Genital tract immune activation, inflammation and sexually transmitted infections in CAPRISA 008 trial participants

By

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PREFACE AND DECLARATION

I, Mr. **SN. Mhlungu**, declare as follows:

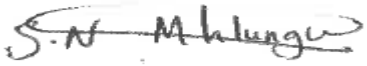
1. The work in this thesis has not been submitted to the University of KwaZulu-Natal (UKZN) or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. My contribution towards this project was as follows: handling of all the specimens, including processing based on the techniques as stipulated in the methodology sections and acquiring data. Also, analysis of data and the writing of this thesis.

3. The contributions of others towards this project were as follows:

Dr. Lenine J. Liebenberg: Supervisor

Dr. Sinaye Ngcapu: Co-supervisor

Signed:  Date: 03-14-2017

DEDICATION

This master's thesis is dedicated to my late mother Ntombizethu Anna Mhlungu. Though she has passed on, she remains forever an important part in my life. I will forever love her and cherish all the good times we had. This work is also dedicated to my family through love we have conquered all.

ACKNOWLEDGEMENTS

I would like to thank Dr L.J Liebenberg, she has been an awesome supervisor and mentor. I am thankful for all her support and guidance over the years. I would like to further thank Dr S. Ngcapu, as my co-supervisor for all the support and motivation. Both have played a major role in refining and shaping me as a young scientific researcher. All the skills I learned will be worthwhile in the future. I would also like to express thanks to the Centre for the AIDS Programme of Research in South Africa (CAPRISA) for hosting me as a fellow student and for providing the funding for this project and the laboratory staff for all help I obtained during the course of this work.

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LIST OF ABBREVIATIONS

AIDS	Acquire immune-deficiency syndrome
APC-H7	Allophycocyanin tandem dye H-7
CMCs	Cervical mononuclear cells
CTACK	Cutaneous T cell-attracting chemokine
CVL	Cervicovaginal lavage
CCR5	Chemokine receptor 5
CD	Cluster of differentiation
CEACAM	Carcinoembryonic antigen-related cellular adhesion molecule
EIA	Enzyme immunoassay
FGT	Female genital tract
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FBS	Foetal bovine serum
FSW	Female sex workers
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HGF	Hepatocyte growth factor
HLA-DR	Human leukocyte antigen-antigen D related

HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
LIF	Leukemia inhibitory factor
M-CSF	Macrophage colony stimulating factor
MCP	Monocyte chemoattractant protein
MIF	Macrophage inhibitory factor
MIP	Macrophage inflammatory protein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction.
PDGF-ββ	Platelet-derived growth factor beta polypepetide
PE	Phycoerythrin
PerCP-Cy5.5	peridinin-chlorophyll protein-cyanine 5.5
RBCs	Red blood cells
RPMI	Roswell Park Memorial Park Institute Medium
SCF	Stem cell factor
SCGF-β	Stem cell growth factor beta
SDF-1α	Stromal cell derived factor 1 alpha
SIV	Simian immunodefiency virus

STI	Sexually transmitted infection
TFV	Tenofovir gel
TNF	Tumour Necrosis Factor
TRAIL	TNF related apoptosis-inducing ligand

ABSTRACT

Background: Data from the CAPRISA 004 trial demonstrated that women with elevated genital inflammatory cytokines have increased risk of HIV acquisition. Other biological factors known to impact HIV risk include, among others, sexually transmitted infections, and immune cell activation. The aim of this study was to determine whether immune cell activation and elevated frequencies of genital $CD4^+CCR5^+$ target cells for HIV infection are associated with (1) increased genital markers of inflammation; and (2) with the presence of sexually transmitted infections (STIs).

Methods: Cervical cytobrushes and cervicovaginal fluids collected from HIV seronegative CAPRISA 008 participants (n=166) were used to measure genital T cell immune activation and inflammation respectively. Flow cytometry was used to identify cellular targets for HIV infection ($CD4^+CCR5^+$), activated cells, and proliferating cells. Dual expression of $CD38^+$/HLA-DR^+ above 75^{th} and below 25^{th} percentile defined women with high and low immune T cell activation respectively. Genital inflammation was defined by elevated levels of at least 5 of any 9 key cytokines as previously described by (Masson *et al.,* 2015). The presence of selected discharge-associated STIs (*N. gonorrhoeae*, *C. trachomatis, T. vaginalis and M. genitalium*) were determined by multiplexed PCR.

Results: This study found that women with high immune activation had significantly higher frequencies of $CD4^+CCR5^+$ ($p<0.0001$) and proliferating $CD4^+CCR5^+$ ($p<0.0001$) T cells compared to women with low immune activation. The prevalence of *C. trachomatis*, *T vaginalis* or any combination of any STI ($p=0.0034$, $p=0.0024$ and $p=0.0002$ respectively) was significantly higher in women with genital inflammation (n=31) compared to those without inflammation (n=134). There was no statistically significant association between cellular markers of immune activation and inflammation. STI were observed more often in the women characterized by high levels of immune activation compared to women with low levels of immune activation.

Although this was not statistically significant, taken together this demonstrates a contribution by STI to immune activation at the genital tract. Furthermore, when the frequency of $CD4^{+}CCR5^{+}$ expressing cells was compared between women with STIs and those without STIs, women infected with *C. trichomonas* or any STI combination had significantly higher frequencies of $CD4^{+}CCR5^{+}$. Taken together, these data demonstrate a contribution by STI to immune activation and inflammation at the genital tract.

Conclusion: While it has been well documented that the genital mucosa provides robust mechanisms that protect against pathogens, STIs play a significant role in the recruitment of HIV target cells, and are potential drivers of the inflammation in the genital tract that fosters an environment conducive to HIV acquisition. It is of great urgency that future biomedical interventions are made to curb HIV acquisition as a result of predisposition to STIs in women.

CHAPTER 1: INTRODUCTION

1.1 Introduction

The female genital tract (FGT) is composed of specialized compartments which include: the lower part (vagina and ectocervix), the transitional endocervix and the upper part (endometrium and fallopian tubes) (Ochiel et al., 2008). In addition to the epithelial lining present at these compartments, the FGT contains mucus which serves as a protective barrier against invading pathogens (Ochiel et al., 2008). The FGT also has defensins, these are small proteins which possess a broad range of activities involved ranging from anti-microbial activity, inducing cell proliferation and modulate innate and acquired immunity (Bowdish et al., 2006). However despite all these active functions to protect itself against invading pathogens, it is considered a hostile environment in which multiple factors can influence the integrity of the genital mucosal epithelial barrier, rendering the female genital tract susceptible to colonization and establishment of an infection (Machado et al., 2014).

Multiple factors are known to influence this natural barrier against infection and an imbalance in mucosal regulation may potentially give way for HIV to penetrate the barrier and initiate a productive infection (Lajoie et al., 2012). An important determinant of infection may be the number and the activation status of potential HIV target cells at the genital tract. Immune activation is a pivotal response against pathogen infections, however it may contribute to HIV susceptibility by increasing the number of HIV target cells to a critical mass for the establishment of an HIV infection. HIV entry into the host $CD4^+$ T cells is dependent on the expression of the C-C chemokine receptors type 5 (CCR5) co-receptors (Cocchi et al., 1995). In a study carried out by Lajoie *et al.* (2014), HIV- uninfected female sex workers (FSW) expressed higher proportions of $CD4^+$ T cells but had lower expression $CCR5^+$ compared to non-FSW who had higher proportion of pro-inflammatory cytokines and $CD4^+$ T cells expressing $CCR5^+$ at the genital mucosa. Chemokines are low molecular weight glycoproteins (8-12 kDa) which are known to regulate cell trafficking. These cytokines participate in

many physiological processes including the regulation of immune and inflammatory responses. Cytokines recruit leukocyte subpopulations selectively into a site of inflammation. Chemokines including Macrophage Inflammatory Protein-1 alpha (MIP-1α), Macrophage Inflammatory Protein-1 beta (MIP-1β) which are produced by macrophages induced by bacterial endotoxins, and play a critical role during infection and inflammation, RANTES (regulated on activation, normal T cell expressed and secreted) and interferon-inducible protein (IP) 10 and have been reported to possess chemotactic activity for T cells with especially those bearing CCR5 receptors (Telda et al., 1996).

A heightened pro-inflammatory state at the genital tract has been hypothesized to enhance HIV susceptibility and transmission (Kahle et al., 2015). In a study carried out by Li et al., (2009) inflammatory cytokines (MIP-1α, MIP-1β and IL-8) in the genital tract of rhesus macaques were associated with establishment of simian immunodefiency virus (SIV) infection. Several reports identify a relationship between elevated concentrations of cytokines or chemokines and increased risk of HIV acquisition at the genital mucosa (Arnold et al., 2016, Masson et al., 2015b). Lajoie *et al.,* (2012) demonstrated that HIV-exposed seronegative individuals had lower concentration of IL-1α, MIG and IP-10 compared to HIV negative or positive groups. MIG and IP-10 also play a role in leukocyte trafficking of natural killer cells and both active and memory T cells. While cytokines play an essential role in cell signaling when mediating an immune response, these studies suggest that HIV is able to take advantage of the immune response to induce a progressive infection. The immune system of the female genital tract is an integrated part of the mucosal system, but is considerably distinct from characteristics observed systemically. Naranbhai *et al.,* (2012) showed that systemic biomarkers of inflammation are associated with HIV acquisition among participants in the CAPRISA 004 trial. MIG, MIP-1α and MIP-1β were found to be significantly higher in the genital mucosa compared to the systemic compartment in FSW who have low basal immune activation (Naranbhai et al., 2012). These findings suggest that observations at the genital mucosa do not parallel those at systemic compartment, therefore since HIV, is mostly transmitted through heterosexual

intercourse, to gain greater and elucidative insights about HIV pathogenicity studies should be carried out using genital samples.

Sexually transmitted infections (STIs) contribute to a great worldwide burden of morbidity and mortality (Tang and Rosenthal, 2010). The high prevalence of STIs has contributed to the disproportionately high HIV incidence and prevalence in Africa (WHO, 2009). Studies have shown that STIs and other genital infections have been associated with increased HIV susceptibility and transmission (Cohen, 2004, Mlisana et al., 2012a). STIs can compromise the integrity of the genital mucosa by causing micro-ulcerations or inflammation, both increasing the amount of exposed HIV target cells at the genital mucosa (Cohen, 2004). The mechanisms that underpin the increase of preferential target cells at the genital tract are of important consideration in HIV acquisition. In this study the prime focus will be on the influence of immune activation, inflammatory responses and co-infections that compromise immunity to HIV infection at the genital tract.

1.2 Study Aim and Objective

1.2.1 Specific aims

a) To determine the relationship between the genital cytokines and the frequency of local activated immune cells.
b) To determine the contribution of STI to levels of cellular and cytokine markers of inflammation.

1.2.2 Study objectives:

- To determine whether elevated frequencies of genital $CD4^{+}CCR5^{+}$ targets for HIV infection are associated with (1) increased concentrations of genital cytokine mediators of inflammation; (2) with T cell immune activation; and (3) with the presence of sexually transmitted infection in the same women.

1.2.3 Hypothesis

- Elevated levels of pro-inflammatory cytokines at the genital tract play a central role in the recruitment and or activation of immune cells into the genital tract. We also hypothesize that sexually transmitted infections in the genital tract prime the responses that trigger genital inflammation.

CHAPTER 2: LITERATURE REVIEW

2.1 HIV epidemic

Almost seventy percent of the 36.7 million people globally infected with HIV-1 reside in sub-Saharan Africa (Figure 1.1), with women accounting for about 60% of these infections (UNAIDS, 2016). Heterosexual contact is the most common route of HIV transmission. Although exposure to HIV through sexual contact is not an efficient route of transmission, there are other aggravating factors that put women at increased risk for sexual transmission of HIV compared to their male counterparts (Gaskins, 1997, Mabala, 2006). In particular, young women (15–24 years) are much more likely to acquire HIV-1 than older women (Stirling et al., 2008). Young women acquire HIV-1 infection 5-7 years before men and they are eight times more likely to be infected than their male counterparts (Abdool Karim et al., 1992, UNAIDS, 2012, UNAIDS, 2016).

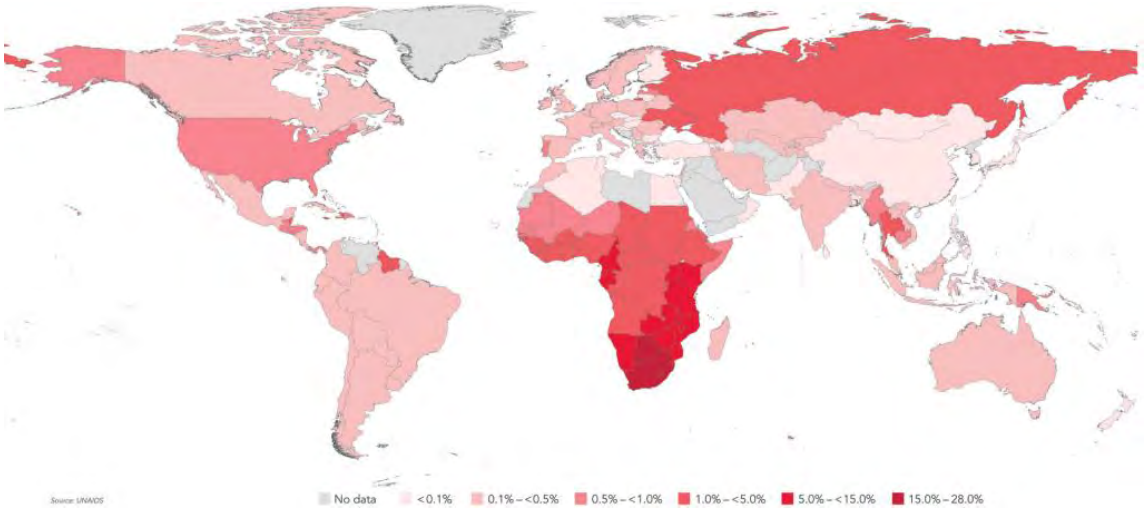

Figure 1.1: The prevalence of HIV in adults and young children (UNAIDS, 2015). People in Eastern and Southern Africa have the highest burden of the HIV epidemic, with more than one million new cases reported in 2015.

