INNATE IMMUNE RESPONSES AMONG HIV-1 HIGHLY EXPOSED SERONEGATIVE (HESN) INDIVIDUALS TO A LIVE ATTENUATED INFLUENZA VACCINE AS A MODEL FOR MUCOSAL VIRAL INFECTION

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In memory of beloved the late Raphaël Obaro Onyango

Missing you Raph

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
ART	Anti-Retroviral Therapy
APC	Antigen Presenting Cells
CBA	Cytometric Bead Array
CFA	Complete Freund's Adjuvant
CTLs	Cytotoxic T lymphocytes
CCR5	Chemokine co-receptor 5
CXCR4	C-X-C motif receptor 4
CVL	cervico-vaginal lavage
CSWs	Commercial Sex Workers
CpG ODN	Cytosine phosphate Guanosine OligoDeoxyNucleotides
DC	Dendritic Cells
DC-SIGN	Dendritic cell- specific intercellular adhesion molecule 3- grabbing nonintegrin
ELISA	Enzyme linked immunosorbent assay
HESN	highly exposed seronegative
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-R	HIV-Resistant
HIV-S	HIV-Susceptible

HPV	Human papilloma virus
HSV	Herpes simplex virus
HSP 40	Heat surface protein 40
IFN	Interferon
IRAK	Interleukin 1 receptor- associated kinase
IRF	Interferon regulating factor
LPS	Lipopolysaccharide
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa beta
NK	Natural killer cells
NOD	Nucleotide binding and oligomerization domain
NLRs	Nucleotide binding and oligomerization domain (NOD)-like receptors
PAMP	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PRRs	Pattern recognition receptors
RIG-I	Retinoid acid-inducible gene I
RLRs	Retinoid acid-inducible gene I (RIG-I)-like receptors
RPMI	Roswell park memorial institute medium

SEB	Staphylococcus Enterotoxin B
SIV	Simian Immunodeficiency Virus
siRNA	Small interfering RNA
SLP-1	Secretory leukocyte protease inhibitor
ssRNA	Single strand RNA
STIs	Sexually Transmitted Infections
TAB	TAK1-binding protein
TAK1	Transforming growth factor (TGF)- β activated kinase
Th1/Th2	T helper cell one/ T helper cell two
TIR	Toll/Interleukin one receptor
TICAM-1	Toll-like receptor adaptor molecule 1
TNF	Tumor Necrosis Factor
TLR	Toll-like receptor
TIRAP	TIR domain containing adapter protein
TRAF6	TNF receptor associated factor 6
TRIF	TIR domain-containing adapter inducing IFN-β
TRAM	TRIF-related adapter molecule
TRAP	Translocon-associated protein
X4/R5	HIV chemokine co-receptor CXCR4 (X4) and CCR5 (R5)
µl	microlitre

ABSTRACT

To better understand the correlates of mucosal protection against HIV among highly exposed seronegative (HESN) which is largely unknown, a viral challenge system to a model viral infection was used. The model consisted of a live attenuated *Influenza* vaccine administered intranasally, to stimulate the mucosal system. The study subjects were HIV-S and HIV-R, plasma, PBMCs and CVL samples were collected from study subjects prior to (day 0, baseline) and following vaccination at day 1, 7 and 30. PBMCs isolated from the subjects were stimulated with TLR3 (Poly I:C) and TLR4 (LPS), TLR9 (CpG ODN), TLR7/8 (ssRNA40) and Brisbane *Influenza* virus vaccine strain. Cytokine and chemokine production in supernatants from stimulated cells was measured using the multiplex assay on Luminex while in plasma and CVL Cytometric bead array (CBA) kit on flow cytometer (LSR II) was used. Following stimulation with TLR 3 and 4 ligands, HIV-R had significantly higher IL-6 levels at baseline compared to HIV-S ($p=0.0317$). Also, significantly higher levels in IL-2 in HIV-R at day 0 ($p=0.0317$) and 30 ($p=0.0079$), as well as high IFN-γ responses at day 0 ($p=0.0079$) and 7 ($p=0.0317$).

Stimulation of PBMCs with TLR 7, 8 and 9 ligands also led to a significant increase in IL-2 production at baseline in HIV-R compared with HIV-S ($p=0.0159$). Also significant increase in IP-10 at day 7 ($p=0.00952$) and 30 ($p=0.0317$) in HIV-R compared to HIV-S was observed. The data obtained from this study suggests that stimulation of PBMCs with TLRs ligands in HIV-R individuals resulted in a more robust release of immunologic factors which can influence the induction of stronger adaptive antiviral immune responses. This may represent a virus exposure induced innate immune protective response against HIV-1 infection.

CHAPTER ONE

1.0 INTRODUCTION

According to World Health Organization (WHO) and United Nations Program on HIV and AIDS (UNAIDS), HIV and AIDS epidemic update December 2010, an estimated 33.3 million [31.4 million - 35.3 million] people were living with HIV. It is estimated that 1.8 million [1.6 million - 2.1 million] deaths due to AIDS-related illnesses occurred worldwide in 2009 (UNAIDS, 2010). The report estimates that 1.9 million [1.6 million - 2.2 million] new infections occur in Sub-Saharan Africa and, with an estimated 22.5 million [20.9 million - 24.2 million] people living with HIV, women and girls continue to be affected disproportionately by HIV (UNAIDS, 2009; UNAIDS, 2010). HIV transmission occurs through sexual intercourse, intravenous drug use, rare occupational transmission and mother to child transmission at birth or through breastfeeding (Awusabo and John, 1999; Simonsen *et al.,* 1988; UNAIDS, 2010).

Although the earliest cases of AIDS in the USA were predominantly found among homosexual men, the major route of transmission is now heterosexual contact which accounts for 85% of all HIV infection, (de Vincenzi, 1994; Simonsen *et al.,* 1988); with the most risky mode of transmission being unprotected receptive anal sex (Hoffmann 2005; Vivianna *et al.*, 2006). It is still not clear whether HIV sexual transmission is mediated mainly by cell-free virus or by infected cells (Ritola *et al.*, 2004). According to UNAIDS report 2010, the incidence of new HIV infections globally declined by 19% between 1999 and 2009. In 33 countries, the HIV incidence has fallen by more than 25%

between; 22 of these countries are in sub-Saharan Africa (UNAIDS, 2010). The decline in number of new infections has been attributed to prevention efforts. For example behavior change campaigns among young people is the most important factor accounting for these encouraging declines in new HIV infections in many countries (UNAIDS, 2010). Also decline in the annual number of new HIV infections has been attributed in part to availability of antiretroviral therapy, postponement of sexual debut, prevention of mother to child transmission, reduction in casual sexual relationship, and consistent and correct condom use (UNAIDS, 2010). In developing nations, single dose anti-retroviral treatment (ART) of HIV-infected pregnant women has proven remarkably effective in preventing maternal–foetal transmission of the virus but this has been held back by inadequate access to antenatal and postnatal services (UNAIDS, 2010). Increased longevity in HIV infected individuals has been attributed to improved access to HIV treatment; the use of multi-drug therapeutic regimens [highly active anti-retroviral therapy (HAART)] has resulted in dramatic improvements in the lifespan of HIV infected patients compared to that of individuals who were infected prior to the availability of HAART (UNAIDS, 2010). Substantial efforts are now focused on development of preventive vaccines and microbicides to stop the continued spread of the infection.

To date billions of dollars have been allocated for HIV vaccine research and numerous HIV-1 vaccines have been developed over the last three decades, but an effective HIV-1 vaccine that can be used for prophylactic or therapeutic (treatment) purposes in humans has not yet been developed. The search for an effective vaccine for example Thai RV144 has encountered unprecedented scientific challenges; failures and limited successes have highlighted the gaps in our knowledge with regard to fundamental immunity against

HIV-1 infection. This has raised a whole new set of important questions that require answers to develop successful vaccine in the future. A deeper understanding of human immune responses is required, in order to design a more effective HIV-1 vaccine (Danushka *et al.*, 2010).

1.1 HIV Infection

The CD4+ T-cell populations are the main targets for HIV infection and the primary immunological pathology is reflected by a progressive depletion of CD4+ T cells in peripheral blood as HIV infection progresses (Moss *et al.*, 1988; Philips *et al.*, 1989) (Figure 1.0). In addition, certain HIV strains will infect monocytes and other cells that have CD4 on their surface. In a normal immune system, CD4+ and CD8+ T cells act together to control infection by pathogens in the body. During HIV infection, the responses from both CD4+ and CD8+ T cells diminish over time, thus promoting the development of opportunistic infection (Young, 2003). Measurement of CD4+ T-cell levels is a routine clinical test used to help evaluate and track the progression of HIV infection and disease as well as response to treatment. CD4+ T-cell depletion also forms part of the defining criteria for HIV infection progression to AIDS (Figure 1.0) (Pantaleo *et al.*, 1996; Stanley *et al.*, 1996). The establishment of infection is dependent on the target cell expression of CD4 receptor and a chemokine co-receptor (CCR).

Although a variety of CCRs can serve as co-receptors *in vitro*, CCR5 and CXCR4 have been documented as the main co-receptors used by HIV-1 *in vivo*, with CCR5 nearly always being the initial target co-receptor for naturally transmitted virus (Alkhatib *et al.*,

1997; Berger *et al.*, 1999). The discovery that CXCR4 and CCR5 serve as coreceptors for HIV-1 on T cells and macrophages, respectively, explained why some strains of HIV-1 preferentially infect T cells (T-tropic strains) while others prefer macrophages (M-tropic strains) (Kuby, 2007).

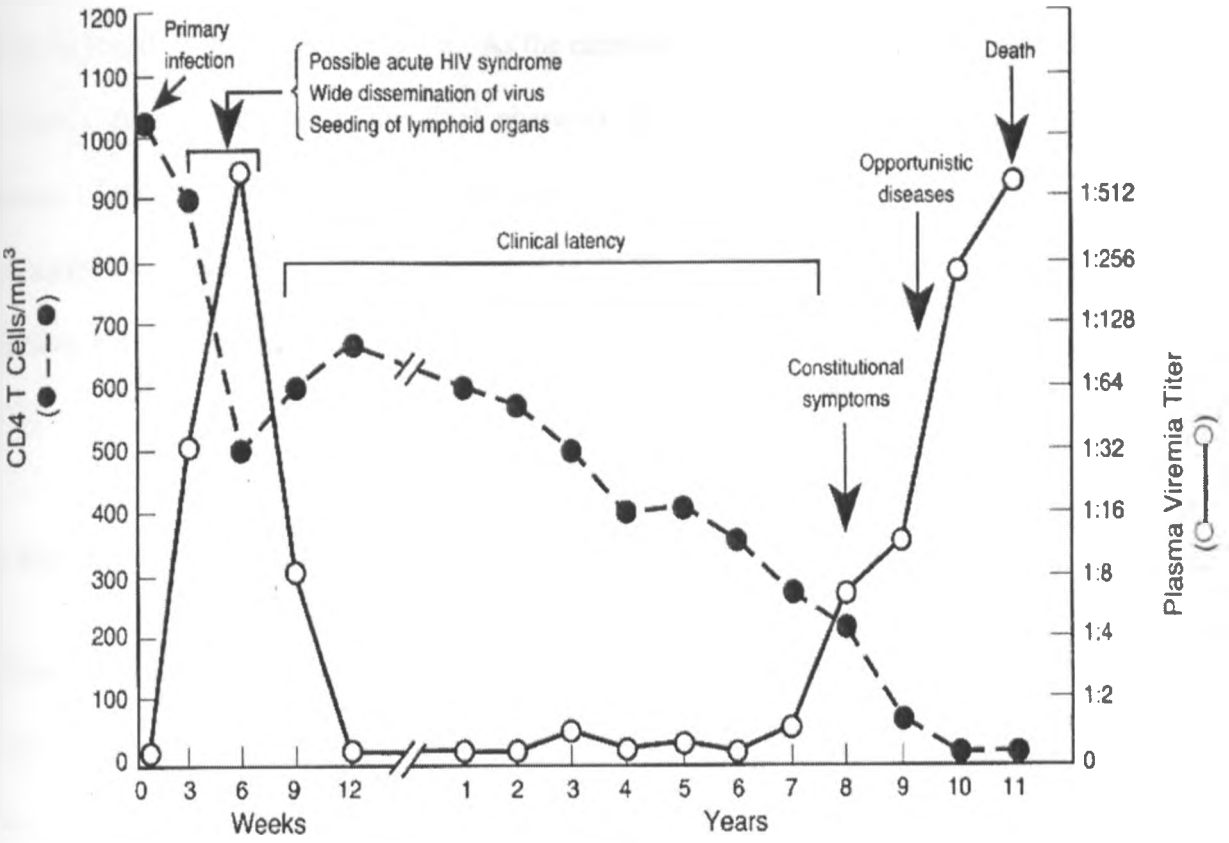


Figure 1: Typical change in CD4+ T-cells count and HIV viral loads over time
(adapted from Fauci, Pantaleo, Stanley and Weissman, 1996)

HIV infection basically occurs in phases: The **acute phase** this is the initial incubation period upon infection, which usually begins 2-4 weeks post-infection, is characterized by patients experiencing temporary flu-like symptoms (fever, rash, lymphadenopathy,sore throat, myalgia). A high viral load is characteristic of acute infection which falls to a lower baseline concentration as anti-HIV CD8+ cytotoxic T cell (CTL) levels rise. Viral levels in the blood drop and reach a "set point". This viral "set point" or baseline level can remain constant for years throughout the clinical latent stage, an **asymptomatic phase** lasting 7-11 years on average. The late stage of HIV infection is characterized by a rapid decline in CD4+ T cell levels due to direct viral destruction of the cells, targeting

of the infected host cells by CD8+ CTLs and apoptosis of the infected T cells this phase varies in length from person to person. As the numbers of CD4+ cells decline below 200 per mm^3, the patient reaches the final phase of HIV infection, termed **AIDS**. In the absence of treatment at this phase patients can easily acquire opportunistic infections and eventually succumb to these AIDS related illnesses. Most common opportunistic infection includes tuberculosis, (Levinson, 2008; Mahungu *et al.*, 2009; Holmes *et al.*, 2003)

1.2 Resistance to HIV Infection

Although all humans encode the CCR5 and CXCR4 receptors that are required for HIV to gain entry into cells, many other factors determines an individual's resistance and susceptibility to infection. Several studies have shown that human populations differ in susceptibility to infection and the course of disease progression caused by microbial agents. Altered susceptibility to infection and disease has been observed in malaria, leprosy, pulmonary tuberculosis and hepatitis B virus (Easterbrook *et al.*, 1999; Hill 1998; Singh et *al.*, 1983; Thursz *et al.*, 1995; Todd *et al.*, 1990). Of those exposed to an infection, not all become infected, and among infected individuals, not all develop disease. It is expected that such variability in susceptibility to infection and disease would apply to infection and disease with HIV-1. Considerable documented evidence has emerged that this is the case (Plummer *et al.*, 1999; Ball *et al.*, 2007; Fowke *et al.*, 1996; Hirbod *et al.*, 2007; Kaul *et al.*, 1999; Kaul *et al.*, 2000; Clerici *et al.*, 1994; Pinto *et a*l., 1995).

The natural course of HIV-1 infection shows considerable inter-individual variability, with some individuals progressing to AIDS rapidly following primary infection, (fast progressors) while other individuals who are infected with HIV, but control the infection without antiretroviral therapy e.g. elite controllers and long-term non-progressors (Pantaleo and Fauci, 1996). Another group of individuals that are HIV exposed seronegative (HESN) also referred to as exposed uninfected (EU) who remain uninfected even after repeated exposure to virus (Paras *et al.*, 2008). The use of HIV-exposed seronegative (HESN) to describe individuals who are exposed to HIV yet remain seronegative was proposed at a workshop on Exposed and Unifected to unify the terminology in the field (Meyers and Fowke, 2010). This group includes discordant couples who practice unprotected sexual intercourse, healthcare workers receiving needle-stick injuries, commercial sex workers (CSWs), hemophiliacs receiving HIV-contaminated blood products, and seronegative infants of HIV-infected mothers who have been exposed *intra partum, in utero*, and during breastfeeding (Fowke *et al.*, 1996; Kaul *et al.*, 2001; Kaul *et al.*,2004; McNicholl *et al.*, 2004; Goh *et al.*,1999; Farquhar *et al.*,2004; Pinto *et al.*, 1995; Clerici *et al.*,1994; Kuhn *et al.*, 2001).

In Nairobi, Kenya, a group of CSWs have been identified who, despite intense repeated sexual exposures to HIV-1, have remained HIV-1 uninfected for periods of >7 years. These women can epidemiologically be defined as resistant to HIV-1 infection based on the following definition; those who have been enrolled in the cohort for at least 7 years, are actively engaged in sex work and have remained seronegative by both PCR and ELISA (Fowke *et al.*, 1996). At the present time the definition has been reviewed to meet changing HIV prevalence. The new definition being CSWs who had been followed up for

>7 years are defined as HIV-R (University of Manitoba/University of Nairobi collaborative HIV Research group). These individuals remain healthy with no clinical evidence of HIV-1 infection and have normal CD4 T cell counts. Studies have shown that decreased susceptibility to infection within this cohort is not due to known chemokine receptor polymorphisms, differences in sexual behavior, condom use, presence of other sexually transmitted infections or a chance phenomenon as indicated by survival modeling of the time to HIV-1 seroconversion (Figure 2) (Fowke *et al.*, 1996). In the real sense all women are equally susceptible to infection; statistically these seronegative women would have been infected with HIV-1 (Anzala *et al.*, 1998; Fowke *et al.*, 1998; Kaul *et al.*, 1999; Fowke *et al.*, 1996).

Numerous studies that have tried to identify correlates of protection against HIV-1 infection in humans have been conducted in this cohort which began in 1985 and continues through present time (Fowke *et al*, 1996). The correlates of protection that confer this unique status to a tiny minority of HIV exposed individuals remain a subject of intense interest. Various mechanisms have been used to explain resistance to HIV infection. Both genetic and immunologic factors have provided clues and help in explaining mechanisms of HIV resistance. Genetically, resistance has been associated with human leucocyte antigen (HLA) polymorphism (Dorak *et al.*, 2004; MacDonald *et al.*, 2000; MacDonald *et al.*, 2001), chemokine and chemokine receptor polymorphism such as CCR5-Δ32 (O'Brien *et al.*, 2000; Marmor *et al.*, 2001; Philpott *et al.*, 2003). Immunologically, both innate (presence of innate immune sensors TLRs, innate immune factors) and adaptive immunity (cellular immunity i.e. CTLs etc) systems have been important in resistance to HIV infection (Kaul *et al.*, 2001; Rowland-Jones *et al.*, 2001).

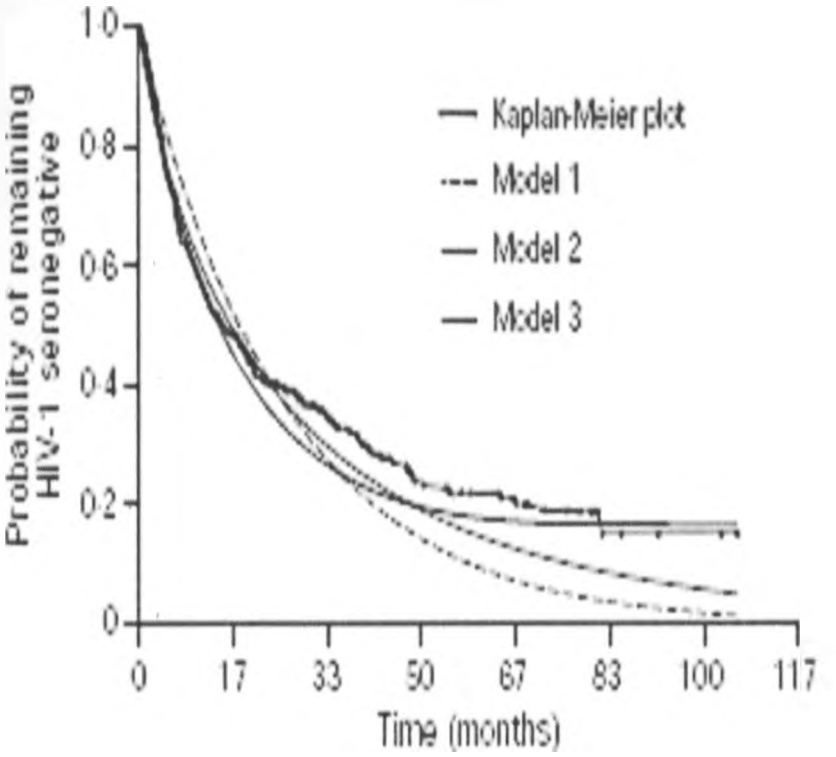


Figure 2: Survival modeling for time HIV-1 seroconversion

Model 1 expected time to seroconversion if seronegative survival time is exponentially distributed; model 2 expected time to seroconversion under a Weibull distribution; model 3 expected time to seroconversion from a mixture model (Fowke *et al.*, 1996).

1.2.1 Immunological correlates of HIV Resistance

There is increasing evidence that HIV specific CD8+ T lymphocytes are key players in protective immunity mediating the relative control of HIV-1 infection (Kaul *et al.*, 2001). Cytotoxic T-lymphocytes (CTLs) are lymphocytes that kill other ("target") cells, and have a vital function in monitoring the cells of the body and eliminating any that display foreign antigen, such as virus-infected cells, tumor cells, and cells of a foreign tissue graft (Kuby 2007). HIV-1 specific CTL responses have been found in HESN individuals (Rowland-Jones *et al.*, 2001; Kaul *et al.*, 2001) targeting epitopes defined in HIV clade B in seronegative individuals (Rowland-Jones *et al.*, 1998). CTLs response thought to be maintained by constant ongoing exposure to HIV has been shown to play a role in resistance to HIV infection (Kaul *et al.*, 2001).

Within this cohort, a subset of women who did seroconvert were found to have had lapses in sex work where seroconversion was attributed to loss of HIV-1 specific CD8+ responses, suggesting that constant boosting of immune memory or frequent exposure to HIV is needed to maintain a protective CTL response (Kaul *et al.*, 2001). Despite the evidence for CTL involvement in resistance to HIV acquisition, the available data does not show causation, leaving the possibility that CTLs are surrogate markers of another, yet undefined mechanism. In this cohort resistance to HIV-1 infection has been associated with presence of HIV-1 specific mucosal IgA (Kaul *et al.*, 1999), elevated RANTES (regulated on activation normal T-cell expressed and secreted) levels (Iqbal *et al.*, 2005) and hyporesponsive IL-4 production (Trivedi *et al.*, 2001) in the genital mucosa.

