PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF CERVICAL AND PERIPHERAL HIV-1 SPECIFIC T CELL RESPONSES

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CONTENTS

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My friends – for my sanity, and finally,

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- even when I couldn't.

DECLARATION

I, LEONIE LIEBENBERG , hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABBREVIATIONS

AIDS	:	Acquired Immunodeficiency Syndrome
CEF peptides	:	Cytomegalovirus (CMV), Epstein Barr Virus (EBV) and influenza virus (Flu) peptides
ELISpot	:	Enzyme-linked immunosorbant spot
FACS	:	Fluorescence-activated cell sorting
FCS	:	Foetal Calf Serum
HAB	:	Human AB Serum
HIV	:	Human Immunodeficiency Virus
ICS	:	Intracellular Cytokine Staining
mAb	:	Monoclonal Antibody
MMC		Mucosal mononuclear cells
PBS	:	Phosphate Saline Buffer
R1	:	1% FCS in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin™
R10	:	10% FCS in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin™
R20	:	20% FCS in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin™
rhIL-2	:	Human recombinant Interleukin-2
SFU	:	Spot-forming units

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ABSTRACT

Distinct HIV variants occur at the genital mucosa compared to in blood, which may similarly result in differences in HIV T cell responses. There have been no studies of the maturational status of HIV-specific T cells present at the female genital mucosa. This study aimed to characterise HIV-specific cervical immune responses and to determine if compartmentalized immune responses occur in chronic HIV infection by comparing the characteristics of T cells at the cervical mucosa to those in blood. This was achieved by evaluating the anti-HIV responsiveness of cervical and systemic T cells *in vitro* using HIV-specific T cell clones. Thirty five HIV^+ women with CD4 counts >300/ul were included in this study. All of these women were screened for HIV Gag-specific responsiveness using IFN-γ ELISpot and a panel of 9 high HIV-1 Gag responders was identified. T cell clones were generated from paired cervical and blood specimens from each of these 9 donors. A total of 74 blood clones (out of 566 plated) and 51 cervical T cell clones (out of the 454 plated) were selected from the 9 donors. Of these 12/74 blood derived clones and 2/51 cervical clones were HIV-specific. Significantly fewer HIV-specific cervical T cell clones were generated than blood-derived clones. None of the T cell clones expanded sufficiently during *in vitro* culture to allow finer mapping of HIV Gag epitope responses. I investigated the maturational status of these cervix-derived and blood-derived T cell clones by assessing CD57 expression of these cells. CD57 is a terminal differentiation or exhaustion marker on T cells that has been associated with a lack of proliferative capacity or replicative senescence. While no difference was observed in the expression of the exhaustion marker CD57 between matched blood and cervical T cells *ex vivo,* CD57 expression was significantly higher in cervical clones than blood clones cultured for similar periods of time. CD57 expression was significantly higher in cervical T cell clones than cervical T cells assessed directly *ex vivo*, a trend not observed in blood-derived T cells. It is clear that further investigation into T cell proliferative and functional impairment is necessary to improve the efficiency of the generation of HIV-specific T cell clones by limiting dilution in order to determine if compartmentalisation of HIV-specific T cell responses exists between the blood and the cervix. This is the first study to have investigated CD57 expression of cervical-derived T cells particularly under conditions of clonal expansion and clearly this has important implications for mucosal vaccine design.

Chapter 1

Literature Review

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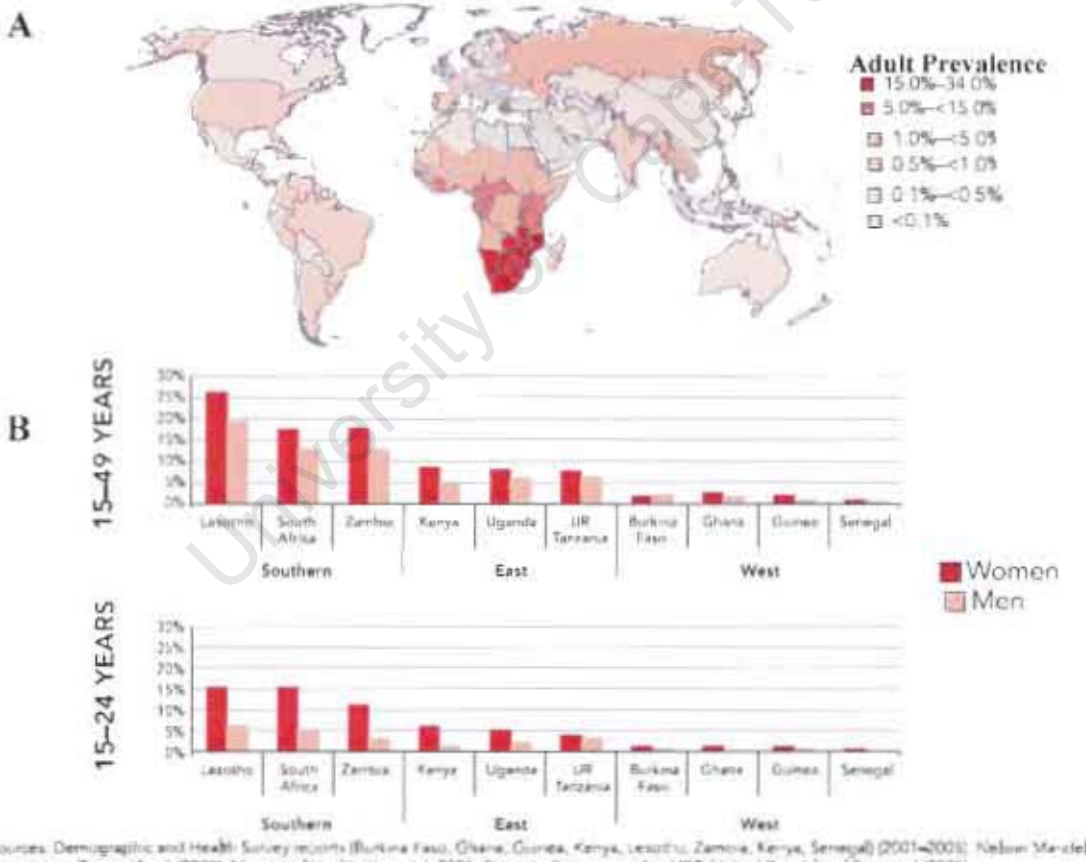
Literature Review

1.1.1. Introduction

More than two decades have passed since the first reported incidents of Acquired Immunodeficiency Syndrome (AIDS). In 1983, the causative agent of AIDS, the Human Immunodeficiency Virus (HIV) was identified (Barre-Sinoussi *et al.*, 1983). Today, the number of people infected with HIV has reached pandemic proportions. In 2005 nearly 40 million people were living with HIV, with about 25-million of this HIV-infected population hailing from sub-Saharan Africa (Figure 1.1A; UNAIDS Report on the Global AIDS epidemic, 2006). Of these HIV-infected individuals, women outnumbered men by 2-fold (Figure 1.1B). Since women are particularly vulnerable to heterosexual transmission of HIV for a number of reasons including socioeconomic, biological and physiological factors (UNFPA, 2007), these statistics clearly emphasise the need to prevent HIV infection at the site of sexual transmission in women, the genital mucosa, and the importance of studying immune responses at this site.



Sources: Demographic and Health Survey reports (Burkina Faso, Ghana, Guinea, Kenya, Lesotho, Zambia, Kenya, Senegal) (2001–2005); Nelson Mandela Foundation (South Africa) (2005); Ministry of Health (Uganda), 2005; Tanzania Commission for AIDS (United Republic of Tanzania) (2005)

Figure 1.1. A global view of HIV infection, 2005. (**A**) HIV prevalence according to geographic region. The darker colours indicate regions with the higher prevalence whereas the lighter colours indicate regions with the lower international prevalence. About 15-34% of the 40 million people infected with HIV worldwide hailed from Sub-Saharan Africa. (**B**) HIV prevalence (%) is described by gender in various African countries (2001-2005). In most countries, the number of HIV-infected women outnumbered the HIV-infected men (adapted from UNAIDS, 2006).

The importance of the mucosal immune system in HIV infection and pathogenesis has only recently been recognized. The mucosal system not only plays a fundamental role in HIV pathogenesis by serving as a portal of entry during heterosexual intercourse (Kozlowski and Neutra, 2003; Neutra *et al.*, 1996) but also as the predominant site of virus replication and $CD4^+$ T cell depletion (Veazey *et al.*, 1998). This has led several investigators to acknowledge that although HIV has a systemic phase of infection that is easily assessable; it is largely a disease of the mucosal immune system (Veazey and Lackner, 2005). The interaction of the mucosal immune system from the female genital mucosa with HIV is the focus of the current study.

1.1.2. HIV subtype C

Three groups of HIV-1 have been identified: groups M, N and O; Figure 1.2; Thomson *et al.*, 2002). The most prevalent group, M, is subdivided into eight subtypes (Myers *et al.*, 1992, 1994). HIV subtype C is the predominant type found in Africa and Asia; HIV subtype B in North America and Europe; while HIV subtypes A and D commonly affect Africa (Hemelaar *et al.*, 2006). HIV subtype C accounts for 50% of all HIV infections worldwide and is currently the predominant subtype affecting South Africa (Figure 1.3; Hemelaar *et al.*, 2006; van Harmelen *et al.*, 1999; Novitsky *et al.*, 2002). The HIV subtype C epidemics are commonly defined by high prevalence rates in the adult population (Hemelaar *et al.*, 2006), with high likelihoods of vertical transmission (Renjifo *et al.*, 2001), high viral loads (Neilson *et al.*, 1999), preferentially infecting cells bearing the chemokine receptor CCR5 (R5-tropic; (Abebe *et al.*, 1999), and by displaying great viral diversity (van Harmelen *et al.*, 2001).

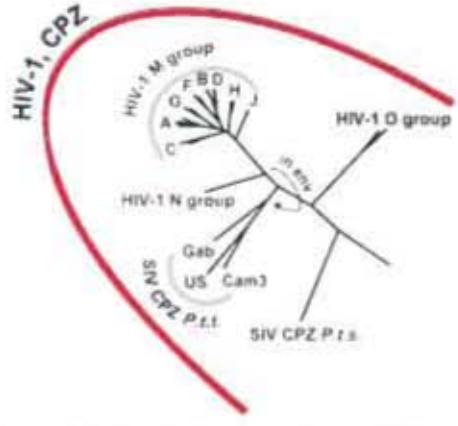


Figure 1.2. Phylogenetic tree of selected primate lentiviruses HIV and SIVcpz. Three groups of HIV-1 have been identified (groups M, N and O) with the most prevalent group (M) being subdivided into eight distinct subtypes (A, B, C, D, F, G, H, and J). HIV is closely related to SIV, in particular to SIV specific to chimpanzees (SIVcpz; adapted from Kuiken *et al.*, 1999).

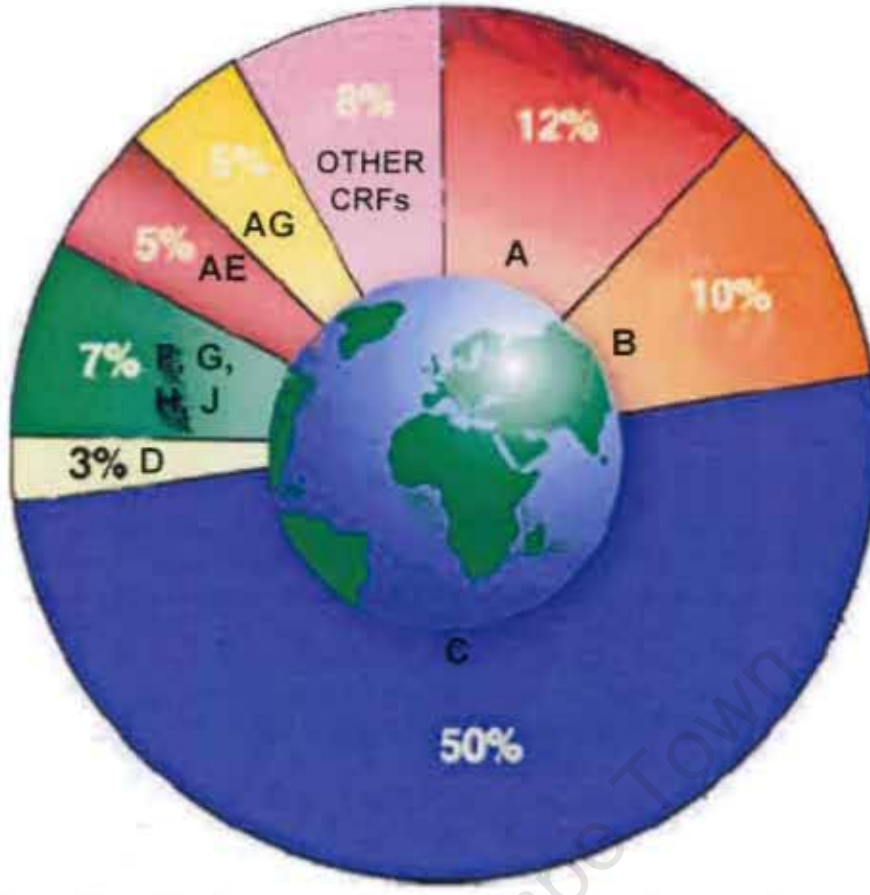


Figure 1.3. Global distribution of HIV-1 subtypes and recombinants in 2004. The number of infections caused by HIV-1 subtypes and recombinants are represented as a proportion of the global total number of individuals living with HIV-1 (adapted from Hemelaar *et al.*, 2006)

1.1.3. HIV-1 genomic organization and structure

The HIV genome is comprised of 9 genes encoding various necessary structural (Gag, Pol and Env; Luciw, 1996), accessory (Nef, Vif, Vpu and Vpr; Anderson and Hope, 2004) and regulatory (Tat and Rev; Addo *et al.*, 2001; Bell *et al.*, 2007) proteins (reviewed in Freed, 1998). A summary of the arrangement and function of each of these 9 genes is shown in Figure 1.4. The arrangement of these gene products in the mature HIV particle is shown in Figure 1.5. The *gag* gene products are responsible for the formation of the viral capsid, nucleocapsid and matrix proteins (Melamed *et al.*, 2004). Processing of the *env* gene products results in the surface glycoprotein gp120 and transmembrane protein gp41 (Rizzuto *et al.*, 1998; Lu *et al.*, 1995). HIV *pol* encodes for the enzymes (protease, reverse transcriptase and integrase) necessary for the completion of the viral replication cycle in the host cell (Luciw, 1996). The regulatory proteins Tat and Rev are essential for transcription of the viral genome and regulation of late gene expression respectively (Malim, 1989; Kao *et al.*, 1987). The accessory proteins Vpu, Vpr, Vif and Nef are collectively responsible for the infectivity, reproduction and pathogenesis of the virus (Emerman and Malim, 1998).

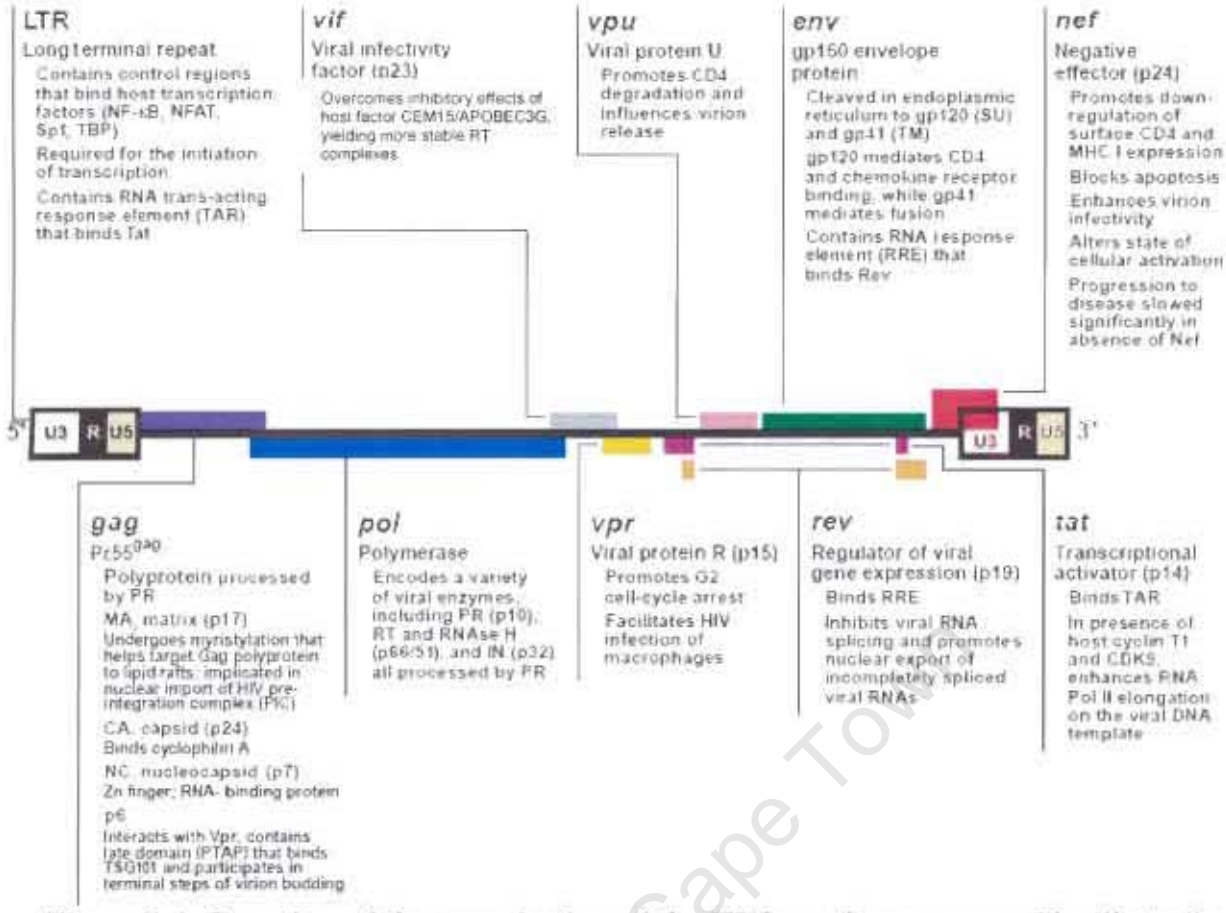


Figure 1.4. Overview of the organisation of the HIV provirus genome. The illustration summarises the arrangement of the 9 HIV genes and the functions of their 15 protein products (taken from Green and Peterlin, 2002; Sheehy *et al*., 2002). Also depicted are the long terminal repeat regions flanking the proviral genome at the 5' and 3' ends.

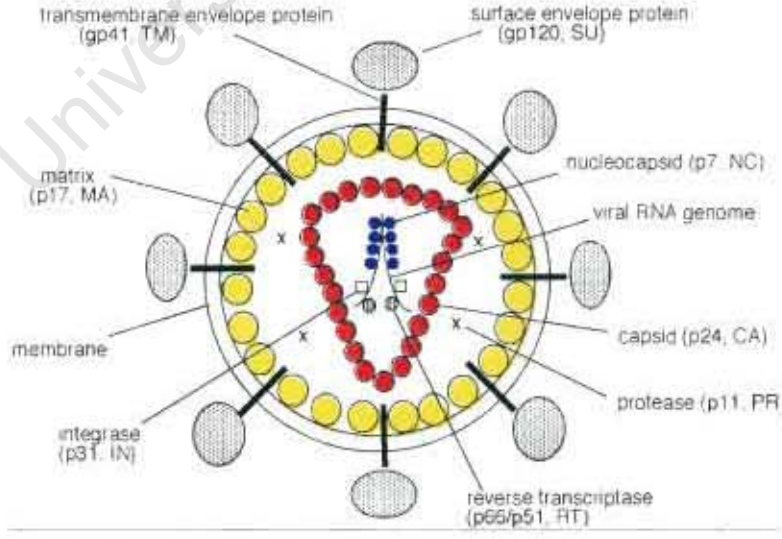


Figure 1.5. Schematic representation of the arrangement of HIV-1 gene products in the mature virion. The HIV-1 virion, indicating the approximate location of Gag proteins, the Env glycoproteins, and the *pol*-encoded enzymes IN, RT, and PR. Colours in the virion correspond to the location of the proteins in the Gag precursor (adapted from Freed, 1998).

The *gag* gene provides the basic structural elements of the virus and comprises the matrix protein (MA) p17; capsid protein (CA) p24; nucleocapsid (NC) p7; p6 involved in late viral assembly (Freed, 2002); and spacer proteins p2 and p1 which separate CA from NC and NC from p6 respectively (Mervis *et al.*, 1988; Figure 1.5; Figure 1.6).

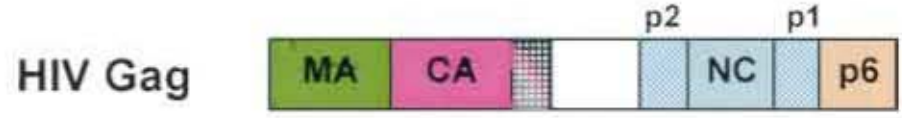


Figure 1.6. Arrangement of HIV Gag cleavage products within the Gag polyprotein (adapted from Göttlinger, 2001).

The p24 capsid protein is the most conserved of all Gag regions (Buseyne *et al.*, 1992). Several studies have shown that point mutations in this gene result in the prevention of virion assembly (Liang *et al.*, 2003; Reicin *et al.*, 1995; Srinivasakumar *et al.*, 1995) and early stages of infection (Tang *et al.*, 2003). The matrix protein (p17) domain is required for virion assembly as well as for membrane binding (Ono *et al.*, 1999, 2000) and the nucleocapsid protein (p7) is required for binding to viral RNA (Zhang *et al.*, 1997) and subsequent encapsidation during virion assembly (Dorfman *et al.*, 1993). As a result, the *gag* gene harbours highly conserved regions (Buseyne *et al.*, 1992). For example, a highly conserved glycine residue serves as a myristylation site at the N terminus of p17 (Ono *et al.*, 2000); 20 conserved residues in p24 are responsible for the hydrophobic core of the protein (Gamble *et al.*, 1997); a conserved zinc finger motif in p7 binds RNA (Zhang *et al.*, 1997). Recently, 11 residues at the C-terminus of p24 were identified as being highly sensitive to point mutations and, as a result, involved in the correct assembly and discharge of HIV virions (Melamed *et al.*, 2004). As a result, numerous studies are focused on the development of drugs and vaccines directed at these conserved regions in HIV Gag (Bolesta *et al.*, 2005; Ondondo *et al.*, 2006; Xu *et al.*, 2006; Tobery *et al.*, 2006; Tavel *et al.*, 2007).

1.1.4. The HIV-specific cellular immune response

1.1.4.1. The importance of $CD8^+$ T cells in the control of HIV

A number of studies have provided strong evidence to suggest that $CD8^+$ T lymphocyte responses (cytokine and lytic) are important in controlling viral replication in HIV-infected individuals. Human studies have shown that in the early phase of infection, the presence of HIV-specific $CD8^+$ T lymphocyte and not $CD4^+$ T cells or neutralizing antibody responses are temporally associated with the decline of plasma viremia (Figure 1.7; Appay *et al.*,

2002; Borrow *et al.*, 1994; Gray *et al.*, 1999; Koup *et al.*, 1995). Strong HIV-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses are maintained in long term non-progressors (Migueles *et al.*, 2002; Betts *et al.*, 2006) and have also been demonstrated in several cohorts of highly exposed yet persistently seronegative individuals (Kaul *et al.*, 2000, 2001; Rowland-Jones *et al.*, 1997, 1998). Most convincingly, $CD8^+$ lymphocyte depletion studies in SIV-infected macaques have shown greater viral replication and more rapid disease progression in the absence of $CD8^+$ cytotoxic T lymphocytes (Jin *et al.*, 1999; Schmitz *et al.*, 1999).

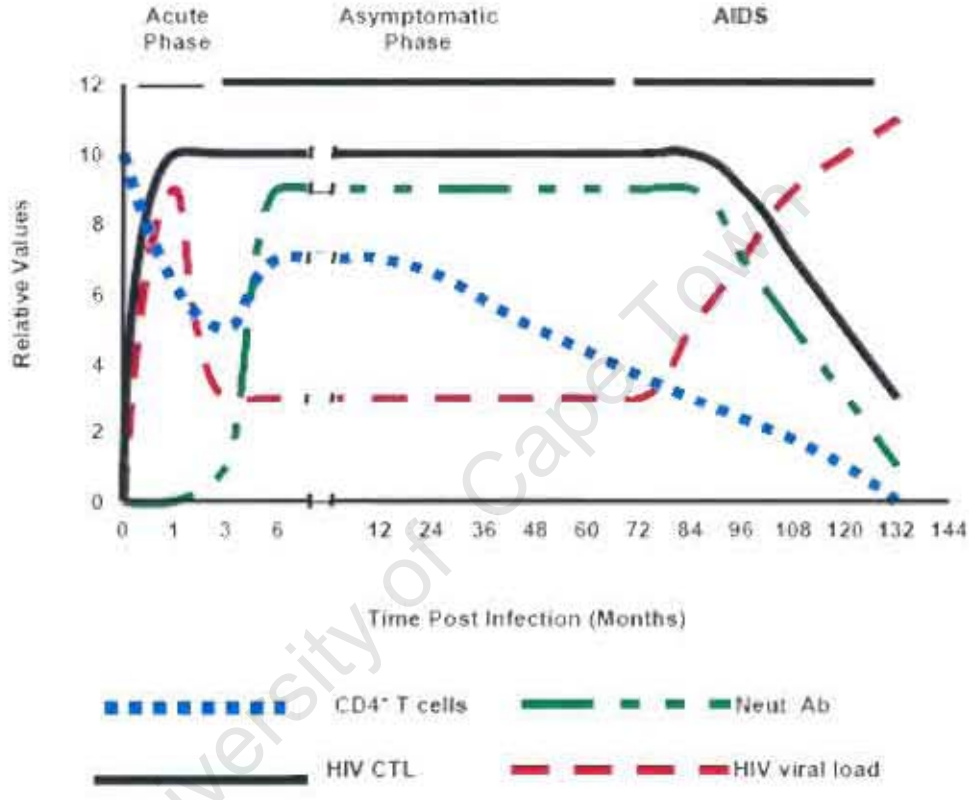


Figure 1.7. Temporal association between decline in HIV viral load and emergence of HIV-specific CD8 T cell number (but not CD4 T cell or neutralizing antibody concentrations) during the course of HIV infection. HIV primarily infects $CD4^+$ T cells and characteristically results in $CD4^+$ T cell depletion. The presence of $CD8^+$ T lymphocytes is temporally associated with the decline of plasma viremia (adapted from Alimonti *et al.*, 2003).