It is thought that young women become infected with HIV-1 after fewer acts of unprotected sex (Glynn et al., 2001, Pettifor et al., 2005), and several biological risk factors that may predispose women to become infected with HIV-1 have been proposed

(Chersich and Rees, 2008). The surface area of the lower reproductive tract exposed during sexual intercourse in women is greater than the reproductive tissue of men, which may increase surface area exposed, time in contact with infectious fluids post-coitus, and exposure of intraepithelial HIV-1 target cells to pathogens (Yi et al., 2013). Microabrasions in the genital tract, in the comparatively more fragile and less keratinized epithelial barrier, may be more common in women than men and provide a portal for pathogen entry (Stanley, 2009). Cervical ectopy (particularly to younger women) may facilitate increased exposure of HIV-1 target cells to pathogens in the lower reproductive tract during sex (Critchlow et al., 1995). Finally, the presence of co-factors such as sexually transmitted infections (STIs), often asymptomatic and therefore untreated in women, also increase the risk of HIV-1 infection (Cohen, 2004, McKinnon et al., 2011).

2.2 The female genital tract

The female genital tract is a unique environment that has the ability to respond sharply to infections that are generally transmitted in seminal plasma or during sexual contact, whilst at the same time being tolerant to allogenic spermatozoa (Haase, 2010, Haase, 2011). The female genital tract (FGT) represents components of the common mucosal system, but with features which are distinct from other mucosal tissues, these include; adaptions of dealing with the presence of residential microbiota as well as responding to challenges imposed by bacteria, yeast and viruses (Gupta & Kumar, 2012). The FGT is composed of specialized components which include: upper reproductive tract part which is composed of a single layer of columnar epithelium, transitional endocervix and the lower reproductive tract (vagina and ectocervix) composed of multilayered stratified squamous epithelium. (Figure 1.2).

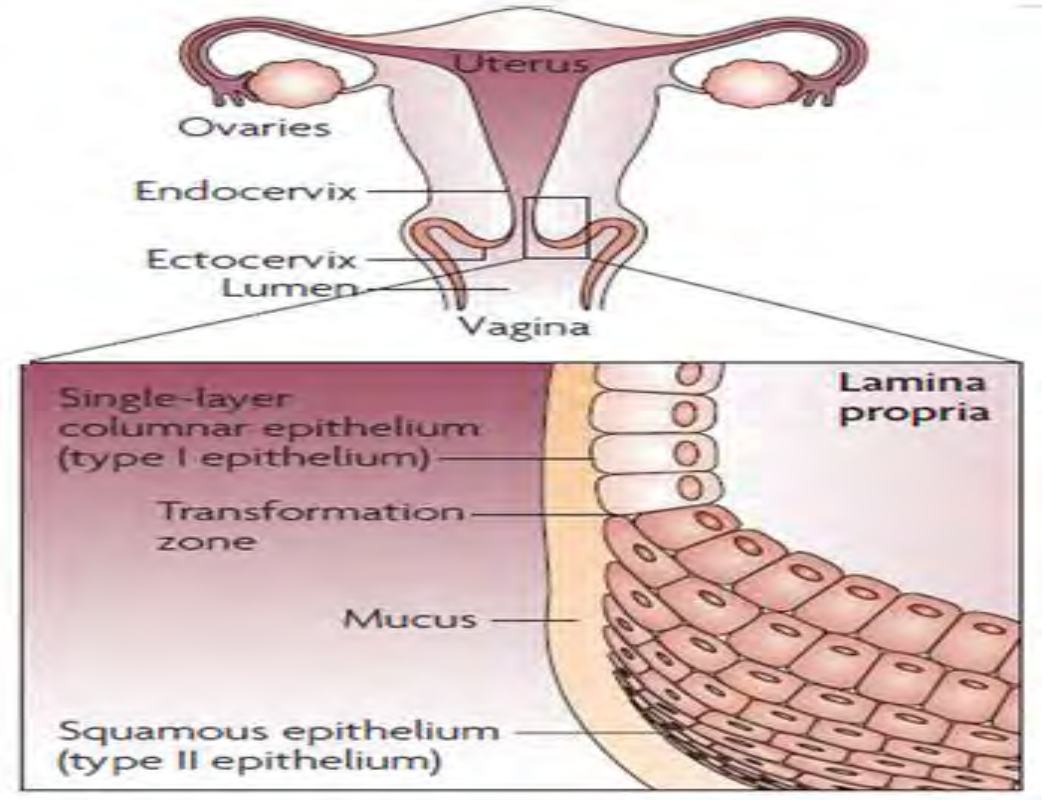

Figure 1.2: The anatomical structure of the female genital tract. Components of the FGT include; the lower reproductive tract which consist of the vagina and the ectocervix. The upper reproductive tract which is composed of both the uterus and cervix. (Iwasaki, 2010).

The susceptibility of these components within the female genital tract to infections differs. Between the ectocervix and endocervix, is the transformation zone. Within this zone the squamous epithelium changes to a single layer of the columnar epithelium. This region is described as being more vulnerable to infection due to a high number of activated lymphocytes and lymphoid aggregates present in the presence of various pathogens (Weisesenbacher et al., 2013). Immune T cells are unevenly distributed in the female genital tract (Johansson et al., 1999). Compared to peripheral blood, the female genital tract contains more $CD8^+$ T cells than $CD4^+$ T cells. Other immune cells present include macrophages, dendritic cells, neutrophils and natural killer cells which are part of the innate immune system in the genital mucosa (Amjadi et al., 2014). This means

that the female genital tract is an active site equipped to defend itself against invading pathogens

2.3 HIV-1 transmission across the female genital tract

The mucosal epithelium of the lower female reproductive tract provides the first line of defense against pathogen entry and mediates the initial host immune response against STIs, including HIV-1 (Kaushic, 2011, Wira et al., 2005a, Wira et al., 2005b). This is also the surface through which HIV-1 is transmitted from an HIV-1 infected woman to her sexual partner (Yi et al., 2013). The genital mucosa is also thought to serve as a discrete site for HIV-1 infection, replication and pathogenesis by providing the virus with a steady supply of susceptible target cells (Zhang et al., 1999). The epithelial barrier of the genital mucosa is also thought to be influenced by hormones, inflammation and infection (Murphy et al., 2014). Acute HIV-1 infection has been associated with increased genital tract inflammatory cytokine responses in women (Roberts et al., 2012), that may result in increased permeability of the mucosal epithelial barrier, allowing HIV-1 easier passage across the mucosal epithelium (Nazli et al., 2010). Cell-free virus has also been shown to move between the upper layers of the "leaky" squamous epithelial barrier (Hladik and Hope, 2009). It has been suggested that transcytosis of HIV-1 may occur through polarized columnar epithelial cells and squamous epithelial cells (Hladik and Hope, 2009). However, other studies have found that HIV-1 does not penetrate through intact cervical explants (Shattock and Moore, 2003).

2.3.1 Mucosal immune system and HIV

The immune system is a host defense mechanism to eradicate infections and pathogens. The mucosal system is an integrated part of the body's immune system, is however distinct in that, it acts both as a natural barrier and interacts with the external environment.

Different factors influence the host susceptibility to HIV infection. HIV infection is dependent on the hosts' chemokine co-receptor $CCR5^+$ on $CD4^+$ T cells to initiate a productive infection. It has been shown that individuals with mutations on the $CCR5^+$ gene are highly resistant to HIV infection (Samson et al., 1996). Furthermore, high-risk seronegative individuals with suppressed T cell immune activation demonstrated low HIV susceptibility (Koning et al., 2005).

Immune activation, characterized by T cell activation and elevation of pro-inflammatory cytokines has been suggested as a hallmark of HIV infection (Lawn et al., 2001). In a study conducted by Naranbhai *et al.,* (2012), though the Tenofovir gel showed 39% efficacy, it was shown that women with high immune activation and inflammation were associated with HIV acquisition. Immune activation, is pivotal in eradicating pathogens and infectious agents. However, in the case of HIV infection, it may contribute to HIV susceptibility by increasing the number of HIV target cells to a critical mass for the establishment of an HIV infection (Lajoie et al., 2014). Cytokines play a crucial role modulating pro-inflammatory and anti-inflammatory immune responses. Elevated concentration of pro-inflammatorty cytokines including chemokines have been show to be associated with HIV acquisition at the genital mucosa (Arnold et al., 2016, Li et al., 2009a, Masson et al., 2015a). The common route of HIV transmission is through heterosexual contact. The presence of HIV target cells and the role of cytokines in the genital mucosa suggest their significance in HIV transmission.

2.4 Genital inflammation and HIV risk

Understanding mucosal risk factors in the female genital tract are critical for understanding STI/HIV susceptibility. In the CAPRISA 004 1% tenofovir microbicide trial, women who became HIV-infected during the trial had significantly elevated genital tract inflammatory markers prior to HIV-infection (evidenced by increased concentrations of MIP-1α, MIP-1β, IL-8, and IP-10, determined by logistic regression and principle component analysis), independent of study arm and age (Masson et al.; 2015b). Furthermore, detection of either MIP-1α or MIP-1β in cervicovaginal lavage

(CVL) was associated with >3 fold increased odds of HIV infection (Masson et al., 2015b). A recent study demonstrated high levels of inflammatory cytokines (including IL-1α, IL-6, IL-1β and IP-10) in endocervical secretions of women with chlamydial infection compared to treated women (Sperling et al., 2013). In the CAPRISA 002 Acute Infection cohort we demonstrated that several inflammatory cytokines (including MIP-1β, RANTES and TNF-α) were significantly associated with both symptomatic and asymptomatic STIs, and that these inflammatory cytokines were independent predictors of HIV acquisition in prospective follow-up, after accounting for multiple comparisons (Masson et al., 2014, Mlisana et al., 2012b). However, STIs accounted for only 20% of genital inflammation associated with HIV acquisition risk in the CAPRISA trial (Masson et al., 2015b). Therefore, investigating and understanding other causes and consequences of mucosal inflammation is essential in developing prevention interventions.

2.5 Genital inflammation, immune activation, HIV shedding and disease progression

HIV infection has been associated with increased mucosal and systemic inflammatory cytokine responses in women (Roberts et al., 2012). Inflammatory responses have also been shown to enhance HIV pathogenesis, by recruiting highly activated HIV target cells to the site of initial viral infection and replication (Li et al., 2009b, Meier et al., 2007, Abel et al., 2005). In macaques infected with SIV, cervicovaginal concentrations of MIP-1α, MIP-1β and MIP-3α resulted in recruitment of $CCR5^{+}$ and CD4+ T cells in the endocervical epithelium (Li et al., 2009b). Furthermore, spread of SIV-infected cells from the genital mucosa to ensure a productive systemic infection, correlated with this pro-inflammatory cytokine gradient (Li et al., 2009b). In humans, concentrations of IL-1β, IL-6, and IL-8 in CVLs from HIV acutely infected women correlated inversely with CD4+ T cell counts in blood, suggesting the level of both mucosal and systemic inflammation present during acute infection predicted adverse CD4 decline during acute infection (Bebell et al., 2008). Furthermore, increased recruitment of activated HIV target cells to the genital epithelium was shown to facilitate local HIV replication and

result in increased local genital HIV shedding from HIV infected women, potentially placing their male partners at higher risk of HIV infection (Gordon et al., 1999, Rottman et al., 1997). In both humans and non-human primates studies, higher levels of T cell activation in blood and plasma viral loads during acute infection predicted faster rates of mucosal and systemic CD4 cell decline and more accelerated disease progression (Douek et al., 2009, Hazenberg et al., 2003, Brenchley et al., 2004, Silvestri et al., 2003). A better understanding of how higher local and systemic viral loads increase risk for partner infection could lead to better interventions aimed at blocking HIV-1 transmission from an infected women to her partner. In addition, it would also identify therapeutic targets to block male-to-female transmission.

2.6 Consequences of Sexually transmitted infections in the female genital tract

Multiple factors are known to influence the genital mucosal barrier. Sexually transmitted infections (STIs) at the genital mucosa are among these factors, and represent one of the most widespread infectious diseases and pose a major health burden in morbidity and mortality (Tang and Rosenthal, 2010). According to the WHO (2016), it is estimated that over a million STIs are acquired per day globally (Figure 1.3). In addition, there is an estimated 357 million new STIs; *C. trachomatis* (131 million), *N. gonorrhea* (78 million), *T. pallidum* (6 million) and *T. vaginalis* (142 million) among young people between the age of 15-49 globally per year. Although measures have been put into place to fight STIs, they still continue to contribute disproportionately to high HIV incidence and prevalence in Africa (WHO, 2009). Studies have shown that STIs and other co-infections in the genital tract are associated with increased HIV susceptibility and transmission (Cohen et al., 2010, Mlisana et al., 2012b, Ramjee et al., 2015). STIs can compromise the integrity of the genital mucosa by causing micro-ulcerations or inflammation, both increasing the amount of exposed HIV target cells at the genital mucosa (Cohen, 2004)

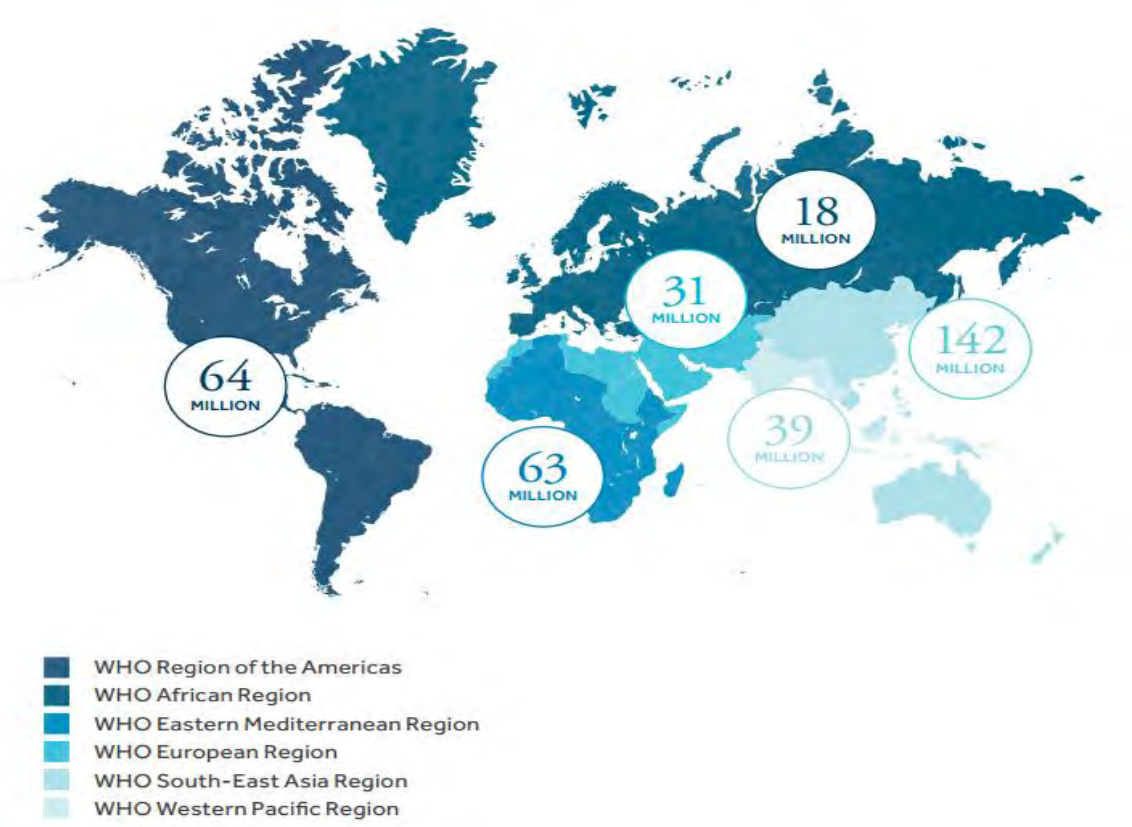

Figure 1.3: Global estimates of new cases of curable STIs; *syphilis, trichomoniasis, chlamydia and gonorrhea* (WHO. 2016). Africa is among the regions with the highest number of STIs cases.

