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HIV-1 Negative Female Sex Workers Sustain High Cervical IFNε, Low Immune Activation and Low Expression of HIV-1 Required Host Genes

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Abstract

Sex workers within high HIV endemic areas are often a target population where anti-HIV prophylactic strategies are tested. We hypothesize that in women with high levels of genital exposure to semen changes in cervicovaginal mucosal and/or systemic immune activation will contribute to a decreased susceptibility to HIV-1 infection. To address this question, we assessed sexual activity, immune activation status (in peripheral blood), as well as cellular infiltrates and gene expression in ectocervical mucosa biopsies in female sex workers [FSW] (n=50), as compared to control women [CG] (n=32). FSW had low to absent HIV-1 specific immune responses with significantly lower CD38 expression on circulating CD4+ or CD8+ T-Cells (both: $p<0.001$) together with lower cervical gene expression of genes associated with leukocyte homing and chemotaxis. FSW also had increased levels of Interferon-ε gene and protein expression in the cervical epithelium together with reduced expression of genes associated with HIV-1 integration

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and replication. A correlative relationship between semen exposure and elevated type-1 IFN expression in FSW was also established. Overall, our data suggest that long-term condomless sex work can result in multiple changes within the cervicovaginal compartment that would contribute to sustaining a lower susceptibility for HIV-1 infection in absence of HIV-specific responses.

Introduction

Women comprise more than 50% of HIV-1 infected individuals, with heterosexual transmission representing the leading route for infection[1]. Understanding the factors that contribute to HIV-1 transmission in the cervicovaginal compartment is essential for the development and improvement of HIV vaccines and/or pre-exposure prophylaxis approaches. Several factors have been associated with higher rates of HIV infection in women. These include pre-existing inflammation, as well as repeated exposure via commercial sex work [2,3]. However, studies in high HIV prevalence areas have also identified female sex workers (FSW) that remain seronegative despite long-term sexual work and low condom use (highly-exposed seronegative, HESN). These observations led to the hypothesis that these women may have developed intrinsic or adaptive mechanism(s) of resistance [4]. Cell-mediated adaptive responses in blood [4] and mucosal anti-HIV IgA [5] titers are frequently absent or weak in HESN FSW, and in one study did not exclude subsequent seroconversion[6]. Thus, adaptive anti-HIV-1-specific responses are unlikely to be solely responsible for maintaining an HESN status. Recent studies, largely supported by data in peripheral blood or cervicovaginal secretions, suggest that HESN FSW may sustain a state of immune quiescence or reduced immune activation that can counter infectivity in spite of repeated acute HIV-1/semen exposures [7–10]. Importantly, it has remained unknown to what extent semen versus other factors (e.g., repeated HIV-1 exposure) may contribute to these observed changes of the cervical tissue microenvironment and in systemic immune modulation. Additional mechanisms proposed to maintain HESN status include genetic polymorphism [11], increased peripheral T-regulatory cell frequency [7], and an increase in anti-proteases within the cervico-vaginal mucosa [12].

Low condom use consistently exposes HESN FSWs to semen and HIV-1. Human semen, more than an inert vehicle for HIV-1 virions within the female reproductive tract, has been shown to be immunologically pleiotropic. Studies have shown that semen can acutely upregulate inflammatory cytokines and chemokines (e.g.: IL-6, IL-8, CCL20 and CXCL3) in cervico-vaginal tissue-derived epithelial cell lines and induce infiltration of immune effectors into cervico-vaginal tissue shortly after coitus [13,14]. However, semen also includes factors able to mediate a tolerogenic Th2 profile (e.g., T-Regulatory Cell induction) driven by high-levels of IL-10, TGF-β, and Prostaglandin E2 in semen, which has been hypothesized to have an important role during fertilization [15–18]. Aside from effects of repeated signaling by IL-10 or PGE2 on myeloid and T-cell in cervix, the presence of Th2-polarized CD4s alone would not exclude infection as both Th1 and Th2 CD4 cells are susceptible to HIV-1 [19]. However, although seminal fluid-derived amyloid fibrils have been shown to have variable results in affecting HIV-1 infectivity *in vitro*[20] no marked *in vivo* effects were noted when tested in non-human primates (NHP) for acute effects on SIV infection[21]. Independently of semen, work in non-human primate models have shown that

acute exposure to high-titer SIV can also induce recruitment of CD4 T-cells, plasmacytoid dendritic cells and macrophages into the cervical and vaginal epithelium which if followed by infection can result in a greater depletion of CD4 T-cells[22,23]. In spite of the potential acute effects of semen and/or viral particle exposure, analysis of ectocervical tissue from HESNs has shown a steady-state of reduced rather than increased inflammation [24], suggesting that the tissue microenvironment alterations resulting from chronic semen or viral particle exposure (e.g.: from long-term sex work) may differ from those associated with acute semen/viral- exposure.

In order to assess the contribution of sex work in groups with low HIV exposure incidence, we hypothesized that in women with high levels of genital exposure to semen both localized tissue (i.e.: cervicovaginal mucosa) and/or systemic immune activation changes will contribute to a lower susceptibility for HIV-1 infection. Furthermore, as no report outside Africa has yet described blood-based and cervical tissue changes in long-term sex worker cohorts, it also remains unknown if reported HESN or long-term sex work protective mechanisms, such as immune quiescence, are attributable to differential genetics or regional sex worker practices. To address our stated hypothesis and reproducibility of mechanisms associated with long-term sex work, we analyzed a cohort of seronegative long-term FSW (and a comparator group of women with low semen exposure, as determined by self-reported frequency of unprotected intercourse) recruited from a geographical distinct region, San Juan, Puerto Rico. HIV-1 prevalence amongst FSW in San Juan has been previously reported at 4.8% [25], which is 5–15 fold lower than in sub-Saharan Africa [26]. Although Puerto Rican FSW cannot be directly compared to HESN-FSW African cohorts, if semen (and not HIV exposure itself) is responsible for the mucosal changes observed in HESN-FSW in Africa, we would expect that FSW with no HIV-specific immune responses but high levels of condomless sex (semen exposure) will have comparable peripheral and mucosal changes as described in HESN FSW.

Results

Study population

The cohort characteristics are reported in Table 1. The median ages of the female sex workers (FSW, n=50) and comparator group (n=35) were 35.50 and 32.00 years, respectively (p=0.439; Table 1). 44 (88%) FSW reported a sex work duration of 3 years or greater. Comparator or control group women were sexually active women in stable monogamous relationships attending a women's health clinic at the study site. In the week prior to sample collection, FSW reported a median of 3 events of condomless vaginal intercourse, compared to 0 amongst control group women (p<0.0001). Only 2 (4%) of FSW reported use of oral hormonal-based contraceptives, as compared to 15 (43%) of the control group (p<0.0001). Only one women within the control reported use of injectable contraceptives. Despite this difference in contraceptive use, serum levels of estradiol and progesterone were not significantly different between the two groups. While intravenous drug users were excluded from recruitment, 27 (54%) FSW reported other drug use (marijuana). While symptomatic sexually-transmitted infections (STI) were disqualifiers for the study, enrollment screening identified 8 (16%) FSW with asymptomatic STIs as

summarized in Table 1. Thirty-four (75.6%) FSW versus 21 (61.7%) of the control group had a vaginal pH of 4.5 or greater. There were no significant differences in HPV infection or abnormal cytology rates between the two groups.