Numerous studies have described the emergence of HIV variants with specific mutations in the precise genomic regions targeted by $CD8^+$ CTL that lead to the escape of that viral variant from CTL detection. This suggests selective immune pressure is placed on the virus by CTLs (Borrow *et al.*, 1997; Goulder *et al.*, 1997). Furthermore, certain Human Leukocyte Antigen (HLA) alleles have been observed to correlate with HIV disease control or progression, suggesting varying degrees of presentation and functional activity by $CD8^+$ T lymphocytes (Kaslow *et al.*, 1996; Carrington *et al.*, 1999). Not all potential escape mutations in HIV gene products are harmless to viral fitness. For example, more escape

variants are observed in regions tolerating more sequence variability (Nef, Tat, Env) than in more conserved regions (Gag; Johnson *et al.*, 1993; Yusim *et al.*, 2002). HIV Gag p24 is the most conserved region of the *gag* gene (Buseyne *et al.*, 1992). CTL escape mutants in this region have been observed to occur as a result of mutation in only a single site (Barouch *et al.*, 2002; Goulder *et al.*, 1997), most likely due to a high cost to viral fitness on greater sequence variation. In contrast, escape mutants described from Nef and Env regions (regions with generally more tolerance for sequence variability than Gag p24) have been observed to arise at multiple sites (Evans *et al.*, 1999; Borrow *et al.*, 1997).

1.1.4.2. Correlates of protection in the control of HIV infection

Numerous studies have provided conflicting data on the association between HIV viral load and the $CD8^+$ T cell response. While some studies have demonstrated no direct correlation between overall HIV-1-specific IFN-γ production by $CD8^+$ T cells and plasma viremia or $CD4^+$ T cell counts (Addo *et al.*, 2003; Edwards *et al.*, 2002), others confirm that $CD8^+$ T cell responses directed against HIV Gag have the ability to reduce viral set-point and are therefore important in controlling HIV infection (Patke *et al.*, 2002; Geldmacher *et al.*, 2007). Several studies have demonstrated inverse correlations between viral load and the magnitude and breadth of HIV Gag p24-specific CTL responses (Kiepiela *et al.*, 2007; Masemola *et al.*, 2004; Novitsky *et al.*, 2003; Edwards *et al.*, 2002). Furthermore, targeting of certain HIV Gag epitopes, particularly within the highly conserved p24 region, can drive strong selection pressure on the virus, evidenced by lower viremia (Kiepiela *et al.*, 2007; Martinez-Picado *et al.*, 2006). Recent studies have emphasized the importance of polyfunctionality in T cell function (the ability of T cells to secrete multiple cytokines and perform multiple functions) in the control of persistent HIV and other chronic viral infections (Casazza *et al.*, 2006; Betts *et al.*, 2006). Betts *et al.* (2006) showed that the ability of these HIV-specific CTLs to exhibit multiple functions - not just IFN-γ production alone - best predicted control of viral replication.

1.1.4.2. The importance of mucosal associated lymphoid tissue in HIV transmission and pathogenesis

It is widely accepted that HIV infection is essentially a disease of the mucosal immune system (Veazey and Lackner, 2005; Johnson and Kaur, 2005). The major route of sexual transmission of HIV is through mucosal exposure to cell free and cell-associated virus, primarily at the mucosa lining the rectum and vagina (Neutra *et al.*, 1996; Kozlowski and Neutra, 2003), and it is the mucosa of the gastrointestinal tract that serves as the primary

site of virus replication (Veazey *et al.*, 1998; Brenchley *et al.*, 2004; Mehandru *et al.*, 2004). The target cells for HIV infection, effector memory $CCR5^+$ $CD4^+$ T cells, are abundant in the mucosa, predominantly in the intestine, but are rare in the peripheral blood (Veazey *et al.*, 2000; Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Li *et al.*, 2005). As a result, large scale depletion of these susceptible cells has been observed in the mucosa of the vagina (Veazey *et al.*, 2003), lung (Vajdy *et al.*, 2001) and predominantly in the gastrointestinal tract (Mehandru *et al.*, 2004; Brenchley *et al.*, 2004), before significant depletion within the periphery and lymph nodes (Mattapallil *et al.*, 2005; Li *et al.*, 2005; Brenchley *et al.*, 2004; Mehandru *et al.*, 2004). About 30 – 60% of intestinal $CD4^+$ T cells are infected during acute HIV infection. Of this infected $CD4^+$ T cell population, about 60 – 80% is depleted within 4 days of infection (Mattapallil *et al.*, 2005; Li *et al.*, 2005). Since $CD4^+$ T cells are essentially responsible for generating help for immune responses (Noelle and Snow, 1990; Meyaard *et al.*, 1994; Fogelman *et al.*, 2000; Autran *et al.*, 1997) and are therefore an integral component of the mucosal immunological barrier against invading pathogens, it is likely that their extensive depletion significantly affects the proper function of the mucosal barrier (Johnson and Kaur, 2005). In light of evidence of the mucosal system being the fundamental site of entry and virus replication, strategies to develop effective AIDS vaccines and therapies are increasingly focusing on stimulating mucosal immune responses (Belyakov and Berzofsky, 2004; Lekkerkerker *et al.*, 2004; Ranasinghe *et al.*, 2007; de Souza *et al.*, 2007).

1.1.4.4. HIV-specific CTL responses at the mucosa

A number of studies have demonstrated that the mucosal compartment is important in controlling HIV infection (Bomsel *et al.*, 2007; Kaul *et al.*, 2000; Belyakov *et al.*, 1998). In a murine model of HIV transmission, Belyakov *et al* (1998) showed that it is mucosal CTL responses and not systemic responses that are necessary to prevent HIV-1 transmission. Similarly in macaques, SIV-specific CTL responses at the rectal mucosa were shown to be important for protection against intra-rectal challenge with SIV (Murphey-Corb *et al.*, 1999). In humans, HIV-specific CTL have been identified in various mucosal compartments including the cervicovaginal mucosa (Musey *et al.*, 1997, 2003), semen (Quayle *et al.*, 1998), rectum (Shacklett *et al.*, 2000b), human breast milk (Sabbaj *et al.*, 2002); and even in the mucosa of the lung (Plata *et al.*, 1987). However, inherent technical difficulties associated with sampling of mucosal tissues have limited the understanding of mucosal HIV-specific cellular immune responses in humans.

1.1.4.5. HIV-specific CTL responses at the female genital tract

Several studies in both humans and non-human primates have presented evidence for HIV-specific CTL responses in the genital mucosa (Lohman *et al.,* 1995; Reynolds *et al.,* 2005; Kaul *et al.,* 2000, 2003; Shacklett *et al.,* 2000b; Ibarrondo *et al.,* 2005; Quayle *et al.,* 2007).

SIV-specific CTL response in the genital tract of female macaques after intravaginal inoculation of SIV was first documented by Lohman *et al.* (1995). More recently, Stevceva *et al.* (2004) identified SIV Gag-specific $CD8^+$ T cells in samples from the rectum, colon, jejunum, and vagina of most of their SIV-infected female macaques (Stevceva *et al.,* 2004). These cells expressed the activation marker CD69 and produced IFN-γ (Stevceva *et al.,* 2004). Although a robust SIV-specific $CD8^+$ T lymphocyte response in primate reproductive tissues is clearly demonstrable, Reynolds *et al.* (2005) showed that this strong response was only observed after the peak in SIV replication and was of insufficient magnitude at early time points to prevent infection and systemic dissemination.

There have been a number of studies which have described HIV-specific T cell responses in chronically HIV-infected women (Musey *et al.,* 1997; 2003; Kaul *et al.,* 2000; Ibarrondo *et al.,* 2005; Shacklett *et al.,* 2000b; Quayle *et al.,* 2007). Musey *et al.* (1997) identified human HIV-specific cervical T cells that mediated HLA class I-restricted HIV-specific cytolysis. The cytolytic activity of the cervical T cells was directed to one or more epitopes within the *env-*, *gag-*, or *pol*-encoded HIV proteins (Musey *et al.,* 1997). The HIV-specific $CD8^+$ CTL isolated from the genital mucosal were shown to produce perforin and favour the granule exocytosis method of destruction of HIV-infected target cells rather than the apoptotic Fas/Fas-ligand mechanism (Musey *et al.,* 2003). Furthermore, cervical HIV-specific CTL have been shown to secrete IFN-γ (Kaul *et al.,* 2000).

HIV-specific CTL have also been identified in the genital tract of highly exposed yet HIV seronegative individuals (Kaul *et al.,* 1998, 2000, Shacklett *et al.,* 2000). The presence of HIV-specific $CD8^+$ T lymphocyte responses was demonstrated in the cervix of HIV-1-resistant Kenyan sex workers in the absence of detectable systemic HIV infection (Kaul *et al.,* 2000). In a follow-up study, it was concluded that a major factor associated with late seroconversion and HIV infection was a break from sex work, implying that continuous antigenic exposure was needed to maintain the CTL response at the cervix (Kaul *et al.,* 2000).

There is still much not understood about mucosal immunity in humans; primarily because of difficulties associated with sampling mucosal tissues, isolating T lymphocytes from these samples, and generating mucosal T lymphocytes in culture (Musey *et al.,* 2003). Most knowledge of the HIV-specific immune response is therefore based on studies in blood.

1.1.5. Organization of the female genital tract

The lower genital tract in women is comprised of four distinct regions: (1) the keratinised introitus (vaginal opening), (2) the vaginal mucosa, (3) the ectocervix, and (4) the endocervix (Figure 1.8, Pudney *et al.,* 2005). The abrupt transition between the ectocervix and endocervix, known as the transformation zone, contains the largest number of lymphocytes in the female lower genital tract (Edwards *et al.,* 1985). Dendritic cells and their specialized mucosal counterparts, Langerhans' cells, appear equally in the endo- and ectocervix as well as vaginal opening regions but not in the vaginal mucosa (Pudney *et al.,* 2005). Other immune cells such as macrophages and granulocytes have also been identified in the cervix and vagina (White *et al.,* 1997). High concentrations of $CD8^+$ cells (CTL and natural killer cells) and antigen-presenting cells in the ectocervix and transformation zone suggest the possibility of these sites being the predominant locations for the induction of effector CTL responses in the lower genital tract (Pudney et al., 2005). The endocervix also contains numerous IgA^+ and IgM^+ cells, rendering it the predominant site for local humoral immune responses (Kutteh, 1999).

1.1.5.1. Early events during sexual transmission of HIV to women

Intact vaginal epithelium can serve as an efficient barrier to viral penetration (Shattock and Moore, 2003; Miller *et al.,* 2005). Furthermore, cervical mucous can strengthen this defence by trapping virions in the vaginal lumen (Maher *et al.,* 2005; Miller *et al.,* 2005). Nevertheless, the presence of large quantities of dendritic cells (DCs) and intraepithelial lymphocytes at the transformation zone (Edwards and Morris, 1985; Pudney *et al.,* 2005) has highlighted the possibility of this being the primary site of HIV transmission as both of these cell types have been implicated in HIV infection (Hu *et al.,* 2000; Spira *et al.,* 1996). Although the predominant mode of infection remains to be determined, several different routes of HIV infection have been demonstrated. HIV can cross the cervicovaginal barrier either as a result of epithelial damage (from trauma-related abrasions or lesions due to sexually-transmitted infections); transcytosis across an intact epithelial barrier (Bomsel, 1997); or by capture and transfer by intraepithelial dendritic cells (Lee *et al.,* 2001). The

latter method of infection is facilitated by dendritic cells that cross the cervicovaginal barrier

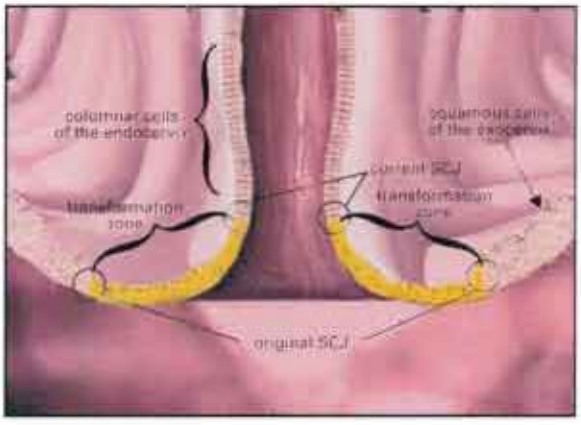


Figure 1.8. Organization of the female genital tract. The lower female genital tract is comprised of the vaginal opening (not shown here), the vaginal mucosa (not shown here), the ecto- or exocervix, and the endocervix. Shown are the columnar cells of the endocervix, the squamous cells of the ecto- or exocervix, and the squamocolumnar junctions (SCJ) and transformation zone between the ectocervix and endocervix (from: http://www.merckmedicus.com/pp/us/hcp/diseasemodules/hpvd/natural-history.jsp)

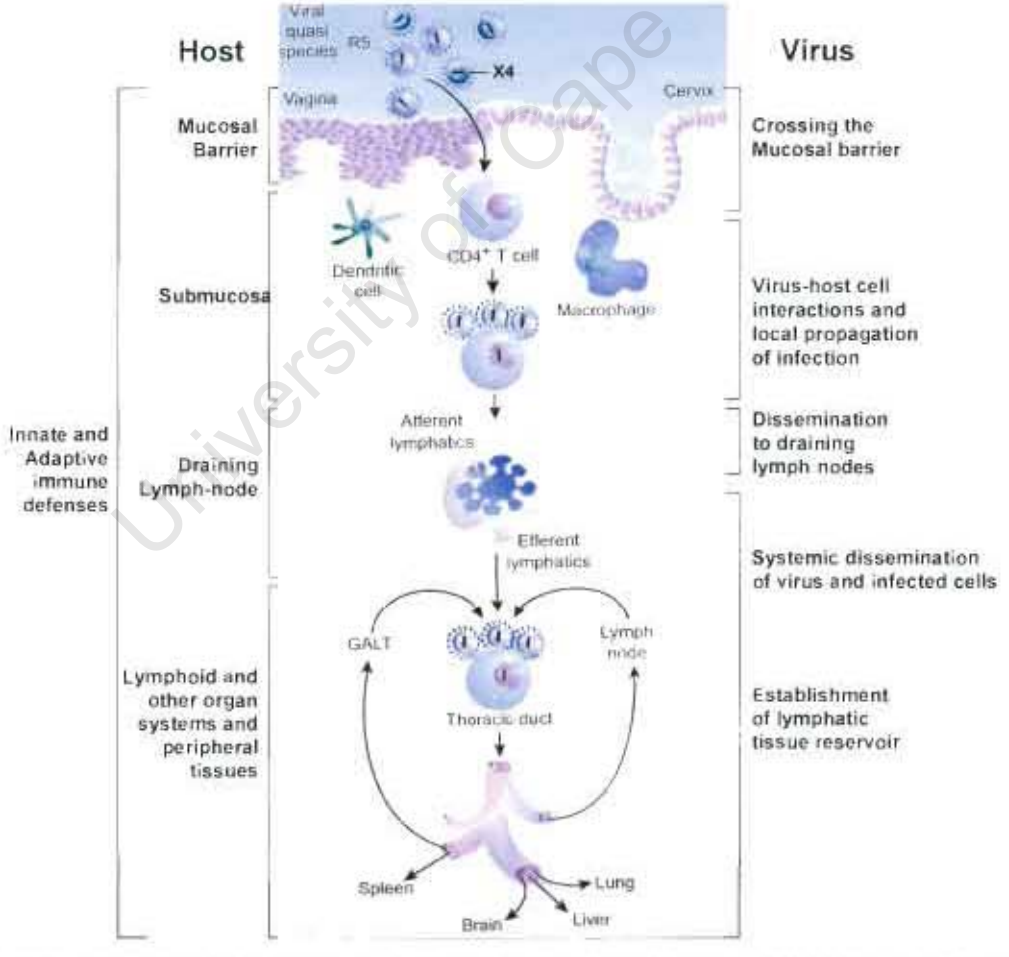


Figure 1.9. Early events during sexual transmission of HIV and acute infection in the female genital tract. R5 viruses are selectively transmitted. After crossing the cervicovaginal mucosal barrier, dendritic cells, $CD4^+$ T cells and macrophages in the underlying submucosa are infected. Infection is subsequently propagated and disseminated, thereby establishing the lymphatic tissue reservoir that spreads infection to other organs

and peripheral tissues. Innate and adaptive host defences (left column) are directed at the different stages to prevent transmission and contain infection (from Pope and Haase, 2003).

either as a result of epithelial damage (from trauma-related abrasions or lesions due to sexually-transmitted infections); transcytosis across an intact epithelial barrier (Bomsel, 1997); or by capture and transfer by intraepithelial dendritic cells (Lee *et al.,* 2001). The latter method of infection is facilitated by dendritic cell surface-expression of CD4, chemokine coreceptor, and C-type lectin receptors. R5 viruses are selectively transmitted (Meng *et al.,* 2002). Several studies have suggested that DC-bound virions traversing the epithelial layer could either infect and complete their replication cycle within DC or be liberated from the DC and infect CD4-bearing T helper cells, macrophages and dendritic cells (McDonald *et al.,* 2003; Pope *et al.,* 1994, 1995). Once HIV has gained entry, virus is transported via the afferent lymphatics to $CD4^+$ T helper cell-rich lymph nodes to further disseminate the infection (Miller *et al.,* 2005). Figure 1.9 illustrates the early events during sexual transmission and acute infection in the female genital tract.

1.1.6. Compartmentalization and HIV genetic diversity in the genital tract

Studies of viral evolution between and within HIV subtypes often compare the envelope gene sequence, as its high degree of genetic variation is commonly observed in its product's (gp120) ability to determine cell tropism (Fisher *et al.,* 1988; O'Brian *et al.,* 1990; Shioda *et al.,* 1991) and escape from the host immune response (Borrow *et al.,* 1997; Goulder *et al.,* 1997; Wei *et al.,* 2003). Comparisons of HIV envelope genes in blood and in the genital secretions of HIV-infected women identified genetic differences as a result of the insertion of new potential glycosylation sites in the variable regions of the envelope glycoprotein (Overbaugh *et al.,* 1996), a property known to affect the immunogenicity of gp120 (Wei *et al.,* 2003). Furthermore, the HIV isolates from the genital mucosa were closer related to only a minor subset of PBMC variants (Poss *et al.,* 1995; 1998). This result highlights the possibility that distinct viral species may arise independently in response to unique tissue-specific selection pressures (such as availability of $CD4^+CCR5^+$ or $CD4^+CXCR4^+$ bearing cells) or as a result of the selective migration of one or more HIV-infected cells between the blood and mucosa, followed by the local expansion and evolution of the virus in that region (Poss *et al.,* 1998). These reports therefore emphasise the importance of understanding immune control mechanisms at the site of heterosexual transmission.

In addition, Ellerbrock *et al.* (2001) showed clearly that cellular replication of HIV-1 does occur in vaginal secretions. They demonstrated by direct sequencing of HIV-1 gp120 envelope in matched blood plasma, vaginal lavage samples and cervical mucous that the major species of cell-free HIV-1 in cervical secretions were more similar to each other than those found in blood plasma (Ellerbrock *et al.,* 2001). Analysis of the HIV-1 drug resistance-associated region of HIV-1 *pol* in cell-free HIV RNA in matched plasma and vaginal lavage samples showed that a drug resistance-associated mutation in plasma may not predict the same in vaginal secretions, and therefore suggested that viral replication between the two compartments is largely independent (Ellerbrock *et al.,* 2001). This is further supported by the observation that replicating proviral populations and quasispecies in the female genital tract are less genetically diverse and divergent than those in blood (Sullivan *et al.,* 2005). A study of full-length RNA genomes derived from the genital tract and blood in the same individuals further supported the concept of viral compartmentalisation (Philpott *et al.,* 2005). HIV-1 recombinants composed of alternating viral sequences of the distinct viral populations were identified in the blood and genital tract of the same HIV-infected woman, suggesting that intra-patient recombination between different anatomical compartments could be another major source of HIV-1 evolution (Philpott *et al.*, 2005).

Taken together, the increasing body of evidence for compartmentalisation of HIV-1 between blood and the genital tract suggests the possibility of a similar compartmentalisation of HIV-specific immune responses. Since blood-derived viral populations may not mirror those in the genital tract, further study on local mucosal virus populations and the HIV-specific immune response is crucial for the design of vaccines and drugs to prevent sexual transmission of HIV.

1.1.7. Compartmentalisation of HIV-specific T cell responses

The mounting evidence for the independent evolution of viral populations between the genital mucosa and the periphery (Poss *et al.,* 1995; Overbaugh *et al.,* 1996, Ellerbrock *et al.,* 2001) increases the likelihood of the concomitant compartmentalisation of the HIV-specific immune response. There is however little evidence to support this hypothesis. Musey *et al.* (1997) observed cytolytic activity by cervical T cells to various HIV-1 epitopes. Furthermore the HIV-1 epitope specificities and HLA class I restriction patterns of CTL clones from the cervix and blood of the same individual were similar (Musey *et al.,* 1997). This implied that the CTL repertoire is common in those two anatomically distinct

compartments of HIV-infected women (Musey *et al.*, 1997). In addition, the HIV-specific CTL response was directed to a narrower spectrum of HIV-epitopes in the mucosa than the blood (Musey *et al.*, 1997). This observation could be attributed to, among others, the possible diversity in the virus population from genital mucosa and blood (Poss *et al.*, 1995). Subsequently, Musey *et al.* (2003) sequenced the Vβ repertoire of the T cell receptors of $CD8^+$ CTL from the blood, cervix, rectum and semen of HIV-1-infected individuals to compare clonally derived lymphocyte populations between the compartments. They found identical HIV-1-specific CTL clones in different compartments in the same individual suggesting that some blood and mucosal HIV-specific CTL can be of common origin and can traffic between the blood and the mucosa (Musey *et al.*, 2003). There is increasing evidence to support the occurrence of overlapping HIV-1 epitope specificities between blood and mucosal CTL (Kaul *et al.*, 2000; Ibarrondo *et al.*, 2005). In a recent comparison of blood and mucosal CTL responses across the full HIV genome, concordant responses between the cervix and blood compartments was observed in 85.1% of the screened HIV peptide pools implying that this overlap between compartments is not absolute (Ibarrondo *et al.*, 2005). There have also been contradictory reports of differing HIV epitope-specificities between cervical and blood-derived CTL (Shacklett *et al.*, 2000). The need for a greater understanding of cervical T cell responses to HIV is evident.

1.1.8. Summary

In conclusion, there is compelling evidence to suggest that distinct HIV variants occur in the mucosa compared to the periphery, which may result in differences in HIV-specific T cell responses. There is, however, conflicting evidence to support this. While most studies have focused exclusively on comparing HIV specificity between compartments, no studies have described the differentiation status of T cells at these two anatomical sites.

Accumulating evidence for HIV-specific CTL responses at the mucosa stresses the importance of understanding local immune responses to HIV to aid the design of efficient treatments and mucosal vaccines to prevent the transmission of HIV at mucosal surfaces. Since most infections are acquired through heterosexual transmission, with the risk of women becoming infected being 2-fold higher than men (Mertens and Burton, 1996), there is an urgency to develop effective preventative measures for those currently most at risk.

1.1.9. Project aim

The aim of this project was therefore to compare the HIV-specific T cell function and phenotype in the genital tract and in peripheral blood. I focused on expanding HIV-specific cervical T lymphocyte populations by either polyclonal expansion or T cell cloning by limiting dilution. The functional characteristics of HIV-specific T cell clones at the cervix of HIV-infected women were compared with that of T cell clones generated from their peripheral blood in order to determine whether compartmentalisation of the HIV-specific immune response occurs between the periphery and female genital tract. In addition, the maturational status of HIV-specific *ex vivo* T cells and T cell clones were compared across compartments.

1.2. Project strategy

HIV-specific T-cell responses at the site of heterosexual transmission in women, the genital mucosa and cervix, is poorly understood. The low yield of T cells generally obtained from the genital tract by popular non-invasive methods (Coombs *et al.*, 2003) hampers in-depth functional analysis of HIV-specific T cells in the genital tract.

Various techniques are available to study antigen-specific responses in circulating T cell populations. ELISpot, intracellular cytokine staining, and MHC-tetramer staining and flow cytometry allow for detection of responses in populations with extremely low frequencies and are therefore commonly used in studies of cellular immunity (Ogg and McMichael, 1999). The IFN-γ ELISpot assay is widely used to measure the frequency of T cells capable of antigen-specific cytokine secretion (Lalvani *et al.*, 1997). It does not, however, have the advantage that both intracellular cytokine staining and MHC-tetramer staining have in the ability to simultaneously phenotype those antigen-specific T cells (Gillespie *et al.*, 1999). MHC-tetramer staining has a further advantage in differentiating between T cells with high and low affinity for specific epitopes as this allows efficient purification of the most effective T cells for study or treatment purposes (Dunbar *et al.*, 1998). This study has focused on the use of IFN-γ ELISpot and intracellular cytokine staining and flow cytometry to assess both the magnitude and specificity of T cell responses at the cervix and in blood to HIV Gag in women with chronic HIV infection.

Cytobrush-mediated sampling of the cervix is a relatively non-invasive technique that yields adequate numbers of T cells for limited analysis of functional HIV-specific activity of $CD8^+$ T lymphocytes (Musey *et al.*, 1997; Shacklett *et al.*, 2000b; Kaul *et al.*, 2000, 2003; Prakash *et al.*, 2004). For more in-depth studies of cytolytic function, epitope specificities, and T cell receptor clonotypes of T cells isolated from cervical cytobrush specimens, *in vitro* expansion techniques are increasingly being employed (Musey *et al.*, 1997, 2003; Ibarrondo *et al.*, 2005). Polyclonal *in vitro* expansion of antigen-specific memory T cells has often been applied in studies in blood (Jones *et al.*, 2002), and has achieved the same success in mucosal studies by allowing the study of T cell subsets that would not, by virtue of their initial frequency *in vivo* and low sampling-associated cell recovery, easily be observed directly *ex vivo* (Musey *et al.*, 1997, 2003; Shacklet *et al.*, 2003). Similarly, T cell cloning by limiting dilution allows the further study of virus-specific cellular interactions at a single cell level (Musey *et al.*, 1997, 2003; Appay *et al.*, 2000) and shows great promise in allowing a greater understanding of HIV-specific T cell

homing patterns, ontogeny, and specific function in samples with low frequencies of a cell subset of interest. The present study has compared various strategies to assess HIV Gag-specific responses. These included *ex vivo* assessment as well as *in vitro* polyclonal expansion of cervical mononuclear T cells. Because of the severe sample size restrictions of *ex vivo* (which are only partially overcome by short-term *in vitro* expansion), this study has largely focused on T cell cloning by limiting dilution as a mechanism to precisely evaluate single-cell phenotype and function during HIV responses at the cervix.

