Human Immunodeficiency Virus Type 1–Infected Women Exhibit Reduced Interferon-γ Secretion after *Chlamydia trachomatis* Stimulation of Peripheral Blood Lymphocytes

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Epidemiologic, animal, and in vitro models suggest an important role for interferon (IFN)–γ in the clearance of *Chlamydia trachomatis* infection. IFN-γ in the supernatants of in vitro–stimulated peripheral blood mononuclear cells (PBMC) from 22 human immunodeficiency virus type 1 (HIV-1)–infected and 73 uninfected women at high risk for *C. trachomatis* acute pelvic inflammatory disease (PID) was studied. PBMC were stimulated with *C. trachomatis* purified major outer membrane protein (MOMP) and whole elementary bodies (EBs) from the 4 predominant serovars (E, F, K, and L2) that circulate in Nairobi. PBMC IFN-γ secretion after stimulation with *C. trachomatis* EBs was significantly decreased in HIV-1–infected women. Among HIV-1–infected women, CD4 T cell depletion was associated with lower IFN-γ secretion from PBMC stimulated with either *C. trachomatis* MOMP or EB antigen. Decreased antigen-specific IFN-γ production may enhance the susceptibility of HIV-1–infected women to *C. trachomatis* PID.

Chlamydia trachomatis is the most common cause of pelvic inflammatory disease (PID) in the United States [1] and is an important cause of female infertility worldwide [2, 3]. Much remains to be discovered about the human immune response to *C. trachomatis* infection; most of our knowledge derives from animal or in vitro models. Because T cells accumulate at the site of infection, they probably play critical roles in controlling infection and modulating tissue-damaging effects from infection [4]. Recent tissue-based studies in nonhuman primates demonstrated induction of mRNA for interferon (IFN)–γ, interleukin (IL)–2, IL-6, and IL-10, but not for IL-4, after single and repeated chlamydial infection of salpingeal tissue [5, 6]. These findings suggest that Th1 and Th2 cytokines may play a role in the clearance of *C. trachomatis* and in regulating tissue damage. In parallel murine experiments, anti–IFN-γ antibody treatment resulted in significantly prolonged infection, whereas passive administration of IFN-γ in chronically infected homozygous nude mice caused resolution of infection [4]. IFN-γ gene knockout mice infected with *C. trachomatis* develop disseminated infection and altered tissue immunopathology [7, 8]. By using a mouse pneumonitis strain of *C. trachomatis,* other investigators demonstrated that clearance from the genital mucosal was mediated by an IL-12–dependent, IFN-γ–independent mechanism; however, follow-up studies showed that human strains were much more sensitive to the inhibitory actions of IFN-γ [9]. Subsets of CD4 and CD8 T cells that secrete different patterns of cytokines are probably important in *C. trachomatis* immunity and immunopathology.

Epidemiologic evidence supports an important role for CD4 T cells in the pathogenesis of *C. trachomatis* infection and disease in women. In a previous investigation, human immunodeficiency virus type 1 (HIV-1) infection was associated with an increased incidence of *C. trachomatis* infection, and CD4 T cell–deficient HIV-1–infected women had an increased risk of acquiring *C. trachomatis* PID [10, 11]. Furthermore *C. trachomatis*–associated infertility was correlated with specific HLA DQ alleles, which suggests a role for CD4 T cells in disease pathogenesis for tubal infertility [12]. We conducted the present investigation among women with clinical evidence of PID, to determine the effect of HIV-1 infection and CD4 T cell deple-

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Written informed consent was obtained from each study participant. The protocol was approved by institutional review boards for human subjects at the University of Washington, University of Manitoba, and Kenyatta National Hospital. Human experimentation guidelines of the US Department of Health and Human Services were followed.

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tion on IFN-γ and IL-10 production after stimulation of peripheral blood mononuclear cells (PBMC) by *C. trachomatis* antigens.

Patients and Methods

Patients. From June 1997 through January 1999, we recruited women 18–40 years old from the Special Treatment Clinic in Nairobi, Kenya, who complained of low abdominal pain for ≤14 days. Women were excluded if they were pregnant or breast-feeding, gave a history of surgery, delivery, or abortion in the prior 6 weeks, or reported receipt of ≥2 days of antibiotics in the preceding 2 weeks.

