DETERMINATION OF CD8+T-CELL RESPONSES IN A HIGH RISK HIV NEGATIVE POPULATION

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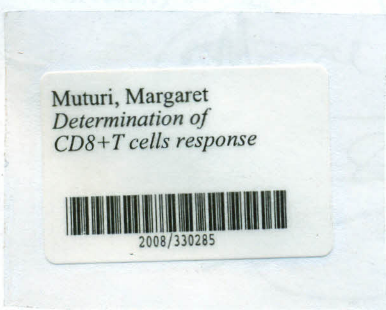
Thesis submitted in partial fulfillment of the requirements for the award of a Ph.D. degree in Immunology in the School of Pure and Applied Sciences, Kenyatta University.



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DECLARATIONS

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

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DEDICATION

I dedicate this work to the people infected and affected by HIV/AIDS and to all those in one way or another are contributing to the search of a lasting solution to this scourge.

ACKNOWLEDGEMENT

Glory, honour and praise, be to God and to my Lord Jesus Christ for the grace and strength to complete this work.

I greatly appreciate my supervisors; Dr Charity Gichuki and Prof. Omu Anzala for their guidance in experimental design and thesis editing, without which, this work would not have been completed.

I thank the International Aids Vaccine Initiative (IAVI) and the Kenya Aids Vaccine Initiative (KAVI) for sponsoring this project. I acknowledge the KAVI staff and in particular, the KAVI laboratory and Kangemi Clinic teams as well as volunteers for their individual contributions towards this study.

I thank Dr Kihoro of JKUAT for criticizing and guiding me in data analysis, and Kenyatta National Hospital Ethics and Research Committee (KNH-ERC) for clearance to carry out this research. I am also thankful to Kenyatta University for time and waiver of fees, and my colleagues in the Department of Biochemistry & Biotechnology for moral support during the study period.

Finally, I really appreciate the encouragement and support that was accorded to me by my family throughout the study period.

ABSTRACT

Naturally acquired cellular immunity in individuals who have been exposed to HIV-1 but have remained uninfected may hold clues for the design of an effective HIV vaccine. IFNγ Elispot has emerged as one of the widely used assay to monitor HIV-specific immune responses. It is becoming the assay of choice for evaluation of HIV-vaccine-induced cell-mediated immune responses in many clinical trials. The objective of this study was to investigate the CTL responses of high risk HIV seronegative individuals to HIV A and RENTA vaccine peptides. The study further sought to investigate whether it was possible to recruit, sample, counsel and follow-up a cohort of high risk seronegative volunteers over a duration time in preparation for vaccine trials. To achieve these objectives, 30 volunteers filled a questionnaire, were counseled, tested for HIV status, recruited and enrolled in a 15 month study.The thirty exposed seronegative (ES) volunteers reported frequent unprotected sex with people of unknown HIV-1 status at enrollment. Every 3 months the volunteers were seen at the KAVI Kangemi clinic where blood samples were taken for the determination of the CTL responses, their HIV status was re-checked, filled questionnaire to assess the changes in their risky sexual behaviour. It was possible to recruit and follow-up the 30 volunteers for the entire duration of the study. All the thirty samples did not show HIV-1 specific T cell responses to both RENTA and HIV-A peptides using the *ex vivo* Elispot assay during the four time points (months 0, 3, 6 and 9). To investigate whether these results were truly negative, samples from 5 seronegative discordant couples were used. There were no HIV-1 specific CD8+ IFNγ T cell responses in the HIV negative spouse. To investigate whether the *ex vivo* Elispot was unable to detect the responses, cultured Elispot assay was applied to the samples. They all tested positive with variations between peptide pools and individuals. The fact that cultured Elispot detected the responses from the 5 seronegative spouses of HIV infected partners and from 12 of the thirty means that the *ex vivo* Elispot assay was not sensitive enough to detect responses to the tested vaccine peptides. Cultured Elispot expands the memory CTL thus enhancing the detection of the responses. Using this method it was possible to demonstrate that HIV-1 specific CD8+ IFNγ T cell responses exist in high risk exposed seronegative individuals. Pool 90 gave positive responses with all the samples. It would appear that combining the pools of peptides would elicit consistent CD8+ IFNγ T cell responses and therefore make a better vaccine candidate. The results suggest that there is need to exercise very stringent criteria for enrolling high risk exposed seronegative participants to any study group meant to investigate immunological parameters related to HIV exposure.

LIST OF ABBREVIATIONS

AIDS	Aquired immunodefiency syndrome
CE	Cultured Elispot
CMV	Cytomegalovirus
CSW	Casual Sex Worker
CTL	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISPOT	Enzyme linked immunospot
ES	Exposed seronegative
EV	*Ex vivo* Elispot
FEC	Flu Epstein-Barr Cytomegalovirus
HAART	Highly active anteretroviral
HEPS	Highly exposed but persistently seronegative
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
IAVI	International Aids Vaccine Initiative
ICA	Intracellular cytokine Assay
KAVI	Kenya Aids Vaccine Initiative
LTNPs	Long-term non-progressors
MAB	Monoclonal antibody
MHC	Major histocompartibility complex
MIP	Macrophage nflammatory protein
MVA	Modified vaccinia Ankara
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS	Phosphate Buffered Saline
PCP	Pneumocytis pneumonia

PHA	Phytohaemagglutinin
RANTES	Regulated upon activated normal T cell expressed and secreted
RNA	Ribonucleic acid
SFC	Spot forming cells
SIV	Simian immunodeficiency virus
STCL	Short-term cell lines
TCLA	T-cell line adapted
UNAIDS	Joint United Nation Programme on HIV/AIDS
VDP	Vaccine Development Project
WHO	World Health Organisation

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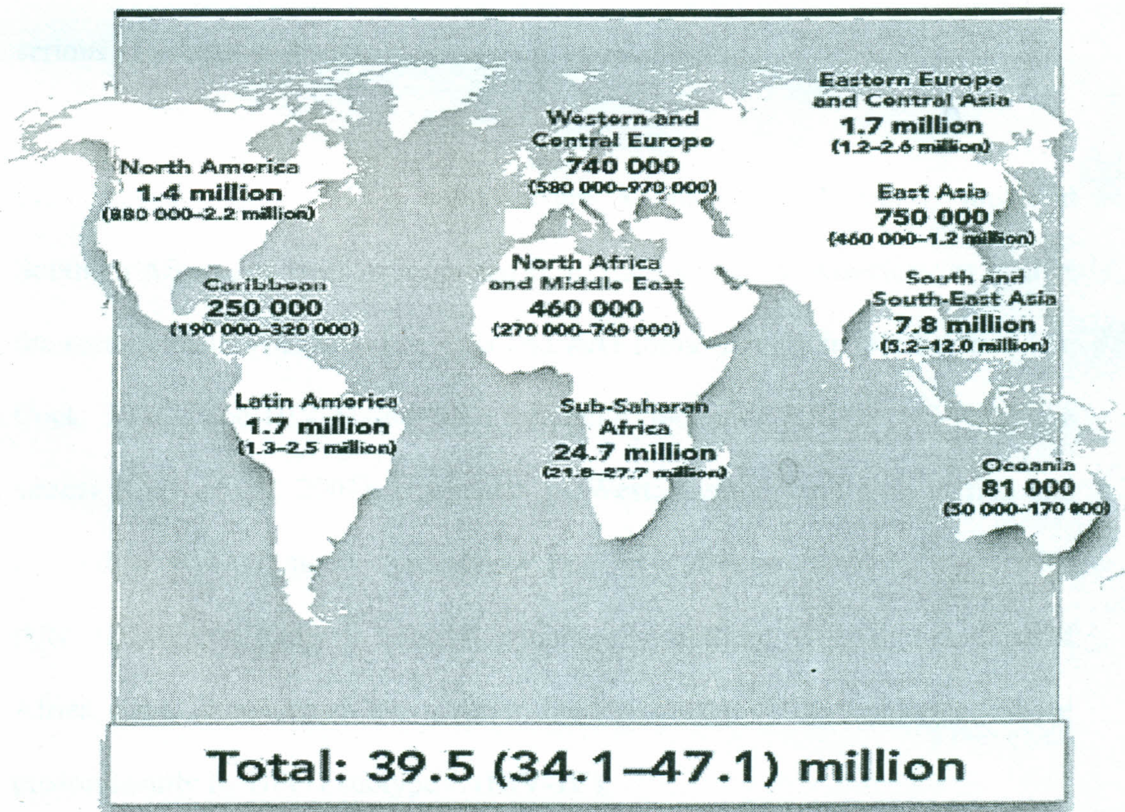
CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Nearly 40 million people were estimated to be living with the HIV virus around the globe in 2006, according to the World Health Organization and UNAIDS, 2006 (Figure 1).

Figure 1:The distribution of adults and children estimated to be living with HIV



Source: 2006 UNAIDS /WHO report on global AIDS epidemic

That number is 2.6 million more than in 2004, and the number of new infections reached 4.3 million in 2006. Two thirds of those infected 24.7 million people, lived in sub-Saharan Africa and this region also accounted for almost 75 per cent of death-2.1 million out of the global toll of 2.9 million in 2006 (UNAIDS/WHO, 2006). However, the estimated number of persons living with HIV worldwide in 2007 was 33.2 million (30.6-36.1 million), a reduction of 16% compared with the estimate of 2006. This was mainly as a result of the advances in the methodology of estimation of HIV epidemics and to a lesser extent due to decline in the number of new infections in six countries: Angola, India, Kenya, Mozambique, Nigeria and Zimbabwe (UNAIDS, 2007). Nevertheless, HIV/AIDS remains the most serious of infectious disease challenges in public health.

The dominant global HIV-1 subtypes are A, mainly in Africa; C found in Southern Africa and parts of India and B, in Europe and the Americas; as well as the recombinants A/E found in Asia and A/G found largely in West Africa (De Cock, 2001). The HIV-1 subtype A epidemic, including CRF02_AG-IbNg-like strains (Carr *et al.*, 2001), dominates in West, Central, and East Africa and reached a plateau at the prevalence rate of 1.5% to 14% by the 1990s (UNAIDS/WHO, 2000). In contrast, countries in southern Africa and the horn of Africa have experienced the greatest burden of the AIDS epidemic caused predominantly by HIV-1 subtype C (HIV-1C).

In recent years, promising developments have been made in global efforts to address the AIDS epidemic, including increased access to effective treatment and prevention programmes. However, the fact that 2.6 million more people were living with AIDS in 2006 than in 2004 indicate that the number of people living with HIV continues to grow, as does the number of deaths due to AIDS.

A total of 39.5 million (34.1million–47.1 million) people were living with HIV in 2006-2.6 million more than in 2004. This figure includes the estimated 4.3 million adults and children who were newly infected with HIV in 2006, which is about 400 000 more than in 2004. In many regions of the world, new HIV infections are heavily concentrated among young people (15–24 years of age) accounting for 40% of new HIV infections in 2006 (UNAIDS/WHO, 2006).

Sub-Saharan Africa continues to bear the brunt of the global epidemic. Although the percentage prevalence has stabilized, continuing new infections (even at a reduced rate) contribute to the estimated number of persons being greater than ever before. The estimated number of deaths due to AIDS in 2007 was 2.1 million worldwide, of which 76% occurred in sub-Saharan Africa. Among the 2.5 million new infections estimated in 2007, 68% occurred in sub-Saharan Africa (UNAIDS, 2007). In 2006, Southern Africa accounted for one third (32%) of the people with HIV and 34% of all deaths due to AIDS globally.

Declines in national HIV prevalence are being observed in some sub-Saharan African countries, but such trends are currently neither strong nor widespread enough to diminish the epidemics' overall impact in this region. In sub-Saharan Africa, almost 61% of the adults living with HIV in 2007 were women (UNAIDS/WHO, 2007).

Kenya is still contending with a serious AIDS epidemic with 1.3 million people (1.1 million–1.5 million) currently living with HIV, despite evidence of declining prevalence among pregnant women (Baltazar, 2005; Cheluget *et al.*, 2006; WHO, 2005a). Kenyan national adult HIV prevalence fell from 10% in the late 1990s to about 7% in 2003 (Ministry of Health Kenya, 2005), just over 6% (5.2–7.0 %) in 2005 (UNAIDS, 2006) and to 5% in 2006 (Ministry of Health Kenya, 2005; Kaiser Daily HIV/AIDS report, 2007). There has also been a steep drop in infection levels among pregnant women at a majority of antenatal sites. HIV prevalence in women attending antenatal clinics fell from 28% in 1999 to 9% in 2003, among 15-49 year old women and from 29% in 1998 to 9% in 2002 among those aged 15-24 years (Hallett *et al.*, 2006). In others it declined from 15% in 2001 to 4.3% in 2004 (Cheluget *et al.*, 2006).

Major HIV prevention efforts were mounted in Kenya from the year 2000 onwards, and there is evidence that more people have been delaying their sexual debuts, that condom use rates have increased and that a smaller percentage of

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adults have multiple sex partners. However, new HIV infections appear to have peaked in the mid-1990s, before the scale-up of prevention programmes occurred. This suggests that other factors, including increasing AIDS mortality and the saturation of infection among people most at risk, provided most of the impetus for the reduction in HIV prevalence observed in the past several years (Cheluget *et al*., 2006). It is to be hoped that the recently observed changes in behaviour will maintain the declining trend. A new concern, however, is the emergence of injection drug abuse as a factor in Kenya's epidemic. For instance 50% injection drug users were found to be HIV-infected in Mombasa (Ndetei, 2004) and 53% in Nairobi (Odek-Ogunde, 2004).

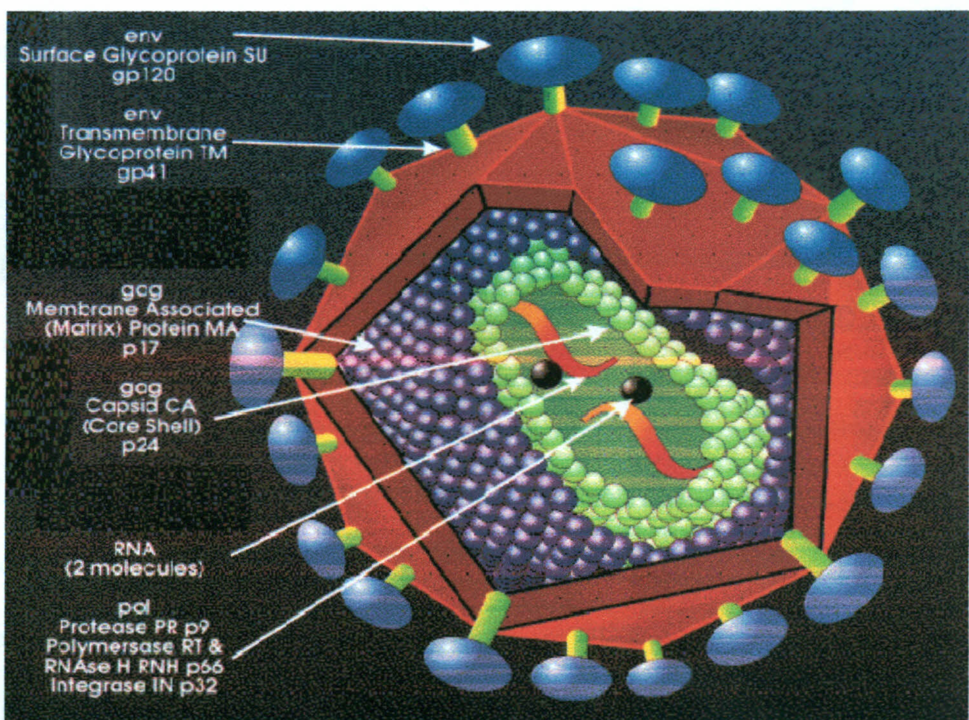
1.2 Literature Review

1.2.1 The human immunodeficiency virus type 1 (HIV-1), mechanisms of infection and replication

The human immunodeficiency virus type 1 (HIV-1) is the primary cause of the acquired immunodeficiency syndrome (AIDS). It is a lentivirus belonging to the retrovirus family (Coffin *et al*., 1986; Sowadsky, 1999). HIV is a retrovirus composed of two copies of positive single stranded RNA that codes for the virus nine proteins, Gag, Pol, Env, Tat, Rev, Nef, Vif, Vpu and Vpr of which only the former five are essential for viral replication *in vitro*. HIV codes for the enzyme reverse transcriptase, which transcribes the viral genomic RNA into a DNA copy

that ultimately integrates into the host cell genome (Fauci, 1988). The viral RNA is enclosed by a conical caspid of viral protein P24. The rest of the structure is shown in Figure 2. HIV is an RNA virus that codes for the enzyme reverse transcriptase, which transcribes the viral genomic RNA into a DNA copy that ultimately integrates into the host cell genome (Fauci, 1988).

Figure 2: The basic structure of Human Immunodeficiency Virus-1



Source: ARIC's AIDS Image Gallery

HIV is a highly polymorphic virus and the two forms: HIV-I and HIV-2 are responsible for the AIDS epidemic. HIV-1 is associated with the worldwide pandemic while HIV-2 has been basically restricted to Africa. HIV-2 illustrates

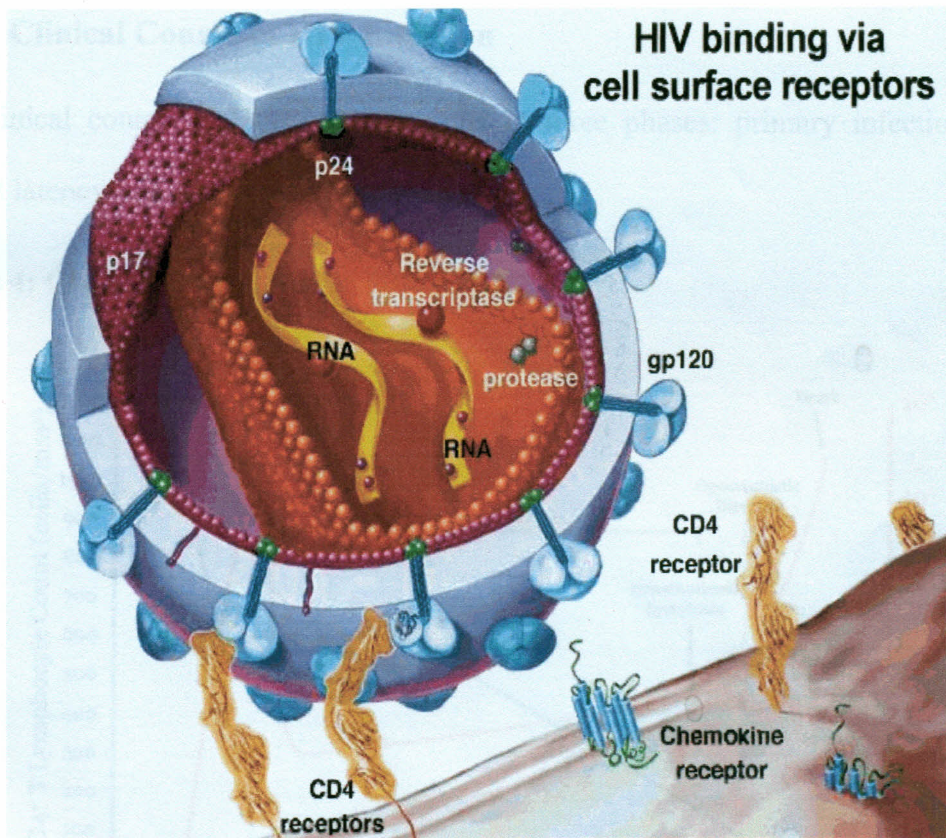
the impact of inherently lower transmissibility compared with that of HIV-I. Phylogenetic analyses of virus samples from different geographical regions have revealed that HIV-I can be classified into 3 groups: M, N and O with M being the pandemic form. HIV is closely related to SIV, as evidenced by viral protein cross-reactivity and genetic sequence similarities (Myers *et al.*, 1992).

Most retroviruses that are capable of replication contain only three genes, *env*, *gag* and *pol* (Varmus, 1988). However, HIV contains not only these essential genes but also the complex regulatory genes *tat, rev, nef,* and auxiliary genes *vif, vpr* and *vpu* (Greene, 1991). The actions of these additional genes probably contribute to the profound pathogenicity that differentiates HIV from many other retroviruses. For example nef down-modulates CD4-T cell and major histocompatibility class I (MHC-I), disrupts cell signaling and increases viral infectivity (Kestler *et al.*, 1991). The gene *rev* is essential for cytoplasmic transport of unspliced and singly spliced viral transcripts. Thus, it is required for the expression of viral structural proteins and establishment of productive infection (Hope, 1999).

HIV-1 primarily infects CD4+ T-lymphocytes and monocytes/macrophages, but also astrocytes and microglial cells of the central nervous system are targets. HIV gains entry into susceptible cells by fusion of the viral membrane with plasma membrane (Chan and Kim, 1998). This process is mediated by binding first to the

CD4 receptor which induces conformational change in gp120, and then to a seven transmembrane domain G-protein coupled receptor which exposes the fusogenic epitopes of gp41 and permits fusion with host cell membrane and subsequent entry into the cell (Berger *et al*., 1999) (Figure 3).

Figure 3: HIV Binding via Cell Surface Receptors



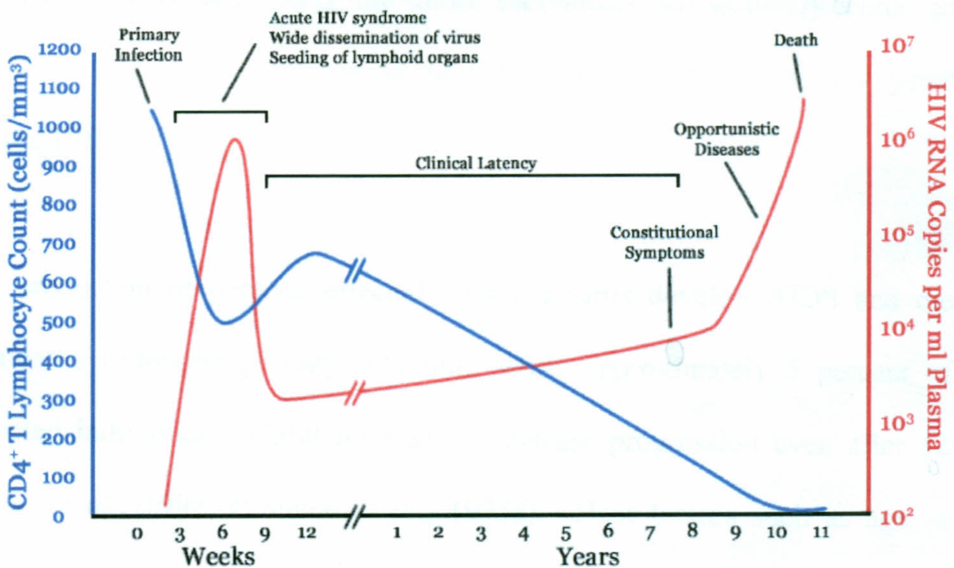
Source: ARIC's AIDS Image Gallery

CD4+ T cells, the cells depleted in AIDS patients, are primary targets of HIV because of the affinity of the gp120 glycoprotein component of the viral envelope for the CD4 molecule (McDougal *et al.*, 1986, 1985a). The infection spreads to the lymphatic tissue that contains follicular dendritic cells that may act as a storage place for latent viruses. Over time, virus replication leads to a slow and progressive destruction of the immune system.

1.2.2 Clinical Course of HIV Infection

The clinical course of HIV infection runs in three phases: primary infection, clinical latency and AIDS-defining illness (Figure 4).

Figure 4: Clinical Progression of HIV to AIDS



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Primary HIV infection may be associated with mononucleosis like syndrome characterized by a burst of HIV viremia, high levels of virus expression and

copies of HIV DNA in CD4+ T lymphocytes (Pantaleo *et al.*, 1993a, 1994). There is also a decline in the number of circulating CD4+ T cells during primary infection attributable to HIV-mediated cell killing and to re-trafficking of cells to the lymphoid tissues and other organs (Fauci, 1993a).

