 3H proliferation and dual-color ELISpot assays, respectively. Experimental assays revealed that PBMCs stimulated with 1ug/mL of chlamydial antigens and incubated for 48 hours was optimal for stimulating T-cells from a seropositive donor to proliferate more robustly and to display more specific secretion of IFN-γ than T-cells from a seronegative donor. In a follow-up study with a small cohort of adolescent women ($13 \leq M_{age} \leq 25$) at high risk for *Chlamydia* infection, statistical analyses of experimental assays suggested that alamar blue T-cell proliferation and ELISpot assays were less accurate at diagnosing *Chlamydia* than current PCR diagnostic methods. Furthermore, analyses revealed that experimental assays were unable to predict immune status differences and to detect pathology in adolescent women. Although, exploratory ROC analyses suggested that experimental assays may have the potential to detect pathology among patients, such that AUC values for experimental assays did better than chance (AUC > 0.5) at predicting pathology when CD4+ T-cells were stimulated with chlamydial antigens.

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Introduction

Chlamydia trachomatis (CT) genital infection is the most prevalent bacterial sexually transmitted infection (STI) worldwide[6]. Infection induces mostly short-term immunity that is strain (serovar) specific[3], which increases both the risk of re-infection and tissue damage[9]. Although *Chlamydia* infection can be treated effectively with antibiotics, infections may persist asymptomatically, damaging tissue even when appropriate antimicrobial therapy is administered. In addition, early treatment of infections may inhibit the development of protective immune responses and promote an increased prevalence of infection[2]. The development of a preventative vaccine may be the only effective means to reduce the spread and morbidity associated with this pathogen, which requires a deeper understanding of the inflammatory cell-mediated immune responses in humans associated with *Chlamydia* infection.

Recently published studies in humans suggest that Interferon-γ-producing CD4+ Th1 cells play a protective role against incident *C. trachomatis* infection[7]. In a study completed with female commercial sex workers in Kenya, Brunham et al. found that IFN-γ production by peripheral blood cells stimulated with chlamydial heat shock protein 60 (HSP60) was strongly correlated with protection against incident *C. trachomatis* infection[2]. In contrast to IFN-γ production, polarization of CD4+ effector T-cells towards an Interleukin-10-producing CD4+ Th2 response does not protect against *Chlamydia* infection and may increase genital tract bacterial load by inhibiting protective Th1 responses[2]. Most significantly, Gaczynska et al. found that IL-10 production by peripheral blood cells stimulated with HSP60 was associated with an increased risk of incident *C. trachomatis* infection in Kenyan sex workers[4]. Furthermore, recently discovered Interleukin-17-producing CD4+ Th17 cells have shown to be powerful inducers of tissue inflammation, preventing CD4+ regulatory T-cells from performing their role as inhibitors of tissue inflammation[1]. Although little is known about the function of IL-17-producing CD4+ Th17 cells in *C. trachomatis* infection, the major role of IL-17 in tissue inflammation warrants exploration of this cytokine. Thus, measurement of IFN-γ, IL-10, and IL-17 cytokine production by T-cells following stimulation with a number of *C. trachomatis*-specific antigens should elucidate the human immune responses that correlate and contribute to either protective immunity or disease pathogenesis.

Specific Aims and Hypotheses

The primary objective of this study is to define the local and systematic immune responses to *Chlamydia* infection of the genital tract in adolescent females and to identify future health implications for protective immunity or disease pathogenesis. In order to determine the *Chlamydia*-specific cellular immune responses associated with protection and incidence of reproductive pathology, we propose to optimize ELISpot and proliferation assay methods useful for the quantification and analysis of cytokine secretion and proliferation in human CD4+ T-cells. In a tritiated thymidine T-cell

proliferation assay, we hypothesize that T-cells isolated from the heparinized blood of a seropositive donor will proliferate more robustly in response to chlamydial antigen stimulants (Serovar D EB & CHSP60-1) than T-cells isolated from a seronegative donor. Furthermore, we hypothesize that T-cells isolated from a seropositive donor will have more specific secretion of IFN-γ and IL-10 in response to chlamydial antigen stimulants in an ELISpot assay than T-cells isolated from a seronegative donor. Finally, we broadly hypothesize that longer cell incubations, greater numbers of T-cells per experimental well, and higher antigen concentrations will be optimal for elucidating the expected differences between seropositive and seronegative donors. Upon optimization of cell incubation times, chlamydial stimulant concentrations, and specimen collection, we hope to use the experimental assay methods in a study with adolescent women at high risk for *Chlamydia*.

In the adolescent CARE study, we propose to analyze the CD4+ T-cell immune responses to chlamydial genital tract infection in a small subset of healthy adolescent women visiting the Adolescent Medical Clinic (AMC) in Children's Hospital of Pittsburgh for a routine or problem-prompted gynecological examination and/or STI testing. In general, we desire to illustrate the usefulness of standardizing experimental assays in adolescent clinics by exploring the predictive and diagnostic capabilities of the assays in comparison to current non-specific PCR diagnostic methods for *Chlamydia*. We hypothesize that ELISpot and alamar-blue T-cell proliferation assays will be able to diagnose *Chlamydia* with greater than or equal sensitivity (94.2%) as current PCR methods, based upon significantly different CD4+ T-cell proliferation and cytokine responses to chlamydial antigens in patients with different current infection statuses (no CT or present CT). Furthermore, we hypothesize that ELISpot will be a useful addition to standardized diagnostic methods because they will allow us to more specifically explore immune responses to *Chlamydia* and help us predict current outcomes of protection or disease pathogenesis by illustrating significantly different cytokine secretion in favor of Th1, Th2, or Th17 pathways. More specifically, we hypothesize that positive IFN-γ response by CD4+ T-cells responding to stimulation with chlamydial antigens will be associated with protection against incident infection, positive IL-10 will be associated with greater risk of incident infection, and positive IL-17 response will be associated with worse baseline clinical presentation (discharge and/or abdominal pain). Finally, we hypothesize that alamar-blue T-cell proliferation assays will be useful for detecting pathology in adolescent patients, such that different CD4+ T-cell proliferation responses among adolescents with different baseline clinical symptoms will be associated with higher IL-17 secretion in the IL-17 ELISpot assay and worse baseline clinical presentation. We hope that upon follow-up testing of these adolescent women, the future outcomes of protection and disease pathogensis will correspond with what was predicted by the experimental assays in this baseline study.

Background

Chlamydia: Structure and Pathogenesis

Chlamydia consists of highly specialized prokaryotic bacteria that exhibit a unique biphasic developmental cycle[5], which alternates between an infectious but metabolically

inactive elementary body (EB) and an intracellular metabolically active reticulate body (RB). *Chlamydia* is morphologically and structurally similar to gram-negative bacteria, such that both bacteria contain a trilaminar outer membrane containing lipopolysaccharide (LPS). However, *Chlamydia* uniquely lacks peptidoglycan molecules that contribute significantly to the structural stability of gram-negative bacterial cell walls. In place of the peptidoglycan molecules, chlamydial bacteria contain elementary bodies (EBs), which exhibit extensive disulfide cross-linking both within and between outer membrane proteins[5]. The EBs are the extracellular form of the infection which are critical to the structural and infection capabilities of *Chlamydia,* such that EBs can easily be transmitted to host T-cells by attaching to host epithelial cells and inducing self-phagocytosis. Once inside epithelial cells, chlamydial EB cell walls inhibit fusion of host T-cell phagosomes and lysosomes and thus transform into the intracellular form of the infection, the reticulate body (RB)[5]. The RB can then grow and replicate by obtaining nutrients and high-energy phosphate compounds from the host T-cell. The RB avoids destruction by host T-cell machinery by remaining hidden within a protective membrane-bound vesicle called an inclusion[5]. Following complete multiplication, RBs can re-condense into EBs, which can then be released and permitted to infect future host T-cells. Thus, extracellular (EB) and intracellular (RB) forms of chlamydial infection work to invade host T-cells, resist antibody and cell-mediated defenses, and persist within a host T-cell despite phagocytic host T-cell defense machinery (Fig. 1).
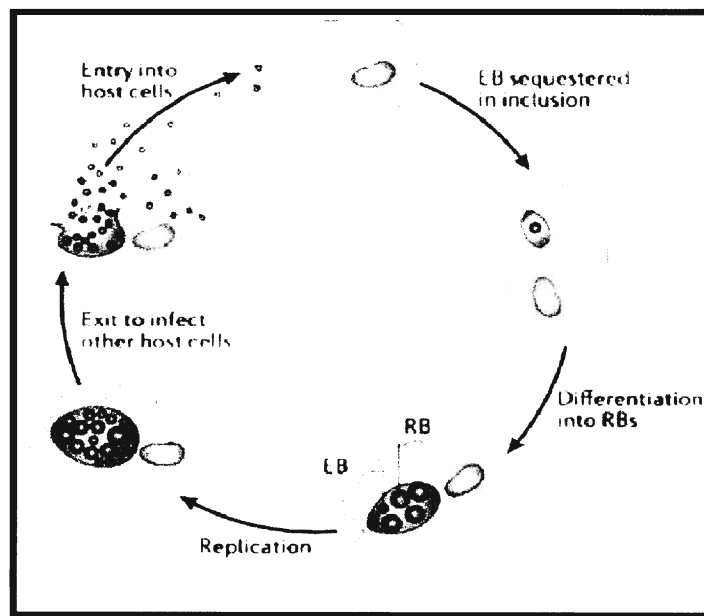

FIGURE 1 *Chlamydia trachomatis* can only replicate inside eukaryotic host T-cells. It has a unique developmental cycle with spore-like elementary bodies (EBs) that infect host T-cells and develop into replicative reticulate bodies (RBs) within a membrane-bound inclusion. RBs redifferentiate into EBs 24–48 hours after infection and the EBs are eventually released by lysis of the host T-cell[10].

The degree of persistence of infection within the host T-cell significantly influences the clinical implications of the pathogen and is directly correlated with the type of host T-cell invaded, the type of chlamydial species or biovar, and the state of the *Chlamydia* upon uptake into the host T-cell[5]. Studies show that the chlamydial biovars with the greatest persistence within host T-cells are related to the most widespread chronic diseases associated with this pathogen. Pathogenesis is characteristically limited to epithelial surfaces of the ocular or genital tract where infection is asymptomatic and often repeated. The chlamydial biovar associated with the most widespread chronic diseases is *C. trachomatis*. Various common infections with *C. trachomatis* include ocular infection with *C. trachomatis* serovars A, Ba, or C associated with infectious blindness, *C. trachomatis* serovars L1, L2, and L3 associated with the rare sexually-transmitted disease, LGV, and *C. trachomatis* serovars D to K associated with the world's most common sexually transmitted bacterial pathogens[5]. Other common chlamydial biovars include *Chlamydial pneumoniae* causing pneumonia, pharyngitis, and bronchitis in humans, and *Chlamydia psittaci* causing veterinary disease and infecting humans only accidentally[5]. For the purposes of this study, we will explore *C. trachomatis* as it contributes to the leading cause of genital tract infection in humans.

C. trachomatis Infection: Genital Tract Disease

C. trachomatis serovars D to K is the most prevalent bacterial STI in the United States, with the highest rates reported among adolescent females. The annual cost of chlamydial infections exceeded $2 billion in the United States in 2000 and has only become more prevalent in sexually active adolescent women[5]. In females, *C. trachomatis* correlates to increased incidences of tubal factor infertility, ectopic pregnancies, and morbidity due to complications of pelvic inflammatory disease (PID). On the other hand, *C. trachomatis* in males causes urethritis and epididymitis. Screening studies in clinics have shown that approximately 10% of sexually active asymptomatic males are infected, and anywhere ranging from 8% to 40% of sexually active females are infected[5]. Furthermore, a study of 106 adolescent females completed by Katz et al. suggests that even when the infection is appropriately diagnosed and treated, chlamydial pathogens can persist for up to 3 months in adolescent females[5]. To this point, Katz and colleagues found that sexually active adolescent women who reported abstinence or 100% condom use maintained high infection rates, suggesting the highly persistent nature of this pathogen[5].

C. trachomatis Infection: Establishing Persistence

One important means by which *C. trachomatis* establishes persistence is the capability of EB molecules to evade fusion of host T-cell lysosomes and phagosomes. Under normal cellular conditions, the host T-cell relies on the fusion of lysosomes and phagosomes in order to fight intracellular parasites. By fusing the lysosomes and phagosomes, the host T-cell releases acid hydrolases into the resulting phagolysosomes to destroy foreign pathogens. However, when EBs attach and enter host T-cells, EBs have inhibitory structures within their cell walls which inhibit the fusion facilitated by the host T-cell defense mechanism. Once the EB has transformed into the intracellular RB form of the infection, the bacteria can grow and propagate by remaining hidden inside an intricately designed inclusion membrane.

The inclusion membrane is the second important quality of *C. trachomatis* that allows the chlamydiae to establish and maintain persistence within the host T-cell by continuing to evade host T-cell lysosomal fusion. Previous research suggests that the inclusion membrane is formed when *C. trachomatis* EBs transform the properties of endocytic vesicles such that they no longer interact with endocytic pathways but instead begin to intercept and fuse with sphingolipids from an exocytic pathway[5]. Sphingolipids are critical to normal cell functioning because they are a class of lipids that play important roles in signal transmission and cell recognition. The EBs work to actively modify the inclusion membrane via chlamydial protein synthesis to establish interactions with the sphingolipids exocytic pathway. The sphingolipids are directly delivered from the host Golgi apparatus and subsequently become incorporated into the cell walls of the chlamydiae inclusion. Incorporation of the sphingolipids into the inclusion allows the host T-cell to recognize the inclusion as a secretory or exocytic vesicle that is not destined to fuse with lysosomes. Thus, RBs can replicate within an inclusion membrane by remaining hidden in a membrane that mimics the membranes of host T-cellular organelles (Fig. 2).
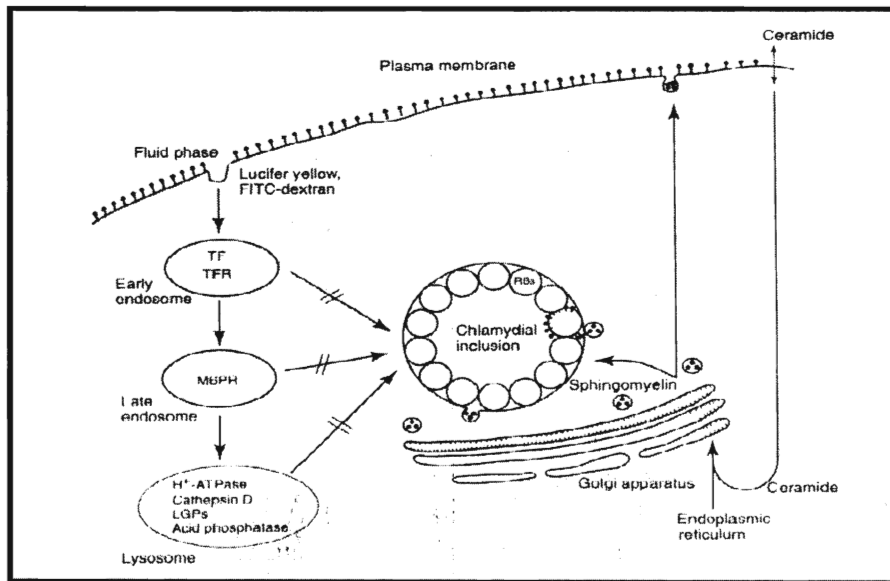

FIGURE 2 Model for the vesicular interactions of the chlamydial inclusion. By 2 h postinfection, in a process that requires early protein synthesis, endocytosed *C. trachomatis* EBs transform the properties of the endocytic vesicles such that they no longer interact with endocytic pathways but begin to intercept sphingolipids from an exocytic pathway. Fluid phase markers or markers for early and later endosomes or lysosomes are not associated with the chlamydial inclusion. Instead, the chlamydial inclusion fuses with a subset of sphingomyelin-containing vesicles in transit to the plasma membrane. Fusion of these vesicles exposes the sphingomyelin on the luminal surface of the inclusion membrane, from which it is adsorbed by the chlamydial RBs and incorporated into their cell walls[5].

A final important mechanism by which *C. trachomatis* establishes persistence is by resisting host T-cell effector T-cell cytokines such as IFN-γ. In response to chlamydial infection, host T-cells activate CD4+ and CD8+ T-cells in a cell-mediated immune response. CD8+ T-cells activate naïve CD8+ T-cells to cytoxic effector T-cells in order to directly lyse infected target T-cells. On the other hand, CD4+ T-cells have a generalized helper function of secreting cytokines that activate other cells in the immune system to achieve a more effector T-cell function. IFN-γ is one such CD4+ T-cell cytokine released by a Th1 pathway response that has been shown to reversibly block the growth of chlamydiae by inducing an enzyme that breaks down tryptophan, an essential amino acid for chlamydiae growth[5]. Thus, IFN-γ is shown to play a protective role in chlamydial infection, such that IFN-γ interferes with normal chlamydial growth by inducing indoleamine 2,3-dioxygenase to catalyze the degradation of tryptophan[5]. However, the ability of some serovars to grow rapidly despite tryptophan metabolism enables *C. trachomatis* to establish persistence. Previous research outlining the role of CD4+ T-cell signature cytokines is the hallmark for this research project. The intricate cell-mediated immune responses are not completely understood and thus have great clinical implications, such that an understanding of the human immune responses to *C. trachomatis* can potentially tell us about persistence and resolution of infection.

C. trachomatis Infection: Host Cell Immune Responses

Once an infection begins, dendritic cells existing in the infected epithelial surfaces of the genital tract actively uptake and transport the *C. trachomatis* pathogens to the secondary lymphoid tissues via a draining lymphatic vessel. In order for the T-cells to participate in an adaptive immune response, the naïve CD4+ and CD8+ T-cells must first encounter a specific antigen and then be activated to proliferate and differentiate into effector T-cells capable of eliminating foreign antigens. Naïve CD4+ and CD8+ T-cells migrating via the blood capillaries to the secondary lymphoid tissues encounter specific antigens presented by MHC class I and II molecules on dendritic cells[8]. Naïve CD4+ and CD8+ T-cells recognize the MHC-peptide complex specific for their antigen on the same dendritic cell and bind via a T-cell receptor. The binding of T-cell receptors activates CD4+ T-cells and leads to T-cell proliferation and acquisition of effector CD4 T-cell functions via activated CD4+ T-cell IL-2 secretion. CD4+ T-cell secretion of IL-2 induces the dendritic cell to increase its level of co-stimulators and thus drives the activation of naïve CD8+ T-cells to proliferate and differentiate into mature CD8+ T-cells. Thus, activated CD4+ T-cells can function to activate naïve CD8+ T-cells in the T-cell mediated immune response.

C. trachomatis: CD8+ vs. CD4+ T-Cell Proliferation and Differentiation

Towards the end of proliferation, activated T-cells acquire the ability to synthesize proteins, which allows them to perform specialized functions as effector T-cells. Cytotoxic CD8+ T-cells are sent to infected target tissues sites to kill cells that have become infected by viruses or intracellular pathogens. CD8+ T-cells represent a powerful defense of the host T-cell-mediated immune response to lyse and destroy infected host T-cells. On the other hand, CD4+ T-cells play more of a helper role in host T-cell immune responses. The proteins acquired during CD4+ T-cell activation are cell-

surface molecules and various cytokines, which activate and help both macrophages and B-cells fight foreign pathogens[8]. Following CD4+ T-cell proliferation, CD4+ T-cells can differentiate into CD4+ Th1 or CD4+ Th2 cells. Cytokines released by CD4+ Th1 cells lead to macrophage activation, inflammation, and the production of antibodies, which enhance the phagocytosis of foreign pathogens[8]. Cytokines released by the CD4+ Th2 cells lead to B-cell differentiation and the production of antibodies.

The differentiation pathway destined by the naïve T-cell is likely to depend on the types of cytokines already present in the secondary lymphoid tissue, the type of professional antigen-presenting cell, and the abundance of MHC-peptide complexes on the surface of the antigen-presenting cell[8]. Differentiation favoring Th1 pathways triggers a cell-mediated immune response, relying on effector cells to fight foreign pathogens. On the other hand, differentiation biased towards a Th2 pathway triggers a humoral immunity response, which relies on releasing antibodies specific to the foreign pathogen present. Th1 and Th2 pathways work antagonistically to reinforce either one of the pathways, such that differentiation of effector Th1 cells suppresses the differentiation of Th2 cells. A final, less understood type of CD4+ helper T-cell involved in *C. trachomatis* infection is the CD4+ Th17 cell. Studies show that cytokines released by CD4+ Th17 cells induce tissue inflammation at the site of an infected host T-cell during an innate immune response[1]. Released cytokines work to recruit, activate and migrate neutrophils, which amplify inflammatory reactions and play a key role in defense against foreign pathogens. Although little is known about the true role of Th17 cytokines, studies suggest that Th17 cytokines also work to inhibit CD4+ regulatory T-cells from performing their role as inhibitors of tissue inflammation[1], which insists that Th17 cells could play a role in tissue pathology if tissue inflammation is unregulated. Although CD8+ and CD4+ T-cells collectively represent the human immune response to *Chlamydia*, previous studies in humans suggest that CD4+ T-cell cytokine secretion dominates the immune response to *C. trachomatis* infection.

C. trachomatis Infection: CD4+ T-Cell Cytokine Secretion

Research completed by Brunham et al. in 2009 with 299 female commercial sex workers in Kariobangi City Council Clinic of Nairobi, Kenya, has shown that CD4+ and CD8+ T-cells play a protective role in *C. trachomatis* immunity[3]. Although, evidence from this sex worker study supports the notion that CD4+ T-cells are the dominant effector T-cell in protection against chlamydial infection[2]. Studies with non-human primates have shown induction of Th1 cytokines following single and repeated chlamydial infection of tissue, thus suggesting that Th1 cytokines may promote immune mediated clearance of infection[2]. More specifically, IFN-γ Th1 cytokines have shown to play a significant role in mediating immunity to chlamydial infection, where studies with IFN-γ knock out mice illustrated impaired mouse immunity to *Chlamydia*[2]. On the other hand, IL-10 Th2 cytokines have shown to play a significant role in suppressing immunity to chlamydial infection, such that studies with IL-10 knock out mice showed accelerated IFN-γ Th1 cytokine responses with enhanced immunity to incident infection[2]. Research with the mouse model of infection has illustrated the dominant role of CD4+ T-cell immune responses to chlamydial infection and the varied protective outcomes provided by differing Th1 and Th2 pathways. Furthermore, preliminary non-human studies with

CD4+ Th17 cells have shown that Th17 cytokines are powerful inducers of unregulated tissue inflammation[1]. Thus, developing a vaccine to prevent *C. trachomatis* relies heavily on understanding the intricate protective and pathogenic pathways involved in the CD4+ T-cell immune response to chlamydial infection (Fig. 3).
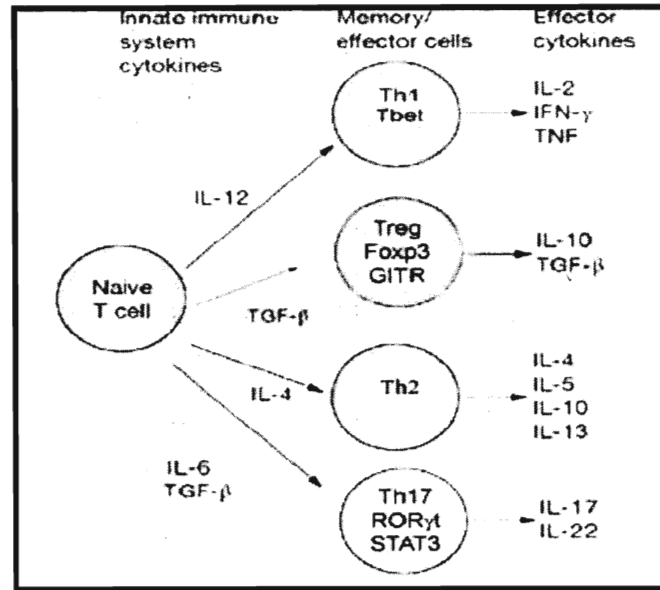

FIGURE 3   The maturation of naïve T-cells into effector or memory populations and their characteristic cytokine expression profiles.

C. trachomatis Infection: Quantifying CD4+ T-Cell Cytokine Secretion

Most often researchers rely on tittering specific antigens specific for chlamydial infection to study *C. trachomatis* in humans. These antigens are powerful in eliciting specific immune responses if patients are currently infected or have been infected with *Chlamydia* in the past. However, a key quality of chlamydial infection that makes it useful for studying immune response trends in greater depth is the various immunogenic proteins found in both the EB and RB of the pathogen. Chlamydial infection begins when the infectious EB comes into contact with the host T-cell epithelial surface. The pathogen attaches its infectious EB by utilizing various protein adhesions on the surface of the EB. Chlamydial heat shock protein 60 (HSP60) is one such immunogenic protein that is found in both the EB and RB of the chlamydial pathogen. There are three known genes that encode for HSP60—Ct110, Ct604, and Ct755—which are all expressed independently during active *C. trachomatis* infection[2]. Expression of Ct110 has been associated with synovial inflammation in patients with chlamydial-associated arthritis, Ct604 has been associated with the maintenance of persistent chlamydial infection, and gene expression of Ct755 has been associated with maintaining active chlamydial infections[2]. Thus, researchers often stimulate peripheral blood mononuclear cells from human patients with HSP60 to study immune responses to chlamydial infection because

previous studies have shown that HSP60 will induce immune responses of interest in patients who have built up an immune response to chlamydial infection. Furthermore, the use of whole EBs from various *C. trachomatis* serovars D to K have shown to be useful in elucidating immune responses to infection. More specifically, Cohen and colleagues determined that Serovar D EB stimulated CD4+ T-cells more robustly than those stimulated with the immunogenic antigen, CHSP60-1[4]. Finally, previous research with *C. trachomatis* infection has focused efforts on the mouse model of genital tract infection because of the immunologic and pathologic similarities to human genital tract infection. Researchers use Nigg antigens isolated from the *C. muridarum* strain of the infection to elucidate immune responses to infection in mouse populations that are comparable to immune responses in humans. Thus, the well-studied structure and pathogenic nature of *Chlamydia* provides researchers with a very powerful tool for studying specific immune cell responses to chlamydial infection.

C. trachomatis Infection: Previous Research on CD4+ T-Cell Cytokine Secretion

Despite alarming CDC estimates which project that approximately 2,291,000 non-institutionalized U.S. females ages 14-39 are infected with *Chlamydia* based on the U.S. National Health and Nutrition Examination Survey[11], very few studies within the U.S. population have experimentally analyzed chlamydial genital tract infection in the human model. Although, recent studies with non-U.S. females at high risk for chlamydial genital tract infection have consistently shown the specific roles of IFN-γ and IL-10 in protective immunity. In 2008, Agrawal and colleagues sought to analyze female patients attending a gynecological outpatient department in New Delhi, India. Females were categorized into groups based upon their infection statuses: CT negative (control group), CT positive-fertile, and CT positive-infertile (current infertility or MSA). The researchers obtained cervical swabs to obtain cervical cells stimulated with the chlamydial antigen Serovar D EB. The numbers of cytokine-secreting cells (SFCs) were quantified with an ELISpot assay procedure specific for IFN-γ and IL-10. Analysis of the ELISpot results revealed that there were a larger number of IL-10 SFCs in the CT positive-infertile group than the other two groups, whereas the CT negative control group followed by the CT positive-fertile group had the largest number of IFN-γ SFCs (Fig. 4).