Study patients were administered a demographic and clinical questionnaire and were given a standardized physical examination. Vaginal specimens were obtained for *Trichomonas vaginalis* culture and were Gram's stained for diagnosis of bacterial vaginosis (BV). Cervical specimens were obtained for *Neisseria gonorrhoeae* and *C. trachomatis* DNA detection by polymerase chain reaction (PCR). Blood was obtained for HIV-1 and chlamydial heat-shock protein 60 (hsp60) serology and for CD4 and CD8 T lymphocyte enumeration. All women were given empiric antibiotic treatment: a single 500-mg dose of ciprofloxacin, followed by 100 mg of doxycycline and 500 mg of metronidazole twice daily for 10 days.

We cultured *T. vaginalis* with InPouch (BioMed Diagnostics, San Jose, CA), diagnosed BV by Gram's stain criteria by the scoring system of Nugent et al. [13], and detected *N. gonorrhoeae* and *C. trachomatis* from the cervix and endometrium by a commercial PCR assay (Roche Diagnostic Systems, Somerville, CA). We tested serum for HIV-1 antibodies with a synthetic peptide immunoassay (ELISA; Detect HIV-1; Biochem ImmunoSystems, Montreal). Those positive on the initial screening test had a second confirmatory test (Recombigen; Cambridge Biotech, Dublin).

C. trachomatis *hsp60 antibodies.* Specific serum antibodies to *C. trachomatis* hsp60 were measured by modified ELISA, as described elsewhere [14]. In brief, 1:200 diluted serum samples were added to 96-well plates coated with recombinant chlamydial hsp60. Antibodies bound to the coated antigen were detected by using biotin-conjugated anti–human IgG, followed by the addition of streptavidin and substrate (Sigma, St. Louis). The positivity criterion for chlamydial hsp60-specific antibodies was ≥0.2 of an optical density value at 405 nm.

PBMC purification and T cell subsets analysis. PBMC were purified from blood with ficoll-hypaque. Absolute T cell subsets (CD4 and CD8 cells) were enumerated by FACS analysis by standard procedures (Becton Dickinson, Sunnyvale, CA).

Antigen preparations. *C. trachomatis* serovars E, F, K, and L2 were propagated in HeLa cells, and elementary bodies (EBs) were purified on discontinuous gradients of Renografin-76 (Squibb Canada, Montreal), as described elsewhere [15]. EBs were inactivated with UV light irradiation for 30 min before use as antigens. No infectious EBs were identified when a dose equivalent to 10^6 inclusion-forming units of UV-inactivated EBs was inoculated onto HeLa cell monolayers in a 96-well plate [15]. Chlamydial major outer membrane protein (MOMP) was purified from EBs, as described elsewhere [16], in 3 steps: EBs were extracted with 2% sarkosyl, the sarkosyl-insoluble fraction then was extracted with 2% sarkosyl–10 m*M* dithiothreitol (DTT), and the sarkosyl-DTT insoluble fraction was further extracted with 2% octylglucoside–10 m*M* DTT. The purity of octylglucoside–10 m*M* DTT soluble fraction, designated as MOMP antigen, was confirmed by SDS-PAGE. The EB protein and MOMP were quantitated by the method of Lowry et al. [17]. Tuberculin purified protein derivative (PPD; Connaught Laboratories, Willowdale, Canada) and phytohaemagglutinin (PHA-P; Sigma, St. Louis) were used as PBMC recall antigen and mitogen, respectively.

PBMC proliferation. PBMC were cultured on round-bottomed 96-well plates at 5×10^5/mL in RPMI 1640 supplemented with 10% fetal calf serum, 50 m*M* 2-mercaptoethanol, 100 U/mL penicillin, and 12 μg/mL gentamicin in the presence or absence of various antigens: UV EBs of individual or equally mixed serovars E, F, K, and L2 in a total protein concentration of 1 μg/mL; 0.5 μg/mL of MOMP(s) extracted from EBs of individual or equally mixed serovars E, F, K, and L2; 8 μg/mL PHA-P; or 5 U/mL PPD. Detergents, used for the extraction of MOMP at the last step, and medium alone were used as controls for MOMP and EB stimulation, respectively. After 5 days of culture, PBMC were pulsed for 12 h with 0.5 μCi/well of [^{3}H]thymidine (ICN, Irvine, CA). Cells were harvested with a PHD cell harvester (Cambridge Technology, Watertown, MA) and were counted with a liquid scintillation counter (model LS5000; Beckman Instruments, Palo Alto, CA). Results were expressed as stimulation index equivalent to geometric mean counts per minute (test) divided by the geometric mean counts per minute (control).

