DENDRITIC CELL FREQUENCY AND STIMULATED CYTOKINE PRODUCTION AMONG MAJENGO COMMERCIAL SEX WORKERS

BY

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Thesis submitted in part fulfillment of the Degree of Master of Science in Medical Microbiology, University of Nairobi

2008
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DEDICATION

This project is dedicated to the women of Africa who had had to bear the burdens of poverty and diseases and forced into commercial sex work as a means of survival.
ACKNOWLEDGEMENTS
I wish to thank my supervisors for their invaluable time spent from the inception of finalizing this project. First, Professor Omu Anzala, the Chairman of Medical Microbiology and Program Director at Kenya Aids Vaccine Initiative who has not only been my project supervisor but who has been my teacher and a working colleague for many years in the field of HIV research.

Secondly, I also thank Dr. Richard Lester who assisted in designing and supervision of this project from the initial stages. This work would not have been possible without his support through his research grant.

I acknowledge the help I got from the late Professor Job Joab Bwayo who until his death in February 2007 supervised this. His advice and personal notes offered to me have remained a source of inspiration to the completion of the project.

My thanks also go to Professor Walter Otieno Jaoko, the immediate chairman of the Department of Medical Microbiology, for allowing this work to be undertaken in his Department. I am grateful for his encouragement at the times when I would develop fatigue while pursuing my career.

I also wish to thank my colleagues in the University of Nairobi, where this work was conducted: Dr. Joshua Kimani and Mr. Tony Kariri and entire clinic staff who have tirelessly worked in the Majengo cohort since its inception almost three decades ago. Also, those from KAVI – Mr. Bashir Farah, Mr. Micah Oyaro, Mr. Simon Ogola, Mr. Jackton Indangasi and Miss Stella Njoroge for their technical support at the time this project was being carried out. Special thanks go to Fredrick Oyugi who helped me in data analysis.

I cannot forget to thank members of my family, my wife Elizabeth Mulili Obila and our children Kathy R. A. Obila, Dorsi J.O. Obila and George Melchizedeck Ogosi Obila for their unrelenting support.
Many thanks also go to the ladies in the Majengo cohort for their co-operation and participation in the study, without which this study would not have been possible.

This study was sponsored by a research grant from the Bill Melinda Gates Foundation under the joint University of Manitoba and University of Nairobi research initiative. Laboratory investigations were performed at Kenya Aids Vaccine Initiative, in the University of Nairobi also supported by Bill Mellinda Gates Foundation and International Aids Vaccine Initiative.
# Table of Contents

Supervisors ........................................................................................................ i
Dedication ........................................................................................................... ii
Summary ............................................................................................................. x
Background ........................................................................................................ x
Methods ............................................................................................................ x
Results .............................................................................................................. x

CHAPTER 1 ........................................................................................................ 1
1.0 Introduction ................................................................................................. 1

CHAPTER 2 ........................................................................................................ 7
2.1 Literature Review ....................................................................................... 7
2.2 Toll like receptors ....................................................................................... 8
2.3 Core components of the NF-κB activation pathways ......................... 11
2.4 Endocytic and scavenger recognition receptors .................................. 12
2.5 Viral recognition by toll like receptors .................................................... 13
2.6 Recognition of dsRNA ............................................................................. 14
2.7 Recognition of viral glycoprotein ............................................................. 14
2.8 Products of recognition ......................................................................... 14
2.9 Cytokines ................................................................................................. 15

CHAPTER 3 ....................................................................................................... 21
3.0 Problem Statement .................................................................................... 21
3.2 Hypothesis ................................................................................................. 21

CHAPTER 4 ....................................................................................................... 24
4.0 Materials and Methods ........................................................................... 24
4.1 Research Design and Methods ............................................................... 24
LIST OF FIGURES

Figure 1.0: Decline in HIV-1 Incidence with follow up in the Majengo Cohort... 2
Figure 2.0: Location of Toll-Like Receptors on cells........................................ 10
Figure 2.1: Stimulation with LPS........................................................................ 12
Figure 2.2: Antiviral Action of Interferon.............................................................. 17
Figure 4.0: Side scatter (SSC) and forward scatter (FSC) in leukocyte separation. 27
Figure 4.1: Distribution of leukocytes in a live gate............................................. 27
Figure 4.2: Distribution of CD11c^{positive} Dendritic cells (mDCs)...................... 28
Figure 4.3: Distribution of CD123^{High} Dendritic cells (pDCs)........................... 28
Figure 5.1a and 5.1b: CD4^{+} T-lymphocyte absolute counts and percentages...... 33
Figure 5.1c and 5.1d: CD4^{+} T-lymphocyte absolute counts and percentages...... 33
Figure 5.2: Percentages of myeloid and plasmacytoid dendritic cells................. 35
Figure 5.3: Percentages of mDCs and pDCs after stimulation............................... 36

LIST OF TABLES

Table 4.1: Plate set up................................................................. 30
Table 5.1: Characteristics of the women......................................................... 32
Table 5.2: Cytokine profile in PBMCs......................................................... 37
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>ARDS</td>
<td>Adult Respiratory Distress Syndrome</td>
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<tr>
<td>CBA</td>
<td>Cytometric Bead Assay</td>
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<td>CCR</td>
<td>Chemokine Receptor</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CpG-DNA</td>
<td>Cytosine –Guanine denucleotide sequence</td>
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<td>CTL</td>
<td>Cytotoxic T- Lymphocyte</td>
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<td>DCs</td>
<td>Dendritic cells</td>
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<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded Deoxy ribonucleic acid</td>
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<tr>
<td>dsRNA</td>
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<td>Florescein Isothiocynate</td>
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<td>Forward Side Scatter</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
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<tr>
<td>HAART</td>
<td>Highly Acting Anti Retroviral Treatment</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
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<tr>
<td>HIV1 and 2</td>
<td>Human Immunodeficiency virus type 1 and 2</td>
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<td>HLA</td>
<td>Human Leukocyte Antigens</td>
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<td>HRP</td>
<td>Horse Radish Peroxidase</td>
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<td>HSV 1 and 2</td>
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<td>Interferon</td>
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<td>Lipopolysaccharide</td>
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<td>mDCs</td>
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<td>MHC I and II</td>
<td>Major Histocompatibility Complex I and II</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MIP</td>
<td>Macrophage Inhibitory Protein</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa Beta</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
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<tr>
<td>NOD</td>
<td>Nucleotide Binding Oligomerization domain</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral Mononuclear Cells</td>
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<tr>
<td>pDCs</td>
<td>Plasmacytoid Dendritic cells</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNAse</td>
<td>Ribonuclease</td>
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<tr>
<td>ssRNA</td>
<td>Single stranded Ribonucleic acid</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded Deoxy ribonucleic acid</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, Normal T Expressed and Secreted</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Syncitial Virus</td>
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<tr>
<td>SSC</td>
<td>Side Scatter</td>
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<tr>
<td>SIRS</td>
<td>Systemic Inflammatory Response Syndrome</td>
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<tr>
<td>μL</td>
<td>Microlitre</td>
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<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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SUMMARY

Background: Investigation was carried out to determine frequencies of dendritic cells and stimulation responses of peripheral blood mononuclear cells of female sex workers in the Majengo cohort in Nairobi, Kenya.

Methods: Blood samples from female commercial sex workers were investigated to determine frequencies of dendritic cells in peripheral circulation and their responses to different tolllike receptor ligands. Stimulation responses leading to cytokine production and cell proliferation were measured using flow-cytometric analysis.

Results: From the study: We enrolled thirty two (n=32) female commercial sex workers and used their blood to determine the frequencies of dendritic cells, we also screened them for HIV-1 antibodies. From this analysis, there was significant reduction in both plasmocytoid and myeloid dendritic cell in HIV infected females compared to HIV non infected females. Similarly, there was reduced proliferation capacity of dendritic cells in HIV infected female compared to HIV negatives.

Conclusion: Our study showed a significant decrease in circulating dendritic cells in patients infected with HIV-1, a reduced proliferation capacity, as well as reduced type I cytokine responses leading to low production of IFN-γ production upon stimulation with TLR ligands. This may lead to poor outcome of antiretroviral therapy since the IFN-γ cytokine is an important antiviral agent that lead to anti-retroviral activity in patients and hence its reduced production may lead to poor prognosis in HIV infected patients.
CHAPTER 1

1.0 INTRODUCTION

1.1 Resistance to HIV

Infection with human immunodeficiency virus type 1 and 2 (HIV-1 and 2) commonly occurs through sexual exposure. The virus infects host cells by integrates into the host’s deoxyribonucleic acid (DNA). Subsequently HIV utilizes the host’s synthetic machinery for its replication leading to death as a result of overwhelming immunodeficiency disease (Apostolakis S.et. al. 2005).

Certain unique individual human hosts have been described who remain uninfected by the virus despite after repeated sexual exposure to infected individuals. Such individuals have been described in a cohort of female commercial sex workers in Nairobi Kenya who have remained HIV-1 negative even after repeated exposure through sex work to HIV infected individuals (Plummer F. et.al.1999). It has been shown that these women could resist viral infection through production of immune mediators which inhibit viral attachment, penetration, integration, and replication processes which are very vital for infection (Chaplin D., 1996). Earlier studies carried out in these women showed that there were no differences in their demography compared to those of other women in the cohort who acquire HIV infection and develop disease and eventually die. These studies have estimated the use of condoms to approximately eighty percent in all sexual encounters but the risk of acquiring HIV-1 infection remains high as the women report more than sixty unprotected sexual exposures annually. Despite this intense exposure, the risk of HIV-1 seroconversion in the cohort gradually declines such that, the longer a woman remains uninfected in sex work, the less likely she is to seroconvert (figure 1). As a result, a small number has remained uninfected for over a period of 20 years and are perceived to be “resistant” (Plummer F. et.al.1999).
To reinforce the theory of resistance to HIV is a finding that the incidence of other sexually acquired diseases among these women perceived to be resistant remains similar to those who are HIV infected.

These observations have made the research team for this cohort conclude that these women are protected against HIV-1 through some as yet unidentified biologic mechanism. An explanation to this paradox is that, in a highly exposed population, with given a range of susceptibility to HIV-1 over time, the most susceptible women would be rapidly infected with HIV-1 and, through death or illness, be removed from the population (figure 1) resulting in a population enriched for women who are less susceptible or relatively resistant to HIV infection.

Figure 1.0 Decline in HIV-1 Incidence with follow up in the Pumwani Cohort.
Apart from these women, HIV resistance phenomena have been shown to exists in other special risk groups, including health care workers encountering accidental parenteral exposure to infected blood or other body fluids, infants born to HIV infected mothers, people sharing needles for intravenous drugs use and HIV discordant couples engaged in unprotected sexual intercourse. In these groups, T-cell mediated immunity has been demonstrated and is currently considered to be the main essential element of resistance.

T-helper cell function characterized by high production of interleukin-2 (IL-2), or high lymphocyte proliferation in response to HIV derived peptides as well as presence of HIV specific cytotoxic T-lymphocytes (CTL) have been demonstrated in these groups including the female commercial sex workers in Majengo. Thus, it has been postulated that the presence of HIV-specific T cell responses in the absence of detectable antibody production may be due to a low or insufficient antigenic dose that is not able to induce viral replication and hence lack of presentation by through major histocompatibility class I (MHC class I) molecules. Together with this, a strong cytokine-mediated T-helper-1 (Th-1) immune responses has been postulated to upregulate cellular effector functions, and down regulate T-cell help for B-Cells to develop into humoral response (Fowke K. et al 2000).

Similarly the same phenomena have been described in individuals who resist rapid progression to a condition of Acquired Immunodeficiency Syndrome (AIDS) after infection with HIV. In those who rapidly progress to AIDS, HIV specific CTL activity is lacking, while those who fail to progression rapidly to AIDS have been shown to develop elaborate specific CTLs, which are thought to contribute in controlling viremea during the asymptomatic phase and may protect non infected cells from attack leading to protective immunity (Rowland-Jones R. et al 1998).