Table 2 Frequency of spot forming cells (SFCs) among stimulated cervical cells by ELISPOT assay

Patients	IL-4	IL-10	IL-12	IFN-γ
Controls	6 (0–23)	66 (37–103)	68 (44–86)	134 (77–221)[***]
CT positive fertile women	9 (0–37)	64 (59–87)	344 (277–521)[*]	133 (119–184)[***]
CT positive with infertility	16 (4–89)[*]	96 (79–235)	241 (115–450)	59 (39–159)
CT positive with MSA	13 (3–75)[*]	102 (79–206)[*]	235 (125–464)[**]	65 (39–172)

Note. Median number of cytokine spot forming cells per 2×10⁵ cervical mononuclear cells at 18 h post stimulation using ELISPOT. Figures in parenthesis depict range of SFCs for each group. CT, *Chlamydia trachomatis*; MSA, multiple spontaneous abortions.
Mann Whitney U test shows:
* P=0.01 compared to the other groups.
** P<0.05 compared to controls.
*** P<0.05 compared to women with fertility disorders.

FIGURE 4 Frequency of SFCs among stimulated cervical cells from females visiting the gynecological department in New Delhi, India by use of ELISpot assay[12].

From these ELISpot assay results, Agrawal and colleagues then concluded that larger numbers of IL-10 secreting cells in response to Serovar D EB may lead to an incomplete clearance of infection of bacteria, the creation of a favorable environment for bacteria to persist, and thus lead to the development of fertility disorders (infertility or MSA)[12]. On the other hand, the results illustrated that large numbers of IFN-γ secreting cells in response to Serovar D EB may lead to protection against CT infection and the inhibition of clinical pathological symptoms (fertility disorders)[12]. Agrawal and colleagues concluded that immune responses to chlamydial genital tract infection favoring Th2 responses (IL-10) down-regulate pro-inflammatory cytokines (IFN-γ) characteristic of the Th1 responses, thus inhibiting protection against infection and pathological symptoms.

In 2009, Brunham and colleagues utilized the results from the Agrawal study to explore the immune responses to *Chlamydia* in Kenyan sex workers (18-35) who had a past medical history of *Chlamydia* infection. As opposed to Agrawal's study, Brunham et al. were interested in the immune responses of past, rather than current, *Chlamydia* infection. Participants had an average of 3 years of prostitution (11 clients per week) and were admitted to the study only if they were not currently infected with *Chlamydia*. Following the collection of heparinized blood samples and endometrial biopsies, PBMCs and endometrial cells were both isolated to obtain a population of both CD4+ and CD8+ depleted T-cells. CD4+ and CD8+ T-cells were stimulated with chlamydial antigens, Serovar D EB and CHSP60 (1-3), and the number of IFN-γ Th1 cytokine secreting cells were quantified with an ELISpot assay. Analysis of ELISpot results illustrated that specific secretion of IFN-γ by CD4+ T-cells in response to stimulation with chlamydial antigens was significantly greater than cytokine secretion by CD8+ T-cells, thus demonstrating the more prominent role of CD4+ T-cells in the immune response to *Chlamydia*[3]. Furthermore, the researchers found that the frequency of *C. trachomatis* EB-specific IFN-γ immune responses was higher in PBMCs than in EMCs[3], which suggests that PBMCs should respond more robustly to stimulation with chlamydial antigens in our study population. Finally, Brunham et al. found that CHSP60-1 was more immunogenic than CHSP60-2 and -3[3], which highlights the importance of using CHSP60-1 in our future studies to stimulate T-cells.

In accordance with this study, Cohen and colleagues explored the protective role of CD4+ T-cell cytokines in Brunham's sex worker population. Cohen et al. analyzed the immune responses of the Kenyan sex workers by obtaining PBMCs and endocervical cells that were isolated into CD4+ and CD8+ depleted T-cell populations. Similar to Brunham, Cohen and colleagues utilized ELISpot assays to quantify the number of IFN-γ Th1 cytokine-secreting cells and the number of IL-10 Th2 cytokine-secreting cells upon stimulation of the cells with CHSP60-1. Upon conducting a hazard rate analysis utilizing a cox proportional hazards model to test for associations between *C. trachomatis* infection and levels of cytokine producing cells within the ELISpot data, the researchers found that a larger proportion of women who were protected against infection had positive IFN-γ production over a 20-month period (Fig. 5, A)[4]. On the other hand, a larger proportion of women infected with *Chlamydia* had positive IL-10 production over a 20-month period (Fig. 5, B)[4]. Thus, the hazard rate for infection decreased in women with a higher specific secretion of IFN-γ-secreting CD4+ T-cells.
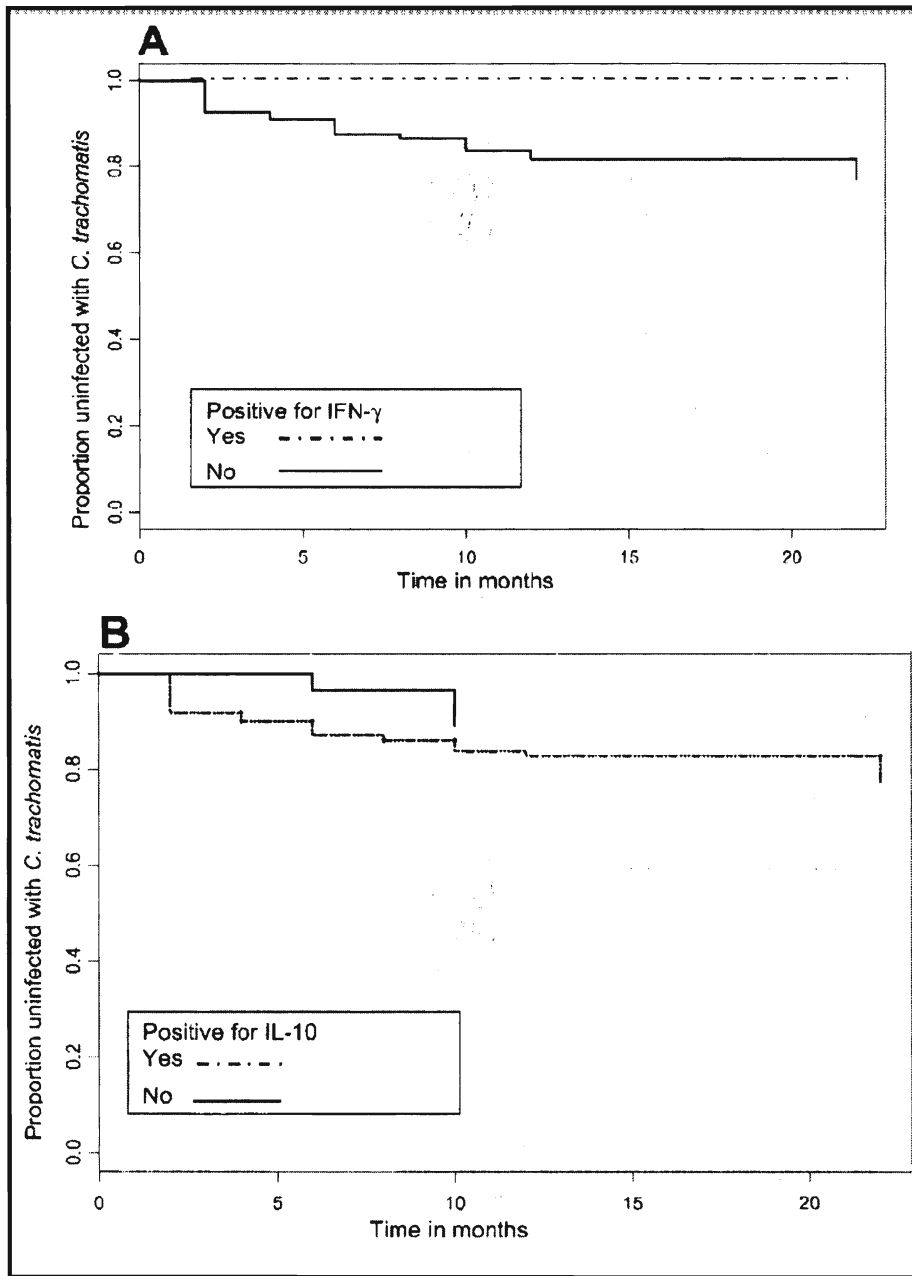
FIGURE 5
Comparison of time to first *Chlamydia trachomatis* infection in women with and without an (IFN)-g response by peripheral blood mononuclear cells (PBMCs) stimulated with Chlamydia heat-shock protein (CHSP) 60 (A); an interleukin (IL)-10 response by PBMCs stimulated with CHSP60 (B)[4].

The following three studies explore immune responses to *Chlamydia* in female populations. The results of these studies represent strong methodological and statistical references for our study with adolescent women. Although the sexual histories of these women may vary significantly from our adolescent population, we found these studies useful models for our current study. We hope that incorporating some of the methods in these studies in our optimization and use of proliferation and ELISpot assays will generate comparable results.

Methodology and Materials

The research completed can be divided into two interrelated projects. In the first part of this study we optimized ELISpot and proliferation assays for our current longitudinal study (CARE) characterizing the CD4+ T-cell responses to *Chlamydia*. Published studies in the United States that explore CD4+ T-cell cytokine secretion in adolescent women outside of the sex worker population are very limited. Prior to working with patients in the AMC who presented with a variety of past medical histories of STI and sexual activity, it was important to determine if ELISpot and proliferation assays would work to detect immunity differences between patients with different statuses of infection and clinical symptoms. Prior to optimization of these assay methods, the Darville laboratory has focused its efforts on exploring the mouse model of *Chlamydia* infection because mouse disease pathogenesis is similar to the human model. To experiment with the effectiveness of experimental assay methods, we collected heparinized blood samples from a seropositive donor who has built up an antibody response to infection through exposure to the *C. trachomatis* pathogen, and heparinized blood samples from a seronegative donor who has an immune system unresponsive to chlamydial antigens. The results from these optimizations will show that experimental assays can be successful in detecting differences between patients if the assays are able to detect subtle differences in women who have never actually been infected with *C. trachomatis*. Thus, the first part of this research project is extremely methodological, such that we will be determining optimal antigen concentrations, optimal cell incubation times, and optimal specimen collection for our current study with human patients who are at high-risk for infection with *Chlamydia*.

The second part of this project involved putting to use the protocols outlined from the optimized ELISpot and proliferation assays. We relied on optimal protocols to collect specimens from adolescents in the clinic and then to compare CD4+ T-cell proliferation and cytokine secretion responses found with the seropositive and the seronegative donor. The long-term goal of this longitudinal adolescent study is to characterize protection and pathology patterns in women over a period of four follow-up clinic visits. For the specific goals of this paper, we are interested in determining if ELISpot and proliferation assays will be a useful addition to current PCR diagnosing methods and should thus be a standardized procedure in adolescent clinics. We hoped that experimental methods would accurately detect the status of infection (positive or negative) as well as the health status of patients (protection or pathology) for future clinic visits by detecting differences in CD4+ T-cell proliferation and cytokine secretion.

Part One

Study Population

After obtaining written informed consent, 2 donors affiliated with the Darville Laboratory volunteered to be in this optimization experiment. Prior to volunteering for this experiment, heparinized blood samples from both donors were tested with a Western Blot protocol to confirm their statuses as seropositive and seronegative donors. Chlamydial proteins were detected upon antibody analysis with cells from the seropositive donor,

thus illustrating that the seropositive donor had built up an immune response to infection through prolonged laboratory exposure to the *C. trachomatis* pathogen. Contrastingly, chlamydial proteins remained undetected in the cells from the seronegative donor, which revealed that the seronegative donor had not built up an immune response to *Chlamydia* infection. Following western blot analyses, heparinized blood samples were collected from the seropositive donor and the seronegative donor. Since variations in immune responses are influenced by the health status of participants, samples were collected from donors pending no current infection or use of antibiotics.

Collection of Peripheral Blood Mononuclear Cells (PBMCs)

For collection and isolation of PBMCs from heparinized whole-blood samples, six BD Vacutainer® glass blood collection tubes (~ 60mL) were collected from each donor. The mononuclear layer in whole blood contains both lymphocytes (CD4+/CD8+ T-cells, natural killer T-cells, B-cells) and macrophages. PBMCs are thus useful for analysis of immune system responses to *Chlamydia* infection because the mononuclear layer in whole blood contains the CD4+ population of interest in CT infection. Immediately following collection of heparinized whole blood, both donor samples were diluted 1:2 with 1X Isotonic Phosphate Buffered Saline (PBS) at room temperature for ease of cell counting in later steps. To retrieve a layer of mononuclear cells, diluted whole-blood samples were layered slowly on top of lymphocyte separation medium (Histopaque®-1077), and underwent density-gradient centrifugation at 400xg for 30 minutes (Fig. 6). Plasma cell layers were removed via gentle aspiration and mononuclear cell layers were extracted for each donor with a Pasteur pipette. We thus extracted a T-cell population that could be pelleted and counted for use in a proliferation and dual-color ELISpot assay.
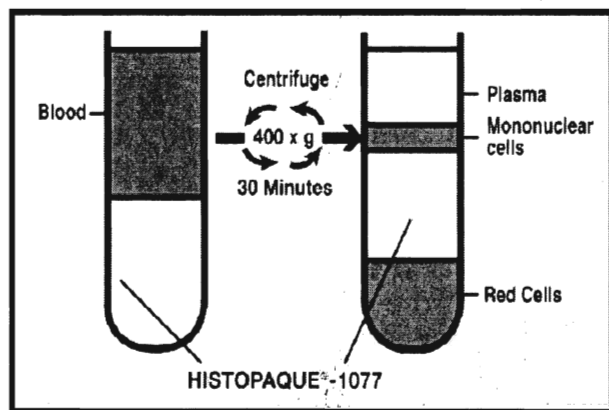

FIGURE 6 Diagram of PBMC separation with Histopaque®-1077 separation medium. Following density-gradient centrifugation, a small translucent layer of mononuclear cells can be obtained.

Treatment of T-Cell Populations

Mononuclear cell layers extracted in PBMC isolation steps for both donors were diluted with complete cell media prior to density centrifugation at 250xg for 10 minutes. Complete cell media provided nutrients for cells received in a cell pellet following centrifugation. Supernatants were discarded and cell pellets were re-suspended again

with complete cell media prior to a final centrifugation at 250xg for 10 minutes. Final cell pellets were re-suspended in complete media and a small proportion (10uL) of each T-cell population was removed for counting purposes. Following the addition of Trypan blue (90uL) to the counting samples, cells were counted with a dissecting microscope at 10X objective and the total number of mononuclear cells per milliliter was determined. Cells were then diluted with complete cell media to create a dilution of cells suitable to plate 100uL of 1E5 cells/well and 2E5 cells/well for treatment conditions in triplicate for ELISpot and tritiated thymidine proliferation assays. Cells of both dilutions were then added in triplicate onto round-bottom 96-well microplates for a thymidine proliferation assay and in triplicate onto a 96-well Millipore PVDF plate for an ELISpot assay. Cells on both plates were then stimulated with media (negative control), ConA (positive mitogen control), Serovar D EB (human chlamydial antigen), CHSP60-1 (human chlamydial antigen), and Nigg (mouse chlamydial antigen) prior to the incubation of cells in a humidified 5% CO_2 incubator at 37°C.

Proliferation Quantification with Tritiated Thymidine T-Cell Proliferation Assay

During T-cell proliferation, PBMCs are separated by density-gradient centrifugation and undergo cell division, differentiation, and new DNA synthesis. We used the T-cell proliferation assay to evaluate the rate of proliferation (counts/minute) when T-cells are exposed to a media control, ConA, Serovar D EB, CHSP60-1, and Nigg. Three 3H T-cell proliferation assays were completed in this optimization experiment to test the effects of different concentrations of stimulants. In the first proliferation assay, cells from only a seropositive donor were stimulated with a media control, 2.5, 5, and 10ug/mL of ConA, and 2.5, 5, and 10ug/mL of Nigg. In the second proliferation assay, cells from both a seropositive and a seronegative donor were stimulated with a media control, 2.5 and 5ug/mL of ConA, and 1 and 2.5ug/mL of Serovar D EB. Finally, a third proliferation assay stimulated cells from only a seropositive donor with a media control, 2.5 and 5ug/mL of ConA, .5, 1, and 2ug/mL of Serovar D EB, and 1ug/mL of CHSP60-1.
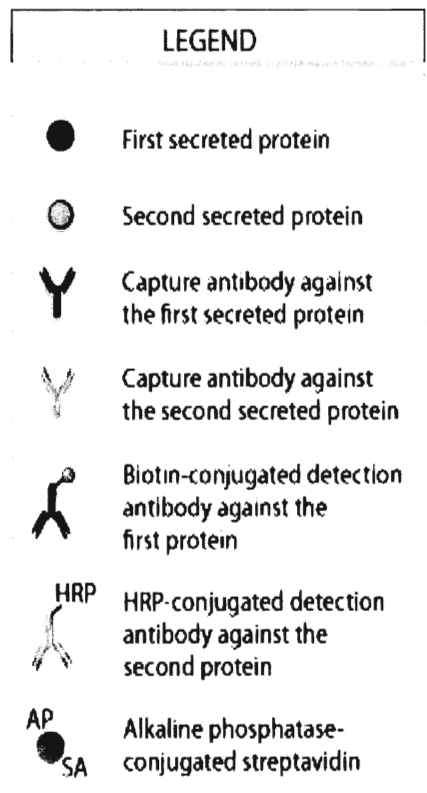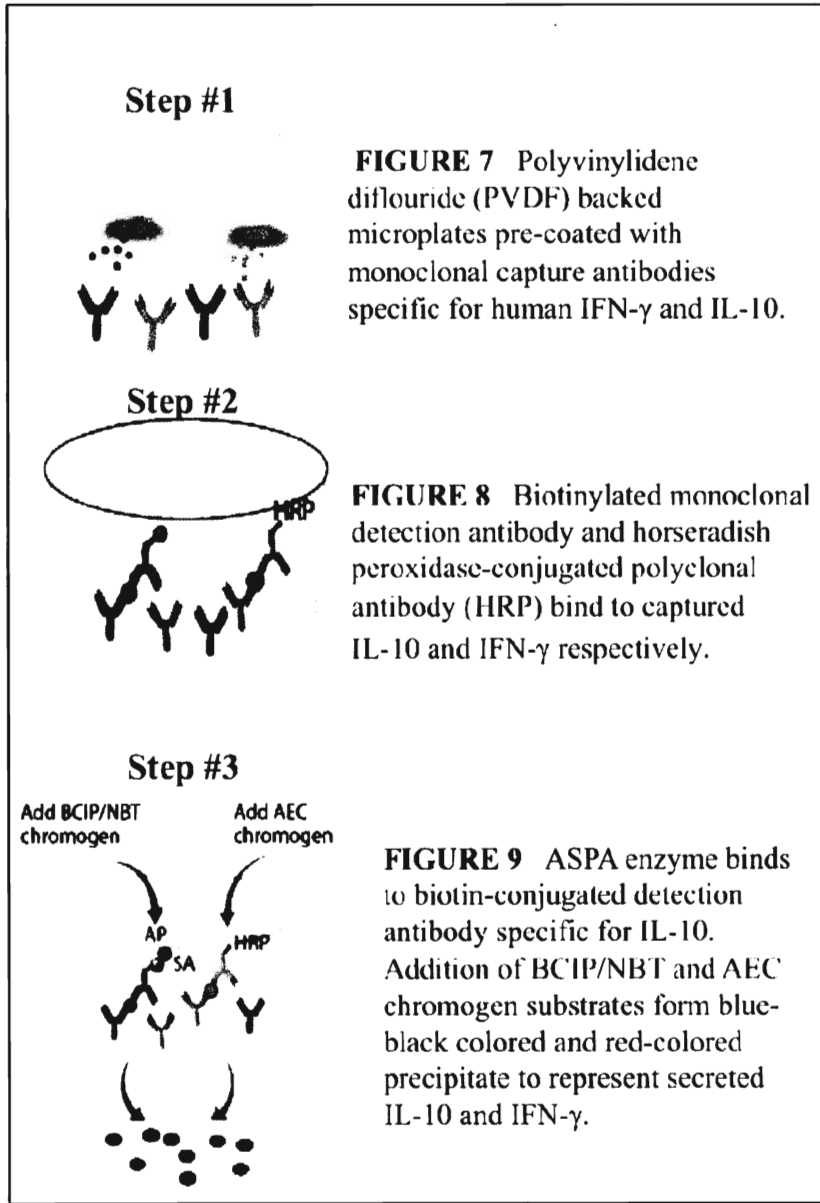
Following stimulation with the different treatment conditions in each proliferation assay, cells were incubated for 72 hours as is outlined in the Hirsh Laboratory for T-cell proliferations. Following the incubation, cells were centrifuged and supernatants (60uL) were removed, stored at -80°C, and replaced with 60uL of tritiated thymidine (3H). Supernatants would be useful for future experiments that analyze cytokine secretion in T-cell supernatant populations. Cells were then incubated again at 37°C overnight upon addition of tritiated thymidine. Following successful incubations at -80°C and 37°C, cells were harvested with a plate harvester which utilizes a vacuum system to remove cells from wells and align well contents on self-aligning filters. Upon addition of Ultima Gold LLT scintillation fluid for detection of 3H, a gamma counter was used together with a Top Count NXT program to detect the number of proliferating cells in counts per minute. The amount of proliferation is quantified by increases in DNA synthesis, and a gamma counter is then used to detect the amount of DNA synthesis by recognizing a 3H label that is incorporated into proliferating cells. We used this technique to evaluate the quality of our antigens and to determine a concentration of antigen that would work to stimulate T-cells of seropositive subjects significantly greater than media controls.

Cytokine Analysis with Dual-Color ELISpot Assay

The dual-color ELISpot assay counts the number of IFN-γ and IL-10 producing cells in PBMCs isolated from a seropositive and a seronegative donor stimulated with varying concentrations of ConA, Serovar D EB, CHSP60-1, and a media control. The ELISpot assay is used to detect the number of IFN-γ and IL-10 secreting T-cells. It was important for us to quantify the number of IFN-γ and IL-10 secreting T-cells because increased IFN-γ responses have been associated with protection against incident infection with *Chlamydia*. On the other hand, increased IL-10 production has shown to down-regulate the number of IFN-γ producing T-cells, which then puts a patient at risk for no protection against incident infection. The dual-color quality of the ELISpot allows us to count the number of cells secreting IFN-γ or IL-10 (SFCs) by distinguishing between red dots for IFN-γ producing cells and blue dots for IL-10 producing cells.

The dual-color ELISpot kit and protocol were produced by R&D systems, and thus all experimental procedures were done according to R&D manufacturer instructions. Prior to addition of cells and stimulants to wells, the 96-well PVDF backed microplates were pre-coated with monoclonal capture antibodies specific for IFN-γ and IL-10 (Fig. 7, Step #1). As cells incubated with stimulants, capture antibodies bound specifically to cells secreting IFN-γ or IL-10. Two dual-color ELISpot assays were completed in this optimization experiment to test different concentrations of antigens and different T-cell incubation times. In both dual-color ELISpot assays, 1E5 and 2E5 dilutions of cells from a seropositive donor and a seronegative donor were stimulated with a media control, 2.5 and 5ug/mL of ConA, 1 and 2ug/mL of Serovar D EB, and 1ug/mL of CHSP60-1. Following the addition of stimulants to cells, one dual-color ELISpot plate was incubated at 37°C for 24 hours and the other dual-color ELISpot plate was incubated for 48 hours to determine an optimal cell incubation time for specific cytokine secretion.

Following the 24- and 48-hour cell incubations, biotinylated monoclonal detection antibody and horseradish peroxidase-conjugated polyclonal antibody (HRP) was added to wells to bind to captured IL-10 and IFN-γ, respectively (Fig. 8, Step #2). Alkaline phosphatase-conjugated streptavidin (ASPA) was added and bound to biotin-conjugated detection antibody specific for IL-10 prior to final plate processing (Fig. 9, Step #3). Finally, addition of BCIP/NBT and AEC chromogen substrates formed blue-black or red colored precipitate representative of IL-10 and IFN-γ (Fig. 9, Step #3). Blue and red spots were then read and counted with a CTL ImmunoSpot® ELISpot reader in which quality controls were utilized to distinguish between true positives and false positives (background noise). Prior to statistical analysis of ELISpot results, cytokine-secreting cell numbers (SFC/200,000 cells) were reported in terms of SFC/1E6 cells and log-transformed in accordance with Brunham et al.'s study with Kenyan sex workers[3].

Step #1

FIGURE 7 Polyvinylidene diflouride (PVDF) backed microplates pre-coated with monoclonal capture antibodies specific for human IFN-γ and IL-10.

Step #2

HRP

FIGURE 8 Biotinylated monoclonal detection antibody and horseradish peroxidase-conjugated polyclonal antibody (HRP) bind to captured IL-10 and IFN-γ respectively.

Step #3

Add BCIP/NBT chromogen   Add AEC chromogen

AP   HRP
SA

FIGURE 9 ASPA enzyme binds to biotin-conjugated detection antibody specific for IL-10. Addition of BCIP/NBT and AEC chromogen substrates form blue-black colored and red-colored precipitate to represent secreted IL-10 and IFN-γ.

LEGEND	
●	First secreted protein
◎	Second secreted protein
Y	Capture antibody against the first secreted protein
Y	Capture antibody against the second secreted protein
⨎	Biotin-conjugated detection antibody against the first protein
HRP	HRP-conjugated detection antibody against the second protein
AP ●SA	Alkaline phosphatase-conjugated streptavidin

Statistical Analysis

For proliferation assay analyses, a one-way ANOVA with repeated measures on one factor was used to predict mean counts per minute for the seropositive and the seronegative donor. Predictor variables included a within-subjects factor for treatment condition (media, ConA, Nigg, Serovar D EB, and CHSP60-1). Follow-up pair-wise comparisons or One-way ANOVAs were used to understand significant main effects of treatment. For dual-color ELISpot analyses, all spot forming cells (SFCs) were analyzed in terms of SFC/1E6 cells and were log-transformed to centralize the data. A One-way ANOVA with repeated measures on one factor was used to predict mean cytokine secretion for the seropositive and the seronegative donor for each cell dilution (1E5 and

2E5). Predictor variables included a within-subjects factor for treatment condition (media, ConA, Serovar D EB, and CHSP60-1). Follow-up pair-wise comparisons of One-way ANOVAs were used to understand significant main effects of treatment.

