



Short communication

Stimulated peripheral blood mononuclear cells from chlamydia-infected women release predominantly Th1-polarizing cytokines

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ABSTRACT

Chlamydia trachomatis infection (chlamydia) is the most prevalent sexually transmitted bacterial infection and causes significant reproductive morbidity in women. Little is known about how immunity to chlamydia develops in women, though animal models of chlamydia indicate that T-helper type 1 (Th1) responses are important for chlamydia clearance and protective immunity, whereas T-helper type 2 (Th2) responses are associated with persisting infection. In chlamydia-infected women, whether the predominant immune response is Th1- or Th2-polarizing remains controversial. To determine the cytokine profiles elicited by peripheral blood mononuclear cells (PBMCs) from chlamydia-infected women, we stimulated PBMCs with *C. trachomatis* elementary bodies and recombinant *C. trachomatis* Pgp3 and measured supernatant levels of select cytokines spanning Th1- and Th2-polarizing responses. We found that stimulated PBMCs from chlamydia-infected women secreted cytokines that indicate strong Th1-polarizing responses, especially interferon-gamma, whereas Th2-polarizing cytokines were expressed at significantly lower levels. In chlamydia-infected women, the predominant cytokine responses elicited on stimulation of PBMCs with *C. trachomatis* antigens were Th1-polarizing, with interferon-gamma as the predominant cytokine.

1. Introduction

Chlamydia trachomatis (Ct) causes “chlamydia”, the most prevalent bacterial sexually transmitted infection (STI) worldwide [1], and is associated with significant reproductive morbidity in women [2]. A Ct vaccine is urgently needed to curb rising Ct rates, but the knowledge gap in our understanding of systemic immune responses in humans hinders its development [3]. The majority of our knowledge of immune responses to chlamydia has been obtained using challenge studies in animal models of urogenital chlamydia. Animal models demonstrate that T-helper type 1 (Th1) cells strongly correlate with immunity against chlamydia re-challenge, although the T-cell type responsible appears to differ by species [4]. The Th1 cytokine IFN- γ plays a major role in protective immunity, although other cytokines are likely contributory [5]. One potential cytokine that may act in synergy with IFN- γ is TNF- α , which has been shown to have anti-chlamydial properties, including strongly accentuating the inhibitory effect of IFN- γ on Ct growth *in vitro* [6]. Further, in mice, the presence of CD4⁺ T-cells producing both TNF- α and IFN- γ correlated with protection against chlamydia challenge [7], which highlights the importance of studying

multiple cytokines to determine the contribution of different cytokine profiles in Ct infection.

In humans, studies of immune responses to chlamydia are limited and the dominant Th response to natural Ct infection remains insufficiently understood. A small study of adolescent women demonstrated that IFN- γ production by CD4⁺ T-cells trended towards a reduced risk for reinfection [8]. Another study showed an increased in the frequency of IFN- γ -producing CD4⁺ T-cells in women without incident CT infection compared to women with CT infection [9]. Further support for the protective role of IFN- γ comes from an immunoepidemiologic study of commercial sex workers that showed a reduced risk for incident Ct infection was associated with the Th1 cytokine IFN- γ and also the Th2 cytokine IL-13 [10]. Another study found that the predominant peripheral blood mononuclear cell (PBMC) response in Ct-infected women was polarized towards Th2, characterized by IL-4 production [11]. Given the conflicting findings from these studies, the predominant response in Ct-infected women remains to be fully elucidated.

We previously showed in a cohort of Ct-infected women that TNF- α is the predominant Th1 cytokine produced by peripheral CD4⁺ T-cells from chlamydia-infected women, not IFN- γ , with approximately one

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third of TNF- α -positive T-cells co-expressing either IFN- γ or IL-2 [12]. Surprisingly, IFN- γ responses were infrequent in that study, though our analysis was limited to CD4⁺ and CD8⁺ T-cells and only evaluated the Th1 cytokines IFN- γ , TNF- α , and IL-2 by intracellular cytokine staining. In this pilot study of cytokine responses in Ct-infected women, we expand our previous cytokine evaluation to include the Th1-polarizing cytokine IL-12 and Th2 cytokines, and we used multiplex cytokine analysis to measure cytokine levels directly from supernatants collected from PBMCs stimulated with Ct antigens. The primary study objective was to expand our understanding of which Th cytokine profile (Th1 vs. Th2) is dominant in stimulated PBMCs from Ct-infected women and whether specific cytokines within a Th profile contributed more than others.

