The Impact of Sex Work on the Susceptibility to HIV Infection among HIV Resistant Sex Workers

Kenneth Odiwuor Omollo

A dissertation submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in Medical Microbiology from the University of Nairobi

DECLARATION

I hereby declare that this research project is my original work and has not been presented for the award of a degree or any other award in any university.

Sign:……..Date: ……

KENNETH ODIWUOR OMOLLO
Registration No: H56/65303/2013
Msc. Medical Microbiology Candidate
University of Nairobi
Phone: +254 -725608484
oduorkenneth@gmail.com

Supervised by:

Sign:……Date: ……

DR. JULIUS O. OYUGI
Lecturer, Department of Medical Microbiology,
University of Nairobi
P.O. Box 19676-00202 Nairobi, Kenya.
Phone: +254 -713898564.
julias_oyugi@hotmail.com

Sign: ……. Date: ……

DR. KEITH FOWKE
Department of Medical Microbiology
University of Manitoba,
539-745 Bannatyne Avenue,
Winnipeg, Canada.
Tel: +1-204-789-3818
Keith.Fowke@med.umanitoba.ca

DEDICATION

Lovingly Dedicated to the Memory of

Grace Awino Ogola
1950 – 2013

A woman of many talents and strengths

I know I'll see you again.

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Above all, I thank God – the custodian of all knowledge- for His mercies endure forever.

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ABBREVIATIONS

AIDS	- Acquired Immune Deficiency Syndrome
ANOVA	- Analysis of Variance
BD	- Beckton Dickinson
CCR5	- C-C Chemokine Receptor 5
CD	- Cluster of Differentiation
CMC	- Cervical Mononuclear Cell
DC	- Dendritic Cell
FACS	- Fluorescence-Activated Cell Sorting
FGT	- Female Genital Tract
FSW	- Female Sex Workers
GALT	- Gut Associated Lymphoid Tissues
HESN	- HIV-Exposed Seronegative
HIV	- Human Immunodeficiency Virus
IFN	- Interferon
IL	- Interleukin
LAMIQ	- Longitudinal Assessment of Mucosal Immune Quiescence
MHC	- Major Histocompatibility Complex
MIG	- Monokine Induced by Interferon-Gamma
NK	- Natural Killer
PBMC	- Peripheral Blood Mononuclear Cell
PGE	- Prostaglandin E
PSA	- Prostate Specific Antigen
RNA	- Ribonucleic Acid
STI	- Sexually Transmitted Infection
TGFβ	- Transforming Growth Factor beta
UNAIDS	- Joint United Nations Program on HIV/AIDS
UNITID	- University of Nairobi Institute of Tropical and Infectious Diseases

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ABSTRACT

Background: Commercial sex workers are at high risk of HIV acquisition especially in countries with high HIV prevalence. In Kenya, over 10% of all new HIV infections have been attributed to sex workers and their clients. Unprotected sex results in exposure of the female genital tract to sex-derived antigens present in semen. Studies have reported a post-coital inflammatory response to these antigens in animals and humans. *In vivo*, such inflammatory response leads to recruitment of activated leukocytes including T cells from the peripheral circulation to the genital mucosa where they can be infected by HIV. There is need to explore the impact of sex work on the activation of the immune system and its role in the transmission of HIV. HIV-exposed seronegative (HESN) female sex workers have been shown to have lower peripheral and mucosal immune activation than their HIV-positive counterparts or HIV-uninfected low risk women. This reduced immune activation state is thought to be protective against HIV infection. It is, therefore, thought that HESN sex workers are able to down regulate sex-work-induced immune activation by a yet unknown mechanism.

Hypothesis: This study hypothesized that sex work results in exposure to very strong immune stimulants which can drive systemic immune activation and that HESN women are able to down regulate this sex work-driven immune activation more efficiently than HIV susceptible controls.

Objective: The study was designed to characterize the impact of sex work on activation and phenotype of peripheral T cells, among female sex workers at the Pumwani Clinic, Nairobi.

Methods: Thirty female sex workers were recruited; 10 in each arm of HESN, HIV-positive, and New Negatives. Blood samples were obtained from them when in active sex work, during a sex break and upon return to sex work. PBMCs were extracted from the blood and stained with a 10-colour monoclonal antibody panel for phenotypic (CD3, CD4, CD8, CD45RA, CD161), trafficking (CCR7, CCR5) and activation markers (CD69, HLA-DR, CD95) of T cells. Expression of these markers was then evaluated by flow cytometry. Statistical analyses were done to compare the expression of T cell activation markers and memory phenotypes within each group during sex work, sex break and upon return to sex work. The difference in response to interruption of sex work was also assessed across groups.

Results: A reduction in CCR5 expression on CD8+ T cells in HIV-positive sex workers was observed upon taking a break from sex work. A trend toward increased expression of CD161 on

CD4+ T cells was also observed in the HIV-positive. In New Negatives, we observed a trend towards decreased naïve CD8+ T cells during the sex break and upon return to sex work. It was also observed that there is no difference in the pattern of response to the interruption of sex work between HESN female sex workers and their HIV-positive and New Negative counterparts. Overall, HIV-positive women had higher frequency of effector memory T cells and CD95 expressing CD8+ T cells and a lower frequency of naïve CD8+ T cells than HESN and New Negatives.

Conclusion: Sex work had a subtle effect on the expression of T cell activation markers and memory phenotypes in the peripheral compartment. Interruption of commercial sexual activity had a greater impact on T cell activation in HIV-positives. In HESN and New Negatives, sex work had no discernible effect on peripheral immune activation. No apparent difference in the way HESN, HIV-positive and New Negative sex workers respond to interruption of sex work was observed, which may be due to various limitations of this study. Therefore, the impact of sex work could be confined to the FGT mucosa and not be reflected in the peripheral compartment.

1 INTRODUCTION

The Joint United Nations Program on Human Immunodeficiency Virus and Acquired Immune Deficiency Syndrome (HIV/AIDS) estimates that more than half of all people living with HIV/AIDS are women. It is estimated that, in Africa, women are twice as likely to contract HIV during vaginal intercourse compared to men and are thus considered to be a vulnerable population [1]. Recently, preliminary findings of the Kenya AIDS Indicator Survey showed that women are still disproportionately affected by AIDS compared to men, with a HIV prevalence of 6.9% compared to 4.4% for men [2].

Among the affected women are commercial sex workers, who are at very high risk of acquiring HIV, especially those residing in countries where HIV prevalence is high, such as Kenya. Based on a mathematical model, the 2009 Kenya Modes of Transmission study estimated that 14% of new infections in the country were attributable to female sex workers, their clients and other partners of the clients [3]. A recent World Bank global assessment also shows that HIV prevalence among female sex workers in Kenya remains high at approximately 45%[4]. These statistics show that sex work is a significant contributor to the HIV epidemic in Kenya.

Sex work, especially when unprotected, exposes both women and their clients to the risk of acquiring HIV and other sexually transmitted infections. Unprotected sex is the main mode of HIV acquisition. Moreover, unprotected sex is thought to influence the immune milieu in the female genital tract, which in turn can influence susceptibility to HIV.

Based on previous *in vitro* studies, sex work can be a powerful immune stimulant. Exposure to seminal fluid can cause the release of chemokines, cytokines and other innate factors in the female genital tract [5]. Seminal plasma can induce the release of pro-inflammatory Interleukin (IL)-8 from cervical explants [6]. Unprotected sex also results in the exposure to client-derived immune and sperm cells. Studies have shown that the immune stimulatory capacity of non-self-allogeneic antigens (alloantigens) is exceptionally strong [7, 8]. It is not known what the stimulatory capacity of allo-semen would be in the case of multiple partners. It is therefore hypothesized that the immune responses observed during intercourse can lead to the activation and recruitment of T cells to the genital mucosa and serve as targets for HIV infection.

Susceptibility to HIV could therefore be influenced by how individuals respond to sex induced T cell activation. Differences in the susceptibility to HIV infection are observed between individuals. Despite being at high risk of HIV acquisition some individuals remain HIV negative and are referred to as HIV-exposed seronegatives (HESN). Studies performed on HESN from Nairobi, Kenya have suggested that the level of immune activation among HESN is lower than in HIV-uninfected women newly practicing sex work and low risk women not involved in sex work [9, 10]. This increased frequency of resting cells with lower levels of activation markers been termed as Immune Quiescence- a model which proposes that HESN have a decreased levels of T cell activation and host proteins required for HIV-1 replication which limits the number of available HIV target cells and therefore makes HIV infection more difficult [9, 11].

Immune quiescence has been described in both the mucosal and peripheral compartments [12]. The role of sex work in driving immune activation, or quiescence is still not clear. There is a gap in the literature about the systemic immune activation following extended exposure to sexual activity and its importance in susceptibility to HIV infection. HESN sex workers have been showed to have lower mucosal immune activation [9-12] and it is therefore hypothesized that they have better control of sex-induced immune activation compared to HIV-infected sex workers and those that have been in sex work for a shorter period.

This study evaluated the impact of sex work on the immune cells, specifically on the activation of T cells from peripheral blood. Active sex workers enrolled in the Pumwani Commercial Sex Worker Cohort were recruited upon giving informed consent and blood samples were collected from them before taking a break from sex work, during an interruption of their commercial sexual activity and 2 weeks after resuming it. Peripheral blood mononuclear cells were extracted, stained with monoclonal antibodies and assessed using flow cytometry for expression of cell surface markers of T cell phenotypes and activation.

2 LITERATURE REVIEW

2.1 HIV infection and immune activation

The hallmark of HIV infection is a gradual loss of CD4+ T cells, with subsequent loss of immune competence, susceptibility to opportunistic infections and persistent systemic immune activation resulting in the acquired immune deficiency syndrome (AIDS) [13, 14]. HIV-induced chronic activation of the immune systemhas been associated with disease progression[14]. In fact, levels of immune activation predict CD4+ T-cell decline better than, and independently of, viral load [15].

Over time, it has become evident that this immune activation status encompasses many cells and tissues, including CD4+ and CD8+ T-cells, monocytes and macrophages as well as plasma soluble inflammatory molecules produced by these cells. However, despite intense research efforts it is still not clear what mechanisms drive this aberrant immune activation. Various non-mutually exclusive, and probably synergistic, mechanisms have been proposed. One is the translocation of microbial products across the gut following disruption of the gut barrier due to intense HIV replication in the Gut Associated Lymphoid Tissues (GALT) [16]. Involvement of cytokines like inteferons (IFN) and other pro-inflammatory mediators has also been proposed, through direct innate recognition of viral Ribonucleic Acid (RNA) and subsequent activation of the type I interferon response [17]. This is supported by the finding by Rempel et al that monocyte gene expression in individuals with HIV-1 viremia is predominantly due to IFN-alpha [18]. A third possible cause of activation in the T cell compartment is the classical direct T-Cell Receptor-mediated recognition of HIV antigens and signaling through CD4 and it co-receptor CCR5[19], and finally through bystander activation[20]- a phenomenon whereby heterologous T cells are activated by non-specific mechanisms, including cytokine-driven stimulation and cross-reactivity, during an antigen specific T cell response[21].

High levels of T cell activation is accompanied by elevated levels of T cell apoptotic death and hyper-responsiveness. This has negative influence on immune responses [22]. Another way in which chronic immune activation can exert suppressive effects on the immune system is by direct inhibition of the normal functions of B cells, NK cells, dendritic cells, and monocytes, leading to less efficient viral control[23, 24]. A role of immune activation in premature aging of

the immune system [25], and destruction/dysregulation of tissues crucial for T-cell homeostasis and function, such as the bone marrow, thymus, and lymph node [26] has also been documented.