Rare HIV-1 Specific Responses in FSW with Low T-Cell CD38 expression

Gag-specific T-Cell responses and cervico-vaginal HIV-1 specific IgA/IgG antibody levels were measured as indicators of HIV-1 exposure in absence of infection [5,27]. As expected due to low HIV prevalence in area, anti-HIV-1 cellular responses as assessed by intracellular staining for interferon gamma and extracellular staining for CD107a after peptide stimulation to a consensus gag sequence was extremely low (2 of 47 FSW) (Supplemental Figure 1A). With regards to humoral anti-HIV responses, only 2 FSW had detectable mucosal HIV-1 specific IgA or IgG as assessed by having anti-HIV specific activity for >2 HIV-1 specific antigens in the binding antibody multiplex assay (Supplemental Figure 1B). Taken together, less than 10% of FSW, but no control had a HIV-1 specific response.

In contrast to HIV-specific responses, flow cytometry-based analysis of activation and functional responses in circulating T-Cell, NK and dendritic cells evidenced a lower expression of CD38 in both CD4+ ($p<0.00001$) and CD8+ T-cell subsets ($p=0.0006$) (Table 2; Supplemental Table 1), indicating that the T-cell compartment was modulated in the FSW cohort. Importantly, the difference in CD38 expression remained significant after adjustment for confounding factors (drug use, oral contraception, sterilization, marijuana use; see supplemental Table 2) supporting its association with long-term sex work. A lower frequency of regulatory CD4+ T-cells and an increased expression of maturation marker CD83 on mDC were also detected in FSW (Table 2). We did not observe any significant between-groups difference in NK cell (lysis of K562 cells) or myeloid (ex vivo TLR 3, 4 or 9-mediated cytokine responses by PBMC) functional responses (not shown). Taken together, FSW showed changes in T-cell subsets indicative of peripheral T-cell modulation of CD38 expression as described in HESN cohorts [7,28,29].

Higher Immune Cell Infiltrates in Ectocervical Mucosa Biopsies from FSWs

We next examined CD4+ T cells, CD123+ plasmacytoid Dendritic Cells (pDC) and CD68+ macrophage infiltrates in 29 ectocervical biopsies [targeted to the squamo-columnar junction (SCJ) region of the cervix] from a subset of FSWs (n=16) and comparator group (CG, n=13). Subject variables for this subset were comparable to the full cohort (Supplemental Table 3) with the exception that no difference in STI frequency was detected.

CD4+cells (Figure 1A–C) were over 2× more abundant in FSW biopsies as compared to controls (median: FSW 1958 vs. CG 609.3 cells/mm^2; $p=0.0400$). Interestingly, the CD4+ T-cell infiltrate was predominantly located in the lamina propria, with many cells adjacent to the basal membrane.

CD68 positive cells, consistent with macrophage infiltrates, (Figure 1D–F) were also increased in FSWs (median: FSW 829.6 vs. CG 294.0 cells/mm^2; $p=0.0384$), and were broadly distributed in both the lamina propria and within the epithelial layer.

Finally, FSWs had significantly higher amounts of CD123+ cells (pDCs, Figure 1G–I) within the lamina propria (median: FSW 461.1 vs. CG 219.8 cells/mm^2; p=0.0127). Taken together, our data indicate that FSWs had significantly increased levels of cervicovaginal mucosa immune CD4 T cell and myeloid infiltration.

Gene Expression in Ectocervical Biopsies

To assess the tissue microenvironment associated with the observed cell infiltrates, we measured global gene expression in ectocervical biopsies from a subset of 26 women (11 control and 14 FSW), using HumanHT-12 v4 BeadChips microarrays. Of the 29,377 probes expressed in the samples, 873 probes were found to be significantly different between control and FSW groups (false discovery rate (FDR) <20%). Over 70% of the differentially expressed genes were up-regulated in FSWs. Principal Component Analysis (PCA) confirmed that differential gene expression from FSW and control group clustered into two distinct sample groups (Figure 2A). Ingenuity Pathway Analysis (IPA) of the 873 differentially expressed genes did not reveal an enrichment of immune activation gene networks within the FSW cohort and instead indicated the presence of a functional inhibition of leukocyte homing (p=0.00036) or chemotaxis (p=0.00029) (Table 3) suggesting resident regulation against added cell recruitment.

Genes associated with resistance to viral infection (33 Genes-Figure 2B) or susceptibility to viral infection (60 Genes-Figure 2C) were differential between FSW and CG with analysis indicating gene modulation within FSW favoring viral susceptibility (Figure 2). To further target our analysis to interferon-mediated signatures, we compared our significant gene expression data to the Interferome database [30]. Of the 873 significantly modulated genes, 95 (10%) were identified as Interferon-Regulated Genes (IRG). Of these, 39% (37 genes) were associated with a Type I interferon stimulation (Supplemental Figure 2), documenting the presence of a Type I interferon signature yet without evidence for a highly polarized response. A similar result was observed upon examination of the 102 Type II IFN IRGs in the gene dataset. Of these, only 35% were associated with an increased IFNγ response (data not shown).

Taken together, our tissue-based RNA microarray data indicate that FSWs do not have a localized enhanced immune activation signature despite the observed greater mucosal immune cell infiltrates; this is consistent with an immune quiescent cervicovaginal microenvironment.

Interferon-Epsilon (IFNε) Staining of Ectocervical Biopsies

Amongst viral susceptibility genes (Figure 2B/3B), we noted that interferon epsilon (IFNε) was significantly over-expressed (2.2× times) in biopsies from FSW as compared to the control group. IFNε is a novel Type I interferon preferentially produced by epithelial cells of the female reproductive tract, and implicated in protection from both viral and bacterial STIs [31,32]. Immunohistochemical staining confirmed the elevated IFNε protein expression in FSW; this was restricted to the basal and parabasal layers of stratified squamous epithelium of the ectocervix (Figure 3A). In biopsies that captured columnar cells of the SCJ, IFNε was also present within the columnar cells (data not shown). Quantification of IFNε protein

expression within the epithelial layer indicated that FSWs had protein levels 2× higher than controls (p=0.0336; Figure 3C). IFNε protein levels were positively correlated with levels of gene expression (r=0.6954, p=0.0019; Figure 3D).

In contrast to other Type I interferons induced by pattern recognition receptors, IFNε expression has been reported to be regulated by estrogen levels [31]. However, we were unable to detect any significant difference in plasma estrogen levels between our study groups, and we found no significant correlation between estrogen levels and IFNε gene expression (r=0.352, p=0.108) or protein levels (r=0.305, p=0.19). IFNε gene expression has also been shown to be regulated by semen exposure in murine models [31] and human ectocervical cell lines [14]. We therefore reanalyzed data by stratifying our study participants based on self-reported condom use in the week prior to sampling (irrespective of prior FSW or control group assignment). The reanalysis resulted in detection of a greater difference with women who reported unprotected intercourse in the prior week showing higher (~ 3×) IFNε expression than women reporting abstinence or consistent condom use (p=0.001; Figure 3E) and showed that self-reported condomless sexual activity correlated with IFNε expression (r=0.4907, p=0.024). Of interest to acute effects of semen, we did detect higher levels of IL-1β, IL-6 and IL-8 in cervico-vaginal secretions from women grouped by condomless sex criteria (Supplemental Figure 3); of note, these differences were not noted comparing FSW versus control groups as there were no differences detected among all variables tested (data not shown). Reanalysis of all previously described significant variables by this new grouping did not result in detection of differences across all variables except IFNε tissue expression consistent with their association with long-term sex work rather than short-term semen exposure (Supplemental Table 2). Similarly, a re-analysis of gene expression data based on condomless sex did not detect a single gene as significant below the 20% FDR cutoff. To allow for the inference as to whether or not semen exposure could increase IFNε RNA and protein expression in human-derived cervical epithelium, we tested the effects of *in vitro* semen exposure on the ectocervical cell line ECT1. A 6hr incubation with 5% seminal plasma (SP) resulted in a 2.5 fold increase in IFNε gene expression (p=0.0023; Supplemental Figure 4A) in conjunction with higher protein expression (Supplemental Figure 4B). Taken together, semen-inducible IFNε expression is present at a higher level within the epithelial layer of the cervix in women reporting condomless vaginal intercourse.

