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Cervicovaginal inflammation and HIV target cell activation in adolescent girls and young women with *Chlamydia trachomatis* infections

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ABSTRACT

Background Adolescent girls and young women (AGYW) in sub-Saharan Africa are disproportionately affected by *Chlamydia trachomatis* (CT) infections, a leading cause of pelvic inflammatory disease, infertility and increased HIV susceptibility. CT-induced genital inflammation disrupts mucosal barriers and activates HIV target cells, compounding immunological risks. We investigated CT infection in AGYW from two South African regions, focusing on genital inflammation and immune activation.

Methods An observational cohort of 298 sexually active AGYW (aged 16–22 years) from Cape Town and Johannesburg was enrolled. Participants in Johannesburg attended one visit, while those in Cape Town were followed longitudinally (6–8 months). Cytokine profiling of cervicovaginal samples assessed inflammatory responses, while cervical cytobrush-derived CD4+ T cells were analysed for CD38, Ki67 and C-C chemokine receptor type 5 expression. CT *ompA* genotyping examined strain diversity.

Results CT prevalence was 2.4-times higher in Cape Town (41.6%) than Johannesburg (17.4%). CT infection was associated with upregulated interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α, with responses appearing stronger in Johannesburg, although low CT cases at this site limited regional comparisons. Genotyping suggested regional variation: genovar D, predominant in Cape Town, was associated with lower interferon (IFN)-γ concentrations, whereas genovar E, more common in Johannesburg, was associated with higher IFN-γ concentrations. AGYW with CT infection across multiple visits did not exhibit elevated genital tract cytokine levels compared with those infected at a single visit. However, they showed increased activated cervical CD4+ T cells.

Conclusions CT-associated immune responses in AGYW appear to reflect a combination of pathogen diversity, regional context and host immune history.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ *Chlamydia trachomatis* (CT) infection is common among adolescent girls and young women in sub-Saharan Africa and is associated with increased genital inflammation and HIV acquisition risk; however, the relative contribution of CT strain diversity and host factors to these inflammatory responses remains unclear.

WHAT THIS STUDY ADDS

⇒ CT infection was associated with increased genital inflammation and activation of cervical HIV target cells in South African adolescent girls and young women.

⇒ Despite similar CT genovar distributions between sites, inflammatory responses differed geographically, suggesting that host and environmental factors may be more important drivers of immune activation than CT genotype alone.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings reinforce the importance of identifying and treating asymptomatic CT infections in young women and highlight the need for future studies that integrate pathogen, microbiome and host immune data to better understand sexually transmitted infection-associated mucosal vulnerability and HIV risk.

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INTRODUCTION

Women in sub-Saharan Africa face disproportionately high risks of HIV infection, driven by biological and socio-behavioural vulnerabilities.[1] Genital inflammation is a critical factor that increases the risk of HIV acquisition.[2] Sexually transmitted infections (STIs), including *Chlamydia trachomatis* (CT), are major drivers of genital inflammation. CT infections are frequently asymptomatic in women, leading to undertreatment despite associations with pelvic inflammatory disease, infertility, ectopic pregnancy and increased HIV risk due to subclinical inflammation.[3]

CT infection induces genital cytokine responses that play a key role in pathogenesis.[4] Pro-inflammatory mediators such as interleukin (IL)-1β, IL-6 and interferon gamma-induced protein 10 can exacerbate tissue damage and increase HIV

susceptibility through mucosal barrier disruption and CD4+ T cell activation.[2 5 6] Persistent CT infection can further amplify inflammation, contributing to immune dysregulation and impaired clearance.[6]

CT exhibits substantial genetic diversity that influences transmission dynamics, tissue tropism and host immune responses. Urogenital CT infections in women, including adolescent girls and young women (AGYW) in sub-Saharan Africa, are dominated by a limited number of *ompA*-defined genotypes, particularly E, D and F, with genotypes J, G and K contributing smaller but consistent proportions across regions.[7 8] Variation within and between *ompA* genotypes has been associated with differences in immune activation, persistence and clinical outcomes, underscoring the relevance of characterising circulating CT genotypes in high-burden African AGYW populations.[9]

AGYW are particularly vulnerable to CT infection due to socio-behavioural and biological factors. The adolescent cervix has a larger area of ectropion,[10] exposing columnar epithelium that is more permissive to bacterial adhesion and invasion than the stratified squamous epithelium in mature women. Together with delayed diagnosis and reinfection, these factors increase the risk of prolonged inflammation and reproductive complications in AGYW.[11]

Given these challenges, it is important to investigate interactions between CT genotypes, host inflammatory responses and mucosal immune activation in AGYW. We examined how CT genotypes influence genital inflammation and immune activation in AGYW from two South African regions.

MATERIALS AND METHODS

Cohort description

Between November 2013 and February 2015, AGYW (aged 16–22 years) were recruited from Cape Town (n=149) and Johannesburg (n=149) through the Desmond Tutu HIV Centre and the Perinatal HIV Research Unit, respectively.[12] The study was cross-sectional in Johannesburg while the cohort from Cape Town arm was followed longitudinally for up to three visits, using a targeted enrolment strategy with predefined sample sizes.[12] For the longitudinal cohort, study visits were 3 monthly unless participants were using Net-EN, in which case it was 2 monthly. AGYW using the injectable contraceptives (DMPA or Net-EN) had visits scheduled 2 weeks postinjection, while non-users were scheduled during the luteal phase (days 14–28) of their menstrual cycle. Participants were excluded if they were HIV-positive, pregnant, sexually inactive, douched or used spermicides in the past 2 days, or took antibiotics in the past 2 weeks.