To detect CTL activity in low frequencies of antigen-specific CTL the sample is often clonally expanded *in vitro* by co-culturing with inactivated (irradiated) antigen presenting cells. Due to the low yield of recoverable T cells from cervical specimens, this method is currently essential in the study of HIV-specific CTL at the female genital tract. However, this process requires prolonged *in vitro* stimulation which is likely to distort the frequency (and potentially the phenotype) of effector CTL, and therefore provides inadequate approximation of the *in vivo* conditions under which virus-specific lysis may occur. The polyclonal expansion of CTL is a qualitative technique useful mostly for the detection of HIV-specific CTL. It becomes more quantitative when combined with limiting dilution analysis (LDA; serial dilutions of the cell sample to set up bulk CTL cultures; Koup *et al.,* 1991; Carmichael *et al.,* 1993). LDA involves serial dilutions of the cell sample to set up bulk CTL cultures. Based on the lowest dilution from which CTL could be detected after *in vitro* stimulation, mathematical techniques (Poisson distribution) are applied to determine the CTL effector frequency in the primary sample. However, LDA has often been argued to underestimate the actual effector cell frequency (Gotch *et al.,* 1990) and to correlate poorly with effector frequencies estimated using HLA-peptide tetramer technology or ELISpot analyses (Tan *et al.,* 1999). This is probably due to LDA measuring only those virus-specific cell subsets that are able to proliferate under limiting dilution conditions, thereby missing those CTL which, have poor proliferative capacity e.g. either effector T cells, terminally differentiated, apoptotic in tissue culture (Lewis *et al.,* 1994; Lloyd *et al.,* 1997; Monteiro *et al.,* 1996). This poses a problem in studies of immune responses in chronic HIV-infection. However, polyclonal expansion is particularly useful in its sensitivity as it is able to detect antigen-specific responses to a frequency of $1/1x10^5$ cells (Goh *et al.,* 1999).

Characterisation of strong memory CTL responses to antigen at the clonal level is common in studies in blood (Weekes *et al.,* 1998, 1999; Wills *et al.,* 1999). Due to the low yield of T cells generally obtained from the genital tract, the merits of clonal expansion of T cells from the cervix are increasingly being recognised (Musey *et al.,* 1997, 2003). After

polyclonal expansion of CTL, HIV-specific CTL can be cloned by limiting dilution to allow further studies of HIV-specific CTL responses at an individual cell level. These more in-depth studies often involve the analysis of T cell receptor usage by the CTL.

Because compartmentalization of function and specificity of HIV-specific T cell responses between the genital tract and blood remains unresolved, the aim of this project was to (i) generate and characterize T cell clones from the cervix and blood of chronically HIV-infected women and (ii) determine if compartmentalization in phenotype and specificity was evident in this cohort. This study focused exclusively on the Gag region of HIV because (i) previous studies have shown that the majority of individuals respond to epitopes in this region of HIV in blood and cervical compartments (Musey *et al.*, 2003); and (ii) low cell yields preclude extensive genome mapping so the most conserved region of HIV was selected.

The first objective of the study was to screen 35 chronically HIV-1 infected women for responses to HIV-1 Gag in peripheral blood by IFN-γ ELISpot to identify women with high frequencies of HIV-specific T cells in blood. The second objective was to investigate HIV-specificity in cervical mononuclear cells after polyclonal *in vitro* expansion with anti-CD3 and to the largely conserved HIV-1 Gag to determine whether HIV-specific T cell responses could be detected after culture in mucosal specimens. Finally, the third objective of this study was to generate matched cervical and blood-derived T cell clones by limiting dilution to compare both the phenotype and specificity of HIV-specific T cell responses between the blood and the genital mucosa at the clonal level.

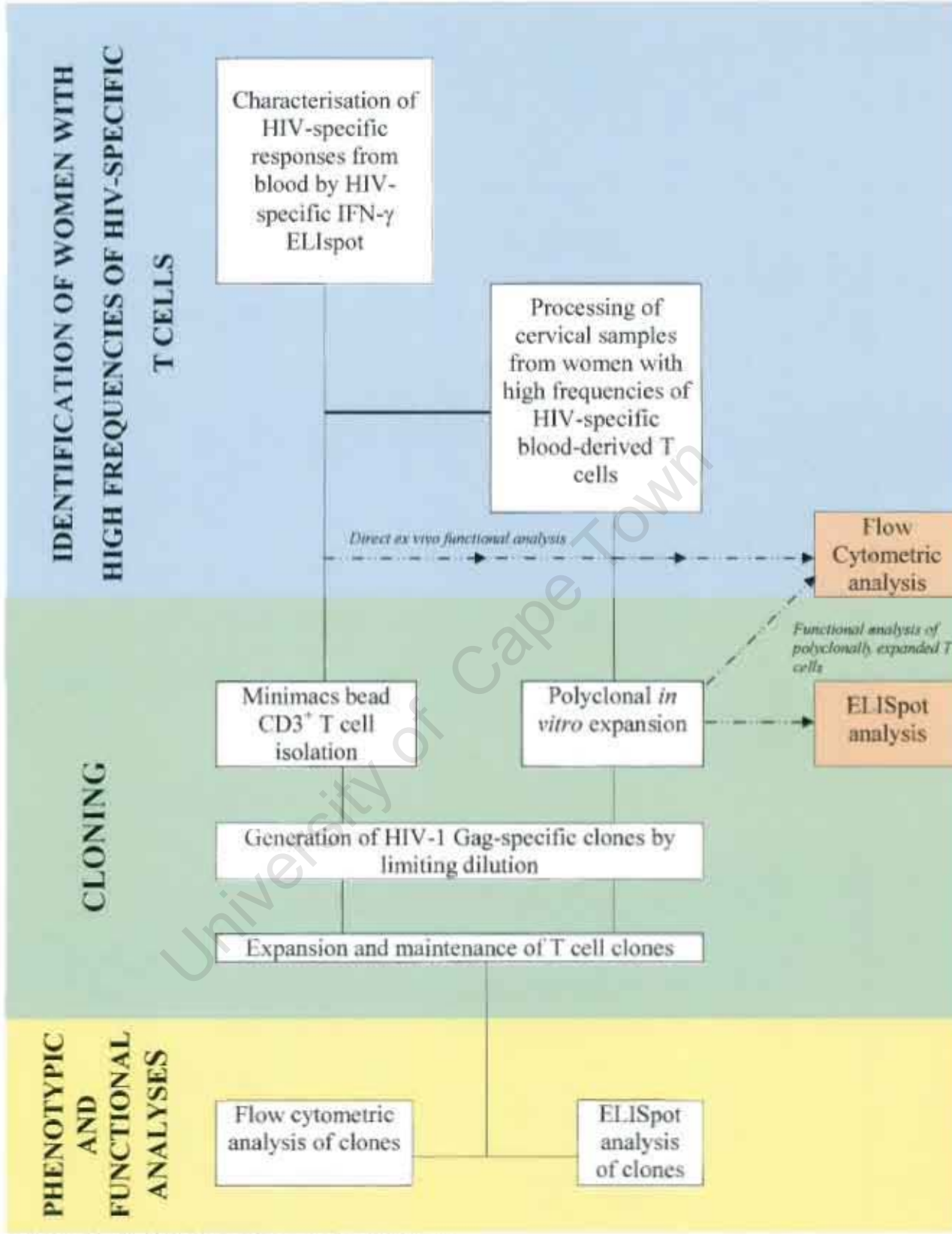


Figure 1.10. Outline of the project strategy.

Chapter 2

Characterisation of peripheral blood HIV-specific responses to HIV Gag by IFN-γ ELISpot in chronically HIV-infected women

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2.1.

Introduction

2.1. Introduction

In 2004, infections with HIV-1 subtype C accounted for 50% of all HIV infections worldwide and is currently the predominant clade affecting Sub-Saharan Africa (Hemelaar *et al.*, 2006; van Harmelen *et al.*, 1999; Novitsky *et al.*, 2002). The HIV subtype C epidemics are commonly defined by high prevalence rates in the adult population (Hemelaar *et al.*, 2006), high likelihoods of vertical transmission (Renjifo *et al.*, 2001), high viral loads (Neilson *et al.*, 1999), being preferentially R5-tropic (Abebe *et al.*, 1999), containing several unique subtype signatures across the viral genome (Novitsky *et al.*, 1999) and by displaying great viral diversity (van Harmelen *et al.*, 2001). As a result, numerous studies have focused on the comprehensive analysis of HIV-1 subtype C-specific immune responses in blood of HIV-infected individuals (Novitsky *et al.*, 1999, 2002-2003; Masemola *et al.*, 2004; Kiepiela *et al.*, 2007).

Several studies have demonstrated the importance of virus-specific $CD8^+$ T cell responses in controlling SIV and HIV infection (Jin *et al.*, 1999; Klein *et al.*, 1995; Walker *et al.*, 1986). Strong cytotoxic T lymphocyte (CTL) responses are associated with a decline of plasma viremia in acute HIV infection (Borrow *et al.*, 1994; Koup *et al.*, 1994) and with viral control (Ogg *et al.*, 1999). Similarly in SIV models of infection, the presence of $CD8^+$ T cells was associated with control (Jin *et al.*, 1999; Schmitz *et al.*, 1999). However, CTLs fail to prevent infection, and efficient CTL responses are continually challenged by viral escape from immune recognition (Borrow *et al.*, 1997; Goulder *et al.*, 1997, Price *et al.*, 1997) as well as increasing viral genetic diversity (Coffin, 1995; Ho, 1996; Philpott *et al.*, 2005; Robertson *et al.*, 1995).

Numerous studies have provided conflicting data on the association between HIV viral load and the $CD8^+$ T cell response. While some studies have demonstrated no correlation between overall HIV-1-specific IFN-γ production by $CD8^+$ T cells and plasma viremia or $CD4^+$ T cell counts (Addo *et al.*, 2003; Edwards *et al.*, 2002), others confirm that $CD8^+$ T cell responses directed against HIV Gag have the ability to reduce viral set-point and are therefore important in controlling HIV infection (Patke *et al.*, 2002; Geldmacher *et al.*, 2007). Several studies have demonstrated inverse correlations between viral load and the magnitude and breadth of the HIV Gag p24-specific CTL response (Kiepiela *et al.*, 2007; Masemola *et al.*, 2004; Novitsky *et al.*, 2003; Edwards *et al.*, 2002). Furthermore, targeting of certain HIV Gag epitopes, particularly within the highly conserved p24 region, can drive

strong selection pressure on the virus, evidenced by lower viremia (Kiepiela *et al.*, 2007; Martinez-Picado *et al.*, 2006).

Various sensitive immunological methods have recently been developed to allow more comprehensive assessment of HIV-specific cellular immune responses, including ELISpot and flow cytometry. For more in-depth investigation of the breadth and magnitude of T cell responses to HIV, many studies have utilised arrays of overlapping peptides based on clade consensus sequences of HIV or sequences modelled on laboratory isolates. However, it is now clear that use of peptides based on consensus or laboratory isolate sequences underestimates the frequency of responses directed against variable regions of these viral sequences compared to autologous virus (Draenert *et al.*, 2002; Altfeld *et al.*, 2003). The use of consensus sequence peptides resulted in failure to detect 28% of peptide-specific T cell responses recognised by autologous virus sequences (Altfeld *et al.*, 2003). Furthermore, 66% of these missed responses were located in the more variable regions of the virus (Vpr and Tat; Altfeld *et al.*, 2003). It is therefore likely that the observed poor correlation between HIV-1-specific T cell responses and viral load or $CD4^+$ T cell count (Addo *et al.*, 2003; Edwards *et al.*, 2002) may be confounded by the use of reference strains of HIV instead of autologous virus. It is important to note, however, that the strong correlation reported by Kiepiela *et al.* (2007) between the magnitude of Gag-specific immunity and lower viral set-point was only significant in a cohort exceeding 400 participants indicating the importance of large cohorts to accurately define these correlates of protection.

The primary aim of this Chapter was to define both the magnitude and breadth of HIV Gag-specific in peripheral blood of chronically HIV-infected women. Numerous studies have focused on the comprehensive analysis of HIV-1 subtype C-specific immune responses in the blood of HIV-infected individuals as HIV-1 subtype C infections account for the largest proportion of HIV infections worldwide (Hemelaar *et al.*, 2006; van Harmelen *et al.*, 1999; Novitsky *et al.*, 2002). More relevant to this study, HIV-1 subtype C is also currently the predominant clade affecting Sub-Saharan Africa (Novitsky *et al.*, 1999, 2002-2003; Masemola *et al.*, 2004; Kiepiela *et al.*, 2007).This study has focused on Gag as this has previously been shown to be one of the most immunodominant regions of HIV with preferential targeting by both peripheral blood T cells (Addo *et al.*, 2003; Ramduth *et al.*, 2005; Masemola *et al*, 2004; Novitsky *et al.*, 2002; Kiepiela *et al.*, 2007) as well as mononuclear cells associated with the genital mucosa (Musey *et al.*, 2003). In addition to providing insight into the frequency and targeting of peripheral blood T cell responses to HIV Gag in this cohort, results presented in this chapter will be central to selection of

appropriate donors for inclusion into mucosal studies (Chapter 3). HIV-infected women with the highest magnitude of HIV-1 Gag-specific T cell responses in blood will be used in subsequent studies of associated HIV-specific immunity at the cervix.

Chapter 2

Characterisation of peripheral blood HIV-specific responses to HIV Gag by IFN-γ ELISpot in chronically HIV-infected women

2.2.

Materials and Methods

2.2.1. Study population

Thirty-five chronically HIV-infected women with CD4 counts >300 cells/ul were enrolled in a longitudinal study of the impact of HIV infection on abnormal cervical cytology (in collaboration with Prof Lynette Denny, Dept Obstetrics and Gynaecology, University of Cape Town). In the study, each woman was followed-up 6 monthly at which blood $CD4^+$ T cell counts were monitored using BD Trucount CD3/CD4/CD8 reagents and FACS Caliber flow cytometry. Plasma HIV-1 RNA levels were determined at enrolment into the study and at eighteen months after their first visit using the Amplicor Monitor® system, according to the manufacturer's instructions.. The study has been approved by the University of Cape Town Human Research Ethics Committee (UCT REC ref: 106/2002). Only women who gave informed consent were entered into the study.

2.2.2. Blood collection and processing of PBMC

At each scheduled study visit, 40 ml Acetate Citrate Dextran (ACD) anti-coagulated whole blood was collected into BD vacutainer tubes by venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation within 3 – 6 hours of venipuncture using Leucosep® tubes (Greiner Bio-one). This method separates by density the lymphocytes from red blood cells, granulocytes, erythrocytes, and platelets. Lymphocytes and platelets have a lower density than red blood cells and granulocytes and are therefore collected on top of the Ficoll-Hypaque layer (Figure 2.2.1) while red blood cells and granulocytes migrate through the Ficoll cushion to pellet at the bottom. The lymphocytes are further purified from platelets by subsequent washing steps (Kanof *et al.*, 1994).

To prepare the Leucosep® tubes for PBMC isolation, a 15ml volume of Histopaque (Sigma®) was poured onto the porous inert barrier of each Leucosep® tube and the tubes were then centrifuged for 1 minute at 1000xG (2300rpm using a Heraeus 1.0R Megafuge) at room temperature to force the Histopaque through the porous barrier. The presence of a barrier served to stabilize the gradient interface and to prevent the PBMC contacting both the separation medium and erythrocytes after centrifugation. A volume of 40ml of fresh HIV-infected blood was split equally into 2 Leucosep® tubes containing Ficoll and the gradients were then centrifuged for 15 minutes at 1000xG (2300rpm using a Heraeus 1.0R Megafuge) at room temperature with the centrifuge brakes inactivated.

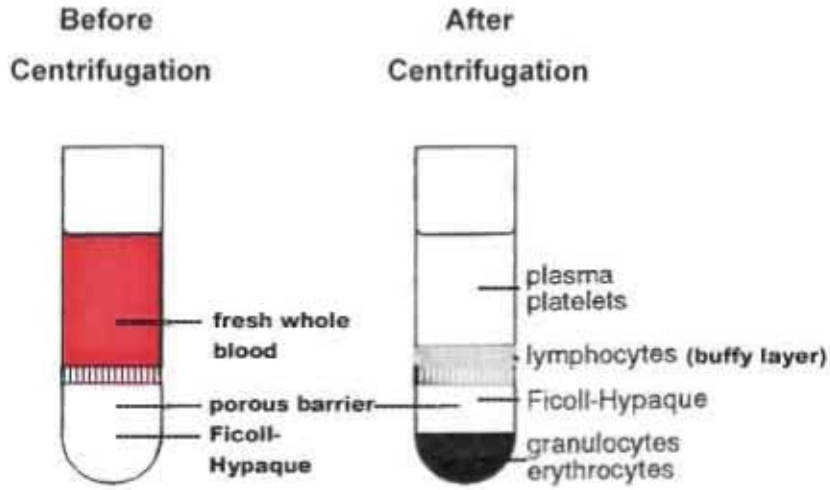

Figure 2.2.1. Isolation of PBMC by Ficoll-Hypaque gradient centrifugation. Whole blood was poured onto a porous barrier above a layer of separation media in a Leucosep® tube and then centrifuged (shown in left diagram). During centrifugation, peripheral blood mononuclear cells are separated from the rest of the components of blood by virtue of their density (shown in right diagram; adapted from Kanof *et al.*, 1994).

The buffy layer enriched for lymphocytes (Figure 2.2.1) was carefully removed with a plastic graduated Pasteur pipette (Copan Innovation) and transferred to a clean 50ml tube (Cellstar®). The PBMC buffy layer was then washed twice in a volume of 35ml **R1** [1% Foetal Calf Serum (FCS) (Delta Bioproducts) in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)] at 320xG (1300rpm using a Heraeus 1.0R Megafuge) for 10 minutes at room temperature. Finally the cell pellet was resuspended in 5ml R1 and a volume of 50µl was removed for either automated cell counting using a Coulter counter (Beckman Coulter MDI 18) or for manual counting. The cell counts were done in duplicate and the mean of the two readings was calculated.

2.2.3. Quantification of lymphocytes in suspension by Trypan Blue staining

A 0.4% dilution of Trypan Blue stain (Sigma®) was used to distinguish living from dead cells based on cell membrane permeability. Since dead or dying cells lose the integrity of their cell membranes, Trypan Blue (Sigma®) can enter these more permeable cells making dead cells appear blue under a light microscope. Manual cell counts were performed using Fast-Read counting chambers (BioSigma). These clear plastic disposable slides consist of 10 separate counting chambers each with a chamber volume of $0.1mm^3$ (Figure 2.2.2).

Freshly isolated or thawed cryo-preserved PBMCs were diluted 1:1 with Trypan Blue (Sigma®). Stained cells were placed in a plastic counting chamber for counting by Trypan Blue (Sigma®) exclusion and counted within 5 minutes of staining (Figure 2.2.2).

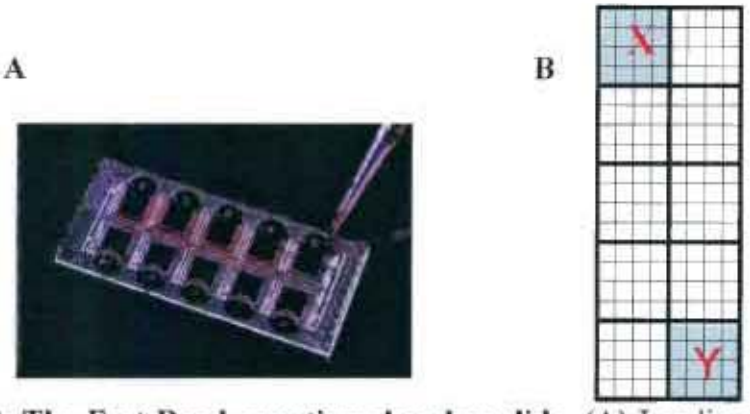

Figure 2.2.2. The Fast-Read counting chamber slide. (A) Loading of the Trypan Blue-cell suspension into the counting chambers **(B)** Each clear plastic slide consists of 10 x $0.1mm^3$ counting chambers each containing 16 x $1mm^3$ squares. Two large squares (X, Y) were counted.

The counting chamber is made up of 10 counting chambers each containing 16 x $1mm^2$ squares. Using a standard light microscope, cells are counted in the top left square (Figure 2.2.2.B block X) and diagonally opposite bottom right square (Figure 2.2.2.B block Y). The final cell concentration was determined using the following equation :

$$\text{Number of cells/ml} = \frac{\text{Blocks X + Y}}{\underset{\text{(i.e. 2)}}{\text{Number of squares counted}}} \times \underset{\text{(i.e. 2)}}{\text{Dilution factor}} \times 10^4$$

2.2.4. Cryopreservation of PBMC

To cryopreserve the PBMC for long-term storage, cells were adjusted to $1x10^7$ cells per 1 ml freezing medium [10% Dimethyl Sulphoxide (DMSO) (Sigma®) in FCS (Delta Bioproducts)]. One ml of cells in freezing medium was then transferred to each clearly labelled cryovial (Greiner Bio-one) which were then immediately transferred into pre-cooled (4˚C for 1hr) Mr Frosty® (Nalgene) containers to facilitate overnight freezing at 1°C/min at -80°C. Cells stored at -80°C overnight were transferred to liquid nitrogen the following day for long term storage.

2.2.5. Thawing of cryopreserved cells

Cryo-preserved PBMCs were retrieved from liquid nitrogen and were placed in a water bath at 37°C until almost completely thawed. The cells were quickly resuspended in 1ml warmed R1 [1% FCS (Delta Bioproducts) in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)]. The solution was then made up to 10ml in a 50ml tube (Cellstar®) with R1 added drop wise. The tube was filled with 25ml R1 and centrifuged at 320xG (1300rpm using a Heraeus 1.0R Megafuge) for 10 minutes. The supernatant was discarded and the pellet resuspended in 500ul 0.002% DNase (Roche) in RPMI 1640 (Gibco™) for 2 minutes to prevent clumping of cells. The tube was again filled with 25ml R1 and the washing step was repeated. The supernatant was discarded and the pellet was resuspended in 2ml **R20** [20% FCS (Delta Bioproducts) in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)].

2.2.6. Preparation of the HIV-1 subtype C Du422 Gag overlapping peptide pools

Lyopholised overlapping Gag peptides derived from HIV-1 subtype C Du422 sequence were kindly provided by Dr Clive Gray (NICD, Johannesburg, South Africa). Sixty six 15-20-amino acid long Gag peptides (overlapping by 10 amino acids) spanning the entire HIV-1 subtype C Du422 Gag protein were used in this study. These 66 individual lyopholised Gag peptides were diluted in 50µl DMSO (Sigma®) to provide a final peptide concentration of 20mg/ml and these were stored in 5 µl aliquots as stock solutions at -80°C. From these stocks, individual peptides were combined into 5 pools. Pools 1 to 4 comprised 14 peptides each while Pool 5 contained only 10 peptides. The final concentration of each peptide in a pool (working stocks) was 80µg/ml. Pool 1 and the beginning of Pool 2 span HIV-1 Gag matrix protein p17; the end of pool 2, the whole pool 3, and beginning of pool 4 span the highly conserved HIV-1 Gag capsid protein p24; while the end of pool 4 and the whole of pool 5 span HIV-1 Gag nucleocapsid protein p15. The arrangement of these overlapping Gag peptides and pools is clearly illustrated in Figure 2.2.3.

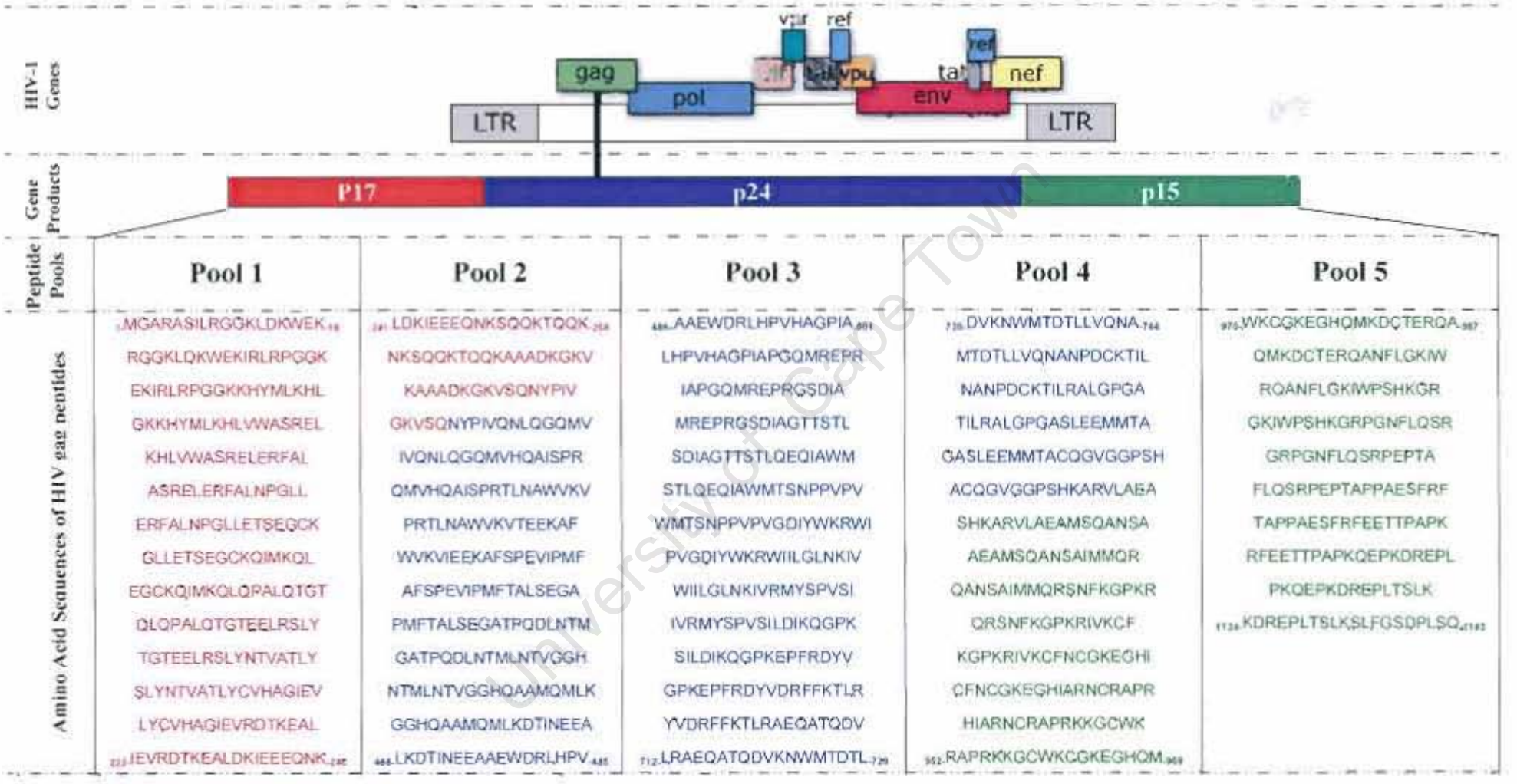

Figure 2.2.3. Amino acid sequences of the individual HIV-1 C Du422 Gag overlapping peptides making up the various pools used in the IFN-γ ELISpot assay. The entire HIV-1 Subtype C Gag protein was divided into 66 15-20-mers that overlap by 10 amino acids. These 66 peptides were grouped into 4 pools of 14 peptides (pools 1-4) and 1 pool of 10 peptides (pool 5). Pools 1 and 2 span HIV-1 Gag p17, pools 2,3 and 4 span HIV-1 Gag p24, and pools 4 and 5 span HIV-1 Gag p15.