Not all STI are symptomatic. In developing countries such as South Africa, 50% of infections are asymptomatic (Wilkinson et al., 1999) and untreated STIs can result in such dire consequences to women as pelvic inflammatory diseases and tubular factor infertility. In developing countries, they rely on syndromic management of STIs. In such resource-poor settings etiological diagnosis is expensive and requires specialized expertise (WHO, 2011). A randomized trial in Tanzania demonstrated that STI treatment reduced HIV incidence by 40% within the population (Kamali et al., 2003; Gregson et al, 2007; Celum et al 2008). Better understanding of the genital mucosal immune system is of paramount importance since STIs have been shown to increase HIV risk acquisition and disease progression, and likely facilitate a crucial role in the recruitment and activation of HIV target cells in the genital tract.

CHAPER 3. RESEARCH DESIGN AND METHODOLOGY

3.1. Study Participants

This study investigated biomarkers of inflammation in a subset (n=166) of participants of the CAPRISA 008 open-label randomized controlled trial that assessed the implementation effectiveness and safety of 1% Tenofovir (TFV) gel. The random convenience sample of CAPRISA 008 trial participants were all above the age of 18, were HIV negative at screening, and within 3 months prior to screening had at least one coital act. All the participants previously participated in the CAPRISA 004 study, and none of the women were pregnant. The study was conducted at the urban CAPRISA eThekwini and rural CAPRISA Vulindlela Clinics, and other public sector family planning services in KwaZulu-Natal, South Africa. The study was conducted in accordance with conditions stipulated by the ethics and regulatory committees upon approval.

3.1.1 HIV testing at screening and enrolment

During screening and enrolment, participants underwent voluntary counselling prior to HIV testing. For HIV-1 screening blood samples were collected for rapid HIV antibody tests. Two antibody HIV tests were conducted at screening and enrolment, a confirmatory HIV enzyme immunoassay (EIA) (BEP 2000; Dade Behring, Marburg, Germany) was performed for participants who had discordant result and based on the result obtained participants were classified into the HIV-negative cohort or HIV follow-up care services.

3.2 Biological Specimen collection and Processing

All specimens in this study were collected at enrolment, approximately 1 month after screening for inclusion into the CAPRISA 008 trial. None of the participants were using 1% TFV gel at the time of sampling. Cervical cytobrushes and cervicovaginal lavage (CVL) specimens were collected for the assessment of immune activation profiles in the

genital tract. Vulvovaginal swabs were collected for testing the presence of sexually transmitted infections. Specimens were not collected at the time of menstruation or if women were pregnant. All specimens were kept on ice and transported to the CAPRISA laboratory within approximately 4 hours.

3.2.1 Cervicovaginal Lavage

CVL specimens were collected and processed as previously reported (Bebell *et al.*, 2008). A volume of 10ml of sterile saline was used to bathe the cervix. The resulting fluid at the posterior fornix was aspirated using a sterile plastic bulb pipette and was transferred to sterile conical tubes. CVLs were transported to the CAPRISA laboratory, where they were centrifuged at 800 x g for 10 minutes and the supernatant was stored in 1ml aliquots at -80°C.

3.2.2 Vulvovaginal Swab

Vulvovaginal swabs were collected at the vulva using sterile cotton wool swabs (Dacron) and were placed into 1.5ml cryovial tubes that contained 0.4ml of PBS. Specimens were stored at -80°C.

3.2.3 Cytobrushes

Cervical mucosal mononuclear cells (CMC) were collected using a Digene Cervical sampler. A single 360^0 rotation of the cytobrush was required to collect cells from the cervical os. The cytobrushes were placed in a 15ml tube (Griener) and immersed into RPMI (Roswell Park Memorial Park Institute Medium 1640, SIGMA-ALDRICH) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and 5mM glutamine, penicillin and streptomycin, hence forth referred to as R10 media. Specimens contaminated with visible blood were discarded. The cytobrushes were processed in Biosafety Laminar Flow Hoods (ESCO class II, BS, LABOTEC) using

aseptic techniques. First, the presence of contaminating sperm was investigated by placing a drop of the sample's medium on a glass slide, covering with a slip and viewing under a light microscope at 100x magnification (Olympus, model CVL31RBSF). Cells from the cytobrush were dislodged by pipetting vigorously (up and down at least 10 times) using a Pasteur Pipette. The cytobrush was washed similarly for a further 5 times with 2ml R10 media and the dislodged cells were collected by centrifugation at 1400rpm (400xG) for 10 minutes at 4^0C.The supernatant was discarded and the pellet resuspended in 500µl of R10 media for counting of cells before staining for flow cytometry.

3.2.4 Cell counting using Trypan Blue Exclusion Method

From the 500µl cell suspension, a manual counting technique was used to count the number of viable mononuclear cells. Trypan blue staining technique was used to selectively distinguish between viable and dead cells. A volume of 15µl of the cell suspension was mixed with 15µl of Trypan Blue (WhiteSci, PTY. Ltd.) and cells were rested in the dye for 2 minutes to allow for optimal staining. The solution was transferred into the counting chambers of Fastread slides (Davis Diagnostic PTY LTD.) and viewed under a light microscope at 400x magnification. Viable cells have an intact cell membrane and because the dye does not transverse through the membrane (Strobe, 2001), viable cells will appear colourless. In contrast dead cells do not have an intact cell membrane therefore the stain transverses the membrane and they will be stained blue. In each counting chamber two squares were selected from the grid and were used to count the average number of lymphocytes.

3.3 Investigation of cellular activation by flow cytometry

3.3.1 Surface staining of cellular immune markers

Cells isolated from cytobrushes and whole blood were transferred to wells of a V-bottom 96 well plate for staining. The cell pellets were resuspended using 150µl PBS

and the plate was centrifuged at 2100rpm (833xG) for 3min at 4^0C. The supernatant was discarded by quickly and firmly flicking liquid contents onto absorbent paper in a bucket. Steps involving antibody-conjugated fluorochromes were conducted in the dark as these compounds are light sensitive. Live/dead stain Fixable Violet Dead cell stain kit (Molecular probes, Life technologies, USA) was used to differentiate between live and dead cells in a respective well. A working stock was prepared by diluting the vivid in de-ionised water to make up a 1:40 dilution. This was further diluted 1:20 in PBS and a final volume of 50µl vivid was added to the cell solutions in their respective wells of the 96-well plate. The cells suspensions were incubated at 4^0C for 20 minutes to allow for optimal antibody binding. Pellets were washed with 200ul/well cold wash buffer 1% FBS in phosphate-buffered saline (PBS) and the pellets were resuspended by mixing. The plate was centrifuged at 2100rpm (833xG) for 3min at 4^0C and the supernatant was discarded by flicking.

The mononuclear cells were then stained extracellularly with a cocktail of the antibody-conjugated fluorochromes: Pacific Blue–labelled anti-CD14 (BD Bioscience) and Pacific Blue labelled anti-CD19 (BD Bioscience), fluorescein isothiocyanate (FITC)-labelled anti-CD8 (BioLegend), allophycocyanin tandem dye (APC-H7)-labelled anti-CD3 (BD Bioscience), peridinin-chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-labelled anti-CD4 (BD Bioscience), eF605-labelled anti-CD16 (eBioscience) and phycoerythrin cyanine 7 labelled anti-CD56 (BD Bioscience). The antibodies were pre-tritrated and added at a volume of 2µl/well for all besides anti-CD4, in which only a volume of 1µl constituted to the total volume (13µl) of the antibody cocktail added per well. The plate was incubated for 20mins at 4ºC in the dark to allow for antibody binding.

3.3.2 Intracellular staining of cellular immune markers

After incubation, excess surface antibody was removed by washing the cells with a volume 200µl wash buffer and centrifuged at 2100rpm (833xG) for 3min at 4^0C The

supernatant was discarded and the pellets were resuspended in 100µl Cytofix/Cytoperm^{TM} (BD Bioscience) prior to incubation at 4ºC for 20min in the dark. A volume of 150µl Perm-Wash Buffer (BD) was used to resuspend cells, prior to centrifugation at 2100rpm (833xG) for 3min at 4^0C. The supernatant was flicked out and subsequently a cocktail of intracellular staining antibodies was added to each respective well. A volume of 2µl of each of the following antibodies was added per well: T cell activation markers eF655-labelled anti-CD38 (eBioscence) and phycoerythrin (PE)-labelled anti-HLA-DR (eBioscience), and proliferation marker Brilliant violet 700 (BV700) labelled anti-Ki67 (BioLegend). Finally, a volume of 10µl of allophycocyanin (APC) labelled anti-CCR5 which is a chemokine receptor was added to make up a total volume of 16µl/well of the intracellular antibody cocktail.

After 20 minutes of incubation at 4ºC, a volume of 200µl Perm-Wash Buffer was used to resuspend the cells. The plate was then centrifuged at 2100rpm (833xG) for 3min at 4^0C and the resulting supernatant discarded by flicking. Cells were finally resuspended in 100µl CellFix (BD) before being transferred into a clean, labelled FACS tubes (BD). The wells were washed with a further 50µl of BD CellFix and added to the corresponding FACs tube prior to data acquisition using an LSRII flow cytometer (BD immunocytometry Systems).

3.3.4 Preparation of compensation beads

Compensation beads (BD Bioscience) were prepared for each antibody-conjugated fluorochrome used. Briefly, the compensation beads were vortexed and 1 drop of the negative and positive beads were added to each of the FACs tube. The same volume of antibody used for extracellular and intracellular staining was added to the appropriately labelled FACS tube for that antibody.

3.3.5 Data acquisition

The samples were acquired on a LSRII flow cytometer (BD immunocytometry Systems), and data was analysed using FlowJo Software (Tree Star). Appendix, Figure 2 depicts a representative schematic of the gating strategy. Fluorescence minus one (FMO) controls were prepared using unrelated PBMCs from liquid nitrogen. FMOs contain all the markers in the panel except the one of interest and in flow cytometery are ideal for showing gating boundaries. Briefly PBMCs were thawed and stained with flourochrome-conjugated antibodies as follows: one FACs tube contained all the antibodies in the panel and the other three subsequent FACs tubes had all the antibodies (did not have either anti-CD38, anti-CCR5 or anti-HLA-DR) in the panel except for one antibody. Specimens with a CMCs CD3 T-cell event count below a threshold level of 100 were excluded.

3.4 Measurement of genital tract cytokine concentrations

Cytokine measurements were conducted on these specimens using the Bio-Rad 27 and 21-Plex Pro Cytokine, Chemokine, and Growth (Bio-Rad, U.S) kits as per manufacturer's instruction. In total a panel of 48 cytokines was used to assess cytokine/chemokine profiles these included pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12p40, IL-18, MIF, TNF-α, TNF-β and TRAIL), chemokines (CTACK, Eotaxin, GRO-α, IL-8, IL-16, IP-10, MCP-1, MCP-3, MIG, MIP-1α, MIP-1β, RANTES, INF-α2), growth factors (B-NGF, FGF-basic, G-CSF, GM-CSF, HGF, IL-3, IL-7, IL-9, LIF, M-CSF, PDGF-BB, SCF, SCGF-β, SDF-1α, VEGF, adaptive cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-13, IL-15, IL-17, IL-2Rα) and anti-inflammatory cytokines (IL-10 and IL-1Rα).

3.4.1. Multiplex ELISA plate preparation

Thawed CVLs and plasma specimens were filtered by centrifuging at 10,000rpm for 10 minutes in Spin-X® 22µm centrifuge tubes (Costar®, USA). A total volume of 300µl per filtered sample was placed in wells of a v-bottomed 96-well (Costar®, USA)

"master plate" and kept at 4^0C until use. Samples were plated on the master plate to limit variability in time of sample exposure to magnetic beads. A total of 6 master plates were prepared, 6 plates for the 21 and 27-Plex Pro, Chemokine and Growth kits. Each Luminex plate included 4 duplicates genital samples to investigate reproducibility of measurements within a plate. Four genital samples were each replicated on a subsequent plate to investigate reproducibility of cytokine measurements across plates. A representative luminex plate is depicted in table 1 below.

