Toll-Like Receptor Expression and Function in the Genital Tract of HIV-1 Resistant Commercial Sex-Workers.

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A thesis submitted in partial fulfilment for the degree of master of science in molecular medicine in the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

This is my original work and has not been presented for a degree in any other university.

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DEDICATION

In memory of my father the Late Joshua Fredrick Omange (the greatest scientist in my world), and dedicated to my mother Jane Peninah Omange, and my siblings- Judy, Steve, Cathy, Vicky, Edward, Suzy and George.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
AP-1	Associated protein one
CpG ODN	Cytosine phosphate Guanosine OligoDeoxyNucleotides
CTL	Cytotoxic lymphocytes
DC-SIGN	Dendritic cell- specific intercellular adhesion molecule 3- grabbing nonintegrin
ELISA	Enzyme linked immunosorbent assay
HIV-1	Human immunodeficiency virus type 1
HSP 40	Heat surface protein 40
IFN	Interferon
IRAK	Interleukin 1 receptor- associated kinase
IRF	Interferon regulating factor
LN	Lymph nodes
LPS	Lipopolysaccharide
LTR	Long terminal repeats
MAP	Mitogen associated protein
mDC	Myeloid dendritic cells
MDM	Monocyte Derived Macrophage
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa beta
NK	Natural killer cells

QRT-PCR	Quantitative real time polymerase chain reaction
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
Poly I: C	Polyinosinic: polycytidylic acid (poly I:C)
PRR	Pattern recognition receptors
SIV	Simian immunodeficiency virus
ssRNA	Single strand ribonucleic acid
Th1/Th2	T helper cell one/ T helper cell two
TIR	Toll/Interleukin one receptor
TIRAP	TIR domain containing adapter protein
TIRAP	TIR domain containing adapter protein
TNF	Tumor Necrosis Factor
TLR	Toll-like receptor
TRAF6	TNF receptor associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR domain-containing adapter inducing IFN-β
X4/R5	HIV chemokine coreceptor CXCR4 (X4) and CCR5 (R5)

ABSTRACT

Toll-Like receptors (TLR) are pattern recognition receptors; a component of the innate immune system that are involved in the recognition of pathogen-associated molecular patterns (PAMPs). Recent studies have show that TLRs are able to recognize microbial components for pathogens causing sexual transmitted infections that increase the risk for acquiring HIV-1 virus. TLR7/8 was recently shown to recognize guanine and uridine rich ssRNA with sequence homology to ssRNA from the HIV-1 virus. This study tested the cytokine responses to TLR agonists by cervical mononuclear cells (CMCs) from the human female genital tract. The hypothesis for the study was that HIV-R women (HIV negative >3years) have different cytokine responses in CMCs after TLR stimulation compared to HIV susceptible (HIV negative <3 years) and HIV positive sex workers. The TLR agonists used were *Escherichia coli* Lipopolysaccharide (TLR4 ligand), ssRNA40/LyoVec (TLR 7/8 ligand) and Imiquimod (TLR7 ligand). The amount of cytokine produced was measured from culture supernatants using cytokine bead arrays and ELISA techniques. The main responses detected were TNF-α, IL-10, IFN-γ and IFN-α, with minor levels of IL-6, IL-5, IL-4, and IL- 2 responses. HIV-R women had significantly lower TNF-α responses to *E. coli* LPS compared to HIV susceptible women p=0.0309, significantly lower TNF-α to ssRNA40/LyoVec compared to HIV-P women p=0.0043. A trend for lower TNF-α responses in the CMCs from HIV-P women compared to HIV-S women p=0.0752 was observed. Imiquimod stimulated significantly higher TNF-α responses in HIV-S compared to HIV-P women p=0.0226. HIV-R women had significantly lower IL-10 responses to *E. coli* LPS compared to both HIV-P

p=0.0137 and HIV-S p=0.0196. HIV-R also had significantly lower IL-10 responses to ssRNA40/LyoVec compared to HIV-P p=0.0276 and lower IL-10 responses to Imiquimod compared to HIV-S p=0.0316. The IFN-γ response in HIV-P women was significantly higher than HIV-S following stimulation with ssRNA p=0.0179 and Imiquimod p=0.0032, and higher than HIV-R after Imiquimod stimulation p=0.0446. Overall, CMCs from the genital tract of HIV-1 resistant women had lower proinflammatory responses to TLR ligands compared to HIV susceptible women. The two TLR7 signaling ligands ssRNA and Imiquimod stimulated different cytokine profiles, which indicate the possibility of use of different TLR signaling agents in immune-modulation to alter the cytokine profiles in the genital tract. These results help improve the understanding of the innate cytokine responses by CMCs in the genital mucosa, and may thus point to innate mechanisms that influence susceptibility to HIV-1 infection.

CHAPTER ONE

1. INTRODUCTION

1.1 History of HIV /AIDS

1.1.1 Discovery of Acquired Immunodeficiency Syndrome (AIDS)

In 1981, a clinic in New York city recorded a eight cases of Kaposi's sarcoma a rare skin cancer in young homosexual men, a disease known to occur in elderly people (Hymes 1981). This was accompanied by a sudden rise in number of cases of a rare pulmonary infection, *Pneumocystis cranii* in the same population of homosexuals from California, an observation by the Center for Disease Control in 1981 (CDC 1981). Around this time a number of theories were developed about the possible cause of these opportunistic infections and cancers. Because there was very little that was known about the new contagion, many theories were proposed on probable modes of transmission, many of which were soon thereafter dispelled. After a few months it became clear that this new disease could be transmitted to other populations other than homosexuals, when transmission was observed to occur in intravenous drug users (Masur H. 1981).

1.1.2 Etymology of A.I.D.S

For a while there was no name for the new disease, various groups referred to it in different ways. The CDC generally referred to the new contagion using the ailments that were associated with it such as lymphadenopathy (swollen glands), although on some occasions it was referred to it as KSOI (Kaposi's Sarcoma and opportunistic infections) (CDC 1982; CDC 1982). There was no formal nomenclature for this new contagion; an

article in The Lancet referred to it as “Gay compromised syndrome”(Brennan 1982), others called it GRID (gay-related immune deficiency), AID (acquired immunodeficiency disease), "gay cancer" or "community-acquired immune dysfunction"(Altman 1982; Blade 1982). In June 1982 a report on gay men in Southern California was released showing that the disease might be transmitted sexually. Later that month, the first reports were released of the disease occurring outside of the USA in Haiti, and in a group of haemophiliacs who had received blood transfusions at one point in their lives (CDC 1982; CDC 1982). The occurrence of the disease in non-homosexual populations meant that names such “Gay Compromised Syndrome”, GRID, Gay Cancer had to be dropped.

The acronym AIDS was developed in a meeting in Washington, D.C. in July 1982 (Kher 1982). The CDC went on to describe the syndrome in September 1982 (CDC 1982). The acronym AIDS was appropriate since people acquired the infection rather than inherited it, after acquisition of the infection, it resulted in a deficiency within the immune system; and because it was a syndrome, with a number of manifestations, rather than a single disease. Thus the new disease finally was named the Acquire Immune Deficiency Syndrome abbreviated AIDS and SIDA in spanish.

1.1.3 Isolation and Naming of the virus causing AIDS

In May 1983, doctors at the Institute Pasteur in France reported that they had isolated a new virus, which they suggested might be the cause of AIDS (Barre-Sinoussi F. 1983).

Little notice was taken of this announcement at the time, but a sample of the virus was sent to the CDC. A few months later the virus was named lymphadenopathy-associated virus or LAV, a sample of LAV was sent to the National Cancer Institute (Connor 1988). In direct contradiction to this pronunciation, the CDC then published a report showing that the cause of AIDS had not been clearly determined, but it appeared that the causative agent was transmitted sexually, through contaminated blood transfusion, through contaminated needles, or, less commonly, by percutaneous inoculation of infectious blood or blood products, and the occurrence of the infection in young infants suggested transmission from an affected mother to a susceptible infant before, during or shortly after birth (CDC 1983). In 1984, CDC researchers were able follow a single man who was infected with AIDS then named patient "O" in whom they determined as the source of infection for a number of his homosexual partners, and announced that they had isolated the causative agent for AIDS from patient "O" (Auerbach 1984). The virus isolated from patient "O" was LAV which had been identified a few weeks prior by researchers at the Pasteur Institute. A day later, US Secretary for Health and Human services announced that Dr Gallo, had named the virus Human T-lymphotropic III virus. HTLV-III (Altman 1984). A number of detailed reports were published immediately thereafter, and by Jan 1985 it was clear that LAV and HTLV-III, were actually the same virus. Commercial companies sought patent rights for development of a testing kit for LAV / HTLV III, which started a patent rights war between The Pasteur Institute and Dr. Gallo's team. This war was resolved in May 1986 when the International Committee on Taxonomy of viruses ruled both names out and settled for a moderate name, Human

Immunodeficiency Virus HIV (Coffin J. 1986). The name HIV has since been adapted to be the name for the virus causing AIDS.

1.1.4 Extent of the AIDS epidemic

By 2007, an estimated 25 million deaths had occurred due to HIV/AIDS since the first description of AIDS in the homosexual men in 1981. The UNAIDS report titled, "2008 Report on the global AIDS epidemic," estimated that 30-36 million people were living with HIV/AIDS at the end of 2007. Majority of the infected, 67% were living in Sub-Saharan Africa, an estimated 22million or 37% of world total percentage. The report estimated that 1.9 million new infections would occur in Sub-Saharan Africa and 1.5 million or 38% more deaths due to AIDS would occur in the region. Women accounted for half of all people living with HIV/AIDS worldwide, by then and nearly 60% of these HIV infections occurred in women found in Sub-Saharan Africa. On a global scale the rates of new infections had leveled off since 2000, but the favourable trends were offset by increases in new infections in other countries. In virtually all regions other than Sub-Saharan Africa, HIV disproportionately affects intravenous drug uses, men who have sex with men and sex workers (UNAIDS 2008).

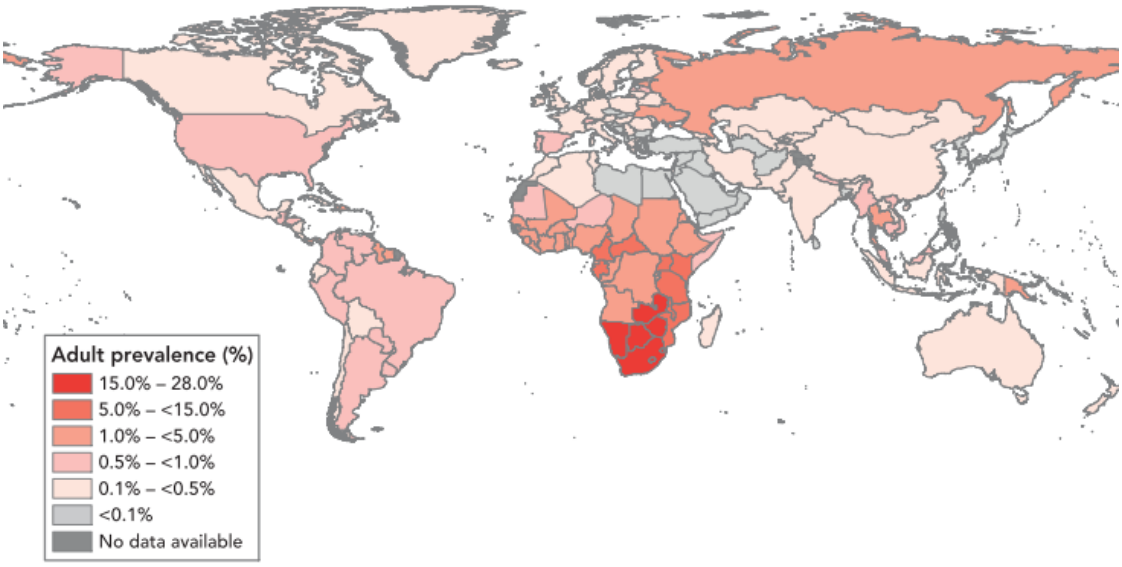


Figure 1.1: A global view of HIV infection, 2007 (UNAIDS)

1.1.5 The different HIV epidemics worldwide

Following the description and identification of the causative agent for the AIDS, researchers embarked on a quest to understand the modes of transmission of HIV, as the epidemic spread to other parts of the world. Reports from Europe suggested that two rather separate AIDS epidemics were occurring. In the UK, West Germany and Denmark, the majority of people with AIDS were homosexual, and many had a history of sex with American nationals. However in France and Belgium AIDS was occurring mainly in people from Central Africa or those with relationship links to the area. This represented the first description of existence of two patterns in the HIV epidemic (Weller 1984). From then, a clearer picture of the different regional HIV epidemics around the world has developed. The main difference in the HIV epidemics by region is in the risk

factors for HIV transmission. To highlight the differences in the importance of risk factors per regional, two epidemics are described below that is, the sub-Saharan Africa and Asia HIV epidemics.

1.1.5.1 The Sub-Saharan HIV Epidemic

In Sub-Saharan Africa which has the highest number of people living with HIV/AIDS, majority acquire the infection through heterosexual transmission. The high rate of sexual transmission has given rise to the world largest population of children living with HIV and orphaned by AIDS. Heterosexual transmission of HIV is related to discordancy in couples, where only one of the partners in the relationship is infected, and this phenomenon is becoming more prevalent in Sub-Saharan Africa. A recent survey in five African countries (Burkina Faso, Cameroon, Kenya, Uganda and Tanzania) found that two thirds of the infected couples were discordant. Strikingly, about 30%-40% more women than men were found to be infected in the discordant relationships (de Walque 2006). Sex work plays an important role but less central to heterosexual transmission in Sub-Saharan Africa, where exceptionally high background prevalence results in substantial transmission during intercourse unrelated to sex work. Injecting drug use is a factor to some extent in several of the HIV epidemics in East and southern Africa, including Mauritius, where the use of contaminated injecting equipment is the main cause of HIV infection (Sulliman 2004). Several recent studies suggest that unprotected anal sex between men is probably a more important factor in the epidemics in sub-Saharan Africa than is commonly thought. In the Kenyan port city of Mombasa, 43% of

men who said they had sex only with other men were found to be living with HIV (Sanders 2007). The main contributor for the new HIV infections is heterosexual sex that is not due to sex work with more and more women getting infected. “It appears that HIV epidemic in sub-Saharan Africa is assuming a female face,” Koffi Annan 2001.

1.1.5.2 The Asian HIV epidemic

In Asia, an estimated 5.0 million people were living with HIV in 2007, including the 380,000 people who were newly infected that year. Approximately 380 000 died from AIDS-related illnesses. There are several modes of transmission which made the Asian epidemic one of the most diverse. Injecting drug use was a major risk factor in the epidemics of several Asian countries. In some countries such as Vietnam, an overlap in transmission existed between intravenous drugs usage and unprotected sex between injecting drug users, the two accounting for most new infections with HIV. Unprotected sex (commercial and otherwise) is the most important risk factor for the spread of HIV in several parts of Asia. Sex-trafficked women and girls face especially high risks of HIV infection. However, in countries where sex work was the main driver of the epidemic, introduction of condom usage reduced the incidence rates dramatically. As with other regions, the importance of men who have sex with men could not be underscored but remained under-researched (UNAIDS 2008).

1.1.5.3 The other HIV epidemics in other parts of the World

The estimated number of people living with HIV/AIDS in Eastern Europe and Central Asia rose to 1.5 million in 2007; almost 90% of those infected lived either in The Russian Federation (69%) or Ukraine (29%). Each of the HIV epidemics in this region were concentrated largely among injecting drug users, sex workers, and their various sexual partners (UNAIDS 2008).

An estimated 230 000 people were living with HIV in the Caribbean in 2007 (about three quarters of them in the Dominican Republic and Haiti). The main mode of HIV transmission in the Caribbean was unprotected heterosexual inter-course, paid or otherwise. However, sex between men, although generally denied by society, was also a significant factor in several national epidemics (UNAIDS 2008).

In Latin America, new HIV infections in 2007 totalled an estimated 140 000, bringing to 1.7 million the number of people living with HIV in this region. HIV transmission in this region occurred primarily among men who had sex with men, sex workers, and (to a lesser extent) injecting drug users (UNAIDS 2008).

1.1.6 Heterogeneity in acquisition of HIV virus

A possible explanation for the variation in transmission modes per region around the world may be immunological or genetic differences in the populations residing in the regions which may be exerting a strong influence on HIV transmission. The earliest

understanding of HIV transmission lead to a belief that exposure of an uninfected individual to the HIV virus through unprotected sexual encounter, infected blood or infected breast milk, led to a life long infection with the HIV virus. However, there is indirect evidence that exposure to HIV virus does not always lead to a persistent infection eventually resulting in death through AIDS. This heterogeneity in acquisition of HIV may be due to protective immunity, specialized immune escape mechanisms or hereditary factors.

There are groups of individuals who in spite of single or multiple exposures to the HIV virus, remain uninfected. These special groups include, commercial sex workers who have multiple sexual partners with high risk exposure during their sex work (Fowke 1996) without seroconverting; HIV negative infants born to infected mothers (Kuhn L 2001; Farquhar C 2004); seronegative intravenous drugs users exposed to HIV contaminated needles (Pinto 1995) and haemophiliacs transfused with HIV contaminated blood (Barretina 2000). A second group of individuals who do not progress to AIDS after acquiring the HIV infection, known as "long term non-progressors" appear to be able to control HIV viral replication. The criteria for defining the long-term non-progressors has been based on the duration of survival with HIV infection (>7 years), with consistently low levels of HIV-1 RNA and little or no loss of HIV primary target cells CD4 bearing T lymphocytes. Long-term non-progressors have been identified in various groups, including homosexual men, women, injection drug users and even in children (Easterbrook 1999).

Vaccines imitate immunity acquired after natural infection, and the identification of these special groups has renewed hope that the much needed HIV-1 vaccines are possible. To identify models of naturally acquired immunity to HIV-1, proof of individuals who are protected against infection is integral. Testing this kind of protection is a complex scientific problem. Ethical considerations bar a scientific demonstration of the definitive resistance, by use of experimental HIV-1 challenge. The only feasible scientific approach is to observe natural experiments of groups of individuals who are exposed to HIV-1 and who appear to be protected against infection.

