Comparison of HIV-1 specific T cell immunity in the female genital tract and blood of HIV-infected women: Impact of *in vitro* T cell expansion on HIV-specific T cell specificity, maturational status and functional complexity

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

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June 2010

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DEDICATION

This thesis is dedicated to Prof. D.S and Mrs S Tevera for being my inspiration and my source of encouragement throughout this work and beyond. Thank you for your faith in me even when I lost confidence, for giving me a home away from home, for your sincere love and all that you have been and always will be.

To My Role Models, My Mentors

This is it Vazaya!!

ACKNOWLEDGEMENTS

My sincere gratitude goes to my supervisor, Dr Jo-Ann Passmore for her absolute commitment in ensuring that I reach my goals. I am appreciative of the effort, support, inspiration, and endless patience she put into grooming me into a young scientist. To my co-supervisor, Dr Wendy Burgers, I thank you for your willingness to supervise, encouragement and advice on flow cytometry work. In addition, my heart goes out to all the women from the Nyanga Day Hospital who kindly participated in the study, Prof. Lynette Denny and Sister Janine Jones for facilitating the recruitment of study participants and collection of necessary specimens used in this study. I gratefully acknowledge the provision of recombinant human IL-2 and HIV subtype C Gag peptides by the NIH AIDS Research and Reference Reagent Program.

I am also indebted to Prof. Barbra Shacklett for hosting me in her Lab during my visit to the University of California, Timothy Hayes and Bridget McLaughlin for being extremely helpful with LSRII training. I would also like to thank the SATVI group for allowing me to use their LSRII machine, in particular Associate Professor Willem Hanekom and Nazma Manson.

To my colleagues in the HIV immunology group, thank you for all your support; Sister Phume and Nono for their constructive criticism, Prinola for helping me with flow cytometry panel optimization, Lindi for assisting me with statistical analysis and Cobus for helping me edit my work. Agano, Amy and Shameem, thanks for the much needed destruction, you were such good friends. Hoyam and Tracey, for being exceptionally helpful in the Lab and assisting with ELISpot assays. Many thanks to Lenine and Lycias for holding my hand throughout this whole PhD journey, we came a long way and I love you guys!

I would like to thank my family and friends who were there to support me when I most needed it. To my wonderful parents, thank you for your incessant prayers and for believing in me. My brothers and sisters, you know what we have been through and what this means, I love you all and I know that even “Mai Pati’ is very proud in her resting place. In my heart you will always remain. To Gamuchirai Madzima, thanks for giving me added strength, hope and encouragement throughout, even in tough times. Bhuru you know the “history”, thanks for being a little brother. Chimaini “Vee Vee”, thanks for disturbing me and to Delgado.... Zhelele for life.

Lastly and most important, I would like to thank the Lord for giving me hope and the strength to face each day.

This work was supported in part by grants from the Welcome Trust, Poliomyelitis Research Foundation (PRF), The Medical Research Council of South Africa (MRC), University of Cape Town (UCT), South African vaccine initiative (SAAVI), Doris Duke Charitable Foundation HIV Pathogenesis Program (HPP), Columbia University-Southern African Fogarty AITRP Program and The Beit Student Fund.

LIST OF ABBREVIATIONS

ACD	Acid citrate dextrose
AIDS	Acquired Immune Deficiency Syndrome
ARV	Antiretroviral drug
ART	Antiretroviral therapy
APC	Allophycocyanin
APCs	Antigen presenting cells
APC-H7	Allophycocyanin-H7
CCR4	Chemokine receptor 4
CCR5	Chemokine receptor 5
CCR7	Chemokine receptor 7
CCL19	Chemokine ligand 19
CCR10	Chemokine receptor 10
CD	Cluster of differentiation
CLR	C-type lectin receptor
CMCs	Cervical Mononuclear Cells
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CVL	Cervicovaginal lavage
DC-SIGN	Dendritic cell-specific-Inter-cellular adhesion molecule 3-grabbing non integrin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
Env	Envelope glycoprotein
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothyocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
FSC-H	Forward scatter-height
Gag	Group specific antigen

GALT	Gut-associated lymphoid tissue
HAB	Human AB serum
HAART	Highly active antiretroviral therapy
HEPS	Highly exposed persistently seronegative
HIV-1	Human Immunodeficiency type 1
HRP	Horse-radish peroxidase
HSV-2	Herpes simplex virus type 2
h	Hour
ICAM3	Intercellular adhesion molecule 3
ICS	Intracellular cytokine staining
IL	Interleukin
IQR	Interquartile range
LCMV	Lymphocytic Choriomeningitis Virus
LTNP	Long-term nonprogressor
LT-1	Liable toxin-1
MALT	Mucosa-associated lymphoid tissue
MFI	Median fluorescent intensity
MHC	Major Histocompatibility Complex
MIP-1β	Macrophage inflammatory protein 1 beta
ml	Millilitre
NK	Natural killer
NICD	National Institute for Communicable Diseases
nm	Nanometer
PBMC s	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PE	Phycoerythrin
PerCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
PID	Patient identity
PMA	Phorbol myristate acetate
Qdot	Quantam dot
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
RNA	Ribonucleic acid
RPMI	Rosewell Park Memoral Institute Medium
SDF	Secreted cell derived factor

SEB	Stapylococcal enterotoxin B
SFU	Spot forming units
SHIV	Simian Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SLPI	Secretory leukocyte protease inhibitor
SSC	Side scatter
STI	Sexually transmitted infection
TCR	T cell receptor
TNF- α	Tumour necrosis factor alpha
USA	United States of America
Vivid	Violet-fluorescent reactive dye
Vpr	Viral protein R
µg	Microgram
µl	Microlitre
^{0}C	Degrees Celsius

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Abstract

Background. Previous studies have detected HIV-specific T cells in the female genital tract of chronically HIV-infected and highly exposed but persistently seronegative (HEPS) women using cells isolated by cervical cytobrushing. A major limitation of these studies has been the low cell yields obtainable by cervical mucosal sampling from the female genital tract. There have been few studies exploring the utility of *in vitro* expansion of T cells derived from the female genital tract to overcome these limitations. The aim of this study was to investigate the feasibility of polyclonal *in vitro* expansion to improve yields of T cells recoverable from cervical cytobrush samples during HIV infection; to characterize the impact of *in vitro* expansion on the frequency, specificity and quality of cervical mucosal T cell responses pre- and post-expansion; and to compare these cervical responses with responses detected in blood.

Methods. Cervical cytobrush-derived and blood-derived T cells from 68 chronically HIV-infected women were examined either *ex vivo* (n=5) or following *in vitro* expansion (n=63) for the magnitude, epitope targeting, maturational status and polyfunctionality in response to stimulation with HIV Gag Du422 peptides. Seven different expansion methods were compared, including anti-CD3, anti-CD3 in combination with anti-CD28, or Dynal anti-CD3/CD28 beads at differing bead to target cell ratios (3:1, 1:1, 1:5) in the presence of varying combinations of growth promoting cytokines (IL-2, IL-7 and IL-15). Initial experiments focused on measuring HIV-specific responses by IFN-γ ELISPOT while later experiments used polychromatic flow cytometry to simultaneously evaluate four T cell functions (CD107a, IFN-γ, TNF-α, and MIP-1β). T cell maturational status pre- and post-expansion was determined by differential staining with CD45RO, CCR7 and CD27. Functional HIV-specific T cell responses in blood and in the female genital tract before and after *in vitro* expansion were compared to the HIV clinical status of the women (CD4 counts in blood and plasma viral loads).

Results. This study found that T cells derived from the female genital tract of HIV-infected women were predominantly effector memory in phenotype, which have limited proliferative but rapid effector function capacity. Despite this, i*n vitro* expansion of cytobrush-derived T cells was achieved in 54/63 (85.7%) of HIV-infected women. Of the 7 alternative expansion methods compared, Dynal beads (1:1) in the presence of IL-2, IL-7 and IL-15 resulted in the highest yields compared to *ex vivo* in both blood (7.1-fold) and at the cervix (5.6-fold) but resulted in an accumulation of central memory T cells following expansion. In contrast, anti-CD3 in the presence of IL-2 resulted in conservation of effector memory dominated T cell memory profile following expansion but yielded significantly fewer cervical T cells than Dynal bead expansion (1.9-fold lower than Dynal). Following anti-CD3 expansion, HIV Gag-specific cervical T cell

responses were detected in mucosal T cell lines from HIV-infected women and both the magnitude ($p=0.002$; $R=0.7$) and specific HIV Gag regions targeted by cervical T cell lines ($p<0.0001$; $R=0.5$) correlated significantly with those detected in blood. Similarly, following Dynal bead expansion, the functional profile (CD107a, IFN-γ and TNF-α) in response to Gag stimulation by CD8+ T cells in blood and at the cervix correlated significantly ($p<0.05$). While the majority of Gag-specific T cell responses in the female genital tract and blood were mono-function, low frequencies of HIV Gag-specific polyfunctional (median of all 2+, 3+ and 4+ responses) CD8+ T cells were detected at the cervix (median net frequency of 1.96%) and blood (median net frequency of 1.08%) of HIV-infected women and the frequency of polyfunctional CD8+ T cell responses were significantly associated between blood and cervix, irrespective of expansion methods tested ($p<0.05$). A significant inverse relationship was observed between plasma viral load and CD8+ T cells producing CD107a in fresh blood, expanded blood and expanded cervical lines ($p<0.05$). In addition, the polyfunctionality of cervical Gag-specific CD8+ T-cells was positively related to blood CD4 count ($p=0.03$) and inversely related to plasma viral load ($p=0.04$).

Conclusion. This study shows that HIV-specific cervical T cells can be isolated by cytobrushing and *in vitro* polyclonal expansion is a useful approach to increase the number of T cells available from mucosal sites. Dynal beads (1:1) in the presence of IL-2, IL-7 and IL-15 resulted in the best yields of cervical T cells while anti-CD3 in the presence of IL-2 best conserved the ex vivo T cell profile. Expanded T cell lines, irrespective of expansion method used, generally maintain their cytokine response profile to HIV antigens. This study shows that HIV Gag-specific blood and cervical T cells were largely mono-functional with polyfunctional T cells being detected in women with high blood CD4 count and low plasma viral load. Most importantly, this study confirms that HIV-specific Gag T cell responses detected in the polyclonal expanded female genital tract T cells are associated with those measured in blood during HIV infection. Studies of mucosal T cell responses are technically challenging and an important contribution of the present study is the development and comparison of *in vitro* expansion methods to improve the quality of the assessment of these mucosal T cell responses.

This study has resulted in the following publication:

Bere, A., Denny, L., Burgers, W.A. and others. 2010. Polyclonal expansion of cervical cytobrush-derived T cells to investigate HIV-specific responses in the female genital tract. *Immunology.* 130(1):23-33.

Bere, A., Denny, L., Hanekom, W. and others. 2010. Comparison of polyclonal expansion methods to improve the recovery of cervical cytobrush-derived T cells from the female genital tract of HIV-infected women. *Journal of immunological methods.* 354(1-2):68-79.

CHAPTER 1

LITERATURE REVIEW

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1.1 The HIV pandemic

Acquired immunodeficiency syndrome (AIDS), discovered more than twenty five years ago, has caused an estimated 25 million deaths worldwide (UNAIDS, 2008). AIDS is caused by the Human Immunodeficiency Virus type 1(HIV). Recent estimates suggest that nearly 33 million people are infected worldwide (UNAIDS, 2008). More than 90% of new HIV infections occur in developing countries, with Sub-Saharan Africa being the most highly impacted region (Figure1.1). Furthermore, approximately 67% of all people living with HIV are situated in this sub-region and more than 75% of all AIDS deaths in 2007 occurred here (UNAIDS, 2008). Sexual transmission of HIV accounts for the majority of new infections worldwide, with young women being at a higher risk of getting infected than young men (UNAIDS, 2008).

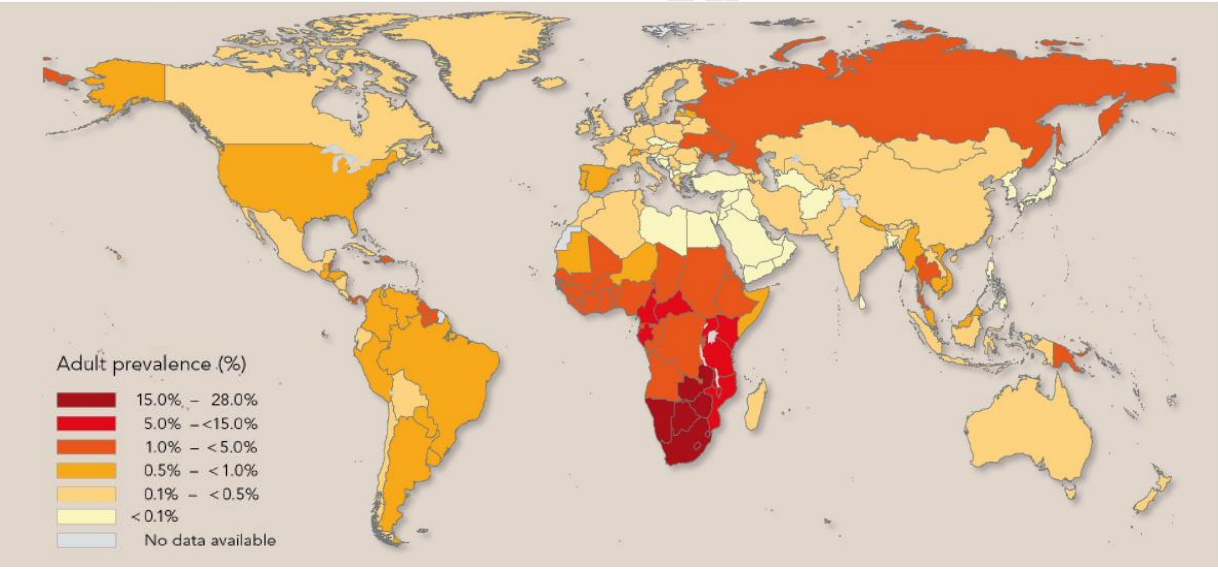


Figure 1.1. The global view of adults and children living with HIV around the world (UNAIDS, 2008). It is estimated that 33 million people (30-36 million) were living with HIV in 2007. Sub-Saharan Africa is the most impacted region (Taken from UNAIDS, 2008)

1.1.1 The HIV burden in Sub-Saharan Africa

Although Sub-Saharan Africa generally has the highest HIV disease burden internationally (Figure 1.1), epidemics within Sub-Saharan African countries differ considerably from country to country in terms of prevalence and mode of transmission (Figure 1.2). The region as a whole has the highest rates of heterosexual

transmissions of HIV, although injectable drug use is an increasingly important factor in some of the HIV epidemics in sub-Saharan Africa (including those in Kenya, Mauritius, South Africa and the United Republic of Tanzania). Mother to child transmission remains the epidemic's driving force in children in these regions (UNAIDS, 2008) and an estimated 90% of children (younger than 15 years) in Sub-Saharan Africa became infected with HIV in 2007 either during pregnancy, birth or breastfeeding.

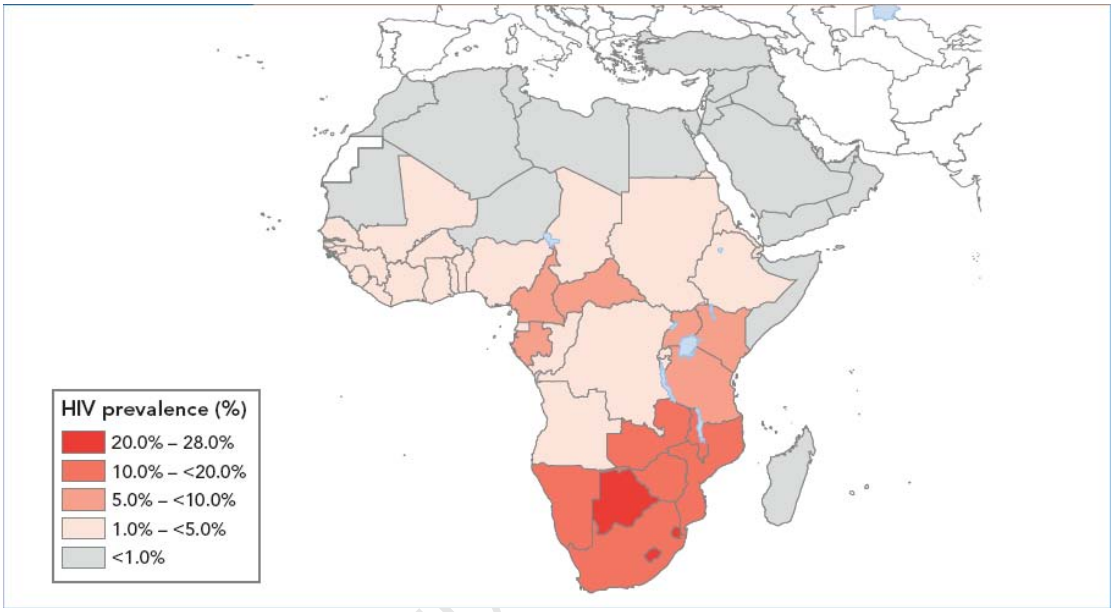


Figure 1.2. The estimated HIV prevalence in adults (15-49 years). Sub-Saharan Africa generally has the highest HIV disease burden (taken from UNAIDS, 2008).

1.1.2 The HIV burden in South Africa

South Africa currently has the largest number of people living with HIV in the world. It is estimated that 5.7 million South Africans lived with HIV in 2007 (representing about 10% of the South African population) and an estimated 1.8 million people have died of AIDS-related disease since the epidemic began (UNAIDS, 2008). In South Africa, HIV is disproportionately associated with lower-income areas with 29% of people living with HIV residing in urban informal settlements despite only 9% of the South African population aged 2 years and older living in these areas (UNAIDS, 2008). Young women aged between 15–24 years in particular in South Africa face greater risks of becoming infected than men, accounting for about 90% of new HIV

infections among this age group. HIV incidence among 20–29 year old women in 2005 was approximately 5.6%, which is more than six times higher than for men in the same age group (0.9%) (UNAIDS, 2008). Currently, the HIV prevalence in South Africa in women attending government antenal clinics is stabilizing, with prevalence rates of 30% being recorded in 2005 compared to 29% in 2006 (UNAIDS, 2008). There was a decrease in the percentage of young pregnant women (15–24 years) found to be infected with HIV, suggesting a possible decline in the annual number of new infections. The drop in prevalence rate among adolescents in South Africa might be due to large scale information campaigns and condom distribution nationally, although there are huge concerns about the consistently high and rising prevalence among older antenatal clinic attendees (UNAIDS, 2008).

The identification of biological cofactors that promote or inhibit transmission of HIV in the female genital tract and understanding the modes of viral dissemination to blood is fundamental since it will provide insight into the development of an effective vaccine or microbicide against this mucosally acquired infection.

1.2 HIV Genetic diversity and variability

There is a huge genetic diversity of HIV. Phylogenetic analysis reveals that two genetically different types of HIV exist and these are HIV-1 and HIV-2 (Robertson *et al*., 2000). HIV-1 is classified into three genetic groups, namely a major group (group M), an outlier group (group O) and a non-M/non-O group (group N) (Robertson *et al*., 2000). HIV-1 group M, which is responsible for the global pandemic, is further subdivided into nine different genetic subtypes or clades designated by the letters from A-D, F-H, J and K (Robertson *et al.*, 2000). These different subtypes show regional distribution, raising the question whether regional subtype-matched vaccines are necessary. It has been shown that strains belonging to the same subtype can differ by up to 20% in the *env* gene and inter-subtype genetic distances can reach up to 35% (Shankarappa *et al*., 1999). HIV is continuously evolving, and factors such as mutations and recombination causing the epidemic to become more complex.

1.2.1 HIV-1 variability

There is a mixture of closely related yet genetically distinct genomes within an individual and these are referred to as quasispecies (Eigen, 1993; 1996). HIV-1 strains within an individual can differ by as much as 10% in sequence (Shankarappa *et al.*, 1999), while the intersubtype distances can be as much as 35% in the *env* gene. There are two mechanisms that result in the vast variability of HIV-1 and these are mutation and recombination. The high variation among gene sequences of HIV-1 strains belonging to different lineages is mainly caused by high mutation, recombination and replication rates, as well as host immune pressure.

1.2.2 Mutation

HIV has an enormous evolutionary capability due to a high rate of point mutations, namely $3x10^{-5}$ substitutions per site per generation (Mansky and Temin, 1995). The high error rate of HIV RT results in misincorporation of nucleotides and is a major source of mutations throughout the viral genome and a determinant for rapid viral evolution (Ji *et al*., 1994; Ji and Loeb, 1994). Rapid evolution of HIV-1 has been shown to correlate with rapid disease progression (Mikhail *et al.*, 2005; Mullins & Jensen, 2006), although it is not clear whether the virus is evolving due to high viral loads or whether it is the evolution resulting in loss of control that lead to high viral loads.

1.2.3 Recombination

Recombination occurs frequently and a circulating recombinant form (CRF) is a virus that carries sections of two or more subtypes in a mosaic genome (Fang *et al*., 2004). A recombinant lineage is designated a CRF when related forms are found in multiple epidemiologically unlinked individuals. Recombination occurs at an average rate of 3 events per genome per round of replication, ranging from 2-20 crossovers (Hu & Temin, 1990). Other studies have reported that recombination rates of 2.8 per genome per viral replication cycle (Zhuang et al., 2002). Detectable recombination requires infection with more than one virus at the cellular level. Recombination can occur between viruses of the same subtype (intra-subtype) (Philpott *et al*., 2005) or viruses of different subtypes (inter-subtype). Inter-subtype recombination is a result of the co-packaging of viral genomes from two genetically distinct viruses into a single viral particle in the next cycle of infection. Intra-subtype recombination has been found in

47% of sequences in a subtype C epidemic (Rousseau *et al*., 2007). Recombination is most frequently identified when it is between subtypes (Charpentier *et al.*, 2006; Yirrell *et al.*, 2002).

1.2.4 Host immune selection pressure

One of the major challenges facing the development of an effective vaccine is the extensive global diversity of HIV. Host immune pressure and availability of target cells are both factors driving variability of the virus**.** The rapid turnover of HIV-1 is also a contributor, as HIV-1 has been shown to produce 10^8 to 10^9 virions per day (Ho *et al.*, 1995; Wei *et al.*, 1995), although it is estimated that 27-66% are defective (Sanchez *et al.*, 1997). Previous studies have suggested that HIV shows stronger effects of positive Darwinian selection (a phenomenon whereby there is a selective pressure forcing change) than any other organism studied so far (Rambaut *et al.*, 2004). There is evidence of positive selection exerted both by neutralizing antibodies (Greenier *et al.*, 2005; Richman *et al.*, 2003) and by the host immune cellular immune response (Allen *et al*., 2000; Barouch *et al*., 2005; Borrow *et al*., 1997). The role of immune selection pressure in driving the global sequence variability of HIV is evident in studies on the adaptation of HIV-1 to CD8+ T cell responses at the population level (Moore *et al*., 2002). However, the level of this sequence variability is different for different HIV genes and this is because certain mutations in some genes such as *gag*, a structural gene, pose a fitness cost to the virus. In support to this, there are studies which have shown the reversion of HIV-1 virus to wild type when transmitted to HLA mismatched individuals. This suggests a cost to the viral replicative capacity that may be incurred by acquisition of the relevant escape mutation (Barouch *et al.*, 2005; Fernandez *et al.*, 2005; Matano *et al.*, 2004). Conversely, the *env* gene is a structural gene but there is rapid evolution which is noticeably associated with strong autologous neutralizing antibody responses (Liang *et al*., 2003; Price *et al*., 1997; Wei *et al*., 2003). Consequently, greater variability of HIV is formed when the virus is trying to escape from immune pressure. The more the viral regions targeted by HIV-specific T cells, the more the virus will mutate in trying to escape from the immune pressure leading to more variability of the virus within an individual as well as within populations when these variants are transmitted.