2. Materials and methods

2.1. Study population and procedures

In this study, we evaluated cytokines in Ct-infected women at an enrollment visit, which was part of an ongoing longitudinal study that has been previously described [12]. Briefly, females ≥ 16 years of age returning to a STD clinic for treatment of a positive screening Ct nucleic acid amplification test were enrolled. At enrollment, participants were interviewed and blood was collected for isolation of PBMCs. All participants received azithromycin 1 g for Ct treatment. The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board and the Jefferson County Department of Health.

2.2. PBMC isolation

PBMCs were isolated at the UAB Center for Clinical and Translational Sciences Specimen Processing and Analytical Nexus by centrifugation through lymphocyte separation medium (Mediatech, Inc, Manassas, VA). PBMCs were frozen in 1 mL aliquots in 90% FBS + 10% DMSO and cryopreserved in liquid nitrogen until used for immunological studies.

2.3. Stimulation assays

Thawed PBMCs were rested overnight and then 1×10^5 cells/well were added to a flat-bottom 96-well culture plate in RPMI-10 media (RPMI 1640 medium containing 10% human AB serum, penicillin/streptomycin [50 U/mL], HEPES [25 mM] and L-glutamine [2 mM]) in the presence of Ct antigen and 10 U/ml IL-2 and incubated for 72 h at 37 °C in 5% CO₂. After 72 h, the cells were removed by centrifugation and the supernatant was stored at -80 °C. Ct antigens used were: recombinant Ct Pgp3 (5 μ g/ml, Biorbyt, San Francisco, CA), formalin-fixed Ct elementary bodies (4 μ g/ml, EB, pooled serotypes D, F and J; obtained from Dr. Richard Morrison from the University of Arkansas for Medical Sciences, Little Rock, AR), and phytohemagglutinin (10 μ g/ml, PHA) as the positive control. RPMI-10 was used to determine background cytokine responses.

2.4. Multiplex cytokine assays

Cytokines were measured using a validated multiplex cytokine assay, V-PLEX (Meso Scale Discovery [MSD], Rockville, MD), according to the manufacturer's instructions. Briefly, supernatants from PMBC stimulations were added in triplicate to 96-well V-PLEX plates and incubated on a rotary shaker for 2 h at room temperature. The plates were then washed, cytokine-specific secondary antibodies (MSD, Rockville, MD) added, incubated for 90 min, washed, and read using a V-PLEX plate reader. A serial dilution of known cytokine standards was used to calculate the supernatant cytokine concentrations using MSD Discovery Workbench software (v4.0, MSD, Rockville, MD), after subtracting the background (blank wells). Supernatant cytokine concentrations are

reported as pg/ml. Cytokine concentrations that were below the level of detection were replaced with the lowest measurable value.

2.5. Statistical analysis

Descriptive statistics were used to summarize demographical and clinical characteristics. Antigen differences in frequencies of positive cytokine responses (defined as > 2 standard deviations above the mean background [unstimulated] supernatant cytokine level) were evaluated by McNemar's chi-square test. For the quantitative analysis of positive cytokine responses, log₁₀-transformed cytokine levels were evaluated with mixed model analyses of variance with antigen as a main effect and participant as a random effect, which accounted for clustering of data within individual participants. Only cytokines with ≥ 5 positive responses were analyzed. Differences were considered significant at a *P*-value < 0.05 . Analyses were performed with SAS software, version 9.3 (SAS Institute, Cary, NC).

3. Results

Seventy-seven chlamydia-infected women were included in this study (Table 1). The median age was 22 (range 16–38), 96% were African American and 99% non-Hispanic. 38% used hormonal contraception and the median number of sex partners in the last 3 months was one (range 1–9). 51% of women had a prior history of chlamydia (based on self-report or review of prior Ct test results). 56% of women were asymptomatic on enrollment, and co-infections present included bacterial vaginosis (in 23%), trichomoniasis (3%), and vulvovaginal candidiasis (14%).

