Association of Sex Work With Reduced Activation of the Mucosal Immune System

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Background. Unprotected intercourse and seminal discharge are powerful activators of the mucosal immune system and are important risk factors for transmission of human immunodeficiency virus (HIV). This study was designed to determine if female sex work is associated with changes in the mucosal immunity.

Methods. Cervicovaginal lavage and plasma from 122 HIV-uninfected female sex workers (FSW) and 44 HIV-uninfected low-risk non-FSW from the same socioeconomic district of Nairobi were analyzed for evidence of immune activation (IA). The cervico-mononuclear cells (CMC) were analyzed for cellular activation by flow cytometry.

Results. Lower IA was observed in FSW compared to the low-risk women as demonstrated by the lower level of MIP-3α (P < .001), ITAC (P < .001), MIG (P < .001), IL-1α (P < .001), IL-1β (P < .001), IL-1Ra (P < .0002), IL-6 (P < .001), IL-8 (P < .001), IL-10 (P < .01), IP-10 (P < .0001), MDC (P < .001), MIP-1α, (P < .001), MIP-1β (P = .005), MCP-1 (P = .03), and TNF-α (P = .006). Significant differences were noted as early as 1 year following initiation of sex work and increased with duration of sex work.

Conclusion. This study showed that sex work is associated with important changes in the mucosal immune system. By analyzing chemokine/cytokine levels and CMC activation, we observed a lower mucosal IA in HIV-uninfected FSW compared to low-risk women.

Keywords. female sex work; cytokines; chemokines; immune activation; HIV; HESN; immune quiescence; female genital tract.
intercourse within the last 3 days, there was a predominance of CD4+ T cells in cervical mononuclear cell (CMC) populations compared to women who did not have sexual intercourse [5]. The induction of cytokine/chemokines and the altered distribution of immune cells (increases of DC and CD4+ T cells) that is observed after sexual activity may increase the risk of HIV acquisition in women. However, there is a gap in the literature about the mucosal activation following extended exposure to sexual activity.

This study was designed to compare the mucosal immune compartment of HIV-uninfected female sex workers (FSW) to low-risk HIV-negative women in order to determine if sex work was modifying the mucosal immune system.

**METHODS**

**Study Population**

The Pumwani Commercial Sex Worker Cohort (Nairobi, Kenya) was established in 1985. Over 4000 women have been enrolled in this open cohort. One hundred and twenty-two HIV-1-negative FSW coming for the annual resurvey (January 2006–2011) were included in this study. Forty-four low-risk women who have never been involved in sex work (non-FSW) from the same socioeconomic district of Nairobi were enrolled from a mother child health clinic. The HIV status was determined by enzyme-linked immunosorbent assay (ELISA) and rapid test. Each participant answered a questionnaire regarding sociodemographics, sexual behaviour, duration of sex work, number of sex clients, condom use, number of regular partners, and reproductive history. University of Manitoba and University of Nairobi ethic board approved this study, and written inform consent was obtained from all participants. Exclusion criteria included pregnancy within the last 12 months. Vaginal specimens were obtained to test for presence of bacterial vaginosis (BV), Neisseria gonorrhea (GC), and Chlamydia trachomatis (CT). GC and CT were tested by polymerase chain reaction (PCR) and presence of BV by Nugent score.

**Cervico-vaginal Lavage (CVL) Sample Collection**

CVL from 122 HIV-uninfected FSW and 44 HIV-uninfected low-risk women were obtained. Samples were collected at mid-cycle. Briefly, the endocervix was washed with 2 mL of sterile 1× phosphate buffered saline (PBS), and the lavage was collected from the posterior fornix. Samples were placed into a 15 mL conical tube, centrifuged to remove cellular debris, and the supernatant stored at −70°C.

**Plasma Sample Collection**

Peripheral blood was collected in heparin tubes. Plasma samples were frozen at −80°C and shipped by liquid nitrogen dry shipper to Winnipeg, Manitoba, Canada, where they were stored at −70°C until used. Plasma and CVL samples were collected on the same day. Plasma and CVL samples were analysed for chemokine and cytokine expression.

