The Predominant CD4+ Th1 Cytokine Elicited to Chlamydia trachomatis Infection in Women is Tumor Necrosis Factor-Alpha, not Interferon-Gamma


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**ABSTRACT**

*Chlamydia trachomatis* infection is the most prevalent bacterial sexually transmitted infection and can cause significant reproductive morbidity in women. There is insufficient knowledge of *C. trachomatis*-specific immune responses in humans, which could be important in guiding vaccine development efforts. In contrast, murine models have clearly demonstrated the essential role of Type 1 T helper cells (Th1), especially interferon-gamma (IFN-γ) producing CD4+ T-cells, in protective immunity to chlamydia.

To determine the frequency and magnitude of Th1 cytokine responses elicited to *C. trachomatis* infection in humans, we stimulated peripheral blood mononuclear cells from 90 chlamydia-infected women with *C. trachomatis* elementary bodies, Pgp3, and major outer membrane protein and measured IFN-γ, tumor necrosis factor-alpha (TNF-α)-, and interleukin 2 (IL-2)-producing CD4+ and CD8+ T-cell responses using intracellular cytokine staining. The majority of chlamydia-infected women elicited CD4+ TNF-α responses, with frequency and magnitude varying significantly depending on the *C. trachomatis* antigen used. CD4+ IFN-γ and IL-2 responses occurred infrequently, as did production of any of the three cytokines by CD8+ T-cells. About one-third of TNF-α-producing CD4+ T-cells co-produced IFN-γ or IL-2. In summary, the predominant Th1 cytokine response elicited to *C. trachomatis* infection in women was a CD4+ TNF-α response, not CD4+ IFN-γ, and a subset of the CD4+ TNF-α-positive cells produced a second Th1 cytokine.
INTRODUCTION

*Chlamydia trachomatis*, an obligate intracellular bacterium, causes mucosal infection (chlamydia) of the genital, anorectal, and oropharyngeal surfaces in humans. Chlamydia is the most prevalent bacterial sexually transmitted infection (STI) worldwide (1), and genital chlamydia is associated with significant reproductive morbidity, including tubal factor infertility. Despite over two decades of national screening efforts in the U.S., the prevalence of chlamydia continues to rise (2). Availability of a chlamydia vaccine could improve chlamydia prevention efforts (3, 4), but vaccine development efforts have been hindered in part by insufficient knowledge of the immune responses to *C. trachomatis* infection in humans that contribute to protective immunity (5).

Because of the ethical concerns in performing *Chlamydia* challenge or natural history studies in humans, animal models have played a critical role in identifying the mechanisms of protective immunity to chlamydia (6). In murine models of genital chlamydia, protective immunity is largely mediated through CD4+ T helper type 1 (Th1) cell responses, with interferon-gamma (IFN-γ) playing an essential role (7). Another Th1 cytokine, tumor necrosis factor alpha (TNF-α), has also been implicated in protective immunity to chlamydia in murine studies (8-10). TNF-α is a pleiotropic proinflammatory cytokine released from monocytes, macrophages, and lymphocytes that stimulates a cascade of other cytokines (11). In the murine chlamydia model, the influence of TNF-α on chlamydia clearance appears to be specific to the mucosal site infected. For example, in the mouse pneumonitis model (which uses *C. muridarum*), TNF-α deletion significantly accelerates mortality and increases organism burden in the lung (8, 9), suggesting a protective role for TNF-α. However, TNF-α depletion seems to have no effect on *C. muridarum* clearance from the genital tract (12-14), although its expression...
in the genital tract increases within days of chlamydia infection (12, 15), suggesting TNF-\(\alpha\) alone is not sufficient for chlamydia clearance. Interestingly, several studies demonstrate TNF-\(\alpha\) may exert its effects in synergy with other cytokines (16) or interleukins (17). One murine study demonstrated that CD4\(^+\) T-cells producing both IFN-\(\gamma\) and TNF-\(\alpha\) correlated with protection against chlamydia challenge (16). While the role of TNF-\(\alpha\) in mediating protective immunity remains unclear, murine studies have reported this proinflammatory cytokine may contribute to upper genital tract pathology (13).

Translating immune mechanisms of chlamydia protection from animal studies to humans is difficult, as mechanisms can differ by animal model (7, 18). For example, mice deficient in CD8\(^+\) T-cells still resolve primary infection (7, 19), yet a non-human primate model demonstrated a key role for CD8\(^+\) T-cells in mediating protection against chlamydia (20). The dominant mucosal mononuclear cell type response to chlamydia also differs by species with CD4\(^+\) T-cells being more numerous in the genital tract in mice (21) and CD8\(^+\) T-cells in the non-human primate trachoma model (22).

