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

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ABSTRACT

Chlamydia trachomatis infection is the most prevalent bacterial sexually transmitted infection and can cause significant reproductive morbidity in women. There is insufficient knowledge of *C. trachomatis*-specific immune responses in humans, which could be important in guiding vaccine development efforts. In contrast, murine models have clearly demonstrated the essential role of Type 1 T helper cells (Th1), especially interferon-gamma (IFN-γ) producing $CD4^+$ T-cells, in protective immunity to chlamydia. To determine the frequency and magnitude of Th1 cytokine responses elicited to *C. trachomatis* infection in humans, we stimulated peripheral blood mononuclear cells from 90 chlamydia-infected women with *C. trachomatis* elementary bodies, Pgp3, and major outer membrane protein and measured IFN-γ-, tumor necrosis factor-alpha (TNF-α)-, and interleukin 2 (IL-2)-producing $CD4^+$ and $CD8^+$ T-cell responses using intracellular cytokine staining. The majority of chlamydia-infected women elicited $CD4^+$ TNF-α responses, with frequency and magnitude varying significantly depending on the *C. trachomatis* antigen used. $CD4^+$ IFN-γ and IL-2 responses occurred infrequently, as did production of any of the three cytokines by $CD8^+$ T-cells. About one-third of TNF-α-producing $CD4^+$ T-cells co-produced IFN-γ or IL-2. In summary, the predominant Th1 cytokine response elicited to *C. trachomatis* infection in women was a $CD4^+$ TNF-α response, not $CD4^+$ IFN-γ, and a subset of the $CD4^+$ TNF-α-positive cells produced a second Th1 cytokine.

INTRODUCTION

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

Because of the ethical concerns in performing *Chlamydia* challenge or natural history studies in humans, animal models have played a critical role in identifying the mechanisms of protective immunity to chlamydia (6). In murine models of genital chlamydia, protective immunity is largely mediated through $CD4^+$ T helper type 1 (Th1) cell responses, with interferon-gamma (IFN-γ) playing an essential role (7). Another Th1 cytokine, tumor necrosis factor alpha (TNF-α), has also been implicated in protective immunity to chlamydia in murine studies (8-10). TNF-α is a pleiotropic proinflammatory cytokine released from monocytes, macrophages, and lymphocytes that stimulates a cascade of other cytokines (11). In the murine chlamydia model, the influence of TNF-α on chlamydia clearance appears to be specific to the mucosal site infected. For example, in the mouse pneumonitis model (which uses *C. muridarum*), TNF-α deletion significantly accelerates mortality and increases organism burden in the lung (8, 9), suggesting a protective role for TNF-α. However, TNF-α depletion seems to have no effect on *C. muridarum* clearance from the genital tract (12-14), although its expression

in the genital tract increases within days of chlamydia infection (12, 15), suggesting TNF-α alone is not sufficient for chlamydia clearance. Interestingly, several studies demonstrate TNF-α may exert its effects in synergy with other cytokines (16) or interleukins (17). One murine study demonstrated that $CD4^+$ T-cells producing both IFN-γ and TNF-α correlated with protection against chlamydia challenge (16). While the role of TNF-α in mediating protective immunity remains unclear, murine studies have reported this proinflammatory cytokine may contribute to upper genital tract pathology (13).

Translating immune mechanisms of chlamydia protection from animal studies to humans is difficult, as mechanisms can differ by animal model (7, 18). For example, mice deficient in $CD8^+$ T-cells still resolve primary infection (7, 19), yet a non-human primate model demonstrated a key role for $CD8^+$ T-cells in mediating protection against chlamydia (20). The dominant mucosal mononuclear cell type response to chlamydia also differs by species with $CD4^+$ T-cells being more numerous in the genital tract in mice (21) and $CD8^+$ T-cells in the non-human primate trachoma model (22). Additionally, differences in *Chlamydia* species tested, time to infection clearance, estrous cycle duration, and variable susceptibility to upper genital tract infection make correlation to humans difficult (7, 18). In humans, evidence for protective immunity to chlamydia is mainly based on epidemiological studies, which have shown that a history of prior chlamydia, spontaneous resolution of chlamydia, and persons with a high likelihood of repeated chlamydia exposures (e.g., commercial sex workers) are associated with a lower reinfection risk (23-25). Although immune mechanisms that mediate protection against *C. trachomatis* in humans remain to be fully elucidated, sparse evidence from two prospective human studies that measured *C. trachomatis*-specific IFN-γ production by ELISA or ELISpot revealed IFN-γ was associated with

96 protective immunity (26, 27).