**Chemokine and Cytokine Measurement**

Chemokine levels were determined using the microbead array assay Milliplex MAP multiplex kit (Human Cytokine/Chemokine I, II from Millipore, Billerica, MA) and analyzed on the BioPlex-200 (Biorad, Mississauga, ON, Canada). CVL were analysed according the manufacturer overnight protocol, whereas plasma was analysed according to the 2-hour incubation protocol. Lower detection limit (LDL) was 10.6 pg/mL for fractalkine, 40.6 pg/mL for IFN-α2, 0.3 pg/mL for IFN-γ, IL-8, and IL-17, 6.4 pg/mL for IL-1α and MIP-1α, 0.7 pg/mL for IL-1β, IL-6, IL-15; 5.5 pg/mL for IL-1ra, 0.6 pg/mL for IL-2, 0.5 pg/mL for IL-10, 2.2 pg/mL for IP-10, 1.6 pg/mL for MCP-1, 3.7 pg/mL for MCP-3, 6.9 for MDC, 8.9 pg/mL for MIP-1β, 7.7 pg/mL for sIL-2Rα, 9.0 pg/mL for sCD40L, 19.4 PG/ML FOR MIG, 2.9 for MIP-3α, 0.8 for ITAC and 0.1 pg/mL for tumor necrosis factor α (TNF-α). Samples below the LDL were assigned a value of half of the lower detection limit in pg/mL.

**Evaluation of Cervical Mononuclear Cell Population by Flow Cytometry**

CMCs were collected using a cytobrush and cervical scraper. During January 2011 annual resurvey, CMCs were obtained from 50 HIV-1 negative FSW and 20 low-risk HIV-negative women. Briefly, CMCs were collected under speculum examination by inserting the cytobrush and the scraper into the endocervical os, rotating 360° and immediately placing in 5 mL of PBS. Cytobrush samples with visible blood contamination or overly low cell counts were excluded from further analysis. Due to the low number of cells in the CMC samples, results from only 7 low-risk HIV-negative women and 32 FSWs were available for analysis. Samples were kept on ice and transported from the clinic to the laboratory where the cytobrush and scraper were vortexed and cells were flushed out of the brush. Two washes were performed (first with 5 mL of RPMI, second with 5 mL of PBS) each followed by a 10 min centrifugation. Fresh CMCs were labeled with a cocktail containing the following mouse anti-human monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-CCR5; phycoerythrin (PE) anti CXCR3; PE-cyanine (Cy)7-conjugated anti-HLA-DR; PE-Cy5-conjugated anti-CD69, allopheocyanin-conjugated anti-CD56; allopheocyanin H7 conjugated anti-CD16; Alexa fluor 700-conjugated anti–CD4; V500 anti-CD8 and V450 anti-CD3 (Becton Dickinson [BD] Biosciences, Mississauga, ON, Canada). Dead cells were identified with live-dead red (PE-Texas Red; Invitrogen, Burlington, ON, Canada). Data acquisition of all events per sample was performed on a BD LSR II cytometer (Becton Dickinson), and the analysis was done using FlowJO software (version 9.3.1; Tree Star, Ashland, OR).
Statistical Analyses
Statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software; La Jolla, CA). A χ² test was used to assess the significance of the associations between categorical variable, Gaussian distribution was tested by D’agostino and Pearson omnibus normality test, and Shapiro-Wilk normality test. One-way analysis of variance (Kruskal–Wallis test) and Mann–Whitney U test were used for variables that were not normally distributed between 3 groups and 2 groups, respectively. Mann–Whitney U tests were performed when a significant difference was observed between more than 2 groups by the Kruskal–Wallis test. Wilcoxon Matched Pairs test was used to compare cytokine/chemokine expression in the CVL and plasma samples. Spearman test was performed for correlation between age of participant and the cytokine/chemokine expression.