3.1. Ct antigen-stimulated PBMCs from the majority of Ct-infected women secreted the Th1-polarizing cytokines IFN- γ , TNF- α , and IL-12

As shown in Fig. 1A, the majority of women had positive Th1-polarizing IFN- γ , TNF- α , and IL-12 responses and Th2 IL-10 responses to either Pgp3 or EB. Only one woman (1.2%) had a positive IL-4 response to Pgp3; all women had negative IL-4 responses to EB. Less than 10% of women had positive IL-13 responses. Overall, compared to Pgp3, EB-stimulated PBMCs were significantly more likely to elicit cytokine-positive responses.

Table 1
Study Participant Characteristics (N = 77).

Participant Characteristics, N (%)	
Median age (range)	22 (16–38)
Race	
African American	74 (96%)
Caucasian	3 (4%)
Ethnicity	
Non-Hispanic	76 (99%)
Hispanic	1 (1%)
Hormonal contraception ^a	29 (38%)
Median prior sex partners	
Last 3 mo. (range) ^b	1 (1–9)
Prior Chlamydia ^a	39 (51%)
Asymptomatic	43 (56%)
Co-Infections	
HIV infection	Excluded
Gonorrhea	Excluded
Bacterial vaginosis	18 (23%)
Trichomoniasis	2 (3%)
Candidiasis	11 (14%)

^a Data missing for one participant.