1.2.5 Availability of target cells

HIV has been shown to predominately use the CCR5 coreceptor during early stages of infection (Connor et al., 1997) and virus which utilises this coreceptor are referred to as the R5 virus. However, during the late stages of infection, the virus switches to shows increased tropism of CXCR4 and this phenotypic switch has been shown to coincides with the first immunological and clinical signs of disease progression (Connor et al., 1997). The switch from X5 to X4 and the availability of different cells expressing receptors for HIV-1 infection are evidence for genetic variation (Overbaugh and Bangham, 2001). However in some individuals, virus may attain dual tropism characteristics, with X4 viruses still retaining their ability to use CXCR5 coreceptor (Doms and Peiper, 1997).

1.3 HIV-specific T cell responses in blood

HIV-1 specific CD4+ T helper (Th) cells and CD8+ cytotoxic T cells (CTLs) have been shown to play an important role in the immune control of HIV (Brander and Walker, 1999; Goulder et al., 1999; McMichael and Rowland-Jones, 2001). The magnitude and frequency of HIV-specific CD8+ T cells are greater than CD4+ T cells (Betts et al., 2001; Ramduth et al., 2005). Despite this, CD4+ T cells are important in initiating functional CD8+ T cell responses as well as for the development of effective CD8+ memory responses (Janssen et al., 2003; Sun et al., 2004) in both mouse studies (Janssen *et al*., 2003; Sun *et al*., 2004) and studies in HIV infection (Lichterfeld *et al*., 2004). This is mainly because CD4+ T cells primarily secrete IL-2 cells upon antigen recognition (Lichterfeld *et al*., 2004), which is a growth factor for antigen-specific cells, and is responsible for their proliferation.

T cells are also important in secreting cytokines such as IFN-γ, an antiviral cytokine and has been implicated with control of diseases. IFN-γ is a T_H 1 cytokine that is key for cell-mediated immunity (as reviewed in Schroder *et al*., 2004). It is an antiviral cytokine that induces a number of anti-viral proteins (such as Mx, dsRNA protein kinase, ribonucleic acid nuclease, and nitric oxide), which in turn inhibit viral replication, degrade single stranded RNA, and inhibit protein synthesis (as reviewed in Chesler *et al*., 2002). T cells have also been implicated in TNF-α production,

which is required for the recruitment of neutrophils and monocytes, while MIP-1β is a chemokine, and is responsible for the recruitment of lymphocytes and monocytes (as reviewed in Clore and Gronenborn, 1995). MIP-1β binds the HIV co-receptor CCR5, thus may compete with HIV for binding to $CD4^+$ T cells expressing this molecule. CD107a is a surrogate marker for cytotoxicity. During the degranulation process, CD107a is transiently expressed on the inner membrane of the cytotoxic granule (Suni *et al*., 2005), which then becomes exposed during degranulation and it can be detected on the surface of the cell (Betts *et al.,* 2003).

Primary HIV infection is associated with a rise in HIV viremia and a decline of CD4+ T cells in the peripheral blood (Borrow et a., 1994; Koup et al., 1994; Borrow et al., 1997). The decline in numbers of circulating CD4+ T cells (lymphopenia) is due to both HIV-mediated cell killing and to re-trafficking of cells to the lymphoid tissues and other organs (Fauci, 1993a). Following the emergence of HIV-specific CD8+ CTLs, the plasma viral load subsequently falls to a level of steady-state (set point) and the level of this is believed to predict the rate of disease progression to AIDS (Fauci, 1993a). A period of clinical latency follows after primary infection and, during this period, the immune system slowly deteriorates due to continuous HIV viral replication (Pantaleo *et al*., 1993a). The correlation between blood CD4 T cell loss and the emergence of opportunistic infections is a well-defined indicator used for many years by clinicians as a measure of disease progression in HIV infected individuals (Egger et al, 2002). However, recent findings have suggested that measuring CD4 counts in blood alone may not be representative of the rapid and severe effect of HIV observed on the immune system (Brenchley et al, 2004, Douek et al, 2003). HIV disease, however, is not uniformly expressed in all individuals and small proportions of individuals infected with the virus develop AIDS and die within months following primary infection (Mellors, 1997). The rate of disease progression is affected by host and viral factors such as virulence of the individual strain of virus, host genetics such as human leukocyte antigen (HLA) alleles, host immune response and co-infection with other viruses (Pantaleo *et al*., 1993a; Fauci, 1993a).

1.3.1 Evidence for the role of protective T cell immunity in blood

Evidence for the importance of HIV-specific CTL responses in the control of HIV come from acute and longitudinal studies in humans (Klein et al., 1995, Pantaleo et

al., 1995, Pitcher et al., 1999, Rosenberg et al., 1997) and primate models of HIV infection (Jin et al., 1999; Metzner et al., 2000; Schmitz et al., 1999; Kuroda et al., 1999). In rhesus macaques, depletion of CD8+ T cells resulted in viral re-emergence in animals that were previously controlling simian immunodeficiency virus (SIV) infection (Jin et al., 1999; Metzner et al., 2000; Schmitz et al., 1999). The magnitude of anti-HIV CTL responses correlated significantly with control of HIV in humans (Kalams et al., 1999; Rosenberg et al., 1997) and SIV in macaques (Hel et al., 2002). Furthermore, several studies have shown that immunologic pressure mediated by SIV- and HIV-specific CTL often resulted in HIV escape mutations in the HIV epitopes being targeted (Goulder et al., 1997; 2001; Price et al., 1997; Leslie et al., 2004; Chopera et al., 2008). The emergence of HIV-specific CD8+ T cells in acute untreated individuals was found to be temporally associated with the initial decrease of viremia and inhibition of viral replication (Koup et al., 1994; Borrow et al., 1994; Yang et al., 1997). In addition, robust HIV-specific CTL responses with enhanced proliferative capacity have been reported in long term non-progressors compared to individuals with a more typical progression (Klein et al., 1995, Pantaleo et al., 1995, Pitcher et al., 1999, Rosenberg et al., 1997). The expression of certain HLA class I alleles has been strongly correlated with lack of escape and non-progressive HIV infection (Goulder et al., 1997; Migueles et al., 2000; Altfeld et al., 2003). In macaques, current HIV vaccine strategies which induce high frequencies of anti-viral CD8+ T cells have been shown to be effective in the control of pathogenic SHIV (Amara et al., 2001; Casimiro et al., 2005; Shiver et al., 2002) and SIV challenges (Johnson et al., 2005; Wilson et al., 2006).

Most HIV-infected individuals, however, progress to AIDS in the absence of effective antiretroviral therapy despite strong CTL responses (McMichael and Rowland-Jones, 2001). This has been partially attributed to the impairment of CD4+ T helper cell function during chronic HIV-infection (Rosenberg et al., 2000) and exhaustion of CD8+ CTL (Appay et al., 2000; Wherry et al., 2003; Brenchley et al., 2003). These studies point to the idea that vaccines that stimulate T helper and CTL responses to HIV may be able to control the virus early in infection, thus increasing the chances of delaying disease progression.

1.3.2 Magnitude and breadth of Gag-specific T cell responses

Studies have shown that HIV-specific T cell responses against all HIV proteins can be detected in blood from HIV-infected individuals, although Gag and Nef are the most commonly recognized by both CD4+ and CD8+ T cells (Betts et al., 2001; Ramduth et al., 2005; Addo et al., 2003; Kaufmann et al., 2004; Zuniga et al., 2006). Of all HIV proteins, Gag in particular (especially the p24 region) is highly conserved by HIV and important in the packaging of the capsid (McMichael and Rowland-Jones, 2001). The immunodominance of Gag-specific CD4+ and CD8+ T cells has been documented in both subtype C and B HIV infections (Kiepiela et al., 2007; Addo et al., 2003; Kaufmann et al., 2004).

There has been some controversy surrounding the direct role of HIV-specific T cells in the control of HIV. HIV-specific CD8+ T cell responses that preferentially target Gag have been associated with better control of HIV (Ramduth et al., 2005; Masemola et al., 2004; Zuniga et al., 2006), in particular the p24 region of Gag (Zuniga et al., 2006). No correlation was, however, found between the magnitude and breadth of total HIV-specific T cell responses (including the whole genome) and viral load (Ramduth et al., 2005; Masemola et al., 2004; Zuniga et al., 2006). Conversely, studies in HIV-infected untreated individuals reported a positive correlation between total magnitude of HIV-specific T cell responses and viral load indicating that generally the magnitude of T cell responses to HIV were tracking viraemia and that the magnitude and breadth of total HIV-specific T cells might only be a reflection of high antigen load (Masemola et al., 2004; Betts et al., 2001). Furthermore, Gray et al. (2009) found that the magnitude of early HIV-specific T cell IFN-γ responses did not play a role in determining viral set-point at 12 months post infection. A longitudinal study conducted on chronically HIV-infected therapy naive individuals showed that Gag-specific IFN-γ responses declined over time (Geldmacher et al., 2007b). This observation is mainly as a result of T cell exhaustion due to extended exposure of the immune system to high antigenic loads.

These studies confirm the importance of measuring T responses against highly conserved Gag regions which may affect replicative capacity of the virus (Leslie et al., 2004; Fernandez et al., 2005; Martinez-Picado et al., 2006; Prado et al., 2009).

Because Gag is a structural protein, it is likely that few mutations will be compatible with viable progeny with the result that the virus cannot mutate to escape immune pressure (Yusim et al., 2002). Because there are so many conflicting studies on the association between the magnitude and breadth of IFN-γ T cell responses to HIV and markers of HIV disease progression (viral load and CD4+ counts), it is likely that control of HIV infection cannot be explained by measuring IFN-γ responses alone. The importance of Gag p24 T cell responses in blood in the control of viremia suggests that this region of HIV might be the most attractive region to focus research on and include in a vaccine candidate.

1.3.3 Polyfunctional T cell responses in HIV

Because measurement of IFN-γ alone (as a correlate of protection against HIV) yielded conflicting results, there is an urgent need for identification of immunological markers associated with protection against HIV. T cells are capable of secreting a broad range of cytokines and chemokines and T cells capable of simultaneous secretion of multiple cytokines are referred to as ‚polyfunctional' (Betts et al, 2006). In addition to being able to secrete multiple cytokines and exhibit multiple functions, polyfunctional T cells have been shown to produce more of each individual cytokine per cell than a monofunctional cell (Precopio et al., 2007; Darrah et al., 2007; Duvall et al., 2008). Several recent studies have shown a significant association between the frequency of HIV-specific polyfunctional CD4+ and CD8+ T cells and control of HIV (Boaz et al., 2002; Kannanganat et al., 2007b; Betts et al., 2006; Almeida et al., 2007).

The association between polyfunctional CD4+ T cells to HIV and long-term survival was first demonstrated by Boaz et al. (2002) and later by Kannanganat et al. (2007b). Betts et al. (2006) found that long-term non-progressors had significantly higher frequencies of polyclonal CD8+ T cells compared to chronically HIV-infected individuals, characterized by simultaneous expression of IFN-γ, TNF-α, IL-2, MIP-1β and CD107a. Similarly, the control of HIV-1 replication in individuals who were HLA-B27+ (an HLA allele associated with long-term survival) was associated with the ability of B27-HIV-specific CD8+ T-cells to accumulate multiple effector functions (Almeida et al., 2007).

The cause and effect relationship between polyfunctional T cell responses and control of viraemia remains to be determined however. Some have argued that association obtained between polyfuction and HIV control is a consequence rather than the cause of lower viral loads (Rehr et al., 2008; Streeck et al., 2008). In support of this, in HIV-infected individuals who had initiated anti-retroviral therapy (ART), immune reconstitution in the absence of high levels of viraemia was associated with the emergence of polyfunctional CD4+ T cells producing both IL-2 and TNF-α 3 months after therapy started (Rehr et al., 2008). Streeck et al (2008) similarly reported partial restoration of T cell functionality in HIV-infected individuals initiating ART (Streeck et al., 2008a). In this study, a switch from polyfunctionality to monofunctionality was noticed after primary HIV-infection which was absent in CTL responses to other viral infections such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Influenza (Streeck et al., 2008a). It has therefore been proposed that impairment in T cell polyfunction may be a consequence of ongoing viral replication from acute to chronic HIV infection (Streeck et al., 2008a; 2008b).

In vaccine studies, rhesus macaques immunized with live attenuated chimeric virus between SIV and HIV (SHIV) 89.6 and challenged with SIVmac239 mounted polyfunctional SIV-specific CD8+ T cells (Genesca et al., 2007; Sun et al., 2008). Recently, HIV candidate vaccines based on recombinant DNA and the poxvirus vector NYVAC (Harari et al., 2008) and DNA prime formulation (5-valent env and monovalent *gag*) followed by a 5-valent Env protein boost (Bansal et al., 2008) have been shown to elicit both CD4+ and CD8+ polyfunctional responses against the inserts but protection against infection has not been assessed. The finding that T cell polyfunction may be an important correlate of protection against HIV has emphasized the importance of the quality rather than quantity of responses generated (Seder et al., 2008). The importance of polyfunctional T cells has also been documented in other infections (Precopio et al., 2007; Darrah et al., 2008). In humans, immunization with recombinant vaccinia virus generated polyfunctional virus-specific CD8+ T-cells (Precopio et al., 2007) and mice challenged with leishmania polyprotein induced Th type 1 cells that were able to produce multiple cytokines (Darrah et al., 2007).

1.4 T cell differentiation during chronic HIV infections

Robust CD8 T-cell responses have been observed in humans during the acute phase of HIV infection (Pantaleo et al., 1997) and in other viral infections such as EBV (Callan et al., 1998). It is estimated that at the peak of T cell clonal expansion, antigen-specific CD8 T cells can divide approximately every 6 to 8 hours with an estimated 10^4- to 10^5-fold expansion in humans (Wherry and Ahmed, 2004). Along with this dramatic proliferation, CD8 T cells also undergo activation and differentiation. A summary of CD8+ T cells differentiation in response to an acute viral infection (where virus is cleared) is shown on Figure 1.3.

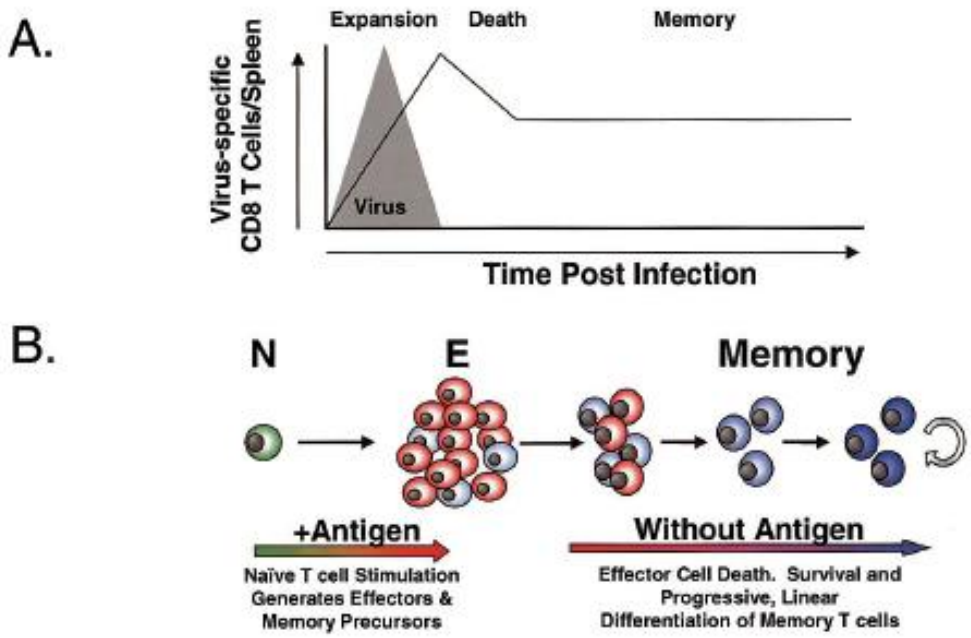


Figure 1.3. The dynamics of a CD8 T-cell response to acute viral infection. (**A)** CD8 T-cell response to an acute viral infection undergoes an expansion phase, resulting in the generation of effector CD8 T cells and viral clearance. The expansion phase is followed by a death phase, when 90 to 95% of the effector T cells die. The surviving effector CD8 T-cell pool further differentiates and generates a memory T-cell population that is maintained long term in the absence of antigen. **(B)** Memory CD8 T-cell generation following a linear model of differentiation. Antigenic stimulation causes naïve CD8 T cells to proliferate and acquire effector functions. The effector T cells that survive the death phase further differentiate, giving rise to memory T cells that continue to differentiate in the absence of antigen and acquire the ability to persist in the absence of antigen via homeostatic turnover (Taken from Wherry and Ahmed., 2004)

1.4.1 Generation and Characteristics of Memory T Cells

It was previously thought that the differentiation of memory T cells is through a linear differentiation model where memory cells are derived from effector T cells (Wherry and Ahmed., 2004., Ahmed et al., 2009). This assumption was largely based on the idea that differentiation is a unidirectional process and that T cells that are well differentiated are unlikely to revert to a less differentiated state (Ahmed et al., 2009). In the linear differentiation model, T cells either become senescent terminally differentiated T cells, which die by apoptosis soon after the pathogen is clear, or differentiate into effector memory T cells that express low levels of CD62L (also known as L-selectin), do not express CC-chemokine receptor 7 (CCR7) or home to lymphoid tissues, and are replication incompetent. These cells can give rise to long-lived central memory T (TCM) cells that express CCR7 and CD62L, and undergo homeostatic proliferation in lymphoid tissues. This clonal burst of naïve cells is largely driven by the strength of signals that a naïve T cell receives via the T cell receptor (TCR) together with co-stimulatory molecule CD28 (Gourley et al., 2004; Tan et al., 2001). However, it is possible that the less differentiated T cells might represent a divergent pathway of differentiation (bifurcative differentiation). This model proposes that following antigen recognition, one T cell can give rise to two daughter cells with different differentiation fates: the distal daughter cell gives rise to TCM cells and the proximal daughter cell gives rise to TEM cells and effector T cells, which undergo apoptosis following pathogen clearance. This type of differentiation could be achieved through asymmetric cell division or other mechanisms capable of generating heterogeneity. A third possible model (referred to here as the self-renewing effector model) suggests that a naive T cell can develop into a TCM cell or an effector T cell that can self-renew. These cells can home to lymphoid tissues and undergo homeostatic proliferation. It is proposed that these cells give rise to TEM cells that can migrate to sites of infection but do not self-renew. Senescent terminally differentiated effector T cells are proposed to derive from these TEM cells. APC, antigen presenting cell; TCR, T cell receptor (Taken from Ahmed et al., 2009)

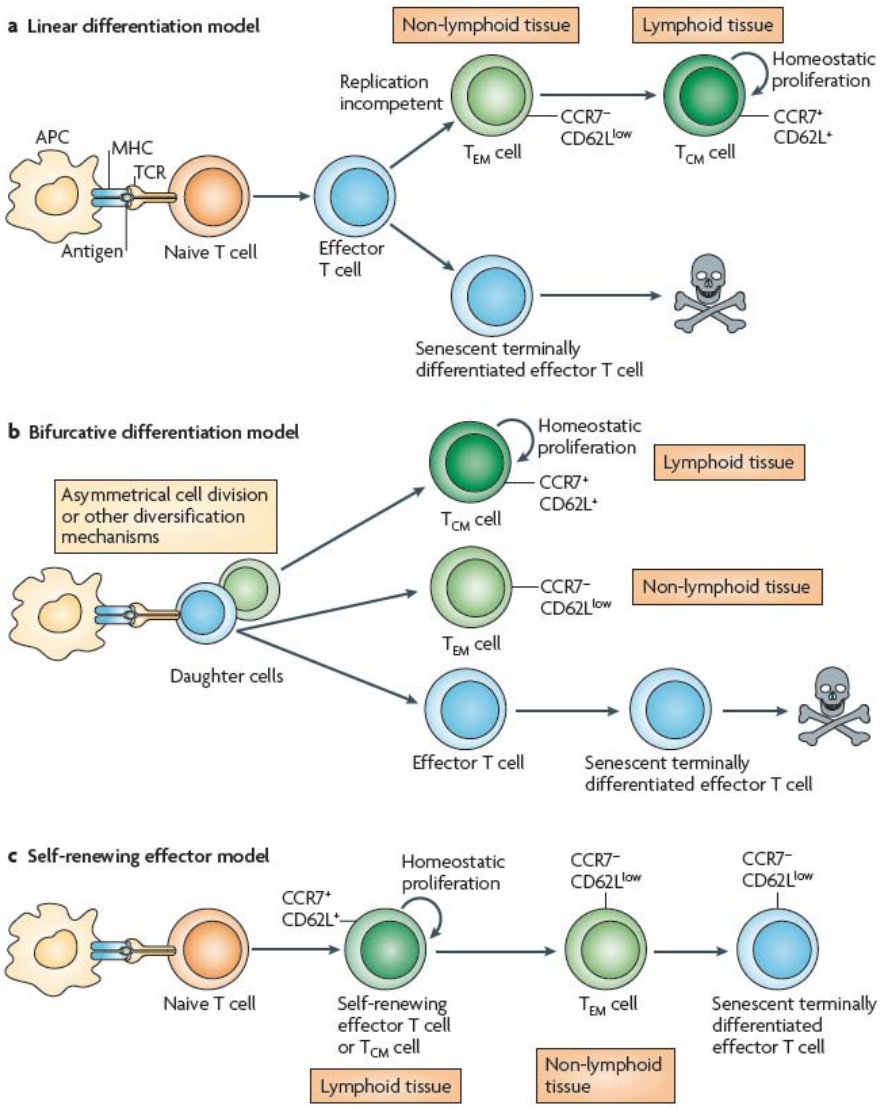


Figure Possible models of memory T cell differentiation. (A) In the linear differentiation model, stimulation of naive T cells with antigen (in the presence of co-stimulatory signals and inflammatory cytokines) results in the generation of effector T cells. Effector T cells either become senescent terminally differentiated T cells, or home to lymphoid tissues, and are replication incompetent. **(B)** The bifurcative differentiation model proposes that following antigen recognition, one T cell can give rise to two daughter cells with different differentiation fates. (**C)** The self-renewing effector model suggests that a naive T cell can develop into a TCM cell or an effector T cell that can self-renew. (Taken from Ahmed et al., 2009)

Homeostatic cytokines are likely to play a major role in driving T cell proliferation, differentiation and survival (Sallusto et al., 2004; Rochman et al., 2009). Previous studies have implicated the common γ-chain family cytokines IL-2, IL-7, and IL-15 as important general regulators of peripheral T cell homeostasis (reviewed in Kovanen and Leonard, 2004). IL-2, a product of activated T cells, supports expansion of antigen-activated CD4+ and CD8+ T cells (reviewed in Malek et al., 2004). IL-7 is produced by nonlymphoid cells and plays a crucial role in promoting expansion of both thymic and peripheral T cell populations, and both the naive and memory

compartments. IL-15, also produced by nonlymphoid cell types, directs NK cell and NK-T cell development, as well as CD8+ memory T cell function and homeostasis (reviewed in Kovanen and Leonard, 2004). In addition to IL-2, IL-7 and IL-15, IL-4 and IL-9 can also act as T cell growth factors (Kanegane et al., 1996). It is critical to investigate T cell differentiation in HIV infection to determine whether there are particular subsets of cells that are involved in viral control.

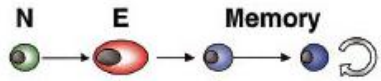
1.4.2 Properties of memory T cells

Memory T cells with distinct functional properties can be identified based on the expression of certain cell surface Wherry and Ahmed, 2004). The expression of co-stimulatory molecules CD27 and CD28 and differing isoforms of CD45 have been used to dissect the heterogeneity of memory T cells (Michie et al., 1992; Sallusto et al., 1999). Naïve T cells express CD45RA and the transition from naive to memory status is connected to the loss of CD45RA expression and gain of CD45RO expression (reviewed in Esser et al., 2003). Upon *in vitro* activation of naïve CD45RA+CD45RO- T cells, a subset of transitional CD45RO-CD45RA- T cells can be observed before conversion to a CD45RO+CD45RA- memory T cell phenotype (Prince et al., 1992). Memory T cells are predominantly divided into central memory and effector memory T cells (Sallusto et al., 1999).