The median period of time between infection with HIV and the onset of clinical disease is approximately 10 years (Alcabes *et al.*, 1993a; Hessol *et al.*, 1994; Pantaleo *et al.*, 1993a). HIV infection runs a variable clinical course. In a minority of the patients, primary HIV infection is sub-clinical but in 50% to 70%, acute clinical symptoms occur 2-4 weeks after infection which lasts 1-2 weeks (Tindall and Cooper, 1991). In some cases the patient may progress into AIDS very rapidly (Groenink *et al.*, 1991) but most individuals subsequently enter an asymptomatic phase and progression to AIDS occurs only after 1-3 years (Tersmette, 1989).

A small proportion of persons infected with the virus develop AIDS and die within months following primary infection, while approximately 5 percent of HIV-infected individuals exhibit no signs of disease progression even after 12 years (Cao *et al.*, 1995; Pantaleo *et al.*, 1995a). Host factors such as age or genetic differences among individuals, the level of virulence of the individual strain of virus, as well as influences such as co-infection with other microbes may

determine the rate and severity of HIV disease expression in different people (Fauci, 1993a; Pantaleo *et al.*, 1993a).

As the disease progresses, increasing amounts of infectious virus, viral antigens and HIV-specific nucleic acids in the body correlate with a worsening clinical course (Allain *et al.*, 1987; Furtado *et al.*, 1995). Increased expression of HIV mRNA in peripheral blood mononuclear (PMN) cells has also been shown to precede clinically defined progression of disease (Saksela *et al.*, 1994).

In the period following primary infection, HIV disseminates widely in the body; an abrupt decrease in CD4+ T cells in the peripheral circulation is often seen. An immune response to HIV ensues, with a decrease in detectable viraemia. A period of clinical latency follows, during which CD4+ T cells counts continue to decrease, until they fall to a critical level below which there is a substantial risk of opportunistic infections (Pantaleo *et al.*, 1993a), see figure 4.

Homosexual and bisexual men, for whom the time of sero-conversion had been documented, had increasing levels of both plasma and intracellular HIV RNA, and their CD4+ T cell numbers declined as the disease progressed (Gupta *et al.*, 1993; Mellors *et al.*, 1995). Men who remained asymptomatic with stable CD4+ T cell numbers maintained extremely low levels of viral RNA. These findings suggest that plasma HIV RNA levels are a strong, CD4-independent predictor of

rapid progression to AIDS. A study of exposed individuals has revealed latent HIV in the resting memory CD4+ cell pool (Rowland-Jones *et al.*, 1995).

Moreover, HIV-1 specific memory CD4+ cell responses and cytotoxic CD8+ T lymphocytes are found in HIV-exposed, seronegative health care workers. However, only HIV specific CTL responses are found in uninfected infants born to infected mothers (Wasik *et al.*, 1999). Some individuals exhibit partial genetic resistance to infection that is related to CCR5 (δ35) (Martinson *et al.*, 1997; Anzala *et al.*, 1998). This prevents cell surface expression of CCR5 chemokine receptor, the primary coreceptor for primary HIV isolates (Liu *et al.*, 1996). These findings indicate that acquired resistance can occur in human, but the immunologic mechanism by which it occurs is not understood.

1.3 Factors predisposing to HIV High-risk behaviour

1.3.1 Drug use and high-risk behaviour

In many parts of the world, sex work and injecting drug use are intricately linked: drug users resort to sex work to fund their habit, while sex workers turn to injecting drugs to escape the pressures of their work (Tran *et al.*, 2004). Sex workers who also inject drugs are at further risk, not least because the combination of their work and drug taking puts them beyond the protection of the law and so opens them to exploitation and abuse, including sexual violence and

harm, and incapacity to negotiate condom use. Research has shown that drug-using sex workers in Viet Nam were about half as likely to use condoms compared with those who did not use drugs (Tran *et al*., 2004).

High rates of HIV and sexually transmitted infections have been found among sex workers in countries with large populations of injecting drug users (sullinman *et al*., 2004). In China, Indonesia, Kazakhstan, Ukraine, Uzbekistan and Viet Nam, the large overlap between injecting drug use and sex work is linked to growing HIV epidemics (UNAIDS, 2005a).

In Manipur, India, which has a well-established HIV epidemic driven by injecting drug use, 20% of female sex workers said they injected drugs, according to behavioral surveillance (MAP, 2005). In Ho Chi Minh City, in 2002, 49% of sex workers who reported injecting drugs were found to be HIV-positive compared to 19% of sex workers who used drugs without injecting them and 8% of those who did not use drugs at all.

1.3.2 HIV-Specific protective immunity

The response to HIV comes in two forms: cellular and humoral. The cellular response refers to the activity of the CD4 and CD8 T-cells, while the humoral response refers to antibody production and activity (Goepfert, 2003).

1.3.2.1 Humoral Immune Responses

The assessment of neutralizing antibodies for HIV-1 has generated considerable controversy. Glycoprotein 120 (gp120) on the outer coat or envelope of the virus contains the CD4 binding site, the region that attaches to human cells. Therefore most neutralizing antibodies in HIV-infected people are directed against gp120 (Burton, 1997). For these reasons, vaccines based on genetically engineered HIV envelope proteins gp120 and a larger molecule, gp160, have been the best studied to date.

Recent advances in the knowledge of the structure of the HIV-1 envelope provide a rational basis for assessing the results of neutralization assays (Burton, 1997; Sattentau, 1996). The HIV-1 envelope is initially synthesized as a precursor (gp160) that is subsequently cleaved to yield an extracellular domain (gp120) that is noncovalently associated with a transmembrane protein (gp41) (figure 2).

Several features of the HIV-1 envelope limit its ability to be neutralized by antibodies. The crystal structure of gp120 goes a long way towards explaining why (Wyatt *et al.*, 1998). The coat protein, gp120, is one of the most heavily glycosylated proteins identified to date, and these N-linked glycosylation sites are believed to limit the ability of antibodies to bind to gp120 (More and Burton, 1999). In addition, the HIV-1 envelope exists as an oligomer of three gp120-gp41 molecules. Oligomerization of the HIV-1 envelope blocks the ability of antibodies

that bind to epitopes present on monomeric forms of envelope to bind to the oligomeric form (Burton, 1997). In fact, the majority of antibodies present in HIV-infected people recognize forms of envelope other than the mature oligomeric protein, including monomeric gp120 and unprocessed gp160 (termed viral debris by Burton) (Burton, 1997).

A final distinction critical to understanding neutralizing antibodies involves the difference between T cell line adapted (TCLA) and primary HIV-1 isolates (Burton, 1997). The process of culturing HIV-1 generally leads to a selection of virus variants that are not representative of the original input population (Meyerhans *et al.*, 1989). Growth of HIV-1 in T cell lines results in selection of HIV-1 strains that are generally more easily neutralized than primary virus isolates. This property appears to result from the greater accessibility of several epitopes, including the V3 loop and the CD4 binding site, to antibody neutralization in TCLA HIV-1 strains (Burton, 1997).

As a result of the factors just listed, antibodies that are able to neutralize primary virus isolates are relatively rare (Sattentau, 1996 Burton, 1997). Primary HIV strains-neutralizing antibodies generally arise between 2 and 6 months following primary infection, after the development of HIV-1-specific CTL activity and virus-binding antibodies (Koup *et al.*, 1994; Moore *et al.*, 1994). Early neutralizing antibodies tend to be of low titer, neutralize a limited range of

isolates and are not present in all patients (Moore *et al.*, 1994). Even following many years of HIV infection, most infected subjects mount only weak neutralizing responses against primary HIV-1 isolates (Sattentau, 1996; Burton, 1997).

1.3.2.2 Cellular Immune Responses

The reason why only humans develop AIDS in response to HIV infection is that only humans respond to the infection with a proliferation of cytotoxic T lymphocytes (CTL) that indiscriminately kill human CD4+ T cells, including healthy, uninfected CD4+ T cells (Zarling *et al.*, 1990).

HIV depletes its primary target, CD4+ T cells, by accelerating the division of existing T cells resulting in an increase in the rate of CD4+ T-cell death (Rosenberg *et al.*, 1999). When patients are treated with antiretroviral drugs, T-cell proliferation and death of the patient slow down. Thus, the increases in CD4+ T-cell counts seen following highly active antiretroviral therapy (HAART) are not due to a boost in the production of new T cells but by a slowdown in the loss of existing T cells (NIAIDS News December 10, 2001).

In the first few weeks after infection, the number of CD8+ T-cells increases to up to 20-fold above the normal range, whilst CD4+ T-cell numbers fall sharply (figure 4). There is a decline in the immune functions which are governed by CD4+ T-cells, sometimes leading to the appearance of infections such as *Candida*

(thrush), herpes and *Pneumocystis* pneumonia (PCP) during seroconversion illness. Six months after infection, CD4+ T-cell function improves except in relation to HIV-specific antigen (Musey, 1999). In most individuals, HIV-specific CD4+-T cells are thought not to be functioning correctly as they can only produce interferon gamma, and this is associated with a lack of viral control (Boaz *et al.*, 2002; Palmer, 2004).

Cytotoxic T lymphocytes (CTL) are a major host defense mechanism against viral infection (Moore *et al.*, 1994). Although both CD4+ and CD8+ CTL exist, CD8+ CTL are the dominant effector cells responsible for defending the host against viral infections (Zinkernagel and Doherty, 1979). The cytotoxic CD8+ lympocytes (CTL) recognize and lyse virus-infected cells following binding of the T cell receptor to a viral peptide presented by a class I HLA molecule on the infected cell. The HLA type of an individual thus plays a major role in determining whether an individual will generate a CTL response to a given epitope. Presentation of viral proteins to CD8+ CTL generally requires synthesis of antigen within the cell.

Most HIV-infected individuals develop a relatively strong virus-specific CD8+ CTL response, as measured by a variety of *in vitro* assays (Rowland-Jones *et al.*, 1997). HIV-1-specific CTL activity can often be detected in unstimulated peripheral blood, in contrast to most other viral infections, in which *in vitro*

stimulation is required to expand CTL precursors. A study employing fluorescent HLA tetramers complexed with HIV peptides to directly identify HIV-specific CTL demonstrated that HIV-1-specific CTL may account for up to 2% or more of all CD8+ T cells (Ogg *et al.*, 1998).

The range of HIV CTL epitopes recognized by infected individuals varies. Some individuals recognize as many as 13 different CTL epitopes, although most individuals recognize 3 to 4 epitopes (Johnson and Walker, 1994). Based on assessment of the spectrum of the T cell receptor repertoire in CD8+ T cells during primary HIV infection, it appears that a more broadly directed CTL response correlates with slower disease progression, presumably by limiting the development of escape mutants (Pantaleo *et al.*, 1997). Efforts to show a correlation between HIV-specific CTL activity and clinical outcome have been limited by the difficulty in precisely quantifying CTL responses.

Recent data involving HLA tetramers, however, have shown a statistically significant inverse relationship between the frequency of HIV-specific CTL and plasma viremia (Ogg *et al.*, 1998) providing strong evidence for the conclusion that HIV-1 specific CTL suppress viral replication *in vivo*. Similarly, inverse correlations between disease progression and HIV-1-specific CTL activity have been reported in two different cohorts of infected subjects (Pantaleo *et al.*, 1997;

Several other HIV-specific cellular immune responses have been described. CD8+ T cells from infected subjects can inhibit HIV replication *in vitro*, a mechanism that is in part mediated by the production of soluble factors (Walker *et al.*, 1986). A number of different factors are believed to mediate this inhibition, including the chemokines; RANTES (regulated upon activation, normal T cell expressed and secreted), macrophage inflammatory protein 1 (MIP-1α), and MIP-1β (Cocchi *et al.,* 1995), macrophage derived chemokine, (Pal *et al.*, 1997), IL-16 and a distinct but still unidentified CD8+ cell antiviral factor (Levy *et al.*, 1996).

The role of HIV-specific CTL in mediating inhibition of HIV replication *in vitro* remains controversial. CTL appear to play a major role in suppression of HIV replication in some *in vitro* systems, but non-cytolytic cells may contribute as well (Levy, 1996). Like CD8+ CTL, CD8+ T cell antiviral activity appears to correlate with clinical outcome. Increased CD8+ T cell antiviral activity has been described in HIV-1 long-term non-progressors (Mackewicz *et al.*, 1991), and is temporally associated with control of viremia in subjects with primary HIV infection (Mackewicz *et al.*, 1994). Conversely, CD8+T cell antiviral activity is lower in patients with advanced HIV infection (landay *et al.*, 1993). A study of seven long-term non-progressors has found that they had relatively low levels of HIV-1-specific CD8 T-cells targeting HIV's Gag and Env proteins, but high levels of CD8 precursor cells. Three of six long-term non-progressors had high HIV-1 p24-specific CD8 T-cell responses (Greenough, 2000).

CD8+ T-cells of non-progressors divide and proliferate more in addition to producing higher levels of perforin which assists in the destruction of HIV-infected cells (Migueles, 2002). Furthermore, CD8+ T-cells of long term non-progressors produce alpha-defensins which have some, albeit minimal HIV-inhibiting properties (Zhang, 2002).

1.3.3 HIV/AIDS Vaccine Development

The development of a safe and effective HIV-1 vaccine remains a global priority. Several approaches have been used to develop HIV vaccines. To induce protection a prophylactic vaccine would have to induce both humoral and cell mediated immune responses. Efforts to develop envelop based vaccines inducing neutralizing antibodies have proved to be nearly impossible (McMichael and Hanke, 1999). Although some new promising approaches to induction of neutralizing antibodies are being developed the emphasis of many vaccine designer have shifted to the induction of cell mediated immunity.

There is growing evidence that CD8+ T cells are an important component of the antiviral responses in both HIV infected people and the simian immunodeficiency virus (SIV) infected macaque model of AIDS (McMichael *et al.*, 2002). *In vivo* CTL may be able to destroy a small number of infected cells before HIV spreads (Hanke and McMichael, 2000). This might explain detection of HIV specific CTL responses in exposed but uninfected individuals (Rowland-Jones *et al.*, 1995; Rowland-Jones *et al.*, 1998).

Several vaccines have been developed that induce CTL responses. However, the responses have been shown to wane with time (Robbison *et al.*, 1995). Plasmid DNA vaccines alone have produced weak CD8+ responses in macaques, but have primed for much stronger responses when gene components have been inserted into viral vector (Hanke *et al.*, 1999). A number of viral vectors have been developed as recombinant DNA HIV vaccines, including canarypox (Boyer *et al.*, 2000) and fowl pox (Kent *et al.*, 1998). vCP205, made from a weakened canarypox virus is used as a carrier for specific HIV genes. Canarypox virus enters human fibroblasts and undergoes one round of replication that enables delivery of HIV genes.

The canarypox vaccine is the first product to result in some HIV-specific CTL responses (Walther-Jallow *et al.*, 2001). Four doses of the vaccine have been found to elicit Gag-specific CTL responses in approximately 50% of the subjects; with either Env or Gag-specific responses observed in more than 50% of human volunteers (Walther-Jallow *et al.*, 2001). The responses observed in subjects have been relatively broad, including cross-clade killing for HIV-1 Clades. CTL activity against different HIV Clades is evident. Canarypox vaccines tested in chimpanzees without protein boosts were able to protect against homologous HIV challenge, but not against challenge with different strains of HIV (Walther-Jallow *et al.*, 2001). Recombinant canarypox SIV or HIV-2 vaccines show some protection against SIV or HIV-2 challenges in monkeys but only after protein

boosting (Santra *et al.*, 2002). Recombinant viral protein vaccines tested to date appear safe.

The vector used for HIV-1 vaccine trials in Nairobi and Oxford was a modified vaccinia virus Ankara (MVA). Vaccine candidates based on MVA have induced SIV-specific (Seth *et al.*, 2000) and HIV specific CD8+ T cell responses in rhesus macaques (Hanke *et al.*, 1999) and enabled protection against SIV disease in macaques (Seth *et al.*, 2000). These results supported development of several DNA and MVA vectored vaccine constructs (Smith *et al.*, 2004).

Two AIDS vaccine constructs, HIVA and RENTA were developed by the Oxford-Kenya vaccine development project (VDP) which together formed the candidate vaccine for efficacy trials. The vaccine constructs were derived from HIV 1 sub type A, a major strain of HIV circulating in Kenya (Hanke and McMichael, 2000). However the group focused on HIVA constructs and conducted trials both in Oxford and Kenya between 2000 and 2005 (Mwau *et al.*, 2004).

The Kenyan HIVA DNA trials were a landmark event, representing the first time that human trials in Africa were conducted using candidate HIV vaccine specifically designed from a primary strain of HIV circulating in Africa.

1.3.3.1 HIVA Vaccine construct

The immunogen designated HIVA was tailored designed for a phase II and III efficacy trial in Nairobi, Kenya. It was derived from the sequences of HIV clade A, the most frequent strain in Kenya. It consisted about 73% of gag protein fused to a string of 25 partially overlapping CTL epitopes. The vaccine was designed to induce HIV-1 specific CTL responses to gag and selected epitopes from other HIV-1 proteins. It was delivered first as naked DNA (the prime) and then in a modified vaccinia Ankara (MVA) vector (boost) (Hanke and McMichael., 2000).

1.3.3.2 RENTA Vaccine construct

The second vaccine construct, RENTA is a fusion protein derived from the consensus HIV A clades sequences of *tat*, reverse transcriptase, *nef*, and *env* (gp41). It consisted of HIV subtype A with the inclusion of additional genes (*pol*, which encode for HIV transcriptase), and regulatory genes, (*tat* and *nef*), which have been mutated to inactivate biological activity. It also includes a synthetic sequence based on two most immunogenic regions of the protein (*env* which contains over 90% of known epitopes for gp41). The vaccine was found to be immunogenic in mice (Nkolola *et al*., 2004) and was to be used in combination with HIVA/DNA in human clinical trials.

1.4 Detection of CD8$^+$ cytotoxic T Lymphocytes (CTL) activity

Detection of CD8 CTL activity has traditionally relied on the chromium release assay (McElrath *et al*, 1997). This assay has been used in numerous vaccine studies but has several disadvantages: Optimal detections of antigen-specific CD8+ T-cells by these methods required fresh rather than cryopreserved, peripheral blood mononuclear cells (PBMC).

Real-time assays become problematic in the conduct of multicenter international trials. Moreover, quantification of cytolytic activities currently relies on the use of limiting dilution assays, which requires laborious *in vitro* stimulation and expansion steps that may be less reflective of the *in vivo* state (Russel *et al.*, 2003). Quantification of virus specific CD8+ T cells by techniques such as IFN-γ Elispot, MHC-peptide tetramer staining and flow cytometric analysis of intracellular cytokine production offer advantage of improved sensitivity and more rapid delineation of class I MHC- restricted epitopes compared with that of chromium release CTL assay.

In addition, these approaches are simpler to perform, require fewer cells, and obviate the need to expand and clone antigen-specific T-lymphocytes and establish antologous B, lymphoblastoid cell lines (Tan *et al.*, 1999; Smith *et al.*, 2001). The IFN-γ Elispot offers an advantage over the tetramer staining by providing a functional analysis of the HIV-1 specific responses in populations

spanning multiple class 1 HLA types without prior knowledge of epitope recognition. Elispot is cheaper, making it more feasible in a field setting (Russel *et al.*, 2003). In addition, the IFN-γ Elispot assay can provide a quantitative measurement of the breath of HIV-specific CD8+ T-cell responses and can define HIV-1 epitopes (Russel *et al.*, 2003). This highly sensitive and specific approach appears to be feasible for application in large-scale HIV-1 vaccine clinical trials, to determine the role of vaccine induced CD8+T-cells in conferring protection against infection.

To understand the persistence of CTL in exposed seronegative individuals (ES), a study was initiated to assay the CD8+ CTL cell responses in a well-characterized cohort of high-risk individuals. The cohort was followed-up longitudinally for duration of up to 9 months. The standard *ex vivo* IFN-γ Elispot assay was initially employed to assess responses to HIV-1 Env, Gag, Pol, and Nef epitopes. The IFN-γ Elispot procedure had undergone vigorous quality control analysis and validation for implementation into phase I and II vaccine trials (Russell *et al.*, 2003).

1.5 Statement of the Problem

HIV/AIDS is a pandemic infecting nearly 40 million people in the world. Seventy five percent of these infections are in Africa. HIV/AIDS pandemic infection is invariably fatal if untreated. The current treatment uses anti-retroviral drugs, does not guarantee cure, is often accompanied by severe side effects and is too costly. This problem is aggravated by the fact that HIV is multiclade and has an unstable genome leading to frequent mutations. This makes it difficult to identify the protective immunity.

Most studied have concentrated on HIV clades B and C which occur in the America and Europe despite the fact that most infections are in Africa. The correlates of immunity, even to these clades, are unknown. Most vaccine development studies have not come up with a protective vaccine construct. Although many studies show that HIV infected persons mount a humoral and cellular immune response, vaccine studies targeting antibody production have not been successful in giving protection against HIV infection.

Cell mediated immune responses against HIV infection are currently being studied, with the hope of identifying a protective immune response. Therefore, there is an urgent need for the development of prophylactic vaccines to prevent infection with the HIV.

1.6 Justification

Accruing data from a variety of studies indicate that IFN-γ producing CTL responses are sufficient to control the levels of the virus in HIV infected individuals (Schmitz *et al.*, 1999). It is expected that eliciting IFN γ producing CTL responses would lead to the development of protective immunity to HIV infection. Towards this end, two AIDS vaccine constructs, targeting HIV clade A, HIVA and RENTA, were developed by the Oxford-Kenya vaccine development project to induce HIV-1 specific CTL responses.

These vaccine candidates induced SIV-specific and HIV-1 specific CD8+ T cell responses in rhesus macaques and enabled protection against SIV disease in macaques. It was not known whether the same would happen with human cells. Since the vaccine would target the high risk group, there was a need to investigate whether HIV A vaccine peptides would stimulate CTL responses from exposed seronegative individuals. In addition, it was important to investigate whether the responses mounted by CTLs from high risk seronegative individuals was different from the one mounted by CTLs from low risk seronegative individuals.

The study reported in this thesis, investigated whether HIV A and RENTA vaccine constructs would induce protective CTL responses in a high risk seronegative cohort to provide background data for planning HIV-1 phase II and III vaccine trials. In order to plan for vaccine trials it was also important to

investigate whether it was possible to recruit, follow up, counsel and monitor volunteers for a considerable duration of time. This study sought to investigate this over 15-month duration.

1.7 Study Objectives

1.7.1 General Objective:

To determine the CTL responses in High Risk HIV-1 negative individuals in Kangemi, Kenya.

1.7.2 Specific Objectives

1 Determine the ability to recruit, counsel and follow-up high-risk HIV negative individuals.

2 Determine the presence and the quantity of CD8+ T cells specific to HIV vaccine peptides *by ex vivo* Elispot and cultured Elispot in high-risk HIV negative individuals.

3 Determine the difference in HIVA and RENTA peptides in eliciting CD8+ T cells specific responses in high-risk HIV negative and low risk Individuals.

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CHAPTER TWO

2.0 MATERIALS AND METHODOLOGY

2.1 Demographic Study

This was carried out to investigate whether it was possible to recruit and follow up a cohort of high risk seronegative volunteers over a period of 15 months in preparation for HIV vaccine trial. A vaccine study is usually done over a long period of time, therefore it requires adherence by the volunteers. It was also necessary to counsel the volunteers and take blood samples from them to assess the CTL responses to the vaccine peptides to determine the base line HIV specific CD8+ T cell responses. The demographic study investigated whether counseling led to positive behavioural change.

2.1.1 Study site

Kangemi is a suburb about 15km West of Nairobi City centre. It is a low income informal settlement, with the majority of the residents living below the poverty i.e. on less $1 a day. Most inhabitants derive their livelihoods from temporary informal employment, usually acquired daily and involving doing odd jobs. One such commercial activity is buying and selling sex. Unlike the Pumwani (Majengo) cohort where sex was sold for cash, Kangemi residents had an intricate sexual behaviour where sex was also sold in kind. This made the Kangemi site unique for the development of a vaccine trial cohort.

2.1.2 Study population

The study population consisted of thirty high risk exposed seronegative individuals adult males and females. For the purpose of this study high risk individuals were defined as individuals who engaged in unprotected sex with multiple (more than one) sexual partners of unknown HIV status. The study sought to investigate whether such individuals also engaged in other activities that would aggravate their risky sexual behaviour including having had STD and/or engaged in drug abuse. With a HIV prevalence rate of 12% at Kangemi, the high risk individuals, who tested negative on an ELISA HIV test were define as exposed seronegative individuals. To ascertain that they were HIV negative throughout the study period, these individuals had an HIV ELISA test at every visit.