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

107

108 **MATERIALS AND METHODS**

109 **Study Population and Procedures.** The study population was comprised of females
110 ≥16 years of age who presented to the Jefferson County Department of Health (JCDH)
111 STD Clinic in Birmingham, Alabama, USA for treatment of a recent positive *C.*
112 *trachomatis* screening nucleic acid amplification test (Hologic Aptima Combo 2 ® [AC2];
113 Hologic, Inc., Marlborough, MA). Patients did not receive empiric chlamydia therapy at
114 the time of screening because they had no cervicitis findings and no other chlamydia
115 treatment indications. Patients interested in the study provided written consent and were
116 enrolled. Women who were pregnant, had a prior hysterectomy, were co-infected with
117 HIV, syphilis, or gonorrhea (tested at screening), were immunosuppressed, or had
118 received antibiotics with anti-chlamydial activity in the prior 30 days were excluded.

119 At enrollment, participants were interviewed and demographical, clinical, and
120 behavioral data were collected. A pelvic examination was performed in which a vaginal

121 swab specimen was collected for wet mount microscopy and an endocervical swab
122 specimen for chlamydia and gonorrhea testing by the AC2 per the manufacturer's
123 instructions. Blood was collected for isolation of peripheral blood mononuclear cells
124 (PBMCs). All participants received directly observed therapy with azithromycin 1g and
125 were advised to refer all sexual partners for treatment, if not already treated. PBMCs
126 from five healthy, low-risk *C. trachomatis*-seronegative women (tested with a *C.*
127 *trachomatis* elementary body-based ELISA assay [28]) without a history of chlamydia
128 were used as negative controls. The study was approved by the University of Alabama
129 at Birmingham (UAB) Institutional Review Board and JCDH.

130 **PBMC Isolation.** At the UAB Center for Clinical and Translational Sciences
131 Specimen Processing and Analytical Nexus, PBMCs were isolated from blood by
132 centrifugation through lymphocyte separation medium (Mediatech, Inc, Manassas, VA).
133 Upon isolation, cells were counted and examined for viability. PBMCs were frozen in
134 1mL aliquots in 90% FBS+10% DMSO and cryopreserved in liquid nitrogen until used
135 for immunological studies.

136 **Intracellular Cytokine Staining (ICS).** PBMCs were stimulated with *C. trachomatis*
137 antigens and analyzed for cytokines as previously reported with modifications (29).
138 Briefly, 2.5×10^5 cells were incubated for two hours at 37°C with 5% CO_2 in RPMI-10
139 media (RPMI 1640 medium containing 10% human AB serum, penicillin/streptomycin
140 [50 U/mL], HEPES [25 mM] and L-glutamine [2mM]) in the presence of co-stimulatory
141 antibodies CD28 and CD49d (BD Biosciences, San Diego, CA) and antigen, followed by
142 a five-hour incubation in the presence of Brefeldin A and Monensin (both from BD
143 Biosciences, San Diego, CA). Antigens used were: recombinant *C. trachomatis* Pgp3 (5
144 µg/ml) (Biorbyt, San Francisco, CA), pooled Major Outer Membrane Protein (MOMP)
145 peptides (5 µg/ml) (VS1 75-92, VS2 132-151, VS2 145-163, VS4 300-318, VS4 308-