2.2.7. IFN-γ ELISpot to detect HIV Gag-specific T cell responses

Cryo-preserved PBMC isolated from HIV-infected women were thawed and incubated overnight at 37°C before assessing IFN-γ production. HIV Gag peptide pool-specific IFN-γ secretion was assessed by IFN-γ ELISpot to characterize the frequencies of HIV Gag-specific T cells in the blood of these chronically infected women. Donors with the highest frequencies of blood HIV responsive cells were included in HIV-specific mucosal studies (Chapter 3).

Nitrocellulose-backed 96-well plates (Millipore) were coated with 50µl/well IFN-γ capturing monoclonal antibody 1-D1K (5µg/ml; Mabtech) in PBS (Gibco™). The plates were sealed with self-adhesive plastic sealers (Fasson®) and kept at 4°C overnight to allow binding of the antibody to the nitrocellulose membrane. The plates were washed three times with 200µl/well sterile PBS (Gibco™) to remove excess coating antibody. A volume of 100µl **R10** [10% FCS (Delta Bioproducts) in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)] was added per well and the plates were kept in the dark at room temperature for 2 hours to allow for the saturation of the remaining binding sites with R10 (blocking).

A representative template of an ELISpot plate is illustrated in Figure 2.2.4. Each plate included triplicate wells containing PBMC and HIV Gag peptide pools, in addition to the mitogen phytohaemagglutinin as a positive control (PHA; FLUKA), and 0.14% DMSO (Sigma®) in RPMI as the negative control. The quantity of DMSO included in the negative control wells matched the final concentration of DMSO present in the Gag peptide cultures. For detection of HIV Gag pool-specific responses, a volume of 50µl PBMC ($2x10^6$/ml) containing $1x10^5$ cells were plated per well with a volume of 50µl of each of the respective HIV-1 subtype C Gag peptide pools 1 – 5 (final concentration 2µg/ml). For detection of background IFN-γ production, negative control wells contained a volume of 50ul of PBMC ($2x10^6$/ml) with 50ul 0.14% DMSO (Sigma®) in R10. As a positive control, a volume of 50ul of the mitogen PHA (FLUKA) in R10 (final concentration 0.008mg/ml) was added to 50ul PBMC ($2x10^6$/ml) per well.

To monitor inter-plate and assay variability, each plate also included quality control wells containing PBMC from a HIV negative donor with well characterized response

frequencies to CEF peptides [Cytomegalovirus (CMV), Epstein Barr Virus (EBV) and influenza virus (Flu) immunodominant peptides]. Each ELISpot plate developed in this study had to fall within 9% CV of the mean CEF response determined for this quality control donor. To do this, each plate included wells with PBMC ($2x10^6$/ml) from this HIV-seronegative donor (with known ELISpot-derived IFN-γ-specific responses to **CEF** peptides) stimulated with either CEF peptides (final concentration 1μg/ml), 0.14% DMSO (Sigma®) in R10, or PHA (FLUKA) (final concentration 0.008mg/ml). CEF peptides are derived from three common human viral pathogens. The immunodominant epitopes making up the pool are restricted by 11 common HLA class I molecules (Currier *et al.*, 2002). It is therefore likely that a large proportion of the population would generate memory responses to this peptide pool. Lyopholised CEF peptides were kindly donated by Dr Clive Gray (NICD). The peptides were reconstituted to 5ug/ml in 100ul DMSO (Sigma®) to produce a stock, and were diluted further 1:125 in DMSO (Sigma®) for a working stock of 40ug/ml. The CEF peptides were used at a final concentration of 1ug/ml in the ELISpot assays.

To identify a suitable CEF-responsive quality control donor for this study, PBMCs were isolated from ten buffy packs obtained from the Western Province Blood Transfusion Service (Pinelands, Cape Town, South Africa) and were screened for responses to CEF peptides by the IFN-γ ELISpot assay. Each donor's PBMC was plated at $1x10^5$ cells/well with CEF peptide (final concentration 1ug/ml) in replicates of 20 to determine the standard CEF response frequency. The donor with the highest mean CEF response frequency (out of the 10 donors investigated) was selected for the quality control experiments. To ensure that inter plate variations were not contributing to the observed difference in magnitude measured in the 35 donors, each of the IFN-γ ELISpot plates developed to determine HIV-specific responses in the 35 donors also included quality control wells with PBMC from a donor with a well-characterised CEF-specific IFN-γ response and CEF peptides. For a plate to be considered for inclusion in the study, the CEF response frequency on the QC sample had to be 2157 SFU/10^6 PBMC ± 9% CV (range 1963.6 – 2350.4 SFU/10^6 PBMC).

Following addition of HIV Gag peptides, cells, positive and negative control reagents to respective wells, the plates were incubated at 37°C with 5% CO_2 for 24 hours to allow for cytokine secretion.

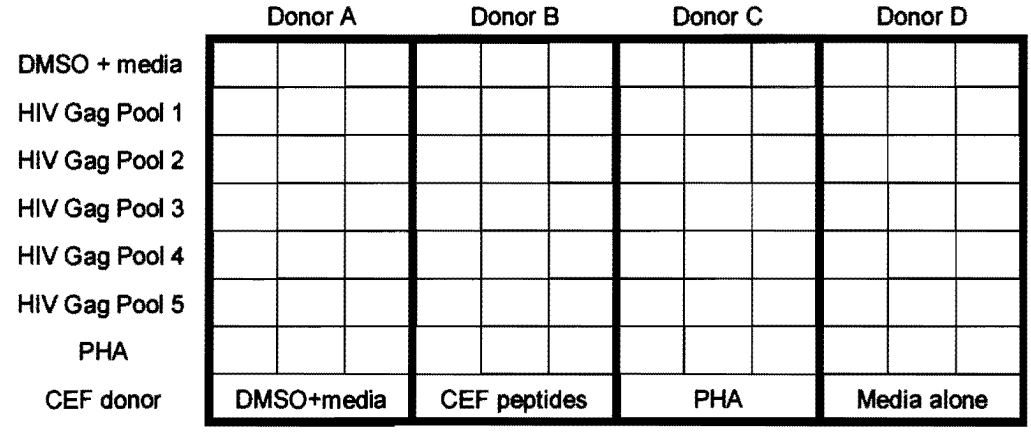

Figure 2.2.4. Representative layout of the IFN-γ ELISpot plates used to investigate HIV Gag peptide-specific T cell response frequencies in chronically HIV-infected women. Each plate included PBMC isolated from 4 donors plated at $1x10^5$/well in triplicate wells containing HIV Gag peptides pools (final concentration 2ug/ml), wells containing 0.14% DMSO in R10 (negative control), and 0.008mg/ml PHA (positive control). Quality control wells contained PBMC from a HIV negative but CEF positive individual and 0.14% DMSO in R10, CEF peptides (final concentration 1ug/ml) and 0.008mg/ml PHA were included on each plate. Three wells containing media alone were included to determine background IFN-γ production in the media.

After 24 hours of incubation in the presence of peptide antigens and PHA, incubation was halted and cells removed by six washes with 200µl/well PBS-Tween [10mM PBS pH 7.4; 0.05% Tween 20 (Sigma ®) dissolved in 1l RO water] using an EL_x50 Auto Strip Washer (Bio-Tek Instruments, Inc.). Biotinylated IFN-γ-detection monoclonal antibody 7-B6-1 (1ug/ml; Mabtech) in 10% FCS (Delta Bioproducts) in PBS (Gibco™) was added to the plates at a volume of 50µl/well. The plates were then kept at room temperature in the dark for 2 hours. The plates were washed six times with PBS-Tween using an EL_x50 Auto Strip Washer (Bio-Tek Instruments, Inc.) to remove excess antibody. A volume of 100µl/well of Streptavidin-HRP (BD Pharmingen™) diluted 1:500 in 10% FCS (Delta Bioproducts) in PBS (Gibco™) was added and the plates were kept at room temperature for 1 hour.

Finally, the plates were washed six times with PBS-Tween using an EL_x50 Auto Strip Washer (Bio-Tek Instruments, Inc.) and 100µl/well Nova Red™ substrate (Vector®) was added. The reaction proceeded for 6 minutes at room temperature in the dark. The plates were rinsed with cold tap water to stop the reaction.

The plates were air-dried in the dark overnight and the resultant spots (spot forming units; SFU) were counted with an ImmunoSpot® Series 3B Analyzer (Cellular Technology Ltd.) using ImmunoSpot® Version 3 software. Background IFN-γ production was assessed in wells containing the PBMC in R10 but with no stimulus.

This was subtracted from the SFU detected in experimental Gag peptide-containing wells and the difference was normalised to SFU/10^6 PBMC plated as per the formula:

$$\text{Net SFU} = \frac{A - B}{1} \times \frac{10^6}{\text{number of cells plated}}$$

where A is the average number of spots in the experimental wells, and B is the average number of spots in the negative control wells.

2.2.8. Statistical analysis

Medians were compared using the nonparametric Mann-Whitney test. Correlations between variables were determined using the nonparametric Spearman rank correlation test. Associations between variables in different groups were determined by two-factor ANOVA test. P-values less than 0.05 were considered significant. Data analysis was conducted using Microsoft® Excel Analysis ToolPak and Prism (version 2.0b, GraphPad Software, San Diego, CA) statistical software.

Chapter 2

Characterisation of peripheral blood HIV-specific responses to HIV Gag by IFN-γ ELISpot in chronically HIV-infected women

2.3.

Results

2.3.1. Clinical details of HIV-infected women included in the study

Thirty five chronically HIV-infected treatment naïve women were included in this study. Table 2.3.1 describes the clinical characteristics of these 35 women. The cohort had a mean $CD4^+$ T cell count of 498.2 ± 199.9 cells/µl and a mean viral load of 62366.31 ± 228657.5 copies/ml. Their average age was 30.3 ± 6 years. Seven of the 35 (20.0%) women had CD4 counts >600 cell/ µl. All of these HIV positive women were enrolled in 2002 in a longitudinal study with 6 monthly follow-up. All women had been confirmed HIV positive for at least 2 years with a mean time of follow up since enrolment being 26 months (± 2 months). No correlation was observed between plasma viral load and $CD4^+$ T cell count (Spearman r = -0.1186, p=0.5180) (Figure 2.3.1).

Table 2.3.1. Clinical description of the 35 chronically HIV-infected women included in this study

Donor ID	Age[a]	Number of $CD4^+$ T cells[a] (Cells/ul)	Amount of HIV-1 RNA (Copies/ml)	Month [b] In Study
G014	31	387	68	32
NY038	31	368	3760	30
NY040	26	414	9500	30
NY055	35	335	9100	29
NY062	26	312	ND	25
NY064	22	371	1100	25
NY078	39	327	11778	24
NY079	23	341	14500	24
NY094	34	383	468	31
NY099	21	468	25318	24
NY100	30	545	15800	24
NY106	33	1053	ND	25
NY107	31	393	41700	25
NY110	36	424	5200	24
NY130	23	322	39811	31
NY138	27	607	4013	27
NY139	33	407	27250	24
NY150	31	572	3500	24
NY155	33	451	30000	24
NY157	43	467	88000	25
NY162	32	461	7100	25
NY165	25	363	9942	24
NY167	27	639	29562	24
NY172	32	411	4000	25
NY194	29	466	1818	24
NY199	36	641	ND	24
NY229	28	698	1800	26
NY230	26	1206	3400	24
NY233	45	573	10987	26
NY237	30	572	3485	25
NY240	23	339	47762	25
NY263	32	400	1300000	25
NY284	37	533	190000	24
NY299	20	834	28000	24
NY302	27	354	27000	24
Mean ± SD	30.3 ± 6	498.2 ± 199.9	62366.31 ± 228657.5	26 ± 2.0

[a] The ages and CD4 counts of the women were determined at the visit from which PBMC was isolated for IFN-γ ELISpot screenings. The tabulated CD4 counts shown correspond with the patients' most recent viral load measurement at the time of PBMC isolation for IFN-γ ELISpot screenings. [b]The time from enrolment into the study until blood sampling for the ELISpot assay.

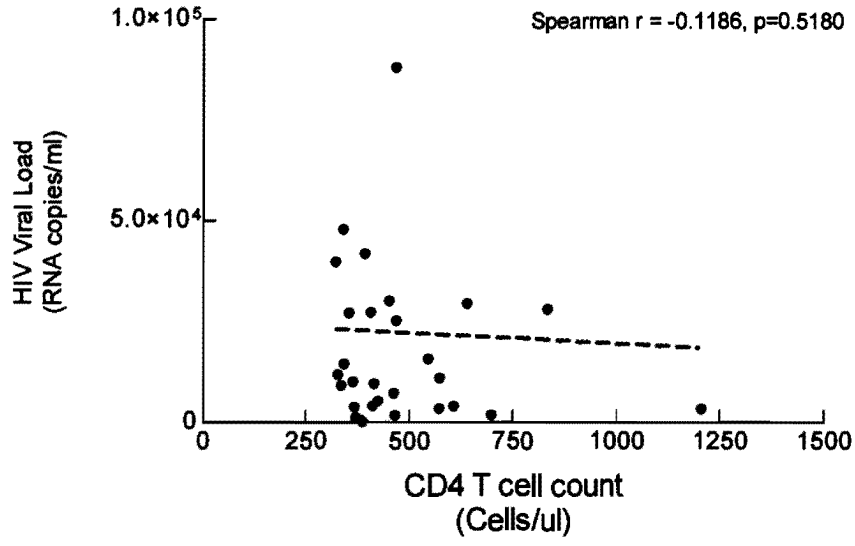

Figure 2.3.1 Correlation between plasma viral load and CD4 T cell count in chronically HIV-infected women. Each data point represents the matched coordinates to CD4 count and plasma viral load in each participant. R and P-values were calculated using Spearman Ranks test for correlation. The goodness of fit (F) and P-value is also shown for the linear regression model.

2.3.2. HIV-specific IFN-γ responses in HIV-negative individuals

In order to establish a cut-off for positive responses and to confirm the specificity of the IFN-γ ELISpot assays, PBMC from ten HIV-seronegative donors (obtained from the Western Province Blood Transfusion Service, Pinelands, Cape Town, South Africa) were stimulated with HIV Gag peptide pools and assessed by IFN-γ ELISpot (Figure 2.3.2). Assuming no previous exposure to HIV, the level of response to HIV peptides in these donors was taken as a reliable measure of background reactivity to these peptide pools in this assay. The assay was performed in triplicate and included positive control wells containing the mitogen PHA and negative control wells containing no antigen in 14% DMSO (Sigma®)in R10 (Figure 2.3.2). The average cumulative HIV Gag pool-specific response observed in the seronegative group was 50.8 ± 2.5 SFU/10^6 PBMC. The cut-off for considering an HIV Gag-response as positive in the HIV-infected cohort was therefore determined to be twice the average number of spot-forming units (SFU) per HIV Gag pool detected in these HIV seronegative donors or 100 SFU/10^6 PBMC.

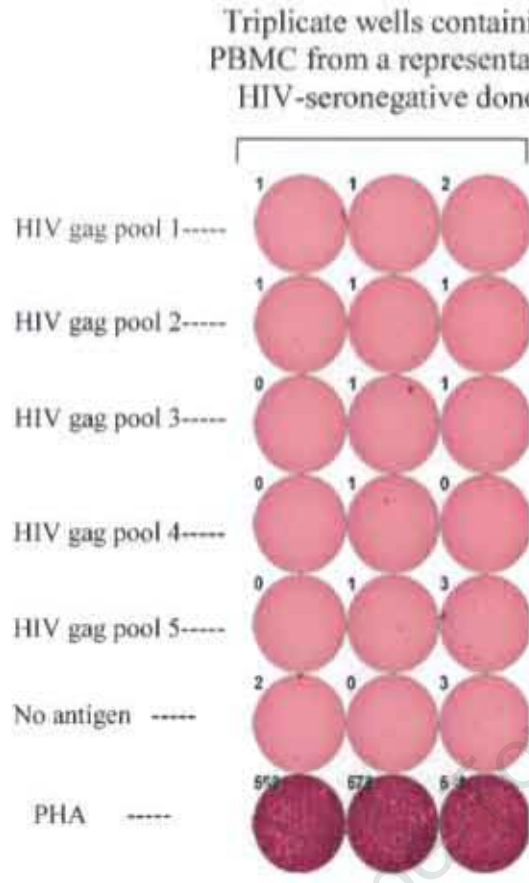

Figure 2.3.2. Representative IFN-γ ELISPOT for an HIV-seronegative individual used to calculate cut-off for positive HIV-specific IFN-γ responses. The figure shows a representative ELISpot plate of one HIV-seronegative donor to the 5 Gag peptide pools, the positive PHA control and negative control. The average number of SFU/10^6 PBMC plated was calculated per pool and then added to determine the cumulative response to HIV Gag by the HIV-seronegative donors. Each spot represents an individual IFN-γ secreting cell in each well. Numbers in the left hand corner of each well represent the number of spots counted per well. Spots were counted using an automated counter [ImmunoSpot® Series 3B Analyzer (Cellular Technology Ltd.]

2.3.3. Evaluation of inter-assay and inter-plate variation by inclusion of quality control samples responsive to CEF peptides

Because the IFN-γ ELISpot assessments were not all done on a single plate or a single day, there was a need to monitor inter-plate and experiment variations. To do this, each IFN-γ ELISpot plate developed (n=10) included triplicate quality control wells with PBMC from an HIV-seronegative donor with a well characterized IFN-γ ELISpot response to CEF peptides. Initially, 10 HIV seronegative donors were screened for response magnitudes to CEF peptides using IFN-γ ELISpot (data not shown). The HIV seronegative donor with the highest IFN-γ response was selected for inclusion in all subsequent ELISpot experiments as the quality control sample. From 20 replicates, the

average CEF-specific response magnitude for the HIV seronegative donor selected was determined to be 2157 ± 193.5 SFU/10^6 PBMC. All subsequent plates developed with this quality control sample included had to fall within this pre-determined 2157 SFU/10^6 PBMC ± 9% CV (range 1963.6 – 2350.4 SFU/10^6 PBMC). For determination of HIV-specific responses in the 35 chronically HIV-infected women, 10 independent IFN-γ ELISpot plates were used. The quality control values from all of the 10 plates evaluated fell within this pre-determined range indicating that the results from individual experiments were comparable with one another (Figure 2.3.3.). The mean determined among all the quality control samples during IFN-γ ELISpot screening of the 35 HIV-infected women was 2312 ± 145.3 SFU/10^6 PBMC (range 1977 – 2347 SFU/10^6 PBMC; Figure 2.3.3.). No significant difference in the median responses was observed between the experimental and previously-determined standard quality control plates (p = 0.6129, Mann-Whitney test; Figure 2.3.3.).

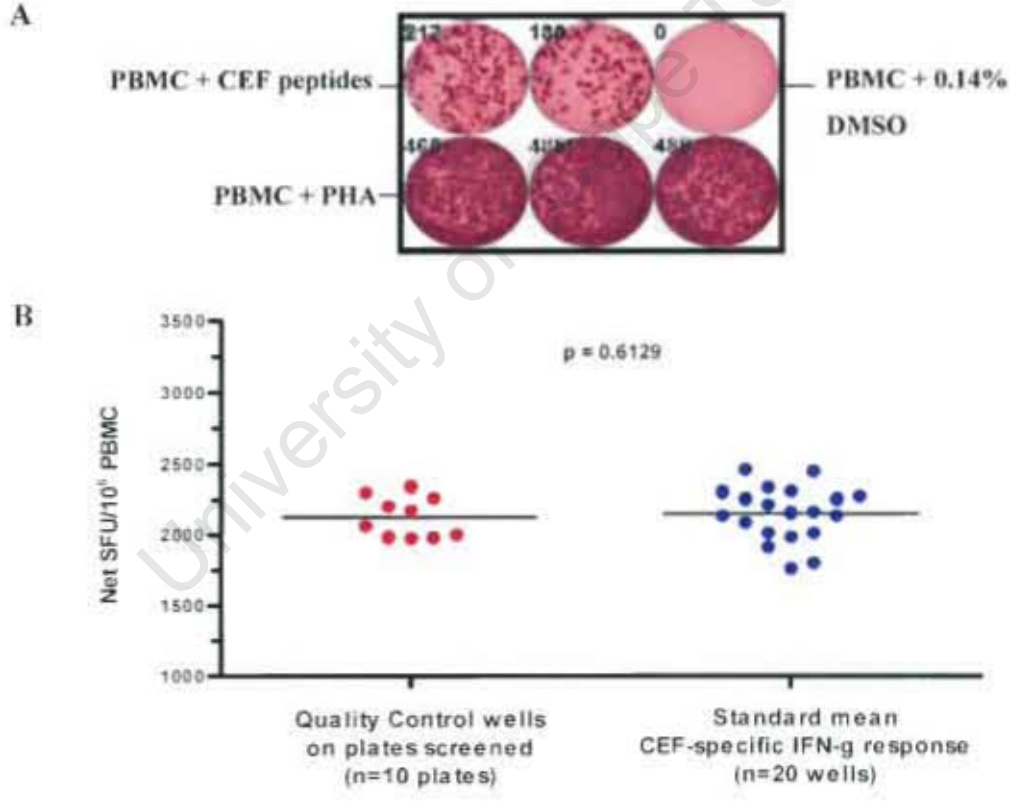

Figure 2.3.3. Variability among the CEF peptide quality control wells per plate. Each plate included triplicate wells of PBMC and CEF peptide (final concentration of 1μg/ml), PBMC and 0.14% DMSO in media, and PBMC and 0.008mg/ml PHA. **(A)** Representative example of the CEF-containing, PHA-containing and media-containing wells. **(B)** No difference was observed in the median CEF-specific IFN-γ responses in the 20 replicates of the CEF QC donor (blue dots) and the 10 previously-determined standard (red dots) quality control plates (p = 0.6129; Mann-Whitney test).

2.3.4. Characterisation of HIV Gag peptide pool-specific IFN-γ responses in 35 chronically HIV-infected women

The frequency of HIV Gag-specific T cells in blood from each of the 35 chronically HIV-infected women included in this study was determined by IFN-γ ELISpot (Figure 2.3.4). Only responses above a cut-off of 100 SFU/10^6 PBMC were considered as positive. The net cumulative HIV Gag-specific IFN-γ responses in the cohort ranged from undetectable to 10371 SFU/10^6 PBMC (Figure 2.3.4), with a mean net cumulative response of 3155 ± 2387 SFU/10^6 PBMC for all 35 HIV-infected women (Figure 2.3.4). Eight of the 35 women (22%) had responses >5000 SFU/10^6 PBMC, 20/35 women (57%) had responses <5000 but >1000 SFU/10^6 PBMC, and 7/35 (20%) had responses <1000 SFU/10^6 PBMC.

The highest magnitudes of responses in this HIV-infected cohort were directed against HIV Gag pools 2 (mean 1268 ± 1575 SFU/10^6 PBMC) and 3 (mean 813 ± 1159 SFU/10^6 PBMC), and to a lesser extent towards pools 1 (mean 473 ± 623 SFU/10^6 PBMC), 4 (mean 408 ± 832 SFU/10^6 PBMC) and 5 (mean 191.42 ± 442 SFU/10^6 PBMC) (Figure 2.3.5). This confirms that the HIV-specific responses detected in this study were directed predominantly towards the p24 region of Gag which has previously been shown to be one of the more conserved regions of HIV Gag (Buseyne *et al.*, 1992; Kiepiela *et al.*, 2007).

Only 4/35 (11%) of the women responded to all 5 HIV Gag pools (Table 2.3.3). The majority of women responded to only 2 pools (13/35, 37%). In these women, the 2 Gag pool-specific responses were more likely to be directed towards HIV Gag pools 3 (69% of the women) and 2 (61% of the women).