Downregulation of Genes Required for HIV-1 Infection in sex workers

As the host profile of gene expression required for HIV-1 infection and replication has been well studied, we assessed whether the ectocervical tissue of FSW would express differential levels of target genes as previously defined by various functional genomic screens or genome-wide association studies [33343536]. Our analysis identified 23 genes, differentially expressed in FSW as compared to controls that overlapped with results from these prior studies (Figure 4). The degree of overlap was significantly higher than by random chance alone as determined by hypergeometrical testing for 3 of 4 studies examined (Supplemental Table 4); moreover, we found that the degree overlap with genes identified in 2 or more of the screens was highly significant (p<0.0001; Supplemental Table 4). These downregulated genes included CD4 and Nucleoporin 153 (NUP153), shown to be critical for nuclear import

of HIV and integration [37]. Overall, target gene expression indicates that lower levels of genes required for HIV infection are present in ectocervical tissue from FSWs.

Discussion

We show that in long-term HIV seronegative female sex workers (FSW) three distinct, non-adaptive mechanisms may contribute to decreasing susceptibility to HIV-1 infection: lower mucosal tissue immune activation, enhanced epithelial IFNε expression, and lower expression of HIV-permissive host genes. In regards to enhanced epithelial IFNε expression, we further show that semen exposure can contribute to the induction of IFNε in cervical cells in vitro, connecting for the first time a feature of long-term sex work (repeated semen exposure through condomless intercourse) with the induction of a highly localized type I interferon in the cervico-vaginal epithelium. Our results indicate that IFNε mRNA and protein are co-expressed in the human female reproductive tract and are up-regulated in women with high exposure to semen, suggesting semen components may regulate gene and protein expression of IFNε (otherwise regulated by estrogen levels in females[31])in this compartment inclusive of transcriptional induction. Our analysis identifies a previously uncharacterized immunological impact of condomless sexual activity of relevance to high-risk groups where HIV-1 prophylactic interventions are usually first tested.

Our observations on CD38 expression on T-cells within PBMC of long-term FSW provides similar results as previous reports [based on African and European cohorts [28,29]] of lowered T-cell activation (i.e.: CD38 expression) in HESN FSW. Importantly, we now document that this change in circulating T-cell is not unique to HESN FSW cohorts, but associated with long-term sexwork. The mechanism mediating a reduced peripheral blood T-cell CD38 expression in FSWs remains to be elucidated. In HESNs, CD38 expression on CD4+ T-cells has been reported to be negatively correlated with condom use [28]. Thus, it is tempting to speculate that semen may impact systemic T-cell activation. The concept that semen can have systemic effects is supported by other observations that semen exposure can drive gene regulation and systemic changes outside of the female reproductive tract [38]. However in our cohort, analysis performed based on recent semen exposure (i.e.: recent condomless intercourse) did not efficiently segregate CD38 expression on circulating CD4+ or CD8+ T-cells. We hypothesize this systemic change may be due to long-term rather than short-term sex work (or long term semen exposure). Indeed, after adjusting for confounders present at baseline, differences in CD38 remained significant when groups were defined as sex workers vs. controls (Supplemental Table 2). We interpret that repeated exposure to semen may induce chronic conditioning effects that differs from the acute effects of semen in induction of a potent inflammatory-like response [13,14] in the FRT. It remains to be determined if the reduction of immune-activation associated gene expression observed in FSW is the result of a counter-regulatory response to repeated semen-induced inflammatory changes or due to IL-10, PGE2 and TGF-b in semen[15–18]. Of interest, we detected higher cervicovaginal fluid IL-1β, IL-6 and IL-8 in women reporting recent condomless sexual activity (Supplemental Figure 3) supporting our interpretation between short-term effects of semen as not associated with chronic gene expression as measured in tissue. While the biological mechanisms underlying the observed lower activation in FSWs remain unknown, exposure to semen

induced IFNε *in vitro* supporting the correlation between recent semen exposure and IFNε expression in vivo.

The detection of IFNε amongst FSWs in our study population establishes for the first time that this type I interferon is expressed in human mucosal tissues. Despite the apparent elevated expression of IFNε in epithelium layer, overall tissue RNA analysis did not show an increase in IRGs, supporting that its activity may be restricted to cells proximal to the basal membrane as opposed to affecting gene expression across the entire stroma (i.e., largest area within biopsy). While addressing the direct antiviral effect of semen-induced IFNε expression within the cervicovaginal mucosa is beyond the scope of this report, we hypothesize that IFNε might target cells crossing the basal membrane into the epithelium such as T-cells, dendritic cells, and macrophages. Apart from IFNε expression or lower immune activation, our data also indicate that expression of genes required for HIV infection [36] [including CD4 (receptor for HIV-1), CXCR4 (co-receptor for HIV) and nucleoporin 153 (required for HIV-1 nuclear import and integration) [37]]are downregulated in FSWs, introducing another potential barrier to a productive infection. Lower immune activation and lower expression of genes required for HIV-1 infection together with the increased expression of epithelial Interferon epsilon (IFNε) suggests that the cervico-vaginal tissue of FSW may provide for a higher inherent level of resistance to HIV-1 infection. Although direct comparisons of our cervical biopsy gene array data to gene expression from isolated cervical mononuclear cells (CMC) obtained by cytobrush of the ectocervix (Majengo FSW cohort) is limited by different tissue site collection [9,10], similar inferences are possible between studies. Our data and that from the Majengo FSW both indicate a lower immune activation state in leukocytes from FSW cohorts [10]. Future studies from tissue-derived CD4+ and CD68+ cells will be needed to establish direct evidence of HIV resistance within target cells.

Although at a population level condomless sex work does increase HIV seroconversion risk [39,40], it remains to be determined if factors identified here limit the potential frequency of HIV infection as suggested by earlier unconfirmed reports where previously characterized HESNs became infected following a lapse in sexwork [6]. Our work raises the hypothesis that a lapse in continued maintenance of factors described here (i.e., stop semen exposure) may contribute to a change in susceptibility for HIV infection [6]. As the role of semen as agonist or antagonist to SIV/HIV-1 transmission remains controversial [21,41], studies based on NHP models and/or longitudinal human HESN FSWs cohorts will be needed to directly address the role of semen and factor described here in mediating and sustaining resistance against SIV-1/HIV-1 infection.