Cytokine determination. PBMC for cytokine assays were cultured under conditions identical to those described above. At day 3, supernatants from PBMC culture wells were harvested and were stored at −80°C for IFN-γ and IL-10 ELISA. Paired antibodies for human IFN-γ and IL-10 ELISA were purchased from PharMingen (San Diego). Cytokines were measured in picograms per milliliter.

Data analysis. We used SPSS software (version 9.0 for Windows; SPSS, Chicago) for analysis. Univariate analysis was performed by using χ^2 and Fisher's exact tests for categorical variables, Mann-Whitney *U* test for interval, and Student's *t* test for continuous variables. Linear regression was used to determine the association between 2 continuous variables. Dependent (e.g., IFN-γ produced by stimulated PBMC) and independent variables (e.g., CD4 cell count) were log transformed to fulfill the assumptions of the model.

Results

Selected patient characteristics. We recruited 351 women with recent onset of low abdominal pain suggestive of acute PID. We also studied cytokine production by stimulated PBMC in a subgroup of 95 women chosen from the overall study population, after enriching for women with chlamydial and/or gonococcal infection. Only 25 women (26%) had histopathologic evidence of endometritis on endometrial biopsy, although microbiologic evidence of sexually transmitted infection was much more prevalent (table 1): of the 99 women, 45 (47%) had bacterial vaginosis, 18 (19%) had trichomoniasis, 29 (30%) had gonococcal infection, and 16 (17%) had chlamydial infection.

Table 1. Selected characteristics of 95 women with clinical pelvic inflammatory disease (PID).

Characteristic	HIV-1 infected (n = 22)	HIV-1 uninfected (n = 73)	P
Age, years (mean, SD)	26.8 (5.4)	26.6 (4.9)	.86
Marital status			
Single	8 (36)	28 (38)	
Married	6 (27)	36 (49)	
Divorced/separated	7 (32)	9 (12)	
Widowed	1 (5)	0	.03
Gravidity (mean, SD)	1.9 (1.6)	2.0 (1.7)	.79
Parity (mean, SD)	1.6 (1.5)	1.7 (1.5)	.79
Miscarriage (mean, SD)	0.3 (0.4)	0.4 (0.7)	.36
History of infertility	3 (14)	13 (18)	.76
Prior PID	10 (46)	24 (33)	.28
Current contraceptive use			
Depoprovera	6 (27)	9 (12)	
Oral	4 (18)	21 (29)	
Other	2 (9)	9 (12)	
None	10 (46)	34 (47)	.54
Age at 1st sexual intercourse (mean, SD)	16.9 (2.3)	17.6 (2.6)	.27
Lifetime sex partners (median, range)	4 (1–10)	3 (1 to >997)	.29
Past sex partners (median, range)			
3 Months	1 (1–3)	1 (1–30)	1.0
1 Year	1 (1–4)	1 (1 to >997)	.41
Commercial sex worker	0	3 (4)	1.0
Endometritis on biopsy	13 (81)	12 (25)	<.001
Bacterial vaginosis	12 (57)	33 (49)	.46
Trichomonas vaginalis culture	6 (27)	12 (16)	.26
Neisseria gonorrhoeae only	3 (13)	20 (27)	.16
Chlamydia trachomatis only	4 (18)	6 (9)	.24
Both gonorrhea and chlamydia	1 (4)	5 (7)	1.0
CD4 cell count, cells/μL			
<200	1 (4)	0	
200–399	10 (44)	3 (4)	
400–599	8 (35)	16 (23)	
⩾800	4 (17)	51 (73)	<.001
Chlamydial hsp60 antibody titers			
Median (range)	0.13 (0.04–0.32)	0.12 (0.02–1.2)	.30
No. >0.2 ODU	6 (32)	12 (23)	.54
No. >0.5 ODU	0	2 (4)	1.0

NOTE. Data are no. (%) unless otherwise indicated. hsp60, Heat-shock protein 60; ODU, optical density units.

In all, 34 (36%) of the 99 women had a prior history of PID, (16) 17% were infertile, and 22 (23%) were HIV-1 seropositive. HIV-1–infected women were similar to HIV-1–uninfected women in most characteristics, although they were more likely to have histopathologic evidence of endometritis on biopsy and fewer CD4 T cells.