2.1.3 Recruitment

Healthy male or female adult volunteers, 18 to 60 years of age, at risk for HIV infection were encouraged to come to KAVI Kangemi clinic through community mobilization and peer-leader recruitment. Interested parties in the community given contact information were encouraged to visit KAVI Kangemi clinic. One thousand adults volunteered for study. A questionnaire to determine the risky sexual behaviour and the suitability of the volunteers for the study was administered (Appendix II). In addition, an HIV ELISA test was carried out to determine the HIV sero-status of the volunteers. Those found HIV infected, were

referred to a clinic of their choice for care. HIV exposed seronegative individuals were enrolled in this study. . Thirty of those volunteers met the eligibility criteria described in sections 2.1.4 and 2.1.5, and were recruited into the study. Control blood samples of low risk volunteers were picked from a previous study on vaccine trials.

2.1.4 Inclusion criteria

- Healthy males and females.
- Age at least 18 years and not more than 60 years on the day of enrolment.
- Able and willing to provide informed consent and sign a consent form (Appendix I).
- At risk of HIV infection, willing to complete a risk assessment questionnaire, HIV counseling and receive results.
- Available for follow-up.
- Able and willing to provide adequate locator information for tracking purposes.

2.1.5 Exclusion criteria

- Unlikely to return for a follow up visit if required.
- Had any condition that in the opinion of the investigator would preclude provision of informed consent, make participation in the study unsafe, or otherwise interfere with achieving the study objectives.

2.1.6 Enrolment visit

HIV negative individuals were encouraged to enroll. Informed consent was obtained prior to conducting any study procedures. At the enrolment visit, the nurse counselor explained all aspects of the study to the volunteers. This visit served as a screening visit to establish the eligibility of the volunteers. Eligible volunteers were subjected to the following procedures:

- Locator information was obtained (the information used to locate where the volunteers live).
- The risk assessment questionnaire was administered to ascertain the responses from previous administration (Appendix II).
- Medical history and ascertainment of current medications was attained.
- Symptoms directed physical examination was performed.
- Post HIV test counseling was done.

The study objectives were explained and the volunteers were given a date for next visit. Volunteers were encouraged to visit the clinic in between the visit dates should they have any illness.

2.1.7 Study design

The following procedures and tests were carried out at every visit:

	M0	M3	M6	M9	M12	M15
Physical examination	√	√	√	√	√	√
Rapid HIV test (Dipstick)	√	√	√	√	√	√
Counseling	√	√	√	√	√	√
Blood collection	√	√	√	√	√	√
HIV Elisa	√	√	√	√	√	√
Elispot	√	√	√	√	√	√

M0 – M15 = Month zero to Month 15

2.1.8 Follow up and study visits

Volunteers were followed up every 3 months for 9 months. At each follow-up visit, a questionnaire was administered. At each follow-up visit interim medical history was obtained, directed physical examination was performed, rapid HIV test was done and HIV negative blood was taken for CTL response determination. For those with discordant results by rapid HIV test, blood was taken for confirmation test by Enzyme Linked Immunosorbent Assay (ELISA). After the phlebotomy and HIV testing the volunteers were counseled and the date for the next visit set.

Volunteers who tested positive during follow up were encouraged to enroll in a study to establish the baseline viral load and HIV incidence and subtype and enrolled in antiretroviral therapy programme. All individuals enrolled in the study were HIV negative by plasma ELISA except for the HIV positive spouses.

2.1.9 Study procedures

2.1.9.1 Informed consent

All volunteers gave their consent to participate in the study by filling in a form that was discussed thoroughly and reviewed with them before enrolment into the study. The volunteer's consent to participate was obtained by signing or marking, and dating the informed consent form witnessed by a nurse counselor or clinical doctor. A copy of the signed and dated informed consent form remained at the clinic and the other copy was offered to the volunteer to take home. The consent form described the purpose of the study, the procedure to be followed, the risks and the benefits in participating in the study (Appendix I).

Volunteers were informed prior to consenting, that they were free to withdraw from the study at any time without any penalty or loss of benefits that otherwise, they would have been entitled to receive. This included, but was not limited, to social and medical benefits, and future care at the Kangemi clinic.

2.1.9.2 Risks

Study volunteers were checked for any discomfort during the drawing of blood. Those that were uncomfortable with phlebotomy were counseled before the procedure was carried out.

2.1.9.3 Benefits

There were direct benefits to volunteers through counseling and treatment of minor ailments at the clinic, other than knowing their HIV status. In addition, they and others may benefit in the future from information learned generated by this study.

2.1.9.4 Risk assessment questionnaire

The nurse counselor assessed the volunteers for past and current risk information for selected HIV risk factors during the risk assessment questionnaire (Appendix II). Those that were illiterate were provided with the necessary assistance to enable completion of the questionnaire by an interviewer who was not a study counselor.

2.1.9.5. History Taking and Examination

At enrolment a complete medical history, including history of sexually transmitted diseases, prior smallpox vaccination, presence or absence of scar from

smallpox vaccination, exposure to wild-type vaccinia and contraceptive practices were recorded. Additionally, a symptom directed physical examination was conducted including vital signs, pulse, respiratory rate, blood pressure, temperature and weight. At enrolment and follow-up volunteers found to have minor ailments were treated at the clinic. Those with major ailments such as tuberculosis or diabetes, were referred to an appropriate clinic of their choice.

2.1.9.6 Study samples

Briefly, 60 ml of blood was collected from the volunteers with heparinized vacutainers. Blood was taken for the separation of peripheral blood mononuclear cells (PBMC) and CTL studies by Elispot.

2.1.9.7 Rapid HIV test

The rapid HIV tests Kits used in the study were Determine, (Abbott, The Netherlands) and Unigold, (Biotech, USA). The test kits comprised of Immunochromatographic strips that incorporated both antigen and signal reagent into a nitrocellulose strip. The specimen (whole blood, plasma or serum) was applied to an absorbent pad from which it was wicked, combined with signal reagent, and migrated through the strip. A positive reaction resulted in a visual line on the membrane where HIV antigen had been applied. A control line that detected immunoglobulin G was applied to the strip beyond the HIV-antigen line. A visual line at the test and control sites indicated a positive test result, a line only

at the control location indicated a negative test result, and the absence of a line at the control site meant that the test was invalid. The test results were obtained in less than 15 minutes.

2.1.9.8. Enzyme linked immunosorbent assay (ELISA)

In this study Vironostika, 4th-generation ELISA kit was used. Inactivated HIV antigens were pre-coated onto an ELISA plate. Upon the addition of a diluted test specimen containing antibodies to HIV-1 to microwells, immune complexes were formed through the interaction between anti-HIV in the specimen and HIV antigens coated on the microwells in the plate.

Following incubation, the specimen was aspirated and the plate was washed with buffer. Subsequently, anti-human immunoglobulin (goat) conjugated with horseradish peroxidase (HRP) was added which bound to the anti-HIV-antigen complex during a second incubation. Following a wash and incubation with ABTS (2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate]) substrate, a green color was produced. The enzyme reaction is stopped by the addition of a fluoride solution. The amounts of antibodies to HIV present in the specimen were qualitatively proportional to color intensity. Results were read by an ELISA plate reader (Diareader ELX800G, Dialab).

2.2 *Ex vivo* Elispot Methods

Measurement of cell mediated immunity relies on detection of antigen specific cytokines production by CD8+ T cells. PBMC cells were separated from the volunteers and the cells were use to measure cell mediated immunity.

2.2.1 Blood collection and processing

Heparinized blood samples were obtained, from adults at high-risk to HIV infection. PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque 1.077 (Sigma, St. Louis, USA). Cells were washed first with Hank's balanced salt solution (Sigma-Aldrich, Irvine, UK) and then washed with 10% fetal bovine serum (FBS) (RPMI 1640; Sigma-Aldrich, Irvine, UK (R10). The cells were counted using an automated cell counter (Beckman Coulter).

2.2.2 Cryopreservation of PBMC

PBMC were re-suspended in an ice-cold freezing medium (10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Irvine, UK) and 90% fetal bovine serum at 10 $\times$ 10^6 cells/ml, and 1 ml of the cell suspension was transferred into a 2 ml cryovial and frozen in a strata cooler box that was pre-chilled at 4°C. The strata cooler box with the cryovials was kept in -80°C freezer overnight. The cryovials were then transferred directly into liquid nitrogen and stored.

2.2.3 Thawing and overnight rest of frozen PBMC

To maximize frozen cell recovery and use of viable cells in the assay, the cells were thawed and rested overnight at 37°C, 5% CO_2 incubator. The vials were retrieved from the liquid nitrogen and placed in a water-bath at 37°C. At the earliest evidence of thawing, the cells were transferred into a pre-labeled 15 ml tube. The media R20 (RPMI 1640 supplemented with 20% FBS, glutamine, sodium pyruvate and penicillin-streptomycin) was added drop by drop until a total volume of 9 ml were added; this was centrifuged at 1400rpm (250g) for 10 minutes. The supernatant was decanted and the pellet flicked to loosen it. The cells were re-suspended in 4 ml of R20 and 2 ml of each transferred to each of the two wells of a sterile 24-well plate. On the day of the assay set-up, the cells were transferred into a 15 ml tube and 5 ml of R10 was added at room temperature. The cells were centrifuged at 1400rpm (250g) for 10 minutes. The supernatant was decanted, the tube bottom was tapped to loosen the pellet and 3 ml of R10 was added. The cells were counted and viability determined. The volume was adjusted with R10 to final concentration of 2×10^6 viable cells/ml.

2.2.4 Vaccine peptides

The vaccine peptides used in this study were designed to target CD8+ T cells. CD8+ T cells recognize foreign peptides of bout 9-11 amino acids in length bound to MHC class 1. Measurement of cell mediated immunity relies on detection of antigen specific cytokines production by CD8+ T cells. CD8 + T

cells when stimulated by antigen specific peptides 15-20 amino acids in length overlapping by 10-11 amino acids produce cytokines one of which is interferon-γ (IFN-γ).

The HIV A is a fusion protein, which consists of a consensus clade A sequence of gag $p24^{gag}$ and $p17^{gag}$. The IAVI Core Laboratory at Imperial College, London, UK provided the synthetic HIV-1 15 mer peptides with 11-aa overlaps. These included 87 gag portion of HIV A peptides and 68 known CTL epitope peptides from the HIV A polyepitope were also included. These peptides are generally pooled to represent the various HIV proteins. HIV A peptides were combined into six pools, of which pools 1 to 4 covered the gag part of HIV A and pool 90 was a combination of the four pools and pool 9 included the known CTL epitopes.

Taking advantage of the fact that most human volunteers are likely to have CTL memory to the influenza, Epstein-Barr virus and cytomegalovirus, a pool of 24 peptides corresponding to well-defined CTL epitopes of these viruses, designated FEC, was used as a quality control. For the negative control, a mock peptide solution (4.49% dimethyl sulfoxide in culture medium) was used while phytohaemagglutinin (PHA) was used as a positive control. The RENTA peptides like HIV A were 15 mer peptides. These peptides were combined into six pools i.e pools 1-6.

2.2.5 *Ex-vivo* IFN-γ Elispot assay

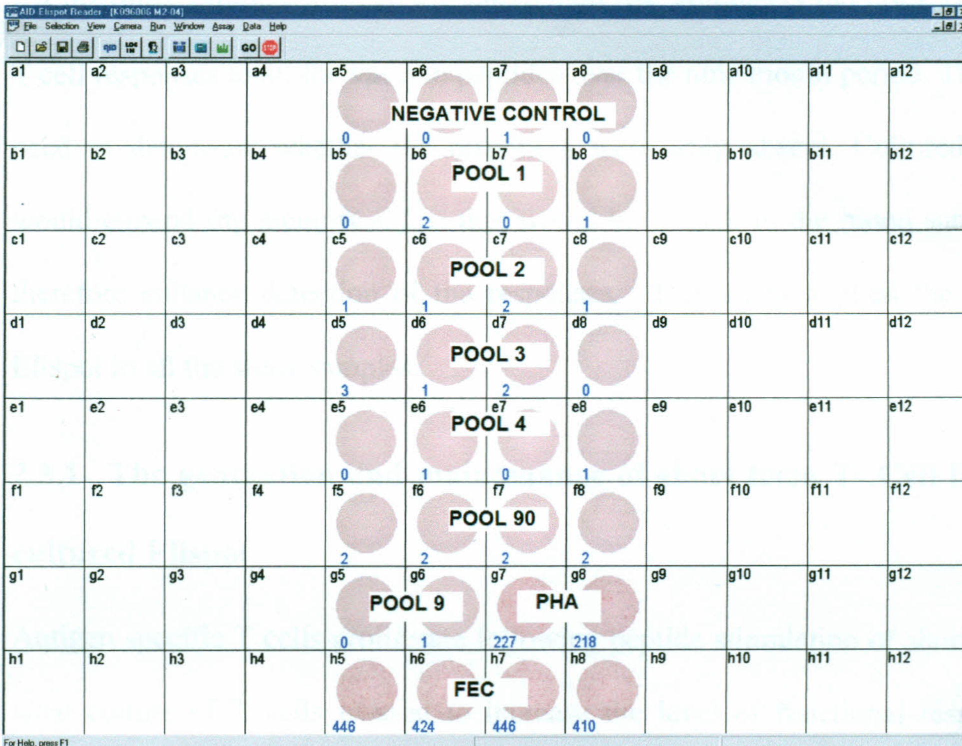
A 96 well nitrocellulose plate (Millititer, Millipore, Bedford, MA) was pre-wetted with 50 μl of 70% ethanol and incubated at room temperature for 5 minutes. The ethanol was discarded and the plate washed three times with sterile PBS (Sigma-Aldrich, Irvine, UK). The plate was coated overnight at 4°C with 100 μl of 10 μg/ml anti-IFN- γ monoclonal antibody (MAb; 1-D1K, mouse IgG1; Mabtech. The plate was then washed three times with sterile phosphate buffer saline (PBS) and blocked with R10 for at least 2 hours at 37°C, in 5% CO_2. After this, 100 μl PBMC suspended in R10 were added to the pre-coated plates at concentrations of 2×10^5 cells/well in quadruplicate and incubated with PHA, no peptide or with peptides, at a final concentration of 2 μg/ml and incubated at 37° C, in 5% CO_2.

The plates were washed with PBS containing 0.5% Tween-20, and incubated at room temperature for 2 hours with a secondary biotinylated anti-IFN-γ MAb at 1 g/ml (7-B6-1, mouse IgG1; Mabtech. Avidin biotinylated enzyme complex (Vectastain ABC Elite Kit, PK-6100; Vector Laboratories was added and incubated at room temperature for 1 hour, followed by 3-amino-9-ethyl carbazole (AEC) peroxidase substrate (Vectastain). After developing plates for 4 minutes, the reaction was stopped by washing with water, and the plates were air-dried and kept in the dark. Colored spot-forming units (SFUs) were counted using an automated AID Eispot reader (Autoimmun Diagnostika Straberg, Germany).

Positive and negative controls were included for each sample and for the whole assay. For the negative controls, cells which had not been stimulated with any antigen were used; to control for the reagents, R10 was added to one of the wells to monitor if the reagents were contaminated or not. PHA, a mitogen was used as a positive control to test for ability of T cells to produce the cytokines to provide a qualitative answer as to whether the assay works; however, they do not test antigen-specific T-cell activation.

The use of antigen-specific stimulation rather than mitogen stimulation is also good for standardizing the IFN-γ secretion kinetics and therefore a previously described pool of peptides within the Flu, EBV and CMV (FEC) was used to stimulate memory T cells. The use of these peptide pools, in addition to PHA to elicit IFN-γ, is useful as a control for the Elispot assay in experiments where HLA type is not necessarily already known at the time of the assay. Flu, EBV and CMV are commonly encountered viruses for which known CD8+ T-cell responses have been demonstrated and for which CD8+ T cell restricted epitopes have been identified. The use of the peptide pool enabled the examination CD8+ T-cell responses in the majority of volunteers previously exposed to Flu, CMV and EBV viruses.

Figure 5: A 96 well plate showing the assay layout



2.2.6 Cut-off points *ex vivo* Elispot assay

The response was positive if the SFU count was greater than 50 SFU/10^6 PBMC above the background (mock) and more than twice the background SFU count for *ex vivo* Elispot. The mock was expected to be less than 25 SFU per million PBMC.

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2.3 Cultured Elispot Methods

All the thirty samples from HIVnegative high risk volunteers did not show CD8+ T cell responses to all the vaccine peptides over the nine month period. There was need to determine whether the responses were truly absent. Cultured Elispot would expand the memory CTLs that would be present in the blood sample and therefore enhance detection of the responses. This study applied the cultured Elispot to all the study samples.

2.3.1 The generation and maintenance of short term T- Cell lines for cultured Elispot

Antigen specific T cells proliferate following peptide stimulation of short term *in vitro* culture of T cells is used to increase the level of functional response in cultured Elispot. PBMC were thawed as described in section 2.10 and where possible they were directly cultured fresh after separation and counted using the Vi cell coulter to determine the viable cells. 16×10^6 cells were placed in a 15ml tube and spun for 10 minutes at 250 -300g. The supernatant was discarded and the tube flicked to re-suspend the cell pellet in 3.2 ml of R10. 400µl of the cells in R10 were dispensed into 8 different wells so that each well contained 2×10^6 PBMC and 100µl of peptides were added into each well as in table 1 below:

Table 1: The antigen peptides, respective number of cells in the different wells

Antigen (peptides)	Total number of PBMC	Number of cultured wells
Pool 90	$10x10^6$	5
Pool 9	$4x10^6$	2
FEC	$2x10^6$	1

Each well carried $2x10^6$ PBMC. Pool 90, which was a cocktail of 4 peptide pools (P1, P2, P3 and P4), was spread over 5 wells, each with 2 million cells which would add up to the figure of $10x10^6$ PBMC. The rest of the wells were filled with PBS to prevent evaporation. The plate was then swirled gently to mix the peptides with the cells and incubated at 37° C, 5% CO_2 in a humidified incubator. This was noted as day 0. On the third day, 1µl of 1800U/ml IL-2 was added into each well. The plate was swirled to ensure mixing of IL-2. On the seventh day a further 1ul of 1800U/ml IL-2 and 1 ml R10 are added into each well. If the media happens to yellow on day 8 or 9 a further 300µl of R10 was added and on the tenth day the cells were harvested as follows:

- Pool 90 was harvested in a 50 ml tube since the volume of the cells was large,
- Pool 9 was harvested into 15ml tube and

- FEC was harvested into a 15ml tube.

The tubes were filled with PBS and centrifuged for 250-300g for10 minutes. The supernatant was discarded, the pellet re-suspended and the cells were washed again. The cells were re-suspended in R10 in the following volumes: Pool 90, 5ml per tube, Pool 9, 2ml and FEC, 1ml. The cells were left at 37° C, 5% CO_2 for 20 to 30 hours before setting the Elispot assay. On day 11, the cells were spun and the pellet was resuspended in R10 to achieve a single cell suspension for accuracy.

2.3.2 Cultured IFN-γ Elispot assay

Short-term cell lines (STCL) were produced for each volunteer and then stimulated with HIV A peptides. They were analysed using IFN- γ Elispot assay using pre aliquoted peptide plate. Specifically, $2x10^6$ cells were stimulated with pool 90, pool 9 or FEC. Cells were cultured at 37°C, 5% CO_2 for 11 to 13 days in R10, receiving a saturating concentration of IL-2 (1800U/ml) at days 3 and 7. 1ml of R10 per well was added at day 7 plus additional R10 at days 8 to 10 as needed. Early on day 10, cells were washed 3 times with sterile PBS and then left in fresh R10 30 to 35 hours at 37°C, in 5% CO_2. On day 11, cells ($4x10^4$/well for pool 90 and pool 9 STCL) were set up in IFN-γ Elispot as described in section 2.12.

2.3.3 Cut off point for cultured Elispot

The response was considered positive if the SFU count was greater than 300 SFU/10^6 PBMC for cultured Elispot. Mock was supposed to be less than 250 SFU/10^6 PBMC. The positive response to pool 90 in the pool 90 STCL was expected to be greater than 250 spots per million to be considered 'positive'.

2.4 Statistical Analysis

Absolute SFU were reported per 10^6 peripheral blood mononuclear cells and after subtraction of background IFN-γ secretion in the cultures containing the control peptides. Differences between cultured and *ex vivo* Elispot assays were tested for statistical significance using t- test at 95% confidence interval [CI].

CHAPTER THREE
3.0 RESULTS

3.1 Demographic and risk factor profiles of HIV Exposed seronegative study populations

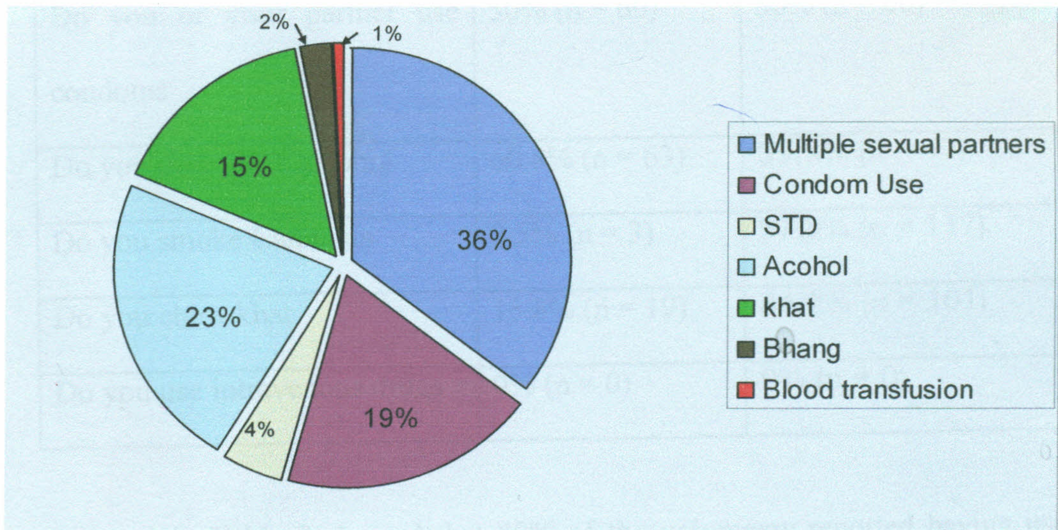
Two groups of HIV exposed but seronegative cohorts were investigated for evidence of protective immunity. To begin with, 30 healthy male and female HIV negative volunteers at high risk of HIV infection (Cohort A) were recruited. These volunteers were followed up for a period of 9 months. All the thirty volunteers selected for follow up came for each scheduled clinic and each volunteer donated 60ml of blood. Some volunteers experienced discomforts while others felt dizzy during the drawing of blood. A few developed a swelling where the needle was inserted. However they were reassured by the clinicians and they continued with the study. The follow-up was 100% since each of the volunteer came for all the scheduled visits.

3.1.1 The High Risk Behavior of the Cohort

The factors that were considered as contributing to high risk behaviour included: multiple sexual partners of unknown HIV status, failure to use condom during sexual contact including inconsistent use of condoms, contraction of sexually transmitted disease, having had a blood transfusion, alcohol and other drugs of abuse. It was found that the greatest indicator of increased HIV infection risk in

this cohort was the multiple sexual partners, abuse of alcohol, bhang and khat and inconsistent or lack of condom use. These responses to high risk activities constituted 36%, 23%, 20%, 15% and 19% of the total volunteers' responses respectively. Blood transfusion, STD and Bhang did not play a major role in the determination of high risk behaviour. Only 4% and 1% had had response to STD and blood transfusion respectively. Those who smoked Bhang (*Cannabis sativa*) were only 2% (Figure 6).

Figure 6: The Percentage of volunteers' responses to the various HIV risk activities



The definition of unsafe sex, for the purpose of this study was; 'unprotected sexual intercourse with a partner whose HIV status was either positive or not

known'. Although the circumstances in which unsafe sex occurred and its frequency over the period of reporting varied considerably, these risky behaviors were dichotomized into Yes or No variables (Table 2).

