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Relationship between female genital tract infections, mucosal interleukin-17 production and local T helper type 17 cells

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Summary

T helper type 17 (Th17) cells play an important role in immunity to fungal and bacterial pathogens, although their role in the female genital tract, where exposure to these pathogens is common, is not well understood. We investigated the relationship between female genital tract infections, cervicovaginal interleukin-17 (IL-17) concentrations and Th17 cell frequencies. Forty-two cytokines were measured in cervicovaginal lavages from HIV-uninfected and HIV-infected women. Frequencies of Th17 cells ($CD3^+$ $CD4^+$ $IL\text{-}17a^+$) were evaluated in cervical cytobrushes and blood by flow cytometry. Women were screened for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Trichomonas vaginalis* and herpes simplex virus 2 by PCR, and candidal infections and bacterial vaginosis by Gram stain. Women with bacterial sexually transmitted infections (STIs), specifically chlamydia and gonorrhoea, had higher genital IL-17 concentrations than women with no STI, whereas women with candidal pseudohyphae/spores had lower IL-17 concentrations compared with women without candidal infections. Viral STIs (herpes simplex virus 2 and HIV) were not associated with significant changes in genital IL-17 concentrations. Genital IL-17 concentrations correlated strongly with other inflammatory cytokines and growth factors. Although Th17 cells were depleted from blood during HIV infection, cervical Th17 cell frequencies were similar in HIV-uninfected and HIV-infected women. Cervical Th17 cell frequencies were also not associated with STIs or candida, although few women had a STI. These findings suggest that IL-17 production in the female genital tract is induced in response to bacterial but not viral STIs. Decreased IL-17 associated with candidal infections suggests that candida may actively suppress IL-17 production or women with dampened IL-17 responses may be more susceptible to candidal outgrowth

Keywords: Female genital tract; interleukin-17; sexually transmitted infection candidiasis; T helper type 17.

Abbreviations: BV, bacterial vaginosis; CI, confidence interval; CMCs, cervical mononuclear cells; CVL, cervicovaginal lavage; EGF, epidermal growth factor; FGF, fibroblast growth factor; Flt3L, Fms-like tyrosine kinase-3 ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GRO, growth-related oncogene; HAART, highly active antiretroviral therapy; HSV-2, herpes simplex virus type 2; IFN, interferon; IL, interleukin; IP-10, interferon-γ-induced protein; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; sCD40L, soluble CD40 ligand; sIL-2Rα, soluble interleukin-2 receptor α; STI, sexually transmitted infection; TGF, transforming growth factor; Th17, T helper type 17; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor

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Introduction

The female genital tract is exposed to diverse bacterial, viral and fungal pathogens as well as commensal organisms. T helper type 17 (Th17) cells, a distinct subset of $CD4^+$ T cells, have been suggested to play an important role in regulating immunity against fungal and bacterial pathogens in the female genital tract and other mucosal sites, including the gut and lung.[1–5] T helper type 17 cells are lymphocytes characterized by interleukin-17 (IL-17) production, although they also secrete other cytokines including IL-21 and IL-22.[6] Interleukin-17 can be pro-inflammatory, inducing the expression of other pro-inflammatory cytokines and chemokines by a broad range of cell types.[6] The Th17 cells help to maintain mucosal barrier integrity by promoting the formation of tight junctions between epithelial cells and production of gut-associated mucin.[7,8] In mice, IL-17 is produced by T cells in response to *Neisseria gonorrhoeae* infection and, in the absence of IL-17 responses, infection is prolonged.[5] The role of Th17 cells and IL-17 production in *Chlamydia trachomatis* infection is not well understood. Mouse models of chlamydial infection have shown that Th17 cells are activated and IL-17 is produced in response to chlamydial infection and suggest that disease severity and duration are reduced in the absence of IL-17.[3] However, following vaccination of mice, IL-17 appears to be important for protection against chlamydia.[3] These findings suggest an important role for this T helper subset in response to bacterial sexually transmitted infections (STIs). T helper type 17 cells also play an essential role in defence against fungal pathogens, including *Candida albicans*, and Th17 deficiency (due to rare genetic disorders or HIV infection) is associated with chronic candidiasis.[4,9] However, under certain conditions Th17 cells and IL-17 production are associated with the pathogenesis of inflammatory and autoimmune conditions.[10,11] T helper type 17 cells in the female genital tract have further been implicated as key targets for HIV replication and increased Th17 activation may result in increased susceptibility to HIV acquisition.[12,13]

Previous studies have shown that $CD4^+$ Th17 cells are depleted from the gut and female genital tract during HIV infection.[2,12,14] It has been suggested that this depletion at mucosal surfaces could compromise mucosal defences; in the case of the gut, this leads to translocation of bacterial and commensal products to the systemic compartment, resulting in immune activation.[2] HIV infection is associated with increased occurrence of vulvovaginal candidiasis and bacterial vaginosis (BV),[15,16] which may be partly attributed to Th17 depletion from this compartment.

Understanding the intersection between Th17 cells, genital inflammation, and vaginal bacterial or candidal burden may provide important insight into the role of these cells in genital tract mucosal immunity. The aims of this study were to investigate changes in Th17 cell frequencies and IL-17 concentrations in the female genital tract associated with bacterial, viral and candidal infections, and determine the relationships between IL-17 production and inflammatory and regulatory cytokines in the genital tract.

Methods

Description of study participants

This study included 227 HIV-uninfected and 39 highly active antiretroviral therapy (HAART)-naive HIV-infected women from Durban, South Africa,[17] and 18 HIV-uninfected and 33 HIV-infected women (21/33 using HAART) from primary health-care clinics in Cape Town, South Africa. Women who were menstruating at the time of sampling, who were postmenopausal, or who had undergone a hysterectomy were excluded from the study. The study was approved by the Research Ethics Committees of the University of Cape Town and the University of Kwa-Zulu Natal, South Africa, and written informed consent was obtained from all women before their participation.