Hence, immune activation proves to have deleterious sequelae. Not only does immune activation influence HIV progression, it may also have an important impact on susceptibility to infection. It is a major stimulus to HIV replication. Activation, resultant proliferation, and differentiation of naive and memory CD4+ T cells lead to increased CCR5 expression that makes these cells more susceptible to infection [27]. The coincident presence of high levels of CD4+ T-cell activation and HIV (which preferentially infects and kills activated CD4+ T cells) may help to sustain a vicious cycle, in which infection stimulates activation and activation stimulates infection [28]. In a study that assessed the role of pre-existing immune activation in HIV acquisition among women, it was observed that those with higher baseline immune activation levels were at a higher risk of acquiring HIV independent of whether they used an antiretroviral vaginal gel or placebo. This underscores the impact of immune activation in susceptibility to HIV [29].

However, immune activation is a two-edged sword. Immune activation stimulates the immune response against foreign pathogens and inflammatory stimuli. It is a normal response to pathogen invasion which activates both the innate and adaptive components of the immune system in a coordinated manner to fight pathogens [30]. However, when prolonged and uncontrolled, immune activation may have negative consequences [31]. It is therefore of high importance to target immune activation in a bid to reduce its negative impact on the immune system.

2.2 Sex work and the immune system

A majority of HIV infections start at mucosal sites. In sexual transmission, the genital mucosa is the primary site where infectious HIV particles are shed by the infected partner and come into contact with the epithelial barrier of the non-infected partner. The virus must then cross the epithelial barrier and mucus layer to reach target cells and establish infection. In the female genital tract, HIV can gain entry through the vaginal, ectocervical and endocervical mucosa [32]. The vagina and ectocervix are covered by a multilayered non-keratinized stratified squamous epithelium which when intact, provides a good degree of mechanical protection against HIV. On the other hand, the endocervix is lined by a single-layer columnar epithelium. Within and

beneath the epithelial linings are Dendritic cells, Langerhans cells, Macrophages and CD4+ T cells which are initially infected by the virus and play a significant role in disseminating the virus leading to systemic viremia [33]. It has been proposed that the region where the ectocervix transforms into the endocervix is enriched with CD4+ T cell populations and is thus a particularly susceptible site to HIV infection [32]. It is therefore plausible that, in women, susceptibility to HIV can be enhanced by factors that increase the number of HIV target cells in the female genital tract.

Studies in animals show that apart from spermatozoa, seminal plasma also delivers to the female an array of signaling molecules that interact with epithelial cells lining the FGT to trigger local cellular and molecular changes that resemble an inflammatory response [34]. Semen contains several antigens, including class Ia, class Ib, and class II Major Histocompatibility Complex (MHC), associated either with sperm, seminal leukocytes and desquamated genital tract epithelial cells, or present in the extracellular fraction [35]. Spermatozoa are, as alloantigen, thought to be immunogenic and pro-inflammatory in the FGT [6]. The immune stimulatory capacity of non-self-allogeneic antigens is exceptionally strong [7, 8].

Sex antigens have been shown to have an impact on the systemic compartment of the immune system. In an *in vitro* study conducted about a decade ago, seminal plasma and its constituent prostaglandins, prostaglandin E_2 (PGE_2) and 19-hydroxyprostaglandin E (19-hydroxy PGE) stimulated release of proinflammatory IL-8 from peripheral blood and cultured cervical explants [6]. More recently, Kingsley et al have identified specific peripheral CD4+ and CD8+ T cell responses, and even tolerance, to partners' cells in both homosexual and heterosexual monogamous couples practising unprotected intercourse [36]. This study also demonstrated greater *ex vivo* inhibition of HIV infectivity of peripheral blood mononuclear cells (PBMCs) in monogamous couples practising unprotected sex suggesting that regulatory T cells generated in response to sex antigens may inhibit CD4+ T cell activation [36].

A postcoital leukocytic response has been described in mice and other mammals. De et al have demonstrated that in mice, within hours of mating, macrophages, dendritic cells (DCs), and granulocytes are recruited into the endometrial stroma and lumen [37]. Very few studies have

comprehensively explored the immune response to seminal fluid in humans. Sharkey et al have recently examined ectocervical biopsies taken after unprotected vaginal coitus and reported changes in expression of proinflammatory cytokines and chemokines, a robust recruitment of macrophages, dendritic cells, and memory T cells accompanied by elevated expression of proinflammatory cytokine genes [38]. These responses did not occur without coitus or in condom-protected coitus.

Evidently, these immune responses observed during intercourse can lead to the recruitment and activation of T cells which serve as targets of infection by HIV. There is therefore need to explore the influence of coitus on the immune response in the human female reproductive tract and its possible impact to the systemic environment as well as its potential importance in transmission of sexually transmitted pathogens such as HIV.

2.3 Immune Quiescence in Highly Exposed Seronegative Individuals

As discussed above, sex work presents a risk of HIV infection, especially to the women. However, several studies have identified and described a minority of individuals across the globe that are persistently exposed to HIV, by sexual, parenteral or vertical routes, but remain HIV seronegative and do not exhibit any signs of immunodeficiency. These are referred to as HIV Exposed Seronegative (HESN) individuals. This reduced susceptibility has generated scientific interest as a model on which novel HIV prevention methods can be based. Both genetic and immunological factors have been identified as correlates of such natural protection to HIV [39]. However, none of these factors singly accounts for the reduced susceptibility. This section will explore some of the mechanisms that have been proposed to explain the HESN phenotype.

Based on epidemiological models, about 5-10% of the women in the Pumwani sex worker cohort have been identified as resistant to HIV after several years of follow-up [40]. Recent studies performed within this cohort have found lower levels of both systemic and mucosal immune activation in these individuals. Reviewed by Card et al [12], results from these studies have pointed to markedly reduced baseline immune activation levels in HESN, which has been proposed as protective from infection in this cohort, by limiting the pool of HIV target cells.

T cell activation, evaluated by expression of surface markers and cytokine production is one of the most studied mechanisms of protection in HESN. Card et al observed reduced CD69+CD4+ and CD8+ T cells in HESN compared with HIV-negative controls with similar exposures, indicating low levels of T cell activation [41]. Using microarrays, Songok et al profiled the expression and transcription of genes involved in metabolism and immune function in unstimulated CD4+ T cells. They found lower levels of genes encoding T Cell receptor (TCR) function, glycolysis pathways, proteasomes and natural killer cell cytotoxicity in HESN sex workers than in their HIV-negative counterparts [42], suggesting a quiescent state. Some in vitro studies have demonstrated that quiescent T cells can be infected by HIV but viral replication inside these cells is inefficient [43], so the virus would preferentially establish infection in activated T cells which express the host factors required for viral replication [44]. It is therefore reasonably thought that reduced T cell activation has a role in resistance to HIV infection.

HESN female sex workers have been found to have a unique pattern of mucosal and systemic chemokine/cytokine expression. Lajoie et al [9] have recently reported lower expression of monokine induced by interferon- γ (MIG), interferon- γ-induced protein 10 (IP-10), and interleukin-1α (IL-1α) in their genital mucosa. This study analyzed expression of proinflammatory proteins in cervical mononuclear cells (CMC) of the women in the Pumwani cohort and demonstrated reduced expression of these proteins. Lower expression of MIG, IP-10 and other proinflammatory cytokines would result in lower infiltration of activated T cells to the FGT [9]. Likewise, a similar study demonstrated reduced production of IL-17 and IL-22 by stimulated peripheral lymphocytes from HESN relative to low-risk HIV-negative controls, suggesting blunted Th17 responses in these individuals [45].

A subset of T cells referred to as T regulatory cells (T-regs), are known to suppress T cell activation and responses. T-regs play a central role in controlling inflammation caused by chronic pathogens [46]. T-regs are known to suppress immune activation through multiple mechanisms which include direct contact with activated cells and by production of anti-inflammatory cytokines. Reviewed by Dorian et al, some of the mechanisms proposed include production of inhibitory cytokines like IL-10 and Transforming Growth Factor beta (TGFβ),

secretion of granzymes which mediate cytolysis of activated cells, metabolic disruption of T cell proliferation and inhibition of antigen presenting cell function[47].T-regs have been found in elevated levels in HESN compared to high-risk HIV-negative controls correlating with lower peripheral T cell activation [41]. Therefore elevated T-regs in HESN could play a role in protection against HIV by limiting activation of CD4+ T cells and maintaining a low activation state.

Antiproteases are a class of innate proteins that have anti-inflammatory activity. One of these antiproteases, Elafin, has been identified as a correlate of protection in HESN [48]. The presence of Elafin, and indeed other proteases, in the FGT may counter inflammation and cellular activation. Antiproteases are usually induced in the presence of inflammatory cytokines and chemokines [12]. Consistent with this, Lajoie et al demonstrated a distinct correlation between cytokine expression and antiprotease levels in cervicovaginal lavages from HESN in the Pumwani cohort [9] leading to speculation that a synergistic effect between cytokines and antiproteases decreases the abundance of activated target cells for propagation of infection.

As discussed above, immune activation provides an impetus for HIV infection of and replication in T cells. HESN individuals have been shown to have various factors that regulate immune activation and limit the pool of activated target CD4+ T cells that are susceptible to infection. This model of reduced cellular activation has been termed 'Immune Quiescence'[12] and is proposed to reduce productive infection in HESN, permitting cytotoxic T lymphocytes (CD8+) to clear low grade infection.

2.4 Markers of T cell Activation and Memory Phenotypes

Evaluation of T cell activation has classically been done by assessing expression of cell surface markers. CD69 is a marker of early activation since it is up-regulated in the early stages of the cell cycle, signifying transcriptional activation and increased cellular susceptibility to HIV infection [49]. On the other hand, HLA-DR is a marker of chronic activation - it is up-regulated late in the cell cycle and is not informative for discriminating between quiescent cells that are resistant to infection and recently activated cells that are susceptible to HIV infection [41]. CD95 (Fas) is also expressed on activated T cells and mediates activation-induced cell death which is implicated in the control of immune cell homeostasis and termination of the immune response

[50]. CCR5 and CCR7 are chemokine receptors which play a role in the migration of antigen specific effector and memory T cells to inflamed tissues and secondary lymphoid tissues. CCR5 also plays an important role as the HIV co-receptor in CD4+ T cells for R5 tropic viruses [51]. Together with CD45RA - an isomer of CD45, the common leukocyte antigen [19]– CCR7 has been also used to define T cell memory subsets in several studies [52, 53].

3 PROBLEM STATEMENT

Immune activation may have negative consequences to the immune system. In the context of HIV, immune activation fuels disease progression and feeds infection and replication of the virus in activated T cells. Activated T cells are the preferential targets for HIV. It is therefore imperative that this phenomenon be exploited in a bid to reduce its negative impact on the immune system. Unprotected sex leads to exposure to strong immune stimulants like alloantigens of semen, bacterial products and viral/bacterial antigens which results in an inflammatory response in the FGT where semen is deposited. This inflammatory response can lead to the recruitment and activation of T cells, from proximal lymph nodes and peripheral blood, to the FGT mucosa and serve as ready targets of HIV infection. HESN female sex workers from the Pumwani Cohort have been shown to have unique immune characteristics systemically and in the genital tract with lower levels of immune activation that may limit the pool of activated T cells available for HIV infection. This low level of immune activation is referred to as Immune Quiescence. It is hypothesized that HESN sex workers are able to down regulate sex related antigen-induced immune activation by a yet unknown mechanism and this contributes to their phenotype of protection from HIV infection.