Table 2: Profile of volunteers' responses showing high-risk activities

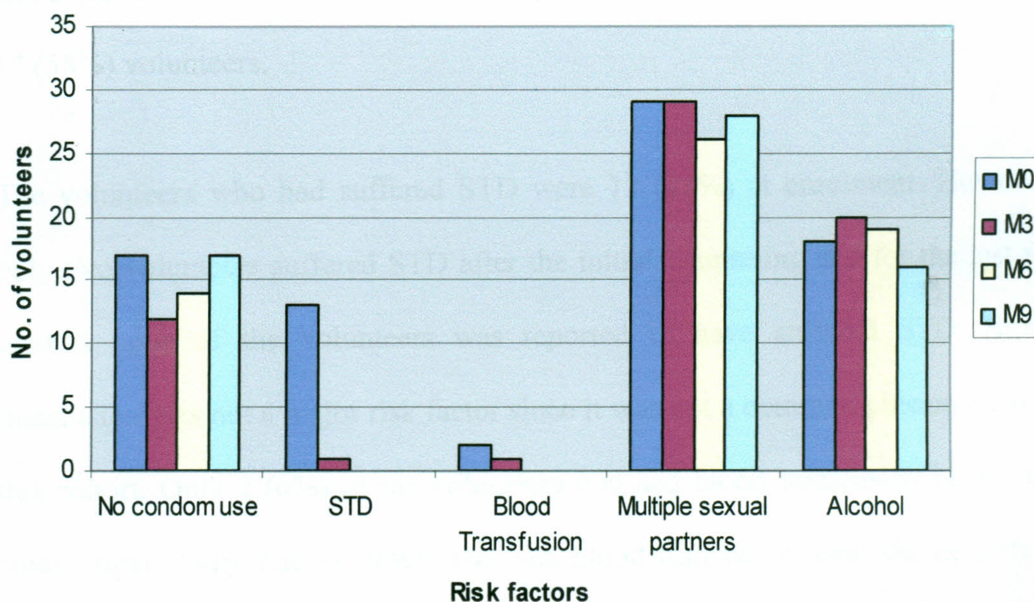
Question	Yes	No
Have you ever suffered STD	11.7% (n = 14)	88.3% (n = 106)
Have you ever received any blood transfusion	2.5% (n = 03)	97.5% (n = 117)
Did you have more than one sexual partner	93.3% (n = 112)	6.7% (n = 8)
Do you or your partner use condoms	50% (n = 60)	50% (n = 60)
Do you ever drink alcohol	60.8% (n = 63)	39.2% (n = 47)
Do you smoke Cannabis	2.5% (n = 3)	97.5% (n = 117)
Do you chew khat	15.8% (n = 19)	84.2% (n = 101)
Do you use intravenous drugs	0% (n = 0)	0% (n = 0)

The report in Table 2 showed that 50% of the volunteers reported having used condoms while the other 50% never used condoms at all. Over the study period 93.3% of the participants reported that they had multiple sexual partners and that

50% of them did not use condoms. 11.7% had STD but only 2.5% of the reponses were pointing to blood transfusion.

having had a blood transfusion. The risky activities at various time points over the 9 months period are shown in Figure 7.

Figure 7: Reported high risk sexual activities for 30 HIV exposed seronegative (ES) volunteers shown at 4 time points for a period of 9 months



Each bar shows the number of individuals that reported the specific high-risk activity over the 9 months period. Counseling was done at each visit to encourage the volunteers to avoid risky behaviour. Positive change in behaviour was expected from each volunteer. However, this was not the case for the majority of the volunteers. The greatest indicators of increased HIV infection risk in this

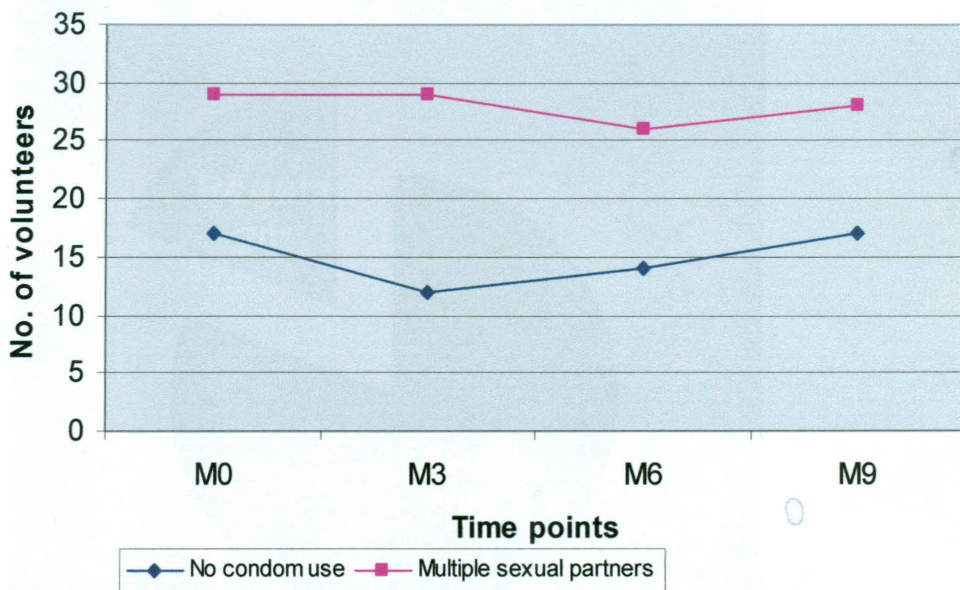
the volunteers. The greatest indicators of increased HIV infection risk in this cohort were lack of condom use, multiple sex partners and abuse of alcohol. There was inconsistency in each risky sexual behaviour trend over the different time points. At the beginning of the study only 17 (58%) of the volunteers were using condoms. After the first counseling, the number of volunteers using condom decreased to 12 (40%) of the volunteers. The trend changed after the second visit (M 3) where the number increased to 14 (47%) over the following three months and continued to increase over the subsequent three months to the initial number, 17 (58%) volunteers.

The volunteers who had suffered STD were 13 (43%) at enrolment. However, only two volunteers suffered STD after the initial counseling and for the last six months none of the volunteers was reported to have suffered STD. Blood transfusion was not a major risk factor since it was not a common phenomenon in this cohort. Only 2 (6%) of the volunteers had had blood transfusion before the study began. Only one volunteer received blood transfusion over the next three months after enrolment. In the last two time points (M6 and M9), none of the volunteers had blood transfusion.

In this cohort only a small variation in the number of volunteers that had multiple sexual partners the volunteers was observed over the study period. In this cohort (29) 97% of the volunteers had multiple sexual partners at the beginning of the

in the following three months. Following the third visit this figure dropped slightly to 26 (87%) and then increased again to 28 (93%) at the last visit (M9). The abuse of alcohol was also key risk factor. Like most of the other risk factors there was little variation in number of volunteers abusing alcohol during the study period. The figures varied between 53 and 67% throughout the study period.

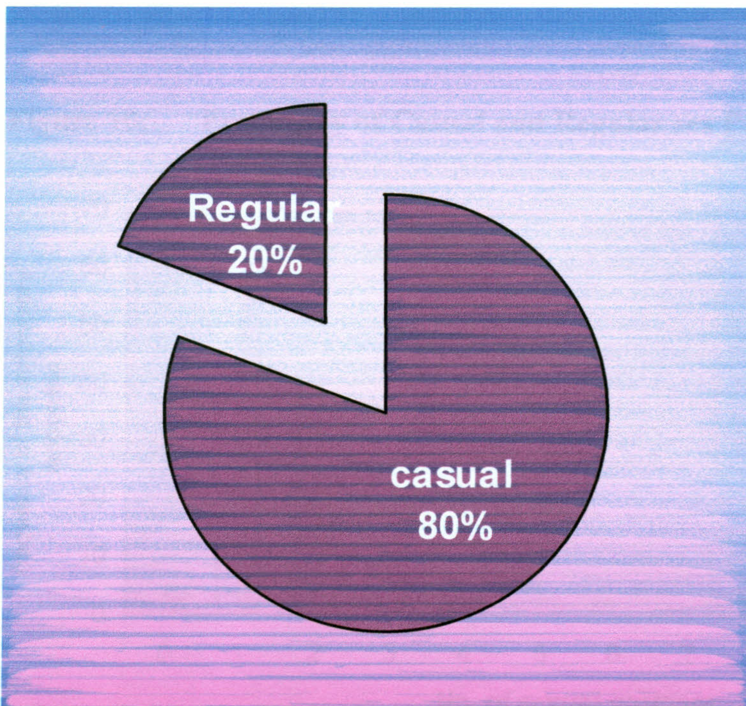
Figure 8: Variations in the use of condom and the number of sexual partners over the study period



Generally, condom use was not popular in this group of volunteers. There was no correlation between the number of volunteers who did not use condoms and those that had many sexual partners. The volunteers had high numbers of sexual

partners at enrolment. Three months into the study the number remained the same while those who did not use condoms decreased significantly. After the third visit the number of volunteers not using condom increased consistently up to month 9 (M9) while there was a slight increase in the number of multiple sexual partners at the same time point. Multiple sex partners were categorized into casual as well as regular partners.

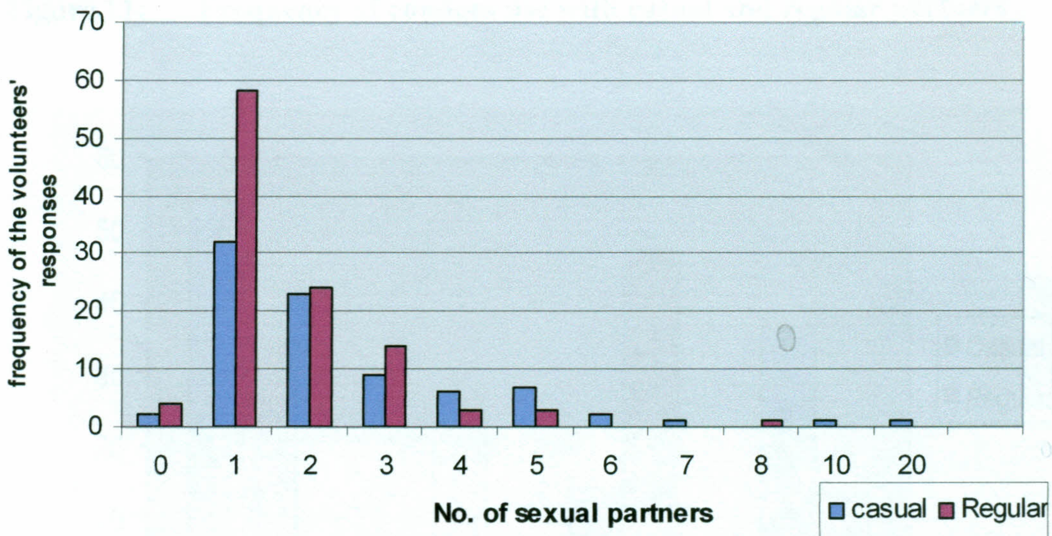
Figure 9: Total percentages of regular and casual sex partners



The volunteers who had casual sexual partners formed 80% while those who had regular sexual partners were 20% (Figure 9) indicates high-risk behaviour within the cohort.

However the total number of casual and regular sexual partners per individual kept on varying during the study period (Figure 10). Majority of the volunteers had one regular and one casual sexual partner. About 23% of the volunteers had two regular and two casual sexual partners. The individuals reporting five or more different casual and regular partners were few (4.1%).

Figure 10: Frequency of Casual and Regular sex Partners

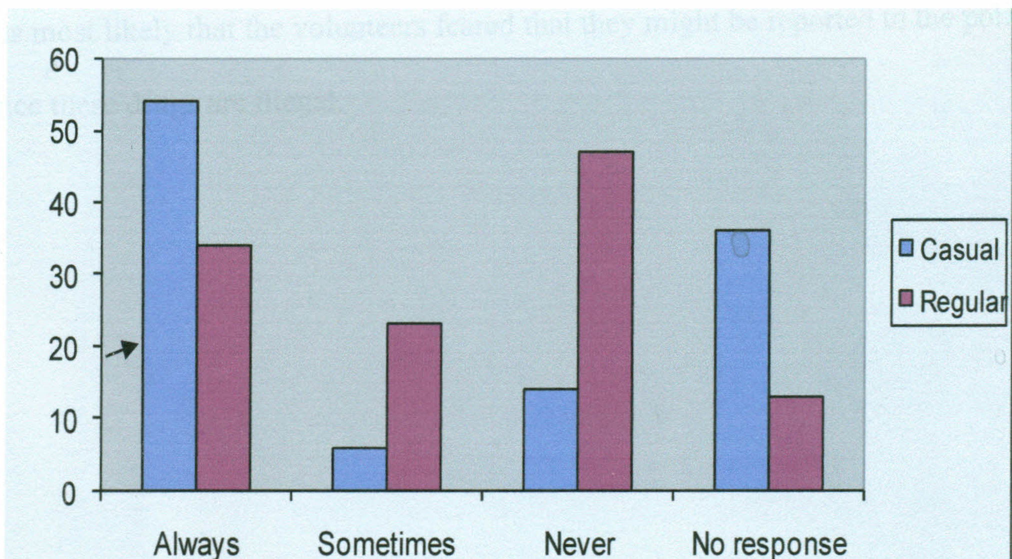


Regular sex partner were usually known to the volunteers and in most cases they paid for sex in kind, meaning that they paid or provided for the upkeep of their partner in exchange for sex. Casual sex partners paid cash for sex and in most

cases they were not known to the volunteer. In this study 28 out of 30 (93.3%) reported that they had multiple sexual partners. Of these 55% had one regular partner who was special in that he did not pay cash for sex. Instead he paid for rent and provided food and other necessities to the volunteer.

Although 50% of the total volunteers reponses indicated that they usd condoms at one time or another during the study period, the use was not consistent. The volunteers reported increased use of condoms with the casual sex partners than with the regular sex partners. Most of the volunteers did not use condoms with the regular sexual partner, even when such regular partners were many. The frequency of condom use is shown in figure 11.

Figure 11: Frequency of condom use with casual and regular partners



Majority of the volunteers never used condoms at all with regular sexual partners. The reasons given for not using condom were that the regular person was trusted and there was no need for protection. In some cases the partner refused to use condoms because it could interfere with his pleasure while sometimes they forgot when they got drunk.

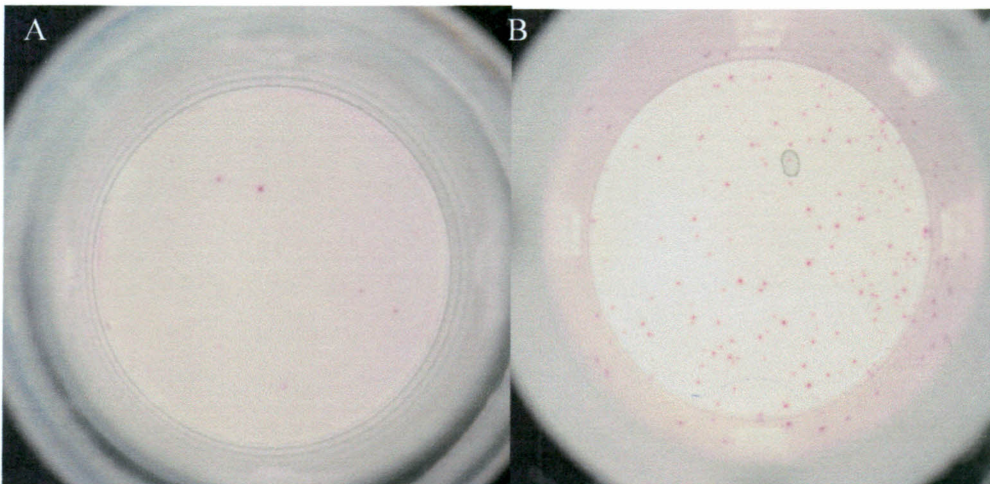
Among the volunteers 60.8% reported that they drank alcohol, 15.8% chew Khat and only 2.5% admitted to smoke bhang (*Cannabis sativa*). Of those who drank alcohol only 15% admitted to use it daily. Nobody owned up to the use of any of the hard drugs like heroine. Given that the KAVI Kangemi clinic is situated in a poor socio-economic area of Nairobi suburbs it is obvious that the volunteers are predominantly poor with little or no education compared to the general population of the Nairobi. They are also unfamiliar with the conduct of medical research thus it is most likely that the volunteers feared that they might be reported to the police since these drugs are illegal.

3.2 HIV-1-specific CD8+ IFN-γ T cell responses

3.2.1 *Ex vivo* Elispot assay

Blood samples were obtained from the enrolled 30 high-risk HIV exposed seronegative individuals showed no evidence of HIV infection by serology (HIV rapid tests and ELISA). The samples were first assessed for HIV specific responses using *ex vivo* IFN-γ Elispot method to a panel of 6 pools HIV A and 6 pools of RENTA peptides. A sample was considered to have a positive response if it met the criteria, that is, if the SFU count was greater than 50 SFU/10^6 PBMC above the background and more than twice the background.

Photograph 1: Elispot Assay (A) A well showing negative results. (B) A well showing positive results.



Photograph 1 two microwells, A and B. Each well shows spots as they appear after an Elispot assay.

Each of the spot in the photographs represents 5 SFU/10^6 PBMC since each well had 200,000 PBMC. Well A show 2 spots equivalent to 10 SFU/10^6 PBMC while well B shows 80 spots equivalent to 400 SFU/10^6 PBMC). Well A is well below the cut off and was considered negative while well B is above the cut off and it was considered positive.

All the thirty samples did not show HIV-1 specific CD8+ IFN-γ T cell responses to both RENTA and HIV-A peptides using the *ex vivo* Elispot assay during the four time points (months 0, 3, 6 and 9). These results are shown in Table 3 and Appendix III. Table 3 shows the mean HIV-1 CD8+ IFN-γ specific responses to both HIVA and RENTA peptides in SFU per million PBMC.

Table 3: Mean Spot forming Units per Million PBMC for each peptide using *ex vivo* Elispot assay

Peptides	Frequency	Min SFU	Max SFU	Mean SFU	Std. Deviation
MOCK	120	0	9	.97	1.758
P1	120	0	9	1.19	1.997
P2	120	0	11	1.20	2.168
P3	120	0	13	1.31	2.274
P4	120	0	16	1.63	3.040
P9	120	0	20	1.57	3.040
P90	120	0	14	1.53	2.547
PHA	120	0	3225	918.64	710.960
FEC	120	0	7138	266.78	791.895
R1	120	0	16	1.17	2.171
R2	120	0	16	1.53	2.520
R3	120	0	15	1.67	2.894
R4	120	0	13	1.31	2.414
R5	120	0	19	1.56	3.201
R6	120	0	25	1.86	3.501

Key

Mock- Negative control, FEC-Positive control, PHA – Positive control

The first column represents the individual volunteers by their identification numbers. The first row shows the various peptides and the controls. Each pool of peptide was tested in quadruplicate and the average calculated. For all the samples

the minimum SFU/10^6 PBMC for each of the peptides was zero while the maximum SFU/10^6 PBMC for HIV A peptides was 20 and for RENTA was 25. HIV-1 specific CD8+ responses for all the samples in all the four time points were less than 25 SFU/million PBMC far less than the cut off (50 SFU/10^6 PBMC) The highest average mean responses for both RENTA and HIV-A peptides were less than 2 SFU/10^6 PBMC. The highest SFU/10^6 PBMC for HIVA peptides was as a result of response to pool 4 (20 SFU/10^6 PBMC), while that for RENTA was 25 SFU/10^6 PBMC due to responses to R6. Both responses were negative since they were all below the cut-off. This means that the responses were altogether absent.

In all the results of *ex vivo* Elispot assay the mean mock reading for both HIV A and RENTA peptides was 1 SFU/10^6 PBMC far below 50 SFU/10^6 PBMC. The standard deviation for mock was 2. The mean readings for the positive controls, PHA and FEC were 919 and 267 SFU/10^6 PBMC respectively. The standard deviations for all the HIV A and RENTA peptides were between 1and 4. This means that the performance of the assay was of the right standard to yield quality results.

These results indicated that either the assay was not sensitive enough to detect any of the responses or the responses were altogether absent. To investigate whether the assay was sensitive enough, a cultured Elispot assay was performed to

enhance signal detection by expanding the reactive memory T cells within the blood samples. To address this, demographic data for this cohort was analyzed to identify individuals with higher risky behaviour than the rest. The samples of these volunteers were used to assay for the HIV-1 specific responses to HIVA peptides using cultured Elispot assay. Twelve such samples were identified.

At this point it was not necessary to follow-up the volunteers up to the 15 months period as was initially stated in the proposal since the results were consistently negative for all the peptides. A decision to terminate the volunteer follow-up was made by IAVI in conjunction with KAVI. So no samples were collected at month 12 and 15.

These results cast doubts on whether the cohort was truly exposed high risk seronegative. It was important to confirm the absence of the responses in samples from proven exposed seronegative individuals and to compare them with HIV positive samples. To address this, 5 discordant couples (one spouse HIV positive) were tested using both *ex vivo* and Cultured Elispot assays. Those who enrolled were ascertained to be exposed since they were couples who had unprotected sex their HIV positive spouses. The HIV negative spouse was then considered exposed seronegative. Only HIV A peptides were used because by this time the RENTA constructs had been withdrawn from circulation.

3.2.2.1: HIV-1 specific CD8+ IFN-γ T cell responses from the high-risk HIV-1 negative individuals by *ex vivo* and culture Elispot

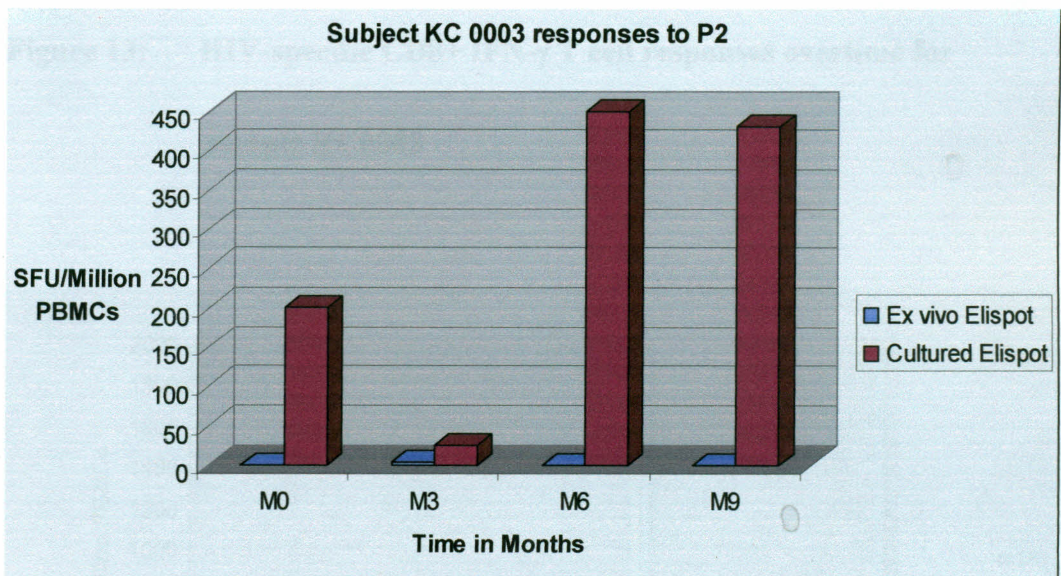
Samples from the selected twelve high risk volunteers were thawed and short-term cell lines (STCL) were produced for each. The cells were stimulated with HIVA peptides in culture media supplemented by IL-2. Cultured Elispot was performed using HIV A peptides. The response was declared positive if the SFU count was greater than 300 SFU/10^6 PBMC, the mock reading less than 250 SFU/10^6 PBMC. The mean reading for mock for all these samples was 35 SFU/10^6 PBMC. The mean readings for PHA and FEC were 5977 and 2895 SFU/10^6 PBMC respectively. They were much higher compared to those in *ex vivo* Elispot.

Five out of the twelve (42%) high-risk seronegative individuals showed HIV-1 specific CD8+ IFN-γ T cell responses to different peptides at different time points. Among these positive responses only three out of twelve showed responses to Pool 9, the CD8+ T cell string of HIVA immunogen. These results indicated the responses were too weak to be detected by the *ex vivo* Elispot assay.