Similar evidence has also been reported in some monkeys: despite simian immunodeficiency virus (SIV), these monkeys remain healthy for a long time without symptoms (Schimitz A. 1999). In general, the antiviral effect in these circumstances is thought to be mediated by CTL’s recognition of processed viral proteins on the surfaces of the infected cells leading to subsequent lyses of the target cells. The CTLs may also produce antiviral factors such as chemokines and
cytokines that help to contain the infection (Hanekom W. 2004). Thus, resistance to HIV-1 infection in exposed seronegatives has been attributed to specific cellular immune responses, structural changes in the host cell receptors, production of antiviral factors and unique stimulation states of target cells.

To date, the role which innate immunity play in this puzzle is still not clearly understood. If studied it may further help in understanding factors controlling this immunity to HIV. Dendritic cells, an integral part of the innate immunity are very important in defense against infectious microbes. As professional antigen presenting cells (APCs), they are essential for initiating primary antigen specific immune responses by inducing specific immune recognition which is a key characteristic of adaptive immunity. Like other cells of the immune system, they originate mainly from the bone marrow, where their precursors migrate towards the primary lymphoid organs and into submucosal tissue of the gut, the genital urinary tract system and the respiratory tract, where they pick and process antigens and then, migrate to secondary lymphoid organs where they activate antigen specific T-lymphocytes. Because of this crucial role, they have been used as vaccine adjuvants by allowing them to ingest antigens and express the peptides on their surface in conjunction with MHC class II antigens and present these to specific T cells (Kodowaki N. et al 2004).

Classification of dendritic cells is very complex. They represent a heterogeneous family expressing diverse phenotypic markers and diverse functional capacities depending on the local microenvironment and the stage of maturation. Immature dendritic cells may have the capacity to pick up and process foreign antigens but lack the T cell stimulatory capacities found in mature dendritic cells. Dendritic cells in tissues and Langerhans cells in the skin and mucosal areas represent an immature phenotype and may take up antigens and migrate to the lymphoid tissue where they develop into a more mature phenotype. Induction by viruses at these sites may induce substantial amounts of interferons which are antiviral in nature (Beginon A; 2005). These interferons are the link between innate and adaptive immune system. Additionally, the processed antigens are co expressed together with co-stimulatory markers that induce T cell activation. The processed antigens are presented through MHC class I molecule on APCs which then allow
activation of CD8\(^+\) T cells but can also activate CD4\(^+\) T cells when presented through MHC class II molecules on nucleated cells. This process of stimulating T cells also depends on secretion of stimulatory cytokines such as interleukins which are responsible for generation and activation of T helper and natural killer (NK) cells.

Recently, molecules designated Toll-like receptors (TLRs) were discovered in mammalian cells as expressed surface molecules on cells near mucosal portals of entry, including macrophages, dendritic cells, and lung epithelial cells. TLRs are believed to serve an important role in the early host defense response of innate immunity by discrimination of "self" from "non-self" in the early response to infectious challenge. They act through recognition of conserved molecules derived from microbial pathogens (such as lipoteichoic acid, peptidoglycan, bacterial lipoproteins, and lipopolysaccharide (LPS). TLRs represent critical molecules in the first line of host defense to microbes such as pathogenic bacteria (Medzhitov and Janeway, 2000) and their role may be critical in HIV resistance. TLRs are both intracellular and extracellular, when intracellular, they are found residing predominantly or entirely within the endosomes, including TLR3, -7, -8, and -9, which detect nucleic acids. TLR1, -2, -4, and -6 are at least largely expressed on the cell surface, although their presence within the phagosome and later components of the endocytic pathway is not excluded. Viruses were the most recent pathogens found to be recognized by TLRs (Tabeta K, et al. 2006). HIV like other viruses are primarily, sensed by TLRs dedicated to the detection of unmethylated DNA, dsRNA, and ssRNA. As such, the challenge of self-/non-self-discrimination is greater because host nucleic acids are only slightly different from virally encoded nucleic acids. ssRNA is abundant in host cells, and most RNA species have at least some dsRNA regions. Some CpG dinucleotides within the mammalian genome are unmethylated and should therefore be recognized by TLR9.

These findings have led to a belief that autoimmune diseases are fueled in part by TLR signaling, stimulated by host nucleoprotein complexes (Leadbetter E.A. et al 2002). At the very least, viruses test the limits of self-/non-self-discrimination, and why TLRs are so effective in recognizing them is still not entirely clear. TLRs 3, 7, 8, and 9 not only detect structural differences between viral nucleic acids and host nucleic acids, but also must engage the nucleic acids within endosomes, a
compartment from which host nucleic acids are normally excluded. The viral envelope glycoprotein may also be detected by TLRs. For example in mice infected with mammary tumor virus, envelope glycoproteins are recognized via TLR4 (Rassa J.C. et al 2002). Since HIV has in it similar structural components, then their recognition should therefore follow the same pathway similar to these viruses. Interests in the pathways involving TLR recognition and stimulation are increasingly gaining attention in HIV vaccinology, with focus being put in employing these pathways as potentiating factor in vaccine delivery to the cells.
CHAPTER 2

2.1 LITERATURE REVIEW

The innate immune system is at the front line in the host's defense against microbial invasion. The system comprises of many biological mediators and cellular components including natural killer cells and dendritic cells. The dendritic cells are critical effectors of innate immunity since they act both as sentinels that detect the presence of pathogens and as key antigen-presenting cells that prime and regulate the adaptive immune response. There are two main populations of human dendritic cells; one is the myeloid DCs (mDCs) which is HLA-DR+ CD3- CD4+ CD11c$^{\text{high}}$ and CD123$^{\text{low}}$ and resides as immature cells in the epithelia of skin and mucosa from where they remain until they encounter a "danger signal", such as microbial invasion. Myeloid DCs express TLR-1, -2, -3, -4, -5, -6, -8 and -10, while plasmocytoid DC express TLR-1, -6, -7, -9 and -10 (Kadowaki et al., 2001, Kapsenberg, 2003 and Iwasaki and Medzhitov, 2004). Such an encounter initiates maturation that involves migration to draining lymph nodes by expression of the lymph node homing molecule, chemokine receptor 7 (CCR7), and uptake of antigens and subsequent processing and presentation of antigen peptides on their surfaces, expression of T-cell co-stimulatory molecules, CD80 and CD86, and production of cytokines that prime adaptive immune cells (Banchereau and Steinman, 1998).

The second, the Plasmacytoid Dendritic Cells (pDCs), are also known as professional type-1 interferon-producing cells because of their capacity to express 100–1000-fold more interferon-α (IFN-α) than any other blood cells upon stimulation by viral agents (Siegal et al., 1999 and Fitzgerald-Bocarsly, 2002). They are HLA-DR+CD3−CD4+CD11c$^{\text{low}}$ and CD123$^{\text{high}}$; they are considered to originate from lymphoid organs and exist primarily in the peripheral blood until they become activated, upon which they express CCR7 causing their rapid homing to lymph nodes (Megjugorac N.J et al., 2004). The antigen-presenting role of the latter cells is less clear than that of mDC; however, pDC can exert potent paracrine effects on mDC via the secretion of immune regulatory cytokines, including IFN-α (Fonteneau et al., 2004).
DCs sense the presence of pathogens via a system of pathogen recognition receptors located either on their surfaces or intracellular within the endosomal compartment (Janeway, 1989 and Banchereau et al., 2000). These receptors bind conserved molecular motifs, known as pathogen-associated molecular patterns (PAMP), present in pathogens, but not their mammalian hosts, thus allowing for the detection of the various classes of microbes. The chief mechanism of pathogen recognition among DC is via TLRs which share an interleukin-1 receptor-like structural motif (Medzhitov and Janeway, 2000). To date, 10 functional human TLRs have been identified, each with specificity for molecular patterns present in bacteria, fungi, viruses and parasites (Mazzoni and Segal, 2004). Examples of microbial TLR ligands are lipopolysaccharide (LPS) and lipoteichoic acid in the cell walls of gram negative and gram positive bacteria, respectively. Other such motifs are unmethylated CpG DNA sequences (characteristic of prokaryotes) and double-stranded viral RNA, a “signature” of viral infection.

DC maturation is thus triggered by microbes via Toll like receptors (TLR), making them critical for the induction and regulation of protective T-cell immunity against infectious agents. DCs, like other APCs, activate the adaptive immune response by migrating from the infection site to the regional lymph node where they present microbe derived antigens to naïve CD4+ T Cells. At the same time, activated DCs express co stimulatory molecules essential to T-Cell activation and can instruct the differentiation of naïve CD4+ Tcells into T helper 1 (Th1) cells or Th2 Cells. The Th1 cells produce interferon γ (IFNγ) and mediate the elimination of bacterial and viral infection, while Th2 cells which produce IL4 and IL 13 are in the responses against helminthic infections. Stimulation of most TLRs leads to Th1 rather that Th2 differentiation, thus innate immunity is the key element in the inflammatory responses as well as the immune response against pathogens.

2.2 Toll like receptors

TLRs are conserved from worms to mammals in evolution. Toll was initially identified as a gene product essential for the development of embryonic polarity in Drosophila and later, was shown to play critical role in the antifungal response in flies. To date 12 members of the TLR family have been identified in mammals (Yamamoto et. al 2000). TLRs are type integral membrane
glycoproteins characterized by their contents. They contain varying number of leucine rich 
repeat (LRR) cytoplasmic signaling domains homologous to that of interleukin-1 receptor (IL- 
1R) also termed Toll/IL-1R homology domain. The LRR domains are composed of 19-25 
tandems, each with 24-25 amino acids in length. Each LRR consists of a beta strands and an 
alpha- helix connected by loop.

Based on their primary sequences TLR can be further divided into several sub families, each of 
which recognizes related PAMPS: the TLR2 and TLR6 subfamilies recognize lipids, whereas the 
closely related TLR7, TLR8 and TLR 9 recognize nucleic acids. However the TLR’s are unusual 
in that some can recognize several structurally unrelated ligands (*Janeway C. A Jr 2002*). TLR4 
for example recognizes a very divergent collection of ligands such as lipopolisaccharides, heat 
shock proteins, fibronectin and the plant diterpene paclitaxel, all of which have different 
structures. TLRs are expressed on various immune cells, including macrophages, dendritic cells 
(DCs), B cells, specific type T cells, and even non immune cells such as fibroblasts and epithelial 
cells. Expression of TLRs is not static but is modulated rapidly in response to pathogens variety, 
cytokines and environmental stresses.

While certain TLRs (TLR 1,2,4,5 and 6) are expressed on the cell surface, others (TLRs 3,7,8 
and 9) are found almost exclusively in intracellular compartments such as endosomes, the 
ligands for TLR3,7,8 and 9 are mainly nucleic acids which require internalization to the 
endosome before signaling is possible. The trans-membrane and membrane proximal regions are 
important for the cellular compartmentalization of these receptors (*Akira et. al 2004*). TLRs 
activate the same signaling molecules that are used for IL-1R signaling. Stimulation of cells with 
a TLR ligand recruits adaptor proteins containing a TLR domain such as myeloid differentiation 
factor 88 (myD88) to the cytoplasmic portion of the TLR through hydrophilic interaction. This 
results in the triggering of downstream signaling cascades and production of proinflammatory 
cytokines and chemokines (*Akira et. al 2004*).

TLRs are capable of recognizing approximately $10^3$ molecular patterns. In bacteria for example, 
they can recognize lipopolysaccharide (LPS) from the gram-negative cell wall, peptidoglycan
found abundantly in the gram-positive cell wall (and to a lesser degree in the gram-negative cell wall) lipoteichoic acids found in the gram-positive cell wall, mannose-rich glycans (common in microbial glycoproteins and glycolipids but are not found in humans), flagellin found in bacterial flagella and pillin from bacterial pili, and bacterial and viral nucleic acids. Bacterial and viral genomes contain a high frequency of unmethylated cytosine-guanine dinucleotide sequences while mammalian DNA has a low frequency of cytosine-guanine dinucleotides which are mostly methylated. N-formylmethionine, an amino acid is common in bacterial proteins, while double-stranded RNA is unique to most viruses. Lipoteichoic acids, glycolipids, and zymosan on the other hand are found in yeast cell walls, while phosphorylcholine and other lipids are common to microbial membranes. The location of various TLRs on cells is as shown in Figure 2.0 below.