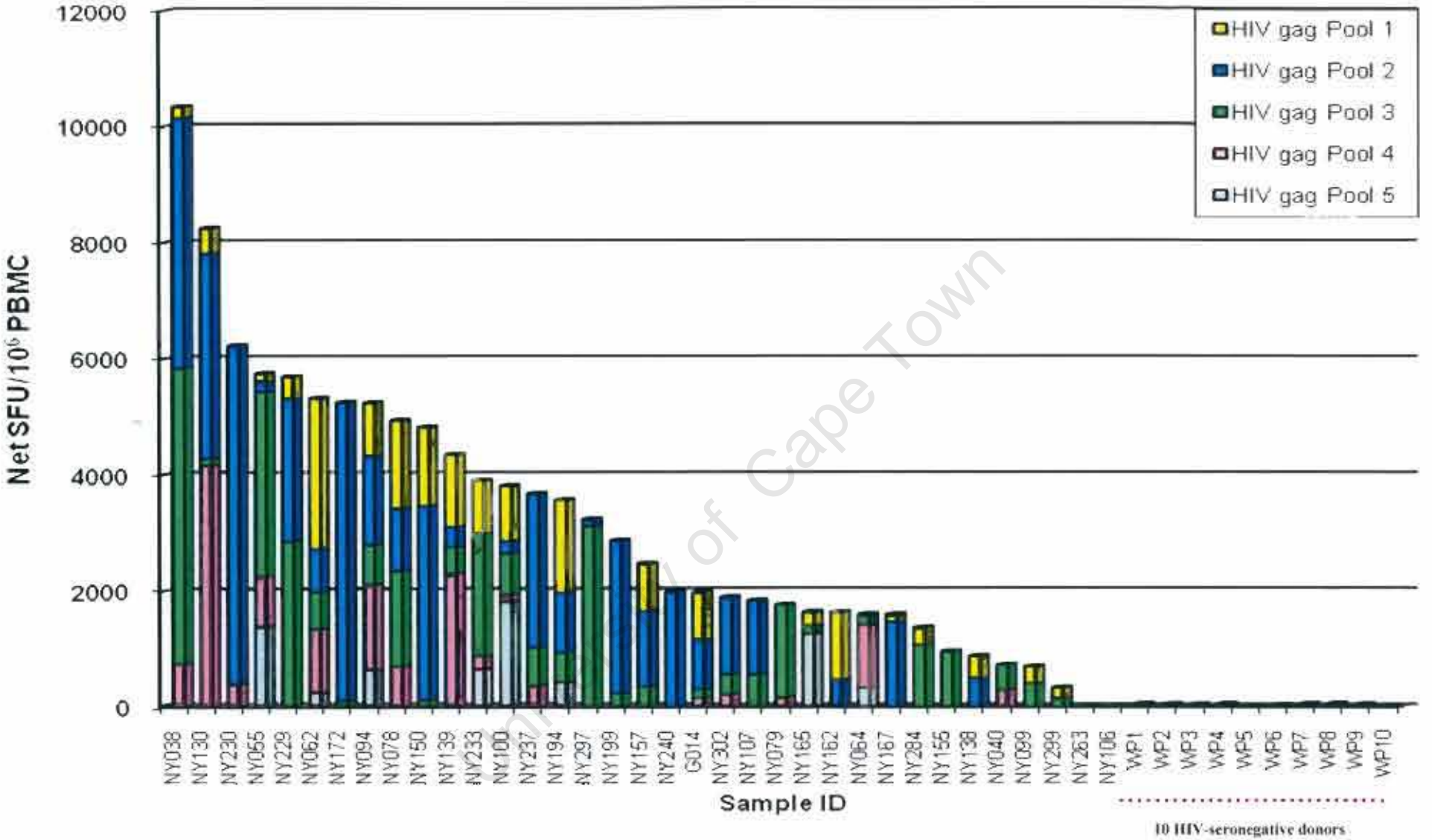

Figure 2.3.4. Cumulative HIV-1 Gag-specific IFN-γ responses in the 35 chronically HIV-infected women included in this study. Thirty-five HIV-infected women were screened by IFN-γ ELIspot to determine their response magnitudes to overlapping peptides spanning HIV-1 subtype C Du422 Gag. Only cumulative responses above 100 SFU/10^6 PBMC were considered positive. The HIV Gag pool-specific responses of the 10 HIV-seronegative donors are included on the graph to further emphasise the stringency of the cut-off value. [▪] represents responses against Gag Pool 1, [▪] Gag Pool 2, [▪] Gag Pool 3, [▪] Gag Pool 4, and [▪] Gag Pool 5.

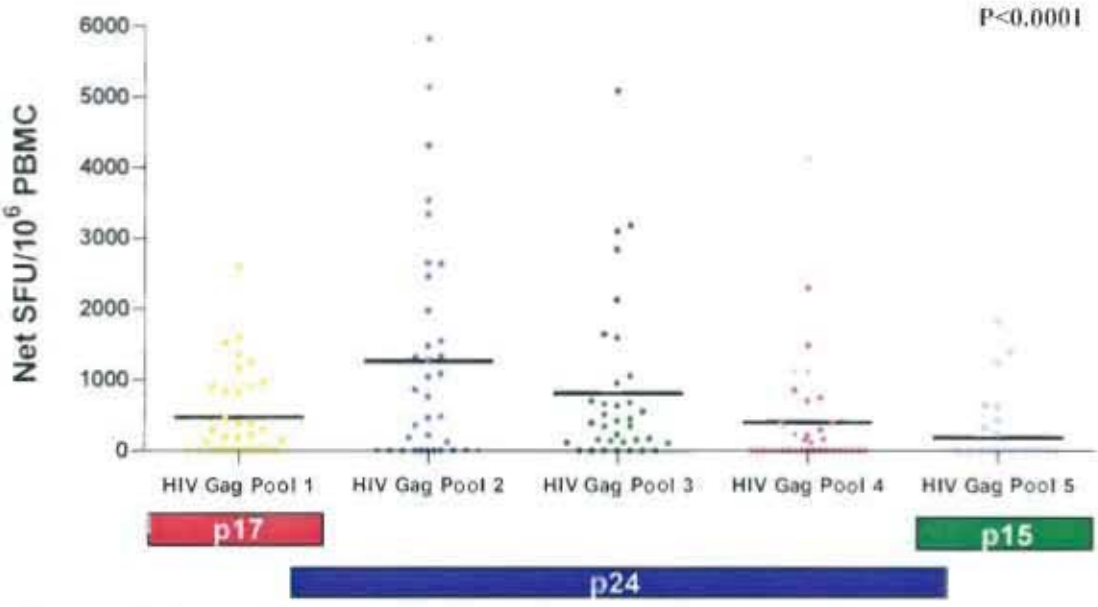

Figure 2.3.5. Breadth and magnitude of HIV Gag pool-specific IFN-γ responses in the 35 HIV-infected women. Each data point represents an individual donor's response to each Gag Pool, with the median response for each Gag pool represented by a black line. The IFN-γ responses have been expressed as net SFU/10^6 PBMC indicating that the background response for each donor has been subtracted. The solid bars (**red** for p17, **blue** for p24 and **green** for p15) under the graph represent the general Gag regions correlating with the individual peptide pools. A significant difference was observed between the respective median HIV-specific responses directed to the HIV Gag pools ($P < 0.0001$; single factor ANOVA test).

Table 2.3.2. The breadth and frequency of HIV Gag pools recognised by the 35 HIV-infected women

Number of pools recognised	Number of women	Gag Pools recognised									
		Pool 1		Pool 2		Pool 3		Pool 4		Pool 5	
		Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%
0 pools	2	0/2	0.00%	0/2	0.00%	0/2	0.00%	0/2	0.00%	0/2	0.00%
1 pool	2	0/2	0.00%	1/2	33.33%	1/2	33.33%	0/2	0.00%	0/2	0.00%
2 pools	13	6/13	46.15%	8/13	61.53%	9/13	69.23%	3/13	23.07%	0/13	0.00%
3 pools	7	4/7	57.14%	5/7	71.427%	7/7	100.00%	3/7	42.85%	2/7	28.57%
4 pools	7	6/7	85.71%	6/7	85.71%	7/7	100.00%	7/7	100.00%	2/7	28.57%
5 pools	4	4/4	100.00%	4/4	100.00%	4/4	100.00%	4/4	100.00%	4/4	100.00%
Sum	35	20/35	57.14%	24/35	68.57%	28/35	80.00%	17/35	48.57%	8/35	22.85%

Although most of the women recognised pool 3 (28/35; 80%; Table 2.3.2), the average specific IFN-γ response magnitude was lower (813 ± 1159 SFU/10^6 PBMC) than that directed towards pool 2 (1268 ± 1575 SFU/10^6 PBMC; Figure 2.3.5). Furthermore, although similar numbers of women recognised pools 1 and 2 (21/35 for pool 1 compared to 24/35 for pool 2; Table 2.3.2.), the average specific IFN-γ response was of higher magnitude in pool 2 than in pool 1 (1268 ± 1575 SFU/10^6 PBMC for pool 2 compared with 473 ± 623 SFU/10^6 PBMC for pool 1). In addition, HIV Gag pools 4 and 5 are more likely to be recognised in women recognising more pools (Table 2.3.2).

No correlation was observed between the cumulative magnitude of the HIV Gag-specific responses and plasma viral load (Spearman r = -0.3006, p = 0.1066) or $CD4^+$ T cell count (Spearman r = -0.2018, p = 0.2849) in this small cohort of chronically HIV-infected women (Figure 2.3.6). No association was observed between the number of pools targeted and viral load (Spearman r = -0.2453; p = 0.1833; data not shown).

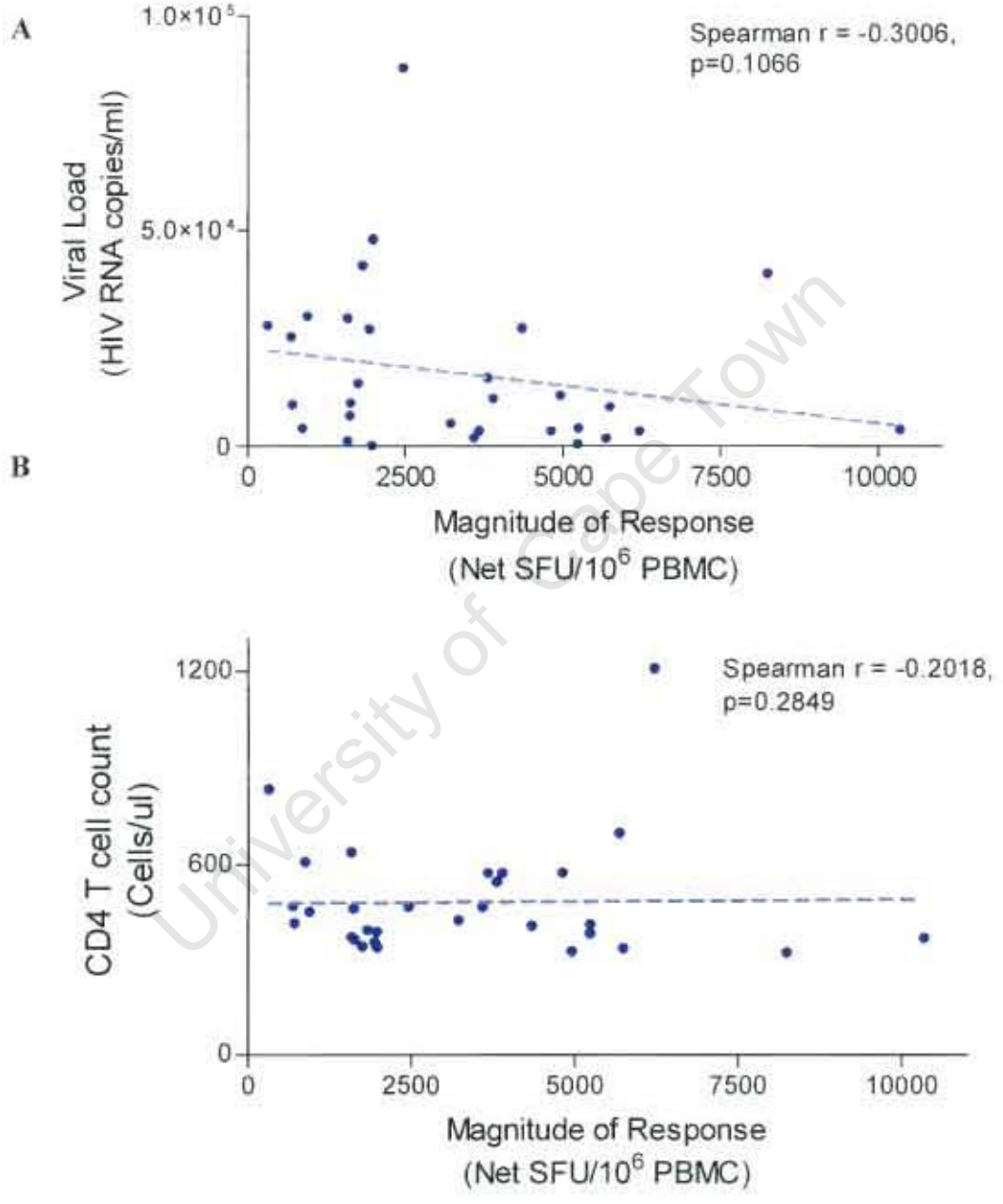

Figure 2.3.6. Relationship between the magnitude of HIV Gag-specific IFN-γ responses, plasma viral load and CD4 T cell count in chronically HIV-infected women. IFN-γ-specific response (measured by ELISpot) to overlapping peptides spanning HIV-1 subtype C Gag were compared with paired viral loads (**A**), and CD4 T cell counts (**B**) in these 35 women. Each square data point represents an individual patient's response to Gag versus viral load (**A**) or CD4 count (**B**). The solid line represents the linear regression curve for each correlation. Spearman rank test was used to determine the significance of the correlation and r and P-values for each comparison is shown on the graph.

2.3.5. Selection of HIV-infected women with the highest frequencies of HIV Gag-specific T cells in blood for T cell cloning experiments

To increase the probability of cloning HIV Gag-specific T cells by limiting dilution, 9 donors with the highest net cumulative response to HIV Gag were selected for T cell expansion experiments. The median HIV-specific IFN-γ response of the 9 selected individuals was 5399 SFU/10^6 PBMC (range 3813-8267 SFU/10^6 PBMC). This was significantly greater than the median of 1931 SFU/10^6 PBMC (range 63 – 1037 SFU/10^6 PBMC) observed in the remaining 26/35 HIV-infected women ($p<0.0008$; Mann-Whitney test; Figure 2.3.7). However, no difference was observed between the median viral loads ($p = 0.1938$) or CD4 T cell counts ($p = 0.5972$) between the nine women selected for T cell cloning and the remaining 26 HIV-infected women (Figure 2.3.7). The selected 9 women had a mean CD4 T cell count of 551.9 ± 275.1 cells/ul and a mean viral load of 17895 ± 28926 RNA copies/ml (Table 2.3.3).

Table 2.3.3. Net cumulative HIV Gag-specific responses of the 9 HIV-infected women selected for T cell cloning experiments

ID	Count (Cells/ml)	(RNA copies/ml)	Response	Pool1	Pool2	Pool 3	Pool 4	Pool 5
NY157	467	88000	3813.32	960	216.66	703.33	110	1823.33
NY130	322	39811	8267	437	3540	110	4160	20
NY233	573	10987	3900	910	0	2127	223	640
NY055	335	9100	5747	143	180	3187	850	1387
NY172	411	4000	5399	63	5140	103	93	0
NY237	572	3485	3816	50	2653	660	360	93
NY230	1206	3400	6264	17	5833	27	387	0
NY229	698	1800	5855	383	2463	2843	83	83
NY094	383	468	5237	913	1543	677	1477	627
Mean	551.89	17894.56	5366.48	430.67	2396.52	1159.70	860.33	519.26
±Std Dev	275.12	28925.91	1439.91	399.82	2146.23	1228.39	1318.37	674.56

* The mean ± standard deviation of the triplicate wells was calculated for each pool-specific response. The mean background was subtracted from the mean of the experimental wells and the difference was multiplied by ten to normalise the value to SFU/10^6 PBMC plated.

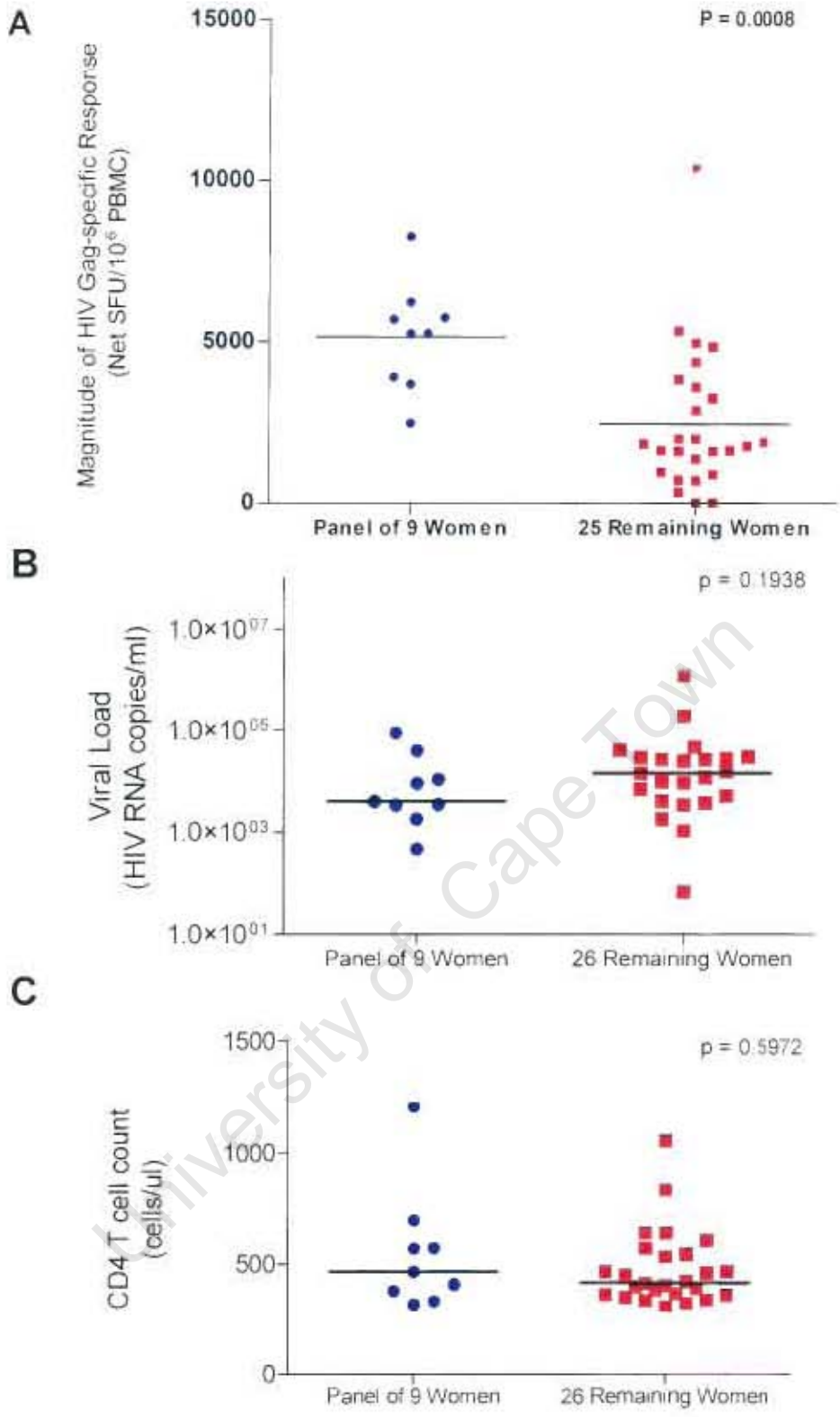

Figure 2.3.7. Comparison of the HIV Gag-specific IFN-γ responses, viral load and CD4 T cell counts between the 9 women selected for T cell expansion experiments and the remaining 26 HIV-infected women in the cohort. Nine women with the greatest net HIV Gag-specific IFN-γ responses were selected for T cell expansion experiments. (**A**) The median HIV Gag-specific response observed in the 9 women selected for T cell expansion experiments was significantly greater than that observed in the remaining women ($p < 0.0008$). (**B**) No difference was observed between the medians of the viral loads ($p = 0.1938$) or (**C**) CD4 T cell counts ($p = 0.5972$) between the two groups. Mann-Whitney U tests were applied to compare these non-parametric variables. Blue dots indicate women with the highest HIV Gag-specific responses while red dots indicate the women not selected for T cell expansion experiments.

HIV-specific IFN-γ responses in these 9 women were predominantly directed towards Gag pools 2, 3 and 4, the pools spanning the highly conserved gene for HIV Gag p24 (Figure 2.2.3). Responses to pools 2 and 4 were significantly higher in these 9 women than in the remaining 26/35 women (Figure 2.3.8.A). Despite the significant difference between the cumulative magnitudes of HIV Gag-specific responses between the selected group of 9 women and the remaining women in the cohort ($p < 0.0008$) (Figure 2.3.7), no significant difference was observed in the breadth of the HIV Gag pool-specific responses between the two groups ($p<0.3678$; Figure 2.3.8B).

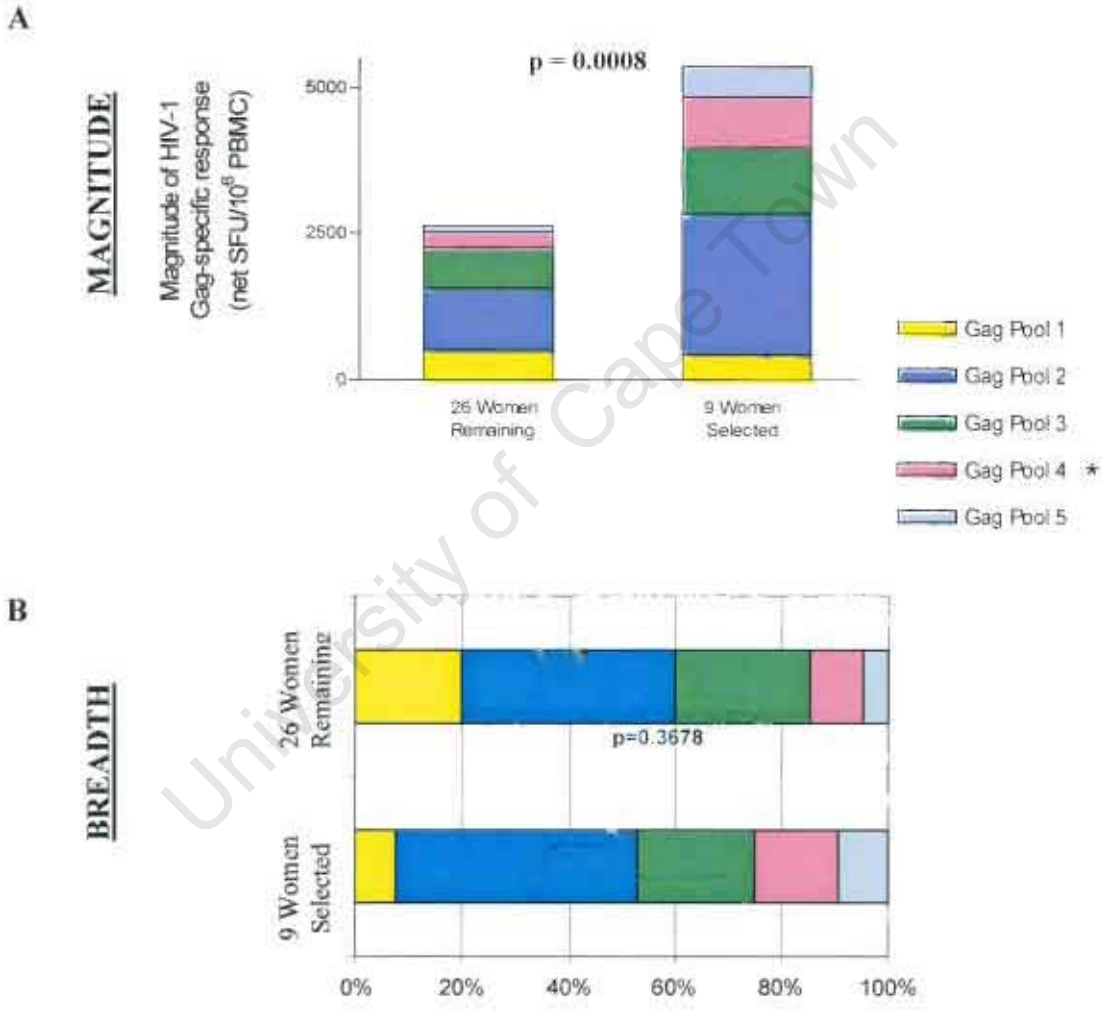

Figure 2.3.8. Comparison of the breadths and magnitudes of IFN-γ responses directed towards individual HIV Gag peptide pools in the 9 women selected for T cell expansion experiments and the remaining 26 women in the cohort. The magnitude (**A**), but not the breadth (**B**) of the HIV-1 Gag-specific response was significantly different between the two groups of women. P values were determined by the Mann-Whitney nonparametric t-test (**A**) and two-factor ANOVA (**B**).

2.3.6. Assessment of variation of breadth and magnitude of HIV-specific responses between 6 monthly visits

The IFN-γ ELISpot assay was used to identify HIV-infected women with high frequencies of HIV-specific T cells in blood to increase the probability of success in limiting dilution studies where HIV-specific T cells from the blood and cervix were subjected to *in vitro* expansion (Chapter 3). Selection of high frequency responders and cloning of HIV-specific T cells have been performed at 2 consecutive 6 month visits for each participant. To determine whether the observed frequency of HIV-specific T cells in blood would be maintained at the subsequent visit, HIV Gag-specific PBMC responses were determined at two consecutive visits in 4 donors (Figure 2.3.9). No significant difference was observed between the mean magnitudes of the HIV-specific responses detected at each visit ($p = 0.8410$; two-factor ANOVA; Figure 2.3.9 A). Two of the four donors had similar magnitudes over the 6-month period. One of the four had a 2.7-fold increase in magnitude of HIV-specific response, and another had a 2.6-fold decrease in magnitude. Despite variation in the overall magnitude of responses to the various pools in these donors, the breadth of the responses during the 6-months was comparable in 3/4 donors. Only 1/4 donors, NY107, had a significant difference in HIV Gag pool-specific response by two-factor ANOVA ($p = 0.0043$). This donor exhibited an expanded response to pool 3, a new response to pool 1, and a reduced response to pool 2. Although there seems to be variation in magnitude over time, the breadth of the response at one point can give an indication of the response at another time point.