146 324; UAB Peptide Core, Birmingham, AL), formalin-fixed *C. trachomatis* elementary
147 bodies (4 µg/ml) (EBs, pooled serotypes D, F and J; obtained from Dr. Richard Morrison
148 from the University of Arkansas for Medical Sciences, Little Rock, AR), and
149 *Staphylococcus* enterotoxin B (10 µg/ml) (SEB, Toxin Technologies, Carasota, FL) as
150 the positive control. RPMI-10 supplemented with anti-CD28 and anti-CD49d antibodies,
151 but without antigen, was used to determine background T-cell responses. Cells were
152 subsequently labeled with LIVE/DEAD fluorescent reactive dye (Life Technologies,
153 Eugene, OR), stained with surface antibodies against CD3-Pacific Blue (BD
154 biosciences, San Diego, CA), CD4-Qdot 655, and CD8-Qdot 605 (both from Invitrogen,
155 Carlsbad, CA), fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences, San Diego,
156 CA), and stained with antibodies for intracellular cytokines IFN-γ-Alexa 700, TNF-α-PE-
157 Cy7, and IL-2-PE (all from BD Biosciences, San Diego, CA). Approximately 100,000
158 events were acquired on a LSRII (BD Immunocytometry Systems, San Diego, CA) and
159 data was analyzed using FlowJo software (v9.8.5, TreeStar, Ashland, OR). All
160 responses are reported after subtracting the background responses (media + co-
161 stimulatory antibodies).

162 **Statistical analysis.** Analyses were performed with SAS software, version 9.3
163 (SAS Institute, Cary, NC). Descriptive statistics were used to summarize demographical
164 and clinical characteristics. We evaluated quantitative and qualitative differences in
165 cytokine responses, the latter to allow comparison with prior studies (26-27); cytokine
166 responses >0.05% and twice the background response (media + co-stimulatory
167 antibodies) were considered positive for our qualitative analyses. Differences in
168 frequencies of each $CD4^+$ cytokine response and each $CD8^+$ cytokine response were
169 evaluated by McNemar's chi-square test. Differences in frequency of a specific cytokine
170 response based on *C. trachomatis* antigen used for the stimulation were also evaluated

171 by McNemar's chi-square test. Differences in the magnitude of $CD4^+$ TNF-α responses
172 (enumeration of cytokine-producing cells) by *C. trachomatis* antigen were evaluated
173 with the Friedman and Wilcoxon signed-rank tests. Differences in the magnitude of
174 cytokine-positive responses between chlamydia-infected cytokine-positive, chlamydia-
175 infected cytokine-negative, and negative control groups were evaluated with the
176 Wilcoxon rank sum test. Associations of subjects' demographical or clinical
177 characteristics with specific cytokines responses were evaluated with the Pearson's chi-
178 square, Fisher's exact, or Wilcoxon rank sum tests as appropriate. Differences were
179 considered significant at a *P*-value <0.05.
180

181 **RESULTS**

182 Ninety women with chlamydia infection at the time of enrollment were investigated in
183 this study (Table 1). The median age was 21 (range 16 – 32), 97% were African
184 American, and 1% were Hispanic. 54% of women were asymptomatic and 40% were
185 diagnosed with co-infections bacterial vaginosis, trichomoniasis, or vaginal candidiasis.
186 57% of women had a prior history of chlamydia, based on self-report and/or
187 documentation of prior positive chlamydia test results.

188 **Of the three Th1 cytokines tested, the predominant $CD4^+$ cytokine elicited by**
189 **stimulated PBMCs from chlamydia-infected women was TNF-α, not IFN-γ.** Fig. 1A
190 shows the gating scheme utilized for the analyses of $CD4^+$ T-cells from a representative
191 chlamydia-infected subject showing Pgp3-stimulated flow cytometry plots. $CD8^+$ T-cells
192 were gated similarly. A $CD4^+$ TNF-α response comprised the majority of *C. trachomatis*-
193 specific $CD4^+$ Th1 cytokine responses to any antigen, with 79% of women mounting a
194 positive $CD4^+$ TNF-α response to at least one antigen, compared to 16% and 12% of
195 women having a $CD4^+$ IFN-γ or IL-2 response, respectively (Fig. 1B, *P* <0.001). There

196 was no difference in the proportion of women with a positive CD4$^+$ IFN-γ vs. IL-2
197 response. CD8$^+$ T-cell IFN-γ, TNF-α, and IL-2 responses occurred less frequently than
198 CD4$^+$ Th1 cytokine responses, with 19-28% of subjects' CD8$^+$ T-cells eliciting a positive
199 cytokine response for any cytokine to at least one antigen (Fig. 1C). There was no
200 difference in the proportion of women with a positive CD8$^+$ IFN-γ, TNF-α, or IL-2
201 response.

