Published in final edited form as:

Am J Reprod Immunol. 2012 December ; 68(6): 499–506. doi:10.1111/aji.12008.

Transient detection of chlamydial-specific Th1 memory cells in the peripheral circulation of women with history of *Chlamydia trachomatis* genital tract infection

Rodolfo D. Vicetti Miguel, MD^a, Seth D. Reighard, BS^a, Jean M. Chavez, MS^a, Lorna K. Rabe, BS^b, Samantha A. Maryak, BS^a, Harold C. Wiesenfeld, MD^c, and Thomas L. Cherpes, MD^a

^aDepartment of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, United States

^bMagee-Womens Research Institute and Foundation, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, United States

^cDepartment of Obstetrics, Gynecology, and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, United States

Abstract

Problem—Development of safe and effective *Chlamydia trachomatis* vaccines requires better understanding of the host immune responses elicited by natural infection.

Method of study—Peripheral blood mononuclear cells isolated from women with or without history of genital tract chlamydial infection were stimulated with inactivated *C. trachomatis* elementary bodies (EB) in ELISPOT assays that enumerated frequencies of cells producing interferon (IFN)- γ or interleukin (IL)-17.

Results—IFN- γ -positive cells were highest among women sampled 30–60 days after diagnosis of *C. trachomatis* infection and treatment initiation, while the numbers of IFN- γ -positive cells were equally low among uninfected women and women sampled < 30 or > 60 days after diagnosis of infection. Conversely, IL-17-positive cell numbers were uniformly low among all participants.

Conclusions—Dramatically reduced numbers of *Chlamydia*-specific Th1 memory cells in the peripheral circulation of study participants sampled more than 2 months after diagnosis and initiation of treatment provides new insight into the results from *C. trachomatis* vaccine trials in which immunization with EB provided only short-lived protection. Our results also suggest that an effective vaccine against this weakly antigenic intracellular pathogen will need to generate immunological memory more durable than that elicited by natural infection.

Keywords

Chlamydia trachomatis; ELISPOT; interferon- γ ; interleukin-17; peripheral blood mononuclear cells

Introduction

Chlamydia trachomatis infection is an important risk factor for ectopic pregnancy and tubal factor infertility.¹ Although vaccination is the strategy most likely to protect women from these sequelae of infection, development of a safe and effective vaccine requires better understanding of the character and durability of *Chlamydia*-specific adaptive immune responses elicited by natural infection. The host responses that protect against *C. trachomatis* genital tract infection are also incompletely defined, but trafficking of interferon (IFN)- γ -producing CD4⁺ T cells to sites of infection is considered essential. This conclusion is based on *in vitro* studies that show the IFN- γ -induced enzyme (indoleamine 2, 3-dioxygenase) depletes tryptophan and suppresses *C. trachomatis* growth,^{2, 3} and data from *C. muridarum* murine models that indicate IFN- γ -producing CD4⁺ T cells promote clearance of the primary infection and decrease susceptibility to re-infection.⁴⁻⁶ Likewise, clinical investigation has identified a correlation between enhanced protection from incident genital tract *C. trachomatis* infection and the production of IFN- γ by peripheral blood mononuclear cells (PBMC) stimulated *ex vivo* with chlamydial antigen.⁷

Although polymorphonuclear leukocytes may dominate acute inflammatory responses elicited by endocervical *C. trachomatis* infection, the role of Th17 immunity is underexplored. Also poorly defined is the durability of *Chlamydia*-specific memory after genital tract infection is eradicated by antimicrobial therapy. As resolution of these uncertainties will help guide *Chlamydia* vaccine development, herein, PBMC from women with no history of chlamydial infection or nucleic acid amplification test (NAAT)-confirmed history of *C. trachomatis* infection were used in ELISPOT assays that identified the number of cells that produced IFN- γ or IL-17 in response to inactivated *C. trachomatis* elementary bodies (EB). We report that cells producing IL-17 in response to EB were infrequently detected among women with history of *C. trachomatis* infection. Conversely, the frequency of cells producing IFN- γ in response to EB stimulation was highest among women that were sampled 30-60 days after the diagnosis of *C. trachomatis* genital tract infection, while women sampled more than 2 months after diagnosis demonstrated numbers of IFN- γ -producing cells that were indistinguishable from the numbers seen among women who denied history of chlamydial infection. Identification of this transient *C. trachomatis*-specific memory provides insight into the high incidence of genital tract re-infection seen in young women, and suggests that *Chlamydia* vaccine research may need to develop methods that generate immunologic memory more durable than that elicited by natural infection.