Central memory T cells express CD62L (L-selectin) and the chemokine (C-C motif) receptor 7 (CCR7) which is the receptor that binds CC chemokine ligand 19 (CCL19) but is also the receptor associated with central memory retention in secondary lymphoid organs (reviewed in Salusto et al., 2004). Central memory T cells can circulate from the blood to secondary lymphoid organs and maintain their ability for secondary proliferation (Geginat et al., 2003a; 2003b; reviewed in Sallusto et al., 2004). In contrast, effector memory T cells express low levels of CD62L and do not express CCR7 or home to lymphoid tissues (Geginat et al., 2003a; 2003b; reviewed in Salusto et al., 2004). Effector memory T cells are unlikely to divide in response to a secondary infection, but are capable of producing large quantities of perforin and granzyme B as well as being able to migrate to sites of inflammation, such as the skin and gut (Sallusto et al., 1999). Although central memory cells express little perforin and granzyme, their ability to acquire these functions rapidly upon secondary encounter with antigen makes this subset more effective at mediating protective

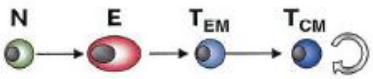
immunity for persisting infections (Burgers et al., 2009). Studies in mice, however, demonstrated that central and effector memory T cells were equally efficient at cytotoxic killing upon reactivation *in vivo* (Barber et al., 2003). The phenotypic changes that occur from naïve to memory transition are summarized on Figure 1.4. Other T cell markers such as CD57 and CD127 (IL-7 receptor) have been used to identify and characterise memory T cells (reviewed in Ma *et al*., 2006). CD127 is a marker for homeostatic survival of T cells. In addition, CD127 is mainly expressed on naïve T cells. CD57 identifies senescent T cells and both *in vivo* and *in vitro* studies have shown that cells that express this surface molecule are incapable of proliferating after antigen-specific stimulation and undergo activation-induced apoptosis (Brenchley et al., 2003).

A



		N	E		Memory
Homing to:	2° Lymphoid Organs	++++	-/+	-/+	+++
	Peripheral Tissues	-/+	++++	++	+
Ex Vivo Effector Functions:	CTL	-	++++	++	-/+
Rapid Recall:	IFN-γ / TNF-α	-/+	++++	++++	++++
	IL-2	++	-/+	-/+	+++
Proliferation:	Antigen Driven	+++	+	++	++++
	Homeostatic	-	-	-/+	++++

B



		N	E	T_{EM}	T_{CM}
Antigen Experienced T Cells					
CD44	Adhesion Molecule	Lo	Hi	Hi	Hi
CD11a	LFA1, α Integrin; Adhesion Molecule	Lo	Hi	Hi	Hi
Ly6C	GPI-linked; unknown function	Lo	Hi	Hi	Hi
CD122	IL-2/IL-15 receptor β chain	Lo	Hi	Hi	Hi
IL-15Rα	IL-15 receptor α chain	Lo	Hi	Hi	Hi
Associated with Effector T Cells					
CCR5	Chemokine Receptor	Lo	Hi		Lo
Perforin	Cytotoxic granule protein	Lo	Hi		Lo
Granzyme B	Cytotoxic granule protein	Lo	Hi	Int	Lo
Dynamically Regulated during Memory T Cell Development					
CD62L	Lymph node homing receptor	Hi	Lo	Lo	Hi
CCR7	Lymph node homing chemokine receptor	Hi	Lo	Lo	Hi
CD27	TNF receptor superfamily; costimulation	Hi	Lo/Int	Lo/Int	Hi
CD127	IL-7 receptor α chain	Hi	Lo	Lo/Int	Hi
Bcl-2	Anti-apoptotic/cell survival	Int	Lo	Int	Hi

Figure 1.4. The phenotypic changes that occur from naïve to memory transition. (A) Memory CD8 T-cell properties that change during the naïve to effector to memory transition are listed. **(B)** Phenotypic changes that occur during the naïve to effector to memory transition are listed, including differences between the effector memory (TEM) and central memory (TCM) subsets of memory CD8 T cells (taken from Wherry and Ahmed., 2004).

1.4.3 T cell differentiation in HIV infection

During HIV infection, the host immune system is chronically activated, which leads to immune exhaustion and dysfunction and ultimately progression to AIDS in the absence of successful antiretroviral therapy (Papagno et al., 2004). Details of how HIV-1 infection leads to chronic HIV infection are covered in section 1.7. The association between T cell exhaustion and HIV/AIDS disease progression is still not well understood.

The skewing towards an effector memory T cell phenotype has been observed for both HIV-specific CD4+ and CD8+ memory T cells during HIV infection (Tilton et al., 2007; Champagne et al., 2001). Recent studies showed that terminally differentiated HIV-specific CD8+ T cells (CD45RA+CCR7-) cells, known as terminally-differentiated effector cells expressing RA or TEMRA) were associated with viral control in HIV infection (Northfield et al., 2007, Addo et al., 2007). In early HIV infection, the presence of these cells has been associated with lower viral set point (Northfield et al., 2007) and a greater frequency of these cells was observed in individuals controlling HIV infection compared to those HIV progressors (Addo et al., 2007). In contrast, Burgers et al. (2009) found that less differentiated phenotypes of both total and HIV-specific CD8+ T cells response profile correlated with a lower viral set point while a more differentiated profile correlated with a higher viral load set point. The frequency of central memory and intermediate populations of CD8+ T memory cells (CD45RO-CD27-CCR7-) in both total and HIV-specific memory CD8+ T cells were inversely associated with viral set point (Burgers et al., 2009). HIV controllers were shown to have a greater percentage of HIV-specific CD4+ central memory cells than HIV viremic individuals (Potter et al., 2008). Similarly, Ladell et al. (2008) reported reduced levels of total CD4+ central memory cells associated with higher viral loads indicating that maintanence of CD4 central memory cells are important for control of HIV disease progression. The preservation of total CD4+ central memory cells has also been shown in elite controllers compared to HIV-infected treated individuals (van Grevenynghe et al., 2008).

These studies demonstrate a link between HIV disease progression and the presence of a particular memory T cell phenotype. Overall, the extent of T cell immune

activation may be critical in driving memory cell differentiation. In contrast to blood of HIV-infected women, there is limited information available on T cell differentiation and maturational status in the female genital tract.

1.5 Organization of the female genital tract

The lower genital tract in women is comprised of four discrete anatomical regions including (a) the vaginal opening, (b) the vaginal mucosa, (c) the ectocervix, and (d) the endocervix (Figure 1.5). It also consists of two main types of epithelium including the squamous and columnar epithelium.

1.5.1 Vagina

The female vagina can be subdivided into the introitus (vaginal opening) which is covered by a keratinized stratified squamous epithelium resembling skin, and the distal and proximal vagina which are covered by non-keratinized stratified squamous epithelium (Pudney et al., 2005). The vaginal epithelial barrier changes markedly at different stages of the menstrual cycle and in response to exogenously administered hormones such as progesterone (Marx et al., 1996). Hormonal cycling has been shown to affect lymphocyte and plasma cell densities in the vagina (Wira et al., 2000; 2003) and, in the acidic environment that predominates here, this compartment has been shown to inhibit lymphocyte proliferation (Hill and Anderson, 1992).

1.5.2 Ectocervix

The ectocervix consists of five layers of epithelium completely covered with an extension of the vaginal stratified squamous epithelium (reviewed in: Coombs et al., 2003; Pope and Haase 2003). This multilayered epithelium acts as a physical barrier against invading pathogens (reviewed in Shacklett et al., 2009b). Compared with the vagina, the ectocervical mucosa contains higher numbers of CD4+ T cells and CD1a+ dendritic cells (Pudney et al., 2005). Immune cells present in this region have been implicated in the sexual transmission of HIV-1, and there is strong evidence that HIV infection is more common in the ectocervix than in the vagina in women without inflammatory conditions (reviewed in Coombs et al., 2003).

1.5.3 Cervical Transformation Zone

The transformation zone represents an abrupt transition situated at the intersection between the ectocervix and the endocervix (Pudney et al., 2005; Figure 1.5). This zone is constantly altered in response to hormonal fluctuation, pregnancy and aging (Jacobson et al., 1999). This site is a rich source of lymphocytes and antigen presenting cells containing more immune cells than any other region within the outer female genital tract (reviewed in Coombs et al., 2003). Accumulation of CD8+ T cells at this site provide evidence that the transformation zone functions as an immunologically dynamic barrier to invading pathogens (Kobayashi et al., 2002). As the transformation zone also harbors higher concentrations of CD4+ T cells, this site has been implicated in both in HIV-1 transmission and HPV clearance (Pudney et al., 2005).

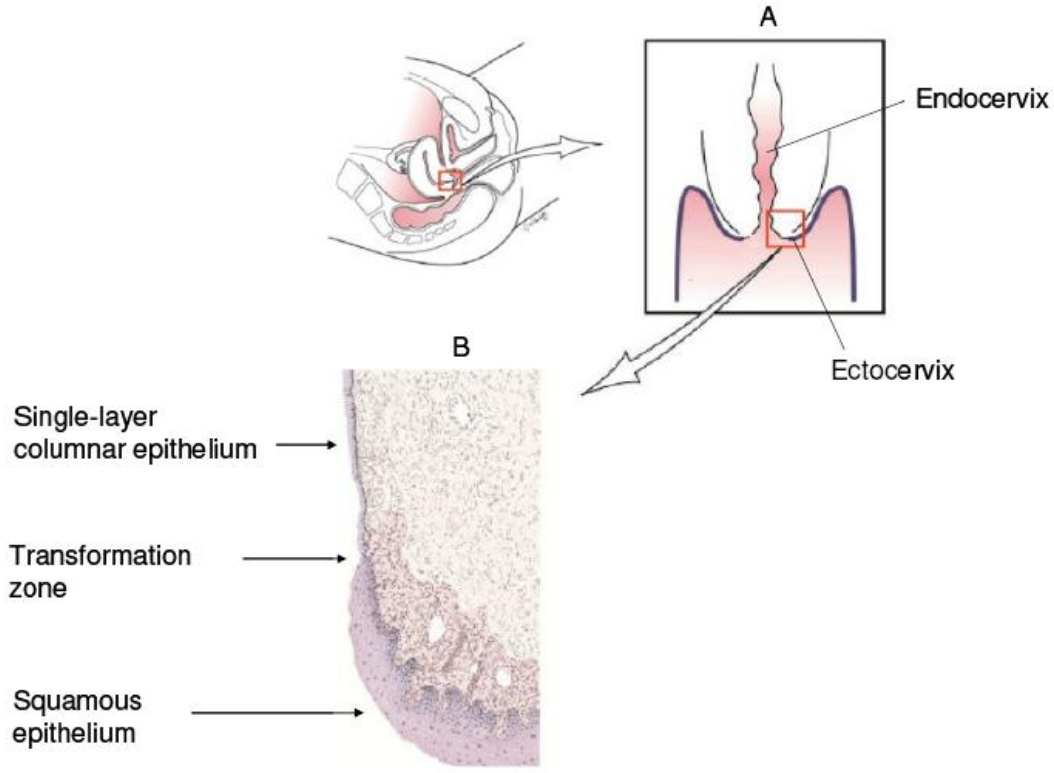


Figure 1.5. A representative figure of an adolescent healthy cervix showing the endocervix, and ectocervix (A) and a magnified section shows the single-layer columnar epithelium, transformation zone and the squamocolumnar junction **(B)** [modified from Coombs et al., 1999].

1.5.4 Endocervix

The endocervical canal is approximately 2.5 cm long and consists of a columnar epithelial monolayer that rests on a continuous, extremely thin basement membrane

(reviewed in Coombs et al., 2003). Although this section of the genital tract contains the lowest number of T cells and macrophages, it is lined with mucus, innate defences including compounds such as secretory leukocytes protease inhibitor (SLPI) and Lactoferrin (reviewed in Shacklett et al., 2009b).

1.6 HIV transmission in the female genital tract

HIV transmission across the genital mucosa involves several steps including survival of the virus or HIV-infected cells in mucosal secretions, transport across or passage through the epithelial barrier and infection of target cells within the mucosa (reviewed in Kozlowski and Neutra, 2003). The strategies that HIV may use to infect cells in the mucosa depend of the site of infection within the female genital tract.

1.6.1 HIV Infection and the female genital tract

The mucosa surface of the female genital tract serves as the site of entry for a variety of bacterial and viral pathogens (Prakash et al., 2001). Higher densities of immune cells in the lower genital tract (ectocervix and transformation zone) compared with the upper genital tract suggest that most infections will take place in this region (Cohen et al., 1999., Bulmer *et al*., 1998; Pudney et al., 2005). Knowledge of the type and distribution of cells in the lower genital tract, including those responsible for the inductive and effector arms of cellular immunity are important to understanding the pathogenesis of HIV and other important sexually transmitted infections in women (Prakash et al., 2001) and in critical to designing appropriate prevention strategies to induce local protective immunity or block mucosal infection (Pudney et al., 2005).

1.6.2 Susceptibility of the female genital tract to HIV-1 infection

Studies have demonstrated that susceptibility to HIV infection following sexual contact is likely to be influenced by multiple factors, including the presence of other pathogenic infections (Chlamydia, Herpes simplex virus type 2 (HSV-2), Human papillomavirus (HPV) and other sexually transmitted infections; reviewed by Pope and Haase (2003). In particular, conditions or infections that disrupt an intact mucosa barrier such as cervical dysplasia, pelvic inflammatory disease, and neoplasia have been implicated in increased risk for HIV-1 transmission (Plummer, 1998; Grosskurth

et al., 1995a; 1995b; Kreiss et al., 1994; Augenbraun et al., 1994). Elevated numbers of CCR5+ Langherhans cells are present in the cervicovaginal area of women with sexually transmitted infections (Zaitseva et al., 1997; Patterson et al., 1998).

The amount of HIV present in the challenge inoculum (viral load in transmitting partner's genital secretions) and the effectiveness of the local mucosal immune system throughout the female reproductive tract have been shown to influence the infectiousness of HIV-1 (Lee et al., 1996). Furthermore, efficiency of heterosexual transmission of HIV in humans has been associated with higher viral loads in blood of the transmitting partner (Lee et al., 1996; Quinn et al., 2000).

Susceptibility to HIV-1 infection is also increased by the use of hormonal contraceptives which has been shown to result in thinning of the genital epithelium (Martin et al., 1998; Mostad et al., 1997). Changes occurring before, during and after ovulation which affect epithelial morphology and cellular densities have also been implicated in increased susceptibility to HIV (Wright et al., 1994). The expression of HIV receptors and co-receptors (CCR5 and CD4, respectively) by T cells and epithelial cells peak in the upper reproductive tracts during ovulation (reviewed in Wira and Fahey, 2008). Following ovulation and during the secretory phase of the menstrual cycle, CTL and Natural Killer cell activity in the uterus are suppressed (reviewed in Wira and Fahey, 2008). Furthermore, small mucosal lacerations produced in the vaginal mucosa during sexual intercourse or mucosal irritants such as repeated exposure to Nonoxynol-9 (reviewed in Pope and Haase, 2003) also increase permeability of the tissue and facilitate entrance of HIV-1 locally in susceptible cells (Rasheed et al., 1996; reviewed in Pope and Haase, 2003; Marx et al., 1996). Thinning and breaches in the mucosal barrier could quickly expose susceptible cells in the submucosa to HIV facilitating viral entry. This would increase the likelihood of establishing and rapidly disseminating infection (reviewed in Pope and Haase, 2003)

The prospect that trauma associated with heterosexual intercourse may be associated with shedding or recruitment of T lymphocytes into the genital tract lumen has also been suggested (Bardeguez et al., 1997). Although micro-trauma is likely to be a involved in transmission of HIV, non-traumatic uptake of virus has also been documented in primates (Milush et al., 2004) and in cervical tissue explants (Hu et al.,

2004; Maher et al., 2005; reviewed in Hladik and Hope, 2009). A study on non-traumatic oral SIV inoculation of rhesus macaques indicated that the mucosal and lymphoid tissues in the head (oral mucosa, esophagus, submandibular and cervical lymph nodes) contained the majority of SIV infection foci (Milush et al., 2004) indicating that infection can take place by passive viral diffusion. The stratified squamous epithelium in the oral and esophageal mucosa is similar to the epithelium present within the vaginal mucosa suggesting a similar mechanism of entry at each of these mucosal sites (Milush et al., 2004). Factors that can increase the risk of infection with HIV are summarized in Figure 1.6.

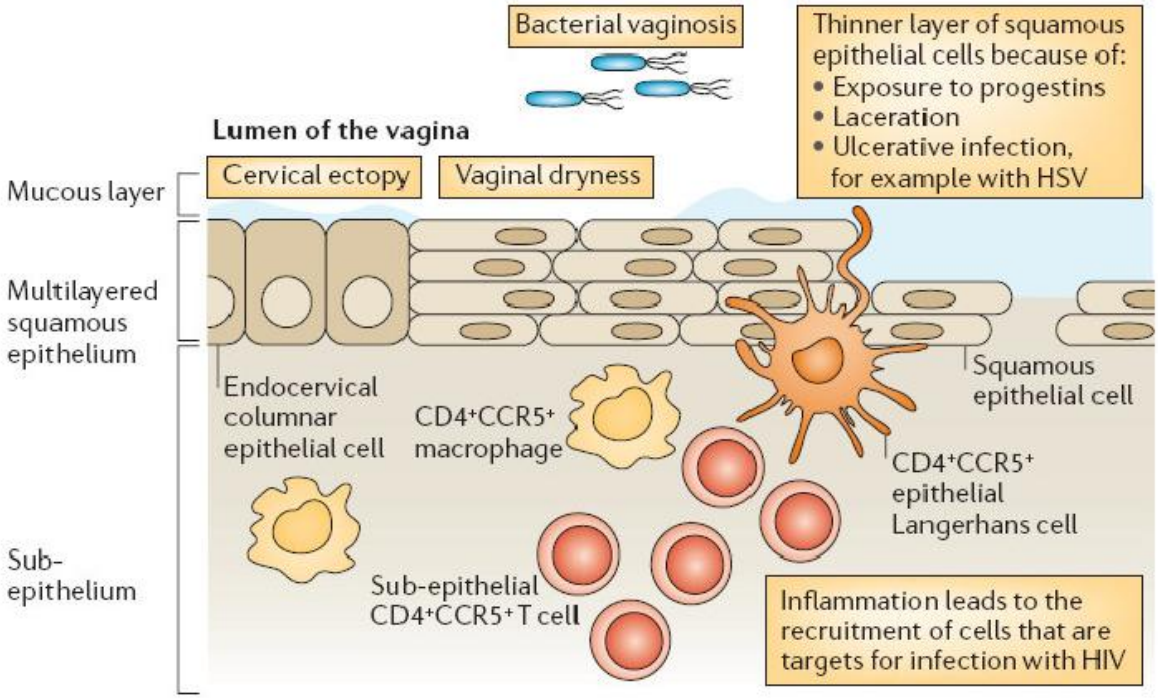


Figure 1.6. Factors that can increase the risk of infection with HIV include: ectopic protrusion of endocervical columnar epithelium into the ectocervix; thinning of the squamous epithelial layer; bacterial vaginosis; vaginal drying; inflammation, which leads to an increase in the number of target cells in the submucosa; trauma; ulcerative infections that might allow more ready access of virus to sub-epithelial dendritic cells (DCs) expressing C-type lectins, such as DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing non-integrin (DC-SIGN), and CD4+ T cells (taken from Lenderman et al., 2006).

1.6.3 Mechanisms used by HIV to cross the female genital mucosal barrier

Upon exposure to HIV, cervical mucus has been shown to be very effective in trapping HIV-infected seminal cells or free virus (reviewed in Lenderman et al., 2006). Despite this, cell-free HIV gains access to its target cells; which include Langerhans cells, intra-epithelial CD4+ T cells, and CCR5+ Dendritic cells. Lederman et al. (2006) argued that it would be more difficult for cell-associated HIV to breach this barrier. Dendritic cells also express DC-SIGN, a member of the C-type lectin receptors (CLRs) family, which are involved in HIV attachment and internalisation into non-degrading endosomal compartments (Turville et al., 2001). Dendritic cells are predominantly specialized Langerhans cells, expressing Langerin but not DC-SIGN and mannose receptor. It has been suggested that HIV-1 infection can be initiated by transmission to intraepithelial Langerhans cells in the stratified squamous epithelium/ectocervix either as cell free virus or bound to Langerhans cells in the epithelium without infecting them (reviewed in Kozlowski and Neutra, 2003). Figure 1.7 summarizes the possible pathways of HIV invasion in the mucosa of the vagina and uterine ectocervix.

Alternatively, Milush et al. (2004) has suggested that HIV transmission may be occurring across microscopic breaks between squamous epithelium cells that provide access for the virus to infect the CD4+ cells and CCR5+ cells present in the mucosa and submucosa (Figure 1.7). Squamous epithelial cells of the exocervix are connected by desmosome muscles which are the weakest form of intercellular junctions (Kress et al., 2004). This keeps the cellular membranes 30nm apart from one another (Kress et al., 2004). Although still significantly smaller than a HIV particle (100-150 nm), it is possible that few viral particles in the inoculum may be able to permeate through the entire epithelium into the cervical canal and initiate an infection (Spira et al., 1996).

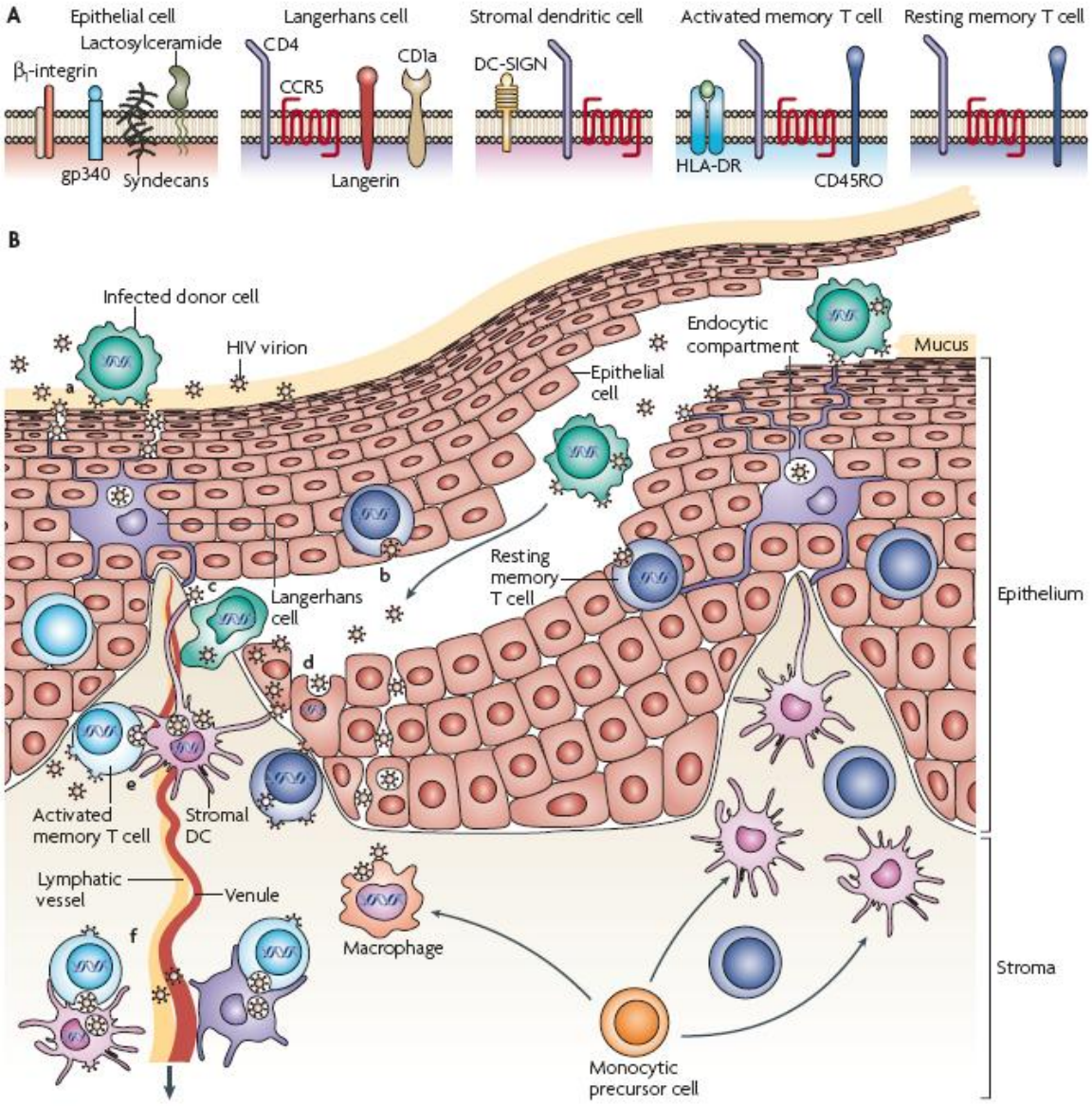


Figure 1.7 Pathways of HIV invasion in the mucosa of the vagina and uterine ectocervix. Characteristic phenotypic cell receptors and receptors relevant for HIV binding and infection are shown on the top of the figure (**A**). The possible pathways of HIV penetration are summarized in **B**. (**a)** Free HIV virions or HIV-infected donor cells are trapped in mucus, resulting in penetration of the free virions into gaps between epithelial cells or attachment of HIV-infected donor cells to the luminal surface of the mucosa and secretion of virions on contact. (**b)** HIV can also fuse with the surface of intraepithelial CD4+ T cells, followed by productive infection of these cells. (**c)** Infected donor cells or free virions can immigrate along physical abrasions of the epithelium into the mucosal stroma. (**d)** Virions can transcytose through epithelial cells near or within the basal layer of the squamous epithelium (**e)** Once within the stroma, virions can productively infect stromal DCs or be internalized into the endocytic compartments of DCs and pass from the stromal DCs to CD4+ T cells (**f)** Productively infected CD4+ T cells and stromal DCs, and stromal DCs or intraepithelial LCs harboring virions in endocytic compartments, can emigrate into the submucosa and the draining lymphatic and venous microvessels (Taken from Hladik and McElrath, 2008).