Part Two

Study Population

All previously healthy adolescent women between the ages of 13 and 25 who presented to the Adolescent Medical Clinic (AMC) in Children's Hospital of Pittsburgh in need of routine or problem gynecological examination and testing for STIs were offered enrollment in the study. In addition to this group, all adolescent women diagnosed with *Chlamydia trachomatis* infection in our AMC who were scheduled for a follow-up appointment were contacted by phone and invited to enroll in the study. All participants were enrolled by obtaining written informed consent. Parental consent was not required for participants who were minors. Enrolled participants completed baseline questionnaires assessing sexual, contraceptive, STI, pregnancy, and gynecological history as well as demographics. Following questionnaire completion, routine physical and gynecological examinations were given and specimens were collected for laboratory diagnoses and experimental analyses. Specimens included: two endocervical swabs to be used to culture and diagnose *N. gonorrhea* and *Chlamydia trachomatis*, blood samples for the experimental analysis of ELISpot and proliferation assays, and a urine sample to detect pregnancy. All participants received $30 as compensation for their participation.

Following baseline procedures, participants will be asked to return for four follow-up visits (6-week, 3-month, 6-month, and 9-month follow-up). During these sessions, participants will self-report resolution of gynecological symptoms, onset of new symptoms, side effects of any therapy, and interim sexual behaviors. Urine samples will be collected for laboratory diagnosis of current *Chlamydia* infection. For participants who tested positive for *Chlamydia* during a prior visit, heparinized blood samples will be collected. Exclusion criteria for follow-up assessments include current or past pregnancy, gynecologic surgery within 2 months of the assessment, systematic or vaginal antibiotics in the preceding 2 weeks, douching or use of vaginal creams or suppositories within 2 days of assessment, and vaginal sex within 2 days of assessment. Exclusion criteria were selected based on plausible influence on cytokine concentrations and immune cell populations. Each participant who completes a follow-up assessment will receive an additional $30.

Collection and Processing of Specimens

Heparinized blood samples were collected in three 10mL heparinized blood tubes via venipuncture. These tubes were then labeled with unidentifiable patient codes. Samples were stored at room temperature and processed within 24 hours of collection. Heparinized blood samples were then used to collect PBMCs by Histopaque density gradient centrifugation in accordance with PBMC collection in Part One of this study. Two endocervical swabs for the culture of *N. gonorrhea* and *Chlamydia* were collected by introducing an endocervical swab into the endocervical canal and gently rotating the

swab for 15 to 30 seconds for specimen collection. The endocervical clinical specimens were then processed by the AMC clinical microbiology laboratory for routine determination of the presence of *C. trachomatis* (CT) and *N. gonorrhea* (NG) via PCR amplification of CT and NG target DNA using base pair (bp) plasmid primers specific for CT and NG.

Isolation and Treatment of CD4+ T- Cell Populations

T-cell populations in isolated PBMCs were depleted of CD8+ T-cells via a mini MACS© or Quatro MACS© magnet as per Miltenyi Biotec MACS© manufacturer instructions. Following the depletion of CD8+ T-cells, PBMC T-cell populations would only contain CD4+ T-cells, macrophages, B-cells, and NK T-cells. Depletion of CD8+ T-cells allows for specific quantification of CD4+ T-cell immune responses that represent the dominant defense mechanism against *Chlamydia* infection. PBMC suspensions were first counted in accordance with Part One of this experiment and then CD8 microbeads specific for CD8+ T-cells were added to the PBMCs re-suspended with MACS© buffer per 10^7 total cells. Following incubation of PBMCs with CD8 microbeads, cell washing, density gradient centrifugation, and re-suspension with MACS© buffer, CD8+ T-cells within the PBMC population were magnetically labeled with CD8 microbeads. When the PBMC cell suspension with labeled CD8 T-cells was added to the MACS© column with MACS© magnetic field separator, the magnetically labeled CD8+ T-cells were retained within the column as the rest of the cell suspension mixture was eluted.

Thus, a final elution mixture theoretically contained a CD4+ T-cell population free of most CD8+ T-cells. CD8+ depleted T-cells eluted in the column were then counted to determine the number of CD8+ depleted T-cells per mL of heparinized blood. Final cell suspensions were then diluted with complete cell media to create a dilution of cells suitable to plate 100uL of 2E5 cells/well for treatment conditions in triplicate for ELISpot and alamar-blue proliferation assays. Cells of both dilutions were then added in triplicate onto round-bottom 96-well microplates for an alamar-blue proliferation assay and in triplicate onto a 96-well Millipore PVDF plate for an ELISpot assay. Cells on both plates were then stimulated with media (negative control), ConA (positive mitogen control), Serovar D EB (chlamydial antigen), and CHSP60-1 (chlamydial antigen) prior to the incubation of cells in a humidified 5% CO_2 incubator at 37°C.

Proliferation Quantification with Alamar-Blue T-cell Proliferation Assay

Upon determining that the cell harvester used for the tritiated thymidine proliferation assays in Part One of this experiment was unavailable for use because of instrumental problems, alamar-blue assays were used as a comparable method to determine the amount of CD4+ T-cell proliferation. Alamar-blue proliferation assays indicate the amount of T-cell proliferation by detecting the level of oxidation during aerobic host T-cell respiration. The alamar-blue indicator detects host T-cell oxidation as it is reduced by cytochromes (NADH and $FADH_2$) in the electron transport chain during aerobic respiration. The indicator undergoes a colometric change from blue to pink (fluorescence) in response to cellular reduction. Furthermore, the amount of color absorbed by living host T-cells corresponds to the amount of metabolic activity or host T-

19

cell proliferation, such that increased absorbance of fluorescence by host T-cells corresponds to a higher percentage of reduction, which is then directly proportional to a larger number of cells proliferating. Thus, alamar-blue proliferation results are reported in percent reduction per experimental well, where larger percents are indicative of more host T-cell proliferation due to stimulation with chlamydial antigens.

CD4+ T-cell populations isolated from PBMCs were stimulated in triplicate with 100uL of a media control, 5 uL of 2.5ug/mL ConA, 1ug/mL Serovar D EB, and 1ug/mL CHSP60-1 as is shown to be optimal for cell stimulation in Part One of this experiment. Following stimulation with the different treatment conditions, cells were incubated for 72 hours as is outlined in the Hirsh Laboratory for T-cell proliferations. Following the incubation, methods and percent reduction formulas were used in accordance with AbD Serotec manufacturer instructions for calculating alamar-blue in spectophotometry with different filters. Alamar-blue was first added to wells in an amount equal to 10% of the volume in each well, and plates were then returned to the 37°C incubator for one hour. The absorbance of alamar-blue in each treatment condition was measured with the SPECTRA Max M_2 ROM at 570nm to represent the lower wavelength (LW) filter and also at 600nm to represent the higher wavelength (HW) filter. Upon determining the absorbencies of treatment wells without alamar-blue (blank wells) at 570nm and 600nm, we were able to calculate a correction factor for the low and high wavelengths. With the values for the correction factor, absorbencies with alamar-blue, and absorbencies of blank wells at 570nm and 600nm, we could then determine the percentage reduction of alamar-blue for each treatment condition. Thus, higher percentage reduction values corresponded to greater CD4+ T-cell proliferation due to chlamydial antigen stimulation.

Cytokine Analysis with ELISpot Assay

Following the use of dual-color ELISpot assays in Part One of this experiment, we found it useful from a cost-efficient standpoint to switch to eBioscience ELISpot assays specific for IFN-γ, IL-10, and IL-17 separately. The principle of the assay remains the same as the dual-color ELISpot assay, with the exception that all cytokine-secreting cells reveal only red spots for each cytokine. As per eBioscience manufacturer instructions, 96-well PVDF membrane plates were coated in the laboratory with capture antibody specific for IFN-γ, IL-10 or IL-17 in specified and separate wells on the 96-well plate and incubated at 4°C overnight. Following overnight incubation, we plated 100uL of 2E5 cells/well for each treatment condition in triplicate for IFN-γ, IL-10, and IL-17. Cells were stimulated with 100uL of a media control, 5 uL of 2.5ug/mL ConA, 1ug/mL Serovar D EB, and 1ug/mL CHSP60-1. Following the addition of stimulants, cells were incubated at 37°C for 48 hours as was determined to be optimal in Part One of this experiment. Upon removing the plates from the incubator, cells were treated with detection antibodies similar to cells in the dual-color ELISpot assays in Part One of this experiment. However, the one color nature of this ELISpot required us to add AEC chromogen substrate solution only to retrieve red-colored spots (SFCs) for IFN-γ, IL-10, and IL-17. Red spots were then counted with a CTL ImmunoSpot® ELISpot reader in which quality controls were utilized to distinguish between true positives and false positives (background noise). Prior to statistical analysis of ELISpot results, cytokine-secreting

cell numbers (SFC/200,000 cells) were reported in terms of SFC/1E6 cells and log-transformed in accordance with Brunham et al.'s study with Kenyan sex workers[3].

Statistical Analysis

Methodological Analysis

For this analysis, participants were separated into two groups. The first group was made up of participants with past medical history of CT infection or present CT infection, and the second group included participants who have never had CT infection. For both alamar-blue proliferation and ELISpot assays, a Two-way ANOVA with repeated measures on one factor was used to predict mean alamar-blue reduction percentage and mean cytokine secretion. Predictor variables included a within-subjects factor for treatment condition (media, ConA, Serovar D EB, and CHSP60-1) and a between-subjects factor for CT status (past/current CT infection and never infected with CT). Follow-up pair-wise comparisons or One-way ANOVAs were used to understand significant main effects and interactions.

Predictive Analysis

To determine the immunological predictive capabilities of ELISpot assays, participants were separated into three groups based upon CT statuses. The first group included participants with present CT infection, the second group contained participants with past CT infection, and the third group was made up of participants with no CT infection. Three Two-way ANOVAs with repeated measures on one factor were used to predict mean cytokine secretion for IFN-γ and IL-10 only. Predictor variables included a within-subjects factor for treatment condition (media, ConA, Serovar D EB, and CHSP60-1) and a between-subjects factor for CT status (present, past, and no CT infection). Follow-up pair-wise comparisons or One-way ANOVAs were used to understand significant main effects and interactions.

Diagnostic Analysis

To quantify the diagnostic capabilities of proliferation and ELISpot assays, participants were separated into two different groups based upon CT status. The first group included participants with present CT infection and the second group was made up of participants with past CT infection or no CT infection. For the alamar-blue proliferation assays, a Two-way ANOVA with repeated measures on one factor was used to predict mean alamar-blue reduction percentage. Predictor variables included a within-subjects factor for treatment condition (media, ConA, Serovar D EB, and CHSP60-1) and a between-subjects factor for CT status (present CT infection and past/no CT infection). Follow-up pair-wise comparisons or One-way ANOVAs were used to understand significant main effects and interactions. For the ELISpot assays, three Two-way ANOVAs with repeated measures on one factor were used to predict mean cytokine secretion for IFN-γ and IL-10 only. Predictor variables included a within-subjects factor for treatment condition (media, ConA, Serovar D EB, and CHSP60-1) and a between-subjects factor for CT status (present CT infection and past/no CT infection). Follow-up pair-wise comparisons or One-way ANOVAs were used to understand significant main effects and interactions.

Upon examination of a significant interaction (p < .05), ROC curves were generated for each chlamydial antigen treatment condition (Serovar D EB and CHSP60-1) to determine the sensitivity of proliferation and ELISpot assay methods in comparison to current PCR methods.

Pathology Analysis

To determine the pathology-detection capabilities of experimental assays, participants were separated into two different groups based on present pathology symptoms. The first group was made up of participants with present clinical symptoms of abdominal pain and/or discharge, and the second group included participants with no present clinical symptoms. For the alamar-blue proliferation assays, a Two-way ANOVA with repeated measures on one factor was used to predict mean alamar-blue reduction percentage. Predictor variables included a within-subjects factor for treatment condition (media, ConA, Serovar D EB, and CHSP60-1) and a between-subjects factor for pathology status (current pathology and no current pathology). Follow-up pair-wise comparisons or One-way ANOVAs were used to understand significant main effects and interactions. For the ELISpot assays, a Two-way ANOVA with repeated measures on one factor was used to predict mean IL-17 secretion. Predictor variables included a within-subjects factor for treatment condition (media, ConA, Serovar D EB, and CHSP60-1) and a between-subjects factor for pathology status (current pathology and no current pathology). Follow-up pair-wise comparisons or One-way ANOVAs were used to understand significant main effects and interactions. Upon analysis of a significant interaction (p < .05), ROC curves were generated for each chlamydial antigen treatment (Serovar D EB and CHSP60-1) to determine the sensitivity of proliferation and ELISpot assays methods at detecting pathology.

Part One Results

Collection of Peripheral Blood Mononuclear Cells (PBMCs)

Upon isolation of PBMCs from a seropositive donor and a seronegative donor, counting protocols revealed that six BD Vacutainer® glass blood collection tubes (~60mL) yielded an average of 2.2E7 total cells across proliferation and ELISpot assay trials. Furthermore, we estimated the total number of PBMCs needed from one donor for one proliferation assay (plating 2E5 cell per well) to be 4.8E6 total cells. The total number of cells was determined by assuming twelve experimental wells would be needed for four treatment conditions in triplicate for each experimental assay. Experiments were planned based upon plating 2E5 cells per well because a follow-up pair-wise comparison of the 48-hour ELISpot assay results (Fig. 15) illustrated that 2E5 T-cell dilutions stimulated with chlamydial antigens secreted IFN-γ more robustly than 2E5 T-cell dilutions stimulated with the media control. Given the total number of PBMCs needed from one donor, we were able to estimate that three BD Vacutainer® glass blood collection tubes (~30mL) would yield more than enough T-cells for one proliferation and ELISpot assay in Part Two of this study.

Analysis of Tritiated Thymidine T-Cell Proliferation Assays

3H T-cell Proliferation Assay #1

Proliferation of peripheral blood T cells
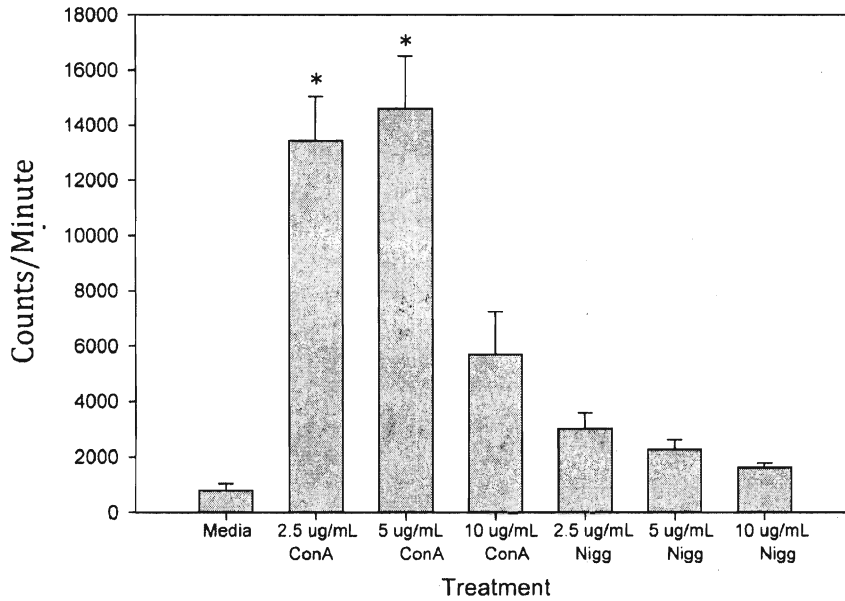from a *Chlamydia* seropositive donor
in response to Nigg or ConA

FIGURE 10 PBMCs isolated from a seropositive donor stimulated with chlamydial mouse antigen, Nigg, do not proliferate more than PBMCs stimulated with a media control. One-way RM ANOVA. *$p<.05$ for ConA versus media for each condition.

A significant main effect for treatment type, $F(6,14) = 76.53$, $p < .001$, revealed that measured T-cell proliferation was different across the seven treatment conditions for the seropositive donor. This effect is illustrated in figure 10. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (13446.33, 1593.74), stimulated T-cells from a seropositive donor to proliferate greater than the media control (800.00, 240.16), $t(5) = 13.60$, $p < .001$. Similarly pair-wise comparisons revealed that 5ug/mL of ConA (14601.17, 1916.22), stimulated T-cells from a seronegative donor to proliferate greater than the media control (800.00, 240.16), $t(5) = 14.85$, $p < .001$. However, there was no difference in T-cell proliferation between the media control condition and 10ug/mL of ConA, p = ns. Finally, there was no difference in T-cell proliferation between the media control condition and all concentrations of Nigg, p = ns.

Proliferation of peripheral blood T cells from a *Chlamydia* seropositive and a *Chlamydia* seronegative donor in response to human Serovar D or Concanavalin A
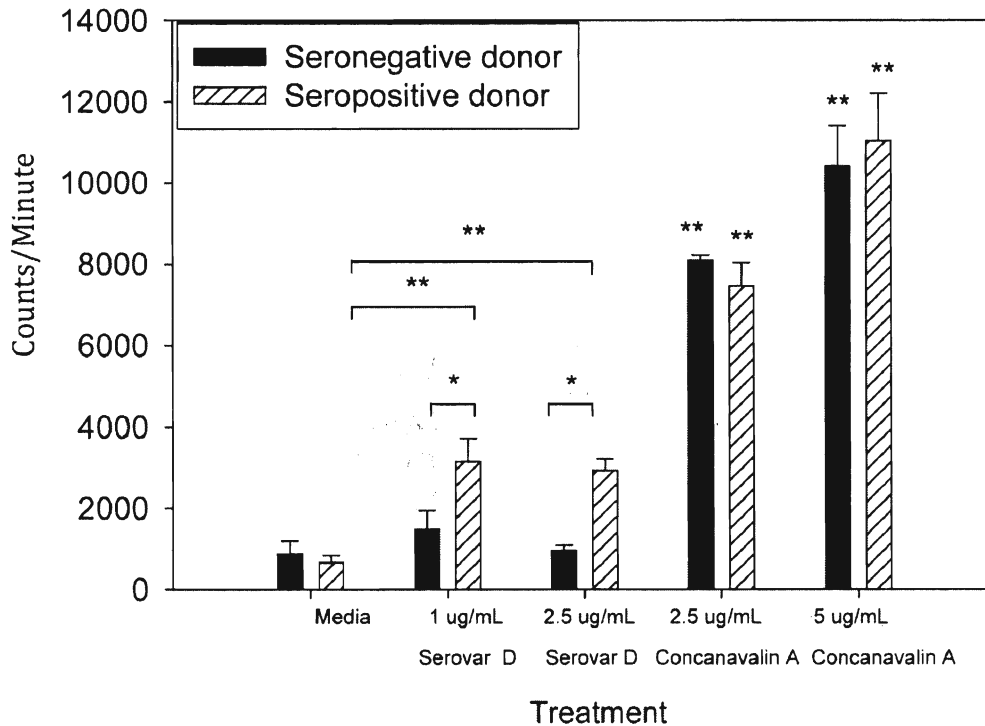
FIGURE 11 PBMCs isolated from a seropositive donor proliferate more robustly in response to chlamydial antigen, Serovar D EB, than PBMCs isolated from a seronegative donor. One-way RM ANOVA. **$p<.003$, *$p<.005$ for indicated pairs or for ConA versus media for each condition.

As predicted, a significant main effect for treatment type, $F(9,20) = 137.40$, $p < .001$, revealed that measured T-cell proliferation was different across the seven treatment conditions for the seropositive donor and the seronegative donor. This effect is illustrated in figure 11. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (7456.67, 580.95), stimulated T-cells from a seropositive donor to proliferate greater than the media control (656.67, 182.66), $t(7) = 13.37$, $p < .003$. Similarly pair-wise comparisons revealed that 2.5ug/mL of ConA (8082.33, 130.14), stimulated T-cells from a seronegative donor to proliferate greater than the media control (873.67, 318.45), $t(7) = 14.17$, $p < .003$. Furthermore, 5ug/mL of ConA (11027.67, 1174.08), stimulated T-cells from a seropositive donor to proliferate greater than the media control (656.67, 182.66), $t(7) = 20.38$, $p < .003$. Additionally, 5ug/mL of ConA (11027.67, 1174.08), stimulated T-cells from a seronegative donor to proliferate greater than the media control (873.67, 318.45), $t(7) = 19.96$, $p < .003$. For the chlamydial antigen stimulant, Serovar D EB, pair-wise

comparisons revealed that 1ug/mL of Serovar D EB (3147.00, 569.74), stimulated T-cells from a seropositive donor to proliferate greater than the media control (656.67, 182.66), $t(7) = 4.895$, $p < .005$. Furthermore, 2.5ug/mL of Serovar D EB (2929.33, 285.22), stimulated T-cells from a seropositive donor to proliferate greater than the media control (656.67, 182.66), $t(7) = 4.467$, $p < .005$. However, there was no difference in T-cell proliferation between the media control condition and 1 and 2.5ug/mL of Serovar D EB for the seronegative donor, $p = $ ns.

3H T-Cell Proliferation #3

Proliferation of peripheral blood T cells from a *Chlamydia* seropositive donor in response to Serovar D, ConA, or HSP60
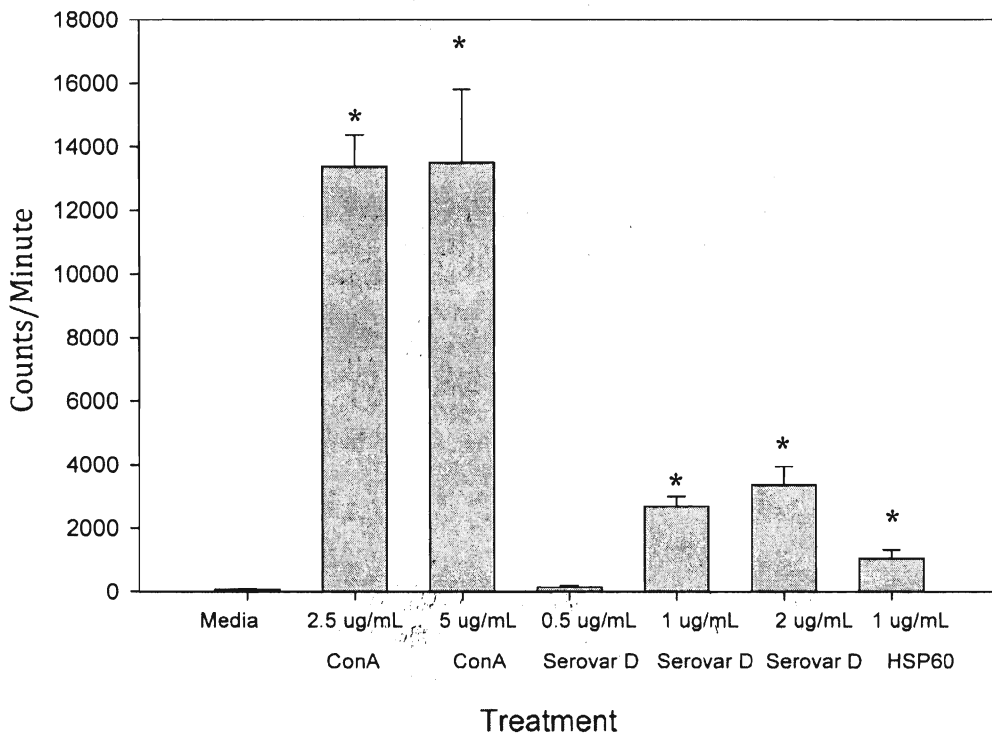
FIGURE 12 PBMCs isolated from a seropositive donor proliferate more robustly when stimulated with chlamydial-specific antigens, Serovar D EB and CHSP60-1, than the media control. One-way RM ANOVA. *$p<.05$ for each treatment condition versus media.

A significant main effect for treatment type, $F(6,14) = 109.48$, $p < .001$, revealed that measured T-cell proliferation was different across the seven treatment conditions for the seropositive donor. This effect is illustrated in figure 12. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (13386.00, 998.31), stimulated T-cells from a seropositive donor to

proliferate greater than the media control (60.67, 8.51), $t(5) = 16.50, p < .001$. Similarly, pair-wise comparisons revealed that 5ug/mL of ConA (13506.67, 2311.37), stimulated T-cells from a seropositive donor to proliferate greater than the media control (60.67, 8.51), $t(5) = 16.65, p < .001$. For the chlamydial antigen stimulant, Serovar D EB, pair-wise comparisons revealed that 1ug/mL of Serovar D EB (2690.00, 317.73), stimulated T-cells from a seropositive donor to proliferate greater than the media control (60.67, 8.51), $t(5) = 3.26, p < .05$. Additionally, 2ug/mL of Serovar D EB (3362.67, 578.51), stimulated T-cells from a seropositive donor to proliferate greater than the media control (60.67, 8.51), $t(5) = 4.09, p < .05$. For the chlamydial antigen stimulant, CHSP60-1, pair-wise comparisons revealed that 1ug/mL of CHSP60-1 (1041.33, 268.82), stimulated T-cells from a seropositive donor to proliferate greater than the media control (60.67, 8.51), $t(5) = 2.21, p < .05$. However, there was no difference in T-cell proliferation between the media control condition and 0.5ug/mL of Serovar D EB for the seronegative donor, $p = $ ns.

Analysis of Dual-Color ELISpot Assays

Dual-Color ELISpot Assay #1
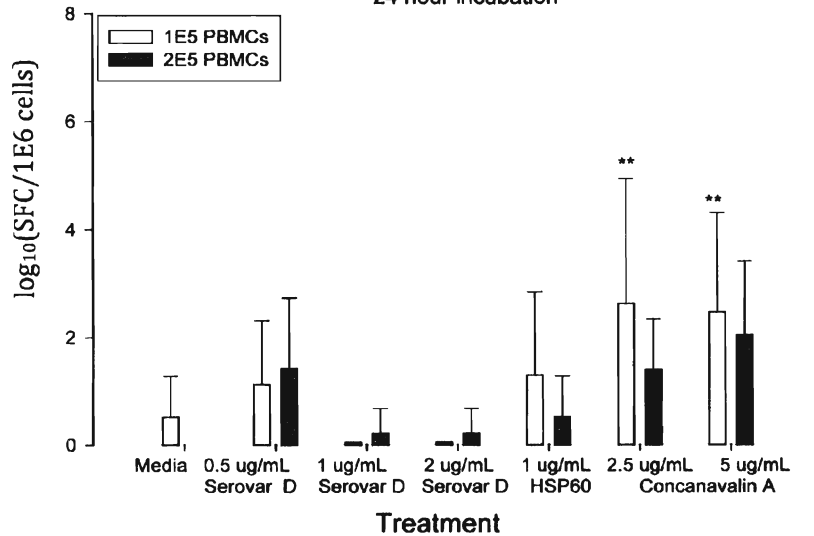
FIGURE 13
Peripheral blood T-cells isolated from a seropositive and a seronegative donor display specific secretion of IFN-γ in response to ConA during a 24-hour incubation. One-way RM ANOVA. **$p<.007$, for each treatment condition versus media.

26

A significant main effect for treatment type, $F(3,8) = 9.11$, $p < .05$, revealed that measured IFN-γ secretion was different across the seven treatment conditions for the seropositive donor. Furthermore, a significant main effect for treatment type, $F(4,10) = 11.40$, $p < .001$, revealed that measured IFN-γ secretion was different across the seven treatment conditions for the seronegative donor. These main effects are illustrated in figure 13. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (2543.33, 1285.94), stimulated the 1E5 dilution of T-cells from the seropositive donor to secrete IFN-γ greater than the media control (0.00, 0.00), $t(11) = 3.80$, $p < .05$. Similarly pair-wise comparisons revealed that 5ug/mL of ConA (2420.00, 1020.93), stimulated the 1E5 dilution T-cells from the seropositive donor to secrete IFN-γ greater than the media control (0.00, 0.00), $t(11) = 3.61$, $p < .05$.