1.2.2 Genetic correlates of HIV Resistance

Host genetic variability plays a major role in determining individual susceptibility or resistance to potentially pathogenic infections. Several reports have been published to associate host genetics and susceptibility to HIV infection. For example resistance to HIV and slower disease progression have been associated with certain specific HLA (Fowke *et al.*, 1998; MacDonald *et al.*, 2000; MacDonald *et al.*, 2001). HLA are a group of genes belonging to the human major histocompatibility complex (MHC) and play an important role in activating the immune system to response to pathogens differentiating between 'self' and 'non-self' (Kuby, 2007). Resistance has been linked to specific HLA class I and II alleles (MacDonald *et al.*, 2000). Specifically, the HLA A2/6802 supertype family (A*0202, A*0205, A*0214, and A*6802), and the allele DRB1*01, have been associated with reduced risk of seroconversion. An important role for HLA has also been shown in a study that was done on mother and infants where it was found that viral transmission was more likely to occur with greater HLA class I concordance between mother and child (MacDonald *et al.*, 1998).

Polymorphism in the genes encoding chemokines and chemokine receptors has been observed to be important for both susceptibility to HIV infection and disease progression. CCR5-Δ32 is a polymorphism in the gene encoding the CCR5 chemokine receptor in which a 32-base pair region has been deleted (O'Brien *et al.*, 2000 Dean *et al.*, 1996; Michael *et al.*, 1997; Zimmerman *et al.*, 1997). This leads to a non functional CCR5 receptor hence HIV cannot bind to CCR5 protein to enter the cell this leads to individuals who are resistance to HIV infection by R5 viruses progress more slowly to AIDS than

individuals with normal CCR5 but are susceptible to X4 usually 2-4 years delayed progression (O'Brien *et al.*, 2000 Dean *et al.*, 1996; Michael *et al.*, 1997; Zimmerman *et al.*, 1997; Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996). The frequency of the CCR5-Δ32 allele in northern European Caucasians has been shown to be 5–15% whereas it is completely absent in Africans. This has also been shown to be absent in HIV-R women from the in Pumwani cohort and among Asians it varies between 0% and 12% (O'Brien *et al.*, 2000; Martinson *et al.* 1997; Michael *et al.* 1997; Libert *et al.* 1998; Stephens *et al.* 1998; Fowke *et al.*, 1998). However it stills remains unclear whether these responses (immunological) are responsible for protection in HESN individuals or they are merely a marker for exposure or are germ-line encoded (genetic).

1.2.3 Innate immunity and HIV resistance

Increasing evidence suggests that innate responses are key determinants of the outcome of HIV infection, influencing critical events in the earliest stages of infection including the efficiency of mucosal HIV transmission. To help clarify the role of innate immunity in HIV infection, researchers are studying different groups of individuals HESN (Biasin *et al.*, 2010). Some scientists have theorized that innate immunity may explain the ability of HESN to avoid HIV infection. A recent study indicates that mucosal innate immune factors are capable of modulating HIV-1 infection *in vitro* (Cummins *et al.*, 2006). For example a number of innate mucosal factors that are secreted in high levels into the lumen of the female genital tract of HESN have been found to have anti-HIV activity *in vitro*, these include SLP-I (Secretory leukocyte protease inhibitor), Trappin-2, α-defensins, and serpin proteases (Iqbal *et al.*, 2008; Iqbal *et al.*, 2009; Burgener *et al.*,

2008). SLPI has been observed in oral and genital tract secretions appear to inhibit *in vitro* infection with HIV in several studies (McNeely *et al.*, 1995; 1996). Of importance is the activation of innate immune sensors - Toll Like Receptors (TLRs) which represent the first line of defense against invading pathogens. Reports have described the possible role of TLRs in innate immunity and susceptibility to HIV infection (Biasin *et al.*, 2010; Montoya *et al.*, 2006; Kebba *et al.*, 2005; Barton 2007; Scott-Algara *et al.*, 2003).

During an infection, one of the principal challenges for the host is to detect the invading pathogen and activate a rapid defensive response which is paramount to the survival of the host. Rapid innate immune defenses against a variety of infections often involve the recognition of invading pathogens by specific pattern recognition receptors (PRRs) recently attributed to the family of TLRs (Akira *et al.*, 2001). TLRs represent the most studied family of PRRs. However, growing evidence suggests that other non-TLR families of innate receptors, such as C-type lectin-like receptors, nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), and retinoid acid-inducible gene I (RIG-I)-like receptor (RLRs), also play critical roles in innate sensing of pathogens and induction of inflammatory responses (Pulendran and Ahmed, 2006; Saito and Gale, 2007; Fritz *et al* ., 2006).

Mucosal surfaces serve as portals of entry for pathogens and exposure to HIV is almost exclusively through the genital mucosa (Akiko, 2010). A variety of innate immune cells at the mucosal surface express several members of the TLR family receptors, that are central to innate immune defenses (Jorunn *et al.*, 2006; Akira *et al.*, 2001; Akira *et al.*, 2006). The expression and activation of TLRs contribute to host defense against infection

by triggering the release of cytokines, chemokines and the differentiation of immature to mature dendritic cells, enabling the innate immune system to instruct the adaptive immune response (Ghosh and Alkan, 2006; Beutler *et al.*,2006; Akira *et al.*, 2006). Because of the diversity and ability of TLRs to modulate adaptive response, they are seen to have potential usefulness as immunological adjuvant for HIV vaccine particularly in the context of peptide, protein, and DNA vaccines (Manicassamy and Pulendran, 2009). For example potent adjuvants such as Complete Freund's Adjuvant (CFA) and bacille–Calmette-Guérin (BCG) are sensed by multiple TLRs (Ishii and Akira 2007). The live attenuated yellow fever (YF-17D) vaccine activates multiple innate immune sensors at least four different TLRs and RIG-I (Querec *et al.*, 2006; 2009).

In this study, the aim was to understand the earliest events in mucosal responses to viral infection (Flumist). An *in vivo* viral challenge system model was used, consisting of measured dosage of a live attenuated *Influenza* vaccine, which was administered intranasally. The advantages of this model are: that it is safe, it is a live mucosally administered immune challenge that we could assess the pre and post-exposure responses. *Influenza* is a major respiratory pathogen causing annual outbreaks, which results in a considerable economic burden due to loss of productivity, hospitalization and cost of treatment of the disease (Dong *et al.*, 2009; Glezen *et al.*, 1980). Annual vaccination is the main method of *Influenza* prophylaxis. Several studies have reported the ability of attenuated *Influenza* vaccine to induce mucosal immune responses compared to trivalent inactivated vaccine which is administered intramuscularly.

Peripheral blood mononuclear cells (PBMCs) collected from study participants were stimulated with different TLR ligands and cytokine /chemokine production in the cellular supernatants were quantified using Luminex to determine if flu vaccination affects TLR responsiveness. Responses to TLR stimulation pre and post vaccination among the different groups of people (HIV resistant and susceptible) were compared. Cytokines and chemokines in plasma and CVL were quantified using Cytometric Bead Array analysis (CBA) using Flow cytometry (LSR II from BD), to determine if innate responses differ between different groups (HIV Resistant and susceptible). It is hoped that this model will improve our understanding of the early events that occur at the site of initial response to mucosal infection. This study will also provide information on how natural immunity limits the early events in HIV-1 infection and contribute to resistance to HIV infection which can provide critical information towards HIV vaccine and microbicide development.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Innate Immunity and Toll Like Receptors (TLRs)

Immunity is the state of protection from infectious disease and has both a non specific (innate) and specific component (acquired immunity). These two aspects of the immune system interact to shape the overall response to an invading microbe. The innate immune system represents an ancient system of host defense with striking structural and functional similarities among various organisms such as insects and humans (Kuby, 2007). The innate immune system is known to play an important role in the activation of an immune response that activates antigen specific acquired immunity (Beutler and Rietschel, 2003).

The recognition of invading microorganisms is paramount to the survival of the host, and the innate immune system has evolved as the first line of defense. Most components of innate immunity are present before the onset of infection and constitute a set of disease-resistance mechanisms that are not specific to a particular pathogen but rather include cellular and molecular components that recognize classes of molecules peculiar to frequently encountered pathogens (Kuby, 2007). Innate immunity can be seen to comprise four types of defensive barriers: anatomic, physiologic, phagocytic, and inflammatory. The innate immune system is composed of a variety of cell types, including dendritic cells (DCs), macrophages, natural killer cells (NK), mast cells, neutrophils, B cells, γδ T cells and non immune cells (e.g fibroblasts and epithelial cells), as well as many soluble components such as cytokines, chemokines and lectin-binding

proteins MBLs (Mannose binding lectin) (Alfano and Poli, 2005; Levy, 2001). The identification of invading pathogen is by three families of sensing molecules collectively termed as PRR which include 1) Toll-like receptors (TLRs), 2) retinoid acid-inducible gene I (RIG-I)-like receptor (RLRs) and 3) cytosolic nucleic acid sensors and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) mediate immune response and function as a bridge between the innate and adaptive responses (Taro and Shizuo, 2009).

In 1989, Charles Janeway proposed that innate immune recognition relies on a set of germ-line encoded, non-clonal receptors (Janeway, 1989). These have evolved to detect specific and broad conserved structures shared by a large group of microorganisms referred to as pathogen-associated molecular patterns (PAMPs) (Taro and Shizuo, 2009). PAMPs are essential for replication and/or survival of the microorganism, and were not believed to be ever expressed by the host. Recognition of PAMPs allows the innate immune system to distinguish infectious non-self (pathogen) from non infectious self (Janeway, 1989; Medzhitov, 2002). TLRs represent evolutionary conserved class of PRRs (Janeway, 1989; Medzhitov, 2002), they are type I transmembrane receptors characterized by leucine-rich repeats (LRRs) in the extracellular portion and an intracellular Toll/interleukin (IL)-1 receptor (TIR) domain, which is homologous to the intracellular domain of IL-1 receptor family members (Medzhitov, 2001). TLR plays an important role in recognizing specific microbial components derived from pathogens, including bacteria, fungi, protozoa, and viruses (Janeway, 1989; Medzhitov, 2002).

Many insights into innate immunity have been obtained from studies in *Drosophila*. In mid-1990s *Drosophila* protein Toll was shown to be critical for defending the flies against fungal infections; this observation opened the way for the subsequent description of similar proteins, called Toll-like receptors (TLRs) in mammalian cells (Lemaitre *et al.*, 1996). To date 13 distinct mammal TLRs have been identified, 10 of which function in humans (TLRs 1–10) and 11 in mice (TLR 1-7, TLR 9, TLR 11-13) (Ghosh *et al.*, 2006; Beutler *et al.*, 2006; Uematsu *et al.*, 2006). TLRs 7, 8, and 9 appear to be "phylogenetic neighbors", they reside in the endoplasmic reticulum membrane with their ligands binding ecto domain pointing toward the lumen of these organelles (Du *et al.*, 2000).

TLRs are the best characterized and most studied family of PRRs (Cook *et al.*, 2003; Iwasaki and Medzhitov, 2004; Staros, 2005), able to trigger activation of adaptive immune responses of several effector classes, including immunoglobulin M (IgM), IgG, and IgA antibody responses; T helper cell 1 (TH1) and TH17 CD4+ T cell responses; and CD8+ T cell responses (Akiko and Medzhitov, 2010). Recognition of ligands by TLRs leads to a series of signaling events including the activation of nuclear factor-kappa B (NF-kB) a transcription factor that is involved in the expression of many inflammatory cytokines, chemokines, co-stimulatory proteins, and adhesion molecules (Medzhitov *et al.*, 1997; Chow *et al.*, 1999). Table 1 below gives a summary of Human TLRs, their ligands and location of TLR in human cell (Akira *et al.*, 2001).

Table 1: Human TLRs, their ligands and location of TLRs in cells (Akira *et al.*, 2001; Were, 2009)

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

2.1.1 Roles of TLRs in pathogen recognition. Toll-like receptor ligands

TLRs can be divided into subfamilies primarily recognizing similar PAMPs; TLR1, TLR2, TLR4, and TLR6 recognize lipid structures, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids. Most TLRs are predominantly expressed on the cell surface (TLR1, 2, 4, 5, 6, and 10) and mainly recognize hydrophobic molecules unique to microbes and are not produced by the host. In contrast others are expressed in endosomal compartments (TLR3, 7, 8, and 9) (Janeway, 1989; Janeway and Medzhitov, 2002).

Toll-like receptor 2 recognizes a wide range of PAMPs derived from various pathogens, including bacteria, fungi, parasites and viruses (Akira *et al.*, 2006). These ligands include triacyl lipopeptides from gram positive bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, porin from *Neisseria*, lipoarabinomannan from mycobacteria, zymosan (containing β-glucan, mannans, chitin, lipid and protein) from fungi, *Trypanosoma* GPI-mucin (tGPI-mucin) and hemagglutinin protein from measles virus. TLR2 generally forms a heterodimer with TLR1 or TLR6 (Taro and Shizuo, 2009).

It is clear that TLR3 responds to the artificial dsRNA mimic, polyiosinic-polycytidylic acid (polyI:C), a synthetic analogue of double stranded (ds) RNA widely used to mimic viral infection when it is provided extracellularly. Double-stranded RNA is known to be generated by RNA viruses during infection as a replication intermediate for ssRNA viruses or as a by-product of symmetrical transcription in DNA viruses (Akira *et.al.*, 2006). While TLR3 is clearly a receptor for dsRNA/poly (I:C), its particular role in antiviral innate immunity *in vivo* is as yet undefined (Bowie, 2007).

TLR4 is a pattern recognition receptor involved in the innate immune response to various microorganisms and other exogenous and endogenous stress factors. TLR4 appears to be the major receptor for bacterial lipopolysaccharide (LPS) or endotoxin produced by all gram negative bacteria which is expressed on the surface of myeloid cells and is the best characterized of the human TLRs (Dabbagh *et al.*, 2002). TLR4 ligands promote phagocytosis of virus-infected cells (Mitchell *et al.*, 2008). Interestingly, both TLR2 and TLR4 are involved in the recognition of fungal pathogens such as *C. albicans* or *Aspergillus fumigatus*, suggesting that the same pathogens may activate distinct signalling pathways simultaneously (Tada *et al.*, 2002).

It is now clear that TLR7 and TLR8 recognize single stranded RNA (ssRNA) and induce innate immune responses to ssRNA-viruses. TLR7 and TLR8 are highly homologous and play important roles in the recognition of viruses. TLR7 was originally shown to be responsible for mediating the antiviral effects induced following delivery of imidazoquinoline compound such as imiquimod and resiquimod (R-848) and guanine analogues such as loxoribine, which have anti-viral and anti-tumor properties (Hemmi *et al.*, 2002). This is mainly by inducing IFN-α and other cytokines, which are largely responsible for its acute antiviral and antitumor effects. TLR7 ligands have been shown to increase viral clearance (Mitchell *et al.*, 2008). Guanosine-rich and uridine-rich ssRNA derived from HIV is recognized by TLR7/8 and stimulate DCs and macrophages to secrete IFN-α and pro-inflammatory cytokines this was the first study to show a direct link between HIV and innate PRRs (Heil *et al.*, 2004). *Influenza* virus, synthetic polyuridine ssRNA and certain siRNAs were subsequently identified as ligands for TLR7. (Diebold *et al.*, 2004). During *Influenza* infection, TLR7 has been shown to be important

in triggering recognition of viral ssRNA, leading to robust induction of type 1 interferons (IFN) by plasmacytoid DCs (pDCs) which have high levels of TLR7 expression (Gilliet *et al.*, 2008; Diebold *et al.*, 2004). One of these imidazoquinoline compounds, imiquimod, has been approved for the treatment of certain cancers and for Human Papiloma virus (HPV) infection (Hemmi *et al.*, 2002).

TLR9 is expressed intracellularly by numerous cells of the immune system such as dendritic cells, B lymphocytes and natural killer (NK) cells. It was originally identified to recognize unmethylated 2'-deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs that are frequently present in bacteria and some viral nucleic acids but are rare in vertebrates, it was observed that TLR9-deficient mice did not respond to CpG DNA challenge, including proliferation of splenocytes, production of pro-inflammatory cytokines, maturation of dendritic cells (Hemmi *et al.*, 2000). It is now clear that TLR9 is the principal means by which HSV-1 and HSV-2 (Herpes Simplex Virus) stimulate type I IFNs in pDCs (plasmacytoid dendritic cells) *in vivo* (Krug *et al* ., 2004; Lund *et al.*, 2003). Cp ODNs (oligonucleotides) has been shown to be effective adjuvant in clinical trials in humans against hepatitis B (Sarah and Mills, 2010).

2.2 TLR Signalling Pathways

TLRs recognize microbial products during infection and initiate intracellular signaling pathway such as NF-kB and increased expression of several genes involved in innate immune response and the initiation of adaptive immunity (Takeda and Akira, 2005). TLR signaling is initiated by the ectodomain-mediated dimerization of TLRs, which then facilitates the recruitment of intracellular specific adaptor proteins. Individual TLR

family members induce different signalling pathways and can be grouped based on their use of the known TLR adaptors (Takeda and Akira, 2005).

TLR signaling pathways consist of a Myeloid differentiation primary response gene 88 (MyD88) dependent pathway that is common to all TLRs and a MyD88-independent pathway that is peculiar to the TLR3- and TLR4 signaling pathways (Akira *et al.*, 2001). As shown in Figure 3 most TLRs signal through the MyD88-dependent pathway or Toll/Interleukin one receptor (TIR) domain-containing adapter inducing IFN-β (TRIF) also known as Toll-like receptor adaptor molecule 1 (TICAM-1) pathway (Figure 3). TLR3 triggers a signalling pathway via a TRIF while TLR4 uses both adaptors (Takeda and Akira, 2005). TIRAP (also known as Mal) functions downstream of TLR2 and TLR4. Overall, ligand engagement of PRRs leads to activation of a pro and anti-inflammatory and antimicrobial response by triggering signal transduction pathways involving various transcriptional factor such as nuclear factor (NF)-κB, activating protein-1 and IFN regulatory factors (IRF), ultimately resulting in activation of gene encoding cytokines, chemokines, cell adhesion molecules and antiviral type I IFN (Taro and Shizuo, 2009; Baccala *et al.*, 2009; Takeda, 2004).

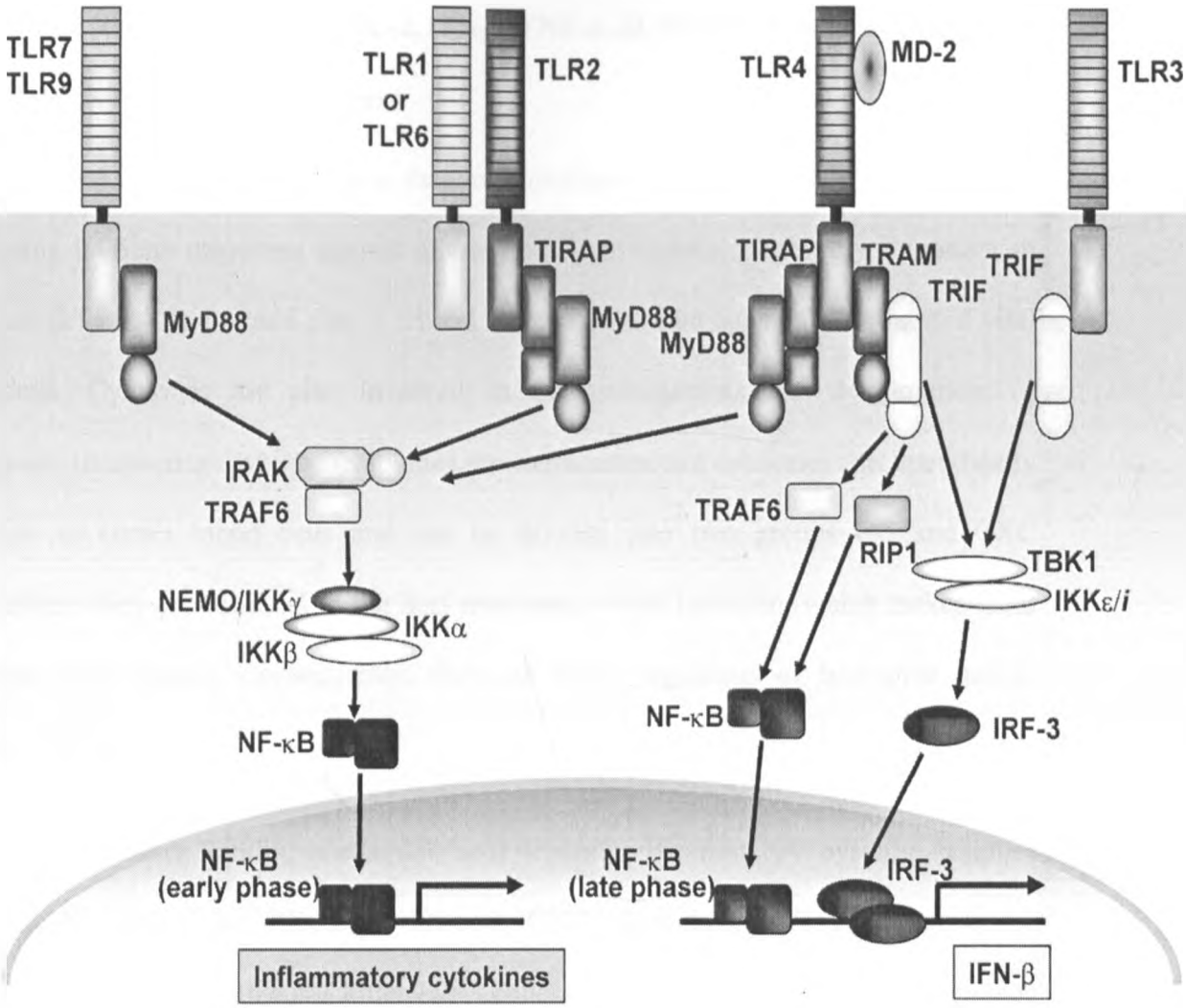


Figure 3: TLR signaling pathways

Recognition of a pathogen-specific molecular pattern, which upon ligation by their specific TLR leads to differentially activating distinct signalling pathways mediating diverse immune responses. TLR signaling can also result in the recruitment and activation of macrophages, NK cells, and dendritic cells, key agents in the presentation of antigen to T cells. The links to T cells and cytokine release shows the intimate relationship between innate and adaptive responses (Takeda and Akira, 2005)

2.2.1 Cytokines (IL-12, IL-10, IL-2, IFN-γ, TNF-α, IL-6) and chemokines (RANTES, IP-10) of interest

Cytokines are small secreted proteins that are important immunomodulatory molecules in regulating immune responses against microorganisms. Cytokines are very important in the host defense system, and play a critical role in protection against bacterial and viral infections. Cytokines are also involved in the pathogenesis and development of symptoms in infections while chemokines are chemoattractant cytokines that specifically function to attract blood cells and can be divided into two groups CC and CXC chemokines they are essential to the host response to viral infection, which makes them common viral targets, Consequently, they are major regulators of leukocyte traffic. (Kuby, 2007).