Sample collection

At enrolment, participants completed a socio-demographic questionnaire.[12] At each visit, cervicovaginal fluid was collected using a Softcup menstrual cup, inserted for 1 hour. During the clinical speculum examination, a Dacron vulvovaginal swab was taken for STI testing, a FLOQSwab collected lateral vaginal wall samples for bacterial vaginosis (BV) screening (Nugent scoring[13]) and cervical mononuclear cells were obtained using a Digene endocervical cytobrush.

Testing for STIs and BV

Vulvovaginal samples were tested for discharge-causing STIs (CT, *Neisseria gonorrhoeae* (NG), *Trichomonas vaginalis* (TV), *Mycoplasma genitalium* (MG)), as well as ulcer-causing STIs (herpes simplex virus (HSV)-1, HSV-2, *Haemophilus ducreyi* (HD), *Treponema pallidum*), using real-time multiplex PCRs.[14] BV was diagnosed by Nugent score.[13] CT-infected cases were treated with azithromycin (1 g orally, single dose), NG with ceftriaxone (250 mg intramuscular injection, single dose) and BV (Nugent 7–10) or TV with metronidazole (400 mg orally, twice daily for 7 days), according to South African national guidelines at the time. AGYW with CT/NG were given partner notification letters and referred to a local clinic.

Chlamydia *ompA* genotyping

OmpA genovar diversity of CT was analysed by MLST genotyping.[8 15] Sequence data were analysed using the CT database hosted on PubMLST (https://pubmlst.org/bigsdb?db=pubmlst_chlamydiales_seqdef). The *ompA* genovars were assigned by comparison with an offline reference database described previously.[8 15]

Measurement of cytokine concentrations

Softcups were collected (in 50 mL Falcon tubes), maintained at 4°C after collection (as previously described[16 17]) and processed at a site-adjacent laboratory within 4 hours for storage at −80°C. Processing and elution of cervicovaginal fluid involved inverting the Softcup membrane using a sterile disposable wooden spatula in a BSL2 hood, followed by centrifugation (1500 rpm for 10 min). The volume of secreted fluid was calculated, as previously described.[17] The Softcup was discarded. Assuming 1 g=1 mL, secretions were diluted 10 times with phosphate-buffered saline (PBS) and stored immediately at −80°C in aliquots. All Softcup samples were transported to the University of Cape Town laboratory on dry ice, aliquots were thawed in batches and cytokines were measured by Luminex using kits from the same lot number.[16] Prior to Luminex, all samples were filtered by centrifugation (1950 g, 10 min, 4°C) using SPIN-X 0.2 μm filters. Two different kits were used: the Human Cytokine Group I 27-plex (fibroblast growth factor-basic, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor, interferon (IFN)-γ, interleukin (IL)-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor-BB, RANTES, tumour necrosis factor (TNF)-α and vascular endothelial growth factor) and the Human Cytokine 21-plex kit (IL-1α, IL-2RA, IL-3, IL-12(p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, macrophage colony-stimulating factor, MIF, MIG, NGF, SCF, SCGF-β, SDF-1α, TNF-β and TRAIL) (Bio-Rad). Experiments followed manufacturer instructions. Samples from both sites were randomly allocated on the different assay plates, with the same 10 samples included in all plates. Interplate and intraplate correlations (Spearman's correlations) between these 10 samples were carried out for each cytokine among all the plates. IL-15 was excluded as the values did not pass the quality control. The cytokines IL-2, IL-5 and RANTES were not included in the analysis as 76.0%, 64.2% and 52.6% of the values, respectively, were below detectable range. For the rest, detection limits ranged from 1 to 307 pg/mL and concentrations were determined using 5PL regression. Values below detection limits were recorded as half the lowest measured concentration.

Flow cytometry

CMC samples from Cape Town participants were collected and analysed via flow cytometry at each visit. Cervical cytobrush samples were processed as previously described,[16] with the cell

pellet resuspended in PBS, transferred to a 96-well plate, centrifuged at 300 g for 3 min at 4°C and washed before staining. An eight-colour flow cytometry panel was used to assess C-C chemokine receptor type 5 (CCR5) (APC; BD Biosciences), activation markers CD38 (PE-Cy7; eBioscience) and human leucocyte antigen-DR (PE; BD Biosciences), and the proliferation marker Ki67 (fluorescein isothiocyanate; BD Biosciences) in CD4+CD3+ (APC-H7; BD Biosciences) and CD8+CD3+ (QDot605, Invitrogen) T cells. CD8+ T cells is not shown. A dump channel (LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen), CD14 (PacBlue, BD Biosciences), CD19 (PacBlue, Invitrogen)) excluded dead cells, B-cells and monocytes, respectively. Cells were first incubated with the live/dead marker for 20 min at room temperature, washed twice and stained with extracellular markers. After 30-minute ice incubation, cells were washed, fixed and permeabilised using Cytofix/Cytoperm (BD Biosciences). Intracellular markers CD3 and Ki67 were added, and cells were incubated for 30 min on ice. The pellet was resuspended in BD CellFIX, transferred to a FACS tube, wrapped in foil and stored at 4°C until acquisition on the BD LSRFortessa. Data were acquired within 48 hours, with compensation tubes prepared for each run. FlowJo (V.10.0.8; Treestar) was used for analysis, with gating based on FMO controls.