Various studies in such groups have demonstrated the existence of HIV-1 specific immunologic responses, such as HIV specific helper T responses present in peripheral circulation and in the genital tract (Fowke 2000), IgA2 responses in genital tract (Kaul 1999) and cytotoxic lymphocyte responses in peripheral circulation (Rowland-Jones 1998) and in the genital tract (Kaul 2000). The mucosal and plasma HIV-1 specific IgA from commercial sex workers in Majengo, has been shown to inhibit HIV-1 transcytosis in epithelial cells (Devito 2000) and to neutralize HIV-1 isolates *in vitro* (Devito 2000). Cytotoxic CD8+ T Lymphocyte responses have been shown to neutralize HIV-1 isolates in *ex vivo* and *in vitro* cultures (Rowland-Jones, Dong et al. 1998).

Immunogenetic studies also show that there are specific MHC I and II genes which are associated with resistance to acquisition of HIV-1 infection and delayed disease progression (Fowke 2000). Some of these MHC genotypes, alleles and haplotypes are

inherited in families where they provide protection or delayed progression to AIDS (Fang 2004).

There are only a few epidemiologic studies that provide evidence that there may be protection against infection with HIV virus in some individuals (Plummer, Ball et al. 1999). Declining incidence of HIV-1 infection in spite of increased risk of exposure to the virus owing to the longer duration of commercial sex work has been observed in a group of sex workers in Nairobi. The declining incidence of new HIV infections was because a small group of women remained persistently seronegative (Fowke 1996). Survival modeling of the time to HIV-1 seroconversion (Figure 1.2) indicated that this was not a chance phenomenon and that if all women were equally susceptible to infection, statistically, these seronegative women would have been infected with HIV-1. HIV-2 virus infected commercial sex workers were later reported to have partial protection against HIV-1 infection (Travers 1995).

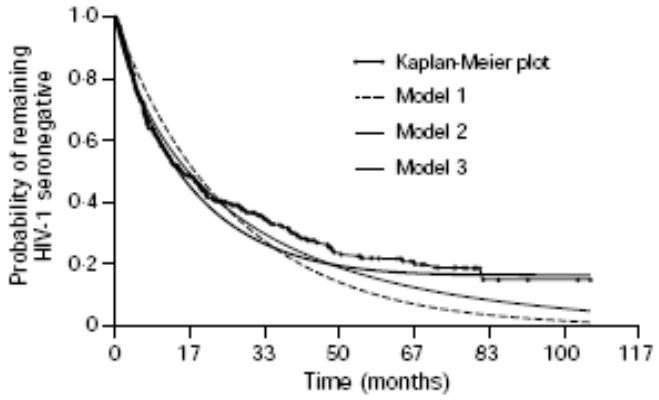


Figure 1.2: Survival modeling for time HIV-1 seroconversion
Model 1 expected time to seroconversion if seronegative survival time is exponentially distributed; model 2expected time to seroconversion under a Weibull distribution; model 3 expected time to seroconversion from a mixture model.
(Fowke, Nagelkerke et al. 1996)

More than a decade has lapsed since the first description of heterogeneity of infection with the HIV-1 virus, a number of correlates for resistance have been identified, some of which have been mentioned above. The search for a vaccine, more effective therapies which contain viral replication and that provide protection against infection with HIV virus continues. One major discovery has been antiretroviral therapy, which has improved the quality of life of HIV infected people and prolonged the duration of life after acquisition of the HIV virus and onset of AIDS.

1.1.7 Understanding HIV heterosexual transmission

Most HIV infections are acquired through heterosexual transmission; there is a trend for increased transmission of HIV to women than men. Women constitute nearly 60% of new infections in Africa, the epicentre of the pandemic. There is an urgent need to

develop effective microbicides, vaccines and other preventive measures for those who are most at risk (Haase 2005).

The time-frame for the crucial interactions that occur between virus and host during vaginal transmission and in the earliest stages of infection is so short that these interactions, for practical and ethical reasons, cannot be seen in HIV-infected individuals. At diagnosis, most HIV infected individuals are already in the later stages of infection often displaying severe flu-like symptoms of acute retroviral infection. The crucial antecedent events have therefore been investigated by studying a highly relevant non-human primate model (Miller 1989; Miller 1994). In this model, monkeys, mainly Indian rhesus macaques, are exposed vaginally to SIV. This monkey model reasonably approximates the anatomy (Hendrickx 1987), target-cell populations (Ma 2001) and immunology (Miller 2003) of a human host. Moreover, similar to HIV infection of humans, the lymphoid tissues are the reservoir of virus production, virus persistence, CD4+ T-cell depletion, and pathology (Reimann 1994; Fox 1992; Reinhart 1997)

The target cells for HIV or SIV virus are located in the lamina propria of the female genital tract. The cell free or cell-bound SIV virus in the genital tract have been shown to be separated by a barrier effect exerted by the genital mucosa against SIV virions crossing the mucosal tissues to infect the target cells in the lamina propria. Most infection studies in the Indian Rhesus Macaque require an inoculum of close to a billion SIV virions for each successful infection. Most of the virions from the inoculum are

trapped in the cervico-vaginal mucous, and the concentration of inoculum has been observed to be diluted with the spread of the virions in the genital tract once the inoculum is administered (Miller 2003). Most of the virions get trapped around the cervical region hence the mucous. In addition to this entrapment, dilution and clearance of virion, there are other mechanisms all contributing to the barrier effect such as low pH, hydrogen peroxide, presence of secretory proteins with antiviral activity (such as secretory leukocyte protease inhibitor) and the epithelial lining (Shattock 2003). The founder populations of infected cells that cross the mucosal barriers are therefore limited to extremely small numbers. The infected cells occur in rare foci, which are detectable in cervicovaginal tissues during the first 3–4 days after exposure to virus (Miller 2003; Zhang 1999; Hu 2000). After the cell bound or free virions cross the epithelial barrier an eclipse phase follows, where SIV RNA is present in the lamina propria and yet the viral-RNA-positive cells are not. This signifies infection but the number of infected cells is too few for detection.

This small founder population of infected cells must now spread the infection to the relatively small number of spatially dispersed susceptible target cells in the mucosa (compared with the lymphoid tissues) that is, 'resting' and activated CD4+ T cells, DCs and macrophages — and must maintain an R^0 greater than or equal to 1, or infection will die out (Gray 2001). These limitations probably account for the low rate of 'success' for sexual transmission of HIV, which is ≈1 infection per 1000 exposures through vaginal intercourse (Gray 2001). Effects of inflammation is important to the rate of transmission,

since destruction of the barrier integrity in increases the rate of transmission (Cohn 2001), as seen in individuals with sexually transmitted or genital ulcer diseases (Serwadda 2003), with bacterial vaginosis (Gray 1997; Martin 1999; Sewankombo 1997), or after the use of some microbicides, such as nonoxynol-9 (van Damme 2002). This early vulnerability of the HIV virus after crossing the epithelial barrier and infecting enough cells to sustain infection, is the first major opportunity to prevent infection. This could be achieved by further hampering the virus with microbicides and, perhaps, anti-inflammatory agents, which would increase the barrier function and decrease the availability of activated CD4+ T cells for infection.

For HIV or SIV to spread from this small founder population there must be a large number of CD4+ T cells or SIV/HIV target cells at mucosal sites. This pool of cells appear to be 'resting' yet may consist of CD4+ T cells recently activated by infections yet appear quiescent, and those that are activated due to presence of a large number of microbial products and co-infective agents. The presence of co-infection agents and their products, may lead to inflammation responses by innate cells stimulated to produce cytokines through germ-line encoded receptors (pattern recognition receptors) that recognize microbial products or pathogen associated molecular patterns PAMPs, such as Toll-like receptors, mannose receptors and so on. The recently activated and now 'resting' cells express the HIV viral entry co-receptor CCR5 (Berger 1999; Pierson 2001), sufficient nucleotides for viral transcription, transcription activators for later stages of viral transcription (Korin 1999; Zack 1990) which are necessary for infection

of cells with the SIV or HIV virus. The absence of these perquisite receptors and factors would prevent a productive SIV or HIV infection from establishing, as is the case with actual resting cells with no previous activation history. Although, infected 'resting' CD4+ T cells once infected produce small amounts of virus per cell, they are numerous, seven times more than activated CD4+ T cells and they out number DCs and macrophages (Zhang 2004). The CD4+ T cells in the lamina propria occur in focal aggregates which allows for rapid propagation of the viral infection.

Taken together, infection of 'resting' and activated CD4+ T cells allows SIV to establish and expand small, infected founder populations at the point of entry, generating sufficient numbers of virions and infected cells to establish a persistent systemic infection throughout lymphoid tissues.

1.2 Problem Statement

The role of the innate immune cells in the lamina propria of the genital tract during HIV transmission is very poorly understood. Thus there is a missing gap in knowledge between the initial events leading to HIV infection and the development of adaptive immune responses that control viral replication at later stages of HIV infection. Innate effectors mechanisms are responsible for inflammatory responses, which have been shown to contribute to the activation of CCR5 or CXCR4 CD4+ cells, driving infection and subsequent depletion of CD4+ T cells with successive HIV viral replication cycle. The rapid depletion CD4+ T cells is further fueled by persistent immune activation,

which is triggered very early in the HIV infection, probably by interaction of the virions or products of HIV replication with non-clonal germ-line receptors such as TLRs found on innate immune cells. Evidence of this has been recently demonstrated in PBMCs which are able to recognize GU rich ssRNA analogous to HIV RNA sequences, through TLR7/8 leading to production of proinflammatory cytokines by NF-κB. This study was one of the first to compare the cytokine responses to TLR stimulation in cervical mononuclear cells from the genital tract of HIV-resistant women with those of susceptible women (HIV-positive and normal HIV negatives).

1.3 Justification

This study was part of a larger study, which has so far demonstrated differential levels of expression of TLRs on peripheral blood mononuclear cells (PBMCs) from women with different HIV status after TLR stimulation, and with HIV disease progression in a group of commercial sex-workers. This variation in TLR expression was accompanied by increased PBMC responsiveness, which was dependant on the HIV status (Lester 2008). This study sought to analyze the cytokine profiles of CMCs from genital tract, stimulated by different TLR agonists, from HIV resistant women, HIV-susceptible women and HIV positive women, from the same cohort. This study will contribute to the better understanding of the initial innate responses in the genital tract, with the hope that it will help in development of new microbicides and an HIV vaccine.

1.4 Hypotheses

1.4.1 Null Hypothesis

The expression and function of TLRs on CMCs in the genital tract of HIV resistant women does not differ from that of HIV susceptible and HIV positive women in Majengo sex-worker cohort.

1.4.2 Alterative hypothesis

The expression and function of TLRs on CMCs in the genital tract of HIV resistant women does differ from that of HIV susceptible and HIV positive women in the Majengo Sex-worker cohort.

1.5 Objectives

1.5.1 General Objective

The overall objective of this study was to quantify and compare the level of cytokine production in CMCs following TLR ligand stimulation, in three groups of women (HIV-1 resistant; HIV-1 susceptible; HIV-1 positive).

1.5.2 Specific Objectives

This study had the following specific objectives:

1) To access and compare the level of cytokine production following stimulation of CMC obtained from the three groups (HIV-1 resistant; HIV-1 susceptible; HIV-1 positive).

2) To compare the level of cytokine production with TLR expression in CMCs in the three groups of women (HIV-1 resistant; HIV-1 susceptible; HIV-1 positive).

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Innate Immunity

Innate immunity is the initial, rapidly induced immune response of multi-cellular organisms; it utilizes non-clonal germ line-encoded receptors for pathogen recognition and the activation/ recruitment of phagocytic and other cells. Innate immune system is phylogenetically conserved and is present in all multicellular organisms (Medzhitov 1997). Metchnikoff first described the innate immune system more than a century ago, recent work, however, has yielded substantial insight into the composition and function of innate immunity, and it is now apparent that the innate immune system utilizes an intricate network of recognition and effectors mechanisms to clear or moderate pathogen replication until the adaptive immune system can mount a more specific and robust response.

2.1.1 Innate Immune Effectors

The components of the innate immune system can be classified into four categories: physical and chemical barriers, cellular component (phagocytes-dendritic cells, macrophages and NK cells); blood proteins (complement proteins and inflammatory proteins).

The initial events during transmission of HIV virus, involves an interaction between the cell bound or free virus and the innate immune system. These are important in

understanding establishment of infection and the subsequent development of the generalized immune activation of chronic HIV-1 infection. The immune activation has been accepted to be the best predictor of HIV-1 disease progression (Fahey 1998). Thus, understanding the events in the innate immune compartment at the gateway of HIV into the human body, especially at the mucosal surfaces (where most transmission occurs), is integral for the identification of immune correlates necessary for development of vaccines, and other antimicrobial agents such as microbicides. TLRs agonists such as CpG ODN, ssRNA and others are currently under trial for combination with vaccines, and for use as adjuvants which are able to stimulate effective mucosal, humoral and cell mediated immune responses to HIV.

2.1.2 Innate Immune Recognition

In 1989, Charles Janeway proposed that innate effector mechanisms are initiated via the specific detection of microbes by germ line-encoded, non-clonal receptors, which are essential for the immediate detection and control of infection in mammals (Janeway 1989). These molecules, termed pattern recognition receptors (PRRs), primarily function in recognizing microbial structures referred to as pathogen-associated molecular patterns (PAMPs), each being critical for pathogen replication and/or survival, thus enabling the immune system to identify different pathogens as foreign. These molecules, PAMPs, include: components of bacterial cell walls such as lipopolysaccharides (LPS) and lipoproteins; viral and bacterial replication intermediates such single strand RNA, double strand RNA; fungal components; and protozoan and other antigenic material found on

pathogens. The number of germ-line genes which encode for the PAMPs are relatively few in number and their expression in cells of the innate immune system is not accompanied by clonal expansion, and as such the innate immune system can only recognize a few PAMPs. Usually, this initial recognition by the host innate immune system is sufficient to prevent pathogenic establishment of most microbes, however, those which are able to escape the innate immune recognition, encounter the subsequent adaptive immune response, which is able to undergo somatic rearrangements and T lymphocyte expansion or antibody production, which in most cases is able to contain the infection or completely eradicate the infectious organism. Both mechanisms of the innate and adaptive immune system appear to be insufficient in containing infection with HIV-1 virus in most individuals. However, heterogeneity exists in transmission of HIV-1 virus, where high risk exposure to HIV-1 virus does not always result in persistent viral infection. This unique class of individuals are referred to as highly exposed and persistently seronegative (HEPS) or HIV-1 resistant individuals (Fowke 1996).

2.1.3 Toll-Like Receptors

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) involved in the recognition of a wide range of highly conserved microbial molecular structures termed PAMPs (Medzhitov 1997). TLRs are thought to have a common historical origin with the *Toll gene* (product) in *Drosophila*, which is responsible for defense against fungal infection and dorsi-anterial development in the fly (Lemaitre 1996). They share a Toll/Interleukin-one Receptor (TIR) domain required for intracellular signaling and the

extracellular leucine-rich repeats for ligation to microbial PAMP. To date, 13 TLR have been identified in mammals, 10 TLRs occur in humans (Hoffmann 2002) and 12 are found in mice (Table 2.1).

Table 2.1: Human TLRs, their ligands and location of TLRs in cells

Toll-like receptor	TLR ligands	Location of TLR in cell
TLR 1	Tri-acyl lipopeptides (bacteria, mycobacteria) Soluble factors (Neisseria meningitides)	Transmembrane protein
TLR 2	Lipoprotein/lipopeptides Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (*Mycobacteria*) A phenol-soluble modulin (*Staphylococcus epidermidis*) Glycoinositolphospholipids (*Trypanosoma Cruzi*) Glycolipids (*Treponema maltophilum*) Porins (*Neisseria*) Zymosan (fungi) Atypical LPS (*Porphyromonas gingivalis*) HSP70 (host)	Transmembrane protein
TLR 3	Double strand RNA (bacteria)	Endosomal protein
TLR 4	LPS (Gram-negative bacteria) Taxol (plant) Fusion protein (RSV) Envelope proteins (MMTV) HSP60 (*Chlamydia pneumoniae*) HSP60 (host) HSP70 (host) Type III repeat extra domain A of fibronectin (host) Oligosaccharides of hyaluronic acid (host) Polysaccharide fragments of heparan sulfate (host) Fibrinogen (host)	Transmembrane protein
TLR 5	Flagellin (bacteria)	Transmembrane protein
TLR 6	Di-acyl lipopeptides (*Mycoplasma*)	Transmembrane protein
TLR 7	Imidazoquinoline (synthetic compounds) Loxoribine (synthetic compounds) Bropirimine (synthetic compounds) ssRNA40/LyoVec	Endosomal protein
TLR8	ssRNA40/LyoVec	Endosomal protein
TLR 9	CpG DNA (bacteria)	Endosomal protein
TLR 10	Not yet described	

(Akira 2001)

These TLRs recognize and bind to a variety of conserved bacterial, viral, fungal, and protozoan PAMPs which upon ligation of the ligands to their specific TLR, a signaling cascade is initiated (Figure 2.1).

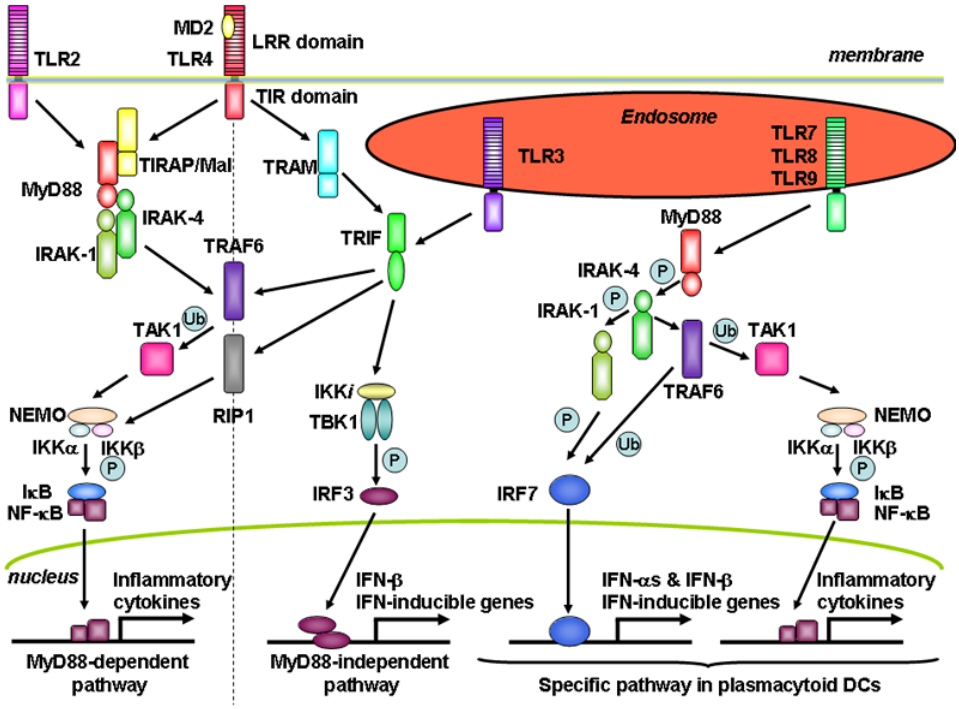


Figure 2.1: TLR signaling pathways

(Takeda 2004)

The TLR signaling cascades (figure 2.1), which involve the recruitment of the adaptor molecule myeloid differentiation primary response gene 88 product (MyD88) and in certain cases TIR domain-containing adapter inducing IFN-β (TRIF), TRIF-related adapter molecule (TRAM), and TIR domain-containing adapter protein (TIRAP),

followed by the binding of IL-1R-associated kinase (IRAK) family members and the activation of TRAF-6, ultimately leads to the activation of NF-κB and mitogen-activated protein (MAP) kinases (Takeda 2004). Later the activation of transcription factors leads to transcription of genes encoding for proinflammatory cytokine production and other gene products responsible for cellular activation (Hazenberg 2003). In addition, TLR signaling can lead to direct NF-κB dependent HIV Long Terminal Repeats expression by the activation of other transcription factors such as AP-1 that have been shown to lead to HIV-1 virion production (Pereira 2000; Rohr 2003).