The single layer of columnar epithelium that lines the endocervix contain CD4+ bearing cells that express the necessary HIV co-receptors (CCR5 and CXCR4) required for infection (Howell et al., 1997; Figure 1.7). The columnar epithelial monolayer associated with the endocervix represents a less formidable barrier for HIV to cross than the multilayer squamous epithelium of the ectocervix or vagina (Pope et al., 2003). Although the architecture of the cervix possesses a number of potential routes by which infection can take place, cervical mucous largely provides an excellent barrier by trapping most of the virus (Miller et al., 2005). The ultimate goal of an HIV vaccine would be to prevent initial mucosal infection or the establishment of persistent HIV-infection.

While various studies have implicated Langerhans cells in transmitting HIV to neighbouring cells and T cells (reviewed in Kozlowski and Neutra, 2003; Lenderman et al., 2006; Turville et al., 2001). It has been recently demonstrated that the C-type lectin langerin receptor expressed on Langerhans cells can act as a natural barrier to HIV transmission by sequestering and targeting viral particles for degradation (deWitte et al, 2007). It has now emerged, however, that activated Langerhans cells are prone to HIV infection while immature Langerhans cells clear invading HIV via langerin (de Jong and Gientenbeek, 2009). It is important to identify the immune cells that reside in the lower genital tract and understanding the processes they are involved in would be beneficial in developing therapeutic strategies.

1.6.4 Local mucosal propagation of HIV and dissemination

Once HIV has successfully crossed the mucosal barrier, the first cells thought to be infected are intraepithelial and subepithelial dendritic cells, local subepithelial CD4+ T cells and macrophages (reviewed in Hladik and McElrath, 2008). These cells subsequently migrate to local draining lymph nodes and transmit the virus to CD4+ T cells present in these lymph nodes (Spira et al., 1996), in the form of an „infectious synapse (McDonald et al., 2003) [Figure 1.7 and Figure 1.8].

Because of the high volumes of lymphocytes that traffic through lymph nodes each day, HIV would be capable of rapidly spread from these regional nodes throughout the body and infect new cells in a relatively short period of time, most efficiently to the memory subset, which is the chief producer of virus in vivo (reviewed in Hladik

and McElrath, 2008). In carrying out their normal function of conveying pathogens and antigens to the draining lymphatic tissues to induce an immune response, virus-carrying DCs can also disseminate infection to large numbers of CD4+ T cells to amplify and further disseminate the infection (reviewed in Pope and Haase, 2003). It is this rapid dissemination of the virus to lymph nodes that is likely to compromise the ability of the host's immune response to contain or eradicate the infection at the mucosal site of exposure (Pope et al., 2003).

Studies of vaginal transmission in the SIV–rhesus macaque model point to opportunities at the earliest stages of infection in which a vaccine or microbicide might be protective, by limiting the expansion of infected founder populations at the portal of entry (Haase et al 2005; Miller et al., 2005). A recent study in macaques has identified the initial influx of CD4 T cells into the genital mucosa as being a key to dissemination of infection since CD4 T cell recruitment to this site is followed by a secondary inflammatory process, probably driven by RANTES and other chemokine-producing cells within inflammatory infiltrates (Li et al., 2009). Li et al. (2009) showed that the influx of CD4 T cell target cells to the genital mucosa is preceded by production of MIP-3α by the endocervical epithelium in response to virus which recruits plasmacytoid Dendritic cells. In turn, plasmacytoid Dendritic cells produce MIP-1α and MIP-1β to recruit CCR5+ CD4 T cell which serve as targets for HIV infection (Li et al., 2009).

Despite the prediction that systemic HIV infection is rapid, key studies with SIV in primates have demonstrated that this only occurs few days after initial infection and all viral replication is local for the first week of infection (Miller et al., 2005; Reynolds et al., 2005). Theses findings collectively suggest that productively HIV infected cells during the first few days are mainly found in cervicovaginal tissues.

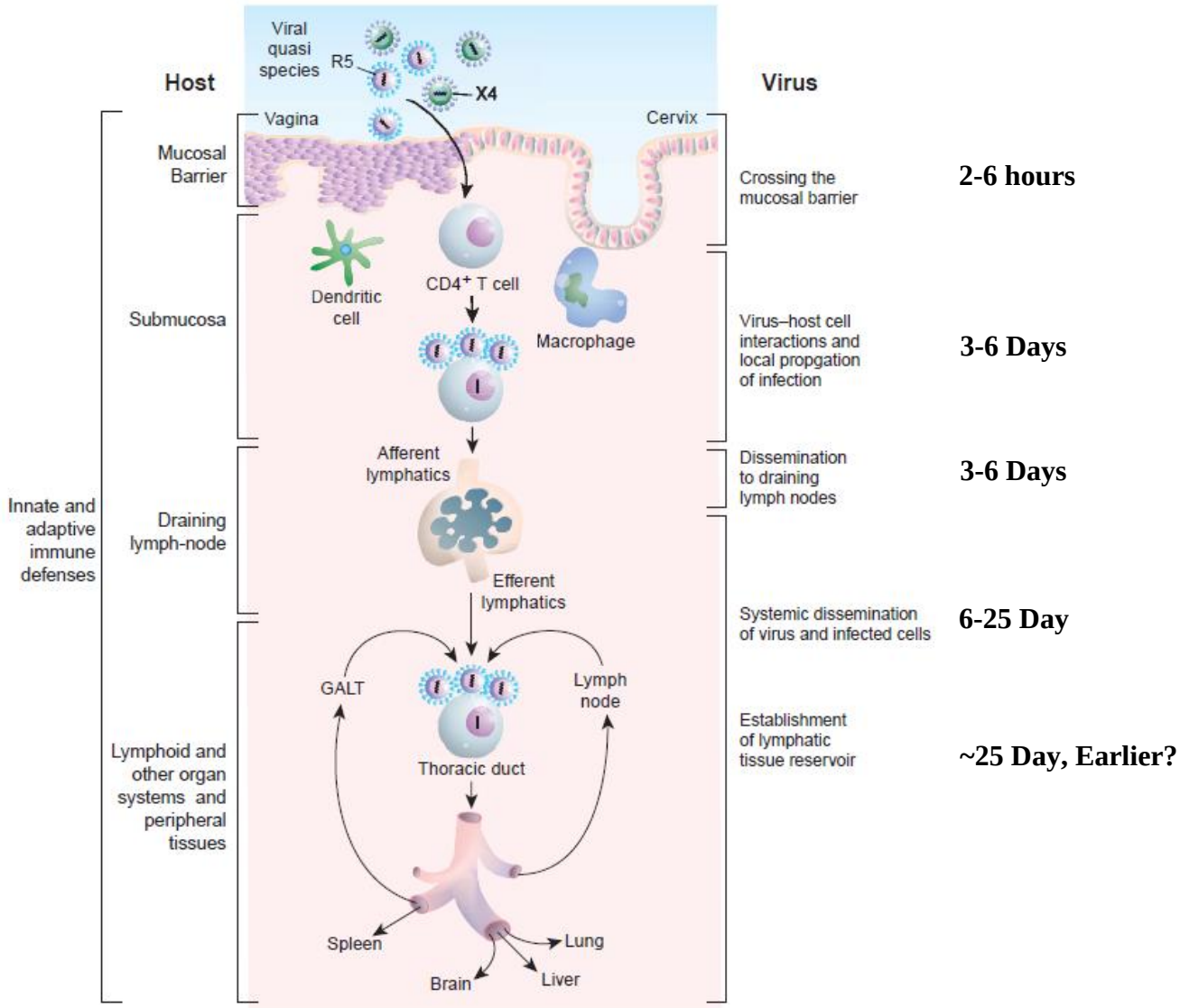


Figure 1.8 Overview of sexual transmission of HIV to women and events during acute HIV infection. After crossing the cervicovaginal mucosal barrier, DCs, CD4+ T cells and macrophages in the underlying submucosa are infected. Infection is subsequently propagated and disseminated, thereby establishing the lymphatic tissue reservoir that spreads infection to other organs and peripheral tissues. Innate and adaptive host defenses (left column) are directed at the different stages to prevent transmission and contain infection (taken from Pope and Haase, 2003; adapted from Haynes and Shattock, 2008).

1.6.5 Factors associated with protection from HIV infection in the female genital tract

The female genital tract is equipped with a variety of physical barriers and innate defences (reviewed in Coombs et al., 2003; Wira et al., 2005; Shacklett et al., 2008; 2009). Physical defences include low vaginal pH, multi-layered vaginal epithelium,

local flora and a mucous layer. Secretory leukocyte protease inhibitor (SLPI) and lactoferrin, a milk protein, are found in a variety of secretions and exhibit anti-HIV activity in vitro (Ma et al., 2004; Berkhout et al., 2004). The chemokine RANTES and several defensin family members (Levinson et al., 2009) have also found in the female reproductive tract (reviewed in Shacklett et al., 2008). HIV-1-specific antibodies, particularly those capable of virus neutralization, and cytotoxic T cells have been considered in prevention and local limitation of viral infection at mucosal sites of HIV-1 or SIV entry (Shacklett et al., 2009; reviewed in Mestecky et al., 2009).

Reduced HIV susceptibility has been associated with a number of genetic polymorphisms (reviewed in: Iqbal and Kaul, 2008; Kulkarni et al., 2003). The CCR5-Δ32 mutation confers near complete resistance to sexual HIV acquisition (Reviewed in Kulkarni, 2003). Natural HIV resistance has also been linked to polymorphisms of innate immune molecules, such as an SDF-1 3' UTR mutation in a group of HEPS individuals, and with human leukocyte antigen (HLA) haplotypes including HLA-B18, HLA-A2 / 6802, HLA-DRB1*01, and HLA-A11 (reviewed in Iqbal and Kaul, 2008).

Studies estimating the risk of HIV transmission per unprotected heterosexual sex act have suggested that HIV transmission risk can range from 3 to 50 per 10,000 unprotected exposures (reviewed and Galvin and Cohen, 2004), with a probability of transmission per coital act is approximately 0.1–1% (Gray et al, 2001). This means that the protective efficacy of the genital mucosal barrier is 99–99.9%, which is much higher than that for preventative vaccines available for almost any infection, much less for HIV (reviewed in Iqbal and Kaul, 2008). However, various factors such as higher viral load and genital ulceration are thought to mainly influence infectivity (Gray et al., 2001). Clearly, both the innate and adaptive responses constitute "protective" immunity against HIV infection and HIV disease progression (reviewed by Shacklett et al., 2009; Mestecky et al., 2009). A summary of factors that might decrease the risk of infection with HIV are shown in Figure 1.9.

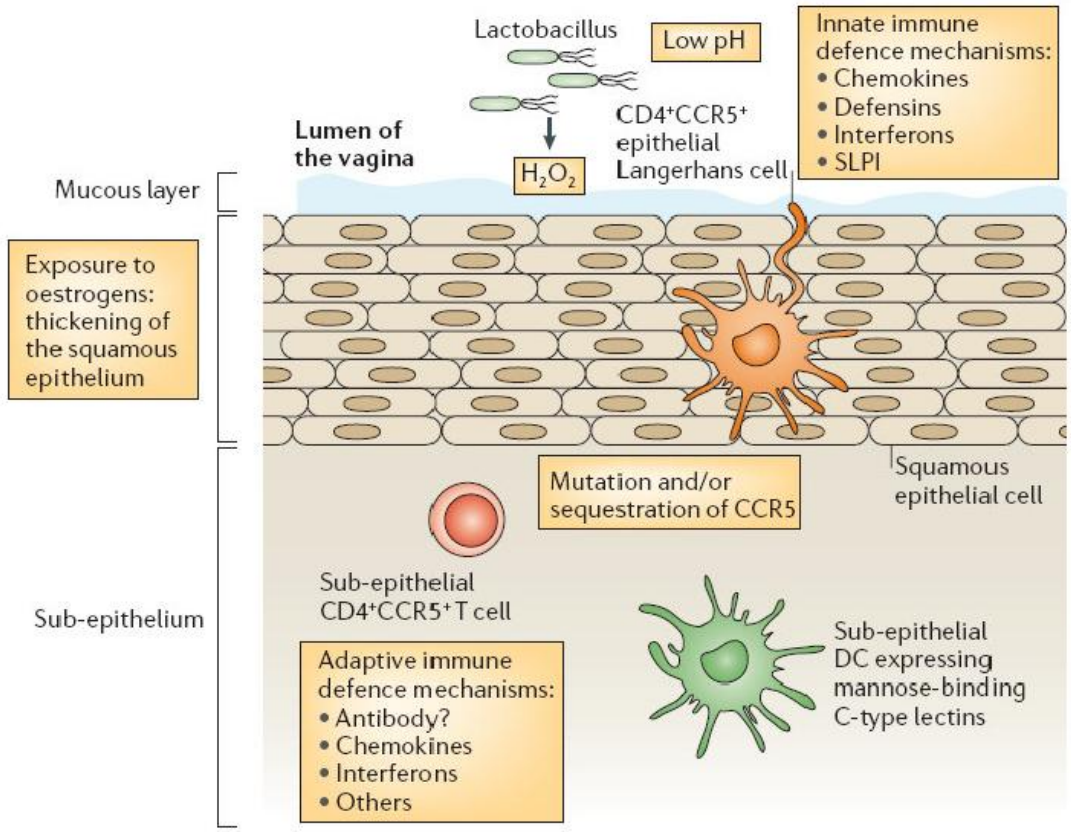


Figure 1.9 A summary of factors that might decrease the risk of infection with HIV include: a thicker squamous epithelium; a mucous layer that physically traps virus; and physical properties leading to HIV inactivation such as low pH and peroxides. These include hydrogen peroxide (H_2O_2) from lactobacilli; innate immune defense molecules, such as defensins, interferons, secretory leukocyte protease inhibitor (SLPI); absence of CC-chemokine receptor 5 (CCR5), either genetically [through homozygosity for a 32-base-pair deletion in the coding region of CCR5 (known as the CCR5Δ32 allele)] or induced (by endogenous chemokine ligands) [Taken from Lederman et al., 2006]

1.7 HIV preferentially targets the mucosal immune system

While the vaginal and rectal mucosa are the predominant sites of HIV entry, recent studies demonstrated that the gastrointestinal mucosa is the site of initial viral replication (Derdeyn et al., 2005; Mehandru et al., 2005; Mattapallil et al., 2005; Brenchley et al., 2006). Studies of sexual transmission of SIV via the female genital mucosa in rhesus macaques have confirmed that CD4+ CCR5+ T cells become rapidly infected after virus inoculation (Spira et al., 1996). In addition, there is a rapid and massive depletion of memory CD4+CCR5+ T cells (>80%) which reside in the gastrointestinal, vaginal and respiratory tracts within the first few days of viral entry (Veazey et al., 1998; 2000; 2003) and this occurs before significant depletion in

peripheral blood or lymph nodes. This is believed to be due to the fact that large populations of activated CD4 T cells expressing the CCR5 receptor reside at this site (Li et al., 2005; Guadalupe et al., 2003).

Productive HIV-1 infection of T lymphocytes requires cellular activation because activated CD4+ T cells express CCR5 (Veazey et al., 1998; Mattapallil et al., 2005). Resting CD4+ T cells infected with SIV or HIV-1 have about five times fewer copies of viral RNA than do infected activated CD4+ T cells (Zhang et al., 1999). Furthermore, T lymphocytes that traffic to the cervical epithelium of normal healthy women were found to exhibit higher activation states compared to blood T cells (Prakash et al., 2001).

Human mucosal surfaces are estimated to cover about 400 m^2 whereas the surface of the skin is only 2.3 m^2 (Holmgren et al., 2005). The frequency of HIV-infected CD4+ T cells in the chronic phase of SIV/HIV infection is too low (0.001-1% of CD4+ cells) to account for the ongoing depletion by viral infection (Mattapallil *et al*., 2004; Douek et al., 2002; Haase et al., 1999; Lassen et al., 2004).

It has been postulated that the extent of early memory CD4+ T-cell depletion in the mucosal tissues sets the stage for the course of HIV disease progression (Derdeyn et al., 2005). Despite rapid depletion of mucosal T cells during acute infection, studies have shown that primates that were able to partially reconstitute mucosal CD4+ T cells progress to AIDS at a slower rate than those that were unable to reconstitute their CD4+ T cell population (reviewed in Picker and Watkins 2005). The mucosal immune system is therefore the reservoir for viral replication in chronic infection; persistence and CD4+ T cell loss in the HIV-1 infected individuals (Derdeyn et al., 2005; Mattapallil et al., 2005; Macal et al 2008). The integrity of the mucosal immune system and the ability to maintain CD4+ T cells above a certain threshold in particular seems to the determining factor for progression to AIDS (Brenchley et al., 2006). It was proposed that this massive depletion of T cells in the gut allows disease-causing bacteria that live in the intestines to leak out and circulate more widely in the body, further burdening the immune system. This factor contributes to the higher levels of immune activation in HIV-infected individuals. Mucosal HIV replication continues in the presence of ARVs despite effective control systemically indicating that ARVs

are not able to control mucosal replication (Guadalupe et al., 2003; 2006; Brenchley et al., 2004; Mehandru et al., 2006; Macal et al., 2008).

1.8 Evidence for the protective role of T cells in cervicovaginal mucosa

While there is clear evidence that CTLs in blood play an important role in controlling HIV replication systemically, little is known about CTL responses to HIV in the genital tract.

1.8.1 Cervicovaginal CTL responses during acute HIV infection

Studies of the kinetics of CTL induction in genital tissues have mainly been conducted in animal models as these are difficult to address in humans (Miller et al., 2005; Reynolds et al., 2005). Miller et al. (2005) showed that intravaginal inoculation of rhesus macaques with 10^9 copies of SIV RNA per ml produced systemic infection in more than 80 % of the inoculated animals 6-10 days post-inoculation. Inoculations were conducted without damaging the mucosal lining. This high viral dose resulted in ~10^4 copies of SIV RNA per μg of cervical and vaginal tissue from two animals at 2 hours and five of six animals 1 day after intravaginal inoculation (Miller et al., 2005). In another study, Reynolds et al. (2005) found that viral dissemination peaked in all tissues and blood between 10-14 days after infection following vaginal exposure of macaques to SIVmac239 and SIVmac251. Despite the high dose intra-vaginal SIV challenge used in these experiments (10^9 copies of viral RNA), relatively low concentrations of SIV RNA were detected in the cervical and vaginal tissues of infected macaques, restricted to only a few foci and most of the virus was trapped and cleared in cervicovaginal fluids (Miller et al., 2005) [Figure 1.8]. Following infection, there was a period of early local mucosal viral replication preceding systemic dissemination. These studies suggest that HIV-specific CTL present at the local site of infection during initial exposure could control or prevent infection before it spreads systemically (Belyakov et al., 2005).

Notably, high frequencies of both mucosal and systemic SIV-specific CTL responses only emerged at 20 days after the peak of virus production, leading to the conclusion

that the CTL response to HIV in mucosal tissues after initial infection was "too little and too late" to contain viral replication and dissemination (Reynolds et al., 2006).

1.8.2 Cervicovaginal CTL during chronic HIV infection

HIV-1 specific CTLs in the female genital tract have also been detected in chronically HIV-infected women (Gumbi et al., 2008; Musey et al., 1997; 2003; Kaul et al., 2003, Shacklett et al., 2003). Although direct control of HIV by these cells has not been demonstrated, cervical HIV-specific CTL have been shown to secrete the antiviral cytokine gamma interferon (IFN-γ) and display cytolytic function in response to various HIV epitopes in chronically HIV-infected individuals (Musey et al., 1997; 2003; Kaul et al,. 2003; Shacklett et al., 2000a; 2003).

The concept that mucosal cytotoxic T cells are important in controlling viral replication within local tissues and eliminating HIV-infected T cells at the sites of viral entry was suggested in studies of highly exposed but persistently HIV seronegative (HEPS) sexworkers (Kaul et al., 2000). Kaul et al. (2000) reported that ~69% of HIV-1–resistant sex workers in Nairobi had HIV-specific CD8+ T cells at their cervices. The epitope specificity of HIV-specific cytotoxic T cells detected in the genital tract of Kenyans HEPS was shown to differ from that of blood CTL and maintenance of mucosal CD8 response appeared to be dependent on the persistence of HIV exposure (Kaul et al., 2001b). Several HEPS women were shown to subsequently seroconvert in the absence of detectable CTL escape and this was most strongly correlated with a break from sex work (Kaul et al., 2001b). Break from sex work and late sero-conversion was also associated with a loss in HIV-specific CD8+ responses in the genital tract and a switch in the epitope specificity of theses CD8+ responses (Kaul et al., 2001c; Kaul et al., 2001d). Kaul et al. (2003) further showed that mucosal T cell responses specific for HIV-1 subtype C peptides could be detected in 80% of chronically HIV-infected women. Genital CD8+ cell responses to HIV were present at similar or higher frequencies than responses detected in blood (Kaul et al., 2001d). In addition to cytokine production, various chemokines (including MIP1β and RANTES) and antimicrobial peptides are secreted in the reproductive tissues (Fahey et al., 2006) and higher levels of β-chemokines were detected in cervical

secreations of HIV-untreated sex workers compared to low risk women (Iqbal et al., 2005).

The importance of genital tract CTL in protecting against sexually acquired HIV infection is also described in mice (Belyakov et al., 1998a; 1998b; 1998c). Mice vaccinated with recombinant vaccinia virus expressing HIV gp160 had detectable mucosal HIV-specific CTL and these CTLs were found to be essential for resistance to mucosal challenge with this recombinant virus (Belyakov et al., 1998a). Belyakov et al. (1998b) suggested that these mucosal HIV-specific CD8+ T cell might play a more direct role than systemic CTL responses in immune mediated protection against viral challenges in these animals. Studies in primates infected with SHIV-ku2 demonstrated that intrarectal immunization of rhesus macaques with a peptide vaccine adjuvanted with E. coli heat liable toxin mutant LT (R192G) was more effective than systemic immunization in reducing the set point plasma viral load subsequent to SHIV challenge and this was largely due to reduction of mucosal viral reservoir seeding the bloodstream (Belyakov et al., 2001).

In macaques, Murphey-Corb et al. (1999) showed that only animals which developed strong SIV env-specific CTLs in the jejuna lamina propria after exposure to SIV intra-rectally were protected against a mucosal challenge with SIV. Another study showed that immunization of macaques with an adjuvanted SIV gp120 and p7 subunit vaccine induced SIV-specific CD4+ T cell proliferative responses in the iliac lymph node draining rectal and vaginal mucosal surfaces and protected macaques against rectal challenge with $SIV_{mac}32HJ5$ (Lehner et al., 1998).