**Figure 2.0: Location of Toll-Like Receptors on cells**

**Key**

- TLR-1/TLR-2: pair bind uniquely to bacterial lipopeptides and glycoprotein anchored on parasites;
- TLR-2/TLR-6: pair bind lipoteichoic acid from gram-positive cell walls and zymosan from fungi;
- TLR-4/TLR-4: pair bind lipopolysaccharide from gram-negative cell walls;
- TLR-5: bind bacterial flagellin;
- Other TLRs are found in the membranes of the endosomes within the cells used for degradation of pathogens originating from intracellular environment of the cells, these include;
  - TLR-3: binds double-stranded viral RNA;
  - TLR-7: binds uracil-rich single-stranded viral RNA such as in HIV;
  - TLR-8: binds single-stranded viral RNA;
  - TLR-9: binds unmethylated cytosine-guanine dinucleotide sequences (CpG DNA) found in bacterial and viral genomes.
The transcription factor NF-κB is the master regulator of TLR induced responses. All TLR signals converge on NF-κB and its activation is critical for TLR function. NF-κB has been shown to comprise a family of proteins with pleiotropic effects and play a role in a wide range of human diseases. There are five NF-κB genes, \(NFKB1\), \(NFKB2\), \(RELA\), \(c-REL\) and \(RELB\), which in turn code for seven proteins: p105, p50, p100, p52, p65, c-Rel and RelB. Protein50 and p52 are generated from limited proteasomal processing of the p105 protein as p100 protein respectively. The p50 and p52 proteins lack the transactivational domain found in the C-terminal region of the other NF-κB proteins. Their homodimers are considered repressors of transcription. All of the NF-κB proteins contain the so-called Rel homology domain (RHD) which comprises approximately 300 amino acids of the N-terminal region and is responsible for NF-κB dimerisation, DNA binding and interaction with the inhibitory IκB proteins. Individual NF-κB proteins form hetero- and homo-dimmers with other NF-κB subunits to produce 15 possible dimers. NF-κB DNA binding sites consist of 10 base pairs with the consensus sequence designated as 5'-'GGGRNWYYCC-3', where R is a purine, N is any base, W is an adenine or thymine and Y is a pyrimidine. The number of NF-κB target genes is now in the hundreds, which include a large number of immunomodulatory factors such as cytokines and chemokines.

2.3 Core components of the NF-κB activation pathways

Although a diverse range of stimuli may activate NF-κB, almost all pathways converge on the same core components of the NF-κB activation apparatus, the IKK complex. The IKK complex represents a rapid response system for the induction of NF-κB, which in the uninduced state is maintained in the cytoplasm through interaction with the inhibitory IκB proteins. Activation of the IKK complex induces phosphorylation of the IκB proteins, leading to their K48-polyubiquitination and proteasomal degradation. The degradation of IκB protein allows for translocation of the liberated NF-κB dimers to the nucleus where they bind their cognate sites in DNA and activate gene transcription.
2.4 Endocytic and scavenger recognition receptors

Endocytic pattern-recognition receptors are found on the surface of phagocytes and promote the attachment of microorganisms to phagocytes for subsequent engulfment and destruction. Examples include mannose binding receptors which bind to terminal mannose and fucose groups on microbial glycoproteins and glycolipids. Scavenger receptors which bind bacterial cell wall components including LPS, peptidoglycan and teichoic acids. There are also scavenger receptors for certain components of other types of microorganisms. Signaling is effected by binding of ligands to TLRs either directly or indirectly. The binding of a microbial molecule to its TLR transmits a signal to the cell nucleus, inducing expression of genes coding for the synthesis of regulatory cytokines. The cytokines, in turn, bind to cytokine receptors on other defense cells. Many TLRs, especially those that bind to bacterial and fungal cell wall components, stimulate the transcription and translation of cytokines such as interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), and interleukin-8 (IL-8) that trigger innate immune defenses leading to inflammation, fever, and phagocytosis of invading microbe. Viral components on the other hand trigger the synthesis of interferons that block viral replication within infected host cells and
interleukin-6 (IL-6) and interleukin-12 that promote activity of both B-lymphocyte and T-lymphocyte respectively.

2.5 Viral recognition by toll like receptors

Viruses contain genetic material composed of DNA or RNA (but not both) that encode for viral structural components, nucleic acids and replication enzymes. Various structural components including viral DNA double stranded RNA (dsRNA), single stranded RNA (ssRNA) and surface glycoprotein are recognized as PAMPs by TLRs and other PRRs. Among the TLR family members TLR3, TLR7, TLR8 and TLR9 are involved in the recognition of viral components through PRRs and commonly stimulate type 1 IFN production, that, can activate target cells in both autocrine and paracrine manners (Beignon A. S 2005) DNA viruses including herpes simplex virus 1 (HSV1), HSV2 and Cytomegalovirus (CMV), contains genomes that are rich in CpG-DNA motifs and they activate inflammatory cytokines and type 1 secretion by stimulation of TLR9.

The TLR9 mediated response to HSV1 and HSV2 is cell type specific and limited to pDCs a sub-population characterized by their ability to secrete high levels of IFN in response to viral infection. Recognition of HSV2 by pDCs does not require virus replication. Indeed both live and inactivated HSV2 induce identical secretions from pDCs. Recognition of ssRNA by TLR 7 and TLR 8 on the other hand, are expressed within the endosomal membrane indicating that the accessibility of ssRNA may be a key factor for cell activation via these receptors. Many enveloped virus traffic into the cytosol through the endosomal compartment. There is highly acidified environment containing abundant degradation enzymes that may damage the viral particles leading to ssRNA release and recognition by TLR7 and TLR 8. Furthermore, when virus infected apoptotic cells are taken up, viral RNAses may be released from these cells in the phagolysosome. Unlike virus particles whose genomes are sheltered in the capsid, ssRNAs are subject to degradation by extracellular RNAses when they are released from the cell and rarely reach the endocytic compartment (Mila et. al 2005) thus, they are very important in HIV recognition.
2.6 Recognition of dsRNA

Double stranded RNA (dsRNA) along with synthetic analogues, are potent producer of IFN and are recognized by TLR3. dsRNA can be generated during viral replication as an intermediate for ssRNA virus or as a byproduct of symmetrical DNA viruses. TLR 3 is specifically expressed in conventional DCs (cDCs) the dying cells, but not in pDCs. TLR3 is also expressed in a variety of epithelial cells including airway, uterine, corneal, vaginal, cervical, biliary and intestinal epithelial cells which function as efficient barriers to infection. Expression of TLR3 is rapid and dramatically up regulated by treatment with PolyIC or IFN2/b. Uterine or cornea epithelial cells express a wide range of TLRs; only polyIC stimulates these cells. Unlike DCs these epithelial cells appear to express TLR3 on their cell surface, furthermore TLR3 is strongly expressed in astrocytes and glioblastoma celllines, indicating a specific role in the brain and/or in the response to viruses. Since dsRNA is a universal viral PAMP it has been suggested that TLR3 has a key role in antiviral immunity (Norimitsu K. et al 2005).

2.7 Recognition of viral glycoprotein

Some viral envelope proteins can be recognized by TLR2 or TLR4. Detection mostly results in the production of pro-inflammatory cytokines, but not type IFNs, implying that the response leads to the inflammation rather than specific antiviral responses. For instance, the fusion of protein from Respiratory syncitia virus (RSV) has been identified as a viral component that activates TLR4 is also produced.

2.8 Products of recognition

In addition to the cell surface pattern-recognition receptors there are also secreted pattern-recognition receptors. These bind to microbial cell walls and enable them to be recognized by the complement pathways and phagocytes. For example, mannan-binding lectin is synthesized by the liver and released into the bloodstream where it can bind to the carbohydrates on bacteria, yeast, some viruses, and some parasites. This in turn, activates the lectin complement pathway and results in the production of C3b, a molecule that promotes the attachment of microorganisms to phagocytes.
2.9 Cytokines

Cytokines are low molecular weight, soluble proteins that are produced in response to an antigen. They function as chemical messengers for regulating the innate and adaptive immune systems. They are produced by virtually all cells involved in innate and adaptive immunity, but especially by T helper lymphocytes. The activation of cytokine-producing cells triggers them to synthesize and secrete their cytokines. The cytokines, in turn, are then able to bind to specific cytokine receptors on other cells of the immune system and influence their activity in some manner.

Cytokines are pleiotropic, redundant, and multifunctional.

- Pleiotropic means that a particular cytokine can act on a number of different types of cells rather than a single cell type.
- Redundant refers to the ability of a number of different cytokines to carry out the same function.
- Multifunctional means the same cytokine is able to regulate a number of different functions.

Some cytokines are antagonistic in that one cytokine stimulates a particular defense function while another cytokine inhibits that function. Other cytokines are synergistic wherein two different cytokines have a greater effect in combination than either of the two would by themselves.

There are three functional categories of cytokines:

1. Cytokines that regulate innate immune responses,
2. Cytokines that regulate adaptive immune responses
3. Cytokines that stimulate hematopoiesis.

Cytokines that regulate innate immunity are produced primarily by mononuclear phagocytes such as macrophages and dendritic cells although they can also be produced by T-lymphocytes, NK cells, and other cells. They are produced primarily in response to pathogen-associated molecular patterns such as LPS, peptidoglycan monomers, teichoic acids, and double-stranded DNA. Most act on leukocytes and the endothelial cells that form blood vessels in order to promote and control early inflammatory responses.
Examples include Tumor necrosis factor-alpha (TNF-α) which a principal cytokine that mediates acute inflammation. In excessive amounts it becomes the principal cause of systemic complications such as the shock cascade. It promotes inflammation and coagulation stimulating endothelial cells to produce selectins and intergrins ligands for leukocyte diapedesis. It also stimulates endothelial cells and macrophages to produce chemokines that contribute to chemotaxis and the recruitment of leukocytes into the sites of inflammation. It act on macrophages to secrete interleukin-1 (IL-1) used in redundancy. It also promotes extracellular killing by neutrophils and is cytotoxic for some tumor cells. In addition, it stimulates liver cells to produce acute phase proteins and act on muscles and fat to initiate catabolism. It interacts with the hypothalamus to induce fever and sleep and promote synthesis of collagen and collagenase for scar tissue formation. TNF is produced by monocytes, macrophages, dendritic cells, T\(_{h1}\) cells, and other cells all of which it stimulate.

An important group of cytokine is chemokines. They enable the migration of leukocytes from the blood vessels to infection sites. They increase the affinity of integrins on leukocytes for ligands on the vascular wall during diapedesis, regulate the polymerization and depolymerization of actin in tissues at the site of inflammation for movement and migration, and function as chemoattractants for leukocytes. In addition, they trigger some WBCs to release their killing agents for extracellular killing and induce some WBCs to ingest the remains of damaged tissue. Chemokines also regulate the movement of B-lymphocytes, T-lymphocytes, and dendritic cells through the lymph nodes and the spleen. Certain chemokines have been shown to suppress HIV, probably by binding to the core receptors for HIV on CD4\(^+\) cells. When produced in excess amounts, chemokines can lead to damage of healthy tissue as seen in such disorders as rheumatoid arthritis, pneumonia, asthma, adult respiratory distress syndrome (ARDS), and septic shock. Examples of chemokines include IL-8, MIP-1\(_{α}\), MIP-1\(_{β}\), MCP-1, MCP-2, MCP-3, GRO-\(_α\), GRO-\(_β\), GRO-\(_γ\), RANTES, and exotaxin. Chemokines are produced by many cells including leukocytes, endothelial cells, epithelial cells, and fibroblasts. Another very important cytokine is interleukin-1 (IL-1). IL-1 functions almost similarly to TNF-α in that it mediates acute inflammatory responses, a role it plays synergistically play with TNF. IL-1 is produced by monocytes, macrophages, dendritic cells, and a variety of other cells in the body. Another
cytokine, called interleukin-12 (IL-12) is a primary mediator of early innate immune responses to intracellular microbes. It promotes synthesis of interferon-gamma by T-lymphocytes and NK cells which increases the killing activity of CTLs and NK cells. It also stimulates the differentiation of naive Th cells into interferon-gamma producing T_h1 cells. It is produced mainly by macrophages and dendritic cells.