Only a few studies have explored the impact of semen exposure on the immune response in humans, and even fewer have looked at the impact of sex work. It is therefore important to comprehensively study how sex work influences the immune response in the FGT and its possible impact to the systemic compartment as well as its potential importance in the transmission of HIV.

4 RATIONALE

There currently lacks comprehensive research, as evidenced by limited information in the literature, on the effect of sex work on the immune system, and specifically on the activation of T cells, and how this would affect susceptibility to HIV. Activated T cells can be recruited from the systemic compartment to the genital mucosa and serve as potential HIV targets. Previous studies have shown that HESN women from the Pumwani Cohort have unique characteristics, both at the genital mucosa and systemically, that accounts for their resistance to HIV. This study investigated the variation in systemic immune activation during active sex work, upon taking a break from sex work in newly involved in sex work susceptible HIV-negative, HIV-positive and HESN female sex workers.

5 HYPOTHESIS

We hypothesized that sex work results in exposure to very strong immune stimulants, such as client-derived allo-antigens, immune modulators, and bacterial products which can drive systemic immune activation. We also hypothesized that HESN women are able to down regulate this sex work-driven immune activation more efficiently than HIV susceptible controls.

6 OBJECTIVES

6.1 Broad Objective

To characterize the impact of active sex work on the systemic immunity among female sex workers at the Pumwani Cohort, Nairobi.

6.2 Specific Objectives

1. To compare the levels of T cell activation among female sex workers during active sex, abstinence and upon return to sex work.
2. To compare the expression of T cell markers of activation among HIV-uninfected susceptible, HIV-infected and HESN FSWs.

7 MATERIALS AND METHODS

7.1 Study Site

This study was carried out at the Majengo Clinic in Nairobi, Kenya. The clinic is located within one of Nairobi's informal settlements. It is run under the collaborative University of Nairobi/Manitoba's Research program and provides free HIV care and treatment for sex workers. A sex worker cohort was set up and has been followed since 1984 when the collaborative program begun. Some of the services offered at the clinic include: HIV testing, counselling for prevention and STI/HIV, provision of free condoms, laboratory testing, and reproductive health services. To date, about 3,000 sex workers have been enrolled in the cohort.

Laboratory analysis of the samples was done at the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID), Functional Immunology Laboratory. This laboratory is situated at the College of Health Sciences and has a well-equipped flow cytometry section where samples from this study were processed.

7.2 Study Design

This was a prospective observational cohort study.

7.3 Study Cohort

The study was conducted among female sex workers (FSW) from the Pumwani Sex Worker Cohort. This cohort is well-characterized and has been adequately described in previous studies [22, 40, 54].

7.4 Definition of cases and controls

Cases were HESN (group active in sex work for at least 7 years) and controls were the 'New Negatives' (FSW with less than 3 years of sex work) and HIV infected groups.

Epidemiological studies in the Pumwani cohort have shown that FSW are likely to seroconvert within 3 years of initiating sex work but those who remain seronegative after 7 years of sex work are classified as HESN [40].

7.5 Inclusion and exclusion criteria for study participants

Participants were considered eligible for enrolment in this study if they fulfilled all the inclusion criteria and none of the exclusion criteria as defined below.

7.5.1 Inclusion Criteria

All participants signed the informed consent form and were:

1. Active in sex work
2. Enrolled in the Pumwani Cohort
3. Aged 18 years or older
4. Willing to take a month compensated and essential sex break
5. In general good health, and for HIV infected women CD4 counts must be over 350/mm^3.

7.5.2 Exclusion criteria

1. Women under the age of 18 years or more than 50 years.
2. Being positive for any sexually transmissible disease and bacterial vaginosis at week 1
3. Being in menopause
4. Pregnant at recruitment or while in the study.
5. Breast feeding
6. Pregnant in the last 12 months
7. Women no longer involved in sex work
8. For HIV positive women CD4<350 or not on anti-retroviral therapy

7.6 Sampling Procedure

The Pumwani Sex Worker Cohort has about 3,000 participants who can be stratified according to HIV status and duration of sex work. To detect a 10% difference in the frequency of each immune activation marker between the groups and assuming an alpha error of 5% and a beta error of 20% and an active cohort of 1000 participants the study required 10 to 40 individuals in each group. Therefore, this study randomly recruited 30 participants, 10 in each arm, upon fulfillment of the inclusion criteria.

7.7 Specimen Collection and Handling

This particular study was nested in the Longitudinal Assessment of Mucosal Immune Quiescence (LAMIQ) study. The study was approved by the University of Nairobi and University of Manitoba Ethics Boards **(KNH-ERC #P224/04/2012)**. The LAMIQ study is investigating the correlates of protection against HIV in the mucosal compartment and the effect of the menstrual cycle and sex work in such protection. Among the samples collected for the study are cervical mononuclear cells (CMC), Cervicovaginal Lavage (CVL) and a cervical biopsy from each

participant. During the two weeks given to allow the biopsy wound to heal, the women were asked to abstain from sex to ensure they are not being exposed to HIV or any other sexually transmitted infections. Abstinence is confirmed using a rapid test for detection of Prostate Specific Antigen. The comprehensive LAMIQ sampling schedule is outlined below:

	Menses	Follicular	Luteal	Menses	Follicular	Luteal	Menses	Follicular	Luteal
Sex Practice	Sex Work			Sex Break			Return to Sex Work		
Study Visit		**1**	2		**3**	4		**5**	6
Samples Taken		CMC	CMC		CMC	CMC		CMC	CMC
		CVL	CVL		CVL	CVL		CVL	CVL
		Blood	Blood		Blood	Blood		Blood	Blood
			Biopsy		Biopsy				

Samples were collected at all visits as shown above. However, for this particular study, we report results for samples collected at three (3) time points: **Visit 1** constituted the baseline sample prior to biopsy and during active sex work. **Visit 3** samples were taken during the period when the participants were on sex break. The women then went back to active sex work. Samples taken at **Visit 5** were in the context of active sex work. For the most part, samples were taken during the follicular phase of the menstrual cycle. Samples collected at the other visits were not included in this report since only not all participants had completed all follow ups.

Twenty millilitres of venous blood was collected at each visit by venipuncture performed by a qualified study nurse and immediately transferred into Sodium Heparin anticoagulant tubes. Samples were then transported to the UNITID labs where PBMCs were extracted for immunophenotyping.

7.8 Laboratory Assay

PBMC were extracted from whole blood using the Ficoll-Hypaque technique. The cells were then counted and 10^6 cells were resuspended in 50 µl of FACS Buffer (Phosphate buffered saline with 2% Fetal Bovine Serum) and transferred into a v-bottom 96 well plate. The cells were then washed with 100 µl of FACS Buffer, centrifuged at 1600 rpm for 10 minutes at 4°C and supernatant removed. A cocktail of 10 fluorochrome conjugated monoclonal antibodies from Beckton Dickinson (BD) Biosciences was then added in a total volume of 90 µl of FACS buffer. The antibodies were Anti-CD3-PECy5: 10 µl, Anti-CD4-FITC: 5 µl, Anti-CD8-V500: 1 µl, Anti-CD45RA-Alexa700: 5 µl, Anti-HLA-DR-APC-H7: 2 µl, Anti-CD69-PECy7: 1 µl, Anti-CCR7- PE-CF594: 2.5 µl, Anti-CCR5-V450: 2.5 µl, Anti-CD161-APC: 10 µl and Anti-CD95-PE: 2 µl. The cells were then incubated for 30 minutes at 4°C then washed with 100 µl of FACS Buffer by centrifuging at 1600 rpm for 10 min at 4°C. The supernatant was then removed. The stained cells were then resuspended in 300µl of Fix solution (1% Paraformaldehyde: 1 part of BD Fix solution and 3 FACS buffer) before acquisition on the cytometer.

Expression of the markers to which the antibodies are directed were then analysed by flow cytometry on the LSR II cytometer (BD Biosciences) using the BD FACSDiva software version 6.1.3 (BD Biosciences).

7.9 Data Management and Analysis

Flow cytometry data were analyzed using FlowJo Software version 10.0.7 (Tree Star, Inc.) to identify T cell phenotype and frequency of expression of activation markers, given as percentages (Gating strategy shown on Figure 1). Gaussian distribution was tested by the D'agostino and Pearson Omnibus test. The Kruskal-Wallis test was used for variables that were not normally distributed. One Way ANOVA was used for normally distributed variables. Comparison of expression levels between time points was done using the Tukey's multiple comparison test and Dunn's multiple comparison test.

Comparison of expression levels between the three groups and time point was done using 2-way ANOVA, and if statistically significant, the Dunn's multiple comparison test was used. The Holm-Sidak's test was also used as a parametric post-test.

Information on sociodemographic, sexual behavior and clinical factors that can influence the immune activation was obtained through a questionnaire administered at every study visit. The Chi-square test was used to assess significance of categorical variables while ANOVA was used for continuous variables. For the HIV-positive group CD4 counts at different visits were tested using Friedman's test.

Statistical significance was accepted if the p value is ≤ 0.05. Statistical analyses were performed using GraphPad Prism software version 6.0.

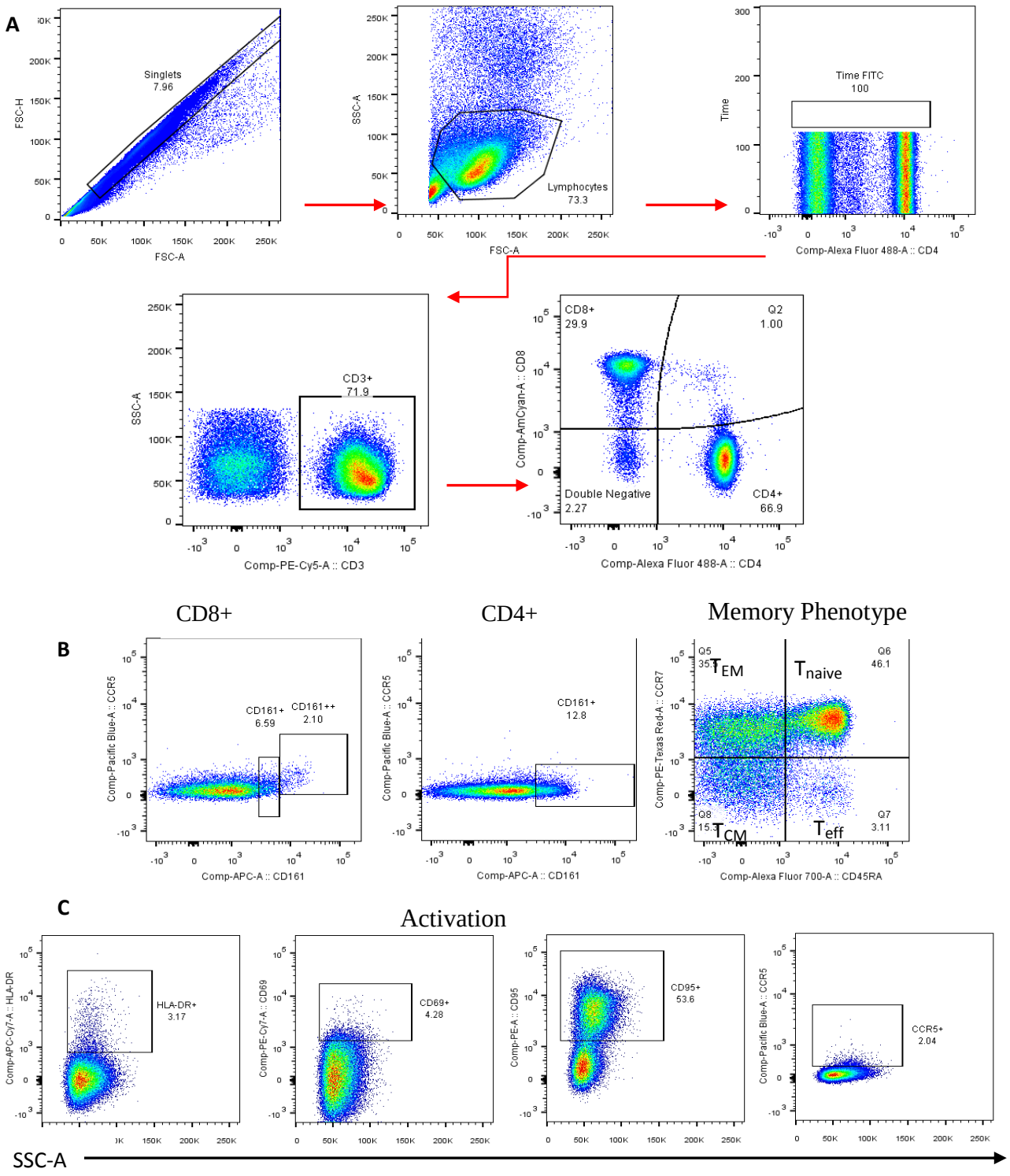
7.9.1 Gating Strategy on FlowJo Software