Additionally, differences in Chlamydia species tested, time to infection clearance, estrous cycle duration, and variable susceptibility to upper genital tract infection make correlation to humans difficult (7, 18). In humans, evidence for protective immunity to chlamydia is mainly based on epidemiological studies, which have shown that a history of prior chlamydia, spontaneous resolution of chlamydia, and persons with a high likelihood of repeated chlamydia exposures (e.g., commercial sex workers) are associated with a lower reinfection risk (23-25). Although immune mechanisms that mediate protection against C. trachomatis in humans remain to be fully elucidated, sparse evidence from two prospective human studies that measured C. trachomatis-specific IFN-\(\gamma\) production by ELISA or ELISpot revealed IFN-\(\gamma\) was associated with
protective immunity (26, 27).

To advance our understanding of *C. trachomatis*-specific immune mechanisms that contribute to protective immunity in humans, a more in depth evaluation of the *C. trachomatis*-specific T-cell effector responses and T-cell phenotypes is needed. To address this knowledge gap, we have established a cohort of chlamydia-infected women from which we have characterized clinical manifestations and are conducting immunological experiments to help unravel the key *C. trachomatis*-specific immune mechanisms and their contribution to protective immunity. Here, we present findings from our initial investigation of the *C. trachomatis*-specific Th1 cytokine responses (IFN-γ, TNF-α, and IL-2) in chlamydia-infected women prior to treatment using intracellular cytokine staining.

**MATERIALS AND METHODS**

**Study Population and Procedures.** The study population was comprised of females ≥16 years of age who presented to the Jefferson County Department of Health (JCDH) STD Clinic in Birmingham, Alabama, USA for treatment of a recent positive *C. trachomatis* screening nucleic acid amplification test (Hologic Aptima Combo 2 ® [AC2]; Hologic, Inc., Marlborough, MA). Patients did not receive empiric chlamydia therapy at the time of screening because they had no cervicitis findings and no other chlamydia treatment indications. Patients interested in the study provided written consent and were enrolled. Women who were pregnant, had a prior hysterectomy, were co-infected with HIV, syphilis, or gonorrhea (tested at screening), were immunosuppressed, or had received antibiotics with anti-chlamydial activity in the prior 30 days were excluded.

At enrollment, participants were interviewed and demographical, clinical, and behavioral data were collected. A pelvic examination was performed in which a vaginal
swab specimen was collected for wet mount microscopy and an endocervical swab specimen for chlamydia and gonorrhea testing by the AC2 per the manufacturer’s instructions. Blood was collected for isolation of peripheral blood mononuclear cells (PBMCs). All participants received directly observed therapy with azithromycin 1g and were advised to refer all sexual partners for treatment, if not already treated. PBMCs from five healthy, low-risk C. trachomatis-seronegative women (tested with a C. trachomatis elementary body-based ELISA assay [28]) without a history of chlamydia were used as negative controls. The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board and JCDH.

PBMC Isolation. At the UAB Center for Clinical and Translational Sciences Specimen Processing and Analytical Nexus, PBMCs were isolated from blood by centrifugation through lymphocyte separation medium (Mediatech, Inc, Manassas, VA). Upon isolation, cells were counted and examined for viability. PBMCs were frozen in 1mL aliquots in 90% FBS+10% DMSO and cryopreserved in liquid nitrogen until used for immunological studies.