For the seronegative donor, pair-wise comparisons revealed that 2.5ug/mL of ConA (416.67, 205.51), stimulated the 1E5 dilution of T-cells to secrete IFN-γ greater than the media control (3.33, 5.77), $t(11) = 5.14$, $p < .05$. Furthermore, comparisons revealed that 5ug/mL of ConA (290.00, 70.00), stimulated the 1E5 dilution of T-cells from the seronegative donor to secrete IFN-γ greater than the media control (3.33, 5.77), $t(11) = 3.56$, $p < .05$. However, there was no difference in T-cell IFN-γ secretion between the media control condition and all concentrations of chlamydial antigen stimulants, Serovar D EB and CHSP60-1, for both the seropositive and the seronegative donor, $p = $ ns.
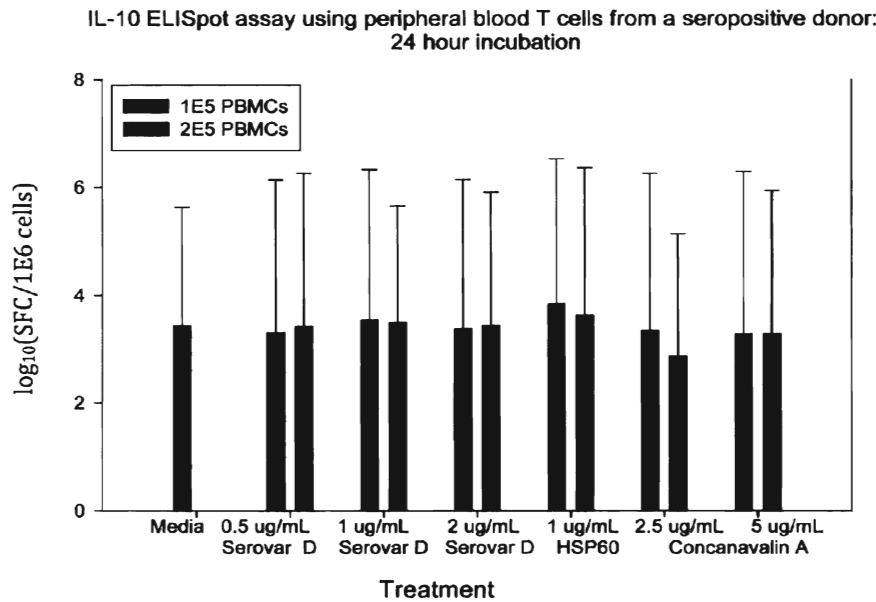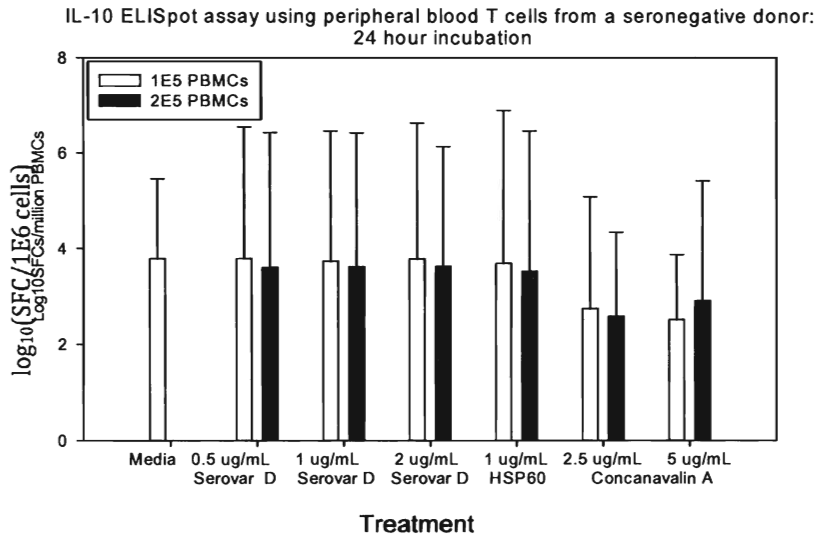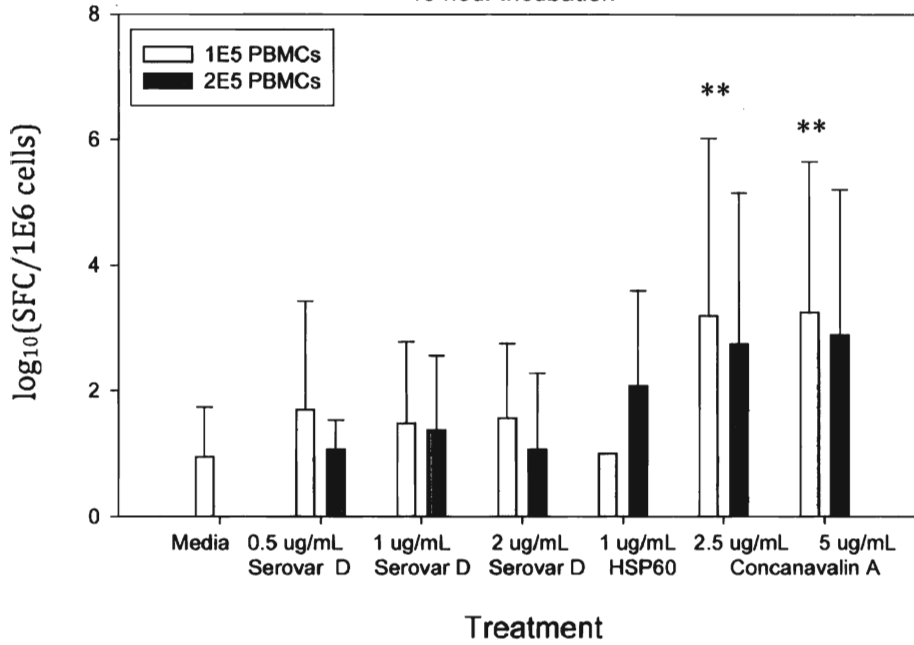
IL-10 ELISpot assay using peripheral blood T cells from a seronegative donor: 24 hour incubation

IL-10 ELISpot assay using peripheral blood T cells from a seropositive donor: 24 hour incubation

FIGURE 14 Peripheral blood T-cells isolated from a seropositive donor and a seronegative donor do not exhibit chlamydial antigen-specific secretion of IL-10 post-24-hour incubation.

The data lacked a significant main effect for treatment type across 1E5 T-cell dilutions for the seropositive donor, $F(6,14) = .015$, $p =$ ns, revealing that measured IL-10 secretion was not significantly different across the seven treatment conditions. Similarly, the data lacked a significant main effect for treatment type across 1E5 T-cell dilutions for the seronegative donor, $F(6,14) = .15$, $p =$ ns. The data also lacked a significant main effect for treatment type across 2E5 T-cell dilutions for the seropositive donor, $F(6,14) = .91$, $p =$ ns. Finally, the data lacked a significant main effect for treatment type across 2E5 T-cell dilutions for the seronegative donor, $F(6,14) = .91$, $p =$ ns. The lack of significant main effects is shown in figure 14.

28

Dual-Color ELISpot Assay #2

Interferon-γ ELISpot assay using peripheral blood T cells from a seronegative donor:
48 hour incubation

Interferon-γ ELISpot assay using peripheral blood T cells from a seropositive donor:
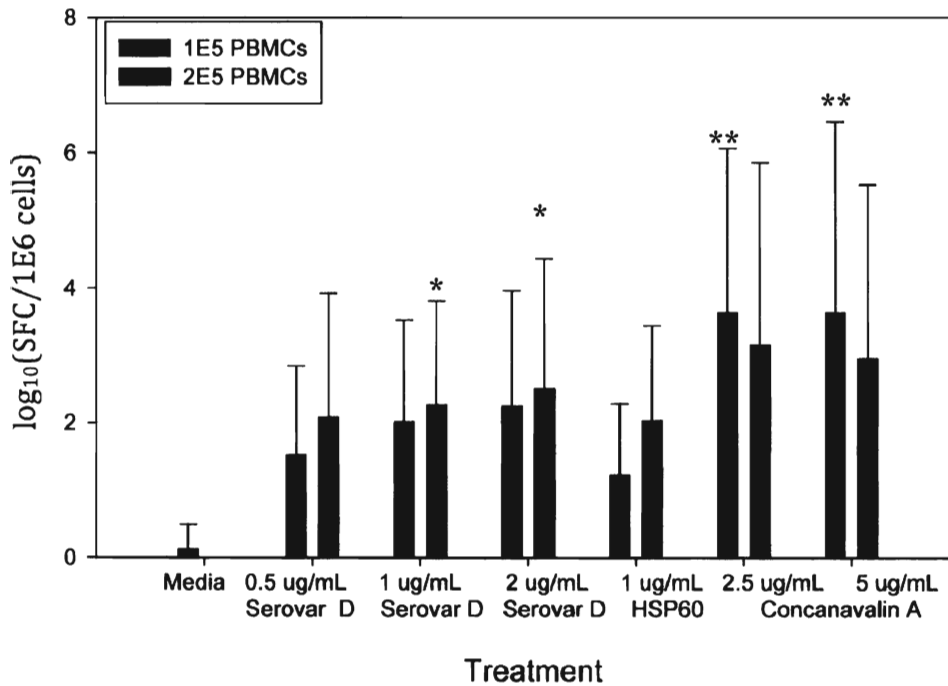48 hour incubation

FIGURE 15   Peripheral blood T-cells isolated from a seropositive donor display chlamydial antigen-specific secretion of IFN-γ in response to Serovar D EB and CHSP60-1 during a 48-hour incubation, while those from a seronegative donor do not. One-way RM ANOVA. *$p < .05$, **$p < .003$ for each treatment condition versus media.

A significant main effect for treatment type, $F(6,14) = 173.16$, $p < .001$, revealed that measured IFN-γ secretion was different across the seven treatment conditions for the seropositive donor. Furthermore, a significant main effect for treatment type, $F(6,14) = 22.89$, $p < .001$, revealed that measured IFN-γ secretion was different across the seven treatment conditions for the seronegative donor. These main effects are illustrated in figure 15. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (4336.67, 266.33), stimulated the 1E5 dilution of T-cells from a seropositive donor to secrete IFN-γ greater than the media control (1.33, 2.31), $t(11) = 19.48$, $p < .003$. Similarly pair-wise comparisons revealed that 5ug/mL of ConA (4280.00, 667.01), stimulated the 1E5 dilution T-cells from a seropositive donor to secrete IFN-γ greater than the media control (1.33, 2.31), $t(11) = 19.23$, $p < .003$.

For the seronegative donor, pair-wise comparisons revealed that 2.5ug/mL of ConA (1553.33, 684.13), stimulated the 1E5 dilution of T-cells to secrete IFN-γ greater than the media control (9.00, 6.08), $t(11) = 6.83$, $p < .003$. Furthermore, comparisons revealed that 5ug/mL of ConA (1633.33, 254.82), stimulated the 1E5 dilution of T-cells from a seronegative donor to secrete IFN-γ greater than the media control (9.00, 6.08), $t(11) = 7.19$, $p < .003$. For Serovar D EB treatment conditions, pair-wise comparisons revealed that 1ug/mL of Serovar D EB (103.33, 30.15), stimulated the 2E5 dilution of T-cells from a seropositive donor to secrete IFN-γ greater than the media control (1.33, 2.31), $t(11) = 0.46$, $p < .05$. However, there was no difference in T-cell IFN-γ secretion between the media control condition and 1ug/mL of CHSP60-1 for the seropositive donor, $p = $ ns. Similarly, there was no difference in T-cell IFN-γ secretion between the media control condition and all concentrations of chlamydial antigen stimulants, Serovar D EB and CHSP60-1, for the seronegative donor, $p = $ ns.

IL-10 ELISpot assay using peripheral blood T cells from a seronegative donor: 48 hour incubation

IL-10 ELISpot assay using peripheral blood T cells from a seropositive donor: 48 hour incubation
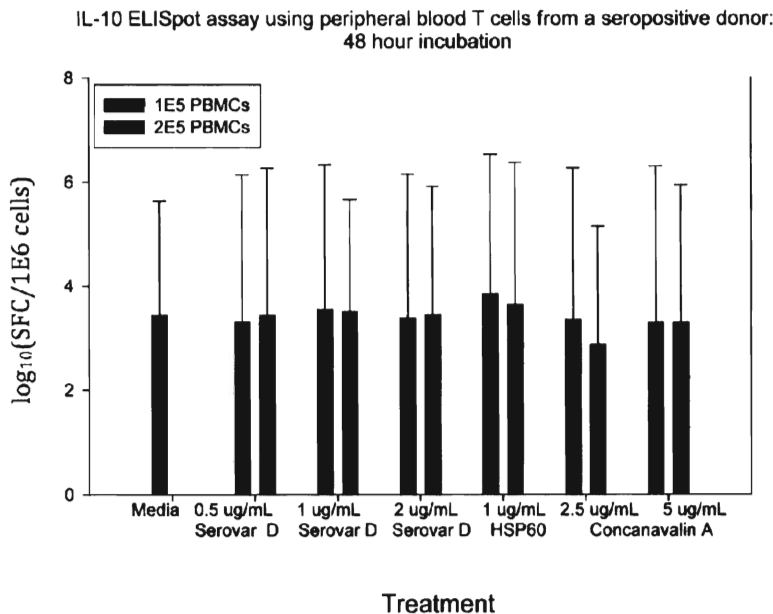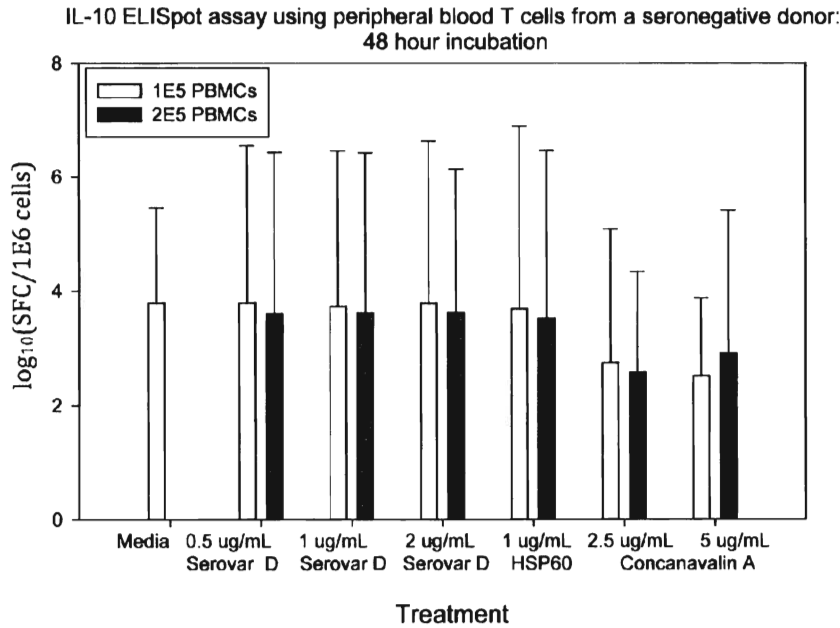
FIGURE 16 Peripheral blood T-cells isolated from a seropositive donor and a seronegative donor do not exhibit chlamydial antigen-specific secretion of IL-10 post-48-hour incubation.

The data lacked a significant main effect for treatment type across 1E5 T-cell dilutions for the seropositive donor, $F(6,14) = .063$, p = ns, revealing that measured IL-10 secretion was not significantly different across the seven treatment conditions. Similarly, the data lacked a significant main effect for treatment type across 1E5 T-cell dilutions for the seronegative donor, $F(6,14) = .15$, p = ns. The data also lacked a significant main effect for treatment type across 2E5 T-cell dilutions for the seropositive donor, $F(6,14) =$

1.04, p = ns. Finally, the data lacked a significant main effect for treatment type across 2E5 T-cell dilutions for the seronegative donor, $F(6,14)$ = .91, p = ns. The lack of significant main effects is shown in figure 16.

Exploratory Summary of ELISpot Results Across Part One Trials:

Table 1	Fold increase in Interferon-γ above media control for stimulated PBMCs in 24 hour incubation.					
	0.5 ug/mL Serovar D	1 ug/mL Serovar D	2 ug/mL Serovar D	1 ug/mL HSP60	2.5 ug/mL Concanavalin A	5 ug/mL Concanavalin A
Seropositive Donor	1	1	20	10.46	68.11	67.68
Seronegative Donor	2.39	0.1	0.1	2.49	5.01	4.71

Note. In the 24-hour incubation, 2ug/mL of Serovar D and 1ug/mL of HSP60 had the greatest fold increase in IFN-γ secretion over the media control for the seropositive donor. ConA is a useful control in the 24-hour incubation because it stimulated cells to secrete IFN-γ the most over the media control.

Table 2	Fold increase in Interferon-γ above media control for stimulated PBMCs in 48 hour incubation.					
	0.5 ug/mL Serovar D	1 ug/mL Serovar D	2 ug/mL Serovar D	1 ug/mL HSP60	2.5 ug/mL Concanavalin A	5 ug/mL Concanavalin A
Seropositive Donor	12.19	16.13	17.99	9.78	29.12	29.07
Seronegative Donor	1.78	1.55	1.64	1.05	3.34	3.41

Note. In the 48-hour incubation, all concentrations of Serovar D and HSP60 had the greatest fold increase in IFN-γ secretion over the media control for the seropositive donor. The smaller fold increases over media control for the seronegative donor are even more pronounced in this table, such that IFN-γ secretion was not specifically greater than the media control across all chlamydial stimulants.

Table 3	Fold increase in IL-10 above media control for stimulated PBMCs in 24 hour incubation.					
	0.5 ug/mL Serovar D	1 ug/mL Serovar D	2 ug/mL Serovar D	1 ug/mL HSP60	2.5 ug/mL Concanavalin A	5 ug/mL Concanavalin A
Seropositive Donor	0.96	1.03	0.98	1.12	0.97	0.96
Seronegative Donor	1	0.99	1	0.97	0.72	0.66

Note. In the 24-hour incubation, PBMCs isolated from a seropositive donor and a seronegative donor did not secrete specific IL-10. For both donors, most chlamydial antigens do not have a fold increase greater than one as compared to the media control.

Table 4 *Fold increase in IL-10 above media control for stimulated PBMCs in 48 hour incubation.*

	0.5 ug/mL Serovar D	*1 ug/mL Serovar D*	*2 ug/mL Serovar D*	*1 ug/mL HSP60*	*2.5 ug/mL Concanavalin A*	*5 ug/mL Concanavalin A*
Seropositive Donor	0.92	1.01	0.96	1.15	0.97	0.95
Seronegative Donor	1	0.99	1	0.95	0.71	0.62

Note. In the 48-hour incubation, PBMCs isolated from a seropositive and seronegative donor did not secrete specific IL-10. For both donors, most chlamydial antigens do not have a fold increase greater than one as compared to the media control.

Treatment of T-Cell Populations

Upon determining in proliferation and ELISpot assays that 1ug/mL of chlamydial antigens (CHSP60-1 & Serovar D EB) and 2.5ug/mL of experimental mitogen (ConA) was optimal for stimulating T-cells greater than media controls for a seropositive donor, the following dilution factor table was created for use in Part Two of this study. The dilution factor table describes how much media should be added to current concentrations of antigens, and then how much of these diluted stimulants should be added to each experimental well to achieve optimal stimulant concentrations for plating 2E5 cells/well.

Table 5 Dilution factors and stimulant addition volumes for various concentrations of chlamydial antigens and proteins.

	1ug/mL CHSP60-1 *(0.559ug/uL)*	1ug/mL Serovar D EB *(1ug/3uL)*	2.5ug/mL ConA *(1ug/2uL)*
Master Mix	3.578uL HSP60 + 96.422uL media	6.06uL D + 93.9uL media	6uL ConA + 54uL media
Amt. Add to Each Well	5uL	5uL	5uL

33

Part Two Results

Study Population

Table 6 Proportion of AMC participants with various CT infection statuses, gynecological histories, and clinical symptoms.

Characteristic	% of Sample [a]
Present CT	23%
[b]PMHx of CT	36%
No Present/[b]PMHx of CT	41%
Present STI	14%
[b]PMHx of STI	36%
Abdominal pain at enrollment	23%
Discharge at enrollment	9%

Note. [a]The sample size for all percentages calculated is 22.
[b]PMHx = Past Medical History

Table 7 Demographic characteristics, sexual histories, and contraceptive histories of AMC participants.

Characteristic	Value [a]
Age, Mean +/- SD	17 +/- 1.7
Race	
Black	17
White	2
Asian	0
Biracial	3
Number of Sexual Partners	
0	0
1-4	18
5-9	4
Contraceptive Method	
None	2
Diaphragm	0
Oral Contraceptives (the pill)	6
The "Ring" (Nuvaring)	1
Male Condoms	13

Note. [a]The sample size for all values is 22.

Average Percentage Reduction for Participants Grouped
Into Infection Statuses Across Treatment Conditions
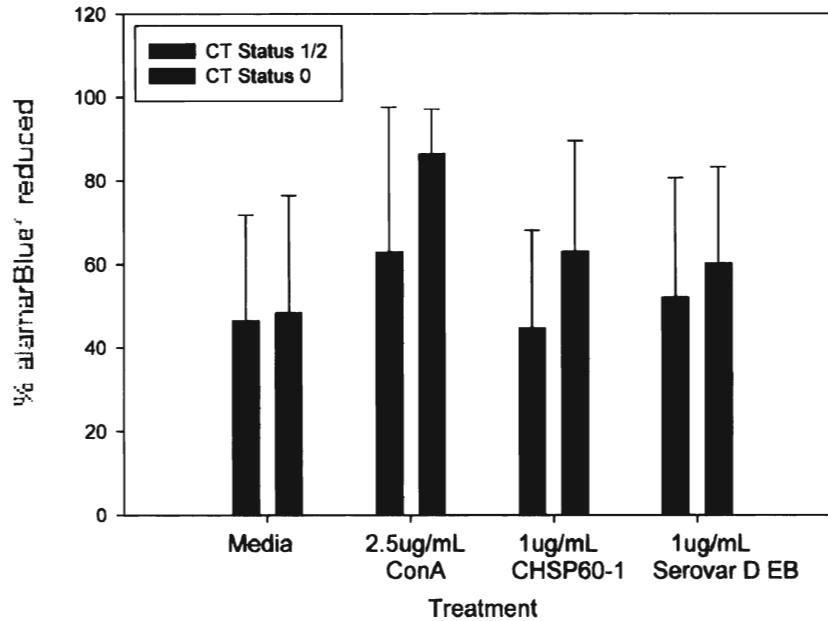
FIGURE 17 Average percentage reductions of PBMCs at 72-hour post stimulation with chlamydial antigens, mitogens, and media controls using an alamar-blue T-cell proliferation assay. Bars represent median percentage reductions +/- standard deviations. CT Statuses 1/2 correspond to participants with Past medical history of CT and Present CT (n = 11), and CT Status 0 corresponds to No Present CT (n = 7).

As predicted, a significant main effect for treatment type, $F(3,48) = 13.09$, $p < .001$, revealed that measured CD4+ T-cell proliferation was different across the four treatment conditions. This effect is illustrated in figure 17. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (72.04, 29.67) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.67), $t(17) = -4.18$, $p < .05$. Furthermore, pair-wise comparisons revealed that 1ug/mL of Serovar D EB (55.21, 26.17) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.63), $t(17) = -2.491$, $p < .05$. However, there was no difference in CD4+ T-cell proliferation between the media control and 1ug/mL of CHSP60-1. The data lacked a significant main effect of *Chlamydia* status, $F(1,16) = 1.27$, $p = $ ns, illustrating that measured CD4+ T-cell proliferation did not differ across the two CT statuses (0 and 1/2). Finally, the data lacked a significant interaction between treatment type and *Chlamydia* status, $F(3,48) = 2.27$, $p = $ ns, revealing that measured CD4+ T-cell proliferation was not significantly different across the four treatment conditions for participants with different CT statuses.

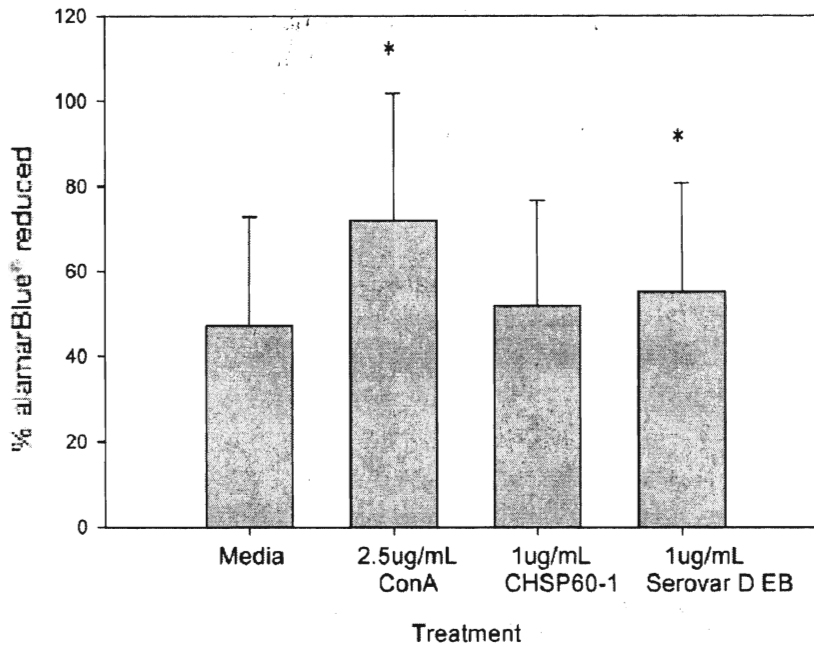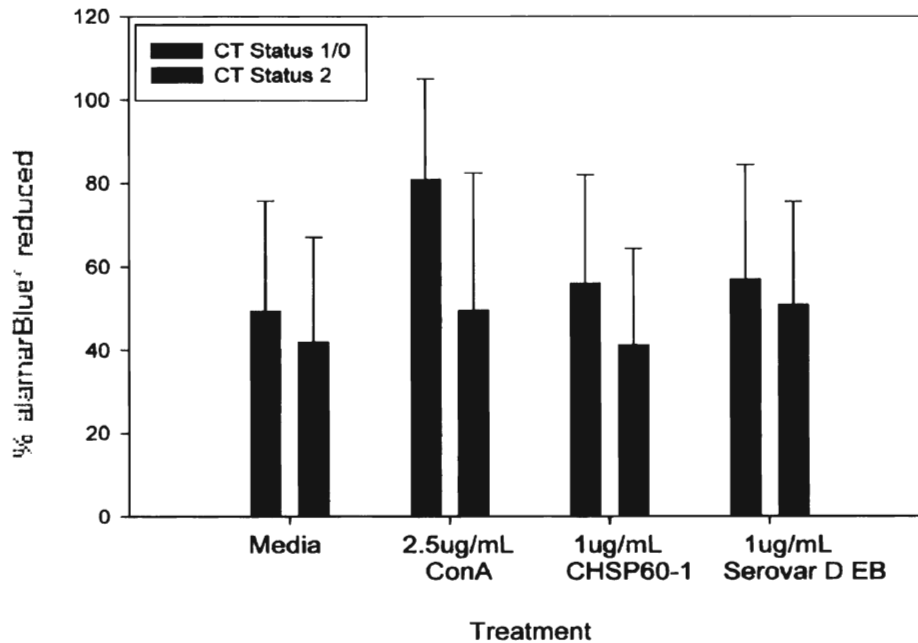Average Percent Reduction
Across Treatment Conditions

FIGURE 18 Average percentage reductions of PBMCs at 72-hour post-stimulation across all participants with chlamydial antigens, mitogens, and media controls using an alamar-blue T-cell proliferation assay. Bars represent median percentage reductions +/- standard deviations for all participants regardless of CT infection or pathology status. Serovar D EB and ConA stimulated PBMCs of participants to proliferate more robustly than media controls. Paired samples t-tests versus a media control illustrate a significant treatment effect. * $p < .05$ versus media control.