3.2.2.1 Variation of HIV-1 specific CD8+ IFN-γ T cell responses over time points

The sample KC 0003 was one of the samples that responded strongly to HIVA antigens by cultured Elispot assay.

Figure 12: Sample KC0003 showing HIV-1 specific CD8+ IFN-γ T cell responses to P2 by *ex vivo* and cultured Elispot assays

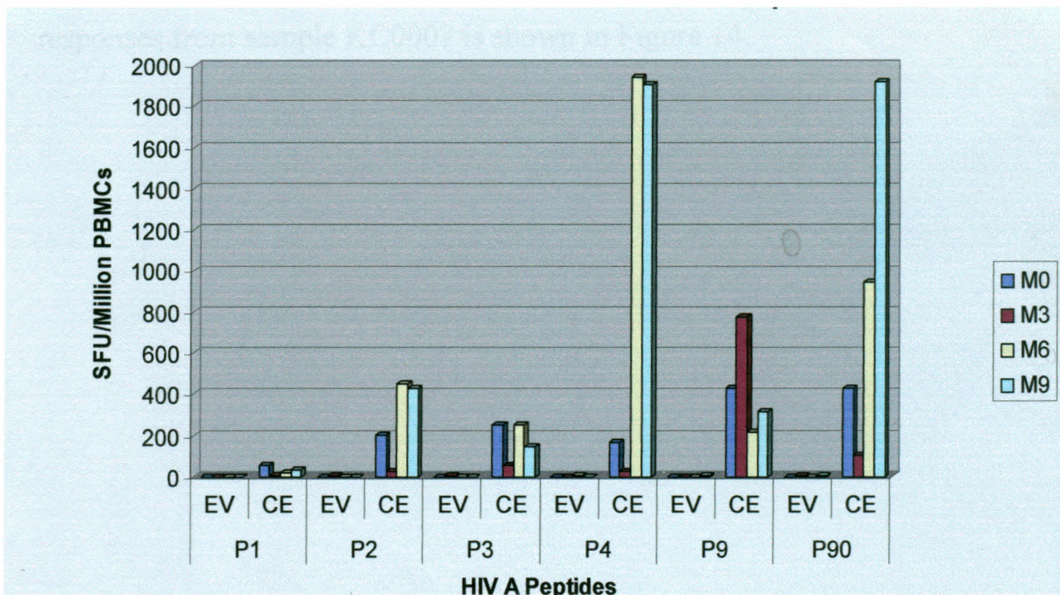


In all the time points' HIV-1 specific CD8+ IFN-γ T cell responses by cultured Elispot were higher than by *ex vivo* Elispot. However, positive HIV-1 specific CD8+ IFN-γ T cell responses by cultured Elispot were detected only in month 6 (M6) and month 9 (M9). The positive responses were above cut off, 300 SFU/10^6

PBMC. Responses in months 0 and 3 were below cut off. Therefore responses to the peptides varied from one month to another.

HIV-1 specific CD8+ IFN-γ T cell responses were found to vary from one time point to time point as well as from individual to individual. The sample KC 0003 was one of the samples that responded strongly to HIVA antigens by cultured Elispot assay. Figure 13 shows HIV-1 specific CD8+ IFN-γ T cell responses to all HIV A peptides for sample KC 0003.

Figure 13: HIV-specific CD8+ IFN-γ T cell responses overtime for sample KC0003



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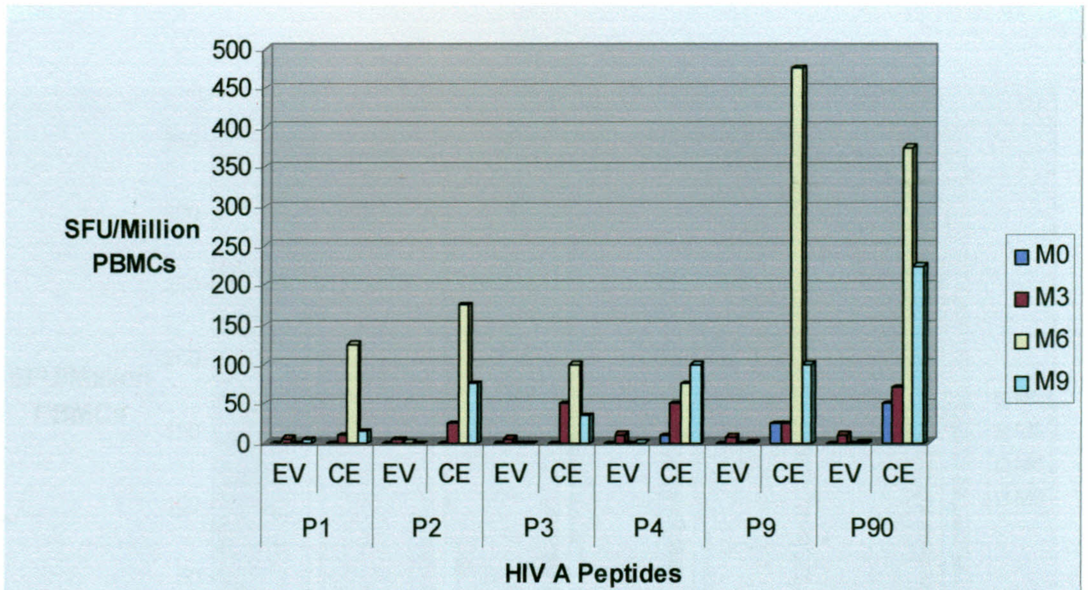
The x axis shows the HIVA peptides, pools 1, 2, 3, 4, 9 and 90 while the y axis shows the HIV 1 CD8+ IFN-γ T cell responses in SFU/million PBMC. On each figure is a key showing the four time points when the assays were performed at months 0, 3, 6, and 9.

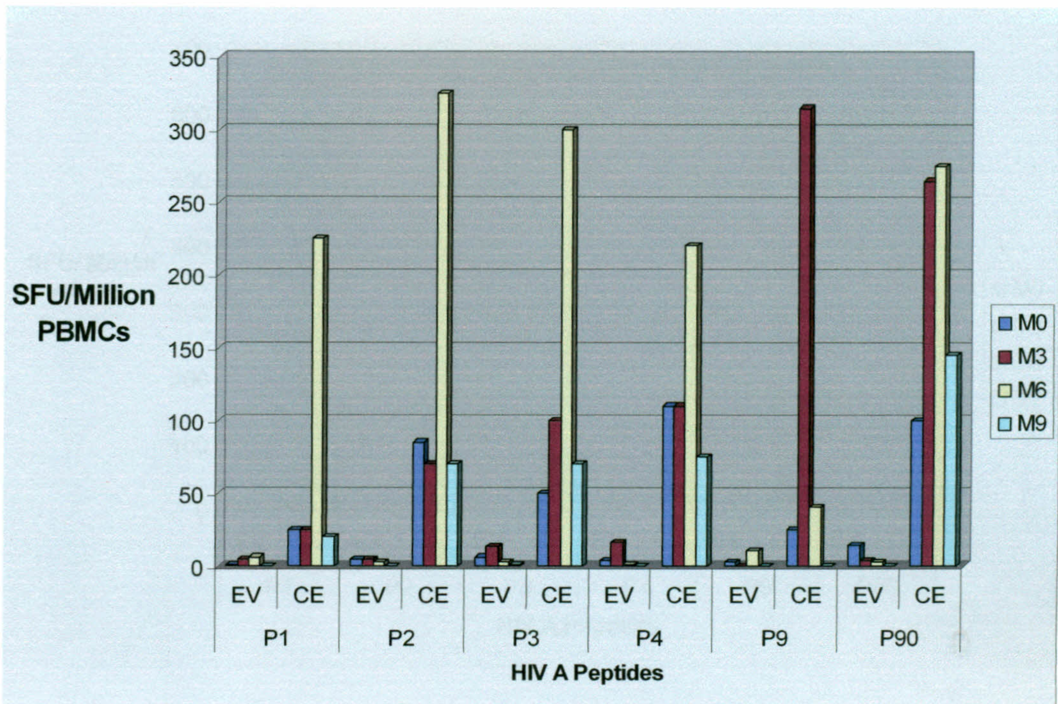
There were positive HIV 1 CD8+ IFN-γ T cell responses by cultured Elispot to most peptides except poo1 and pool 3 from volunteer KC 0003. In Month 0, there were HIV- 1 specific CD8+ IFN-γ T cell responses to P9 and P 90. However, there were strong responses to the peptide pool P90 in month 9. The only response detected at the month 3 time point was to P9, CTL epitopes. However, in month 6, the HIV- 1 specific CD8+ T cell responses were to P2, P4 and P90 with month 9 time point showing similar results with an additional response to P9. Variation was evident within peptides and time points. HIV 1 CD8+ IFN-γ T cell responses from sample KC0007 is shown in Figure 14.

Figure 14: HIV-specific CD8+ IFN-γ T cell responses for sample KC0007



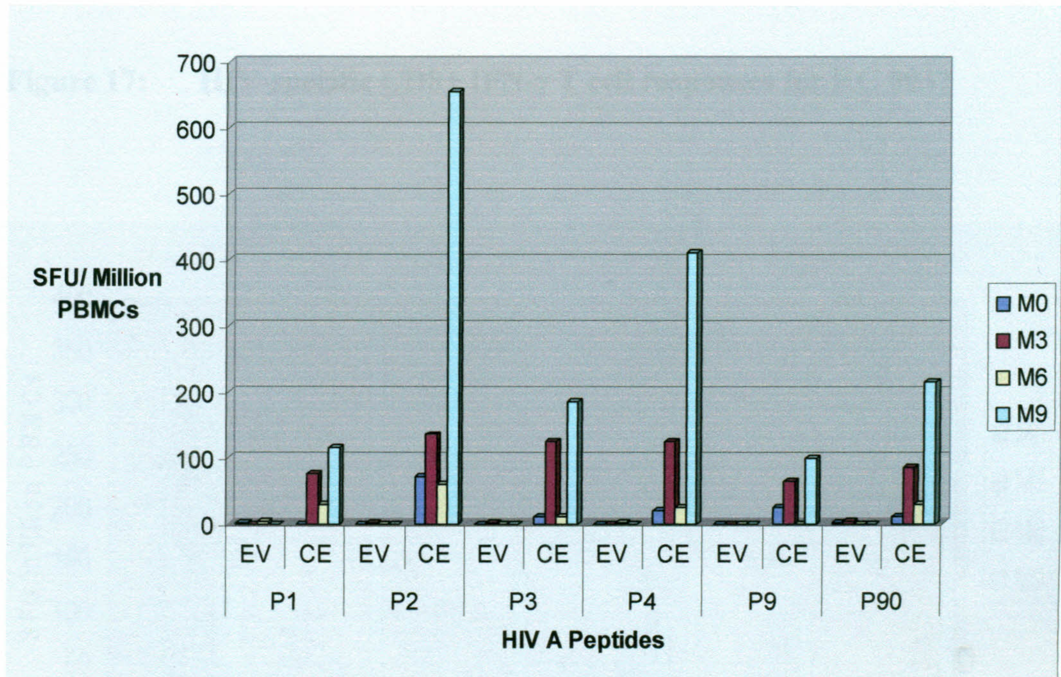
The HIV 1 CD8+ IFN-γ T cell responses by cultured Elispot were higher while those for *ex vivo* Elispot assay to all of the peptides were absent However the positive responses were detected in months 6 and 9 for pools 9 and pool 90 respectively. There were no significant HIV-specific CD8+ IFN-γ T cell responses to the peptide pool 1, 2, 3 and 4 (gag). However, the summation of weak responses of an average 100SFU/million PBMC for these peptide pools gave strong responses for Pool 90 in month 6 only. This variation is also seen in volunteer sample KC0024 in figure 15.

Figure 15: Sample KC0024 HIV-specific CD8+ IFN-γ T cell responses



Generally, the overall HIV 1 CD8+ IFN-γ T cell responses for volunteer KC 0024 were very weak for cultured Elispot. Positive responses exist only at month 6 for pool 2 and pool 9. However, the responses were weak being slightly above the cut off (300) SFU/million PBMC. HIV-specific CD8+ IFN-γ T cell responses for volunteer KC 0026 by cultured Elispot were better than those of KC 0024.

Figure 16: HIV-specific CD8+ IFN-γ T cell responses for KC0026

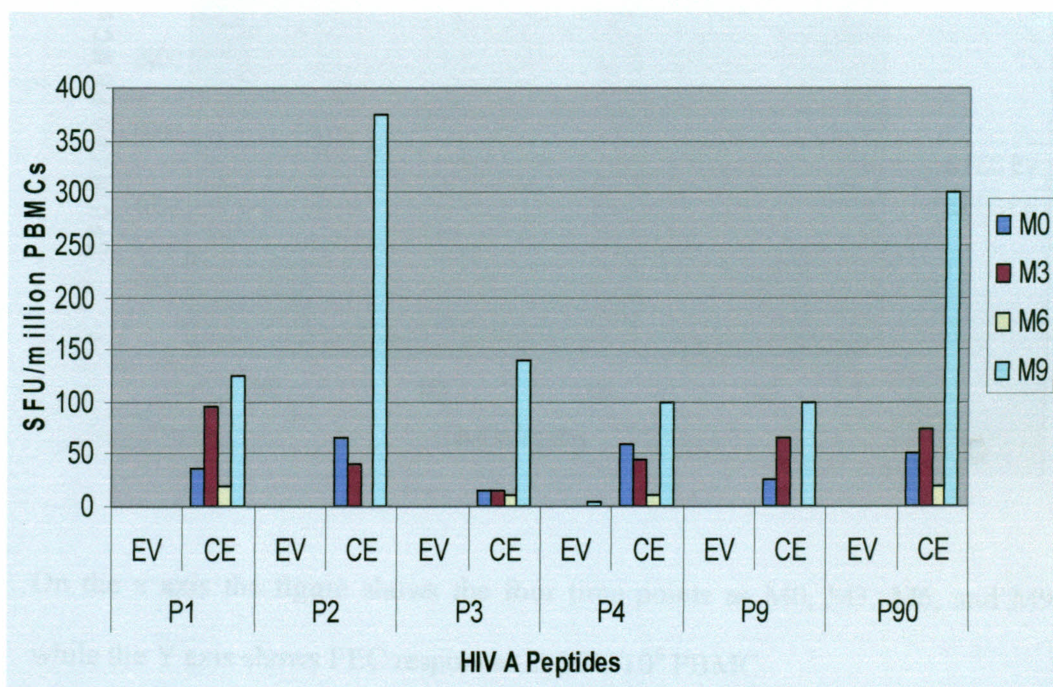


HIV-specific CD8+ IFN-γ T cell responses for volunteer KC 0026 were only in one time point, month 9 to peptide pools 2 and 4. The response to pool 2 was 650 SFU/10^6 PBMC and to pool 4 was 400 SFU/10^6 PBMC. The responses for the other peptides were considered absent falling below 200 SFU/10^6 PBMC. Figure 17 shows HIV-specific CD8+ T cell responses for volunteer KC 0037.

The samples from volunteer KC 0037 showed negative HIV-specific CD8+ IFN-γ T cell responses to almost all the peptides over all the time points. However, there

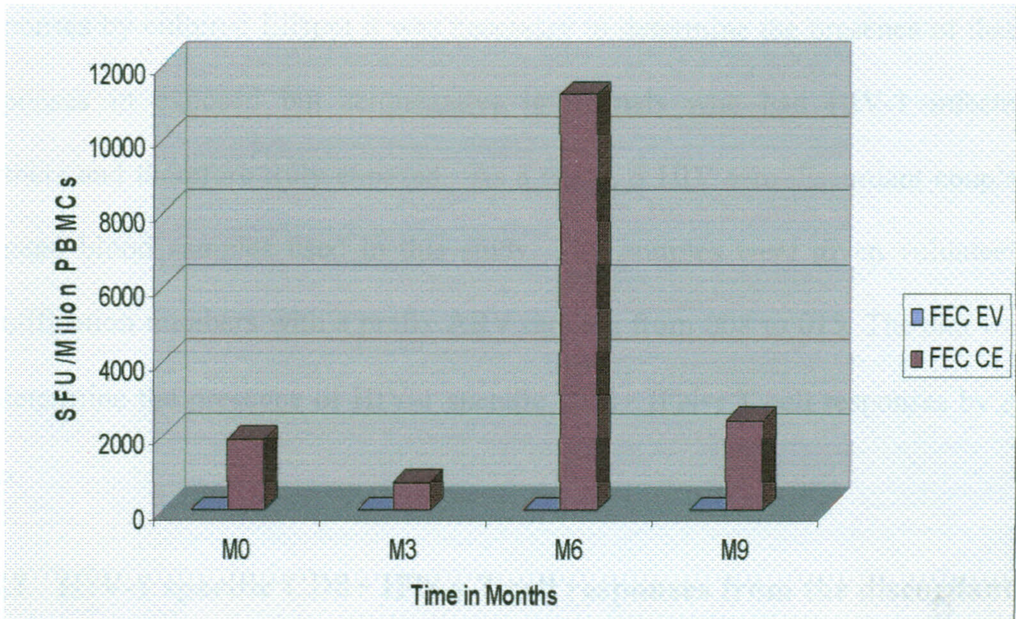
was a weak positive response to pool 2 at month 9 at 370SFU/10^6PBMC. These results are shown in figure 17.

Figure 17: HIV-specific CD8+ IFN-γ T cell responses for KC 0037



Most human volunteers were likely to have been exposed to and therefore have had memory cells to Influenza, Epstein Barr virus and Cytomegalovirus. A pool of FEC peptides corresponding to specific CTL epitope of these viruses was used in this study for quality control. A majority of volunteers (over 70%) had positive response to FEC (Figure18).

Figure 18: Mean FEC CD8+ T cell responses to FEC peptides in *ex vivo* and cultured Elispot for all the volunteers



On the x axis the figure shows the four time points as M0, M3, M6, and M9) while the Y axis shows FEC responses in SFU/10^6 PBMC.

The FEC specific CD8+ IFN-γ responses by both *ex vivo* and cultured Elispot were compared. It was evident that FEC specific CD8+ IFN-γ responses Elispot for cultured were stronger than those of *ex vivo* Elispot ($P<0.001$).

3.3 Discordant Couples cohort

In the high-risk cohort the sexual partners were of unknown HIV status. Since only a few individuals showed the presence of HIV-1 specific CD8+ IFN-γ T cell responses by cultured Elispot it was necessary to determine the presence of these responses in exposed but seronegative individuals who had HIV-1 infected partners and therefore truly exposed. As a result, 5 HIV sero-discordant couples donated blood samples used in this study. The samples were given volunteers identification numbers with a prefix ARV ranging from 008 to 015. The aim was to determine the presence of HIV-1 specific CD8+ IFN-γ T cell responses by *ex vivo*.

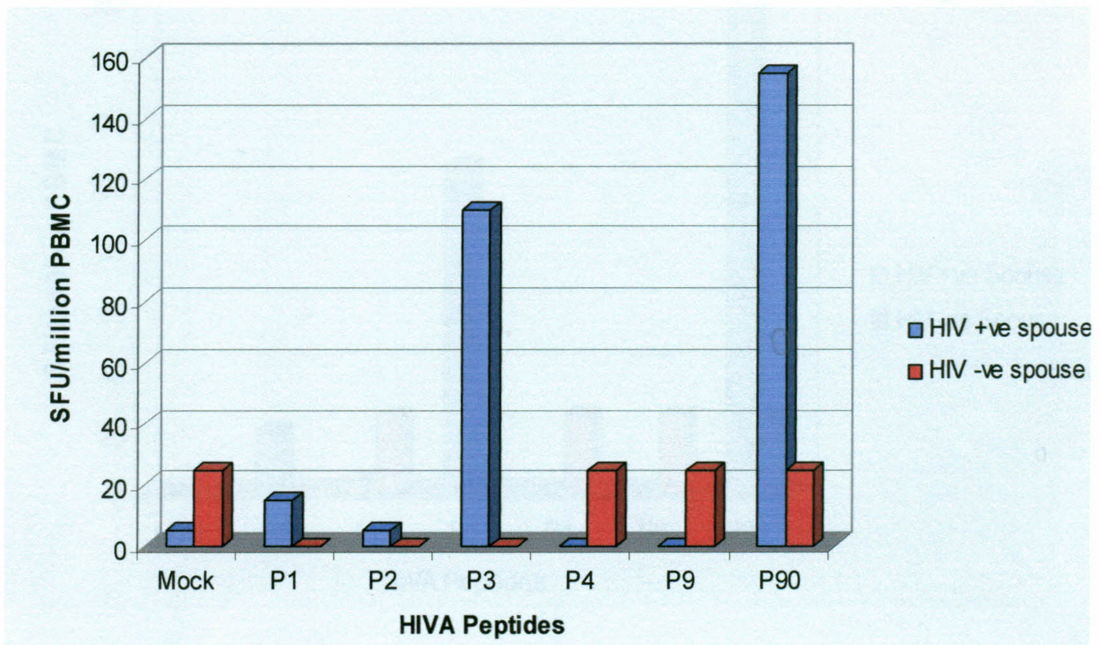
3.3.1 HIV-1 specific CD8+ IFN-γ T cell responses from the discordant couples by *ex vivo* Elispot assay

Ex vivo Elispot assay was performed with all the five samples from HIV seronegative discordant spouses to assay for HIV-1 specific CD8+ IFN-γ T cell responses to HIV A peptides. A sample was considered to have a positive response if it met the criteria for *ex vivo* Elispot assay. That is if the SFU count was greater than 50 SFU/10^6 PBMC above the background and more than twice the background.

The results showed that HIV-1 specific CD8+ IFN-γ T cell responses were below cut off. Therefore HIV-1 specific CD8+ IFN-γ T cell responses could not be

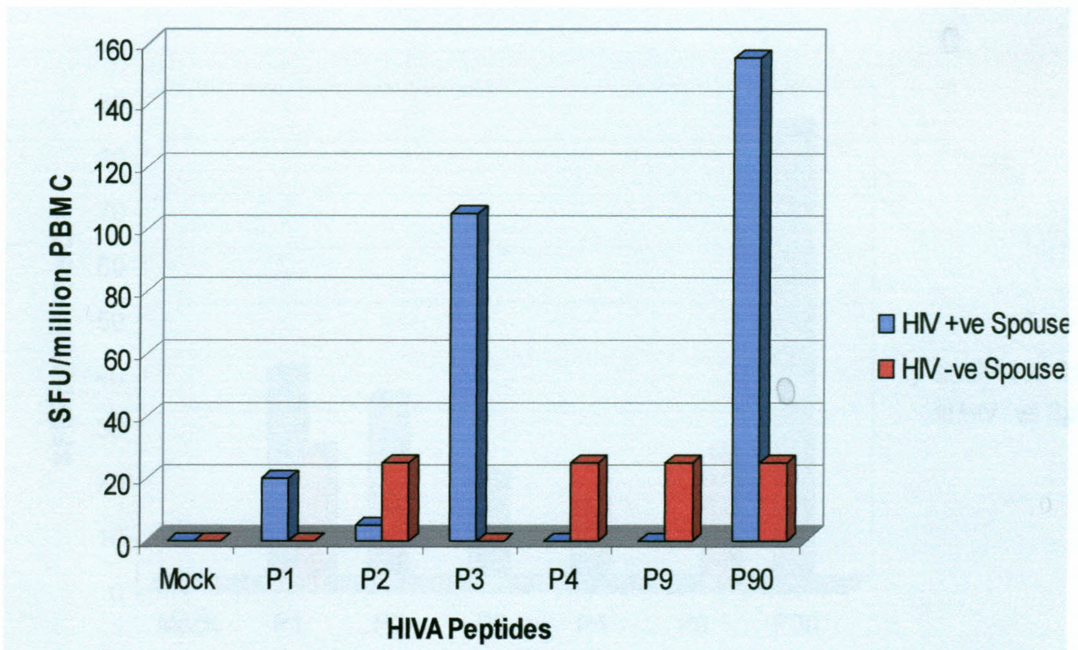
detected by *ex vivo* Elispot assay. The fact that HIV seronegative discordant spouses were most likely exposed to HIV infection, made the assay doubtful. At this point it was necessary to use HIV positive samples to confirm that the *ex vivo* Elispot could detect the responses. *Ex vivo* Elispot assay was therefore performed with samples from the HIV positive spouses. The results were compared with those of HIV negative spouses. The results are shown below. Figure 19 shows result from the ARV 008 couple.

