Frequency and Magnitude of *Chlamydia trachomatis* Elementary Body– and Heat Shock Protein 60–Stimulated Interferon γ Responses in Peripheral Blood Mononuclear Cells and Endometrial Biopsy Samples from Women with High Exposure to Infection

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Background. Cellular immune responses characterized by interferon γ (IFN-γ) production enhance clearance and confer protective immunity against *Chlamydia trachomatis* infection but have not been simultaneously investigated in systemic and mucosal compartments.

Methods. With use of the IFN-γ enzyme-linked immunosorbent spot assay, we investigated immune responses to *Chlamydia* elementary body (EB) and 3 genotypically variant heat shock protein 60 (CHSP60) antigens using peripheral blood mononuclear cells and endometrial mononuclear cells obtained from a female sex worker cohort with high levels of exposure to *C. trachomatis*.

Results. Although we observed a marginally higher frequency of IFN-γ responses to EB in peripheral blood mononuclear cells, compared with the frequency in endometrial mononuclear cells, the magnitudes of systemic and mucosal responses were similar except for preferential targeting of CHSP60 type 2 by endometrial mononuclear cells. Systemic and mucosal responses were highly correlated for EB and CHSP60 types 1 and 2 but not type 3. The frequency and magnitude of systemic responses specific for EB and CHSP60 type 1 were greater for $CD4^+$ T cells than they were for $CD8^+$ T cells, whereas preferential targeting by CHSP60 types 2 and 3 was undetectable. IFN-γ response to CHSP60 type 1 by peripheral blood mononuclear cells was inversely correlated with systemic antibody titers to CHSP60 type 1.

Conclusion. Systemic and mucosal IFN-γ responses are correlated, with preferential systemic targeting of $CD4^+$ T cells. Furthermore, CHSP60 type 1 response is largely $CD4^+$ T cell mediated and follows discrete T helper 1 and T helper 2 pathways.

There is enormous evidence for diverse antibody and T cell responses during *Chlamydia trachomatis* infection; however, this evidence has not been translated into improvements in public health through the development of vaccines or immunodiagnostic assays [1–5]. In fact, persistent infection and reinfection remain too common and often lead to immunopathology [6]. Clearly, our understanding of *C. trachomatis* immunobiology re-

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mains incomplete, yet establishing protective immune responses is important to the development of effective and sustainable interventions to prevent infection and related sequelae.

Cellular immune responses characterized by interferon γ (IFN-γ) production confer high levels of protective immunity in animal models of genital *C. trachomatis* infection [2, 7–9] and prevent severe conjuctival scarring in humans with trachoma [7]. Both $CD4^+$ and $CD8^+$ T cells have a protective role in *C. trachomatis* immunity [10–13], although evidence supports $CD4^+$ T cells as the dominant effectors [3, 14–19]. Tissue-based studies involving nonhuman primates have demonstrated induction of T helper 1 (Th1) cytokines mRNA after single and repeated chlamydial infection of salpingeal tissue [20–22], which suggests that Th1 cytokines may promote immune-mediated clearance of infection. In murine models, anti–IFN-γ antibody treatment resulted in significantly prolonged infection, whereas passive administration of IFN-γ to chronically infected nude/nude mice caused resolution of infection [10]. IFN-γ gene knockout or IFN-γ receptor gene knockout mice showed impaired immunity to *Chlamydia* species and developed disseminated infection, whereas interleukin (IL)–10 gene knockout mice exhibited accelerated Th1 cytokine responses with enhanced immunity and reduced tissue pathology [23].

In vitro studies of peripheral blood mononuclear cells (PBMCs) obtained from human subjects with and without trachoma suggest that $CD4^+$ T cells that preferentially express Th1 cytokines are important in preventing trachomatous scarring [24]. Furthermore, IFN-γ responses to *Chlamydia* heat shock protein 60 (CHSP60) are significantly associated with reduced risk for infection [25], whereas repeated chlamydial infections and/or pelvic inflammatory disease (PID) are associated with lower CHSP60-specific IFN-γ responses [26]. Thus, antigen-specific $CD4^+$ T cells that secrete IFN-γ could be important in mediating *C. trachomatis* immunity, and subunit vaccine development will depend on identifying chlamydial antigens that elicit such protective responses.

CHSP60, which is one of the most immunogenic *C. trachomatis* proteins, is found in the elementary and reticulate bodies, where it is encoded by 3 genes that are expressed independently and differentially in active versus persistent *C. trachomatis* infections (i.e., Ct110, Ct604, and Ct755 encoding CHSP60-1, CHSP60-2, and CHSP60-3, respectively) [27]. Ct110 is associated with synovial inflammation in chlamydia-associated arthritis [28], Ct755 is abundantly expressed during active infection, and Ct604 is associated with establishment and/or maintenance of persistent infection. Because of molecular mimicry, chronic antigenic stimulation, or overproduction during persistent infection, CHSP60 is speculated to incite immune responses that induce immunopathology [29–31] associated with persistent *C. trachomatis* upper genital tract infection [32–35]. In particular, high titers of serum antibodies to CHSP60 correlate with increased risks for PID [36], scarring trachoma [37], and tubal infertility [38–41]. A central role for CHSP60 in chlamydia-associated immune-mediated inflammation is supported by studies that correlate the most-severe *C. trachomatis* disease (PID, fallopian tube obstruction, and ectopic pregnancy) with the highest levels of anti-CHSP60 antibodies [42, 43]. Thus, it is surprising and somewhat paradoxical that IFN-γ cellular immune responses to CHSP60-1 have also been associated with protection against cervical infection and against PID.