2.1.3.1 TLR expression of innate immune cells

Although innate immune cells express the highest levels of TLRs, their mRNA expression profile differs from one cell type to another. For instance, human blood-derived myeloid dendritic cells (mDC) express TLR 2, ~3, ~4, ~5 and ~7 whereas plasmacytoid dendritic cells (pDC) express TLR 7 and 9 only. Myeloid dendritic cells are more prevalent than pDCs and are found throughout the body, mainly in the skin and mucosal tissues (Langerhan cells). Plasmacytoid DCs are more sparsely distributed and are normally found only in blood, lymph nodes, and thymus but are recruited to sites of inflammation under pathological conditions (Cella 1999; Farkas 2001)**.** Both DC subsets have antigen-presenting capacity but differ in their expression of TLRs and some important functional aspects. In general, mDCs are characterized by their ability to secrete high levels of interleukin 12 (IL-12), whereas pDCs produce high levels of alpha interferon (IFN-α) in response to TLR-induced activation and maturation (Akira 2001).

The two subsets of dendritic cells also show differential TLR mRNA expression patterns, which allow for differential stimulation and selection of the two subsets. Peripheral myeloid DC produce cytokines in response to TLR ligands, such as peptidoglycan and lipopeptides (TLR2), poly I: C (TLR3), LPS (TLR4) and flagellin (TLR5), but do not respond to CpG DNA (TLR9) (Teleshova 2004). On the other hand, pDC produce cytokines in response to CpG DNA, but do not respond to PGN, Poly I: C, LPS and flagellin. Therefore one can evaluate the specificity of TLR agonists from different populations of cells with different TLR expression patterns.

There are studies that reveal a variation in patterns of expression of TLRs in different cell populations found in the female reproductive system. Immortalized human cervical and vaginal epithelial cells expressed mRNA for TLR 1, ~2, ~3, ~5 and ~6 but failed to express TLR 4 and MD2, both of which are involved in innate responses to LPS (Fichorova 2005; Wira 2005). This could be an explanation for the unresponsiveness of the innate components of the vaginal wall to gram negative bacteria which populate the lower female genital tract. Uterine cells express TLR 1-9 with the exception of TLR 10 (Schaefer 2004). However, only TLR 2, TLR 4 and TLR 9 stimulated the expression of IL-6, IL-8 and Mononuclear chemotactic protein 1 MCP-1 in uterine cells (Schaefer 2004).

2.1.3.2 TLR Stimulatants and Viral Inhibitors

Low molecular synthetic compounds called imidazoquinolines have been shown to be potent activators of immune cells with anti-viral and anti-tumor properties (Weeks

1994). The anti-viral activity of one of the imidazoquinolines- Imiquimod, was first shown in guinea pigs infected with herpes simplex virus (Harrison 1994). TLR 7 ligands Imiquimod and Resquimod have been previously demonstrated to have the ability to stimulate IFN-α cytokine responses in immune competent mice (Zuber 2004). These agents along with others currently under trial seek to modify host innate immune recognition or indirectly control the replication of HIV-1 virus by regulating TLR signaling pathways.

2.1.3.3 Effects of Innate Recognition and Signaling

Chronic HIV-1 infection is characterized by chronic immune activation which currently provides the best determinant for HIV-1 disease progression (Fahey 1998; Hazenberg 2003). Current research efforts are directed toward identification of the trigger for the generalized immune activation observed in chronic HIV-1 infection. Probable causes of immune activation have been identified, but a complete picture is still elusive.

The innate immune system has been shown to recognize components of HIV-1 virus which are able to induce intracellular signaling resulting in activation of transcription genes such as NF-κB and AP-1 encoding for proinflammatory cytokines downstream (Antoni 1994). The proinflammatory cytokines produced such as TNF-α lead to activation of cells of the mononuclear lineage and X4/R5 CD4+ T cells through CD40/CD40L binding between activated DCs and T lymphocytes and NK cells (Caux 1994). This activation of transcription factors that lead to production of proinflammatory

cytokines has been show to trigger expression of HIV-1 LTRs and leading of production HIV virions. This accelerates the rate infection of activated HIV-1 target cells, leading to a very rapid establishment of a HIV infection (Antoni 1994).

2.1.3.4 Cytokines as Effectors of the Innate Immune System

Table 2.2 outlines the role of cytokine mediators and regulators of innate immunity, with special focus on their role in HIV-1 disease.

Table 2.2: Cytokines, chemokines and defensins in HIV infection and replication

Cytokine / Chemokine	Producer cell	Effect on HIV infection/ replication
IL-2	Activated T cells, myeloid dendritic cells mDC	Up-regulation via induction of pro-inflammatory cytokines
IL-4	Activated T cells, Th2 cells	Bimodal: inhibition of cytokine-mediated virus replication (monocytes),but enhancement of HIV expression (Mø)
IL-7	Epithelial cell	Enhancement of HIV transcription
IL-10	DC, activated T cells	Bimodal: inhibition of cytokine-mediated virus replication (Mø), but enhancement of HIV transcription. Inhibition of helper T cell function.
IL-12	mDC, APC	Enhancement of HIV replication (PBMC)
IL-13	Mø	Post-transcriptional inhibition HIV replication in Mø
IL-15	Mø, PDC, NK	Up-regulation via induction of pro-inflammatory cytokines
IFN-α/β	Monocytes, Mø, PDC	Inhibition of multiple steps of the virus life cycle
IFN-γ	NK, NKT, $\gamma\delta$ T, CTL, PDC, activated T cells, Th1 cells	Bimodal: enhancement of HIV transcription, but inhibition of R5 virus entry and virion release
TGF-β	Haemopoietic and endothelial cells, connective tissue, Mø, Th3 cells	Bimodal: both enhancement and suppression of HIV replication as a function of time of stimulation vs. infection (Mø)
CCL3, CCL4, CCL5	NK, DC, $\gamma\delta$ activated T cells, Mø	I
CCL2	Monocytes, Mø	nhibition of R5 HIV entry
CXCL8	Monocytes, activated T cells, NK, fibroblasts, endothelial and epithelial cells	Enhancement of HIV replication Enhancement of HIV replication
Defensins	Neutrophils, Mø, epithelial cells, Paneth cell, NK, B cell, T cell	Inhibition of (X4) HIV entry and virion infectivity
TNFα	TNF is produced by a large range of cells including lymphocytes and mononuclear cells	Homing of neutrophils and eosinophils on endothelial surfaces and in mucosal tissue Activation of mononuclear phagocytes and Ag stimulated NK cells, T cells and Mast cells

(From -Immunobiology 5th Editon by Charles A. Janeway Jnr. pg 65)

2.1.3.4.1 Tumour Necrosis Factor (TNF or TNF-α)

TNF is the principle mediator of the acute inflammation response to gram-negative bacteria and other infectious microbes (Rollof 1990). Upon secretion it is capable of exerting local effects (on neutrophils and endothelial cells) or systemic effects (cachexia and sepsis) and this is augmented by the presence of TNF receptors on most cell types in the body (von Asmuth 1991). The main cellular source of TNF- is activated mononuclear phagocytes, and occasionally antigen stimulated T cells, NK cells, and mast cells. The most potent stimulus of TNF-α production in macrophages is TLR engagement with TLR ligands or PAMPs. *In vitro*, increased expression of HIV-1 Long terminal repeats (LTR) by activation of NF-κB, AP-1, IRF-3 and IRF-7 genes has been connected to TLR signaling initiated by recognition of PAMPs from a variety of microbes. This increased viral shedding due to mixed infections in HIV-1 disease has been thought to speed up HIV disease progression terminating in AIDS.

Biological Role of TNF-α

TNF-α recruits neutrophils and monocytes to infection sites, and activates these cells to eradicate invading microbes. TNF-α induces vascular endothelial cells to express adhesion molecules selectins and integrins, making these surfaces more adhesive to leukocytes, later to neutrophils and monocytes. TNF-α stimulates endothelial cells and macrophages to secrete chemokines that enhance the affinity of integrin receptor for their ligands and induce leukocyte chemotaxis and recruitment. TNF-α also induces production of IL-1, which functions much like itself.

If innate recognition stimulus is intense, a large amount of TNF-α is produced, and it exerts systemic effects causing clinical and pathologic abnormalities. TNF-α has been show to act on hypothalamus to induce fever and is therefore an endogenous pyrogen; the fever is caused by cytokine induced hypothalamic cell production of prostaglandins. TNF-α acts on hepatocytes to increase serum protein production (serum amyloid A and fibrinogen) which in turn stimulates other cytokines to form an acute phase inflammatory response. The prolonged production of TNF-α causes wasting of muscle and fat cells, called cachexia; this is due to TNF-α suppression of lipoprotein lipase. These and other systemic effects of TNF-α have pathologic importance which may contribute to some clinical manifestations of chronic HIV infection. Septic shock caused by high levels of endotoxin in blood causes vascular collapse, disseminated intravascular coagulation and metabolic disturbances, a number of the effects described above.

TNF-α linkage to Adaptive immune response

There are many cytokines and membrane proteins belonging to TNF-α receptor families. Two TNF-α family membrane proteins expressed on activated T lymphocytes are CD40 ligand and Fas ligand. CD40 ligand mediates activation of macrophages and B cells. Fas ligand is involved in killing of some cells types. BAFF and APRIL are TNF-α family members that play a critical role in B cell survival and differentiation. Glucorticoid-induced TNF-related (GITR) ligand and OX40 ligand are TNF family molecules that are involved in regulation of T cell responses.

2.1.3.4.2 Interlukin-12

Interleukin 12, a potent inducer of T_H1 cell polarization and IFN-γ production, it is a heterodimeric cytokine produced by antigen presenting cells (APC) such as macrophages and DC (Sartori 1997; Trinchieri 1998; Trinchieri 1998). IL-12 exerts proliferative and stimulatory activity on T and NK cells, and favors the maturation of CTLs (Sartori 1997). HIV-1 p17 matrix antigen induces IL-12 production from purified NK cells (Vitale 2003), whereas *nef* and p55 Gag proteins are internalized by immature dendritic cells (iDC), therefore inducing IL-12 secretion and DC maturation (Wilson 1999; Quaranta 2003; Tsunetsugu-Yokota 2003). IL-12 expression in LN was found to be up regulated during primary SIV infection and this was correlated to the extent of viral replication (Khatissian 1996). In contrast, an impaired production of IL-12 from PBMC has been reported in HIV^+ individual (Chehimi 1993; Clerici 1993; Marshall 1999) and in SIV_{mac251} infected macaques (Clerici 1993; Poaty-Mavoungou 2002). In vitro, IL-12 has shown inductive effects on HIV replication (Foli 1995; Kinter 1995; Bayard-McNeeley 1996; Al-Harthi 1998) but also inhibition of virus replication (Akridge 1996) correlated to CCR5 down-regulation if cells were stimulated with the cytokine before infection (Wang 1999). *In vitro*, IL-12 was shown to rescue proliferation and IFN-γ production by T and NK cells, Ag presentation and accessory functions of macrophages and DC, and cytolytic capacity of both CTL and NK cells in either HIV infected individuals or SIV infected animals(Chehimi 1993; Clerici 1993; Chehimi 1994; Sirianni 1994; Marshall 1999; Poaty-Mavoungou 2002). Administration of exogenous IL-12 did not affect either the virus load or the frequency of circulating

infected lymphocytes in chronically SIV-infected rhesus monkeys (Watanabe 1998; Villinger 2000). Although, it increased the proliferative response to multiple HIV Ags in chimpanzees vaccinated with a DNA-based vaccine (Boyer 2000). IL-12 administration to either SIV-infected macaques (Villinger 2000) or HIV-infected individuals (Boyer 2000) in the early stages of infection increased the frequency and activity of circulating NK cells. These observations were contrary to an earlier report that had documented decrease of NK cell activity in IL-12 treated HIV+ individuals (Kohl 1996).

2.1.3.4.3 Type 1 Interferons (IFNs)

Among the anti-viral cytokines, type I IFNs (IFN-α/β) play an important role. IFN-β production in PBMC is mostly carried out by monocytes and plasmacytoid dendritic cells pDC, and it regulates many immune responses like antibody (Ab) production, enhancement of T and NK cell cytotoxicity, inhibition of T suppressor/regulatory cells, and anti-tumor activity (Belardelli 1996; Belardelli 2002).

IFN-α, has shown potent anti-HIV activities *in vitro* by inhibiting multiple steps of the virus life cycle, including reverse transcription and viral expression from integrated provirus in acutely infected primary cells (Poli 1994; Popik 2000), HIV replication in primary monocyte-derived macrophages (MDM) (Weiden 2000) and HIV virion release from chronically infected cell lines (Poli 1989).

Up regulation of type II IFN-γ mRNA has been demonstrated on PBMCs, Langerhan cells, and broncho-alevolar mononuclear cells in primary SIV infection in Macaques (Khatissian 1996; Cheret 1999; Villinger 2000), and intestinal lamina propria lymphocytes where IFN-α has been associated with the containment of viral replication (Smit-McBride 1998). IFN-α and IL-10 production has been shown to increase during the interaction of infected DC and T cells leading to HIV spreading (Ludewig 1996).

2.1.3.4.4 Interleukin 10 (IL-10)

IL-10 is a cytokine mostly secreted by DC and T_H2 cells (Banchereau 2003). It inhibits the production of all proinflammatory cytokines and chemokines and the expression of DC-costimulatory molecules, therefore shutting-off T cell activation (Moore 2001). IL-10 has been found to upregulate *in vitro* CXCR4 expression and X4 HIV infectivity of DCs, although this did not affect the efficiency of viral transmission to autologous CD4+ T cells, an event involving DC-SIGN rather than conventional viral receptors (Ancuta 2001). Depending on the concentration of IL-10, both inhibitory and inductive effects on HIV replication have been observed. Inhibition of viral replication in monocyte derived macrophage MDM, was correlated to the prevention of the synthesis and release of endogenous TNF-α and IL-6 (Weissman 1994). However, lower concentrations of IL-10 resulted in the enhancement of HIV replication in MDM, an effect that has been correlated to the cooperation with the released TNF-α and IL-6, as demonstrated in terms of induction of HIV expression in U1 cells (Weissman 1995; Barcellini 1996; Rabbi 1998). In addition to IL-4 (Schuitemaker 1992; Weissman 1994; Foli 1995), IL-10

upregulated the expression of CCR5 and R5-HIV replication in freshly isolated monocytes (Sozzani 1998). In contrast, others have shown that IL-10 inhibits *in vitro* HIV infection in macrophages (Kollmann 1996; Schols 1996). IL-10 levels are elevated, particularly in lymph nodes of HIV-infected individuals (Graziosi 1994; Graziosi 1996; Fakoya 1997), whereas progression to AIDS has been correlated with an IL-10 promoter variant associated with a decreased IL-10 expression (Shin 2000).

2.1.3.4.5 Other cytokines of Innate Immunity

IL-6 is a cytokine that functions in both the innate and adaptive immunity. It is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts and other cells in response to microbes and to other cytokines, notably IL-1 and TNF, and some activated forms of T cells.

In innate immunity it stimulates the synthesis of acute-phase proteins by hepatocytes and thus contributes to the acute phase response. In adaptive immunity, IL-6 stimulates the growth of B lymphocytes that have differentiated into antibody producers. IL-6 similarly acts as a growth factor for neoplastic plasma cells (myelomas). IL-6 has also been shown to promote growth of monoclonal antibody- producing hybridomas, which are derived from myelomas. There is also emerging evidence that IL-6 promotes cell-mediated immune reactions by stimulating production of some proinflammatory cytokines (IL-17).

IL-15 is produced by macrophages and several other cell types. It serves as an important growth stimulating factor and enhances survival functions of T cells and NK cells, these functions being similar to those of IL-2. HIV-1 matrix protein p17 has been shown to up-regulate IL-15 in activated NK cells (Vitale 2003). IL-15 was shown to enhance HIV replication in acutely infected PBMC or T cells with a predilection for R5 HIV strains(Kinter 1995; Kinter 1995; Patki 1996; Al-Harthi 1998). No correlation was found between *in vitro* HIV infection of PBMC and IL-15 production (Chehimi 1997). As previously shown for IL-2 (Kinter 1995), this effect was caused by the induction of pro-inflammatory cytokines including TNF-α, IFN-γ and IL-1-β and by the up-regulation of CCR5 (Zou 1999; Weissman 2000; Yang 2001).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study site

This study was carried out at University of Manitoba laboratories located at the University of Nairobi medical school.

3.2 Study Design

The study was nested within on-going HIV studies in commercial sex workers, who are part of a well-established cohort attending the Majengo clinic in Nairobi. All subjects were sampled during routine research visits and had no features of overt systemic illness during the time of sample collection. Sample collection was done between November 2007 and January 2008 based on a schedule for patient visit to the Majengo clinic. The samples used for experiment had to have cells, and thus number of study participants whose CMC responses were studied varied compared to the actual number who were sampled.

Informed consent for demographic, behavioral surveys and use of biological samples in immunological assays was obtained from all study participants. The study protocol and design was approved by the institutional review boards of the University of Manitoba (Protocol number 37879), and the University of Nairobi through the Kenyatta National Hospital Ethical Review Committee (Protocol number P211/09/2006) and KEMRI and National Ethical Review Committee (review number 1353). All clinical investigation was conducted according to the principles of the Helsinki Declaration.