Singlets were gated on based on Forward Scatter Area and Height after which lymphocytes were identified based on their Forward Scatter and Side Scatter properties. A time gate was then done on the lymphocytes to exclude any flaws that may have occurred in the flow of cells during sample acquisition on the cytometer. CD3+ T cells were then gated on based on Side Scatter and PE-Cy5 fluorescence. From the CD3+ T cells, CD8+ and CD4+ T cells were identified through fluorescence of AmCyan and Alexafluor 488 respectively (Figure 1A). Memory phenotypes for both CD8+ and CD4+ T cells were gated on based on CCR7 and CD45RA expression (Figure 1B). CD161+ populations were gated on based on APC fluorescence on both T cell subsets (Figure 1B). Cells expressing the activation markers (HLA-DR, CD69, CD95 and CCR5) were gated on based on Side Scatter and fluorescence of the corresponding fluorochrome (Figure 1C).

8 ETHICAL CONSIDERATIONS

Witten informed consent was obtained from each participant. Ethical approval for this study was obtained through the KNH/UoN Ethical Review Committee **#P30/1/2014.**

Figure 1. Gating Strategy on Singlets, Lymphocytes, CD3+, CD4+ and CD8+ Populations



A. Gating strategy on Singlets (first at the left), Lymphocytes, Time gate that excludes flaws in cell flow, CD3+, CD4+ and CD8 T cells. **B.** Gating for CD161 expression on CD8+ and CD4+ T cells, and T cell memory phenotypes based on CCR7 and CD45RA expression. T_{EM}: effector memory, Tnaive: naïve T cells, T_{CM}: Central memory, Teff: terminally differentiated T cells. **C.** Gating on activation markers: HLA-DR, CD69, CD95, CCR5.

9 RESULTS

9.1 Socio-demographics and Clinical Characteristics

A total of 30 female sex workers were enrolled into the study. They were divided into three groups; HESN (n=10), HIV positive (n=10) and New Negatives (n=10) according to their HIV status and their reported duration of sex work. HESN are seronegative for HIV and active in sex work for more than 7 years. New Negatives were testing negative for HIV and reported 3 years or less of active sex work. Blood samples were collected from participants at three time points: V1, V3 and V5. V1 constituted the period at which the women were in active sex work (SW). Samples at V3 were collected when the women were 2 weeks into the sex break (SB) and V5 samples were collected 2 weeks after the women had resumed active sex work.

The participants were matched for age; the median age being 37.5 for HESN, 38.0 for HIV-positive and 34.5 for the New Negatives. Because of the criterion used to define the length of follow up for HESN and New Negatives, there was significant difference in the median duration of sex work for the three groups ($p = 0.0003$) with a median duration of 11.5 years of sex work for HESN, 3 years for New Negatives and 7 years for HIV-positive. CD4 counts were done for all participants at recruitment, and as expected there was a significant difference ($p = 0.0059$), with the lowest median counts of 610 cells/µl observed in women with HIV infection. The CD4 counts for the HIV-positive women did not change significantly between visits throughout the study ($p = 0.2781$).

Samples were mainly collected at the follicular phase of the menstrual cycle to control for the possible effect of reproductive hormones on immune activation. For a 28 day cycle, the follicular phase is 5–14 days after menses. There was no difference in the number of days since the women had their menses during the sex break. However, a trend towards difference was observed at V1 ($p = 0.0525$) with the samples collected at a median of 11 days post-menses for HIV-positive, 8.5 days for HESN and 8 days for New Negatives. At V5, the samples were collected at a median days post-menses of 19 days for HESN while HIV-positives and New Negatives were 7.5 and 7.25 days ($p = 0.0889$).

Across the three groups, the women reported similar frequencies of unprotected sex in the week prior to sampling. However, when compared across time points, a change in reported unprotected

sex was observed for all groups during active sex work and the sex break ($p = 0.0392$ for HESN, $p = 0.0126$ for HIV, $p = 0.0891$ for New Negatives). The women reported a lower frequency of unprotected sex at V3 than V1. It was also observed that all the groups had similar usage of condoms with clients and similar numbers of regular partners. The HIV-positive women had a median of 12 clients in the 7 days prior to sampling at V1, significantly more than HESN and New Negatives who had a median of 5 and 2 respectively.

During the sex break, abstinence was confirmed using a rapid test for detection of Prostate Specific Antigen (PSA) which gives an indication of exposure to semen in the female genital tract. PSA was detected in 2 HESN women despite the fact that they reported not having sex during the sex break.

Table 1 summarizes the sociodemographic and clinical characteristics of the participants that were of interest in this study.

Table 1. Sociodemographic and Clinical Characteristics in HESN, HIV-Positive and New Negative Female Sex Workers

		HESN n=10		New Negative n=10		HIV-Positive n=10		p-value
Age		37.5 (34.5 - 44.25)		34.5 (32 - 39.5)		38 (34 - 45.25)		0.4708
Duration of Sex Work (years)		11.5 (9.5 - 13.75)		3 (2 - 3)		7 (3 - 12)		**0.0003**
CD4 Count at Recruitment (cells/µl)		1011 (800 - 1111)		1033 (921.3 - 1193)		610 (545 - 745)		**0.0059**
Days Post-menses	**SW:**	8.5 (7 - 13)		8 (6 – 9)		11 (8 - 14.5)		0.0525
	SB:	10 (7.25 - 18.25)		9 (5.75 - 25.25)		12.5 (8 - 19.25)		0.834
	RSW:	19 (9.75 - 23.25)		7.25 (10 - 23)		7.5 (4.75 - 15)		0.0889
No. of clients in the last 7 days								
	SW:	5 (2.75 – 18)		2 (0.75 – 7.25)		12 (5.75 – 20)		**0.0338**
	SB:	0		0		0		NA
	RSW:	4 (0.75 – 8)		2 (0.5 – 8.5)		2.5 (0.75 – 10)		0.8077
Report always using a condom with clients in the last 7 days		**YES**	**NO**	**YES**	**NO**	**YES**	**NO**	
	SW:	7	3	6	2	8	2	0.8752
	SB:	NA	NA	NA	NA	NA	NA	
	RSW:	8	2	7	2	9	0	0.328
Report having a regular sex partner		**YES**	**NO**	**YES**	**NO**	**YES**	**NO**	
	SW:	5	5	7	3	5	5	0.581
	SB:	5	5	5	5	5	5	1.000
	RSW:	5	5	4	6	4	6	0.8731
Report unprotected sex with a client/partner in the last 2 weeks		**YES**	**NO**	**YES**	**NO**	**YES**	**NO**	
	SW:	5	5	4	6	5	5	0.8747
	SB:	0	10	0	10	0	10	NA
	RSW:	3	7	2	6	1	9	0.5292
PSA Test Results during the sex break period		**POS**	**NEG**	**POS**	**NEG**	**POS**	**NEG**	
		2	8	0	10	0	9	0.1299

Data are presented as Median (Interquartile Range); duration of sex work (years) and number of clients. Abbreviations: SW, Sex Work; SB, Sex Break; RSW, Return to Sex Work. One-way ANOVA was used to test for significance of days post menses at the three time points. Chi-Square test was used for categorical variables.

9.2 T-Cell Activation and Memory Phenotypes

To assess the impact of sex work on peripheral immune activation, we measured the level of expression of activation markers on T cell subsets. T cell activation was defined by the single expression of CCR5, CD95, CD69 or HLA-DR on CD4+ and CD8+ T cells. We also characterized T cell memory phenotypes to assess whether sex work drives their generation and the impact of interruption of sex work on their frequencies. Memory phenotypes were defined by expression of CCR7 and CD45RA on T cells (Section 9.2.3)

9.2.1 CD4+ and CD8+T cells expression of activation markers

We quantified the frequency and intensity (median fluorescence intensify (MFI) of expression of CCR5, CD95, CD69 and HLA-DR on both CD4+ and CD8+ T cells for each group at the three time points. No difference in expression of these markers could be observed between active sex work, sex break and return to sex work within the groups in the CD4+ T cell population. The results are summarized in Table 2. These results suggest that sex work does not affect the level of peripheral CD4+ T cell activation.

Table 2. CD4+ T-Cell Activation Markers among HESN, New Negatives and HIV-positives

		HESN	New Negative	HIV-positive
%CCR5	SW	2.99 (1.96 - 2.97	2.44 (1.075 - 3.865)	2.99 (2.358 - 3.815)
	SB	2.92 (1.955 - 5.845)	2.255 (1.84 - 3.375)	1.405 (1.028 - 2.655)
	RSW	3.26 (1.015 - 4.9)	3.525 (1.013 - 3.758)	1.65 (1.1 - 3.028)
	***p* value**	0.8539	0.7103	0.1872
%CD95	SW	62.3 (40.9 -71.1)	55.55 (49.6 - 63.75)	53.75 (43.3 - 63.05)
	SB	52.45 (42.68 -70.23)	50.65 (48.4 - 60.13)	48.15 (39.8 - 63.05)
	RSW	63.15 (44.9 - 71.83)	55.35 (45.05 - 60.93)	53.4 (41.98 - 59.45)
	***p* value**	0.4897	0.2474	0.9185
%CD69	SW	1.54 (0.65 - 2.61)	1.22 (0.935 - 3.018)	3.16 (1.38 - 3.908)
	SB	0.905 (0.72 - 3.16)	2.935 (1.77 - 4.51)	2.745 ((1.35 - 4.97)
	RSW	1.36 (0.935 - 3.025)	2.48 (1.108 - 2.898)	3.79 (2.29 - 3.79)
	***p* value**	0.9712	0.6543	0.6013
%HLA-DR	SW	1.79 (1.5 - 3.035)	2.975 (1.933 - 3.558)	2.375 (1.048 - 5.993)
	SB	1.575 (1.208 - 2.923)	2.84 (1.565 - 4.14)	2.045 (1.403 - 6.945)
	RSW	1.645 (1.323 - 2.625)	2.175 (1.18 - 3.878)	2.765 (1.958 - 6.733)
	***p* value**	0.2781	0.6013	0.7103

In the CD8+ subset, a significant difference in expression of CCR5 was seen over time only in the HIV-positive group ($p = 0.0095$). CCR5 is a chemokine receptor that has a role in the migration of T cells to extra lymphoid sites of inflammation [19]. We observed a significant decrease in the levels of CCR5 on CD8+ T cells in the HIV-positive group after two weeks of interruption of sexual activity (Figure 2). Upon resumption of sex work, the median frequency of expression increased but this was not significant.