Interferons on the other hand modulate the activity of virtually every component of the immune system. Type I interferons include more than 20 types of interferon-α, interferon-β, interferon-ω, and interferon tau. There is only one type II interferon, interferon-gamma. Type I interferons, produced by virtually any virus-infected cell, provide an early innate immune response against viruses. Interferons induce uninfected cells to produce enzymes capable of degrading mRNA. These enzymes remain inactive until the uninfected cell becomes infected with a virus. At this point, the enzymes are activated and begin to degrade both viral and cellular mRNA. This not only blocks viral protein synthesis, it also eventually kills the infected cell. They also promote body defenses by enhancing the activities of CTLs, macrophages, dendritic cells, NK cells, and antibody-producing cells. Type I interferons also induce MHC-I antigen expression needed for recognition of antigens by CTLs; augment macrophage, NK cell, CTL, and B-lymphocyte activity; and induce fever. Interferon-alpha is produced by T-lymphocytes, B-lymphocytes, NK cells, monocytes/macrophages; interferon-beta by virus-infected cells, fibroblasts, macrophages, epithelial cells, and endothelial cells. The figure below demonstrates the production of the interferon in which viral components produced through replication stimulate interferon secretion.
Interleukin-6 (IL-6) also stimulates the liver to produce acute phase proteins which promote proliferation of B-lymphocytes and increases neutrophil production. IL-6 is produced by many cells including T-lymphocytes, macrophages, monocytes, endothelial cells, and fibroblasts.

A part from cytokines which heightens inflammatory responses, (IL-10) does suppressory role by inhibiting activated macrophages and dendritic cells and therefore regulate innate immunity and cell-mediated immunity. IL-10 inhibits production of IL-12 and co-stimulator molecules as well as expression of MHC class II molecules which are critical in cell-mediated immunity. Production of IL-10 is mainly by macrophages, and T\textsubscript{h}2 cells.

Interleukin 15 (IL-15) and Interleukin-18 (IL-18) stimulates NK cell proliferation and of interferon-gamma by NK cells and T-lymphocytes and thus induces cell-mediated immunity. It is produced mainly by macrophages.
2.10 Harmful Effects Associated with Pattern-Recognition Receptors and Cytokine Production

There are a number of harmful effects that are known to occur as a result of overreaction and underactive innate immune response. Systemic inflammatory response syndrome (SIRS) is an example of over reactive innate immune response. Inflammatory cytokines as TNF-α, IL-1, (IL-8) are known to promote inflammation by enabling white blood cells to leave the blood vessels and enter the surrounding tissue. This triggers release of extra cellular mediators. In addition, these cytokines activate the complement pathways as well as the coagulation pathway. The products of the coagulation pathway lead to the clotting of blood hence can stop bleeding and arrest localized infection. Therefore at moderate levels, inflammation products of complement activation and products of the coagulation pathway are essential to body defense. However, excessive amounts can cause considerable harm to the body especially severe infection with very large numbers of bacteria where high levels of cell wall components are present may lead to excessive cytokine production with detrimental results of death due to shock cascade. These conditions are commonly seen in individuals with various defects including TLR genes such as:

a. People with an under reactive form of TLR-4, the toll-like receptor for bacterial LPS, have been found to be five times as likely to contract a severe bacterial infection over a five year period than those with normal TLR-4.

b. Most people that die as a result of Legionnaire's disease have been found to have a mutation in the gene coding for TLR-5.

c. People with the autoimmune disease systemic lupus erythematosus have an altered form of TLR-9 that reacts with the body's own DNA.

d. Mutations in the gene coding for NOD2 that prevent the NOD2 from recognizing muramyl dipeptide make a person more susceptible to Crohn's disease.

2.11 Therapeutic Possibilities

Researchers are now looking at various ways to either artificially activate TLRs in order to enhance immune responses or inactivate TLRs to lessen inflammatory disorders. Examples of agents being evaluated in clinical studies include:
a. TLR-4 and TLR-9 activators: as vaccine adjuvants to activate the immune system.
b. TLR-7 activator: as an antiviral against hepatitis C.
c. TLR-4 inhibitor: as an antisepsis agent against SIRS.
d. General TLR inhibitors: to treat autoimmune disorders.

A number of human cytokines produced by recombinant DNA technologies are now being used to treat various infections or immune disorders. These include:

1. Recombinant interferon α-2a (*Roferon-A*): a cytokine used to treat Kaposi’s sarcoma, chronic myelogenous leukemia, and hairy cell leukemia.
2. Peginterferon alfa-2a (*Pegasys*): used to treat hepatitis C (HCV).
3. Recombinant interferon-alpha 2b (*Intron A*): a cytokine produced by recombinant DNA technology and used to treat Hepatitis B; malignant melanoma, Kaposi’s sarcoma, follicular lymphoma, hairy cell leukemia, warts, and Hepatitis C.
4. Peginterferon α -2b (*PEG-Intron; PEG-Intron Redipen*): used to treat hepatitis C (HCV).
5. Recombinant Interferon α -2b plus the antiviral drug ribavirin (*Rebetron*): used to treat hepatitis C (HCV).
6. Recombinant interferon- α n3 (*Alferon N*): used to treat warts.
7. Recombinant interferon alfacon-1 (*Infergen*): used to treat hepatitis C (HCV).
9. GM-CSF (granulocyte-macrophage colony stimulating factor): for hematopoietic reconstruction after bone marrow transplant in people with lymphoid cancers. TLRs also participate in adaptive immunity by triggering various secondary signals needed for humoral immunity (the production of antibodies) and cell-mediated immunity (the production of cytotoxic T-lymphocytes and additional cytokines). Without innate immune responses there could be no adaptive immunity.
CHAPTER 3

3.0 PROBLEM STATEMENT

3.1 The Majengo cohort
The Department of Medical Microbiology in the University of Nairobi has had a long standing research interest in sexually transmitted diseases in slums in Nairobi dating from early 1980s. Initially, their goal mainly targeted bacterial infections causing genital ulcers and gonococcal diseases in female sex workers. On realizing through serological studies that, up to sixty per cent of the female commercial sex workers in the study had HIV-1 antibody (Plummer F.A, 1994), a cohort was set up and has since been followed-up to the present, with the aim of understanding risks that predispose these women to HIV acquisition. One of the finding of the cohort study is that there exists resistance to HIV infection in a special group of women constituting about one percent of women presently enrolled in the study. As a result, there is still an interest in understanding mediators of this immunity in order to use it as a model for HIV vaccine developed.

Studies seeking to characterize cellular responses to antigens at the female genital tract and immune activation between the so called ‘HIV-resistant’ and HIV infected have shown that there is an altered immune activation and T Cell regulatory levels. Similarly, studies on the same women revealed that, HIV-resistant women had significantly reduced expression of the innate immune sensors TLR 7 and TLR 8. These findings suggest that biologic differences in innate factors and are responsible for HIV resistance. This project seeks to compare the dendritic cells in HIV positive and HIV negative women in the study.

3.2 Hypothesis
Effector CTL and IgA are believed to play a major role in HIV resistance, and it is apparent in women in Majengo cohort. However, mechanisms responsible for this resistance need elucidation. Correlation between T-helper responses and HIV-1 specific recognition responses in the resistant women would help elucidate the immunologic pathways utilised to induce these somewhat obscure immunologic phenomena. Furthermore, correlation of TLR activation and
expression may help to determine what is responsible for driving the polarity of T-helper responses. Linking the adaptive response to expression of germ line encoded factors may help explain inheritance of resistance, and provide long sought after markers for resistance in this cohort. Perhaps most importantly such correlation would have significant importance because new therapeutic manipulations through the use of vaccine adjuvant are already available. This study sought to determine if there is difference in dendritic cell frequencies and stimulation responses in female commercial sex workers leading to altered cytokine production

3.3 Justification

Potential theories as to the origin of this response are varied, and control of the cytokine milieu may be influenced by known innate immune system receptors, such as Toll-like receptors on dendritic cells, which are germ line encoded. Variable expression in such receptors could account for inheritance of resistance, and this will ultimately provide a target for drug or vaccine manipulation. It is further hypothesize, that variable expression in these receptors on DCs in peripheral circulation may lead to variable expression of cytokine and may correlate with HIV resistance in this group.

If adaptive immune responses such as HIV-1 specific CTL and IgA responses contribute to HIV-1 resistance, and there appears to be a genetic component to resistance, then it is reasonable that known inducible genetic factors capable of regulating these responses should be sought. With our growing understanding of germ-line encoded pattern recognition receptors (part of the innate immune system), data suggests that, these receptors are inducible and capable of orchestrating the T helper response to antigen.

Toll-like receptors (TLRs), of which there are currently 10 known human examples, have specificity to a limited range of molecular patterns that are evolutionarily conserved among pathogens, as well as being capable of induction by host cell stress. Stimulation of these receptors activates a cascade which mediates proinflammatory cytokine production through NF-kβ mediated transcription. In most cases a Th1 type response is always favoured taking IL-12
and IFN-γ axis. Current vaccine adjuvants utilize these receptors, and they are a natural system to evaluate in the search for determinants of HIV resistance.

3.4 Objectives

3.4.1 General objective

To determine frequencies and stimulation responses of dendritic cells in peripheral blood of female in a cohort of commercial sex workers in Majengo, Kenya.

3.4.2 Specific objectives

1. To determine the frequencies of dendritic cells in whole blood of HIV infected female commercial sex workers

2. To determine the frequencies of dendritic cells in whole blood of HIV negative female commercial sex workers

3. Determine frequency of expression of TLR2 and TLR4 on dendritic cells

4. To compare cytokine profile in HIV infected and HIV negative female sex workers
4.0 MATERIALS AND METHODS

4.1 Research Design and Methods

In order to meet the objectives of this project, women in the Majengo commercial sex worker cohort who presented in the clinic for their follow up visits were requested to participate in this nested study. The women were allocated two groups depending on their HIV seroprevalence. Thirty two women consented to participate in the nested study. The study was approved by the National ethics and scientific review committee of Kenyatta National Hospital [appendix vii].

The consenting women were subjected to physical examination by the clinic physician and specimens including endo-cervical swabs and blood were collected. Blood specimens were used to conduct enumeration assays of T-lymphocytes and dendritic cells, stimulation assays, serological detection antibodies to HIV-1 and syphilis. 10 mL of blood were drawn directly into 10 ml “green-capped” vacutainer™ tubes, containing 143 USP units of freeze dried sodium heparin as anticoagulant, and were gently rocked at room temperature until processing, generally within 6 hours of collection.

4.2 Staining of T-lymphocyte subsets

For each participant sample, a 12x75mm Tru count tube was labeled, 20 µl of multitest CD3, CD8, CD45/CD4 reagent were dispensed into the bottom of the labeled tubes and 50µl of well mixed, anticoagulated whole blood were added, the tubes were capped and vortexed gently to mix. The tubes were then incubated for 15 minutes at room temperature (20-25 °C) in the dark. 450µl (1xFACs lysis solution was then added into each tube and the tubes were then capped and then incubated for 15 minutes in the dark at room temperature (20-25°C). The samples were then analyzed in the flow cytometer.
4.3 Analysis of T-lymphocyte subsets using flowcytometry

The PMT and sensitivity of the machine was checked by using CaliBRITE 3 and APC beads (BD catalog Nos. 340486 and 340487 respectively) and control blood was used to optimize instrument setting. The samples were run after attaining a brighter CD45 versus Side scatter (SSC) dot plot separation of lymphocytes. The lymphocytes appeared as bright compact cluster with low SSC as shown in figure 3, 4 and 5. Approximately 1500 cells were enumerated. Absolute counts of the lymphocytes and percentages were determined in cells per μl and percent respectively.