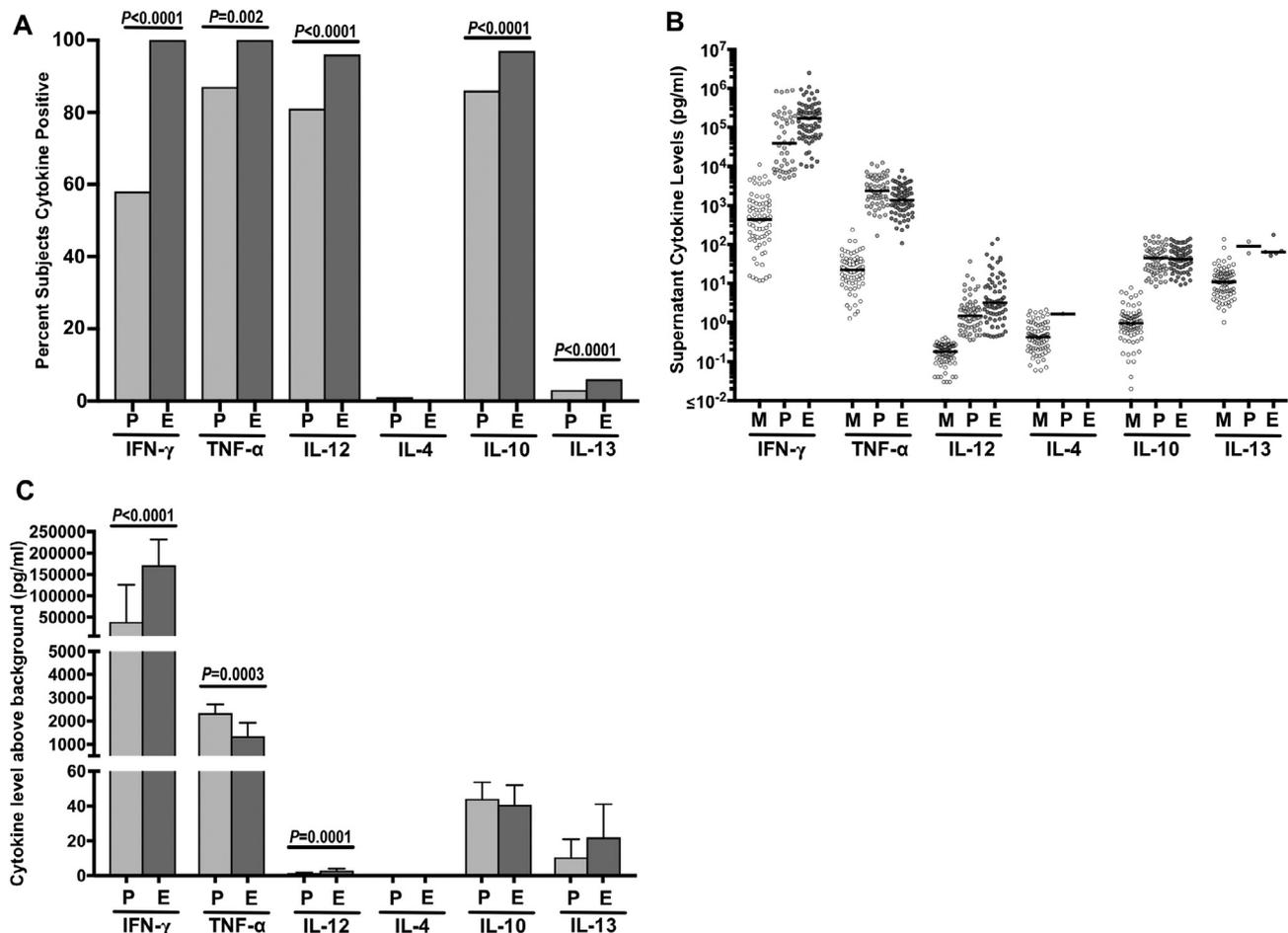


Fig. 1. Cytokines secreted from stimulated PBMCs from *Chlamydia trachomatis* (Ct)-infected women are predominately Th1-polarizing. PBMCs from Ct-infected women were stimulated with Ct elementary bodies (EB) and recombinant Ct Pgp3 and levels of six secreted cytokines, spanning Th1 and Th2 pathways, were measured by multiplex cytokine analysis. (A) Qualitative positive cytokine responses (defined as > 2 standard deviations above the mean background [unstimulated] supernatant cytokine level) are illustrated. The majority of subjects did not elicit positive Th2 cytokines IL-4 and IL-13 responses. (B) Quantitative cytokine levels in unstimulated PBMCs and in stimulated PBMCs in those with a positive cytokine response (pg/ml). (C) Quantitative antigen-stimulated cytokine levels above background. The highest cytokine levels were seen for Th1 cytokines, especially IFN- γ , in which levels were highest with EB-stimulated PBMCs compared with Pgp3-stimulated PBMCs. Th2 cytokines were expressed at lower levels with the lowest being IL-4, in which supernatant levels of antigen-stimulated PBMCs were not significantly different compared to background. The horizontal lines denote the median cytokine level and the whiskers denote the 95% confidence interval. M, P and E denote media, Pgp3, and EB, respectively. Significance was evaluated by McNemar's Chi-square or mixed model analysis of variance. Non-significant comparisons are not labeled. Only cytokines with ≥ 5 positive responses were analyzed.

3.2. Th1-polarizing cytokines were the dominant response by stimulated PBMCs in Ct-infected women

Among the positive cytokine responses, the highest levels of cytokines were seen with the Th1-polarizing cytokines IFN- γ and TNF- α , which were significantly elevated above background levels (Fig. 1B). Fig. 1C shows cytokine levels for each antigen after subtracting the background (unstimulated cells). We also found differences in cytokine levels based on the Ct antigen used in the PBMC stimulation. IFN- γ had the highest levels of all cytokines studied, and the IFN- γ level produced in response to EB stimulation was significantly higher than to Pgp3 (median 171,166 pg/ml vs 39,248 pg/ml, respectively, $P < 0.001$). Conversely for TNF- α , Pgp3-stimulated cells produced higher TNF- α levels than EB (2343 pg/ml vs 1350 pg/ml, respectively, $P = 0.002$). Although the majority of women generated positive IL-12 responses, the magnitude of IL-12 levels were low with the response to EB slightly higher than Pgp3 (3 pg/ml vs 1 pg/ml, $P = 0.0001$).

Th2 cytokines were produced at lower levels by stimulated PBMCs than the Th1 cytokines IFN- γ and TNF- α . The single positive IL-4 response was ≤ 0.01 pg/ml. IL-10 production was the highest of the Th2 cytokines, with levels produced to both Ct antigens of approximately

43 pg/ml. IL-13 responses were infrequent but, when present, were approximately 15 pg/ml. IL-10 or IL-13 responses did not differ by antigen.