Because *C. trachomatis* is an obligate intracellular pathogen of epithelial cells, both mucosal and systemic immune responses are relevant for immunity and immunopathobiology. However, these 2 immune compartments have not been simultaneously tested in humans. This study was designed to simultaneously investigate the frequency and the magnitude of IFN-γ cellular immune response to *C. trachomatis* elementary body (EB) and CHSP60 with use of endometrial mononuclear cells (EMCs) and PBMCs collected from a cohort of women with high levels of exposure to *C. trachomatis.*

MATERIALS AND METHODS

Study population. The study protocol was approved by the institutional review boards for human subjects' research at the University of Nairobi (Nairobi, Kenya), University of Washington (Seattle, Washington), and University of California, San Francisco. All procedures were followed in accordance with ethical standards for human experimentation established by the Declaration of Helsinki of 1975 (revised in 1983). A cohort of 299 female commercial sex workers, aged 18–35 years, was established in the Kariobangi City Council Clinic of Nairobi to study the epidemiological and immunobiological profiles of sexually transmitted infections. After written, informed consent, collection of demographic and clinical data and a general physical and pelvic examination were done. Evaluation of incident sexually transmitted infections was done every 2 months. At the initial visit, cervical specimens were obtained for detection of *C. trachomatis* and *Neisseria gonorrhoeae,* plasma and endocervical mucus samples were obtained for measurement of EB and CHSP60 antibodies, and serum samples were obtained for human immunodeficiency virus type 1 (HIV-1) serological testing. Heparinized blood and endometrial aspirate samples were collected for IFN-γ enzyme-linked immunosorbent spot (Elispot) assay. Plasma and PBMC samples for evaluation of antibody and cellular immune responses to *C. trachomatis* were obtained from a convenience sample of 46 women. A convenience sample refers to the maximum number of samples that could be adequately processed for functional T cell assays, taking into consideration the availability of resources and manpower at the time of the study. We obtained sufficient endometrial biopsy samples from 31 of the 46 women to conduct T cell assays. Thus, comparisons of systemic and mucosal cellular immune responses are limited

to 31 women from whom both PBMCs and sufficient EMCs were obtained.

C. trachomatis *antigens.* *C. trachomatis* antigens were isolated and purified at the British Columbia Centre for Disease Control, Canada, as described elsewhere [25]. EB was harvested from serovars E, F, K, and L2 of *C. trachomatis,* were subjected to ultraviolet radiation for 30 min, and were used at a final concentration of 2×10^6 inclusion-forming units per mL. CHSP60-1, CHSP60-2, and CHSP60-3 antigens were cloned in *Escherichia coli* on the basis of the gene sequences from serovar D. The genes were cloned in pET vector (NOVagen), the histidine-tagged recombinant protein expressed in *E. coli* BL21, and purified by use of a nickel-nitrilotriacetic acid agarose column (Qiagen), which only binds the histidine-tagged proteins. The columns were thoroughly washed to eliminate any possible contamination with bacterial lipopolysaccharide. CHSP60 antigens were used at a final concentration of 1.0 mg/mL.

Isolation of CD4⁺ and CD8⁺ T cells from PBMCs. PBMCs were isolated from heparinized blood by density gradient centrifugation over Ficoll-hypaque Lymphoprep (Nycomed Pharma). $CD4^+$ and $CD8^+$ T cell depletions were performed using anti-$CD4^+$ or anti-$CD8^+$ T cell mAbs conjugated to ferrous magnetic Dynal beads (Dynabeads M-450; Dynal) in accordance with the manufacturer's specifications.

Isolation of mononuclear cells from endometrial tissue. Endometrial biopsy samples were collected using an endometrial pipele (Unimar), which is a minimally invasive, standard gynecological procedure that allows sampling of endometrial tissues by suction and has been shown to have a sensitivity of 97.5% for the diagnosis of endometrial cancer. Nevertheless, one limitation of this method is that only a fraction of the endometrial tissue is obtained. Although endometrial biopsies were performed for all 299 patients who were recruited into the study, cell numbers sufficient to perform immune assays were obtained from only 31 women. The tissue specimens were washed with phosphate-buffered saline by centrifugation at 1500 rpm for 10 min, transferred into sterile 35-mm mesh pore Medicon (Becton Dickinson), and homogenized for 5 min in a Medimachine (Becton Dickinson). The homogenized tissue suspension was layered onto ficoll-hypaque, and EMCs were obtained as described above for PBMCs.