Follow-up pair-wise comparisons of aggregate data over all participants revealed that 2.5ug/mL of ConA (72.04, 29.67) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.63), $t(17) = -4.18$, $p < .05$, as well as 1ug/mL of Serovar D EB (55.21, 26.17) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.63), $t(17) = -2.491$, $p < .05$. These significant comparisons are illustrated in figure 18.

Average Percentage Reduction for Participants Grouped Into Infection Statuses Across Treatment Conditions

FIGURE 19 Average percentage reductions of PBMCs at 72-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an alamar-blue T-cell proliferation assay. Bars represent median percentage reductions +/- standard deviations with CT infection statuses grouped for diagnostic analysis. CT Statuses 1/0 correspond to combined average percentage reductions for participants with No Present CT and Past medical history of CT (n = 13). CT Status 2 corresponds to participants with Present CT (n = 5).

As predicted, a significant main effect for treatment type, $F(3,48) = 6.17$, $p < .05$, revealed that measured CD4+ T-cell proliferation was different across the four treatment conditions. This effect is illustrated in figure 19. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (72.04, 29.67) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.67), $t(17) = -4.18$, $p < .05$. Furthermore, pair-wise comparisons revealed that 1ug/mL of Serovar D EB (55.21, 26.17) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.63), $t(17) = -2.49$, $p < .05$. However, there was no difference in CD4+ T-cell proliferation between the media control and 1ug/mL of CHSP60-1. The data lacked a significant main effect of *Chlamydia* status, $F(1,16) = 1.44$, $p =$ ns, illustrating that measured CD4+ T-cell proliferation did not differ across the two CT statuses (2 and 1/0). Finally, the data lacked a significant interaction between treatment type and *Chlamydia* status, $F(3,48) = 2.76$, $p =$ ns, revealing that

37

measured CD4+ T-cell proliferation was not significantly different across the four
treatment conditions for participants with different CT statuses.

ROC Analysis of Alamar-Blue Proliferation Assays When
CD4+ T-Cells Are Stimulated with CHSP60-1 (CT Infection Status Grouping)
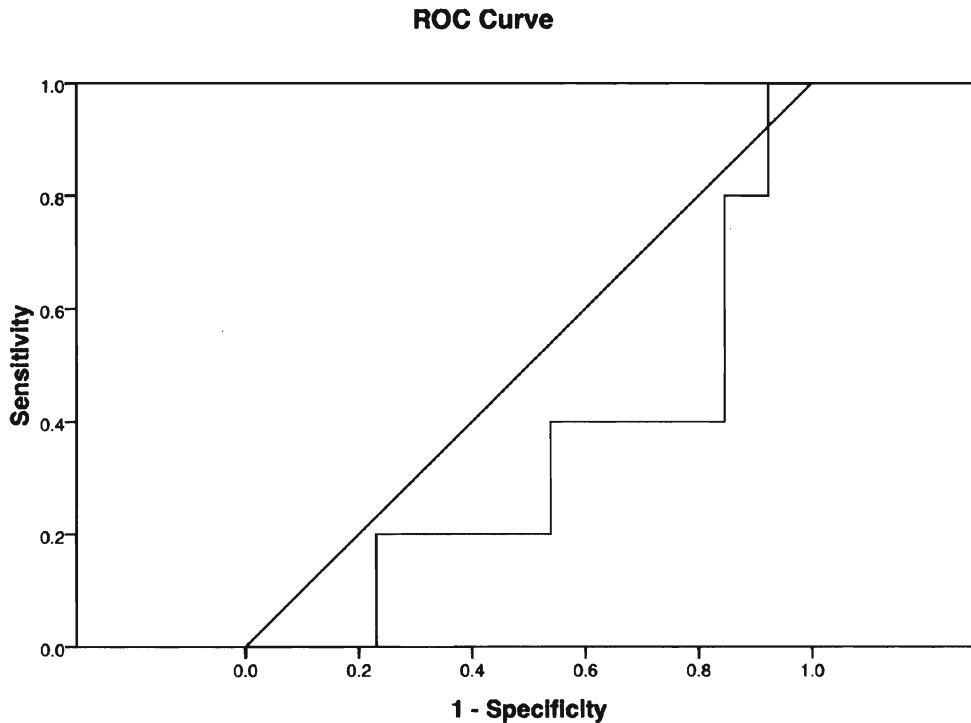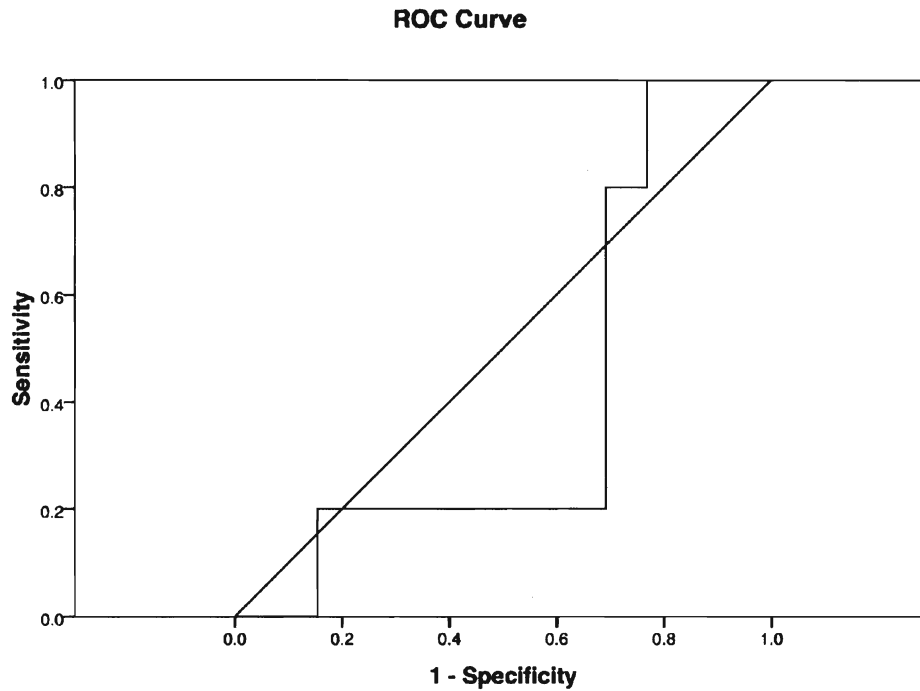
ROC Curve

FIGURE 20 Exploratory ROC analysis to determine the accuracy of alamar-blue T-cell
proliferation assays at diagnosing CT infection based upon differences in CD4+ T-cell
proliferation responses of participants in non-infected groups (CT Status 1/0) and
currently infected groups (CT Status 2) when CD4+ T-cells are stimulated with 1ug/mL
of CHSP60-1. The probability that alamar-blue T-cell proliferation assays detect CT in
adolescent women populations correctly is less than chance (AUC = 0.323).
Note. Sensitivity = true positive rate (woman has CT infection and is correctly diagnosed
with CT infection) and 1-Specificity = false positive rate (woman does not have CT
infection and is incorrectly diagnosed with CT infection). The green line represents the
chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false
positive rates at various percentage reduction cut-off values.

ROC Analysis of Alamar-Blue Proliferation Assays When
CD4+ T-Cells Are Stimulated with Serovar D EB (CT Infection Status Grouping)

ROC Curve

FIGURE 21 Exploratory ROC analysis to determine the accuracy of alamar-blue T-cell proliferation assays at diagnosing CT infection based upon differences in CD4+ T-cell proliferation responses of participants in non-infected groups (CT Status 1/0) and currently infected groups (CT Status 2) when CD4+ T-cells are stimulated with 1ug/mL of Serovar D EB. The probability that alamar-blue T-cell proliferation assays detect CT in adolescent women populations correctly is less than chance (AUC = 0.400).
Note. Sensitivity = true positive rate (woman has CT infection and is correctly diagnosed with CT infection) and 1-Specificity = false positive rate (woman does not have CT infection and is incorrectly diagnosed with CT infection). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at various percentage reduction cut-off values.

Average Percentage Reduction for Participants Grouped Into
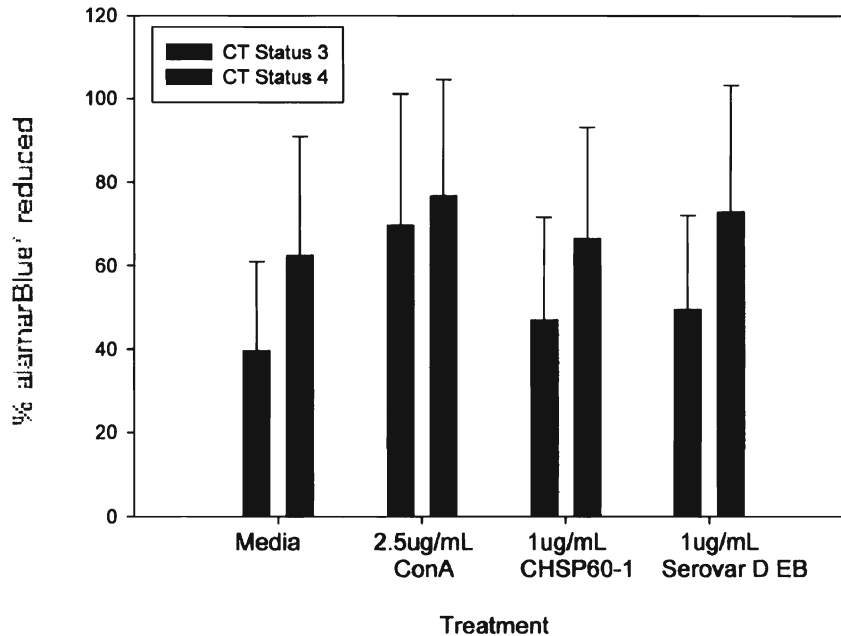CT Pathology Statuses Across Treatment Conditions

FIGURE 22 Average percentage reductions of PBMCs at 72-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an alamar-blue T-cell proliferation assay. Bars represent median percentage reductions +/- standard deviations with CT pathology statuses grouped for predictive analysis. CT Status 3 corresponds to combined average percentage reductions for participants with No Present clinical symptoms (no discharge/abdominal pain) (n = 12). CT Status 4 corresponds to participants with Present clinical symptoms (n = 6).

As predicted, a significant main effect for treatment type, $F(3,48) = 8.01$, $p < .05$, revealed that measured CD4+ T-cell proliferation was different across the four treatment conditions. This effect is illustrated in figure 22. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (72.04, 29.67) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.67), $t(17) = -4.18$, $p < .05$. Furthermore, pair-wise comparisons revealed that 1ug/mL of Serovar D EB (55.21, 26.17) stimulated CD4+ T-cells to proliferate greater than the media control (47.21, 25.63), $t(17) = -2.49$, $p < .05$. However, there was no difference in CD4+ T-cell proliferation between the media control and 1ug/mL of CHSP60-1. The data lacked a significant main effect of pathology status, $F(1,16) = 1.73$, $p =$ ns, illustrating that measured CD4+ T-cell proliferation did not differ across the two pathology statuses (3 and 4). Finally, the data lacked a significant interaction between treatment type and pathology status, $F(3,48) = .606$, $p =$ ns, revealing that measured CD4+ T-cell proliferation was not significantly different across the four treatment conditions for participants with different pathology statuses.
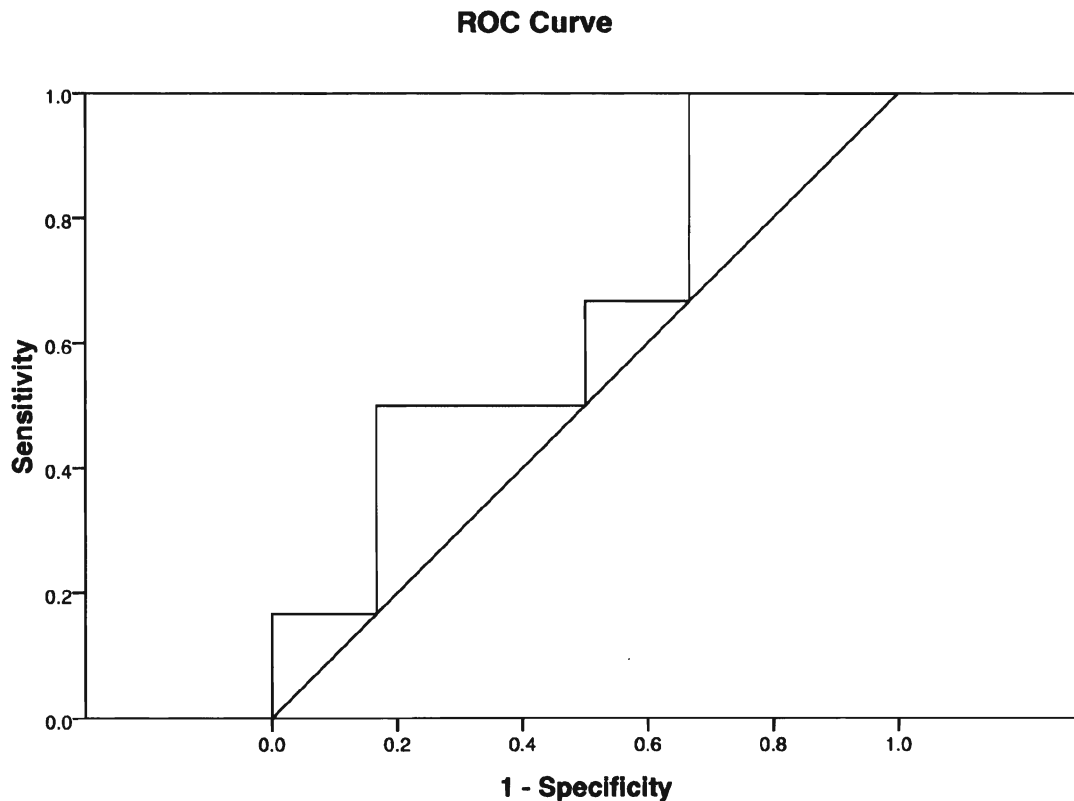
40

ROC Curve

FIGURE 23 Exploratory ROC analysis to determine the accuracy of alamar-blue T-cell proliferation assays at detecting pathology based upon differences in CD4+ T-cell proliferation responses of participants with No Present clinical symptoms (CT Status 3) and with Present clinical symptoms (CT Status 4) when CD4+ T-cells are stimulated with 1ug/mL of CHSP60-1. The probability that alamar-blue T-cell proliferation assays detect pathology in adolescent women populations correctly is greater than chance (AUC = 0.639).

Note. Sensitivity = true positive rate (woman has pathology and is correctly predicted as having pathology) and 1-Specificity = false positive rate (woman has no pathology and is incorrectly predicted as having pathology). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at various percentage reduction cut-off values.
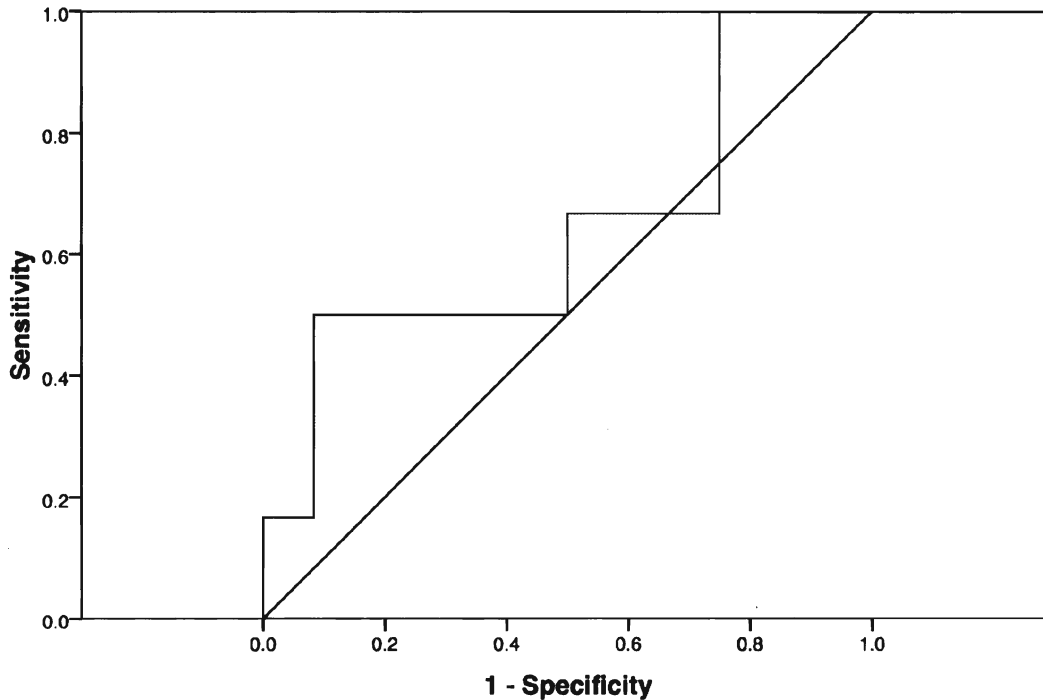
ROC Curve

FIGURE 24 Exploratory ROC analysis to determine the accuracy of alamar-blue T-cell proliferation assays at detecting pathology based upon differences in CD4+ T-cell proliferation responses of participants with No Present clinical symptoms (CT Status 3) and with Present clinical symptoms (CT Status 4) when CD4+ T-cells are stimulated with 1ug/mL of Serovar D EB. The probability that alamar-blue T-cell proliferation assays detect pathology in adolescent women populations correctly is greater than chance (AUC = 0.639).

Note. Sensitivity = true positive rate (woman has pathology and is correctly predicted as having pathology) and 1-Specificity = false positive rate (woman has no pathology and is incorrectly predicted as having pathology). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at various percentage reduction cut-off values.

Average SFCs/1E6 cells for Participants Grouped Into
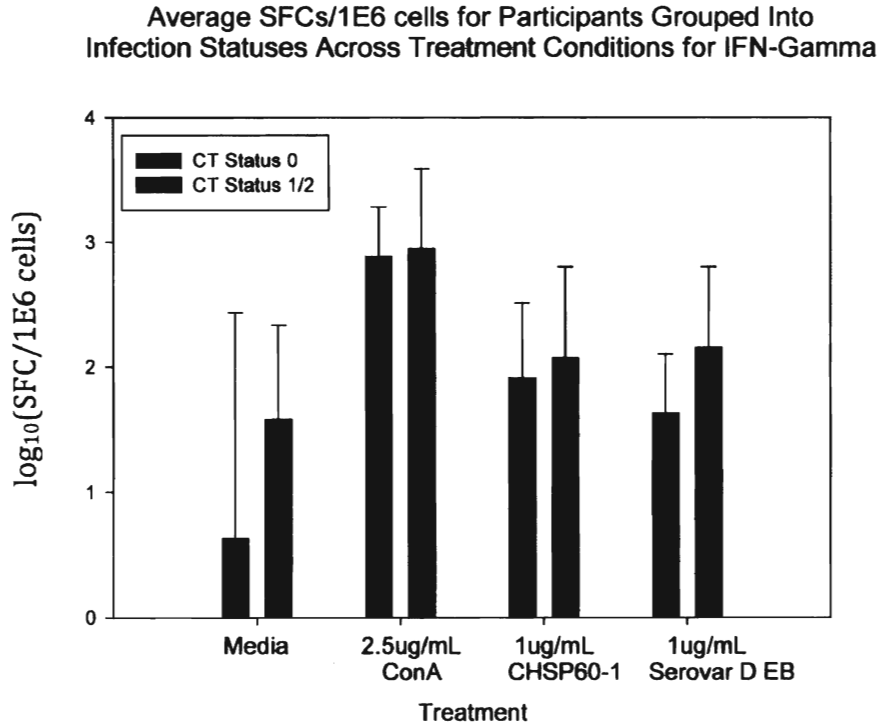Infection Statuses Across Treatment Conditions for IFN-Gamma

FIGURE 25 Average SFCs/1E6 PBMCs for IFN-γ at 48-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an ELISpot assay. Bars represent median percentage reductions +/- standard deviations. CT Statuses 1/2 correspond to participants with Past medical history of CT and Present CT (n = 9), and CT Status 0 corresponds to No Present CT (n = 4).

As predicted, a significant main effect for treatment type, $F(3,33) = 9.17$, $p < .05$, revealed that measured IFN-γ secretion was different across the four treatment conditions. This effect is illustrated in figure 25. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (2.89, .84) stimulated CD4+ T-cells to secrete IFN-γ greater than the media control (1.36, 1.27), $t(12) = -3.12$, $p < .05$. However, there was no difference in CD4+ T-cell IFN-γ secretion between the media control and the chlamydial antigen treatment conditions, 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB. The data lacked a significant main effect of *Chlamydia* status, $F(1,11) = 1.75$, $p = $ ns, illustrating that measured CD4+ T-cell IFN-γ secretion did not differ across the two CT statuses (0 and 1/2). Finally, the data lacked a significant interaction between treatment type and *Chlamydia* status, $F(3,33) = 1.90$, $p = $ ns, revealing that measured CD4+ T-cell IFN-γ secretion was not significantly different across the four treatment conditions for participants with different CT statuses.

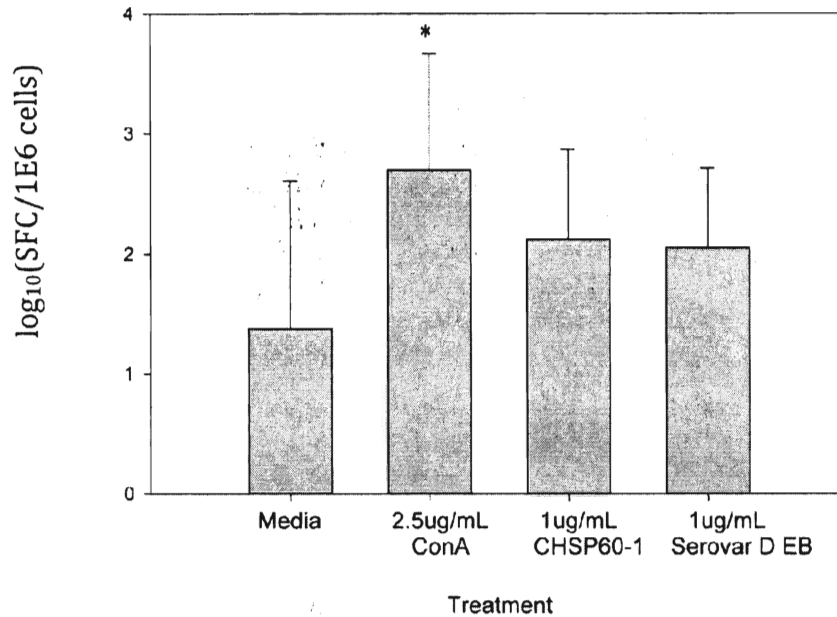Average SFCs/1E6 cells Across
Treatment Conditions for IFN-Gamma

FIGURE 26 Average SFCs/1E6 PBMCs for IFN-γ at 48-hour post-stimulation across all participants with chlamydial antigens, mitogens, and media controls using an IFN-γ ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations for all participants regardless of CT infection or pathology status. ConA stimulated PBMCs of participants to secrete IFN-γ more robustly than media controls. Paired samples t-tests versus a media control illustrate a significant treatment effect. * $p < .05$ for each treatment condition versus media.

Follow-up pair-wise comparisons of aggregate data over all participants revealed that 2.5ug/mL of ConA (2.89, .84) stimulated CD4+ T-cells to secrete IFN-γ greater than the media control (1.36, 1.27), $t(12) = -3.12$, $p < .05$. Significant comparisons are illustrated in figure 26.

Average SFCs/1E6 cells for Participants with Various CT Infection Statuses Across Treatment Conditions for IFN-Gamma
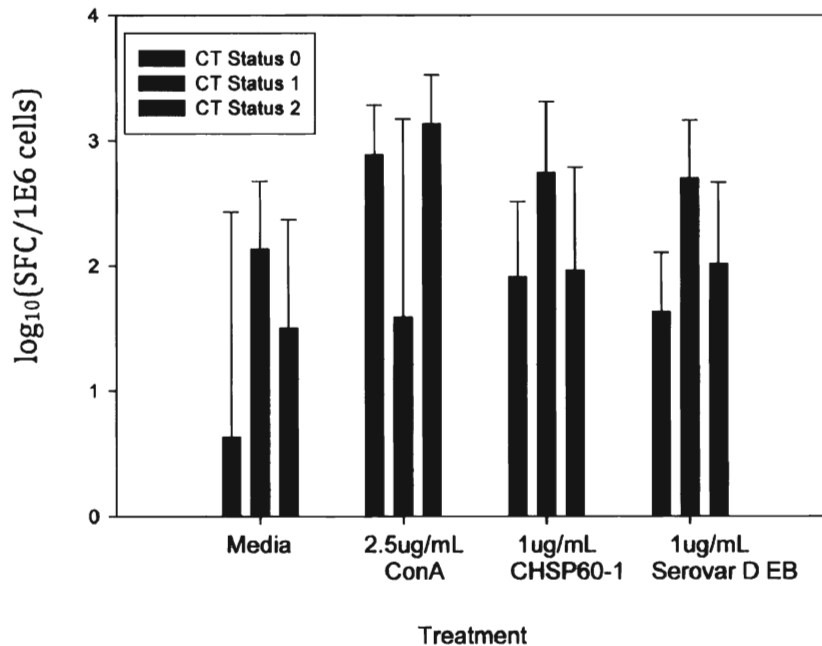
FIGURE 27 Average SFCs/1E6 PBMCs for IFN-γ at 48-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations. CT Statuses 0-2 correspond to participants with No Present CT (n = 4), Past medical history of CT (n = 3), and Present CT (n = 6), respectively.