Table 1. Representative diagram for plate design showing how standards, blanks, controls and CVLs were plated.

	1	2	3	4	5	6	7	8	9
A	S1	S1	S9	S9	CVL-5	CVL-13	CVL-21	Samples from the previous plate	Samples for the next plate
B	S2	S2	S10	S10	CVL-6	CVL-14	CVL-22		
C	S3	S3	Blank	Blank	CVL-7	CVL-15	CVL-23		
D	S4	S4	Control	Control	CVL-8	CVL-16	CVL-24		
E	S5	S5	**CVL-1**	**CVL-1**	CVL-9	CVL-17	CVL-25		
F	S6	S6	**CVL-2**	**CVL-2**	CVL-10	CVL-18	CVL-26		
G	S7	S7	**CVL-3**	**CVL-3**	CVL-11	CVL-19	CVL-27		
H	S8	S8	**CVL-4**	**CVL-4**	CVL-12	CVL-20	CVL-28		

3.4.2 The Multiplex ELISA Assay

Figure 2.1 depicts a flow diagram summarizing the assay procedure. Briefly assay buffer, wash buffer and standard were brought to room temperature (RT), while other kit reagents including detection antibody, detection antibody diluent, streptavidin-phycoerythrin and the magnetic beads were kept at 4ºC until use. Briefly, a volume of 50µl/well 10x magnetic beads were added to a 96 well flat-bottomed plate and washed twice with 100µl of Bio-Plex™ Wash Buffer using Bio-Plex Pro™ II (BIO-RAD) automated washing station. Once prepared, the standards and blanks were each vortexed (VORTEX GENIE 2, Scientific Industries) for a further 10 seconds before adding 50µl to an appropriate respective well on the flat-bottomed plate. Samples from the master plate were resuspended using a pipette before transferring 50µl/sample to the designated

well. Once sealed and covered with foil to protect from light, the plate was incubated at RT in the dark on an Orbital shaker SSL1 (Stuart®) at 850rpm for 30minutes. Thereafter samples were washed 3 times with 100ul Bio-Plex™ Wash Buffer using Bio-Plex Pro™ II (BIO-RAD) automated washing station. A volume of 25µl detection antibody was added per well and the plates were sealed and covered with foil prior to being incubated at RT on a mechanical shaker at 850rpm for 30minutes.

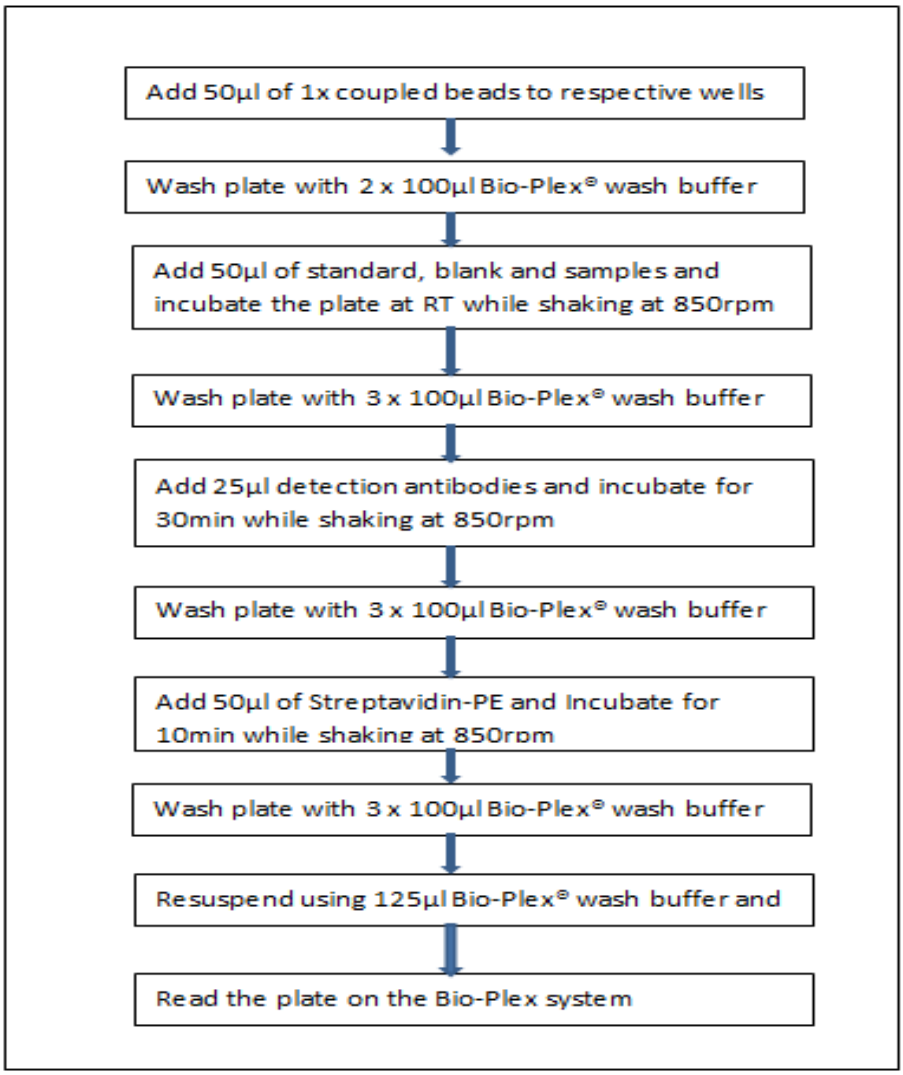

Figure 2.1. Summary of the assay procedure (adapted from the manufacturer's protocol; BIO-RAD).

Plates were washed 3 times as before, and a volume of 50ul PE-conjugated streptavidin was then added/well. The plates were incubated at RT on a mechanical shaker at 850rpm for 10minutes. Thereafter plates were washed 3 times as before, and a volume of 125ul/well Assay buffer was added in preparation for data acquisition using the Bio-Plex® MAGPIX™ MultiPlex Reader (XMAP TECHNOLOGY, BIO-RAD).

3.4.2 Standard preparation

Briefly, a volume of 500µl of the standard diluent was added into the lyophilized standard and vortexted intensively for 3 seconds before being incubated on ice for 30 minutes. A set of 10 polypropylene tubes (BD Falcon™) were used for the standards. A volume of 72µl was added to the first tube and subsequently a volume of 150µl of the standard diluent was added to the remaining tubes. After incubation the reconstituted standard was vortexed and 1:4 serial dilutions was performed by transferring 128µl to the first tube and subsequently transferring a volume of 50µl between each tube.

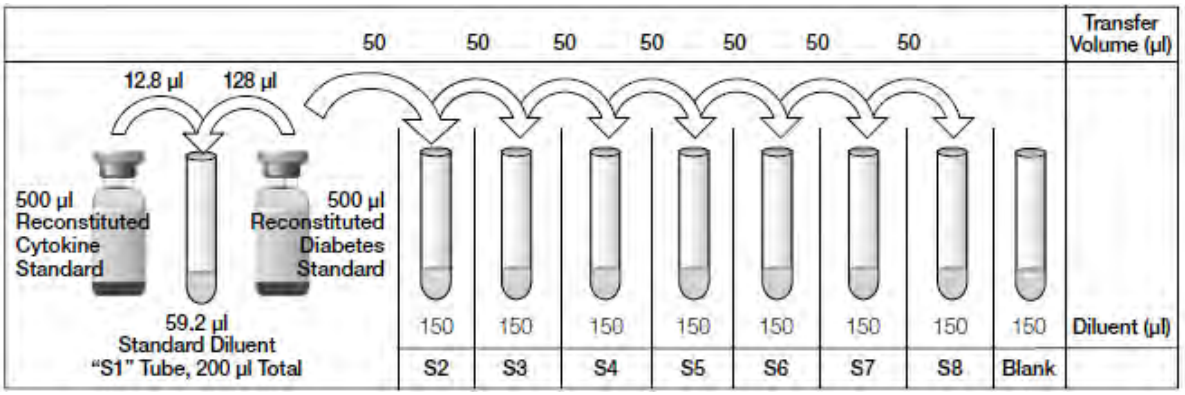

Figure 2.2: Preparation of standards (adapted from the manufacturer's protocol; BIO-RAD).

3.4.3 Bead preparation

Coupled beads were prepared by adding a volume of 575µl of 10X beads to a volume of 5175µl of assay buffer to make up a total volume of 5750µl. The working bead

solution stock was vortexed at medium speed for 20 seconds and 50µl was added to each well.

3.4.4 Detection antibody preparation

A detection antibody solution was diluted by adding a volume of 300µl of 10X detection antibody to 2700µl of the detection antibody diluent. The working antibody stock was vortexed for a period of 15-20 seconds before transferring 25µl to each well.

3.4.5 Streptavidin-PE preparation

A working stock of Streptavidin-PE was prepared by adding a volume of 60µl 100x beads to a volume of 5940µl of the assay buffer. A volume of 50µl/well vortexed Streptavidin-PE working stock was added to each well.

3.5 STI testing

Swabs were collected and screened at National Institute for Communicable Diseases (NCID; Johannesburg, South Africa) to determine the STI prevalence of *N. gonorrhoeae, C. trachomatis, T. vaginalis* and *M. genitalium* within the cohort using multiple polymerase chain reaction (multiplex PCR).

3.6 Statistical Analysis

Statistical tests were performed on GraphPad prism version 5. Mann–Whitney U tests and Chi square tests were performed for independent sample comparisons. Adjustment for multiple comparisons was performed using a step-down approach. All tests were 2-tailed, P value < 0.05 were considered significant

CHAPTER 4. RESULTS

4.1 Clinical and socio-behavioral characteristics of the study participants

The baseline socio-demographic and clinical characteristics of the participants are summarized in Table 2A. and 2B. below. A total of 166 participants were included in this study. The mean age of women in this study was 29 years, and had experienced their first sexual encounter at the mean age of 18 years. Less than 5% of women attained a tertiary qualification. All but one participant had genital swabs available for STI diagnosis (n=165). The overall prevalence of laboratory diagnosed STIs (*N. gonorrhoeae*, *T. vaginalis*, *C. trachomatis* and/or *M. genitalium*) was 18.7%. within women in this cohort (n=165). *C. trachomatis* had the highest prevalence at 6.7% (n=11), followed by M. *genitalium* at 4.8% (n=8) and N. *gonorrhoeae* and T. *vaginalis* both at 3% (n=5). In addition, condom usage was substantially below 50%. The overall immune T cell activation status was measured on $CD3^+$, $CD4^+$ and $CD8^+$ population using flow cytometry in 120 eligible women. Activation markers $Ki67^+$, $CCR5^+$ and dual expression of $CD38^+$/HLA-DR^+ were measured on these T cell subsets. The frequencies of $CCR5^+$ and $CD38^+$/HLA-DR^+ activation markers were higher on $CD4^+$ T cell sub populations, with median (IQR) frequencies of 35.3% (0.30-51.53%) and 2.40% (0.5-15.95%) observed respectively.

Table 2A. Baseline clinical and socio-behavioral characteristics in the CAPRISA 008 study.

Characteristic	**Value**
Number of participants (N)	166
Age (mean±SD)	29.65±5.61
Education complete [n (%)]**:**	
Less than primary school	40.5%,(66)
Primary school complete	2.5% (4)
High school complete	52.2% (85)
Tertiary complete	4.9% (8)
Sexual behavior:	
Age (year) at first sexual encounter [median (IQR)]	18 (16-19)
Condom usage [% (n)]:	
Always	37.4% (61)
Sometimes	49.7% (81)
Never	12.9% (21)
STIs tested [%, (n =165)]**:**	
Prevalence of *chlamydia trachomatis*	6.7% (11/165)
Prevalence of *Neisseria gonorrhoeae*	3% (5/165)
Prevalence of *Mycoplasma genitalium*	4.8% (8/165)
Prevalence of *Trachomonas vaginalis*	4.8% (8/165)
Overall sexually transmitted infections (%)	18.07%

Abbreviations: SD: standard deviation, IQR: interquartile range, STIs: Sexually transmitted infections

Table 2B. Baseline activation in the $CD3^+$, $CD4^+$ and $CD8^+$ population from live lymphocytes in the genital mucosa of CAPRISA 008 participants (n=120).

Cellular phenotype	Median % (IQR)
Frequency of live $CD3^+$ from lymphocytes	11.65 (5.53-21.53)
$CD3^+$ T cell population	
$CD3^+Ki67^+$	30.2 (0.371-48.05)
$CD3^+CCR5^+$	33.05 (20,08-59.25)
$CD3^+CD38^+$/HLA-DR^+	2.17 (0.40-6.78)
$CD4^+$ T cell population	
$CD3^+CD4^+$	45 (33.45-55.38)
$CD4^+Ki67^+$	32.25 (0.30-51.53)
$CD4^+CCR5^+$	38.3 (16.78-60.40)
$CD4^+$CD38/HLA-DR^+	2.40 (0.50-15.95)
$CD8^+$ T cell population	
$CD3^+CD8^+$	26.3 (16.18-36.33)
$CD8^+Ki67^+$	44.5 (0.00-71.35)
$CD8^+CCR5^+$	33.75 (17.8-55.18)
$CD8^+$CD38/HLA-DR^+	1.44 (0.00-11.45)

4.2 Cellular activation and inflammation in CAPRISA 008 participants.

Healthy women from sub-Saharan Africa are known to have increased levels of immune activation compared to women in the United States (Cohen *et al.,* 2010). Immune activation has been found to be a significant factor in HIV acquisition (Naranbhai *et al.,* 2012) and disease progression (Hazenberg *et al.,* 2003). The presence of activated $CD4^+$ T cell targets for HIV infection at the genital tract may partially explain the increased risk of HIV acquisition in these women. In this study and others, high levels of T cell activation are defined by the dual expression of $CD38^+$ and HLA-DR^+ activation markers (Jarrett *et al.,* 2015, Jaspan *et al.,* 2011). To compare women with low and high levels of T cell activation at the genital tract, women with high levels of immune activation were defined as those with $CD38^+$/HLA-DR^+ frequencies above the 75^{th} percentile (n=30), while those below the 25^{th} percentile (n=30) were defined as having a low activation status (Figure 3.1 A, B & C).