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

209 **The proportion of women with a positive cytokine response varied with the *C.*
210 *trachomatis* antigen used for stimulation.** Stratifying the ICS responses by *C.*
211 *trachomatis* antigens used for stimulating PBMCs, we assessed whether there was a
212 difference in the proportion of women with a positive cytokine response elicited by
213 different *C. trachomatis* antigens. Fig. 2A shows representative flow cytometry plots for
214 a chlamydia-infected subject and a chlamydia-negative control subject. As shown in Fig.
215 2B, all three antigens generated predominantly CD4$^+$ TNF-α qualitative responses, but
216 these responses were most often generated with Pgp3 stimulation when compared with
217 stimulation with MOMP (64% vs. 34%; P <0.001) or EB (64% vs. 51%; P = 0.035). The
218 proportion of women with a positive CD4$^+$ TNF-α response to EB was significantly
219 higher than to MOMP (51% vs. 34%; P = 0.011). There was no significant difference in

220 the proportion of women with a positive $CD4^+$ IFN-γ or IL-2 response between the
221 antigens. In contrast to $CD4^+$ responses, the proportion of women with positive $CD8^+$
222 IFN-γ, TNF-α, or IL-2 responses across different antigens was lower, occurring in 6-15%
223 of women (Fig 2C). The proportion of women with a positive $CD8^+$ IFN-γ response when
224 stimulated with Pgp3 was significantly lower compared with EB (6% vs. 14%; $P = 0.02$),
225 otherwise there was no significant difference in the proportion of women with positive
226 $CD8^+$ cytokines responses between the antigens.

227 **In subjects with a positive Th1 cytokine response, a higher magnitude of TNF-**
228 **α- and IFN-γ-producing $CD4^+$ T-cells was seen to all antigens, with Pgp3 eliciting**
229 **the highest magnitude of TNF-α-producing $CD4^+$ T cells.** Although we expected an
230 increase in the number of cytokine-producing $CD4^+$ T-cells in subjects with positive
231 cytokine responses to the various antigens when compared to cytokine-negative
232 chlamydia-infected women (based on our qualitative analysis cutoff described in the
233 methods), we wanted to compare the magnitudes of those responses. To evaluate the
234 differences in the magnitudes (measured by percent cytokine-positive cells) of the
235 responses between these two group of women and chlamydia-negative controls (all
236 who had a negative response for all Th1 cytokines we tested), we stratified the data into
237 chlamydia-infected cytokine positive, chlamydia-infected cytokine negative, and
238 chlamydia-negative control groups. Given the low proportion of women with positive
239 $CD8^+$ cytokine responses, we focused our quantification analyses of cytokine producing
240 cells on $CD4^+$ T-cells. As shown in Fig. 3, comparison of the magnitude of the cytokine-
241 positive $CD4^+$ responses between antigens showed that Pgp3 elicited a 40% higher
242 $CD4^+$ TNF-α response (median TNF-α-producing cells 0.11%) than MOMP or EB

243 (0.08% for both, P = 0.0017 and P = 0.0016, respectively). IFN-γ or IL-2 CD4$^+$ cytokine
244 positive responses were not significantly different between antigens.