IFN-γ Elispot assay. Elispot assays were performed according to the manufacturer's instructions (Mabtech). In brief, 96-well nitro-cellulose bottomed Microtiter plates (Maipn45; Millipore) were coated with 15 mg/mL of anti-human IFN-γ monoclonal antibody (Mabtech) for 2 h at 37°C and were blocked with Roswell Park Memorial Institute 1640 medium; 2×10^5 cells were cultured in duplicate, either in the presence of antigen, positive control (10 μM phytohemagglutinin), or negative control (phosphate-buffered saline). We tested the following cell preparations: total PBMCs (undepleted), $CD4^+$ T cell–depleted PBMCs (representing a $CD8^+$ T cell response), $CD8^+$ T cell–depleted PBMCs (representing a $CD4^+$ T cell response), and total (undepleted) EMCs. The plates were incubated for 15–18 h at 37°C, and spots were detected after incubation with biotinylated anti-human IFN-γ monoclonal antibody (mAb 7-B6–1 biotin; Mabtech) and streptavidin conjugated to alkaline phosphatise. Spots were enumerated on an AID Elispot reader and were expressed as spot-forming units per 10^6 cells. A positive result was defined as ⩾50 *C. trachomatis* spot-forming units per 10^6 cells, with the number of cells at least 3 times higher than the number of the unstimulated cells.

Measurement of antibodies to EB and CHSP60 antigens. Antibodies to EB and CHSP60 were measured with a modified enzyme-linked immunosorbent assay, as described elsewhere [34]. In brief, 1:200 diluted plasma samples were added to 96-well plates coated with recombinant CHSP60-1, CHSP60-2, or CHSP60-3. Antibodies bound to the antigen were detected with biotin-conjugated anti-human IgG, followed by addition of streptavidin and the substrate (Sigma). An optical density⩾0.2 at 405 nm was considered to indicate a positive result.

Statistical analyses. Statistical analyses were performed using Stata software, version 9.0 (Stata). R, version 2.2.0, was used for a portion of graphical output. Comparisons between the convenience sample and the rest of the parent population were assessed using Student's *t* test. Comparison of PBMC and EMC binary responses to the 4 antigens was analyzed using conditional (fixed-effects) logistic regression. Differences in the log-transformed values of IFN-γ responses to the antigens between PBMCs and EMCs and between $CD4^+$ and $CD8^+$ T cell populations were determined using Student's *t* test. A *P* value ⩽.05 was considered to be statistically significant. Comparisons of antibody and IFN-γ responses were completed using McNemar test, assuming a lack of independence from each other.

RESULTS

Cohort characteristics. Table 1 compares the sociodemographic, behavioral, and microbiological characteristics of the convenience sample of 46 women who were tested for IFN-γ responses in PBMCs with those of the remaining 253 women, and it also compares the characteristics of 31 women tested for IFN-γ responses in both PBMCs and EMCs with those of the remaining 268 women in the original cohort (table 1). Overall, these characteristics were statistically similar between the groups. The cohort comprised young, unmarried women (mean age, 23 years) with a mean of 3 years of prostitution and reporting a mean of 11 clients per week. The baseline prevalences of *C. trachomatis* and *N. gonorrhoeae* were 4% and 2%, respectively, and 22% of the cohort (10 of 45 patients) had test results positive for HIV-1. All incident *C. trachomatis* infections occurred prior to collection of PBMC and EMC specimens and no subject was infected at the time of mononuclear cell collection.

Table 1. Sociodemographic characteristics, sexual history, and laboratory results at baseline in a cohort of commercial sex workers in Nairobi, Kenya.

Variable	Women from whom PBMCs were obtained (n = 46)	Women from whom PBMCs were not obtained (n = 253)	P[a]	Women from whom PBMCs and evaluable endometrial biopsy samples were obtained (n = 31)	Women from whom PBMCs and evaluable endometrial biopsy samples were not obtained (n = 268)	P[b]
Age, mean ± SD, years	23.7 ± 5.5	23.9 ± 5.5	.81	24.6 ± 6.2	23.8 ± 5.2	.38
Duration of prostitution, mean ± SD, years	3.3 ± 2.7	4.0 ± 3.2	.17	3.4 ± 3.0	4.0 ± 3.2	.32
Clients per week, mean ± SD, no. of clients	11.6 ± 7.3	11.7 ± 7.7	.95	10.5 ± 6.3	11.8 ± 7.7	.37
Marital status						
Single (never married)	31/46 (67)	174/252 (69)	.87	20/31 (65)	184/267 (69)	.62
Married	1/46 (2)	0	.02	1/31 (3)	0	.003
Widowed, divorced, or separated	14/46 (30)	78/252 (31)	.90	10/31 (32)	83/267 (31)	.89
Frequency of condom usage						
<75% of sexual acts	28/46 (61)	148/251 (59)	.75	18/31 (58)	157/266 (59)	.92
75%– 99% of sexual acts	6/46 (13)	51/251 (20)	.25	6/31 (19)	51/266 (19)	.98
Always	12/46 (26)	53/251 (21)	.45	7/31 (23)	58/266 (22)	.92
HIV-1 infected	10/45 (22)	80/243 (33)	.17	8/30 (27)	80/258 (31)	.60
Chlamydia trachomatis infection	2/45 (4)	22/251 (9)	.33	2/30 (7)	22/266 (8)	.76
Neisseria gonorrhoeae infection	1/45 (2)	17/251 (7)	.24	1/30 (3)	17/266 (6)	.51