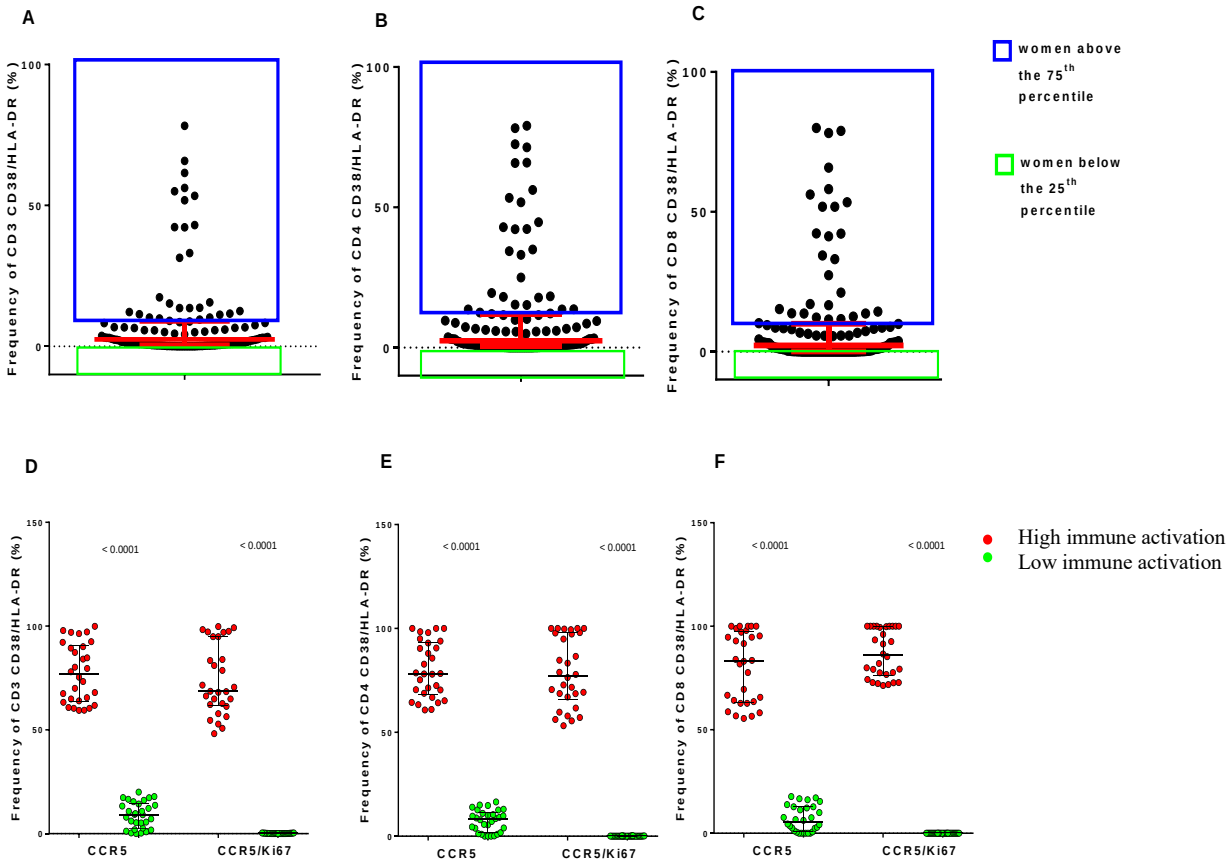

Figure 3.1: The distribution frequency of activated T cells defined by dual expression of CD38$^+$ and HLA-DR$^+$ on each patient and the comparison of genital immune T cells between women with high and low activation status. A, B & C is a scatter plot showing women who had high (blue box) and low (green box) T cell activation status on CD3$^+$ and CD4$^+$ (n=30 both 75th and 25th percentiles) and CD8$^+$ [n=30 (75th percentile), n=35 (25th percentile] in the cervix (Figure 3.1A, B & C respectively). A scattered dot plot showing the frequency of activated (CD38$^+$/HLA-DR$^+$); CD3$^+$, CD4$^+$ and CD8$^+$ T cell subpopulations expressing either CCR5$^+$ or both Ki67$^+$/CCR5$^+$ between women with high (red dots corresponding with the blue box; 75th percentile CD38$^+$HLA-DR$^+$) and women who had low (green dots corresponding with the green box; 25th percentile CD38$^+$HLA-DR$^+$) immune activation status (Figure 3.1D, E & F). The dark solid lines represent the median, upper and lower quartile range T-cell activation status which were compared using the Mann-Whitney U test. The median and interquartile ranges are represented by a solid line. P values ≤ 0.05 were considered significant

Women with high T cell activation status had elevated frequencies of $CD4^+$ T cells expressing $CCR5^+$ ($p<0.0001$) compared to women with low T cell activation status (Figure 3.1B). Similarly, women with high activation status had significant proportions of proliferating ($p<0.0001$) $CD4^+CCR5^+$ T cells at the genital tract (Figure 3.1D). These data identify women with high levels of activation as potentially more at risk for HIV infection by virtue of their increased frequencies of activated and replicating targets for HIV infection. The same trend was observed in $CD3^+$ and $CD8^+$ T cell populations, where women who had high T cell activation status had significant proportions of $CD3^+$ $CCR5^+$ ($p<0.0001$) and $CD8^+CCR5^+$ ($p<0.0001$) T cells. Proliferating $CD3^+CCR5^+$ ($p<0.0001$) and $CD8^+$ $CCR5^+$ ($p<0.0001$) T cell were also significantly elevated in women who had T cell activation status (Figure 3.1E & F).

To assess differences between women with high and low T cell immune activation status, cytokine/chemokine profile and the prevalence of STIs were measured in each group. There were no overwhelming significant observations in cytokine/chemokine concentration expression profile between women with high immune and low immune activation at the genital tract. However, women with highly activated overall $CD3^+$ T cell status had significantly higher concentrations of pro-inflammatory cytokines MIF ($p=0.0217$) and IL-12p70 ($p=0.0173$), the chemokine MCP-1 ($p=0.0024$), and the regulatory cytokine IL-10 ($p=0.0064$) compared to women with a low T cell activation status (Appendix: Table 3.3.2 A). Only the growth factor M-CSF, and the adaptive cytokine IL-17 were reduced in women with high activation status relative to those with low levels of $CD3^+$ T cell activation (Appendix: Table 3.3.2 A). In women with highly activated $CD4^+$ T cell populations, significantly higher concentration of cytokines Basic FGF ($p=0.0491$), IL-10 ($p=0.0362$), MCP-3 ($p=0.0180$), IL-13 ($p=0.0468$), and MIF ($p=0.0235$) were observed relative to women with low levels of activation; and lower levels of chemokine M-CSF and b-NGF were observed (Figure 3.2; Appendix: Table 3.3.2. B). The cytokines MIF ($p=0.0056$), IL-10 ($p=0.0168$), IL-12p70 ($p=0.0145$),

MCP-1 (p=0.0326) and IL-5 (p=0.0123) were elevated in women with high levels of $CD8^+$ T cell activation, while concentrations of M-CSF (p=0.0229), b-NGF (p=0.0186) and IL-15 (p=0.0203) were reduced relative to women with low levels of $CD8^+$ T cell activation (Appendix: Table 3.3.2 C). However, after adjustment for multiple comparisons none of the observations maintained statistical significance. Interestingly, none of the cytokines associated with $CCR5^+$ recruitment (MIP-1a, MIP-1b, RANTES, IP-10) differed in concentration between women with high and low levels of cellular activation. Further, there was no correlation between $CCR5^+$ frequencies and concentrations of these cytokines (data not shown).

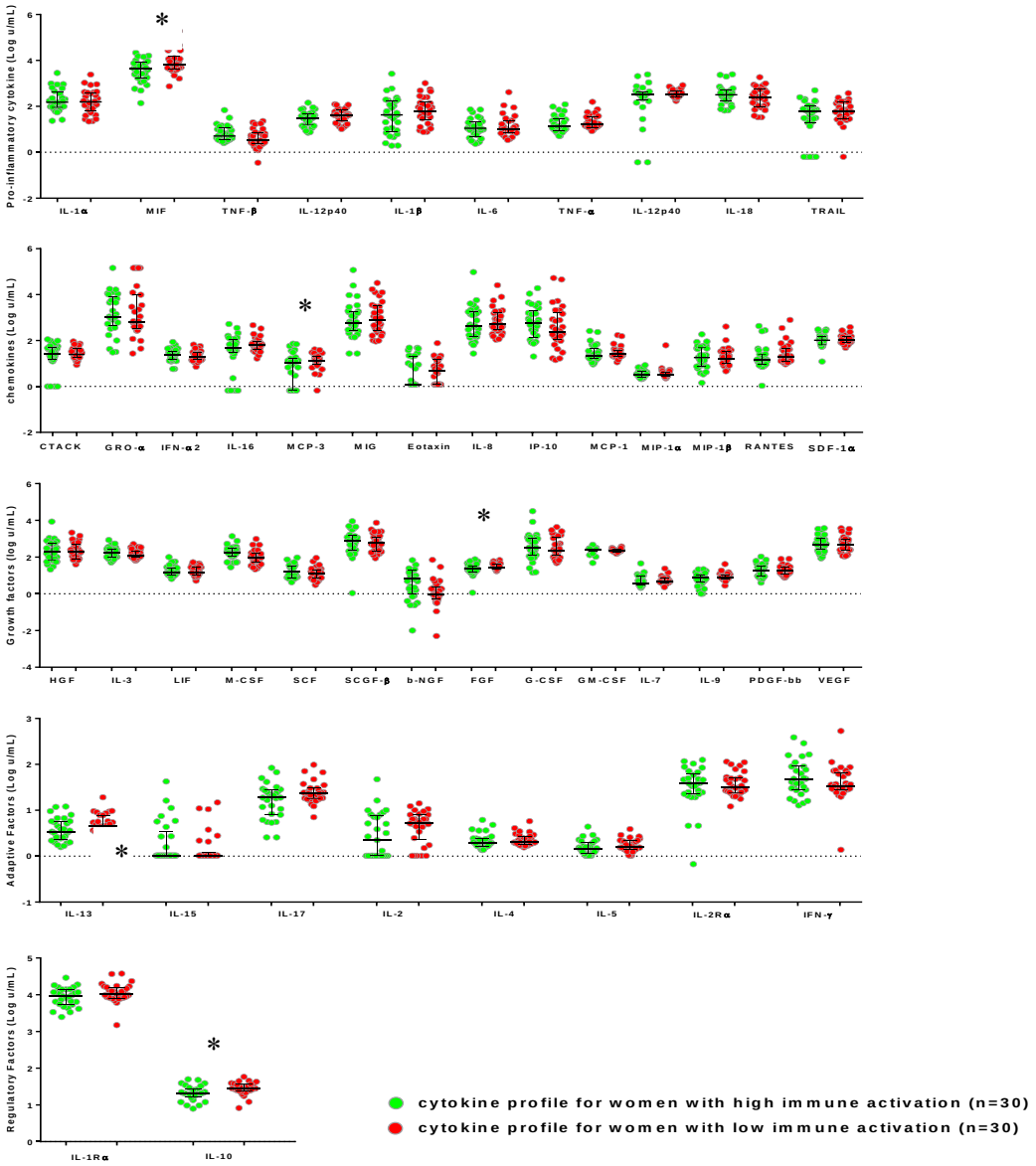

Figure 3.2. Representative diagram showing the cytokine/chemokine expression between women with high and low T cell immune activation status in the CD4$^+$ T cell population. A scatter plot of cytokine profile between women with high immune (green dots) and low immune (red dots) activation at the genital tract. The dark solid lines represent the median, upper and lower quartile range. The cytokine profile between women with high and low immune activation was compared using the Mann-Whitney U test. An asterisk (*) represent significant P values ≤ 0.05 were significant prior to adjustment for multiple comparisons, however none of the observations upheld multiple comparison testing.

Another biological factor that might explain high incidence of HIV rates in young South African women include genital inflammation (Arnold *et al.,* 2016, Masson *et al.,* 2015). Cytokines recruit leukocyte subpopulations selectively into a site of inflammation (Turner *et al.,* 2014). Chemokines, including MIP-1α, MIP-1β, RANTES and IP-10, have been reported to possess chemotactic activity for T cells (Telda *et al.,* 1996). While cytokines play an essential role in cell signaling when mediating an immune response, these studies suggest that HIV is able to take advantage of the immune response to induce a progressive infection (Kahle *et al.,* 2015, Li *et al.,* 2009).

Previous data from CAPRISA showed that the risk of HIV acquisition was significantly higher in women with inflammation at the genital tract (Masson *et al*., 2015), so genital inflammation was investigated in women with high and low levels of cellular activation in this study. Genital inflammation was defined by elevated levels of at least 5 of 9 pro-inflammatory (IL-1α, IL-1β, IL-6 and TNF-α) and chemotactic (MIP-1α, MIP-1β, IP-10, IL-8 and MCP-1) cytokines above the 75th percentile as described by (Masson et al., 2015). By this definition, 17.5% (n=20) of women had genital inflammation compared to 82.5% (n=94) of women who had no genital inflammation.

Furthermore, when the relationship in the frequency of activated CD4^{+} T cell population was compared between women with inflammation (n=20) and those without inflammation (n=94), data shows that there was no statistical significant difference between the two groups, Figure 3.3.