In the α chemokine family, one amino acid separates the first two cysteine residues (cysteine-X-cysteine, or CXC). In general, CXC chemokines are chemotactic for neutrophils, T-cells and natural killer (NK) cells. Members of the CXC family include interleukin-8 (IL-8), IFN-γ inducible protein, 10 kDa (IP-10) etc. The β chemokine family is characterized by the first two cysteine residues being adjacent to each other (cysteine-cysteine, or CC). Members of the CC family include macrophage inflammatory protein (MIP)-1a, RANTES etc (Jae-wook *et al.*, 1999).

IL-12 cytokine is produced by antigen presenting cells (APC) such as macrophages and dendritic cells (DCs) and stimulates production of IFN-γ by T cells, NK cells, and enhances cytolytic functions of CD8+ T cells and NK cells. (Sartori, 1997; Trinchieri

1998a and Trinchieri 1998b). IL-12 has been shown to induce the differentiation of the pro-inflammatory T_H1 subset (Kuby, 2007).

IL-10 is an anti-inflammatory cytokine mostly secreted by DCs and T_H2 cells (Banchereau 2003).It has also been shown to interfere with antigen presentation by monocytes /macrophages and dendritic cells to T cells by reducing their expression of MHC class II thus indirectly inhibiting T cell activation and reduction of IFN-γ. In addition, it exerts some direct effects on T lymphocytes (Asadullah *et al.*, 2003). Specifically, IL-10 inhibits the antigen-specific proliferation of CD4+T cells and downregulate cytokine production (de Waal Malefyt *et al.*, 1993; Del Prete *et al.*, 1993).

IL-6 cytokine is produced by T and B cells, monocytes and vascular endothelial cells. IL-6 functions in both the innate and adaptive immune systems, and it is responsible for T-cell function and differentiation of cytotoxic T-cells (Kishimoto *et al.*, 1999). IL-6 has also been linked to the severity of *Influenza* symptoms (Mitchell *et al.*, 2008).

Interferons are group of inducible cytokines that have a central role in innate antiviral immune responses because they prevent viral replication and prevent the spread of virus between cells (Randal *et al.*, 2008; Stetson and Medzhitov, 2006).They are grouped into three classes according to their amino acid sequence, chromosomal location and receptor specificity (Pestka *et a.,l* 2004).

IFN-γ is the only known member of the type II cytokine family produced by activated immune cells mainly T cells, antigen presenting cells, NK cells and to a lesser extent B-cells. It plays a pivotal role in defense against viruses and intracellular pathogens and also induction of immune mediated inflammatory responses (Stark *et al.*, 1998). One of the

most striking effects of IFN-γ is its ability to activate macrophages. Activated macrophages exhibit increased expression of class II MHC molecules, increased cytokine production, and increased microbicidal activity compared with non-activated macrophages. Thus, they are more effective in antigen presentation and killing of intracellular microbial pathogens (Kuby 2007). It has also been shown to increases NK cell function by promoting the differentiation of T and NK cells (Belardelli, 2002). IFN-γ has been used clinically as an agent for the treatment of chronic granulomatous disease (CGD) (Kuby, 2007).

Tumor Necrosis Factor (TNF-α) is a pro-inflammatory cytokine and is one of the principal cytokines secreted by activated macrophages (Kuby 2007). It has been shown to induce replication of HIV-1 in cells (Vassalli 1992). It has also been shown to induce inflammation and apoptosis thereby inhibiting viral and bacterial infections (Benedict *et al.*, 2003; Rahman and Mcfadden, 2006).

IL-2 is a naturally occurring cytokine that can stimulate proliferation and improve functioning of T cells. It also stimulates natural killer cells (Smith, 2001). IL-2 mediates its effects by binding to IL-2 receptors, which are expressed by lymphocytes, the cells that are responsible for immunity. IL-2 is normally produced by the body during an immune response (Cantrell and Smith, 1984).

RANTES (Regulated Upon Activation Normal T cells Expressed and secreted) is a pro inflammatory chemokine belonging to CC family of chemokines. It is produced and secreted from T cells, endothelial cells and platelets (Kuby, 2007). The natural affinity of

RANTES for CCR5 is of particular interest since CCR5 is a co receptor for HIV. RANTES has been shown to suppress infection of R5 strains of HIV-1 by blocking CCR5 (Paxton *et al.*, 1996).

IP-10 (Interferon gamma-induced Protein 10 kDa) chemokine has been observed to be a major chemoattracttant for activated T-cells; this may result in recruitment of HIV-1 target cells CD4+ T cells. IP-10 has also been shown to be important in promoting responses IFN-γ (Gangur *et al.*, 1998).

2.3 *Influenza* Virus and immune responses

2.3.1 *Influenza* virus-types and structure

Influenza infection *("flu")* is a highly contagious, acute, febrile, respiratory disease caused by *Influenza* virus which is a segmented enveloped RNA virus, from the family *Orthomyxoviridae (*Brockwell-Staats *et al.*, 2009*)*. Three subtypes of *Influenza* viruses exist A, B and C. All the three infect humans with *Influenza* A being the most capable of causing pandemics as it infects both humans and animals (Susan and John, 2003). *Influenza* viruses cause seasonal epidemics of acute respiratory disease with most severe consequences in pediatric and geriatric populations (Dong *et al.*, 2009). Disease severity ranges from mild self-limited illness to a more severe illness leading to mortality. Which is due to either the virus infection directly or indirectly from infection with secondary pathogens. This can lead to pandemic situations with serious public health consequences (CDC 2010). Pandemics are caused by *Influenza* viruses which contain surface protein(s)

for which the human population has little or no immunity, spread easily amongst the human population (Hoimoto and Kawaoka, 2005).

Influenza virions contain two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA) which are known conveyors of virulence. The genome is comprised of eight negative sense single stranded RNA segments which encode eleven proteins (Brockwell-Staats *et al.*, 2009). There are 16 types of HA antigen and nine types of NA antigen, and there are many subtypes of each (Brockwell-Staats *et al.*, 2009).

2.3.2 *Influenza* virus mutation (antigenic shift and drift)

From time to time a virus emerges with a new combination of HA and NA genes as a result of reassortment of gene segments which can lead to development of a pandemic strain (Gatherer, 2009). The hosts of *Influenza* A viruses are principally birds that frequent aquatic habitats (Michaelis *et al.*, 2009). The birds (e.g. ducks, geese, gulls) acquire infections by ingestion or inhalation and the viruses infect their intestinal and/or respiratory tracts. Infection with most virus strains results in few or no signs of disease, but some strains, for example H5N1 are highly pathogenic and can kill their avian hosts. Like most RNA viruses, *Influenza* lacks an inherent proof reading ability during replication which can result in formation of novel strains (Steinhauler and Skehel, 2002). Pandemic *Influenza* emerge as a result of a process called "antigenic shift". Antigenic shift causes an abrupt or sudden, major change in certain proteins (HA or NA) (Michaelis *et al.*, 2009).

Antigenic shift can occur when a single cell in same host is infected with more than two different virus subtypes leading to reassortment (exchange of gene segments HA or NA) (Youri, 1994; Michaelis *et al.*, 2009; Brockwell-Staats *et al.*, 2009). Certain antigenic shifts may allow the virus to become more easily transmissible and more "contagious". This has played an important role in the evolution of *Influenza* A virus. Thus, each antigenic shift finds the population immunologically unprepared, resulting in major outbreaks of *Influenza*, which sometimes reach pandemic proportions (Kuby, 2007). A good example of this is the recent 2009 flu pandemic of new strain of H1N1 which contains gene segments of both swine and human *Influenza* viruses (Brockwell-Staats *et al.*, 2009) usually referred to as swine flu. The 2009 H1N1 pandemic virus derives six genes from triple-reassortant North American swine virus lineages and two genes (encoding neuraminidase and matrix proteins) from Eurasian swine virus lineages (Garten *et al.*, 2009). Antigenic shift is most dangerous when it occurs in a virus that has demonstrated high lethality, such as the H5N1 bird flu.

Between pandemic-causing antigenic shifts, the *Influenza* virus undergoes Ántigenic drift which occurs as a result of mutation of surface glycoproteins enabling the virus to evade the host immune response which results in a new strain of the virus (Brockwell-Staats *et al.*, 2009). The immune response has been shown to contribute to the emergence of these different *Influenza* strains. As individuals infected with a given *Influenza* strain mount an effective immune response, the strain is eliminated (Kuby, 2007).

According to WHO, antigenic and genetic analyses are used in combination with epidemiologic information to define emergent antigenic variants and their spread which

help to select the most appropriate viruses to recommend for inclusion in vaccines to combat antigenic drift and shift. Relationships between circulating antigens are evaluated mainly by haemagglutination inhibition (HI) tests using post-infection ferret antisera against egg and/ or cell-culture grown viruses (WHO, 2009). Hemagglutinin plays a key role in virus cell entry by binding to cell surface receptors. *In vitro* binding of the *Influenza* virus to red blood cells leads to hemagglutination; this can be observed as a layering of agglutinated red blood cells at the bottom of a tube or well. The HI test measures the presence and quantity of antibodies directed against the hemagglutinin component of the *Influenza* virus and and HIA titers >40 correlate with protection (Paul *et al.*, 2010). In the HI test, antibodies directed against hemagglutinin block the *Influenza* virus from binding to red blood cells, thus inhibiting the hemagglutination reaction.

Phylogenetic analyses of HA and NA genes help to define the genetic relatedness of antigenic variants to their predecessors and to elucidate the molecular basis for antigenic drift. The incidence of antigenic variants associated with *Influenza* outbreaks in different countries is also an important criterion for selection of epidemiologically relevant vaccine virus candidates. This explains why a seasonal *Influenza* vaccine needs to be modified each year by a panel of World Health Organization (WHO) experts in the Northern and Southern hemispheres in keeping up with the phenomena of both Antigenic Shift and Drift, this process aims to ensure that the vaccine strains match the circulating strains and provide reliable immunogenic protection (Steinhauler and Skehel, 2002).

2.3.3 Host response to *Influenza* virus infection.

After initial exposure to a novel *Influenza* strain, it takes 5 to7 days before specific

antibodies and T cells arrive in the lung to definitively clear the virus; hence, this defines the time window in which innate immunity is critical (Mitchell *et al.*, 2008). Antibody specific for the HA molecule is produced during an *Influenza* infection. This antibody confers protection against *Influenza*, but it is specificity is strain-specific and readily bypassed by antigenic drift (Kuby, 2007). Infection with the *Influenza* virus elicits an immune response resulting in generation of interleukin (IL-6) and interferon (IFN- α) that peak early in the course of infection and contribute to symptom development. A host defense response against *Influenza* also Tumor necrosis factor (TNF-α) where levels increase later as viral shedding and symptoms subside during the later days of the illness (days 4 to 6) (Hayden *et al.*, 1998). Interleukin (IL-8) levels rise, correlating with the onset of lower respiratory tract symptoms. The development of *Influenza*-specific cytotoxic T lymphocytes is important for clearance of the virus and recovery from illness; in individuals with T-lymphocyte immunodeficiency states, clearance of virus is delayed (Hayden *et al.*, 1998).

Innate immunity is crucial in the early containment of viral (Flu) infection and relies on recognition of Flu antigens by PRRs. During *Influenza* infection the TLR system involves recognition of viral RNA in endosomal compartments through either TLR3 (dsRNA) or TLR7 (ssRNA) which is critical for the activation of these responses (Mitchell *et al.*, 2008). Signaling occurs through both the PI3K/Akt signaling pathway and TRIF-dependent pathway, resulting in the activation of INF-β and IFN-inducible genes (IL-1, IL-6, TNF-α, IL-8 and RANTES) from macrophages and dendritic cells (Meylan and Tschopp 2006; Koyama *et al.*, 2007; Le Goffic *et al.*, 2007; Mitchell *et al.*, 2008) these pathway are important in the recognition of the RNA virus. Several studies have shown

the influence of HLA alleles on variation of immune response to measles, mumps, rubella, vaccinia and other vaccines (Poland *et al.*, 2001; Jacobson *et al.*, 2001; Ovsyannikova *et al.*, 2008; Ovsyannikova *et al.*, 2005; Gioia *et al.*, 2008; Oseroffv *et al.*, 2005). Studies have shown that HLA-Bw35 specifically may influence early response to *Influenza* A antigens (Cunningham *et al.*, 1979; Mackenzie *et al.*, 1977).

Another important component of the innate immune response to *Influenza* involves Natural Killer (NK) cells. NK cells produce a number of immunologically important cytokines, they play important roles in immune regulation and influence both innate and adaptive immunity. In particular, IFN-γ production by NK cells can affect the participation of macrophages in innate immunity by activation of the phagocytic and microbicidal activities. IFN-γ derived from NK cells can influence the T_H1 versus T_H2 commitment of helper T cell populations by its inhibitory effects on T_H2 expansion, and stimulate T_H1 development via induction of IL-12 by macrophages and dendritic cells (Natarajan *et al.*, 2002). The importance of NK cells has been confirmed in many studies such as a study done by Gazit *et al.*, 2006 showing an increase in mortality rate during *Influenza* infection in mice lacking the NKp46 receptor.

2.3.4 *Influenza* vaccination - Live Attenuated *Influenza* Vaccine (LAIV)

Influenza vaccines contains viral surface antigens (HA and NA) of strains produced in embryonated hen eggs (WHO, 2006). Immunity to these antigens, especially to HA response directly increases the number of specific B and T cells available to respond against a live infection encountered at a later time - reducing the likelihood of infection and lessening the severity of disease if infection occurs. For a vaccine to be effective, it

must be able to elicit both an antibody and cellular immune response at the sites of virus entry and replication (Hiroi *et al.*, 1998). Currently two *Influenza* vaccines have been licensed in the United States: trivalent inactivated vaccines (TIV) and live attenuated vaccines (LAIV).

Micro organisms (infectious pathogens) are attenuated so that they lose their ability to cause significant disease (pathogenicity) but retain their capacity for transient growth within an inoculated host (Kuby, 2007). LAIV has been seen as an alternative to inactivated *Influenza* vaccines. They are widely accepted because of several factors including ease of administration, i.e. intranasal rather than an intramuscular route of administration, induction of a broad immune response including mucosal, systemic and cell mediated responses (Nichol, 2001). In contrast, studies have shown that parenteral inactivated *Influenza* vaccine does not effectively stimulate local immunity which may be an important mode of prevention of initial infection with virus. LAIV has also been shown to provide cross protection against drift variants (Brokstad *et al.*, 1995; Brokstad *et al.*, 2002; Slepushkin *et al.*, 1993; Naikhin *et al.*, 2002). Because of their capacity for transient growth, such vaccines provide prolonged immune system exposure to the individual epitopes on the attenuated organisms, resulting in increased immunogenicity and production of memory cells. Consequently, these vaccines often require only a single immunization, eliminating the need for repeated boosters and may lead to superior cross protective responses (WHO, 2010). However a major disadvantage of attenuated vaccines is the possibility that they will revert to a virulent form (Kuby, 2007). The nasal-spray flu vaccine is approved for use only in healthy people 2-49 years of age who are not

pregnant. The vaccine should not be given to person with history of hypersensitivity to eggs, persons with a history of Guillain-Barré syndrome (Belshe *et al.*, 1998).

Much of the research on HIV is based on research of the influenza virus (Manal and O'Shaughnessy, 2009). The fusion mechanism for HIV and the *Influenza* virus is very similar. Structurally, both viruses are enveloped viruses with lipid bilayer membranes. Immune responses during *Influenza* virus infection is similar that of HIV. For example during the early course of *Influenza* infection elicit immune responses resulting in generation of IL-6 and IFN-α this contribute to symptom development whereas during the later stages of *Influenza* virus infection TNF-α levels increases (Hayden *et al.*, 1998). During HIV-1 infection viral replication results in activation of pro-inflammatory cytokines and type I interferon (IFN), including IFN-α and IFN-β cytokines. TNF-α , IL-6 has been observed to affect the rate of HIV-1 replication once infection has been established. Increase in TNF-α production has been observed to be proportional to viral replication (Edana *et al*, 2006). The development of *Influenza*-specific cytotoxic T lymphocytes is important for clearance of the virus similarly in HIV-1 infection CD8+ T cell-mediated control of viraemia is mediated by cytotoxic killing of productively infected cells.

The aim of this study was to understand the earliest event in mucosally transmitted HIV-1 infection in a group of women whose exposure to HIV is almost exclusively through the genital mucosa. To better understand the correlates of mucosal protection which are unknown, a viral challenge system model was used to mimic HIV. The model consisted of a Live attenuated *Influenza* vaccine administered intranasally to stimulate the mucosal system. Genital secretions (CVL), blood, and plasma were collected at baseline (day 0)

and after vaccination (days 7 and 30). Chemokines and cytokines were quantified in CVL, and plasma. PBMCs were isolated from blood, and stimulated with TLRs agonists as well as *Influenza* virus Brisbane (Vaccine strain). Supernatants were tested for presence of chemokines and pro and anti-inflammatory cytokines.

2.4 Problem statement

Altered susceptibility to HIV infection has been observed in individuals who after repeated exposure to the virus have remained uninfected for a long period of time (Fowke *et al.*, 1996). Studies on these individuals have shed considerable light on correlates of protection against HIV, but our understandings of these factors are incomplete (Marmor *et al.*, 2006). One approach toward furthering our understanding of the immune response to HIV and why it fails in most people lies in examining the few individuals who appear to be resistant either to acquisition of the virus or disease progression. Although resistance to HIV has been associated with numerous immunological and genetic factors, the mechanism and composition of this natural resistance remains unclear.

For nearly three decades, analysis of HIV immunity and correlates of HIV-1 protection has historically focused on HIV-1 adaptive immune response such as specific T and B-cell lymphocyte responses, CD4 and CD8 T cells, and humoral factors. This has led to a gap in our understanding of an alternative arm of the immune system; the innate immune response in HIV infection. Given the difficulties in inducing protective B and T cell immunity in previous HIV-1 vaccine trials, this emphasizes the need to look beyond mechanisms of adaptive immunity.

In order to understand immune events at the time of exposure and following exposure to a pathogen, a model Live Attenuated *Influenza* Vaccine (LAIV) was used to challenge HIV-R individuals. The model will add to the existing knowledge by studying a known timing and dose of mucosal exposure. It is hoped that this study will improved our understanding of the important role of the innate immune response in the generation of adaptive immune responses in HIV exposed but uninfected individuals, and the mechanisms of HIV infection across mucosal surfaces. Identification of immune correlates of protection is likely to be important in providing clues for effective HIV-1 vaccine research, design and development.

2.5 Justification

So far, the search for an elusive HIV-1 vaccine has been met with much disappointment for nearly three decades, but the recent success of the human HIV-1 clinical trials in Thailand showed partial efficacy of about 31% protection against infection, which offers optimism that a protective HIV-1 vaccine is possible (Supachai Rerks *et al.*, 2009).

Innate immunity has been shown to play a key role in inhibiting HIV and other infection may be critical to inhibiting HIV as it may be activated directly at the time of HIV-1 transmission, inhibiting early HIV-1 replication and stimulation of adaptive immunity to deal with the virus effectively. The innate immune system has been shown to reduce viral replication in models of herpes simplex virus, hepatitis C and *Influenza* infection in animal studies (Takeda and Akira, 2008; Lehner *et al.*, 2008; Milush *et al.*, 2007; Wu *et al.*, 2007; Horsmans *et al.*, 2005; Gill *et al.*, 2008). It has also been demonstrated that artificial TLR activation could be of therapeutic value in that local mucosal delivery of

CpG oligodeoxynucleotides which is a ligand of TLR 9 to the genital tracts protected mice from a lethal HSV-2 challenge, through induction of dramatic changes in the genital mucosa, the recruitment of innate immune cells and the inhibition of HSV-2 replication (Ashkar *et al.*, 2003). TLR functional differences documented above shows the important role of innate immune sensors. This supports the development of similar approach for HIV thus altered innate immune responses should be explored in HIV-1 HESN individuals.

In this study in order to determine the correlates of mucosal protection from HIV infection, a virus challenge would be necessary. As this is obviously not possible, an *in vivo* viral model was used in HIV resistant and susceptible women to examine how the innate immune systems responds to a mucosal infectious insult and what role innate responses play in the development of protection. Therefore, to mimic as practically as possible this situation, a licensed, safe, live attenuated *Influenza* vaccine model delivered through the anterior nares was used to generate mucosal immune response. It is hoped that this study will lead to a better understanding of the earliest innate mucosal immune responses. Particularly innate immune sensors in recognition of Pathogen associated molecular patterns (PAMPs) with the hope that it will help in the development of innovative strategies targeted at inducing and maintaining protective mucosal immune response at the portal entry.

2.6 Hypotheses

2.6.1 Null hypothesis

- HIV-R individuals may not have altered innate immune response to TLRs and these responses may not differ post viral challenge with live attenuated *Influenza* vaccine compared to the HIV susceptible individuals.

2.6.2 Alternative hypothesis

- HIV-R individuals are hypothesized to have altered innate immune responses to TLRs, these heightened responses may differ post mucosal challenge with a live attenuated *Influenza* vaccine compared to HIV susceptible individuals.

2.7 Objectives

2.7.1 General Objective

- To compare the level of cytokines and chemokines at baseline and post vaccination as well as the expression levels of TLRs before and after mucosal challenge with a live attenuated *Influenza* vaccine in HIV-1 resistant and susceptible individuals.

2.7.2 Specific Objectives

- To examine TLR responsiveness before and after flu vaccination between HIV-1 resistant and susceptible subjects.
- To determine the levels of chemokines in CVL and plasma samples of HIV-1 resistant and susceptible subjects before and after vaccine administration.
- To determine the levels of cytokine in CVL and Plasma of HIV-1 resistant and susceptible subjects before and after vaccine administration.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study sites and design

The study was conducted at Majengo clinic and University of Manitoba laboratories located at the University of Nairobi medical school, Nairobi, Kenya and University of Manitoba (Department of Medical Microbiology and Immunology) in Winnipeg, Canada. The study was a prospective cohort study nested within on-going studies on correlates of HIV-1 protection among commercial sex workers.