As predicted, a significant main effect for treatment type, $F(3,30) = 6.17$, $p < .05$, revealed that measured IFN-γ secretion was different across the four treatment conditions. This effect is illustrated in figure 27. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (2.80, .84), stimulated CD4+ T-cells to secrete IFN-γ greater than the media control (1.36, 1.27), $t(12) = -3.12$, $p < .05$. However, there were no differences in IFN-γ between the media control condition and chlamydial antigen treatment conditions, 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB. Furthermore, there was no main effect of *Chlamydia* status, $F(2,10) = .80$, $p =$ ns, revealing that measured IFN-γ secretion was not significantly different across the three CT statuses (0, 1, and 2). Finally, there was no significant interaction between treatment and *Chlamydia* status, $F(6,30) = 2.72$, $p =$ ns, revealing that measured IFN-γ was not significantly different across the four treatment conditions for participants with different CT statuses.

Average SFCs/1E6 cells for Participants Grouped Into
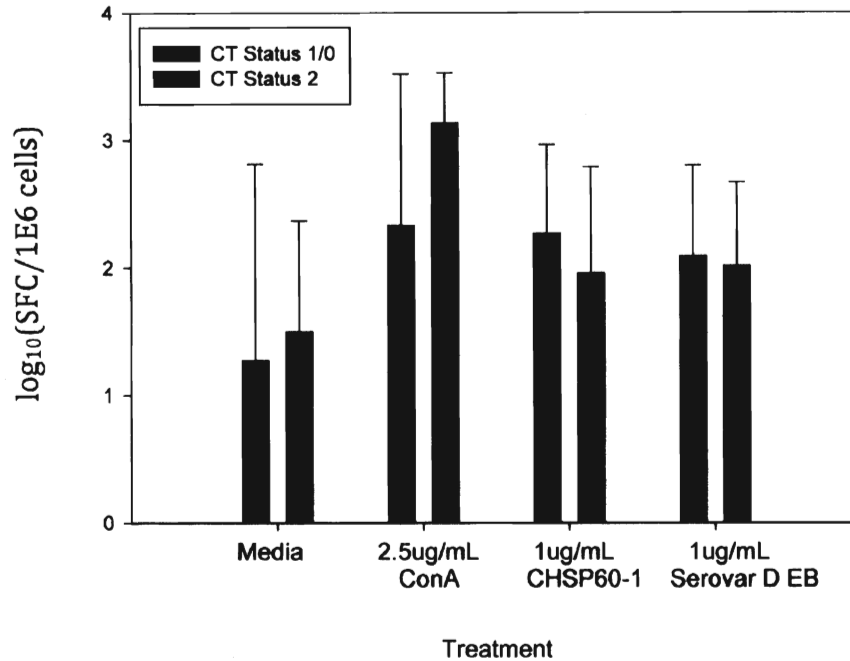Infection Statuses Across Treatment Conditions for IFN-Gamma

FIGURE 28 Average SFCs/1E6 PBMCs for IFN-γ at 48-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations with CT infection statuses grouped for diagnostic analysis. CT Statuses 1/0 correspond to combined average SFCs/1E6 cells for participants with No Present CT and Past medical history of CT (n = 7). CT Status 2 corresponds to participants with No Present CT (n = 6).

The data lacked a significant main effect for treatment type, $F(3,33) = 3.65$, $p =$ ns, revealing that measured IFN-γ secretion was not different across the four treatment conditions. Furthermore, there was no main effect of *Chlamydia* status $F(1,11) = .60$, $p =$ ns, revealing that measured IFN-γ secretion was not different for the two CT statuses (2 and 1/2). Finally, there was no interaction between treatment type and *Chlamydia* status $F(3,33) = .43$, $p =$ ns, illustrating that measured IFN-γ secretion did not differ across the four treatment conditions for participants with different CT statuses. The lack of significant main effects is illustrated in figure 28.
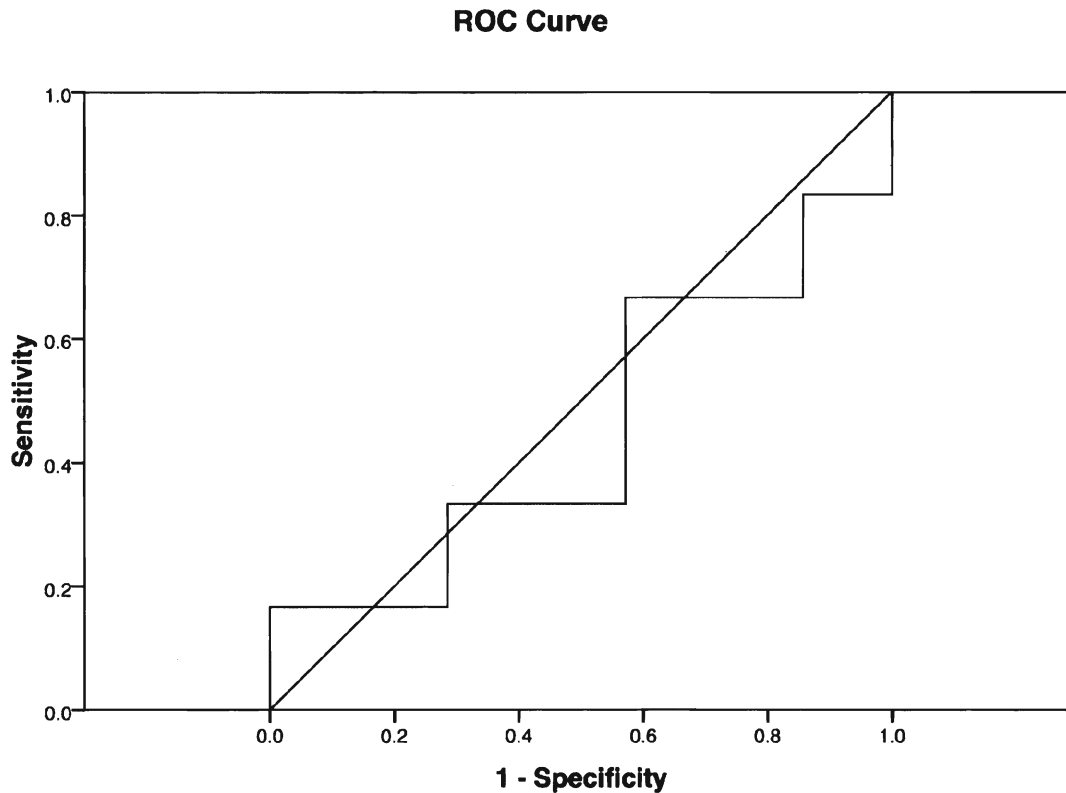
ROC Curve

FIGURE 29 Exploratory ROC analysis to determine the accuracy of ELISpot assays with IFN-γ at diagnosing CT infection based upon differences in CD4+ T-cell cytokine secretions of participants in non-infected groups (CT Status 1/0) and currently infected groups (CT Status 2) when CD4+ T-cells are stimulated with 1ug/mL of CHSP60-1. The probability that ELISpot assays with IFN-γ detect CT in adolescent women populations correctly is less than chance (AUC = 0.452).

Note. Sensitivity = true positive rate (woman has CT infection and is correctly diagnosed with CT infection) and 1-Specificity = false positive rate (woman does not have CT infection and is incorrectly diagnosed with CT infection). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at cut-off rates representing various numbers of CD4+ T-cells that are secreting IFN-γ.

ROC Analysis of ELISpot Assays with IFN-γ When
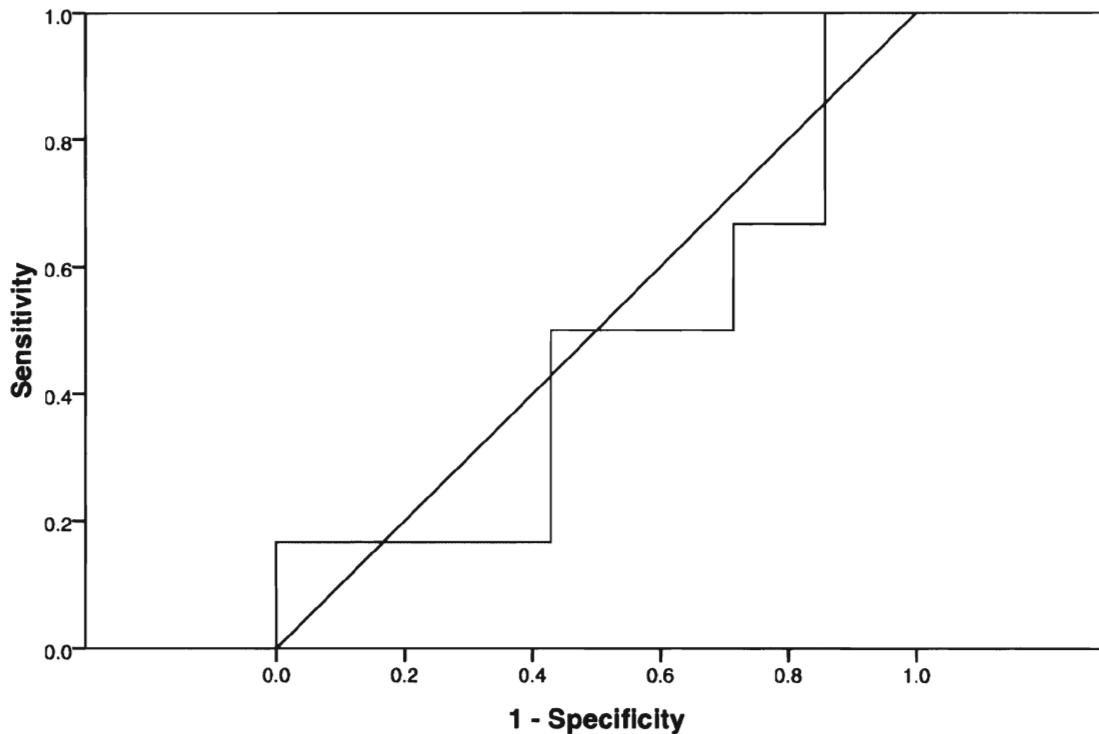## CD4+ T-Cells Are Stimulated with Serovar D EB (CT Infection Status Grouping)

ROC Curve

FIGURE 30 Exploratory ROC analysis to determine the accuracy of ELISpot assays with IFN-γ at diagnosing CT infection based upon differences in CD4+ T-cell cytokine secretions of participants in non-infected groups (CT Status 1/0) and currently infected groups (CT Status 2) when CD4+ T-cells are stimulated with 1ug/mL of Serovar D EB. The probability that ELISpot assays with IFN-γ detect CT in adolescent women populations correctly is less than chance (AUC = 0.452).

Note. Sensitivity = true positive rate (woman has CT infection and is correctly diagnosed with CT infection) and 1-Specificity = false positive rate (woman does not have CT infection and is incorrectly diagnosed with CT infection). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at cut-off rates representing various numbers of CD4+ T-cells that are secreting IFN-γ.

Average SFCs/1E6 cells for Participants Grouped Into Infection Statuses Across Treatment Conditions for IL-10
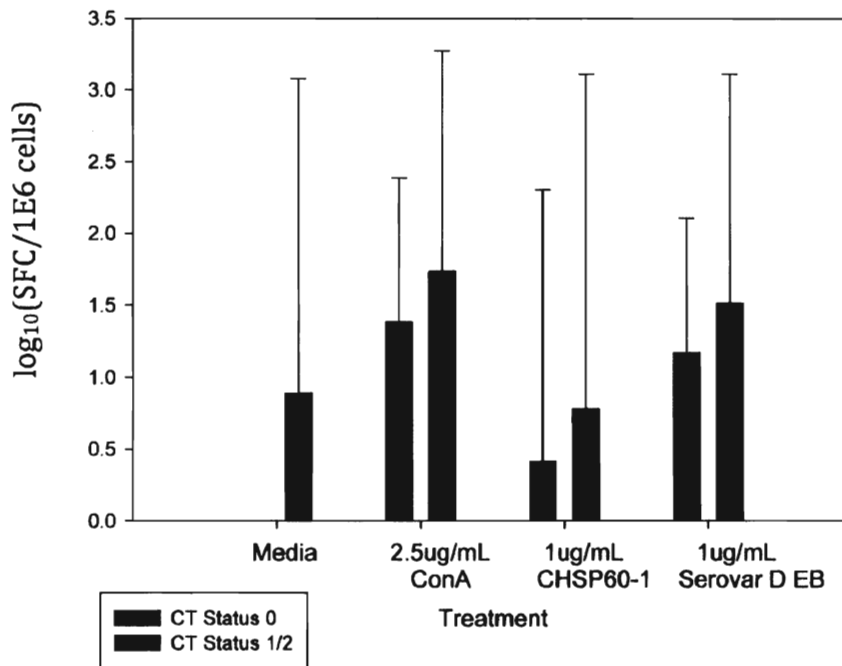
FIGURE 31 Average SFCs/1E6 PBMCs for IL-10 at 48-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an ELISpot assay. Bars represent median percentage reductions +/- standard deviations. CT Status 1/2 corresponds to participants with Past medical history of CT and Present CT (n = 5), and CT Status 0 corresponds to No Present CT (n = 3).

As predicted, a significant main effect for treatment type, $F(3,18) = 3.83$, $p < .05$, revealed that measured IL-10 secretion was different across the four treatment conditions. This effect is illustrated in figure 31. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (2.42, .78) stimulated CD4+ T-cells to secrete IL-10 greater than the media control (.53, 2.54), $t(7) = -2.31$, $p < .05$. Furthermore, pair-wise comparisons revealed that 1ug/mL of Serovar D EB (2.09, 1.14) stimulated CD4+ T-cell to secrete IL-10 greater than the media control (.53, 2.54), $t(7) = -3.08$, $p < .05$. However, there was no difference in CD4+ T-cell IL-10 secretion between the media control and 1ug/mL of CHSP60-1. The data lacked a significant main effect of *Chlamydia* status, $F(1,6) = .91$, $p = $ ns, illustrating that measured CD4+ T-cell IL-10 secretion did not differ across the two CT statuses (0 and 1/2). Finally, the data lacked a significant interaction between treatment type and *Chlamydia* status, $F(3, 18) = .38$, $p = $ ns, revealing that measured CD4+ T-cell IL-10 secretion was not significantly different across the four treatment conditions for participants with different CT statuses.

Average SFCs/1E6 cells Across
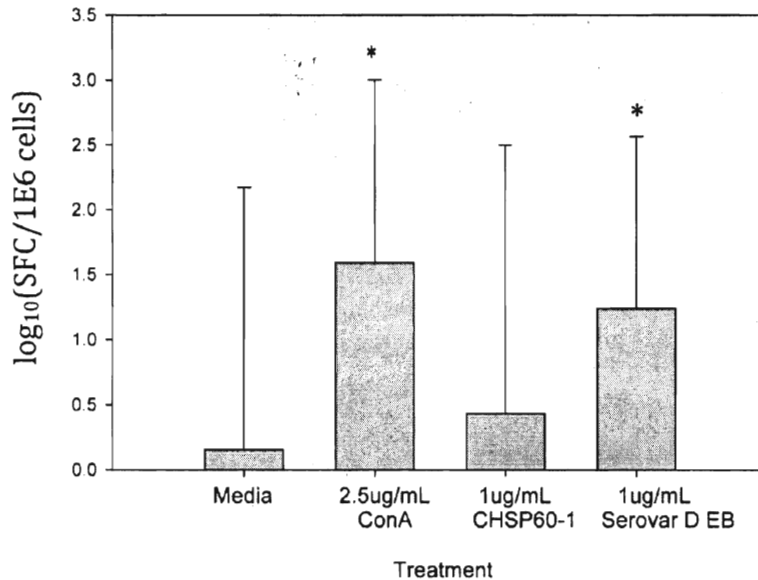Treatment Conditions for IL-10

FIGURE 32 Average SFCs/1E6 PBMCs for IL-10 at 48-hour post-stimulation across all participants with chlamydial antigens, mitogens, and media controls using an IL-10 ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations for all participants regardless of CT infection or pathology status. ConA stimulated PBMCs of participants to secrete IL-10 more robustly than media controls. Paired samples t-tests versus a media control illustrate a significant treatment effect. * $p < .05$ versus media control.

Follow-up pair-wise comparisons of aggregate data over all participants revealed that 2.5ug/mL of ConA (2.42, .78) stimulated CD4+ T-cells to secrete IL-10 greater than the media control (.53, 2.54), $t(7) = -2.31$, $p < .05$, as well as 1ug/mL of Serovar D EB (2.09, 1.14) stimulated CD4+ T-cells to secrete IL-10 greater than the media control (.53, 2.54), $t(7) = -3.08$, $p < .05$. These significant comparisons are illustrated in figure 32.

FIGURE 33 Average SFCs/1E6 PBMCs for IL-10 at 48-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations. CT Statuses 0-2 correspond to participants with No Present CT (n = 3), Past medical history of CT (n = 1), and Present CT (n = 4), respectively.

The data lacked a significant main effect for treatment type, $F(3,15) = 1.42$, $p =$ ns, revealing that measured IL-10 secretion was not significantly different across the four treatment conditions. Similarly, the data lacked a significant main effect of *Chlamydia* status, $F(2,5) = .51$, $p =$ ns, illustrating that measured CD4+ T-cell IL-10 secretion did not differ across the three CT statuses (0,1, and 2). Finally, the data lacked a significant interaction between treatment type and *Chlamydia* status, $F(6,15) = .69$, $p =$ ns, revealing that measured CD4+ T-cell IL-10 secretion was not significantly different across the four treatment conditions for participants with different CT statuses. The lack of significant main effects and interactions is illustrated in figure 33.

Average SFCs/1E6 cells for Participants Grouped Into
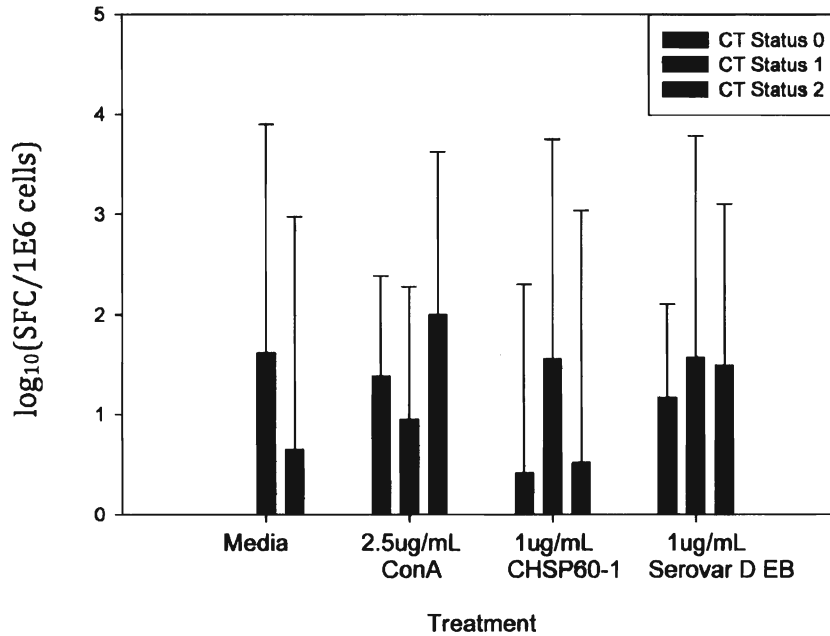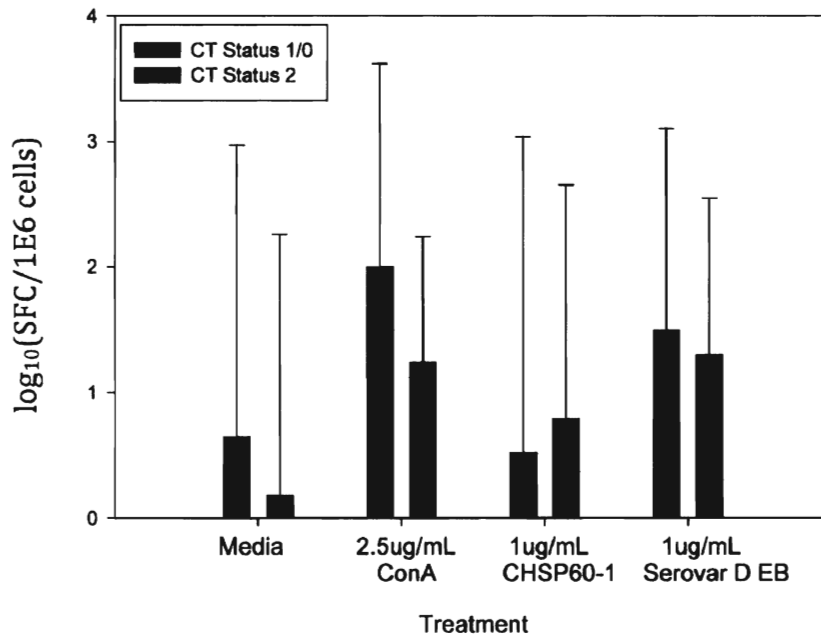Infection Statuses Across Treatment Conditions for IL-10

FIGURE 34 Average SFCs/1E6 PBMCs for IL-10 at 48-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations with CT infection statuses grouped for diagnostic analysis. CT Status 1/0 corresponds to combined average SFCs/1E6 cells for participants with No Present CT and Past medical history of CT (n = 4). CT Status 2 corresponds to participants with Present CT (n = 4).

As predicted, a significant main effect for treatment type, $F(3,18) = 3.80$, $p < .05$, revealed that measured IL-10 secretion was different across the four treatment conditions. This effect is illustrated in figure 34. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (2.42, .78) stimulated CD4+ T-cells to secrete IL-10 greater than the media control (.53, 2.54), $t(7) = -2.31$, $p < .05$. Furthermore, pair-wise comparisons revealed that 1ug/mL of Serovar D EB (2.09, 1.14) stimulated CD4+ T-cell to secrete IL-10 greater than the media control (.53, 2.54), $t(7) = -3.08$, $p < .05$. However, there was no difference in CD4+ T-cell IL-10 secretion between the media control and 1ug/mL of CHSP60-1. The data lacked a significant main effect of *Chlamydia* status, $F(1,6) = .15$, $p = $ ns, illustrating that measured CD4+ T-cell IL-10 secretion did not differ across the two CT statuses (2 and 1/0). Finally, the data lacked a significant interaction between treatment type and *Chlamydia* status, $F(3,18) = .56$, $p = $ ns, revealing that measured CD4+

T-cell IL-10 secretion was not significantly different across the four treatment conditions for participants with different CT statuses.

ROC Analysis of ELISpot Assays with IL-10 When CD4+ T-Cells Are Stimulated with CHSP60-1 (CT Infection Status Grouping)

ROC Curve

FIGURE 35 Exploratory curve analysis to determine the accuracy of ELISpot assays with IL-10 at diagnosing CT infection based upon differences in CD4+ T-cell cytokine secretions of participants in non-infected groups (CT Status 1/0) and currently infected groups (CT Status 2) when CD4+ T-cells are stimulated with 1ug/mL of CHSP60-1. The probability that ELISpot assays with IL-10 detect CT in adolescent women populations correctly is less than chance (AUC = 0.167).

Note. Sensitivity = true positive rate (woman has CT infection and is correctly diagnosed with CT infection) and 1-Specificity = false positive rate (woman does not have CT infection and is incorrectly diagnosed with CT infection). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at cut-off rates representing various numbers of CD4+ T-cells secreting IL-10.

53

ROC Analysis of ELISpot Assays with IL-10 When CD4+ T-Cells Are Stimulated with Serovar D EB (CT Infection Status Grouping)
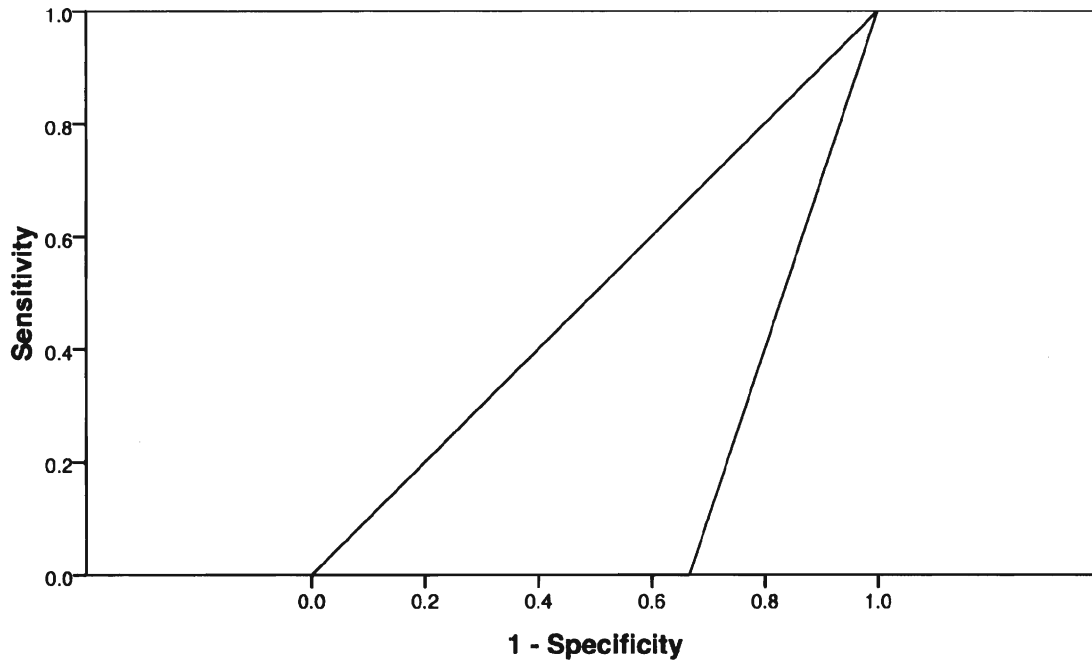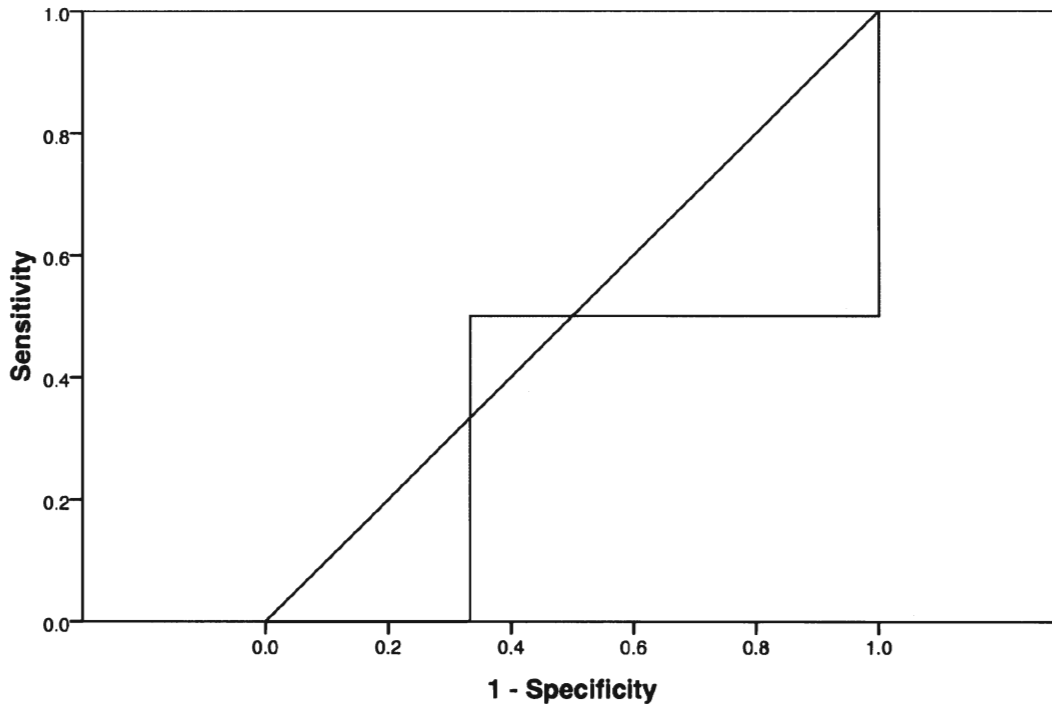
ROC Curve

FIGURE 36 Exploratory curve analysis to determine the accuracy of ELISpot assays with IL-10 at diagnosing CT infection based upon differences in CD4+ T-cell cytokine secretions of participants in non-infected groups (CT Status 1/0) and currently infected groups (CT Status 2) when CD4+ T-cells are stimulated with 1ug/mL of Serovar D EB. The probability that ELISpot assays with IL-10 detect CT in adolescent women populations correctly is less than chance (AUC = 0.333).
Note. Sensitivity = true positive rate (woman has CT infection and is correctly diagnosed with CT infection) and 1-Specificity = false positive rate (woman does not have CT infection and is incorrectly diagnosed with CT infection). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at cut-off rates representing various numbers of CD4+ T-cells secreting IL-10.