Intracellular Cytokine Staining (ICS). PBMCs were stimulated with C. trachomatis antigens and analyzed for cytokines as previously reported with modifications (29). Briefly, 2.5x10^5 cells were incubated for two hours at 37°C with 5% CO₂ in RPMI-10 media (RPMI 1640 medium containing 10% human AB serum, penicillin/streptomycin [50 U/mL], HEPES [25 mM] and L-glutamine [2mM]) in the presence of co-stimulatory antibodies CD28 and CD49d (BD Biosciences, San Diego, CA) and antigen, followed by a five-hour incubation in the presence of Brefeldin A and Monensin (both from BD Biosciences, San Diego, CA). Antigens used were: recombinant C. trachomatis Pgp3 (5 µg/ml) (Biorbyt, San Francisco, CA), pooled Major Outer Membrane Protein (MOMP) peptides (5 µg/ml) (VS1 75-92, VS2 132-151, VS2 145-163, VS4 300-318, VS4 308-
324; UAB Peptide Core, Birmingham, AL), formalin-fixed C. trachomatis elementary bodies (4 µg/ml) (EBs, pooled serotypes D, F and J; obtained from Dr. Richard Morrison from the University of Arkansas for Medical Sciences, Little Rock, AR), and *Staphylococcus* enterotoxin B (10 µg/ml) (SEB, Toxin Technologies, Carasota, FL) as the positive control. RPMI-10 supplemented with anti-CD28 and anti-CD49d antibodies, but without antigen, was used to determine background T-cell responses. Cells were subsequently labeled with LIVE/DEAD fluorescent reactive dye (Life Technologies, Eugene, OR), stained with surface antibodies against CD3-Pacific Blue (BD biosciences, San Diego, CA), CD4-Qdot 655, and CD8-Qdot 605 (both from Invitrogen, Carlsbad, CA), fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences, San Diego, CA), and stained with antibodies for intracellular cytokines IFN-γ-Alexa 700, TNF-α-PE-Cy7, and IL-2-PE (all from BD Biosciences, San Diego, CA). Approximately 100,000 events were acquired on a LSRII (BD Immunocytometry Systems, San Diego, CA) and data was analyzed using FlowJo software (v9.8.5, TreeStar, Ashland, OR). All responses are reported after subtracting the background responses (media + co-stimulatory antibodies).

**Statistical analysis.** Analyses were performed with SAS software, version 9.3 (SAS Institute, Cary, NC). Descriptive statistics were used to summarize demographical and clinical characteristics. We evaluated quantitative and qualitative differences in cytokine responses, the latter to allow comparison with prior studies (26-27); cytokine responses >0.05% and twice the background response (media + co-stimulatory antibodies) were considered positive for our qualitative analyses. Differences in frequencies of each CD4+ cytokine response and each CD8+ cytokine response were evaluated by McNemar’s chi-square test. Differences in frequency of a specific cytokine response based on C. trachomatis antigen used for the stimulation were also evaluated.
by McNemar’s chi-square test. Differences in the magnitude of CD4⁺ TNF-α responses (enumeration of cytokine-producing cells) by C. trachomatis antigen were evaluated with the Friedman and Wilcoxon signed-rank tests. Differences in the magnitude of cytokine-positive responses between chlamydia-infected cytokine-positive, chlamydia-infected cytokine-negative, and negative control groups were evaluated with the Wilcoxon rank sum test. Associations of subjects’ demographical or clinical characteristics with specific cytokines responses were evaluated with the Pearson’s chi-square, Fisher’s exact, or Wilcoxon rank sum tests as appropriate. Differences were considered significant at a P-value <0.05.

RESULTS

Ninety women with chlamydia infection at the time of enrollment were investigated in this study (Table 1). The median age was 21 (range 16 – 32), 97% were African American, and 1% were Hispanic. 54% of women were asymptomatic and 40% were diagnosed with co-infections bacterial vaginosis, trichomoniasis, or vaginal candidiasis. 57% of women had a prior history of chlamydia, based on self-report and/or documentation of prior positive chlamydia test results.

Of the three Th1 cytokines tested, the predominant CD4⁺ cytokine elicited by stimulated PBMCs from chlamydia-infected women was TNF-α, not IFN-γ. Fig. 1A shows the gating scheme utilized for the analyses of CD4⁺ T-cells from a representative chlamydia-infected subject showing Pgp3-stimulated flow cytometry plots. CD8⁺ T-cells were gated similarly. A CD4⁺ TNF-α response comprised the majority of C. trachomatis-specific CD4⁺ Th1 cytokine responses to any antigen, with 79% of women mounting a positive CD4⁺ TNF-α response to at least one antigen, compared to 16% and 12% of women having a CD4⁺ IFN-γ or IL-2 response, respectively (Fig. 1B, P <0.001). There
was no difference in the proportion of women with a positive CD4+ IFN-γ vs. IL-2 response. CD8+ T-cell IFN-γ, TNF-α, and IL-2 responses occurred less frequently than CD4+ Th1 cytokine responses, with 19-28% of subjects' CD8+ T-cells eliciting a positive cytokine response for any cytokine to at least one antigen (Fig. 1C). There was no difference in the proportion of women with a positive CD8+ IFN-γ, TNF-α, or IL-2 response.

We further investigated whether a positive response for any of the CD4+ Th1 cytokines was associated with demographical or clinical characteristics of subjects, especially age and history of prior chlamydia, as both have been found to be associated with a reduced risk of reinfection (23, 30). There were no significant associations found between CD4+ Th1 cytokine responses and age, symptom status, prior history of chlamydia, number of sexual partners, hormonal contraception, cervicitis, or co-infections (data not shown).