3.2 Study Populations

The Pumwani Sex Worker Cohort: This cohort was established in 1985 to study the epidemiology, biology and immunobiology of HIV-1 and STIs. As of January 2011, the cohort consists of more than 3000 women out of these approximately 700 are on follow up. New enrolment continues at the rate of approximately 100 women per year. At enrollment, women are asked about several factors including demographic information, sexual behavior, duration of prostitution, number of sex partners per day, number of regular partners, condom use, and reproductive history. These women have an extremely high risk of HIV-1 infection. Despite this intense exposure, approximately 10% of these women remain persistently seronegative, with approximately 60% of women being HIV-1 seropositive at enrollment. The enrolled women are resurveyed twice yearly when biological samples (i.e. blood, genital tract swabs) are collected and epidemiological and behavioral information is gathered. Study participants are free to attend the study clinic

for any acute conditions at any time. The cohort remains one of the few studied to explain resistance to HIV-1 infection (Kreiss *et al.*, 1986; Simonsen *et al.*, 1990). The women are classified as: - HESN, HIV-S

HESN, or resistant (HIV-R) are long term highly exposed women who can be epidemiologically defined upon meeting the following definition: 1) HIV seronegative by serology and PCR 2) still active in sex work and 3) followed for greater than 7 years modified from survival modeling for time HIV-1 seroconversion (Fowke *et al.*, 1996).

HIV-S are short term highly exposed women, these are HIV negative women who were enrolled and followed in the cohort for less than 3 years. They are also classified as HIV new negatives or HIV susceptible (Fowke *et al.*, 1996). In this study the HIV-S women were used as the control group since the used of low risk women as control groups was not approved due to ethical issues.

Inclusion and exclusion Criteria

- The study only included women who are and have been part of the Pumwani sex worker cohort for more than one year.
- The women included in the study were 18years and above.
- The study subject volunteered out of their own consent.
- Only women who tested negative for sexually transmitted infections (STIs) of the genital tract at the time of sample collection and follow up visits were included in the study.
- They should not be applying any microbicide (vaginal) gels.
- The study excluded women who tested HIV positive.
- No sampling was done on women who were not part of the cohort.

- The study excluded women who have been previously received flu vaccine.

Sample size

A total of 60 women (30 HIV-R and 30 HIV-S) were included in the study based on inclusion and exclusion criteria and number of HIV-R women that could be age matched with the HIV-S individuals. Due to limited funding available quantification of cytokines and chemokine level in plasma and CVL samples were only able to be done in 10 HIV-R and 10 HIV-S while in supernatants from stimulated PBMCs 5 HIV-R and 5 HIV-S

3.2.1 Ethical Consideration

The study protocol and cohort was approved by the Institutional review boards (National Ethical and Scientific Review Committee) of the University of Manitoba, and Kenyatta National Hospital Ethical Review Committee. All clinical investigations were conducted in accordance with the principles of the Helsinki Declaration. Taking part in this study was on voluntary basis; subjects had the right to leave the study at any time. Leaving the study did not result in any penalty or loss of benefits to which they are entitled. If the participation in the study results in illness, the study team provided medical care for the problem for free. Efforts were made to keep subjects personal information confidential, for identification a special number was used. The number is only known to the clinic staff and the subject.

3.3 Methodology

3.3.1 LAIV seasonal Flumist® Vaccine Administration

The subjects were vaccinated using the 2009-2010 *Influenza* seasonal LAIV (FluMist®) which is commercially made by MedImmune. The vaccine is composed of HA (Hemagglutinin) of A/Brisbane/59/2007 (H1N1)-like virus, an A/Brisbane/10/2007 (H3N2)-like virus, B/Brisbane/60/2008-like virus.

After giving informed consent, the study subjects provided baseline samples immediately prior to vaccination. Administration was done by administering 0.25ml of the vaccine into each nostril. The follow-up visits were at day 1, day 7, day 30 and 4-6 month post vaccination. This was based on previous experience with *Influenza* vaccination as a model of immune activation (Fowke *et al.*, 1997). Day 7 and 30 were chosen to investigate the link between innate and adaptive immunity. Whereas innate immunity is first adaptive immunity comes later following exposure to pathogens or vaccines. At day 0 (baseline) and the four follow-up visits, 21 ml blood, a nasal swab and cervical vaginal lavage secretions were obtained for assessment of immune responses.

Sample collection

Blood: heparin-treated blood (21ml) was collected from the subjects by venipuncture method.

CVL: Cotton tipped swab was rotated at 360° in the cervical os, a second swab collected secretions from posterior vaginal fornix. The swabs were transferred into 15 mL conical tubes containing 2mL of 1X phosphate buffered saline (PBS).

3.3.2 Preparation of Plasma, PBMCs and Cervico Vaginal Lavage (CVL)

Plasma was separated by centrifugation of heparin treated blood at 1600g for 10 minutes. This was collected in 15ml tubes that were spun for another 10 minutes at 1600g. Plasma was then aspirated and collected into vials and stored at -80^{0}C till quantification of cytokine and chemokines assay using Cytometric Bead Array (CBA).

PBMCs from the subjects were isolated by Ficoll Hypaque (Histopaque-1077) density gradient centrifugation from heparin-treated blood within 3 hours after venipuncture. This was done by re-suspending the remaining blood after plasma separation with 20ml of Phosphate buffered saline (PBS) and layered on 10ml of Ficoll Hypaque. This was then spun at 1600RPM without breaks for 20 minutes. Cells were then harvested from the interface using transfer pipette into 50ml tubes containing 10ml PBS. This was centrifuged at 2000RPM for 10 minutes with breaks on low. Supernatant was poured off, cells pellets were re-suspended by dragging the tube on the rack, 10ml RPMI solution was added. Viable harvested cells were counted by Trypan Blue (Sigma-Aldrich) exclusion technique using a haemocytometer. Remaining cells were resuspended in culture medium (RPMI 1640 supplemented with 5% FCS, 1% glutamine and 1% penicillin/streptomycin (Gibco). Cells were stored in freezing media (90% heat inactivated fetal calf serum/10 % dimethylsulfoxide) and later shipped to Winnipeg. Stimulation assay was not done on fresh samples due to unavailability of Luminex.

CVL: To separate the content from the sponge the tubes were centrifuged then transported on ice to the laboratory. To remove the cellular debris the tubes were spun down at 1000g and cryopreserved -80^{0}C till the quantification assay.

3.3.3 Quantification of cytokine and chemokine level and production

3.3.3.1 Plasma and CVL

The BD™ Cytometric Bead Array (CBA) immunoassay is a flow cytometry application that allows users to quantify multiple proteins simultaneously. The BD CBA system uses the broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads to efficiently capture analytes. Each bead in the array has unique fluorescence intensity so that beads can be mixed and run simultaneously in a single tube. This method significantly reduces the amount of sample required for testing and time to results in comparison with traditional ELISA and Western blot techniques. An important characteristic of the assay system is that several components (the calibrators, the Ab–bead reagent and the second Ab–PE detector antibody reagent) are made of a mixture of all cytokines or chemokines. Several standard curves (standard ranging from 0 to 5000 pg/ml) are obtained from one set of calibrators and several results are obtained on one test sample.

BD™ Cytokine Bead Array (CBA) Human Th1/Th2/Th17 Kit which measure IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, IL-17A was used to quantify cytokines from plasma and CVL samples while BD CBA Human Chemokine Kit was used to quantitatively measure five chemokines; Interleukin-8 (CXCL8/IL-8), RANTES (CCL5/RANTES), Monokine-induced by Interferon-γ (CXCL9/MIG), Monocyte Chemoattractant Protein-1 (CCL2/MCP-1), and Interferon-γ-induced Protein-10 (CXCL10/IP-10).

Assay procedure

These assays were done as per manufacturer's instructions. Briefly, the beads (chemokines/cytokines) were mixed with samples (plasma or CVL followed by the addition of phycoerythrin (PE) detection reagent. The tubes were incubated for 3 hours at room temperature in the dark to allow formation of sandwich complexes. At the same time the LSR II flow cytometer was calibrated using cytometer setup beads. The tubes were then centrifuged at 1000g for 10 minutes. 1mL of wash buffer was added to each assay tube and centrifuged at 200g for 5 minutes. Supernatants were carefully aspirated and discarded from each assay tube. To resuspend the bead pellet 300µl of wash buffer was added to each assay tube. Samples were acquired on the BD LSR II flow cytometer using BD CBA acquisition template. The concentrations of analytes were determined using FCAP Array software (developed by Soft Flow Inc.)

3.3.3.2 PBMCs Stimulation Assay

Thawed PBMCs were in a suspension containing $1x10^6$ cell/ml in RPMI 1640 1640 supplemented with 5% FCS, 1% glutamine and 1% penicillin/streptomycin (Gibco). 100µL or 100,000 PBMCs in suspension was pipetted to each well (sterile 96 well plates). The stimulation were as follows 10µL of 5µg/ml LPS (TLR4), 10µL of 5µg/ml poly I: C (TLR3), 10µL of 5µg/ml ssRNA (TLR7/8), 10µL of 20uM ODN2006 and ODN2116 (TLR 9) and Bris (Flu Antigen) - 10µL of log 10^{-6} HA 1:512. Unstimulated cells served as negative controls (media only) while Staphylococcus aureus enterotoxin B (SEB) served as positive control. Stimulations for all assays were conducted at 37°C, under 5% CO_2 conditions. Supernatants were harvested after 18 hours.

3.3.3.3 PBMCs Culture supernatants

Cell culture supernatants were harvested from stimulated PBMCs and multiplex analysis of 18 cytokines and chemokines (interleukins IL-1α, IL-1β, IL-1ra, IL-2, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-15, IL-17, sIL-2ra, IFN- γ (TNF-α), monocyte chemoattractant protein-1 (MCP-1), monocyte chemoattractant protein-3 (MCP-3, macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β) and IFN-γ-inducible 10-kDa protein (IP-10) quantification was done using Millipore MILLIPLEX® human cytokine and chemokine kit.

The supernatants were thawed briefly, followed by incubation of 25µl of each sample with antibody coupled beads overnight at 4^0C on a plate shaker. Complexes were washed twice with 200µl/well of wash buffer, wash buffer was removed with the use of a vacuum filtration between each wash. 25µl of Biotinylated detector antibody was added into each well and incubated with agitation for 1 h at RT on a plate shaker. 25µl of Streptavidin-phycoeryrhrin (RPE) was added into each well containing 25µl of detection antibodies, this was followed by incubation with agitation on a plate shaker for 30 min at RT. The plate was then washed 2 times with 200µl/well wash buffer, wash buffer was removed with the use of a vacuum filtration between each wash. 150µl/well of sheath fluid was added to resuspend the beads on a plate shaker for 5 minutes before detection on the Luminex 200TM instrument.

Data acquisition and analysis was performed using Bioplex manager software version 2.0 (Bioplex; Bio-Rad), standard curves were plotted through a five parameter logistic curve

setting. Cytokines and chemokines that were below detection level were excluded from analysis.

3.3.4 Statistical Analyses

Analysis of data was performed using GraphPad Prism 4 software. (Version 3.02 for Windows, GraphPad Software, San Diego CA). The nonparametric Mann-Whitney *U* test was used for comparisons of group means cytokine and chemokine concentrations. A probability value of less than 0.05 ($p<0.05$) was considered as statistically significant.

CHAPTER FOUR

4 RESULTS

Quantification of chemokines and cytokines in CVL and plasma samples were done using CBA Human Th1/Th2/Th17 cytokine and Human chemokine kit. These assays were done according to the manufacturers Instruction. The samples were then acquired on LSR (II). CVL samples were obtained pre and post vaccine administration. Five chemokines were quantitatively measured: XCL8/IL-8, RANTES, MIG, MCP-1 and IP-10. Among the five chemokines measured three chemokines had detectable expression in CVL both at baseline and after vaccination.

Based Time-Response curve for different chemokine in CVL samples from HIV-R and HIV-S combined. There was a gradual increase in IP-10 from baseline (day 0) and following vaccine administration (day 1 and 30). IL-8 level decreased gradually after vaccine administration. MIG level in CVL increased gradually after vaccination (Figure 4).

In CVL samples IL-8 level was the most robust compared to MIG and IP-10 chemokines (Figure 4.0). No significant differences in IL-8 level were observed in CVL of HIV-R and HIV-S women at baseline ($p=0.1903$), and following vaccination day 1 ($p=0.5787$) and day 30 ($p=0.8534$) (Figure 5). At baseline ($p=0.4647$) and prior vaccination, day 1 ($p=0.7884$) and 30 ($p=0.7017$) no significant differences were observed in MIG CVL levels between HIV-R and HIV-S (Figure 6).

No significant differences were observed in IP-10 levels in CVL samples both at (day 0) ($p=0.6490$), and following vaccination day 1 ($p=0.4051$) and day 30 ($p=0.9087$) in HIV-

R and HIV-S (Figure 7). RANTES level in CVL was detected in two samples only, the rest fell below detectable limit both at baseline and after vaccination whereas MCP-1 was only observed in some women at day 0 and 30 therefore could not be analyzed hence (data not shown).

4.0 Chemokine Time response in CVL samples from HIV-R and HIV-S

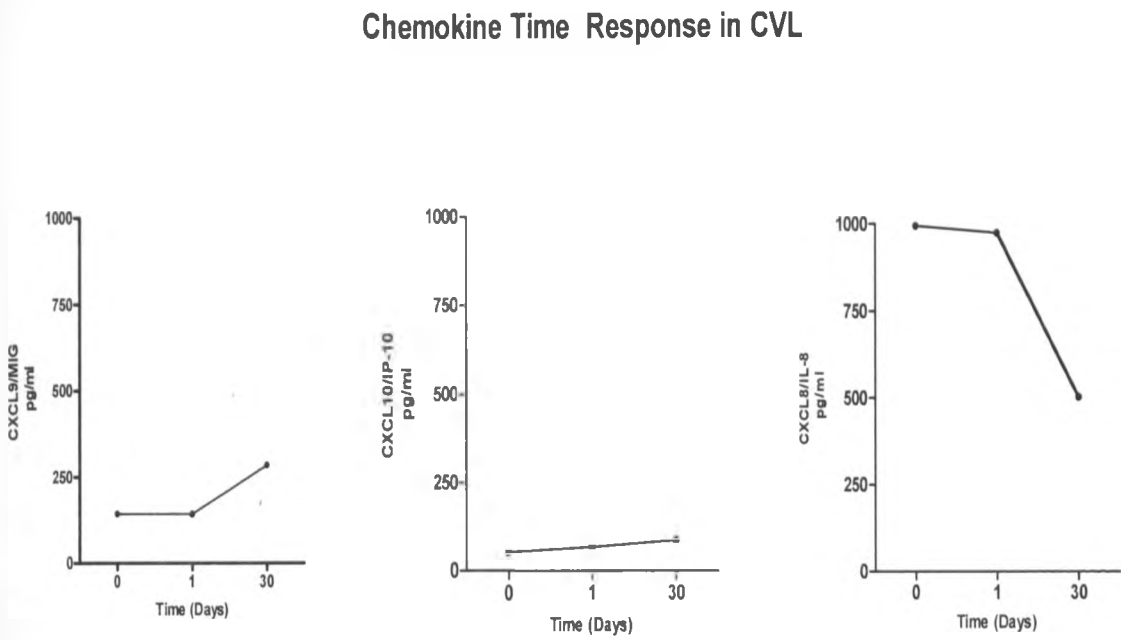


Figure 4: Overall Time Response Curves for chemokine level in CVL from HIV-R and HIV-S

The figure above represents the results from both HIV-R and HIV-S. Based on selected Time-Response curve for different chemokine in CVL samples from HIV-R and HIV-S there was a gradual increase in IP-10 from baseline (day 0) and following vaccine administration (day 1 and 30). IL-8 level decreased gradually after vaccine administration. MIG level in CVL increased gradually after vaccination.

4.1 Comparison chemokine level in CVL from HIV-R and HIV-S

4.1.1 IL-8 Chemokine level in CVL

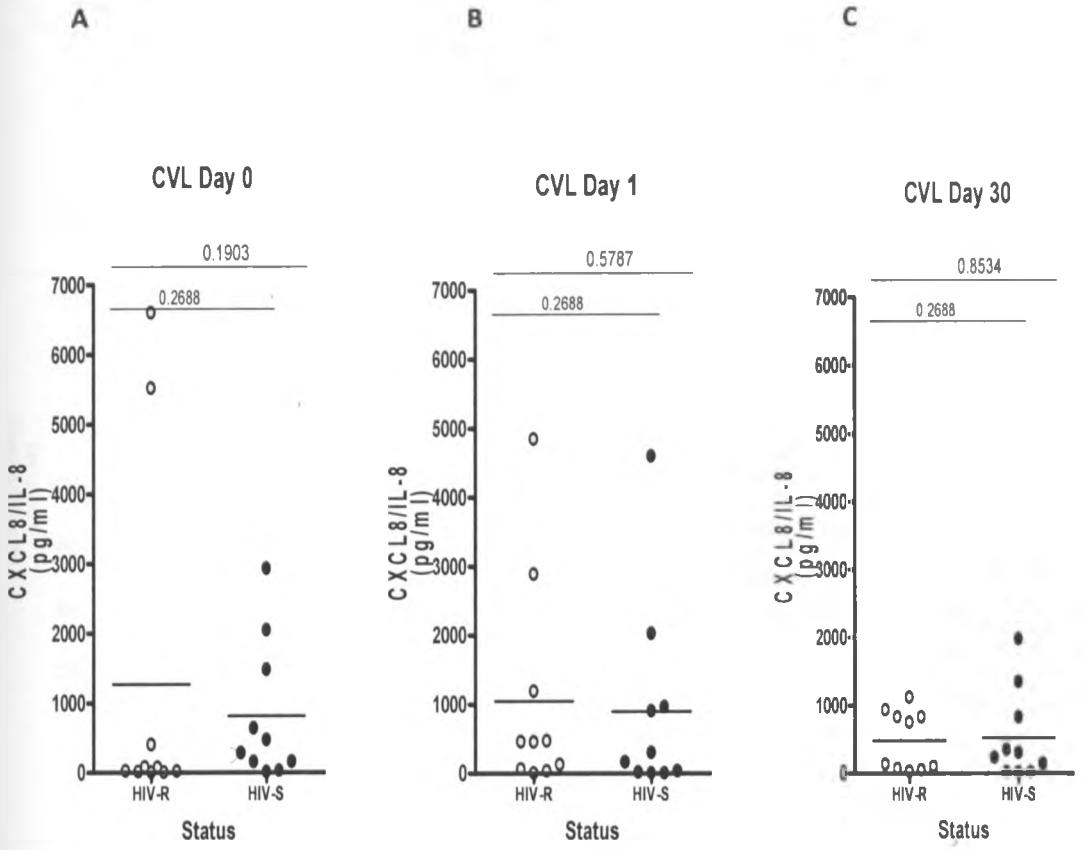


Figure 5: IL-8 level in CVL samples from HIV-R and HIV-S women before and after FluMist Vaccination

Levels of IL-8 in CVL samples collected from study participants prior to (A) and following (B, C) vaccination with FluMist were measured and compared. IL-8 levels (pg/ml) are depicted along the y-axis and HIV status along the x-axis. The HIV resistant (HIV-R) (n=10) represented in grey and HIV susceptible (HIV-S) (n=10) women represented in black. No significant differences were observed between HIV-R and HIV-S women at baseline (p=0.1903), and following vaccination day 1 (p=0.5787) and day 30 (p=0.8534).

4.1.2 MIG Chemokine level in CVL

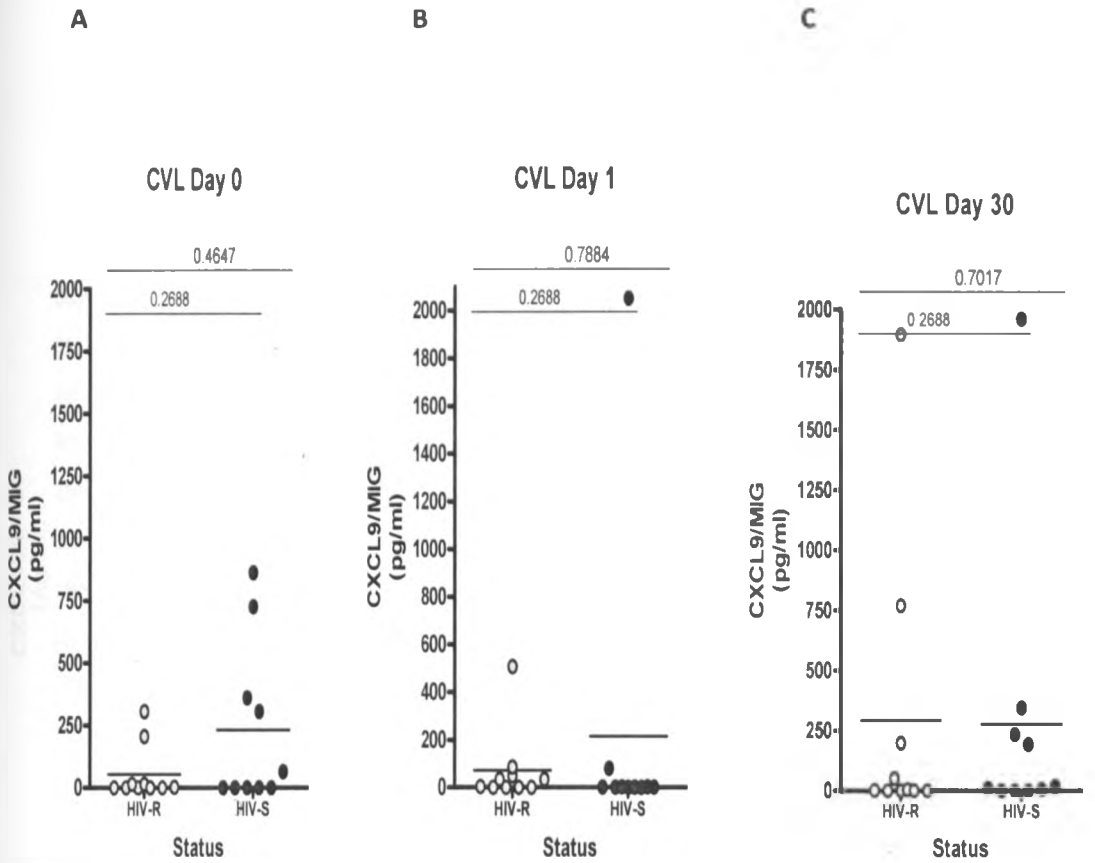


Figure 6: MIG level in CVL samples from HIV-R and HVI-S women before and after to FluMist vaccination

Levels of MIG in CVL samples collected from study participants prior to (A) and following (B, C) vaccination with FluMist were measured and compared. MIG levels (pg/ml) are depicted along the y-axis and HIV status along the x-axis. The HIV resistant (HIV-R) (n=10) represented in grey and HIV susceptible (HIV-S) (n=10) women represented in black. No significant differences were observed between HIV-R and HIV-S women at baseline (day 0) (p=0.4647), and following vaccination day 1 (p=0.7884) and day 30 (p=0.7017).