The role played by CTL in controlling local HIV-1 infection and prevention of viral transmission clearly needs to be determined. HIV-specific T cells are capable of destroying HIV-infected cells and their presence in the genital tract may serve to control local viral replication and thus reduce the likelihood of transmission to sexual partners. Vaccine induced cellular responses and innate immunity in the mucosal tissues should complement systemic immunity in controlling viral replication, reducing the viral reservoir and preventing virus transmission at mucosal entry sites (reviewed in Shacklett, 2008; 2009).

1.8.3 *Polyfunctional HIV-specific T cell responses in mucosal compartments*

Polyfunctional HIV-1 specific T cells have previously been identified at mucosal sites such as in lung (Brenchley et al., 2008) and rectal tissue (Critchfield et al., 2008; Ferre et al., 2009). HIV-specific polyfunctional T cell responses were present at higher frequencies in the lung compared to blood (Brenchley et al., 2008). Critchfield et al. (2008) showed that polyfunctionality in rectal mucosal Gag-specific CD8+ T-cell responses was associated with the extent of CD4 depletion and inversely related to plasma viral load during chronic infection. They found no difference in the frequency of rectal and blood polyfunctional responses (defined as triple positive for CD107a, IFN-γ and TNF-α). Ferre et al. (2009) reported that the frequency of polyfunctional responses in rectal mucosa in HIV controllers was significantly higher than non-controllers and that the frequency and complexity of mucosal responses was generally elevated at the mucosa of controllers compared to blood. The existence and function these cells in the female genital tract compartment have not been determined.

1.9 HIV diversity between compartments

Previous studies have shown that HIV is readily detectable in genital secretions (Sullivan et al., 2005; Ellerbrock et al., 2001; Poss et al., 1998). The amount of HIV detectable in cervical secretions were found to correlate with viral load in blood (Chakraborty et al., 2001; Vettore et al., 2006; Hart et al., 1999) as does the efficacy of transmission (Quinn et al., 2000). There has been no reported incidence of HIV transmission in individuals with blood viral loads ≤ 1,500 copies per ml (Quinn et al., 2000). This implies that blood-derived CTL and the impact these have on systemic viremia has indirect impact on the amount of virus available at the genital mucosa during transmission.

Within chronically HIV-infected individuals, substantial genetic diversity in plasma-derived HIV exists, with each HIV variant being a closely-related but genetically distinct quasispecies (Gupta et al., 2000; Philpott et al., 2005). Comparison of HIV phylogenetic relatedness between genital and blood-derived HIV during chronic infection has demonstrated distinct phenotypic and genetic differences between

compartments, suggesting that distinct anatomical sites are unique microenvironments for HIV diversification (Poss et al., 1998; Ellerbrook et al., 2001; Overbaugh et al., 1996; Philpott et al., 2005). Recombination between cervical and blood strains of HIV were shown to contribute to the rapid evolution of viral sequence in distinct compartments (Philpott et al., 2005). Although differences between viral species present in each compartment were identified, Poss et al. (1998) suggested that the genital and blood compartments are only partially independent of one another and that there is a continual process of migration of a restricted population of HIV-infected cells to mucosal sites from systemic circulation, followed by localized HIV expansion and independent evolution of the new quasispecies. They suggest that the genital mucosa is continually challenged with new viral variants which might be better adapted to the mucosal sites (Poss et al., 1998). Understanding changes in HIV species between these two compartments is crucial for studying the effects of systemic or antiviral therapy and the epidemiology of viral transmission (Coombs et al., 2001).

Compartmentalisation in HIV genotype and phenotype has also been described in brain and tissue lymphocytes (Kober et al., 1994; Wong et al., 1997) during chronic but not acute HIV infection (Wong et al., 1997). Zhang et al. (2002) the relatively homogenous viral strains present during acute HIV-infection diversifies under selective and distinct immune pressure in the different compartments as HIV disease progresses. HIV replication may also be affected by local conditions in each compartment, including the presence of different immune and other cell types, cytokine milieu, and inflammatory signals (Philpott et al., 2005).

Compartmentalisation could also arise as a result of independent migration of HIV-specific HIV-infected memory T cells homing to tissue compartments in which they were initially primed. These mucosal homing T cells make a small and transient contribution to the systemically circulating pool of lymphocytes which would reduce the likelihood that they would be detected in peripheral blood assessment (Mackay et al., 1992; Poss et al., 1998). The mechanism by which HIV species are selectively transmitted and subsequently undergo sequestration, and evolution in different tissue compartments will be important for understanding early immune responses against sexually transmitted HIV and the development vaccine therapies (Poss et al., 1995).

1.10 CTL diversity between compartments

Because of the presence of detectable compartment-specific diversity in HIV genotype, many groups have speculated that HIV-1 specific immune responses at the genital mucosa are also likely to differ from those detectable in blood (Musey et al., 2003). There are, however, conflicting reports about similar HIV-specific T cell compartmentalization.

Studies in macaques have shown that, during acute SIV infection, there are significant differences between mucosal and peripheral blood HIV-specific CTL frequencies that reflect early differences in HIV infection kinetics and spread (Reynolds et al., 2005). Reynolds et al. (2005) showed that HIV-specific immune responses following genital infection predominate in genital tissue and draining lymph nodes, and are of greater magnitude compared to blood responses even after one month of infection (Reynolds et al., 2005).

In contrast, while some studies have reported differences in HIV-specificity between CTL responses at the cervix and in blood (Shacklett et al., 2000), others have found that these compartments are largely overlapping in their HIV specificity (Musey et al., 2003; Kaul et al., 2000). In the most comprehensive study conducted to date, HIV-specific CTL responses were found to be overlap in the majority of HIV regions investigated (comprehensive HIV genome scan; Ibarrondo et al., 2005). Ibarrondo et al. (2005) found that the discordant CTL responses were found only in those that were of a low frequency in either compartment (Ibarrondo et al., 2005). They found concordant negative or positive results between compartments in 85% of screened peptide pools following whole HIV genome scan. A limitation of this data is that genomic regions were separated into fairly large peptide pools, which would significantly impact on the resolution of the HIV specificities which can be differentiated.

While HIV clearly has a systemic phase of infection and CTL responses directed against Gag in the blood do correlate with better disease outcome (Kiepiela et al., 2007), it can be argued that the impact of systemic immunity on transmission and mucosal viremia may not yield a comprehensive understanding of the factors involved

in transmission. It is therefore important to compare responses detected in blood with those detected at the genital mucosa.

1.11 Memory T cell subsets in mucosal compartments

The generation and maintenance of early differentiated cells capable of self-renewal and survival abilities in HIV infected individuals is essential in the establishment of a "protective" immune response (reviwed in Woodland and Kohlmeier et al., 2009; Ahmed et al., 2009). The importance of maintaining the GALT CD4+ central memory pool has been described during SIV infection (Veazey et al 1998; 2000; 2003; Picker et al., 2004) and in HAART-treated HIV-infected individuals (Macal et al., 2008). Macal et al. (2008) showed that GALT is predominantly populated with central memory phenotypes with a minor proportion of effector memory irrespective of HIV status. In addition, CD4+ T-cell restoration following initiation of HAART was shown to be associated with the frequency of central memory T cells in GALT. In addition, the frequency of CD4+ central memory T cells in GALT were found to be associated with better mucosal CD 4 + T -cell restoration (Verhoeven et al., 2008).

More differentiated T cell phenotypes are largely expected at the cervicovaginal area due to increased bacterial flora in this compartment compared to peripheral blood system (Nkwanyana et al., 2009). A recent study by Nkwanyana et al. (2009), reported that ex vivo cytobrush T cells were mostly of the effector memory phenotype (Nkwanyana et al., 2009).

1.12 Challenges in conducting mucosal studies

Technical and operational difficulties associated in sampling tissue from mucosal sites remains a major obstacle to the broad inclusion of mucosal assessment into studies of HIV pathogenesis. The invasiveness of mucosal sampling procedures and the limits imposed by low cell yields also makes the comprehensive assessments of mucosal site by standard laboratory analysis difficult. The difference in tissue composition at distinct mucosal sites (gut versus genital tract) also impacts on assay standardization and assessment. Variability in cell types and number from one mucosal sample to

another also makes standardization and assessment difficult, especially in the female genital tract where T cell yields are under the influence of hormones (Prakash et al., 2001).

While cervical mucosal sampling is largely invasive, several sampling methods of the cervix and lower gastrointestinal tract have been developed and were found to be well tolerated (Anton et al., 2000; Shacklett et al., 2000a; Kaul et al., 2003). With all of these methods, viable cell recovery is low and this has resulted in few studies being published comparing the breadth, magnitude and specificity of T cell responses in mucosal tissues and blood (Shacklett et al., 2000; Kaul et al., 2000; Gumbi et al., 2008). All of these studies encountered high frequencies of spontaneous IFN-γ production measured by ELISpot (Shacklett et al., 2000) and intracellular cytokine staining (Gumbi et al., 2008). There is a continuing need to develop and optimise protocols for accessing mucosal specimens and for performing the broadest range of immunological analysis using the limited samples recoverable. Polyclonal expansion of T cells provides a useful tool in combating limitations of T cell availability especially at mucosal sites.

1.13 Polyclonal T cell expansion

There have been few published studies focusing on use of polyclonal expansion of mucosal T cells to improve mucosal cell yields in order to do more comprehensive analysis of HIV-specific responses at mucosal sites (Ibbarondo et al., 2005). Bulk expansion of T cells in vitro have been widely and effectively used to increase yields of T cells to study the pathogenesis of various diseases such as malaria particularly in the context of low cell yields or magnitude responses (Wang et al., 2004; Winstone et al., 2009; Goonetilleke et al., 2006; Hanke et al., 2007; Keating et al., 2005; Todryk et al., 2009, Shacklet et al., 2003b).

Techniques used to polyclonally expand T cells include: (i) immobilised anti-CD3 (Yang et al., 1996); (ii) immobilised anti-CD3 with anti-CD28 (Azuma et al., 1992; Levine et al., 1996); (iii) anti-CD3 and anti-CD28 covalently linked to super-paramagnetic beads (Dynabeads; Hippen et al., 2008; Onlammon et al., 2006; Tricket

et al., 2002) or bi-specific monoclonal antibodies directed at both CD3 and CD4 or CD8 (Jones et al., 2003). The latter reagent is, however, not currently commercially available. Addition of IL-2, IL-4, IL-7, IL-9 and IL-15 has also been shown to be important T cell growth factors during polyclonal expansion (Kanegane et al., 1996; Chen et al., 2006; Geginat et al., 2001; Kalamasz et al., 2004). IL-7 has been shown to be essential for naive T-cell survival and homeostatic proliferation (Lalvani et al., 1997; Tan et al., 2001; Schluns et al., 2000). IL-15 has been shown to be essential for memory T cell in vivo renewal (Tan et al., 2002).

Polyclonal expansion of biopsied rectal material using a bispecific antibody has been used to generate sufficient cells within 2-3 weeks for comprehensive analysis by IFN-γ ELISpot, using pooled overlapping peptides spanning the entire HIV-1 genome (Shacklett et al., 2003b). Ibbarondo et al. (2005) reported 75-85% concordance between HIV-specific IFN-γ responses pre- and post expansion of biopsy-derived rectal mucosal T cells. They showed that expansion does not alter phenotype or specificity of antigen-specific T cell population as majority of T cell populations remained dominant following expansion. There is an urgent need for the development of methods that can similarly be applied to cervical cytobrush samples to improve cell yields rapidly in order to conduct simultaneous, high-resolution determination of T cell responses against various viral genes and multiple viral infections.

Although polyclonal expansion offers the advantage of improving cell yields from mucosal samples, this approach also presents challenges due to the increased susceptibility of these cells to microbial contamination from mucosal compartments such as intestines (Shacklett et al., 2003b) or the genital tract (Kaul et al., 2001). Contamination can be eliminated by the use of antibiotics targeted at combating mucosal compartment-specific microbes (Shacklett et al., 2003b).

Some authors have identified expansion bias and epitope skewing introduced by expansion which may favour outgrowth of certain populations at the expense of others as an important challenge imposed by polyclonal expansion (Shacklett et al., 2000; Jones et al., 2003; Ibarrondo et al., 2005). The differences in T cell maturational status between blood and cervix (Nkwanyana et al., 2009) complicate the comparison of

these compartments following expansion, as distinct memory subsets also have differing capacities to expand (Sallusto et al., 2004).

Previous studies have shown that distinct memory subsets of T cells differ in their ability to clonally expand and become activated following stimulation. Naïve T cells have higher activation thresholds and proliferate less vigorously than memory T cells (Sallusto et al., 2003). In addition, naive T cells require co-stimulation with anti-CD28 whereas memory cells do not (Gourley et al., 2004; Tan et al., 2001). A loss of naive cell populations (CD45RA+) with expansion of memory cell populations (CD45RA-) has been reported following expansion (Roth et al., 1994).

The lack of robust and validated assays that directly measure mucosal immune responses is a serious hurdle blocking progress in the field. This makes development and optimization of such assays an urgent priority. This thesis explores the use of *in vitro* polyclonal expansion to bulk up cytobrush-derived cervical T cells making it possible to asses the breath and specificity of T cells using fewer mucosal cells. It is critical to evaluate various techniques available to expand T cells and determine which population of T cells is favourable following expansion. This is pivotal in assessment of antigen specific responses in compartments such as blood and mucosa were different *ex vivo* phenotypes are present.

1.14 Aims and objectives

Overall aim of this dissertation

To investigate the feasibility of polyclonal *in vitro* expansion to improve yields of T cells recoverable from cervical cytobrush samples during HIV infection and then to compare the frequency, specificity and quality of cervical mucosal T cell responses with those detected in blood of chronically HIV-infected women.

Rationale for assessing mucosal immune responses

There have been few studies investigating the frequency, specificity or function of HIV-specific T-cell responses in the female genital tract of HIV-infected women. The predominant route of HIV transmission in Sub-Sahara Africa is via the genital mucosa (UAIDS, 2008) and >40% of HIV-infected women from this region are shedding HIV in their genital secretions (Gumbi et al., 2008; Nkwanyana et al., 2009). The female genital tissues release distinct HIV-1 quasispecies that are different from the populations present in the blood (Ellerbrook et al., 2001; Overbaugh et al., 1996; Philpott et al., 2005). Previous findings indicate that compartmentalization is maintained through immune response pressures on viral selection (Gupta et al., 2000). Understanding HIV transmission in the female genital tract and the immune pressures molding this is vital to creating better treatment and prevention regimens.

Rational for using polyclonal T cell expansion

An important limitation of all mucosal sampling methods, but cervical cytobrushing in particular, is the small number of cells obtainable from mucosal sites which makes it difficult to conduct in depth analyses on freshly isolated mucosal cells. Cervical cytobrushing is well-tolerated and relatively non-invasive compared to cervical or rectal biopsy. It is also more informative at the cellular level than cervical lavage since it targets the cervical transformation zone which is rich in T cells and macrophages (Passmore et al., 2006). This is also the zone implicated in HIV transmission (reviewed in Pope and Haase 2003; Coombs et al., 2003). Cervical cytobrushing is, however, constrained by the small number of cell it yields. Previous work from this laboratory has shown that a single cervical cytobrush yields an average of 100 000 CD3+ T lymphocytes (Gumbi et al., 2008; Nkwanyana et al.,

2009), a quantity insufficient to perform comprehensive mapping of HIV-1-specific CD8+ T-lymphocyte responses even with cell-sparing assays such as ELISpot (Shacklett et al., 2000). Polyclonal in vitro expansion methods have been effectively used to overcome the technical barrier of small numbers of cells available from other mucosal sites such as the gut (Shacklett et al., 2003b Ibarrondo et al., 2005).

Specific objective 1

To evaluate HIV-1-specific CTL responses from cytobrush-derived cervical mucosal T cells in chronically HIV-infected women following polyclonal *in vitro* expansion with anti-CD3 in the presence of IL-2 (Chapter 2).

Hypothesis

Polyclonal anti-CD3 expansion is a well-characterized technique in expanding T cells derived from blood and it expands most T cell clonotypes (Yang et al., 1996). It was hypothesized that polyclonal anti-CD3 expansion would improve yields of T cells recoverable from cytobrush and allow comprehensive mapping of the cervical T cell responses to HIV Gag peptides. Since Musey et al. (2003) has previously shown that >60% of HIV-specific responses in the female genital tract were directed against Gag and since the aim of this study was to map individual HIV epitopes in detail, this study focused on Gag only.

Specific objective 2

To optimize and compare polyclonal T cell expansion protocols based on anti-CD3 and IL-2 to improve the frequency of cervical samples capable of being expanded; and increase the yield of cervical T cells obtainable for study (Chapter 3).

Hypothesis

Since robust expansion of T cells is central to the success of these studies, there is an urgent need for the development of expansion methods that can generate the highest yield of T cells, within the shortest period of time in order to conduct simultaneous, high-resolution studies of T cell responses to HIV. Distinct memory subsets of T cells differ in their ability to clonally expand and become activated following stimulation (Sallusto et al., 2004). The hypothesis for this Chapter is that T cells from the cervix,

representing a more differentiated phenotype than those present in blood, will have reduced proliferative capacity and therefore ability to expand.

Specific objective 3

Using the optimized T cell expansion protocols developed in Chapter 3, the aim of this final Chapter was to compare HIV Gag-specific T cell responses at the cervix and in blood of chronically HIV-infected women using multiparameter flow cytometry (Chapter 4).

Hypothesis

Evaluation of HIV-specific immune responses at the genital mucosa will provide important information for the better understanding of virus–host interaction during HIV infection and transmission. Recent studies revealed that polyfunctional T cells are capable of producing more of each individual cytokine per cell than a monofunctional cell (Precopio et al., 2007; Darrah et al., 2007; Duvall et al., 2008) and there is an added value in measuring multiple functions compare to a single functional assessment of IFN-γ (Betts et al., 2006; Crithfield et al., 2008). Studies by Chritchfield et al. showed that rectal CD8+ responses are robust compared to PBMC difference and are related to blood CD4 count and plasma viral load between compartments in polyfunctionality. Brenchley et al. (2008) showed that lung had more polyfunctional HIV-specific T cells compared to blood. Gumbi et al. (2008) showed that the frequency of HIV Gag-specific T cell responses were not associated with protection from HIV shedding in chronically HIV-infected women. The hypothesis of this last Chapter was that cervical mucosal T cells would exhibit higher frequencies of polyfunctional responses to Gag than detected in the blood and this would be associated with better protection from local HIV shedding.

CHAPTER 2

Polyclonal expansion of cervical cytobrush-derived T cells to investigate HIV-specific responses in the female genital tract

Table of Contents

2.1 Introduction

Sexual transmission of HIV accounts for the majority of new infections worldwide (UNAIDS, 2008). In women, the mucosal surface of the female genital tract is ultimately the site of HIV infection and transmission (Prakash et al., 2001). While there is clear evidence that HIV-specific cytotoxic T lymphocytes (CTLs) in blood play an important role in controlling HIV replication systemically (Addo et al., 2003; Betts et al., 2001; Edwards et al., 2002; Kaufmann et al., 2004; Ramdaduth et al., 2005), there is comparatively little known about both CTL responses to HIV in the genital tract, and the factors that govern these responses. HIV-specific mucosal CTLs have been detected in men and women during chronic HIV-infection (Musey et al., 2003; Ibarrondo et al., 2005; Kaul et al., 2000a;2003; Shacklett et al., 2000; Gumbi et al., 2008; Sheth et al., 2005). The potential importance of such CTLs in protection from HIV is highlighted by the fact that they have also been detected in individuals who, despite frequent exposure to HIV-1, fail to become productively infected (Kaul et al., 2000a). In chronically HIV-infected men (Sheth et al., 2005) and women (Gumbi et al., 2008), the detection of HIV-specific T cells in the genital tract was independent of HIV shedding and viral load in genital secretions.

There is an urgent need for reliable, validated and non-invasive methods for investigating mucosal immune responses in the female genital tract. Such methods would be particularly valuable in HIV vaccine trial settings where intensive mucosal sampling is not an option. To date, however, the HIV-specific mucosal CTL literature has been dominated by biopsy approaches to isolating lymphocytes from rectal and gastrointestinal mucosal tissue (Ibarrondo et al., 2005; Anton et al., 2000; Guadalupe et al., 2003; Shacklett et al., 2003a; Critchfield et al., 2007; Musey et al., 1997). Fewer approaches are available for sampling mucosal tissue from the female genital tract (cervical lavages and cervical cytobrushes). The usefulness of both these methods are, however, constrained by the low numbers of *ex vivo* lymphocytes they yield (Kaul et al., 2000a; Shacklett et al., 2000; Gumbi et al., 2008; Sheth et al., 2005).

Here we investigate the feasibility of polyclonal *in vitro* expansion of cervical cytobrush-derived T cells to investigate HIV-specific responses in the female genital tract. We show that cervical T cells sampled from women with chronic HIV-1 infection can be expanded and that the magnitude of expansion is significantly associated with initial *ex vivo* cell yield and viability. Following *in vitro* expansion, we found both the magnitude and breadth of HIV-specific T cell responses at the cervix correlate significantly with those detected in blood. Cervical responses were, however, generally only detectable in women with corresponding blood HIV-specific T cell responses above 1000 SFU/10^6 cells.

2.2 Materials and Methods

2.2.1 Study population

Twenty-seven women with chronic HIV infection were enrolled. All women had CD4 counts >300 cells/µl and were antiretroviral therapy naïve at the time of study. Samples were not collected if participants were menstruating. All women gave informed consent and the Research Ethics Committee of the University of Cape Town approved all aspects of the study.

2.2.2 Collection and processing of cervical and blood specimens

Cervical samples were collected using a cytobrush as previously described (Gumbi et al., 2008; Passmore et al., 2006). Briefly, a Digene cervical cytobrush was inserted into the cervical os and rotated 360^{o}. The cytobrush was immediately placed in 15 ml tubes containing ice cold transport medium or R10 (RPMI1640 medium, supplemented with 10 % heat-inactivated human AB serum (AB), 5mM L-glutamine, fungizone, 50 U/ml penicillin and 50 µg/ml streptomycin). The cervical samples were kept in a Bench-top cooler (Nalgene Rochester, NY, USA) at 4^{o}C until transport to the laboratory and were processed within 4 h of sampling. Of the 27 samples collected, 5/27 (18.5%) were discarded because they were visibly contaminated with blood. Red blood cell contamination was measured by macroscopic visual inspection of cells in suspension and following centrifugation. A previous study from our group has demonstrated that macroscopic assessment of red blood cell contamination has a threshold of sensitivity equivalent to ≤0.0005% PBMC contamination per cytobrush sample (Passmore et al., 2006).

Cervical cells were isolated from the cytobrush by flushing approximately 20 times using a sterile plastic disposable pipette to dislodge mucus. The cell suspension was centrifuged at 1500 rpm (437g) for 10 min and the pellet resuspended in R10.

Whole blood was collected in ACD Vacutainer tubes [Becton Dickinson (BD) Biosciences, Plymouth, UK by venipuncture]. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich, Egham, Runnymede, UK) and LeucoSep® centrifuge tubes (Greiner Bio-one; Frickenhausen, Germany). Cells concentrations were adjusted to 1–2 x 10^6 cells/ml and incubated overnight at 37°C, 5% CO_2 for use in ELISPOT assays. Mononuclear cells were counted using Typan Blue staining to assess viability.