The proportion of women with a positive cytokine response varied with the C. trachomatis antigen used for stimulation. Stratifying the ICS responses by C. trachomatis antigens used for stimulating PBMCs, we assessed whether there was a difference in the proportion of women with a positive cytokine response elicited by different C. trachomatis antigens. Fig. 2A shows representative flow cytometry plots for a chlamydia-infected subject and a chlamydia-negative control subject. As shown in Fig. 2B, all three antigens generated predominantly CD4+ TNF-α qualitative responses, but these responses were most often generated with Pgp3 stimulation when compared with stimulation with MOMP (64% vs. 34%; P < 0.001) or EB (64% vs. 51%; P = 0.035). The proportion of women with a positive CD4+ TNF-α response to EB was significantly higher than to MOMP (51% vs. 34%; P = 0.011). There was no significant difference in
the proportion of women with a positive CD4+ IFN-γ or IL-2 response between the antigens. In contrast to CD4+ responses, the proportion of women with positive CD8+ IFN-γ, TNF-α, or IL-2 responses across different antigens was lower, occurring in 6-15% of women (Fig 2C). The proportion of women with a positive CD8+ IFN-γ response when stimulated with Pgp3 was significantly lower compared with EB (6% vs. 14%; *P* = 0.02), otherwise there was no significant difference in the proportion of women with positive CD8+ cytokines responses between the antigens.

In subjects with a positive Th1 cytokine response, a higher magnitude of TNF-α- and IFN-γ-producing CD4+ T-cells was seen to all antigens, with Pgp3 eliciting the highest magnitude of TNF-α-producing CD4+ T cells. Although we expected an increase in the number of cytokine-producing CD4+ T-cells in subjects with positive cytokine responses to the various antigens when compared to cytokine-negative chlamydia-infected women (based on our qualitative analysis cutoff described in the methods), we wanted to compare the magnitudes of those responses. To evaluate the differences in the magnitudes (measured by percent cytokine-positive cells) of the responses between these two group of women and chlamydia-negative controls (all who had a negative response for all Th1 cytokines we tested), we stratified the data into chlamydia-infected cytokine positive, chlamydia-infected cytokine negative, and chlamydia-negative control groups. Given the low proportion of women with positive CD8+ cytokine responses, we focused our quantification analyses of cytokine producing cells on CD4+ T-cells. As shown in Fig. 3, comparison of the magnitude of the cytokine-positive CD4+ responses between antigens showed that Pgp3 elicited a 40% higher CD4+ TNF-α response (median TNF-α-producing cells 0.11%) than MOMP or EB
(0.08% for both, \( P = 0.0017 \) and \( P = 0.0016 \), respectively). IFN-\( \gamma \) or IL-2 \( \text{CD}^+ \) cytokine positive responses were not significantly different between antigens.

Comparing the magnitude of the cytokine responses in cytokine-positive chlamydia-infected women to either cytokine-negative chlamydia-infected women or chlamydia-negative control women for each antigen, we found a significant increase in the magnitude of TNF-\( \alpha \)- and IFN-\( \gamma \)-positive CD4\(^+\) T-cell responses for the cytokine-positive chlamydia-infected women compared to the other groups for each antigen, but there was no difference in the magnitude of the IL-2 responses between the groups. The median TNF-\( \alpha \)-positive CD4\(^+\) cell responses ranged from 0.080-0.11% in cytokine-positive chlamydia-infected women vs. 0.010-0.015% in cytokine-negative chlamydia-infected women. The median IFN-\( \gamma \)-positive CD4\(^+\) cell response ranged from 0.043-0.053% in cytokine-positive chlamydia-infected women vs. 0.000-0.004% in cytokine-negative chlamydia-infected women. There was no difference in the magnitude of the cytokine responses between cytokine-negative chlamydia-infected women and negative-control women for any of the cytokines with the exception of Pgp3-stimulated IL-2 responses, which were higher in the control group compared to the chlamydia-infected IL-2-negative group (\( P = 0.02 \)). Taken together, these data suggest that responses to chlamydia antigens are chlamydia-specific and demonstrate the immunogenicity of Pgp3 in generating TNF-\( \alpha \)-positive CD4\(^+\) T-cell responses.