No difference in the expression of other markers was observed during sex work, sex break or upon return to sex work for all groups (Table 3).

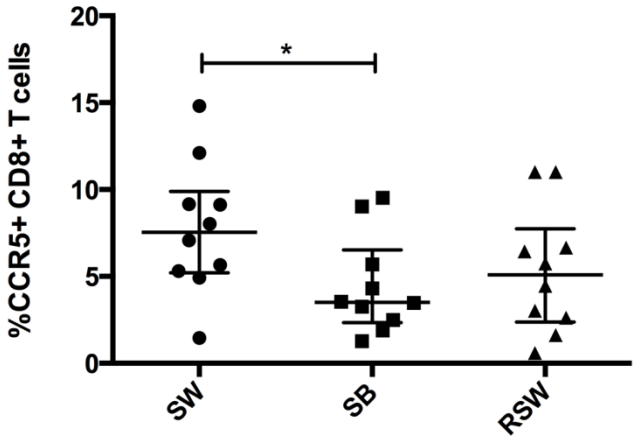


Figure 2. CD8+CCR5+ Expression in HIV-Positive Sex Workers

Expression of CCR5 on CD8+ T cells was compared during Sex Work (SW), Sex Break (SB), and upon Return to Sex Work (RSW) in HIV-positive FSWs

		HESN	New Negative	HIV-positive
%CCR5	SW	4.09 (2.295 -7.015)	5.245 (3.013 - 9.21)	7.545 (5.213 - 9.895)
	SB	5.44 (2.62 - 6.09)	4.975 (2.158 - 11.2)	3.51 (2.34 - 6.523)
	RSW	4.34 (1.36 - 10.09)	2.9 (2.668 - 6.653)	5.1 (2.378 - 7.745)
	p value	0.8135	0.3159	**0.0095**
%CD95	SW	50.2 (35.85 - 71.05)	52.4 (45.35 - 61.28)	74.6 (66.63 - 81.48)
	SB	61.1 (45.95 - 70.9)	48.1 (43.35 - 58)	80.45 (72.33 - 85.2)
	RSW	58.2 (43.9 - 73.15)	53.5 (49.5 - 61.53)	78.75 (72.02 -81.63)
	p value	0.1527	0.1595	0.3256
%CD69	SW	10.4 (2.815 - 21.5)	6.455 (2.268 - 11.8)	5.975 (2.695 - 35.45)
	SB	9.055 (2.18 - 19.13)	4.995 (2.065 - 8.103)	9.395 (1.61 - 14.35)
	RSW	9.86 (2.173 - 19.73)	4.32 (2.27 - 14.28)	7.815 (4.063 - 19.25)
	p value	0.1066	0.9674	0.6013
%HLA-DR	SW	3.58 (1.195 - 5.495)	4.45 (3.168 - 6.168)	2.765 (1.733 - 5.89)
	SB	2.88 (2.123 - 4.328)	5.135 (3.173 - 7.29)	3.24 (1.878 - 14.9)
	RSW	3.84 (2.048 - 6.788)	4.305 (2.83 - 6.778)	4.105 (3.358 - 12.13)
	p value	0.3122	0.9737	0.6013

Table 3. Expression of CD8+ T cell Activation Markers among HESN, New Negatives and HIV-Positives

Having observed the variation in expression of activation markers between time points within each group, we assessed the difference in the pattern of response to the sex break between groups. For this, two-way ANOVA was done and we found no significant interaction between the sex break and the groups in terms of expression of activation markers on both CD4+ and CD8+ subsets. This implies that HIV-positive, New Negative and HESN do not differ significantly in their response to the interruption of sex work in terms of activation marker expression. However, independent of groups, we observed a trend towards an effect of interruption of sex work on expression of CD95 on CD8+ T cells (ANOVA p value 0.0691, Figure 6C). Overall this suggests that interruption of sex work may have an effect on the frequencies of CD95 expressing CD8+ T cells

9.2.2 Expression of CD161 – a phenotypic marker for Th17 cells

Expression of CD161, a phenotypic marker, on both CD4+ and CD8+ T cells was analyzed among the three groups at the different time points. Consistent with previous studies [55, 56], a single CD4+ CD161+population expressing intermediate levels of CD161 and two CD8+CD161+ T cells – one with intermediate (CD161+) and another with high CD161 (CD161++) expression were identified as earlier shown in Figure 1. CD161 expressing CD4+ T cells are Th17 cells which secrete Interleukin-17 and have been reported to be the preferential targets for HIV infection and replication within the gut mucosa[57, 58]. CD8+CD161+ also produce IL-17 while CD8+CD161++ T cells are Mucosa Associated Invariant T cells (MAIT) (also IL-7 secreting cells) which are highly inflammatory and have a role in cytotoxicity against cells infected with bacterial and fungal pathogens[59] This population is depleted in HIV-positive individuals.

Having identified these CD161 expressing T cells, their proportions were analyzed in each group for the three time points in order to asses if interruption in sex work could lead to a variation in the potential HIV targets cells at periphery. A trend towards an increased CD161 expression was observed in the CD4+ subset between sex break and upon return to sex work ($p = 0.0548$) in the HIV-positive (Figure 3). There was no change in frequencies of CD4+CD161+ cells in the HESN and New Negatives between sex work, sex break and upon return to sex work (Table 4).

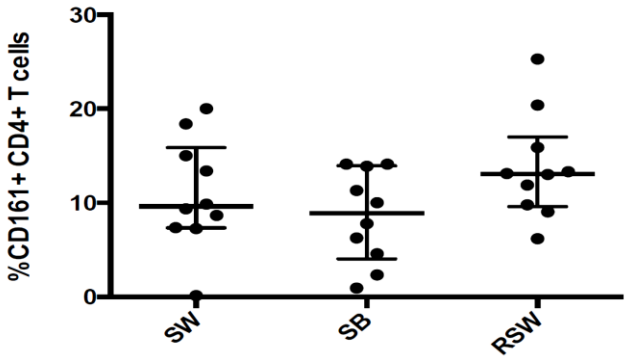


Figure 3. CD161 Expression on CD4 T cells in HIV-positive Sex Workers

Expression of CD161 on CD4+ T cells was compared during Sex Work (SW), Sex Break (SB), and upon Return to Sex Work (RSW) in HIV-positive FSWs

		HESN	New Negative	HIV-positive
%CD161	SW	11.9 (6.465 - 15.8)	11.55 (10.15 - 14)	9.62 (7.343 - 15.85)
	SB	9.62 (5.705 -25.03)	13.2 (10.68 - 18.05)	8.895 (4.033 - 13.95)
	RSW	11.85 (7.595 - 16.4)	12.45 (10.21 - 18.55	13.05 (9.603 - 17.03)
	***p* value**	0.6842	0.4372	**0.0548**

Table 4. CD161 Expression on CD4+ T Cells

In the CD8+ population, no difference was observed in both CD161+ and CD161++ subsets among the three groups at each time point (Table 5).

		HESN	New Negative	HIV-positive
%CD161	SW	3.86 (2.555 - 9.35)	4.945 (3.028 - 6.335)	2.69 (2.17 - 6.09)
	SB	4.17 (3.265 - 7.318)	4.815 (3.118 - 6.558)	3.085 (1.448 - 5.235)
	RSW	4.85 (3.128 - 7.04)	4.99 (3.325 - 8.285)	3.82 (2.665 - 4.833)
	p value	0.8074	0.3832	0.3675
%CD161++	SW	2.93 (1.73 - 4.42)	3.11 (2.19 - 5.478)	1.415 (0.2725 - 3.033)
	SB	1.9 (1.045 - 4.305)	4.72 (1.898 - 6.675)	0.92 (0.1863 - 2.74)
	RSW	3.51 (1.64 - 3.51)	2.89 (1.678 - 9.845)	1.01 (0.535 - 2.663)
	***p* value**	0.6539	0.7103	0.4362

Table 5. Expression of CD161 on CD8+ T cells

Overall, when assessing the impact of an interruption in sex work on the levels of activation markers expression on T cells, we observed that in HESN, a break from sex work did not affect the activation levels of both CD4+ and CD8+ T cells nor did it have an impact on the frequency of Th17 cells in blood. In the HIV-positive, we observed that interruption of sex work caused a decrease in CCR5 expression on CD8+ T cells and that two weeks after resumption of sex work, these women tend to have increased levels of Th17 cells.

9.2.3 Memory Phenotypes

Unprotected sex leads to exposure of the female genital tract to sex antigens from the male partner which can elicit an inflammatory response in the FGT mucosa. This exposure to sex antigens can lead to the expansion and differentiation of antigen specific T cells to confer immediate protection and also mount recall/secondary responses against these antigens on subsequent exposure [38]. We therefore hypothesized that sex work drives generation of memory T cells and that an interruption in sex work would lead to a change in the frequencies of memory T cells circulating in blood.

Memory T cells were described based on expression of CCR7 and CD45RA. Naïve T cells are CCR7+CD45RA+ and are mature T cells that have not encountered their cognate antigen [19]. Central memory T cells (T_{CM}) are defined as CCR7+CD45RA-. These are long lived memory cells that home to lymphoid organs and have little or no effector function but upon antigenic stimulation, differentiate into effector cells [53]. Effector memory T cells (T_{EM}) are defined as CCR7-CD45RA- and are characterized by rapid effector function in response to antigens, usually the first targets of HIV infection and depletion [53]. Terminally differentiated T cells (T_{EFF}) are described as CCR7-CD45RA+. T_{EFF} are short lived cells that are generated in response to continued antigenic stimulation of T cells [60].

To investigate the frequencies of these phenotypic subpopulations of memory T cells, the expression of CCR7 and CD45RA in CD4+ and CD8+ T cells was analyzed in each group across the three time points (Table 6). A significant difference in the frequency of terminally differentiated CD4+ T cell subset (T_{EFF}) was observed in the HIV-positive group (ANOVA, $p = 0.0355$), with an increase of these cells during the sex break and reduction upon resumption of sex work. Figure 4 illustrates this observation.