Screening for STIs, BV and candidal infections

Vulvovaginal swabs from HIV-uninfected and -infected women from Durban were screened for *C. trachomatis*, *N. gonorrhoeae*, *Mycoplasma genitalium*, *Trichomonas vaginalis* and herpes simplex virus type 2 (HSV-2) by PCR, BV using Nugent's criteria and candidal pseudohyphae or spores using microscopy.[18] Cervical samples from Cape Town study participants were screened for *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis* by PCR and the Fungitell® kit was used to detect (1→3)-β-D-glucan produced by *Candida albicans*. (1→3)-β-D-glucan is a major constituent of the cell walls of most medically important fungi, including *Candida* and *Aspergillus*, although it cannot discriminate between these infections (manufacturer's brochure).

Sample collection and processing

Cervicovaginal lavages (CVLs) were collected from the women in Durban using sterile normal saline (10 ml). Saline was used to repeatedly bathe the cervix and allowed to pool in the posterior fornix, where it was then aspirated into a plastic bulb pipette. The CVLs were centrifuged and the supernatant was stored at −80°. Before cytokine measurements, CVLs were pre-filtered by centrifugation using 0·2-μm cellulose acetate filters (Sigma-Aldrich, St Louis, MO). Cervical mononuclear cells (CMCs) were collected from the endocervical canal of the women in the Cape Town cohort using a Digene cervical sampler and samples without blood contamination were processed within 3–6 hr of collection as described by Gumbi *et al.*[19] Blood was collected by venepuncture into sterile Acid Citrate Dextrose (ACD) anticoagulated Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient (Ficoll-Hypaque) centrifugation (Sigma-Aldrich, St Louis, MO, USA).

Measurement of cytokine concentrations

The concentrations of 42 cytokines were measured in CVLs collected from 227 HIV-uninfected and 39 HIV-infected Durban women using High Sensitivity and Human Cytokine LINCOplex Premixed kits (LINCO Research, St Charles, MO): IL-17, IL-1α, IL-1β, IL-1Ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, epidermal growth factor (EGF), eotaxin/CCL11, fibroblast growth factor (FGF)-2, fms-like tyrosine kinase-3 ligand (Flt3L), fractalkine/CX3CL1, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage (GM)-CSF, growth-related oncogene (GRO) family (CXCL1-CXCL3), interferon-α (IFN-α), IFN-γ, IFN-γ-induced protein (IP)-10/CXCL10, monocyte chemotactic protein (MCP)-1/CCL2, MCP-3/CCL7, macrophage-derived chemokine (MDC)/CCL22, macrophage inflammatory protein-1α (MIP-1α)/CCL3, MIP-1β/CCL4, platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB, regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5, sCD40L, soluble IL-2 receptor α (sIL-2Rα), transforming growth factor-α (TGF-α), tumour necrosis factor-α (TNF-α), TNF-β, and vascular endothelial growth factor (VEGF). The lower limit of detection of these kits ranged between 0·01 and 27·65 pg/ml for each of the 42 cytokines measured. In cervical cytobrush supernatants and plasma samples collected from the Cape Town women, Luminex (LINCO Research) was used to measure the concentrations of IL-1β, IL-6, IL-8, IL-10 and IL-12p40 and commercial ELISA was used to measure the concentrations of IL-17, IL-23 and TGF-β (R&D Systems, Minneapolis, MN). Luminex data were collected using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®, Hercules, CA) and a 5 PL regression formula was used to calculate cytokine concentrations from the standard curves (version 4; Bio-Rad Laboratories). Cytokine concentrations that were below the lower limit of detection of the assay were reported as the mid-point between the lowest concentration measured for each cytokine and zero.

Intracellular cytokine staining and flow cytometry for IL-17 production

Intracellular cytokine staining of cervical cytobrush-derived and blood cells was performed to analyse the frequency of IL-17-producing Th17 cells.[20] The CMCs and PBMCs were transferred into two BD Falcon tubes at ~2 × 10^5/500 μl/tube for CMCs (all of the cytobrush cells isolated) and 1 × 10^6 PBMC/500 μl/tube. The CMCs and PBMCs were stimulated with PMA/ionomycin [1 μg/ml PMA and 50 μg/ml ionomycin (Sigma-Aldrich, St Louis, MO, USA)] or left unstimulated (negative control). Cells were incubated for a total of 6 hr in a humidified incubator (37°, 5% CO_2). Brefeldin A (12·5 μg/ml; Sigma-Aldrich) was added after 1 hr of incubation. Cells were first stained with Vivid (Invitrogen, Carlsbad, CA) for 20 min at room temperature. Cells were then stained with CD8-Peridinin chlorophyll protein-Cy5.5 (BD Biosciences, San Jose, CA), CD4-FITC (BD Biosciences), CCR7-allophycocyanin (R&D Systems) and CD45RA-phycoerythrin-Cy7 (BD Biosciences). Cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and stained intracellularly with IFN-γ-Alexafluor 700 (BD Biosciences), IL-17-phycoerythrin (BioLegend, San Diego, CA) and CD3-allophycocyanin H7 (BD Biosciences). Cells were then re-suspended in 1× CellFix (BD Biosciences). Cell fluorescence was measured by flow cytometry using an LSR2 Flow Cytometer (BD Immunocytometry Systems, San Jose, CA) and FlowJo (FlowJo, LLC, Ashland, OR) was used for data analysis. A

minimum of 1×10^6 total events were captured per analysis for PBMCs and the entire cytobrush sample was captured for CMCs.