Statistical analysis

Statistical analyses and linear regressions were carried out using STATA V.12.0 and R V.3.6.0. Graphs and figures were generated using Prism 6 and R. Unpaired and paired continuous variables were compared using the Mann-Whitney U test and Wilcoxon signed-rank test, respectively, and categorical variables using two-sided χ^2 tests. Analyses were adjusted for multiple comparisons using false discovery rate step-down procedures.

RESULTS

A total of 298 sexually active AGYW (median age: 18 years; IQR: 17–20) from Cape Town and Johannesburg were included.[12 16] Johannesburg participants had a single-visit study, while Cape Town participants were followed over three visits spanning 6–8 months. Contraceptive use differed by site: in Johannesburg, 63.8% (77/121) of AGYW reported condom use alone, whereas in Cape Town 70.5% (105/149) used injectable hormonal contraception (DMPA or Net-EN). CT was the most prevalent STI (29.5%, 88/298), with CT prevalence being significantly higher in Cape Town (41.6%, 62/149; 95% CI 33.6% to 49.9%) than in Johannesburg (17.4%, 26/149; 95% CI 11.8% to 24.3%; p<0.0001). Among those with CT, 71.6% (63/88) had another STI or BV.

Impact of CT infection on genital inflammation in AGYW

The genital cytokine responses to CT infection in AGYW at baseline were assessed by multivariate linear regressions, adjusted for contraceptive use, co-infections with STIs and BV (figure 1A). AGYW infected with CT exhibited significant upregulation of cytokines across all functional classes compared with uninfected individuals. These included IL-1β (β-coefficient=0.36; p=0.022), IL-6 (β=0.21; p=0.038), TNF-α (β=0.23; p=0.015), IP-10 (β=0.29; p=0.035), MIG (β=0.27; p<0.0001), G-CSF (β=0.28; p=0.018), HGF (β=0.27; p=0.011), SCF (β=0.13; p=0.034), IFN-γ (β=0.17; p=0.025) and IL-4 (β=0.17; p=0.015), after

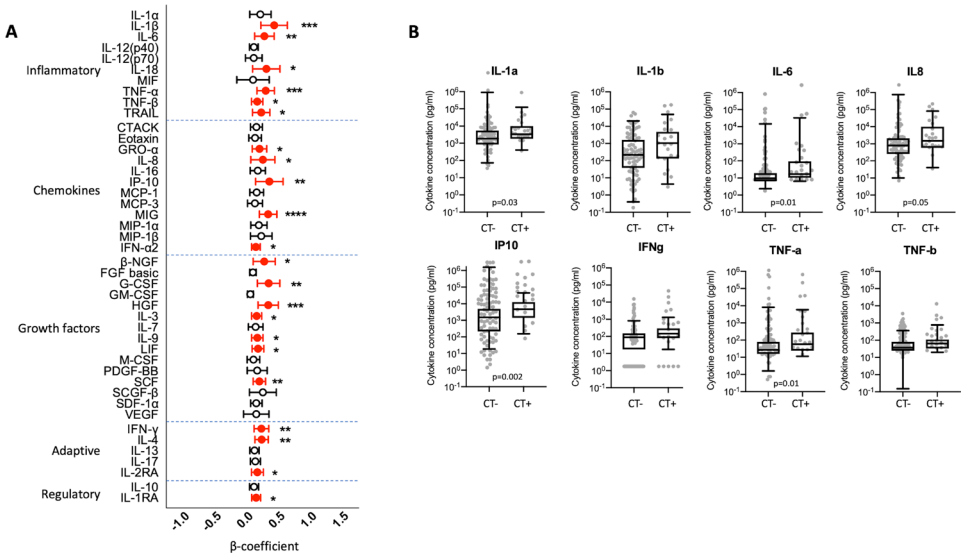

Figure 1 Impact of CT infection on cervicovaginal cytokine profiles in South African AGYW at baseline (only visit 1 for Cape Town AGYW). (A) Multivariate linear regression analysis illustrating associations between cervicovaginal cytokine levels and CT infection status, adjusted for participant age, hormonal contraceptive use, other co-infections, BV and semen exposure. Each association is represented by a β-coefficient, with error bars indicating the 95% CIs. Statistically significant associations (p≤0.05 after correction for multiple comparisons) are denoted with filled symbols. (B) Concentrations of cervicovaginal cytokines associated with inflammation (IL-1α, IL-1β, IL-6, IL-8, IP-10, TNF-α, TNF-β) and protection (IFN-γ) are compared between AGYW with CT infections (n=26; with no other STIs or BV) and those without CT (n=62; no STIs or BV). Group comparisons were performed using Mann-Whitney U tests with statistical significance set at p≤0.05 after adjustment for multiple comparisons. For women in Cape Town for which multiple visits were available, only the baseline visit was included (no repeated measures). AGYW, adolescent girls and young women; BV, bacterial vaginosis; CT, *Chlamydia trachomatis*; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon gamma-induced protein 10; MCP, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; STI, sexually transmitted infection; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