Evidence for C. trachomatis *infection.* Detection of *C. trachomatis* DNA by PCR was similar in HIV-1–infected and –uninfected women (24% vs. 17%; P = .52). Antibody to *C. trachomatis* hsp60 was detected in 6 (32%) of 22 HIV-1–infected and 12 (23%) of 73 HIV-1–uninfected women (P = .46). In neither group was there a significant relationship between mean CD4 cell count ($\log_{10}$) and detection of *C. trachomatis* hsp60 antibody (P = .86 and P = .46, respectively).

Cytokine and proliferation assays. We compared cytokine production by stimulated PBMC in the 22 HIV-1–infected women with that in the 73 HIV-1–uninfected women. We focused attention on cellular immune responses to chlamydial antigens among HIV-1–infected and –uninfected women because of the pronounced effect of HIV-1 on susceptibility to chlamydial infection and PID risk in a prior study [10, 11].

Secretion of IFN-γ stimulated by a pool of mixed serovars of *C. trachomatis* EBs was more impaired in HIV-1–infected women than in HIV-1–uninfected women. A similar trend was detected for IFN-γ secretion after stimulation with *C. trachomatis* MOMP, although this finding was of borderline statistical significance (table 2). We further limited our analysis to the 17 women (5 HIV-1 infected and 12 HIV-1–uninfected) diagnosed with PCR-positive *C. trachomatis* infection and stratified by HIV-1 serostatus. Similar to our analysis of the unselected population, PBMC from HIV-1–infected women stimulated with chlamydial EB antigen produced less IFN-γ than did PBMC from HIV-1–uninfected women (459 ± 558 pg/mL vs. 3229 ± 5434 pg/mL, respectively; P = .04, $\log_{10}$ scale). Surprisingly, we did not detect a significant change in IFN-γ secretion after stimulation of PBMC by PHA or PPD when stratified by HIV-1 serostatus. IL-10 secretion induced by *C. trachomatis* antigen-stimulated PBMC was also not affected by HIV-1 serostatus. However, PHA-stimulated PBMC of HIV-1–infected women produced significantly less IL-10 than that of uninfected women (table 2).

We used linear regression analysis to determine the potential

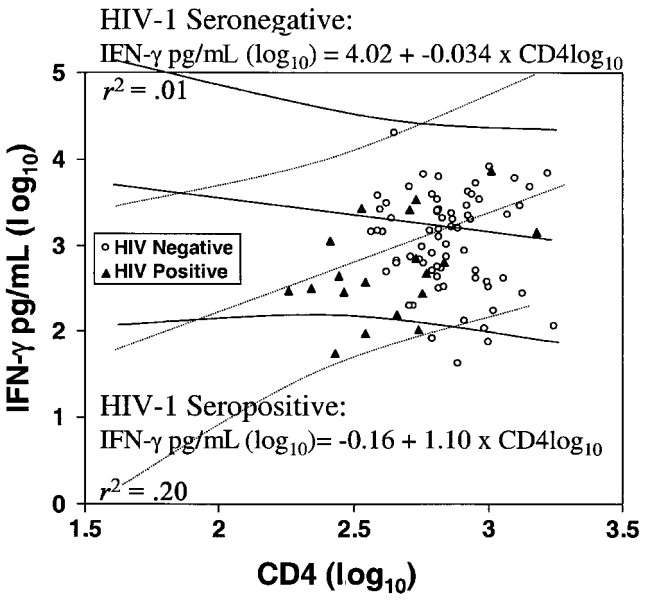

Figure 1. Regression of interferon (IFN)–γ secretion by peripheral blood mononuclear cells stimulated by *Chlamydia trachomatis* mixed elementary body antigen by CD4 cell count for human immunodeficiency virus type 1 (HIV-1)–seronegative (r^2 = .01; P = .58) and –seropositive (r^2 = .20; P = .05) women.

Table 2. Interferon (IFN)–γ and interleukin (IL)–10 secretion by stimulated peripheral blood mononuclear cells (PBMC), by human immunodeficiency virus type 1 (HIV-1) serostatus.