Statistical analysis

Data analysis was performed using GraphPad Prism (version 5; GraphPad Software, La Jolla, CA) and STATAtm (version 11; StataCorp, College Station, TX). Mann–Whitney *U*-test was used for unmatched comparisons, Wilcoxon Signed Rank test for matched comparisons and Spearman Rank test for correlations. *P*-values were adjusted using a false discovery rate step-down procedure to reduce false-positive results when multiple comparisons were made.[21] Cytokine signalling networks were evaluated using Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com). Gene identifiers were substituted for each cytokine name and mapped to their corresponding objects in the Ingenuity® Knowledge Base. These objects, called Network Eligible molecules, were overlaid onto a global molecular network and networks were algorithmically generated based on their connectivity. Median cytokine concentrations in CVL from women who had chlamydia or gonorrhoea that were more than twofold above the median in the no STI/BV group were considered up-regulated.

Results

Cervicovaginal IL-17 concentrations were elevated in women with bacterial STIs, but lower in those with candidal infections

In order to evaluate the relationships between soluble IL-17 production in the female genital tract and bacterial, candidal and viral infections, cytokine concentrations were measured in CVLs from HIV-uninfected and HIV-infected women from Durban, South Africa who were screened for common bacterial (*C. trachomatis*, *N. gonorrhoeae* or *M. genitalium*), protozoal (*T. vaginalis*), viral (HSV-2 and HIV) and candidal infections and BV (Table 1; Fig. 1). Interleukin-17 concentrations were significantly elevated in CVLs from women with bacterial infections, specifically *C. trachomatis* and *N. gonorrhoeae*, compared with women who did not have an STI or BV (adjusted $P = 0{\cdot}0005$ and $P = 0{\cdot}0047$, respectively). Although most of the women who had *C. trachomatis* or *N. gonorrhoeae* infections had other co-infections (Table 1), IL-17 remained significantly associated with chlamydial infection after adjusting for co-infections using logistic regression [odds ratio (OR) 10·16; 95% confidence interval (95% CI) 1·65–62·41; $P = 0{\cdot}012$], but not gonorrhoea (OR 2·98; 95% CI 0·95–9·28; $P = 0{\cdot}060$). Women with BV, *T. vaginalis* infections or BV/*M. genitalium* co-infections (each of the three women who had *M. genitalium* infections had BV) had similar levels of IL-17 compared with women who did not have BV or an STI.

Table 1. Prevelance of sexually transmitted infections and bacterial vaginosis in women from Durban, KwaZulu Natal

Durban women	HIV-uninfected $n = 227$	HIV-infected $n = 39$
Age in years, median (interquartile range)	36 (24–42)	24 (21–36)
Race, number of black women/total (%)	224/227 (98·7)	39/39 (100)
Injectable hormone contraceptive use, *n*/total (%)	63/226 (27·9)[1]	13/39 (33·3)
Combined oral contraceptive use, *n*/total (%)	5/226 (2·2)[1]	0/39 (0·0)
No STI or bacterial vaginosis, *n*/total	85/227	2/37
Trichomonas vaginalis, *n*/total (no. of single infections)	47/227 (13)	4/37 (1)
Neisseria gonorrhoeae, *n*/total (no. of single infections)	13/227 (2)	8/37 (2)
Chlamydia trachomatis, *n*/total (no. of single infections)	10/227 (3)	5/37 (2)
Mycoplasma genitalium, *n*/total (no. of single infections)	3/227 (0)	2/37 (0)
HSV-2, *n*/total (no. of single infections)	6/227 (0)	2/37 (0)
Candidal infection, *n*/total (no. single infections)	19/172 (10)[2]	ND
Bacterial vaginosis, *n*/total (no. of women with BV only)	120/227 (73)	28/37 (16)

BV, bacterial vaginosis; HSV-2, herpes simplex virus type 2; ND, not done; STI, sexually transmitted infection.
[1]One woman declined to answer.
[2]A total of 172 slides were available for candida analysis.

Interestingly, detection of candidal pseudohyphae and spores in genital secretions was associated with reduced IL-17 concentrations compared with women who did not have detectable fungal infections (Fig. 1b). This association remained significant after adjusting for STIs and BV using logistic regression (OR 0·49; 95% CI 0·26–0·92; $P = 0{\cdot}026$). This suggests that candidal infection may suppress IL-17 production or that lower levels of IL-17 in the female genital tract may render women more susceptible to candidal outgrowth. Viral infections, specifically HSV-2 ($P = 0{\cdot}219$) and HIV ($P = 0{\cdot}455$; Fig. 1c, d), were not associated with significant changes in IL-17 concentrations, although very few women ($n = 6$) were shedding HSV-2 and this analysis may have lacked statistical power.

Women who were using injectable hormonal contraception had reduced genital IL-17 concentrations compared with those not using injectable contraceptives (OR 0·69; 95% CI 0·47–0·99; $P = 0{\cdot}046$). Adjusting for injectable hormonal contraceptive use did not influence the