Figure 19: HIV-1 specific CD8+ IFN-γ T cell responses in the discordant Couple ARV 008 by *ex vivo* Elispot assay



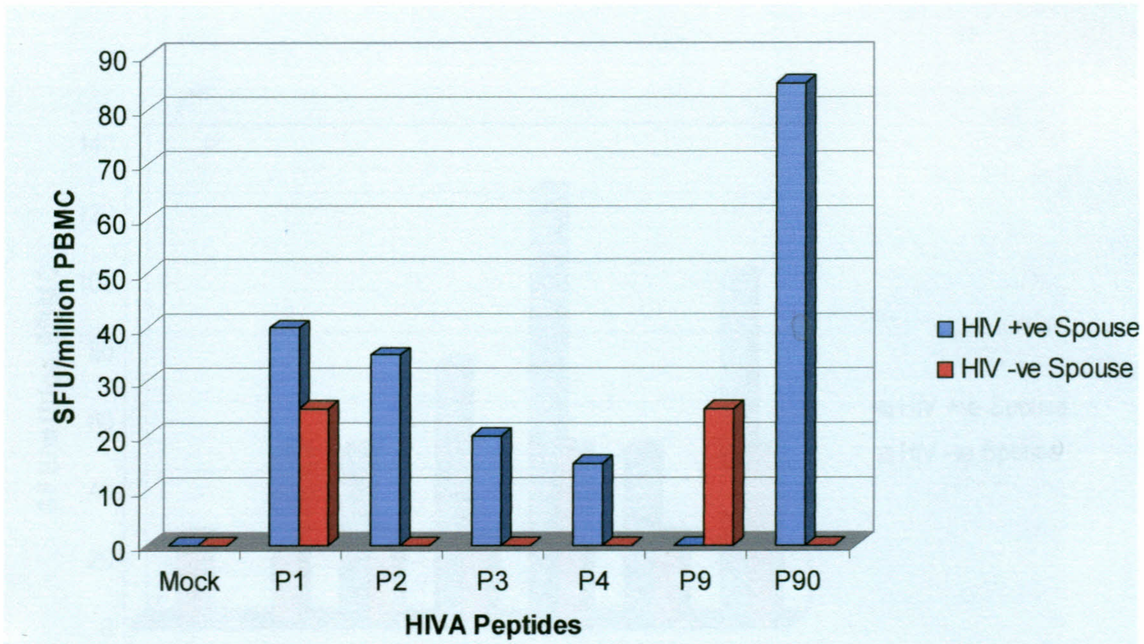
There were no HIV-1 specific CD8+ IFN-γ T cell responses in the HIV negative spouse by the *ex vivo* method. The responses detected were below the cut-off 50 SFU per million PBMC. However the results for HIV positive spouse revealed the presence of HIV-1 specific CD8+ IFN-γ T cell responses to a pool 3 and pool 90 of HIVA peptides by *ex vivo* Elispot assay. The presences of these responses were above the cut off. The couple ARV 009 displayed similar result (Figure 20).

Figure 20: HIV-1 specific CD8+ T cell responses in the discordant couple ARV 009 by *ex vivo* Elispot assay



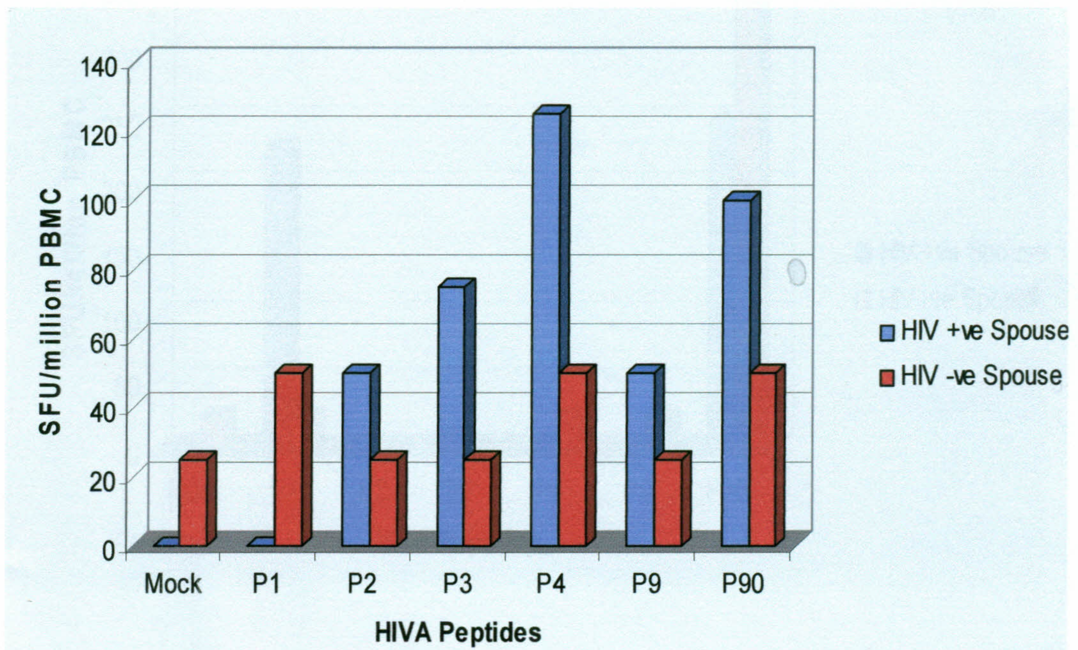
ARV 009 HIV negative spouse had no positive HIV-1 specific CD8+ IFN-γ T cell responses. All the negative responses from either of the spouse were below 20SFU/10^6 PBMC at the cut off of 50SFU/10^6 PBMC. The positive responses were far above cut off. ARV 009 HIV positive spouse had HIV-1 specific CD8+ IFNγ T cell responses to pool 3 (100 SFU/10^6 PBMC) and Pool 90 (150 SFU/10^6 PBMC). However, couple ARV 012 displayed positive responses only to pool 90. Figure 21 shows the response of couple ARV 012.

Figure 21: HIV-1 specific CD8+ IFN-γ T cell responses in the discordant Couple ARV 012 by *ex vivo* Elispot assay



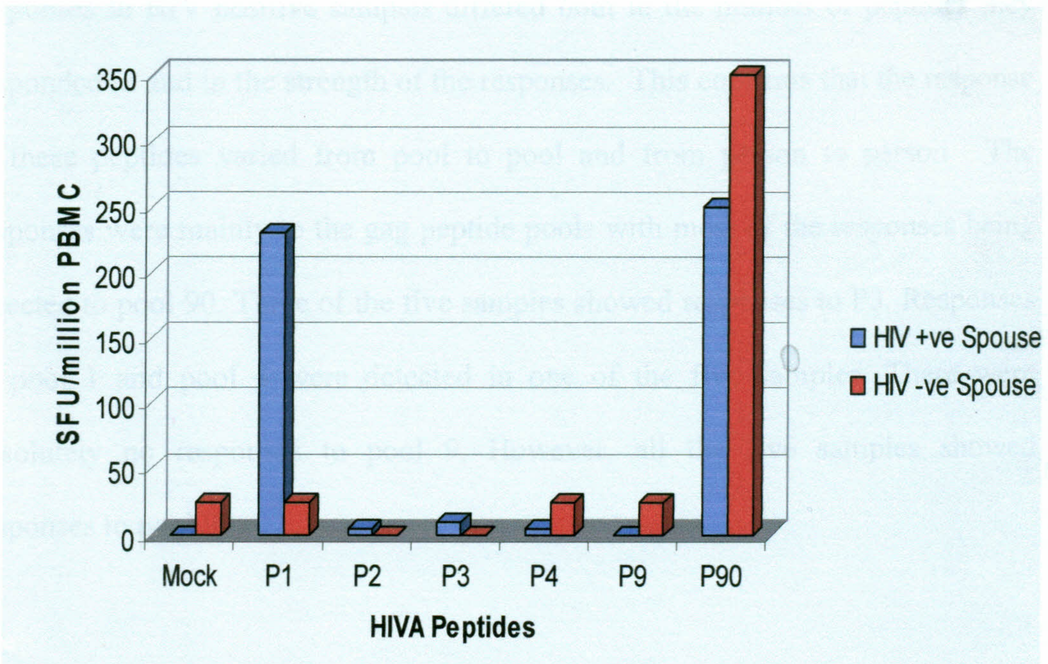
HIV-1 specific CD8+ IFN-γ T cell responses for ARV 012 negative spouse did not make HIV-1 specific CD8+ IFN-γ T cell responses to any of the HIV A peptides. However, the HIV positive spouse responded only to pool 90. All the negative responses were below 40 SFU per million PBMC. The summation of pool 1 to pool 4 HIV-1 specific CD8+ IFN-γ T cell responses could have contributed to the strong response seen for pool 90 since this pool comprised pool to pool 4. ARV 015 had very strong responses to three peptide pools, peptides pools3, 4 and 90, as shown in figure 22.

Figure 22: HIV-1 specific CD8+ IFN-γ T cell responses in the discordant Couple ARV 015 by *ex vivo* Elispot assay



ARV 015 negative spouse had no positive HIV-1 specific CD8+ IFN-γ T cell responses to any of the HIV A peptides. However most of these responses were just slightly below the cut off point (50 SFU/million PBMC). HIV-1 specific CD8+ IFN-γ T cell responses in the HIV positive spouse were above the cut off for pool 3 (70) pool 4 (120) and pool 90 (90). It was surprising to have no response to pool 1 at all.

Figure 23: HIV-1 specific CD8+ IFN-γ T cell responses for discordant couple ARV 019 by *ex vivo* Elispot assay



ARV 019 HIV negative spouse did not respond positively to HIV A peptides except for pool 90. These negative responses were very weak (less than 30SFU per million PBMC). Unlike in the other entire HIV negative spouses the pool 90 was unusually strong. The positive spouse responded strongly to pools 1 and 90 as well. Both positive responses were above 200 SFU per million PBMC which was very strong positive for *ex vivo* Elispot assay.

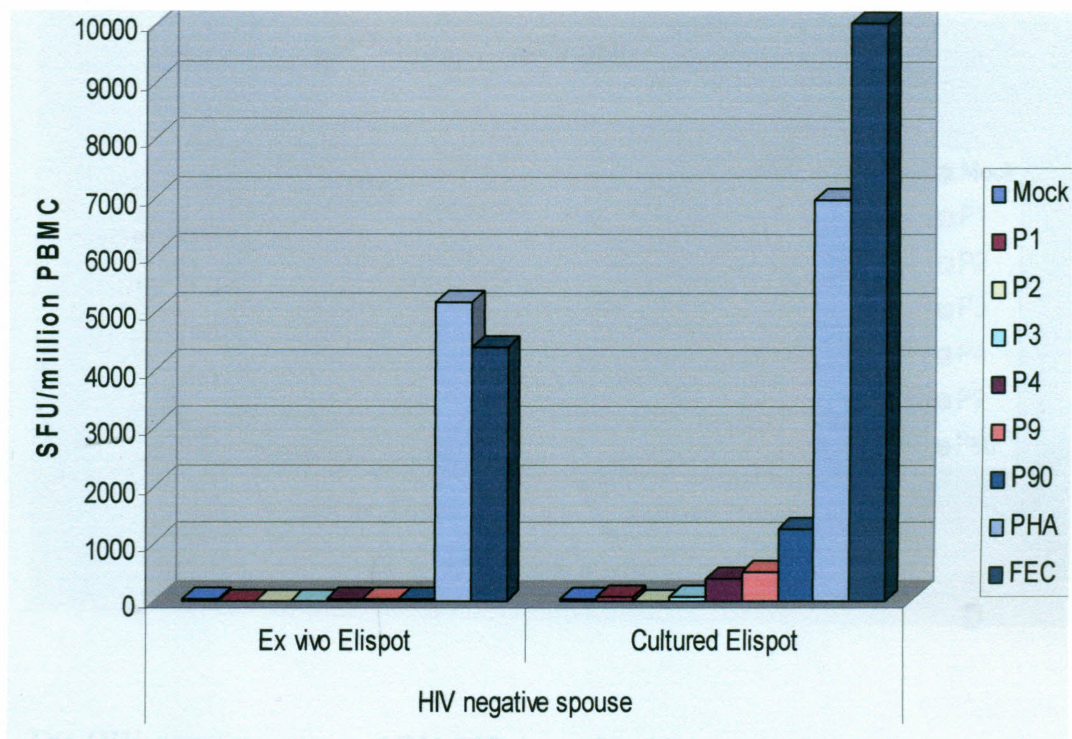
These results confirm that HIV seronegative and exposed individuals displayed no responses to the vaccine peptides using the *ex vivo* Elispot assay. However, HIV-1 specific CD8+ IFN-γ T cell responses to a number of HIVA peptides were detected in all the five HIV positive spouses. HIV-1 specific CD8+ IFN-γ T cell responses in HIV positive samples differed both in the number of peptides they responded to and in the strength of the responses. This confirms that the response to these peptides varied from pool to pool and from person to person. The responses were mainly to the gag peptide pools with most of the responses being directed to pool 90. Three of the five samples showed responses to P3. Responses to pool 1 and pool 4 were detected in one of the five samples. There were absolutely no responses to pool 9. However, all the five samples showed responses to pool 90.

3.3.2 HIV-1 specific CD8+ IFN-γ T cell responses for discordant Couples by Cultured Elispot

The positive responses from HIV positive samples confirmed that the assay could detect the responses to HIV A peptides if present. It was noted that the responses from HIV serongative sample ARV 015 were very close to the cut off and that there was a strong response to P90. Therefore, cultured Elispot assay was performed on the PBMC from the HIV seronegative spouse to enhance detection of HIV-1 specific CD8+ IFN-γ T cell responses. The results were compared with those of *ex vivo* Elispot assay.

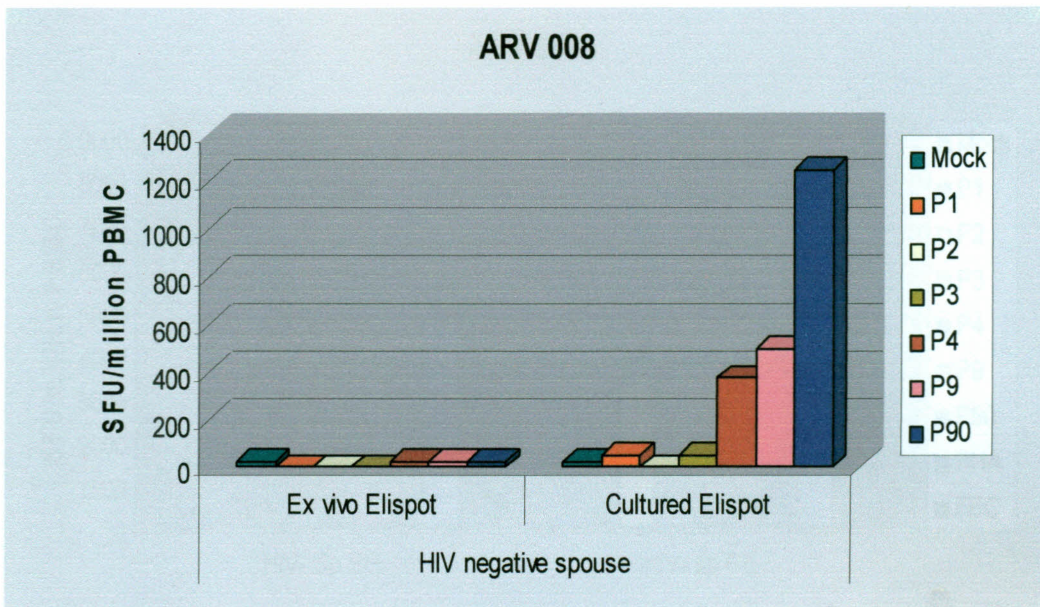
The positive cultured Elispot HIV-1 specific CD8+ IFN-γ T cell responses were more than 15 times higher than those of the *ex vivo* Elispot assay. In all the samples both positive controls, FEC and PHA were also enhanced. Figure 24a shows HIV-1 specific CD8+ IFNγ-T cell responses for ARV 008 with the positive control while Figure 24b shows the same results without positive control.

Figure 24 a: HIV-1 specific CD8 + IFN-γ T cell responses in the ARV 008 HIV negative spouse by *ex vivo* and cultured Elispot assay



ARV 008 HIV negative spouse had positive responses to pool 4 (375 SFU/10^6 PBMC), pool 9 (500 SFU/10^6 PBMC) and pool 90 (1250 SFU/10^6 PBMC) ($P<0.001$). The volunteer, ARV 008 HIV negative spouse did not show significant HIV-1 specific CD8+ T cell responses to all HIV A peptides by *ex vivo* Elispot assay. The HIV-1 specific CD8+ IFN-γ T cell responses were below the cut-off 50 SFU per million PBMC. Figure 24b shows the same response without PHA and FEC, the positive controls. The visual impact is now more apparent showing the responses to P4, P9 and P90.

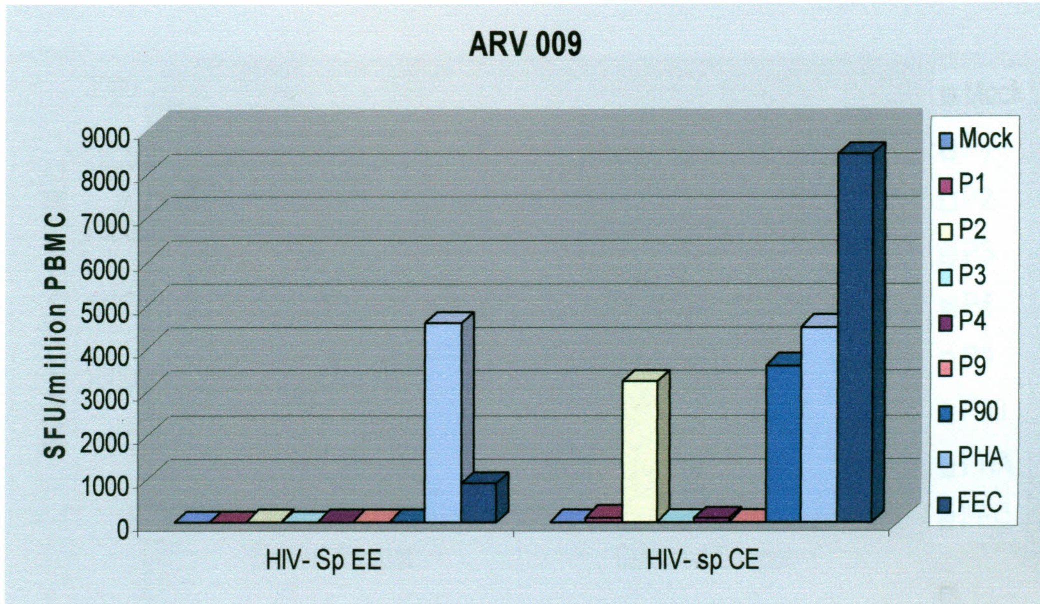
Figure 24 b: HIV-1 specific CD8+ IFN-γ T cell responses in the ARV 008 HIV negative spouse by *ex vivo* and cultured Elispot assay



The HIV negative spouse ARV 008, has a significant ($P<0.001$) response to P4, P9 and P90 by cultured Elispot.

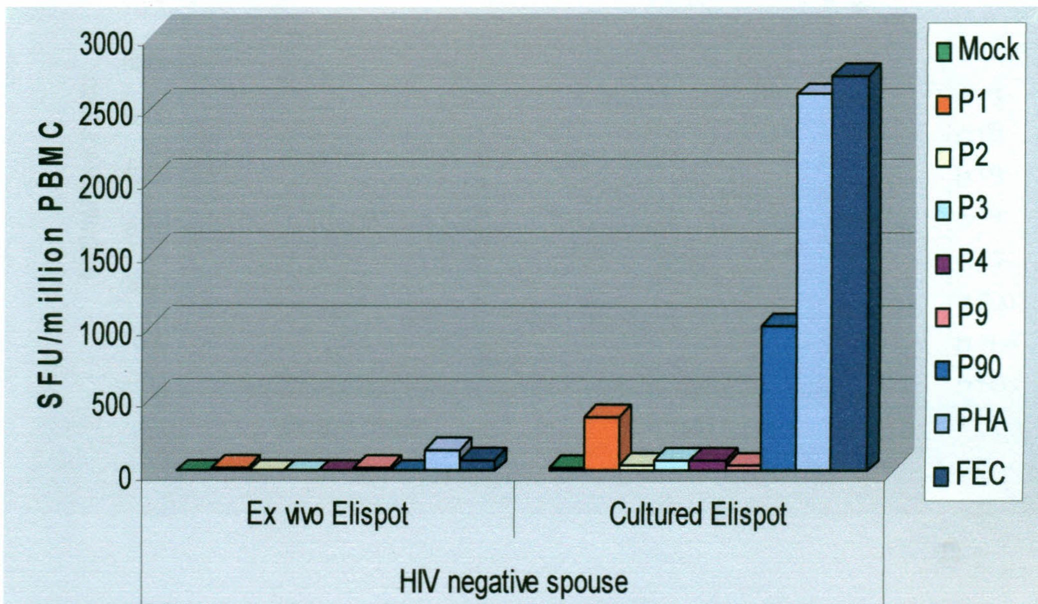
The HIV negative spouse ARV 009 had positive HIV-1 specific CD8+ IFN-γ T cell responses to pool 2 and pool 90 (Figure 25). The responses to both peptides were above 3000 SFU/10^6 PBMC.

Figure 25: HIV-1 specific CD8+ IFN-γ T cell responses in HIV negative spouse ARV 009 by *ex vivo* and cultured Elispot assay



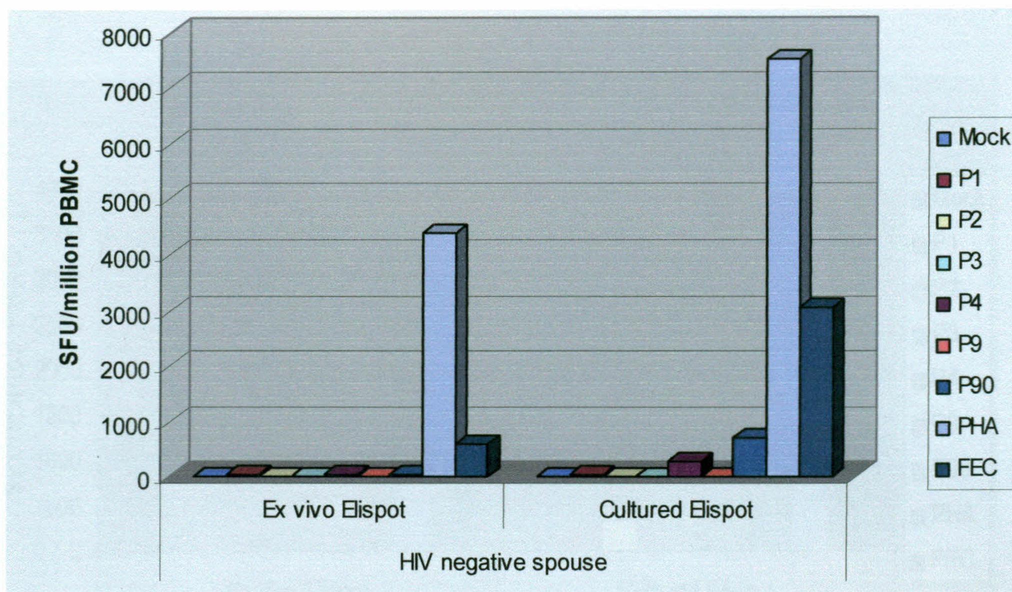
ARV 012 HIV negative spouse had positive HIV-1 specific IFN-γ CD8+ T cell responses to pool 1 and pool 90. The *ex vivo* Elispot responses for the positive controls were very low; FEC (75 SFU/10^6 PBMC) PHA (145 SFU/10^6 PBMC). However, they were enhanced by cultured Elispot; FEC (2725 SFU/10^6 PBMC) PHA (2600 SFU/10^6 PBMC) as shown in figure 26.