NOTE. Data are proportion (%) of patients, unless otherwise indicated. HIV-1, human immunodeficiency virus type 1; PBMCs, peripheral blood mononuclear cells; SD, standard deviation.

[a] Women from whom PBMCs were obtained versus women from whom PBMCs were not obtained.

[b] Women from whom PBMCs and evaluable endometrial biopsy samples were obtained versus women from whom PBMCs and evaluable endometrial biopsy samples were not obtained.

Frequency of* C. trachomatis *antigen–specific IFN-γ responses in PBMCs and EMCs. To determine the frequency and magnitude of IFN-γ secretion by mononuclear cells, PBMCs and EMCs were stimulated with *C. trachomatis* EB and the 3 CHSP60 antigens with use of an IFN-γ Elispot assay. The number of women with an antigen-specific IFN-γ response to EB in PBMCs was greater than the number with a response in EMCs (odds ratio, 2.5; 95% confidence interval [CI], 0.78–8.0), which indicates a potentially greater concentration of *C. trachomatis* IFN-γ–responsive cells in PBMCs than in EMCs (figure 1).

To determine whether IFN-γ responses to CHSP60 antigens were different in the systemic and endometrial compartments, we compared IFN-γ responses to these antigenic variants among women with a positive IFN-γ response to EB in PBMCs and EMCs, respectively. Among those individuals with a positive IFN-γ response to EB, there were subsets in both systemic and mucosal compartments that had positive responses to ⩾1 of the 3 CHSP60 antigens (figure 1). Table 2 summarizes the proportional differences in the frequency of response to each of the CHSP60 antigens, given a positive response to EB. Although there were no statisticallly significant differences between the proportions of positive responses to either CHSP60-1, CHSP60-2, or CHSP60-3 in PBMCs, compared with the response in EMCs, EMCs tended to have a positive IFN-γ response after stimulation with each of the 3 CHSP60 antigens more often than did PBMCs. However, because there were women with a positive response to CHSP60 antigens who did not have an EB response, these proportional differences do not reflect the overall differences in frequency of response to CHSP60 antigens in the PBMC and EMC compartments, because they are conditional to having a positive EB response.

Magnitude of IFN-γ responses in PBMCs and EMCs. To compare the magnitude of antigen-specific responses elicited between the systemic and mucosal compartments, we analyzed log-transformed values of the IFN-γ spot-forming units obtained after stimulation of PBMCs and EMCs with the 4 *C. trachomatis* antigens. The magnitude of IFN-γ responses to EB, CHSP60-1, and CHSP60-3 in PBMCs and EMCs were statistically indistinct, whereas the level of response to CHSP60-2 was greater in EMCs ($P = .05$), suggesting preferential targeting of CHSP60-2 by EMCs, compared with the response in PBMCs (figure 2*A*). However, IFN-γ responses to EB in PBMCs and EMCs ($r = 0.52$; $P = .002$), CHSP60-1 ($r = 0.42$; $P = .02$), and CHSP60-2 ($r = 0.49$; $P = .005$)—but not CHSP60-3 ($r = 0.24$; $P = .19$)—were highly correlated (figure 2*B*). The preferential targeting of CHSP60-2 by EMCs, as seen in figure 2*A*, is not consistent with the significant correlation of EMC and PBMC responses to this antigen, as depicted in figure 2*B*. In addition, the lack of correlation of EMC and PBMC responses to