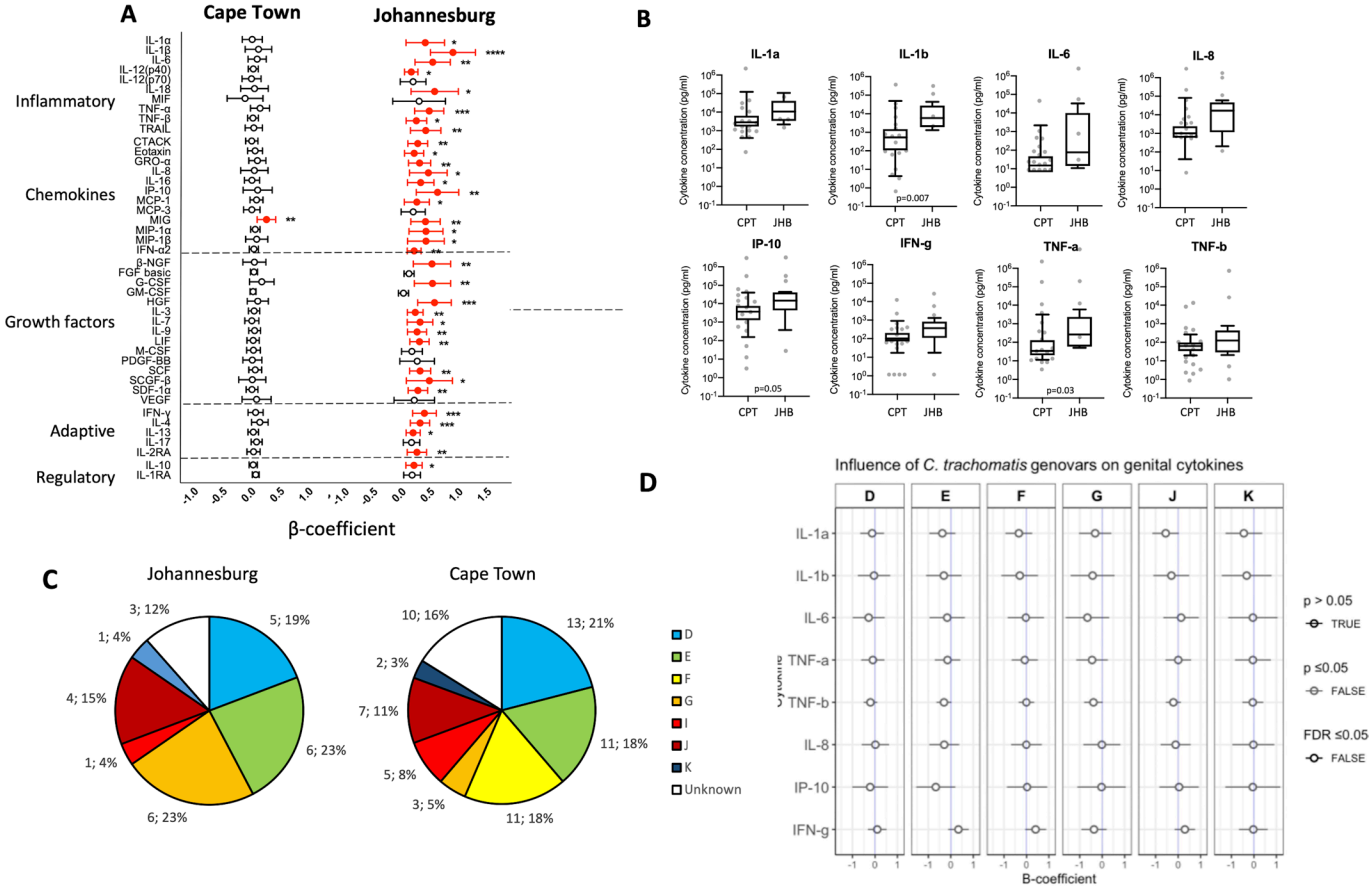

Figure 2 Investigating the impact of CT genovar on inflammatory responses in AGYW from CPT and JHB at baseline. (A) Multivariate linear regression analysis comparing cervicovaginal cytokine levels associated with CT infection in AGYW from CPT (left panel) and JHB (right panel), adjusted for participant age, hormonal contraceptive use, other co-infections, BV and semen exposure. Associations are represented by β-coefficients, with error bars indicating 95% CIs. Statistically significant associations (p≤0.05 after correction for multiple comparisons) are marked with filled symbols. (B) Cervicovaginal cytokine concentrations associated with inflammation (IL-1α, IL-1β, IL-6, IL-8, IP-10, TNF-α, TNF-β) and protection (IFN-γ) are compared between AGYW with CT infections in CPT and JHB (n=26; with no other STIs or BV). Group comparisons were conducted using Mann-Whitney U tests, with significance defined as p≤0.05 after adjustment for multiple comparisons. For women in CPT for which multiple visits were available, only the baseline visit was included (no repeated measures). (C) Distribution (n, percentage) of CT *ompA* genotypes in AGYW from JHB (left pie chart) and CPT (right pie chart). Genovar D is represented in blue, E in green, F in yellow, G in orange, I in bright red, J in dark red, K in purple and unknown genotypes in white. (D) Multivariate linear regression analysis of associations between cervicovaginal cytokine levels and CT *ompA* genovars (D–K), adjusted for participant age, hormonal contraceptive use, other co-infections, BV and semen exposure. Associations are represented by β-coefficients with 95% CIs. None of the associations were statistically significant (p≤0.05) after correction for multiple comparisons. AGYW, adolescent girls and young women; BV, bacterial vaginosis; CPT, Cape Town; CT, *Chlamydia trachomatis*; FDR, false discovery rate; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon gamma-induced protein 10; JHB, Johannesburg; MCP, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; STI, sexually transmitted infection; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