4.1.3 IP-10 Chemokine level in CVL

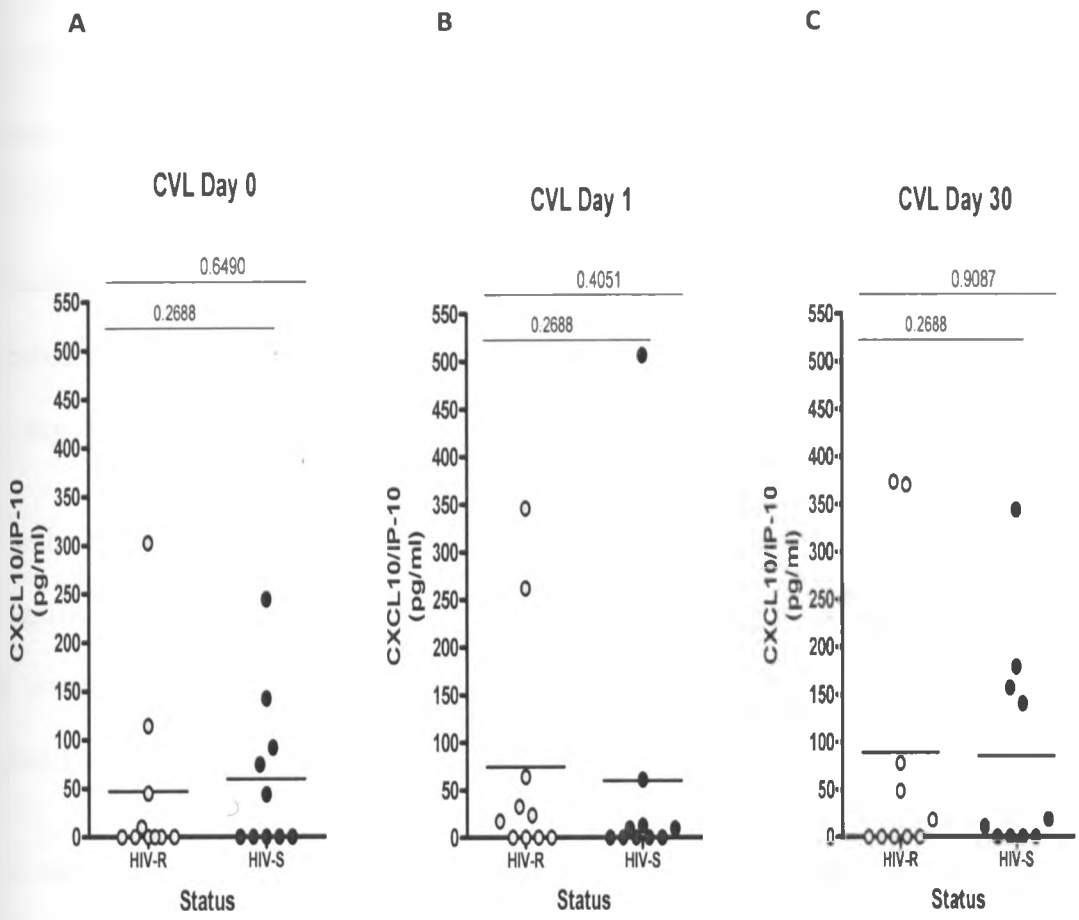


Figure 7: IP-10 level in CVL samples from HIV-R and HIV-S women before and after FluMist vaccination

Levels of IP-10 in CVL samples collected from study participants prior to (A) and following (B, C) vaccination with FluMist vaccine. IP-10 levels (pg/ml) are depicted along the y-axis and HIV status along the x-axis. The HIV resistant (HIV-R) (n=10) represented in grey and HIV susceptible (HIV-S) (n=10) women represented in black. No significant differences were observed between HIV-R and HIV-S women at baseline (day 0) (p=0.6490), and following vaccination day 1 (p=0.4051) and day 30 (p=0.9087).

In Plasma samples five chemokines were quantitatively measured: IL-8, RANTES, MIG, MCP-1, and IP-10.

IL-8 was only observed in some women at baseline and 30, majority of day 7 plasma samples were below detectable range (data not shown).

Based on selected Time-Response curve for different chemokine in plasma samples from HIV-R and HIV-S combined there was an increase from baseline and following vaccine administration in MCP-1 level. Whereas for IP-10 and MIG in plasma there was a decrease in chemokine level following vaccine administration, an increase was observed at day 30 (Figure 8).

On the other hand RANTES level in plasma was above standard range (data not shown).

Significant difference in MIG level in plasma was at day 7 ($p=0.0231$) observed in HIV-R compared to HIV-S women. No significant differences were observed at baseline ($p=0.1981$.) and at day 30 ($p=0.5693$) between the two groups (Figure 11).

No significant differences were observed in IP-10 plasma level between HIV-R and HIV-S women both at baseline (day 0) ($p=0.8534$), and following vaccination day 7 ($p=0.5787$) and day 30 ($p=0.6842$). A gradual decrease in IP-10 level was observed from day 0 to 7 among HIV-R compared to HIV-S (Figure 9).

No significant differences were observed in plasma MCP-1 level at baseline (day 0) ($p=0.2668$), and following vaccination day 7 ($p=0.1641$) and day 30 ($p=0.8181$) between the two groups (Figure 10).

4.2 Chemokine Time response in plasma samples from HIV-R and HIV-S

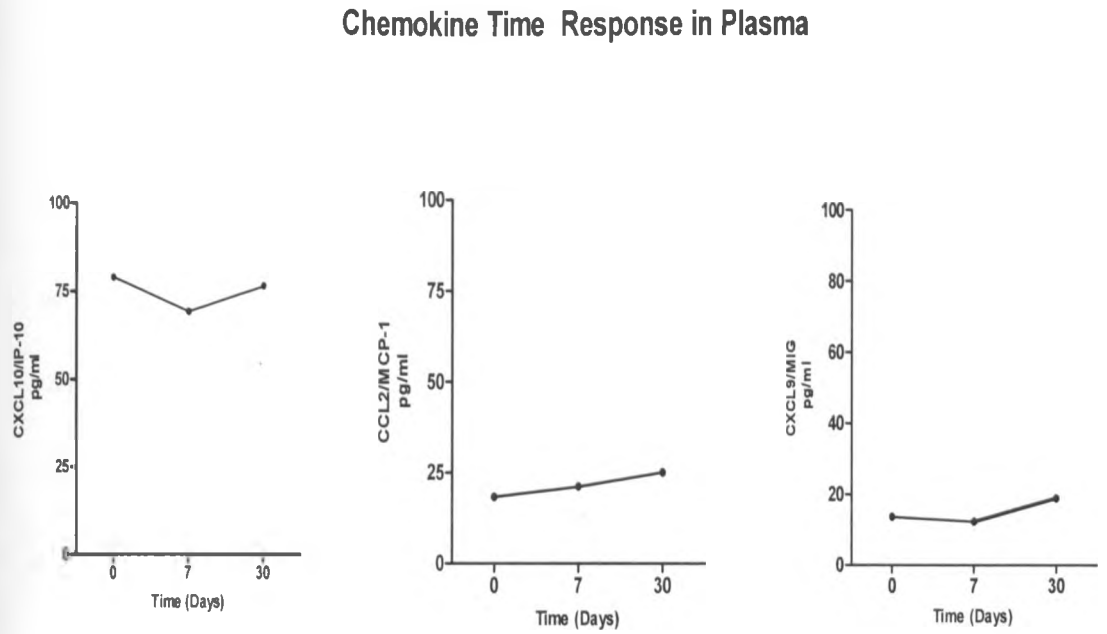


Figure 8: Overall Time Response Curves for chemokine level in Plasma from HIV-R and HIV-S

The figure above represents the results from both HIV-R and HIV-S. Based on selected Time-Response curve for different chemokine in plasma samples there was an increase from baseline and following vaccine administration in MCP-1 level. Whereas for IP-10 and MIG in plasma there was a decrease in chemokine level following vaccine administration, an increase was observed at day 30.

4.3 Comparison of Chemokine level in Plasma from HIV-R and HIV-S

4.3.1 IP-10 level in Plasma

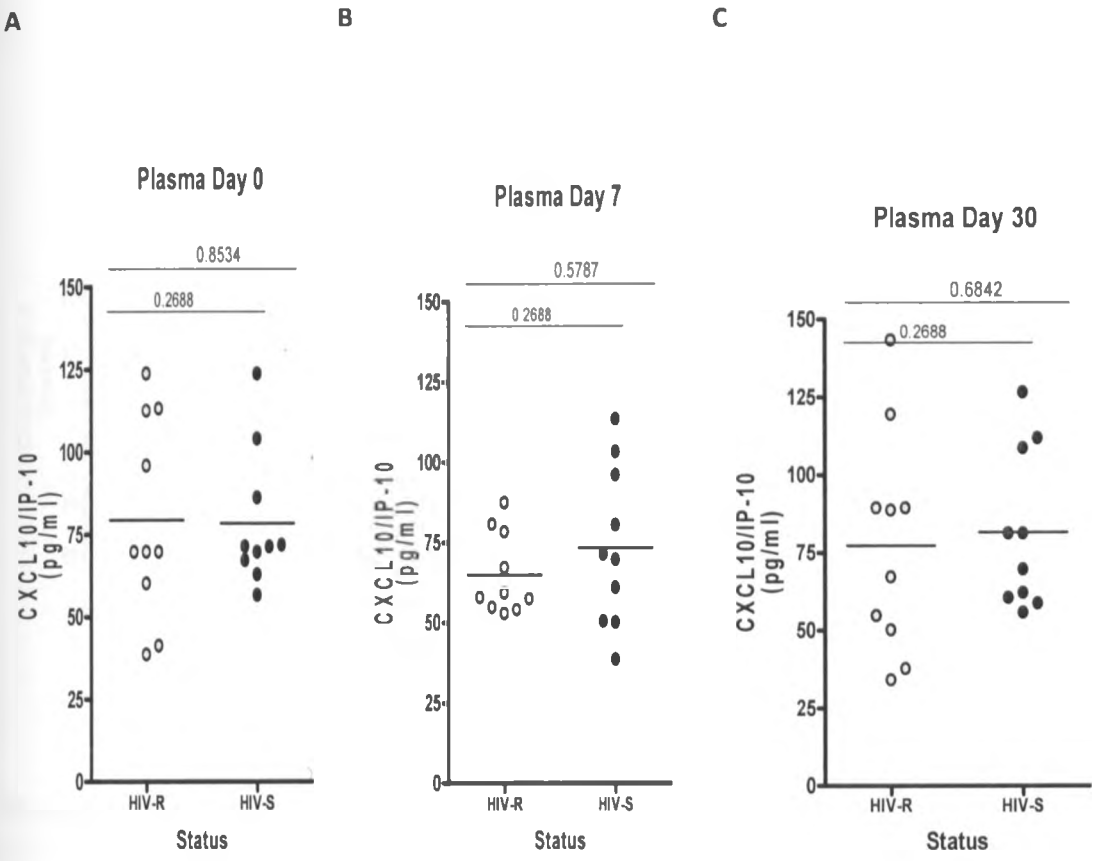


Figure 9: IP-10 level in Plasma samples from HIV-R and HIV-S women before and after FluMist vaccination

Levels of IP-10 in Plasma samples collected from study participants prior to (A) and following (B, C) vaccination with FluMist vaccine. IP-10 levels (pg/ml) are depicted along the y-axis and HIV status along the x-axis. The HIV resistant (HIV-R) (n=10) represented in grey and HIV susceptible (HIV-S) (n=10) women represented in black. No significant differences were observed between HIV-R and HIV-S women at baseline (day 0) (p=0.8534), and following vaccination day 7 (p=0.5787) and day 30 (p=0.6842).

4.3.2 MCP-1 Chemokine level in Plasma

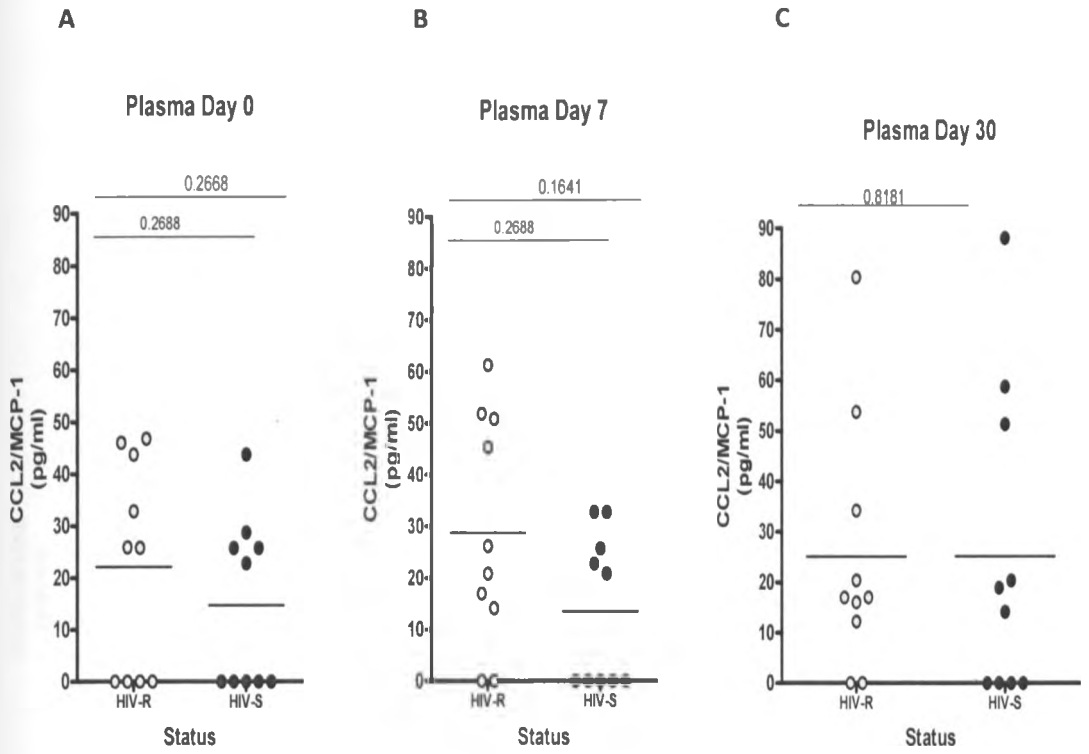


Figure 10: MCP-1 level in Plasma samples from HIV-R and HIV-S women before and after FluMist vaccination

Levels of MCP-1 in Plasma samples collected from study participants prior to (A) and following (B, C) vaccination with FluMist vaccine. MCP-1 levels (pg/ml) are depicted along the y-axis and HIV status along the x-axis. The HIV resistant (HIV-R) (n=10) represented in grey and HIV susceptible (HIV-S) (n=10) women represented in black. No significant differences were observed between HIV-R and HIV-S women at baseline (day 0) (p=0.2668), and following vaccination day 7 (p=0.1641) and day 30 (p=0.8181).

4.3.3 MIG Chemokine level in Plasma

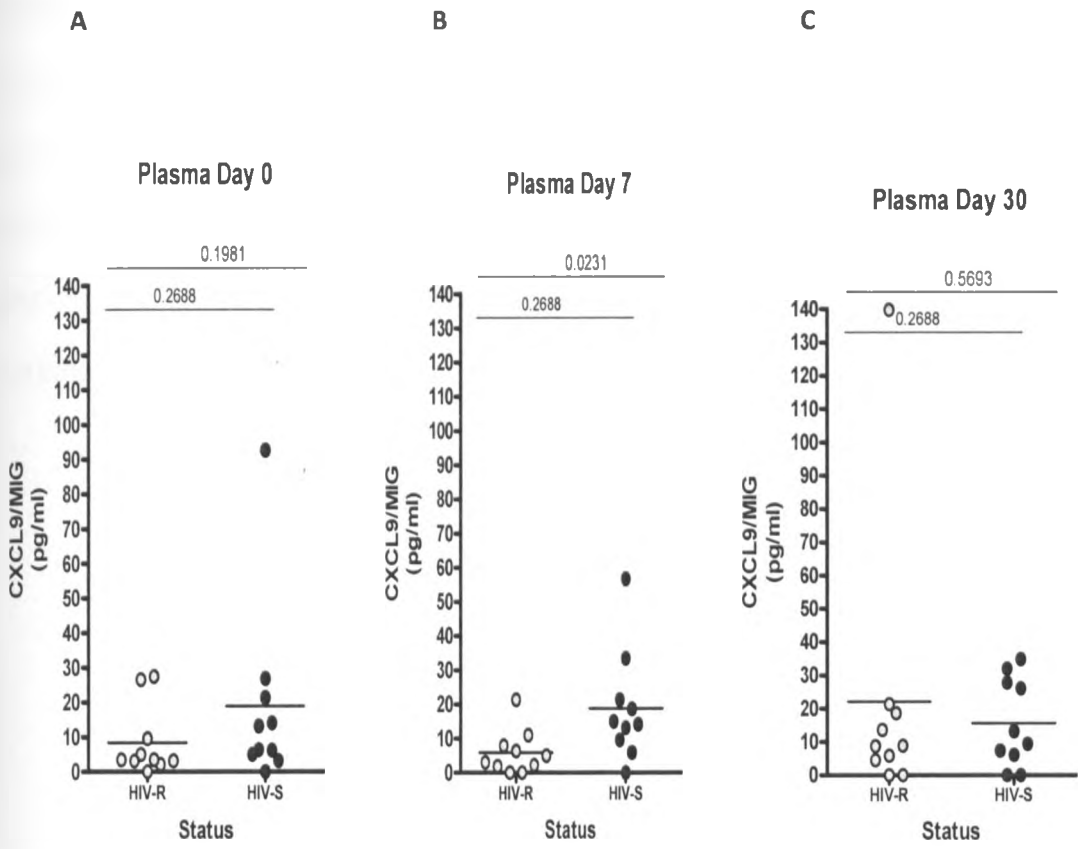


Figure 11: MIG level in Plasma samples from HIV-R and HIV-S women before and after FluMist vaccination

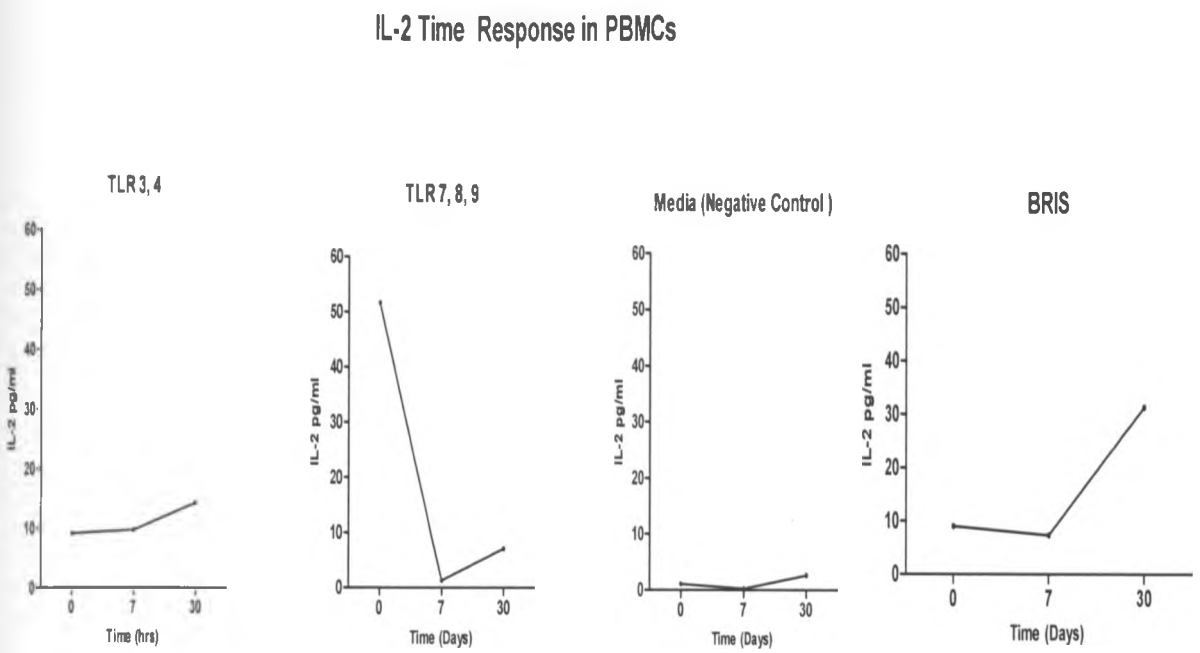
Levels of MIG in Plasma samples collected from study participants prior to (A) and following (B, C) vaccination with FluMist vaccine. MIG levels (pg/ml) are depicted along the y-axis and HIV status along the x-axis. The HIV resistant (HIV-R) (n=10) represented in grey and HIV susceptible (HIV-S) (n=10) women represented in black. Significant difference in MIG level was observed between HIV-R and HIV-S women at day 7 (p=0.0231). No significant differences were observed at baseline (p=0.1981.) and at day 30 (p=0.5693) between HIV-R and HIV-S.