	IFN-γ		IL-10	
Variable stimulating PBMC	HIV-1 infected (n = 22 [23%])	HIV-1 uninfected (n = 73 [77%])	HIV-1 infected (n = 22 [23%])	HIV-1 uninfected (n = 73 [77%])
C. trachomatis mixed EBs	1201 (1789)	2180 (2847)[a]	1244 (1874)	896 (693)
C. trachomatis mixed MOMP	763 (1146)	978 (1093)	897 (1595)	583 (528)
PHA	10427 (7107)	10165 (6491)	1337 (1143)	2027 (1476)[a]
PPD	570 (1621)	794 (2119)	107 (133)	170 (250)

NOTE. Data are mean, SD in picograms per milliliter. Data were compared by Student's t test using $\log_{10}$ values. EBs, Whole elementary bodies; MOMP, major outer membrane protein; PHA, phytohemagglutinin; PPD, purified protein derivative.

effect of peripheral blood CD4 T lymphocyte count on the secretion of cytokines by stimulated PBMC in both HIV-1–seropositive and –seronegative women. In HIV-1–infected women, but not in the HIV-1–uninfected women, production of IFN-γ by *C. trachomatis* MOMP-1 and EB-stimulated PBMC was significantly correlated with reduced CD4 cell count and was lowest in HIV-1–infected women with the fewest CD4 cells (figures 1 and 2). CD4 cell count remained inversely correlated with chlamydial antigen-specific IFN-γ production, after adjusting for CD8 cell count. Secretion of IFN-γ by PHA- and PPD-stimulated PBMC was not associated with CD4 cell count in either HIV-1–infected or –uninfected women. Similarly, no significant correlation was observed between secretion of IL-10 by *C. trachomatis* antigens and PHA- or PPD-stimulated PBMC and CD4 cell count stratified by HIV-1 serostatus (table 3).

Because the secretion of IFN-γ could be affected by the degree of PBMC proliferation in HIV-1–infected versus –uninfected women, we measured lymphocyte proliferation after stimulation with *C. trachomatis* MOMP, *C. trachomatis* EB, and PHA. Stratified by HIV-1 serostatus, *C. trachomatis* MOMP (mean $\log_{10}$ stimulation index ± SD: 0.36 ± 0.43 vs. 0.27 ± 0.39; $P = .33$) and EB (0.63 ± 0.38 vs. 0.64 ± 0.43; $P = .89$) stimulated cell proliferation on a $\log_{10}$ scale were similar for HIV-1–seropositive and –seronegative women. Of interest, PBMC stimulated with PHA from HIV-1–infected women had greater proliferation than PBMC from HIV-1–uninfected women (1.61 ± 0.53 vs. 1.22 ± 0.52; $P < .005$). We also explored the possibility that CD4 T cell count might correlate with PBMC proliferation. In separate linear regression models for HIV-1–infected and –uninfected persons, we failed to find a significant relationship between cell proliferation after *C. trachomatis* MOMP, *C. trachomatis* EB, and PHA stimulation with CD4 T cell count (data not shown). Furthermore, background median IFN-γ secretion in the absence of antigen or mitogen stimulation was not associated with HIV-1 serostatus (15 vs. 10 pg/mL; $P = .49$). Thus, it appears unlikely that secretion of IFN-γ from stimulated PBMC resulted from different degrees of cell proliferation in HIV-1–infected versus –uninfected women.

Discussion

We previously reported a higher incidence of *C. trachomatis* PID in HIV-1–infected women with low CD4 T cell counts [10]. For the current investigation, we hypothesized that this finding may be due, in part, to diminished IFN-γ secretion from PBMC after *C. trachomatis* antigen stimulation in HIV-1–infected women. The following findings support this hypothesis.

First, incubation of PBMC with *C. trachomatis* MOMP and EB in HIV-1–infected patients led to significantly less IFN-γ secretion than in HIV-1–uninfected women. Second, this finding was most pronounced in HIV-1–infected women with the lowest CD4 cell count. Third, the finding was independent of degree of mononuclear cell proliferation, CD8 T cell count, and current or past evidence of *C. trachomatis* infection. Fourth, IFN-γ secretion by PBMC was unaltered by general mitogen (i.e., PHA or PPD stimulation), which suggests that decreased IFN-γ secretion in chlamydial antigen-stimulated PBMC was not due to an overall depressed T cell response. Because antigen-specific IFN-γ production measures effector and/or memory T cell responses, these data suggest that HIV-1 causes depletion of CD4 T cells preferentially from the effector and/or memory compartment, rather than from the naive T cell compartment. Because chlamydial PID appears to be a disease of reinfection [10], protection is probably dependent on rapid responses of

Table 3. Linear regression models to evaluate the effect of CD4 T lymphocyte count ($\log_{10}$) on interferon (IFN)–γ and interleukin (IL)–10 secretion by stimulated peripheral blood mononuclear cells (PBMC) for human immunodeficiency virus type 1 (HIV-1)–infected and –uninfected women with clinical pelvic inflammatory disease.