4.4 Detection of dendritic cells

The assay for detecting different subsets of peripheral blood dendritic cells (DCs) CD 123⁺ (Anti IL 3Ra⁺) and RD 11α⁺ was performed using flow cytometric technology. These cell populations were identified using a combination of multiple markers since CD 11c and CD 123 are not DC specific. Additional staining panel was employed. DCs populations express high levels of HLA-DR and low levels lineage markers for monocytes, lymphocytes and NK cells. Identification of DCs therefore depends on low detection expression of these lineage markers without discrimination between individual antibodies, the lineage markers are labeled with a single fluorochrome. The lineage-1 (Lin 1) cocktail used in the assay contain CD3, CD14, CD16, CD19, CD20 and CD56. In addition, the assay allows identification of basophilic granulocytes on basophils, which are lineage negative and express similar levels of CD123 as the CD123⁺ DCs subset. These basophils can be discriminated upon by negative selection since they lacked HLA DR antigens.

Reagents used in the study were from Beckman Dickinson Company (BDCS). The reagents are immunostaining fluorochrome – conjugated with monoclonal antibodies for detecting dendritic cells. These included lineage contain (Lin 1) FIFC catalog No. 340546. The cocktail contains CD3, CD14, CD16, CD20 and CD56. CD123 (Anti IL3Ra) PE catalog No. 340545, Anti HLA DR PerCP (Catalog No. 347364), CD 11c APC (catalog 340 544), Mouse IgG1 PE (Catalog No. 349043) and Mouse IgG2α APC (Catalog No. 340473).
Monoclonal antibodies were added to pre-labeled tubes as shown in the table. 1.5μl of CD123 PE, CD11c APC, Mouse IgG1 and Mouse IgG2α were added into appropriate labeled tubes. 10μl of anti HLA DR was added into all the tubes followed by 20μl of Lin 1 FITC. TLR 2 and TLR1 PE were added into tube number 3 and 4 respectively. 100ul of heparinized whole blood was used for surface staining was added into the tubes and were mixed by volex at 200rpm.

The mixture was then incubated in the dark at room temperature for 15 minutes. Lysing solution was then added into each tube to lyse RBCs and the tubes were centrifuged at 300g for 5 minutes for separation. The sediment was then washed in isotonic buffer and then palleted through centrifugation at 300g for 5 minutes at room temperature. The cell pallet was then fixed in 1% paraformaldehyde and was acquired in a four colour BD Facscalibur.

4.5 Cell acquisition and analysis

Four color brand BD Facscalibur was used to acquire the cells. CaliBRITE™ beads and FacsCOMP™ software for adjusting the photomultiplier tube (PMT) voltages and fluorescence compensation were used to check the sensitivity of the instrument. Cellquest™ software with a threshold on FSC to exclude debris was used in acquisition. 100,000 events were acquired from each patient sample. The acquired cells were discriminated from debris by selecting the live cells through forward scatter, side scatter (FSC/SSC) dot plot (unigated) on R1 as shown in figure 1. Lineage 1 (Lin 1 dim) and negative events were gated on R1 giving gate R2. Gating on Anti HLA-DR/CD 123 dot plot and anti HLA-DR/CD11c dot plot each gated from gate 3. These are cells that are Lin 1/dim/negative. Basophils were discriminated by being Anti HCA-DR/CD123+ region 3 and R4 to define CD123+ (Anti HLA-PR+/CD123+) ad region R5 to define CD11c+ (Anti HLA-DR.CD11c+)
Figure 4.0: Side scatter (SSC) and forward scatter (FSC) in leukocyte separation (Gated cells are PBMCs)

Figure 4.1: Distribution of leukocytes in a live gate (Gated cells are Lineage 1 negative and HLA-DR positive)
Figure 4.2:  Distribution of CD11c$^{\text{positive}}$ Dendritic cells (mDCs) (Gated cells are mDCs)

Figure 4.3:  Distribution of CD123$^{\text{High}}$ Dendritic cells (pDCs) (Gated cells are pDCs)
4.6 Isolation and stimulation of peripheral mononuclear cells (PBMCs)

PBMC were isolated by Ficoll-Paque density centrifugation method, washed, counted and re-suspended at concentration of 11 million PBMC per mL in 11 ml R10 media for PBMC stimulation. Baseline PBMC (from 11 ml suspension) 1x10^6/ml were used as follows:

1) Two, 200 µl PBMCs were put in FACS tubes for to PBMC subset and DC staining. 200 µl PBMC were stained for DC using Dc monoclonal antibodies (lin cocktail FITC, CD123-PE, HLA-DR-PerCP, CD11c-APC) (CD123 and CD11c DC markers, isotype controls were used to provide background test.

2) 200µl of PBMC were plated in 96 round bottom well Tissue culture plates and were incubated with 2 µl LPS (10 µg/ml) or ssRNA40 (100 µg/ml) to appropriate wells for t0, the cell were incubated at 37°C and 5% CO2 for 16 hour. After incubation cell were harvested and re-stimulated with appropriate ligands at t1 for 16 hours. After incubation, 180µl of supernatants were then collected and transferred into new 96 well non-sterile plate and label with time of stimulation such as “t1 supernatants”, the plates were then sealed and stored at -80C Freezer for analysis of cytokines. Dislodged cell pellets were centrifuged 1600rpm for 5 minutes before collecting the supernatant.

The cells were replenished with 180 µl of RPMI-1640 for washing cells to remove dead cells were then centrifuged at 1600rpm for 5 minutes. The buffer was then decanted in the waste bucket and plates were then replenished with fresh 180µl fresh RPMI-1640. Cells were then transferred into FACS tubes labeled for mDC and pDC staining. Remaining cells were resuspended with stimulants and incubated for another 8 hours (t2). The plates were incubated and 180µl of supernatant removed from the plate wells. The supanatant were then from frozen as for t1 and labeled with a “t2 supernatant” prefix, and were stored at -80C for analysis of cytokine. Flow cytometric analysis was performed on fresh samples at t0, t1, t2. Cells were stained cells using Lin-1 Neg-FITC, CD123-PE, HLA-DR-PerCP, and CD11c-APC. Cytokine assays using a cytometric bead array were performed on the supernatant at t1 and t2 supernatants.
using (CBA) Th1/Th2 kit. The analysis were performed for each patient at 3(x2) =6 t1 conditions, and 9(x2) = 18 t2 conditions, totaling 24 conditions for CBA per individual.

Table 4.1 Plate set up: Columns 1-3 are t1, 4-12 are t2. A-D = One individual and E-H another set of PBMC.

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KEY

C= Culture media (control), L=LPS (0.1μg/ml), R=ssRNA40 (1μg/ml). The first stimulant in hyphenated letter is t0 to t1 (0-16hours ‘prime’), then the second stimulation is t1 to t2 (16-24 hours).
CHAPTER 5

5.0 RESULTS

Thirty two women (n=32) were selected from a cohort of female commercial sex workers to participate in this nested study. From our serological studies for HIV antibodies, twelve women (n=12) tested positive for HIV-1 antibodies while twenty women (n=20) were categorized as HIV seronegative at the time of the study. The women were subjected to physical examination by the clinic physician and were further screened for presence of sexually transmitted infections such as gonorrhea, Chlamydia, syphilis, chancroid and trichomonas parasites. From the investigation 9.3 percent had trichomoniasis while other venereal illnesses were not detected at the time of investigation.

Age distribution of the women was 34 ±20 years with median of 34 years. HIV infection was 37.5 percent. Those who had HIV infection had CD4+ T-helper cell counts ranging between 180 cells per µL of blood and 600 cells per µL. Six of those classified as HIV positive were on Septrin™ prophylactic treatment. There was no significant differences observed in terms of age and sexual practices between these groups.

Women with HIV infection had a significant leukocytosis (7,300 to 12,600/µl, p < 0.001) compared to HIV women (6,700 to 9000/µl). There was decrease in lymphocyte counts (34±12 %) in HIV-1 infected women than in HIV-1 negative women (45±10 %). There was no significant difference in other laboratory parameters as shown in table 5.1.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV positive (n=12)</th>
<th>HIV negative (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>31±6.6</td>
<td>29.9±5.6</td>
</tr>
<tr>
<td>Duration of prostitution (Years)</td>
<td>4.8±4.7</td>
<td>4.6±5.2</td>
</tr>
<tr>
<td>Contraceptive use:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>58.3 %</td>
<td>30.0 %</td>
</tr>
<tr>
<td>Condom</td>
<td>≥70±10 %</td>
<td>≥69±12 %</td>
</tr>
<tr>
<td>Blood profile:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cells</td>
<td>9.0±4.0 x 10^3/µL</td>
<td>6.4±2.2 x 10^3/µL</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>4.2±3.0 x 10^3/µL</td>
<td>5.0±3.0 x 10^3/µL</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>34±12 %</td>
<td>45±10 %</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>11.2±2.05 g/dL</td>
<td>10.1±2.18 g/dL</td>
</tr>
<tr>
<td>CD4 cells ≥200 Cells/µL</td>
<td>484.8±200 Cells/µL</td>
<td>882±180 Cells/µL</td>
</tr>
<tr>
<td>CD4 cell counts ≤200 Cells/µL</td>
<td>155±25 Cells/µL</td>
<td>None</td>
</tr>
</tbody>
</table>

(Table 5.1): Comparison of the characteristics of the women in the study

Analysis of T lymphocytes subsets revealed that, HIV-1 seronegative women had a mean CD4+ T helper cell count of 882 cells/µL in their blood and CD8+ Cytotoxic/Suppressor T cell counts of 531 cells/µL in blood. This comprised 42.6 and 25.3 per cent of lymphocytes respectively (4.1a and 4.1b).

The HIV-1 seropositive women on the other hand, had mean cell counts of 484.8 CD4+ T Helper cells/µL of blood and 1090.9 CD8+ Cytotoxic/Suppressor T cell counts cells/µL of blood. This comprised 21.2 and 48.0 per cent of lymphocytes respectively (figure 4.1c and 4.1d). The cell counts of CD4+ T helper in HIV -1 seronegative woman were within the normal range (500-2000 cells/µL) as compared with those of similar women of the same age and socio-demography from this region. Their CD8+ T Cytotoxic/suppressor cells were also within normal range (270-1500 cells/µL) as compared to other women in this region (Stevens W.et al, 2008: report sent for publication). These are shown in figure 5.1a, 5.1b, 5.1c, and 5.1d.
Figure 5.1(a) and 5.1(b): CD4 + T-lymphocyte absolute counts and percentages

Figure 5.1(c) and 5.1(d): CD4 + T-lymphocyte absolute counts and percentages
Circulating myeloid dendritic cells (mDCs) was identified by the fact that they display strong HLA-DR MHC class II antigen molecules and high CD11c molecule, which were detected by monoclonal antibody staining. Upon staining they displayed HLA-DR\textsuperscript{high}CD11c\textsuperscript{high}CD123\textsuperscript{low} molecules, while plasmocytoid dendritic cells (pDCs) were identified on the basis that they of expressed high HLA-DR\textsuperscript{high}CD123\textsuperscript{high}, and low CD11c\textsuperscript{low} on their surfaces. Unlike other leucocytes they both lacked expression of linage markers.

This investigation revealed that, plasmocytoid dendritic cells (Anti HLA-DR\textsuperscript{high}CD123\textsuperscript{high}, CD11c\textsuperscript{low}) had higher percentage compared to myeloid dendritic cells (Anti HLA-DR\textsuperscript{high}CD11c\textsuperscript{high} CD123\textsuperscript{low}). Plasmocytoid dendritic cell counts were 1.38 ± 1.16 percent with an absolute count of (105 ± 38 cells/µL), and a median count of 1.28 percent.

Myeloid dendritic cells on the other hand had counts of 1.13±0.97 percent and an absolute count of 63.84 ± 40 cells/µL of blood, with median count of 0.97 percent. These values were within previously described range (Pacanowski et al 2000).

Higher percentages of circulating myeloid DCs were however observed in HIV seronegative commercial sex workers as opposed to HIV seropositive women. The mean myeloid DCs in HIV seropositive women were significantly decreased (p value =0.017), with counts of 0.18 ± 0.08 per cent and absolute counts of 56 ± 30 cells/µL and versus 1.08 ± 1.04 percent, and absolute counts of 110 ± 26 cells/µL, in HIV-1 seronegative negative women. The low counts reported in our study had been observed in similar studies, for example Jérôme Pacanowski and co-workers had reported a decrease in cell numbers of mDCs in HIV chronic infection, which was consistent with our findings (Pacanowski et al 2000). The reduced mDC counts was however seen in individuals with CD 4\textsuperscript{T} helper cell less than 200 cells/µL, and were also not on antiretroviral treatment for HIV.