3.3 Study Populations

The study participants from the Majengo clinic, were classified into three groups based on their HIV status that is, HIV Resistant (n=25), HIV positive (n=25) and HIV susceptible or new negative women (n=20).

3.3.1 HIV Positives (HIV-P)

The HIV status was confirmed using ELISA (Vironostika^{TM} Kit) and polymerase chain reaction techniques (Roche HIV-PCR) the two HIV-1 serologic tests being taken every six months. All HIV infected subjects were in the chronic phase of infection if they had CD4+ T cell counts of greater than 200 cells per μl with no signs of overt illness. Subjects were defined as having 'AIDS' if their $CD4^{+}$ T cell counts were less than 200 cells per μl and were excluded from the study.

3.3.2 HIV Resistant women (HIV-R)

Study subjects were classified as being HIV resistant if they were HIV negative at the time of enrollment into the cohort, and had remained HIV negative for more than 3 years of follow-up in the Majengo cohort (Fowke 1996), during which time they should have been active in commercial sex work. Confirmation of their seronegativity was done using ELISA (Vironostika) and PCR (Roche) testing techniques at the time of sample extraction.

3.3.3 HIV susceptible (HIV-S)

HIV negative women, who were enrolled and followed in the cohort for less than 3 years, were classified as new negatives or being HIV susceptible. The duration of their follow up was shorter than what epidemiological studies had shown sufficient for exposure to HIV virus to occur with continued commercial sex work (Fowke 1996). They are considered to be susceptible until the 3 year period lapses, after which they are considered to be HIV resistant.

In all the women who enrolled in this study no sexual transmitted infections other than HIV was detected at the time of genital sample collection. The women were not applying any vaginal gels with anti-inflammatory properties.

3.4 Procedures

3.4.1 Sample Acquisition

The cervical mononuclear cells (CMC) samples were obtained from the genital tract of the study subjects using two techniques to optimize CMC cell numbers. These methods have also been shown to be disruptive to the vaginal epithelium and gaining access to the vaginal stroma (lamina propria) which contains the CMCs. The techniques used were; cytobrushing (Iversen 1998; Hart 1999; Coombs 2001) and ectocervical sampling with a plastic spatula protocol to optimize maximum cell recovery. The cytobrush and plastic spatula were then placed in 10 ml of sterile PBS at a pH= 6.9-7.0 (Dulbecco's phosphate buffered), and placed in a single 50 ml sterile tube on ice for transport to the laboratory

for functional assays. The cervical samples were obtained by medically trained personnel and samples stored for laboratory analysis.

3.4.2 Sample Preparation for Culture

Cellular samples were visually assessed for blood contamination using a semi-quantitative scale (0-4). Samples exhibiting excess blood contamination (2+ or higher) were discarded as per HIV resistance studies (University of Manitoba) protocol. In order to obtain the cellular mass with the CMCs from the cytobrush and scraper, the 50ml tubes were vortexed for 25 seconds at low speed, the cytobrush and scraper washed with RPMI media (containing 5%amphotericin penicillin and 10% Foetal Bovine Serum heat deactivated at 56^0) and discarded. The cellular mixture in PBS and media was then separated by Ficol-Histopaque (Lymphoprep- Axis-Bird Cat No 3773) density centrifugation; the centrifugation was done at 1500 rpm for 15 minutes without brakes. Upon density centrifugation the CMCs segregated to the PBS- Ficol histopaque interface, with the denser epithelial cells precipitating at the bottom of the centrifuge tube. The CMCs were then obtained from the PBS- Ficol histopaque interface, washed twice using RPMI media containing antibiotic-antimycotic and deactivated FBS, and the number viable of CMCs counted using 0.4% Trypan blue (SIGMA Cat No. T8154) exclusion technique using a haemocytometer. The volume of isolated cells was then adjusted to give a final concentration of 1×10^6 CMCs/ml. If the viable CMC count was lower than 1×10^6 CMCs /ml the cells were span down at 1600rpm for 10 with brakes on low, and an adjusted volume of media used to resuspend the CMCs to give the required

concentration of 1 x 10^6 CMCs /ml.

3.4.3 *In vitro* Stimulation

Freshly isolated CMCs were in a suspension containing 1x10^6 cell/ml in HyQ® RPMI 1640 (HyClone Cat. No. sh-30027.61), 0.5% Penicillin and Amphotericin B (Invitrogen Cat No. 15240-00) and 10% Fetal Bovine Serum (Invitrogen Cat No. 12483-020) were then transferred to a culture plate. 100µL or 100,000 CMCs in suspension was pipetted to each well in a sterile round-bottomed culture plate, TLR agonists were the added to the CMC suspension as follows: 10µL of 0.01ug/ml LPS (Invivogen Cat. No. tlrl-hodab), 10µL of 1mM ssRNA40/LyoVec40 (Invivogen Cat. No. tlrl-irna40), or 10µL of 1mM Imiquimod TLR7-R837 (Invivogen Cat. No. tlrl-imq) or 10µL of RPMI 1640 Media containing antibiotic-antimycotic and foetal bovine serum(unstimulated). The TLR agonist concentrations used were determined by a protocol previously developed by the Dr Richard Lester 2006. The TLR agonists were added to the specified wells on a sterile 96-well round bottomed plate to the cells and cultured overnight. Overnight, the CMCs which had been in suspension settled to the rounded bottom of each well forming a cellular pellet. The supernatant was harvested from each well while tilting the pipette tip to an angle of 60^0, to avoid aspirating the cellular pellet. The harvested supernatants were batched and stored at -80^0 C in sealed 96-well plates. Owing to low number of CMCs in most of the samples (which is a problem experienced by many in other similar studies), the stimulations were done in singly per patient and not in duplicate as would be appropriate.

The supernatants were later assayed for cytokines using cytometric bead array (BD Biosystems) on a FACScan flow cytometer (BD) with the data being obtained using Cell quest software, and HuIFN-α ELISAs to test for IFN-α in supernatants. Cell pellets which remained on the culture plates were preserved in Trizol (phenol) and frozen at -85^{o}C for TLR mRNA quantification by QRT-PCR.

3.4.4 Quantification of Proinflammatory cytokine production from Culture Supernatants

The level of proinflammatory cytokines produced by the CMCs was quantified from culture supernatants using the following techniques:

A. Cytokine Bead Array (BD Biosciences)

Principle of Assay

A Cytometric Bead Array (CBA), commonly referred to as a multiplexed bead assay, is a series of spectrally discrete particles that can be used to capture and quantitate soluble analytes (such as cytokines). The analyte is then measured by detection of a fluorescence-based emission and flow cytometric analysis.

The basic "sandwich assay" schema for the CBA is shown in figure 3.1**.** The BD CBA generates data that is comparable to ELISA based assays, but in a "multiplexed" or simultaneous fashion. Concentration of unknowns is calculated for the cytometric bead array as with any sandwich format assay, i.e. through the use of known standards and plotting unknowns against a standard curve.

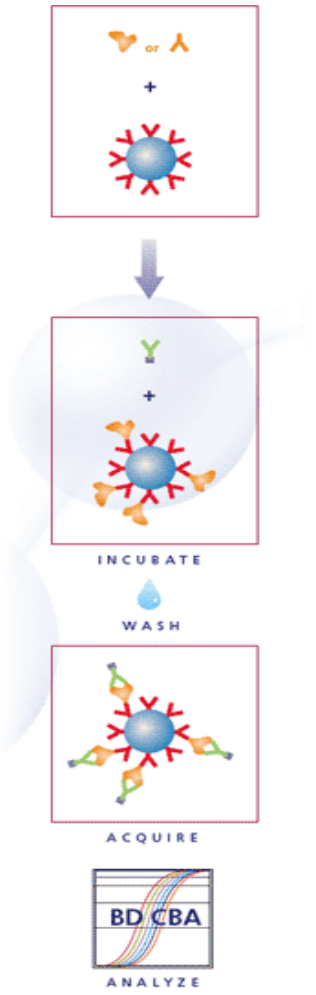


Figure 3.1: Basic "Sandwich Assay" Technique for the CBA Immunoassays (http://www.bdbiosciences.com/pharmingen/products/display_product.php?keyID=9)

The beads used provide a capture surface for a protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture beads mixture is in a suspension to allow for the detection of multiple analytes in a small volume sample.

The BD CBA Kit I was used to evaluate IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α; and BD CBA Kit II for IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α from CMC cell culture supernatants. These assays were done as per BD CBA Th1/Th2 Instruction manual by BD Biosciences.

Acquisition of Data from the flow cytometer was done using Cellquest software and transfered into a personal computer using BD CBA acquisition software™, into Microsoft Excel ™.

B. Human IFN- α Multi-Subtype (Hu-IFN- α) ELISA Kit (Cat log No. 41105-1)

This kit was used to quantify Hu IFN-α (αA, α2, αA/D, αB2, αC, αD, αG, αH, αI, αJ, αK, α4b and αWA in media using a sandwich immunoassay. The kit is based on an ELISA with anti-secondary antibody conjugated to horseradish peroxidase (HRP), tetramethyl-benzidine was the substrate. These HuIFN-α experiments were done as per manufacturers protocol (**PBL Biomedical Labs, NJ USA. Cat No. 41105-1**).

3.5 Statistical Analysis

GraphPad Prism 4.0 software was used for generation of graphs and group comparisons. HuIFN-α data was stored using MS Excel and analyzed using GraphPad Prism 4.0 software. The HIV group comparison of CMC cytokine responses was done using Mann-Whitney t-test. Paired comparisons were done using Fischer's exact t-test.

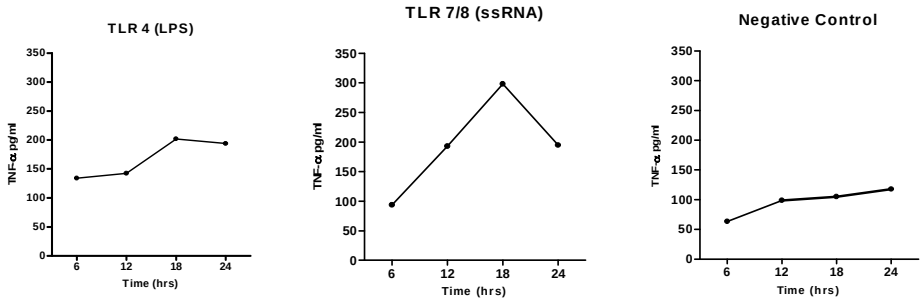
CHAPTER FOUR

4 RESULTS

4.1 Cytokine responses due to TLR ligands stimulation of CMCs

The *in vitro* stimulation of CMCs with TLR agonists *E. coli* LPS (TLR4), ssRNA40/LyoVec (TLR7/8) and Imiquimod (TLR7) induced significantly detectable production TNF-α, IFN-γ, IL-10 and IFN-α (TLR7 Imiquimod). These broadly represent cytokines produced by innate immune cells after stimulation with TLR agonists.

A



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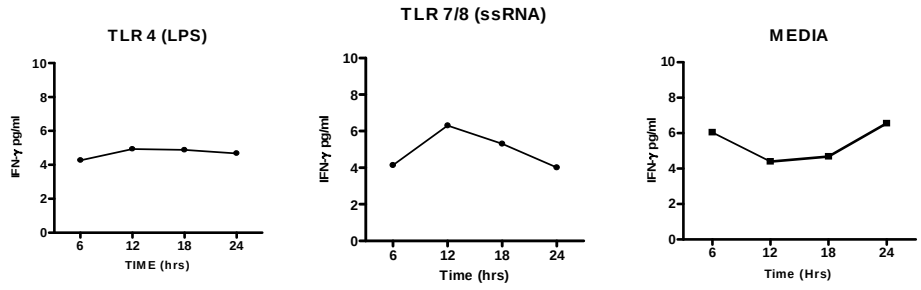

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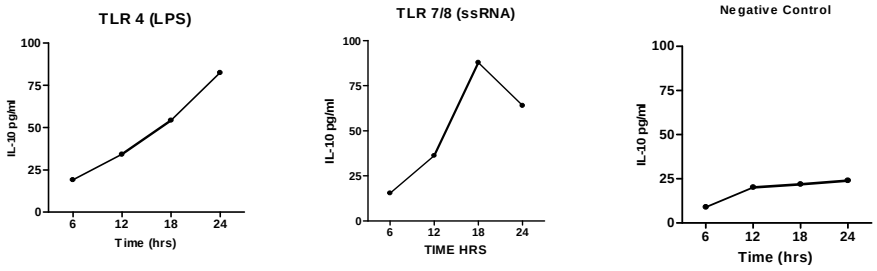

Figure 4.1: Time Response Curves for cytokine production
The group of graphs in A shows TNF-α response (pg/ml) to *E. coli* LPS, ssRNA40 and media as a negative control. Group of graphs in B show IFN-γ in response to *E. coli* LPS, ssRNA40 and media as a negative control. The group of graphs in C shows IL-10 response to *E. coli* LPS, ssRNA40 and media as a negative control.

Based on the Time response optimization experiments (figure 4.1), the peak period for cytokine production after overnight stimulation with TLR ligands was observed to occur at about 18 hours of culture. The optimal time chosen for CMC culture experiments in this study was 18-20 hours.

The dose-response experiments had been conducted earlier by Richard Lester to establish optimal dosages for CMC stimulation with TLR ligands, and the dosages selected were; 0.01ug/ml for *E. coli* LPS (TLR 4), 1.0 μg/ml ssRNA40/LyoVec for (TLR 7/8) ssRNA40/LyoVec and 1.0 μg/ml of TLR 7 (Imq) (data not shown).

4.1.1 TLR 4 (Lipopolysaccharide) cytokine responses in CMCs

The cytokine responses by CMCs to *E. coli* LPS (TLR 4) had significant production of TNF-α, IFN-γ, and IL-10 , and no IL-2, IL-4, IL-5 and IL-6 was detected.

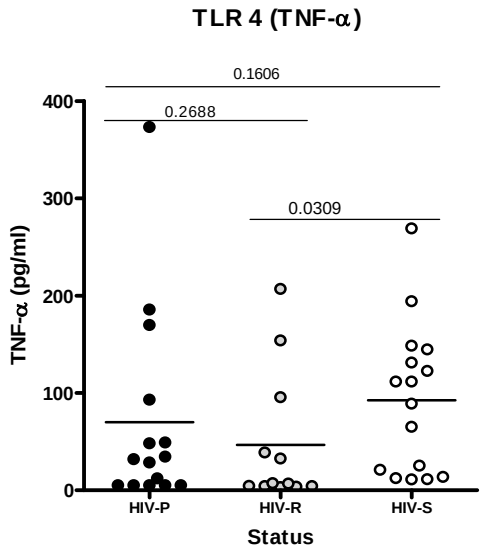

Figure 4.2: TNF-α Responses to *E. coli* LPS or TLR4
Production of TNF-α (y-axis pg/ml) by CMCs of women with different HIV status (X-axis) following stimulation with TLR4 ligand *E. coli* LPS. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

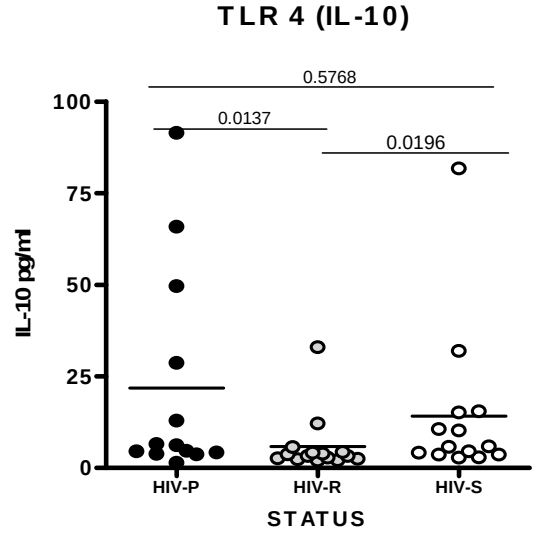

Figure 4.3: IL-10 Responses to *E. coli* LPS or TLR4
Production of IL-10 (y-axis pg/ml) by CMCs of women with different HIV status (x-axis) following stimulation with TLR4 ligand *E. coli* LPS. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

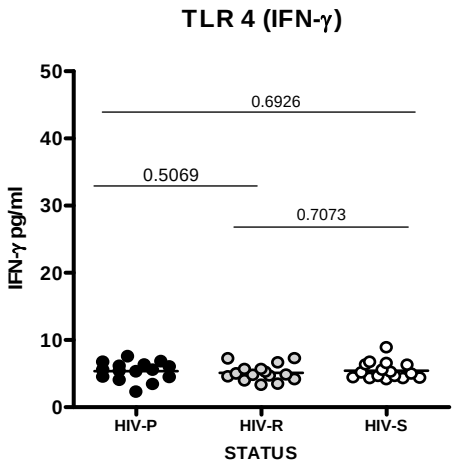

Figure 4.4: IFN-γ Responses to *E. coli* LPS or TLR4
Production of IFN-γ (y-axis pg/ml) by CMCs of women with different HIV status (x-axis) following stimulation with TLR4 ligand *E. coli* LPS. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

TNF-α response to TLR 4 (*E. coli* LPS) (Figure 4.2) was the most robust in CMC culture supernatants, as compared to the other detected cytokines. The production of TNF-α in HIV-1 resistant women was observed to be significantly lower compared to HIV susceptible p=0.0309 (Figure 4.3). The IL-10 responses in HIV resistant women was significantly lower than both HIV susceptible p=0.0137 and HIV positive p=0.0196 (Figure 4.3). The production of IFN-γ after *E. coli* LPS stimulation was undetectable (Figure 4.4).

4.1.2 TLR 7/8 Single Stranded RNA (ssRNA40)

The cytokine responses to ssRNA40/LyoVec in CMCs, were mainly TNF-α, IL-10 and IFN-γ.