		HESN	New Negative	HIV-positive
T_{CM}	SW	39.8 (34.2 - 44.85)	40 (33.65 - 44.9)	41.85 (36.5 - 46.73)
	SB	43.6 (35.15 - 52.23)	39.8 (34.13 - 46.63)	40.15 (30.18 - 49.85)
	RSW	46.25 (37.9 - 53.03)	44.9 (37.4 - 5.28)	39.9 (31.33 - 45.43)
	***p* value**	0.4591	0.3159	0.7103
Naïve	SW	29.6 (20.3 - 50.05)	36.8 (27.2 - 41.58)	34.65 (30.93 - 40.7)
	SB	39.7 (23.6 - 49.3)	32.65 (21.68 - 44.75)	32.4 (29.15 - 46.35)
	RSW	33.3 (22.2 - 47.1)	32.9 (21.23 - 40.03)	36.85 (31.85 - 47.38)
	***p* value**	0.1301	0.3475	0.1873
T_{EFF}	SW	1.69 (0.945 - 2.33)	1.04 (0.71 - 2.55)	1.17 (0.7675 - 2.415)
	SB	1.655 (1.363 - 1.965)	2.185 (1.178 - 5.35)	2.095 (1.32 - 3.413)
	RSW	1.005 (0.2225 - 4.085)	1.33 (0.7925 - 2.963)	0.84 (0.56 - 3.39)
	***p* value**	0.6854	0.2223	0.0355
T_{EM}	SW	24.7 (11.8 - 34.7)	20.05 (13.33 - 25.25)	20.35 (13.5 - 27.88)
	SB	15.75 (10.75 - 21.05)	19.2 (16.58 - 26.45)	17.05 (12.88 - 28.18)
	RSW	17.5 (14.95 - 25.53)	24.85 (18.53 - 27.48)	15.9 (13.13 - 20.05)
	***p* value**	0.2385	0.3675	0.7632

Table 6. Frequencies of CD4+ T cell Memory Phenotypes

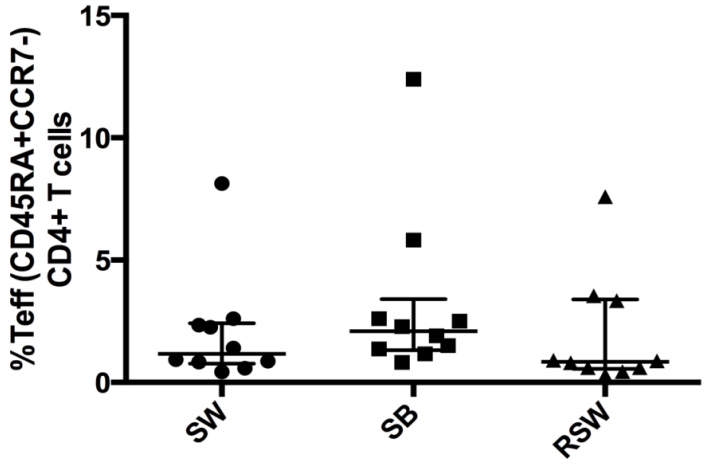


Figure 4. Frequency of CD4+ T_{EFF} cells in HIV-positive Participants

Frequency of terminally differentiated CD4+ T cells was compared during Sex Work (SW), Sex Break (SB), and upon Return to Sex Work (RSW) in HIV-positive FSWs

In summary the frequency of CD4+ memory T cells did not significantly change within the three groups across all time points (Table 6), except for the T_{EFF} which increased in HIV-positive women during the sex break and then showed a reduction two weeks resuming sex work (Figure 4).

In the CD8+ population, no difference in the frequencies of memory T cells in all groups across the three time points was observed. (Table 7)

		HESN	New Negative	HIV-positive
TCM	SW	10.7 (5.435 - 34.95)	8.685 (5.863 - 10.62)	7.26 (5.53 - 9.223)
	SB	11.95 (5.718 - 29.00)	9.235 (5.223 - 11.93)	6.16 (1.673 - 7.833)
	RSW	12 (7.833 - 33.7)	8.005 (5.65 - 14.35)	8.27 (3.858 - 10.83)
	p value	0.3322	0.7103	0.1352
Naïve	SW	44.9 (27.55 - 54.15)	47.45 (33.3 - 53.05)	21.9 (16.2 - 29.95)
	SB	49.25 (27.4 - 60.85)	43.1 (31.88 - 50.1)	18 (14.24 - 27.45)
	RSW	38.15 (27.28 - 45.83)	41.95 (27.9 - 46.13)	22.95 (15.1 - 30.73)
	p value	0.2861	**0.0924**	0.6043
TEFF	SW	14.6 (1.36 - 30.9)	19.15 (17.73 - 27.15)	28.95 (18 - 34.1)
	SB	14.75 (1.72 - 31)	20.3 (15.83 - 28.63)	30.5 (26.93 - 37.25)
	RSW	22.95 (1.65 - 35.78)	25.55 (15 - 31.7)	28.5 (18.1 - 37.48)
	p value	0.7801	0.1352	0.1869
EM	SW	20.8 (9.305 - 30.35)	21.15 (17.78 - 27.18)	41.5 (29.73 - 45.6)
	SB	15.75 (10.75 - 21.05)	26.3 (20.45 - 30.88)	43.65 (23.3 - 46.48)
	RSW	16.55 (13.03 - 21.63)	21.7 (17.55 - 34.43)	38.25 (32.03 - 50.8)
	p value	0.8729	0.1352	0.7103

Table 7. Frequencies of CD8+ T cell Memory Phenotypes

However, a p value of 0.0924 for the New Negative group suggests a trend towards a decrease in the frequencies of Naïve T cells in this group both during the sex break and upon resumption of sex work as shown in Figure 5 below. In addition, we observed that independent of groups, interruption of sex work has an effect on the frequencies of CD8+ naïve T cells (ANOVA p value, 0.048. Figure 6B).

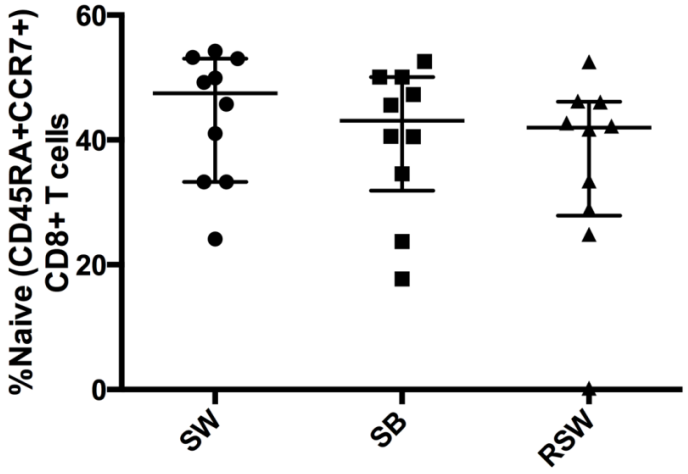


Figure 5. Frequency of CD8+ Naive T cells in New Negatives

Frequency of Naïve CD8+ T cells was compared during Sex Work (SW), Sex Break (SB), and upon Return to Sex Work (RSW) in New Negative FSW

9.3 The Impact of HIV on memory T cells and activation markers

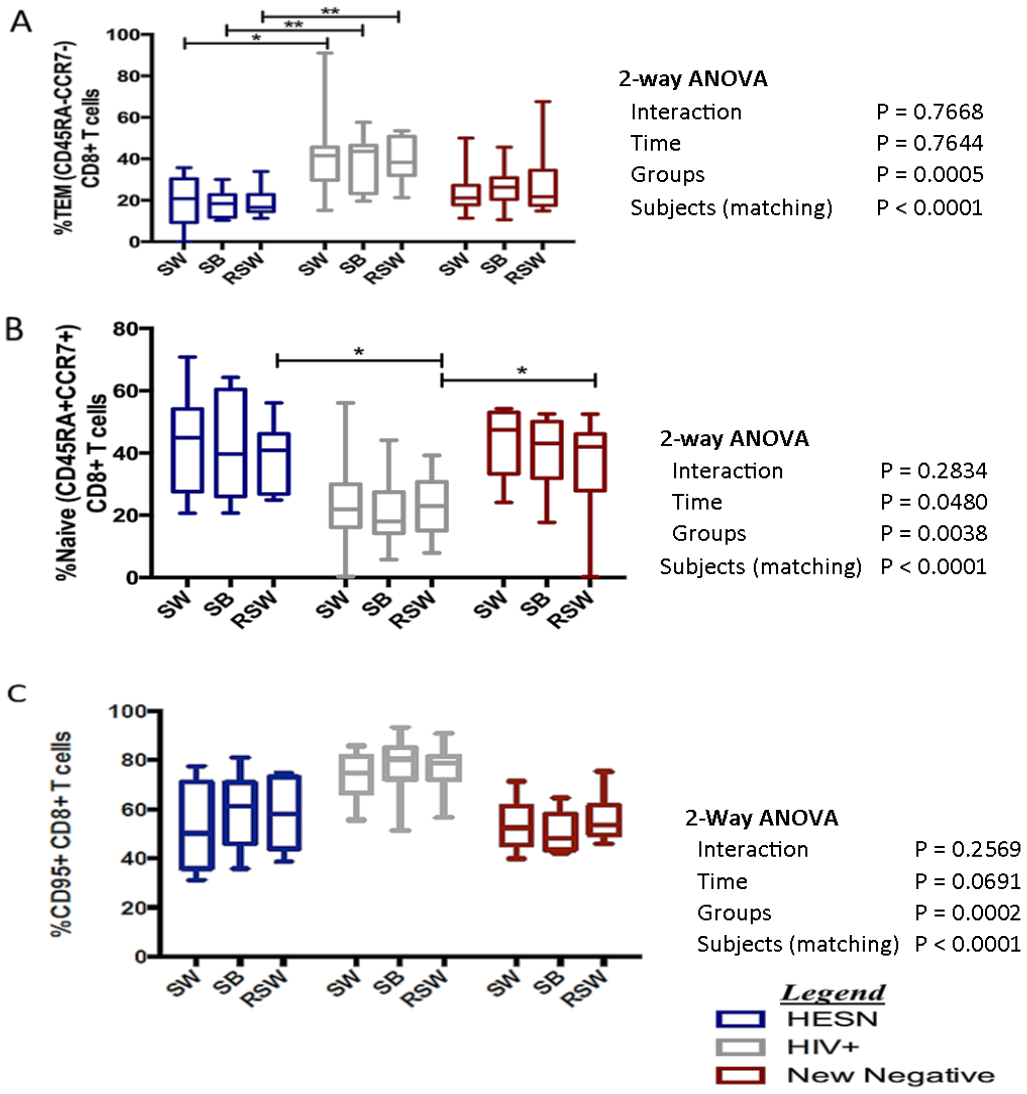
Some interesting observations, consistent with previous studies [61, 62], were also made on CD8+ memory T cell frequencies and activation markers when comparing the three groups. Two-way ANOVA was done to compare the interaction between the sex break and the three groups in their expression levels of memory phenotypes and activation markers. No difference in the pattern of expression over time points was observed, however, we observed significant differences between groups accounting for the infection with HIV.

We observed that HIV-positive female sex workers had higher frequencies of CD8+ effector memory T cells (T_{EM}) ($p = 0.0005$) as Figure 6A illustrates, along with lower frequencies of naïve CD8+ T cells ($p = 0.0038$) than HESN and New Negatives (figure 6B). The HIV-positive group also expressed higher levels of CD95 on CD8+ T cells ($p = 0.0002$) than did HESN and New Negatives as illustrated in Figure 6C.

Other studies have reported alterations in T cell memory phenotypes during HIV infection. Frequencies of naïve CD8+ T cells have been reported to be lower in HIV infected patients than in healthy controls [61]and also lower in chronically infected persons than in primary infections

[63]. On the other hand, expansion of CD8+ effector memory T cell numbers have been reported in HIV-positive individuals [64, 65]. CD95 (Fas) is a receptor that mediates activation induced cell death (AICD) of lymphocytes to control immune cell homeostasis[50]. Consistent with what we observed in this study (Figure 6C), CD95 expression on CD8+ T cells has been shown to be up-regulated in HIV infection [62, 66].