Figure 26: HIV-1 specific CD8 + IFN-γ T cell responses in the HIV spouse ARV 012 by *ex vivo* and cultured Elispot assay



The sample from ARV 015 responded different to all the other samples (Figure 27).

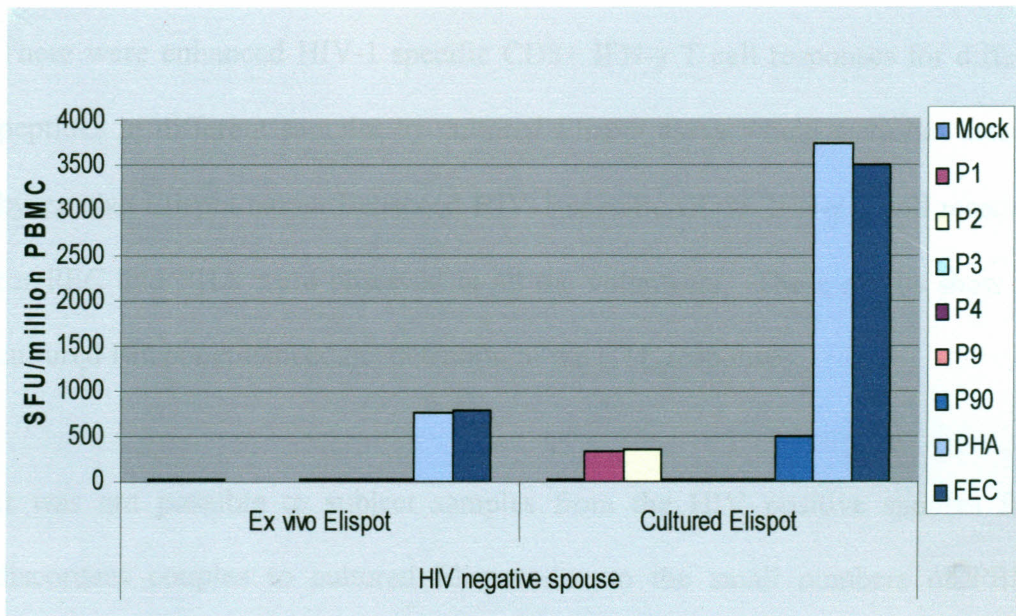
Figure 27: HIV-1 specific CD8+ IFN-γ T cell responses in the discordant Couple ARV 015 by *ex vivo* and cultured Elispot assay



ARV 015 HIV negative spouse had weak HIV-1 specific CD8+ IFN-γ T cell responses. The response to pool 4 was at the boarder line (300 SFU/10^6 PBMC) and it was considered negative. The only response was to pool 90 (725 SFU/10^6 PBMC) which were weak compared to responses in the other samples.

ARV 019 HIV negative sample had HIV-1 specific CD8+ IFN-γ T cell responses the gag peptide pools by cultured Elispot assay (Figure 28).

Figure 28: HIV-1 specific CD8+ IFN-γ T cell responses in the HIV negative spouse ARV 019 by both *ex vivo* and cultured Elispot assay



HIV-1 specific CD8+ IFN-γ T cell responses to HIV A peptides were generally weak. This sample showed weak responses to pools 1, 2, and 90. Responses to pool 9 were completely absent.

HIV-1 specific CD8+ IFN-γ T cell responses specific for HIV A were detected in all the five HIV negative spouses by cultured Elispot. Each of the volunteers responded to one or two of peptides. All the samples gave strong HIV-1 specific CD8+ IFN-γ T cell responses to pool 90; each volunteer responding to two different peptides. Two samples responded to pool 2 and only one sample had

responses to pool 1. However, none of the volunteers gave responses to pool 3. The CD8+ T cell string of HIV A epitopes were detected in only one volunteer, ARV 008with 500 SFU/10^6 PBMC and were not detected in the other four volunteers.

There were enhanced HIV-1 specific CD8+ IFN-γ T cell responses for different peptides in different samples by cultured Elispot assay which were not detected by *ex vivo* Elispot assay. Enhanced HIV-1 specific CD8+ IFN-γ T cell responses for FEC and PHA were observed in all the volunteers. These results show that cultured Elispot enhanced the detection of the CTL responses.

It was not possible to subject samples from the HIV positive spouses from discordant couples to cultured Elispot due to the small numbers of PBMC harvested.

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CHAPTER FOUR

4.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Demography

The reported study sought to determine the ability to recruit, counsel and follow-up high-risk HIV negative individuals in preparation for HIVA HIV/AIDS phase II and III vaccine trials in Kenya. Thirty HIV seronegative high risk volunteers were recruited into the study at KAVI Kangemi Clinic. All the thirty volunteers adhered to the follow-up procedures on month 3, 6 and 9 after recruitment. The follow-up was 100% successful due to counseling and community mobilization that took place through peer leaders. IAVI regulations demanded that volunteers be given monetary compensation for the time spent at the clinic throughout the study. Thus it is also possible that the 500 shillings that was given to volunteers to compensate for the time they spent at the clinic acted as an incentive for adherence to the study.

A majority of people who live in Kangemi live below the poverty line, earning less than one dollar a day. Most of them are hired on day to day basis, to work in certain industries including domestic help, construction sites, and matatu touting. This means that they do not have a reliable source of income. So the monetary

incentive given to the volunteers might have enticed some of the low risk individuals to pretend to enroll in this study.

However, a number of challenges were encountered such as low literacy levels, poverty, stigma, fears and mistrust about the expected vaccine. In most African, and in particular, Kenyan cultures, having many sexual partners outside marriage is considered immoral. Such behaviour is stigmatized and individuals are likely not to own up to them (Nichols, 1989). It was therefore surprising that the Kangemi KAVI clinic had 100% overwhelming adherence during the course of the study. This might be attributable to the fact that Kangemi is an informal settlement with many known commercial sex workers, who might have considered themselves to be of bad reputation to start with. In addition, Kangemi being peri-urban has been exposed to the aggressive HIV/AIDS awareness campaigns which might have lead the volunteers desire to know their HIV status. Furthermore, many residents found being involved in a study which is a more acceptable way for getting tested for HIV. This implied that one not suspected of having the dreaded HIV/AIDS. This might have positively influenced the response to participate in this study.

Considering that the volunteers were counseled during each visit, the observation that this did not lead to change in sexual behavior was surprising. It might have been possible that because of poverty, commercial sex workers were not in

control of the choices their clients made as far as condom use was concerned. In addition, the participants' perception of multiple sexual partners as regular customers might have led to the belief that if they had not infected these people by now they were unlikely to do so in the future. However, results from a study done among the youth in Kenya have shown that perception of risk and high knowledge about HIV/AIDS does not necessarily translate to behavioral change (Toroitich-Ruto, 2005).

Even in the face of existing perception and knowledge of self-risk, (multiple partners, sex with high risk partners, no condom use, healthy-looking person does not have HIV/AIDS, etc) risky sexual behavior is still high (Toroitich-Ruto, 2005). Public AIDS awareness campaigns in Kenya emphasize change in sexual behaviour, including abstinence, condom use, monogamy and reduction of sexual partners.

The assumption is that people will respond to risk in a way that reflects their HIV/AIDS awareness levels. Often, risky behaviour is defined as sexual intercourse without a condom whereas the present study defined it as 'unprotected sexual intercourse with a partner whose HIV status is unknown'. The rates of risk behaviour reported in the study are lower than expected at the beginning of the study. This might have probably been due to this sample being drawn from a group of people who have not been in a scientific study group before. Another

reason could be that the volunteers were not sure how the information they disclosed would be used. Again there is a possibility that powerful prevailing messages within the commercial sex community and media about the unacceptability of unprotected sex and the promotion of condoms led to the under reporting of risky sexual behavior.

The questionnaire used in this study was based on guidelines designed to increase the rates of disclosure of stigmatized behaviors. However, the volunteers being from an African setting where open discussion on risky sexual behaviour is a taboo, it was hard to collect all the information regarding risky sexual behavior. In fact, some questions in the questionnaire were left unanswered. Thus it remains difficult to ascertain the relationship between the reported and actual rates of risky sexual behaviour.

In the absence of a vaccine or a cure for AIDS, changing risky sexual behavior is the only means available to reduce the sexual transmission of HIV infection. Consistent use of condoms reduces the risk of infection by blocking exchange of the virus and may reduce the efficacy of transmission by lowering the prevalence of a cofactor for HIV transmission and other sexually transmitted diseases. This study reported use of condoms in 50% high risk HIV negative individuals. These are individuals who accepted that they used condom but the consistent use of condom was hard to determine. Significant behaviour change was not evident in

this study group after counseling. However two individuals (6%) who reported non use of condom at enrolment were seen to change their behaviour by using condoms consistently. It was noted that there was inconsistent condom use in greater than 30% of the volunteers throughout the study. This is due to the fact that some sexual partners interpret the request to use a condom as insulting, mistrustful, and a barrier to sexual fulfillment. One of the reasons given for not using condom was that there was trust between the sexual partners and the volunteers in 25.8% of the cases.

The definition of whether a participant had risky sexual behaviour was based on multiple sexual partners of unknown HIV status. In choosing this definition, it was assumed that the participants' reports about the frequency of condom use would be reliable. The current data shows that there were 21.6% cases of condom use with the regular partner as compared to 45% with the casual partners. This finding indicates that condoms would be used with partners who were considered casual partners but less so with partners who were considered regular.

In the group studied at Kangemi, a number had multiple partners that they considered regular. Such participants did not consider themselves commercial sex workers (prostitutes). This might be a risky sexual behaviour, usually not considered in studies on HIV/AIDS. Indeed, 30.8% denied having casual sex partners, meaning that some of these volunteers (37.5%) had more than one

regular sexual partner. Considering that a number of participants were commercial sex workers, the low use of condoms with casual sex partners might indicate poor negotiation powers on the side of the volunteer as far as condom use was concerned.

The study found widespread alcohol use at Kangemi. Alcohol usage has been associated with risky sexual behaviour in both men and women including, unprotected sex, multiple sexual partners and paying for sex (Robertson and Plant, 1988). The findings in the current study are consistent with this observation. Indeed, alcohol consumption was the common reason why volunteers did not use condoms with the majority of volunteers reporting that they forgot to use condoms when drunk. Over sixty percent (60.8%) of the volunteers admitted that they drunk alcohol with 15% admitting that they drunk alcohol daily. This might have have raised the other risk factors in this population.

Only two out of thirty (6%) of the volunteers admitted smoking *Cannabis sativa* (bhang) and nobody owned up to intravenous drug use such as heroine, cocaine and others despite the fact that the use of these drugs is rampant in Kangemi. Heroin injection has been reported as a serious problem in Kenya (Jochelson *et al.*, 1991). Considering that the use of bhang and intravenous drugs are illegal in Kenya, it was not surprising that very few people admitted to their use. It is most likely that the volunteers feared the consequences of owning up to the use of these

illegal drugs. They may have thought that they could be reported to the law enforcers. This is another area where the volunteers might have underreported their risky sexual behaviour.

Research has demonstrates that the presence of untreated STDs significantly increases the risk of contracting HIV (Moses *et al*., 2001). Kangemi, was chosen because of its low social economic status and the intricate sexual behaviour of the majority residents (Ngugi E, personal communications). It was expected that majority of the volunteers would have suffered STD. On the contrarily only very few, three out of thirty volunteers (10%) reported to have suffered from an STD. It has been noted that there is reduction in the cases of the STDs country wide and also in Kangemi over the last 10 years (Anzala, personal communications). This might explain why there was low reporting of incidences of STDs.

Low social economic status of the people make them focus more on their daily survival than their health and are stymied by a crushing sense of powerlessness which leads to hopelessness and often to risky sexual behaviour, including prostitution. Some individuals, mainly women, become sexually involved with numerous male friends or clients in exchange for financial support. The Kangemi volunteers were not different. In the absence of a cure for HIV or an inoculation against the virus, prevention of infection remains the most important public health strategy. Gold (1995), suggests that preventative strategies must acknowledge that

100% condom use is not the norm within the population of sexually active gay men. It would then appear that there was a high incidence of risky sexual behaviour even amongst the heterosexuals.

Elsewhere, it has been found that many high risk individuals are practicing and negotiating lower-risk sex with casual sex partners (Ekstrand *et al.*, 1999). Health information strategies need to acknowledge these factors and the 'high value placed on sexual expression' by populations at high risk of HIV infection. Information strategies should promote the disclosure of testing, disclosure and negotiated safety as part of safer sex strategies rather than the simplistic and unrealistic '100% condom use' message favoured in the majority of campaigns.

The link between substance misuse and sexual risk taking could also be made explicit. Giving vulnerable individuals positive messages about negotiating safer sex may enable them to be more assertive about condom use. Previous research has indicated a link between substance use and risk behaviours and this might also be a suitable area for preventative strategies. HIV infection resulting from blood transfusion has been documented repeatedly since the first case reported in late 1982 (Curran *et al.*, 1984).

In the United States, almost all cases were due to blood transfused before March 1985, when HIV antibody testing became available to screen donated blood (CDC

HIV Statistics., 2003) As of December 2001, an estimated 14,262 persons had been diagnosed with AIDS as a result of transfusing contaminated blood or blood products (CDC HIV Statistics., 2003). No HIV-infected but persistently seronegative transfusion recipients have been identified (Moore *et al*., 2001). The 90% probability of seroconversion is independent of the age or sex of the recipient, the reason for transfusion, and the type of component. However, this has reduced drastically. A study in Kenya by the Ministry of Health in a collaborative multicentre assessment, to establish the risk of HIV transmission by transfusion estimated that 2.0% of transfusions transmitted HIV, primarily because of erroneous laboratory practices (Moore *et al*., 2001).

In this study, only two out of thirty volunteers had blood transfusion. It has been shown that the groups of people at highest risk are those with malaria and anaemia, women with pregnancy-related haemorrhage or anaemia, victims of trauma and subjects with sickle-cell disease. The low number of people who have been transfused could be due to the fact that Kangemi is malaria free zone. The other causes of transfusion are rare. Blood transfusion may not be a serious risk factor in this population.

4.2 HIV-1specific CD8+ IFN-γ Responses by Elispot assay

4.2.1 *Ex vivo* Elispot assay

Individuals who remain seronegative despite several exposures to the virus represent an extremely valuable study population in determining important mechanisms of protection from HIV infection. Steps towards understanding potential mechanisms of natural immunity have focused on observational studies of persons who resist infection despite repeated viral exposure. There has been contradicting information on the presence of HIV-1 specific IFN-γ secreting CD8+T cells in high risk individuals exposed but not infected with HIV (Hladik *et al.*, 2003; Rowland-Jones *et al.,* 1998).

To determine the presence of such HIV-1 specific immune responses, the quantity of HIV-1specific IFN-γ secreting CD8+T cells in a group of highly exposed seronegative heterosexual individuals and HIV negative spouses of discordant couples was evaluated using HIV A and RENTA peptides. The results demonstrated that there were no specific HIV-1specific IFN IFN-γ secreting CD8+T cells to HIV A and RENTA peptides in all of the 35 samples assayed by *ex vivo* Elispot assay. Thirty samples were from the high risk seronegative group while the other five were from HIV negative discordant couples. The HIV-1 specific CD8+ responses for all the samples in all the four time points were less than 25 SFU/million PBMC, which was far below the cut off (50 SFU/10^6 PBMC). In fact, the HIV specific CD8+ IFN-γ T cell responses were not different

from those of a negative control group and HIV specific CD8+ IFN-γ T cell responses of a lower risk control group. These results are consistent with those of Hladik *et al*. (2003) where 46 highly exposed seronegative men having sex with men (MSM) were evaluated for HIV-1 specific CD8+ IFN-γ T cells. These men demonstrated no or very low numbers of HIV-1 specific CD8+ IFN-γ T cells. specific CD8+ IFN-γ T cells remained infrequent, and the proportion of responders was not significantly different from that in a lower risk seronegative control cohort.

The results of the study reported here were also consistent with the results from parallel studies carried out using the HIV vaccine candidate from which the peptides tested in this study were derived (Peters *et al.,* 2007). The candidate vaccine was based on modified vaccinia virus Ankara with and without DNA priming. HIV-1 specific responses were evaluated by *ex vivo* Elispot assay. The result revealed that there was little evidence of detectable *ex vivo* IFN-γ Elispot response to the vaccine (Peters *et al.,* 2007). In this study IFN-γ-secreting memory T cells recognizing HIV-1 epitopes were altogether absent. However, similar studies (Rowland-Jones *et al*., 1998; Goh *et al*., 1999); Kaul *et al*., 2001; Hladik *et al*., 2003; Peters *et al.,* 2007) had shown the presence of specific HIV-1 specific IFN-γ-secreting CTL response in a small number of volunteers. It is therefore impossible to rule out the presence of these responses.

HIV positive spouse HIV-1 specific CD8+ IFN-γ T cell responses to a number of HIVA peptides were detected in all the five HIV positive spouses using the *ex vivo* assay. It is possible that CD8+ cells from HIV infected persons were mounting responses because they were constantly in contact with the virus. It would appear that constant exposure to the virus leads to an immune response within the patients. It would also appear that when the virus is not present, then such responses would decrease. This might explain the lack of response in exposed seronegative individuals. Indeed this is on of the ways the virus evades destruction by the host immune system (Korber *et al.*, 2001).

The results of this study indicate HIV-1 specific CD8+ IFN-γ T cell responses in HIV positive samples differed both in the pool of the peptides they responded to and the intensity of the responses. It is possible that the antigenic variation of the virus in infected persons was selecting for certain clones of the CD8+ cells leading to down-regulation of some receptors for certain HIV A peptides. This might explain the variation in responses to different peptides by different HIV positive individuals.

4.2.2 Cultured Elispot

The results reported in such studies and the ones found during the course of this work would make the sensitivity of *ex vivo* Elispot assay doubtful. To clear the doubt it was important to use the more sensitive cultured Elispot assay using the

samples from the most high risk individuals from the demographic survey. Twelve Exposed seronegative (ES) individuals were assayed for CTL responses by cultured Elispot.

The results revealed that some of the samples that had not shown CTL responses by *ex vivo* Elispot, now exhibited HIV-1 specific IFN-γ-secreting CD8+ T cells by cultured Elispot assay. It is possible that even low levels of HIV-1 specific IFN-γ-secreting CD8+ T cells may have contributed to rapid containment of HIV-1 following exposure in these individuals. These results were consistent with those of Goonetilleke *et al*., (2006). The study measured HIV-1 specific immune responses induced by prime boost regimen of DNA and modified vaccinia virus Ankara vectored vaccines. The results revealed that cultured Elispot assay was able to detect T cell responses that were not detected using any other assay including *ex vivo* Elispot assay.

Applying the cultured Elispot to samples from the sero-discordant couples revealed the presence of HIV-1 specific IFN-γ-secreting CD8+ T cells responses to a number of HIVA peptides in the entire five HIV positive and all the five exposed HIV negative spouses. It would appear that the *in vitro* peptide-stimulated culture in this assay expanded the CTL responses which were not detectable in *ex vivo* assays, and showed more than 20 fold increases in frequency of specific T cells responses measured by *ex vivo* IFNγ Elispot assay. The kinetics

of the CD8+ T cell responses observed are consistent with those induced in response to viral infections in which the effector T cell population decays rapidly leaving a small sub set of long lived memory cells. The scarcity of HIV-1 specific IFN-γ-secreting CD8+ T cells in the studied cohort must be reconciled with the findings of other studies that have implicated cellular immunity to be an important factor of resistance in multiply exposed, uninfected individuals (Rowland-Jones *et al.*, 1998; Goh *et al.*, 1999; Kaul *et al.*, 2001).

The results of this study could be compared to results from one of the most studied exposed seronegative cohort, the Pumwani casual sex workers. However, the sexual behaviours of the two cohorts were different in that the Kangemi cohort had intricate sexual behaviour where in almost all cases there was a regular partner who was regarded as special and when present could keep away the casual customers who are believed to be at a higher risk to HIV infection. The Pumwani cohort was an exclusive prostitute community and sexual relationships were mainly casual making this cohort to be at a higher risk of HIV exposure.

The Pumwani cohort was composed of large sample size (91 resistant commercial sex workers) and they had been followed up for a long period of time (mean period of 5.4 years). Therefore they had established the cohort as resistant. The Kangemi cohort was a new study site, and there was no previous existence of a study group. Taking into consideration that the HIV status of their partners was

unknown, chances were that some may not have been exposed. Due to the marked differences in sexual behaviour of the two geographic regions, the female CSW in Pumwani are likely to be exposed to greater numbers of HIV-1-infected partners in their clientele than our ES individuals in Kangemi. Again, high condom use was reported in the study meaning that there was no continuous exposure to stimulate the CTL in the high risk group. It is also possible that some of the high risk volunteers did not give true information about their risky sexual behaviour.

It should be noted that the standard Elispot assay and the statistical interpretation of Elispot data have undergone rigorous evaluation for validation purposes in DNA/MVA phase I and II vaccine trials done in both Nairobi and Oxford. The assay had no varying background and the cut off for the positive was 50 SFU per million PBMC which is far much higher than 20 SFU per million PBMC used in other similar studies. In the study reported here, the positive results were determined with a stringent likelihood ratio statistic that maintains a consistent false positive rate of $P<0.01$ across all assays. This method thus treats all Elispot results with identical stringency and tends to be more conservative for borderline responses than the previously published criteria (Goh *et al.*, 1999). Low IFN γ SFU frequencies that would be considered positive using traditional cut offs are thus more likely to be interpreted as negative by our statistical criteria. This in part may account for why our findings indicate that as a group the ES responses were no different from those of the control subjects.

Despite these caveats, the HIV negative spouses recruited in the study provided a group of ES heterosexual known to have regularly practiced high risk sexual activities with their HIV-1 infected spouses. In this group the *ex vivo* and cultured Elispot assays gave differing reactivity patterns for all individuals. However, despite those limitations our high risk cohort was a good indicator of exposed HIV seronegative individuals who showed HIV specific IFN-γ CTL responses by cultured Elispot assay.

HIV-1 specific IFN-γ secreting CD8+ T cells responses were not consistent, and seemed to disappear or/ and reappear with time. This observation is in agreement with other similar studies (Kaul *et al.*, 2001; Rowland-Jones *et al*; 1998, Goh *et al*., 1999). This could suggest that resistance is not absolute. Kaul *et al* (2001) found that ES could seroconvert despite a variety of pre existing CTL specific responses. Those subjects had a reduction in sex work and presumably HIV exposure prior to seroconversion. There was no correlation between the sexual behaviour and CTL responses in the study reported here, probably due to the relative short follow up period.

However, taking the hypothesis that break from sex and consequently reduction in HIV exposure renders the CTL response undetectable then, I would argue that the individual who showed the CTL responses on any one time were not continuously being exposed to the virus. They might have used condoms at certain times and

also their sexual partners may not have been HIV infected. CTL responses waned below a detectable threshold by cultured Elispot assay during the different time points. None of the volunteers seroconverted throughout the course of the study reported here. It would appear that HIV-1 specific IFN-γ secreting CD8+ T cells are not an 'all or none' phenomenon but inducible during the right kind of antigenic exposure.

Perhaps a three month follow-up period is too long a time and it is likely to miss a number of CTL responses in between. It therefore cannot be concluded that CTL responses were totally missing even in those samples that were negative by cultured Elispot. Future studies should reduce the time between follow-up visits to about a week or a fortnight. This would capture a number of responses, which might have been in this study.

The discordant couple samples provided both HIV positive samples from the HIV positive spouse and ES negative samples from the HIV negative spouse. Both *ex vivo* and cultured Elispot assays were used. The HIV positive samples unlike those from HIV negative spouse were positive with *ex vivo* Elispot assay. This was probably because they were exposed to greater levels of antigen because of their HIV positive status. However, those from the HIV negative spouse were only positive by cultured Elispot assay.

The positive results showed that there was no correlation between the responses in the two assays. This was expected considering that the spouses bear different HLA alleles. The reactivity pattern for each couple was unique. The HIV positive couples responded to mostly pools 3 and 4; only ARV 019 to pool 1 and one of them responded to pool 9, the CTL epitope by *ex vivo* Elispot. There was a positive response to all peptide except pool 3 by cultured Elispot. It would appear that the prolonged culture with peptides and IL-2 in cultured Elispot could have increased the proliferation capacity of the PBMC and hence increased activation of the CTL so that there were responses to all peptide including the CTL epitopes. The examination of the ES responses by the *ex vivo* IFN-γ Elispot methodology was thorough and it was unlikely to miss out any present CTL response.