RESULTS
Sociodemographics
No differences were observed between the 2 groups for age, practice of vaginal douching, presence of BV/GC/CT infection, and number of regular partner (Table 1, Supplementary Figure 1). The FSW group were sex workers with an average of 11 years and reported sex with about 4 clients per day. There was no significant difference in self-reported clients/day and duration of sex work (data not shown).

Cytokine/chemokine Expression in CVL
No differences were observed for CVL expression of fractalkine, IFN-α2, IFN-γ, IL-2, IL-7, IL-17, monocyte chemoattractant protein (MCP)-3, and sCD40L between the 2 study groups (minimum P = .1). Low-risk HIV-negative women had higher mucosal expression of macrophage inflammatory protein (MIP)-3α (P < .0001), Interferon-inducible T-cell alpha (ITAC; P < .0001), monokine induced by IFN-gamma (MIG; P = .0001), IL-1α (P < .0001), IL-1β (P < .0001), IL-Rα (P = .0002), IL-6 (P < .0001), IL-8 (P < .0001), IL-10 (P = .01), IP-10 (P = .0001), MCP-1 (P = .03), MDC (P < .0001), MIP-1α (P < .0001), MIP-1β (P = .005), and TNF-a (P = .006; Figure 1A). The FSWs had higher mucosal level of IL-15 (P = .04) and sIL-2Ra (P = .005; Figure 1B).

Comparison of Mucosal and Systemic Level of Cytokine/chemokine Expression
When comparing together the mucosal and systemic expression, we observed two different chemokine/chemokine gradients between the groups. Overall, the level of fractalkine, ITAC, IL-2, MDC, sCD40L, sIL-2Ra, TNF-a, and IP-10 were significantly higher in the plasma in both study groups, whereas the level of IL-6, IL-1α, IL-1β, IL-1Ra, IL-7, and IL-8 were higher in the CVL compared to the systemic compartment. In the FSW group, the level of MIG (P = .007), MIP-1α (P = .001), MIP-1β (P = .02), MCP-3 (P = .001) was significantly higher in the systemic compartment vs the FGT, but these differences were not observed in the low-risk women (minimum P = .1). Interestingly, the level of IL-15 and MCP-1 was more elevated at the mucosal compartment of FSW (P < .0001 and P = .001, respectively), although they were higher in the systemic compartment for the low-risk women (P = .006 and P = .03, respectively). Mucosal level of MIP-3α was higher in the FSWs (P = .03). This was observed in the low-risk HIV-negative women (Figure 2). Note that the lack of statistical significance in the low risk can be either due to lack of effect or lack of power to detect an effect.

Correlation of Mucosal Cytokine/chemokine Levels According to Duration of Sex Work
As we observed differences in the cytokine/chemokine expression between FSWs and low-risk women, we decided to stratify the FSW group according to self-reported duration of sex work in order to contrast the immediate and long-term impact of sex work. The FSWs were stratified into three groups: sex worker for 3 years or less (recent; n = 29); 4–6 years of self reported involvement in sex work (intermediate) (n = 13); and ≥ 7 years of involvement in sex work (long-term; n = 79).

As shown in Figure 3, some differences were observed between the low-risk HIV-negative women and the 3 FSW groups. Lower expression of MIP-3α (P < .0001), ITAC (P < .0001), IL-1α (P < .0001), IL-1β (P = .0006), IL-8 (P < .0001), MDC (P < .0001), and MIP-1α (P = .0004) were observed between the 3 FSW groups and the low-risk women (Kruskal–Wallis results). The FSW groups had higher level of sIL-2Ra (P = .04). Differences in MIP-3α and MDC expression were observed between the intermediate and long-term FSWs. Overall, a decrease of mucosal immune activation was associated with duration of sex work.