adjusting for multiple comparisons. A focused analysis on cytokines implicated in CT pathogenesis (IL-1α, IL-1β, IL-6, IL-8, TNF-α, TNF-β) and protection (IFN-γ), including AGYW with CT infection alone (n=26; no co-infections or BV) compared with uninfected controls (n=62; no STIs or BV) (figure 1B), further confirmed that CT infection significantly elevated concentrations of inflammatory cytokines, including IL-1α (p=0.03), IL-6 (p=0.01), IL-8 (p=0.05), IP-10 (p=0.002) and TNF-α (p=0.01). Although protective IFN-γ levels were higher in CT cases compared with controls, this was not significant.

Chlamydia diversity and associated inflammation by study site

CT infection appeared more inflammatory in Johannesburg than in Cape Town at baseline (figure 2A). In Cape Town, CT was linked to MIG upregulation (not significant after multiple comparisons), while in Johannesburg, it was associated with increased concentrations of 34/44 cytokines. Among AGYW with CT alone (no other STIs or BV), inflammatory cytokines tended to be higher in Johannesburg (n=4 CT+; no other STIs/BV) than Cape Town (n=22 CT+; no other STIs/BV), although numbers were small (figure 2B).

To assess whether CT strain diversity contributed to differences in inflammatory responses, *ompA* genotyping was performed on 128 samples with sufficient DNA (figure 2C), including 20/26 cases from Johannesburg and 108/121 cases from Cape Town (spanning all three study visits). Eighteen samples were excluded due to low DNA quantity. The distribution of *ompA* genovars did not differ significantly between sites (p=0.1; χ^2 test) and globally predominant urogenital genotypes were represented at