There is a possibility that the correlates of immunity, important in preventing infection in the ES, may not be represented by the identification of IFN-γ secreting T cells by *ex vivo* Elispot. Thus, in ES, the ability to detect very low frequency responses is greater in cultured Elispot than the *ex vivo* Elispot analysis. When the *ex vivo*-Elispot results between this group and the ES cohort, were compared the proportion of subjects in each group who demonstrated HIV-1 cellular immunity was not different. The findings in this study might suggest that HIV-1-specific cellular immunity represented by IFN-γ secreting CD8+ T cells is either absent in some cases or extremely weak in the majority of ES to be detected by *ex vivo* Elispot.

Detection could only be enhanced by cultured Elispot. Since not all of ES negative individuals seroconvert even after the CTL responses wane and become undetectable, cultured Elispot could be used to detect whether the CTL responses are present or are completely absent. This could tell whether the individual who seroconvert have or have not completely lost all CTL responses.

It appears that the low frequency of HIV-1 specific IFN-γ-secreting CD8+ T cells in the ES cohort; argue against a prominent role of HIV-1-specific T cells that secrete IFN-γ in preventing infection in our cohort when *ex vivo* Elispot assay is used. It is clear that the ES represent a unique population in whom mechanisms of HIV-1 protection can be elucidated (Dean *et al.*, 1996), and that it is likely that multiple factors contribute to their relative resistance. These findings emphasize the importance of considering the type and extent of risky activity as well as inclusion of appropriate controls in interpreting findings that may have a role in HIV-1 protection.

Though *ex vivo* Elispot has been used as the gold standard to measure HIV-1 IFN-γ responses in many studies, our data agrees with that of Hladik *et al.*, (2003). They evaluated the quantity and specificity of HIV-1 specific IFN-γ secreting CD8+ T cells in a group of highly exposed seronegative men having sex with men. In this study the high frequency of unprotected anal intercourse and potential HIV-1 exposure, the vast majority of individuals demonstrated no or very low

numbers of HIV-1 specific IFN-γ secreting CD8+ T cells. Even when HIV-1 epitopes were presented by peptide-pulsed autologous dendritic cells in 15 of the highest risk volunteers, HIV-1-specific T cells remained infrequent, and the proportion of responders was not significantly different from that in a lower risk seronegative control cohort. Only PBMC from two individuals who have remained uninfected exhibited distinctly positive responses which rarely persisted over time Hladik *et al*., 2003.

However, it is my opinion that if those researchers in this study had the opportunity to use cultured Elispot they would have had CTL responses in many of their volunteer and their conclusion might have been different. The findings of this study are subject to at least five limitations. First, small sample sizes, including small numbers of respondents with high risk behaviours. Second, the *ex vivo* Elispot methodology was not sensitive enough.

Though the follow-up was intended to be for 15 months, this was not feasible since the results were consistently negative. The follow-up was terminated at 9 months. This lack of response was not surprising because similar results had been obtained by the Oxford Group (Goonetilleke, personal communication). It was possible that, the IAVI study, *ex vivo* Elispot cut-off used for the vaccine study were too high to detect the responses elicited by the peptides in this study.

Considering that these peptides were designed as vaccine peptides, it is possible that a requirement for a very strong, immunogenic response put the cut off points higher than the responses elicited by the peptides and might not mean absence of CTL responses. Indeed, CTL responses were detected on cultured Elispot from some of the individuals sampled during the course of this study.

4.3 Conclusion

The sample of volunteers in this study is broadly comparable to groups of high risk HIV exposed but seronegative individuals in other similar studies. However, the study represents a new cohort during its initial development stage. Rates of risk behaviour were lower than comparative studies, possibly reflecting the fact that the sample was not taken from a sexual health clinic or from people directly buying and selling sex for cash.

Ex vivo Elispot assay could not detect the low CTL responses in the high-risk exposed seronegative individuals. However, cultured Elispot was more sensitive and was able to pick up responses missed by *ex vivo* Elispot. This implies that cultured Elispot is more sensitive than the gold standard Elispot assay.

There was no association between the CTL responses and sexual risk behaviour observed in this study. Although other potential mechanisms of resistance have not been examined in this population there is evidence that HIV-1 specific IFN-γ-secreting CD8+ T cells may play an important role in this cohort. Therefore a comprehensive understanding of the mechanisms of resistance in HIV exposed but persistently seronegative individuals may lead to an understanding of their interactions and provide insight into therapeutic improvement and vaccine development.

Considering that these peptides were designed as vaccine peptides, it is possible that a requirement for a very strong, immunogenic response put the cut off points higher than the responses elicited by the peptides and might not mean absence of CTL responses.

4.4 Recommendations

The demographics of the sample did not consider age, heterosexual anal intercourse by men who had sex with men (MSM), those with higher education and high income. These biases were due to the fact that the studied cohort was more readily available in terms of recruitment and availability. Future studies should consider other sampling methods, specifically targeting groups that this study did not investigate; for example, sampling men who are self-identified as gay, heterosexual men who are known to engage in sex with men.

Anal sexual intercourse is associated with high incidence of STDs and HIV since majority of the individuals do not use condoms. This group now exists publicly in Kenya as seen in the world Social Forum held in Kenya, January, 2007. It is possible that this group, has less access to safer sex information or less safe sex-positive environments, and may be at greater risk. It is likely that this group will be altogether easier to survey in future than before.

Risky behaviours were measured by several factors including having multiple (i.e., two or more) sex partners during the preceding one week, a composite measure of risk that included use of intravenous drugs, treatment for sexually transmitted disease, and sex without a condom during the preceding one week; specific risks were not assessed individually. A number of issues were not

addressed in the questionnaire or, in retrospect, could have been addressed in a more informative manner. It remains difficult to ascertain how the rates of unsafe sex might be measured. The finding, in this study, of very low responsiveness even when cultured Elispot was used might suggest that the reliance on unprotected sex with person of unknown HIV status as an indicator of unsafe sex might have been over inclusive. The different parts of the questionnaire asked about different risky sexual behaviour over different time frames.

However details about the number of times sex with or with out condom took place was not taken into consideration. Thus, the study dealt with the approximate number of partners and not the times that unprotected sex occurred, with no reference to factors such as the partner's, HIV status. It is therefore important that these considerations be taken into account in future studies. It would have been useful to consider those that are really exposed to HIV by determining the HIV status of their partners, especially the regular partners. The reported study followed a strict vaccine trial protocol that did not allow alterations to include this aspect. It is therefore recommended that future studies include this aspect as well as investigate immune response in high-risk individuals based on risk criteria with greater face validity than those used in the present research.

I recommend that interview method should be also be used so that more detailed exploration should be done, such as the accuracy of estimates of partner's HIV

status and perception of risk to HIV infection. This adjustment in the methodology exhausted the entire sample PBMC such that it was not possible to determine the T cell phenotype for samples which tested positive on cultured Elispot. Further studies need to be carried out to investigate the phenotypes of responding cells using ICA.

Third, because the volunteers are of low social economic status, and barely met their basic needs, the project management gave the volunteers Ksh.500 to compensate for the day they reported to the clinic. However this little money may encourage some of the low risk individuals to give false information to be enrolled into the study. Future studies should include a stringent inclusion criterion of selecting the participants.

Finally, future studies should involve large number of volunteers followed over a long period of time in short intervals and new methodologies should be elucidated to enhance detection in heterosexual ES from regions hard hit by HIV scourge. This will enhance the understanding of the role of IFN-γ-secreting T cells and other relevant antiviral mediators that confer protection.

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APPENDIX I
INFORMED CONSENT FORM
(Kangemi, Nairobi)

Consent Information Sheet for Enrollment into Feasibility and Sero-prevalence Study

Title: A Cross Sectional, Observational Feasibility Study To Assess Recruitment And Determine HIV Sero-prevalence Among Volunteers, In A Potential Cohort For A Phase II/ III HIV Vaccine Prevention Efficacy Trial.

Rationale for this Study

Over 40 million people worldwide are infected with human immunodeficiency virus (HIV), the virus that causes AIDS. Even though people are trying to reduce their chance of becoming infected, there are many new cases of HIV every year. Many experts believe that a HIV vaccine offers the only real hope in controlling the spread of disease. At this time there is no HIV vaccine that can protect you against HIV/ AIDS.

Many different HIV vaccines are being developed and tested. In order to find out if such vaccines protect people, it is important to know how many people have HIV and how many do not. This research study aims to find out the number of people who are infected with HIV, the virus that causes AIDS. This information will be useful in designing larger, HIV- Prevention vaccine trials in the future. Also, this study will help the KAVI set up a system to prepare all activities necessary for vaccine trials in the future at Kangemi.

Background

Kangemi is in Westlands division, approximately 10 km from Nairobi center and it is characterized by an extremely high population. Residents are exposed to severe environmental health risks and the majority of households have very low incomes. Morbidity and mortality rates are caused by diseases stemming from poor environmental management owing to poor sanitation, lack of potable water, poor drainage, overcrowding and uncollected refuse. HIV/AIDS preference is also high.

The international AIDS Vaccine Initiative (IAVI), the Sponsor of this Study is an international, scientific, non-profit organization, whose mission it is to develop a safe and effective, preventive vaccine against HIV and to ensure that if such a vaccine is found, that it becomes available to those that need it most.

Selection of Study Volunteers

This study will enroll a maximum of 1,000 healthy, male or female volunteers at risk of HIV infection, aged between 18-60 years, who are well informed about the study and agree to participate in the study.

Duration of the Study

You may have more than one study visit. You may be asked information about yourself and any behaviors that may put you at risk for HIV infection. You may be required to come for a second visit in the next 1-4 weeks if additional testing is required.

Your participation is voluntary

This consent form gives information about the study that will be discussed with you.

If you agree to participate you will sign your name or make your mark on 2 copies of this consent form confirming that you agree to take part - one copy is for you to keep and one will be kept in at the KAVI Kangemi Clinic. If you do not wish to keep the second copy, it will be kept in a safe and secure place.

It is important that you know the following:

- Your participation is voluntary, it is entirely up to you whether you choose to take part in this study or not.
- You may decide not to take part in the study, or to withdraw from the study at any time.
- If you decide not to take part in the study, none of your rights will be compromised, and you will not lose the benefits of care.

Cost to the client

There is no cost to you for being in the study. You will be reimbursed Ksh 500 for travel to and from the study site and for each follow-up visit to the clinic.

Study Procedures

If you decide to take part in the study, your visit will continue today, after you read, discuss and sign or mark this form.

- You will be examined by your study doctor and will be asked questions regarding your medical history, any health problems that you are suffering from and about medicines that you are taking, if any. You will receive a physical examination focused on your complaints and your blood pressure, temperature, pulse, breathing, height and weight will be recorded.
- If you have any medical problems that you require treatment that is not available in the clinic, the study staff will either provide it or send you for medical care to another clinic or hospital of your choice.
- You will be asked questions about your risk of acquiring HIV infection. You may ask study staff for help in answering the questions.

- If you are a woman, your urine will be tested to see if you are pregnant.

- You will have the chance to ask questions and talk with study staff and receive counseling before and after the HIV blood test.

- About 60ml of blood will be drawn from a vein in your arm at this study visit. The blood will be tested for HIV. The remainder of the blood will be stored in case additional testing is required and for other study tests.

This test may take about 1-2 weeks, so you will have to come back to Kavi Kangemi clinic at that time to get the results. Sometimes HIV tests are not clearly positive but also not clearly negative. You may have to have another blood test if it is difficult to tell if you are infected or not. You will be able to ask questions and talk to the study staff about the meaning of your results and how you feel about them.

- If you are not infected with HIV, you will receive counseling to help you decrease your risk of getting HIV. You will also get free condoms. You will be told about another study where you will be seen by study staff every 3 months for up to 2 years, for counseling and testing and encouraged to take part in it. This study will be explained to you in detail in another consent form.

- If you are infected with HIV, you will receive counseling and be referred for care and support in a hospital of your own choice. You will be told about another study where you will be seen by study staff every 3 months for up to 1 year and encouraged to take part in it. This study will measure the amount and type of virus you have in your blood and measure your immune cell (CD4) count. This study will be explained to you in detail in another consent form.

If you are a woman infected with HIV and you are pregnant you will be referred to prenatal care and to a program called Prevention of Mother To Child Transmission (PMTCT) where you may be able to get medicines to prevent your baby from getting HIV.

- If you wish, your partner and/or family members can have counseling with you if they are concerned about your participation in this study.

Storage of Blood

After completion of the HIV tests on your blood specimens, some of the remaining blood will be stored. This blood will only be labeled by number and not your name to ensure that no one, other than people running the study knows who you are. If your test is not clearly positive or negative, some of this stored blood may be used to test your blood again, using special tests, to make sure that your previous test results are correct. If you have HIV, some of this blood will be used to measure the amount of virus in your blood and analyze the type of virus.

Your stored blood may be used in the future to ensure that tests in the laboratory are always done correctly and carefully. Your stored blood may also be sent to other expert laboratories that are not part of the study, for more tests. It is not known at this time to

which laboratories these samples will be shipped or for how long the samples will be stored.

Risks and/or Discomforts

There may be some risk involved in drawing blood from a vein for the HIV tests. Blood drawing sometimes cause pain and bruising where the needle goes into your arm. You may feel dizzy or faint which is not common.

You may become embarrassed, worried, or anxious when discussing your sexual practices, ways to protect against HIV and your test results. You may become worried or anxious while waiting for your test results. If you have HIV or other infections passed during sex, knowing this could make you worried or anxious, a trained counselor will help you deal with any feelings or questions you have. Sexually transmitted diseases will be treated in the clinic; if treatment is not available here, you will be referred for care elsewhere.

We will make every effort to protect your privacy while you are in the study. However, it is possible that others may learn of your participation here, and think you have HIV, or are at "high risk" for HIV. Because of this, others may treat you unfairly or discriminate against you. For example, you could have problems getting or keeping a job, or being accepted by your family or community.

Benefits

You may benefit by taking part in the study by knowing whether you have HIV or not and receiving counseling and some treatment for sexually transmitted diseases. You or others may benefit in the future from information learned in this study. You may get some personal satisfaction from being part of research on HIV. The information that we collect from this study will help to design and develop a larger, HIV vaccine prevention efficacy trial.

If you are a woman, you will have a pregnancy test. If you are pregnant, you will be referred for prenatal medical care and other services that you and your baby may need.

You will get information about your general health, HIV and family planning counseling and testing for HIV. You will get free condoms. If you are infected with HIV, you will be referred for medical care, counseling, and other services available to you. If you are a pregnant, HIV – infected woman, you will be referred to a program for the Prevention of Mother to Child Transmission (PMTCT).

You can bring your partner and family members at KAVI Kangemi clinic for counseling, if they have questions and concerns about your health status.

Circumstances for Withdrawal from the Study

Your participation in the study is completely voluntary. You can withdraw from the study at any time without giving a reason. Withdrawal will NOT compromise any rights you may have or influence any current or future medical care you may need.

You may be removed from the study without your consent for the following reasons:

- If you don't keep your follow-up appointment, if needed
- If the study is stopped or cancelled
- You are not willing to find out your HIV test results
- Other administrative reasons

If you withdraw/discontinue from the study, you will be followed-up to receive additional tests, care and counseling.

New information

You will be told any new information gained during the course of the study. You will be told when the results of the study may be available and how to learn about them.

Confidentiality

Your participation in the study, all information collected about you as well as all results of laboratory tests will be private and not available to others outside the study team members. Your own special identity number known only to you and the clinic staff will identify you. Apart from the study team members that you meet, other staff from National or international government regulatory agencies, members of the Ethics Committee, study monitors, auditors, inspectors, and representatives of the Sponsor (IAVI) will check the records to make sure that the study was conducted properly. They are equally bound to respect your confidentiality. Your identity will not be disclosed in any publication or presentation of this study.

CONSENT FORM

(Name of Subject) ....

of (address) ....

agree to take part in the research project:

Title: A Cross Sectional, Observational Feasibility Study to Assess Recruitment and Determine HIV Sero-prevalence Among Volunteers, In A Potential Cohort For A phase II/ III HIV Vaccine Prevention Efficacy Trial.

I have been told in detail about the study and know what is required of me. I understand and accept them. I understand that my consent is entirely voluntary and that I may withdraw from the research study if I find that I am unable to continue for any reason and this will not affect the legal rights I may otherwise have.

Participant:

Print Name: .... Signature: ....

Date: |__|__|/|__|__|__|/|__|__|__|__| |Time:|__|__|:|__|__| (24 hours).

Person Obtaining Consent:

I have explained the nature, demands and foreseeable risks of the above study to the volunteer:

Print Name: ....Signature: ....

Date: |__|__|/|__|__|__|/|__|__|__|__| |Time:|__|__|:|__|__| (24 hours).

Principal Investigator or Designee

Print Name: .... Signature: ....

Date: |__|__|/|__|__|__|/|__|__|__|__| |Time:|__|__|:|__|__| (24 hours).

APPENDIX II

SELF-ASSESSMENT QUESTIONAIRE

Thank you for expressing interest in this study. This is a preliminary questionnaire to access whether or not you might be eligible for the HIV vaccine study. Please take the time to answer this questionnaire carefully. Ensure that you have answered all the questions and then read the instructions provided at the end of the questionnaire. If you are eligible, you will be invited to a one to one session with a KAVI nurse and you will be able to ask questions.

Section 1 – Medical History

1. Are you younger than 18 years? YES NO

2. Are you older than 60 years YES NO

3. Have you had any serious allergic reactions to any substance (such as tablets injections, food or insect bites) that required admission to hospital or emergency medical care? YES NO

4. Have you ever had a serious adverse reaction to any vaccine? YES NO

5. Have you ever had fits, seizures or convulsions after the age of 5? YES NO

6. Have you ever had treatment for mental illness or depression? YES NO

7. To your knowledge, have you ever been diagnosed with
   a. hepatitis YES NO
   b. syphilis YES NO
   c. tuberculosis YES NO

   (*If you have had hepatitis and are not sure which type it was*, please contact us)

8. Have you any medical condition, which could limit your ability to complete the study (duration18 months), for instance, if you are on the waiting list for an operation? YES NO

9. Have you ever tested HIV positive? YES NO

10. Has your sexual partner or one of your sexual partners has ever tested HIV positive? YES NO

Section 2 – Medication and Treatment

11. Have you been treated with any of the following drugs in the last 6 months?
    - Prednisolone YES NO
    - Hydrocortisone injections YES NO

12. Have you received treatment for cancer in the last 5 years? YES NO

13. Have you had any experimental vaccines within the last 6 months? YES NO

14. Have you received any blood transfusions or blood products in the last 6 months? YES NO

15. Have you been treated with gold salt for arthritis? YES NO

16. Have you participated in any experimental drug study in the past 12 weeks? YES NO

17. **For Females:** Are you pregnant or planning to become pregnant with in the next 7months? YES NO

    For Males: Is your partner (female) planning to become pregnant within the next 7 months? YES NO

Section - 5 Lifestyle and risk

The next few questions are about your lifestyle, some of which are quite intimate.

18. Have you any reason to believe that you might be infected with HIV? YES NO

19. Have you had any Sexually Transmitted Disease (STD) in the last 6 months? YES NO

20. Have you had unprotected sexual intercourse with two or more casual partners within the last 6 months? YES NO

21. Have you ever had unprotected sex with a known HIV infected person? YES NO

22. Have you taken any drugs by injections that were not prescribed by a doctor for the last 1 year? YES NO

23. Do you think you have an alcohol problem YES NO

24. Would you be able to comply with the study vaccination schedule (4 injections over an 18 months period) and follow up procedures? YES NO

25. Have you been a resident of Nairobi Metropolitan Area, with reliable contact address, for the last 2 years with plans of continued residence in the city for at least 18 more months? YES NO

Thank you for completing the questionnaire. Please check your answers.

If there are any questions above about which you are not sure or a question seems inappropriate, please let us know by indicating the number of the question on the Answer Slip provided and we can discuss your eligibility.

Alternatively, please contact us at the **KAVI** centre , Department of Medical Microbiology, University of Nairobi.

Thank you very much for your interest.

ANSWER SLIP

(Part of Self-Assessment Questionnaire)

Today's date: ____ ____ / _____ _____ ____ ____/ ____ ___
Day month year

Name: ____

Phone number (s): ______________________________________

Mailing address: _______________________________________

Please return the Answer slip in the envelope provided. Please do not return the Self-Assessment Questionnaire.

Please note that you do not need to tell us the reason for your decision or to how you answered the questions.

PLEASE MARK YOUR ANSWERS BY TICKING APPROPRIATE BOXES

I have answered: ☐ "NO" to ALL questions

☐ "YES" to AT LEAST ONE question

☐ I have read and completed the Self-Assessment Questionnaire I am interested in learning more about the trial I understand that ticking this box does not commit me to participate in the vaccine trial.

☐ I have read and completed the Self- Assessment Questionnaire I am still interested in the vaccine trial but the following is a list of questions about which I am unsure. Please contact me on my phone number provided above.

Questions about which I am unsure are:

(Write question numbers form the Self-Assessment Questionnaire)

☐ I have read and completed the Self-Assessment Questionnaire. I am not able to participate in the vaccine trial.

Appendix III

Mean HIV specific responses for both HIV A and RENTA vaccine peptides in Spot Forming unit per million PBMCs (SFU/million PBMCs) using *ex vivo* Elispot assay

	Vaccine Peptides														
ID NO	Mock	P1	P2	P3	P4	P9 0	P9	R1	R2	R3	R4	R5	R6	PHA	FEC
KC 0001	0	0	0	1	0	2	1	1	1	1	1	1	2	1083	315
KC 0003	2	0	1	0	0	1	2	3	2	2	1	2	1	693	128
KC 0005	0	2	0	1	2	0	0	1	0	0	1	0	0	1606	0
KC 0007	0	3	3	2	2	3	4	3	3	3	5	1	3	1095	106
KC 0009	0	3	3	4	2	1	3	1	3	4	1	8	2	652	31
KC 0010	1	1	1	1	4	2	1	2	2	1	3	1	4	1047	9
KC 0031	1	1	2	2	1	1	1	2	2	2	2	3	2	778	60
KC 0032	2	3	1	2	1	2	3	2	3	2	4	5	3	868	68
KC 0033	0	2	0	1	1	3	1	2	1	0	2	3	0	1083	0
KC 0034	2	2	1	1	3	3	1	1	3	5	1	4	3	1307	30
KC 0035	2	1	1	0	2	2	2	1	2	1	2	2	2	1477	10
KC 0037	0	0	0	0	2	0	0	0	1	0	0	0	0	224	1
KC 0038	1	0	0	0	0	0	2	0	0	0	1	2	1	937	2
KC 0041	1	2	2	0	1	1	0	1	1	0	0	2	1	807	550
KC 0042	1	0	1	1	0	1	2	2	3	1	1	4	0	753	3
KC 0043	1	1	1	2	2	2	0	0	0	0	0	0	2	1313	2316
KC 0047	1	0	0	0	0	2	0	0	0	0	0	0	0	250	274
KC0011	2	1	2	2	4	2	2	4	3	1	27	1	1	1328	912
KC0012	2	2	3	3	3	0	1	1	2	2	2	2	3	255	146
KC0013	0	0	0	0	1	0	0	1	0	0	1	0	0	541	3
KC0016	0	0	0	2	1	0	0	1	0	0	1	0	0	737	38
KC0017	3	2	3	2	5	7	3	3	5	4	4	2	2	848	2122
KC0018	1	0	1	0	1	0	0	1	0	1	3	1	3	391	1
KC0021	2	4	2	3	3	1	1	4	2	4	2	2	1	1510	46
KC0022	2	2	3	1	2	4	2	2	3	2	2	2	2	1325	43
KC0023	2	1	1	2	1	4	1	2	3	2	1	2	1	767	215
KC0024	1	3	3	6	5	3	1	2	4	2	3	3	5	1266	89
KC0025	2	1	1	0	1	1	1	1	0	0	0	0	0	903	86
KC0026	0	1	0	0	0	0	2	1	1	1	0	2	1	808	40
KC0028	2	0	2	0	1	2	0	4	1	0	5	0	0	912	364

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