**Average SFCs/1E6 cells for Participants with Various
CT Pathology Statuses Across Treatment Conditions for IL-17**

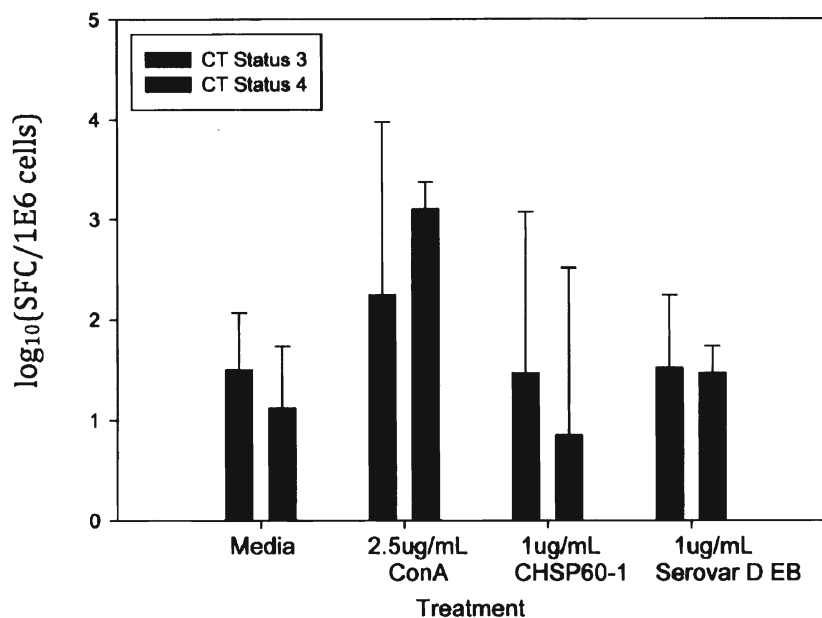**FIGURE 37** Average SFCs/1E6 PBMCs for IL-17 at 48-hour post-stimulation with chlamydial antigens, mitogens, and media controls using an ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations with CT pathology statuses grouped for predictive analysis. CT Status 3 corresponds to combined average SFCs/1E6 for participants with No Present clinical symptoms (discharge/abdominal pain) (n = 5) and CT Status 4 corresponds to participants with Present clinical symptoms (n = 5).

As predicted, a significant main effect for treatment type, $F(3,33) = 4.16, p < .05$, revealed that measured IL-17 secretion was different across the four treatment conditions. This effect is illustrated in figure 37. Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5ug/mL of ConA (2.57, 1.41) stimulated CD4+ T-cells to secrete IL-17 greater than the media control (1.35, .59), $t(12) = -2.37, p < .05$. However, there was no difference in CD4+ T-cell IL-17 secretion between the media control and the chlamydial antigen stimulants, 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB. The data lacked a significant main effect of pathology status, $F(1,11) = .024, p = $ ns, illustrating that measured CD4+ T-cell IL-17 secretion did not differ across the two pathology statuses (3 and 4). Finally, the data lacked a significant interaction between treatment type and pathology status, $F(3,33) = .92, p = $ ns, revealing that measured CD4+ T-cell IL-17 secretion was not significantly different across the four treatment conditions for participants with different pathology statuses.

Average SFCs/1E6 cells Across
Treatment Conditions for IL-17
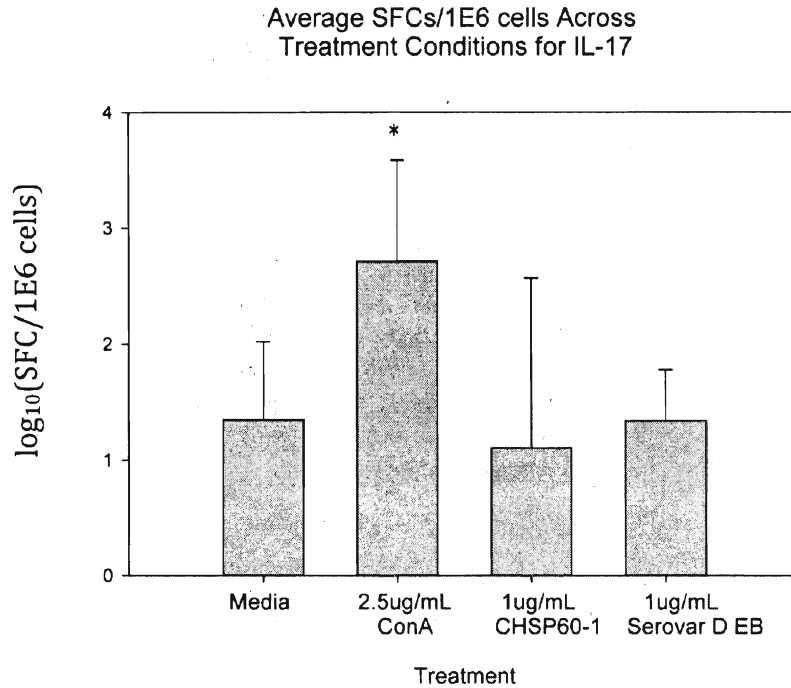
FIGURE 38 Average SFCs/1E6 PBMCs for IL-17 at 48-hour post-stimulation across all participants with chlamydial antigens, mitogens, and media controls using an IL-17 ELISpot assay. Bars represent median SFCs/1E6 cells +/- standard deviations for all participants regardless of CT infection or pathology statuses. ConA stimulated PBMCs of participants to secrete IL-17 more robustly than media controls. Paired samples t-tests versus a media control illustrate a significant treatment effect. * $p < .05$ versus media control.

Follow-up pair-wise comparisons of aggregate data over all participants revealed that 2.5ug/mL of ConA (2.57, 1.41) stimulated CD4+ T-cells to secrete IL-17 greater than the media control (1.35, .59), $t(12) = -2.37$, $p < .05$. Significant comparisons are illustrated in figure 38.

ROC Curve

FIGURE 39 Exploratory ROC analysis to determine the accuracy of ELISpot assays with IL-17 at detecting CT pathology based upon differences in CD4+ T-cell cytokine secretions of participants with No Present clinical symptoms (CT Status 3) and participants with Present clinical symptoms (CT Status 4) when CD4+ T-cells are stimulated with 1ug/mL of CHSP60-1. The probability that ELISpot assays with IL-17 detect CT in adolescent women populations correctly is greater than chance (AUC = 0.625).

Note. Sensitivity = true positive rate (woman has pathology and is correctly predicted as having pathology) and 1-Specificity = false positive rate (woman does not pathology and is incorrectly predicted as having pathology). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at cut-off rates representing various numbers of CD4+ T-cells secreting IL-17.
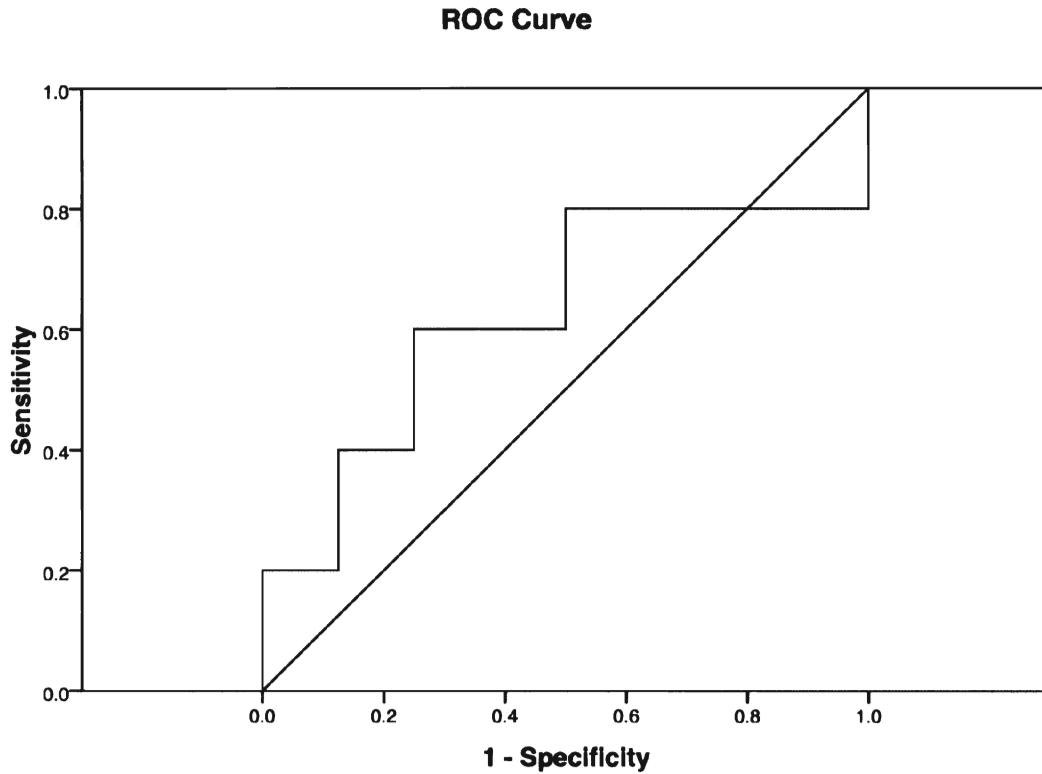
ROC Curve
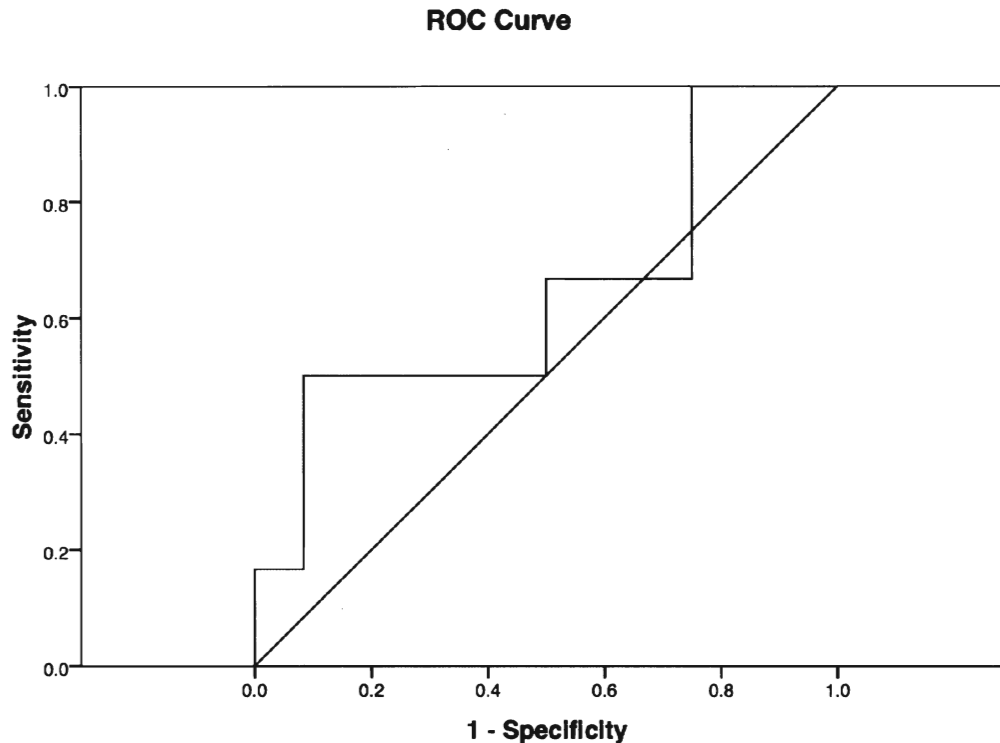
FIGURE 40 Exploratory ROC curve analysis to determine the accuracy of ELISpot assays with IL-17 at detecting CT pathology based upon differences in T-cell cytokine secretions of participants with No Present clinical symptoms (CT Status 3) and participants with Present clinical symptoms (CT Status 4) when CD4+ T-cells are stimulated with 1ug/mL of Serovar D EB. The probability that ELISpot assays with IL-17 detect CT in adolescent women populations correctly is greater than chance (AUC = 0.639).

Note. Sensitivity = true positive rate (woman has pathology and is correctly predicted as having pathology) and 1-Specificity = false positive rate (woman does not pathology and is incorrectly predicted as having pathology). The green line represents the chance line (AUC = 0.5) and the blue line represents the pairs of true positive and false positive rates at cut-off rates representing various numbers of CD4+ T-cells secreting IL-17.

Part One Discussion

3H T-Cell Proliferation Assays: Optimal Antigen Concentrations

The main goal of optimizing the 3H T-cell proliferation assays was to determine optimal antigen concentrations and incubation times for T-cells to proliferate more robustly than media controls. We hoped that optimizing antigen concentrations and incubations would illustrate the usefulness of T-cell proliferations for distinguishing between participants with different CT infection and pathology statuses in Part Two of this study. In the first proliferation assay completed with a mitogen control (ConA) and a mouse chlamydial antigen (Nigg), a One-way RM ANOVA illustrated that there was a significant main effect of treatment type for the seropositive donor (Fig. 10). Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5 and 5ug/mL of ConA stimulated T-cells to proliferate greater than media controls. However, there was no difference in proliferation responses between the media control condition and both the Nigg and the 10ug/mL of ConA treatment conditions. These statistical analyses suggest that 10ug/mL of ConA worked to stimulate T-cells opposite of our original hypothesis. We previously believed that larger concentrations of antigens would stimulate T-cells to proliferate incrementally, such that T-cells stimulated with 10ug/mL of ConA would have the highest counts per minute. However, statistical results imply that concentrations of 10ug/mL or higher of ConA will stimulate T-cells less robustly because T-cells have reached a maximum proliferative state. Therefore, 10ug/mL of ConA was not utilized in future proliferation assays. Furthermore, the low proliferation responses to Nigg suggest that chlamydial mouse antigen should not be used in future proliferation assays because T-cells are unresponsive to Nigg. Despite the similarities between mouse and human *Chlamydia* infection and pathology, statistical results suggest that Nigg does not stimulate T-cells to proliferate greater than media controls.

In the second proliferation assay we wanted to determine if proliferation assays would work with human chlamydial antigens to distinguish between donors with different CT immune statuses. A One-way RM ANOVA revealed that there was a significant main effect of treatment type for both the seropositive and the seronegative donor (Fig. 11). Follow-up pair-wise comparisons between the media control and each of the active treatments revealed that 2.5 and 5ug/mL of ConA stimulated T-cells to proliferate greater than media controls for both the seropositive and the seronegative donor. These results indicate that ConA, our positive control, is working effectively to stimulate all T-cells non-specifically and regardless of CT immune status. Furthermore, pair-wise comparisons revealed that 1 and 2.5ug/mL of Serovar D EB stimulated T-cells to proliferate greater than media controls for the seropositive donor only. These statistical results confirm our hypothesis that T-cells isolated from a seropositive donor will proliferate robustly in response to chlamydial antigen stimulants because the donor has previously built up an immune response to *Chlamydia*. In general, statistical analyses suggest that T-cell proliferation assays are capable of distinguishing between human donors with different immune responses to CT infection. To determine optimal antigen concentrations, we performed one more proliferation assay to test different concentrations of Serovar D EB and another chlamydial antigen, CHSP60-1.

In the final proliferation assay we focused our attention on the seropositive donor because we knew our seronegative donor was successfully unresponsive to our chlamydial antigens. Upon stimulation of T-cells with various concentrations of Serovar D EB, ConA, and 1ug/mL of CHSP60-1, a One-way RM ANOVA revealed that there was a significant main effect of treatment type for the seropositive donor (Fig. 12). Follow-up pair-wise comparisons illustrated that T-cells proliferated greater than media controls when stimulated with all concentrations of chlamydial antigen stimulants, with the exception of 0.5ug/mL of Serovar D EB. Statistical results suggest that either 1 or 2ug/mL of Serovar D EB is optimal for stimulating T-cells to proliferate greater than media controls. Furthermore, results imply that 1ug/mL of CHSP60-1 is also optimal for stimulating T-cells to proliferate greater than media controls. Finally, either 2.5 or 5ug/mL of ConA is optimal for non-specifically stimulating T-cells to proliferate greater than media controls. Based on the statistical results, we decided to aliquot and dilute Serovar D EB and CHSP60-1 to 1ug/mL for Part Two of this study to conserve our limited antigen. Dilutions are consistent with previous research done with female sex workers in Kenya, such that Brunham and colleagues utilized 1ug/mL of Serovar D EB and CHSP60-1 to make successful conclusions about immune responses to CT infection[3]. Finally, 2.5ug/mL of ConA was diluted and aliquoted to conserve the amount of mitogen control. We planned next to further test the optimized concentrations of chlamydial antigens in dual-color ELISpot assays to determine if the same antigen concentrations would work for a different experimental assay method.

Dual-Color ELISpot Assays: Optimal Incubation Times

The main goal of optimizing the dual-color ELISpot assays was to determine if the ELISpot assay with optimized chlamydial antigens would distinguish between seropositive and seronegative donors. Within this optimization experiment, we determined the optimal incubation times for T-cells to secrete cytokines greater than media controls. Thus, we set up two experimental dual-color ELISpot assays with a seropositive and a seronegative donor to analyze 24- and 48-hour incubations. A One-way RM ANOVA with the cytokine secretion data for the seropositive and the seronegative donor post 24-hour T-cell incubation revealed that a 24-hour T-cell incubation was not sufficient for T-cells from a seropositive donor to display chlamydial antigen specific secretion of IFN-γ in response to 1ug/mL of Serovar D EB and 1ug/mL of CHSP60-1 (Fig 13). Follow-up pair-wise comparisons illustrated that 2.5 and 5ug/mL of ConA was the only treatment that stimulated T-cells to secrete IFN-γ greater than media controls for both donors. Although statistical results suggest that our positive controls are working as expected, chlamydial antigen stimulants are not stimulating T-cells to secrete IFN-γ greater than the media control. We hypothesized that chlamydial antigen stimulants would be able to distinguish between seropositive and seronegative donors based upon differences in IFN-γ secretion because of the success of these antigens in the 3H T-cell proliferation assay. However, pair-wise comparisons imply that T-cells need to be incubated longer with chlamydial antigen stimulants. A One-way RM ANOVA with the 48-hour T-cell incubation data uncovered the need for longer T-cell incubations, such that the 48-hour incubation was sufficient for T-cells isolated from a

seropositive donor to display chlamydial antigen-specific secretion of IFN-γ (Fig. 15). Follow-up pair-wise comparisons demonstrated that 1 and 2ug/mL of Serovar D EB and 1ug/mL of CHSP60-1 stimulated T-cells to secrete IFN-γ greater than media controls for the seropositive donor only. Statistical results suggest that 48-hour incubations are sufficient for distinguishing between donors with different CT immune statuses on the basis of IFN-γ secretion because T-cells isolated from a seronegative donor did not secrete IFN-γ greater than media controls. Results confirmed our hypothesis that T-cells isolated from a seropositive donor secrete IFN-γ more robustly than T-cells isolated from a seronegative donor because the seropositive donor has built up an immune response to CT infection. The exploratory summary of the IFN-γ results (Tables 1-2) illustrates the differences in 24- and 48-hour incubations, such that T-cells isolated from the seropositive donor had a greater fold increase of IFN-γ secretion over media controls in the 48-hour incubation.

However, a different result was found with a One-way RM ANOVA of the cytokine secretion data for the seropositive and the seronegative donor in the IL-10 ELISpot assay. The statistical analysis illustrated that there were no significant differences in T-cell IL-10 secretion for the seropositive and the seronegative donor across treatment conditions in the 24- and 48-hour incubations (Figs. 14 & 16). Statistical analyses countered our initial hypothesis that T-cells stimulated with chlamydial antigens would display chlamydial antigen-specific secretion of IL-10 for the seropositive donor only because the seropositive donor has an immune response that is responsive to chlamydial antigens. However, results imply that there are some methodological problems with the IL-10 ELISpot assay because our positive control, ConA, did not non-specifically stimulate all T-cells to secrete IL-10 over media controls. High IL-10 responses were observed for all treatment conditions, which suggests that chlamydial antigens are not stimulating T-cells to secrete IL-10 relative to media controls. The exploratory summary of the ELISpot data of all cytokine secretion for IL-10 over media controls (Tables 3-4) demonstrates that neither the 24- nor the 48-hour incubation are stimulating T-cells to secrete IL-10 greater than media controls regardless of donor status.

Because we established in the IFN-γ ELISpot that our chlamydial antigens were working to stimulate T-cells above media controls, we determined that IL-10 cytokine secretions must be representative of non-specific T-cell cytokine secretion. The high numbers of SFCs/1E6 cells reported for each treatment and media control condition suggest that cytokine secretion is not a product of CD4+ T-cell cytokine secretion alone. Within the PBMC population there are T-cell populations consisting of both CD4+ and CD8+ T-cells. CD8+ T-cells represent an aspect of the human immune response that is not dominant in *Chlamydia* infection. Thus, the large non-specific cytokine secretion for the IL-10 ELISpot assay implies that chlamydial antigens are stimulating all T-cells to non-specifically secrete IL-10 similar to the positive mitogen control (ConA). Although this pattern is found only in the IL-10 ELISpot, we decided to deplete CD4+ T-cells of CD8+ T-cell populations prior to both ELISpot and T-cell proliferation assays in Part Two. We hypothesized that CD8+ T-cell depletion would help us make specific conclusions about CD4+ T-cell immune responses to CT infection. Despite the controversial results found

61

in the IL-10 ELISpot assay, we determined that 48-hour incubations would be optimal for CD4+ T-cells to elicit specific cytokine secretion in response to chlamydial antigens.

Part Two Discussion

Alamar-Blue T-Cell Proliferation Assays: Testing Our Methodology

The first thing to determine in our subset of adolescent participants was if the alamar-blue T-cell proliferation assay methods and chlamydial antigen stimulants were working as predicted in Part One of this study. Although we are unable to make direct comparisons to previous T-cell proliferations completed because we utilized a different 3H T-Cell proliferation in Part One, we would expect to see similar results because the idea behind the T-cell methodologies is the same. Both 3H and alamar-blue T-cell proliferation assays quantify proliferation by larger values, more counts per minute for 3H proliferation assays and more percent reduced for alamar-blue proliferation assays. Thus, we would expect that when participants are organized into a present or past infected group (CT Status 1/2) and a never infected group (CT Status 0), the CT Status 1/2 group would proliferate more robustly in response to chlamydial antigens in concordance with the proliferation responses found with the seropositive donor in Part One. However, a Two-way RM ANOVA illustrated that there were no significant differences in CD4+ T-cell proliferation for the two CT status groups (Fig. 17). These statistical results suggest that the alamar-blue T-cell proliferation assays are not working as predicted in the optimized T-cell proliferation assays in Part One. A paired-samples t-test supports the notion that positive mitogen controls and chlamydial-antigen stimulants are working as expected because ConA and Serovar D EB stimulated CD4+ T-cells greater than media controls (Fig. 18). Although a pair-wise analysis of each treatment condition versus the media control illustrated that CHSP60-1 was not stimulating cells to proliferate greater than media controls, we are not surprised by this finding. In his study with Kenyan female sex workers, Cohen and colleagues determined that CHSP60-1 does not stimulate CD4+ T-cells to proliferate as robustly as Serovar D EB[4].

Alamar-Blue Proliferation Assays: Diagnostic Analysis

In order to determine the diagnostic capabilities of alamar-blue T-cell proliferation assays, it was useful to organize the participant data meaningfully into groups. Although the participants in our population are diverse in terms of their sexual and gynecological histories, there were enough women in the study population for us to group the participants into two groups. One group consisted of women who have no present CT infection or who have had a past medical history of CT infection (CT Status 0/1). The second group consisted of women who are currently infected (CT Status 2). We would expect that if T-cell proliferation assays were as capable as current PCR methods at detecting a current infection, statistical analyses would illustrate that CD4+ T-cell proliferation in response to chlamydial antigens is greater for the group of women who are currently infected (CT Status 2). However, statistical results revealed that there were no significant differences in CD4+ T-cell proliferation between participants who are presently infected and who are not presently infected (Fig. 19). For exploratory purposes we completed an ROC analysis for the data to look at the current diagnostic accuracy of

T-cell proliferations. These results mirrored Two-way ANOVA conclusions. The AUC value of the ROC curve for the alamar-blue proliferation assays when CD4+ T-cells were stimulated with 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB was 0.323 and 0.400 respectively (Figs. 20-21). AUC values of 0.323 and 0.400 indicate that when a randomly selected pair of currently infected and non-infected participants is drawn from the population, the alamar-blue T-cell proliferation assay will predict the status of these participants less often than a random prediction. In conclusion, we can state that alamar-blue T-cell proliferations, as measured in the current analyses, cannot be used to diagnose participants for CT as accurately as PCR diagnosing methods with a sensitivity value of 94.2%. Thus, if experimental methods can contribute a significant predictive benefit to routine gynecological clinic visits, PCR methods must still be used to accurately diagnose patients for CT infection.

Alamar-Blue T-Cell Proliferation Assays: Pathology Analysis

To explore the use of alamar-blue T-cell proliferations to detect and predict pathology, it was necessary to group participants into different groups prior to statistical analyses. Our population includes adolescent women who do or do not present with baseline clinical symptoms such as abdominal pain or vaginal discharge. For the purposes of analyzing pathology detection capabilities, we grouped participants into a CT pathology status with current clinical symptoms (CT Status 4) and a second group with no clinical symptoms (CT Status 3). We predicted that there would be significant differences between these two groups with respect to CD4+ T-cell proliferation, provided that T-cell proliferation assays can detect pathology. However, a Two-way RM ANOVA illustrated that there were no significant differences in CD4+ T-cell proliferation between CT pathology status groups (Fig. 22). Although these results suggest that T-cell proliferation assays have little capabilities as pathology detectors, we followed these analyses with exploratory ROC analysis.