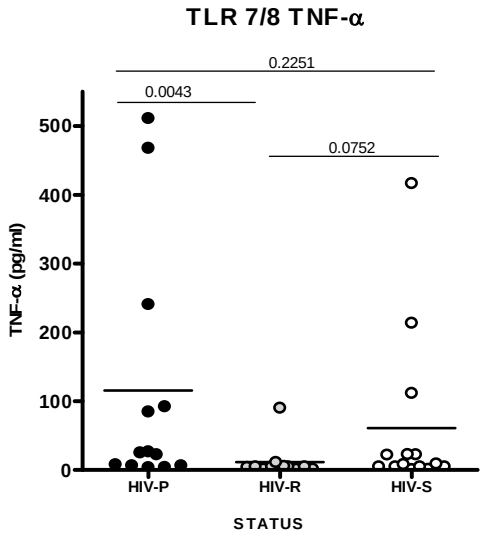

Figure 4.5: TNF-α Responses to ssRNA40 (TLR7/8)
Production of TNF-α (y-axis pg/ml) by CMCs from women with different HIV status (X-axis) following stimulation with TLR7/8 ligand ssRNA/LyoVec. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

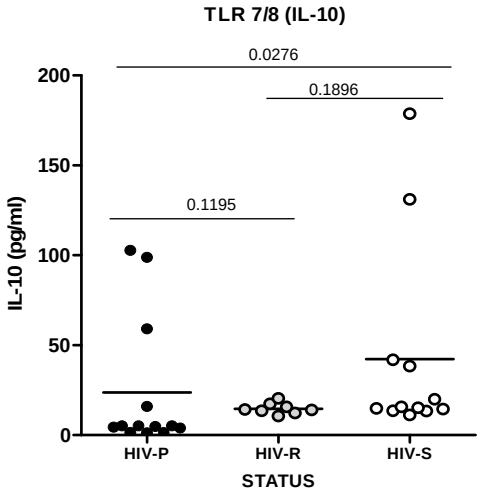

Figure 4.6: IL-10 Responses to ssRNA40 (TLR 7/8)
Production of IL-10 (y-axis pg/ml) by CMCs of women with different HIV status (x-axis) following stimulation with TLR7/8 ligand ssRNA40/LyoVec. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

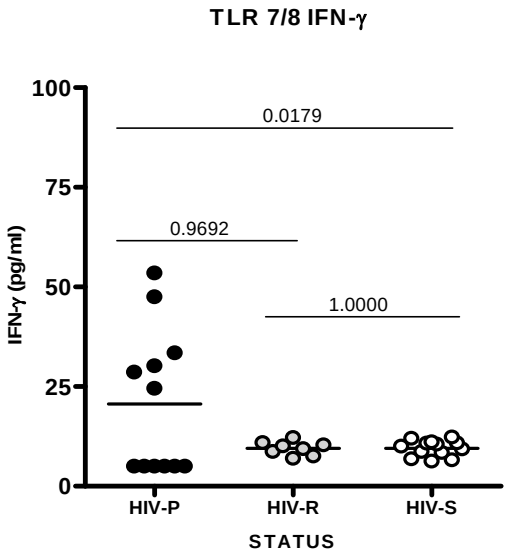

Figure 4.7: IFN-γ Responses to ssRNA40 (TLR7/8)
Production of IFN-γ (y-axis pg/ml) by CMCs of women with different HIV status (x-axis) following stimulation with TLR7/8 ligand ssRNA40/LyoVec. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

A significantly lower response in TNF-α production by HIV-R compared to HIV-P was also observed p=0.0043 (Figure 4.5). The CMCs from HIV-R women had a trend for lowered TNF-α production compared to HIV-S p=0.0756 (Figure 4.5). The production of IL-10 was significantly higher in HIV-S compared to the HIV-P women p=0.0276. A significantly higher production of IFN-γ was observed in a few of the HIV-P individuals, and higher IFN-γ production was observed in HIV-P compared to HIV-S p=0.0179 (Figure 4.7) but no differences were observed in between HIV-P and HIV-R, and between HIV-S and HIV-R.

4.1.3 TLR 7 (Imiquimod –R837)

The cytokine responses to Imiquimod quantified using two techniques; the responses observed in the CBA assay consisted of mainly TNF-α, IFN-γ and IL-10 and ELISA was used to quantify human IFN-α responses.

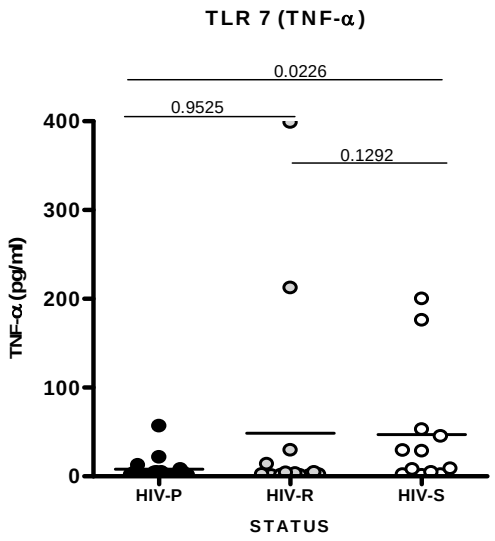

Figure 4.8: TNF-α Responses to Imiquimod orTLR 7
Production of TNF-α in pg/ml (y-axis) by CMCs from women with different HIV status (X-axis), after stimulation with TLR7 ligand Imiquimod. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

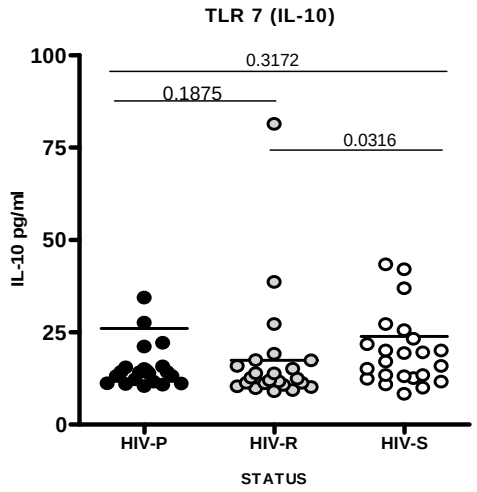

Figure 4.9: IL-10 Responses to Imiquimod or TLR 7
Production of IL-10 in pg/ml (y-axis) by CMCs of women with different HIV status (x-axis), after stimulation with TLR7 ligand Imiquimod. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

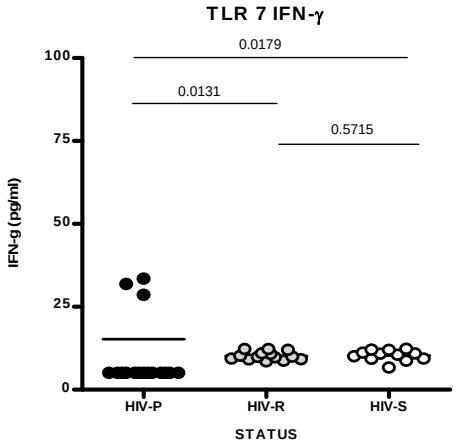

Figure 4.10: TLR7 IFN-γ Responses
Production of IFN-γ in pg/ml (y-axis) by CMCs of women with different HIV status (x-axis) after stimulation with TLR7 ligand Imiquimod. The HIV positive (HIV-P) women represented by black dots, HIV resistant (HIV-R) represented in grey and HIV susceptible (HIV-S) women represented in white.

The TNF-α responses in CMCs of HIV-P women were significantly lower production in comparison to those of HIV-S women p=0.0226, after stimulation of CMCs with

Imiquimod-R837 (TLR7) (Figure 4.8). There was no significant difference in TNF-α responses between HIV-P and HIV-R p=0.9525 and HIV-S p=0.1292 respectively. The level of IL-10 production by CMCs for HIV-1 resistant women was significantly lower compared to IL-10 production in CMCs from HIV-1 susceptible women p=0.0316 (Figure 4.9). Other comparisons of IL-10 production by CMCs from HIV-P to HIV-R or HIV-S showed no significant differences. The level of IFN-γ production by CMCs from a few HIV-P women was significantly higher as compared to both the HIV-R p=0.0131 and HIV-S p=0.0179 respectively (Figure 4.10). Between the two groups of HIV negative women there had little difference in IFN-γ production by CMCs.

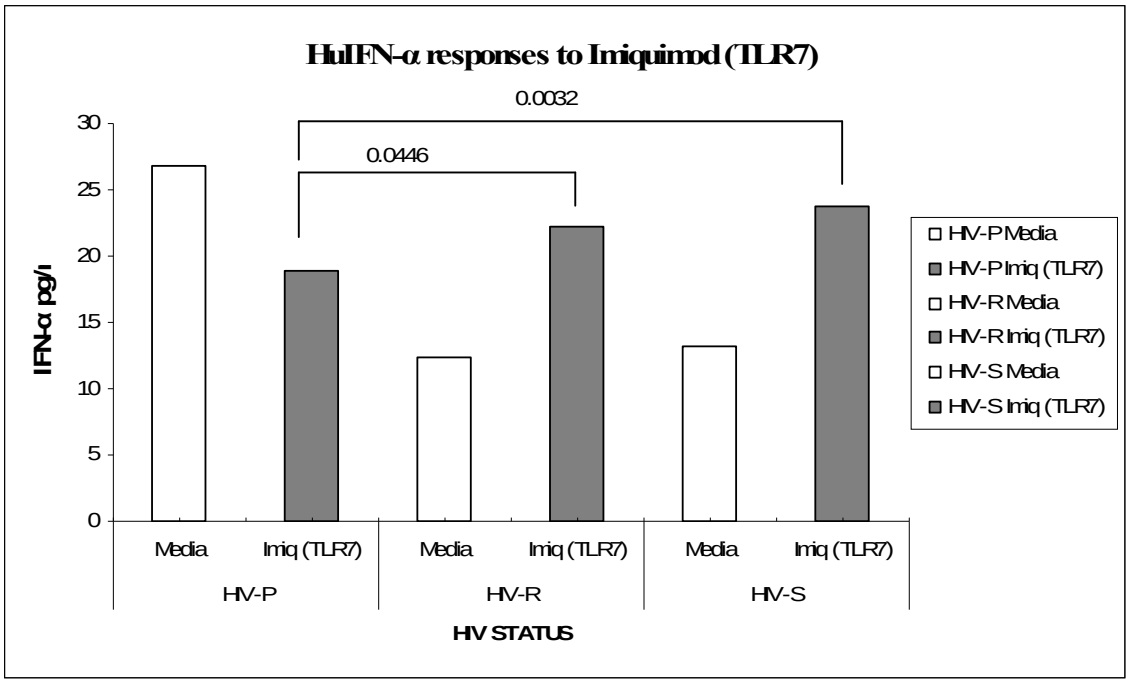

Figure 4.11: Hu IFN-α Responses to Imiquimod (TLR7)
Production of IFN-α (y-axis in pg/ml), by CMCs of women with different HIV status (x-axis), after stimulation with TLR7 ligand Imiquimod (Grey Bars) and in unstimulated media culture (White bars).

A fourth cytokine- HuIFN-α was assayed specifically in the supernatants obtained from CMCs stimulated with TLR 7 (Imiquimod) and supernatants of unstimulated CMCs (Media only) by ELISA. The levels produced due to stimulation TLR 7 was determined by comparing the media production of HuIFN-α (taken to be the baseline production in unstimulated cells) and the difference obtained from the levels of HuIFN-α produced by TLR 7stimulated CMCs. The value obtained was used to plot a comparison graph **(Fig. 12)** between the study participant groups**.** (The negative values are due to the IFN-α in media being higher than Imiquimod TLR7 stimulated cell culture supernatant). IFN-α responses in HIV-P subjects was depressed considerably compared to the HIV resistant's p=0.0446 and HIV susceptible women p=0.0032 respectively.

4.1.4 Other Cytokine Responses

Other cytokines evaluated using BD CBA Th1/Th2 both Kit 1 and Kit 2 were IL-2, IL-4, IL-5 and IL-6. There were no detectable cytokine responses to IL-2, IL-4, IL-5 and IL-6 (No comparison was done for these cytokines, but data is contained in Appendices).

4.2 Comparison of Cytokine responses per TLR ligand

A comparison was made of the cytokine responses in CMCs to each TLR ligand, in the different groups of women divided based of their HIV status.

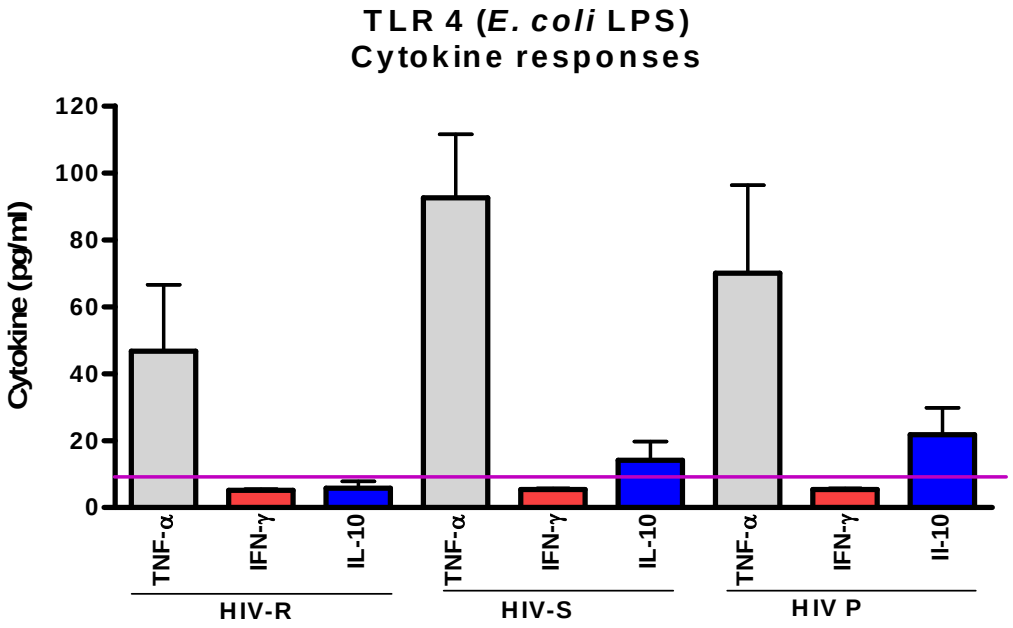

Figure 4.12: Cytokine responses to *E. coli* LPS by CMCs
Cytokine response (y-axis pg/ml) in CMCs from genital tract of women of different HIV status (x-axis). TNF-α (Light Grey bars), IFN-γ (grey bars) and IL-10 (black bars).

All the groups of women had TNF-α or proinflammatory cytokine responses to *E. coli* LPS stimulation (Figure 4.12). The IL-10 reponses to LPS by HIV-R women was undetectable, some IL-10 response was observed in HIV-P and HIV-S. The IFN-γ responses in all three groups of women were undetectable

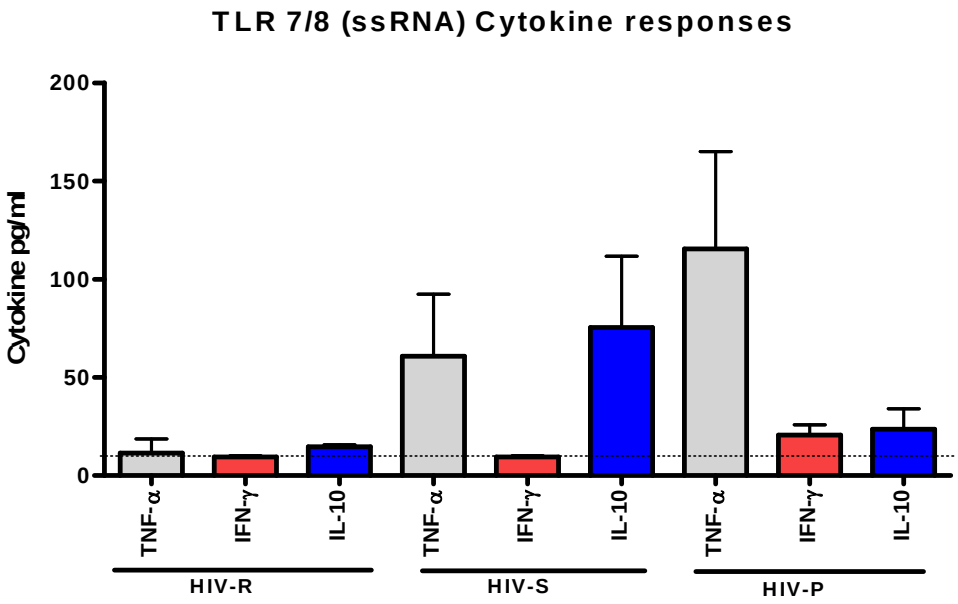

Figure 4.13: Cytokine responses to ssRNA40/LyoVec by CMCs
Cytokine response (y-axis pg/ml) to ssRNA40/LyoVec a TLR 7/8 ligand, by CMCs from genital tract of women of different HIV status (x-axis). TNF-α (light Grey bars), IFN-γ (grey bars) and IL-10 (black bars).

All cytokine responses to ssRNA40/LyoVec a HIV-1 single strand analogue were undetectable in HIV-R women. The TNF-α responses in CMCs to ssRNA40/LyoVec appeared to have an incremental trend with the lowest TNF- α responses occurring in HIV-R, this followed by HIV-S and the highest TNF-α responses were in CMCs from HIV-P women. The IL-10 responses in HIV-R women was undetectable, HIV-P women's CMCs gave low IL-10 responses and HIV-S women had the highest IL-10 responses to ssRNA40/LyoVec. The CMCs from HIV-P women had low IFN-γ responses to ssRNA40/LyoVec as the responses in the other two groups were undetectable.

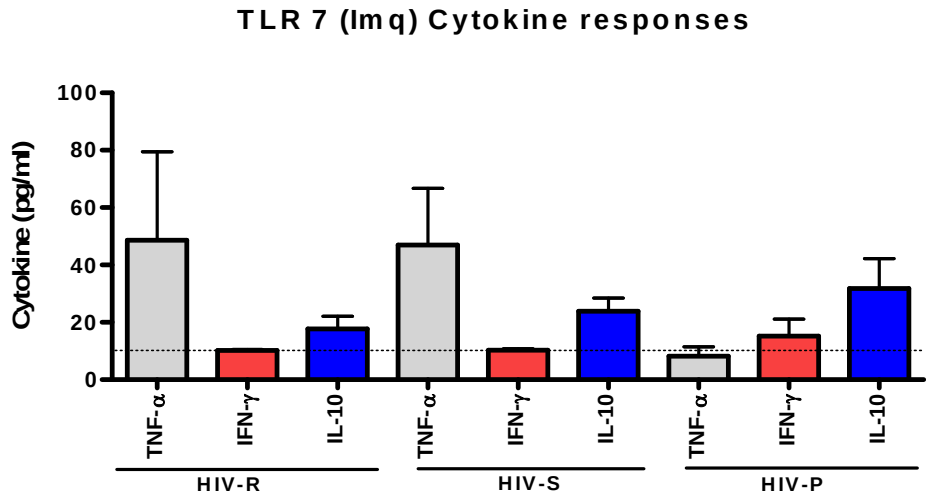

Figure 4.14: **Cytokine responses to Imiquimod by CMCs**
Cytokine response (y-axis pg/ml) to Imiquimod a TLR 7 ligand, by CMCs from genital tract of women of different HIV status (x-axis). TNF-α (light grey bars), IFN-γ (grey bars) and IL-10 (Black bars).