245 Comparing the magnitude of the cytokine responses in cytokine-positive chlamydia-
246 infected women to either cytokine-negative chlamydia-infected women or chlamydia-
247 negative control women for each antigen, we found a significant increase in the
248 magnitude of TNF-α- and IFN-γ-positive CD4$^+$ T-cell responses for the cytokine-positive
249 chlamydia-infected women compared to the other groups for each antigen, but there
250 was no difference in the magnitude of the IL-2 responses between the groups. The
251 median TNF-α-positive CD4$^+$ cell responses ranged from 0.080-0.11% in cytokine-
252 positive chlamydia-infected women vs. 0.010-0.015% in cytokine-negative chlamydia-
253 infected women. The median IFN-γ-positive CD4$^+$ cell response ranged from 0.043-
254 0.053% in cytokine-positive chlamydia-infected women vs. 0.000-0.004% in cytokine-
255 negative chlamydia-infected women. There was no difference in the magnitude of the
256 cytokine responses between cytokine-negative chlamydia-infected women and
257 negative-control women for any of the cytokines with the exception of Pgp3-stimulated
258 IL-2 responses, which were higher in the control group compared to the chlamydia-
259 infected IL-2-negative group (P = 0.02). Taken together, these data suggest that
260 responses to chlamydia antigens are chlamydia-specific and demonstrate the
261 immunogenicity of Pgp3 in generating TNF-α-positive CD4$^+$ T-cell responses.

262 **In TNF-α-positive subjects, the frequency of positive CD4$^+$ TNF-α responses is**
263 **highly variable and antigen dependent.** In subjects who had a positive CD4$^+$ TNF-α
264 response, we evaluated the frequency of inter-antigenic TNF-α-positive responses
265 across each *C. trachomatis* antigen to determine if the tested antigens generated
266 isolated responses (*i.e.* cytokine responses only to that antigen). We had initially

hypothesized that EB, being a whole *C. trachomatis* organism, would elicit the greatest cross-antigenic responses due to a more diverse antigen presentation compared to either Pgp3 or MOMP. Including only women with a positive $CD4^+$ TNF-α response to any antigen (n = 71), we saw significant inter-antigenic variability as expected, with 28% of women mounting TNF-α-positive responses to all three antigens, 21% to Pgp3 only, 13% to EB only, and 3% to MOMP only (Fig. 4).

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

DISCUSSION

With murine chlamydia models convincingly demonstrating the importance of Th1 $CD4^+$ IFN-γ in chlamydia infection clearance (7) and some animal studies demonstrating a potential protective role for TNF-α (8, 33), our study sought to increase our understanding of the frequency and magnitude of *C. trachomatis*-specific Th1-associated cytokine responses in humans. Using intracellular cytokine staining, we

291 analyzed *C. trachomatis*-specific TNF-α, IFN-γ and IL-2 cytokine production from both
292 $CD4^+$ and $CD8^+$ T-cells from chlamydia-infected women before treatment. We report for
293 the first time that the predominant Th1-associated cytokine response in women with
294 chlamydia was a $CD4^+$ TNF-α response, not IFN-γ, with PBMCs from approximately
295 80% of women eliciting a $CD4^+$ TNF-α response to at least one of the three *C.*
296 *trachomatis* antigens we tested. Other $CD4^+$ Th1 cytokine responses, IFN-γ and IL-2,
297 were present, but at much lower frequencies. Also, TNF-α-positive responses had a
298 higher magnitude than IFN-γ-positive responses, by almost 2-fold.

299 In contrast to CD4 responses, the overall frequency of $CD8^+$ T-cell TNF-α, IFN-γ and
300 IL-2 responses were low, occurring in <30% of infected women. Although one might
301 anticipate low $CD8^+$ responses with the use of a recombinant protein and EBs, we also
302 observed weak $CD8^+$ responses with the use of five 17-20mer MOMP peptides, which
303 have been shown to contain strong and functional $CD8^+$ epitopes (45, 46). Further, we
304 did not see any difference in the response between peptide and protein antigens with
305 longer incubation time before golgi transport inhibition, which allowed additional time for
306 antigen processing (data not shown). Therefore, we feel confident that our observed low
307 $CD8^+$ responses are accurate, though we cannot exclude the possibility that epitopes
308 not present in our MOMP peptides may contribute to $CD8^+$ responses.