Table 1. Sociodemographic and Clinical Characteristics in HIV-1 Negative FSW and Low Risk HIV-1 Uninfected Women

<table>
<thead>
<tr>
<th></th>
<th>HIV-1 uninfected FSW (122)</th>
<th>Low Risk HIV-1 Uninfected (44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>39 (21–65)</td>
<td>37 (32–40)</td>
</tr>
<tr>
<td>Duration of sex work, years</td>
<td>11 (9)</td>
<td>NA</td>
</tr>
<tr>
<td>Clients per day</td>
<td>4 (3)</td>
<td>NA</td>
</tr>
<tr>
<td>Regular partner</td>
<td>2 (2)</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>No. of participants who do vaginal douching</td>
<td>122</td>
<td>44</td>
</tr>
<tr>
<td>Bacterial vaginosis</td>
<td>2 (3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Neisseria gonorrhoea/ Chlamydia trachomatis</td>
<td>4 (4%)</td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>

Data are age mean (range); duration of sex work (years); and clients per day mean (standard deviation); bacterial vaginosis, and NG/CT number of positive test (percentage).
Abbreviations: FSW, Female sex worker; HIV, human immunodeficiency virus; NA, not applicable.
Figure 1. Cytokine/chemokines cervico vaginal expression. A, Cytokine/chemokine with higher expression in the low risk HIV-negative women; B, Cytokine/chemokine with higher expression in the CSW group. Data represents mean and standard deviation. Circle are data from CSW and square are data from low risk HIV uninfected women. \( P \) value are the result of Mann–Whitney test.
When comparing the cytokine/chemokine expression between only FSW groups, we observed lower expression of IL-1Ra between the recent and long-term FSWs (Mann–Whitney U P = .05). MDC level was significantly different between recent and intermediate group (Mann–Whitney U P = .03).

In a subanalysis to compare the extreme phenotype, we stratified the recent group to compare only the women who initiated sex work ≤1 year (n = 20) to the more experienced one (>7 years) (n = 73) and to the non-FSW women. Figure 3B shows that within the first year of commercial sex work, significant differences appear in the immune activation compared to non-FSWs. The expression of MIP-3a (P < .0001), ITAC (P = .01), IL-1a (P = .003), IL-1b (P = .04), IL-8 (P = .02), MDC (P = .003), MIP-1a (P = .02) were lower in the ≤1 year group compared to the low-risk group. The levels of MIP-3a, MIG, IL-1Ra, IP-10, MDC (P = .02; P = .04; P = .006; P = .03; P = .05; P = .01, respectively) were higher in the ≤1 year group compared to the long-term FSWs. Those data demonstrated that overall early FSWs had more activated mucosal milieu than the long-term FSWs but a lower activated milieu than non-FSWs.

Cervical Mononuclear Cell Analysis

Previous studies showed that sexual activity alters the cellular population in the FGT ([5–8]). CMCs were analyzed by flow cytometry (Figure 4A). We observed that FSWs had a higher relative proportion of bulk CD4 T cells and CD3-CD56dim natural killer cells (P = .016 and P = .02, respectively; Figure 4B). However, they had lower relative proportion of CD4+ CCR5+ T cells (P = .005), CD8+ T cells (P = .01), CD8+ CCR5+ T cells (P = .04), and CD8+ CD69+ T cells (P = .006) than low-risk HIV-negative women (Figure 4B). We found that in low-risk women there was significantly higher relative proportion of double-negative T cells (CD3+ CD4−CD8−; P = .03), and they tended to be more activated (CCR5+ DN P = .02; CD69+ DN P = .07).