Figure 6. Frequencies of Effector Memory, Naive and CD95+ CD8+ T cells between groups



10 DISCUSSION

The mucosal milieu of the FGT plays an important role in the susceptibility to HIV infection during unprotected sex with a HIV infected partner. Unprotected sex leads to the deposition of semen and its components in the FGT. Semen contains several antigens including bacterial/viral antigens, allo-antigenic spermatozoa, desquamated epithelial cells, seminal leukocytes and MHC molecules associated with these cells [35]. Cellular and molecular changes that resemble an inflammatory response in the FGT following exposure to seminal fluid have been described [34]. A proinflammatory response illustrated by secretion of IL-8 and IL-6 by epithelial cells has also been described [6, 67]. Further, a post-coital leukocytic response has been described in mice[37] and recently in humans[38] showing that macrophages, dendritic cells and memory T cells are recruited to the stromal and luminal layers of the cervix following contact between seminal fluid and cells of the FGT. Sexual activity has therefore been associated with mucosal immune activation and this can affect acquisition of HIV at the mucosa since HIV preferentially infects and replicates in activated T cells [38].

In this study, we hypothesized that sex work results in exposure to immune stimulants which can drive systemic activation of T cells and that HESN FSW are able to down regulate such sex-driven activation better than their New Negative and HIV-positive counterparts. We aimed to compare the levels of systemic T cell activation among HESN, HIV-positive and HIV-uninfected susceptible FSW during active sex work, two weeks of abstinence and upon resumption of sex work. We looked at the expression of activation markers and frequencies of memory T cell phenotypes in each group independently during the three study time points and also compared the expression of T cell activation markers during the time point of interruption of sex work across groups in order to asses if there is a difference in their profile of response.

CCR5 is a chemokine receptor which plays a critical role in the migration of antigen specific effector and memory CD8+ T cells to inflamed tissues and secondary lymphoid tissues [68]. We observed that following interruption of sex work, fewer CD8+ T cells expressed CCR5 in HIV-positive FSW whereas frequencies of effector and memory cells did not change in this subset. This suggests that in the HIV-positive women, taking a break from sex work reduces sex-induced inflammation at the FGT and this is reflected in the systemic compartment.

Notably, even in the context of HIV-induced immune activation, an interruption of commercial sexual activities reduces peripheral expression of CCR5 on CD8+ T cells. This underlines the strong impact of sex-related immune stimulation. We further observed that compared to HESN and New Negatives, HIV-positive sex workers reported a higher number of clients in the week before taking a break from sex. Despite the fact that reported unprotected sex in this period was similar in all groups, the HIV-positive women could still have had higher exposure to multiple seminal fluids while in active sex work. This may explain why an interruption of sex work had a greater impact in CCR5 expression by T cells in this group when compared to the other groups.

Nevertheless, we also have to take into consideration the impact of HIV infection to explain these results. Dysregulation of the immune system is a major consequence of HIV infection [13, 25]. HIV impairs the innate and adaptive components of the immune system [23, 25] and also causes dysregulation of tissues necessary for T cell homeostasis [26]. Since we observe no difference in CCR5 expression by CD8+ T cells in HESN and New Negatives, it may also be possible that interruption of sex work has a greater effect on the HIV-positive women because of pre-existing immune dysregulation.

CD4+ T cells expressing CD161 are Th17 cells – a proinflammatory T cell subset which plays a role in mucosal antimicrobial immune responses [69]. These cells have been shown to be preferentially infected by HIV and depleted from the gut [57] and cervix [70] of HIV infected individuals. In the context of sex work, we observed that HIV-positive sex workers display a trend toward an increased number of Th17 cells in the blood following resumption of sex work. Interestingly, all the HIV-positive reported using condoms after returning to sex work and only one reports unprotected sex with a regular partner. Self-reported condom use may not match actual condom use, and as Zimmerman et al review, studies have questioned the accuracy of self-reported condom use [71]. All the same, the return to sex work for the HIV-positive group does not imply a high re-exposure to sexual antigens. We therefore cannot attribute for sure this increased frequency of Th17 cells to sex work.

Taking the study hypothesis into consideration, it is notable that all participants did not report having unprotected sexual intercourse during the sex break period. However, some studies have cast doubt on the validity of self-reported sexual behavior [72, 73], and indeed, two HESN

participants tested positive for PSA during the sex break period even after having reported abstinence. Nevertheless, we observed that participants had less sex during the abstinence period which was necessary for them to reduce the risk of exposure to HIV and other STIs at the cervix while the biopsy wound healed. Overall, we observed that an interruption in sex work did not affect expression of markers of T cell activation, frequencies of Th17 cells and memory T cell phenotypes in HESN and New Negatives. However, we observed a trend in decreasing frequencies of naïve CD8+ T cells in the blood over time among New Negative women. This could imply that these women that are new in sex work (3 years or less) develop a specific memory against sex antigens over time, decreasing their pool of Naïve T cells. Although we did not observe a concomitant increase in the frequencies of the other memory phenotypes in blood, it is possible that these may populate the secondary lymphoid organs to be differentiated in effector cells capable of migrating to the FGT to perform their effector functions.

Moreover, we report that there is no difference in the pattern of response to the interruption of sex work across all groups. However, independent of the groups, there was a significant difference in the frequency of CD95-expressing and naïve CD8+ T cells between the time points.

An important observation made in this study is that independent of sexual activity, HIV-positive participants exhibited CD8+ T cell memory phenotypes and CD95 expression that is consistent with previous studies. Alterations in the frequencies of CD8+ naïve and effector memory T cells during the course of HIV infection have been described [74] with an expanded T_{EM} cell population [64, 65] and lower frequencies of naïve T cells [61] compared to healthy controls or acutely infected persons. This skewed distribution of memory phenotypes has been associated with immune activation and disease progression [25]. The increased expression of CD95 on CD8+ T cells is also a feature of HIV infection [62, 66] and is evidence of increased apoptosis of memory T cells. Altogether these observations demonstrate that our assay to detect ex vivo T cell activation is reproducible and that the statistical analyses were robust as we can report findings similar to other studies.

10.1 Limitations

A possible confounder in this study is the impact of menstrual cycle on immune activation. Hormones are important immune modulators of the FGT throughout the menstrual cycle. Changes in hormonal expression at the genital mucosa have been shown to influence thickness of the epithelial layer and activation of immune cells [75]. Use of hormonal contraception has also been associated with increased risk of HIV acquisition [76] underlying the importance of hormonal regulation of the immune system. Estradiol and Progesterone can influence the expression of CCR5 on peripheral and mucosal T cells [77]. Thus considering the possible effect of hormones on the activation of T cells, we collected samples mainly at the follicular phase of the menstrual cycle. As earlier illustrated in Table 1, the women had similar number of days after menses but we noticed a trend towards difference between the groups at V1 and V5. We recognize that the impact of menstrual cycle may not have been fully controlled for as menstrual cycle differs among women and some women with shorter or longer cycle were recruited in this study.

One of the limitations in this study is the focus on blood samples. Since semen is deposited in the FGT during coitus, its stimulatory capacity could be more evident in the mucosal compartment. This study focused on PBMCs and not cervical mononuclear cells (CMCs). A comparison of the impact of sex work on both compartments would provide a better picture of sex-induced immune activation. However, the larger study in which this was nested is already investigating the impact of interruption of sex work on the activation of mucosal immune cells.

Another limitation is the fact that two of the participants in the HESN group tested positive for PSA during the sex break – implying that they had sex in the period they should have been abstaining. This may have an impact on the results in this group. No participant in the New Negative and HIV-positive groups tested positive for PSA. We recognize that this may have affected the comparison of the HESN group to the New Negative and HIV-positive groups especially in terms of the response to interruption of sex work.

Finally, the small sample size (10 in each group) of this study is a limitation in our ability to detect a difference in immune activation markers between the groups. This study is on-going and we expect that as more participants are enrolled and with more follow up visits completed, the study will be able to attain a higher statistical power.

10.2 Conclusion

In conclusion this study has demonstrated that in HIV-positive women an interruption of sex work leads to reduction of the frequency of CCR5 expressing CD8+ T cells and that in the same women, an increase in potential Th17 cells occurs upon resumption of sex work. We have also reported that women who have been in sex work for 3 years show a gradual loss in naïve CD8+ T cells possibly due to development of sex antigen-specific memory. We observed no apparent difference in the way HESN, HIV-positive and New Negative sex workers respond to interruption of sex work in the systemic compartment.

Interruption of sex work has subtle effects on peripheral activation of T cells most notably among HIV-positive FSW. Finally, HESN do not seem to display a unique capacity to regulate sex induced peripheral immune activation compared to HIV-positive and New Negative sex workers. Hence, the impact of sex work on T cell activation could be limited to the mucosal compartment and not be reflected in the systemic compartment.

10.3 Recommendations

From the observations made in this study, we propose that further studies are required to provide more insight into the relationship between sex work and peripheral immune activation. Ex vivo studies could pursue to examine the specificity of T cell response to sex antigens. T cells can be stimulated with seminal fluid components, say PSA or sperm cells, to see the antigen specificity of these cells and the magnitude of response. Such a study would elucidate whether the immune response observed post coitus is specific for alloantigens or pathogens present in semen. This can be important in the development of safer microbicides which target HIV and other viruses or bacteria that play a role in the activation of the FGT mucosa.

Regulatory T-cells play a role in suppression of T cell activation. T-regs may play an important role in shaping the immune balance at the mucosal level by limiting allogeneic stimulation and protecting against HIV acquisition. It would be critical to investigate the role of these cells in inducing tolerance to sex antigens and how interruption of sex work would affect their frequencies. It is plausible that T-regs may have an effect on the lack of sex-work induced T cell activation we have observed in HESN and New Negatives. These women may have developed

tolerance to sex related antigens over time. Elucidation of such a mechanism can help in the development of vaccines that can induce such tolerance especially at the mucosal level.

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12 APPENDIX

12.1 QUESTIONNAIRE

1.	Study number		
2.	Signed consent form? *If No, stop completion of the questionnaire immediately*	0. No 1. Yes	
3.	Date of visit	day/month/year	
4.	Identification of the person completing the questionnaire (initials)		
5.	Age		____ years
6.	Country of origin		
7.	Marital status	1. Not married, not living with a man 2. Not married, living with a man 3. Married, not living with a man 4. Married, living with a man	
8.	How many years of school have you completed?		____ years
9.	Are you taking contraceptive	0.No If no go to question 10 1.Yes	
9b	What type of contraceptive are you taking	0. Oral 1. Injection	
10.	How many times have you been pregnant		
10.b	When is the last you were prégnant		
10c.	How many children do you have		
10d	How many children alive		
11	Do you drink alcohol?		
11b	How much and how often		
12.	Do you take prescribe drug? medecine form pharmacist or tradional	0.no 1.yes	
12b	If yes, which one, for which reason		
13.	How many years have you been working as a sex worker?		
14.	When was the last time that you had sex with a client **without** a condom?		___ days ago ___ weeks ago ___ months ago ___ years ago ___ Never
15.	How many clients have you had totally in the last 7 days?		
16.	With how many clients have you used a condom in the last 7 days?		
17.	Have you used a condom with your last client?	0. No 1. Yes	
18a.	Do you have a regular partner?	0. No → Question 1. Yes	
18b.	If Yes, when was the last time that you had sex with your regular partner **without** using a condom?		___ days ago ___ weeks ago ___ months ago ___ years ago ___ Never
18c.	How many times totally have you had sex with your regular partner in the last 7 days?		
18d.	How many times have you used a condom with your regular partner in the last 7 days?		