Methods

Study participants

Peripheral blood was collected from non-pregnant women 18-35 years of age without history of *Chlamydia* infection or with a known date of diagnosis. Among women providing blood in the cross-sectional arm of our investigation, 15 denied history of chlamydial infection and 56 were by self-report at various time points (2-1623 days) after initiation of anti-chlamydial antimicrobial therapy. Among the 7 women in the longitudinal arm, each provided blood on the same day genital tract chlamydial infection was diagnosed and at a follow-up visit 30-60 days after completing a 1-day course of ceftriaxone and azithromycin. At the follow-up visit, *C. trachomatis* eradication was also confirmed by NAAT. In separate studies, paraffin-embedded endometrial tissue specimens from 5 women who tested negative for *C. trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* infections and 5 women with existing endocervical and endometrial *C. trachomatis* infection were used to immunohistochemically evaluate expression of T-bet and ROR γ ; the transcription factors that drive differentiation of Th1 and Th17 immunity, respectively. The University of

Pittsburgh's Institutional Review Board approved this study design, and informed consent was obtained from each woman prior to her participation.

Laboratory procedures

PBMC were isolated from peripheral venous blood samples using density gradient centrifugation, and CD8⁺ cells depleted using anti-human CD8-coated magnetic beads and MACS separation columns (Miltenyi Biotec) in accordance with manufacturer's specifications. As described elsewhere, EB were prepared from *C. trachomatis* serovar D.⁸

Prior to use in ELISPOT or multiplex assays, EB were inactivated by gamma-irradiation and used at a final concentration of 2×10^5 inclusion-forming units (IFU) per ml (loss of infectivity confirmed by the absence of inclusions when doses equivalent to 10^7 IFU were inoculated onto HeLa cell monolayers). According to manufacturer's instructions, IFN- γ and IL-17 ELISPOT assays were performed using commercially available kits (Millipore). PBMC from all 71 women enrolled in the cross-sectional arm were evaluated by IFN- γ ELISPOT assay, but sufficient cell numbers from only 50 women were available for use in the IL-17 ELISPOT assay. For either assay, 10^5 PBMC per well were plated into 96-well ELISPOT plates, followed by the addition of EB, 5 μ g/ml phytohemagglutinin (PHA) (positive controls), or media alone (negative controls). Cells were incubated 24 h at 37°C in a 5% CO₂ atmosphere, and positive cells enumerated by an ImmunoSpot® S5 Macro Analyzer (C.T.L.) were expressed as numbers of IFN- γ or IL-17 spot-forming units (SFU) per 10^6 cells. In separate experiments, 2×10^5 CD8-depleted PBMC from 22 women with NAAT-confirmed *C. trachomatis* infection were plated into single wells of 96-well plates, and incubated 96 h with EB, PHA, or media alone. Supernatants were removed after 24 h and 96 h incubation to determine IFN- γ and IL-17 levels using commercially available ELISA kits (R&D Systems) in accordance with manufacturer's instructions. In these assays, PBMC from all 22 participants responded to PHA stimulation by producing IFN- γ and IL-17 (data not shown). In addition, paraffin-embedded endometrial tissue from women with no identifiable genital tract infection or women with endocervical and endometrial *C. trachomatis* infection were immunostained with commercially available monoclonal antibodies to detect the expression of T-bet (Santa Cruz) or ROR γ (Abcam). To evaluate staining, conventional bright field images were collected using a CRi Nuance spectral analyzer (CRi). These images were used to reconstruct multiple spectral distributions and determine intensity of diaminobenzidine staining per pixel using CRi Nuance software, after which staining intensities were converted to composite false color images.

Statistical considerations

All statistical analyses were performed using Prism® 5 software (GraphPad). Increases in numbers of IFN- γ - or IL-17-producing cells in positive vs. negative controls were compared using one-tailed Wilcoxon signed rank tests. To evaluate durability of chlamydial-specific IFN- γ or IL-17 memory cells in the cross-sectional arm, women with history of *C. trachomatis* infection were placed into 3 groups based on number of days elapsed since starting anti-chlamydial antimicrobial therapy (i.e., < 30, 30-60, and > 60 days). Differences in cytokine-secreting cell numbers were evaluated using Kruskal-Wallis test, while Dunn's test for multiple comparisons was used for post-hoc analysis. Differences in supernatant concentrations of IFN- γ and IL-17 among unstimulated cells and EB-stimulated cells after 24 or 96 h incubation were evaluated using Friedman test and Dunn's test for multiple comparisons. In the longitudinal arm of the investigation, increases in the number of IFN- γ -positive cells detected at enrollment (i.e., current infection) vs. follow-up visits (i.e., after resolution of infection) were evaluated using one-tailed paired *t* tests (*p* values < 0.05 were considered statistically significant).