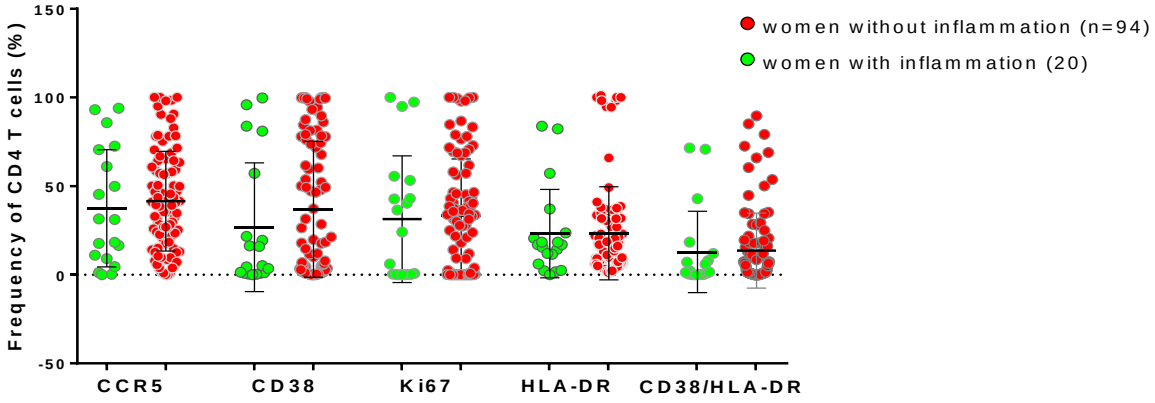

Figure 3.3. Representative diagram of the relationship between activated CD4$^+$ T cell population between women with inflammation and those who did not have inflammation. A scatter plot showing a comparison of activation markers CCR5$^+$, CD38$^+$, Ki67$^+$, HLA-DR$^+$ and dual expression of CD38$^+$/HLA-DR$^+$ on CD4$^+$ T cell population between women with inflammation (green dots) and those without inflammation (red dots). The comparison was done using the Mann-Whitney U test. The black solid lines represent the median, upper and lower quartiles. P value ≤ 0.05 were considered significant

4.3 Sexually transmitted infections as potential drivers of genital immune activation and inflammation.

To investigate an association between inflammation and STIs, the prevalence of common discharge-associated STIs *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis* and *M. genitalium* was measured using an established multiplex PCR assay at the National Institute for Communicable Diseases. The cases of STIs were significantly higher in women with inflammation (n=31) for *C. trachomatis* (19.4%), *T. vaginalis* (16.1%) or any combination of any STI (p=0.0034, p=0.0024 and p=0.0002 respectively) compared to women with no inflammation(n=134) (Table 3), suggesting that inflammation is strongly associated with STI at the genital tract, and fosters an environment conducive to HIV acquisition.

Table 3. Prevalence of STIs in women with and without genital inflammation.

STI	Inflammation present (n=31)	Inflammation absent (n=134)	P-value	Adjusted P value*
N. gonorrhea	6.5% (n=2)	2.2% (n=3)	0.233	1.165
C. trachomatis	19.4% (n=6)	3.7% (n=5)	0.0017	0.0085*
T. vaginalis	16.1% (n=5)	2.2% (n=3)	0.0012	0.006*
M. genitalium	6.5% (n=2)	12.6% (n=6)	0.0645	0.3225
Any STI	41.9% (n=13)	12.7% (n=17)	0.0001	0.0005*

Women who have STIs and inflammation were compared to women with STIs but without inflammation using Chi-square test. P value < 0.05 was significant. After adjustment for multiple comparisons that's values that retained significance are represented by an asterisk (*).

The association between STIs and T cell immune activation status was then investigated by comparing the prevalence of STIs among two groups of women: those who had high immune and low immune activation status. While more women with STI had high levels of $CD3^+$ T cell activation compared to those with low activation (Table 4), this was not statistically significant. Although this observation was not statistically significant, larger studies have shown that there is an association between the status of immune activation and STIs.

Table 4. The association between the presence of STIs and T cell immune activation status.

	STI+ Women with high immune activation	STI+ Women with Low immune activation	P value
CD3 population	23% (n=7/30)	13% (n=4/30)	0.984
CD4 population	23% (n=7/30)	13% (n=4/30)	0.984
CD8 population	27% (n=8/30)	12.9% (n=4/35)	0.72

The level of immune activation was compared in women with STI using Chi-square test. P value < 0.05 was significant.

Furthermore, when the relationship between STIs and the frequency of $CD4^+$ immune T cells expressing $CCR5^+$ observed at the genital tract was investigated, women who had *C. trachomatis* [p=0.0040, (n=8)], *T. vaginalis* [p=0.0105, (n=8)] or Any STI [p=0.0098, (n=22)] had significantly higher frequencies of $CD4^+$ T cells expressing $CCR5^+$ compared to women without STIs. But after adjusting for multiple comparisons, there was no significant difference in the frequency of $CD4^+CCR5^+$ between women infected and none infected with *T. vaginalis* (p=0.0525). There were no significant differences in the frequencies of $CD4^+CCR5^+$ T cells between women who had *M. genitalium* (n=4) and *N. gonorrhea* (n=4) and those who did not have these STIs (Table 5).

Table 5. The relationship between STIs and the frequency of $CD4^+$ immune T cells expressing $CCR5^+$

	$CD4^+CCR5^+$ frequency in STI+ women **Median (IQR)**	**$CD4^+CCR5^+$ frequency in STI- women** **Median (IQR)**	**P value**	**Adjusted P value**
N. gonorrohoeae	49.4 (22.8-78.5)	42.5(18.1-65.5)	0.715	3.575
C. trachomatis	79.3 (47.3-91.8)	39.2 (17.0-63.5)	0.0040	0.020*
T. vaginalis	70.7 (53.2-85.4)	40.8 (17.6-64.6)	0.0105	0.0525
M. genitalium	50.5 (9.21-95.8)	42.5 (20.0-65.5)	0.770	3.850
Any STI	70.7 (35.8-84.8)	43.5 (18.3-66.2)	0.0098	0.049*

Women with and without STIs were compared using Mann-Whitney test. P value < 0.05 was significant. After adjustment for multiple comparisons the values that retained significance are represented by an asterisk (*).

Previous studies have demonstrated that women who had elevated genital inflammatory cytokines has a significantly higher risk of HIV acquisition (Masson *et al.,* 2015). In a study conducted by Mlisana *et al.,* (2012) the presence of STIs at the genital mucosa was both associated with the upregulation of inflammatory cytokine and increased risk of HIV acquisition. Furthermore, Smith & Garber (2015), demonstrated the role of STIs in cellular recruitment of HIV target cells in mice vaginal tissue. In summary, data in this study clearly supports the role of STIs as potential drivers of both immune activation and inflammation at the genital tract.

CHAPTER 5: DISCUSSION AND CONCLUSION

Recent studies have demonstrated that increased concentrations of inflammatory cytokines and heightened immune T cell activation are among the key components in mounting an effective immune response, however in the female genital tract both these interconnected physiological processes are known to potentially increase the risk of HIV acquisition by up to three times (Masson *et al.,* 2015, Naranbhai *et al.,* 2012). In this study, HIV negative women with high T cell immune activation status defined by dual expression of $CD38^+$ and HLA-DR had significantly higher frequencies of $CD4^+$ T cells expressing $CCR5^+$ and proliferating $CD4^+$ $CCR5^+$ T cells at the genital tract compared to women with a low activation status. These data identify women with high levels of activation as potentially more at risk for HIV infection by virtue of their increased frequencies of activated and replicating targets for HIV infection. In addition, based on the definition of inflammation (Masson *et al.,* 2015), some of the women studied had genital inflammation (17.5%) while the majority of women had none (82.5%). Furthermore, this study also found increased prevalence of *C. trachomatis* and *T. vaginalis* in women with genital inflammation compared to those without inflammation. This suggests that infection with either STI potentially drives inflammation in the female genital tract.

Strong evidence has shown that infections elicit an inflammatory response in the genital tract, resulting in the upregulation of pro-inflammatory cytokines and the recruitment of immune effector cells to site of infection. Univariate analyses do not reflect this, as there was no remarkable association observed between individual cytokines and cellular markers of immune activation in the genital mucosa. In addition, there were no notable observations between women with high and low T cell immune activation status attributed to by individual cytokines/chemokines.

Women who had highly activated $CD3^+$ T and $CD8^+$ T cells, had higher concentrations of pro-inflammatory cytokines MIF and IL-12p70.Whereas women who had highly

activated $CD4^+$ T cells had higher concentration of MIF. IL-12p70 is from the IL-12 family cytokines, which play a role in inducing the pro-inflammatory responses (Berger, 2000, Katrina *et al.,* 2009). Counteracting this effect, might have been a high concentration of IL-10 that was observed in the genital tract of women with high immune activation ($CD38^+$/HLA-DR^+: $CD3^+$, $CD4^+$ and $CD8^+$). IL-10 is produced by many cell types including dendritic cells, macrophages, B cells, T cells and also by innate and regulatory cells (Brooks *et al.*, 2010). The anti-inflammatory cytokine IL-10 limits the production of pro-inflammatory cytokines among others including IL-1α, IL-1β, IL-6, IL-18 and TNF α, and also the production of chemokines such as RANTEs, MCP-1, IL-8 and MIP (Joss *et al.,* 2000, Couper *et al.*, 2008).

Interestingly, women with elevated $CD38^+$/HLA-DR^+ on $CD4^+$ T cell population had higher concentrations of monocyte chemotactic protein 3 (MCP-3). MCP-3 plays a dual role in chemo-attracting and activation of T lymphocytes, monocytes and eosinophils among others (Ward *et al.,* 1998). In a study were they investigated the production of MCP-3 in an inflamed mucosa, it was observed that MCP-3 is enhanced in regions where inflammation is present (Wedemeyer *et al.,* 1999). Though the recruitment of immune cells to inflamed areas is imperative for the host defense (Mack *et al.,* 2001). $CD4^+$ T cell bear more than one receptor (Zhang *et al.,* 2010), $CD4^+$ T cells recruited by MCP-3, may have $CCR5^+$ receptors. As a consequence, this recruitment in the genital mucosa may result in inflamed areas rich with HIV target cells. Though the findings in this study were not statistically significant, other factors might be potentially blocking the effect of individual cytokines on immune activation. The role of cytokines is promiscuous and not limited to T lymphocytes they as they also act on other cell types (Ward and Westwick, 1998).

Women who had STIs were mostly found in the group characterized by high immune T cell activation status (highly expressing $CD38^+$/HLA-DR^+: $CD3^+$, $CD4^+$ and $CD8^+$ population) compared to women with low immune T cell activation. Although this

observation was not statistically significant, the presence of STIs at the genital tract potentially plays a crucial in the activation status and HIV pathogenesis.

Viral entry of HIV into $CD4^+$ T cells is dependent on chemokine co-receptors; therefore, expression of CC receptors ultimately influences host susceptibly (Wu et al., 1996). When comparing the frequencies of potential HIV targets on $CD4^+$ $CCR5^+$ bearing T cells in the genital tract between women with STIs and those without STIs, the data showed that women who were infected with *C. trachomatis,* or any combination of STIs similarly had significantly higher frequencies of $CD4^+CCR5^+$ T cells compared to women without STIs in the genital mucosa.

Previous animal studies have shown that high expression of $CXCR3^+$ and $CCR5^+$ by $CD4^+$ T cells was associated with clearance of *C. trachomatis* and that these $CXCR3^+$ and $CCR5^+$ chemokine receptors are essential for promoting the recruitment of immune cells to resolve the infection (Belay et al., 2002, Olive et al., 2011). Even though in this study the number of women who were infected with *C. trachomatis* was low (6.7%), our results are consistent with these findings. Women infected with *C. trachomatis* had significantly higher frequencies of activated $CD4^+$ T cells expressing $CCR5^+$, suggesting that homing of $CD4^+$ T cells is essential in *C. trachomatis*-associated immune responses. In a study conducted by (Wu et al., 1997), reduced infectability of $CD4^+$ T cells correlated with low $CCR5^+$ expression. Hence, women with high frequency of $CD4^+CCR5^+$ are more likely to be susceptible.

Based on a postulated mechanism of chlamydial pathogenesis, a response to a chlamydial infection is initiated and maintained primarily by the host's epithelial cells (Stephens, 2003). The epithelial lining at the genital mucosa serves as a key innate responder by secreting chemokines and cytokines, which results in the influx of immune cells and the formation of immune inductive sites in the submucosa (Darville and Hiltke, 2010). Among the cells present and abundant at the site of infection are the

$CD4^+$ T cells, which serve as targets for HIV infection. As $CD4^+$ T cells seem to play an integral role in the resolution of a chlamydial infection in the genital mucosa, it coherently increases the chances for the establishment of a successful HIV infection in women with a chlamydial infection.

In this study, it was also observed that infection with *T. vaginalis* was associated with inflammation. However, when the relationship between *T. vaginalis* and the frequency of $CD4^+$ immune T cells expressing $CCR5^+$ observed at the genital tract was investigated, data showed that women infected had higher frequencies of $CD4^+CCR5^+$ T cells at the genital. But after adjustment for multiple comparisons this observation lost statistical significance. Immunological responses to T vaginalis infection have been studied both *in vivo* and *in vitro* (Fichorova, 2009). A study by (Kiviat et al., 1985, Fichorova et al., 2006) showed that *T. vaginalis* infection typically induces local cellular immune responses accompanied inflammation in the squamous epithelium in the genital tract. thus our results suggest comparable findings.

However, our study did not find significant differences in $CD4^+$ T cells (expressing CCR5) and inflammatory status in women who were infected with either *N. gonorrhoeae* or *M. genitalium* compared to women not infected by any STI. Previous studies on *M. genitalium* have indicated that this pathogen causes inflammation at the genital tract (McGowin et al., 2009, McGowin et al., 2013). *N. gonorrhea* pathogenesis also results in the induction of an inflamed local environment through the attachment and invasion of epithelial cells at the genital mucosa (McGee et al., 1981). Nonetheless colony opacity-associated (Opa) proteins present on the surface of *N. gonorrhea* are known to bind to the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM)-1, and the binding of these proteins was accompanied by suppression of activation and proliferation of $CD4^+$ T cells (Chen et al., 2001, Boulton and Gray-Owen, 2002). This explains the suppressed immune activation and the influx of $CD4^+CCR5^+$ in women infected with *N. gonorrhea*. The limited evidence to show that there was an association between inflammation and STIs was due to a small sample size. Furthermore, at the time the study was conducted there was no data on bacterial

vaginosis was not available and study did not look ulcerative STIs, as both have been known to cause immune activation and inflammation.