Given the association of IFN- γ with protection against chlamydia in animal model studies and several human studies, we then assessed for any correlation between the frequency or magnitude of secreted IFN- γ and age, presence of symptoms, a prior history of chlamydia, number of partners in the last 3 months, use of hormonal contraception, and presence of co-infections. None of these factors were found to be correlated with either the frequency or magnitude of the IFN- γ response (data not shown).

4. Discussion

Our study demonstrated that, in general, Th1-polarizing cytokine responses (especially IFN- γ and TNF- α) were produced at a higher frequency and magnitude than Th2-polarizing responses in stimulated PBMCs from Ct-infected women. Of the Th1-polarizing cytokines produced, levels were highest for IFN- γ , followed by TNF- α ; IL-12 production was minimal. IFN- γ levels were highest when stimulated with EB, compared to Pgp3, which suggests that cytokine levels are related,

in part, to antigen specificity (more antigens are present on the EB surface compared to the single recombinant antigen Pgp3). Given our previous finding that TNF- α , not IFN- γ , was the predominant cytokine produced by stimulated CD4⁺ T-cells [12], our current study findings suggest that in some Ct-infected individuals, there may be a significant contribution by other cell types to IFN- γ production, possibly natural killer (NK) cells. This hypothesis is further supported by a prior study demonstrating that Ct EB-stimulated PBMCs had high levels of IFN- γ production by NK cells [13]. Interestingly, another study demonstrated that NK cell function (including IFN- γ production) was impaired in some Ct-infected humans [14]. Together, these findings suggest that NK cells may contribute to IFN- γ production in some, but not all, Ct-infected individuals and warrants further investigation.

In our study, positive Th2 cytokines were both infrequent and secreted at low levels. Our results are consistent with a previous study that found IL-4 and IL-10 mRNA was produced at significantly lower levels than IFN- γ on stimulation with EB [15]. Our results appear discordant with the study by Vicetti Miguel et al., which concluded that stimulated PBMCs from Ct-infected women are primarily Th2-polarized [11]. An important distinction is that their study used intracellular cytokine staining to report a high frequency of IL-4-positive CD3⁺ proliferating cells, while our study objective was not limited to replicating CD3⁺ cells, but instead focused on measuring the increase in secreted cytokines (including IL-4) from stimulated PBMCs. Also, the PBMC stimulation length was different (48 h in their study vs 72 h in our study), which may also contribute to differences in cytokine levels.

Interestingly, the secretion of the Th1-polarizing cytokine IL-12 was low in our study which, combined with the high levels of IFN- γ produced in Ct-infected women, suggests that a significant contribution of IFN- γ production may occur through an IL-12-independent mechanism, as has been reported to occur with other intracellular pathogens [16] and may be NK cell-mediated [17]. This implies that Th1 polarization may occur through multiple mechanisms and warrants further study into how specific cell types contribute to Th1-polarized IFN- γ production.

Our study has strengths and weaknesses. To our knowledge, this is the largest study to date of Th1- and Th2-polarizing cytokine responses produced by PBMCs from Ct-infected humans. We also used a recombinant Ct-specific protein (Pgp3) to address potential concerns about cytokine responses being confounded by cross-reactivity of Ct EBs (e.g. from Ct HSP60, etc.) or non-specific responses (e.g., from Ct LPS). We acknowledge that African Americans were over-represented in our study, which could limit generalizability of our findings. We also used whole PBMCs (without monocyte depletion) in our assays and the contribution of non-lymphoid cells (i.e. NK cells, monocytes) to total IFN- γ production remains unknown. Also, cytokine studies using stimulated PBMCs *in vitro* may not completely reflect cytokine production in the mucosal environment. Future studies involving depletion of specific cell-populations in both Ct-infected and Ct-negative women will be important in defining the contribution of Th1-polarizing cytokine production by specific IFN- γ -producing populations (e.g. NK cells).

In conclusion, we showed that stimulated PBMCs from Ct-infected women produce predominantly Th1-polarizing cytokines on exposure to Ct antigens Pgp3 and EB, but not Th2-polarizing cytokines. Future studies that investigate the contribution of individual cell populations to Th1 cytokine production are warranted.

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