Imiquimod stimulated TNF-α responses in both groups of HIV negative- HIV-R and HIV-S women through TLR7 activation. The TNF-α response by CMCs from HIV-P women was undetectable. IFN- γ responses in HIV-P was higher than those observed in HIV-S and HIV-R. IL-10 responses by CMCs to Imiquimod increased from HIV-R, HIV-S to HIV-P who had the highest IL-10 responses.

CHAPTER FIVE

5 DISCUSSION

Discovery of Toll-like receptors recognition and signaling pathways, has led to invigorated research aimed at enhancing the understanding of innate immune responses in infectious diseases. The initial events in HIV transmission involves a complex interaction of the HIV virus and target cells (Hladik 2007). Infection is driven by cytokines and chemokines which may be responsible for recruitment and seeding of HIV susceptible cell populations with either cell free or bound virus, leading to the establishment of HIV infection. The characteristic immune activation that follows this establishment of HIV-1 infection, and that remains present throughout the disease's chronic phase (Hazenberg 2003), may be trigged very early through innate proinflammatory cytokine responses (Norris 2006).

Toll-like receptors expression in the genital tract has previously been characterized in both in the upper (Wira 2005) and lower genital tract (Herbst-Kralovetz 2008). This differential expression of TLRs allows for specific analysis of cytokine function in different innate cells from the genital tract. This study investigated the differences in cytokine production in CMCs following stimulation with different TLR agonists, in three groups of women divided according to their HIV status.

In order to determine optimal time for culture of CMCs with TLR ligands, time-response experiments were done using LPS and ssRNA (Figure 4.1). The TNF-α responses by

CMCs to *E. coli* LPS appeared to peak after 18 hours in culture and level off. The TNF-α responses to ssRNA peaked at 18 hours then started to decline, the IL-10 response to LPS continued to rise after 20hrs while the IL-10 responses to ssRNA peaked at 18hours and declined after that. The declines in the TNF-α and IL-10 responses could have been due to activation induced cell death of CMCs in culture or disintegration of cytokines. The IFN-γ responses by CMCs to LPS and ssRNA appeared to follow no trend and may be not related to TLR4 and TLR 7/8 stimulation of CMCs.

In the main study, the cytokine responses observed due to LPS, ssRNA40 and Imiquimod stimulation of CMCs were TNF-α, IFN-γ, and IL-10. These responses have previously been described in the earliest stages of HIV-1 infection (Norris 2006). A fourth cytokine known for its antiviral properties IFN-α was also assayed in supernatants of CMCs stimulated with Imiquimod (TLR7). HIV-R women had lower cytokine responses when compared to those of the other two groups of women, HIV-P and HIV-S. Lipopolysaccharide from E. coli bacteria stimulated proinflammatory cytokine responses in CMCs with little anti-inflammatory IL-10 responses. IFN-γ response after TLR4 stimulation were absent, an observation that has been described previously.

Single strand RNA and Imiquimod are able to stimulate cytokine production through TLR7. Single strand RNA a HIV analogue, led to quantifiable proinflammatory cytokine responses in HIV-S and HIV-P. The highest proinflammatory or TNF-α response to ssRNA stimulation occurred in CMCs from HIV-P, this was reversed when Imiquimod

an immune-modulator and TLR7 ligand was used. HIV-R women had undetectable TNF-α responses following ssRNA stimulation, but when Imiquimod was used to stimulate CMCs high amounts TNF-α was produced. IL-10 production after Imiquimod stimulation of CMCs led to production of IFN-α in HIV-R and HIV-S, yet in HIV-P women's CMCs the amount IFN-α was lower in Imiquimod stimulated CMCs than in the negative control. If the cytokine responses observed in CMCs after stimulation with ssRNA represent what takes place in the genital tract after innate recognition of HIV virus particle ssRNA, then imiquimod appears to be able to act as an immune-modulator by altering the cytokine responses by CMCs depending on the HIV status of the donor. The biological implications of the shifts in cytokine production due to stimulation of TLR7 signaling pathway using different TLR ligands may be due to difference in immune function of CMCs from the two groups of women, or this may be as a result of differences in the signaling pathways after stimulation by the two ligands. This should be further investigated.

The results of this study showed overall TNF-α hyper-responsiveness by CMCs to stimulation with the TLR agonists tested that is; LPS (TLR4), ssRNA40 (TLR 7/8) and Imiquimod (TLR7). Other studies have shown TNF-α hyper-responsiveness in the genital tract by TLR2 and TLR4 activation in women with bacterial vaginosis (Sha 2005). In a different study, no significant difference was observed in TNF-α levels in cervical lavage samples from genital tract of HIV positive women compared to those of HIV negative women (Sha 1997). Similar observations were made from this study

where no significant difference in TNF-α production was observed between HIV positive women and high risk HIV negatives. Thus the similarity in the proinflammatory (TNF-α) cytokine responses to LPS and ssRNA in CMCs from HIV-P and HIV-S is in agreement with previous observations. However, HIV-Resistant women had significantly lower TNF-α response to LPS TLR4 compared to HIV susceptible women, and lowered responses than HIV-P, a trend for lowered TNF-α response of CMC to ssRNA compared HIV-S was also observed. The difference in proinflammatory responses of CMCs due TLR signaling between HIV-R and the other two susceptible groups of women is indicative of the potential role inflammatory responses play in susceptibility to infection with HIV virus. The inflammatory responses may play a role in recruitment and seeding of different cell population during the early phases of HIV infection accelerating the spread and establishment of the HIV virus in the genital mucosa. This mechanism may be similar to the homing of inflammation cells on the endothelium, where proinflammatory cytokines play an integral role in influencing expression of integrins and selectins on endothelial cells, aiding recruitment and homing of neutrophils and other leukocytes to sites of infection (Jung U 1998).

Stimulation of CMCs with ssRNA40, an analogue of HIV-1 ssRNA, led to the most robust production of TNF-α, indicating the potency of ssRNA as a HIV viral product in the inflammation response leading to HIV-1 viral dissemination, and potentially initiation of immune activation that persists throughout HIV-1 disease. The activation of TLRs by HIV products after infection or by co-infection with pathogen may be

responsible for the maturation and activation of various cell populations. Toll-like receptor signaling has been shown to play a role in the maturation of immature DC (iDC) by enhancing expression of DC-SIGN on iDCs. DC-SIGN receptors on DCs then aid in the transport of cell free HIV virus to target cells. The maturation and activation of different HIV target cells then increases the infection rate R^0 uninfected cells and driving the spread of the HIV virus. TLR signaling also leads to activation of transcription factors such as NF-kβ and AP-1 thereby increasing HIV virions production by increased HIV-1 LTR expression.

Anti-infammatory cytokine IL-10 has been demonstrated in previous studies to block HIV virus replication in monocyte-derived macrophage cultures *in vitro*, and prevent HIV-1 induced TNF-α and IL-6 release (Weissman 1994), it has also been shown to inhibit IFN-γ, IL-2 and IL-4 activity (Pestka 2004). A more recent study, has demonstrated IL-10 produced by CD4+ T cells had an antiviral effect, by diminishing HIV-1 replication (Andrade 2007). In this study, CMCs from HIV positive women produced very little IL-10 following stimulation with ssRNA and through TLR7/8, indicating a possible immune dysfunction of CMCs from HIV positive persons to produce anti-inflammatory cytokines**.** Stimulation through the same TLR7 signaling pathway with Imiquimod, appeared to increase IL-10 while lowering TNF-α production in HIV positive women (Ref. Figure 4.12 and 4.14)**.** Thus it would appear that the "normal" balance in the case of HIV infection, between TNF-α and IL-10 production is as observed in HIV-Susceptible women, who had a higher IL-10 production compared to

TNF-α in response to HIV-1 analogue ssRNA (Figure 4.12). The high IL-10 responses may be able to have a regulatory effect to proinflammatory cytokine production, an effect that has previous been described, with IL-10 being able to suppress other proinflammatory cytokines leading to development of immune dysfunctions such as anergy and inhibition of HIV viral replication (Green D. R. and Beere H.M 2000). Elevated levels IL-10 has also been observed in elderly patients receiving antiretroviral therapy compared to younger patients, with the former demonstrating better treatment outcomes (Andrade 2007). This poses a new question as to the role of the IL-10 response in HIV-1 infection, its advantage or disadvantages in augmenting host immune responses or its predictive value in HIV-1 disease progression.

Imiquimod is an immune response modifier (Testerman 1995) and a TLR7 agonist. Previously, it has demonstrated potent indirect antiviral and anti-tumour activity both *in vitro* and *in vivo* (Miller 1999) due to its ability to induce production interferons that direct the innate and acquired immune response (Tyring 1996). Imiquimod has been shown to be able to induce interferon-alpha and other cytokines through TLR7 signaling (Hemmi 2002). This induction has been shown to lead to maturation of plasmacytoid dendritic cells (Gibson 2002). Interferon-α has also been shown to stimulate IFN-γ production and activate NK cells (Vollstedt 2001). The present study is one of the first to investigate cytokine responses due to Imiquimod stimulation of the innate immune cellular compartment (CMCs) in the genital tract of women. The ability of TLR7 (Imiquimod) to stimulate production of either anti-inflammatory or proinflammatory

cytokines in CMC, provides a new rationale for consideration for its usage in development of therapeutics in HIV, specifically targeting the restoration of a normalcy in cytokine function, control of viral shedding by HIV-1 latently infected cells and promoting production of antiviral interferon's (IFN-α) in mucosal tissues.

Cytokine responses can be broadly classified into T helper 1 /T_H1 (IL-1, IFN-γ or TNF-α), T helper 2/T_H2 (IL-4, IL-5, IL-8, IL-10) or T helper 0/T_H0 (a combination of T_H1 and T_H2). The exact number of cytokine categories remains controversial. Two different attempts have been made at describing the pattern of cytokine production in HIV-1 disease. A first group of investigators reported to observe a skew towards T_H1 cytokine responses early in HIV infection and a later switch toward T_H2 responses with HIV-1 disease progression. Yet others report observing a shift from T_H1 to T_H0 with disease progression. Based on this study's design, the samples collected from the study participants were done at a single time point. As such the data does not give information on the changes in the cytokine production with HIV-1 disease progression which would require sample collection at multiple points. However, the analyzed culture supernatants had undetectable T_H2 responses- IL-2, IL-4, IL-5 and IL-6, with only quantifiable IL-10 responses being detected. Interleukin 10 is considered a T_H2 response of mononuclear cells, and its levels increase late in the HIV-1 disease often with worsening prognostic implications. More recent observations shown that it is elevated in patients with AIDS (Klein 1997). The responses observed in this study tended to have a T_H1 bias, with TNF-α, IFN-γ and IFN-α constituting most of the responses.

In order to understand the relevance of the cytokine responses observed in this study in HIV disease pathogenesis, a comparison was done on TLR expression in CMCs (Lester R.T. and Xiaodan Yao, unpublished data) and cytokine responses to TLR agonists of CMCs. Expression of TLR4 in CMCs did not vary with HIV-1 status however the cytokine responses observed varied significantly between the different HIV groups. Proinflammatory cytokine, TNF-α was by far the most robust response by CMCs to stimulation with *E. coli* LPS (TLR4). Considering there was no significant difference in TLR4 mRNA expression in CMCs from the three groups, the difference in cytokine responses may be due to variation in immunological function of CMCs. This may be due to the differences in cytokine responses in CMCs to LPS from the two HIV negative groups may be related to differences in TLR recognition or signaling pathway, which may affect TLR4 responsiveness. Lipopolysaccharide has been shown to induce tolerance of innate cells when a certain threshold of exposure is reached, tolerization of CMCs could also provide an alternative explanation for the difference in cytokine response by CMCs to LPS between the three groups of women. These differences may also be due to TLR4 gene polymorphisms, which have recently been shown to alter TLR4 function, with two TLR4 Polymorphisms (Asp299Gly and Thr399Ile) being linked to reduced cytokine response and increased susceptibility to gram-negative infections (Ferwerda 2008). Another possible mechanism of non-variant TLR expression in general, may involve proinflammatory cytokines themselves such as GM-CSF (Granulocyte monocyte colony stimulating factor) and IFN-γ. Recent findings

demonstrate that these proinflammatory cytokines specifically regulated the expression of TLR expression in innate immune cells (O'Mahony 2008).

The HIV-1 virus has ssRNA sequences which has GU-rich repeats (Jurk 2002), is recognized by two TLRs, TLR7 and TLR8. The expression of these two receptors on CMCs was observed to increase from HIV-R (lowest) to HIV-S and HIV-P (highest) (Xiaodan Yao Unpublished data). Some of the cytokine responses such as TNF-α production, showed a similar increment production directly related with TLR expression. There appears to be in existence a direct relationship between cytokine production following TLR stimulation in CMCs and TLR mRNA expression in regard to TLR7 and TLR8

HIV resistant women appear to be exposed to HIV-1 virus through heterosexual sex with multiple partners and yet remain persistently seronegative. They are the center of focus for HIV-1 resistance studies as the search for correlates of resistance for an HIV vaccine continues. The commercial sex-workers from the cohort used in this study offer the possibility for studying the mechanism for natural immunity to the HIV-1 virus. It is now apparent that the lowered ability to acquire HIV-1 infection in this group of women may be due to multiple factors and not a single unique mechanism. The results of this study demonstrated, lower proinflammatory cytokine responses by CMCs from HIV resistant women to HIV-1 analogue ssRNA and bacterial component LPS. This reduced responsiveness to LPS or TLR 4 agonist, in HIV-1 resistant women may be due to

failure of recognition due genetic polymorphism in the TLR 4 gene, or alterations in TLR4 signaling and its related pathways. However, responsiveness of CMCs to HIV-1 analogue ssRNA appeared to bear a direct relationship with the level of TLR7 mRNA expressed on CMCs.

In order to accurately describe the differences in functional responses observed in the CMCs of HIV resistant women, in comparison to those in normal HIV susceptible women (HIV-S and HIV-P) the proinflammatory cytokine responses were considered. The proinflammatory responses that were observed to a HIV analogue ssRNA and bacterial LPS in HIV-1 resistant women was low possibly due to an equally low level of activation of components of the TLR signaling pathways. The HIV-1 resistant women showed the lowest level of TLR 7 and TLR 8 gene expression, and similarly showed the lowest production of cytokines in CMCs following stimulation with either Imiquimod or ssRNA (TLR 7 or TLR 8 respectively). These results demonstrate that HIV resistant women have lowered responsiveness to viral ssRNA and which can be related to decreased expression of endosomal TLR 7 and 8.

Taken together, the lowered proinflammatory cytokines production in the female genital tract due to TLR stimulation appears to have an influence on susceptibility to HIV-1 infection. This may be due to variable TLR gene expression in HIV disease, TLR gene polymorphisms or other influences on TLR expression eventually leading to difference in innate function. The immune system is complex and the fact that there may be other

influences for mounting resistance or susceptibility by an individual to the HIV virus other that TLR signaling and recognition, has to be acknowledged. Understanding the overall picture of initial events in the genital tract during HIV infection will help in the development of a protective vaccine or effective cures for HIV disease.

CHAPTER SIX

6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, this study showed that the stimulation of CMCs with *E. coli* LPS (TLR4 ligand), ssRNA40/LyoVec (TLR 7/8 ligand) and Imiquimod (TLR7 ligand), led to variable production of proinflammatory cytokines and antiviral interferons depending on the HIV-1 status of the donor.

The main cytokine responses after stimulation of CMCs with *E. coli* LPS, ssRNA and Imiquimod observed was TNF-α, IFN-γ, IL-10 and IFN-α (TLR7 Imiquimod stimulation). HIV resistant women had lower TNF-α response and IL-10 responses in CMCs to *E. coli* LPS compared to HIV susceptible women. HIV resistant women also had lower TNF-α responses ssRNA compared to HIV positive women and HIV susceptible women. HIV-P women had significantly higher IFN-γ responses to both ssRNA and Imiquimod compared to HIV-R and HIV-S women..

Stimulation of the TLR7 signaling pathway with different ligands (ssRNA and Imiquimod) led to alterations in CMC cytokine production with the changes observed depending on the HIV status of the donor.

The cytokine responses to TLR7 and TLR7/8 ligands corresponded with the TLR7 and TLR8 mRNA expression in the CMCs. The cytokine responses to TLR4 were independent of TLR4 expression.

The study showed that HIV resistant women have different TLR expression and function in their genital tract compared to HIV susceptible and HIV positive women. Therefore the null hypothesis was rejected, and the alternative hypothesis accepted. This study has contributed to the current knowledge of innate immune responses to TLRs by CMCs while demonstrating variability in responses by CMCs depending on the HIV status of the female donor.

6.2 Recommendations

Further comparison and definition of the cytokine responses in the genital tract following TLR ligand stimulation needs to be done especially in HIV-1 positive individuals either receiving ARV therapy and not on ARV therapy.

The cytokine responses by other innate should be observed at different time points, and possibly to enhance the understanding on cytokine profile with HIV-1 disease progression in the genital tract.

More research efforts should be directed towards the discovery of more efficient methods of CMCs sample collection and purification from the genital tract of women. This is considering that the current methods produce low cell numbers and are limited in the number of washes that can be carried out to clean the CMCs which are usually trapped in mucilage or mixed with tissue debris. This will allow for easier quantification of cytokines, RNA or DNA in different experiments, which currently is very difficult due to low cell numbers,

Furrther studies should be done to characterize the immune function of other innate cells such as NK cells, Langerhan cells and Neutrophil in the genital tract of HIV positive versus HIV negative and HIV resistants is needed to further improve on the knowledge the role of innate immunity at the interface of HIV transmission.