Unlike prior studies in HESN FSW cohorts, our data does not support that sexwork alone accounts for previous observations of a higher frequency of FoxP3+ CD4+ T-Regulatory cells (Table 2) or differential cervico-vaginal cytokine levels between control and FSW groups [7,10]. In addition, prior work in the Majengo FSW HESN cohort found higher CD4+ T-cells within cervical scrapings [9,42], and lower CD4+ T-cell infiltration as indicated by staining of ectocervical biopsies[24]. The latter results contrast with our findings of increased CD4+ T-cells, macrophages and plasmacytoid dendritic cells (pDC) in ectocervical tissue biopsies. Taken together, this suggests that other cohort-specific factors (e.g. HIV exposure,

sexual practices [5,43], regional, cultural or genetic factors, etc.) may account for the differences between our and prior studies. For example, differences in reported douching frequency in Africa versus Latin American women [5,43] remains to be evaluated as a potential reason for noted differences. It is also important to note that inclusion criteria excluded women showing active local inflammation. Therefore, our findings are limited to constitutive factors that may be associated with a high degree of semen exposure or active sex work absent an ongoing symptomatic infection. Another important limitation of this and other similar studies is that self-reported behavior is frequently inaccurate as has been shown by assessing condom use in HIV-1 prevention/acquisition in multiple studies [44]; however, studies have also shown that self-reporting of recent coital activity tends to be more accurate [45]. We interpret that self-reporting in our cohort is more accurate per recent coital exposure as reflected by the observation that recent condomless sexual activity correlated with IFNε expression (Figure 3F). We do not address the potential role of semen exposure from multiple donors in FSW as compared to controls that have a single partner (or semen source). In order to assess the importance of sexual frequency or partner specific differences in semen composition as potential determinants for the immunological and mucosal differences ascribed to FSW in this work, future studies will need to define what components of semen or sex work contribute to these changes.

In conclusion, our data brings together for the first time the detection of three separate anti-HIV features independent of antiviral adaptive associated with long-term sexwork independently of high-risk HIV exposure or anti-HIV adaptive responses. Behavior (long-term sex work), chronic semen exposure and induction of interferon ε within human ectocervical tissue are identified as determinants of local mucosal tissue and systemic changes consistent with decreased HIV-1 susceptibility which may in part explain sustained HIV-1 Highly-Exposed Seronegative (HESN) status identified in some HIV-1 high-risk cohorts. We propose a working model (Supplemental Figure 5) suggesting that prolonged sex work can sustain changes in the cervico-vaginal microenvironment that may increase HIV-1 resistance in part dependent on continued semen exposure. Importantly, the indication that sex work is known to be associated with a higher risk for HIV infection due to multiple inter-related factors is not addressed here (co-infections, drug use, etc.) [2,46], therefore limiting our data to documenting host factors that may reduce but not eliminate susceptibility of HIV infection in female sex workers.

Methods

Participants

Ethics Statement—All female recruitment done for this study was according to Institutional Review Board approved protocols at the Wistar Institute (#2908242) and the University of Puerto Rico (#1350210). All participants gave written informed consent before study inclusion.

Cohort—A total of 85 women were recruited. 50 Female sex workers were recruited by the Clinical Research Unit of the Maternal Infant Studies Center (CEMI) at the University of

Puerto Rico (UPR) in San Juan. 35 control group women were recruited from the family planning clinic at CEMI.

Inclusion/Exclusion Criteria—All recruited women were 21 years old or older (21 is the legal lower limit for PR); gave informed consent; not currently menstruating; tested negative for HIV-1 by OraQuick (Orasure tech, Inc., Bethlehem, PA) and/or PCR; refrained from injection drug use; completed sexual behavior questionnaires and sexually transmitted infection screening.

Recruitment—Recruitment and enrollment of female sex workers and women for the control/comparator group was done at the PR study site that was simultaneously recruiting FSW for the HIV Vaccine Trials Network's 907 protocol [25]. Similar recruiting strategies were employed with the exception that high-risk women actively using injection drugs were excluded. Recruited FSW were HIV-1 seronegative women who had maintained self-reported high-levels of sexual activity (condomless sex with multiple partners) for at least 2 years prior to study inclusion (median of 8 years in sex work as noted in Table 1). Women in the control or comparator group were enrolled from women attending the women's health clinic at CEMI and reported being in monogamous relationships.

Study Visit/Sample Collection—All consenting women underwent two study visits. An initial visit to collect sex behavior history information, confirm HIV-1 IgG seronegativity, collect peripheral blood for STIs tests (gonorrhea, syphilis and chlamydia); complete a vaginal pH and a pap smear test to assess the presence of cervical dysplasia and inflammation. At the second visit women were counseled on test results and those confirmed to be seronegative for HIV-1 without the presence of active STIs and without a CIN2 or greater dysplastic cytology diagnosis were recruited to assigned study groups and underwent a sampling for peripheral blood (PBMC archiving and whole blood phenotypic flow cytometric staining) and cervical fluid collection by vaginal wash with 1× PBS (HIV-1 specific IgA quantification and cytokine testing). In a subset of FSW and control group women, additional consent was obtained for a colposcopy to collect a cervical biopsy (3×3mm) targeting the squamocolumnar junction region otherwise routinely taken as part of standard gynecological care. Biopsies were fixed using Safe Fix II (Fisher Scientific, Pittsburgh, PA) according to manufacturer protocol and paraffin embedded. Women were advised to refrain from sexual activity for 1 week until healed; although, previous work has shown that such biopsies can be taken safely from women at risk of HIV-1 infection [47].

Hormone Measurements

Archived sera were used to determine the estradiol concentration of participants at time of blood draw. Hormone concentrations were measured by enzyme-amplified chemiluminescence (Immulite 1000, Siemens) as described by manufacture protocol. The analytical limits of sensitivity of the estradiol and progesterone assays were 15 pg/mL (references range of 20–2,000 pg/mL) and 0.1 ng/mL (reference range of 0.2–40 ng/mL), respectively.

Whole Blood Staining of Lymphocyte Subsets

200 μl of whole blood from FSW and control group women was stained for NK Cell, T-Cell and DC subsets using antibodies obtained from BD Bioscience (San Jose, CA) or Miltenyi (Auburn, CA). Following staining, red blood cells were lysed according to BD FACS Lysing Solution protocol. Cells were suspended in BD Staining Buffer (BD, Bioscience) for flow cytometric analysis.

Peripheral Blood Mononuclear Cell Isolation

Standard density gradient centrifugation was used to isolate PBMC from whole blood derived from FSW and control group participants. Briefly, blood was overlaid on Ficoll-pacque Plus (GE Healthcare, Little Chalfont, UK) and centrifuged at 1,200xG without break. Resulting PBMC were removed and washed in 1× PBS. Cells were diluted in trypan blue to exclude dead cells and contaminating red blood cells and then counted.

HIV-Specific T-Cell Memory

500,000 Peripheral Blood Mononuclear cells from FSW and control group participants were incubated for 17 hrs at 37°C with 15-mer HIV-1 consensus B gag peptides or immunodominant peptides (9-mer) from CMV, EBV and Influenza obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH. For co-stimulation, CD28/CD49 (BD Bioscience, San Jose, CA) was added along with Brefeldin A for intracellular staining. Following incubation, cells were stained for CD3 (SK7), CD4 (SK3), CD8 (RPA-T8) and CD107 (H4A3). Following extracellular staining, cells were fixed and permeabilized according to manufacturer protocol for BD Cytofix/Cytoperm (BD Bioscience). Cells were then intracellularly stained for interferon γ (25723.11). Finally, cells were suspended in BD Staining Buffer (BD, Bioscience) with at least 200,000 single cell events collected per target gating. Analysis was done with FlowJo software (Tree Star Incorporated, Ashland, OR). A positive HIV-1 gag response was defined as 3× the median or greater of IFNg+ CD8+ T-cells from the control or comparator group.