2.2.3 Expansion of cervical T cells

Polyclonal expansion of cervical cells was performed using anti-CD3 (Anti-CD3 monoclonal antibody; Clone UCHT1; Lot no MAB100; R&D Biosystems; Minneapolis, MN, USA) in the presence of recombinant human interleukin-2 (rhIL-2; NIH AIDS Research and Reference Reagent Program, Germantown, MD). UCHT1 is a well characterised IgG1 mouse mAb that identifies a determinant present on all human T cells as and appears to share the same characteristics as OKT3 (Burns et al., 1982). Freshly isolated cervical cells were plated (at 4 x 100 µl per well per cytobrush) into 96-well round-bottomed plates pre-coated with anti-CD3mAb (10µg/ml). Irradiated autologous PBMCs (10^6cells/ml; 100µl/well; irradiated at 40 Gy) were added to each well as feeders. Recombinant human IL-2 was added to each well at a final concentration of 100 IU/ml (kindly provided by the NIH AIDS Reagent Program). Cervical cytobrush T cell lines were incubated at 37^{o}C, 5 % $C0_2$ and cultures supplemented every 2 days with fresh R10 containing rhIL-2 (100 IU/ml). Wells containing irradiated feeder cells alone in the presence and absence of anti-CD3mAb and rIL-2 were included on each plate to control for outgrowth of feeders. T cell lines were re-stimulated again after 14 days with anti-CD3 mAb (10µg/ml) and fresh medium supplemented with rIL-2 (100 IU/ml). Throughout culture, all R10 was supplemented with 2 mg/ml fungizone, 50 U/ml penicillin and 50 µg/ml streptomycin and cell lines were monitored for bacterial and fungal infections, periodically counted and adjusted to 1 x 10^5 cells/well.

Cervical cytobrush cells and T cell lines were analysed for expression of CD4 and CD8 T cell markers before and after polyclonal expansion by flow cytometry. Because the

number of cervical mononuclear cells available on day 0 was limited, only 10% (v/v) of the sample was used for determination of CD4:CD8 ratio on day 0. The antibodies used were CD3-APC, CD8-FITC and CD4-FITC (BD Biosciences, San Jose, CA, USA). Cells were resuspended in 0.5mL of R10 and 50µl-100 µl (depending on cell count) of cell suspension was aliquoted for surface staining. Flow cytometric acquisition was performed using a four colour FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo (Tree Star, Inc; Ashland, OR, USA) software.

2.2.4 HIV-1 Gag peptides

HIV-1 subtype C Du422 Gag overlapping peptides spanning the entire Gag sequence were kindly provided by Dr Clive Gray (National Institute for Communicable Diseases, Johannesburg, South Africa) and consisted of 66 (15- to 18-mer) peptides overlapping by 10 amino acids. Lyophilized stocks of each peptide were dissolved in DMSO at a concentration of 20 mg/ml and stored at -80 ^{0}C. The 66 Gag peptides were divided into five pools each containing 14 peptides (except for pool 5 that had 10 peptides).

2.2.5 IFN-γ ELISPOT

The production of IFN-γ by HIV Gag-specific T cells was detected by the ELISPOT assay. Briefly, 96-well nitrocellulose plates were pre-coated with IFN-γ monoclonal antibody (clone 1-D1K; Mabtech, Stockholm, Sweden). Cervical mononuclear cells were washed twice, counted, and plated into wells either with or without HIV-1 subtype C Gag peptides (1 ug/ml) or in PHA. Fresh PBMC were plated in triplicate at 1 x 10^5 cells/well while expanded mucosal cells were plated at 0.5 x 10^5 cells/ml. Plates were incubated overnight at 37 ^{0}C in a 5 % CO_2 humidified incubator. After washing, bound IFN-γ was detected by a second biotinylated IFN-γ mAb (clone 7-B6-1; Mabtech), followed by streptavidin-bound horseradish peroxidase (HRP) and visualisation was performed using a Nova Red substrate kit (Vector Laboratories, CA, USA). Finally, individual cytokine-producing cells (spots) were counted using a CTL ELISpot reader (Cellular Technology, Jessup, Maryland, MD, USA). Totals for plated wells were averaged and normalized to numbers of IFN-γ spot-forming cells per 1 x 10^6 PBMC (SFU/10^6 PBMCs). Mean values for negative media control wells were subtracted from the mean values of antigen-

stimulated wells to give net SFU/10^6 cells. Assays with a negative control background mean of less than 100 SFU/10^6 cells were considered valid and a positive response was defined as exceeding the mean + 2 standard deviations (Shacklett et al., 2000).

2.2.6 Intracellular cytokine staining of PBMCs following HIV Gag stimulation

To define which T cell subset was contributing to HIV Gag-specific IFN-γ responses detected by ELISPOT, IFN-γ production by PBMCs was also assessed using intracellular cytokine staining. PBMCs (2-3 x 10^6 cell/ml) were stimulated with HIV Gag peptides (1 ug/ml), staphylococcal enterotoxin-B (SEB; 10 μg/ml; Sigma-Aldrich Eghum, Runnymede, UK; positive control) or not stimulated (negative controls) for 6 h at 37^{o}C, 5% CO2. Brefeldin A (10 μg/ml; Sigma, St. Louis, MO, USA) was added after the first hour. Following 6 hours, cells were washed with FACS wash buffer (10 % FCS-PBS containing 0.01 % NaN_3) and centrifuged at 200g for 5 min. The cells were fixed and permeabilised with 2 ml of BD cytofix/cytoperm solution (BD Biosciences-Pharmingen, San Diego, CA, USA) for 10 min at room temperature. Cells were then washed once with 0.1 % w/v Saponin (Fluka;; Biochemica, Mulhouse, France) PBS (containing 5 % FCS and 0.01 % NaN_3) at 200g for 5 min. Cells were then stained with phenotypic markers APC-labelled anti-CD3, PerCP-labelled CD4, and FITC-labelled anti-CD8 (BD Biosciences, San Jose, CA, USA). To detect IFN-γ, cells were simultaneously stained with PE-labelled anti-IFN-γ (BD Biosciences-Pharmingen, San Diego, CA, USA) for 30 min on ice. Cells were washed with 2 ml of FACS wash buffer, centrifuged at 200g for 5 min and fixed with 1 % paraformaldyde in PBS. Stained PBMC were acquired on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed using either Cellquest (BD Biosciences, San Jose, CA, USA) version 4 or FlowJo (Tree Star, Inc) software version 6. To define a cut-off for positive HIV Gag responses by either CD8+ or CD4+ T cells, IFN-γ responses to Gag peptides were initially evaluated in a panel of 10 HIV negative women (Western blood Transfusion Service (WBTS), Cape Town, South Africa). CD8+ or CD4+ T cell responses to Gag >3-fold above background were considered positive.

2.2.7 Impact of polyclonal expansion on Vβ T cell receptor usage by PBMCs

The impact of polyclonal expansion on Vβ T cell receptor (TCR) usage by PBMCs was evaluated using the IOTest Beta Mark flow cytometry kit according to the manufacture's protocol (Immunotech, Marseille, France). The kit contains 8 distinct combinations of conjugated TCRVβ antibodies corresponding to 24 different specificities (Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, Vβ23). Briefly, unexpanded (day 0) or polyclonally expanded (day 28) PBMCs (2-3 x 10^6 cells/ml) from four individuals (PID 23-26) were each divided into 8 BD FACS tubes. The 8 tubes were then stained with a panel of 24 Vβ family-specific antibodies (3 Vβ specificities/tube). While antibodies directed against Vβ specificities were restricted to FITC, PE and APC, we added anti-CD3 PerCP (Beckton Dickinson) to differentiate T cell subset. All tubes were incubated at 4^{o}C for 30 min, then washed twice and fixed by the addition of 500 µl of BD Cell fix. Cells were acquired using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo (Tree Star, Inc) software.

2.2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (Graph Pad San Diego California, CA, USA). The Mann-Whitney U test was applied for independent sample comparison, the Wilcoxon Ranks Test was used for matched non-parametric comparisons and Spearman Ranks or Pearson correlation was applied for correlation comparisons. A p-value of ≤ 0.05 was considered significant. Where indicated, the false discovery rate (FDR) step-down procedure was used to control for false-positive results when performing multiple comparisons (Columb and Sagadai, 2006). However, due to the rarity of the mucosal samples used throughout this study and because the analyses were mainly exploratory, multiple comparison adjustment was not widely performed.

2.3 Results

Twenty two women with chronic HIV infection were investigated for HIV Gag-specific cervical cytobrush-derived T cell responses following *in vitro* expansion (Table 2.1). All the study subjects were under the age of 40 years (median age of 31 years, range 22-39), had a median blood CD4 count of 451 (range 300-1,300) cells/μl and plasma viral load of 10,097 (range 970-1,300,000). Five of the 22 (22.7%) women had visible vaginal discharge or yeast infections at the time the cytobrush was taken.

Table 2.1. Description of HIV-infected women included in the study

Patient ID	Age	CD4 Count (cell/µl)	HIV Viral Load (RNA copies/ml)	Macroscopic findings	Day 0 cervical cell counts	Day 14 cervical cell counts	Day 28 cervical cell counts	Fold Expansion (day 28)
1	33	1396	<50[a]	Vaginal discharge	20,000	100,000	1,600,000	80
2	26	414	9,500	Nil	10,000	100,000	680,000	68
3	23	341	14,500	Nil	10,000	310,000	480,000	48
4	30	428	<50[a]	Nil	14,278	Contaminated	Contaminated	-
5	28	698	1,800	Vaginal discharge	18,520	Contaminated	Contaminated	-
6	28	341	100,573	Nil	40,000	130,000	560,000	14
7	36	572	51,325	Nil	68,640	125,600	1,650,000	24
8	26	607	4,013	Nil	75,800	114,000	Contaminated	-
9	31	482	27,250	Nil	80,000	140,000	990,890	12.4
10	30	545	15,800	Nil	90,000	440,000	1780,000	19.8
11	32	400	1300,000	Cervix inflamed	90,860	Contaminated	Contaminated	-
12	31	393	41700	Nil	100,000	610,000	850,000	8.5
13	22	301	2,200	Nil	100,000	270,000	1,750,000	17.5
14	32	461	7,100	Nil	110,000	490,000	1,040,000	9.5
15	31	788	1926	Nil	120,000	790,000	1,200,000	10
16	33	451	30,000	Nil	120,000	260,000	1,900,000	15.8
17	24	ND	3,649	Nil	135,721	617,600	1,460,000	10.8
18	39	422	5,450	Nil	138,040	652,000	Contaminated	-
19	39	338	<50[a]	Nil	180,000	240,000	1,200,000	6.7
20	43	467	88000	Yeast	218,064	Contaminated	Contaminated	-
21	37	300	10,097	Vaginal discharge	220,000	740,000	2,120,000	9.6
22	38	482	970	Nil	240,000	530,000	2,850,000	11.9
Median	31	451	10,097	5/22 (22.7%)	100,000	290,000	1,330,000	13.2
Range	22-39	300-1,300	970-1,300,000		10,000-240,000	132,500-590,000	480,000 -2,850,000	6.0-80.0

2.3.1 Expansion of cervical cytobrush-derived T cells from women with chronic HIV-infection

A median of 0.1 x 10^6 cells (range 0.01-0.2 x 10^6 cells) was obtained per cytobrush *ex vivo* (Table 2.1). The association between *ex vivo* cervical cell yields and both age and clinical HIV disease status (CD4 counts and viral load) were initially investigated. A significant positive association was observed between the age of women in the study and the yield of cervical cells obtained (p=0.0008; Pearson R=0.66) indicating that older age was associated with increased yield of cervical cells. There was no association between markers of disease status (CD4 count and viral load) and cervical yield (data not shown). However, all women included in this study were recruited during the chronic phase of infection with CD4 counts >300 cell/μl (CD4 counts ranging from 300-1300 cells/μl; Table 2.1). Age and clinical status was not associated with viability of cervical cytobrush cells in this study (data not shown). All women in the study were not menstruating at the time of cytobrush collection although we did not control for menstrual cycle stage. A recent study from our group has shown that *ex vivo* cervical cytobrush mononuclear cell yield are significantly associated with genital tract inflammation (Nkwanyana et al., 2009). While inflammatory cytokine concentrations in genital secretions were not measured in this study, genital inflammatory mediators are likely to have a substantial impact on *ex vivo* cervical mononuclear cell yield.

Cervical T cells were polyclonally expanded for either 28 days (18/22) or 42 days (4/22) to increase the yield of cells from the cervix for the identification of peptide responses to Gag by the IFN-γ ELISPOT assay. Of the 22 cervical T cell lines initiated, 16/22 (72.7%) expanded. A median of 1.3 x 10^6 cells (range 0.5-2.9 x 10^6 cells) was obtained at day 28, which represented a 13-fold increase in T cell yield compared to *ex vivo* cell yields (Table 2.1 and Figure 2.1A). Six of 22 lines (27.3%) were lost to contamination during the first few days of culture. Of these, 3/6 contaminated samples were collected from women with visible cervical discharge.

While CD8 T cells were found to be the dominant T cell subset detected *ex vivo* (CD4:CD8 of 0.5), 28 days of *in vitro* expansion resulted in the ratio equalizing such that

we obtained a CD4:8 ratio of 1 (Figure 2.1B; p=0.006, Wilcoxon Rank Test). Of the cervical lymphocytes isolated *ex vivo*, 90.9 % (± 7.5; mean±SD) were viable (range 70-100 %). A significant positive correlation was found between the absolute number of cells obtained following expansion and both the number of cervical cells isolated ex vivo (p=0.01; R=0.62) and the ex vivo viability of cells (p=0.0002; R=0.71). Conversely, a significant negative correlation was found between fold expansion by 28 days and *ex vivo* cell number (p=0.004; R=-0.68), indicating that *ex vivo* cervical cells with the lowest yield exhibited the highest relative rate of expansion.

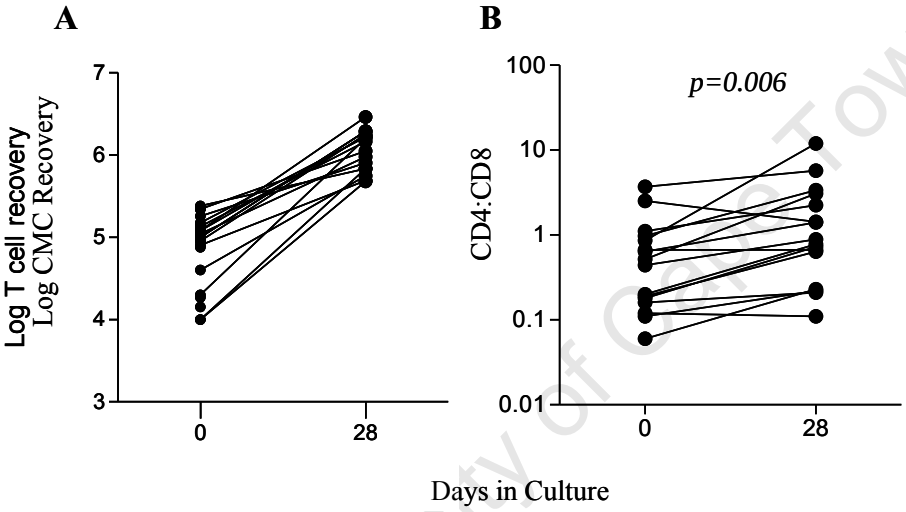


Figure 2.1. Impact of *ex vivo* yield on expansion of cervical cytobrush-derived cervical mononuclear cells (CMC). (A) Comparison between day 0 and day 28 CMC yields in donors whose cervical CD3 cells expanded. **(B)** Comparison of CD4:CD8 ratio *ex vivo* (day 0) and day 28 in expanders. CMC were cultured *in vitro* with anti-CD3 monoclonal antibody in the presence of rhIL-2. CMC counts and viability were performed by Trypan blue counting. CD4:CD8 ratios on cervical cell lines were analysed before and after polyclonal expansion using CD3-APC, CD8-FITC and CD4-FITC staining (Becton-Dickinson, San Jose, CA, USA) on a FACSCaliber flow cytometer and analysis was performed using FlowJo (Tree Star, Inc) software. Wilcoxon Rank Test was applied to compare yield and CD4:CD8 ratios at day 0 and day 28. P-values <0.05 were considered significant. Each data point represents an individual donor.

2.3.2 Gag-specific responses by cervical T cells

HIV-1 Gag-specific responses were mapped by IFN-γ ELISPOT for 16 of 22 cervical T-cell lines (72.7 %) from chronically HIV-infected women. These were compared with similarly identified responses identified in PBMC *ex vivo* of these women (Figure 2.2). Nine of the 16 (56.2 %) cervical lines from the chronically HIV-infected women showed clear evidence of HIV-specific T cell responses to Gag with a cumulative frequency of IFN-γ producing cells ranging from 350-13553 SFU/10^6 cells. There was no correlation between cervical *ex vivo* yield, viability, expansion (day 28) yield, or plasma viral load and the magnitudes of HIV Gag-specific cervical T cell responses (data not shown). Eight of the nine cervical lines that exhibited Gag-specificity were derived from women with clearly detectable Gag-specific blood responses (>600 SFU/10^6 cells; Figure 2.2). There was only one participant (PID 1) who had a detectable cervical response to Gag but no matching blood response.

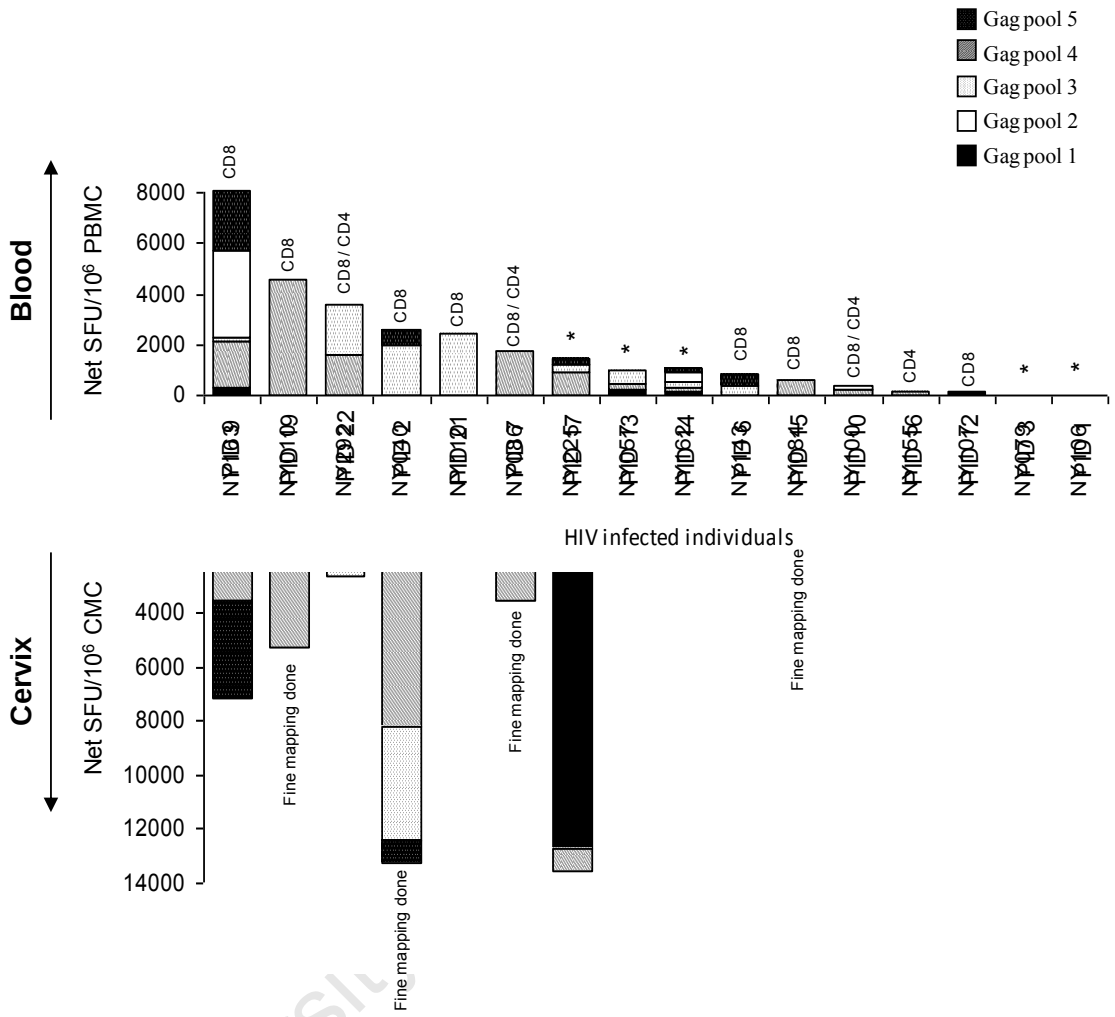


Figure 2.2. Comparison of cervical and blood HIV Gag-specific IFN-γ responses in women with chronic HIV-infection. Blood (top panel) and cervical (bottom panel) IFN-γ responses to HIV-1 subtype C Gag peptide pools 1-5. Net spot forming units (SFU)/10^6 were calculated by subtracting background IFN-γ frequency from Gag peptide pool specific IFN-γ frequencies in 16 women with chronic HIV-infection. Each stacked bar represents the cumulative IFN-γ frequency for all five Gag peptide pools and the subjects have been ordered from highest to lowest blood T cell magnitude to Gag. The contribution of CD4+ and CD8+ T cell subsets to overall IFN-γ responses to HIV Gag peptides was determined by intracellular cytokine staining and FACS. The T cell subset contributing to Gag-specific responses per donor is shown above each stacked bar for blood responses. The CD8 indicates CD8 only response, CD4 indicates CD4 responses only while CD8/CD4 indicates that responses in both subsets were detected by flow cytometry. A * indicates that responses were not evaluated in either the blood or cervical compartment of these individuals. „Fine mapping done' denotes the 4 women for whom this analysis was done.

From the 16 women for which HIV Gag ELISpot mapping was performed (Figure 2.2), there was a significant correlation between the cervix and blood in both the total magnitude of IFN-γ responses to Gag (Table 2.2 and Figure 2.3A; rho=0.7, p=0.002; Spearman Ranks test) and the individual Gag pool magnitudes of IFN-γ responses per individual (Table 2.2 and Figure 2.3B; rho=0.51, p<0.0001; Spearman Ranks test). Despite good maintenance of specific Gag pool targeting between cervical and blood, 4/9 (44.4 %) of women with detectable HIV-specific responses at the cervix had unique pool specificities that were not detected in blood (PID 1, 2, 17, and 19; Figure 2.2 and Table 2.2).

Table 2.2. Comparison between breadth of response at cervix and in blood

Patient ID	Cumulative magnitude (SFU/10^6)		Gag pools	Gag pools differently targeted	
	Blood	Cervix	shared	Blood	Cervix
9	8,040	7,130	1,2,5	3,4	-
19	4,605	5,280	2	-	1
22	3,565	2,640	2,3	-	-
2	2,610	13,240	3,5	-	2
21	2,440	490	3	-	-
7	1,740	3,540	2	-	-
17	1,480	13,553	2	3,5	1
13	1,000	0	-	1,2,3	-
14	1,100	0	-	1,2,3,4,5	-
6	850	0	-	3,5	-
15	600	1,140	-	2	-
10	360	0	-	2,4	-
16	0	0	-	2	-
12	165	0	-	1	-
3	120	0	-	-	-
1	0	350	-	-	5
Median	1,050	420			
Range	0 – 8,040	0 – 13,553			

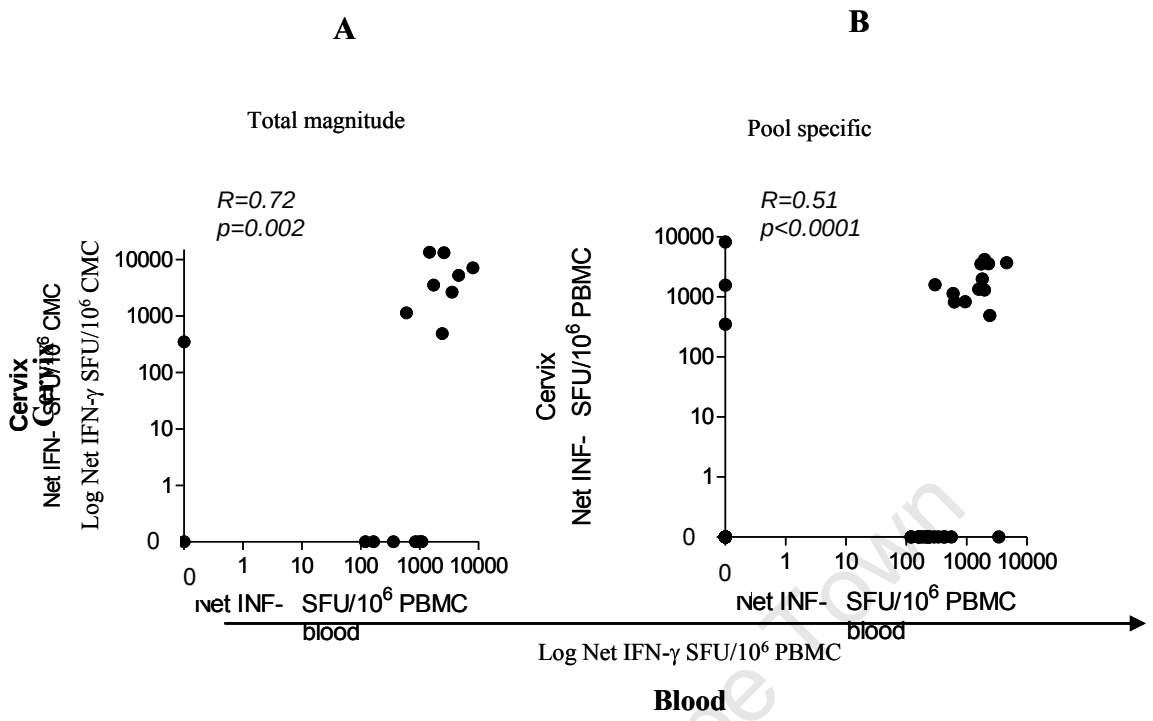


Figure 2.3. Correlation between HIV Gag-specific IFN-γ response magnitudes detected at the cervix and in blood of HIV infected subjects. (A) Correlation between cumulative net IFN-γ responses to Gag pools (sum of 5 pools) per chronically HIV-infected individual in each compartment (n=16 women for whom matched ELISPOT was conducted on cervical lines and PBMCs). **(B)** Correlation between individual Gag pools (1-5) at the cervix and in blood in HIV-infected women. Each data point represents an individual's IFN-γ response at the cervix and in blood. Spearman Rank Test was applied to test correlations and p-values <0.05 were considered significant. Spearman R-value is shown on each plot.