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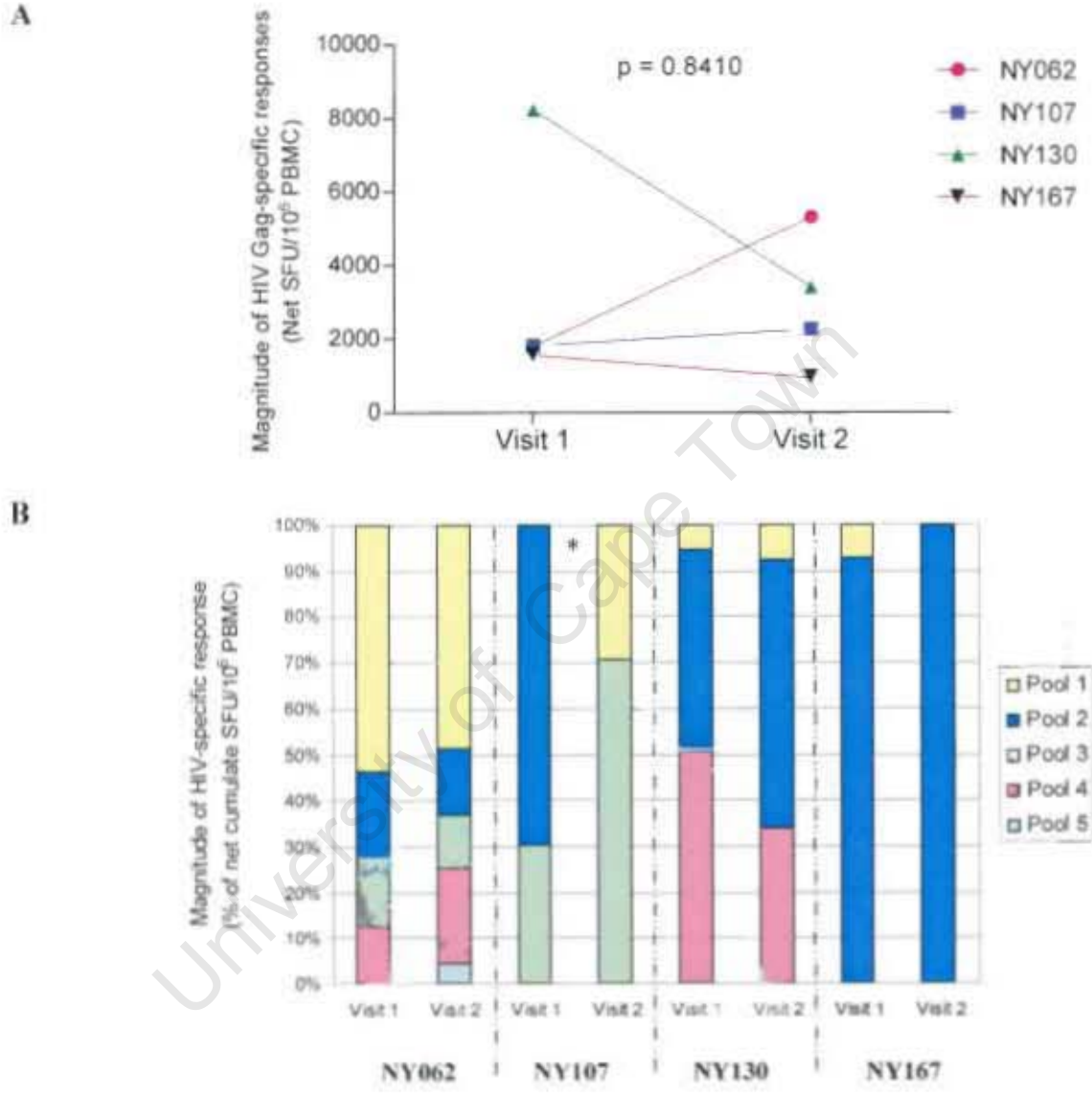

Figure 2.3.9. HIV Gag pool-specific IFN-γ responses of 4 HIV-infected women at two consecutive 6-monthly visits. The magnitude (**A**) and breadth (**B**) of HIV Gag pool-specific IFN-γ responses of 4 women at two consecutive visits were compared using two-factor ANOVA. An asterisk (*) is indicative of P-values <0.001.

Chapter 2

Characterisation of peripheral blood HIV-specific responses to HIV Gag by IFN-γ ELISpot in chronically HIV-infected women

2.4.

Discussion

2.4. Discussion

In this study, 35 chronically HIV-infected women were assessed for HIV-1 subtype C Gag-specific IFN-γ responses by ELISpot. Only 2/35 women screened did not respond to HIV-1 subtype C Gag peptides. Concordant with several other reports, CTL responses were directed predominantly towards the p24 region of HIV Gag (Buseyne *et al.*, 1992; Kiepiela *et al.*, 2007; Masemola *et al.*, 2004; Altfeld *et al.*, 2003). No association was observed between the magnitude of the HIV-1 Gag-specific response of these HIV-infected women and their plasma viral load or $CD4^+$ T cell count. The impact of HIV-specific CTL activity on viral load is highly contentious with a number of conflicting reports (Kiepiela *et al.*, 2007; Masemola *et al.*, 2004; Novitsky *et al.*, 2003; Addo *et al.*, 2003; Migueles *et al.*, 2001; Edwards *et al.*, 2002). While earlier studies have shown no association between CTL responses and viral load (Addo *et al.*, 2003; Migueles *et al.*, 2001; Edwards *et al.*, 2002), others have clearly shown that HIV-specific T cell responses against some regions of the genome have significant impact on reducing viremia (Kiepiela *et al.*, 2007; Masemola *et al.*, 2004; Novitsky *et al.*, 2003). T cells targeting Gag in particular have been implicated in better disease outcome (Kiepiela *et al.*, 2007). The association between T cell responses and reduced viremia was only noted when the cohort exceeded 400 individuals (Kiepiela *et al.*, 2007) indicating that cohort size may have been a restricting factor in the earlier studies. The nature of this association and the factors affecting it remains unknown.

This study has focused exclusively on T cell responses to HIV-1 subtype C Gag peptides since HIV subtype C is the predominant strain of HIV found in Southern Africa (van Harmelen *et al.*, 2003; Novitsky *et al.*, 2002) and several studies have confirmed that Gag is the most common target of HIV-specific responses (Addo *et al.*, 2003; Kiepiela *et al.*, 2003; Masemola *et al.*, 2004). HIV-infected PBMC were screened for IFN-γ responses to pools of peptides spanning the entire HIV-1 subtype C Gag protein. Recent comparisons of the use of consensus strains versus autologous virus have revealed that use of consensus strains underestimates responses directed against variable regions of viral reference sequences compared to autologous sequences such that up to 28% of responses will not be detected using consensus strains (Draenert *et al.*, 2003; Altfeld *et al.*, 2003). Furthermore, as much as 33% of these missed responses can be located in the conserved p24 Gag region (Altfeld *et al.*, 2003). Despite these findings, synthesis of autologous HIV peptides is cost prohibitive for studies of even moderate cohort sizes and would not have been relevant or feasible for the present study. Since the purpose of this study was to identify a panel of

HIV-infected women with high frequencies of HIV Gag-specific T cell responses, the use of the peptides based on the HIV subtype C Gag Du422 strain was adequate to determine the magnitude of responses.

In this study, HIV-specific T cell responses were most frequently directed towards the p24 region (pools 2 and 3) of HIV Gag and this is concordant with previous reports (Buseyne *et al.*, 1992; Masemola *et al.*, 2002). Furthermore, the women in the cohort were more likely to respond to only 2 of the 5 HIV Gag pools, with the targeting of p15 (pools 4 and 5) increasing in frequency with increasing number of pools targeted. In this study, no association was observed between number of pools targeted and viral load as previously reported (Masemola *et al.*, 2004).

Of the 35 HIV-infected women screened by IFN-γ ELISpot, 9 women with the highest HIV-specific IFN-γ responses in blood were selected for T cell cloning experiments (Chapter 3). A significantly higher magnitude of HIV-specific T cell cumulative response was observed in this group compared to the remaining 26/35 women in the cohort, with most significant increases being observed in the p24 region of HIV Gag.

Because I proposed to use magnitude assessment at one visit to predict high responders at the next 6 monthly visits, it was of interest to assess the maintenance of frequency of HIV-specific T cell responses in blood between visits. IFN-γ ELISpot screens performed on four HIV-infected women at two consecutive visits confirmed no significant difference between the magnitudes of responses between the two time points although clear fluctuations were noted.

The robust and sensitive IFN-γ ELISpot assays have widely been used for screening large numbers of samples for HIV-specific T cell responses and are particularly useful in providing information on the immunogenicity of vaccines. However, recent evidence has highlighted the importance of polyfunctional $CD8^+$ T cell responses (the simultaneous expression of IFN-γ, MIP-1-β, TNF-α, IL-2 and degranulation marker CD107a) in suppressing HIV replication (Betts *et al.*, 2006). Because it allows assessment of only one T cell function, the IFN-γ ELISpot assay may not be equipped to determine the functional diversity associated with effective immune control. For the purpose of this study, the measurement of IFN-γ-secretion by IFN-γ ELISpot was sufficient to meet the aim of determining the frequency of HIV-specific T cells in the blood of HIV-infected women.

In summary, this chapter has shown that T cell IFN-γ responses against Gag were common in chronically HIV-infected women with 33/35 (94.3 %) women having clear specificity against the *gag* gene product. These responses were focused predominantly against the capsid protein p24 which is recognized to be the most conserved. Although this is a relatively small study, I found no correlation between T cell IFN-γ responses to Gag and either CD4 count or viral load. From this analysis, a panel of 9 chronically HIV-infected women were identified with high frequencies of HIV-1 subtype C Gag-specific T cells for further studies comparing responses in blood with those detectable at the cervix (Chapter 3).

Chapter 3

Generation and characterization of HIV Gag-specific cervical- and blood-derived T cell clones

Chapter 3

Generation and characterization of HIV Gag-specific cervix- and blood-derived T cell clones

Chapter 3

Generation and characterization of HIV Gag-specific cervix- and blood-derived T cell clones

3.1.

Introduction

3.1. Introduction

Few studies have thoroughly investigated the comparative function of HIV-specific T-cell responses at the genital mucosa with those in blood. One of the primary reasons for this being that sampling of T cells from the genital mucosa generally yields too few T cells to conduct thorough functional analysis. In-depth studies of cytolytic function, epitope specificities, and T cell receptor clonotypes of T cells isolated from the genital mucosa have therefore increasingly employed *in vitro* expansion techniques (Musey *et al.*, 1997, 2003; Ibarrondo *et al.*, 2005). Polyclonal *in vitro* expansion of antigen-specific memory T cells allows the study of T cell subsets that would not, by virtue of their initial frequency *in vivo* and low sampling-associated cell recovery, easily be observed directly *ex vivo* (Musey *et al.*, 1997, 2003; Shacklet *et al.*, 2003). Similarly, T cell cloning by limiting dilution allows the further study of virus-specific cellular interactions at a single cell level (Musey *et al.*, 1997, 2003; Appay *et al.*, 2000) with potential benefits of better understanding of HIV-specific T cell homing patterns, ontogeny, and specific function in samples with low frequencies of a cell subset of interest.

In this study, I investigated the use of T cell cloning by limiting dilution of HIV-specific cells from the genital mucosa. Because this process requires *in vitro* stimulation with cognate antigen which is likely to distort the frequency (and potentially the phenotype) of effector CTL, it provides only an approximation of the *in vivo* conditions under which virus-specific lysis may occur. The polyclonal expansion of CTL is therefore largely a qualitative technique that is useful mostly for the detection of HIV-specific CTL. It can, however, become more quantitative when combined with limiting dilution analysis (LDA; Koup *et al.*, 1991; Carmichael *et al.*, 1993). Poisson transformation of the cloning efficiency can then be applied to determine the CTL effector frequency in the primary sample based on the lowest dilution from which CTL could be detected after *in vitro* stimulation (Taswell, 1981, 1984). Because LDA only measures those virus-specific cell subsets that are able to proliferate under limiting dilution conditions, it has been shown to underestimate the actual effector cell frequency (Gotch *et al.*, 1990). However, polyclonal expansion is particularly useful in its sensitivity as it is able to detect antigen-specific responses to a frequency of 1 in $1x10^5$ cells (Goh *et al.*, 1999).

Due to the low yield of T cells generally obtained from the genital tract, the merits of clonal expansion of T cells from the cervix are increasingly being recognised (Musey *et al.*, 1997, 2003). After polyclonal expansion of CTL, HIV-specific CTL can be cloned by

limiting dilution to allow further studies of HIV-specific CTL responses at an individual cell level. These more in-depth studies are beneficial in characterising memory CTL responses to antigen at the clonal level (Weekes *et al.,* 1998, 1999; Wills *et al.,* 1999) and in understanding T cell homing patterns and ontogeny (Musey *et al.,* 2003).

Various studies have demonstrated that $CD8^+$ T cells are capable of cytokine secretion and cytotoxic lysis but incapable of proliferation in studies of chronic antigenic stimulation such as cytomegalovirus and HIV (Wang and Borysiewicx., 1995; Evans *et al.,* 1999). On first encounter with an antigen, naïve T cells are induced to proliferate and kill infected cells (Topham *et al.,* 1997). Since only certain antigen-specific T cell receptors induce proliferation, the T cell population increases in clonality. Subsequent activation-induced cell death results in the loss of a large number of activated T cells such that only a small pool of memory T cells remain (Ahmed and Gray, 1996; Schmitz *et al.,* 1999). This memory T cell pool will expand more rapidly on re-encounter with the antigen (Ahmed and Gray, 1996). Since T cells have a finite proliferative capacity and eventually become terminally differentiated and senescent, chronic stimulation by antigen such as is the case with HIV infection or cytokine may accelerate replicative senescence (Bestilny *et al.*, 2000; Palmer *et al.*, 1997). Numerous studies have described the expression of CD57 on the cell surface of T cells as a marker of proliferative impairment of T cells (Wang and Borysiewicx., 1995; Weekes *et al.,* 1999) and the expression of CD57 has also been associated with antigen-induced apoptotic death of $CD8^+$ T cells (Brenchley *et al.,* 2003). Generally, CD57 expression on T cells is regarded as a marker of terminal T cell differentiation.

In this chapter I investigated HIV-specificity in cervical mononuclear cells after polyclonal *in vitro* expansion and limiting dilution cloning initially with anti-CD3 and then with HIV-1 Gag to determine whether HIV-specific T cell responses could be detected after culture in mucosal specimens. I generated matched cervical and blood-derived T cell clones from the women identified in Chapter 2 as having high frequencies of blood Gag-specific T cells. These were generated in order to compare HIV-specific T cell responses between the blood and the genital mucosa at the clonal level. In addition to determining their HIV-specificity, I also compared the differentiation status of the cervical and blood-derived T cell clones generated in this study.

Chapter 3

Generation and characterization of HIV Gag-specific cervical and blood-derived T cell clones

3.2.

Materials and Methods

3.2.1. Isolation of T cells from peripheral blood

Blood from the women with high frequencies of HIV-specific blood-derived T cells (identified in Chapter 2; Section 2.3.5) were collected in Vacutainer tubes containing acetate citrate dextran (ACD) (BD Vacutainer). PBMC were isolated by Ficoll-Hypaque density gradient centrifugation according to the method described in Chapter 2 (Section 2.2.2) within 3 – 6 hours of venipuncture. Between 1 - 2 x10^6 PBMC were analysed *ex vivo* for IFN-γ production to HIV Gag by intracellular cytokine staining (Section 3.2.5), or were used as autologous feeder cells (Section 3.2.7.3.2) during the expansion of T cells. Remaining cells were frozen in liquid nitrogen as described in Chapter 2 (Section 2.2.4).

3.2.2. Isolation of T cells from cytobrush specimens

Several studies have employed the relatively non-invasive cytobrush-mediated technique of sampling for cervical T cells (Musey *et al.*, 1997; Shacklett *et al.*, 2000b; Kaul *et al.*, 2000, 2003; Prakash *et al.*, 2004; Quayle *et al.*, 2007). Although well tolerated, this method yields few cells for analysis. Cervical lymphocytes were collected according to the method described by Passmore *et al.* (2002; 2006). Briefly, a Digene cervical sampler was inserted into the cervical os and rotated 360°. It was withdrawn and transferred to a 15ml V-bottomed Falcon tube containing 3ml transport medium [10% FCS (Delta Bioproducts) in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)]. Cervical samples were not taken if the donor was menstruating, had visible sexually transmitted infection or discharge or was pregnant. Cervical samples were discarded if the sample had visible blood contamination. Processing was done within 3 – 6 hours of collection to maximise T cell recovery and to maintain viability.

3.2.3. Processing of cervical T lymphocytes from cervical cytobrush specimens

A plastic disposable Pasteur pipette (Medipack®) was used to flush mucous and cells from the cytobrush by pipetting up and down 20 times in 3ml transport medium. The media containing dislodged cells was then transferred to a fresh conical-bottomed 15ml centrifuge tube and centrifuged at 320xG (1300rpm using a Heraeus 1.0R Megafuge) for 10 minutes. The supernatant was carefully removed using a disposable Pasteur pipette and stored at -80°C. The cytobrush was rinsed a second time with another 3ml transport medium and a disposable Pasteur pipette was used to dislodge any remaining cells. This second wash with 3ml transport medium was added to the cells pelleted from the first wash. These cells were

gently overlaid onto a 4ml Ficoll-Hypaque gradient in a 10ml round bottom tube and centrifuged at 480xG (1600rpm using a Heraeus 1.0R Megafuge) for 30 minutes with the brake turned off. The buffy layer enriched for mucosal mononuclear cells (MMCs) was removed using a disposable Pasteur pipette (Medipack®)and transferred to a fresh 15ml Falcon tube. The tube was filled with **R1** [1% FCS (Delta Bioproducts) in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)] and was centrifuged at 1300 rpm (320xG) for 10 minutes. The supernatant was discarded and cells washed a second time with R1. The supernatant was discarded and the cell pellet was resuspended in 400µl 10% human AB serum in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)]. The cell count was determined manually using Trypan blue exclusion staining (Section 2.2.3).

3.2.4. Preparation of the single pool of 66 HIV Gag peptides

The entire HIV-1 subtype C Gag protein was divided into 66 15-20-mers that overlap by 10 amino acids (described in detail in Section 2.2.6). A single pool of 66 HIV Gag peptides was prepared for use in this Chapter. The single pool of 66 HIV Gag peptides was used at final concentration of 2ug/ml by diluting the stock of 66 HIV Gag individual peptides [concentration of 0.3mg/ml in DMSO (Sigma)] 1:150 in the relevant media for functional analysis or T cell expansion.

3.2.5. Polyclonal *in vitro* expansion of cervical T cells

The cervical T cells recovered from cytobrush specimens (Section 3.2.2) were first cultured in the presence of anti-CD3 mononclonal antibody for 14 days to expand $CD3^+$ T cell populations; and with a single pool of 66 HIV Gag peptides for a further 14 days to expand HIV-specific $CD3^+$ T cells. Cervical MMC from each donor was aliquoted into 4 wells of a 96-well microtitre plate (Nunc) each containing 100 ul. Wells of the 96-well plate were pre-coated with 100ul anti-CD3 monoclonal antibody. These wells had been pre-coated by adding the 100ul anti-CD3 monoclonal antibody at a final concentration of 10ug/ml (R&D Biosystems) in PBS (Gibco™) at 37°C or overnight at 4°C and then washing away excess anti-CD3 antibody 3 times with PBS (Gibco™) before the addition of cervical MMC. The cervical MMC were cultured in the presence of $1x10^5$/well autologous irradiated (4000 rads) PBMC (feeder cells). After 14 days, the four wells were pooled and T cells were counted manually (Section 2.2.3). The cervical MMCs were re-plated at $1x10^5$/well in a new 96-well microtitre plate and stimulated with a single pool of 66 HIV Gag peptides

(section 3.2.4) at a final concentration of 2ug/ml in the presence of fresh autologous irradiated PBMC ($1x10^5$/well) which served as both antigen-presenting cells and feeder cells. The cervical MMC were kept at 37°C with 5%CO_2 throughout the 28-day culture period. Recombinant human interleukin-2 (IL-2; final concentration of 100U/ml; NIH AIDS Reagent Repository, Germantown, MD) in fresh media [10% Human AB serum in RPMI containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)] was added to each well at the initiation of culture and then every second day by replacing 100ul of media in the well. After 14 days of culture with anti-CD3 monoclonal antibody and then 14 days of expansion in the presence of HIV Gag peptides, cervical T cell lines were then used in T cell cloning experiments or assessed for HIV Gag specificity by IFN-γ ELISpot (section 2.2.7).

3.2.6. Assessment of HIV Gag-specificity of cervical T cell lines by IFN-γ ELISpot analysis

Expanded T cells were counted manually after 26-days of culture (section 2.2.3). Cells were removed from IL-2 by centrifuging cells at 425xG (1500rpm Heraeus 1.0R Megafuge) for 5min and resuspending the cells in fresh culture media containing no IL-2 [10% Human AB serum in RPMI supplemented with 50U penicillin/ml, 50mg streptomycin/ml, 50mg glutamine/ml (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)]. The expanded cervical MMC number was adjusted to $1x10^6$cells/ml at 28-days of culture to assay for specificity to the five HIV Gag peptide pools. A volume of 50ul expanded cervical MMC ($1x10^6$/ml; $5x10^4$/well) was added per well on the ELISpot plate and the net IFN-γ response to the HIV Gag peptide pools was presented as SFU/10^6 T cells. The majority of the cervical MMCs were used to generate HIV Gag-specific T cell clones.

3.2.7. Generation of HIV Gag-specific cervical and peripheral T cell clones

$CD3^+$ T cells from PBMC for T cell cloning experiments were purified using MiniMacs (Miltenyi Biotec) magnetic bead purification. The yield of T cells from the cervix was insufficient to be purified using this method and were therefore cloned directly after the 28-day period of polyclonal expansion.

3.2.7.1. Magnetic bead isolation of blood-derived T cells for cloning

3.2.7.1.1. Preparation of microbeads

The microbeads (MACS) provided by the manufacturer were stored in azide that had to be removed before use. A MS Separation Column was placed in to a Minimacs magnet (Figure 3.2.1) and rinsed with wash buffer [500ul 0.5% FCS (Delta Bioproducts) and 2mM EDTA in PBS (Gibco™)]. A volume of 500ul MACS CD3 MicroBeads (Miltenyi Biotec) was passed through the column (attached to the magnet). The column containing the bound anti-CD3 microbeads was washed two times with 500ul wash buffer and the effluent was discarded. The column was removed from the magnet and placed into a sterile Eppendorf tube. The column bound anti-CD3 microbeads were eluted off the column by adding 500ul wash buffer to the top of the column and then plunging the beads off the column using the plunger provided to transfer the magnetic beads to an Eppendorf tube.

3.2.7.1.2. Labelling of PBMC with anti-CD3-coated magnetic microbeads

PBMC ($1x10^7$ cells) were resuspended in 80ul wash buffer [0.5% FCS (Delta Bioproducts) and 2mM EDTA in PBS (Gibco™)]. A volume of 20ul of the washed anti-CD3 magnetic beads was added to 80ul of PBMC. The cells were kept at 4°C for 20 minutes to allow specific binding of the antibody to $CD3^+$ T cells. A control sample of 80ul wash buffer and donor cells in 20ul 0.5% Foetal Calf Serum and 2mM EDTA in PBS were also placed at 4°C for 20min to control for non-specific binding of the PBMC to the column (the unseparated population).

3.2.7.1.3. Separation of $CD3^+$ cells from PBMC

The MACS MS Separation Column attached to the MACS MultiStand magnet was set up as shown in Figure 3.2.1. The column's filter was initially washed with a volume of 500ul wash buffer. Labelled cells were pipetted onto the column in the magnetic field. The tube in which the labelled cells were incubated was washed with 500ul wash buffer and passed over the column to collect residual cells from the tube. The column containing the magnetic beads and attached cells was washed three times with 500ul wash buffer. The effluent was collected as the negative fraction. The column was removed from the magnet and placed in a clean collection tube. A volume of 1ml wash buffer was added to the column and a plastic plunger was applied to the column to flush out the positively labelled cells. FACS analysis was used to confirm the purity of $CD3^+$ T cells in this positive fraction.

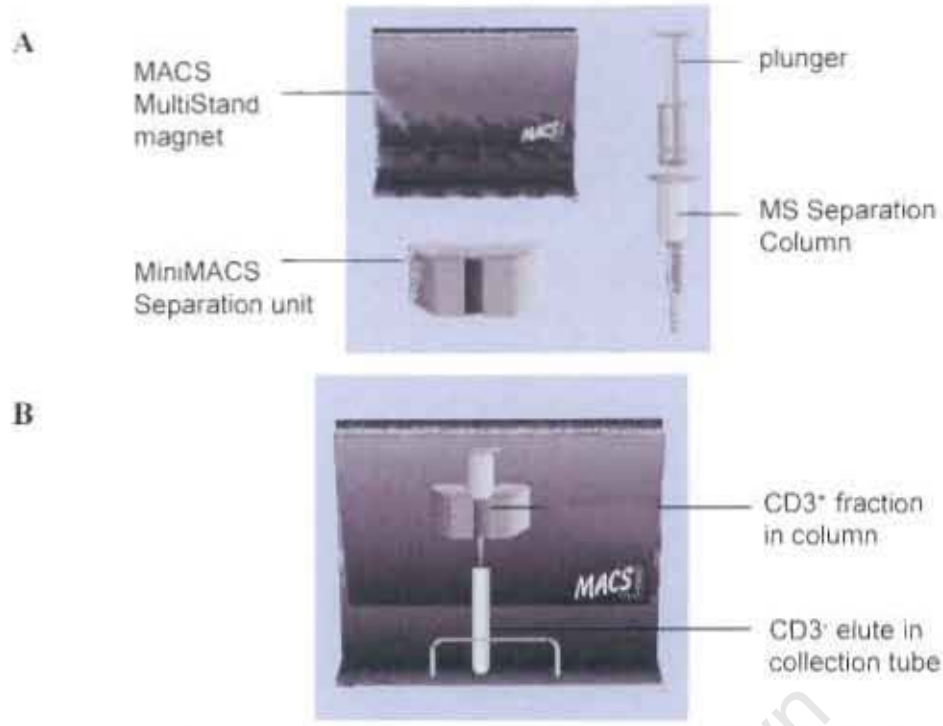

Figure 3.2.1. Assembly of the MS Separation Column and MACS MultiStand. (A) The MACS multistand magnet, the MiniMACS separation unit, the MS Separation column and plunger are indicated in this figure. **(B)** The MiniMACS Separation unit is attached to the MACS MultiStand magnet. The MS Separation Column is then placed in the MiniMACS Separation unit. A collection tube is then placed under the column. A volume of 500ul wash buffer is passed through the column before the immediate addition of the cell sample (adapted from the MACS MS Separation Columns instruction manual, Miltenyi Biotech).