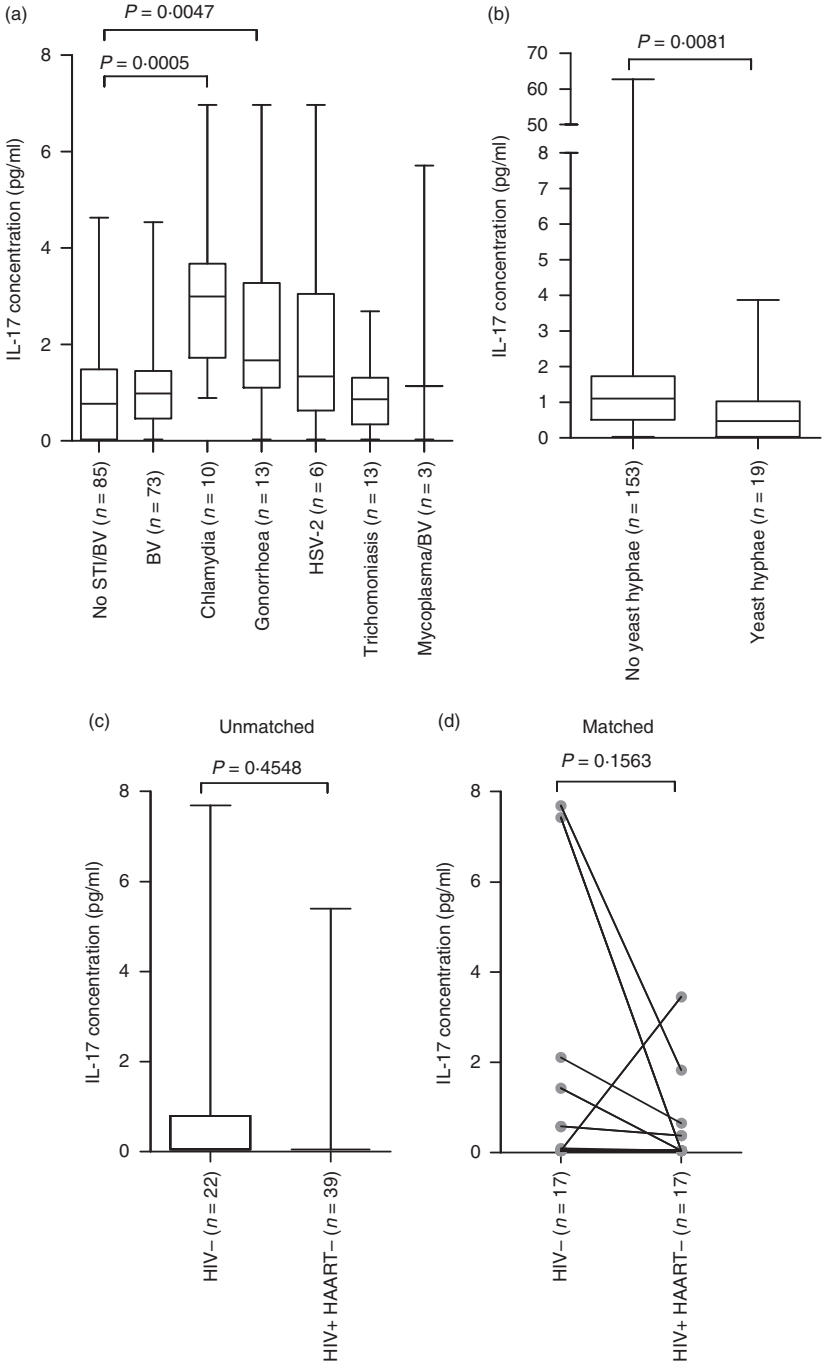


Figure 1. Relationships between interleukin-17 (IL-17) concentrations in cervicovaginal lavages and bacterial, candidal and viral infections. (a) Genital IL-17 concentrations were compared between HIV-uninfected women who were either not infected with a sexually transmitted infection (STI) or bacterial vaginosis (BV), had BV, *Chlamydia trachomatis*, *Neisseria gonnorhoeae*, *Trichomonas vaginalis*, reactivated herpes simplex virus type 2 (HSV-2) or BV/*Mycoplasma genitalium* co-infections (all of the women with *M. genitalium* infections also had BV). (b) Genital IL-17 concentrations were compared between HIV-uninfected women who did not have detectable candidal pseudohyphae and spores in their genital secretions to those with detectable candidal morphotypes. c) Genital IL-17 concentrations were compared between HIVuninfected and HIV-infected women. d) In a matched-pair analysis, genital IL-17 concentrations in HIV-infected women were compared to their IL-17 concentrations prior to HIV acquisition. Box and whisker plots show the median, 90 and 10th percentiles of IL-17 detected in CVL from each group of women. The Mann–Whitney *U* Test was used for unmatched comparisons. *P*-values were adjusted for multiple comparisons using a false discovery rate step-down procedure and adjusted *P*-values < 0·05 were considered significant.

relationships between IL-17 and candidal, *C. trachomatis* or *N. gonorrhoeae* infections.

Interleukin-17 production in the female genital tract was associated with genital inflammation

We compared IL-17 levels with concentrations of inflammatory, regulatory and homeostatic cytokines detected in matched CVLs in HIV-uninfected women from Durban (Fig. 2). Concentrations of IL-17 correlated strongly with growth factors IL-15, IL-9, TGF-α, Flt3L, FGF-2 and EGF ($\rho > 0{\cdot}5$; Fig. 2a) and the inflammatory chemokine CCL4/MIP-1β ($\rho > 0{\cdot}5$). Moderate associations with growth factors IL-3, G-CSF and PDGF-AB/BB and inflammatory cytokines CCL11/eotaxin, CCL3/MIP-1α, IL-12p40 and CX3CL1/fractalkine were observed ($\rho > 0{\cdot}4$; Fig. 2b). Of interest is the particularly strong correlation observed between IL-17 and both IL-15 and IL-9 concentrations, as these cytokines were also found to be down-regulated in the genital tracts of women with