Individual peptide mapping was done on cervical lines derived from 4/9 women with detectable Gag pool responses who had sufficient cervical cell numbers to allow finer mapping (PID 2, 7, 15 and 19; Table 2.3). All 4 women had cervical T cell responses that preferentially targeted peptides in p24 and 3/4 women targeted the same peptide within p24 containing the HLA class I TL9 epitope (^{178}GA**TPQDLNTML**NTVGGH$_{194}$). Although the HLA genotypes for the participants in this study are not known, the peptides recognized by these 4 women had a number of previously characterized HLA class I and fewer HLA class II epitopes embedded within them (Table 2.3;predicted by the Los Alamos Database: http://www.hiv.lanl.gov/content/sequence/ELF/epitope_analyzer.html).

Table 2.3. Epitope prediction from confirmed peptide specificities on cervical samples

PID	HIV protein	Net SFU/million cells	Peptide	HLA I epitope	HLA I type	HLA II epitope
19	p24	880	24	^{170}PMFT**ALSEGATPQDL**NTM$_{187}$		^{170}**PMFTALSEGAT**PQDLNTM$_{187}$
				^{170}PMFTA**LSEGATPQDL**NTM$_{187}$	B42, B*4403	
	p24	4,680	25	^{178}GA**TPQDLNTML**NTVGGH$_{194}$	B*3910, B*4201, B*8101, B*0702, Cw*0802	^{178}GA**TPQDLNTMLNTVGGH**$_{194}$
2	p24	5,600	22	^{155}W**VKVIEEKAF**SPEVIPMF^{172}	B*1503	^{155}WVKVI**EEKAFSPEV**IPMF^{172}
				^{155}WVKV**IEEKAFSPEV**IPMF^{172}	B*4006	^{155}WVKVI**EEKAFSPEVIP**MF^{172}
				^{155}WVKV**IEEKAFSPEVI**PMF^{172}	B*4501	
				^{155}WVKVI**EEKAFSPEV**IPMF^{172}	B*4501	
				^{155}WVKVIE**EKAFSPEV**IPMF^{172}	Cw*0602	
				^{155}WVKVIEE**KAFSPEVIPMF**^{172}	B57, B63	
				^{155}WVKVIEEKA**FSPEVIPMF**^{172}	B63, B57, B58	
				^{155}WVKVIEEKAF**SPEVIPMF**^{172}	B*35	
				^{178}GA**TPQDLNTML**NTVGGH$_{194}$	B*3910, B*4201, B*8101, B*0702, Cw*0802	^{178}GA**TPQDLNTMLNTVGGH**$_{194}$
	p24	2,040	25			
				^{234}SDIAGT**TSTLQEQIAW**M^{250}	B*5701, B*5703, B*5801	^{234}SDIA**GTTSTLQEQIA**WM^{250}
	p24	6,120	33			
				^{414}W**KCGKEGHQMK**DCTERQA^{431}	A11	
	p2p7p1p6	680	57			
15	p24	3,210	25	^{178}GA**TPQDLNTML**NTVGGH$_{194}$	B*3910, B*4201, B*8101, B*0702, Cw*0802	^{178}GA**TPQDLNTMLNTVGGH**$_{194}$
7	p24	3,820	23	^{163}**AFSPEVIPMFT**ALSEGA^{179}		^{163}AF**SPEVIPMFTALSE**GA^{179}
				^{163}AF**SPEVIPMF**TALSEGA^{179}	B63, B57, B58	
				^{163}AFSP**EVIPMFTAL**SEGA^{179}	A*2601, A*2603	

HLA, human leucocyte antigen; HIV, human immunodeficiency virus; PID, patient identity; SFU, spot-forming units.

Although the low cervical cell numbers generated in this study were insufficient to evaluate whether the cervical Gag-specific responses detected (Figure 2.2) were specific to the CD8+ or CD4+ T cell subsets, T cell subset-specific IFN-γ responses to Gag were evaluated in matched blood samples from 11/16 women by intracellular cytokine staining. The criteria we used for scoring Gag-specific CD8+ and CD4+ T cell responses as positive or negative (3-fold background cut-off) was based on analysis of PBMC from 10 HIV negative women. In HIV-infected women, the Gag-specific IFN-γ response frequency by CD8+ T cells ranged from undetectable (0% above background) to 8% above background. A similar analysis in uninfected women showed that Gag-specific IFN-γ responses ranged from undetectable to 1 % above background. The background levels in the CD4+ T cell compartment were lower compared to the CD8+ T cells, ranging from undetectable levels to 5% in HIV-infected women and ranging undetectable to 0.1% above background in HIV negative women. The majority of these women had only CD8+ T cell responses to Gag (7/11; PID 2, 6, 9, 12, 15, 19, 21), 3/11 women had Gag-specific responses in both CD4+ and CD8+ subsets (PID 7, 10, 22) and 1/11 women had only a CD4+ response (PID 16). Of these 11 women, 7 had detectable cervical responses to Gag. Five of these 7 women had exclusively CD8+ T cell responses to Gag in blood while 2/7 had both CD4+ and CD8+ T cell responses. The T cell subset contributing to Gag-specific responses per donor is shown above each stacked bar for blood responses (Figure 2.2).

While it was not possible to confirm which T cell subset was contributing to the cervical T cell responses detected in this study (Figure 2.2), the fact that the individual peptides mapped in 4 individuals (Table 2.3) contained both HLA class I and II epitopes and that flow cytometry on matched blood samples showed both CD8+ and CD4+ responses systemically to Gag implies that both T cell subsets may be involved. The dominance of CD8+ responses in blood together with the number of HLA class I predicted epitopes suggests, however, that CD8+ T cells are likely to be the dominant T cell subset involved in the female genital tract as well.

2.3.3 Impact of expansion on HIV-specificity and T cell Vβ repertoire in blood

To evaluate the impact polyclonal expansion with anti-CD3mAb on skewing of Gag regions targeted by HIV specific T cells, PBMC from 4 HIV-infected individuals with well

characterized *ex vivo* Gag-specific responses were examined before and following expansion by IFN-γ ELISpot (Figure 2.4). The dominant *ex vivo* Gag regions targeted were maintained following *in vitro* expansion in all individuals. Compared to *ex vivo* responses (day 0), there was an overall increase in the cumulative frequency of IFN-γ producing cells following *in vitro* expansion, ranging from 2 to 11-fold. The frequency of Gag pool responses at day 0 were, however, significantly correlated with those following expansion for 28 days (Figure 2.4B; $R=0.81$; $p<0.0001$; Spearman Ranks Test) indicating that the specificity profile was largely maintained following expansion. In a single donor (PID 26), however, a unique specificity to pool 5 (485 SFU/million cells) was observed after expansion that was not detectable *ex vivo*. This highlights the possibility that specificities of low frequency that are not apparent *ex vivo* may become detectable following expansion due to the observed overall increase in cumulative frequency. It is also important to note that only 1/4 unique Gag specificities that we observed in cervical samples (PID 1) had a magnitude of <500 SFU/10^6 cells while unique cervical specificities from the other 3/4 donors (PID 2, 17, 19) had magnitudes of >1,500 SFU/10^6 cells. This implies that the unique cervical specificities we recorded for at least 3/4 cervical donors represent "real" unique responses and are not the result of expansion of previously low frequency events. It should be noted, however, that an extrapolation of blood expansion kinetics with that of cervical T cells might just be a reflection of low frequency events.

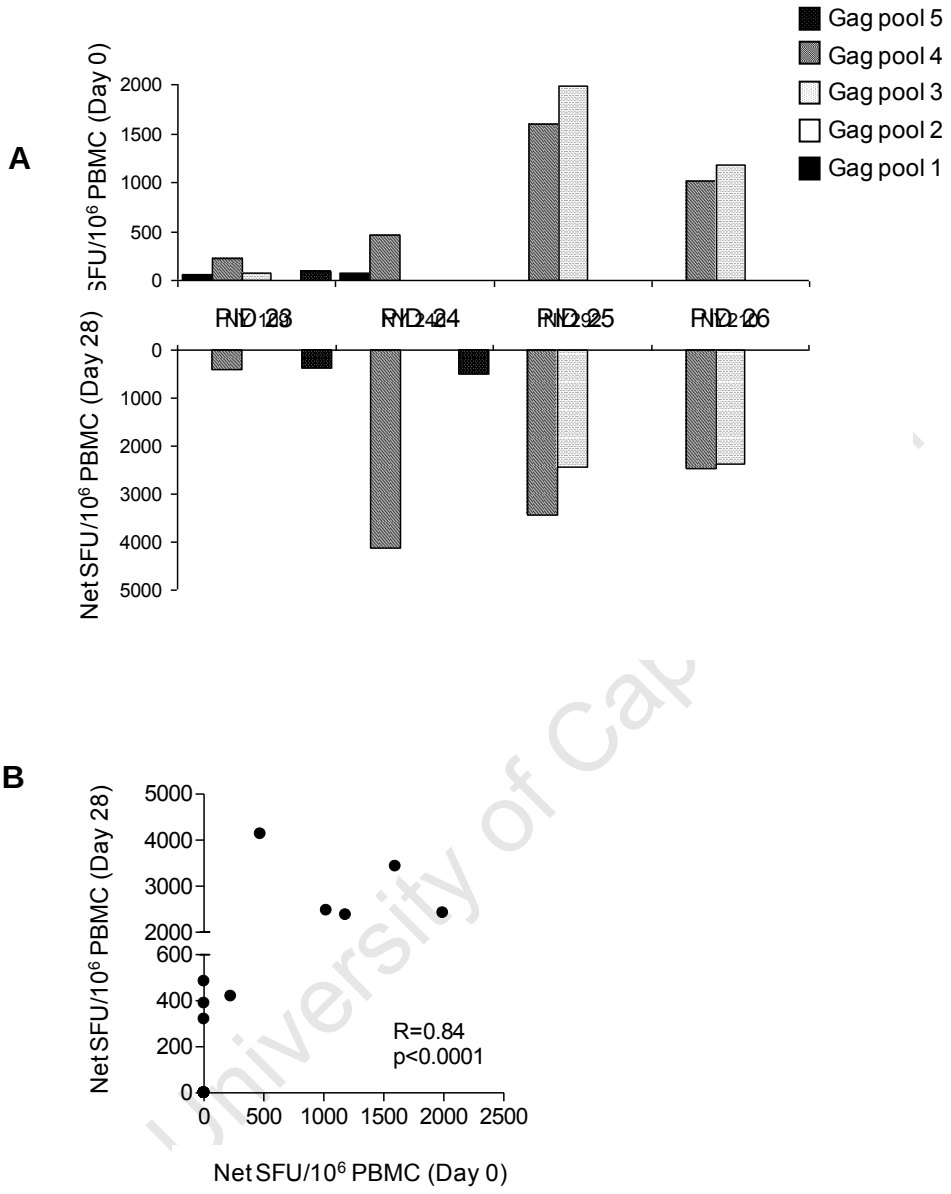


Figure 2.4. Correlation between *ex vivo* and expanded HIV Gag-specific IFN-γ ELISpot responses in blood from chronically HIV-infected subjects. (A) PBMC from four HIV-infected women were assessed for HIV-specific IFN-γ production *ex vivo* (top panel) and following *in vitro* expansion with anti-CD3 monoclonal antibody for 28 days (bottom panel). **(B)** Correlation between IFN-γ responses detected in PBMC *ex vivo* and after 28 days expansion. HIV-1 subtype C Gag overlapping peptides were divided into 5 pools and responses in these donors to each Gag pool were assessed. Net IFN-γ response to Gag was calculated by subtracting background IFN-γ production in each individual from Gag-specific responses. Each dot (B) represents an individual donor's matched IFN-γ response to each of the 5 Gag pools in blood *ex vivo* and following expansion (day 28). Spearman Rank Test was used to test the correlation and a p-value <0.05 were considered significant.

Changes in the T cell receptor (TCR) Vβ repertoire were next evaluated following 28 day expansion of PBMC with anti-CD3 and rhIL-2 in these 4 individuals (Table 2.4). The profile of TCR Vβ frequencies on day 0 in 3/4 the study subjects correlated significantly with profiles following in vitro polyclonal expansion (Patient 26: $R=0.97$, $p<0.0001$; Patient 24: $R=0.69$, $p=0.0002$, Patient : $R=0.84$, $p<0.0001$). In contrast, TCR Vβ frequencies for Patient 23 following expansion did not correlate and this was likely due to expansion of certain Vβ types. In this individual, Vβ4 expanded 9.9-fold, Vβ7.2 expanded 4.6-fold while Vβ13.1 contracted 7.6-fold over 28 days. Unlike in blood, experiments showing fluctuations in cervical TCRVβ specificities during the course of expansion could not be conducted because of limitations in cervical T cell numbers.

Table 2.4. T cell receptor Vβ usage ex vivo and following 28 days of in vitro expansion with anti-CD3 and rhIL-2

	PID 23			PID 24			PID 25			PID 26		
TCR Usage	Day 0	Day 28	Ratio Day 28:0	Day 0	Day 28	Ratio Day 28:0	Day 0	Day 28	Ratio Day 28:0	Day 0	Day 28	Ratio Day 28:0
Vβ1	4.27	2.2	0.52	5.84	5.61	0.96	3.65	5.94	1.63	6.93	6.56	0.95
Vβ2	3.02	0.78	0.26	6.69	5.55	0.83	3.56	6.59	1.85	3.38	5.21	1.54
Vβ3	5.52	6.34	1.15	4.17	5.14	1.23	1.64	2.27	1.38	4.48	5.73	1.28
Vβ4	**0.64**	**6.24**	**9.75**[a]	**1.51**	**3.41**	**2.26**	1.26	1.72	1.37	0.85	0.66	0.78
Vβ5.1	3.22	3.52	1.09	3.85	4.26	1.11	3.12	6.19	1.98	4.5	5.71	1.27
Vβ5.2	1.44	1.43	0.99	0.79	0.91	1.15	1.3	1.59	1.22	1.8	1.56	0.87
Vβ5.3	2.19	0.96	0.44	1.45	1.44	0.99	1.43	1.32	0.92	0.92	1.09	1.18
Vβ7.1	3.42	3.23	0.94	2.23	2.41	1.08	2.17	3.93	1.81	1.73	2.56	1.48
Vβ7.2	**0.97**	**4.47**	**4.61**	1.36	1	0.74	**0.66**	**1.56**	**2.36**	**0.34**	**0.85**	**2.50**
Vβ8	3.86	4.54	1.18	3.96	4.43	1.12	4.24	4.67	1.10	4.6	4.99	1.08
Vβ9	3.4	1.69	0.50	2.18	2.58	1.18	3.56	2.37	0.67	5.09	2.52	0.50
Vβ11	0.95	1.54	1.62	1.06	1.33	1.25	1.07	1.32	1.23	0.4	1.22	3.05
Vβ12	2.17	0.82	0.38	1.47	1.4	0.95	2.17	3.02	1.39	1.53	3.57	2.33
Vβ13.1	6.15	0.81	0.13	2.96	3.69	1.25	3.2	4.82	1.51	4.27	4.98	1.17
Vβ13.2	1.3	1.41	1.08	2.62	2.61	1.00	1.24	1.59	1.28	0.97	1.66	1.71
Vβ13.6	1.97	3.03	1.54	2.31	2.43	1.05	1.74	3.19	1.83	**0.89**	**2.3**	**2.58**
Vβ14	2.72	2.73	1.00	10.55	11.1	1.05	6.27	4.24	0.68	3.15	3.52	1.12
Vβ16	2.36	2.39	1.01	0.93	0.95	1.02	**1.59**	**3.18**	**2.00**	1.27	2.4	1.89
Vβ17	2.54	4.06	1.60	3.57	3.8	1.06	**2.47**	**6.23**	**2.52**	5.96	4.39	0.74
Vβ18	0.64	0.55	0.86	0.36	0.46	1.28	0.56	0.62	1.11	0.81	0.96	1.19
Vβ20	3.03	3.37	1.11	2	2.41	1.21	2.61	3.48	1.33	2.38	3.19	1.34
Vβ21.3	5.42	5.48	1.01	0.55	0.87	1.58	2.64	4.42	1.67	**1.99**	**4.17**	**2.10**
Vβ22	**2.55**	**5.16**	**2.02**	3.87	3.94	1.02	2.43	4.68	1.93	3.54	3.94	1.11
Vβ23	**2**	**4.59**	**2.30**	0.64	0.56	0.88	0.82	1.18	1.44	0.94	1.25	1.33
Mean			1.55			1.14			1.51			1.46
Pearson R-value			0.1820[b]			0.9736			0.6896			0.8408
Pearson p-value			0.3946			<0.0001			0.0004			<0.0001

[a]Changes in any Vβ subset from day 28 to day 0 that were >2 are denoted in bold. [b]The correlation between Vb TCR usage at day 0 and day 28 were tested using a Pearson test and R- and p-values shown. A total of 200, 000 events were acquired on a FACS Calibur (BD Biosciences; San Jose, CA, USA). The false discovery rate (FDR) step-down procedure was used to reduce false-positive results when performing multiple comparisons (Columb and Sagadai, 2006).

2.4 Discussion

Immunological events at the cervix are likely to influence susceptibility to heterosexually-transmitted HIV infection. There is an urgent need for the development of validated, non-invasive methodologies for investigating HIV-specific mucosal immune responses associated with HIV pathogenesis and for vaccine assessment specifically in the female genital tract (Kaul et al., 2000; Shacklett et al., 2003a). Although cervical cytobrushing is relatively non-invasive, low cervical cell yields associated with this approach have significantly impacted on thorough evaluation of HIV-specific T cell responses (Kaul et al., 2000a; Gumbi et al., 2008; Shacklett et al., 2003a). Although *in vitro* expansion of cervical cytobrush-derived T cells would circumvent the problem of low yield, there have been few studies investigating the ability of cervical cytobrush-derived T cells to expand *in vitro* either polyclonally or in response to HIV-1 antigens. This study shows that cervical T cells can be isolated by cytobrushing and polyclonally expanded in the majority of women studied. Further, this study shows that HIV Gag-specific cervical T cell responses can be detected in HIV-infected women and correlate with responses detected in blood.

With one exception, cervical responses to Gag were detected only in HIV-infected women with blood Gag-specific responses >1000 SFU/10^6. Although a 13-fold expansion of cervical cell numbers was achieved, the inability to resolve genital tract responses to Gag in individuals with less than 1000 SFU/10^6 cells in blood suggests that the approach described here may not be sensitive enough for the evaluation of vaccine efficacy. The reason for this is that vaccine induced T-cell responses to Gag are expected to be substantially lower than those found in HIV infected individuals. While the bottleneck imposed by non-invasive cytobrush sampling impinges on the sensitivity achievable in cellular assays such as those described in this study, it is likely that a combination of even moderately improved sampling and cellular expansion methodologies could substantially lower the threshold above which HIV specific cellular responses could be detected.

Cervical cytobrush-derived T cells were polyclonally expanded *in vitro* from 73% of samples analyzed. A median of 13-fold expansion of cervical mononuclear cells was achieved using

anti-CD3 over 28 days. Ibarrondo *et al*. (2005) similarly expanded rectal biopsy-derived mucosal T cells from 12 HIV-infected individuals and reported a 4- to 10-fold expansion of CD8+ T cells over 14 days with bi-specific CD4/3 antibodies. Whereas their method selectively expanded CD8+ T cells, anti-CD3 expansion used in the present study expanded both CD4 and CD8 T cells with an overall 2-fold enrichment of CD4+ cells following expansion. This might be attributed to the fact that the expansion protocol used in this study was polyclonal with no specific T cell subset being targeted. Although the overall relative expansion described here was higher than previous studies (Ibarrondo et al., 2005), rectal biopsy mucosal sampling yielded 16- to 39-fold more cells than were obtained in our study from a single cytobrush. Although this study has focused on a single expansion protocol with anti-CD3mAb and rIL-2, it will be important in future studies to compare cervical T cell expansion kinetics using different expansion protocols, such as bi-specific antibodies (Jones et al., 2003), anti-CD3/28-coated beads (T cell expander; Dynabeads; Invitrogen, Osla, Norway); and addition of alternative or complementary cytokines such as IL-7 and IL-15 (Chen et al., 2006; Kalamasz et al., 2004).

In this study, 27% of cytobrush-derived cervical cultures that were initiated became contaminated within the first 24 hours of culture. Of these cytobrush samples, half were from women with macroscopic evidence of vaginal discharge, cervical inflammation or yeast infection indicating that concomitant cervical infections impact on the sterility of the sample for culture. Although the culture medium for transport of these samples to the laboratory and for processing contains penicillin, streptomycin and amphotericin B, these contaminations could possibly be minimized by including higher concentrations of amphotericin B and antibacterial agents specific for genital tract bacterial infections.

From expanded cervical T cell lines, it was found that HIV Gag-specific cervical T cell responses were detectable in ~50% of women with chronic HIV-infection. From matched blood samples, 88% of these women had systemic responses to Gag. The magnitude of these mucosal responses correlate significantly with the magnitude of Gag-specific responses measured in blood and were largely restricted to women with >1,000 Gag-responsive cells/10^6. Similarly, Kaul et al. (2003) showed concordance between HIV-specific responses in blood and at the

cervix *ex vivo* during chronic infection with 8/10 women studied having matching responses at the cervix as they had in blood. Since Kaul et al. (2003) selected participants based on high response frequencies to Gag in blood; it is possible that this may have impacted on the strong compartmental overlap they described. In comparison, Shacklett et al. (2000) reported only 3/8 chronically HIV-infected women with detectable HIV-specific responses in blood had a corresponding response at the cervix. Shacklett et al. (2000) also focused their study on women with well characterized high frequency responses in blood. In contrast to these studies, Kaul et al. (2000) found in both HIV-infected and apparently HIV-resistant uninfected sex workers that *ex vivo* HIV-specific responses at the cervix were as prevalent as they were in blood, that responses in the two compartments largely correlated and that 95% of women with detectable HIV-specific cervical T-cells responses (40/42) had corresponding blood responses of less than 1000 SFU/million cells.