Binding Antibody Multiplex Assay (BAMA)

Mucosal HIV-1 envelope specific antibodies from FSW and control group CVL samples were measured by a custom HIV-1 binding antibody multiplex assay against a panel of group M consensus and clade B envelope glycoproteins as previously described [48] and included in supplemental methods. Samples were considered positive for HIV-1 specific antibody if they were positive for specific activity for >2 HIV-1 specific antigens.

Cytokine Multiplex Assay

Life Tech Human Cytokine 30-plex Assay (Carlsbad, CA) was used to measure cervico-vaginal cytokine levels according to manufacture protocol. Data was acquired using a Bio-Plex 200 System (Biorad, Inc.). Cutoffs were determined as 50% of the MFI of the lowest point on the standard curve for a given cytokine.

Ectocervical Biopsy Tissue Staining

Briefly, 6-μm sections were cut and adhered to silanized slides from paraffin embedded tissues. Immunohistochemical staining for CD123, CD68, CD4, IFNε and Mx1 were conducted as previously reported [22,23,32]. Quantification of tissue staining was done as described previously [23,32] with complete methods provided in supplemental methods.

Gene and miRNA Expression analyses of Ectocervical Tissue

Total RNA from formalin-fixed paraffin embedded tissues was isolated with High Pure RNA Paraffin Kit (Roche Applied Science, Branford, CT). Total RNA at 500ng was used on Illumina Whole Genome gene expression DASL HT assay (Illumina, San Diego, CA) with the MCS4 cDNA synthesis reagents and Illumina HT12v4 DASL BeadChip. miRNA expression in Exctocervical Tissues were assayed using TaqMan OpenArray RT PCR platform (Applied Biosystems). Methods information on gene expression data analysis is provided in supplemental methods.

Semen Preparation

De-identified, discarded cryopreserved semen samples were received under IRB exception from the Andrology Laboratory of Penn Fertility Care, University of Pennsylvania. 30 individual vials of semen were thawed at 37°C and pooled into a master mix before refreezing and storage at −80°C until rethawed at 37°C. Seminal plasma (SP) was separated from sperm by centrifugation.

ECT1 Culture and Immunofluorescence Staining for Interferon ε

ECT1 Cells (ATCC, Manassas, VA) were cultured until 50% confluent in Keratinocyte-Serum Free Media (KSFM) (Life Technologies, Carlsbad, CA). Cells were then treated with 5% seminal plasma in KSFM (v/v) for 6 hours at 37°C, 5% CO_2. After treatment cells were fixed in 2% PFA for 20 minutes. Cells were blocked with 5% BSA/0.2% Triton X and incubated with IFN-ε antibody (1:500; Atlas Antibodies) overnight. After washing, cells were incubated with an Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody (1:200; Life Technologies). Cells were counterstained with DRAQ5 (1:1000; Biostatus Limited, Shepshed, UK). Images were collected using a TE2000 Inverted Microscope (Nikon, Melville, NY).

ECT1 RNA Isolation and qRT-PCR

Seminal Plasma treated (as above) and untreated ECT1 cells were used for RNA isolation by RNeasy Mini Kit (Qiagen, Valencia, CA). NanoDrop 1000 (ThermoScientific, Waltham, MA) was used to measure RNA quality and quantity. cDNA was prepared from 1 μg total RNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), and diluted 1:15 in RNAse-free water. Each PCR sample was tested in triplicate, and the PCR for the housekeeping gene Beta-Actin and the target gene, IFNε, were run in parallel on the same plate. Primers used and PCR analysis information provided in supplemental methods.

Statistical analysis

Shapiro-Wilk tests were carried out to detect normality distribution of variables and then to determine appropriate statistical tests or procedures. When specified, Fisher's Exact Test was used to analyze contingency tables. Correlations were done using the Pearson or Spearman method depending on the previous normality test of variable distribution. P-values less that 0.05 were considered significant for single test. For comparisons in each data assay (aside from gene expression analysis described above), when multiple testing adjustment needed, Benjamini-Hochberg and Benjamini-Yekutieli adjustment were performed at 10% of the false discovery rate.

To study the robustness of the results to potential confounding variables, we tested for group differences using a novel, weighted version of the Mann-Whitney test. Individuals were weighted in such a way so that the observed difference between groups is not attributable to the measured confounding variables. The method is based on inverse probability weighting [49,50], and a permutation testing approach was used to obtain the p-values. Propensity scoring used to derive inverse probability weighting used confounding variables including marijuana use, vaginal pH, male condom use and oral contraceptive use.

Significance of overlap between any two gene sets were done using hypergeometrical test as a probability of having overlap $\geq m$ between sets of size n_1 and n_2 with total number of available genes N, where N = total number of genes overlapped between two platforms used to generate the two gene lists, n_1 = number of genes in list 1, n_2 = number of genes in list 2, m = number of overlapped genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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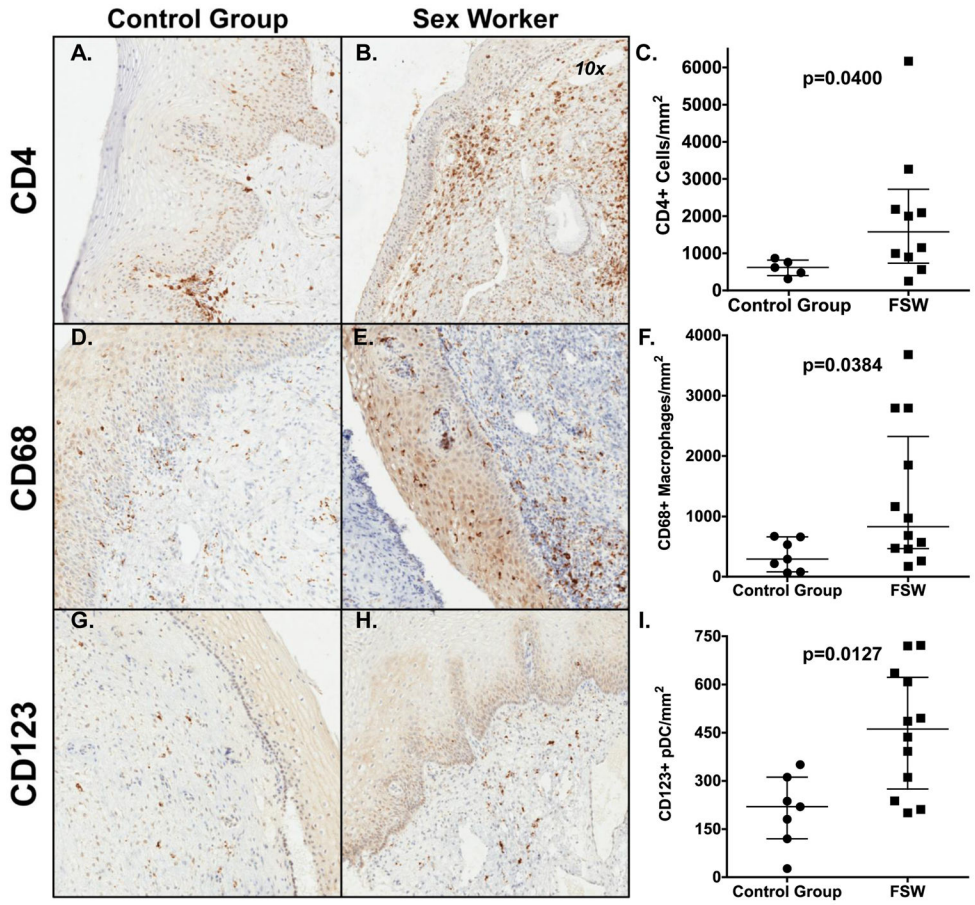

Figure 1.