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APPENDICES

APPENDIX I: DATA TABLES

HU IFN-α ELISA

BATCH ONE

Table 0.1: Batch One Plate layout

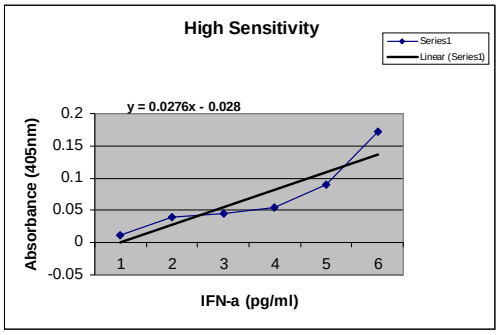
	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	767	2840								
B	BL	BL	1072	1248								
C	S1	S1	1705	1940								
D	S2	S2	1740	2039								
E	S3	S3	2317	2141								
F	S4	S4	2839	2842								
G	S5	S5	2841	0								
H	S6	S6	1956	2314								

Table 0.2: Absorbance at 405nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.003	0.002	0.031	0.045								
B	0.003	0.005	0.027	0.034								
C	0.011	0.034	0.071	0.024								
D	0.039	0.083	0.072	0.014								
E	0.045	0.126	0.022	0.019								
F	0.055	0.249	0.028	0.053								
G	0.089	0.464	0.029	0.012								
H	0.172	0.61	0.033	0.022								

Figure 0.1: Batch One-High and Extended Sensitivity Standard Curve

A



B

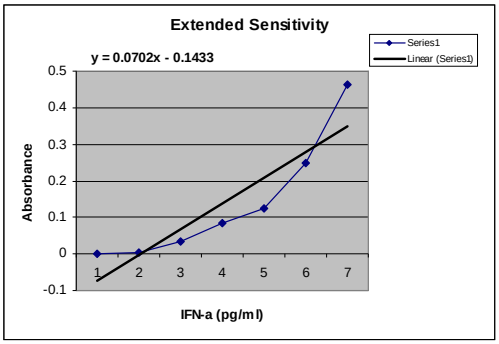


Table 0.3: Concentration of HuIFN-α per sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	14.81501	22.18131								
B	0	0	10.24498	14.46988								
C	12.5	156	34.80817	9.707414								
D	25	312	36.81595	4.147188								
E	50	625	11.48788	9.169321								
F	100	1250	11.84181	25.39391								
G	200	2500	13.59626									
H	500	5000	17.57676	11.31208								

BATCH TWO

Table 0.4: Batch Two Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	24	1854	2051	59	2124	2128	2285	2451	2143	2616
B	BL	BL	2146	2622	2595	2264	2127	2326	2463	1814	1815	1848
C	S1	S1	2182	2442	2751	2682	2151	2367	2652	2408	1907	1930
D	S2	S2	525	2843	2661	2142	2342	2458	2235	2432	2196	2296
E	S3	S3	1846	2842	1694	2323	2202	1067	1996	2587	2346	2561
F	S4	S4	2055	415	1969	2454	2345	2551	2786	2298	887	2259
G	S5	S5	2315	1941	2809	2102	2445	2046	1749	2674	1552	2297
H	S6	S6	1151	2042	2446	2257	1165 28	2237	2137	2302	1952	1152 25

Table 0.5: Absorbance at 405 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.002	0.001	0.091	0.095	0.063	0.275	0.072	0.049	0.165	0.067	0.067	0.075
B	0.003	0.004	0.277	0.076	0.115	0.111	0.042	0.076	0.035	0.026	0.073	0.061
C	0.037	0.194	0.048	0.06	0.055	0.067	0.123	0.405	0.116	0.045	0.043	0.073
D	0.06	0.342	0.048	0.06	0.053	0.033	0.045	0.037	0.041	0.035	0.04	0.08
E	0.094	0.576	0.039	0.048	0.048	0.129	0.141	0.038	0.331	0.031	0.165	0.067
F	0.166	1.167	0.045	0.047	0.084	0.143	0.043	0.047	0.066	0.075	0.063	0.107
G	0.344	1.635	0.05	0.035	0.046	0.016	0.045	0.048	0.15	0.035	0.111	0.044
H	0.634	1.89	0.039	0.082	0.143	0.024	0.04	0.096	0.029	0.027	0.039	0.029

Figure 0.2: Batch Two-High and Extended Sensitivity Standard Curves

A **B**

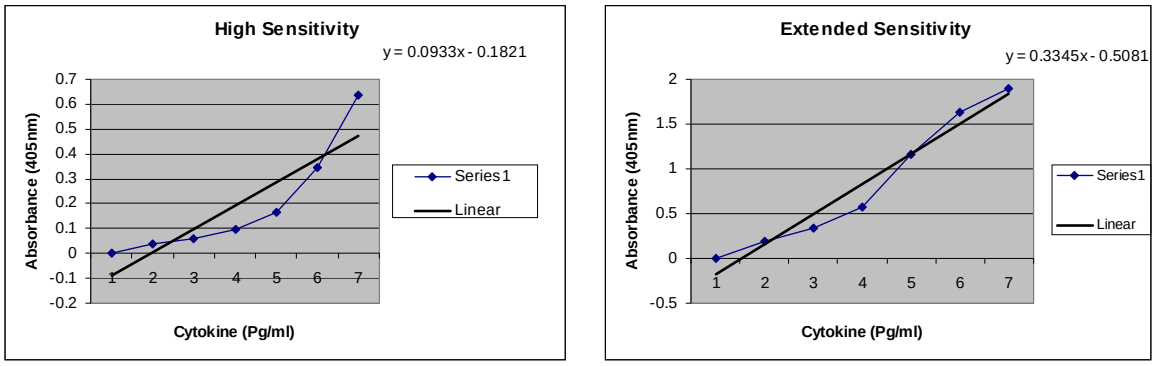


Table 0.6: Concentrations of Hu IFN-α pg/ml

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	14.814	15.508	9.888	45.888	11.487	7.349	27.416	10.600	10.600	12.017
B	0	0	46.229	12.196	18.946	18.262	6.051	12.196	4.725	2.962	11.666	9.529
C	12.5	156	7.165	9.348	8.445	10.600	20.312	68.045	19.118	6.610	6.238	11.666
D	25	312	7.165	9.348	8.082	4.340	6.610	5.108	5.863	4.725	5.675	12.896
E	50	625	5.487	7.165	7.165	21.332	23.366	5.298	55.359	3.952	27.413	10.600
F	100	1250	6.610	6.980	13.596	23.708	6.238	6.980	10.423	12.017	9.888	17.576
G	200	2500	7.532	4.725	6.796		6.610	7.165	24.891	4.725	18.262	6.424
H	500	5000	5.487	13.249	23.708	2.556	5.675	15.684	3.559	3.162	5.487	3.559

BATCH THREE

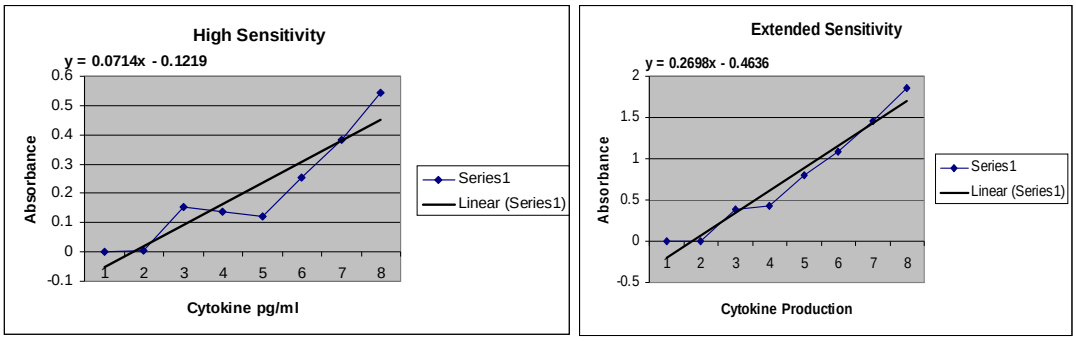
Table 0.7: Batch Three Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	2325	2327								
B	BL	BL	2337	2303								
C	S1	S1	2349	106527								
D	S2	S2	2756	115621								
E	S3	S3	2765	100367								
F	S4	S4	2766	104654								
G	S5	S5	889	108172								
H	S6	S6	1500	1815								

Table 0.8: Absorbance at 405nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.001	0.001	0.131	0.185	0.006	0	0.001	0.001	0.001	0.001	0	0.001
B	0.003	0.002	0.191	0.101	0.003	0	0.001	0.001	0.001	0.001	0	0.001
C	0.153	0.387	0.213	0.068	0.003	0	0.001	0.001	0.001	0.001	0	0.001
D	0.138	0.428	0.124	0.051	0.003	0	0.001	0.001	0.001	0.001	0	0.001
E	0.123	0.799	0.062	0.038	0.002	0	0.001	0.001	0.001	0.001	0	0.001
F	0.255	1.083	0.097	0.319	0.001	0	0.001	0.001	0.001	0.001	0	0.001
G	0.382	1.453	0.127	0.051	0.004	0	0.001	0.001	0.001	0.001	0	0.001
H	0.544	1.851	0.142	0.035	0	0	0.001	0.001	0.001	0.001	0	0.001

Figure 0.3: Batch Three- High and Extended Sensitivity Standard Curves



BATCH FOUR

Table 0.9: Batch Four- Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	1996	2587	2346	2561	2425	2478	2818	1500	2551	115225
B	BL	BL	2786	2298	887	2259	258	2810	2325	2227	2046	106527
C	S1	S1	1749	2674	1552	2297	1535	2014	2337	2303	2237	115621
D	S2	S2	1437	2303	1952	1250	2260	2195	2349	2128	2235	
E	S3	S3	2451	2643	2616	2123	2472	2261	2756	2326	2463	
F	S4	S4	1814	1815	1848	1529	2559	1700	2766	2367	2452	
G	S5	S5	2408	1907	1930	2137	2147	1892	2765	2458	2235	
H	S6	S6	2432	2196	2296	2266	2426	2505	1889	1067	116588	

Table 0.10: Batch Four Absorbances at 405nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.001	0.605	0.122	0.594	0.09	0.103	0.059	0.068	0.053	0.041	0.052
B	0.001	0.002	0.07	0.047	0.051	0.493	0.203	0.056	0.059	0.136	0.064	0.057
C	0.077	0.109	0.407	0.053	0.077	0.076	0.039	0.06	0.074	0.311	0.084	0.048
D	0.105	0.16	0.116	0.06	0.027	0.058	0.056	0.042	0.094	0.093	0.296	0.069
E	0.178	0.25	0.039	0.038	0.044	0.023	0.033	0.215	0.182	0.093	0.076	0.036
F	0.327	0.332	0.101	0.043	0.071	0.036	0.06	0.051	0.053	0.033	0.073	0.052
G	0.674	0.557	0.466	0.049	0.042	0.051	0.055	0.059	0.021	0.022	0.026	0.035
H	1.066	1.057	0.091	0.059	0.092	0.043	0.185	0.04	0.082	0.037	0.034	0.043

Figure 0.4: Batch Four- High and Extended Sensitivity Standard curves

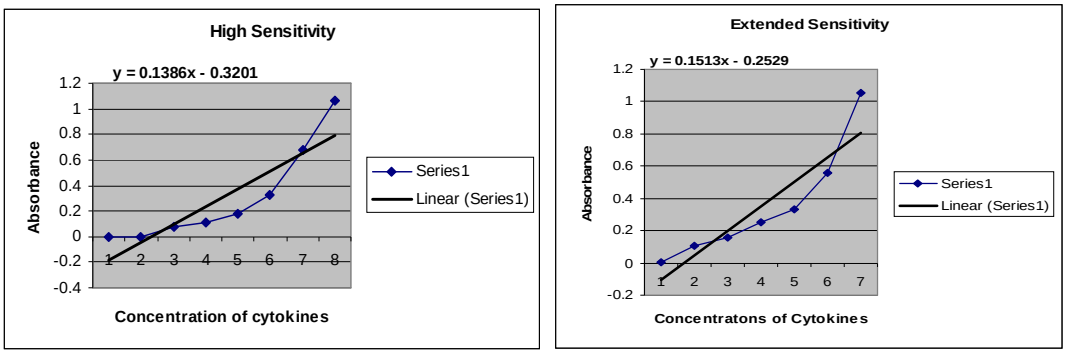


Table 0.11: Batch Four- Concentration of HuIFN-α pg/ml

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.004	0.001	1.873	0.349	1.88	0.284	0.323	0.193	0.218	0.165	0.135	0.168
B	0.002	-0.003	0.206	0.13	0.136	1.541	0.634	0.165	0.175	0.418	0.191	0.165
C	0.232	0.335	1.288	0.151	0.213	0.235	0.121	0.184	0.226	0.992	0.261	0.139
D	0.326	0.498	0.339	0.178	0.093	0.183	0.18	0.132	0.297	0.287	0.95	0.213
E	0.566	0.795	0.123	0.108	0.137	0.073	0.105	0.697	0.586	0.283	0.247	0.113
F	1.026	1.041	0.307	0.12	0.218	0.113	0.19	0.156	0.157	0.101	0.225	0.153
G	2.083	1.733	1.457	0.147	0.125	0.158	0.164	0.154	0.017	0.063	0.081	0.107
H	2.706	2.789	0.288	0.18	0.197	0.13	0.594	0.129	0.243	0.13	0.123	0.153

BATCH FIVE

Table 0.12: Batch Five- Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	767	1956	24	1854	2446	2451	2124	2326	2463	2765
B	BL	BL	1072	2640	2146	2622	59	2661	2127	2367	2652	889
C	S1	S1	1705	1248	2182	2442	2264	2682	2151	2458	2235	1500
D	S2	S2	1740	1940	525	2843	415	2144	2342	1067	2325	2227
E	S3	S3	2317	2039	1846	2842	1941	2323	2202	2551	2337	2303
F	S4	S4	1766	2141	2055	1694	2042	2454	2345	2046	2349	
G	S5	S5	2839	2312	2315	1969	2051	2102	2445	2237	2756	
H	S6	S6	2841	2314	1151	2809	2595	2257	2128	2235	2766	

Table 0.13: Batch Five- Absorbance at 405nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.021	0.003	0.088	0.019	0.1	0.031	0.027	0.026	0.037	0.05	0.042	0.048
B	0.009	0.008	0.036	0.028	0.067	0.024	0.026	0.042	0.035	0.038	0.027	0.022
C	0.024	0.001	0.025	0.037	0.022	0.021	0.062	0.047	0.09	0.031	0.021	0.031
D	0.065	0	0.025	0.019	0.023	0.018	0.027	0.028	0.019	0.014	0.021	0.156
E	0.111	-0.002	0.017	0.016	0.024	0.017	0.022	0.021	0.15	0.018	0.027	0.056
F	0.274	-0.001	0.039	0.025	0.028	0.025	0.021	0.049	0.047	0.008	0.022	0.102
G	0.559	-0.004	0.021	0.027	0.026	0.018	0.022	0.074	0.058	0.057	0.022	0.019
H	0.681	-0.003	0.054	0.029	0.036	-0.005	0.031	0.036	0.034	0.136	0.138	0.046

Figure 0.5 Batch Five- High Sensitivity curve

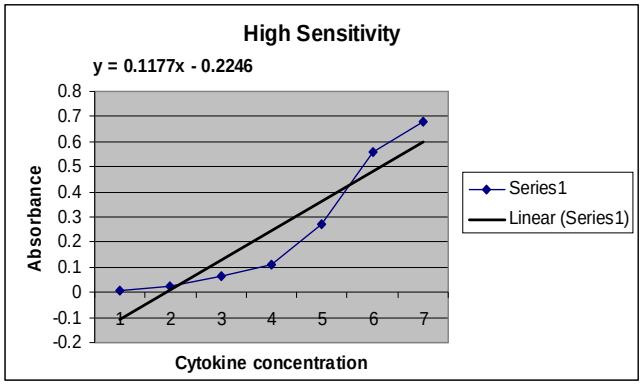


Table 0.14: Batch Five- Concentration of Hu IFN-α pg/ml

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	46.14669	5.373927	49.06841	22.5729	18.44906	17.91842	24.90381	30.75087	27.66223	29.55715
B	0	0	21.51665	14.59559	36.59773	9.926392	10.95816	24.14996	20.85853	22.5729	11.88595	10
C	12.5	156	12.7377	22.78338	10	8.735448	34.72366	28.46799	44.82917	17.64758	10	17.09221
D	25	312	12.31947	10	9.350574	10	14.24635	14.93645	10	10	10	63.81065
E	50	625	10	10	12.7377	10	9.350574	8.735448	60.55799	10	13.51444	31.75778
F	100	1250	23.77537	7.295924	13.51444	13.89021	5.373927	29.40933	28.46799	10	6.424598	48.78586
G	200	2500	11.4334	15.58878	13.89021	10	9.350574	39.99911	33.79767	32.86036	11.4334	7.295924
H	500	5000	33.40572	19.9333	24.14996	10	20.62803	24.5279	21.95302	59.38448	60.55799	

APPENDIX II: TABLE OF STUDY PARTICIPANT COINFECTIONS

DATE	MLNO	CLASS	*Gonococcus sp*	Bacterial Vaginosis	*Trichomonas sp.*
11-Aug-07	24	POS	-ve	Norm	-ve
13-Nov-07	59	POS	-ve	Norm	-ve
12-Nov-07	157	POS	-ve	Norm	-ve
12-Oct-07	274	POS	-ve	Norm	-ve
14-Nov-07	415	POS	-ve	Norm	-ve
11-Sep-07	525	POS	-ve	Norm	-ve
12-Oct-07	915	POS	-ve	Norm	-ve
26-Nov-07	1067	POS	-ve	Norm	-ve
11-Dec-07	1151	POS	-ve	Norm	-ve
11-Jul-07	1248	POS	-ve	Norm	-ve
01-Sep-08	1250	POS	-ve	Norm	-ve
29-Nov-07	1500	POS	-ve	Norm	+ve
01-Oct-08	1529	POS	-ve	Norm	-ve
01-Nov-08	1535	POS	-ve	B.V	-ve
12-Jun-07	1552	POS	-ve	Norm	-ve
11/62007	1740	POS	-ve	Norm	-ve
20-Nov-07	1805	POS	-ve	Norm	-ve
19-Nov-07	1842	POS	-ve	Norm	-ve
1/12/2007	1848	POS	-ve	Norm	-ve
11-Sep-07	1864	POS	-ve	Norm	-ve
11/62007	1940	POS	-ve	Norm	-ve
14-Nov-07	1941	POS	-ve	Norm	-ve
10-Dec-07	1943	POS	-ve	Norm	-ve
12-Jun-07	1952	POS	-ve	Norm	-ve
06-Nov-07	1956	POS	-ve	Norm	+ve
07-Nov-07	2039	POS	-ve	Norm	-ve
15-Nov-07	2102	POS	-ve	Norm	-ve
22-Nov-07	2128	POS	-ve	Norm	-ve
01-Oct-08	2137	POS	-ve	B.V.	-ve
15-Nov-07	2144	POS	-ve	Norm	-ve
13-Jan-08	2147	POS	-ve	Interm	-ve
11/12/2007	2196	POS	-ve	Norm	-ve
30-Nov-07	2227	POS	-ve	Norm	-ve
27-Nov-07	2237	POS	-ve	Norm	-ve
11/14/2007	2250	POS	-ve	Norm	-ve
19-Nov-07	2257	POS	-ve	Norm	-ve
11/13/2008	2264	POS	-ve	Norm	-ve
01-Oct-08	2266	POS	-ve	Norm	-ve
27-Nov-07	2285	POS	-ve	B.V.	-ve
14-Nov-07	2288	POS	-ve	Norm	-ve
1/9/2008	2297	POS	-ve	Norm	-ve
30-Nov-07	2303	POS	-ve	Norm	+ve