The AUC value of the ROC curve for the alamar-blue proliferation assays when CD4+ T-cells are stimulated with both 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB was 0.639 (Figs. 23-24). An AUC value of 0.639 indicates that when a randomly selected pair of participants with and without current pathology is drawn from the population, the alamar-blue T-cell proliferation assay will predict pathology status correctly 63.9% of the time. Tables 8 and 9 below show the expected true positives (participants with pathology who would be predicted to have pathology) and false positives (participants with no pathology who would be predicted incorrectly to have pathology), based upon specific cut-off values representing percentage reduction values for CHSP60-1 and Serovar D EB, respectively. As one example, if 92.41 and 102.86 were chosen as the cut-off values for detecting pathology in a woman with current pathology using CHSP60-1 and Serovar D EB respectively, 16.7% of the women with current pathology will be correctly detected as having pathology and 0% of the women with no current pathology will be incorrectly detected as having pathology. As more liberal cut-off values are chosen for both chlamydial antigen stimulants, the accuracy of the alamar-blue T-cell proliferation at predicting pathology decreases because more woman are predicted as having pathology correctly at the price of more women being incorrectly predicted as having pathology.

Table 8 ROC curve coordinates for alamar-blue T-cell proliferation assays with CHSP60-1.

Positive Percentage Reduction Value	True Positive Rate	False Positive Rate
15.70	100%	100%
34.97	83.3%	66.7%
42.48	66.7%	50%
75.09	50%	16.7%
79.03	33.3%	16.7%
92.41	16.7%	0%
98.10	0%	0%

Table 9 ROC curve coordinates for alamar-blue T-cell proliferation assays with Serovar D EB.

Positive Percentage Reduction Value	True Positive Rate	False Positive Rate
12.50	100%	100%
34.94	83.3%	75%
47.50	66.7%	50%
73.55	50%	8.3%
88.50	33.3%	8.3%
102.86	16.7%	0%
106.75	0%	0%

Based on the analyses above, we failed to find support for the hypothesis that T-cell proliferations would predict pathology in participants with and without baseline clinical symptoms. Although there were no statistically significant differences between groups with pathology and no pathology, exploratory ROC analysis suggested that, with a larger sample size, alamar-blue T-cell proliferation assays could be a useful tool in addition to standardized PCR methods in the adolescent clinic. If alamar-blue proliferation T-cell assays demonstrate differences in CD4+ T-cell proliferation responses among participants with pathological differences, IL-17 ELISpot assays could then confirm these differences and provide a reason (increased IL-17 cytokine secretion) for differences in pathology among participants.

ELISpot Assays: Testing Our Methodology

The first thing to determine in our small subset of adolescent participants was if the ELISpot assay methods and chlamydial antigen stimulants were working as predicted in Part One of this study. In order to compare the results of Part Two to the optimized assays in Part One of this study, we first had to organize the participant data meaningfully. In Part One of this study we found that T-cells isolated from seropositive donors, who have built up an immune response to CT, respond more robustly to chlamydial antigen stimulants than seronegative donors who have never been exposed to the pathogen. Based on these results, we first organized the data into two groups, such

that one group consisted of participants who were presently infected with CT or who had a past medical history with CT (CT Status 1/2), and the second group consisted only of participants who have never been infected (CT Status 0). If IFN-γ and IL-10 ELISpot assays are working correctly, there should be significant differences in cytokine secretion between these groups. In concordance with Part One results, we hypothesized that participants who are presently infected or who have a past medical history with CT would have more specific cytokine secretion of IFN-γ than participants who have never been infected with CT. Given that CD4+ T-cells have been depleted of CD8+ T-cells, we predicted that participants in the CT Status 1/2 group would also have more specific cytokine secretion of IL-10 than participants who have never been infected with CT. However, statistical analyses revealed that there were no significant differences in cytokine secretion among CT infection status groups for both IFN-γ and IL-10 (Figs. 25 & 31). The results indicate that IFN-γ and IL-10 ELISpot assays are not working as predicted in the optimized IFN-γ and IL-10 ELISpot assays in Part One. There is evidence that neither the methods nor the chlamydial-antigen stimulants are working as expected in the optimized ELISpot assays in Part One. Pair-wise comparisons of each treatment condition compared to the media control showed that the positive mitogen control, ConA, was the only treatment condition that differed significantly from media controls across the entire subset of participants for both IFN-γ and IL-10 (Figs. 26 & 32). Although we would expect CD4+ T-cells stimulated with ConA to secrete more IFN-γ and IL-10 than T-cells stimulated with media controls regardless of infection status (ConA serves as a positive control), we also expected to see that chlamydial antigen stimulants (CHSP60-1 and Serovar D EB) stimulated CD4+ T-cells to secrete IFN-γ and IL-10 significantly greater than CD4+ T-cells stimulated with media controls. Although there is evidence that ELISpot assays did not distinguish between participants with different CT statuses, it was important to determine if the ELISpot assays have anything meaningful to contribute to standardized PCR diagnosing methods.

ELISpot Assays: Predictive Analysis

For the predictive analyses, we were interested in determining the extent to which ELISpot assays could describe the current immune status of patients, and thus potentially predict the future immune status for patients at high risk for *Chlamydia* infection. We grouped participants in a way such that their group associations theoretically would predict differences in cytokine secretion responses to chlamydial antigens. We thus found it helpful to first analyze the participant ELISpot responses in terms of three different groups which seemed to pervade significantly in our subset population: no present/no past medical history of CT infection (CT Status 0), past medical history of CT infection (CT Status 1), and present CT infection (CT Status 2). In terms of immunity predictions, we hypothesized that currently infected participants would have higher and more specific secretion of IL-10 than IFN-γ in response to stimulation with chlamydial antigens. This prediction is based on Cohen et al.'s previous research with Kenyan female sex workers, which illustrates that women who are unprotected against incident CT infection have a CD4+ T-cell response polarized towards a Th2 rather than a Th1 response[4]. Thus, participants who are currently infected should theoretically have CD4+ T-cells dominated by Th2 immune responses because they were not protected upon infection with present CT. On the other hand, participants who had CT infection in the

65

past and have resolved infection successfully at their initial baseline visit at the AMC should have more specific secretion of IFN-γ than IL-10 because they have been protected from the CT pathogen. Finally, we predicted that participants who have never had CT infection, but whose unsafe sexual practices put them at high risk for infection, would be more likely to have a specific secretion of IFN-γ than IL-10 because they have been protected. In comparison to participants with past history with CT infection, we expected that participants who have never been infected with CT would also have a specific Th1 immune response upon stimulation with chlamydial antigens. However, we also expected that there would be significant differences between these two CT status groups, such that participants with a past history will have higher specific secretion of IFN-γ because they have seen the CT pathogen previously and thus have an immune system which responds more robustly upon stimulation with chlamydial antigen stimulants.

Contrary to expectation, our analyses did not find differences in CT status groups across treatment conditions (Figs. 27 & 33). Paired samples t-tests completed for IFN-γ and IL-10 ELISpot assays (described above) suggested that chlamydial antigens did not stimulate CD4+ T-cells to secrete IFN-γ or IL-10 greater than media controls. Thus, we can infer from these results that ELISpot assays used in a small subset of adolescent women at high risk for CT infection were not sensitive enough to detect differences in immune responses to CT infection as we would expect with participants with different infection statuses. Furthermore, we failed to find that chlamydial antigens were working in an ELISpot assay to stimulate CD4+ T-cells to secrete IFN-γ and IL-10 significantly over media controls as they have done previously with a seropositive donor in Part One of this study. Thus, based on these preliminary results we cannot state that ELISpot assays tell us any significant information about immune responses to CT infection and thus should not be standardized in adolescent clinics with current PCR diagnosing methods.

ELISpot Assays: Diagnostic Analysis

In order to determine the diagnostic capabilities of ELISpot assays, it was useful for us to organize the participant cytokine secretion responses in a different way prior to statistical analyses. To determine the accuracy of ELISpot assays as diagnostic tools in comparison to current PCR diagnosis tools, we organized participant data into two groups. The first group represented all cytokine secretion responses for participants who are either not infected or were infected only in the past (CT Status 0/1), and the second grouped represented responses for participants who are presently infected (CT Status 2). If the ELISpot assays represent an accurate means for diagnosing, cytokine secretion responses should be significantly different between the two CT status infection groups. However, we failed to find significant differences in cytokine secretion responses for participants who are presently infected and who are not presently infected both IFN-γ and IL-10 (Figs. 28 & 34). For exploratory purposes, we completed an ROC analysis. The results mirrored the lack of significance in the statistical test of variance. The AUC value of the ROC curve for the IFN-γ ELISpot assay when CD4+ T-cells were stimulated with both 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB is 0.452 (Figs. 29-30 & 35-36). An AUC value of 0.452 indicates that when a randomly selected pair of currently infected

and non-infected participants is drawn from the population, the IFN-γ ELISpot assay will classify them correctly 45.2% of the time. Chance performance would classify a randomly selected pair correctly 50% of the time, and therefore, the AUC value suggests that the ELISpot assay diagnoses CT more poorly than chance. The AUC values of the ROC curves for the IL-10 ELISpot assay when CD4+ T-cells are stimulated with 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB are 0.167 and 0.333, respectively. AUC values of 0.167 and 0.333 indicate that when a randomly selected pair of currently infected and non-infected participants is drawn from the population, the IL-10 ELISpot assay will predict the status of the participants correctly 16.7% of the time with CHSP60-1 and 33.3% of the time with Serovar D EB. Thus, the IL-10 ELISpot assay predicts the status of the participants more poorly than chance when using both chlamydial antigen stimulants.

Based on the analyses for the IFN-γ and IL-10 ELISpot assays, there is no evidence to suggest that ELISpot assays can diagnose CT as accurately as current PCR methods. Although, comparisons of AUC values for IFN-γ and IL-10 illustrate that if IFN-γ and IL-10 ELISpot assays were successfully able to distinguish between participants with different CT infection statuses, IFN-γ ELISpot assays would be better at detecting a positive result (CT infection). Therefore, if experimental methods are able to contribute a significant predictive benefit to routine gynecological clinic visits, PCR methods must still be used to accurately diagnose patients for CT infection.

ELISpot Assays: Pathology Analysis

To explore the capabilities of ELISpot assays in predicting current pathology in the form of baseline visit clinical symptoms of discharge and/or abdominal pain, we had to once again organize the participant data into two different CT pathology groups. One group consisted of all participants who do not have present clinical symptoms of discharge or abdominal pain (CT Status 3), and another group consisted of all participants who do have present clinical symptoms at baseline visits (CT Status 4). Based upon Bettelli and colleagues' current studies with Th17 cells[1], we hypothesized that participants with current pathology will have more specific IL-17 secretion than participants with no current clinical symptoms. Moreover, previous research suggests that positive IL-17 should predict worse pathology[1]. Analyses failed to reveal significant differences in IL-17 secretion for participants with different CT pathology statuses across various treatment conditions (Fig. 37). Exploratory follow-up analyses also failed to show that chlamydial antigens stimulated CD4+ T-cells to secrete IL-17 more than media controls for the entire study population (Fig. 38). Although pair-wise comparisons versus media controls verified that our positive mitogen control is stimulating CD4+ T-cells greater than media controls, the chlamydial antigens did not stimulate CD4+ T-cells to secrete IL-17 in our IL-17 ELISpot assay. Despite the lack of significance in the data, an exploratory ROC analysis was completed to explore the potential for IL-17 ELISpot assays to predict or detect pathology in participants.

The AUC values of the ROC curves for the IL-17 ELISpot assay when CD4+ T-cells are stimulated with 1ug/mL of CHSP60-1 and 1ug/mL of Serovar D EB are 0.625 and 0.639, respectively (Figs. 39-40). AUC values of 0.625 and 0.639 indicate that when a

randomly selected pair of women with current pathology and with no current pathology is drawn from the population, the IL-17 ELISpot assay will predict the status of the participants 62.5% and 63.9% of the time when CD4+ T-cells are stimulated with CHSP60-1 and Serovar D EB, respectively. Tables 10 and 11 below show the expected true positives (participants with pathology who would be predicted to have pathology) and false positives (participants with no pathology who would be predicted incorrectly to have pathology), based upon specific cut-off values the number of CD4+ T-cells secreting IL-17 for CHSP60-1 and Serovar D EB, respectively. As one example, if 92.41 and 102.86 were chosen as the cut-off values for detecting pathology in a woman with current pathology using CHSP60-1 and Serovar D EB respectively, 16.7% of the women with current pathology will be correctly detected as having pathology and 0% of the women with no current pathology will be incorrectly detected as having pathology. As more liberal cut-off values are chosen for both chlamydial antigen stimulants, the accuracy of IL-17 ELISpot assays at predicting pathology decreases because more woman are predicted as having pathology correctly at the price of more women being incorrectly predicted as having pathology.

Table 10 ROC curve coordinates for IL-17 ELISpot Assay with CHSP60-1.

Positive # of CD4+ T-cells Secreting IL-17	True Positive Rate	False Positive Rate
15.69	100%	100%
34.97	83.3%	66.7%
42.48	68.7%	50%
75.09	50%	16.7%
79.03	33.3%	16.7%
92.41	16.7%	0%
98.10	0%	0%

Table 11 ROC curve coordinates for IL-17 ELISpot Assay with Serovar D EB.

Positive # of CD4+ T-cells Secreting IL-17	True Positive Rate	False Positive Rate
11.50	100%	100%
34.90	83.3%	75%
45.50	66.7%	50%
72.55	50%	16.7%
85.50	33.3%	8.3%
103.86	16.7%	0%
105.75	0%	0%

Based on the analyses above, we failed to find support for the hypothesis that IL-17 ELISpot assays would predict pathology in participants with and without baseline clinical symptoms. Although there were no statistically significant differences in CD4+ T-cell

IL-17 secretion between groups with pathology and no pathology, exploratory ROC analysis suggested that, with a larger sample size, IL-17 ELISpot assays could be a useful tool in addition to standardized PCR methods in the adolescent clinic. If the IL-17 ELISpot assay can accurately detect pathology based on Th17 immune responses to CT infection, we may be able to shape treatment plans for patients that cater more specifically to prevention of pathology. These exploratory results are in accordance with exploratory ROC results found with alamar-blue T-cell proliferation assays.

Possible Explanations for Contradictory Results

Perhaps the most startling trend found in Part Two of this study was the lack of a significant association between alamar-blue T-cell proliferation and ELISpot assays and *Chlamydia* and pathology statuses. Upon analyzing the results for trends similar to the ones found in Part One of this study, we were surprised to find that most results did not satisfy our initial hypotheses. There is evidence across multiple analyses to suggest that our methods and chlamydial-antigen stimulants are not working as expected in Part One of this study. Furthermore, exploratory ROC analysis suggests the existence of significant interactions within the data, which are shadowed by the sample size of the participant population. Perhaps a final factor to note is the complexity of participants in the study who may need to be more specifically organized in order to yield significant differences in immune responses and pathology.

Instrumental Errors

In order to test our experimental assay methodology, participant data was organized similar to Part One of this study. Participants with a past medical history of CT infection and present CT infection were organized to represent the seropositive donor group in Part One, and participants with no present or past medical history of CT infection were organized to represent the seronegative donor group. Statistical results from the Two-way RM ANOVAs completed for both the alamar-blue T-cell proliferation assay and the IFN-γ/IL-10 ELISpot assays illustrated that the experimental assay methods were not working as expected. A possible reason for these inconsistencies is instrumental errors noted for both experimental assays. For IFN-γ and IL-10 ELISpot assays, a particularly inconsistent aspect of the assay method was the plate-washing ELISpot machine used for the first time in Part Two of this study. In Part One of this study, all plates were hand-washed because time was not a significant factor. However, due to the influx of patient samples needing to be analyzed together, a plate-washing machine seemed logical for expediting the ELISpot assay process. However, the variance in the data suggests that across wells in triplicate for each treatment condition, cytokine secretion responses were not consistent. In the graphical representations of the ELISpot data, one can observe large error bars for each treatment condition which suggest poor consistency across measurement occasions. Although log transformations of the data were used to reduce these variability problems (and centralize the data), considerable variability in CD4+ T-cell secretion responses remained. There is evidence to assume that this variability is due to the inconsistent washing of the ELISpot plates because, upon use of the plate washer with a test plate prior to completion of samples, the plate washer appeared to fill wells with ELISpot wash buffer in inconsistent amounts across wells in triplicate. Wells that

are inconsistently washed can significantly affect the SFCs/1E6 cells recorded by the ELISpot reader because stimulants and capture antibodies inconsistently washed will continue to stimulate and capture cytokine secreting cells. Thus, some wells will be counted for a 48-hour incubation while other wells will be counted for an incubation unknown based upon how much stimulants continue to stimulate cells to secrete cytokines.

In terms of the alamar-blue T-cell proliferation assays, a potential instrumental error was the inconsistent absorbance readings of the SPECTRA Max M_2 ROM plate reader. As is outlined in the manufacturer instructions, percent reductions are calculated in an equation that takes into account the absorbencies of blank wells. Blank wells are experimental wells without treatment conditions, which are read at 570nm and 600nm. Upon reading these blank wells, it appeared that despite our confidence that wells were not incubated with treatments, the SPECTRA Max M_2 ROM plate reader was still detecting redox reactions in these wells. Although we were able to account for these inconsistencies in the SPECTRA Max M_2 ROM program, the existence of these results gives us cause to question the readings determined by this plate reader. Upon noting this trend in several samples in the participant population, laboratory technicians were called to explore these inconsistencies in detail as they are still unknown to us.

Chlamydial-Antigen Stimulants

In addition to evidence suggesting instrumental errors, paired-samples t-tests for both T-cell proliferation and ELISpot assays suggested that chlamydial antigen stimulants were not stimulating T-cells to proliferate or to release cytokines. These results were unexpected because our chlamydial-antigen stimulants stimulated T-cells to proliferate and release cytokines more robustly than media controls in Part One of this study. A possible explanation for this inconsistent trend is that optimized chlamydial-antigen stimulants in Part One of this study were stimulating cells significantly because T-cell populations consisted of more than just CD4+ T-cells. A T-cell population that consists of more T-cells to stimulate could result in proliferation and cytokine secretion greater than media controls. However, these assay results could be significantly greater than media controls merely because enough T-cells are non-specifically being stimulated greater than media. The depletion of CD8+ T-cells in Part Two suggests that we may need to increase the concentration of chlamydial-antigen stimulants because we now need larger concentrations to stimulate smaller populations of CD4+ T-cells. Thus, non-significant statistical tests may be due to the possibility that the smaller concentrations of chlamydial-antigen stimulants were not stimulating T-cells to proliferate or secrete cytokines robustly.

Complexity of Participant Histories

Upon analysis of the participants' sexual and gynecological histories, it appeared that participants had complex histories that could act as a confounding variable in Part Two of this study. For example, some participants had history of other STIs or have had multiple sexual partners with histories of STIs including CT. Variations in gynecological histories could be a confounding variable because participants' immune responses may respond

differently to chlamydial-antigens because their immune system has built up similar inflammatory responses to other STIs. Furthermore, participants who have had a greater number of sexual partners may respond more robustly to chlamydial antigen stimulants because they may have encountered the pathogen more often. Given our small sample, we lacked the power to control for these relevant differences in gynecological history. Our sample size limited us to organize patients into no CT infection, past medical history of CT infection, and present CT infection, which may not fully account for the complexity of the participant immune responses and pathology. Perhaps a larger sample size would yield more patients with similar complexities, such that multiple Two-way RM ANOVAs could be run to test for interactions between each complex CT status and the treatment conditions. Thus, lack of significance of results could potentially be due to the fact that proliferation and cytokine secretion responses are not being organized in ways to elicit meaningful differences in immune responses. Grouping participants into more general statuses that fail to account for the complexity of sexual and gynecological histories could make experimental assay responses look similar across conditions because underlying immune system and pathology differences are not being accounted for appropriately.

Sample Size Considerations

As is highlighted in the discussion of complex participant histories, sample size represents a large hindrance. For the larger goals of the CARE study, we hope to collect T-cell proliferation and ELISpot assay data for approximately 400 adolescent women. For the purposes of this preliminary result project, only 18 and 13 participants were included in the analysis of the T-cell proliferation assay and ELISpot assay respectively. Due to the time limitations of this project, only a small subset of the larger sample population was analyzed over the first 3 months of this CARE study. In addition to time limitations, participant data was excluded prior to analysis because participants either dropped out of the study or were asked not to continue due to pregnancy. Additionally, pronounced instrumental errors required additional data exclusions. With all of these interferences encountered prior to data analysis, the final sample size may have been underpowered to detect the effects we predicted in the T-cell proliferation and ELISpot assays. Some exploratory analysis suggested that proliferation and ELISpot assays may have the potential to predict differences in CT pathology. AUC values greater than 0.5 (greater than chance), suggest that a larger sample size may yield differences in participants with different CT pathology statuses. We hope to gather more data in the future to discover if experimental assays have the ability to provide information about the immune responses to CT infection among adolescent participants with different CT infection histories and current statuses.

Future Directions

Methodological Directions

Prior to the laboratory analysis of any new patients, we plan on making some methodological changes with respect to the alamar-blue T-cell proliferation and ELISpot assays. We would first like to resolve alamar-blue proliferation assay problems found

with the SPECTRA Max M_2 ROM incorrectly reading blank wells as treatment wells. We hope that we can detect the error with the machine and then fix it for future analyses. For ELISpot assays, we would like to run IFN-γ, IL-10, and IL-17 experimental ELISpot assays with seropositive and seronegative donors to optimize chlamydial antigen stimulants with CD8+-depleted T-cells. We found in Part Two of the study that chlamydial antigens were not stimulating T-cells, which could directly be a product of insufficient antigen concentrations for stimulation of CD8+-depleted CD4+ T-cell populations. We hope that experimental assays with seropositive and seronegative donors will allow us to determine optimal antigen concentrations for the stimulation of CD4+ T-cells specifically.

Participant Analysis Directions

A large limitation in this study was the complexity of participant sexual and gynecological histories, which could significantly influence CD4+ T-cell immune responses to *Chlamydia* infection. Upon looking at the participants in our small population, an interesting pattern emerged with regards to participant histories with *Chlamydia* infection. The following four CT statuses existed among the 22 participants in the study population: never infected with CT, past history with CT infection/present CT infection, past history with CT infection/no present CT infection, and present CT infection/no past history with CT infection. The number of participants with these different CT statuses was not large enough for us to organize CD4+ T-cell proliferation and cytokine secretion responses into these four CT statuses. In the future, we would like to organize participants into the four CT statuses that emerged in our small subset of adolescent participants. We hypothesize that organizing participants in these CT statuses will allow us to see more subtle differences in CD4+ T-cell proliferation and cytokine secretion.

Laboratory Directions

Upon review of previous research done by T. Agrawal and colleagues with female patients at a gynecological clinic in India[12], we have found that cervical cells also contain CD4+ T-cell populations similar to T-cell populations in PBMCs. The researchers found that cervical swab specimens collected from patients yielded cervical cells with CD4+ T-cell populations that secreted cytokines characteristic of both Th1 and Th2 responses in response to chlamydial antigen stimulants[12]. Agrawal and colleagues also found that cervical cells with more specific secretion of IFN-γ protected women against incident CT infection[12]. Contrastingly, they discovered that cervical cells with more specific secretion of IL-10 inhibited protective immune responses[12]. Thus, we want to test these previous findings by retrieving cervical cells isolated from cervical swabs that have been collected from adolescent women at clinic visits throughout the study. We have stored these cervical swabs at -80°C after each participant visit, and thus we plan to collect cervical cells from these swabs and analyze CD4+ T-cell populations with newly optimized alamar-blue T-cell proliferation and ELISpot assays. We hope that CD4+ T-cells isolated from cervical cells will demonstrate immunology and pathology differences among participants that we had hoped to find with CD4+ T-cells isolated from PBMCs.

Statistical Analysis Directions

The main hope for the future of the adolescent CARE study is that a larger sample size will allow us to organize data more meaningfully. In terms of statistical analyses, we plan to continue to run Two-way RM ANOVAs and ROC analyses with the data. However, in the future we would like to conduct a hazard ratio analysis in SPSS with the T-cell proliferation and ELISpot assay data collected for baseline and follow-up visits spanning over a 9-month period. Similar to Cohen and colleagues hazard ratio results (Fig. 5)[4], we hope to find that CD4+ T-cell proliferation and cytokine secretion responses will be able to predict the proportion of patients who have been uninfected with *C. trachomatis*. We hope that organizing the data in this fashion will allow us to determine the true role of IFN-γ, IL-10, and IL-17 in the protective immunity and disease pathogenesis associated with *C. trachomatis* genital tract infection.

Works Cited

1. Bettelli, Korn E, M Oukka, and VK Kuchroo. "Induction and effector functions of TH17 cells." *Nature* 453 (2008): 1051-57.

2. Brunham RC, Pourbohloul B, Mak S, White R, Rekart ML. The Unexpected Impact of a Chlamydia trachomatis Infection Control Program on Susceptibility to Reinfection. J Infect Dis 200b5; 192(10):1836-1844.

3. Brunham RC, Rey-Ladino J. Immunology of Chlamydia infection: implications for a Chlamydia trachomatis vaccine. Nat Rev Immunol 2005a; 5(2):149-161.

4. Cohen, CR, KM Koochesfahani, AS Meier, C Shen, K Karunakaran, B Ondondo, T Kinyari, NR Mugo, R Nguti, and RC Brunham. "Immunoepidemiologic profile of Chlamydia trachomatis infection: importance of heat-shock protein 60 and IFN-γ." *J Infect Dis* 192 (2005): 591-599.

5. Darville, Toni. "Chlamydia SPP." *Persistent Bacterial Infections*. Washington, DC : ASM Press, 2000. 229-261.

6. Geneva: WHO. World Health Organization (WHO). Global prevalence and incidence of selected curable sexually transmitted infections: overview and estimates. 2001.

7. Hoplland MJ, Bailey RL, Hayes LJ, Whittle HC, Mabey DC. Conjunctival scarring in trachoma is associated with depressed cell- mediated immune responses to chlamydial antigens. J Infect Dis 1993; 168(6):1528-1531.

8. Parham, Peter. "T-cell-Mediated Immunity." *The Immune System*. 2 ed. New York and London: Garland Science, 2005. 145-178. Print.

9. Patton DL, Kuo CC. Histopathology of Chlamydia trachomatis salpingitis after primary and repeated reinfections in the monkey subcutaneous pocket model. J Reprod Fertil 1989; 85(2):647-656.

10. Peeling, Rosanna W., Mabey, David, Herring, Alan, Hook, Edward W. 3 ed. *Nature Reviews Microbiology* 4, 909-921 (December 2006)

11. "STD Facts - Chlamydia." *Centers for Disease Control and Prevention*. N.p., 20 Dec. 2007. Web. 22 Apr. 2010. <http://www.cdc.gov/std/chlamydia/stdfact-chlamydia.htm#Common>.

12. T. Agrawal, et al., Protective or pathogenic immune response to genital chlamydial infection in women—A possible role of cytokine secretion profile of cervical mucosal cells, Clin. Immunol. (2008).