In TNF-\( \alpha \)-positive subjects, the frequency of positive CD4\(^+\) TNF-\( \alpha \) responses is highly variable and antigen dependent. In subjects who had a positive CD4\(^+\) TNF-\( \alpha \) response, we evaluated the frequency of inter-antigenic TNF-\( \alpha \)-positive responses across each \textit{C. trachomatis} antigen to determine if the tested antigens generated isolated responses (\textit{i.e.} cytokine responses only to that antigen). We had initially
hypothesized that EB, being a whole *C. trachomatis* organism, would elicit the greatest cross-antigenic responses due to a more diverse antigen presentation compared to either Pgp3 or MOMP. Including only women with a positive CD4^+^ TNF-α response to any antigen (n = 71), we saw significant inter-antigenic variability as expected, with 28% of women mounting TNF-α-positive responses to all three antigens, 21% to Pgp3 only, 13% to EB only, and 3% to MOMP only (Fig. 4).

**A subset of stimulated CD4^+^ T-cells demonstrated dual cytokine responses.** Using dual gating, we determined the proportion of TNF-α-producing CD4^+^ T-cells that were dual-positive for either IL-2 or IFN-γ. Representative dual-gated flow cytometry plots are shown in Fig. 5A. As shown in Fig. 5B, dual cytokine-positive CD4^+^ populations were identified in 28% of subjects with positive TNF-α responses, with TNF-α/IL-2 dual-positive responses being more frequently identified than TNF-α/IFN-γ dual positive responses (21% vs. 7%, respectively). Corresponding to the immunodominance observed earlier with Pgp3 eliciting TNF-α responses, Pgp3 elicited a significantly higher frequency of TNF-α/IL-2 dual-positive responses as compared to MOMP (93% vs. 20%, respectively; P = 0.003) and EB (93% vs. 27%, respectively; P = 0.002) (Fig. 5C). No significant difference by antigen was identified for TNF-α/IFN-γ responses.

**DISCUSSION**

With murine chlamydia models convincingly demonstrating the importance of Th1 CD4^+^ IFN-γ in chlamydia infection clearance (7) and some animal studies demonstrating a potential protective role for TNF-α (8, 33), our study sought to increase our understanding of the frequency and magnitude of *C. trachomatis*-specific Th1-associated cytokine responses in humans. Using intracellular cytokine staining, we
analyzed *C. trachomatis*-specific TNF-α, IFN-γ and IL-2 cytokine production from both CD4⁺ and CD8⁺ T-cells from chlamydia-infected women before treatment. We report for the first time that the predominant Th1-associated cytokine response in women with chlamydia was a CD4⁺ TNF-α response, not IFN-γ, with PBMCs from approximately 80% of women eliciting a CD4⁺ TNF-α response to at least one of the three *C. trachomatis* antigens we tested. Other CD4⁺ Th1 cytokine responses, IFN-γ and IL-2, were present, but at much lower frequencies. Also, TNF-α-positive responses had a higher magnitude than IFN-γ-positive responses, by almost 2-fold.

In contrast to CD4 responses, the overall frequency of CD8⁺ T-cell TNF-α, IFN-γ and IL-2 responses were low, occurring in <30% of infected women. Although one might anticipate low CD8⁺ responses with the use of a recombinant protein and EBs, we also observed weak CD8⁺ responses with the use of five 17-20mer MOMP peptides, which have been shown to contain strong and functional CD8⁺ epitopes (45, 46). Further, we did not see any difference in the response between peptide and protein antigens with longer incubation time before golgi transport inhibition, which allowed additional time for antigen processing (data not shown). Therefore, we feel confident that our observed low CD8⁺ responses are accurate, though we cannot exclude the possibility that epitopes not present in our MOMP peptides may contribute to CD8⁺ responses.

TNF-α is an effector cytokine with known anti-chlamydial activity. *In vitro*, recombinant TNF-α alone inhibits *C. trachomatis* in cell culture (31). *C. trachomatis* growth inhibition by TNF-α has been shown to be synergistic with IFN-γ, with a 100-fold increase in TNF-α potency (31). Inhibition by TNF-α likely occurs by enhancing mechanisms of IFN-γ-dependent tryptophan depletion, as inhibition can be reversed by tryptophan supplementation *in vitro* (32). In animal models, the contribution of TNF-α to...
Chlamydia clearance appears tissue and/or species specific. For example, in the genital tract, mice and guinea pig studies show increased TNF-α production during chlamydial infection (15), but the absence of TNF-α does not appear to influence chlamydia clearance (13, 14). However, in the mouse pneumonitis model, TNF-α inhibition increased susceptibility to C. muridarum and increased mortality (8). An in vivo study comparing C. trachomatis and C. muridarum infection of different mouse strains demonstrated that TNF-α-deficient mice had delayed chlamydia clearance, which differed by Chlamydia species (33). This may explain the variation in the influence of TNF-α on Chlamydia growth inhibition and highlights the importance of human studies for understanding mechanisms of chlamydia clearance.