4.4Cytokine and chemokine profiles in supernatants of stimulated PBMCs

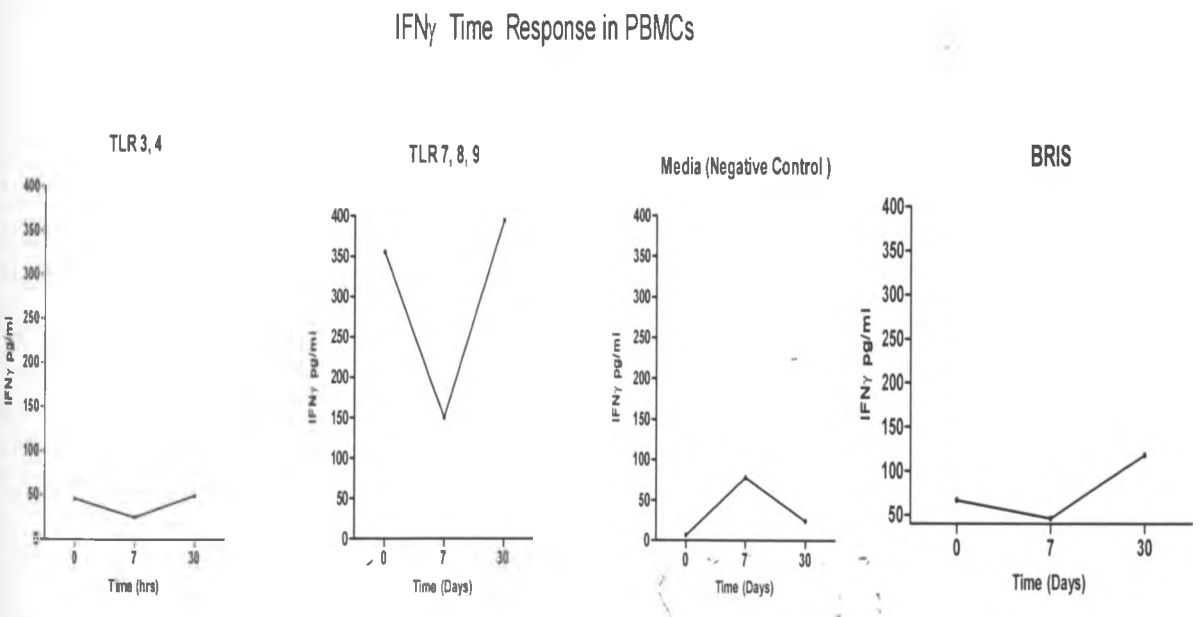
PBMCs were isolated from HIV-R and HIV-S participants, 100,000 or 100µl cells in suspension were stimulated with agonists specific for TLR3 (poly I:C), TLR4 (LPS), TLR7 (imiquimod), TLR7/8 (ssRNA40) and TLR9 (CpG ODN). Unstimulated cells served as negative controls (media only). Supernatants harvested were tested for cytokine and chemokine content using multiplex analysis assay on Luminex as described in the text. Results are presented as median cytokine and chemokine levels in picograms per milliliter (pg/ml).

4.4.1 Time response in PBMCs

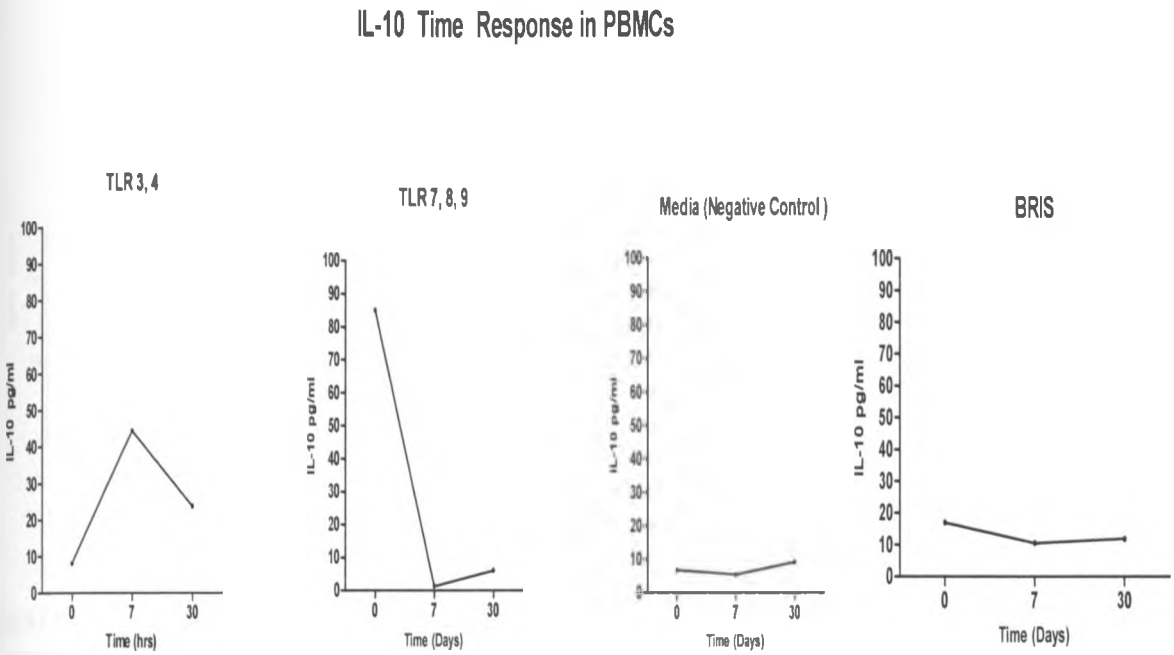
a) IL-2 Time responses in PBMCs



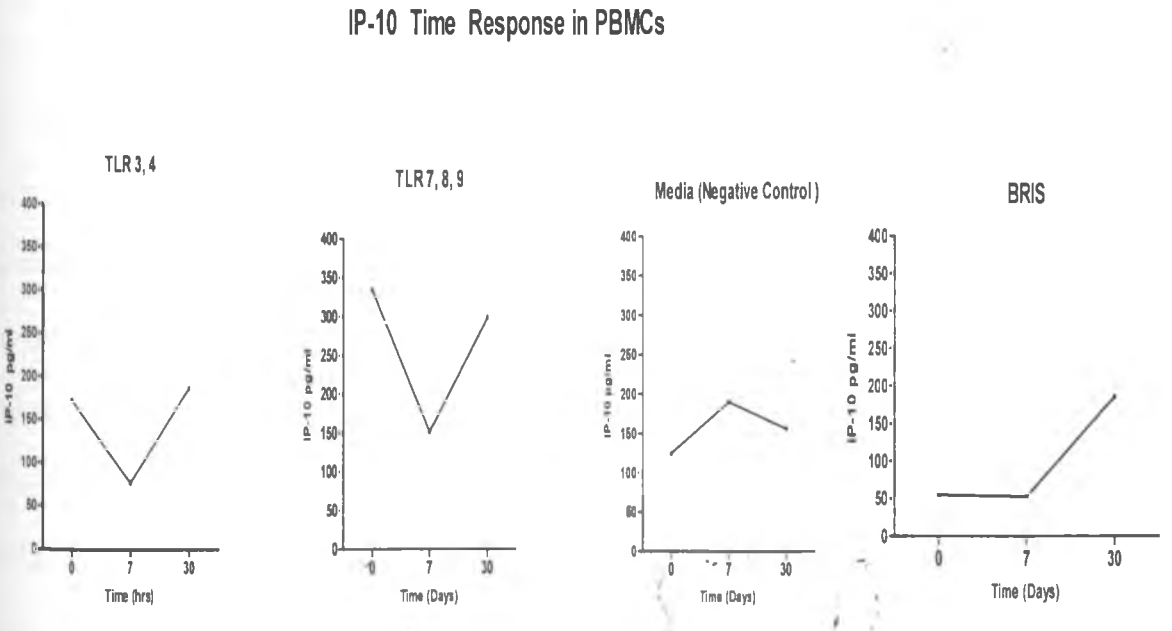
b) IFN-γ Time responses in PBMCs



c) IL-10 Time responses in PBMCs



d) IP-10 Time responses in PBMCs



e) TNF-α Time responses in PBMCs

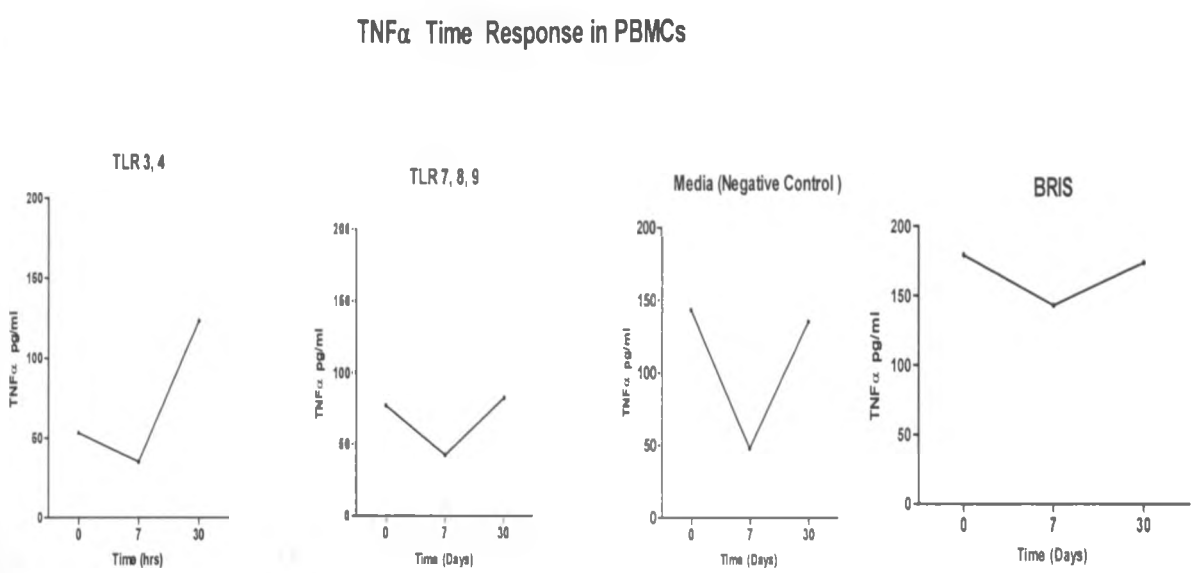


Figure 12: Overall Time Response Curves for chemokine and cytokine from stimulated PBMCs HIV-R and HIV-S women a) IL-2 responses b) IFN-γ responses c) IL-10 responses d) IP-10 responses e) TNF-α responses.

The figure above represents the results from both HIV-R and HIV-S. Based on time response curve IL-2 responses after vaccine administration decreased in unstimulated and PBMCs stimulated with TLR 7, 8, 9, and Brisbane while there was an increase in its level in TLR 3, 4 stimulated PBMCs (a). There was an increase in IFN-γ responses after vaccination in unstimulated PBMCs while there was a decrease after stimulations with TLR 3, 4, TLR 7, 8, 9 agonists and Brisbane (b). Stimulation of PBMCs by TLR 3, 4 ligands showed an increased IL-10 cytokine level after vaccination while there was a decrease in unstimulated and PBMCs stimulated with TLR 7, 8, 9 and Brisbane (c). IP-10 levels after vaccination decreased TLR 3, 4, TLR 7, 8, 9 agonist and *Influenza* virus strain Brisbane while there was an increase in its level in unstimulated PBMCs (d). TNF-α reponses showed a decrease in the negative control (media only) and the three stimulants after vaccine administration (e).

Chemokine and Cytokine responses to TLR 3 and 4 ligands

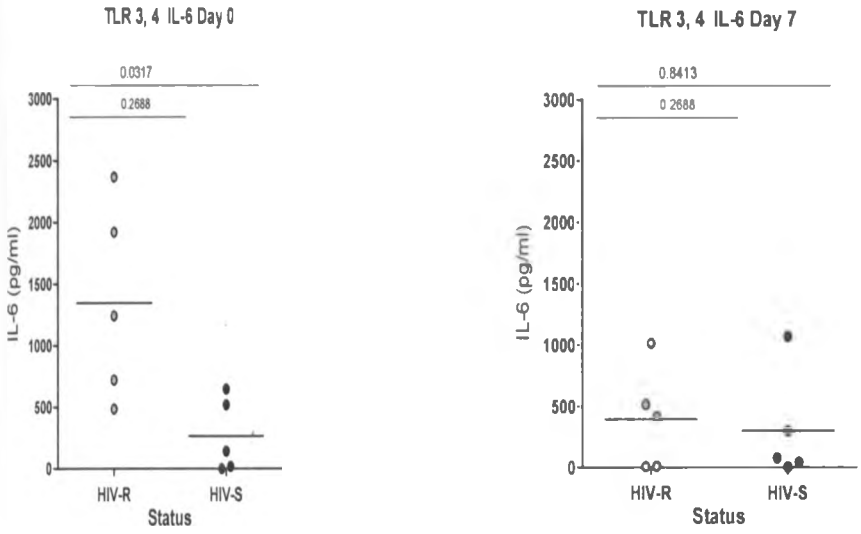
Cytokine responses by PBMCs to TLR 3 and 4 induced levels of IL-6, IFN-γ, and IL-2 responses. No detectable responses were observed in other cytokine and chemokine analyzed. The level of IL-6 at baseline was observed to be significantly higher in HIV-1 resistant women compared to HIV susceptible women ($p=0.0137$). No significant differences were observed post vaccination day 7 ($p=0.8413$) and day 30 ($p=0.1905$) (Figure 13).

The level of IFN-γ in HIV-1 resistant women was significantly higher compared to HIV susceptible women both at baseline ($p=0.0079$) and at day 7 post vaccination ($p=0.0137$) with no significant difference observed at day 30 ($p=0.0952$) though HIV-R appeared to have heightened responses to TLR3,4 ligands than the HIV-S individuals (Figure 15).

IL-2 responses to TLR 3, 4 were observed to be significantly higher in HIV-R than HIV-S at baseline ($p=0.0137$) and day 30 post vaccination ($p=0.0079$), no significant differences were observed at day 7 ($p=0.2904$) (Figure 14).

4.5 TLR 3 and 4 responses in PBMCs from HIV-R and HIV-S

a) b)



c)

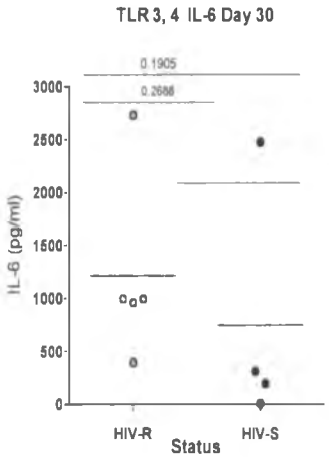
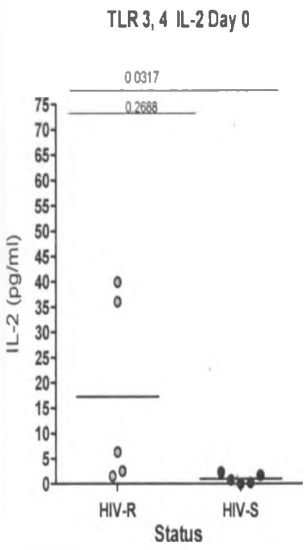


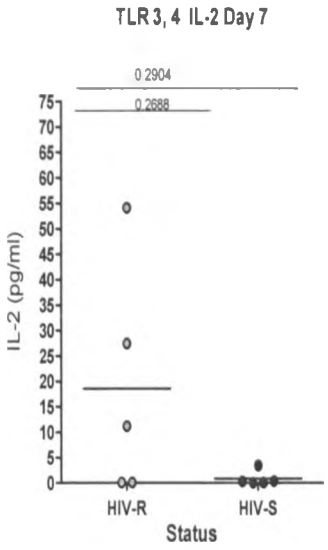
Figure 13: IL-6 Responses following stimulation of PBMCs with TLR 3 and 4 ligands before (a) and after FluMist vaccination (b, c)

Levels of IL-6 are depicted along the y-axis and HIV status along the x-axis. The HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. Significant high IL-6 level was observed at day 0 (p=0.0317) in HIV-R individuals compared to HIV-S, these differences disappeared after vaccination day 7 (p=0.8413) and 30 (p=0.1905).

a)

b)





c)

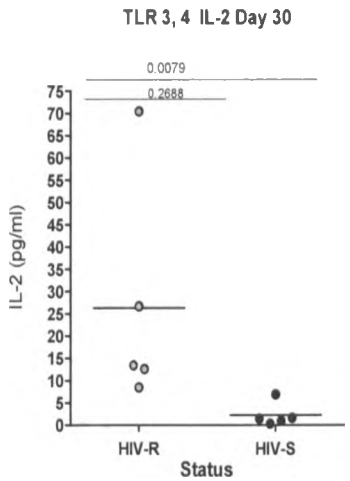
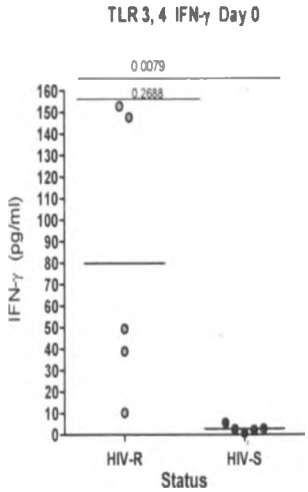


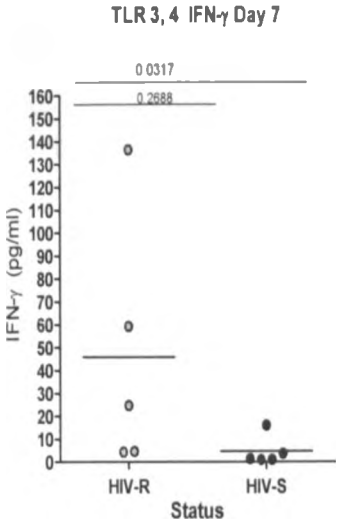
Figure 14: IL-2 Responses following stimulation of PBMCs with TLR 3 and 4 ligands before (a) and after FluMist vaccination (b, c)

IL-2 cytokine levels are depicted along y-axis (pg/ml) and HIV status along the x-axis. The HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. The level of IL-2 was significantly higher in HIV-R compared to HIV-S women at baseline (p=0.0317) and day 30 post-vaccinations (p=0.0079), no significant difference between the two groups was observed at day 7 (p=0.2904).

a)



b)



c)

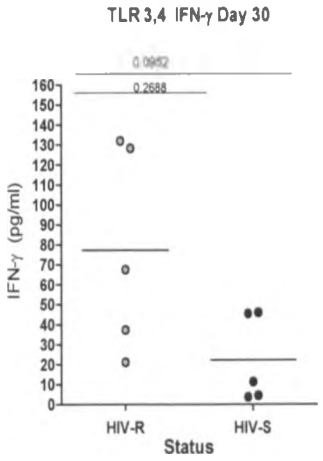


Figure 15: IFN-γ Responses following stimulation of PBMCs with TLR 3 and 4 before (a) and after FluMist vaccination (b, c)

Level of IFN-γ (y-axis pg/ml) and different HIV status (X-axis), the HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. IFN-γ level observed at day 0 (p=0.0079), day 7 (p=0.0317) were significantly higher in HIV-R individuals as compared to HIV-S. There was no significant difference observed between HIV-R and HIV-S at day 30 (p=0.0952). Horizontal line on the graph indicate group median.

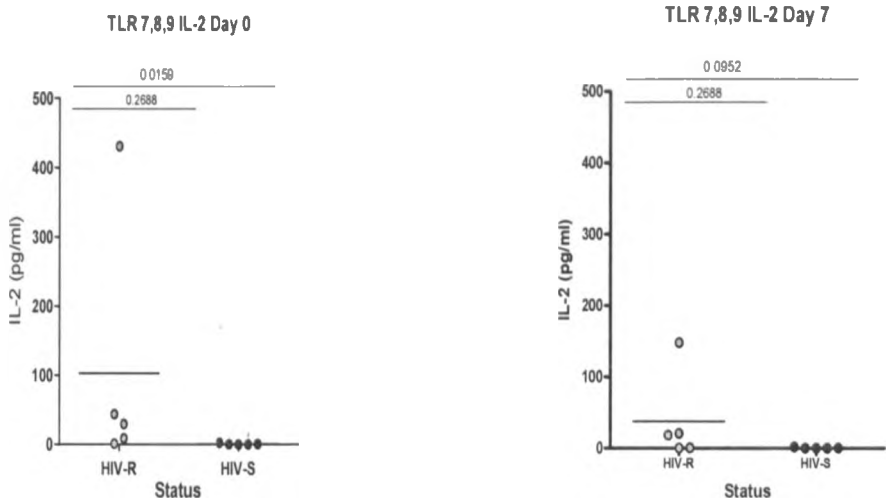
Chemokine and Cytokine responses to TLR 7, 8 and 9 ligands

Following stimulation of PBMCs with TLR 7, 8 and 9 ligands HIV-R women had significantly elevated level of IL-2 cytokine compared to HIV-S at baseline (p=0.0159) and after vaccine administration day 30 (0.0079). Significant difference was not observed day 7 (p=0.0952) post vaccination between the two groups (Figure 16).

The level of IP-10 chemokine by TLR 7, 8, 9 stimulated PBMCs were significantly lower in HIV-1 susceptible women compared to HIV-1 resistant at day 30 post vaccination (p=0.0137) , no significant differences were observed at baseline (p=0.1508) and day 7 (p=0.5476) (Figure 17).

4.6 TLR 7, 8 and 9 responses in PBMCs from HIV-R and HIV-S

a) b)



c)

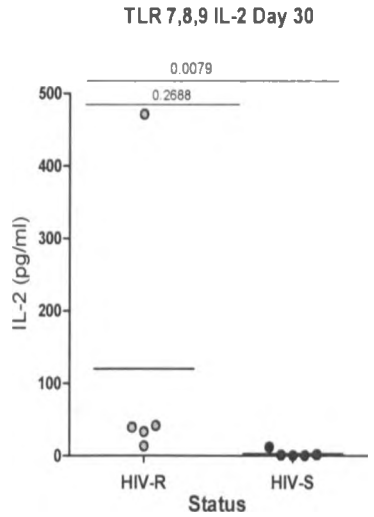
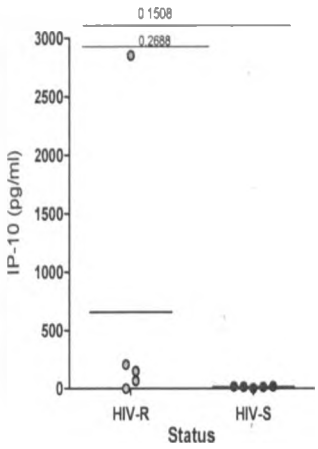


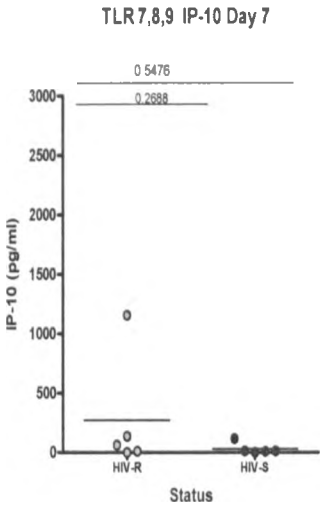
Figure 16: IL-2 Responses following stimulation of PBMCs with TLR 7, 8 and 9 ligands before (a) and after FluMist vaccination (b, c)

IL-2 cytokine level (y-axis pg/ml), HIV status (X-axis) the HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. At baseline (p=0.0159) and day 30 (p=0.0079) post vaccination, IL-2 level was significantly higher in HIV-R compared to HIV-S women, no significant difference was observed at day 7 (p=0.0952) between the two groups.

a)



b)



c)

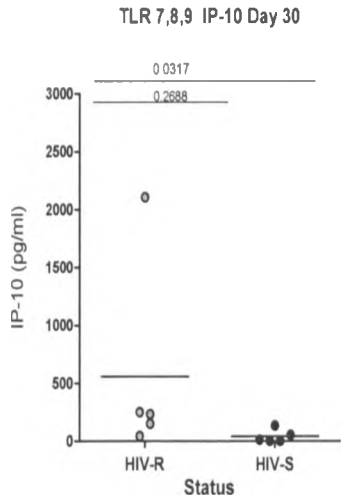


Figure 17: IP-10 Responses following stimulation of PBMCs with TLR 7, 8 and 9 ligands before (a) and after vaccination (b, c)

Level of IP-10 chemokine (y-axis pg/ml) and HIV status (X-axis), the HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. IP-10 level observed at day 30 (p=0.0317) post vaccination were significantly over expressed in HIV-R individuals compared to HIV-S. There was no significant difference observed between the two groups at baseline (0.1508) and day 7 (p=0.5476). Horizontal line on the graph indicate group median.