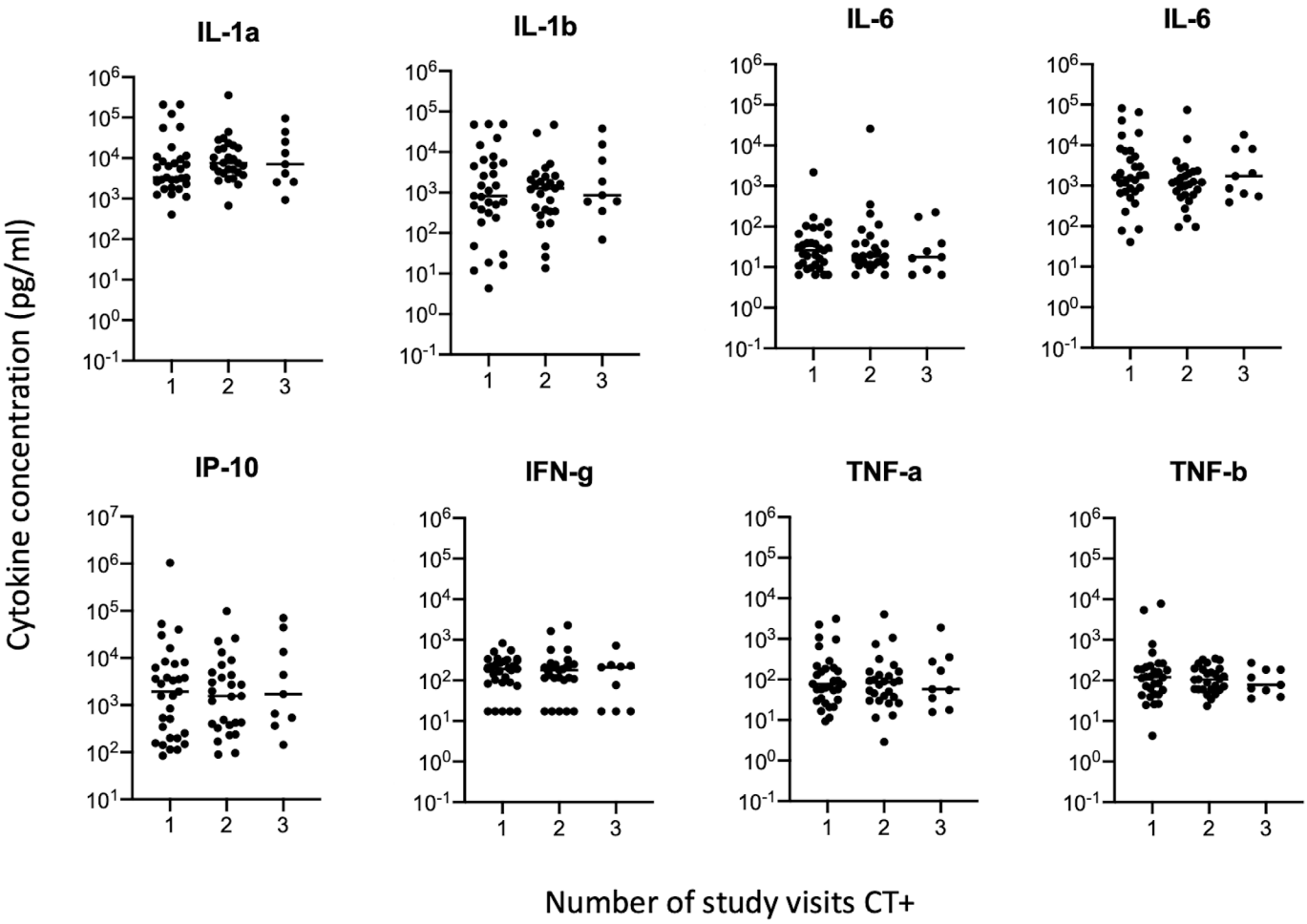

Figure 3 Cervicovaginal cytokine concentrations in AGYW infected with CT over one, two or three study visits. Each dot represents the cytokine response of an individual participant, with black lines indicating the median values for each group. Group comparisons were conducted using Mann-Whitney U tests, with statistical significance defined as p≤0.05. AGYW, adolescent girls and young women; CT, *Chlamydia trachomatis*; IFN, interferon; IL, interleukin; IP-10, interferon gamma-induced protein 10; TNF, tumour necrosis factor.

both sites. In Cape Town, genovar D was the most prevalent (21%, 13/62), followed by genovars E (18%, 11/62) and F (18%, 11/62), while genovar G was detected in 5% of cases (3/62). In contrast, in Johannesburg, genovars E and G were most common (23% each, 6/26), while genovar D was detected in 19% (5/26) of cases and genovar F was not detected. Less common genovars (I, J, K) were observed at low frequencies at both sites. No significant associations were observed between individual CT genovars and inflammatory cytokines, after adjustment for multiple comparisons (figure 2D). However, interpretation of regional differences should be approached cautiously. The number of CT-positive participants in Johannesburg was substantially smaller than in Cape Town (26 vs 62 CT-positive women at baseline), limiting statistical power for site-specific analyses and reducing confidence in estimates of regional inflammatory and genovar-associated profiles. However, interpretation of regional differences should be approached cautiously.

Inflammation associated with persistent CT infections

In Cape Town, where AGYW were followed longitudinally, 33.9% (43/127) were infected with CT at visit 2 and 18.2% (16/88) were infected at visit 3 (online supplemental table 1). Among participants with repeat CT detection, genotyping indicated that the infecting genovar was most often the same across visits: 10 women were CT-positive at all three study visits, of whom 7/10 harboured the same genovar at each visit; 10 were CT-positive at visits 1 and 2 only, all of whom (10/10) had the same genovar at both visits and five were CT-positive at visits 1 and 3 but not visit 2, of whom 4/5 had the same genovar at both positive visits. These findings suggest that repeated detection of CT in this cohort was more frequently associated with persistence or re-exposure to the same strain, which may reflect ongoing exposure from the same sexual partner or incomplete clearance, rather than acquisition of a new genotype. To investigate whether persistent or repeat CT infections were associated with increased inflammation, cervicovaginal cytokine concentrations were compared among women infected at one, two or three visits (figure 3). The analysis revealed no significant differences in the concentrations of any measured cytokines across these groups, regardless of the duration of infection.

Genital tract HIV target-cell activation associated with CT infections

Cervical cytobrush-derived CD3+CD4+ T cells were used to assess the expression of CCR5 (HIV entry receptor on CD4+ cells), CD38 (a marker of cellular activation) and Ki67 (a marker of T-cell proliferation) (Cape Town only). Figure 4A shows a representative figure of the gating strategy. The frequency of activated cervical CD38+ cells among CD3+CD4+ T cells