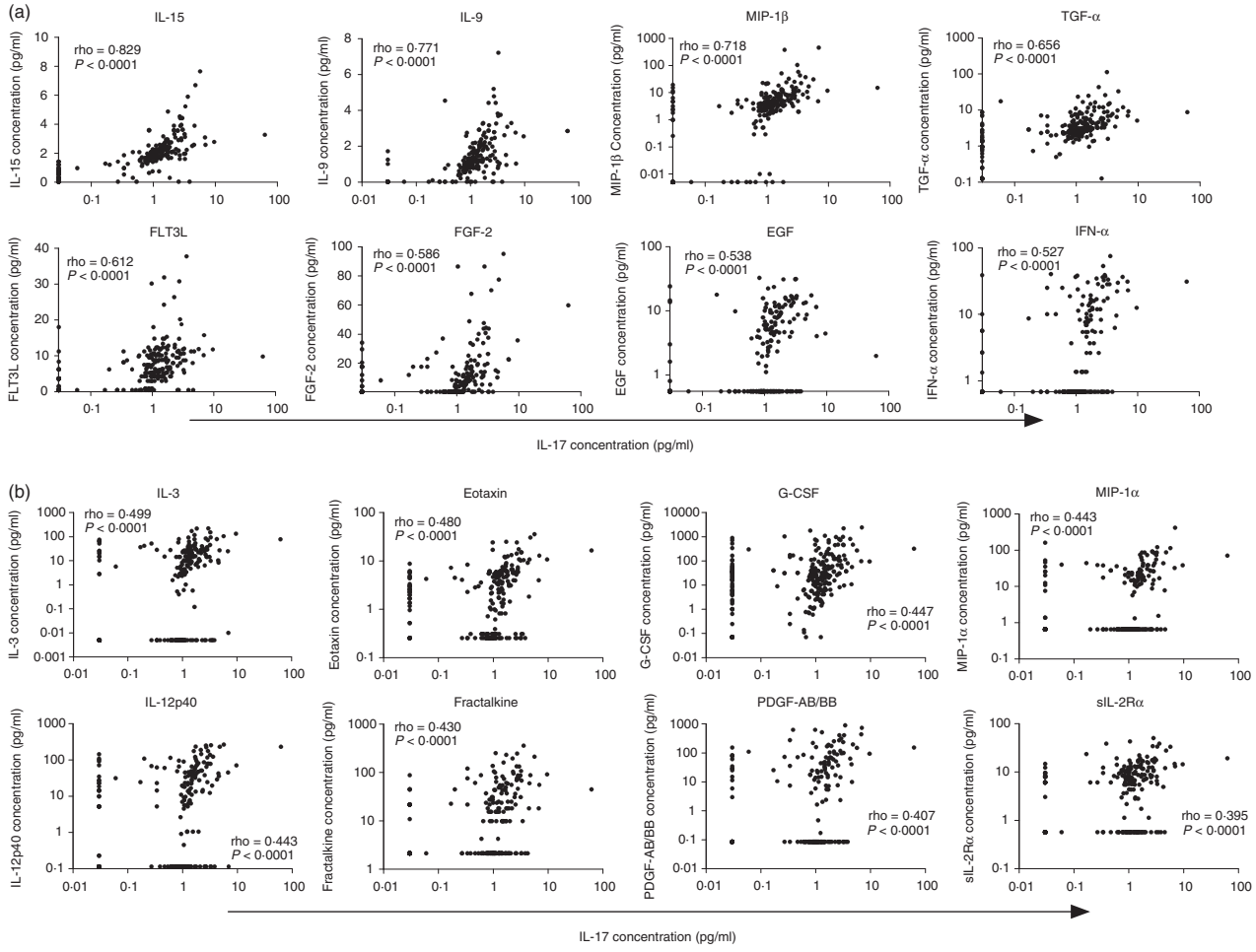


Figure 2. Relationship between interleukin-17 (IL-17) in cervicovaginal lavages and other markers of genital tract inflammation and/or homeostatis. (a) Strong correlations between IL-17 concentrations in cervicovaginal lavage and inflammatory and haematopoietic cytokines ($\rho > 0{\cdot}5$). (b) Moderate correlations between IL-17 and other cytokines ($\rho < 0{\cdot}5 > 0{\cdot}3$).

detectable candidal hyphae in their genital specimens (data not shown). These findings indicate that IL-17 production may be co-regulated with the inflammatory and homeostatic cytokine cascade in the female genital tract.

We used pathway analysis to further investigate the inter-relationships between IL-17 and other cytokines in the data set using the Ingenuity Knowledge Base (Fig. 3). Networks that included the most up-regulated cytokines and with the greatest degree of interconnectivity in women with chlamydia (Fig. 3a) or gonorrhoea (Fig. 3b) included IL-17 and multiple pro-inflammatory cytokines and chemokines. Based on the information contained in the Ingenuity Knowledge Base, IL-17 was found to have several connections with pro-inflammatory cytokines and chemokines. Interleukin-17 expression is indirectly (as indicated by dotted lines) induced by the pro-inflammatory IL-1 family, IL-6 and chemokine CCL2/MCP-1. In turn, IL-17 positively regulates the expression of pro-inflammatory cytokines IL-1, IL-6 and the TNF family and chemokines CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL11/eotaxin and CXCL1/GRO. Additionally, as shown in each network, while IL-17 promotes the production of anti-inflammatory IL-10, IL-17 expression is indirectly inhibited by IL-10. These findings, which are supported by the observed positive correlations between IL-17 and pro-inflammatory cytokines measured in this study, indicate that IL-17 plays a relatively central role in the mucosal inflammatory cytokine cascade, promoting the expression of several other inflammatory mediators.

Th17 cells were depleted in blood during HIV infection, but not in the female genital tract

Differential staining of $CD3^+$ T cells that were either $CD4^+$ or $CD8^+$ with maturational markers CCR7 and CD45RA was used to define four memory T-cell subsets: naive T cells ($CD45RA^+$ $CCR7^+$), effector T cells ($CD45RA^+$ $CCR7^-$), central memory T cells