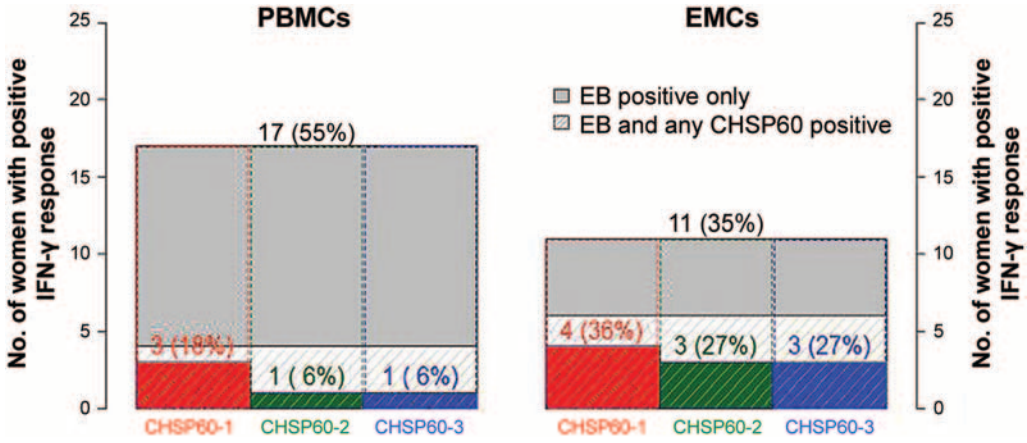

Figure 1. Frequency of interferon (IFN)–γ responses to *Chlamydia trachomatis* antigens. Peripheral blood mononuclear cells (PBMCs) and endometrial mononuclear cells (EMCs) from 31 women were tested for chlamydia antigen–specific IFN-γ secretion by enzyme-linked immunosorbent spot assay. The frequencies of systemic versus localized mucosal IFN-γ response to chlamydia elementary body (EB) and *Chlamydia* heat shock protein 60 (CHSP60) are represented by the heights of the respective bar graphs. Data for women with a positive IFN-γ response in PBMCs are shown in the panel on the left, and data for women with a positive IFN-γ response in EMCs are shown in the panel on the right. Overlaid on this graph are the frequencies of CHSP60-specific IFN-γ response in PMBCs, compared with the responses in EMCs, given a positive response to EB. Red, CHSP60-1; green, CHSP60-2; blue, CHSP60-3. Among individuals in the PBMC group, 1 (6%) had a positive response to both CHSP60-1 and CHSP60-3. Among individuals in the EMC group, 3 (27%) had a positive response to both CHSP60-1 and CHSP60-2, 1 (9%) had a positive response to both CHSP60-1 and CHSP60-3, and 1 (9%) had a positive response to both CHSP60-2 and CHSP60-3. The number of responses to each CHSP60 antigen is listed at the top of each column, with the rounded percentage in parentheses.

CHSP60-3 antigen is not consistent with what is observed in figure 2*A*, where the magnitude of IFN-γ response to this antigen is higher in EMCs than in PBMCs (reaching borderline statistical significance at $P = .06$). These conflicting observations may be attributable to low statistical power with respect to the responses to CHSP60-2 and CHSP60-3 antigens.

Frequency and magnitude of IFN-γ responses in CD4⁺ and CD8⁺ T cells. T cell subset dominance to EB and CHSP60 antigen stimulation was gauged by assessing the frequency and magnitude of responses elicited by $CD4^+$ T cell– or $CD8^+$ T cell–depleted PBMCs. Sufficient EMCs were not available to perform depletion experiments; therefore, this analysis was applied to PBMC samples only (for 46 patients). There was no statistically significant difference between the number of women with a positive IFN-γ response in $CD8^+$ T cell–depleted PBMCs (23 [50.0%] of 46 women) and those with a positive IFN-γ response in $CD4^+$ T cell–depleted PBMCs (19 [41.3%] of 46 women). The Elispot data were log-transformed, and the strengths of the 2 subsets of the T cell–mediated immune response were compared (figure 3). After stimulation with EB and CHSP60-1 antigens, IFN-γ secretion by $CD4^+$ T cells was significantly greater than secretion by $CD8^+$ T cells ($P < .001$ and $P = .02$, respectively), suggesting a predominant role for the $CD4^+$ T helper cells with respect to EB and CHSP60-1 antigen recognition. There were no statistically significant differences in the magnitude of IFN-γ secretion by $CD4^+$ and $CD8^+$ T cells when stimulated with the CHSP60-2 and CHSP60-3 antigens; this demonstrated a lack of preference for one subset over the other for these 2 immunorecessive chlamydial antigens. This finding may, however, be confounded by low statistical power for the CHSP60-2 and CHSP60-3 antigens, because only 9 and 7 PBMC specimens had a positive IFN-γ response to each antigen, respectively.

Correlation of CHSP60-1 antibodies and CHSP60-1–specific IFN-γ responses. Because of the association of CHSP60-1 antibodies with chlamydia disease sequelae and CHSP60-1–specific IFN-γ responses with protection, we compared how

Table 2. Mucosal and systemic proportional differences in interferon (IFN)–γ response to *Chlamydia* heat shock protein 60 (CHSP60), given a positive response to elementary body.

Antigen	Proportion (%) of women		
	With IFN-γ response in PBMCs	With IFN-γ response in EMCs	Difference,[a] % (95% CI)
CHSP60-1	3/17 (17.6)	4/11 (36.4)	19 (−15 to 52)
CHSP60-2	1/17 (5.8)	3/11 (27.3)	21 (−7 to 50)
CHSP60-3	1/17 (5.8)	3/11 (27.3)	21 (−7 to 50)

NOTE. CI, confidence interval; EMC, endometrial mononuclear cell; PBMC, peripheral blood mononuclear cell.