Chemokine and Cytokine responses to Brisbane virus

The level of IL-2 from *Influenza* (Brisbane strain) virus stimulated PBMCs were significantly higher in HIV-R compared to HIV-S at day 30 post vaccine administration (p=0.0137), no significant differences were observed at baseline (p=0.1508) and day 7 (p=0.6905) (Figure 18).

IL-10 level in HIV resistant women was significantly higher than in HIV susceptible (p=0.0362) 7 days after vaccination, no significant differences were observed at baseline (p=0.9166) and at day 30 (p=0.1732) (Figure 19).

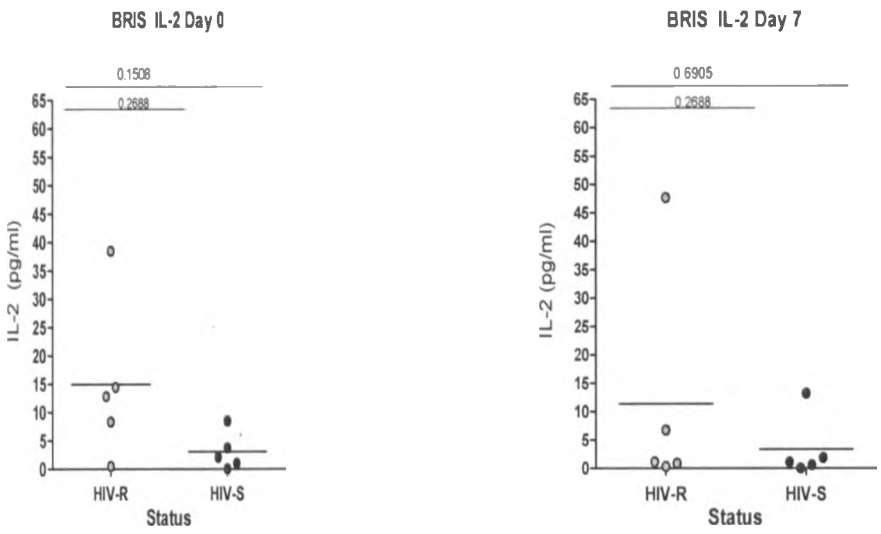
The level of TNF-α responses in PBMCs after stimulation with *Influenza* virus (Vaccine strain Brisbane), in both HIV–R and HIV-S women at baseline appeared to be similar (p=0.2222). Interestingly after 7 and 30 days post vaccination HIV-R women showed heightened responses with a significantly high level of TNFα at Day-7 (p=0.0137) and day 30 (p=0.0137) (Figure 21).

On the other hand IL-12(p40) levels were similar in the two groups before (p=0.1945) and 7 days (p=0.2477) after vaccination. However after 30 days significant high levels were observed among HIV-R women (p=0.0137) (Figure 20).

No significant differences were observed between HIV-R and HIV-S in IL-6 and IP-10 levels at different time point following stimulation of PBMCs with *Influenza* virus (Vaccine strain Brisbane) results not shown.

4.7 BRIS responses in PBMCs in HIV-R and HIV-S

a) b)



c)

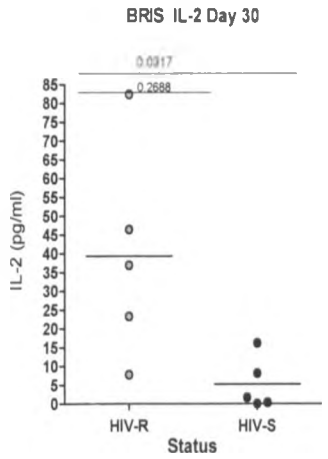
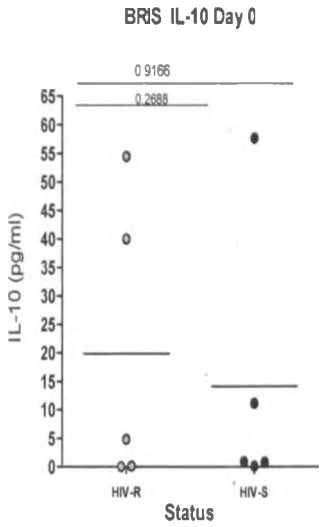


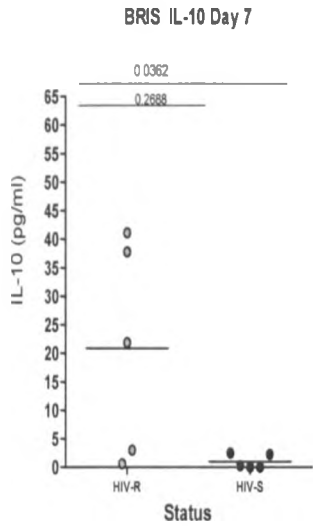
Figure 18: IL-2 Responses following stimulation of PBMCs with Brisbane virus before (a) and after vaccination (b, c)

IL-2 cytokine level (y-axis pg/ml) by PBMCs of women with different HIV status (X-axis) following stimulation with *Influenza* virus strain Brisbane before and after *Influenza* vaccination. The HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. IL-2 levels in HIV-R were significantly higher at day 30 (p=0.0317) post vaccination compared to HIV-S, no significance at day 0 (p=0.1508) and 7 (p=0.6905) between the two groups.

a)

b)





c)

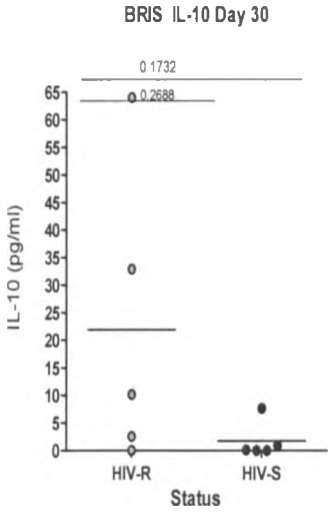
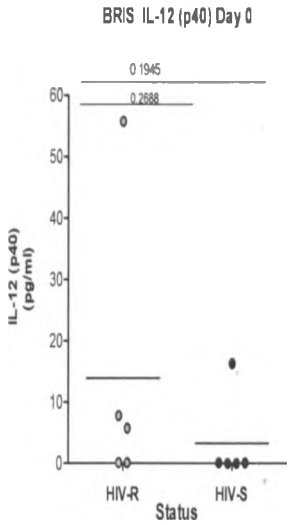


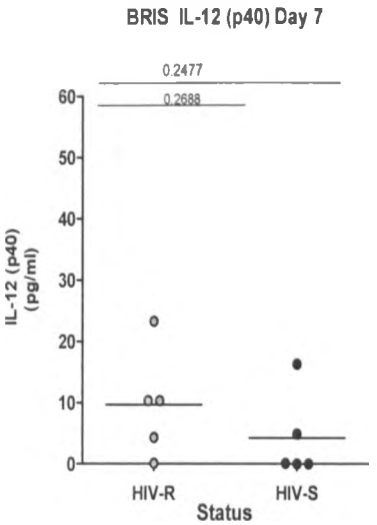
Figure 19: IL-10 Responses following stimulation of PBMCs with Brisbane virus before (a) and after vaccination (b, c)

IL-10 cytokine level (y-axis pg/ml) by PBMCs of women with different HIV status (X-axis) following stimulation with *Influenza* virus strain Brisbane before and after *influenza*-vaccination. The HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. Baseline (p=0.9166) levels were similar with significant difference observed at day 7 (p=0.0362) after vaccination. The significant differences disappeared at day 30 (p=0.1732).

a)



b)



c)

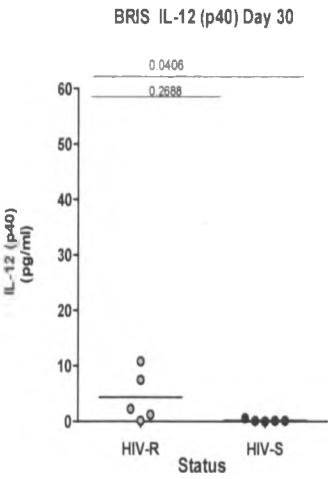
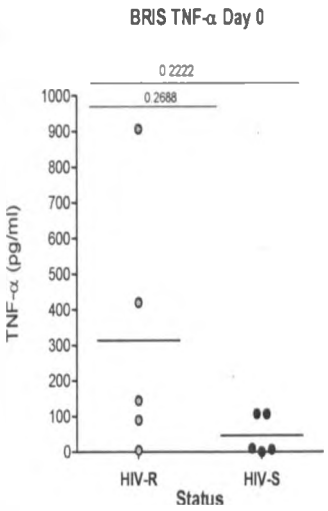


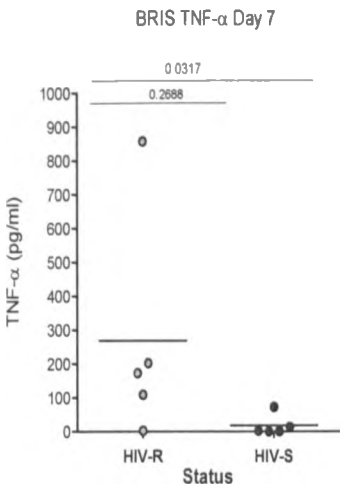
Figure 20: IL-12(p40) Responses following stimulation of PBMCs with Brisbane virus before (a) and after vaccination (b, c)

IL-12(p40) cytokine level (y-axis pg/ml) by PBMCs of women with different HIV status (X-axis) following stimulation with *Influenza* virus strain Brisbane before and after *influenza* vaccination. The HIV resistant (HIV-R) (n=5) represented in grey and HIV susceptible (HIV-S) (n=5) women represented in black. There was no significance observed between HIV-R and HIV-S, observed at baseline (p=0.1945) and 1 week (p=0.2477) after vaccination, significant difference was observed at day 30 (p=0.0406) HIV-R individuals showing heightened responses compared HIV-S.

a)



b)



c)

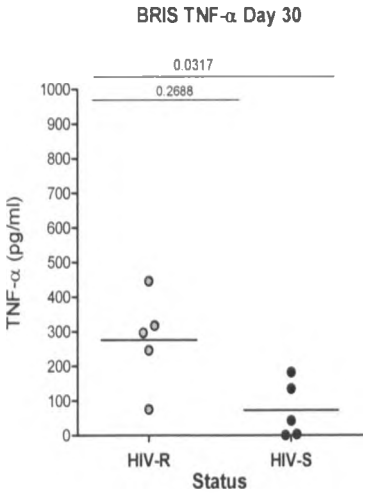


Figure 21: TNF-α Responses following stimulation of PBMCs with Brisbane virus before (a) and after vaccination (b, c)

TNF-α cytokine level (y-axis pg/ml) by PBMCs of women with different HIV status (X-axis) following stimulation with *Influenza* virus strain Brisbane before and after *Influenza* vaccination. The HIV-R (n=5) represented in grey and HIV-S (n=5) women represented in black. Significant high level of TNF-α was observed at day 7 (p=0.0317) and 30 (p=0.0317) with the HIV-R showing heightened responses compared to HIV-S. There was no significant difference observed at baseline (p=0.2222).

CHAPTER FIVE

5. DISCUSSION

Cytokine and chemokines responses following stimulation of PBMCs with TLRs ligands prior to and after vaccination

In my main objective PBMCs isolated from these women were stimulated with different endosomal TLRs that recognize nucleic acids, specifically double stranded (ds) RNA (TLR3 ligand), single stranded (ss) RNA (TLR 7/8 ligand) and unmethylated CpG DNA (TLR9 ligand) as well as transmembrane LPS (TLR4 ligand).

In vitro cytokine production by PBMCs can be an important and reliable measure of immuno-competence and activation since it includes the chemokines and cytokines produced by multiple cell types of the immune system (Friberg *et al.*, 1994). Also, spontaneous *in vitro* release of cytokines by PBMCs isolated from peripheral blood may serve as a measure of their activation *in vivo* (Boehlen *et al.*, 2001; Friberg *et al.*, 1994).

In this study *in vitro* production of cytokines/chemokines important in driving Th1/Th2 responses and immunoregulatory cytokines such as IL-10 in response to TLR-3, 4, 7, 8, 9 ligands in Peripheral blood mononuclear cells (PBMCs) from HIV-1 resistant and susceptible women at baseline and after vaccine administration was compared. In this study we also sought to determine cytokine and chemokine profile from PBMCs, stimulated by different stimulant (TLR ligands and Brisbane virus (vaccine strain). It was noted that most of the previous studies that have been done so far have examined the role of a single TLR pathway in mediating immune outcome this is mainly for simplicity. However, this is not the full picture. In the real world, such interactions are complex,

often encompassing multiple TLRs that interact with distinct signatures in a single virus. This is particularly true in the case of the Herpes simplex viruses (HSV) where HSV interacts with TLRs 2, 3, and 9, while CMV interacts with TLRs 2, 3, and 9 (Zhang *et al.*, 2007; Finberg *et al.*, 2005; Tabeta *et al.*, 2004; Sato *et al.*, 2006). This study is among the first to demonstrate altered innate cytokine/chemokine responses to TLRs ligand stimulation combined in immunologically distinct groups HIV-R and HIV-S.

Toll-like receptor 3 which recognizes double-stranded RNA (dsRNA), a molecular signature of most viruses, and triggers inflammatory responses that prevent viral spread. Although the role of TLR3 in controlling infections is not fully understood, it is clear that TLR3 non redundantly contributes to the prevention of herpes simplex encephalitis in children (Zhang *et al.*, 2007). Recent report of lentivirus vector-induced activation of TLRs su ggests that TLR3 may also be involved in sensing of dsRNA structures during HIV infection (Breckpot *et al.*, 2010).

Following stimulation of PBMCs with TLR 3 and 4 agonists combined there were no significant differences between the HIV-R and HIV-S individuals were observed in IL-10, IL-12(p40), IL-12(p70) and TNF-α. HIV-R showed significant elevated responses in IL-6, IFN-γ and IL-2 cytokines compared to HIV-S individuals.

Elevated IL-10 levels were observed in HIV-R individuals compared to HIV-S 1 week after vaccine administration after stimulation of PBMCs with TLR 3, 4. Interleukin-10 (IL-10) cytokine plays a crucial immunosuppressive role during excessive Th1 responses. Hence it can protect the host from potentially damaging immunopathology. However, excessive IL-10 production suppresses host immune responses and can inadvertently

facilitate the ability of intracellular pathogens to escape host innate immune defenses. IL-10 cytokine being an anti-inflammatory cytokine that suppresses the secretion of pro-inflammatory cytokines it has been shown to inhibit IFN-γ, IL-2 and IL-4 activity (Pestka, 2004). Previous studies have observed that IL-10 block HIV virus replication in monocyte-derived macrophage cultures *in vitro,* and prevent HIV-1 induced TNF-α and IL-6 release (Weissman, 1994). It has also been demonstrated that IL-10 produced by CD4+ T cells had an antiviral effect, by diminishing HIV-1 viral replication and immune dysfunction such as anergy (Andrade, 2007; Green and Beere, 2000). In this study I observed in HIV-R an increase in IL-10 level compared to HIV-S individuals after vaccination with a return to basal level at day 30. On the other hand IL-2 level increased after vaccination and the same trend was observed at day 30. T helper 1 (Th1) cytokine responses are essential for eradicating invading intracellular pathogens where as vigorous pro-inflammatory cytokines induction may cause a cytokine storm that leads to severe immunopathology when appropriately timed feedback mechanism via production of anti-inflammatory cytokines is not observed.

In this study increasing responses of IL-2 observed in HIV-R individuals from day 7 and 30 post vaccination with no parallel feedback mechanism via production of IL-10 observed may cause cytokine storm. This may lead to HIV-R individuals become more susceptible to infection by the activation of HIV-1 susceptible cells hence leads to enhancement of HIV-1 infection. Following stimulation of PBMCs with LPS and dsRNA the HIV-1 resistant women showed significant difference in IL-6 level at day 0 (baseline) compared to HIV-S individuals. No significant differences were observed at day 7 and 30 post vaccination. A significant increase in IL-2 levels was seen at baseline and at day 30

post vaccination in HIV-R compared to HIV-S. IFN-γ levels was found to be significantly higher in HIV-R compared to HIV-S at baseline and at day 7 post vaccine administration, no significant difference was observed between HIV-R and HIV-S at day 30. Increase in IL-2 levels observed in HIV-R after the vaccine challenge despite IL-10 increment may be due to dual stimulation of both T and B lymphocytes; in contrast HIV-S showed a decrease in IL-2 after the vaccine challenge there was a slight increase in IL-10 observed. Inhibition of IL-2 cytokine which plays a central role in containment of viral infection and elimination by activation and proliferation of both CD4+, CD8+ T cells. IL-2 production has been found to be reduced in infected PBMCs as well as HIV infected individuals, this reduction has been correlated to an increased production of anti-inflammatory cytokines IL-10 and IL-4 that inhibits the production of all pro-inflammatory cytokines and chemokines and the expression of DC-costimulatory molecules, therefore shutting-off T cell activation to prevent over-stimulation of helper T cells while still maximizing the production of antibodies, thus inhibition of IL-2 could limit the magnitude of the overall immune response (Barcova *et al.*, 1998; Kawamura *et al.*, 2003a; Klein *et al.*, 1997; Zerhouni *et al.*, 1997; Honda *et al.*, 1989; Klein *et al.*, 1997; Rook *et al.*, 1985; Granucci *et al.*, 2001, 2003; Napolitano, 2003; Moore *et al.*, 2001).

IL-10 has been found to upregulate *in vitro* CXCR4 expression and X4 HIV infection of DCs, although this did not affect the efficiency of viral transmission to autologous CD4+ T cells, an event involving DC-SIGN rather than conventional viral receptors (Ancuta *et al.*, 2001). Studies have shown that Systemic IL-10 production is increased in various human chronic viral infections, such as hepatitis C virus, human immunodeficiency virus

and hepatitis B virus (Clerici *et al.*, 1994; Accapezzato *et al.*, 2004; Woitas *et al.*, 2002; Ameglio *et al.*, 1994). The ability of IL-10 to down-regulate IFN-γ production is a consequence of its ability to inhibit accessory cell function, including production of cytokines and expression of costimulatory molecules that are necessary for optimal stimulation of T cells.

Stimulation of PBMCs with an analogue of HIV-1 ssRNA and unmethylated CpG DNA led to significant differences in IP-10 chemokine and IL-2 cytokine level. HIV-R women showed significant elevated level of interleukin 2 cytokine at baseline (day 0) and day 30 post vaccination compared to the HIV-S individuals. A decrease in its level was also observed one week after vaccine administration (day 7). On the other hand human IFN-gamma-inducible 10-kDa protein (IP-10) a CXCL10 chemokine which is expressed on activated Th1 lymphocytes functions as chemoattractants for human monocytes activation. T cells progenitor maturation, and direct migration of activated Th1 cells (Farber, 1997; Luster *et al.*, 1987). In this study the HIV-resistant women had elevated level of the chemokine at baseline and one week after vaccination compared to the HIV-S. A decrease after vaccination was observed, a significant difference in elevated level in HIV-R was observed at day 30. Previous studies observed that IP-10 may be a critical component present in breast milk transferred to the infants to fight infections (Garofalo and Goldman, 1998; Takahata *et al.*, 2003).

An earlier study from this cohort has shown that HIV-resistant women have elevated IL-10 responses to TLR-2 and 7 ligand ($p=0.001$ and 0.021), elevated IL-12p40 to TLR-4 ligand, ($p=0.009$) and depressed IFN-γ responses to TLR-4 and 7 ligand ($p=0.021$ and 0.013) respectively (Ball *et al.*, 2006). These observations were associated with a

hyporesponsive innate response rather than a preference to generate cellular immune responses (Ball *et al., 2006*). Heil *et al* in 2004 demonstrated that guanine-uridine-rich ssRNA derived from HIV is recognized by TLR7/8 and stimulates DCs and macrophages to secrete IFN-α and pro-inflammatory cytokines, TLR7/8 agonist has also been shown to stimulate both human and rat peripheral blood mononuclear cells to produce IFN-α, TNF-α, and IL-12 p40/70 (Heil *et al.*, 2004).

At baseline following stimulation of PBMC with TLRs 7, 8 and 9 agonists HIV-resistant women had IL-10 responses which were statistically higher compared to HIV-susceptible women. However a decrease in IL-10 was observed in both groups one week after vaccination. The levels of TNF-α between the two groups at baseline and after vaccination were not statistically significant. Statistically elevated responses in IFN-γ were observed in HIV-R compared to HIV-S. IL-6 which was the most abundant cytokine produced. It was elevated in HIV-S compared to HIV-R at baseline and seven days post vaccination.

A more recent study determining whether triggering of TLR results in a unique cytokine/chemokine profiles in TLR agonist-stimulated PBMCs from exposed seronegative (ESN) and healthy controls (HC) showed that; IL-6, TNF-α, and IFN-γ cytokines level were significantly elevated after stimulation with ssRNA40-stimulated (TLR7/8) in ESN compared with HC. IL-6 and TNF-α were significantly increased in LPS-stimulated (TLR4) cultures of PBMCs from ESN individuals compared with HC (Biasin *et al.*, 2010). They also showed that poly I:C (TLR3) stimulation of PBMCs from ESN individuals compared to HC exhibited increased expression of effector molecules (IL-1a, IL-1b, IL-6, IL-8, IL-10, IL-12, TNF-a, CCL2, CSF3,COX2). Thus engagement

of TLR results in the induction of enhanced innate and possibly, adaptive immune responses that in turn interfere with HIV replication and productive infection in ESN individuals (Biasin *et al.*, 2010).