Results

PBMC from all women responded to stimulation with the polyclonal T cell activator PHA (numbers of IFN- γ - and IL-17-secreting cells increased 6-2026 fold compared to unstimulated paired controls) (Fig. 1). Next, stratification of cross-sectional study participants with history of genital tract *C. trachomatis* infection into 3 groups based on the number of days elapsed between diagnosis of genital tract chlamydial infection and the collection of PBMC showed that the frequency of IFN- γ positive cells was highest among the women that provided PBMC 30-60 days after diagnosis (Fig. 2). Interestingly, the number of cells producing IFN- γ in response to stimulation with EB among women from whom PBMC had been collected at earlier (< 30 days) and later (> 60 days) time points after *C. trachomatis* diagnosis were indistinguishable from numbers of IFN- γ positive cells enumerated among women denying history of chlamydial infection. On the other hand, frequencies of IL-17 positive cells detected by ELISPOT assay were uniformly low among women that denied history of infection and previously infected women that were assigned to 1 of 3 groups based on the numbers of days elapsed between diagnosis of infection and PBMC collection (Fig. 3).

To confirm ELISPOT findings that indicated *C. trachomatis* infection was not associated with Th17 memory cell development, we also measured the levels of IFN- γ and IL-17 levels in supernatants of PBMC cultures stimulated with inactivated EB. Although this stimulation dramatically increased the levels of IFN- γ in culture supernatants of women with history of *C. trachomatis* infection, only small, but statistically significant, increases in IL-17 were detected in these supernatants after 96 h incubation (Fig. 4). Moreover, while a portion of endometrial mononuclear cells from women with existing endocervical and endometrial *C. trachomatis* infection expressed the Th1 transcription factor T-bet (representative results shown in Fig. 5), none of these cells were shown to express ROR γ , the transcription factor regulating Th17 immunity (data not shown). Combined, these findings indicate that existing *C. trachomatis* infection does not polarize endometrial mononuclear cells toward Th17 differentiation, and that history of genital tract infection is not associated with the development of durable Th17 memory.

To confirm ELISPOT findings that suggested IFN- γ -producing cell numbers were higher among women 1-2 months after treatment than among women with current *C. trachomatis* infection, we performed a longitudinal investigation in which PBMC were collected from study participants on the day they were diagnosed with endocervical *C. trachomatis* infection and again approximately 1 month later. In each study participant, infection was successfully eradicated by a 1-day course of ceftriaxone and azithromycin that was administered on the day of diagnosis (*C. trachomatis* diagnosis and eradication were both confirmed by NAAT). As predicted by our cross-sectional results, these additional ELISPOT assays showed that IFN- γ -producing cells were significantly higher in PBMC collected at the follow-up visit (i.e., 30-60 days after the diagnosis and initiation of anti-chlamydial antimicrobial therapy) than the numbers seen in PBMC collected from these same women at enrollment ($p = 0.0002$) (Fig. 6).

Discussion

Our data indicate that Th1 immune responses against *C. trachomatis* develop slowly, are altered by antimicrobial therapy, and are impermanent. Such findings are consistent with natural history studies that reveal untreated *C. trachomatis* infection of the female genital tract often requires months to resolve,⁹ and results from trachoma vaccine trials that saw increased protection in the first 3 months after immunization but diminished protection thereafter.¹⁰ Our discovery that the numbers of CD8-depleted PBMC producing IFN- γ in

response to EB were equivalent among women denying history of chlamydial infection and *C. trachomatis*-infected women 2 months after initiation of anti-chlamydial antimicrobial therapy also provides new support for the “arrested immunity” hypothesis, which argues that *C. trachomatis* infection control programs promoting earlier diagnosis and treatment of genital tract infection ultimately lead to increased populational *Chlamydia* disease prevalence and rates of re-infection by interrupting formation of protective immunity.^{11, 12} Our findings, thus, corroborate 2 tenets that were central to formation of this hypothesis; that *Chlamydia*-specific adaptive immunity is slow to develop and diminished by antimicrobial therapy. As mice treated with doxycycline within the first 10 days of primary intravaginal chlamydial infection were less protected from chlamydial reinfection than untreated controls,¹³ our results also support the need for studies that can establish if transient presence of *Chlamydia*-specific Th1 memory in women, regardless if it is sequelae to antimicrobial therapy administration, is similarly linked to increased susceptibility to genital tract re-infection.