[a] Difference in proportions, calculated by subtracting the percentage of women with IFN-γ response in PBMCs from the percentage of women with IFN-γ response in EMCs. Data are rounded to the nearest percentage. Comparisons assume the responses between the 2 compartments to be independent. Conditional (fixed-effects) logistic regression models were used to control for unobserved heterogeneity between the systemic and mucosal compartments.

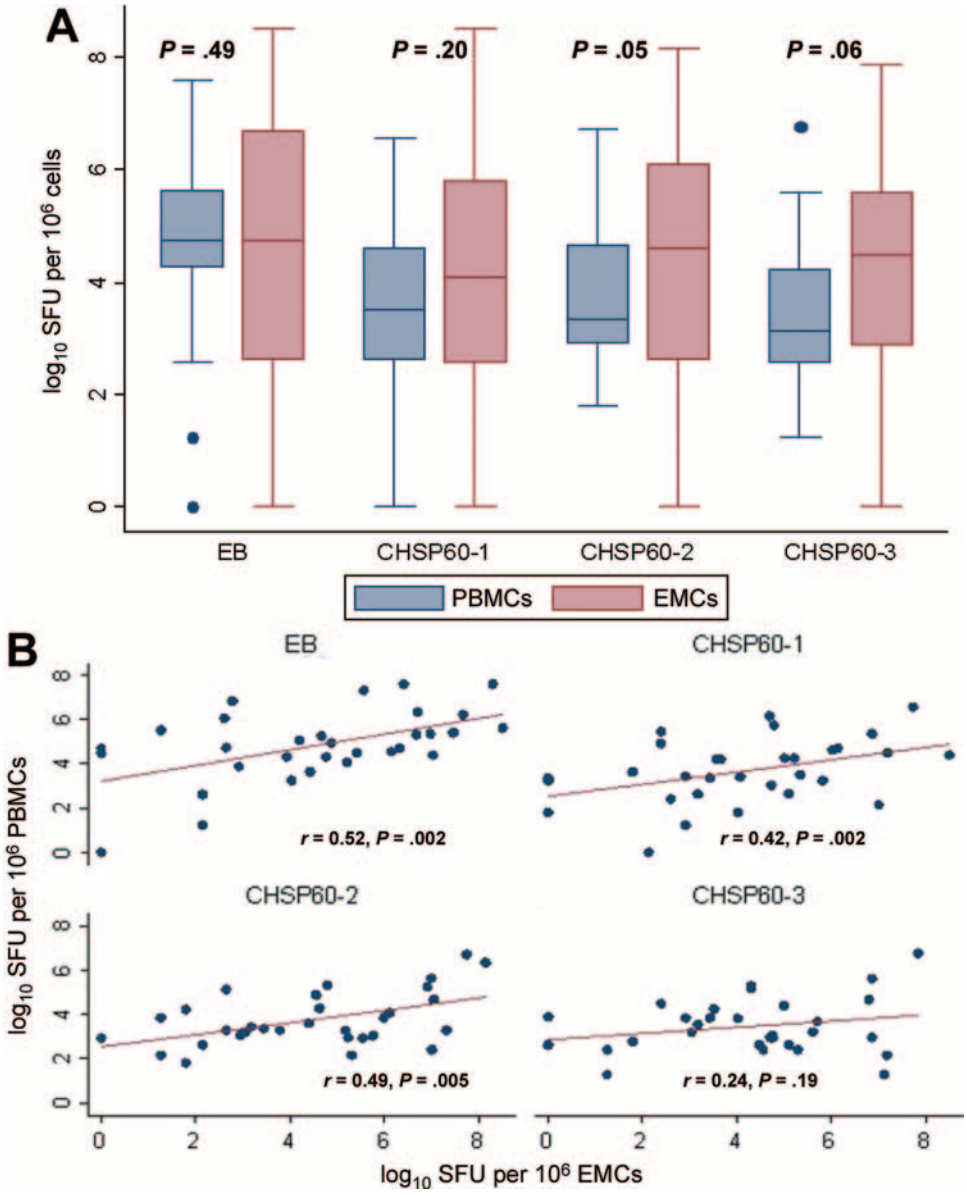

Figure 2. Magnitude and variability of systemic and mucosal interferon (IFN)–γ responses. Data are for 31 women. *A,* Tests for statistical significance were performed after log-transformation of the IFN-γ spot-forming units (SFU) obtained after stimulation of peripheral blood mononuclear cells (PMBCs) and endometrial mononuclear cells (EMCs) with *Chlamydia trachomatis* elementary body (EB), *Chlamydia* heat shock protein 60 (CHSP60)–1, CHSP60-2, and CHSP60-3 in an enzyme-linked immunosorbent spot assay. Horizontal lines in the boxes indicate the median values; the bases and tops of the boxes indicate the 25th and 75th percentiles, respectively; the whiskers indicate the ranges, with outliers represented by single points. *B,* Correlation of the magnitude of PBMC and EMC IFN-γ response for each antigen. Log-transformed values of IFN-γ SFU obtained from PBMCs and EMCs after stimulation with the chlamydia antigens as indicated.

these 2 arms of the immune response are interrelated. In addition to the 31 patients with both PBMC and EMC results, we included 15 women with PBMC data only that were collected during the same period. In this sample of 46 women, CHSP60-1–stimulated IFN-γ response in PBMCs was inversely correlated with CHSP60-1 antibody (odds ratio, 0.08; 95% CI, 0.002–0.51); remaining antigens exhibited no significant correlations of antibody levels with IFN-γ production (table 3).