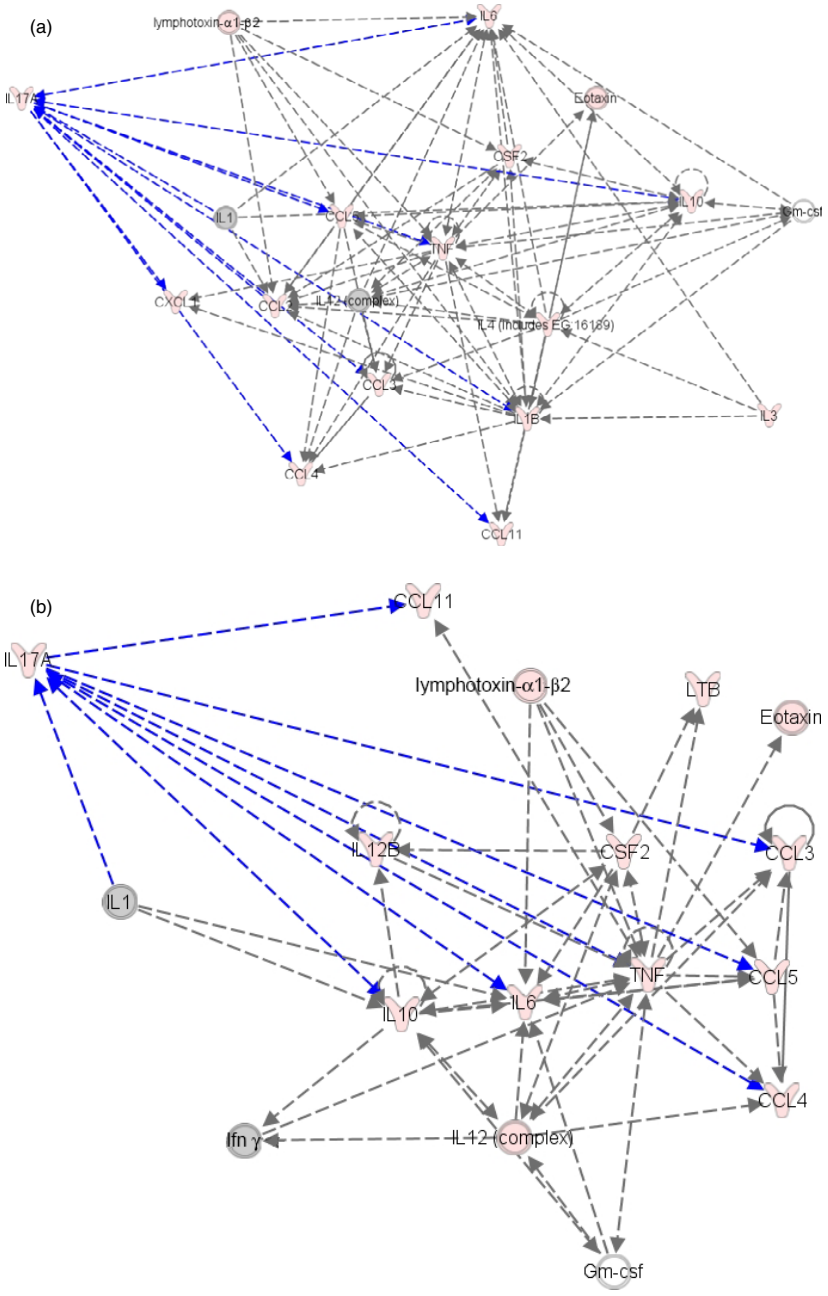
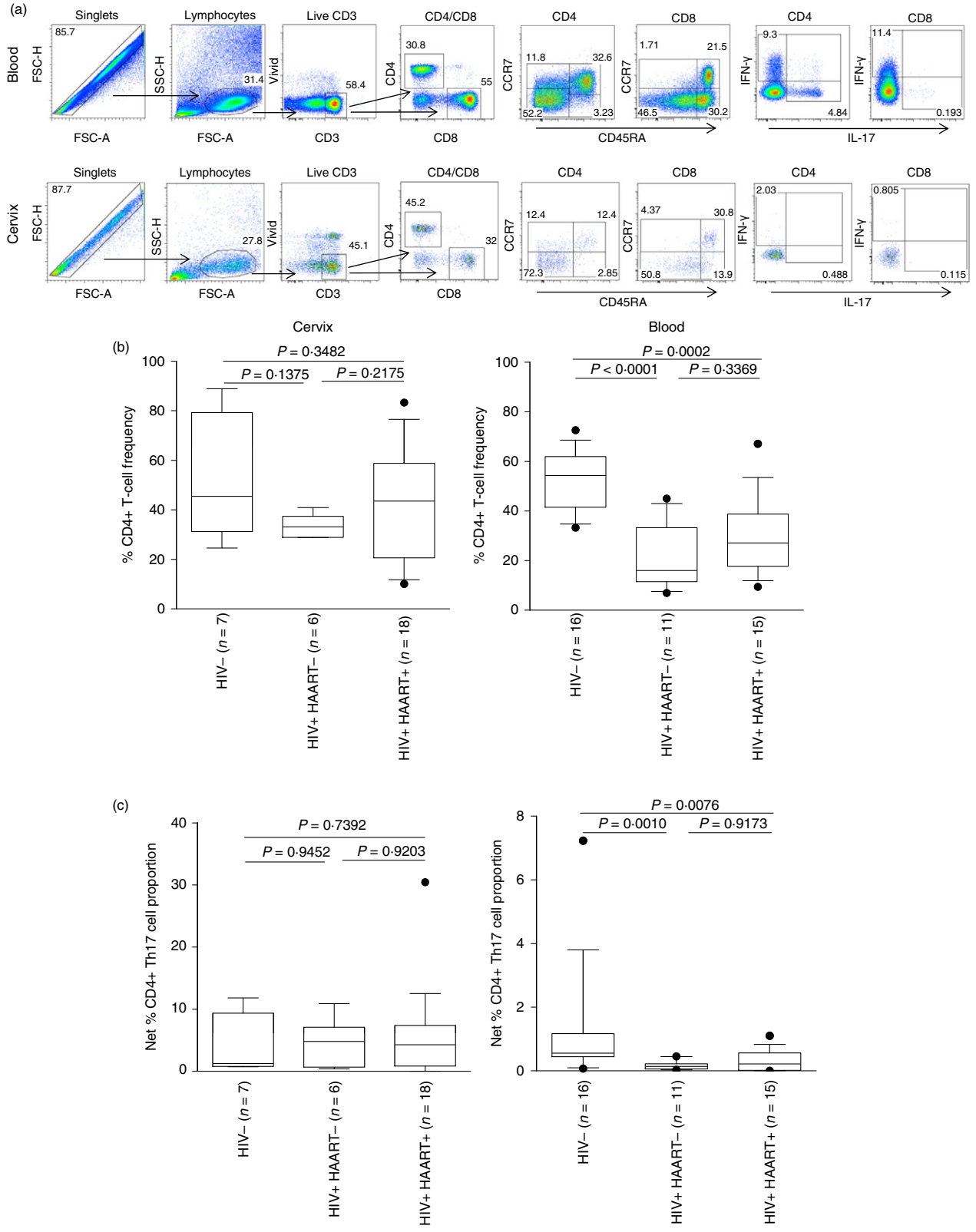