06-Nov-07	2312	POS	-ve	Norm	-ve
15-Nov-07	2323	POS	-ve	Norm	-ve
22-Nov-07	2326	POS	-ve	Norm	-ve
21-Nov-07	2349	POS	-ve	Norm	+ve
11/19/2007	2408	POS	-ve	Norm	-ve
13-Jan-08	2426	POS	-ve	Norm	-ve
11/23/2007	2452	POS	-ve	Norm	-ve
22-Nov-07	2458	POS	-ve	Norm	-ve
01-Nov-08	2472	POS	-ve	Norm	-ve
13-Jan-08	2478	POS	-ve	Interm	-ve
01-Nov-08	2559	POS	-ve	Norm	-ve
11/15/2007	2561	POS	-ve	Norm	-ve
12-Nov-07	2565	POS	-ve	Norm	-ve
04-Dec-07	2587	POS	-ve	Norm	-ve
05-Dec-07	2596	POS	-ve	Norm	-ve
11-Dec-07	2622	POS	-ve	Norm	-ve
14-Nov-07	2661	POS	-ve	Norm	-ve
04-Dec-07	2674	POS	-ve	Norm	-ve
15-Nov-07	2682	POS	-ve	Norm	-ve
14-Nov-07	2751	POS	-ve	Norm	-ve
28-Nov-07	2756	POS	-ve	Norm	+ve
28-Nov-07	2766	POS	-ve	Norm	-ve
11-Dec-07	2842	POS	-ve	Norm	-ve
11-Dec-07	2843	POS	-ve	Norm	-ve
12-Nov-07	751	RES	-ve	Norm	-ve
11/62007	767	RES	-ve	Norm	-ve
06-Dec-07	887	RES	-ve	Norm	-ve
28-Nov-07	889	RES	-ve	Norm	-ve
06-Nov-07	1072	RES	-ve	Norm	-ve
19-Nov-07	1356	RES	-ve	Norm	-ve
19-Nov-07	1437	RES	-ve	Norm	-ve
13-Nov-07	1697	RES	-ve	Norm	-ve
06-Nov-07	1705	RES	-ve	Norm	-ve
03-Dec-07	1749	RES	-ve	Norm	+ve
06-Nov-07	1766	RES	-ve	Norm	-ve
04-Dec-07	1814	RES	-ve	Norm	-ve
05-Dec-07	1815	RES	-ve	Norm	-ve
11-Dec-07	1854	RES	-ve	Norm	-ve
05-Dec-07	1907	RES	-ve	Interm	-ve
12-Nov-07	1930	RES	-ve	Norm	-ve
12-Nov-07	1938	RES	-ve	Norm	-ve
13-Nov-07	1969	RES	-ve	Norm	-ve
30-Nov-07	1996	RES	-ve	Norm	-ve
14-Nov-07	2042	RES	-ve	Norm	-ve
26-Nov-07	2043	RES	-ve	Norm	-ve
26-Nov-07	2046	RES	-ve	Norm	+ve

14-Nov-07	2051	RES	-ve	Norm	-ve
11-Sep-07	2055	RES	-ve	Norm	-ve
11-Aug-07	2083	RES	-ve	Interrm	-ve
01-Sep-07	2123	RES	-ve	B.V	-ve
19-Nov-07	2124	RES	-ve	Norm	-ve
19-Nov-07	2127	RES	-ve	Norm	-ve
11-Jun-07	2141	RES	-ve	Norm	-ve
11/16/2007	2143	RES	-ve	Norm	-ve
11-Aug-07	2146	RES	-ve	Norm	-ve
19-Nov-07	2151	RES	-ve	Norm	-ve
11-Aug-07	2182	RES	-ve	Norm	-ve
28-Nov-07	2235	RES	-ve	Norm	-ve
11-Sep-07	2236	RES	-ve	Norm	-ve
13-Dec-07	2296	RES	-ve	Norm	-ve
04-Dec-07	2298	RES	-ve	Norm	-ve
04-Dec-07	2302	RES	-ve	Norm	-ve
03-Dec-07	2451	SUD	-ve	Norm	-ve
20-Nov-07	2202	SUS	-ve	Norm	-ve
06-Nov-07	2314	SUS	-ve	Norm	-ve
11-Sep-07	2315	SUS	-ve	Norm	-ve
06-Nov-07	2317	SUS	-ve	Norm	-ve
21-Nov-07	2325	SUS	-ve	Norm	-ve
12/5/2007	2327	SUS	-ve	Norm	-ve
1/9/2008	2337	SUS	-ve	Norm	-ve
20-Nov-07	2342	SUS	-ve	Norm	-ve
20-Nov-07	2345	SUS	-ve	Norm	-ve
05-Dec-07	2346	SUS	-ve	Norm	-ve
22-Nov-07	2367	SUS	-ve	Norm	-ve
04-Dec-07	2432	SUS	-ve	Norm	-ve
11-Dec-07	2442	SUS	-ve	Norm	-ve
20-Nov-07	2445	SUS	-ve	Norm	-ve
15-Nov-07	2454	SUS	-ve	Norm	+ve
27-Nov-07	2463	SUS	-ve	Norm	-ve
12/11/2007	2481	SUS	-ve	Norm	-ve
11/14/2007	2487	SUS	-ve	Norm	-ve
12-Nov-07	2526	SUS	-ve	Norm	-ve
26-Nov-07	2551	SUS	-ve	Norm	+ve
30-Nov-07	2552	SUS	-ve	Norm	-ve
1/11/2008	2559	SUS	-ve	Norm	-ve
06-Dec-07	2588	SUS	-ve	Norm	-ve
14-Nov-07	2595	SUS	-ve	Norm	-ve
06-Dec-07	2616	SUS	-ve	Norm	-ve
04-Dec-07	2649	SUS	-ve	Norm	-ve
27-Nov-07	2652	SUS	-ve	Norm	-ve
09-Nov-07	2696	SUS	-ve	Norm	-ve
14-Nov-07	2701	SUS	-ve	Norm	-ve

28-Nov-07	2765	SUS	-ve	Norm	-ve
30-Nov-07	2786	SUS	-ve	Norm	-ve
13-Nov-07	2809	SUS	-ve	Norm	-ve
13-Jan-08	2810	SUS	-ve	Norm	-ve
07-Nov-07	2839	SUS	+ve	Norm	-ve
11/7/2007	2840	SUS	-ve	Norm	-ve
07-Nov-07	2841	SUS	-ve	Norm	-ve
05-Dec-07	106527	SUS	-ve	Norm	-ve
05-Dec-07	115225	SUS	-ve	Norm	-ve
06-Dec-07	115621	SUS	-ve	Norm	-ve
29-Nov-07	116528	SUS	-ve	Norm	-ve

APPENDIX III: CONSENT FORM

Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya

Majengo Research Clinic

Resurvey: Patient Information and Consent Form

This information will be communicated orally in English, Swahili or other Kenyan dialect of potential participant's preference.

Investigators:

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Background information

The University of Nairobi and its collaborators from Canada 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 study because you are:

a) at a very high risk of acquiring an STI or are already infected with an STI or

b) at a low risk of acquiring an STI; or

c) the relative of a person in group a) or b).

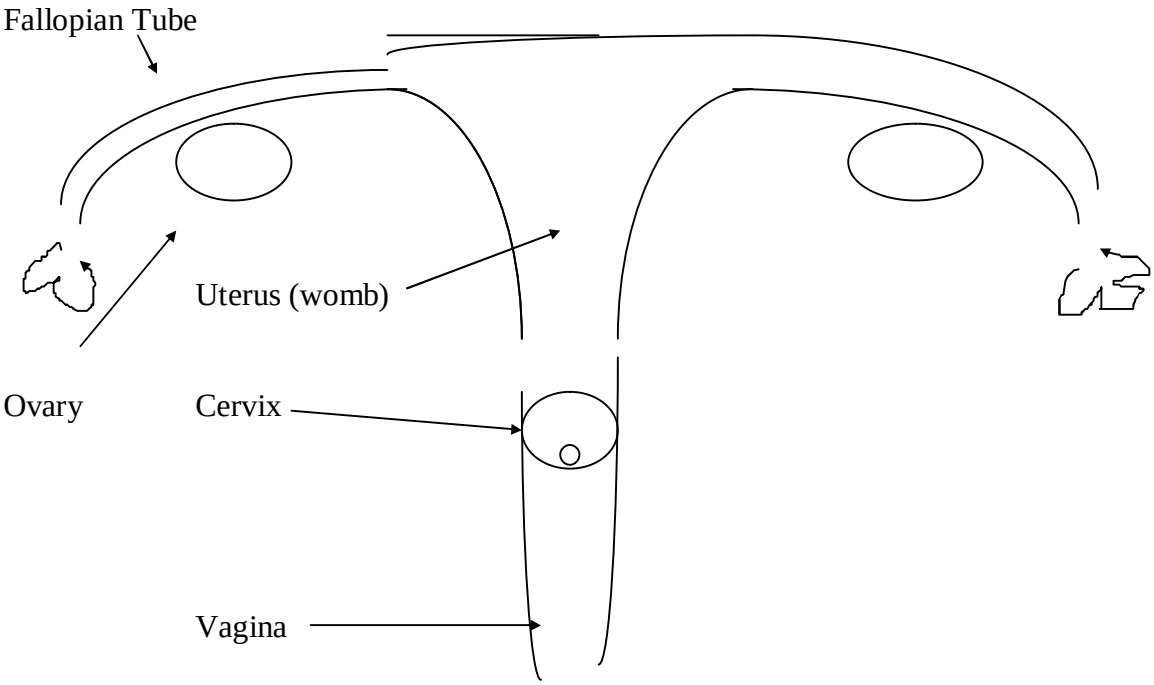
The purpose of this research program is to determine if there are factors that could protect individuals from acquiring sexually transmitted infections (STI) especially HIV. It is important to keep free of other sexually transmitted diseases, as the presence of these infections may increase your risk of becoming infected with HIV. If you have an STI, you should seek treatment for it as quickly as possible. However, sometimes you may have an STD and not know it, because you may not have any symptoms and thus advised to visit the clinic monthly for free check ups.

Why Is This Study Being Done?

This study is being done to find out why some people are more or less likely to get the Human Immunodeficiency Virus (HIV), the virus that causes AIDS. There is more and more evidence that the immune system in some people is able to protect them against infection with HIV. Since most people get HIV through sexual exposure to an HIV infected partner, the first contact with the virus occur in the genital tract, the vagina and cervix in women. We know from some of our previous work that some women, who seem to be protected against HIV, have a special type of immune

response that it not present in women who get HIV. The purpose of this study is to try to find out the targets of this immune response in the vagina, uterus and cervix and to try to find out what is special about the immune system of these few individuals. This work may be helpful in eventually making a vaccine for HIV.

To help you understand what is involved in the study a drawing of the vagina, cervix and uterus of the female genital tract is shown below.



How Many People Will Take Part in the Study

About 3000 participants mainly women will take part in this study.

What Is Involved in the Study?

You have been invited to voluntarily participate in this study because all are at risk of becoming infected with STDs and HIV. Some sexual behavior especially among sex workers or those who use sex as an income generating activity exposes those involved or their partners to a higher risk of contracting HIV. If you agree to participate in the study, you will be given additional counseling, advised on appropriate STIs prevention

strategies and requested to practice safer sex. You can also choose to leave sex work at any time but you will be asked to return to the clinic every month for free check ups. Again, the results of these tests will be ready after one week or less, and you will be informed of the results and given the correct treatment if you have an infection. You will also be encouraged to come to the clinic for examination and treatment at any other time that you feel ill. If you forget to return to the clinic for one of your scheduled visits, a clinic staff member will contact you by phone, SMS or send one of your friends to remind you of the missed appointment**. All study participants will also be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on HIV infection status every three months.** In addition, we will store specimens from your blood for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

Clinic visits

First visit and semi-annual visits (All study participants)

We will ask you general questions about your life, about problems you are having, and about your sexual history.

The doctor will examine your body, including your female parts.

Swab and washing from your vagina to look for germs and to collect samples for studying your immune response.

Swab from your cervix to look from germs and to collect samples for studying your immune response.

A thin plastic tube will be placed in your cervix (opening to your womb) to get some of the mucous your cervix makes.

Urine to look for germs.

Three tablespoons of blood will be taken for testing syphilis, and HIV and for studying your immune response. We will inform you of your results at your one month visit. We also will test your spouse for 0the HIV virus free of charge if he/she wishes.

Monthly visits

Questions will be asked about you, and what problems you are having.

If you have any complaints the doctor will examine your body, including your female parts.

Every third month all study participants will be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on individuals HIV infection status.

Follow-up visits (All study participants)

You will be asked to return 3 to 7 days after every visit to be given you laboratory results.

You will be treated for new infections, free of charge.

How Long Will I Be in the Study?

The study will last 5years. Although we would appreciate if you stayed in the study for the entire period you may choose to leave the study at any time without any penalty to you.

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.

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 have antiretrovirals in the clinic to manage your condition. We will also test one boyfriend for HIV virus if he wants.

Risks of taking antibiotics / Antiretrovirals

If we find that you have an STD or AIDS we will provide you with the appropriate treatment. With any drug there is some potential for side effects. For the antibiotics/ antiretrovirals you might receive, the following side effects are possible.

Very likely:

Sick to your stomach

Headache

Metallic taste in mouth

Diarrhea

If a woman - infection of your vagina by yeast (a white discharge with itching). If this happens, we will give you medicine to put inside your vagina to treat the yeast infection.

Less likely but serious:

Less than 1 person in 100 will have a severe allergic reaction to one of the antibiotics/ antiretrovirals.

Are There Benefits to Taking Part in the Study?

The benefits that you will get from this study are that you will be examined regularly, and if you are found to have an STD 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?

Efforts will be made to keep your personal information 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.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: the researchers, members of the local and international ethics teams and the National Institutes of Health in the United States of America. 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.

Although you will not be paid to participate in the study, you will be offered a small payment of two hundred shillings (KSh 200) for the resurvey visits only to compensate you for your transportation to the clinic.

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 be holding baraza's at the different clinics 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 Dr. Wachihi 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 Bhatt, who is the chairperson of the Ethical Review Committee at the University of Nairobi, by calling 725452, or make an appointment to see her in the Department of Medicine, at the University of Nairobi.

Statement of Consent:

I have read the attached written information and / or received verbal information on the above study. I have been given the opportunity and time to have any questions about the research answered to my satisfaction. I consent to take part in the study and I am aware that my participation is entirely voluntary. I understand that I may withdraw at any time without giving a reason and without this affecting my future care.

By signing this information and consent form I agree that my personal data, may be used as described in this consent form and may be consulted by qualified representatives from sponsor the Ethics Committee or the health authorities.

I understand that the following (check the box only if you fully understand and agree with each statement):

☐ the goals of this research program are to study resistance and susceptibility to sexually transmitted infections

☐ 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 and may be used for genetic studies

☐ any blood specimens previously collected may be used for this study

☐ a portion of my blood, cervical and vaginal specimens will be stored for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

I am willing to participate in the study.

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 ____ ____ ____

Standards of Medical Care for Participants in the Research Clinics

This document outlines the standard of medical care for all participants in the Majengo, MCH Majengo, Kindred, Kibera and Korogocho cohorts, regardless of HIV-1 serostatus. It should be emphasized that any member of the said cohorts may freely decline to take part in any cohort substudy, and that this decision will in no way affect their access to this standard of care. All care outlined is provided free of charge, thereby significantly improving health care access and outcomes for all members of the cohorts... The nature of the medical care will vary depending on HIV-1 serostatus of the participants, as outlined below.

1. **General medical care for all participants, regardless of HIV-1 status**.

HIV and STD prevention services: provision of the male condom, and peer-based and clinic-based counseling regarding safer sexual practices.

Family planning services as directed in the *Kenyan National Family Planning Guidelines*

Rapid and effective treatment of sexually transmitted diseases in accordance with the *Kenya National Guidelines for the Syndromic Management of Sexually Transmitted Diseases*

Medical care for acute and chronic illnesses, both infectious and non-infectious

Access to diagnostic testing in haematology, biochemistry, infectious diseases, immunology, radiology

Prompt referral for specialist consultation and hospitalization when indicated

2. **Management of Opportunistic Infections in HIV-1 Infected Participants**.

Primary Prophylaxis: Trimethoprim-Sulphamethoxazole (Septrin): all participants with a CD4+ T cell count <200/mm^3 or on Anti-TB must use Septrin for prevention of PCP, toxoplasmosis and bacterial infections (bacterial pneumonia, bacteremias, some bacterial diarrhoea), according to *National AIDS/STD Control Program (NASCOP) Guidelines.*

However, all HIV infected individuals with CD4+ <350 should be encouraged to use Septrin according to the latest WHO guidelines (2006)

Secondary Prophylaxis: Septrin: offered to all participants regardless of CD4+ T cell count after an episode of PCP, toxoplasmosis, or severe bacterial infection. Fluconazole: provided for secondary prevention of Cryptococcus

Treatment

Herpes. simplex/Herpes zoster infection: acyclovir

Candidiasis (oral, esophageal, vaginal): nystatin, clotrimazole, Fluconazole

Tuberculosis (pulmonary or extra pulmonary): referral to National TB Programme

Toxoplasmosis: referral for inpatient therapy

Cryptococcus: referral for inpatient therapy

PCP: Septrin (with prednisolone, if severe)

Kaposi's Sarcoma: ARV and referral to Clinical Oncologist

3. **Antiretroviral therapy.**

Antiretroviral therapy rollout in Kenya is supported and directed by NASCOP and The Ministry of Health. Kenya is a recipient of ARVs and infrastructure support through the *Presidents Emergency Plan for AIDS Relief (PEPFAR)* a US government international development initiative.

ARV drugs and infrastructure support has been secured by the University of Manitoba from NASCOP/PEPFAR and CDC PEPFAR to provide HIV basic and ARV care for **all** cohorts members who are eligible as per the *"Guidelines for Antiretroviral Drug Therapy in Kenya"* (NASCOP-2005 edition). Such medical treatment and its requisite follow-up, integrated with the above standard of care, will also be provided at no cost.