309 TNF-α is an effector cytokine with known anti-chlamydial activity. *In vitro*,
310 recombinant TNF-α alone inhibits *C. trachomatis* in cell culture (31). *C. trachomatis*
311 growth inhibition by TNF-α has been shown to be synergistic with IFN-γ, with a 100-fold
312 increase in TNF-α potency (31). Inhibition by TNF-α likely occurs by enhancing
313 mechanisms of IFN-γ-dependent tryptophan depletion, as inhibition can be reversed by
314 tryptophan supplementation *in vitro* (32). In animal models, the contribution of TNF-α to

315 *Chlamydia* clearance appears tissue and/or species specific. For example, in the genital
316 tract, mice and guinea pig studies show increased TNF-α production during chlamydial
317 infection (15), but the absence of TNF-α does not appear to influence chlamydia
318 clearance (13, 14). However, in the mouse pneumonitis model, TNF-α inhibition
319 increased susceptibility to *C. muridarum* and increased mortality (8). An *in vivo* study
320 comparing *C. trachomatis* and *C. muridarum* infection of different mouse strains
321 demonstrated that TNF-α-deficient mice had delayed chlamydia clearance, which
322 differed by *Chlamydia* species (33). This may explain the variation in the influence of
323 TNF-α on *Chlamydia* growth inhibition and highlights the importance of human studies
324 for understanding mechanisms of chlamydia clearance.

325 Our finding that TNF-α is the predominant cytokine elicited to the intracellular
326 pathogen *C. trachomatis* suggests it may be an important component of the systemic
327 effector immune response and is consistent with other studies showing it contributes to
328 the immune response to *C. trachomatis*. For example, in individuals with trachoma,
329 sequence variation associated with the *TNF* locus has been associated with increased
330 risk of trachomatous scarring and trichiasis and elevated TNF responses induced by
331 EBs (34). The presence of the *TNF-α-308A* allele has been associated with an
332 increased risk of scarring trachoma (35) and development of severe adhesions in tubal
333 factor infertility caused by *C. trachomatis* (36). These studies suggest that although
334 TNF-α is an effector cytokine that may contribute to protective immune responses,
335 excess levels of this proinflammatory cytokine can lead to immunopathology after
336 infection (12, 37). Other evidence supporting the importance of TNF-α in immune
337 control of intracellular organisms includes studies demonstrating that TNF-α-inhibitory
338 monoclonal antibodies are strongly associated with an increased susceptibility to

339 infection with the intracellular pathogens *Histoplasma* and *Listeria* and reactivation of
340 latent tuberculosis (38). Whether TNF-α is associated with a reduced risk for *C.*
341 *trachomatis* reinfection remains unknown, and we are conducting studies that will
342 address this important question.

343 Our finding that positive Th1 cytokine responses, especially IFN-γ, are not generated
344 in all chlamydia-infected women is consistent with other studies. Two previous human
345 studies evaluated the frequency of positive IFN-γ responses with PBMC stimulation with
346 *C. trachomatis* antigens, measured by either ELISpot or ELISA, but did not evaluate the
347 frequency of other Th1 cytokine responses including TNF-α (26, 27). In a cohort of
348 commercial sex workers at risk for *C. trachomatis* infection, Cohen et al. reported
349 positive IFN-γ responses to EB occurred in 40% (26). In a cohort of female adolescents
350 with current or prior *C. trachomatis* infection, Barral et al. reported positive IFN-γ
351 responses to EB and MOMP occurred in 62% and 38% of adolescents, respectively
352 (27). In our study, we used ICS rather than ELISpot or ELISA to characterize cytokines
353 responses, which allowed us to determine IFN-γ responses produced specifically by
354 $CD4^+$ and $CD8^+$ T-cells. In our cohort of chlamydia-infected women, a positive $CD4^+$
355 IFN-γ response to any of the 3 antigens occurred in 16% of subjects and positive $CD8^+$
356 IFN-γ response in 22% of subjects. Our lower frequency of positive IFN-γ responses
357 compared with the above studies could, in part, reflect differences in assay cutoff for
358 positivity (with all the studies using different assays) or the fact that we measured IFN-γ
359 produced specifically by $CD4^+$ and $CD8^+$ cells, whereas the above studies likely
360 measured IFN-γ production from all $CD3^+$ T-cells with an unknown contribution from
361 other mononuclear cells (*e.g.*, NK cells, monocytes, *etc*); it is worth noting that the