More evidence supporting the role of TLRs in innate immunity, have shown that engagement of Toll-like receptors (TLRs) on DCs and macrophages stimulates signaling cascades that result in production of cytokines essential for T-cell activation and differentiation. Stimulation of DCs through TLR9, which recognizes CpG motifs, yields high levels of IFN-α in response to HSV-2. It also has been demonstrated that artificial TLR activation could be of therapeutic value in that Local mucosal delivery of CpG oligodeoxynucleotides to the genital tracts protected mice from a lethal HSV-2 challenge.

Through induction of dramatic changes in the genital mucosa by recruitment of innate immune cells and the inhibition of HSV-2 replication (Ashkar *et al.*, 2003). Thus, the induction of TLR9-mediated mucosal innate immunity could provide protection against HSV infection. Similarly, CpG ODN treatment of macrophage cell lines expressing TLR9 *in vitro* significantly protected against HSV-2 infection. Similar observation were made after providing CpG motifs during a vaccine administration was shown to boost both innate and adaptive mucosal immune responses hence prevented infection following SIV challenge (Cafaro *et al.*, 2001; Kang and Compans, 2003). However, mucosal immune activation would likely need to be undertaken in a careful manner, as the application of imiquimod or CpGs to the vaginal mucosa 30 minutes prior to SIV administration resulted in increased plasma viral loads, indicating that the immune activation favoured viral replication (Wang *et al.*, 2005).

Cytokine and chemokines responses following stimulation of PBMCs with *Influenza* virus vaccine strain (Brisbane) prior to and after vaccination

In this study cytokine and chemokines level in response to *ex-vivo* challenge with 2009-2010 seasonal vaccine component *Influenza* virus strain Brisbane, among HIV-R and HIV-S before and after vaccine administration was also compared. HIV-R women had significant higher responses to IL-2, IL-10, and TNF-α compared to HIV-S at day 0, day 7, day 30; following stimulation of PBMCs with *Influenza* virus strain Brisbane. It was observed that vaccination led to a decrease in IL-2 level as well as significant increase in IL-10 cytokine level at day 7 post vaccination in HIV-R a decrease was observed in HIV-S individuals. On the other hand TNF-α levels increased significantly after vaccination in HIV-R in contrast there was a decrease in TNF-α levels in HIV-S, a decrease in IL-2 level observed after vaccination might be due to the increase in production of anti-inflammatory IL-10 hence inhibition of T cell functions. Significant elevated levels of IL-10 observed in HIV-R after vaccination can be explained with the phenomenon of immune quiescence which is as a result of higher levels of regulatory T cells or lower levels of activated T cells (Fowke *et al.*, 2008). The ability of IL-10 to suppress T-cell activation and proliferation is likely also to play a prominent role in its ability to suppress HIV-1 replication *in vivo* (Masood *et al.*, 1994).

Cytokine and chemokines level in plasma and CVL prior to and after vaccination

Second objective quantified the concentration of 7 cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, IL-17A) and 5 chemokines (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, CXCL10/IP-10) directly in the plasma and genital secretions (CVL) at

baseline and after vaccination using the (CBA) Cytometric Bead Array analysis (on LSRII flow cytometer). In this study measurement of cytokines in plasma and CVL posed a huge challenge. The levels of cytokines in plasma and CVL were generally low and were below the detectable levels of this assay. Only the cytokine IL-6 was detectable in CVL while IL-17 was detectable in plasma of some women results majority fell below the detectable limit therefore could not be analyzed hence (results not shown). A number of analytes had detectable levels for example in plasma RANTES and IL-8 in CVL, whereas the other analytes were not found above the detection limit.

There were no significant differences were observed between HIV-R and HIV-S at baseline and after vaccination in chemokines (MIG, IP-10 and IL-8) detected in CVL. The measurement of cytokine levels in body fluids such as plasma presents a huge challenge in that great care has to be taken when collecting, handling and storing the samples to avoid degradation of the cytokines in the sample (Emanuela and Robert, 2010). It has been reported that measurements of cytokine levels in plasma may not adequately reflect the cytokine-producing potential of immune cells because cytokines have short half-lives and various inhibitors are present in human sera. Though cytokine and chemokines production *in vitro* from PBMCs can be a reliable measure of immuno-competence, quantification of cytokines in plasma is still of importance since most of immune cells that participate in immune response are carried within blood.

Generally the levels of many analytes (chemokines) decreased after vaccination; with the lowest levels observed 1 week after vaccination with slight increases 30 days after vaccine administration. There was only a slight increase observed after vaccination in MCP-1 levels in plasma and IP-10 levels in CVL though the increase was not significant.

Previous study has shown that, the serum levels of all the cytokines tested that is IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were lower in HIV-1-infected CSWs compared with those in both HIV-uninfected CSW and non-CSW groups. HIV-1 infected CSWs had significantly higher genital mucosal levels of TNF-α and IFN-γ compared with those in both the HIV-1 uninfected CSW and non-CSW group. The increased production of genital mucosal pro-inflammatory cytokines observed in HIV-1 infected CSWs could possibly reflect susceptibility to HIV-1 infection and disease progression at the initial site of exposure (Lajoie *et al.,* 2008). In contrast study done by Iqbal *et al* 2008 showed that genital levels of IL-2, IL-10, and IL-13 levels were similar in the HIV-1 uninfected, HIV-1 infected, and HIV-1 resistant CSWs and the HIV-1 uninfected non-CSW control subjects. Similar study done by Lenine *et al.,* 2010 compared the concentrations of inflammatory cytokines IL-6, IL-8, IL-1β and TNF-α in genital secretion from women infected with HIV and those who were not HIV infected. The results showed that the HIV infected women had significantly elevated genital tract concentrations of IL-6, IL-8 and IL-1β than in uninfected women (Lenine *et al.,* 2010).

Another study done on a different cohort to quantify chemokine and cytokine pattern in systemic and mucosal compartment showed that HIV-1 infected CSWs had significantly higher systemic and mucosal levels of MCP-3 and MIG compared with those both the HIV-1-uninfected CSW and non-CSW control women, though the level of MIG and MCP-3 was significantly higher in genital mucosa than in the blood (Lajoie *et al.,* 2010). Higher levels of MIG in genital mucosa than in systemic compartment were also observed in the two groups in this study. Depressed IP-10 levels in genital secretion in HESN have been described (Lajoie personal communication). In this study lower IP-10

level observed in HIV-R compared to HIV-S this may lead to few activated susceptible cells hence leading to protection against establishment of infection in HIV-R (Figure 11).

IL-8 also known as neutrophil chemotactic factor (NCF), not only affects the recruitment of neutrophils into the tissues but also the ability of these neutrophils to cross epithelial barriers and to kill bacteria (Godaly *et al.*, 2001; Godaly *et al.*, 1997). In this study no significant differences were observed between the two groups after vaccination. Locally produced chemotactic factors are presumed to mediate the sequence of events leading to expression of pro-inflammatory chemokines in vaginal epithelium, after contact with pathogens that could lead to the attraction of professional immune cells to the sites of infection. In this study elevated levels of RANTES in HIV-R compared to HIV-S were observed in Plasma samples both at baseline and after vaccination. Studies have shown that upon blood collection and centrifugation platelets may spontaneously secrete RANTES. This may explain the high level of RANTES observed (Jen *et al.*, 2007).

In CVL samples only 2 samples had detectable levels of RANTES at different time points. The remaining 58 samples fell below the limit of detection indicating that elevated RANTES levels in HIV-R compared to HIV-S women. Therefore it may not likely to be a requirement for altered susceptibility to HIV infection in HIV-R. However the results from this study do not limit the possibility of RANTES involvement in HIV-Resistance via some other mechanisms or in combination with other innate proteins hence more research needed in this area to establish causation.

The results in this study are contrary to previous study in this cohort done by Iqbal *et al* that showed that the HIV-1 resistant CSWs had a 10-fold increase in RANTES

expression, compared with the HIV-1 uninfected CSWs, other studies that have shown elevated levels of RANTES in CVL from Highly exposed women did correlate increased with increased number of HIV-susceptible cells (CD4 T cells), increased pro-inflammatory cytokines (TNF-α) and increased cellular expression of CCR5 but the common shortfalls has been the small sample size (Iqbal *et al.,* 2005; Noval *et al.*, 2007; Belec *et al.*, 2001; Kaul *et al.*, 2008). Increase in RANTES level may increase susceptibility of HIV infection since it's produced by activated immune cells. Finally lack of successful measurement of cytokines in plasma and genital secretion may be due to presence of interfering factors e.g. heterophilic antibodies.

It was observed that *Influenza* vaccination with LAIV resulted in mucosal immune responses by production of pro and anti-inflammatory cytokines and chemokines. In this study. It was difficult to tell whether *Influenza* vaccination was of value. In some subjects vaccination led to increase in IP-10 and IFN-γ. Based on this production IFN-γ might be protective since it has been shown to play a pivotal role in defense against viruses and intracellular pathogens. While decrease in IL-2 observed after vaccination in some subjects may lead to loss of T-cell function hence become more susceptible to infection.

CHAPTER SIX

6 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Activation of TLRs plays a key role in initiating innate immunity and in regulating the nature of the adaptive immune response characterized by the production of pro and anti-inflammatory cytokines and chemokines.

This study did achieve its objective first and second specific objectives except for the third objective measurement of cytokines in plasma and CVL samples was a challenge, majority of the samples fell below detectable limit hence could not be analysed.

The study findings demonstrate the following:

Both T helper 1 /T_H1 (IL-2, IFN-γ, TNF-α) and T helper 2/T_H2 (IL-6, IL-10) responses were detected, hence responses did not tend to have a T_H1 bias.

TLRs (TLR 3, 4, 7, 8, 9) activation led to the expression of anti-inflammatory cytokine IL-10, with the. IL-6 being the most abundant and robust cytokine response after stimulation in both groups compared to other analytes detected. Generally HIV-R women generally had heightened responses after stimulation compared with HIV-S women. For instance stimulation with TLR 3 and 4 agonist led to significant elevated level in IL-2, IFN-γ and IL-6 cytokines at baseline and after vaccination with the HIV-R having heightened responses compared to the HIV-S. An interesting promiscuity in pro and anti-inflammatory response to TLR3 and 4 agonists was observed in that they not only induce pro-inflammatory cytokines but also comparable levels of IL-10 without inhibiting each

other. TLR 3 and 4 ligands were observed to induce the strongest pro-inflammatory responses compared to TLR 7, 8 and 9 and Brisbane virus *Influenza* vaccine strain.

PBMCs stimulated with TLR Increase in IL-10 levels was observed after stimulation with TLR 3 and 4 agonist while a decrease was observed after stimulation with TLR 7, 8 and 9 agonist after vaccination. Stimulation of PBMCs with TLR 7, 8 and 9 led to a significant elevated level of IL-2 and IP-10 chemokine in HIV-R compared to HIV-S.

Stimulation of PBMCs with *Influenza* virus strain Brisbane led to a significant increase in IL-2, IL-12(p40), IL-10 and TNF-α levels in HIV-R compared to HIV-S after vaccine administration.

Vaccination in HIV-S individuals led to a significant elevated MIG chemokines level in plasma compared to HIV-R

Collectively these data indicate that stimulation of PBMCs with different TLR ligands may activate the cells of the immune cascade in a different way. These data document significant marked differences in TLR responsiveness (cytokine and chemokine profiles) between HIV-Resistance and HIV-Susceptible individuals both at baseline and after *Influenza* vaccine administration. The data obtained suggest that suggest that TLR stimulation of lymphocytes from HIV-R individuals resulted in a more robust release of cytokines that can influence the induction of stronger long lasting adaptive antiviral immune responses. The difference in inflammatory responses of PBMCs due TLR signaling between HIV-R and HIV-S women may be indicative of the potential ability of innate immune system as a key determinant in susceptibility to infection.

6.2 RECOMMENDATIONS

Despite the recognition that innate immunity provides the first line of defense during infection and plays a critical role in preventing infection and in limiting viral replication. Further studies are needed to provide a better understanding of the contribution of specific components of mucosal innate immunity and the mechanisms by which virus may evade these immune strategies.

Further studies should be done to isolate and characterize the function of each innate immune cells e.g. NK cells, DCs, macrophages, Langerhan cells and Neutrophils. Since different cell types express peculiar pattern to in order to identify which one is responsible for observed increased responsiveness. Research on the role of several other biological factors secreted in the Female Genital Tract, such as SLPI, lactoferrin, defensins and trappin/elafin, against HIV-1 infection.

Women in this cohort acquire HIV infections predominantly at the genital mucosa through heterosexual transmission hence a critical determinant of HIV susceptibility, studies need to be done on the important role of the vaginal epithelial cells, and cervical cells in innate host defense. Since the results obtained in this study was from systemic compartment and might not reflect what happens on the mucosal compartment.

Finally one of the short fall of this study was the small sample size, there is a need to use a sample size which is a representative of the population so as to provide a clear observation of the findings, also use of low risk women as control so as to provide a good comparison with the exposed group.

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APPENDICES

A study of Immune Responses to Influenza Vaccine ((**Flumist®**)) Challenge among **Highly Exposed Uninfected Female Sex Workers in Nairobi, Kenya**

Majengo, Korogocho and MCH Pumwani Research Clinics

Patient Information and Consent Form

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

Investigators:

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BACKGROUND INFORMATION

The University of Nairobi and its collaborators from Canada have been working for many years to fight the epidemics of AIDS and other sexually transmitted infections that we are facing in Kenya. This basic science research program is conducting studies to determine the relationship the immune response to a mucosal viral challenge. You are being asked to participate in this study because you are:

a) already enrolled in a study evaluating the mechanism of HIV resistance in Nairobi, Kenya.

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

The purpose of this sub-study is to determine if there are factors that could protect individuals from acquiring HIV. It will study the response of the immune system to the flu vaccine administered intra-nasally.

WHY IS THIS STUDY BEING DONE?

This study is being done to find out why some people are more or less likely to get the Human Immunodeficiency Virus (HIV), the virus that causes AIDS. There is more and more evidence that the immune system in some people is able to protect them against infection with HIV. Since most people get HIV through sexual exposure to an HIV infected partner, the first contact with the virus occur in the genital tract, the vagina and cervix in women. We know from some of our previous work that some women, who seem to be protected against HIV, have a special type of immune response that is not present in women who get HIV. The purpose of this study is to try to find out what happens at the time of exposure and thereafter. The immune response in the vagina, uterus and cervix, nasal mucosa as well as in the blood will be studied. This work may be helpful in understanding the events that occur in the early period after exposure to virus and may eventually assist in making a vaccine for HIV.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY

About 250 women will take part in this study.

WHAT IS INVOLVED IN THE STUDY?

You have been invited to voluntarily participate in this study because you are enrolled in a cohort studying the mechanisms of resistance to HIV. If you now agree to participate in the study, you will be asked several questions about flu and previous reactions to vaccines. You will be asked to receive an intranasal flu vaccine and will be followed-up for 6 months after vaccination. You will also be encouraged to come to the clinic for examination and treatment at any other time that you feel ill. If you forget to return to the clinic for one of your scheduled visits, a clinic staff member will contact you by phone, SMS or send your peer group leader to remind you of the missed appointment. All study participants will also be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on HIV infection status every three months. In addition, we will store specimens from your blood for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

Safety of the vaccine:

The Flumist vaccine (Medimmune inc.) has undergone all the steps of USA FDA approval and it is in use in all states. Since its approval in 2003 over 4.5 million subjects have received the vaccine and many studies conducted to date have documented its effectiveness and safety.

Clinic visits

5 visits: First day (day 0 or baseline), and follow-up visits: day 1, 7, day 30 and day 120 (4 months) -All study participants)

1. We will you general questions about flu, recent and past medical history.
2. The doctor will review the medical history.
3. Swab and washing from your vagina for studying your immune response.
4. Swab from your cervix to collect samples for studying your immune response.

5. A thin plastic tube will be placed in your cervix (opening to your womb) to get some of the mucous your cervix makes.
6. Nasopharyngeal aspirate to collect samples for studying your immune response.
7. Three tablespoons of blood will be taken for testing HIV and for studying your immune response. We will inform you of your results at your one month visit. We also will test your spouse for the HIV virus free of charge if he/she wishes.

Follow-up visits (All study participants)

1. You will be asked to return on day 1, day 7, day 30 and 4 months (day 120) after receiving the influenza vaccine.
2. You will be treated for new infections, free of charge.

HOW LONG WILL I BE IN THE STUDY?

The study will last 6 months. Although we would appreciate if you stayed in the study for the entire period you may choose to leave the sub-study at any time without any penalty to you.

WHAT ARE THE RISKS OF THE STUDY?

Risk of blood cervical sample collection and nasopharyngeal aspirate

This study requires the use of your blood. In order to get the blood we will need to insert a needle into a vein in your arm so that the blood can be removed. There will be some pain associated with the needle stick but this will be only for a short period of time and some bruising around the needle site may occur. Nasopharyngeal aspirate involves introducing a saline solution to your nose through a tube and collecting the fluid into a tube. This may cause some minor discomfort.

HIV test

Non-physical risks:

1. If you are HIV positive, learning so may cause you to become depressed. We will counsel you about your HIV test results if you are negative or positive. If you are HIV positive, we will be able to provide HIV care and ART services free of charge at this clinic. We will also test your husband or boyfriend for the HIV virus if he wants.

Risks of taking the vaccine

Very likely:

1. Runny nose
2. cold-like symptoms
3. headache
4. cough
5. sore throat
6. tiredness/weakness
7. irritability
8. muscle aches

You should not take the vaccine if you have any of the following conditions:

1. allergy to eggs
2. a history of Guillain-Barré syndrome
3. pregnant
4. asthma

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

The benefits that you will get from this study are that you will be examined regularly, and if you are found to have AIDS, you will receive appropriate and effective medication. Medical care will also be provided for other illnesses that you might have as outlined in our standard of care document (attached) for all research participants. You will also receive the flu vaccine that will also decrease the likelihood of contracting flu.

WHAT ABOUT CONFIDENTIALITY?

Efforts will be made to keep your personal information confidential. We will record your information only by a special number assigned to you. The number will only be known to the clinic staff and yourself.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: the researchers, members of the local and international ethics teams. The research results will be published, but your identity will remain secret.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. If the participation in the study results in you becoming ill, the study team will provide you with medical care for the problem for free.

Although you will not be paid to participate in the study, you will be offered a small token of five hundred shillings (Ksh 500) **for every clinic visit to** compensate you for your transportation to the clinic and any other expenses you might incur.

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

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

At the end of every year, we will be holding baraza's at the clinic to give progress reports and share any new findings from the study with all members of the clinic.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, call or contact Drs. Wachihi, Kimani or any one of the researchers named above at the Medical Microbiology Annex at the University of Nairobi. For any urgent medical problems or emergencies while you are in this study please use the following cell phone numbers 0724 255560 / 0721673080 to reach **Drs. Wachihi and Kimani** who will be on call 24 hours daily.

For questions about your rights as a research participant, contact **Professor Bhatt,** who is the chairperson of the Ethical Review Committee at the University of Nairobi, by calling 2726300 ext 44102, or make an appointment to see her at the Department of Internal Medicine, College of Health Sciences, University of Nairobi.

Statement of Consent:

If you agree to participate in the study, please sign below.

I, _____, have read or have had read to me, the consent form for the above study and have discussed the study with _____.

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

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

☐
- enrolment is completely voluntary and I can withdraw from the study at any time

☐
- blood, cervical, vaginal and nasal specimens will be required for this study and may be used for genetic studies

☐
- any blood specimens previously collected for the main study (Comprehensive Mechanisms of HIV resistance) may be re-evaluated in the course of this sub-study

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

☐ a flu vaccine will be given to me through the nose

I am willing to participate in the study.

Name of Study Participant_____

Signature/Thumb print: _____ Date: _____

For clinic staff:

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

Name of Clinic Staff: _____

Signature: _____ Date: _____

Assigned Study Number / Clinic Number ____ ____ ____

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

Standards of Medical Care for Participants in the UOM/UON Research Clinics

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

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

- HIV and STD prevention services: provision of the male condom, and peer-based and clinic-based counseling regarding safer sexual practices.
- Family planning services as directed in the *Kenyan National Family Planning Guidelines*
- Rapid and effective treatment of sexually transmitted diseases in accordance with the *Kenya National Guidelines for the Syndromic Management of Sexually Transmitted Diseases*
- Medical care for acute and chronic illnesses, both infectious and non-infectious
- Access to diagnostic testing in haematology, biochemistry, infectious diseases, immunology, radiology

- Prompt referral for specialist consultation and hospitalization at KNH when indicated free of charges to the patient.

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

- **Primary Prophylaxis:** Trimethoprim-Sulphamethoxazole (Septrin): all participants with a CD4+ T cell count <200/mm^3, for prevention of PCP, toxoplasmosis and bacterial infections (bacterial pneumonia, bacteremias, some bacterial diarrhoea), according to *National AIDS/STD Control Program (NASCOP) Guidelines*
- **Secondary Prophylaxis:** Septrin: offered to all participants regardless of CD4+ T cell count after an episode of PCP, PTB, toxoplasmosis, or severe bacterial infection. Fluconazole: provided for secondary prevention of Cryptococcus

Treatment

- Herpes. simplex/Herpes zoster infection: acyclovir
- Candidiasis (oral, esophageal, vaginal): nystatin, clotrimazole, Fluconazole
- Tuberculosis (pulmonary or extra pulmonary): referral to National TB Programme and co-managed by the research team
- Toxoplasmosis: referral for inpatient therapy
- Cryptococcus: referral for inpatient therapy
- PCP: Septrin (with prednisolone, if severe)
- Kaposi's Sarcoma: ARV and referral to Clinical Oncologist

3. **HIV Care and Antiretroviral therapy.**

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

- ARV drugs and infrastructure support has been secured by the University of Manitoba from NASCOP/PEPFAR and CDC PEPFAR to provide HIV basic and ARV care for **all** cohorts members who are eligible as per the *"Guidelines to Antiretroviral Drug Therapy in Kenya"* (NASCOP-2002). Such medical treatment and its requisite follow-up, integrated with the above standard of care, will also be provided at no cost.

- The UOM/UON Cooperative agreement number U62/CCU024510-03 was signed in 2003 for the next 10 years for HIV Care and Antiretroviral therapy. Our current budgetary level for this standard of care component for all research participants has been adequate and we have had no problems in the past years.