18e.	Did you use a condom the last time that you had sex with your regular partner?	0. No 1. Yes	
19a.	Do you practice oral sex?	0. No 1. Yes	
20a.	Do you practice anal sex?	0. No 1. Yes	
21.	Have you had oral sex (penis in the mouth) or anal sex (penis in the anus) in the **last 30 days**?	0=No 1=Yes (anal) 2=Yes (oral) 3=Yes (both: oral and anal)	
22a.	Have you **already** received a blood **transfusion**?	0. No → Question 23a 1. Yes	
22b.	If Yes, how old were you when you had a blood transfusion for the first time?		____ years
23a.	Except for vaccinations, have you recently received medicinal **injections** to treat a health problem?	0. No → Question 24a 1. Yes	
23c.	Where did you receive this injection?	1. Public clinic 2. Private clinic 3. Traditional healer 4. Other (specify) _____	
23d.	How many injections have you received in the **last year**?		
24a.	Have you already had an STI (give examples)?	0. No 1. Yes	
24b.	If Yes, specify		
25.	During the period of suppose inactive sex work did you have sex	0.No 1.Yes	
25b	If yes with a client	O.No 1.Yes	
25c	If yes with a regular partner	0.No 1.Yes	
25d	How many times did you have sexual activities		
25e	Do you know the HIV status of the partner	0.No 1.Yes	
26	Do you do vaginal douching?	0.No 1.Yes	
26b	DId you do vaginal douching in the last 3 days	0.No 1.Yes	

12.2 LAMIQ INFORMED CONSENT DOCUMENT

LONGITUDINAL ANALYSIS OF MUCOSAL IMMUNE QUIESCENCE STUDY

Majengo, Research Clinics

Patient Information and Consent Form

This information will be communicated orally in English, Swahili or other Kenyan dialect of potential participant's preference. The form is also available written in Swahili.

Investigators:

Dr Keith Fowke University of Manitoba, 745 Bannatyne, room 539, Winnipeg, MB, Canada 1-204789-3818

Dr. Joshua Kimani Medical Microbiology, University of Nairobi, tel. 714851, P.O Box 19676, Nairobi, Kenya

Dr. Kristina Broliden Karolinska Institutet, CMM L8:03, 17176 Stockholm, Sweden 46-(0)8-517 760 45

Background Information

The University of Nairobi and its collaborators from Canada and Sweden have been working for many years to fight the epidemics of AIDS and other sexually transmitted infections that we are facing in Kenya. This basic science research program is conducting studies to determine the relationship between immunity and susceptibility to sexually transmitted infections (STI) with the goal of developing vaccines or treatments for STIs. You are being asked to participate in this sub-study because you are at a very high risk of acquiring an STI.

The purpose of this study is to determine if there are factors in the female genital tract that could protect individuals from acquiring HIV.

Why Is This Study Being Done? This study is being done to try and find new ways to prevent high-risk women from acquiring HIV, the virus that causes AIDS. Our previous studies showed that the immune system of the female genital tract in women who remain HIV negative for many years is less activated and those women expressed lower levels of certain immune proteins. We would now like to expand these experiments and also look at the distribution of HIV "target cells" in the female genital tract to try to find out what is special about the immune system of the few individuals who do not become infected with HIV. This work may be helpful in eventually making a microbicide or vaccine for HIV.

How Many People Will Take Part in the Study?

About 120 women will take part in this study.

What Is Involved in the Study? You are already enrolled in the study called *"Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya"*. This is a part of that larger study but involves three changes to the procedures of the main study. These changes are:

You will be followed every 2 weeks for a period of 3 months for a total of 6 visits

Two tissue samples (called biopsies) each the size of half a grain of rice (3mm x3mm) will be collected from the upper portion of your cervix by using a small biopsy forceps. After the biopsy sampling a vasoconstrictor and haemostatic gel with a fungicidal and bactericidal effect will be applied to the area where the biopsies were taken.

Following the taking of each tissue sample, you will be asked to abstain from ANY vaginal sex (with regular partners or clients) for at least 14 days to allow your body to heal. The two tissue samples will mean that you will have to abstain from sex for a total of 28 days or one month. You will be financially compensated for the loss of income during this period.

What is tissue or biopsy sampling? Cervical biopsy sampling is very common and usually performed when a cervical Pap test indicates significant abnormalities or when an abnormal area is seen on the cervix during a routine pelvic examination. The sampling will be done without any pain relief as it is very quick and most women tolerate it with very little discomfort. A cervical biopsy sampling is done in a health professional's office, a clinic, or a hospital as an outpatient procedure and the patient does not have to spend the night in the hospital. Two days is sufficient for full healing of the cervix after routine biopsies according to our previous studies but the study team recommends having a wider safety margin since you are involved in sex work. Through your sex work you might be at a very high risk of acquiring an STI or are already infected with an STI, therefore, you will be asked to take a mandatory break from having sex for at least 14 days after each procedure. This will allow your body lots of time to heal so when you do return to sex work you will not be an a higher risk of getting an STI.

IMPORTANT FOR STUDY PARTICIPANTS

Statement on Abstaining from Sex

A. ***We request that you abstain from ANY sex (regular partners or clients) for at least 14 days after each cervical biopsy has been taken. If you have sex within 14 days of the biopsy procedure, you will have a much higher risk of becoming HIV-infected or re-infected. If you are HIV- infected and your partner is not, then he will be at much greater risk of becoming HIV-infected too. It is very important that you don't have sex for at least 14 days after the biopsy has been collected. However, if abstaining is absolutely not possible, then please use a male or female condom to help reduce the HIV risk. We will be testing for the presence of male sperm in your vaginal washes to determine if unprotected sex has occurred. To assist in the healing process you should also avoid vaginal douching and the use of tampons.***
B. ***You will be compensated for travel and loss of income as is standard in the Majengo clinic.***
    - ***50 Ksh for travel costs for each visit (6 visits total)***
    - ***400 Ksh for each visit where vaginal samples and blood are taken (6 visits total)***
    - ***700 Ksh for each biopsy (500 for loss of income for that day plus 200 for travel to the clinic at the Kenyatta National Hospital) (2 biopsy total)***
    - ***500 Ksh per day of abstinence from to sex work to compensate for loss of income (28 days in total).***

Clinic visits:

At each visit:

1. We will ask you general questions about your life, about problems you are having, and about your sexual history.
2. The doctor will examine your body, including your female parts.
3. Washing from your vagina to look for germs and to collect samples for studying immune cells and proteins in the vagina. We will also use this wash to confirm you did not have unprotected sex during the abstinence period.
4. Swab and cytobrush specimen (like a PAP test) from your cervix to look for germs and to collect samples for studying immune cells and proteins in the cervix.
5. Two tubes with about 10mls of blood each will be collected from your veins to study immune cells in the blood. As well, we will test for HIV and syphilis

6. At visit two and three, two biopsies the size of half a grain of rice (3x3 mm each) will be taken from the upper portion of your cervix (which is the part the separates the vagina from the womb) will be collected using a small forceps for studying immune cells and proteins in the cervix. The tissue samples are taken using local pain medication and you should not feel any pain.

How Long Will I Be in the Study? The sub-study requires you to come once every two weeks for a total duration of 3 months. This will not affect the regular care that you receive in the clinic in any way.

What Are the Risks of the Study?

Risk of blood and cervical collection

This study requires the use of your blood. In order to get the blood we will need to insert a needle into a vein in your arm so that the blood can be removed. There will be some pain associated with the needle stick but this will be only for a short period of time. There may be some bruising around the needle site and, although we will sterilize the site to minimize infection, there is a very minimal risk of infection at the site. There is also some discomfort associated with taking specimens from your cervix. The biopsy may feel like a pinch each time a tissue sample is taken and may cause some cramping with it. Any pain or cramping occurring during the biopsy may be helped by relaxing and taking a few slow deep breaths. Some vaginal bleeding and a small amount of dark brown discharge are normal after the sampling. We will use procedures to limit the bleeding at the site and we will apply a gel to help prevent infection. Sexual intercourse, douching or use tampons is discouraged for at least 14 days after the cervical biopsies have been collected. ***However, if abstaining is absolutely not possible, then use of a male or female condom to help reduce the HIV risk is a must. Also, please feel free to let the clinic staff know if you have had unprotected sex; it will not affect your participation in this study or the clinic.***

HIV test: Non-physical Risks

If you are HIV positive, learning so may cause you to become depressed. We will counsel you about your HIV test results if you are negative or positive. If you are HIV positive, we will refer you to another clinic for care and treatment. We will also test your husband or boyfriend for the HIV virus if he wants.

Are There Benefits to Taking Part in the Study? If you take part in this sub-study you will have the same benefits provided through the main study. Specifically, you will be examined regularly, and if you are found to have an STI or AIDS, you will receive appropriate and effective medication. Medical care will also be provided for other illnesses

that you might have. You will also be informed about what you are suffering from, and you will be informed about the future implications of these STDs and of HIV.

What about Confidentiality? Your personal information will be kept confidential. We will record your information only by a special number assigned to you. The number will only be known to the clinic staff and yourself. None of the information in study computers or books outside the clinic will contain your name. Organizations that may inspect and/or copy your clinic records for quality assurance and data analysis include the researchers, and members of the local and international ethics teams. This is to protect your welfare. The research results will be published, but your identity will remain secret.

What Are My Rights as a Participant? Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. If the participation in the study results in you becoming ill, the study team will provide you with medical care for the problem for free. You are welcome to ask any questions any time to any member of the study clinic.

Although you will not be paid to participate in the study, you will be offered a small token in consultation with members of the KNH-ERC. This amount will cover your lost work time and transportation to the clinic and any other expenses you might incur.

We will also provide you with any new information and findings from the study that may affect your health, welfare, or willingness to stay in this study.

All information that is obtained will be kept strictly confidential, and your identity will not be known, except to those providing your medical care.

At the end of every year, we will hold a baraza at the Majengo clinic to give progress reports and share any new findings from the study with all members of the different clinics.

Whom Do I Call if I Have Questions or Problems? For questions about the study or a research-related injury, call or contact Drs. Wachihi, Kimani or Makobu or any one of the researchers named above at the Medical Microbiology Annex at the University of Nairobi.

For questions about your rights as a research participant, contact Professor A.N Guantai, who is the secretary of the Ethical Review Committee at the University of Nairobi, by calling 0733220580, or make an appointment to see her in the Department of Pharmacology and Pharmacognosy, at the University of Nairobi.

Analysis of the sample taken

While most of the samples will be analyzed in Nairobi, some studies will need to be performed at the University of Manitoba, Winnipeg, Canada and the Karolinska Institute in Stockholm, Sweden. Therefore, some of the stored samples will be shipped to Winnipeg and Stockholm for analysis.

Statement of Consent:

If you agree to participate in this sub-study, please sign below.

I, _____, have read or have had read to me, the consent form for the above study and have discussed the study with _____. I understand that the following (check the box only if you fully understand and agree with each statement):

- ☐ The goal of this research is to study the immune cells and proteins in my blood and genital tract (vagina and cervix).
- ☐ Enrolment is completely voluntary and I can withdraw from the study at any time
- ☐ Blood, cervical and vaginal specimens will be required for this study
- ☐ I am willing to take a mandatory 4 week break from having sex if I participate in the study.
- ☐ I am aware and give permission that a part of the samples taken will shipped to the University of Manitoba, Winnipeg, Canada and the Karolinska Institute, Stockholm, Sweden for analysis.

Name of Study Participant_____

Signature/Thumb print: _____ Date: _____

For clinic staff:

I, _____, have explained the nature and purpose of the above study to _____

Name of Clinic Staff: _____

Signature: _____ Date: _____

Assigned Study Number / Clinic Number ____ ____ ____

NB: All study participants will be issued with a copy of this information and consent form