DISCUSSION

Susceptibility to HIV infection is multifactorial but is greatly enhanced by preexisting immune activation [10]. The mucosal environment of the FGT has an important influence on the susceptibility and establishment of HIV infection during unprotected intercourse. Sexual activities have been previously associated with mucosal immune activation, and this activation is believed to be an important factor in susceptibility to HIV [5–8]. Here, we reported a different level of mucosal immune activation between women of the general population and those engaged in commercial sex work. More interestingly, these differences appear within the first year of initiation to commercial sex work and become more pronounced as the duration of sex work increases.
Figure 3.  A, Difference in the cytokine/chemokines expression between low risk HIV-negative women and CSW according to self-reported duration of sex work in the female genital tract B, Difference in the mucosal cytokine/chemokine expression between low risk HIV-negative women and CSW with one year of less of involvement in sex work. The mean and SEM are represented FSW ≤1 year: n = 20. FSWs ≤3 years: n = 29. FSWs 4–6 years: n = 13. FSWs ≥7 years: n = 79. Low risk women: n = 44.
In this study, we found that sIL-2Ra and IL-15 were the only 2 cytokines expressed at higher levels in the CVL of FSWs compared to sexually active low-risk women. IL-15 induces natural killer (NK) cell proliferation and helps activate T-cell survival signals that maintain memory T cells in the absence of antigen [11]. Consistent with the higher level of IL-15, we observed a higher proportion of CD56dim NK cells in the FGT of FSWs. IL-15 gradient of expression was also directed toward the mucosal compartment only in the FSWs. This result could suggest that high-risk individuals develop a stronger innate immune response calling innate effectors toward the genital compartment. Higher proportion of activated NK cells with more cytotoxic
Figure 4. Relative percentage of T cells subpopulations among cervico vaginal cells (CMC). A, gating strategy on singlet (first at the left), lymphocyte population, CD3+ cells alive and CD4 and CD8+ T cells. B, Percentage of T cells subsets among cervico vaginal cells (CMC).
capacity have been observed in the blood of high-risk individuals and correlated with protection against HIV [12, 13]. Therefore, continuous exposure to sex antigens through sex work may favour the development and recruitment of a stronger innate response to the FGT and contribute to HIV protection. A decreased proportion of DNT cells in the FSWs compared to the low-risk women was also observed. The DNT population at the FGT of FSWs showed less immune activation. Furthermore, we observed that CMCs from FSWs had a higher proportion of CD4+ T cells but a lower proportion of that subset expressed the HIV co-receptor CCR5. These data are supported by those of Hirbod et al who showed, in the same population, that in cervical biopsy samples the highly exposed seronegative (HESN) FSWs have fewer CD4+ CCR5+ T cells in the tissue compared to low-risk women (Hibord et al, unpublished). Other groups also observed decreased CCR5 expression on CD4+ T cells in high-risk HIV-negative individuals [13, 14].

The immune activation is critical for the response against pathogen invasion. However, in the case of HIV infection, this immune activation contributes to the infection by increasing the number of target cells because activated CD4+ T cells are more susceptible to HIV infection and produce more virus once infected [10]. Herein, we observed that the mucosal immune system of FSWs is not activated to the same extent relative to low-risk HIV-negative women. In FSWs, the mucosal immune activation seemed to favour the activation of the innate system without increasing the number of potential target cells. Indeed, sexually active non-FSW had higher mucosal levels of proinflammatory cytokines/chemokines and a higher proportion of vaginal CD4+ CCR5+ T cells, CD8+ T cells, and CD8+ CCR5+ or CD8+ CD69+ in the FGT. Furthermore, when comparing the gradient pattern of the cytokine/chemokine expression between the systemic and the mucosal environment, we observed the different pattern. Indeed, in FSWs the levels of MIG, MIP-1a, and MIP-1b were significantly higher in the systemic vs the mucosal compartment. These 3 chemokines are important for the trafficking of activated T cell, which suggests that in FSWs there is not strong recruitment of activated CD4+ T cells to the FGT. However, this absence of difference in the low-risk women could also be due to the small sample size of this group. A study of sex workers from Benin comparing HIV-negative FSWs to HIV-positive FSWs showed a similar finding. Indeed, there was less MIP-1a, MIG, and MCP-3 in the FGT of HIV-uninfected FSWs, and the gradient expression in this group was different than the one observed in HIV-positive FSWs [15].