Our finding that TNF-α is the predominant cytokine elicited to the intracellular pathogen C. trachomatis suggests it may be an important component of the systemic effector immune response and is consistent with other studies showing it contributes to the immune response to C. trachomatis. For example, in individuals with trachoma, sequence variation associated with the TNF locus has been associated with increased risk of trachomatous scarring and trichiasis and elevated TNF responses induced by EBs (34). The presence of the TNF-α-308A allele has been associated with an increased risk of scarring trachoma (35) and development of severe adhesions in tubal factor infertility caused by C. trachomatis (36). These studies suggest that although TNF-α is an effector cytokine that may contribute to protective immune responses, excess levels of this proinflammatory cytokine can lead to immunopathology after infection (12, 37). Other evidence supporting the importance of TNF-α in immune control of intracellular organisms includes studies demonstrating that TNF-α-inhibitory monoclonal antibodies are strongly associated with an increased susceptibility to
infection with the intracellular pathogens *Histoplasma* and *Listeria* and reactivation of latent tuberculosis (38). Whether TNF-α is associated with a reduced risk for *C. trachomatis* reinfec­tion remains unknown, and we are conducting studies that will address this important question.

Our finding that positive Th1 cytokine responses, especially IFN-γ, are not generated in all chlamydia-infected women is consistent with other studies. Two previous human studies evaluated the frequency of positive IFN-γ responses with PBMC stimulation with *C. trachomatis* antigens, measured by either ELISpot or ELISA, but did not evaluate the frequency of other Th1 cytokine responses including TNF-α (26, 27). In a cohort of commercial sex workers at risk for *C. trachomatis* infection, Cohen et al. reported positive IFN-γ responses to EB occurred in 40% (26). In a cohort of female adolescents with current or prior *C. trachomatis* infection, Barral et al. reported positive IFN-γ responses to EB and MOMP occurred in 62% and 38% of adolescents, respectively (27). In our study, we used ICS rather than ELISpot or ELISA to characterize cytokines responses, which allowed us to determine IFN-γ responses produced specifically by CD4+ and CD8+ T-cells. In our cohort of chlamydia-infected women, a positive CD4+ IFN-γ response to any of the 3 antigens occurred in 16% of subjects and positive CD8+ IFN-γ response in 22% of subjects. Our lower frequency of positive IFN-γ responses compared with the above studies could, in part, reflect differences in assay cutoff for positivity (with all the studies using different assays) or the fact that we measured IFN-γ produced specifically by CD4+ and CD8+ cells, whereas the above studies likely measured IFN-γ production from all CD3+ T-cells with an unknown contribution from other mononuclear cells (e.g., NK cells, monocytes, etc); it is worth noting that the
Barral et al. study did partially deplete CD8+ cells to limit detection of IFN-γ from that cell source (27), though other mononuclear cells remained present.

Murine studies have revealed that TNF-α can act in synergy with other cytokines, including IL-22 (17) and IFN-γ (16), to modulate the immune response, and the presence of dual-positive TNF-α/IFN-γ CD4+ T-cell responses in mice correlates with protection against chlamydia challenge (16). In our study, we found that one third of TNF-α-positive CD4+ T-cells were able to produce two cytokines, with the majority co-producing IL-2 rather than IFN-γ. IL-2, a pro-survival cytokine, is known to positively modulate TNF-α production (39). IL-2 also has an important role in lymphocyte proliferation and arming effector T-cells, as well as maintaining effector T-cells in the circulation (40). Goon et al. reported a higher frequency of TNF-α/IL-2 dual producing CD4+ T-cells in patients with HTLV-1-associated pathology than asymptomatic carriers with similar viral load (41). Their observation raises an interesting question about the role of TNF-α/IL-2 dual producing CD4+ T-cells in C. trachomatis infection; it would be important to determine whether these dual cytokine-producing CD4+ T-cells contribute to immune protection or pathology.