HIV seronegative women also had a slightly higher plasmocytoid DCs percentage (1.47 ± 1.31per cent, with the absolute counts of 109.32 ± 27 cells/µL,) compared to 1.2 ± 0.62 per cent with absolute counts of 68.4 ± 52 cells/µL obtained from HIV-1 positive women.
Figure 5.2: Percentages of pDCs and mDCs before activation

We further analyzed intensity of surface expression of TLR 2 and 4 on dendritic cells using surface staining techniques. From this analysis we found out that circulating dendritic cells had an overall expression intensity average of $2.29 \pm 1.92$ percent for TLR 2 compared to TLR 4 expressed at an intensity of $0.83 \pm 0.65$ percent on mDCs.

On comparison, HIV seropositive women had considerably lower intensity of TLR2 expression dendritic cells than those from HIV seronegative women. The TLR2 expression on dendritic cells from HIV seropositive women were $1.79 \pm 1.13$ percent and a median of $1.99$ percent. HIV seronegative women on the other hand had a mean of $2.58 \pm 2.2$ percent expression on dendritic cells.

Stimulation of these cells showed very different proliferation pattern. The HIV seropositive women did not show as much increase in cell numbers as those HIV seronegative women after stimulation with TLR ligands. Stimulation with ssRNA produced an increase in plasmocytoid dendritic cells from the original $1.5 \pm 1.26$ percent to $1.86 \pm 1.27$ percent, which in general is equivalent to 18.5 percent increase in HIV sereopositive women, while HIV seronegative women
had an increase from $1.47 \pm 1.31$ percent to $4.32 \pm 1.27$ percent. Upon stimulation myeloid dendritic cell on the other hand did not increase in numbers in blood of HIV positive group, following stimulation with ssRNA, giving counts of $1.52 \pm 1.09$ percent from $1.21 \pm 0.89$ percent count at baseline, while in HIV seronegative women there was an increase in cell numbers from $1.08 \pm 1.04$ percent to $6.34 \pm 3.5$ percent. Stimulation with LPS led to increase in cell numbers in both the plasmocytoid dendritic cells and myeloid dendritic cells which increased from $1.488 \pm 1.31$ percent to $3.50 \pm 1.74$ percent and $1.08 \pm 1.04$ percent to $4.54 \pm 1.27$ percent in HIV seronegative women respectively. In HIV seropositive women cell increased from $1.21 \pm 0.88$ percent to $1.81 \pm 1.9$ percent in plasmocytoid percentage and the myeloid dendritic cell increased from $1.21 \pm 0.88$ percent to $1.22 \pm 1.02$, thus, indicating very minimal change in cell numbers in HIV seropositive women.

![Figure 5.3: Percentages of myeloid and plasmocytoid dendritic cells after cell stimulation](image)

This difference in cell numbers signified difference in proliferation ability in vitro. We further analyzed various cytokines that are important in directing the subsequent innate and adaptive immune responses. Some of the most commonly secreted cytokines in response to TLR ligands include IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ. In this study, mononuclear cells were stimulated with different ligands, LPS (TLR2 and 4), and ssRNA (TLR7, 8 and 9), and the secreted cytokines were measured by Flow cytometry. We investigated the ability of these ligands to stimulate cell from HIV seropositive women and compared the response with that obtained from HIV-1 seronegative women. Results demonstrated that, ssRNA and LPS variably induced...
cytokine production. Single stranded RNA induced more IFN-γ tested in the two groups, but significant IFN-γ (p value=0.007) was produced in HIV negative women than in HIV positive women. LPS on the other hand induced high production of TNF-α in HIV seropositives than in HIV-1 seronegative group but this difference were not statistically significant. Likewise, IL-2, IL-5, IL-10, and IFN-γ production did not show any significant difference in the group. IL-10 was more robust in HIV-1 seronegative women than in seropositive. Stimulation with LPS and ssRNA induced in as twice amounts of IL-10 in HIV negative women as in HIV seropositive women (Table 4.1) IL-5 production was higher in the seropositive women than in seronegative with both ligands, while more IL-2 was produced in the HIV-1 seronegative woman than in seropositive women. IL-4 production did not differ in the two groups with either stimulant.

<table>
<thead>
<tr>
<th>Product</th>
<th>Ligand</th>
<th>HIV seronegative</th>
<th>HIV seropositive</th>
<th>P-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 (pg/mL)</td>
<td>LPS</td>
<td>1.01±1</td>
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<tr>
<td></td>
<td>ssRNA</td>
<td>4.08±2.03</td>
<td>1.08±2.82</td>
<td>0.432</td>
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<tr>
<td>IL-4 (pg/mL)</td>
<td>LPS</td>
<td>1.0±0.8</td>
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</tr>
<tr>
<td></td>
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<td>2.03±1.5</td>
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<td>0.500</td>
<td>No Significance</td>
</tr>
<tr>
<td>IL-5 (pg/mL)</td>
<td>LPS</td>
<td>1.36±1.2</td>
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<tr>
<td></td>
<td>ssRNA</td>
<td>1.44±1.9</td>
<td>3.17±2.1</td>
<td>0.325</td>
<td>No Significance</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>LPS</td>
<td>3587±550</td>
<td>1490±500</td>
<td>0.344</td>
<td>No Significance</td>
</tr>
<tr>
<td></td>
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<td>1172±80.5</td>
<td>325±102</td>
<td>0.84</td>
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<tr>
<td>TNF-α (pg/mL)</td>
<td>LPS</td>
<td>8128±230</td>
<td>13118±554</td>
<td>0.134</td>
<td>No Significance</td>
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<tr>
<td></td>
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<td>306±102</td>
<td>9214±108</td>
<td>0.007</td>
<td>Significant</td>
</tr>
<tr>
<td>IFN-γ (pg/mL)</td>
<td>LPS</td>
<td>11.16±3.4</td>
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<tr>
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<td>254±16</td>
<td>101±26</td>
<td>0.406</td>
<td>No Significance</td>
</tr>
</tbody>
</table>

Table 5.2 Cytokine profile in PBMC upon stimulation
CHAPTER 6

6.0 DISCUSSION

Dendritic cells are able to regulate the immune response to foreign- as well as self-antigens, inducing either an immune response or tolerance. Earlier studies showed that immune activation of adaptive immunity depends on antigen recognition by antigen presenting cells and eventual presentation to lymphocytes (Kadowaki N et al). Dendritic cells play a major role as APCs and therefore, as well, play a key role in immune responses in HIV infection. Three distinct differentiation stages of DCs and their typical migratory route are now well known. Dendritic cells are derived from a common CD34+ progenitor in the bone marrow and are released as circulating DC precursors into the blood. From the bloodstream, they enter various tissues and develop into tissue-resident, immature DC there, which are able to take up antigens. In response to inflammatory signals, their maturation is induced, enabling mature DCs to activate T cells after migration into the lymph nodes. Dependent on the local stimuli such as cytokine environment, tissue-resident immature DCs might alternatively arise from monocytes. In our study, we investigated whether infection with HIV-1 may change the frequencies of both circulating myeloid dendrite cells and plasmocytoid dendritic cells in female commercial sex workers. We also studied stimulation responses of these cells to LPS and ssRNA both TLR ligand in invitro environments.

We showed that, myeloid and plasmocytoid cells were reduced in blood of patients with HIV infection compared to HIV negative women. Additionally, patients with HIV infection were less responsive to stimuli with LPS and ssRNA which are components of gram negative bacteria and some viruses respectively. We also discovered that HIV positive women exhibited a reduction in production of key cytokines including TNF-α and IFN-γ which are anti viral agents and may play a greater role controlling viraemia and spread of HIV-1 to other cells in the host. Similar to our observations in these cell numbers, previous studies showed a decrease in circulating dendritic cell precursors in various autoimmune or infectious diseases. Dependent on the type of the immunological disease, either a predominant decrease in plasmocytoid dendritic in (Sjögren’s syndrome, tuberculosis, and asthma bronchiale) or myeloid dendrite cells (lupus
erythematous, chronic hepatitis B and C) was described. However, it is still yet unclear why different DC precursor subtypes are altered in particular immunological diseases, and this fact might be associated with disease-specific immune mechanisms. The data presented here show that there is reduction of blood plasmocytoid (CD123+) and myeloid CD4+ T-helper cells. In this study, women who had low CD4+ T-cells (≤ 200 cell/μL), had also registered a decrease in number of these two key cell populations. Similar finding had been previously described in chronically ill HIV-infected patients (Pacanowski et al, 2000) in which they detected defects and low numbers of DCs as early as 26 to 57 days after the estimated date of infection, indicating that the loss of these cells were probably related to HIV itself rather than to the chronic activation of the immune system found in later stages of infection. Most interestingly, in their study they were able to restore the cells by antiretroviral treatment in a few patients, and a strong correlation was found between CD123+ DC number recovery and good virologic and immunologic parameters.

Potential causes of reduction in numbers of blood DCs may be attributed either to central or peripheral. In the periphery, DCs may have homed to lymphoid organs and may no longer be in the blood. DC production, differentiation, and survival are supported by T lymphocytes through several interleukins (including IFN-gamma, GM-CSF, and IL-4) and surface molecules (including CD40L, 4-1BB, OX 40, and TRANCE) (reviewed in Banchereau et al). The T-cell defect, which is a main event in HIV infection, may induce the low numbers of DCs found here.

The potential consequences of the decreased numbers of DC populations in HIV infection are related to some of their functions: cytokine production (type I IFN, IL-12) and antigen presentation to naive T lymphocytes. CD123+ DCs are the natural interferon-producing cells. During chronic HIV infection, a defective type I IFN in vitro response of DCs to viral stimuli was shown, where the HIV positive women with low DC and CD4+cells had reduced level of secretion of IFN-γ compared to those with high number of these cells. The kinetics of type I IFN secretion still needs to be studied in HIV+ patients. Then reduction of CD123+ DC numbers might decrease this innate immune response, which was shown to be able to decrease HIV replication (reviewed in Khatissian et al), to promote CD123+ DC survival, to increase TH1-type
responses, and, together with GM-CSF, to mediate in vitro differentiation of DCs with strong HIV antigen presentation capacities in the severe immunodeficiency.

CD11c$^+$ and CD123$^+$ DCs secrete IL-12. Their reduced numbers may participate in the defective responsiveness to inflammatory and viral stimuli that was found in PBMCs from HIV$^+$ patients, and especially if they involve IL-12. This might contribute to the low IL-2 secretion by CD4$^+$ T cells, because IL-12 has been shown to restore in vitro IL-2 production from chronic HIV$^+$ patient cells in response to HIV antigens.

A consequence of low DC numbers may be a decreased ability to stimulate naive HIV-specific T lymphocytes. Indeed, Langerhans cells from HIV$^+$ patients were deficient for allostimulation, hence naive T-cell stimulation, but not memory CD8$^+$ T-cell activation. This would add to the T helper cell defect to explain the low T-cell responses during HIV primary infection. In addition, if DCs are not restored under HAART, naive T cells may not respond to viral antigens when HAART is interrupted. This may also hamper responses to therapeutic vaccination protocols currently undertaken under HAART to restore strong memory T-cell responses to the virus. It might be necessary to restore DC numbers before vaccination. This might be achieved, like CD4$^+$ T-cell restoration, by long-term viral suppression, and might be improved by immune therapy.

In combination with vaccination protocols under HAART, it may be necessary either to supplement DCs by IFN-alpha or IL-12 treatment if toxicity potential was overcome. Alternatively, it may be necessary to try and restore DC numbers using interleukins or molecules triggering DC survival, differentiation, and activation, like FLT3 ligand, GM-CSF, CD40 ligand trimers, IFN-gamma alone or in combination. This might restart the positive feedback loop between DCs and T cells, and hopefully help stimulate stronger HIV-specific CD4$^+$ and CD8$^+$ T lymphocytes able to control HIV infection after HAART interruption. A larger proportion of infected individuals would then join the exposed, non infected individuals or the long-term non progressors in a successful immune control of HIV replication. Overall, at present, there is increasing evidence suggesting an important role of dendritic cells modulating the course of HIV disease in either delaying or accelerating the disease process towards AIDS. However, future
studies will have to determine whether a depletion or specific suppression of DC in function may be a predictor of immunotherapy in immunodeficiency.