Recent studies showed that age positively correlated with chronic immune activation (Hearps et al 2012 Aging cells 11, 867). In a study looking at age and immune activation in FSWs from the same cohort, Siviro et al showed that older FSWs have higher expression of MCP-1 and IP-10 [16]. In our study, we showed that despite the wider range of ages of the FSWs group and the presence of older participants, FSWs had lower immune activation when compared to low-risk women. Furthermore, when comparing the FSWs between ages of 32 and 40 (n = 33) to the low-risk women group, almost the same difference was observed. Only IL-10, TNF-a, MCP-1, and MIP-1b did not reach significance (data not shown).

The results of this study provide 2 important observations. First, dramatic changes in mucosal immune system seem to appear as early as 1 year after initiation of sex work. These results are slightly surprising, in that the early FSW participants exhibited decreased, not increased, levels of immune activation. Ejaculate is known to contain not only proteins but also CD4+ T cells, macrophages, and epithelial cells that express client-derived HLA-antigens and cell-free HLA antigens [2, 17, 18]. In HIV infection, the viral coat also contains HLA proteins. Peters et al showed that the partner’s HLA antigens in seminal fluid could induce strong mucosal allo-immune responses in women during unprotected sex, but in some cases repeated immunization to allo-antigens might lead to tolerance [19]. Our results seem to corroborate this finding, indicating that repeated exposure to different semen and antigens might lead to a certain tolerance as indicated by the lower cytokine/chemokine levels observed in those early in their sex work experience.

The second major observation from the FSW cohort lies in the progressive decline in mucosal immune activation as duration of sex work increases. We have previously described that women who have been exposed to HIV without becoming infected demonstrate an immune quiescent (IQ) phenotype in both the blood and the genital mucosa [20–23]. We have suggested that there may be multiple drivers of this IQ phenotype, including genetics, and together they may be contributing to the protection against HIV acquisition observed in these women [24–26]. On the other hand, our current data suggest that prolonged sex work may also contribute to the mucosal IQ. The direction of causality in vivo cannot be determined through a cross-sectional study, and in reality causation may be bi-directional. That is, FSWs who naturally had a lower basal immune activation and who respond to allo-stimulation with a stronger down-regulation of immune activation would be less likely to become HIV-infected compared to women with inflammatory immune responses in the first years of involvement in sex work. Over time, this selection pressure would enrich the HIV-negative FSWs pool for women with the IQ phenotype, with a stronger bias as duration of sex work increased. This bi-directionality is supported by the fact that when comparing the systemic immune activation between low-risk women and FSWs, significant differences were observed only between the low-risk women and experienced FSWs (data not shown), which seems to indicate that the IQ phenotype associated with HESN women in this cohort is not only due to sex work.

The design of this study cannot determine if the decrease of mucosal immune activation observed in FSWs is due to the
trauma due to intercourse (whether with or without use of a condom) or to the discharge of semen associated with unprotected sex work. We also cannot determine if semen from different men are required or if the same pattern could be observed in low-risk women with a unique partner who have similar number of unprotected intercourse a week. Another limitation of this study may be the $P < 0.05$ cut-off used for the comparisons, which may have introduced a type 1 error. However, given the discovery nature of the study, a $P < 0.5$ cut-off was chosen to avoid a type 2 error, which is missing a real finding.

The mucosal immune activation can be modified by many factors. In this study, we considered a number of confounding factors. However, other confounding factors such as presence of human papillomavirus, herpes (none of the participants show visible sign of infection), or product used for vaginal douching can influence the environment. The presence of mucosal ENV specific immunoglobulin in HIV-uninfected FSWs could also potentially modulates the mucosal immune system by developing a mechanism to control/tolerate HIV. The information about those factors was not available or tested in the current study. We recognized that some of them could have contributed to the change in the mucosal environment.

Overall, this study highlights that sex work significantly affects the mucosal immune system of the FGT as soon as the first year following initiation of sex work. The study shows that sex work itself may be one of the factors contributing to an IQ phenotype observed in the HESN women of the Pumwani cohort. Our study gives new insights that might be useful for the development of a microbicide to limit immune activation in high-risk women and decrease their risk of HIV infection.

### Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

### Notes

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