362 Barral et al. study did partially deplete CD8$^+$ cells to limit detection of IFN-γ from that cell
363 source (27), though other mononuclear cells remained present.

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

378 Our study used a panel of multiple *C. trachomatis* antigens to provide a more
379 comprehensive characterization of *C. trachomatis*-specific T-cell responses. We found
380 that Pgp3 most often elicited a positive CD4$^+$ TNF-α-positive response compared with
381 the other antigens and also more often elicited dual-positive CD4$^+$ cytokine responses.
382 To our knowledge, Pgp3 has never been investigated in prior studies of *C. trachomatis*-
383 specific cytokine cellular immune responses in humans, although it has been
384 demonstrated in a murine model that Pgp3 deficiency significantly attenuates infectivity
385 and pathogenicity of *C. trachomatis* (42). In our study, both single- and dual-positive

386 TNF-α-producing CD4$^+$ T-cell responses to Pgp3 were higher in frequency and
387 magnitude than to MOMP or EB, the latter which contains, as part of its surface, the
388 chlamydial outer membrane complex comprised of over 300 different proteins (43).
389 Although it remains unclear why EB did not elicit cytokine responses as frequently as
390 Pgp3, it could indicate that the key EB antigenic T-cell epitopes are relatively diluted as
391 compared to a single protein like Pgp3, which thereby may blunt the magnitude of the
392 immune response. Although an insufficient EB concentration is another possibility, we
393 think this is unlikely as we used a 4-fold higher concentration of EB in our ICS assays
394 than Barral et al. (27) and we found that EBs generally elicited more frequent CD4$^+$
395 TNF-α responses than MOMP and comparable IFN-γ responses (Fig. 2B-C). Although
396 the difference in percent positive cells elicited by Pgp3 stimulation versus the other
397 antigens may appear insignificant (0.11% versus 0.08%), that represents an
398 approximate 40% increase in circulating TNF-α-positive CD4$^+$ T-cells and a magnitude
399 of 0.1% is consistent with CD4$^+$ T-cell responses reported by others in post-vaccinated
400 subjects vaccinated with Hepatitis B (47), influenza (48), and MMR (49). Additionally,
401 the observation that Pgp3 generated more dual-positive responses than MOMP or EB
402 may be an important observation for Pgp3 vaccine candidacy since TNF-α/IFN-γ dual-
403 positive responses have been shown to be functionally superior and elicit protective
404 responses in murine models (16).

405 Our study had strengths and limitations. This is the largest study investigating
406 human *C. trachomatis*-specific T-cell responses using ICS; a previous study by Miguel
407 et al that used ICS only studied cytokine responses in 14 *C. trachomatis*-infected
408 women and did not present data on the proportion of women with a positive response
409 (44). Because we included multiple *C. trachomatis* antigens in our ICS studies, we
410 were able to investigate inter-antigenic variation of cytokine responses, which revealed

411 significant variability in $CD4^+$ TNF-α responses. Our study is the first to evaluate *C.*
412 *trachomatis*-specific cellular immune responses through incorporation of dual gating
413 into our ICS methodology, which allowed us to differentiate single cytokine producing
414 cells from dual producing cells. One notable caveat with our findings is that the majority
415 of subjects studied were African American, representative of the race/ethnicity of the
416 clinic population, and therefore these findings may not be generalizable to other
417 populations. In this study, we focused on Th1 cytokine responses, which was the aim
418 of our initial investigation into *C. trachomatis*-specific immune responses in humans
419 because of animal models demonstrating the importance of Th1 responses (7). In
420 future studies, we will be evaluating a broader group of cytokines, including Th2-
421 associated cytokines, and will evaluate immune responses at follow-up visits to
422 determine longevity of *C. trachomatis*-specific immune responses and association of
423 cytokine responses (single and dual-functional) with chlamydia reinfection. Ultimately,
424 we hope to identify immune correlates of protection to *C. trachomatis* infection in
425 humans.