DISCUSSION

C. trachomatis infection readily elicits T cell and B cell responses that correlate with specific phenotypes of resistance and susceptibility. In particular, high titers of *C. trachomatis* EB and CHSP60 antibodies correlate with severe forms of disease [36–41], and Th2 cellular immune responses to CHSP60, characterized by IL-4 and IL-10 secretion, are linked to severe trachomatous scarring during trachoma [24]. IFN-γ responses to CHSP60

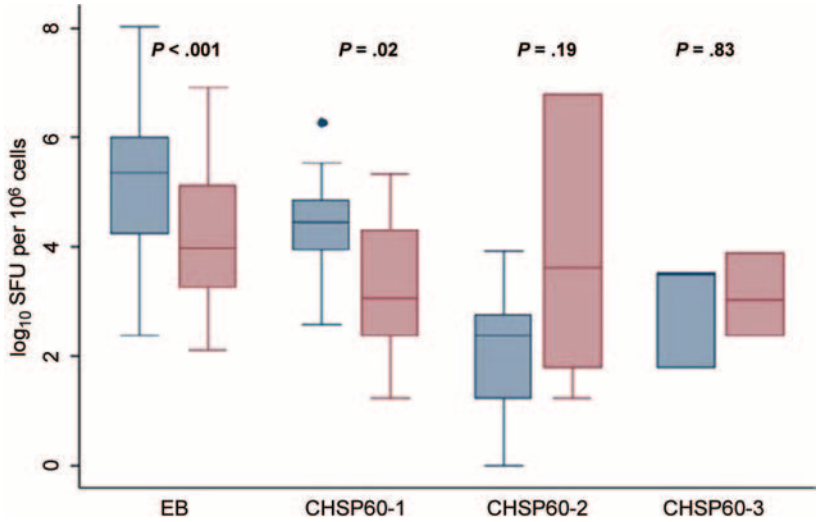

Figure 3. Magnitude of *Chlamydia trachomatis*–specific $CD4^+$ and $CD8^+$ T cell interferon–γ responses in peripheral blood mononuclear cells (PBMCs) obtained from 46 women that were depleted of $CD4^+$ or $CD8^+$ T cells and were then stimulated with the 4 chlamydial antigens. The spot-forming units (SFUs) were log-transformed and plotted on a box-and-whisker graph. Horizontal lines indicate the median values. $CD4^+$ T cell responses *(blue)* are those obtained in $CD8^+$ T cell–depleted PBMCs, whereas $CD8^+$ T cell responses *(red)* are those obtained in $CD4^+$ T cell–depleted PBMCs. CHSP60, *Chlamydia* heat shock protein 60; EB, elementary body.

have also been correlated with protective immunity to *C. trachomatis* [25]. Because *C. trachomatis* immunobiology is expressed at both systemic and mucosal levels, we simultaneously investigated the frequency and magnitude of EB-specific and CHSP60-specific IFN-γ responses in the endometrium and in PBMCs among women with high levels of exposure to *C. trachomatis*, to further illuminate the complex immunobiology of human *C. trachomatis* infection.

The frequency of *C. trachomatis* EB-specific IFN-γ responses was higher among PBMCs than among EMCs, suggesting a higher frequency of antigen-responsive T cells in the systemic compartment. However, because chlamydia infection is focal, this skewing may be confounded by limitations of the endometrial pipele procedure, in which only a fraction of the endometrial tissue is obtained and which might have missed some localized endometrial memory T cells that are specific for *C. trachomatis*. However, EB and CHSP60 antigens stimulated similar magnitudes of IFN-γ responses in PBMCs and EMCs (figure 2*A*), although there was a significantly greater response to CHSP60-2 in EMCs than in PBMCs. These data imply that EB and CHSP60 antigens are immunogenic in both the systemic and mucosal compartments and hint that there may be compartmentalization of EB-specific and CHSP60 IFN-γ responses, with the systemic and mucosal compartments being more responsive to EB and CHSP60, respectively (figure 1). It will be important to determine whether the lower prevalence of EB antigen–responsive cells in the mucosal compartment explains why individuals with previous exposure (with positive systemic responses) remain susceptible to reinfection. In addition, the previously described association of CHSP60-2 with establishment and/or maintenance of persistent chlamydial infection and the apparent preferential targeting of CHSP60-2 by EMC (figure 2*A*) calls for further investigation to determine whether enrichment in mucosal CHSP60-1, CHSP60-2, or CHSP60-3 immune responses correlates with protection or pathology. We also observed statistically significant correlations between the magnitude of EMC and PBMC IFN-γ responses (figure 2*B*) with all of the chlamydial antigens that were tested, except for CHSP60-3. Because this could be relevant to vaccines and therapeutic interventions that target either or both the mucosal and systemic compartments, this observation needs to be studied in detail in larger studies. It is important to note, however, that the findings reported in this study may be a direct result of the absence of active infection, because it was performed for women with a history of *C. trachomatis* infection, rather than for women with