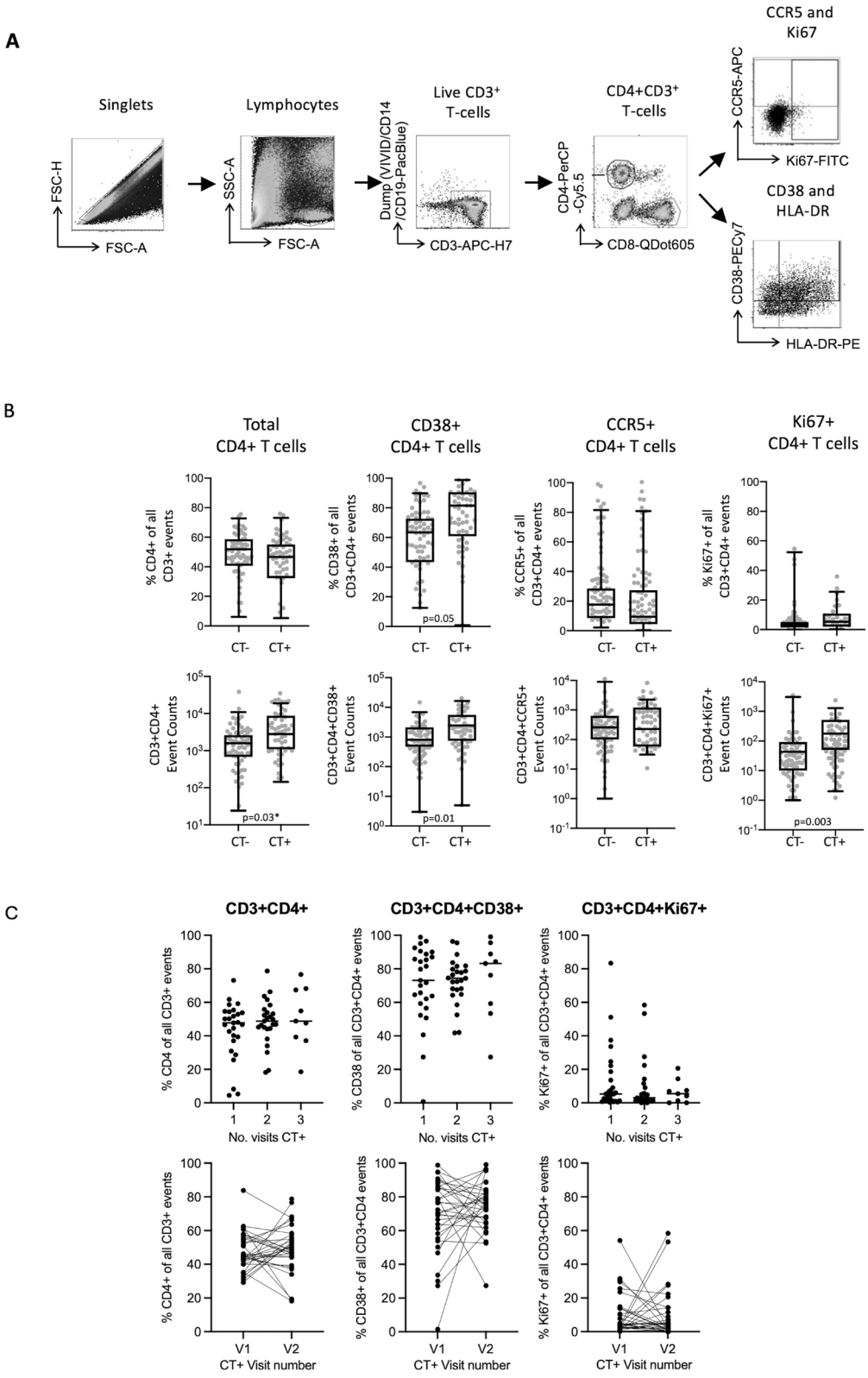

Figure 4 Impact of prevalent or persistent CT infections on cervical cytobrush-derived CD4+ T cell activation. (A) Representative gating strategy for flow cytometry analysis of cervical cytobrush-derived T cells, focusing on live CD3+CD4+ cells expressing activation marker CD38+, proliferation marker Ki67+ or HIV co-receptor CCR5. (B) The frequency (top panel) and absolute number of events (bottom panel) are shown for total CD3+CD4+ T cells (far left), CD3+CD4+ T cells expressing CD38 (middle left), CCR5 (middle right) and Ki67 (far right). Data were collected at visit 1 (study entry) from n=36 controls and n=18 CT cases. (C) Longitudinal analysis of cervicovaginal CD3+CD4+ T cell activation marker expression is presented for AGYW with CT infections spanning one, two or three study visits (top panel). Matched analyses for participants with repeat CT infections across multiple visits are shown in the lower panel. Statistical comparisons were performed using Mann-Whitney U tests for unpaired analyses (B) and Wilcoxon signed-rank test for paired data (C), with significance defined as p≤0.05. AGYW, adolescent girls and young women; CCR5, C-C chemokine receptor type 5; CT, *Chlamydia trachomatis*; FITC, fluorescein isothiocyanate; HLA, human leucocyte antigen.

was significantly higher in CT cases compared with controls (p=0.05; figure 4B, top panel). However, there were no significant differences in the frequencies of CD3+CD4+ T cells or those expressing CCR5 or Ki67. As a technical observation, CT+ cytobrush specimens yielded higher total acquired live events under standardised acquisition settings (figure 4B, bottom panel).

We further compared CD3+CD4+ T cell events in women infected with CT at one, two or three study visits (figure 4C, upper panel). The frequency of activated or proliferating cervical CD3+CD4+ T cells did not differ significantly between women infected at multiple time points and those infected at a single visit. In a paired analysis of women infected at two study visits (n=32), there was no significant difference in the frequency of T cells measured at the second positive visit compared with the first positive visit, and frequencies did not correlate significantly (CD3+CD4+ R^2=0.002 p=0.8; CD3+CD4+CD38+ R^2=0.02 p=0.5; CD3+CD4+Ki67+ R^2=0.02 p=0.5). This suggests that inflammation did not resolve or intensify with persistent or repeated infections (figure 4C, lower panel).