426 In summary, the predominate Th1-associated cytokine response in women with
427 uncomplicated chlamydia was a $CD4^+$ TNF-α response, and $CD4^+$ T-cells producing
428 both TNF-α and IL-2 were not uncommon. The frequency of $CD4^+$ TNF-α responses
429 was influenced by antigen used for stimulating PBMCs, with Pgp3 most often eliciting a
430 $CD4^+$ TNF-α single and dual-positive response.

431

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436

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FIGURE LEGENDS

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

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

FIG. 3. Magnitude of cytokine responses from $CD4^+$ T-cells stimulated with *C. trachomatis* antigens in chlamydia-infected women with a positive or negative cytokine response (qualitative) and in chlamydia-negative control women. Responses were measured by intracellular cytokine staining. A positive cytokine response was defined as >0.05% and twice

619 the background (media + co-stimulatory antibodies). The magnitude of the cytokine response is
620 shown as percentage of total $CD4^+$ T-cells after subtracting the background response, stratified
621 by *C. trachomatis* antigen used. The box denotes the interquartile range and the whiskers
622 denote the 5th and 95th percentiles. Dots denote outliers. The median is shown as the horizontal
623 line in the box. *N* denotes the number of subjects. Significance was evaluated by Wilcoxon
624 signed-rank or rank sum tests. The asterisks denote $P < 0.001$. The Y-axis is $\log_{10}$.
625
626 FIG. 4. Venn diagram demonstrating the overlap of $CD4^+$ TNF-α responses across *C.*
627 *trachomatis* antigens used for PBMC stimulation. Subjects with a positive $CD4^+$ TNF-α
628 response to any antigen were identified (n = 71) and the concordance of positive responses
629 across antigens MOMP, Pgp3, and EB was evaluated. The percentages indicate the proportion
630 of total positive $CD4^+$ TNF-α responses elicited by antigen(s).
631
632 FIG. 5. Dual cytokine-producing $CD4^+$ T-cells from chlamydia-infected women expressing TNF-
633 α and either IL-2 or IFN-γ. (A) Representative flow cytometry plots from chlamydia-infected
634 women with dual-positive $CD4^+$ TNF-α and either IFN-γ or IL-2 responses. (B) Pie chart
635 showing proportion of positive $CD4^+$ TNF-α responses that produced TNF-α alone or produced
636 both TNF-α and IL-2 responses or both TNF-α and IFN-γ responses. (C) Percentage of total
637 dual-positive subjects with a dual positive cytokine response by antigen. n = 15 for a positive
638 $CD4^+$ TNF-α+/IL-2+ response to at least one antigen and n = 5 for a positive $CD4^+$ TNF-α+/IFN-
639 γ+ response to at least one antigen. A positive cytokine response was defined as >0.05% and
640 twice the background (media + co-stimulatory antibodies). The number of positive responses by
641 antigen is indicated above each bar. Significance was determined using McNemar's chi-square
642 test.

Table 1. Study Subject Characteristics (*n* = 90)

Subject Characteristics	*n* (%)
Median age (range)	22 (16-32)
Race	
Black	87 (97)
White	3 (3)
Ethnicity	
Non-Hispanic	89 (99)
Hispanic	1 (1)
Hormonal contraception	41 (46)
Median prior sex partners**	
Last 3 mo. (range)	1 (1-5)
Prior Chlamydia*	51 (57)
Asymptomatic	49 (54)
Cervicitis*	17 (19)
Co-Infections	
HIV infection	0
Gonorrhea	0
Bacterial Vaginosis	26 (29)
Trichomoniasis	3 (3)
Candidiasis	11 (12)

* *n* =89, ***n* =88