Increased levels of immune effectors in FSW ectocervical tissue as compared to control women. Pictured are IHC images from each staining group representative of Control or Sex Worker groups, brown punctate staining represents individually stained cells. (A–C) FSWs had over 2× more CD4+ T-Cells within ectocervical biopsies as compared to biopsies from low-risk controls (median: FSW 1958 vs CG 609.3 cells/mm^2; p=0.0400). (D–F) CD68+ macrophages were also over-represented in FSWs with the highest differential between FSWs and control group women (median: FSW 829.6 vs CG 294.0 cells/mm^2; p=0.0384) (G–I) FSWs had higher levels of CD123+ pDC within ectocervical tissue as compared to controls (median: FSW 461.1 vs CG 219.8 cells/mm^2; p=0.0127). Images at 10x. Median and Interquartile range shown in C, F & I. Groups comparison was done by Mann-Whitney U tests.

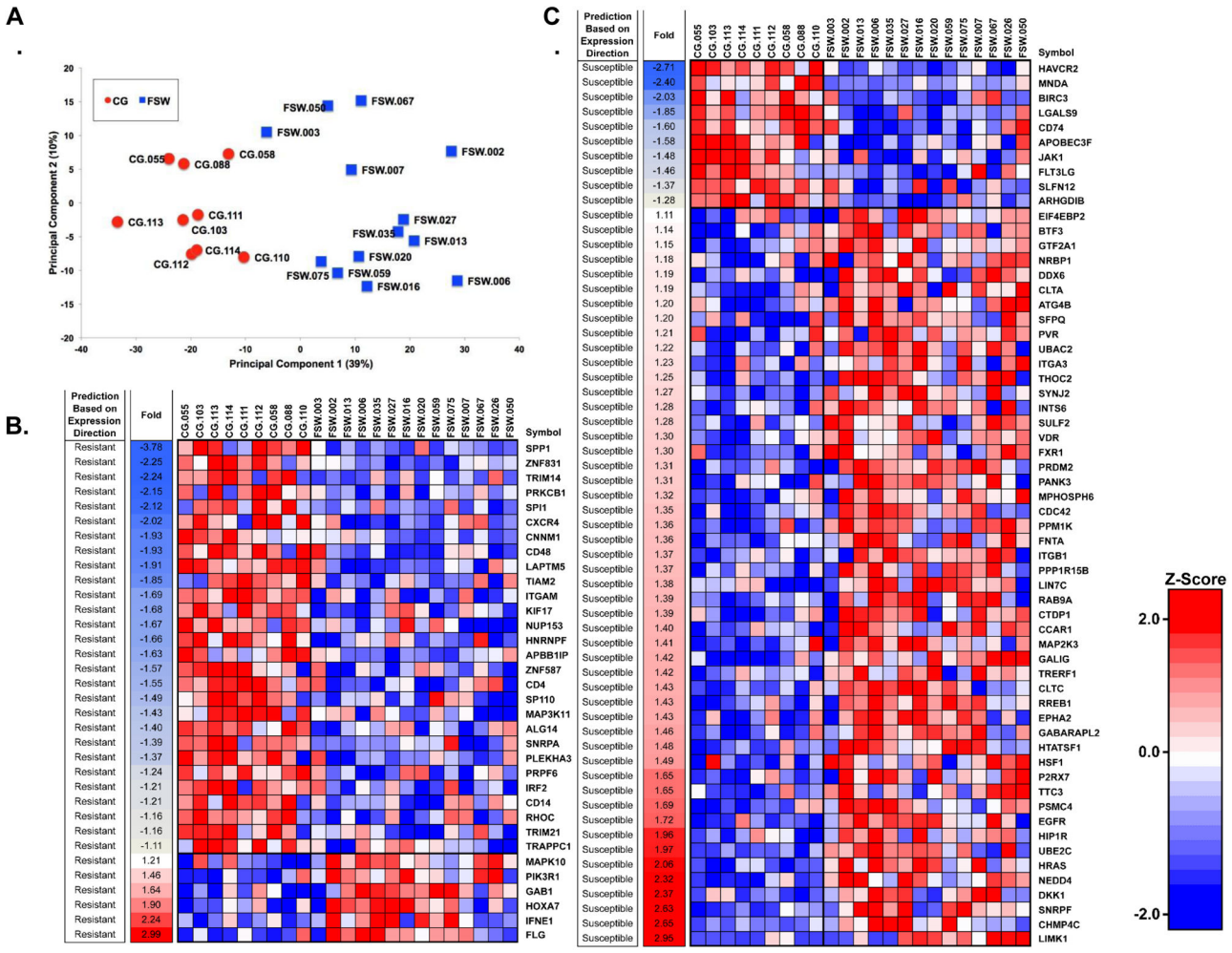

Figure 2.

Gene expression differences in ectocervical biopsies between 11 females from control group (CG) and 14 female sex workers (FSW). (A) Principle component analysis using all 873 significant genes (1.2≥Fold) demonstrates clear segregation of sex workers from control group samples. (B) Of 873 genes, 106 overlapped with a predetermined Ingenuity Pathway Analysis list of genes involved with viral infection. 33 of the 106 genes differentially regulated between FSW and CG had an expression profile in FSW predicted to reduce susceptibility to viral infection as compare to CG. (C) Conversely, 60 of the 106 genes had an expression profile predicted to increase susceptibility to viral infection FSW as compared to CG. Overall, IPA predicted higher viral susceptibility in FSW as compared to CG (Z-score = +3.35).