DISCUSSION

Understanding how asymptomatic CT infection contributes to HIV susceptibility in AGYW remains an important challenge for prevention. Here, we examined the intersection between CT infection, genital inflammation and HIV target cell availability across two South African settings, highlighting a complex interplay between pathogen diversity, host immune history and local mucosal context. Rather than implicating a single explanatory factor, our findings indicate that inflammatory and cellular immune responses to CT reflect multiple determinants, including strain-level variation, prior exposure and site-specific host or vaginal milieu factors (microbiome), helping to explain why inflammatory outcomes may differ across populations despite exposure to common CT lineages.

Genotyping revealed few differences in *ompA* genovar distribution between sites, with genovar D predominating in Cape Town and genovars E and G more frequent in Johannesburg. Although AGYW with CT infection in Johannesburg appeared to exhibit a stronger overall inflammatory signature than those in Cape Town, these findings should be interpreted cautiously given the substantially smaller number of CT-positive participants in Johannesburg, which limited statistical power for regional comparisons. As a result, the drivers of the apparent site-level differences remain uncertain but may extend beyond *ompA* genovar distribution to include host, microbial, environmental or vaginal milieu factors. Differences in CT prevalence, genovar distribution and inflammatory profiles suggest distinct CT epidemiological settings in Cape Town and Johannesburg.

While variation in *ompA* genovars was explored, emerging evidence indicates that host immune history is likely a more dominant determinant of inflammation. In the same cohort, prior CT exposure and the quality of CT-specific CD4+ T cell responses strongly influenced genital immune profiles, with robust systemic type 1 T helper responses inversely associated with mucosal inflammatory mediators.[18] Women with untreated or recurrent CT infection showed increased genital T cell activation alongside impaired CT-specific T cell function, consistent with immune dysregulation following repeated exposure. Together, these findings suggest that adaptive immune history may outweigh strain-level variation and that genovar associations should be regarded as hypothesis-generating rather than causal.

Strain-specific associations nonetheless highlight heterogeneity in CT immunogenicity. The association of genovar D with lower IFN-γ and genovar E with higher IFN-γ supports a role for pathogen genetic variation in shaping host responses. IFN-γ can drive CT into a persistent state via induction of indoleamine-2,3-dioxygenase, promoting immune evasion and chronic infection.[19] Supporting this, Duynhoven *et al*[20] reported greater lower abdominal pain among women infected with F or G group genotypes. Collectively, these findings may reflect variation in immunodominant T-cell epitopes within the major outer membrane protein,[21] reinforcing the value of strain-level characterisation for understanding CT pathogenesis and informing vaccine design.

This study has several limitations. Longitudinal data were only available from Cape Town, limiting analyses of repeat CT infections and cellular activation measurements were similarly restricted. Partner treatment data were not systematically collected and post-treatment cure testing was not performed. Longitudinal *ompA* genotyping in Cape Town showed that repeat CT detection usually involved the same genovar, suggesting persistence or re-exposure to the same strain, although reinfection from the same partner or incomplete clearance cannot be excluded. Genotyping was based on *ompA* sequencing rather than whole-genome sequencing, which may not capture within-genotype diversity[21] or recombination events.[22] The relatively small number of CT-positive participants in Johannesburg limited statistical power for regional comparisons and may have influenced estimates of generalisability of site-specific genotype associations and genotype-associated profiles. Consequently, conclusions regarding geographic differences should be considered hypothesis-generating and require confirmation in larger cohorts specifically designed to assess regional variation.

The high prevalence of CT-associated inflammation in AGYW highlights the need for improved STI screening and integrated sexual and reproductive health services in high HIV-burden settings.[23] Regional differences in CT immunogenicity support tailored prevention approaches, including regionally informed vaccine strategies. In conclusion, our findings show that CT-associated genital immune responses in AGYW reflect the interplay of strain diversity, regional context and host immune history.

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Contributors J-ASP and HBJ conceptualised and designed the study, secured funding, oversaw study implementation, supervised data analysis and contributed to manuscript drafting and revision. SD performed laboratory experiments, conducted data analyses, interpreted results and drafted the manuscript. SB contributed to participant recruitment, laboratory analyses, data analysis, interpretation of findings and manuscript drafting. BK performed CT MLST and genovar analyses, contributed to data interpretation and manuscript preparation. BV performed CT MLST typing, conducted sequence type analyses, interpreted genotyping data and contributed to manuscript preparation. EM and VM performed STI and BV diagnostic testing; EM additionally performed CT MLST typing. EM and VM contributed to data analysis, interpretation and manuscript drafting. DAL led STI diagnostic activities at the National Institute for Communicable Diseases, developed and supervised the STI diagnostic platform, supervised EM and VM, advised on CT genotyping approaches, contributed to data interpretation and revised the manuscript. JD and GG led participant recruitment and clinical activities in Johannesburg, contributed to interpretation of findings and revised the manuscript. KG and L-GB led participant recruitment and clinical activities in Cape Town, contributed to interpretation of findings and revised the manuscript. L-GB additionally supervised SB. MS and DPM contributed to statistical and data analyses, interpretation of findings and manuscript preparation. SMB established the MLST methodology used in the study, supervised BV, contributed to interpretation of results and revised the manuscript. All authors reviewed, revised and approved the final manuscript. J-ASP is the guarantor of the study and accepts full responsibility for the integrity of the work as a whole, including the study design, data collection, analysis, interpretation and decision to submit the manuscript for publication. Artificial intelligence (chatGPT) was used to check language and grammar.

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