STIs have become a global concern, among other reasons is the role they play in facilitating HIV transmission (Sexton et al., 2005, Mlisana et al., 2012, van de Wijgert et al., 2009). The genital tract mucosa is the predominant portal of entry for sexually transmitted infections. The immune response at this site is essential to provide protective aid against invading pathogens that are encountered for the very first time, as well as provide long-term secondary immunity necessary to confer resistance against STIs. The integrity of the female genital mucosal system is important. While it was shown that 99% of unprotected sexual exposure to HIV do not result in a productive infection (Royce et al., 1997), it may be likely that protection against HIV acquisition is offered by an intact epithelial barrier, as well as mucosal immune factors present in the genital tract. Our data suggest that women with STI will potentially have a genital immune milieu characterized by high pro-inflammatory cytokine levels, and high frequencies of activated T cells, including $CD4^{+}CCR5^{+}$ expressing T cell targets for HIV replication (Miller & Shattock, 2003; H. Ward & Rönn, 2010). Taken together, STI can promote an immune environment conducive to HIV replication.

The high HIV incidence rate in young women (UNAIDS, 2016) can be partially explained by behavioral risks. In a health-care system where there is a syndromic management of STIs, the impact of STIs, especially asymptomatic infections on $CD4^{+}$ T cell activation and inflammation in the genital mucosa needs to be urgently addressed. A recent study has shown that there is a high prevalence and incidence of reported STIs among women in rural and urban areas in KwaZulu-Natal, South Africa (Abbai et al., 2013). To alleviate STIs/HIV burden especially on women understanding intricacies of STI pathogenesis and its role in HIV transmission, is vital to curb the HIV pandemic. As much as it is economical in resource poor countries to effectively manage infectious

diseases, which involves diagnosis, prevention and treatment (Ronald et al., 2006), new interventions are required to be established.

In conclusion, our findings suggest discharge-associated STIs in CAPRISA 008 study participants play a role in recruitment of HIV target cells, are potential drivers of inflammation in the genital tract, and may foster an environment conductive to HIV acquisition. In developing countries, the incidence and prevalence of STI rates are generally high and impose a health burden. This is a result of a variety of factors amongst others include poor health service provision and expensive cost of setting up good quality STI services is costly in resource poor settings. Under these prevailing conditions, it is still critical to find measures to curb STI infections by providing medical interventions for those who are currently infected. These data support the concept of reducing HIV risk by limiting STI-associated inflammation and recruitment of HIV target cells at the genital tract.

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APPENDIX

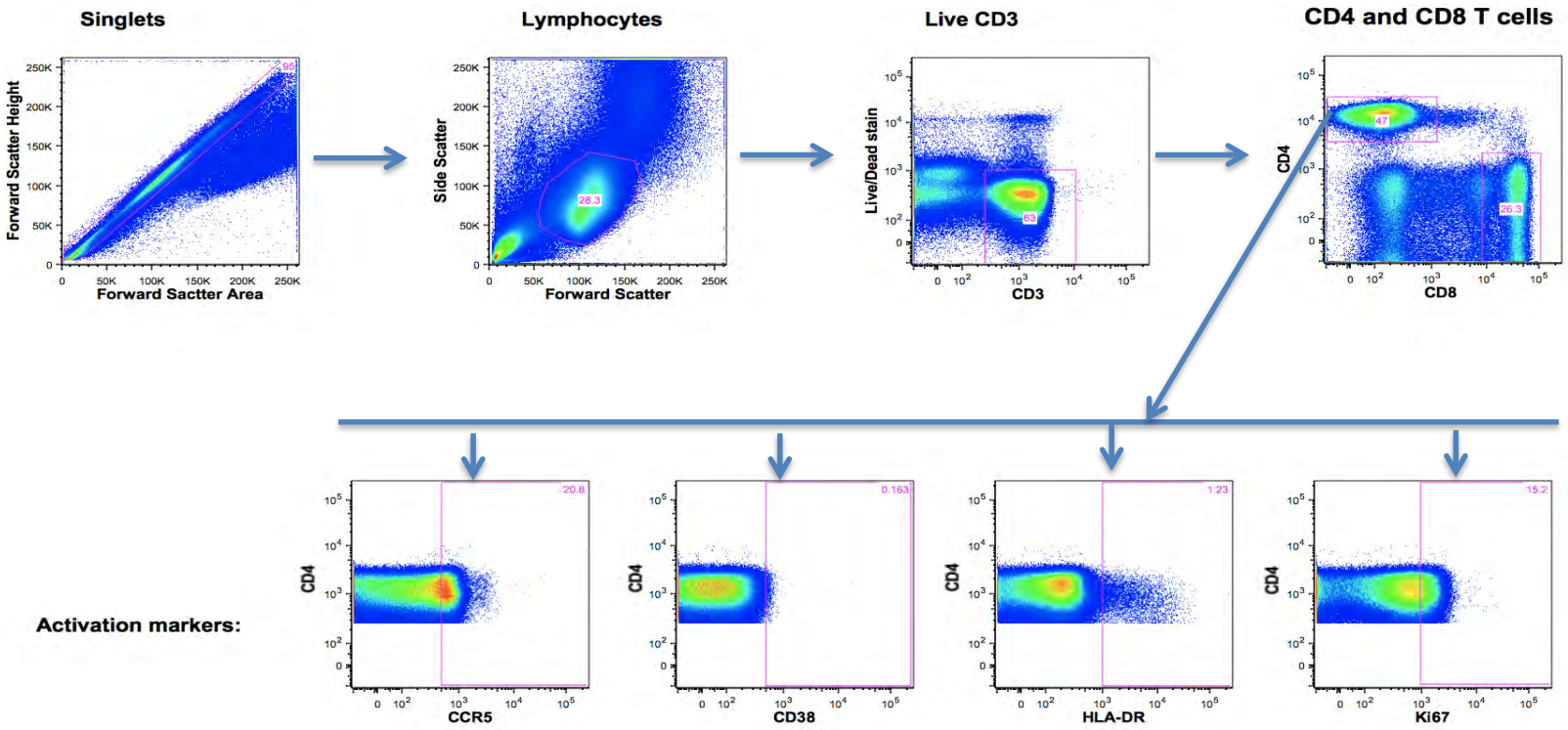

Figure 3.1 Representative plots showing the gating strategy used to define activated T-cell populations by flow cytometry. Peripheral blood mononuclear cells were stained with fluorochrome labeled monoclonal antibodies CD3 (APC-H7), CD4 (PerCP. Cy5.5), CD8 (FITC), CCR5 (APC), Ki67 (BV 700), HLA-DR (PE) and CD14/CD19/Live/Dead stain Fixable Violet Dead Cell Stain (Pacific Blue). A singlet gate was used to exclude cell doublets or cell aggregates. Live $CD3^+$ T cell population was divided into $CD4^+$ and $CD8^+$ subsets. The overall expression of activation markers on $CD3^+$, $CD4^+$ and $CD8^+$ was evaluated. Fluorescence minus one (FMO) were used to determine the positioning of the specific gates.

Table 3.3.2 A: Shows the cytokine/chemokine expression profiles between women with low and high T cell immune activation status in $CD3^+$ population.

Individual cytokine	High immune activation (n=30) Median (IQR)	Low immune activation (n=30) Median (IQR)	P-value
Pro-inflammatoy cytokines			
IL-1α	2.44 (2.04-2.66)	2.17 (1.93-2.46)	0.157
IL-1β	1.9 (1.41-2.25)	1.61 (0.972-2.2)	0.270
IL-6	1.01 (0.861-1.32)	1.01 (0.599-1.44)	0.6182
IL-12p40	2.56 (2.42-2.74)	2.54(2.33-2.79)	0.761
IL-18	2.5 (1.98-2.81)	2.51 (2.23-2.67)	0.766
MIF	3.86 (3.63-4.44)	3.65 (3.27-3.950	0.0217
TNF-α	1.23 (1.08-1.46)	1.07 (0.93-1.45)	0.0905
TNF-β	0.713 (0.468-0.884)	0.841 (0.543-1.16)	0.0822
TRAIL	1.81 (1.6-2.2)	1.82 (1.37-2.23)	0.99
IL-12p70	1.65 (1.46-1.86)	1.49 (1.23-1.66)	0.0173
Chemokines			
CTACK	1.53(1.27-1.68)	1.53(1.21-1.72)	0.826
Eotaxin	0.436 (0.0811-1.16)	0.174 (0.0881-1.29)	0.834
GRO-α	2.84 (2.44-4.72)	3.07 (2.37-3.92)	0.817
IL-8	2.73 (2.44-3.23)	2.72 (2.35-3.240)	0.7986
IL-16	1.86 (1.63 -1.95)	1.7 (1.47-2.1)	0.199
IP-10	2.39 (1.93-3.25)	2.76 (2.06-3.160	0.5202
MCP-1	1.46 (1.34-1.580	1.28 (1.19-1.4)	0.0024
MIP-1α	0.5 (0.43-0.615)	0.534 (0.427-0.647)	0.4932
MIP-1β	1.13 (0.915-1.52)	1.26 (0.806-1.66)	0.756
MIG	2.99 (2.54-3.73)	2.78 (2.15-3.37)	0.162
RANTES	1.32 (1.1-1.74)	1.18 (1.11-1.41)	0.257
INF-α2	1.42 (1.18-1.510	1.42 (1.18-1.6)	0.832
Growth factors			
Basic FGF	1.43 (1.23-1.52)	1.38 (1.23-1.52)	0.131
G-CSF	2.41 (2.22-2.41)	2.45 (1.84-3)	0.732
GM-CSF	2.33 (2.27-2.42)	2.42 (2.29-2.46)	0.092

HGF	2.41 (2.09-2.78)	2.28 (1.73-2.77)	0.352
IL-3	2.23 (2.02-2.35)	2.3 (2.05-2.51)	0.223
IL-7	0.663 (0.605-0.824)	0.592 (0.453-0.958)	0.4532
IL-9	0.886 (0.788-1.04)	0.861 (0.636-1.02)	0.194
LIF	1.39 (1.14-1.47)	1.18 (0.978-1.42)	0.269
M-CSF	2.04 (1.83-2.36)	2.32 (2.04-2.53)	0.0144
PDGF-bb	1.21 (1.07-1.43)	1.28 (1.06-1.55)	0.8381
SCF	1.14 (0.876-1.35)	1.22 (0.869-1.54)	0.5221
SCGF-β	2.71 (2.38-2.99)	2.94 (2.49-3.53)	0.120
SDF-1α	2.1 (1.92-2.21)	2.0 (1.75-2.18)	0.197
VEGF	2.71 (2.51-2.96)	2.62 (2.37-2.98)	0.549
b-NGF	1.45 (1.18-1.510	1.47 (1.18-1.6)	0.832
Adaptive cytokines			
IFN-γ	1.53 (1.45-1.8)	1.71 (1.44-1.98)	0.223
IL-2	0.751 (0.21-0.895)	0.251 (0.0086-0.896)	0.1725
IL-4	0.299 (0.237-0.415)	0.294 (0.225-0.413)	0.675
IL-5	0.217 (0.166-0.323)	0.17 (0.0314-0.284)	0.119
IL-13	0.647 (0.528-0.818)	0.535 (0.362-0.761)	0.109
IL-15	0.647 (0.528-0.818)	0.535 (0.362-0.761)	0.109
IL-17	0.0128 (0.0128- 0.0128)	0.0128 (0.0128- 0.739)	0.0388
IL2Rα	1.65 (1.38-1.81)	1.58 (1.34-1.84)	0.674
Anti-inflammatory cytokines			
IL-10	1.45 (1.39-1.56)	1.32 (1.21-1.46)	0.0064
IL-1Rα	3.99 (3.9-4.16)	4.05 (3.82-4.15)	0.755

The log concentrations of cytokine/chemokine expression profiles in women with low and high which were compared using Mann-Whitney test, an asterisk (*) represents P values ≤ 0.05.

Table 3.3. 2B: Shows the cytokine/chemokine expression profiles between women with low and high T cell immune activation status in $CD4^+$ population.

Individual cytokine	High immune activation (n=30)	Low immune activation (n=30)	P-value
Pro-inflammatoy cytokines			
IL-1α	2.21 (1.94-2.62)	2.17 (1.94-2.36)	0.387
IL-1β	1.69 (1.31-2.34)	1.63 (0.958-2.07)	0.270
IL-6	1.01 (0.833-1.21)	0.991 (0.626-1.23)	0.655
IL-12p40	2.52 (2.42-2.66)	2.54 (2.22-2.65)	0.517
IL-18	2.5 (2.09-2.85)	2.51 (2.24-2.680)	0.924
MIF	3.83 (3.65-4.35)	3.68 (3.25-3.96)	0.0235*
TNF-α	1.21 (1.09-1.36)	1.13 (0.924-1.40)	0.134
TNF-β	0.675 (0.426-0.844)	0.708 (0.539-1.08)	0.173
TRAIL	1.83 (1.6-2.2)	1.7 (1.28-2.04)	0.210
IL12p70	1.61 (1.31-1.82)	1.48 (1.26-1.62)	0.219
Chemokines			
CTACK	1.45 (1.27-1.69)	1.53 1.16-1.75)	0.793
Eotaxin	0.59 (0.0881-1.17)	0.0881 (0.0881-1.30)	0.587
GRO-α	2.82 (2.55-4.87)	3.01 (2.79-3.82)	0.751
IL-8	2.66 (2.43-3.13)	2.64 (2.19-3.26)	0.728
IL-16	1.82 (1.63-1.91)	1.7 (1.42-2.07)	0.878
IP-10	2.30 (2.00-3.11)	2.76 (2.34-3.42)	0.520
MCP-1	1.39 (1.31-1.56)	1.32 (1.22-1.54)	0.119
MIP-1α	0.514 (0.431-0.626)	0.504 (0.395-0.647)	0.858
MIP-1β	1.15 (1.02-1.50)	1.271 (0.950-1.73)	0.405
MIG	2.99 (2.47-3.53)	2.82 (2.51-3.290)	0.741
RANTES	1.27 (1.10-1.60)	1.16 (1.08-1.41)	0.346
IFN-α2	1.4 (1.18-1.51)	1.42 (1.13-1.56)	0.924
MCP-3	1.17 (1.0-1.3)	1.01 (-0.177-1.27)	0.0180*
Growth factors			
Basic FGF	1.42 (1.36-1.61)	1.37 (1.23-1.51)	0.0491*
G-CSF	2.34 (2.04-2.73)	2.38 (1.98-3.04)	0.735
GM-CSF	2.34 (2.28-2.42)	2.41 (2.30-2.46)	0.236
HGF	2.28 (1.94-2.73)	2.28 (1.86-2.74)	0.619