6.1 Conclusion
Our study showed a significant decrease in circulating dendritic cells in HIV-1 infected women, with decreased numbers of CD4$^+$ T cells, reduced IFN-γ production upon stimulation with TLR ligands that could indicate altered type 1 responses. Since dendritic cells play a major role in secretion of cytokines including IFN-γ then, their reduction in numbers may lead to low production of these key immune mediators and may lead to poor control of viraemia and therefore poor outcome of anti retroviral therapy as these mediators augment activity of antiretroviral therapy.

6.2 Recommendation
We recommend that a more detailed study should be carried out to evaluate dendritic cells in HIV infected patients with aim of understanding their nature and functionality. Monitoring of these cells should also be carried out as part of HAART program in order to assess their response to treatment.
APPENDIX I

HIV-1 Plus O Microelisa System

Store between 2-8°C.

Intended Use

The Vironostika® HIV-1 Plus O Microelisa System is an enzyme-linked immunosorbent assay (ELISA) for the qualitative detection of antibodies to Human Immunodeficiency Virus Type 1 (HIV-1), including Group O, in human specimens collected as serum, plasma, or dried blood spots. The Vironostika HIV-1 Plus O Microelisa System is intended for use as an aid in diagnosis of infection with HIV-1. It is not intended for use in screening blood donors.

Summary and explanation of the test

Published data indicate a strong correlation between the acquired immunodeficiency syndrome (AIDS) and a retrovirus referred to as Human Immunodeficiency Virus (HIV). Currently, two HIV serotypes, designated as HIV-1 and HIV-2, have been identified based on the results of serologic and molecular studies. Both HIV serotypes have been isolated from patients with AIDS and AIDS-related complex (ARC), as well as from apparently healthy individuals at high risk for AIDS. Both viruses have the same morphology, lymphotropism, and modes of transmission. Since 1984, reports have indicated that HIV-1 can be isolated from a variety of tissues and body fluids of infected individuals. In 1990, a more divergent strain of HIV-1, which is known as Group O HIV-1, was isolated and characterized. Antibodies specific for group O HIV-1 are difficult to be detected with some HIV antibody detection systems unless these systems use whole viral lysate and/or contain group O specific epitopes as antigens. In addition, an increasing number of variants have been identified with HIV-1 serotype, some of which have arisen from genetic recombinations within a host having infection with multiple types of HIV-1. Such variants may be more difficult to be detected with ELISA systems that are not based on viral lysate. Worldwide distribution of HIV shows prevalence of the serotypes in different areas, with HIV-1 most widely distributed and Group O appearing primarily in West Central Africa. Following infection with HIV, an individual rapidly (e.g. within 4 weeks) develops antibodies to viral proteins, a process known as seroconversion. After seroconversion, HIV specific antibodies
can be readily detected in the blood specimen. A majority of patients who exhibit symptoms of AIDS or ARC have HIV specific antibodies in their blood.

In addition, a significant proportion of apparently healthy individuals at increased risk for AIDS also contain HIV specific antibodies in their blood specimens. Due to close relation of HIV-1 and HIV-2, proteins of the two viruses, especially the core and polymerase proteins, result in serologic cross-reactivity. The Vironostika HIV-1 Plus O Microelisa System assay was designed to be highly sensitive for a spectrum of HIV-1 serotypes, including group O virus. As a result, nonspecific reactions may occasionally be seen in specimens from people who have prior pregnancy, blood transfusion, or exposure to human cells or media containing cultured HIV antigen. Because of these and other potential nonspecific reactions, specimens reactive with the Vironostika HIV-1 Plus O Microelisa System assay should be confirmed with a confirmatory test, e.g., Western Blot testing.

Reactive specimens upon initial testing with the Vironostika HIV-1 Plus O Microelisa System assay should be re-tested in duplicate. Reactivity in either or both of the duplicate tests indicates a potential for the presence of HIV-specific antibody. In individuals at increased risk of infection, such as homosexual men, hemophiliacs, or intravenous drug users, repeatedly reactive specimens are usually found to contain antibodies to HIV by additional, more specific tests. However, when the ELISA is used to screen populations with a low prevalence of HIV infections, nonspecific reactions may be more common than specific reaction. Information about prevalence of HIV infections in persons in various categories of risk, as well as clinical and public health guidelines, are available in each CDC publication of Morbidity and Mortality Weekly Reports. Although information about the degree of risk for HIV-1 infection and the degree of reactivity of the serum are of value in interpreting the test, a diagnosis should not be based only on this information. Therefore, it is appropriate to investigate repeatedly reactive specimens by additional, more specific tests, such as Western Blot, immunofluorescence, radioimmunoprecipitation, viral antigen based immunoassays, peptide ELISAs, or nucleic acid amplification assays.
**Principle of the Test**

This test uses HIV-1 antigens, which are coated onto the wells of microwell plates, for the detection of antibodies specific for HIV-1, including Group O. These antigens include inactivated, purified HIV-1 viral lysate proteins, purified viral envelope proteins, and a synthetic peptide with amino acid sequence corresponding to that of the transmembrane immunodominant domain of the HIV-1 Group O (ANT 70) isolate. Upon the addition of a diluted test specimen containing antibodies to HIV-1 or HIV-1 Group O to a microwell, immune complexes are formed through the interaction between anti-HIV in the specimen and HIV antigens coated on the microwell. Following incubation, the specimen is aspirated and the well is washed with buffer. Subsequently, anti-human immunoglobulin (goat) conjugated with horseradish peroxidase (HRP) is added which binds to the anti-HIV-antigen complex during a second incubation. Following a wash and incubation with ABTS (2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate]) substrate, a green color is produced. The enzyme reaction is stopped by the addition of a fluoride solution. The amounts of antibodies to HIV present in the specimen are qualitatively proportional to color intensity.

**VIRONOSTIKA®**

**HIV-1 PLUS O MICROELISA TEST PROCEDURE**

**Procedural notes**

Test procedure for serum or plasma specimens

1) Fit stripholder with the required number of HIV-1 Plus O Microelisa Strips. If less than twelve Strips are needed, use uncoated strips to complete the plate when using a 96-well washer.

2) Prepare a 1:21 dilution of each serum or plasma test specimen, Calibrator, and Controls. Include three wells of Negative Calibrator, and one well each of HIV-1 Positive Control and HIV-O Positive Control on each run. **Caution:** Use a clean tip for each specimen. Do not pipet specimen into an empty well without Dilsim III. Do not allow the microelisa wells to dry once the assay has begun.

   a) Automated diluter/dispenser: Pipet 10 μl of specimen, Calibrator, or Control with 200 μl Dilsim III into the designated microelisa well.
b) Premixed manual method: Pipet 15 μl specimen, Calibrator, or Control into a clean test tube containing 300 μl Dilsim III. Mix well. Pipet 210 μl of the diluted specimen into the designated microelisa well.

c) Direct manual method: Using a multichannel pipet, add 100 μl Dilsim III to each microelisa well. Pipet 10 μl specimen, Calibrator, or Control into the designated wells. Using a multichannel pipet and clean tips, add an additional 100 μl Dilsim III to each well and repeatedly aspirate and dispense to mix. Note: The addition of a serum or plasma specimen to Dilsim III will cause the specimen addition indicator to turn the dilution to a lavender color.

3) Cover the Strips with adhesive plate sealers or equivalent. Within 60 minutes of specimen/control addition, incubate Strips at 37 ± 2°C for 60-70 minutes.

4) Wash each well four times with Wash Solution (refer to "Wash procedure") using a soak cycle of at least 30-seconds.

5) Pipet 150 μl of reconstituted EnzAbody working solution into each well. Caution: Do not allow EnzAbody to contaminate ABTS Substrate Solution. If the same equipment is used to add both reagents, new disposable tips must be used.

6) Cover the Strips with adhesive plate sealers or equivalents. Incubate at 37 ± 2°C for 60 to 65 minutes.

7) Wash each well four times with Wash Solution (refer to "Wash procedure") using a soak cycle of at least 30-seconds.

8) Pipet 150 μl of ABTS Substrate Solution into each well. Do not mix or agitate. Do not cover the Strips.

9) Incubate at room temperature (15-30°C) for 10 to 13 minutes.

10) Stop the reaction by adding 150 μl of Stop Solution to each well (maintain the same sequence and time intervals used for ABTS Substrate Solution addition). Plates should be read within two hours.

11) Blank the microelisa reader on air (without strip holder and strips) and read the absorbance of the solution in each well at 450 nm.
RAPID PLASMA REAGIN (RPR)

Intended Use
The Macro-Vue™ RPR (Rapid Plasma Reagin) 18 mm Circle Card Test is a nontreponemal testing procedure for the serologic detection of syphilis.

Product Summary
The Macro-Vue RPR Teardrop Card Test (using finger puncture blood) was the original Card Test and was developed for field use where testing could be performed without laboratory equipment. By incorporating machine rotation, ringed test surfaces, and certain other technical changes, the RPR Circle Card Test was developed for use in large scale testing in public health and clinical laboratories.

The RPR 18 mm Circle Card Test is recommended when venous blood collection is employed and a large volume of serum is available, such as generally prevails in public health and clinical laboratories. When a specimen contains antibody, flocculation occurs with a coagglutination of the carbon particles of the RPR Card antigen, which appear as black clumps against the white background of the plastic-coated card. By contrast, nonreactive specimens appear to have an even light-gray color.

In special situations when nontreponemal test results are needed rapidly and the specimen is collected as EDTA plasma, the RPR 18 mm Circle Card Test can be used if the test is performed within 24 h

Use of Quality Control
Quality control requirements must be performed in accordance with applicable local, state and/or federal regulations or accreditation requirements and your laboratory’s standard Quality Control
procedures. It is recommended that the user refer to pertinent CLSI (formerly NCCLS) guidance and CLIA regulations for appropriate Quality Control practices.

Reagents

The ingredients* of the RPR Card antigen suspension are: 0.003% cardiolipin, 0.020 – 0.022% lecithin, 0.09% cholesterol, 0.0125 M EDTA, 0.01 M Na₂HPO₄, 0.01 M KH₂PO₄, 0.1% thimerosal (preservative), 0.02% charcoal (specially prepared, BD), 10% choline chloride, w/v, and deionized/distilled water.

*Adjusted and/or supplemented as required to meet performance criteria.

Warnings and Precautions

For in vitro Diagnostic Use.

Pathogenic microorganisms, including hepatitis viruses and Human Immunodeficiency Virus, may be present in clinical specimens. “Standard Precautions” and institutional guidelines should be followed in handling all items contaminated with blood and other body fluids.