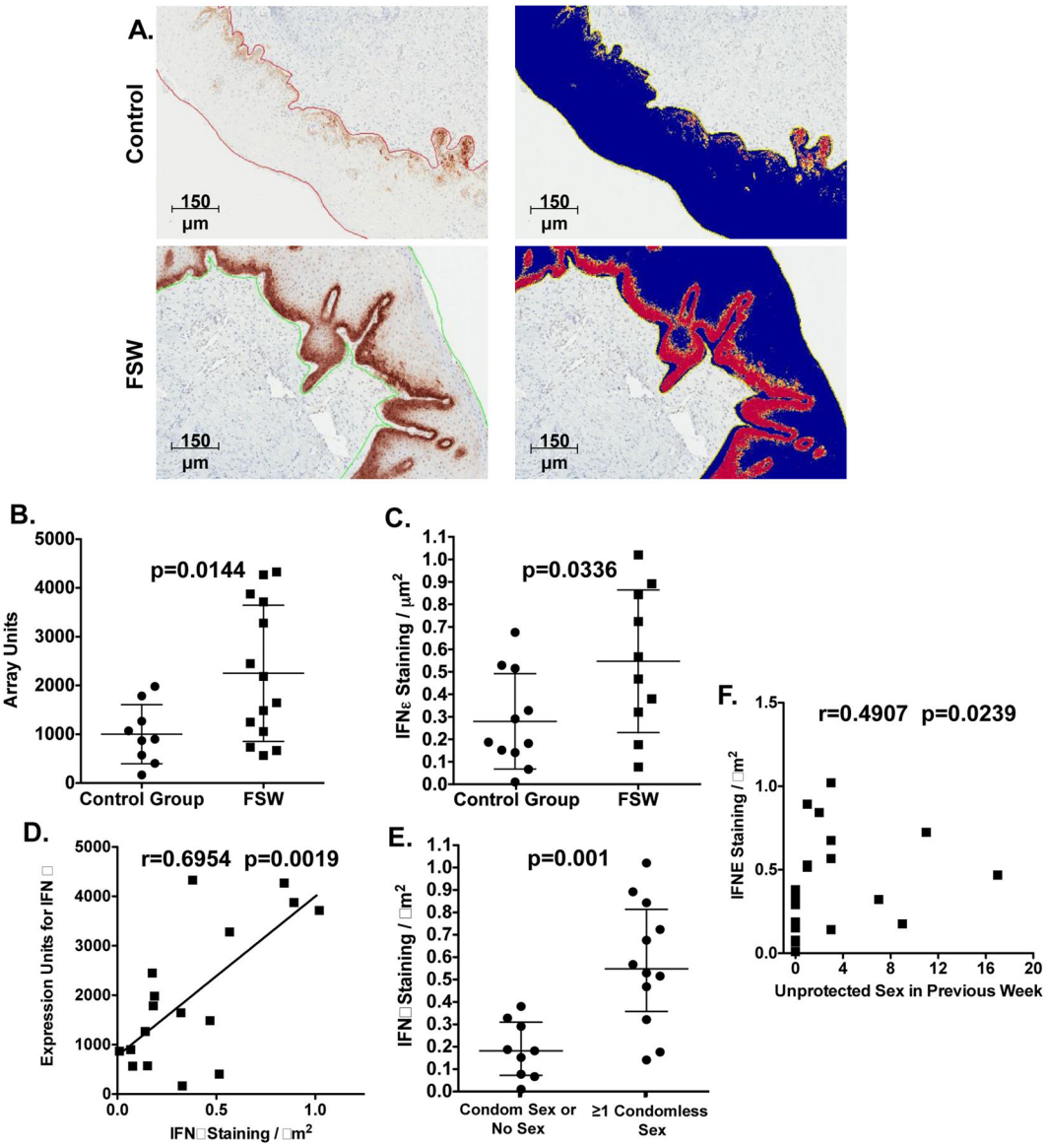

Figure 3.

Increased IFNε in FSW-derived ectocervical tissue as compared to samples from women in the control (A) Interferon ε staining (brown) showed that expression of this Type I interferon was tightly restricted to basal and parabasal stratified squamous epithelium. Staining was also evident in columnar cells but to a much lesser extent (data not shown). Female Sex Workers (FSW - bottom) had much higher expression of IFNε than controls (Top) in general. Both gene expression array analysis **[Right panels are gradient maps to highlight positive staining within the epithelium]** (B) (p=0.0144) and Image quantification (C) (p=0.0336) of tissues showed higher expression of IFNε in FSWs. (D) Protein expression for IFNε strongly correlated with the gene expression array (r=0.6954 p=0.0019) showing that FSWs had higher IFNε expression. (E) Splitting all women in the study based on previous unprotected sex in the week prior to biopsy showed that women who had unprotected sex had 3× more IFNε than those who did not (p=0.001). Median and Interquartile range shown

for panels A, B & E. The difference between groups was tested using Mann-Whitney tests. Correlations were assessed using the spearman test

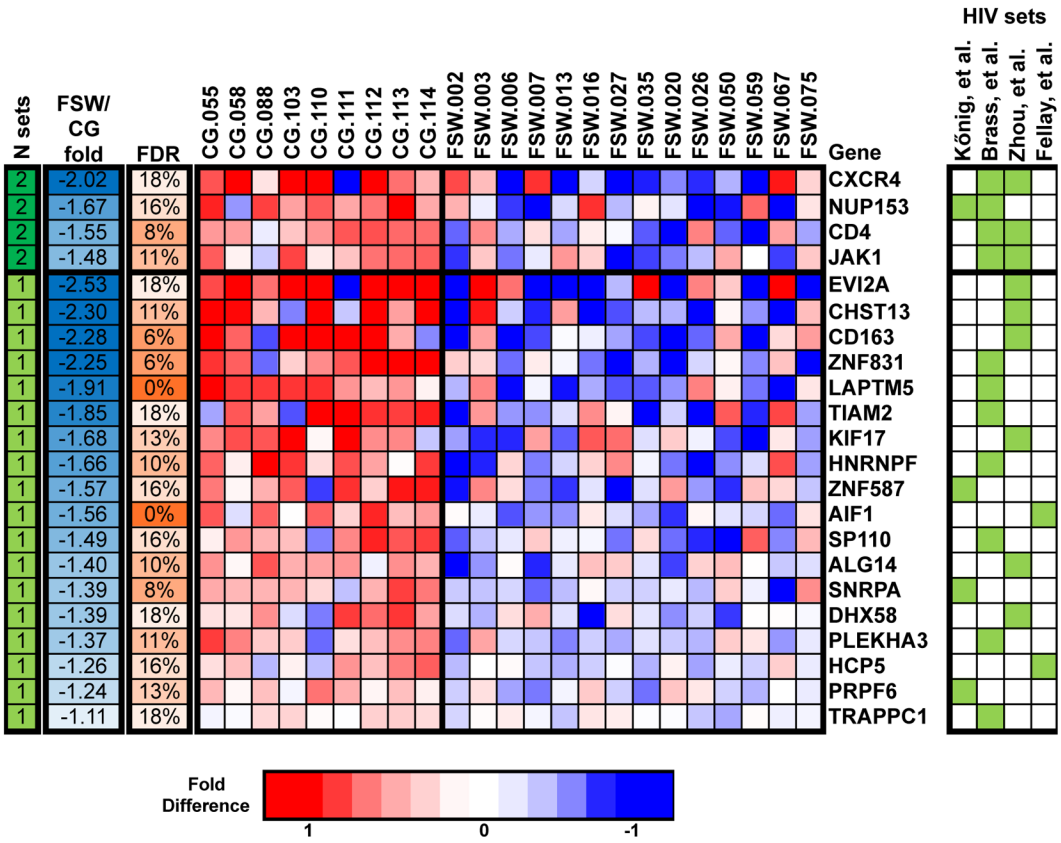

Figure 4.

Heatmap of Dowregulated Genes from FSW/CG Microarray Shown Previously to be Essential for HIV-1 Infection and Replication. As multiple studies have examined required genes for HIV-1 infection/replication, we assessed our data set for overlap with four previously published gene sets [33–36]. 22 genes from our data set overlapped with results from these prior studies (hypergeometrical test: $p<0.0001$) this was more than twice the number of genes as expected from random overlap. Additionally, being present in at least 2 of these prior studies further validated 4 of these genes. Heatmap is presented as fold difference over median expression across all patients for a particular gene. HIV Sets are color coded as green for overlap with a particular data set and white for no overlap.