Figure 3. Cytokine signalling networks in women with chlamdyial infections (a) or gonorrhoea (b) were evaluated using Ingenuity Pathway Analysis. The networks that included the most up-regulated cytokines with the greatest degree of interconnectivity in women with chlamydia or gonorrhoea both included interleukin-17 (IL-17) and multiple pro-inflammatory cytokines and chemokines. Median cytokine concentrations in cervicovaginal lavage from women who had chlamydia or gonorrhoea that were more than twofold above the median in the no sexually transmitted infection/bacterial vaginosis (STI/BV) group were considered up-regulated (coloured red). Relationships between IL-17 and other cytokines are coloured blue. Dotted lines indicate indirect associations.

($CD45RA^-$ $CCR7^+$) and effector memory T cells ($CD45RA^-$ $CCR7^-$).[22] In women from Cape Town, South Africa, we found that, regardless of compartment, Th17 cells were predominantly effector memory in phenotype ($CD45RA^-$ $CCR7^-$). For subsequent analyses, $CD4^+$ memory T cells producing IL-17 in response to PMA/ionomycin were considered to be Th17 cells.

$CD4^+$ T cells ($P < 0{\cdot}0001$) and Th17 cells ($P = 0{\cdot}001$) were found to be depleted in blood during HIV infection (Fig. 4b, c). Administration of HAART resulted in partial restoration of $CD4^+$ T-cell and Th17 cell proportions in

blood compared with therapy-naive HIV-infected women, although both $CD4^+$ T cell and Th17 cell frequencies in the blood of $HAART^+$ women were still significantly lower than in uninfected individuals ($P = 0{\cdot}0002$ and $P = 0{\cdot}0076$, respectively). A similar trend towards a reduction in $CD4^+$ T cells was observed at the cervix in HIV-



infected women compared with uninfected women (Fig. 4b, $P = 0{\cdot}1375$), although no change in the proportion of $CD4^+$ Th17 cells was detected at the cervix of HIV-infected women compared with uninfected women (Fig. 4c). Although $CD4^+$ T-cell frequencies between compartments were significantly associated ($\rho = 0{\cdot}50$, $P = 0{\cdot}0255$), frequencies of Th17 cells in blood did not correlate with cervical Th17 cell frequencies ($\rho = -0{\cdot}05$, $P = 0{\cdot}834$; Spearman Rank test), regardless of HIV status.

As IL-17 is the predominant cytokine produced by Th17 cells, we investigated the relationship between IL-17 concentrations in cytobrush supernatants and Th17 cell frequencies in the female genital tract. The Th17 cell frequencies in the genital tract or blood did not correlate with the amount of IL-17 measured in cytobrush supernatants or plasma, respectively ($\rho = -0{\cdot}12$, $P = 0{\cdot}594$ and $\rho = -0{\cdot}30$, $P = 0{\cdot}120$). In addition, the frequencies of Th17 cells detected at the cervix or in blood were not associated with the concentration of inflammatory cytokines detected in genital secretions or plasma (data not shown). Additionally, the median fluorescence intensities of IL-17 in cervical samples did not correlate with the amount of IL-17 measured in cytobrush supernatants ($\rho = -0{\cdot}09$; $P = 0{\cdot}717$), nor the concentrations of any of the other inflammatory cytokines measured.

Th17 cell frequencies at the cervix were not associated with STIs or candidal infections

As we found that secreted IL-17 concentrations were associated with bacterial STIs and candidal infections, we investigated whether cervical Th17 cell frequencies were altered in women with these infections. Of the 51 women from Cape Town from whom cervical cytobrushes were collected, 31 had sufficient $CD3^+$ T cells for analysis, 25 had samples available for screening for STIs and 27 for fungal detection. None of the women were infected with *C. trachomatis*, and only one woman had *N. gonorrhoeae*, so it was not possible to evaluate the influence of these infections on Th17 cell frequencies. Women who had one of the assessed STIs [either *N. gonorrhoeae* ($n = 1$), *M. genitalium* ($n = 2$) or *T. vaginalis* ($n = 4$)] did not have significantly elevated Th17 frequencies in their genital tracts relative to women who did not have one of these pathogens or chlamydial infection ($n = 19$, Table 2).

Table 2. Impact of sexually transmitted infections on T helper type 17 (Th17) cell frequencies in women

	n/total tested	Th17 cell frequency, median (IQR)
Sexually transmitted infections (STIs)[1]		
No STI	19/25 (76%)[2]	3·5 (0·9–7·6)
Neisseria gonorrhoeae	1/25 (4%)	1·3
Chlamydia trachomatis	0/25 (0%)	–
Trichomonas vaginalis	4/25 (16%)	2·2 (1·3–3·4)
Mycoplasma genitalium	2/25 (8%)	5·7 (5·7–5·8)
Fungal infections		
(1→3)-β-D-Glucan < 300 pg/ml	15/27 (56%)[2]	3·2 (0·8–5·7)
(1→3)-β-D-Glucan ≥ 300 pg/ml	12/27 (44%)	4·9 (1·2–6·9)

[1]Women were tested for discharge-causing STIs as these were the STIs found to be associated with increased interleukin-17 concentrations in CVL.
[2]In all, 25/31 and 27/31 women had cervical samples available for STI and fungal screening, respectively.

The concentrations of (1→3)-β-D-glucan from *Candida albicans* were measured in cervical cytobrush supernatants to evaluate whether candidal infections were associated with Th17 cell frequencies in these women. The concentration of (1→3)-β-D-glucan did not correlate significantly with either cytobrush Th17 frequencies ($\rho = 0{\cdot}09$; $P = 0{\cdot}6484$) or genital IL-17 concentrations ($\rho = -0{\cdot}2598$; $P = 0{\cdot}2429$).