Perhaps more surprising, our findings also indicate that numbers of chlamydial-specific, IFN- γ -producing PBMC were markedly higher 1-2 months after treatment compared to numbers seen among infected women before initiation of anti-chlamydial antimicrobial therapy. This implies that *en masse* antimicrobial-mediated killing of *C. trachomatis* may induce more robust Th1 immunity than gradual eradication of the organism by natural host defense mechanisms, and that anti-chlamydial immunity in the female genital tract may have evolved to control *Chlamydia* without eliciting overly exuberant tissue inflammation. This prospect is consistent with the typically asymptomatic presentation of *C. trachomatis* genital tract infection in women and trachoma vaccine studies among humans and nonhuman primates in which live or formalininactivated EB vaccines exacerbated disease expression and elicited pathologic cell-mediated immune responses¹⁴⁻¹⁷. Thus, our results also support the possibility that a vaccine augmenting IFN- γ production by *Chlamydia*-specific T cells could simultaneously confer protection and promote immunopathological damage in the female genital tract upon repetitive exposure to the organism.

As opposed to IFN- γ ELISPOT results, numbers of IL-17-producing cells were uniformly low among women with or without history of *Chlamydia* infection. This implies that Th17 cells are only infrequently generated by *C. trachomatis* genital tract infection or that chlamydial-specific IL-17-producing cells are less likely than chlamydial-specific IFN- γ producing cells to appear in peripheral circulation. These findings are inconsistent with a recently published study in which women with current *C. trachomatis* genital tract infection showed increased levels of IL-17 in cervical wash samples and in the supernatants from PBMC cultures stimulated with EB¹⁸. Because of these discordant results, we performed additional studies with blood and endometrial tissue from *C. trachomatis*-infected women that seemed to confirm that Th17 immunity is not a significant part of the host response to genital tract infection. However, it is possible that the CD8⁺ PBMC (e.g., T cells and innate-like T cells) we depleted are the cell types primarily responsible for IL-17 production,¹⁹ that numbers of IL-17-producing cells were below the level of detection for our ELISPOT assay, or that Th17 cells require more than the 24 h incubation provided by our ELISPOT assay protocol to elicit detectable IL-17 levels. This latter scenario may be the more plausible, as we found low but detectable levels of IL-17 in PBMC supernatants that were stimulated 96 h with inactivated EB. Conversely, these IL-17 levels were about 500 times lower than the IFN- γ levels detected in these same culture supernatants. Taken together, our results indicate that while Th17 immunity is generated in response to *C. trachomatis* genital tract infection, its contribution to host defense against chronic infection may be relatively minor.

In summary, our results indicate that the numbers of *Chlamydia*-specific Th1 cells increase in the peripheral circulation of women 1 month after initiation of antimicrobial therapy but

diminish thereafter. There are, however, limitations associated with our study. We did not explore cell-mediated immune responses to *C. trachomatis* in cervical mucosal tissue, the initial site of infection in the female genital tract. We are also unable to assess any effects of the duration of infection prior to diagnosis on the persistence of immunological memory; the efficiency of partner notification and treatment; or the correlation between magnitude or durability of anti-chlamydial immune responses and the susceptibility to *C. trachomatis* re-infection. Clearly, no simple relationships exist between *Chlamydia* genital tract infection, its treatment, and treatment-induced alteration of host immunity, but resolution of these uncertainties is paramount to the development of safe and effective *C. trachomatis* vaccines.

Acknowledgments

Work was supported by the University of Pittsburgh's Departments of Obstetrics, Gynecology and Reproductive Sciences and Pediatrics and the National Institute of Allergy and Infectious Diseases (U19AI084024). Authors thank Bridget Leyland for helping develop our ELISPOT assay and Kathleen Cieply for assistance with the CRI Nuance systems. Data in this manuscript was shown at the meeting of the Sexually Transmitted Infections Cooperative Research Centers of the National Institute of Allergy and Infectious Diseases, Chapel Hill, NC, Nov. 1-2, 2011.

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Fig. 1. PBMC from each study participant increased production of IFN- γ and IL-17 in response to polyclonal T cell activation

CD8-depleted PBMC were plated into individual wells (10^5 PBMC/well) of commercially available IFN- γ and IL-17 ELISPOT plates, and PHA ($5\mu\text{g/ml}$) was added. After 24 h incubation, ELISPOT plates were developed in accordance with manufacturer's instructions. Numbers of (A) IFN- γ and (B) IL-17 spot-forming units (SFU) per 10^6 cells were increased 6-2026 fold compared to the numbers of these cell detected among wells not receiving PHA (representative results from 28 study participants displayed).