3.2.7.2. Limiting dilution cloning of HIV-specific cervical and peripheral T cells

Cloning by limiting dilution entails plating cells in wells at a dilution that ensures a high probability that any resulting colony is derived from a single cell (Taswell, 1981). The lower the average number of cells plated per well, the greater the probability that the resultant population is a true clone. Table 3.2.1 summarizes the probability that one would achieve a single cell clone at various plating dilutions ranging from 1 cell per well to 0.1 cells per well. If the average number of cells plated per well is 1, then the probability that the resulting cells expanding from that well would be clonal is only 0.582. In contrast, if the average number of cells plated per well was 0.1, then the probability that any wells showing expansion are clonal is 0.951. In this study, $CD3^+$ PBMC and MMC were plated at 0.1, 0.3 and 1.0 cells/well into 96-well plates.

Table 3.2.1. Manipulation of the Poisson distribution to determine the probability that any cell population picked at random is a true clone

	Average number of cells per well		
	1.000	0.300	0.100
Probability	0.582	0.857	0.951

Adapted from Davis, 1994

The frequency of HIV-specific T cells in PBMC of the 9 donors had previously been determined (Chapter 2) and this was taken into account when calculating the number of HIV-specific $CD3^+$ PBMC that I needed to plate to obtain a frequency of 0.1 HIV-specific T cell/well. Table 3.2.2 summarises the number of cells plated per 100ul to obtain a frequency of 0.1 HIV-specific blood-derived cells per well. From Table 3.2.1, even though the tabulated probabilities are based on the assumption that only single cells are being plated and that populations are randomly selected, at an average of 0.1 cells plated per well there is still an approximate 5% chance that a population will not be clonal. Therefore, further testing by FACS analysis of phenotype, T cell receptor typing, or further limiting dilution (sub-cloning) should be performed to confirm that the selected colonies were indeed clonal.

Table 3.2.2. Plating frequency of blood-derived T cells based on previously-determined HIV-specific T cell frequency in blood

Donor ID	Net Cumulative Magnitude (SFU/10^6 PBMC)	Plating Frequency for 0.1 HIV-specific blood-derived cells/well [a] (cells/100ul)
NY130	8246.67	12.13
NY230	6220.00	16.08
NY055	5746.67	17.40
NY229	5689.99	17.57
NY172	5243.33	19.07
NY094	5236.67	19.10
NY233	3900.00	25.64
NY237	3673.33	27.22
NY157	2466.67	40.54

[a]Where frequency was calculated by the equation: Plating Frequency = [10^6/ magnitude of HIV-specific response observed] x 0.1

3.2.7.3. Maintenance of HIV-specific T cell clones

T cell clones were supplied with growth-promoting human recombinant IL-2 and irradiated autologous feeder cells (PBMC) to provide the media with the appropriate cytokines, chemokines and spatial environment for T cell growth. Since T cells respond to processed antigens when recognised in the context of HLA class I molecules, autologous antigen-presenting macrophages were included in wells to provide appropriate HLA-matched antigenic stimulation to T cells.

3.2.7.3.1. Preparation of antigen-presenting cells for cloning

Autologous PBMC were placed at $5x10^6$/ml (1ml/well) in RPMI (Gibco™) in 24-well plastic plates (Nunc) to allow monocytes to adhere (Kanof et al., 1994). After 90 minutes of culture at 37°C with 5% CO_2, wells containing adherent cells were washed 3 times with warmed (37°C) 10% Human AB serum in RPMI supplemented with 50U penicillin/ml, 50mg streptomycin/ml, and 50mg glutamine/ml (Gibco™)] to remove non-adherent cells. The adherent monocytes were pulsed with the HIV Gag peptides (final concentration 2ug/ml in 500ul) for 3 hours at 37°C and 5% CO_2. The HIV Gag peptide-pulsed macrophages were detached using a rubber scraper and were transferred to a sterile 50ml culture flask. The wells were then washed with 500ul 10% Human AB serum in RPMI and were scraped a second time with the rubber scraper to detach residual macrophages from the wells. The macrophages were counted manually (section 2.2.3) and were adjusted to a volume of $1x10^5$ cells/ml and $5x10^3$ cells were added per well. These were irradiated at 4000 rads and added to the plated PBMC and MMC to allow cloning in the continual presence of HIV Gag peptides.

3.2.7.3.2. Preparation of feeder cells for cloning

When generating antigen-presenting cells for cloning, autologous non-adherent monocytes were collected during the various washing steps to serve as feeder cells. The non-adherent monocytes were counted manually (Section 2.2.3) and adjusted to $1x10^6$ cells/ml. The cells were irradiated at 4000 rads and added to the wells at $5x10^4$cells/well. Wells containing feeder cells alone were also plated to ensure that the irradiated feeder cells were not capable of replication in culture.

3.2.7.3.3. General culture conditions

MMC and $CD3^+$ T cell-enriched PBMC were plated at 0.1, 0.3 and 1 cell/well respectively in the presence of HIV Gag-pulsed macrophages ($5x10^3$cells/well), feeder cells ($5x10^4$cells/well) and a final concentration of 100U recombinant human IL-2/well. The

plates were kept at 37°C with 5% CO_2 to allow expansion of HIV Gag-specific T cells. Being careful not to disrupt the cell pellet, fresh culture media [10% Human AB serum in RPMI supplemented with 50U penicillin/ml, 50mg streptomycin/ml, 50mg glutamine/ml (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)] supplemented with IL-2 (for a final concentration of 100U/well) was added every second day by replacing half the volume in each well. Wells were visually inspected for clusters of proliferating T cells using an Olympus-CKX31 inverted microscope. At a minimum of 14 days of culture, wells with clear signs of proliferation were picked for further culture in the presence of a single pool of 66 HIV Gag peptides for a further 14 days.

Cells were counted every 14 days (section 2.2.3). Once the cell number exceeded $2x10^5$/well in a 96-well plate, the cells were centrifuged at 425xG (1500rpm using a Heraeus 1.0R Megafuge) for 5 minutes. The cell pellet was resuspended in 500ul culture media [10% Human AB serum in RPMI supplemented with 50U penicillin/ml, 50mg streptomycin/ml, 50mg glutamine/ml (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)] and was transferred to wells of 24-well plates (Nunc). Once the cell number in the wells of the 24-well plates (Nunc) exceeded $1x10^6$cells/well, the cells were split into 2 wells.

Cells were maintained in culture until a yield sufficient to conduct functional analysis was achieved ($\geq 3.5x10^5$ cells for HIV Gag pool ELISpot; $\geq 2x10^6$ cells for TCR β-typing). After 4 weeks of culture in the presence of a single pool of HIV Gag peptides, cells were then cultured in the presence of anti-CD3 monoclonal antibody (Section 3.2.3) for a further 4 weeks. The cells were cultured in this fashion for a maximum of 19 weeks and were phenotypically and functionally analysed by flow cytometry.

3.2.8. Functional analysis of cervical and peripheral T cells

3.2.8.1. Flow cytometric analysis of T cell phenotype and function

3.2.8.1.1. Phenotypic screening of T cells

Cells in culture were counted manually by removing 10ul of cells per well and staining with 10ul Trypan Blue (Section 2.2.3). Cells from wells exhibiting growth in the presence of HIV Gag peptides were assessed for cell surface phenotype using FACS. A volume of 50ul cells was combined with 5ul CD3-APC and 5ul CD8-FITC (BD Pharmingen) to identify $CD3^+CD8^+$ T cell populations and $CD4^+$ T cell populations ($CD3^+CD8^-$ T cell populations). The cells were kept on ice for 20 minutes to allow the monoclonal antibodies

to bind to cell surface markers. The cells were washed in a volume of 2ml R10 [10% FCS (Delta Bioproducts) in PBS (Gibco™)] and were centrifuged at 425xG (1500rpm Heraeus 1.0R Megafuge) for 5min. The pellets were resuspended in 100ul R10 and 100ul paraformaldehyde (CellFix, BD Pharmingen) to fix the cells for acquisition of the data on a FACScalibur flow cytometer (Becton Dickinson) and analysis using CellQuest software (Becton Dickinson).

3.2.8.1.2. Assessment of HIV-specificity by intracellular cytokine staining

T cells were assayed for specificity to a single pool of 66 HIV Gag peptides after T cell cloning by limiting dilution. T cell clones (50ul/well) and were stimulated for four hours at 37°C 5% CO_2 with phorbol myristate acetate (PMA) (1ug/ml)/ionomycin (50ug/ml) or with a single pool of 66 HIV Gag peptides (at a final concentration of 2ug/ml) for antigen-specific stimulation. Brefeldin A (at a final concentration of 10ug/ml) was added after the first hour of stimulation to inhibit cytokine secretion. Unstimulated cells served as negative controls for cytokine secretion. These were also incubated for four hours at 37°C and 5% CO_2 and were also treated with Brefeldin A one hour into the incubation period. The cells were then washed once and were resuspended in 500ul Cytofix/Cytoperm (BD Biosciences Pharmingen). The cells were kept in the dark at room temperature for 10 minutes to allow for fixing and permeabilization of cells. The cells were washed once with permeabilization solution [0.1% Saponin (Sigma) + 1% FCS (Delta Bioproducts) + 0.01% NaN_3 in PBS (Gibco™)]. It is important to include saponin in these wash steps to maintain permeability of the cell membrane as this is reversible. The fixed cell pellet was resuspended in a volume 5ul each of the antibodies IFN-γ-PE, CD3-APC, CD8-PeCy5 and CD57-FITC to identify $CD8^+$ and $CD4^+$ T cell populations ($CD3^+$) capable of HIV-specific IFN-γ secretion, and to further define their differentiation status. All antibodies were from Becton Dickenson. The cells were kept on ice for 30min to allow antibody binding to cellular markers. The cells were washed in once in 2ml permeabilization buffer and once with 2ml wash buffer at 425xG (1500rpm Heraeus 1.0R Megafuge) for 5min to remove excess antibody. A volume of 500ul CellFix™ (BD Biosciences) was added to the pellets to fix the cells for acquisition of data on the FACScalibur flow cytometer (Beckton Dickinson) and for analysis using either CellQuest software (Becton Dickinson) or FlowJo (TreeStar).

3.2.8.2. Assessment of HIV Gag-specificity of T cell clones by IFN-γ Pool ELISpot analysis

Once the T cell clones reached >3.5x10^5 cells (enough to conduct an IFN-γ ELISpot assay as well as continue culturing), rhIL-2 was removed from the clones by washing and clones were transferred into fresh culture media containing no IL2 [10% Human AB serum in RPMI supplemented with 50U penicillin/ml, 50mg streptomycin/ml, 50mg glutamine/ml (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen)]. The clones were rested out of IL2 in this way for 48 hours before assessment for IFN-γ production by cultured ELISpot. The T cell clones were assayed for specificity to the five HIV Gag peptide pools according to the protocol outlined in Section 2.2.7. T cell clones (1x10^6/ml; 5x10^4/well) were added to the wells of the ELISpot plate and the net IFN-γ response to the HIV Gag peptide pools was presented as SFU/10^6 T cells.

3.2.9. Statistical analysis

Medians were compared using the nonparametric Mann-Whitney test. Correlations between variables were determined using the nonparametric Spearman rank correlation test. Associations between variables in different groups were determined by two- factor ANOVA test. P-values less than 0.05 were considered significant. Data analysis was conducted using Microsoft® Excel Analysis ToolPak and Prism (version 2.0b, GraphPad Software, San Diego, CA) statistical software.

Chapter 3

Generation and characterization of HIV Gag-specific cervical and blood-derived T cell clones

3.3.

Results

3.3.1. Direct *ex vivo* detection of HIV-1 Gag-specific T cell responses in blood and at the cervix

I initially investigated whether HIV Gag-specific T cell responses could be detected immediately after isolation (*ex vivo*) at the cervix and in blood. Intracellular cytokine staining and flow cytometry was used to examine the *ex vivo* phenotypic and functional properties of blood- and cervix-derived T cells isolated from HIV-infected women. Figure 3.3.1 shows a representative intracellular cytokine response detected by FACS following stimulation of cervical and blood-derived mononuclear cells. In this donor a net HIV Gag-specific IFN-γ response of 0.83% was observed in the cervix and 0.45% in the blood (net IFN-γ responses are defined as responses to Gag following subtraction of responses without stimulation; Figure 3.3.1).

Although HIV-specific T cell responses were detectable at the cervix direct *ex vivo*, the number of $CD3^+$ T cells isolated from the cervix was limiting and only one functional assay could be performed per donor. This confirms that expansion of cervical T cells is necessary for more extensive investigation of HIV-specific T cell function at the genital mucosa.

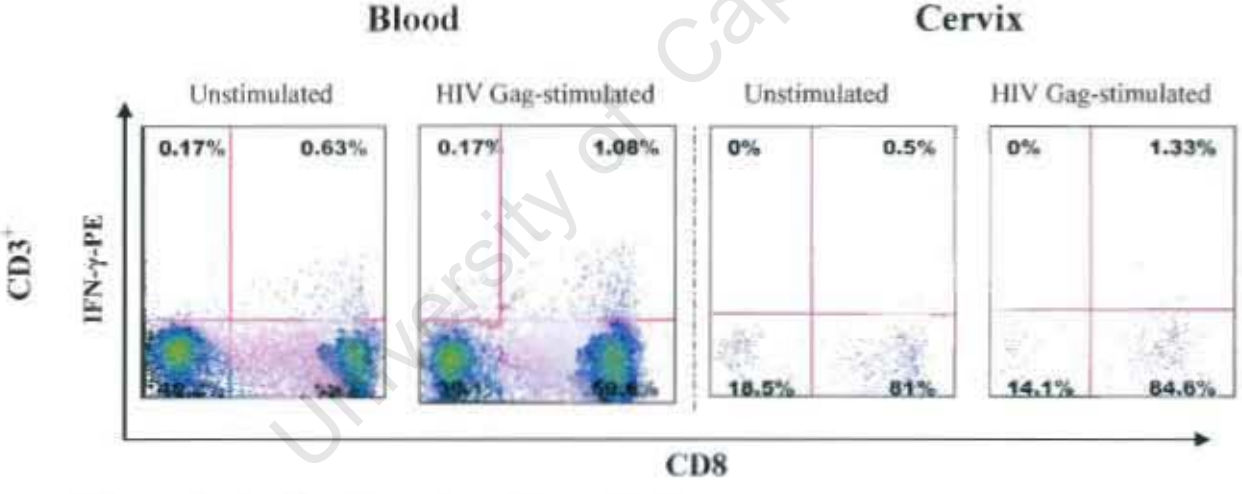

Figure 3.3.1. *Ex vivo* analysis of PBMC- and cervix-derived T cells from a representative HIV-infected woman who had detectable responses to HIV Gag. Intracellular cytokine staining was used to investigate IFN-γ responses to a single pool of 66 HIV Gag peptides in PBMC- and cervix-derived T cells isolated from HIV-infected women. The T cells were stimulated with the single pool of 66 HIV Gag peptides, or were left unstimulated for a period of 5 hours, and were stained with CD3-APC, CD8-PeCY5, and IFN-γ-PE. The proportion of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells was determined by examining IFN-γ expression in the CD3 population. A *net* HIV-specific IFN-γ response of 0.83% was observed in cervical $CD8^+$ T cells, and 0.45% in blood-derived $CD8^+$ T cells. Analysis was performed using FlowJo (kindly provided by TreeStar).

3.3.2. Polyclonal expansion of cervical T cells

Nine women from a cohort of 35 with high frequencies of HIV-specific T cells in blood were identified in Chapter 2 (Table 2.3.3) and included in the study. PBMC and cervical cytobrush-derived mucosal mononuclear cells (MMC) were isolated from each of these women. A mean ± SD of $6.49 \times 10^5 \pm 5.38 \times 10^5$ (range $1.00 \times 10^4 - 1.87 \times 10^6$) T cells was recovered from the cervical cytobrushes of these 9 women. The viability and cervical lymphocyte counts of the 9 women are illustrated in Table 3.3.1.

Table 3.3.1. Characteristics of cervical cytobrush specimens isolated from 9 women with chronic HIV infection

Patient ID	Cervical lymphocyte counts	Viability (%)
NY055	8.40×10^5	97.6
NY094	7.29×10^5	60.7
NY130	9.00×10^4	100
NY157	4.70×10^5	95.3
NY172	7.40×10^5	100
NY229	1.87×10^6	76.9
NY230	5.10×10^5	100
NY233	1.00×10^4	99.5
NY237	5.80×10^5	93.55
Mean (SD)	$6.49 \times 10^5 \pm 5.38 \times 10^5$	91.5 ± 13.68

Cervical T cells isolated from the 9 HIV-infected women were polyclonally expanded in the presence of anti-CD3 monoclonal antibody for 14 days and with HIV Gag peptides for 14 days to expand HIV Gag-specific T cells. Figure 3.3.2 depicts the kinetics of cervical T cell expansion during culture. Cervical MMC from 2/9 (22.2%) of donors did not expand after 28 days (Figure 3.3.2). However, the majority of samples (7/9; 77.8%) increased in number during culture, with a mean of 1.54-fold expansion (range 1.33-fold – 93-fold; n=7; Figure 3.3.2) and a mean count of $1.05 \times 10^6 \pm 8.0 \times 10^5$ cells after 28 days in culture.

After the 28-day polyclonal *in vitro* expansion period, cervical MMCs were assessed for HIV Gag pool-specific IFN-γ responses by IFN-γ ELISpot immediately prior to T cell cloning by limiting dilution in one of these 9 donors (Figure 3.3.3). This representative donor's cervical T cells responded to 4 of the 5 HIV Gag pools tested, with Pool 3 (Gag peptides 29 to 42; corresponding to p24; Chapter 2; Figure 2.2.3) being the most immunodominant region targeted at the cervix in this donor. The
cervical MMC's did not respond to peptides in Pool 2. In comparison, PBMC derived from this donor showed responses to all 5 of the Gag pools tested. Pool 3 was also the most immunodominant region of Gag targeted in blood for this donor. Interestingly, the

magnitude of IFN-γ responses to Gag pool 1 was significantly higher at the cervix in this patient than those found in PBMC. The net cumulative HIV Gag-specific response for this donor at this time point was 7860 SFU/10^6 MMC at the cervix compared to the 5936 SFU/10^6 PBMC detected in the corresponding PBMC sample. The remaining 8/9 donors cervical MMC did not have sufficient cervical yields and were cloned directly.

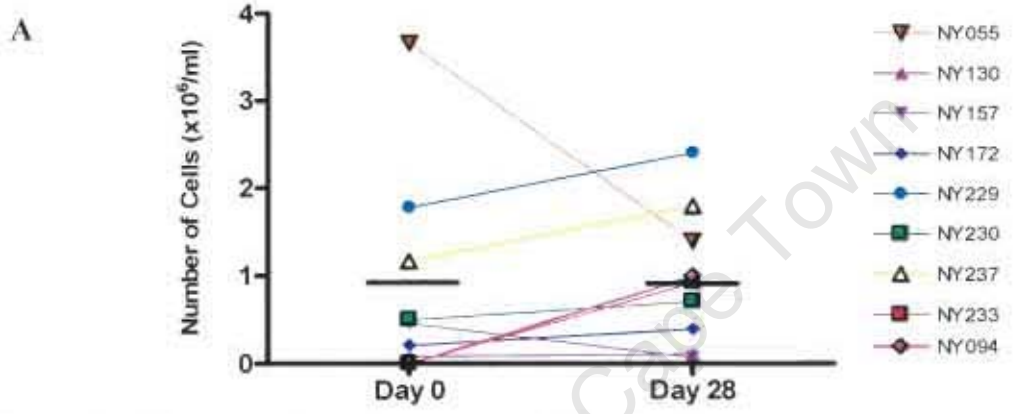

Figure 3.3.2. Growth kinetics of cervical MMC after 28 days of polyclonal expansion with anti-CD3 antibody and HIV-1 Gag peptides. Cervical T cells isolated from HIV-1-infected women were expanded in the presence of anti-CD3 antibody for 14 days and in the presence of HIV-1 Gag peptides for a further 14 days. Cervical MMC from each donor was counted using trypan staining and a haemocytometer at day 0 and at day 28.

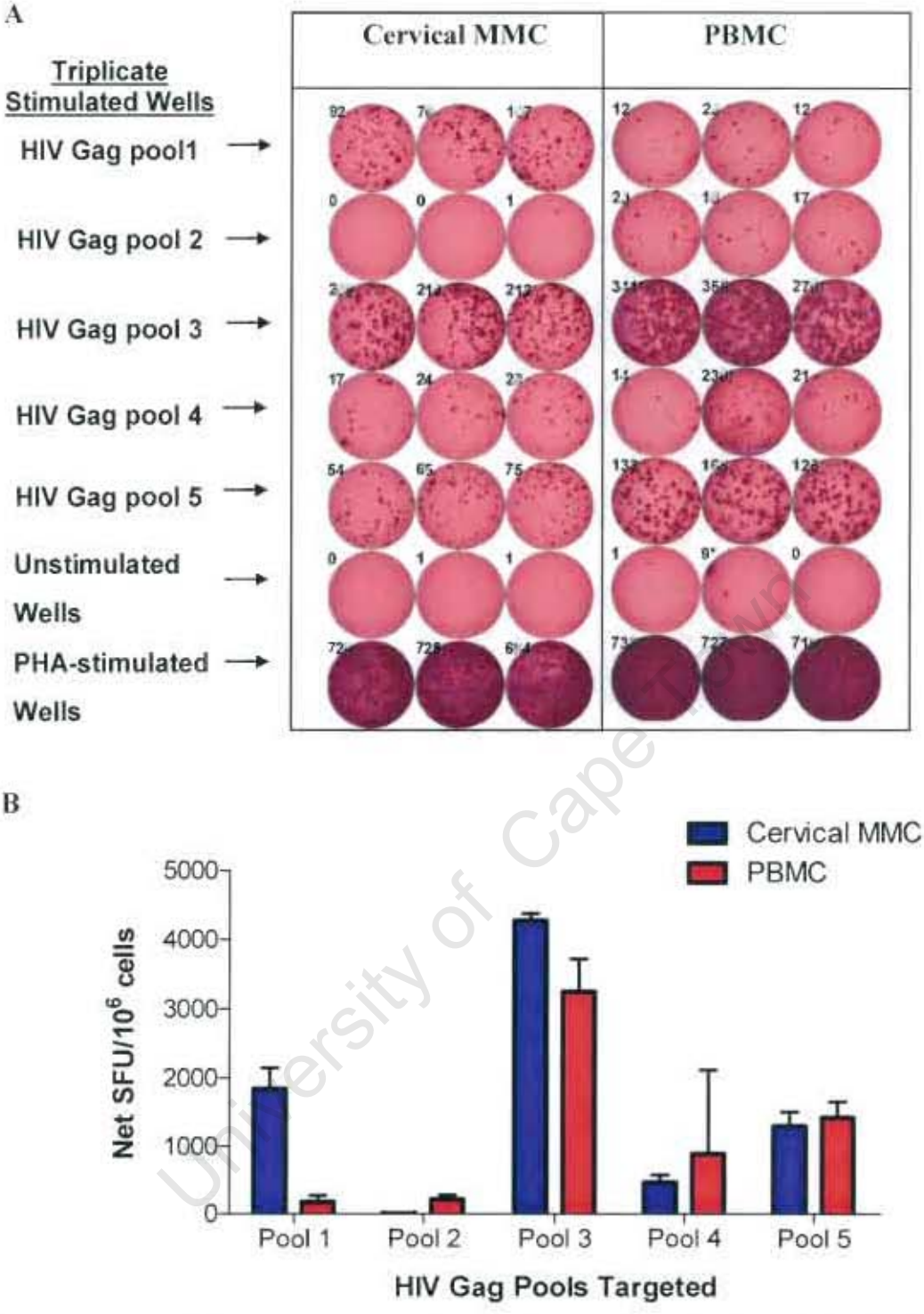

Figure 3.3.3. HIV Gag pool-specificity of cervical MMC and PBMC from a representative donor NY233 after *in vitro* polyclonal expansion. Cervical MMC and PBMC were polyclonally expanded for 14 days in the presence of anti-CD3 monoclonal antibody and for a further 14 days in the presence of a single pool of HIV Gag peptides to promote the growth of HIV Gag-specific T cells. (**A**) Cervical MMC were plated into triplicate wells at 50000 cells/well while PBMC were plated at 100000 cells/ well in triplicate. The cells were stimulated with 5 HIV-1 Gag peptide pools (final concentration 2ug/ml). Net responses greater than 100 SFU/10^6 cells were considered positive responses. (**B**) Bar graph of net IFN-γ responses per pool of PBMC and polyclonally expanded cervical MMC of donor NY233.

3.3.3. Limiting dilution cloning of HIV-1 Gag-specific PBMC- and cervix-derived T cells

Table 3.3.2 summarizes the cloning efficiency for HIV-specific cervical and blood-derived cells. Eight out of the nine donors examined yielded wells with positive growth during the 14 day cloning period. For both cervix and PBMC cloning experiments, the expected picking frequency exceeded the actual number of wells picked (Table 3.3.3) indicating that not every cell plated expanded successfully. Generally, the picking frequency for cervical and blood-derived T cells at this stage was not significantly different (11.2% of total plated for cervical clones compared to 13.1% for blood clones; p=0.373, X^2 test).

Table 3.3.2. Observed T cell cloning efficiency of cervical and blood-derived cells by limiting dilution

		Number of wells Picked/Plated[c]			
		Cervix		Blood	
Donor	Plating Frequency[a]	Picked wells[b]	(%)	Picked wells[b]	(%)
NY055	0.1	2/30	10.42%	1/30	3.33%
	0.3	1/48	20.00%	3/48	6.25%
	1	ND	ND	2/10	40.00%
NY094	0.1	1/21	4.76%	10/70	14.28%
	1	4/21	19.05%	20/20	100.00%
NY157	0.1	2/30	6.67%	0/8	0.00%
	1	18/30	60.00%	8/10	80.00%
NY130	0.1	7/64	4.69%	10/88	11.36%
NY172	0.1	1/20	5.00%	1/68	1.47%
	0.3	1/10	10.00%	1/58	1.72%
NY229	0.1	ND	ND	2/20	10.00%
	0.3	2/10	20.00%	ND	ND
	1	7/20	35.00%	8/10	80.00%
NY230	0.1	1/20	5.00%	2/30	6.67%
	1	2/4	50.00%	ND	ND
NY237	0.1	2/96	2.08%	6/96	6.25%
Sum		51/454	11.2%	74/566	13.1%

Where [a] denotes the frequency at which cells were plated per 100ul well; and [b] the actual number of wells picked from wells containing cervix- and blood-derived T cells respectively. The table represents all wells picked and plated when cloning by limiting dilution at each visit. Donor NY233 is absent as no blood and cervical cytobrush samples were picked from this donor.