Warning: RPR Card Antigen Suspension Contains Mercury. Don’t put in trash. Recycle or dispose as hazardous waste.
APPENDIX III

SOURCES OF REAGENTS:

Cell isolation and media:

- Ficoll-Paque
- Media (RPMI, FCS, antibiotic-antimycotic)
- Sterile endotoxin free PBS
- EasySep Monocyte enrichment [www.StemCell.com](http://www.StemCell.com) starter kit, cat#19048 $2300, or #19058 plus magnet.
- RoboSep buffer (cat#20104)

Tissue culture (stims)

- TC plates with lids [www.nucbrand.com](http://www.nucbrand.com) Cat no. 163320 (large box eg. 50)
- Plate sealers (have plenty)
- 200μl pipette tips (sterile, endotoxin free)
- LPS, ssRNA40, endotoxin free water (Invitrogen)

Cytokine assays

[http://wwwbdbiosciences.ca/ptDatabaseList.jsp](http://wwwbdbiosciences.ca/ptDatabaseList.jsp)

- CBA Th1/Th2 kit 1 (2 kits needed) Cat# [550749](#)

Plasma LAL assays


ELISA plates and reader (have local)

Flow cytometry (Tubes and FACS reagents (local), need calibration beads)

- CD14-FITC [555397](#)
CD4-PE  555347
CD8-PerCP  347314
CD38-APC  555462
CD14-FITC  555397
CD80-PE
CD3-PerCP
Lineage Cocktail 1  FITC (CatNo.340546)
CD123 (Anti–IL-3R □) PE* (CatNo.340545)
Anti–HLA-DR PerCP† (Cat No. 347364)
CD11c APC* (Cat No. 340544)
Mouse IgG1 PE (Cat No. 349043)
Mouse IgG2a APC (Cat No. 340473)
APPENDIX IV

QUESTIONNAIRE FORM
Extracted with permission from the major Cohort study in Majengo

MLNO _______________ DATE _______________ MLNO _______________
NAME __________________________________________

DEMOGRAPHIC:
1. Permanent address ______________________________________
   Contact phone no. ______________________________________
2. District ____________________________________________
   Country ____________________________________________
3. Residence (Estate) ____________________________
   Block & Door No. __________________________
4. Year of Birth ____________________________
   Age (yrs) ______________________________________
5. Prostitution (year started) __________
6. Duration of prostitution (yrs) __________

RELATIVES:
7. Do you have any relatives who come to this clinic? (Yes/No) If yes fill out below
   MLNO _______ Name _______________ Relationship __________
   MLNO _______ Name _______________ Relationship __________
   MLNO _______ Name _______________ Relationship __________
   MLNO _______ Name _______________ Relationship __________

REPRODUCTIVE:
8(a). Gravida: _______ 8(b) Are you pregnant now? (Yes/No) LMP ___/___/___
9. No of Children alive_______ Dead_______ Spontaneous Abortion_______ Therapeutic_______
10. Last pregnancy ______ (yr)
10(a) Have you gone through Menopause? (Yes/No)
   (b) Have you had a hysterectomy? (Yes/No)
CONTRACEPTION:

11. Current: (0) None (5) Condom
(1) Oral (6) Female condom
(2) IUCD (7) Diaphragm
(3) Depo (8) Spermicide
(4) TL (9) Other _________

12. Why do you (A), When don’t you (B) use condoms?

<table>
<thead>
<tr>
<th>Why do you; column (A)</th>
<th>Why don’t you use condom; column (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevent AIDS</td>
<td>Can’t get STD/AIDS</td>
</tr>
<tr>
<td>Prevent STDs</td>
<td>Client refuses</td>
</tr>
<tr>
<td>Contraception</td>
<td>Regular client</td>
</tr>
<tr>
<td>Don’t trust client</td>
<td>Trust/well client</td>
</tr>
<tr>
<td>Client request</td>
<td>Not Available</td>
</tr>
<tr>
<td>Client pay less</td>
<td>Client pays more</td>
</tr>
<tr>
<td>On ARV</td>
<td>On ARV</td>
</tr>
<tr>
<td>Other __________</td>
<td>Client will think I have STD/AIDS</td>
</tr>
</tbody>
</table>

13. Why would you stop condom use?

(0) Never stop
(1) If had to buy
(2) If I developed AIDS???
(3) If AIDS has cure
(4) If vaccine available for HIV
(5) When ARVs are available

14. Who supplies condoms?

(0) Yourself (1) Client (2) Both

15. Sex practices with clients/Casual

<table>
<thead>
<tr>
<th>Never</th>
<th>Some (&lt;50%)</th>
<th>Most (&gt;50%)</th>
<th>Always</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaginal sex</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>During menses</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Condom use</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
16. Do you practice any other forms of sex? (Yes/No)
   (1) Oral Sex  (2) Anal Sex  (3) Other (specify)

17. Clients/Day ________ Clients/week ________ Condoms used/week ________

18(a) Number of Regular partners ________ Condom ________ (Y/N)

18(b) Sex practices (Regular partners)

<table>
<thead>
<tr>
<th>Sex practice</th>
<th>Never</th>
<th>Some (&lt;50%)</th>
<th>Most (&gt;50%)</th>
<th>Always</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaginal sex</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>During menses</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Condom use</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

19. Sexual encounters with regular partners per week ________________

20. Have you stopped doing sex work? (Yes/No) If so, when (yr) ________________

21. Douching? (Yes/No)
   When?: (1) Post coital (2) Daily
   With?: (1) Water only (2) Water + soap (3) Water and bleach
   (4) Herbal (5) Other (specify)

22. Did you douche today? (Yes/No)

SEX WORK BREAK

23. Have you taken a break from sex work over the last one year? (Yes/No)

24. If yes, How long was the break? ______________ (Mo)

25. Why did you take the break? (1) Travel (2) On Leave (3) Unwell
   (4) Got a Baby (5) Married (6) Other (specify)

26. Past Medical Problems

   Number of medical injections past 1 year 0 1-4 >4 Why? __________

   Scarification past 1 year (Yes/No) Where? ________ Why? __________

   Hospital admission 1 year (Yes/No) Where? ________ Why? __________

27. In the past 1-year have you had?

   Weight loss > 10kg (Yes/No)
   Diarrhea > 1 month (Yes/No) Duration ________ wks
   Cough > 1-month (Yes/No) Duration ________ wks
Fever/sweats/chills > 1 month (Yes/No) Duration _______ wks
Itchy skin rash (Yes/No) Duration _______ wks
Painful skin rash (Zoster) (Yes/No) Duration _______ wks

28. Have you had any infections during the last two months (genital discharge or ulcer, cold, flu etc.)? (Yes/No) If yes, provide details of problem__________________________

29. Immunization
   Have you had BCG? (Yes/No)

**DRUGS**

30. Have you been on any doctor/pharmacy prescribed medication(s) in the last 6 months (Yes/No)

31. Type and reason: ____________________________________________

32. Are you addicted to any drug? (Yes/No) If Yes which one?__________________________

33. Ever used recreational injection (IV) drugs/narcotics? (Yes/No)

34. Ever been treated for TB: (Yes/No)

35(a) Ever been on ARV’s? (Yes/No)
   If yes when? (__/__/__)
   Where?__________________________

35(b) Are you currently on ARV’s (Yes/No)
   If yes when? (__/__/__)
   Where __________

36. Are you on Septrin? (Yes/No)

**HERBAL MEDICINE:**

37. History of use: (1) Never (2) Occasionally (3) Always

38. If ever used why?__________________________________________

39. **Hx of Allergies** (Yes/No) If yes specify:__________________________
   Check any that has been a problem: (1) Allergic Rhinitis (2) Urticaria (3) Drug Allergies (3) contact dermatitis (4) Asthma Psoriasis
CURRENT MEDICAL PROBLEM

Weight loss  (Yes/No)____ day
Fever  (Yes/No)____ day   Night sweats  (Yes/No)____ day
Chills  (Yes/No)____ day   Fatigue  (Yes/No)____ day
Wt loss  (Yes/No)____ day   Headaches  (Yes/No)____ day
Dizziness  (Yes/No)____ day   Cough  (Yes/No)____ day
Swollen glands  (Yes/No)____ day   Diarrhea  (Yes/No)____ day
Itchy Rash  (Yes/No)____ day   Genital ulcer  (Yes/No)____ day
Backache  (Yes/No)____ day   Genital warts  (Yes/No)____ day
Vag. discharge  (Yes/No)____ day   Dysuria  (Yes/No)____ day
Abdominal Pain  (Yes/No)____ day   Vulva itch  (Yes/No)____ day
Chest pain  (Yes/No)____ day   Joints itch  (Yes/No)____ day
Painful rash  (Yes/No)____ day   Bubo  (Yes/No)____ day
Other

HIV stage  0  I  II  III  IVA  AIDS

Why AIDS? ______________

LABORATORY RESULTS

Serology  Specimen Date: ___________  Test Date:
EIA1(OD): ___  EIA1(Result): ___  EIA2(OD): ___  EIA2(result): ___
RPR Stat: ___  TPHA: ___
TPHA Titre: ___

Bacteriology/Parasitology  Specimen Date: ___________  Test Date:
GC:  Neg  Pos  Gm Stain  Yeast?  Y  N
TV:  Neg  Pos  Gm Stain  Clue cells?  Y  N
Vaginal pH _____
BV  Normal (1-5)  Intermediate (4-6)  BV >=7
51. **FACSCAN** Specimen Date: ___________ Test Date:

52. CD4 Count: _______ CD4%: _______

53. CD8 Count: _______ CD8%: _______

54. WBC: _______ HB: _______ HCT No.: _______ Platelets: _______

55. Lymphocytes: _______ Ratio: ___________

   RBC: ___________
Informed Consent:
This consent form contains information for screening Toll-Like receptors in HIV-1 exposed, but seronegative women commercial sex workers.

Study Title: “Frequency of dendritic cells and cytokine production in HIV exposed commercial Sex workers in Pumwani, Majengo, Nairobi.”

Rationale: Exposure to HIV-1 have been shown in many studies including, the Research in Pumwani, Majengo that HIV-1 resistance exist. The reasons behind this resistance are probably due to ability of some individuals producing effective Cytotoxic T Lymphocytes, IgA antibodies and perhaps genetic variations in chemokine receptors. These factors must be working in concert with immediate and effective recognition of pathogens through toll like receptors, an event that if studied may lead to understanding the basis of HIV-1 resistance.

This study therefore, will try to demonstrate cytokine production in whole blood obtained from HIV-1 exposed individuals with the ultimate goal of correlating the finding with either type 1 or type 2 immunity and possible eventual design of HIV-1 vaccine and possibly prevent cases of Acquired Immunodeficiency Syndrome (AIDS) which so far is the leading cause of death in Sub-Saharan Africa. To help understand the events following exposure, the study will recruit female volunteers individuals aged between 18 years and above who must provide consent to participate in the study following an explanation during counseling a session. Nine mLs of whole blood samples will be obtained from willing volunteers. Part of the blood will be used to screen for HIV infection and the other portion will be used in the research study for screening for cytokines and expression of Toll-like receptors. Participation in this study is voluntary and refusal to participate will not in any way jeopardize the chances of any individual from obtaining medical services offered in the Majengo clinic.

Contact Numbers: Department of Medical Microbiology, University of Nairobi, telephone numbers 020-2717694, 2714613.
APPENDIX VI

Screening Consent Form:

I, ___________________________ confirm that, I have been explained to the nature of the screening exercise and I confirm that I fully understand the nature of the study. I have also had the opportunity to ask questions about the study and I feel that I have received satisfactory answers to my questions. I am aware that in order to be accepted in the study, I will have to undergo an HIV test before being recruited in the study. I understand that my consent is entirely voluntary and that it does not mean that I have to join the study against my willingness.

Participant:
Print name:............................. Signature...........................................

_ _ / _ _ / _ _

Date: DD/MMM/YYYY Time ___ : ___ (24 hours)

Person obtaining consent:
I have explained the nature, demands and foreseeable risks of the screening process to the participant.

Print name........................................ Signature...........................................

_ _ / _ _ / _ _

Date: DD/MMM/YYYY Time ___ : ___ (24 hours)
Ref: KNH-ERC/ 01/ 4273

3rd May 2007

Mr. Onyango J. I Obila
Dept. of Medical Microbiology,
School of Medicine
University of Nairobi

Dear Mr. Onyango

RESEARCH PROPOSAL: "ROLE OF TOLL LIKE RECEPTORS AND CYTOKINE PRODUCTION IN HIV EXPOSED COMMERCIAL SEX WORKERS IN PUMWANI, NAIROBI" (P166/8/2006)

This is to inform you that the Kenyatta National Hospital Ethics and Research Committee has reviewed and approved your revised research proposal for the period 3rd May 2007 – 2nd May 2008.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimen must also be obtained from KNH-ERC for each batch.

On behalf of the Committee, I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

PROF A N GUANTAI
SECRETARY, KNH-ERC

c.c.   Prof. K.M. Bhatt, Chairperson, KNH-ERC
The Deputy Director CS, KNH
The Dean, School of Medicine, UON
The Chairman, Dept. of Med. Microbiology, UON
Supervisor: Dr. Richard Lester, Dept. of Med. Microbiology, UON
IAVI gratefully acknowledges the generous support provided by the following major donors:


*Founding donors of IAVI

And many other generous individuals from around the world

As of June 2009
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