Fig. 2. Frequency of PBMC producing IFN- γ in response to EB was highest among women enrolled 30-60 days after diagnosis of *C. trachomatis* infection

CD8-depleted PBMC from women denying history of chlamydial infection or with nucleic acid amplification test (NAAT)-confirmed history of *C. trachomatis* genital tract infection were stimulated 24 h with inactivated *C. trachomatis* serovar D elementary bodies (EB), and numbers of IFN- γ -producing cells were measured by ELISPOT assay (n = 71). For analysis purposes, women with history of infection were grouped into those diagnosed with *C. trachomatis* genital tract infection either < 30, 30-60, or > 60 days before PBMC collection. Groups were compared using Kruskal-Wallis one-way analysis of variance and Dunn's test for multiple comparisons. Open circles represent results from women denying history of chlamydial infection, while grey circles represent results from women with history of *C. trachomatis* infection (horizontal bars indicate median values for each group) (** indicates $p < 0.01$ compared to all other groups).

Fig. 3. Frequency of PBMC producing IL-17 cells in response to EB was indistinguishable among women denying history of *C. trachomatis* infection and those previously diagnosed with infection

CD8-depleted PBMC from women denying history of *C. trachomatis* infection or women with NAAT-confirmed history of chlamydial infection were stimulated for 24 hours with inactivated *C. trachomatis* serovar D EB, and numbers of IL-17-producing cells were then measured by ELISPOT assay ($n = 50$). For analysis purposes, women with history of infection were grouped into those < 30, 30-60, or > 60 days post diagnosis of *C. trachomatis* genital tract infection. As evaluated by Kruskal-Wallis one-way analysis of variance, frequencies of IL-17 positive cells were statistically equivalent between groups. Open circles represent results from women denying history of chlamydial infection; grey circles represent results from women with history of *C. trachomatis* infection (horizontal bars indicate median values for each group).

Fig. 4. More IFN- γ than IL-17 was produced by PBMC isolated from women with history of genital tract *C. trachomatis* infection when stimulated *ex vivo* with inactivated EB
 CD8-depleted PBMC isolated from women with NAAT-confirmed history of *C. trachomatis* genital tract infection (n=22) were plated in 96-well plates (2×10^5 PBMC/well) and incubated 96 h in the presence of inactivated EB or complete media. Supernatants were sampled 24 and 96 h after addition of EB, and concentrations of (A) IFN - γ and (B) IL-17 determined using commercially available ELISA kits according to manufacturer's instructions. Cytokine concentrations were compared using Kruskal-Wallis one-way analysis of variance and Dunn's test for multiple comparisons. Open circles represent results from samples that were not exposed to chlamydial antigens, while grey circles represent samples stimulated with inactivated EB (horizontal bars indicate median values for each group) (***) indicates $p < 0.001$ compared to all other groups).

Fig. 5. Existing *C. trachomatis* infection is associated with increased expression of the Th1 transcription factor T-bet by stromal endometrial mononuclear cells

Paraffin-embedded endometrial tissue from women negative for *C. trachomatis*, *N. gonorrhea*, and *T. vaginalis* infection (n=5) or women diagnosed with dual endocervical and endometrial *C. trachomatis* infection (n=5) were used to evaluate stromal cell expression of T-bet by immunohistochemistry. (A) T-bet positive stromal cells were infrequently found in the endometria of uninfected controls. (B-D) Conversely, aggregates of T-bet positive cells were observed in the endometrial stroma of women with *C. trachomatis* infection.

Representative micrographs are shown: top panels show micrographs at $\times 200$ magnification; panel (C) shows $\times 400$ magnification; and panel (D) shows $\times 1000$ magnification. Boxes are used to highlight image areas displayed in subsequent panels. As detailed in Methods, micrographs are composite false color images.

Fig. 6. Significantly fewer PBMC that secreted IFN- γ in response to EB were found among women with existing *C. trachomatis* infection than were detected in these same women 1-2 months after infection was eradicated by antimicrobial therapy

CD8-depleted PBMC that were isolated from women at enrollment (i.e., with current infection) and at follow-up (i.e., 1-2 months after infection had been eradicated by a 1-day course of ceftriaxone and azithromycin) were stimulated 24 h with inactivated EB, and IFN- γ -producing cell numbers were enumerated by ELISPOT assay ($n = 7$). As evaluated by one-tailed paired t test, IFN- γ -positive cell numbers were significantly higher at the follow-up visit compared to the numbers of these cells detected at enrollment (connected circles designate results from individual women).