Table 3. Correlation of systemic peripheral blood mononuclear cell interferon-γ response to baseline plasma antibody response to the chlamydial antigens.

Chlamydia trachomatis antigen	Odds ratio (95% CI)
CHSP60-1	0.08 (0.002–0.512)
CHSP60-2	1.67 (0.32–10.7)
CHSP60-3	0.38 (0.06–1.56)
Elementary body	0.50 (0.17–1.32)

NOTE. Correlation of plasma antibody and interferon-γ responses was assessed for the 46 women for whom both the antibody response and the peripheral blood mononuclear cell interferon-γ response were measured. CI, confidence interval.

active *C. trachomatis* infection. The dynamics of cellular responses in the PBMC and mucosal compartments could be hugely altered by active infection, which recruits specialized immune cells, such as $CD4^+$, $CD8^+$, and dendritic cells, to localized immune-inductive sites in the mucosa [44]. Although these dynamics will differ depending on the type of cellular immune response (Th1 vs. Th2), whether lower or upper genital tract mucosa is involved, whether primary or secondary infection is experienced, and on the chlamydia antigens used, mucosal immune responses have been shown to be higher than PBMC responses during active infection [45]. Studies that compare endometrial and PBMC compartments among women with active chlamydial infection will be paramount in accurately assessing which chlamydial antigens stimulate indistinguishable immune responses in the 2 compartments.

A significant portion of the IFN-γ responses to EB and CHSP60-1 in PBMCs were mediated by $CD4^+$ T cells (figure 3), which is consistent with studies that have shown that cell-mediated immunity to *C. trachomatis* is largely elicited by $CD4^+$ T cells. In fact, recent evidence shows elevated numbers of $CD4^+$ T lymphocytes in the cervical mucosa of women with chyamydia and mucopurulent cervicitis [45]. Thus, the association of CHSP60-1 with $CD4^+$ T cell response may suggest a superior protective role for CHSP60-1. Furthermore, because the levels of CHSP60-1 response are similar in the mucosal and systemic compartments (figure 2*A*), their role in mucosal immunity may be important.

Interestingly, CHSP60-1 IFN-γ responses in PBMCs were inversely related to CHSP60-1 antibody responses. This observation may explain the apparent paradoxical finding that CHSP60-1 immune response can be associated with either protective immunity (if it is IFN-γ mediated) or immunopathology (if it is antibody dominated). Considering the negative correlation that we observed between the serum IgG levels and the cellular IFN-γ response to CHSP60-1, and in view of the recent observations linking the IgG4 isotype with higher levels of Th2 and IL-10 responses in certain diseases, such as cholangitis and autoimmune pancreatitis [46], it would be relevant for future studies to analyze correlations of IFN-γ responses with IgG4 antibody titers in humans infected with *C. trachomatis.*

Finally, we observed that CHSP60-1 appears to be more immunogenic than CHSP60-2 and CHSP60-3. CHSP60-1 is expressed abundantly in active chlamydial infection, and we therefore hypothesize that it stimulates an effective IFN-γ response aimed at clearing infection. On the other hand, CHSP60-2 and CHSP60-3 only expressed at high levels during persistent chlamydial infections may elicit or result from inadequate immune effector functions incapable of clearing infection. Additional studies are needed to delineate the role of these antigens in either mucosal immunity or pathology with respect to persistent chlamydial infections and tubal factor infertility.

In summary, this is the first study, to our knowledge, to simultaneously evaluate cellular immune responses to *C. trachomatis* EB and CHSP60 antigens in both the systemic and mucosal compartments. We found the frequency and magnitude of IFN-γ responses to EB and CHSP60 in PBMCs and EMCs to be significantly correlated, indicating that PBMCs are a good marker for mucosal immune response to chlamydia. Among the CHSP60 antigens, CHSP60-1 was associated with immunodominance, elicited mainly $CD4^+$ T helper responses, and induced reciprocal cellular and humoral immune responses, demonstrating the well-known Th1 versus Th2 counter regulation. The significant positive correlation between EB and CHSP60-1 IFN-γ responses in both the systemic and mucosal compartment raises hopes that chlamydia-associated immunogens could be used as targets to elicit protective systemic and mucosal immunity.

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