	HIV-1 infected (n = 22)		HIV-1 uninfected (n = 73)	
Variable	r^2	P	r^2	P
IFN-γ from PBMC stimulated with				
C. trachomatis MOMP	.23	<.03	.00	.90
C. trachomatis mixed EBs	.20	.05	.01	.58
PHA	.01	.72	.03	.16
PPD	.03	.49	.00	.95
IL-10 from PBMC stimulated with				
C. trachomatis MOMP	.06	.26	.04	.12
C. trachomatis mixed EBs	.06	.33	.02	.27
PHA	.00	.94	.00	.70
PPD	.00	.83	.03	.19

NOTE. Data for all variables $\log_{10}$. EBs, Whole elementary bodies; MOMP, major outer membrane protein, PHA, phytohemagglutinin; PPD, purified protein derivative.

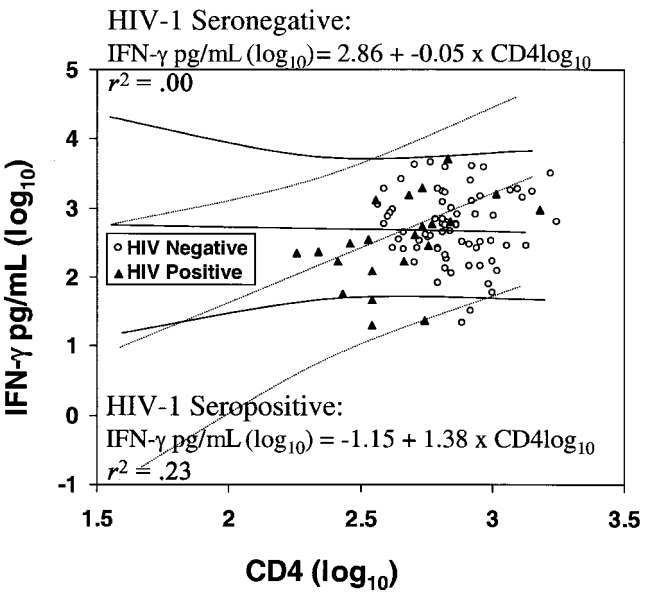

Figure 2. Regression of interferon (IFN)–γ secretion by peripheral blood mononuclear cells stimulated by *Chlamydia trachomatis* major outer membrane protein antigen by CD4 cell count for human immunodeficiency virus type 1 (HIV-1)–seronegative (r^2 = .00; P = .90) and –seropositive (r^2 = .23; P < .03) women.

memory and effector T cells. Our results provide strong evidence for selective elimination of protective immune responses by HIV-1, which may be the underlying mechanism by which HIV-1 enhances susceptibility to chlamydial PID.

Evidence that IFN-γ mediates clearance of *C. trachomatis* derives from animal, in vitro, and a small number of human studies [4–10, 18–20]. Most investigators hypothesize that *C. trachomatis* genital and ocular disease share similar immunopathogenic pathways [21]. In clinical studies that measured Th1 versus Th2 cytokine profiles of PBMC response to *C. trachomatis* in subjects with severe trachomatous scarring, IFN-γ production after *C. trachomatis* MOMP stimulation was greater in control subjects than in persons with trachomatous scarring. Incubation with *C. trachomatis* hsp60 was associated with increased numbers of IL-4–producing cells and decreased production of IFN-γ–producing cells. Such evidence supports the concept that severe *C. trachomatis* disease and its sequelae (i.e., PID, tubal and trachomatous scarring) may be associated with a predominant Th2 immune response. IFN-γ is present in genital tract secretions of many women with cervical *C. trachomatis* infection [19] and as mRNA in the lymphocyte infiltrate of upper genital tract infection in female macaques during acute infection [6]. Some of the strongest evidence to support the importance of IFN-γ in the clearance of infection derives from experiments of mice chronically infected with *C. trachomatis* that were passively administered IFN-γ and cured of infection and by the prolonging of infection by administering anti–IFN-γ [4]. Even more compelling, Wang et al. [8] showed that IFN-γ gene knockout mice exhibited Th2 cytokine-dependent immunopathology together with disseminated chlamydial infection. Other investigators have found that IFN-γ may also play a role in protection against *C. trachomatis* through the CD8 T cell lymphocyte pathway [20]. Although our results provide indirect human data to support an IFN-γ–dependent protective mechanism in *C. trachomatis* PID, determination of the specific role of IFN-γ in immunity to *C. trachomatis* infection will require additional investigation.

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