Table 1

Sexual Practices and Cervico-vaginal Status Amongst Biopsy Contributors

	Category	Control Group (CG) 35 Women	(FSW) 50 women	P-Values
Cohort Descriptors	**Age (years)**	35.5 (29.5 – 41.25)	32 (28 – 38)	**0.439**
	Sexual Partners (1 Month)	1 (1 – 1)	6 (15 – 4)	**<0.0001** *
	Unprotected Sex (1 week)	0 (0 – 3)	3 (2 – 9.7)	**<0.0001** *
	Years Sex Work	(N/A)	8.45 (5 – 11.5)[t]	
	Oral Contraceptives	16 (45.7%)	2 (4.0%)	**<0.0001** *
	Sterilization[1]	4 (17.1%)	27 (54%)	**0.0025** *
	Non-IDU Drug Use	4 (12.9%)	34 (68%)	**<0.0001** *
Vagina Microenvironment	**Chlamydia**[2] **Gonorrhea Syphilis**	0 (0%)	8 (16.0%)	**0.0247** *
	HPV	5 (17.2%)	6 (20.5%)	0.879
	ASC/CIN	6 (24.0%)	14 (28.0%)	0.406
	Vaginal pH (>4.5)	21 (61.7%)	34 (75.6%)	0.2219
Hormone Levels	**Estradiol**[3]	56.65 (20.0 – 158.5)	76.9 (20.0 – 89.75)	0.2097

Parenthetical data presented as range or as percentage.

* Indicates significant variable as measured by Mann-Whitney U or Fisher's Exact Testing

[1] Sterilization was done by tubal ligation

[2] STIs were asymptomatic, symptomatic STIs were apart of the exclusion criteria

[3] For Estradiol: 28 FSW/14 CG measured

Table 2

Selected Significant Variables from Flow Cytometric Analysis of PBMC

Categories	Variables	Control Group	FSW	P-value	BY*
T-Cell Activation	CD38 MFI on CD4+ T-Cells	201.9 (126 – 238)	84.4 (52.4 – 116)	<0.0001	<0.0001
	%CD38 CD4+ T-Cells	53.67 (46.2 – 64.2)	40.3 (33.7 – 47.1)	<0.0001	0.0006
	CD38 MFI on CD8+ T-Cells	101.2 (64.9 – 178)	55.9 (32.3 – 72.1)	<0.0001	0.0006
	%CD38 CD8+ T-Cells	41.95 (34.8 – 50.9)	32.21 (23.9 – 40.0)	0.0029	0.021
CD4 T-Regulatory Cells	%FoxP3+ CD25+ CD4+ T-Cells	1.27 (0.76 – 1.69)	0.87 (0.46 – 1.15)	0.019	0.019
Dendritic Cells	%mDC	0.219 (0.18 – 0.25)	0.17 (0.11 – 0.21)	0.0022	0.022
	CD83 MFI on mDC	67.7 (28.0 – 72.0)	94.4 (59.4 – 127)	0.0030	0.022

Variables listed in order of p-value. Median and IQR values shown. Group comparisons done by Mann-Whitney U tests.

*Benjamini & Yekutieli adjustment (at rate of 10%) was done per examined immune cell compartment or functional test

Table 3

Biological Functions significantly altered based on genes differentially expressed between FSW and Control Group Women.

Diseases or Functions Annotation	P-Value	Predicted Activation State*	Z-Score	Genes in Category
Chemotaxis of Granulocytes	0.0074200	Decreased	−2.22	15
Chemotaxis of Leukocytes	0.0002850	Decreased	−2.38	29
Chemotaxis of Myeloid Cells	0.0011300	Decreased	−2.75	23
Chemotaxis of Neutrophils	0.0033000	Decreased	−2.03	14
Chemotaxis of Phagocytes	0.0007740	Decreased	−2.93	24
Engulfment of Antigen Presenting Cells	0.0062300	Decreased	−2.20	10
Engulfment of Phagocytes	0.0101000	Decreased	−2.41	11
Homing of Granulocytes	0.0032700	Decreased	−2.22	16
Homing of Leukocytes	0.0003570	Decreased	−2.38	30
Homing of Neutrophils	0.0012900	Decreased	−2.03	15
Organismal Death	0.0011900	Decreased	−3.93	98
Phagocytosis by Macrophages	0.0043000	Decreased	−2.20	9
Phagocytosis of Blood Cells	0.0125000	Decreased	−2.07	11
Phagocytosis of Myeloid Cells	0.0096900	Decreased	−2.41	10
Phagocytosis of Phagocytes	0.0096900	Decreased	−2.41	10
Apoptosis of Breast Cancer Cell Lines	0.0029400	Increased	2.43	21
Cell Death of Breast Cancer Cell Lines	0.0018800	Increased	2.82	24
Cell movement of Endothelial Cells	0.0123000	Increased	2.17	22
Cell Movement of Fibroblast Cell Lines	0.0113000	Increased	2.33	12
Cell Viability of Cervical Cancer Cell Lines	0.0029600	Increased	2.07	17
Interphase of Fibroblasts	0.0042900	Increased	2.22	8
Replication of RNA Virus	0.0001860	Increased	2.41	40
Replication of Virus	0.0002020	Increased	2.67	43
Survival of Organism	0.0104000	Increased	2.61	41
Viral Infection	0.0004060	Increased	3.35	106

*Activation state indicates direction of change in FSW compared to control group with the significance of the prediction expressed as Z-score

Table 4

Genes from Sex Worker Ectocervical Biopsy Microarray which Overlap with Genes Required for HIV infection

Gene Names	Fold	P-Value	FDR
Zinc Finger Protein 831 (ZNF831)	−2.25	0.00026	6.0%
C-X-C chemokine receptor type 4 (CXCR4)	−2.02	0.00370	17.6%
Lysosomal-associated protein transmembrane 5 (LAPTM5)	−1.91	0.00006	0.0%
T-cell lymphoma invasion and metastasis 2 (TIAM2)	−1.85	0.00406	17.6%
Nucleoporin 153kDa (NUP153)	−1.67	0.00251	15.5%
Heterogeneous nuclear ribonucleoprotein F (HNRPF)	−1.66	0.00070	10.2%
Cluster of differentiation 4 *(CD4)*	−1.55	0.00054	8.2%
SP110 nuclear body protein (SP110)	−1.49	0.00220	15.5%
Janus Kinase 1 (JAK1)	−1.48	0.00127	10.8%
Four-phosphate-adaptor protein 1 (FAPP1)	−1.37	0.00111	10.8%
Trafficking protein particle complex (TRAPPC1)	−1.11	0.00407	17.6%
THO complex subunit 2 (THOC2)	1.25	0.00168	10.2%
M-phase phosphoprotein 6 (MPHOSPH6)	1.32	0.00529	13.0%
Farnesyltransferase (FNTA)	1.36	0.00484	13.0%
Carboxy-terminal domain, RNA polymerase II, polypeptide A, phosphatase, subunit 1 (CTDP1)	1.39	0.00816	15.5%
Gamma-aminobutyric acid receptor-associated protein-like 2 (GABARAPL2)	1.46	0.00222	10.8%
HIV-1 Tat specific factor 1 (HTATSF1)	1.48	0.00272	10.8%
Epidermal growth factor receptor (EGFR)	1.72	0.00099	8.2%
Huntingtin-interacting protein 1-related protein (HIP1R)	1.96	0.01007	15.5%