Our study used a panel of multiple C. trachomatis antigens to provide a more comprehensive characterization of C. trachomatis-specific T-cell responses. We found that Pgp3 most often elicited a positive CD4+ TNF-α-positive response compared with the other antigens and also more often elicited dual-positive CD4+ cytokine responses. To our knowledge, Pgp3 has never been investigated in prior studies of C. trachomatis-specific cytokine cellular immune responses in humans, although it has been demonstrated in a murine model that Pgp3 deficiency significantly attenuates infectivity and pathogenicity of C. trachomatis (42). In our study, both single- and dual-positive...
TNF-α-producing CD4+ T-cell responses to Pgp3 were higher in frequency and magnitude than to MOMP or EB, the latter which contains, as part of its surface, the chlamydial outer membrane complex comprised of over 300 different proteins (43). Although it remains unclear why EB did not elicit cytokine responses as frequently as Pgp3, it could indicate that the key EB antigenic T-cell epitopes are relatively diluted as compared to a single protein like Pgp3, which thereby may blunt the magnitude of the immune response. Although an insufficient EB concentration is another possibility, we think this is unlikely as we used a 4-fold higher concentration of EB in our ICS assays than Barral et al. (27) and we found that EBs generally elicited more frequent CD4+ TNF-α responses than MOMP and comparable IFN-γ responses (Fig. 2B-C). Although the difference in percent positive cells elicited by Pgp3 stimulation versus the other antigens may appear insignificant (0.11% versus 0.08%), that represents an approximate 40% increase in circulating TNF-α-positive CD4+ T-cells and a magnitude of 0.1% is consistent with CD4+ T-cell responses reported by others in post-vaccinated subjects vaccinated with Hepatitis B (47), influenza (48), and MMR (49). Additionally, the observation that Pgp3 generated more dual-positive responses than MOMP or EB may be an important observation for Pgp3 vaccine candidacy since TNF-α/IFN-γ dual-positive responses have been shown to be functionally superior and elicit protective responses in murine models (16).

Our study had strengths and limitations. This is the largest study investigating human C. trachomatis-specific T-cell responses using ICS; a previous study by Miguel et al. that used ICS only studied cytokine responses in 14 C. trachomatis-infected women and did not present data on the proportion of women with a positive response (44). Because we included multiple C. trachomatis antigens in our ICS studies, we were able to investigate inter-antigenic variation of cytokine responses, which revealed...
significant variability in CD4^+ TNF-α responses. Our study is the first to evaluate C. trachomatis-specific cellular immune responses through incorporation of dual gating into our ICS methodology, which allowed us to differentiate single cytokine producing cells from dual producing cells. One notable caveat with our findings is that the majority of subjects studied were African American, representative of the race/ethnicity of the clinic population, and therefore these findings may not be generalizable to other populations. In this study, we focused on Th1 cytokine responses, which was the aim of our initial investigation into C. trachomatis-specific immune responses in humans because of animal models demonstrating the importance of Th1 responses (7). In future studies, we will be evaluating a broader group of cytokines, including Th2-associated cytokines, and will evaluate immune responses at follow-up visits to determine longevity of C. trachomatis-specific immune responses and association of cytokine responses (single and dual-functional) with chlamydia reinfection. Ultimately, we hope to identify immune correlates of protection to C. trachomatis infection in humans.

In summary, the predominate Th1-associated cytokine response in women with uncomplicated chlamydia was a CD4^+ TNF-α response, and CD4^+ T-cells producing both TNF-α and IL-2 were not uncommon. The frequency of CD4^+ TNF-α responses was influenced by antigen used for stimulating PBMCs, with Pgp3 most often eliciting a CD4^+ TNF-α single and dual-positive response.

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REFERENCES


37. Manam S, Thomas JD, Li W, Maladore A, Schriplema JH, Ramsey KH, Murthy AK. 2015. Tumor Necrosis Factor (TNF) receptor superfamily member 1b on CD8+ T cells and TNF receptor superfamily member 1a on non-CD8+ T cells contribute significantly to upper genital tract pathology following chlamydial infection. J Infect Dis 211:2014–2022.


FIGURE LEGENDS

FIG. 1. Intracellular cytokine staining of PBMCs from chlamydia-infected women that were stimulated with *C. trachomatis* antigens. PBMCs from 90 chlamydia-infected women were stimulated with *C. trachomatis* MOMP peptides, Pgp3, and EB and production of TNF-α, IFN-γ, and IL-2 was measured by intracellular cytokine staining. (A) Representative flow cytometry plots showing intracellular cytokine staining gating strategy for CD4+ T-cells producing TNF-α, IFN-γ, or IL-2. Numbers indicate the percentage of gated cells. CD8+ T-cells were gated similarly. Fig. 1B and 1C shows the proportion of women with a positive CD4+ or CD8+ T-cell cytokine response against at least one antigen. A positive cytokine response was defined as >0.05% and twice the background (media + co-stimulatory antibodies). Significance was evaluated by the McNemar’s chi-square test.

FIG. 2. The percentage of chlamydia-infected women with a positive Th1 cytokine response based on the *C. trachomatis* antigen used for stimulating PBMCs. (A) Representative flow cytometry plots from intracellular cytokine staining of CD4+ T-cells from a chlamydia-negative and chlamydia-infected subject. (B, C) Percentage of women with a positive cytokine response to chlamydia antigens in CD4+ and CD8+ T-cells. A positive cytokine response was defined as >0.05% and twice the background (media + co-stimulatory antibodies). Significance was determined using McNemar’s chi-square test.