Table 3.3.3. Comparison of Expected and Actual picking frequencies of cervix- and PBMC-derived T cells during cloning by limiting dilution

Expected Frequency		Actual Frequency (%) Cervix	PBMC
0.1/well	(10%of wells plated)	5.52 ± 0.03	6.67 ± 0.05
0.3/well	(33.33% of wells plated)	16.7 ± 0.06	3.99 ± 0.03
1/well	(100% of wells plated)	41.0 ± 0.17	75.0 ± 0.25

3.3.4. Functional analysis of PBMC- and cervix-derived T cell clones

From the 74 blood-derived and 51 cervix-derived T cell clones picked, significantly more of the blood-derived clones compared with the cervical T cell clones [12/74 (16.2%) blood and 2/51 (3.9%) cervical clones] were specific for HIV Gag by ELISpot and IFN-γ ICS (Table 3.3.4; p=0.0528; X^2 test). Both of the cervical T cell clones and 1 PBMC clone that showed HIV-specificity were derived from the same donor (NY130). This participant had a CD4 count of 322 cells/ul and a viral load of 39811 at this visit. She was also the second strongest Gag responder from the cohort of 35 (Chapter 2; Figure 2.3.4) that was investigated with a cumulative net response magnitude of 8267 SFU/10^6 cells at the visit immediately prior to the one used in this cloning experiment.

Table 3.3.4. Number of HIV Gag-specific T cells generated from picked wells

Donor	NY229	NY094	NY055	NY130	NY230	NY172	NY157	NY237	Total
PBMC-derived T cells	1*/10	10*/10, 0/20	0/6	1*/10	0/2	0/2	0/8	0/6	11*/20, 1*/54
Cervix-derived T cells	0/9	0/5	0/3	2*/7	0/3	0/2	0/20	0/2	2*/51

Where* denotes HIV Gag-specificity determined by IFN-γ ELISpot, and * by ICS.

Of the 12/74 blood-derived T cells clones that were found to be HIV-specific, the specificity of the majority (11/12) was confirmed by IFN-γ ELISpot while 1/12 was confirmed by IFN-γ ICS. Figure 3.3.4 shows a representative ELISpot for 2 of these clones (NY094 clone 5 and 10) as well as a summary of net IFN-γ responses in the clones assessed.

FACS phenotypic analysis of 62/74 blood and 40/51 cervical clones picked identified those picked clones as $CD3^+$ T cells (data not shown). Of the 62 blood clones that were phenotyped 36/62 (58%) were exclusively $CD8^+CD3^+$ T cells and 19/62 (30%) $CD4^+CD3^+$ T cells (data not shown). Of the 40 cervical clones that were phenotyped, 29/40 (72.5%) were exclusively $CD8^+CD3^+$ T cells and 11/40 (27.5%) $CD4^+CD3^+$ T cells (data not shown). All of the cervical and peripheral T cells that responded to HIV Gag were $CD8^+$ T cells.

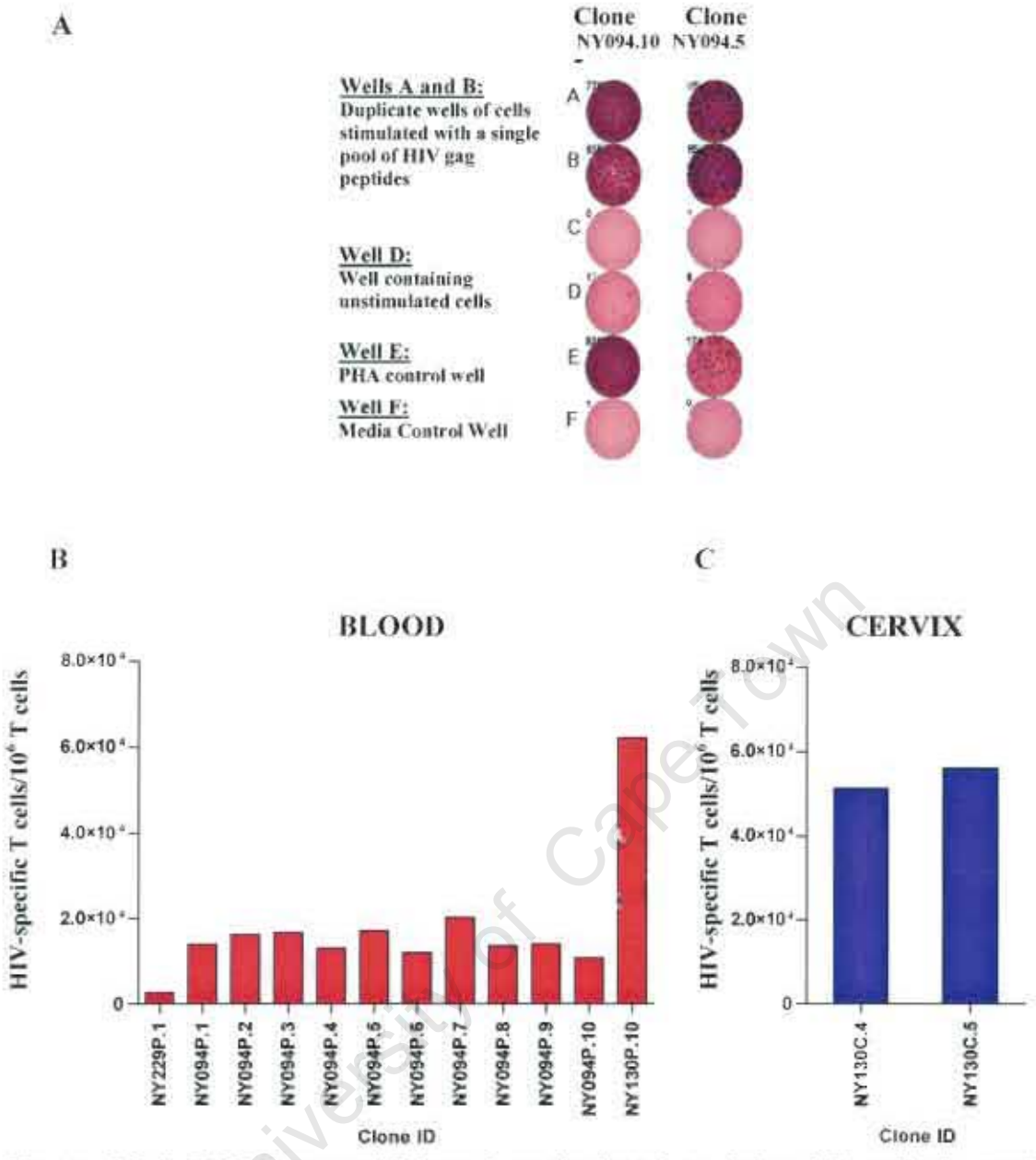

Figure 3.3.4. HIV Gag-specificity of cervical and peripheral T cell clones (A) A representative example of the ELISpot plate result of 2 of the 10 donors assayed for HIV Gag-specific IFN-γ secretion. The cells were plated in duplicate wells of $5x10^4$ cells/well and were stimulated with a single pool of HIV Gag peptides (final concentration 2ug/ml). Both clones responded to the HIV Gag peptide pool, wells A and B. **(B)** The net HIV Gag-specific IFN-γ responses of the 12 PBMC-derived T cell clones, and **(C)** the 2 cervix-derived T cell clones generated by limiting dilution cloning. HIV Gag-specific clones NY130C.4, NY130C.5, and NY130P.10 were identified by ICS. The net percentage of these HIV-specific IFN-γ secreting T cell clones was normalised to net HIV-specific T cell clones/$1x10^6$. The remaining 11 HIV-specific T cell clones were identified by IFN-γ ELISpot. Only HIV-specific IFN-γ responses greater than 100 SFU/10^6 cells were considered positive responses.

3.3.5. Investigation of maturational status of cervix- and PBMC-derived T cell clones

None of the 51 cervical T cell clones picked during limiting dilution culture expanded enough during *in vitro* culture to facilitate IFN-γ ELISpot analysis of HIV Gag-specificity. The cervix-derived T cell clones were cultured for a mean period of 12.8 weeks and PBMC-derived T cell clones for a mean period of 13.8 weeks of culture. Because cervical T cells were derived from genital tissue and therefore were likely to be effector cells while blood derived cells were obtained from the circulating T cell pool, it was of interest to investigate the respective maturational status of clones derived from each site. This study focused on expression of CD57 on clones from blood and cervix because this maturational marker has previously been linked to terminal differentiation and senescence (clonal exhaustion; Brenchley *et al.* 2003). To explore the possibility of T cell exhaustion as a factor underlying the difficulty in generating HIV-specific clones from cervical tissue, the expression of CD57 was investigated in matched cervix- and blood-derived T cells isolated directly *ex vivo* (n=8; Figure 3.3.6.A) and in T cell clones derived from both compartments (n=40 and 47 matched cervix- and blood-derived clones respectively; Figure 3.3.6.B). Firstly, levels of CD57 on $CD3^+$ T cells was not significantly different in the blood of HIV negative individuals, and the blood and cervical samples from chronically HIV-infected women (Figure 3.3.6 A). Secondly, no significant difference was observed in CD57 expression between blood and cervical T cells directly *ex vivo* (Figure 3.3.6.A). But, CD57 expression was significantly higher in cervical clones than blood clones cultured for similar periods of time (Figure 3.3.6.B; $P<0.0001$). Furthermore, CD57 expression was significantly higher in cervical T cell clones than cervical T cells assessed directly *ex vivo* ($P=0.0120$; Mann-Whitney U Test). This trend was not observed in blood-derived T cells.

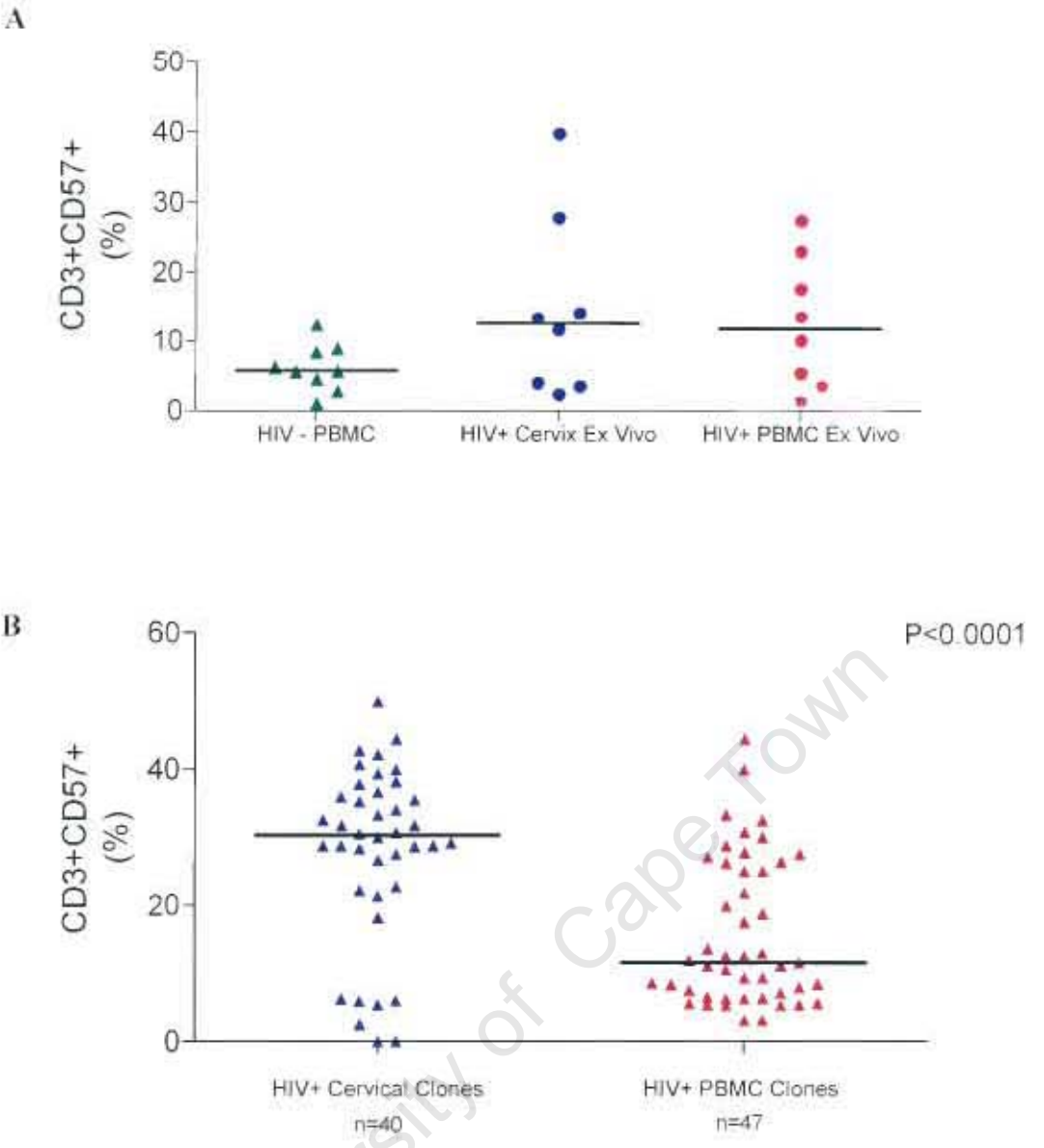

Figure 3.3.5. CD57 expression of blood- and cervix-derived T cell clones and *ex vivo* T cells. PBMC- and cervix-derived T cells from HIV-infected donors were analysed direct *ex vivo* or after periods of cloning by limiting dilution for expression of the T cell exhaustion marker CD57. The T cells were stimulated with a single pool of 66 HIV Gag peptides, or were left unstimulated for a period of 5 hours, and were stained with CD3-APC, CD8-PeCY5, CD57-FITC and IFN-γ-PE. (**A**) CD57 expression by $CD3^+$ T cells in HIV- PBMC (n=9) and direct *ex vivo* HIV^+ cervical T cells (n=8) and PBMC (n=8) are illustrated. No significant difference in CD57 expression is observed between the groups. (**B**) CD57 expression by $CD3^+$ cervical T cell clones (mean 12.8 weeks of culture) was significantly greater than CD57 expression by PBMC-derived T cell clones (mean 13.8 weeks of culture). The proportion of HIV Gag-specific $CD3^+CD8^+$ T cells was determined by examining IFN-γ expression in the CD3 population.

Chapter 3

Generation and characterization of HIV Gag-specific cervix- and blood-derived T cell clones

3.4.

Discussion

3.4. Discussion

A key factor limiting extensive studies of HIV-specific T cell responses at the genital tract of HIV-infected women is the low yield of cervical T cells using well-tolerated sampling methods. Highly sensitive methods of investigating T cell function (IFN-γ ELISpot, intracellular flow cytometry, MHC-peptide tetramer staining, ^{51}Cr release assays to name a few) require cell numbers considerably greater than the $1x10^4$ cells commonly recovered *ex vivo* from a cervix cervical cytobrush. To circumvent the problem of low T cell recovery in this project, I expanded cervical T cells both polyclonally and by limiting dilution cloning to allow more comprehensive investigations of HIV-specific T cell phenotype and function at the cervix. Using 9 women with well characterized and high magnitude T cell responses to HIV Gag, I showed that T cell clones from the peripheral blood and cervix that were specific for Gag could be generated. All of the clones characterized were of a $CD8^+CD3^+$ T cell phenotype. Only a minority of the clones picked from the cervix and blood produced IFN-γ in response to Gag. Most importantly, I showed that the efficacy of generating Gag-specific T cell clones was far lower in cervical samples than in blood. Others in our laboratory have shown that cervical T cells have significantly higher frequencies of Gag-responsive cells than blood-derived T cells ($p=0.0002$; Pamela Gumbi, Manuscript in preparation) and that cervical T cells exhibit significantly higher frequencies of responses to PMA/ionomycin than blood T cells (indicating heightened activation status and viability, Pamela Gumbi, personal communication). The poor efficacy of generating cervical T cell clones in this study was therefore unlikely to be due to poor viability or low Gag-specific T cell frequencies in cervical samples but may be due to poor *in vitro* proliferative capacity of cervical-derived T cell clones compared to blood clones. I went on to determine the maturational status of the cervical and blood T cell clones generated in this study using the terminal differentiation or exhaustion marker CD57 (Brenchley *et al.*, 2003). I showed that while *ex vivo* cervical and blood T cells show similar levels of CD57 expression, cervical T cell clones showed significantly higher levels of this marker compared to blood-derived T cell clones despite similar time in culture.

The nine chronically HIV-infected women used for this study were selected from a cohort of 35 based on high magnitude and breadth of IFN-γ T cell responses to HIV Gag (Chapter 2). The motivation for using high HIV-specific IFN-γ responses in blood as a predictor of HIV-specific T cell frequency at the cervix was underscored by a recent report of similarities in HIV Gag-specific epitope targeting between the blood and mucosa of the

same individuals (Musey *et al.*, 2003) indicating some link between immunological compartments.

Although *ex vivo* FACS analysis clearly has the capacity to detect HIV-specific T cell responses at the cervix and blood as I have shown in this chapter, the number of $CD3^+$ T cells isolated from the cervix remains a significantly limiting factor. One of the major advantages of direct *ex vivo* cytokine flow cytometry is the ability to study co-expression of cytokines on single cells without any potential artefacts induced during long term culture. However, when cell numbers are insufficient to conduct a comprehensive analysis of cell function as a result of low yield of cervical T cells, *in vitro* expansion of T cells is essential.

I next investigated HIV-specificity in cervical mononuclear cells after polyclonal *in vitro* expansion to determine whether HIV-specific T cell responses could be detected after culture in mucosal specimens. Cervical MMC were expanded in the presence of anti-CD3 monoclonal antibody for 14 days and for a further 14 days in the presence of HIV Gag peptides. Cervical MMC from the majority of HIV-infected donors (7/9 donors) increased in number after culturing (median 1.54-fold increase; range 1.33-fold – 93-fold; n=7) yielding a mean count of $1.05x10^6$ ($\pm$ $8.0x10^5$) MMC after 28 days in culture. Similar HIV Gag pools were targeted in the expanded cervical MMC and PBMC samples. From these limited studies, I noticed a broader response in blood than in expanded cervical MMC. HIV specificity could therefore be detected after *in vitro* culture of the mucosal T cells.

Finally, the main objective of this study was to generate matched cervical and blood-derived T cell clones by limiting dilution to compare HIV-specific T cell responses between the blood and the genital mucosa at the clonal level. HIV Gag-specificity was observed in 12/74 blood-derived clones and in 2/51 cervix-derived T cell clones. Although I clearly demonstrated in this chapter that HIV Gag-specific T cell clones could be generated from both the blood and the cervix of chronically HIV-infected women, I consistently found that the cervical MMC clones were generated at significantly lower frequencies than the blood clones and expanded less successfully in culture.

Because of the expansion kinetic differences to Gag that I noted in the blood- and cervix-derived T cell clones, I went on to investigate potential factors contributing to slow *in vitro* growth of cervical clones. I focused on expression of the exhaustion marker CD57. CD57 expression was significantly greater on cervical T cell clones than blood-derived T cell clones. CD57 expression was not significantly different between blood- and cervix-derived

T cells isolated direct *ex vivo*, and when compared to CD57 expression of PBMC from HIV-seronegative donors. CD57 expression was only significantly upregulated on cervical T cell clones compared to cervical T cells assayed direct *ex vivo*, but not between blood-derived T cells assayed direct *ex vivo* and blood-derived T cell clones. It is likely that *in vitro* expansion of cervical MMC during limiting dilution cloning selected for the expansion of a particular subset of memory cells close to maturity that were not detected by FACS directly *ex vivo*. It is clear that further investigation into T cell proliferative and functional impairment is necessary to improve the efficiency of the generation of HIV-specific T cell clones by limiting dilution in order to determine if compartmentalisation of HIV-specific T cell responses exists between the blood and the cervix.

Chapter 4

Discussion

Mucosal immunity has a fundamental role in controlling HIV-1 infection. However, advances in the understanding of HIV-specific cellular immune responses at the site of transmission in women, the genital mucosa, has been hampered primarily by the recovery of insufficient viable T cells by non-invasive procedures. In the present study this problem was circumvented by sampling from the cervical transformation zone of the female genital tract where T cells are more abundant (Pudney *et al.,* 2005), and expanding the HIV-specific T cell populations isolated from cervical cytobrush specimens.

In this study, HIV-1 Gag-specific T cell responses in blood sampled from 35 chronically HIV-infected women were assessed for IFN-γ responses to pools of peptides spanning the entire HIV-1 subtype C Gag protein (Chapter 2). Nine women with the highest frequencies of HIV-1 subtype C Gag-specific T cells were selected for T cell cloning experiments. T cell IFN-γ responses against Gag were common in these chronically HIV-infected women with 33/35 (94.3%) women having clear specificity against Gag. These responses, in accordance with previous reports (Buseyne *et al.*, 1992; Masemola *et al.*, 2002 Kiepiela *et al.,* 2007), were focused predominantly against the capsid protein p24, which is recognized to be the most conserved of Gag (Buseyne *et al.*, 1992). Furthermore, the women in the cohort were more likely to respond to only 2 of the 5 HIV Gag pools, with the targeting of p15 (pools 4 and 5) increasing in frequency with increasing number of pools targeted. No association was observed between either the magnitude of responses or the number of pools targeted and viral load as previously reported (Masemola *et al.,* 2004; Addo *et al.,* 2003; Edwards *et al.,* 2002). This was likely to be result of the limited cohort size used in this study (Kiepiela *et al.*, 2007) as well as the fact that only IFN-γ production by $CD8^+$ T cells was investigated (Betts *et al.*, 2006). Betts *et al.* (2006) reported that measurement of IFN-γ production alone is insufficient to control viremia but instead the ability of CTL to be multifunctional is associated with better control of viral replication. Recently, Kiepiela *et al.* (2007) clearly showed using measurement of IFN-γ alone that CTL specifically recognizing Gag were capable of reducing viremia but only when the cohort exceeded 400 individuals indicating the need for significantly increased populations to make this sort of association.

I show in this study that cervical T cells from HIV-infected women can be expanded both polyclonally and at the clonal level to allow more comprehensive investigations of HIV-specific T cell phenotype and function at the cervix (Chapter 3). Using 9 women with well characterized and high magnitude PBMC responses to HIV Gag, I showed that T cell

clones from the peripheral blood and cervix that were specific for Gag could be generated. All of the clones characterized were of a $CD8^+CD3^+$ T cell phenotype. However, only a minority of the clones picked from the cervix and blood produced IFN-γ in response to Gag. It is therefore possible that the consensus HIV Gag subtype C sequence used in this study, designed from data predominantly accumulated from studies in blood, may fail to elicit specific responses at the cervix. Since there is currently no evidence that HIV variants differ at key epitopes between blood and mucosal tissues it would be beneficial to compare HIV epitope-specific T cell responses between the two anatomically distinct compartments. However, considering that the yield of mucosal T cells is the limiting factor in pursuing this sort of investigation, and that in this study cervical MMC clones were consistently generated at significantly lower frequencies than the blood clones and expanded less successfully in culture I investigated potential factors contributing to slow *in vitro* growth of cervical clones.

The importance of understanding and identifying factors associated with the lack of T cell proliferation has been underscored by the recent finding that upregulation of the inhibitory receptor PD-1 (programmed cell death 1) on the surface of HIV-specific $CD8^+$ T cells is associated with T cell exhaustion and disease progression (Day *et al.*, 2006). Furthermore, the observation that the blockade of signalling pathway of PD-1 and its ligand PD-L1 results in enhanced HIV-specific $CD4^+$ and $CD8^+$ T cell function (Freeman *et al.*, 2006; Trautman *et al.*, 2006) highlighted the need to investigate the renewal of a functionally competent HIV-specific T cell repertoire in an attempt to prevent disease progression. I focused on the expression of the maturation and exhaustion marker CD57. CD57 expression on $CD8^+$ and $CD4^+$ T cells has been associated with a lack of proliferative capacity and with chronic immune activation (Brenchley *et al.*, 2003; Palmer *et al.*, 2005). Furthermore, an increase in $CD8^+CD57^+$ T cell number is frequently associated with persistent viral infections (Weekes *et al.*, 1999; Wang *et al.*, 1993), with these cells displaying a clear propensity for activation-induced apoptosis (Brenchley *et al.*, 2003). In this study, CD57 expression was significantly greater on cervical T cell clones than blood-derived T cell clones. CD57 expression was not significantly different between blood- and cervix-derived T cells isolated direct *ex vivo*, and when compared to CD57 expression of PBMC from HIV-seronegative donors. CD57 expression was only significantly upregulated on cervical T cell clones compared to cervical T cells assayed direct *ex vivo*, but not between blood-derived T cells assayed direct *ex vivo* and blood-derived T cell clones. It is likely that the difference in CD57 expression observed between the blood and cervix was as a result of differences in manipulation in vitro i.e. the initial polyclonal

expansion of cervix-derived but not blood-derived T cells before limiting dilution cloning. Furthermore, the *in vitro* expansion of cervical MMC during limiting dilution cloning selected for the expansion of a particular subset of memory cells close to maturity that were not present at high frequencies *ex vivo*. An increased level of CD57 expression on $CD8^+$ T cells at the cervix is indicative of increased immune cell replicative exhaustion at the site of transmission of HIV. Replicative senescence of CTLs has recently been associated with impairment of lytic and cytokine functions in HIV infection (Dagarag *et al.,* 2003; Yang *et al.,* 2005). Since perforin expression and proliferative capacity are associated with long-term nonprogression in HIV-1-infected individuals (Migueles *et al.,* 2002), the loss of CTL killing potential with replicative senescence raises concern about the effectiveness of cellular immunity at the female genital tract.

This is the first study to have investigated CD57 expression of cervix-derived T cells under conditions of clonal expansion and has important implications for mucosal vaccine design. The ability to mount a sustainable and effective mucosal cellular immune response to an HIV vaccine is one of the fundamental prerequisites to providing prophylactic protection against infection. This study has demonstrated that clonally expanded cervix-derived mucosal T cells directed against HIV Gag express significantly higher levels of CD57 than similarly clonally expanded blood-derived cells. Further investigation is needed to determine whether this is related to the fact that these mucosal cells were derived from women with chronic HIV infection, and is therefore related to the disease status of the donors; or whether this relates to the tissue origin of these cells present at the genital mucosal barrier.

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