IL-3	2.19 (2.01-2.33)	2.3 (2.00-2.44)	0.368
IL-7	0.67 (0.599-0.828)	0.547 (0.455-0.852)	0.162
IL-9	0.889 (0.798-0.988)	0.887 (0.634-1.02)	0.223
LIF	1.18 (1.04-1.45)	1.18 (1.01-1.4)	0.444
M-CSF	1.97 (1.82-2.21)	2.32 (2.03-2.51)	0.0248*
PDGF-bb	1.255 (1.07-1.42)	1.26 (0.927-1.46)	0.748
SCF	1.09(0.874-1.3)	1.22 (0.881-1.53)	0.269
SCGF-β	2.72 (2.33-3.07)	2.94 (2.32-3.22)	0.425
SDF-1α	2.04 (1.92-2.19)	2.01 (1.8-2.16)	0.226
VEGF	2.649 (2.393-2.948)	2.64 (2.56-3.05)	0.584
b-NGF	2.0 (1.75-2.18)	2.23 (2.02-2.35)	0.0491*
Adaptive cytokines			
IFN-γ	1.51 (1.45-1.78)	1.62 (1.44-2.03)	0.326
IL-2	0.706 (0.279-0.879)	0.117 (0.0086-0.856)	0.101
IL-4	0.2956 (0.245-0.393)	0.288 (0.206-0.387)	0.396
IL-5	0.203 (0.147-0.327)	0.173 (0.0492-0.218)	0.131
IL-13	0.666(0.525-0.858)	0.513 (0.378-0.739)	0.0468*
IL-15	0.0128 (0.0128-0.240)	0.0128 (0.0128-0.435)	0.454
IL-17	1.37 (1.25-1.48)	1.27 (0.919-1.43)	0.117
IL2Rα	1.56 (1.39-1.72)	1.6 (1.32-1.83)	0.838
Anti-inflammatory cytokines			
IL-10	1.44 (1.39-1.56)	1.32 (1.20-1.44)	0.0059*
IL-1Rα	4.01 (3.90-4.22)	3.99 (3.8 -4.14)	0.198

The log concentrations of cytokine/chemokine expression profiles in women with low and high which were compared using Mann-Whitney test, an asterisk (*) represents P values $\leq$ 0.05.

Table 3.3.2 C: Shows the cytokine/chemokine expression profiles between women with low and high T cell immune activation status in CD8 population.

Individual cytokine	High immune activation (n=30)	Low immune activation (n=25)	P-value
Pro-inflammatoy cytokines			
IL-1α	2.39 (2.04-2.64)	2.1 (1.78-2.37)	0.0514
IL-1β	1.9 (1.5-2.27)	1.58 (0.788-2.02)	0.270
IL-6	0.963 (0.827-1.29)	0.962 (0.591-1.35)	0.777
IL-12p40	2.52 (2.42-2.72)	2.53 (2.23-2.73)	0.417
IL-18	2.49 (1.98-2.73)	2.48 (2.22-2.67)	0.878
MIF	3.82 (3.62-4.07)	3.51 (3.22-3.86)	0.0056*
TNF-α	1.23 (1.08-1.46)	1.15 (0.975-1.39)	0.198
TNF-β	0.641 (0.468-0.862)	0.843 (0.522-1.16)	0.133
TRAIL	1.81 (1.48-2.2)	1.86 (1.38-2.25)	0.913
IL12p70	1.65 (1.49-1.84)	1.48 (1.16-1.67)	0.0102*
Chemokines			
CTACK	1.47 (1.27-1.64)	1.48 (1.26-1.75)	0.997
Eotaxin	0.575 (0.0881-1.16)	0.925 (0.0881-1.3)	0.475
GRO-α	2.8 (2.44-4.62)	3.06 (2.6-4.05)	0.835
IL-8	2.73 (2.37-3.23)	2.62 (2.19-3.13)	0.468
IL-16	1.8 (1.61-1.92)	1.7 (1.54-2.04)	0.944
IP-10	2.29 (1.65-3.25)	2.75 (1.92-3.13)	0.564
MCP-1	1.38 (1.32-1.62)	1.31 (1.21-1.46)	0.0326
MIP-1α	0.5 (0.436-0.615)	0.526 (0.427-0.629)	0.831
MIP-1β	1.14 (0.915-1.52)	1.21 (0.788-1.65)	0.771
MIG	2.98 (2.5-3.52)	2.75 (2.15-3.24)	0.127
RANTES	1.32 (1.12-1.63)	1.21 (1.11-1.4)	0.229
INF-α2	1.39 (1.18-1.5)	1.41 (1.17-1.62)	0.917
MCP-3	1.18 (0.997-1.31)	1.11 (-0.177-1.31)	0.210
Growth factors			
Basic FGF	1.43(1.37-1.6)	1.38 (1.29-1.5)	0.0992
G-CSF	2.35 (2.08-2.88)	2.42 (1.69-2.96)	0.921
GM-CSF	2.34 (2.28-2.42)	2.38 (2.22-2.46)	0.578
HGF	2.3 (1.98-2.75)	2.22 (1.75-2.76)	0.353

IL-3	2.16 (2-2.35)	2.28 (2-2.51)	0.345
IL-7	0.663 (0.612-0.803)	0.597 (0.415-0.969)	0.644
IL-9	0.891 (0.807-1.04)	0.911 (0.66-1.02)	0.205
LIF	1.2 (1.14-1.49)	1.17 (0.944-1.38)	0.102
M-CSF	2.03 (1.83-2.36)	2.26 (2.01-2.59)	0.0229*
PDGF-bb	1.21 (1.07-1.4)	1.14 (0.927-1.45)	0.906
SCF	1.1 (0.861-1.34)	1.16 (0.844-1.47)	0.782
SCGF-β	2.7 (2.28-2.98)	2.84 (2.43-3.29)	0.179
SDF-1α	2.04 (1.92-2.19)	1.98 (1.83-2.17)	0.182
VEGF	2.71 (2.52-2.96)	2.57 (2.32-2.95)	0.331
b-NGF	2.43(1.37-2.6)	1.98 (1.29-2.02)	0.0186*
Adaptive cytokines			
IFN-γ	1.53 (1.45-1.8)	1.59 (1.25-1.97)	0.521
IL-2	0.751 (0.21-0.897)	0.504 (0.0086-0.856)	0.227
IL-4	0.299 (0.237-0.415)	0.297 (0.22-0.387)	0.750
IL-5	0.225 (0.185-0.33)	0.104 (0.0107-0.217)	0.0014*
IL-13	0.647 (0.528-0.819)	0.556 (0.34-0.76)	0.147
IL-15	0.0128 (0.0128-0.177)	0.0128 (0.012-0.943)	0.0323
IL-17	1.36 (1.25-1.48)	1.32 (1.24-1.43)	0.598
IL2Rα	1.6 (1.38-1.81)	1.59 (1.32-1.83)	0.559
Anti-inflammatory cytokines			
IL-10	1.45 (1.39-1.55)	1.33 (1.25-1.44)	0.0138*
IL-1Rα	4.01 (3.91-4.16)	4.01 (3.75-4.13)	0.383

The log concentrations of cytokine/chemokine expression profiles in women with low and high which were compared using Mann-Whitney test, an asterisk (*) represents P values ≤ 0.05.

Table 3.3 A: The frequency of cellular markers compared between women who have inflammation and women that do not have inflammation.

Cellular markers (%):	Inflammation present (n=20)	Inflammation absent (n=94)	P-value
Live CD3 T cells	12.8 (6.24-20.7)	11.1 (5.42-22.8)	0.664
$CD3^+$ $CCR5^+$	29.0 (14.8-63.2)	35.3 (19.0-59.1)	0.592
$CD3^+$ $CD38^+$	23.4 (3.12-48)	19 (4.69-64.7)	0.408
$CD3^+$ HLA-DR^+	8.71 (3.75-16)	7.33 (4.56-15.4)	0.883
$CD3^+$ $Ki67^+$	27.5 (0.217-40)	32 (0.385-49.5)	0.818
$CD3^+$ $CD38^+$/HLA-DR^+	2.66 (0.326-8.12)	2.43 (0.632-9.68)	0.804
$CD3^+$ $CCR5^+$/$CD38^+$/HLA-DR^+	1.67 (0.129-5.66)	1.02 (0.244-5.31)	0.957
$CD3^+$ Total activation	57.6 (34.7-80.5)	62.6 (45.4-89)	0.370
CD4 $^+$T cells	46.7 (25.7-60.6)	45 (34.5-54.7)	0.912
$CD4^+$ $CCR5^+$	29 (12.4-68.1)	39.4 (14.9-60)	0.613
$CD4^+$ $CD38^+$	16.1 (1.26-55.2)	14.6 (1.5-77)	0.730
$CD4^+$ HLA-DR^+	16.3 (11.6-22.8)	14.1 (6.2-31.6)	0.654
$CD4^+$ $Ki67^+$	37.5 (0.25-51.5)	31.3 (0.236-51.4)	0.612
$CD4^+$ $CD38^+$/HLA-DR^+	4.05 (0.31-12)	2.41 (0.51-18)	0.978
$CD4^+$ $CCR5^+$/$CD38^+$/HLA-DR^+	2.15 (0.095-8.69)	0.71 (0-8.48)	0.423
$CD4^+$ Total activation	59.7 (31.6-94.1)	66.9 (47.1-96.5)	0.412
$CD8^+$ T cells	20.6 (14.3-27.5)	28 (16-38.1)	0.093
$CD8^+$ $CCR5^+$	36.4 (10.6-60.1)	33 (17.6-56)	0.787
CD8 CD38	18.4 (0.526-72.5)	17.4 (1.11-75.2)	0.730
$CD8^+$ HLA-DR^+	8.04 (6.18-25.8)	10.3 (4.29-21.4)	0.898
$CD8^+$ $ki67^+$	43.7 (0.035-50.2)	42.4 (0-72.1)	0.692
$CD8^+$ $CD38^+$/HLA-DR^+	3.71 (0.0217-8.23)	13 (0-13.2)	0.842
$CD8^+$ $CCR5^+$/$CD38^+$/HLA-DR^+	1.35 (0-5.59)	0.494 (0-7.66)	0.929
$CD8^+$ Total activation	63 (34.9-93.7)	75.6 (47-95.1)	0.442

Table 3.3B: The event count of cellular markers compared between women who have inflammation and women that do not have inflammation.

Cellular marker (count)	Inflammation present (n=20)	Inflammation absent (n=94)	P-value
Live CD3 T cells	1008 (475-2641)	909 (346-1584)	0.201
Live CD3 T cells	241 (82-611)	290 (95-688)	0.932
$CD3^+$ $CCR5^+$	129 (40-457)	118 (46.5-557)	0.992
$CD3^+$ $CD38^+$	88 (41-266)	68 (24-164)	0.385
$CD3^+$ $HLA\text{-}DR^+$	197 (3-356)	146 (3-529)	0.905
$CD3^+$ $Ki67^+$	24 (11-111)	17 (5-77)	0.566
$CD3^+$ $CD38^+/HLA\text{-}DR^+$	14 (3-80)	10 (2-46)	0.957
$CD3^+$ $CCR5^+/CD38^+/HLA\text{-}DR^+$	461 (229-1172)	580 (197-1040)	0.809
$CD3^+$ Total activation	312 (203-783)	377 (130-682)	0.644
CD4 CCR5	112 (30-153)	109 (39-303)	0.259
CD4 $^+$T cells	110 (10-200)	95 (175-315)	0.465
$CD4^+$ $CCR5^+$	48 (31-112)	49 (17.8-148)	0.809
$CD4^+$ $CD38^+$	74 (2-149)	63 (2-247)	0.476
$CD4^+$ $HLA\text{-}DR^+$	11 (2-48)	12 (1-57	0.632
$CD4^+$ $Ki67^+$	3 (1-29)	4 (0-24)	0.965
$CD4^+$ $CD38^+/HLA\text{-}DR^+$	224 (108-291)	208 (74-463)	0.806
$CD4^+$ $CCR5^+/CD38^+/HLA\text{-}DR^+$	177 (69-547)	184 (71-507)	0.977
$CD8^+$ CCR5	30 912-95)	67 (16-222)	0.355
$CD8^+$ $CD38^+$	19 (3-76)	15 (4-107)	0.771
$CD8^+$ $HLA\text{-}DR^+$	14 (6-99)	18 (5-50)	0.713
$CD8^+$ $ki67^+$	28 (0-90)	53 (0-238)	0.375
$CD8^+$ $CD38^+/HLA\text{-}DR^+$	3 (0-21)	3 (0-17)	0.830
$CD8^+$ $CCR5^+/CD38^+/HLA\text{-}DR^+$	1 (0-21)	1 (0-10)	0.788
$CD8^+$ Total activation	89 (28-147)	89 (29-374)	0.523
Sum of $CD4^+$ and $CD8^+$ T cells	518 (272-1669)	649 (242-1157)	0.548
$CD4^+/CD8^+$ ratio	2.53 (1.51-2.92)	1.76 (1.09-2.67)	0.203

Cellular markers in women with inflammation (n=20) were compared to those with women who had no inflammation (n=94) using Mann-Whitney test.

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

13 June 2016

Mr S Mhlungu (209504114)
Discipline of Medical Virology
School of Laboratory Medicine and Medical Sciences
sn.mhlungu@gmail.com

Dear Mr Mhlungu

Study Title: Immune activation and inflammation in the genital tract.
Degree: MMedSc
BREC REF NO: BE326/16 (sub-study of BFC237/010 and BE428/14)

The Biomedical Research Ethics Committee has considered and noted your application received on 06 June 2016.

The conditions have been met and the study is given **full ethics approval.**

This approval is valid for one year from **13 June 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **12 July 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: Liebenberg@ukzn.ac.za
cc postgraduate administrator: dudhrajhp@ukzn.ac.za