Discussion

Interleukin-17 concentrations in genital secretions from HIV-uninfected women were found to be significantly higher in women with the bacterial STIs *C. trachomatis* and *N. gonorrhoeae* compared with women with no STI or BV. In contrast, women with candidal pseudohyphae or spores in their vaginal secretions had lower genital IL-17 concentrations compared with women who did not have detectable candidal infections. Interleukin-17 deficiency has been associated with chronic candidiasis,[4] suggesting that reduced IL-17 production in the genital tracts of the women in this study may have allowed for candidal outgrowth. Alternatively, IL-17 expression may be down-regulated by candidal infection, as has previously been demonstrated *in vitro*.[23] Soluble IL-17 concen-

Figure 4. $CD4^+$ T helper type 17 (Th17) cell frequencies at the cervix and in blood of HIV-uninfected, and HIV-infected women. (a) Representative plots showing the gating strategy used to define memory $CD4^+$ and $CD8^+$ T cells in blood (top) and at the cervix (bottom) producing interferon-γ (IFN-γ) and interleukin-17 (IL-17) in response to stimulation with PMA/ionomycin. The proportions of (b) $CD3^+$ T cells that were $CD4^+$ and (c) $CD3^+$ $CD4^+$ T cells that produced IL-17 (Th17 cells) were evaluated in cervical cytobrush samples (left panels) and blood (right panels) from uninfected women, HIV^+ $HAART^-$ women and HIV^+ $HAART^+$ women. Horizontal lines indicate median values while upper and lower limits of the box indicate the 75th and 25th centiles. Whiskers indicate the 10th and 90th centiles and dots indicate outliers. Mann–Whitney *U*-test was used for comparisons and *P*-values < 0·05 were considered significant.

trations did not correlate with cervical $CD4^+$ Th17 cell frequencies in the female genital tract, probably because, in addition to Th17 cells, several other immune cells (including $\gamma\delta$ T cells, natural killer T cells, natural killer cells, neutrophils and eosinophils) have been shown to produce IL-17 and would therefore contribute to total IL-17 concentrations observed.[6] The finding that IL-17 concentrations were elevated in the genital tracts of women with chlamydia or gonorrhoea suggests that IL-17 production may be induced in response to these pathogens, as has been shown in mouse models.[3,5] Although IL-17 has been implicated in protective immune responses to chlamydia and gonorrhoea in mice,[3,5] IL-17 was found to increase disease pathology and duration.[5]

Genital IL-17 concentrations correlated strongly with IL-9 and IL-15 concentrations, which were also significantly down-regulated in women with detectable candidal infections. Interleukin-15 has been shown to induce IL-17 production by PBMCs and synovial fluid cells,[24] whereas IL-9 is produced by Th17 cells and IL-9 attenuation is associated with decreased Th17 cell numbers in mice.[25] Concentrations of IL-17 were also associated with the concentrations of multiple pro-inflammatory cytokines (MIP-1α, MIP-1β, eotaxin, fractalkine and IL-12p40) and growth factors (IL-15, IL-9, TGF-α, Flt3L, FGF-2, EGF, IL-3, G-CSF and PDGF-AB/BB). In the genital tracts of women with chlamydial infection and gonorrhoea, the inflammatory cytokine signalling network, which included IL-17, was found to be strongly induced, with many of the assessed inflammatory cytokines being up-regulated. These findings suggest that IL-17 production may be up-regulated in women with chlamydial infection and gonorrhoea as part of the inflammatory response to these infections, which have been found to be particularly inflammatory.[26] Previous studies have found that progesterone suppresses Th17 responses.[27] Similarly, we found that IL-17 concentrations were marginally lower in women using a progestin-only injectable hormone contraceptive. However, adjusting for hormone contraceptive use did not influence the relationship between IL-17 and candidal, chlamydial or gonorrhoea infections.

Although a strong relationship was observed between secreted genital IL-17 concentrations and bacterial STIs (specifically chlamydial infection and gonorrhoea), as well as candidal infections, cervical Th17 cell frequencies were not associated with STIs (gonorrhoea, trichomoniasis or *M. genitalium* infections) or Candida (1→3)-β-D-glucan concentrations. However, as only one of these women had gonorrhoea, none had chlamydial infection and most of the women who did have an STI had trichomoniasis (which is a protozoal STI and was not associated with changes in secreted IL-17 concentrations), we were not able to evaluate the potential relationship between Th17 cell frequencies and bacterial STIs, chlamydia and gonorrhoea in this study.

Neither HIV infection nor HSV-2 reactivation were associated with changes in genital IL-17 concentrations, and frequencies of cervical $CD4^+$ Th17 cells did not differ between HIV-infected and uninfected women. This is in contrast to a previous study that showed that $CD4^+$ Th17 cells were depleted from the female genital tract during HIV infection.[12] Consistent with other literature, frequencies of both total $CD4^+$ and $CD4^+$ Th17 cells were significantly reduced in the blood of HIV-infected women compared with uninfected women. Differences observed between this and a previous study[12] may be due to differences in the stage of HIV infection of the women included, HAART, study population, STI and BV prevalence. Unlike the present study population, the cohort described by McKinnon *et al.*[12] were female sex workers recently infected with HIV, including both HAART-naive women and women on HAART, with a relatively low STI and BV prevalence.

The findings of this study suggest that IL-17 may play a central role in the inflammatory cascade in the female genital tract, and may be induced in response to bacterial infections chlamydia and gonorrhoea. Lower concentrations of IL-17 observed in women with candidal infections suggest that candida may actively inhibit IL-17 production or alternatively, women with lower IL-17 concentrations may be more prone to candidal outgrowth.

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Disclosure

The authors of this study do not have commercial or other associations that might pose a conflict of interest.

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