FIG. 3. Magnitude of cytokine responses from CD4+ T-cells stimulated with *C. trachomatis* antigens in chlamydia-infected women with a positive or negative cytokine response (qualitative) and in chlamydia-negative control women. Responses were measured by intracellular cytokine staining. A positive cytokine response was defined as >0.05% and twice
the background (media + co-stimulatory antibodies). The magnitude of the cytokine response is shown as percentage of total CD4\(^+\) T-cells after subtracting the background response, stratified by \textit{C. trachomatis} antigen used. The box denotes the interquartile range and the whiskers denote the 5\(^{th}\) and 95\(^{th}\) percentiles. Dots denote outliers. The median is shown as the horizontal line in the box. \(N\) denotes the number of subjects. Significance was evaluated by Wilcoxon signed-rank or rank sum tests. The asterisks denote \(P < 0.001\). The Y-axis is \(\log_{10}\).

\textbf{FIG. 4.} Venn diagram demonstrating the overlap of CD4\(^+\) TNF-\(\alpha\) responses across \textit{C. trachomatis} antigens used for PBMC stimulation. Subjects with a positive CD4\(^+\) TNF-\(\alpha\) response to any antigen were identified (\(n = 71\)) and the concordance of positive responses across antigens MOMP, Pgp3, and EB was evaluated. The percentages indicate the proportion of total positive CD4\(^+\) TNF-\(\alpha\) responses elicited by antigen(s).

\textbf{FIG. 5.} Dual cytokine-producing CD4\(^+\) T-cells from chlamydia-infected women expressing TNF-\(\alpha\) and either IL-2 or IFN-\(\gamma\). (A) Representative flow cytometry plots from chlamydia-infected women with dual-positive CD4\(^+\) TNF-\(\alpha\) and either IFN-\(\gamma\) or IL-2 responses. (B) Pie chart showing proportion of positive CD4\(^+\) TNF-\(\alpha\) responses that produced TNF-\(\alpha\) alone or produced both TNF-\(\alpha\) and IL-2 responses or both TNF-\(\alpha\) and IFN-\(\gamma\) responses. (C) Percentage of total dual-positive subjects with a dual positive cytokine response by antigen. \(n = 15\) for a positive CD4\(^+\) TNF-\(\alpha^+\)/IL-2\(^+\) response to at least one antigen and \(n = 5\) for a positive CD4\(^+\) TNF-\(\alpha^+\)/IFN-\(\gamma^+\) response to at least one antigen. A positive cytokine response was defined as \(>0.05\%\) and twice the background (media + co-stimulatory antibodies). The number of positive responses by antigen is indicated above each bar. Significance was determined using McNemar's chi-square test.
Table 1. Study Subject Characteristics ($n=90$)

<table>
<thead>
<tr>
<th>Subject Characteristics</th>
<th>$n$ (%)</th>
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</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>22 (16-32)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>87 (97)</td>
</tr>
<tr>
<td>White</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Ethnicity</td>
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</tr>
<tr>
<td>Non-Hispanic</td>
<td>89 (99)</td>
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<tr>
<td>Hispanic</td>
<td>1 (1)</td>
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<tr>
<td>Hormonal contraception</td>
<td>41 (46)</td>
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<tr>
<td>Median prior sex partners**</td>
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<tr>
<td>Last 3 mo. (range)</td>
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<tr>
<td>Prior Chlamydia*</td>
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<tr>
<td>Asymptomatic</td>
<td>49 (54)</td>
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<tr>
<td>Cervicitis*</td>
<td>17 (19)</td>
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<tr>
<td>Co-Infections</td>
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<tr>
<td>HIV infection</td>
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<tr>
<td>Gonorrhea</td>
<td>0</td>
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<tr>
<td>Bacterial Vaginosis</td>
<td>26 (29)</td>
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<tr>
<td>Trichomonias</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Candidias</td>
<td>11 (12)</td>
</tr>
</tbody>
</table>

* $n=89$, **$n=88$
% of dual-cytokine positive subjects with positive response by antigen

- MOMP Pgp3: 3
- EB: 4
- MOMP Pgp3: 1
- EB: 2

- TNF-α+IL-2+ (n=15)
- TNF-α+IFN-γ+ (n=9)

- Single TNF-α+ (51%)
- Single IFN-γ+ (15%)
- Single IL-2+ (5%)
- Single (12%)

n = 71