Recently, Gumbi et al. (2008) reported that *ex vivo* frequencies of HIV Gag-specific CD8 responses in blood and at the cervix did not correlate. Unlike Kaul et al. (2003) and Shacklett et al. (2000), study (Gumbi et al., 2008) did not select women with high magnitude responses in blood and this may have impacted on the lack of concordance observed between compartments *ex vivo*. Gumbi et al. (2008) also focused on *ex vivo* functional assessment rather than *in vitro* expansion used in the present study. *Ex vivo* cytobrush-derived T cell effector cell distribution is likely to differ substantially from phenotypes present after *in vitro* expansion, with the former being dominated by effector cells and the latter being dominated by expanded memory cells (Keating et al., 2005). Although the maturational status of *ex vivo* or expanded cervical T cells was not compared, this may play a role in the success of *in vitro* expansion. Naïve and memory CD8+ T cell subsets in humans have significantly different capacities to proliferate and differentiate in response to T cell receptor stimulation or cytokines (Geginat et al., 2003a, 2003b). There are a number of issues that may also impact on the extent of *in vitro* expansion of cytobrush-derived T cells including the stage of the menstrual cycle that cytobrushes were collected, concomitant sexually transmitted infections, and vaginal hygiene practises (Cohn et al., 2001; Iqbal et al., 2005; White et al., 1997).

While *in vitro* polyclonal expansion of T cells is a useful approach to increase the number of cells available from inaccessible mucosal sites, antigen bias and epitope skewing introduced by polyclonal expansion (potentially favouring the outgrowth of certain populations at the expense of others) cannot be entirely excluded. This study shows here that both CD4+ and CD8+ cervical T cells were expanded and that similarly expanding PBMC did not significantly alter HIV Gag targeting nor Vβ T cell receptor repertoire in most individuals. Since some fluctuations were noted in TCR usage and the range of HIV Gag pools targeted in a minority of women, this suggest that some bias may be introduced by expansion in a subset of individuals.

It is critical to determine what constitutes a positive response in T cell response assays. Various way of calculating positive cells have been previously developed and these include using two standard deviations above background responses as a cut-off as well as using a two to three-fold above background cut off (Trigona *et al*., 2003), using a 90th percentile cut-off based on background responses (Duvall *et al*., 2008). Others have resorted to using a ten event cut-off for polyfunctional T cells (Betts *et al*., 2006), or using a threshold value (Shacklett *et al*., 2003). Like in this present study, cut-off values may also be determined by evaluating cytokine responses in HIV-uninfected PBMC samples (Gumbi et al., 2008). Here, given the level of background and variability in specific response frequencies, we decided that an appropriate cut-off for positive HIV Gag responses was 3- fold above background which is much more stringent than previous studies. Kaul et al. (2003) used an arbitrary cut-off of 2-fold above background for IFN-γ ICS of cervical cytobrush samples. Kaul et al (2003) used an arbitrary cut-off of 100 SFU/106 cells for IFN- ELISPOT preformed on cervical cytobrush samples. Similarly, Shacklett et al. (2000) also used the same arbitrary cut-off of 100 SFU/106 cells. In comparison, we use a relatively stringent cut-off of 3-fold above background for considering a response positive.

In summary, this study evaluates the feasibility and efficacy of polyclonal *in vitro* expansion of cervical cytobrush-derived T cells from women with HIV infection. The low cell yields that can be recovered non-invasively from the female genital tract necessitates focused efforts to optimize effective expansion methodologies. Since we do not fully understand what would constitute protective immunity against HIV, and immunity at the genital mucosa is likely to

play an important role in preventing HIV acquisition, the incorporation of mucosal sampling at the female genital tract and understanding HIV-specific events at this site should be a high priority during HIV vaccine trials. The finding that cervical cytobrushing and anti-CD3-mediated polyclonal expansion only enable detection of Gag-specific T cell responses in the genital tracts of women with correspondingly high systemic Gag-specific responses sets a benchmark against which to measure future efforts in this field and highlights the need for more efficient expansion methodologies.

CHAPTER 3

Comparison of polyclonal expansion methods to improve recovery of cervical cytobrush-derived T cells from the female genital tract of HIV-infected women

Table of Contents

3.1 Introduction

Understanding the role of immunity in the female genital tract in the control of HIV transmission is central to our efforts to prevent new infections. Several approaches have been used to isolate mononuclear cells from the genital tract, including cervical biopsy (TOMBOLA Group, 2009) cervicovaginal lavage (CVL) and cytobrushing (Coombs et al., 2001). While cervical biopsies yield the most cells, this approach is invasive and can compromises the integrity of the cervical surface, inducing slow healing ulcerations (TOMBOLA Group, 2009) which might enhance local HIV-1 replication in HIV-infected women (Lawn et al., 2000) or increase susceptibility to infection in uninfected women. Cervical cytobrushing and CVL have a significant advantage of being non-invasive but both are constrained by the low numbers of *ex vivo* lymphocytes they yield (Gumbi et al., 2008; Shacklett et al., 2000; Kaul et al., 2000a). Short-term polyclonal expansion of T cells derived from the cervical compartment would offer a useful approach to overcome the limitation of low cell yields (Iqbal et al., 2005).

Several methods to expand T cells have been used, including immobilised anti-CD3 (Yang et al., 1996), immobilised anti-CD3 with anti-CD28 (Azuma et al., 1992; Levine et al., 1996) or bi-specific monoclonal antibodies directed at both CD3 and CD4 or CD8 (Jones et al., 2003). Bi-specific antibodies are, however, not currently commercially available. Recently, anti-CD3 and anti-CD28 monoclonal antibodies covalently linked to super-paramagnetic beads have been applied to expand cells (Dynabeads) (Hippen et al., 2008; Onlamoon et al., 2006; Trickett et al., 2002).

Distinct maturational phenotypes or 'memory subsets' of T cells differ in their ability to clonally expand and become activated following stimulation. Compared to naïve T cells, memory T cells show lower activation thresholds and proliferate more vigorously (Sallusto et al., 2004). Further, the expansion potential of memory subsets differs between distinct memory classes, with central memory T cells exhibiting the highest proliferative capacity, followed by effector memory and then terminally differentiated memory cells (Sallusto et al., 2004). T cells derived by cytobrush from

the female genital tract are predominantly effector memory in phenotype (Nkwanyana et al., 2009), which is likely to impact on the ability of these cells to expand *in vitro*. While stimulation of T cells via the CD3-T cell receptor (TCR) plays a critical role in determining the fate of T cells (Sallusto et al., 2004), the presence of appropriate homeostatic cytokines is likely to play a major role in driving T cell proliferation, differentiation and survival, both *in vivo* (Rochman et al., 2009; Zhang et al., 1998; Sallusto et al., 2004) and *in vitro* (Onlamoon et al., 2006). Interleukin (IL)-2 is the predominant growth factor used to support proliferation of T cells during *in vitro* expansion, with the use of concentrations ranging from 20 IU/ml (Smith et al., 1995) to 1800 IU/ml described (Winstone et al., 2009). In addition, IL-7 plays an important role in the maintenance and antigen-independent proliferative ability of naïve T cells (Soares et al., 1998). IL-15 is essential for the homeostatic proliferation of memory CD8+ T cells and natural killer (NK) cells (Rochman et al., 2009) and it has also been reported to affect the homeostasis of memory CD4+ T cells in the absence of IL-7 (Purton et al., 2007a, 2007b). Interleukin-15 shares many biological functions with IL-2 (Picker et al., 2006). Interleukin-15 can also drive antigen-independent proliferation and differentiation of central memory to effector memory (Geginat et al., 2003; Picker et al., 2006).

While understanding and defining protective HIV-specific immunity in the female genital tract during HIV infection and transmission is recognized to be important, we and others have shown that few cells can be recovered ex vivo limiting the depth of analysis that can be performed (Nkwanyana et al., 2009; Gumbi et al., 2008; Shacklett et al., 2000; Kaul et al., 2000). The aim of the present study was therefore to compare *in vitro* expansion methods (anti-CD3, anti-CD3/CD28 or Dynal anti-CD3/CD28 beads) and cytokine combinations (IL-2, IL-7 and IL-15) to maximize the yield of T cells derived from the female genital tract of women infected with HIV-1. We identify maturational characteristics of T cells derived from the female genital tract that may limit *in vitro* expansion and investigate conditions that can be applied to overcome this.

3.2 Materials and Methods

3. 2.1 Study participants

Eighteen HIV-infected women from the Nyanga Day Hospital in Cape Town, South Africa were recruited for this study. All women had CD4 counts ≥300 cells/µl and were not on antiretroviral therapy at the time of study. Women menstruating, who were post-menopausal, had undergone a hysterectomy, or had visible or reported evidence of genital tract infections or discharges were excluded from the study. All women gave written informed consent, and the Research Ethics Committee of the University of Cape Town approved all aspects of the study.

3.2.2 Polyclonal expansion methods

The ability of anti-CD3, anti-CD3/anti-CD28 and Dynal anti-CD3/CD28 beads to expand T cells in the presence of differing combinations of IL-2, IL7 and IL-15 were compared. Table 3.1 summarizes the expansion methods and cytokine combinations used in this study. Expansions were performed in 96-well microtitre round bottomed plates (Greiner Bio-one; Frickenhausen, Germany). Wells were coated overnight at 4°C with immobilized anti-CD3 (final concentration 10µg/ml; MAB100; R&D Biosystems, Minneapolis, MN, USA), either alone or in combination with anti-CD28 [final concentration of 10µg/ml; L293, Becton Dickinson (BD) Biosciences-PharMingen, San Diego, CA, USA; (Azuma et al., 1992), in a final volume of 50µl/well in phosphate buffered saline (GIBCO® PBS, Invitogen™, Carlsbad, CA, USA). The coated plates were washed three times with PBS to remove unbound or excess antibody. Alternatively, Dynal magnetic beads (Dynabeads®) coated with anti-CD3 and anti-CD28 antibodies (T-cell expander; Invitrogen Dynal, AS, Oslo, Norway; (Trickett et al., 2002)) were added to wells at varying bead-to-target cell ratios (1:5, 1:1 and 3:1) in a final volume of 200µl/well [cell suspension + R10 medium (RPMI1640 medium, supplemented with 10 % human AB (HAB) serum, 5mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (GIBCO® Invitrogen™) and 2mg/ml fungin® (Invivogen, San Diego, CA, USA)]. Differing combinations of recombinant IL-2 (200 IU/ml; NIH AIDS Reagent Repository,

Germantown, Maryland, MD, USA), IL-7 (20 ng/ml; R&D Biosystem) and IL-15 (20 ng/ml; R&D Biosystems) were added to cultures (Table 1).

Table 3.1. Summary of the polyclonal expansion methods used

Stimulus	Cytokine cocktail			References
	IL-2 (IU/ml)	IL-7 (ng/ml)	IL-15 (ng/ml)	
Anti-CD3 immobilized	200	-	-	Yang et al. (1996)
Anti-CD3 immobilized	200	20	20	Liu et al. (2006)
Anti-CD3/anti-CD28 immobilized	200	-	-	Levine et al. (1996)
Dynal anti-CD3/CD28 beads (1:5)*	200	-	-	Kalamasz et al. (2004)
Dynal anti-CD3/CD28 beads (1:1)	200	-	-	Onlamoon et al. (2006)
Dynal anti-CD3/CD28 beads (3:1)	200	-	-	Kalamasz et al. (2004)
Dynal anti-CD3/CD28 beads (1:1)	200	20	20	Onlamoon et al. (2006)

* Anti CD3/CD28 bead-to-T cell ratios

3.2.3 Isolation of peripheral blood mononuclear cells (PBMCs)

Whole blood was collected in ACD anti-coagulated vacutainer tubes (BD Biosciences, Plymouth, UK). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich, Egham, Runnymede, UK) and LeucoSep® centrifuge tubes (Greiner Bio-one, Frickenhausen, Germany).

3.2.4 Polyclonal expansion of PBMCs

Broad comparison of *in vitro* expansion methods were initially performed using PBMCs because *ex vivo* cervical T cell yield precluded comprehensive comparisons of methodology. The two most efficient methods for expanding PBMCs were then applied to expand fresh cervical cytobrush-derived mucosal cells. Briefly, PBMCs from HIV-infected women were subjected to the seven different expansion conditions described in Table 3.1. PBMCs were added to respective wells at $1x10^5$ PBMC/well in R10 medium. Expansion with each method was performed in triplicate. Cells were cultured in a 5% CO_2 at 37^oC for 7 days and fresh R10 medium with cytokines was replenished after every 2 days. Cells were removed for cell counting on days 3, 5 and 7. When the cell density exceeded 2 x 10^6 cells/ml or when the medium became

yellow, cultures were split to a density of 0.5 x 10^6 cells/ml. Comparison of cell yields and T cell viability was determined by Guava automated cell counting (Guava Technologies, Hayward, CA, USA) and Trypan staining using a Fast Read Haemocytometer by microscopy (Sigma-Aldrich, Irvine, UK), respectively.

3.2.5 Collection and processing of cervical cytobrush specimens

Cervical cytobrush samples were collected from the female genital tract of all women under speculum examination using a Digene cervical sampler as previously described (Gumbi et al., 2008; Iqbal et al., 2005; Nkwanyana et al., 2009; Passmore et al., 2006). A Digene cytobrush was inserted into the cervical os and rotated 360^o and immediately placed into 3ml ice transport R10 medium. The cervical cytobrushes were transferred to 4^oC in a Nalgene (Rochester, NY, USA) bench-top cooler until transported to the laboratory. Cervical cytobrushes with visible blood contamination were discarded (Passmore et al., 2006). Cells were processed within 4h of collection by flushing the cytobrush ~30 times with R10. The cell suspension was then transferred to a clean 15ml tube and centrifuged at 1000 x g for 10 min. The pelleted cells were resuspended in 500µl R10. The absolute number of CD3+ T cells in each cytobrush sample was counted using a Guava automated cell counter (Guava Technologies) according to the method described by Nkwanyana et al. (2009). Viability of cervical mononuclear cells was determined by Trypan staining (Sigma-Aldrich, Irvine, UK) using a Fast Read Haemocytometer.

3.2.6 Polyclonal expansion of cervical cytobrush-derived T cells

Cervical T cells were expanded using either anti-CD3/IL-2, Dynal (1:1)/IL-2 or Dynal (1:1)/IL-2/IL-7/IL-15. Cervical cells were resuspended in R10 at 0.5-1 x 10^6 cells/ml and added to each well at 100 ul/well. Due to limited cell numbers, cervical cell expansions per donor were performed from multiple donors, rather than as replicates. Cervical cells were cultured as for PBMC.

3.2.7 Assessment of T cell maturational status by flow cytometry

Distinct memory T cell subsets were identified by differential staining with fluorescent antibodies directed against CD45RO, CCR7 and CD27. Cells were phenotyped on days 0 and day 7 after expansion. The following antibodies and fluorochromes were used in this study: CD3-Allophycocyanin-H7 (CD3-APC-H7), CD4-FITC, CD8-PerCp-Cy5.5, CD27-PE (all BD Biosciences, San Diego, CA, USA), CCR7-APC (R&D Systems, Minneapolis, MN, USA), CD45RO-Texas Red-PE (Beckman Coulter, Marseille, France), CD14-PacBlue (BD Biosciences San Diego, CA, USA), and CD19-PacBlue (Invitrogen, Carlsbad, CA, USA). Cells were stained with antibodies directed at phenotypic (CD3, CD4 and CD8) and maturational markers (CD45RO, CCR7 and CD27). Violet amine reactive dye ('ViVid'; Invitrogen™ Molecular Probes™, Eugene, OR, USA) was included in the staining protocol. Cells were stained for 30 min and washed in wash buffer (1% FCS in PBS). Approximately 200 000 events were acquired on an LSRII flow cytometer (BD Biosciences; San Jose, CA, USA). Data analysis and colour compensation were performed using FlowJo software v8.5.3 (Tree Star, Inc; Ashland, Oregon, OR, USA). Dead cells ($ViVid^+$), monocytes (CD14+), and B cells (CD19+) were excluded from the analysis. Fluorescence minus one (FMO) controls were used to set gates.

3.2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5® (GraphPad Software, San Diego California USA). The Mann-Whitney U test was applied for independent sample comparison, the Wilcoxon Ranks Test was used for matched non-parametric comparisons and Spearman Ranks correlation was applied for assessing the associations. P-values of ≤ 0.05 were considered significant. Due to the rarity of the

mucosal samples used throughout this study and because the analyses in this Chapter was mainly exploratory, multiple comparison adjustment was not widely performed.

3.3 Results

3.3.1 Characteristics of women

Eighteen HIV-infected women were included in this study to compare methods for polyclonal expansion of T cells from the female genital tract and blood (Table 3.2). Their median age was 34 years (range 26-43). They had a median blood CD4 count of 396 cells/μl (range 302-811 cells/μl) and a median plasma viral load of 6000 RNA copies/ml (range undetectable-170,000). These women were the same group of women as those studied in Chapter 2.

Table 3.2. Clinical characteristics of HIV-infected women included in this study

Group	PID	Age	CD4 count (cells/μl)	HIV viral load (RNA copies/ml)	*Ex vivo* cervical cell count
Anti-CD3/IL-2	1	26	703	<50	117,320
	2	26	384	13,000	96,800
	3	33	465	170,000	110,800
	4	42	601	<50	98,050
	5	31	302	120,000	94,800
	6*	34	552	13,000	112,356
Dynal(1:1)/IL-2	7	33	473	<50	118,800
	8	34	310	8,100	121,800
	9	43	605	<50	94,800
	10	32	811	<50	156,000
	11	36	389	<50	91,320
	12*	40	353	160,000	128,560
	13*	37	306	6,000	132,000
Dynal(1:1)/IL-2/IL-7/IL-15	14	41	396	1,600	124,800
	15	41	312	<50	112,800
	16	31	302	120,000	92,800
	17	40	401	22,000	142,000
	18	36	308	16,000	101,320
Median		34	396	6,000	112,800
Range		26-43	302-811	<50-170,000	91,320-156,000

* Cervical cytobrush samples became contaminated during expansion.

3.3.2 Comparison of polyclonal expansion methods for expanding T cells from blood

PBMCs from HIV-infected women were initially used to compare a broad panel of *in vitro* expansion methods because *ex vivo* cervical T cell yield did not allow for such comprehensive and parallel comparisons of methodology. The performances of seven different expansion protocols (Table 3.1) were compared to expand T cells from blood of five donors (PID 1-5 of Table 3.2; Figure. 3.1). Anti-CD3 and IL-2 treatment resulted in a median of 4.5-fold (range 3.7–5.3) expansion of CD3+ T cells in blood in 7 days (compared to day 0). Inclusion of anti-CD28 or addition of IL-7 and IL-15 to this combination did not significantly improve expansion (4.8-fold in the presence of anti-CD28 and 5.1-fold in the presence of IL-7 and IL-15; p=0.33 and p=0.18, respectively).

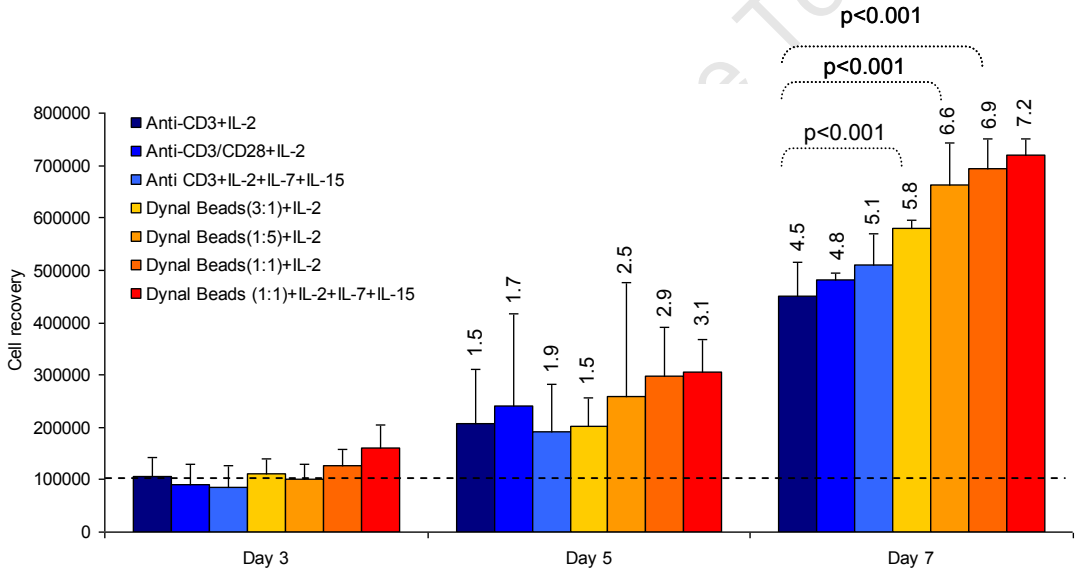


Figure 3.1. Comparison of polyclonal expansion methods for expanding T cells from blood. PBMC were expanded using seven different combinations of expansion protocols and cytokine cocktail: anti-CD3/IL-2 (dark blue), anti-CD3/anti-CD28/IL-2 (medium blue), anti-CD3/IL-2/IL-7/IL-15 (light blue), Dynal anti-CD3/CD28 beads (3:1)/IL-2 (yellow), Dynal anti-CD3/CD28 beads (1:5)/IL-2 (light orange), Dynal anti-CD3/CD28 beads (1:1)/IL-2 (dark orange), and Dynal anti-CD3/CD28 beads (1:1)/IL-2/IL-7/IL-15 (red). Bars represent the median of 5 donors cell recovery after 3, 5 and 7 days. Error bars represent the interquartile range for measurements taken from five donors. The figures on top of the bars represent median fold expansion after culturing. Wilcoxon Rank Test was used for comparison of day 0 and day 7 yields.

Dynal beads were tested at three bead-to-target cell ratios (3:1, 1:1, and 1:5) and in the presence or absence of IL-7 and IL-15 (Figure 3.1). At all bead-to-target cell ratios tested, Dynal bead expansion significantly improved yields compared to immobilized anti-CD3 and IL-2 ($p=0.008$). The greatest improvement in T cell yields was observed when an equal number of Dynal beads-to-T cells (1:1) was used (6.7-fold expansion with Dynal 1:1 compared to 4.5-fold for anti-CD3; $p=0.008$). Addition of IL-7 and IL-15 in the presence of Dynal beads (1:1) improved expansion yields slightly (7.1-fold; $p=0.3$ compared to Dynal beads 1:1 and IL-2 alone).

None of the expansion methods assessed significantly impacted on the viability of PBMCs compared to day 0. Compared to expansion with anti-CD3 and IL-2, expansion with Dynal beads (1:1) and IL-2 both in the presence and absence of additional IL-7 and IL-15 resulted in improved PBMC viability [$p=0.008$ for Dynal+IL-2 alone and $p=0.06$ for Dynal+IL-2+IL-7+IL-15] (Table 3.3).

Table 3:3. Impact of *in vitro* expansion methods on viability of PBMC

Stimulus	Cytokines Cocktail	Percentage viability following expansion					
		Day 3		Day 5		Day 7	
		Median	IQR	Median	IQR	Median	IQR
Anti-CD3 immobilized	IL-2	86.2	(67.0-86.3)	83.9	(74.3-85.9)	79.2	(77.4-83.7)
Anti-CD3 immobilized	IL-2, IL-7, IL-15	85.9	(66.4-86.0)	84.0	(75.7-84.6)	82.7	(79.8-82.8)
Anti-CD3/anti-CD28 immobilized	IL-2	73.1	(70.5-74.6)	81.4	(80.6-87.9)	85.3	(82.3-87.9)
Dynal anti-CD3/CD28 beads (1:5)*	IL-2	84.4	(70.8-86.9)	84.1	(72.6-84.2)	81.0	(80.9-81.3)
Dynal anti-CD3/CD28 beads (1:1)	IL-2	86.4	(85.6-87.4)	84.3	(83.1-84.3)	93.3	(76.5-95.7)
Dynal anti-CD3/CD28 beads (3:1)	IL-2	84.8	(82.7-84.8)	74.1	(74.0-74.2)	71.8	(69.4-73.7)
Dynal anti-CD3/CD28 beads (1:1)	IL-2, IL-7, IL-15	92.9	(90.8-94.1)	81.3	(79.2-81.7)	94.4	(87.1-95.0)

* Anti CD3/CD28 bead-to-T cell ratios. Median PBMC viability at day 0 was 94.5%.

From these experiments, we concluded that Dynal beads at a ratio of 1:1 in the presence of IL-2, IL-7 and IL-15 generated the greatest increase in CD3+ yield in 7 days.

To investigate the impact of HIV infection on *in vitro* expansion kinetics, we compared the expansion kinetics of PBMCs from 10 uninfected women (from Western Province Blood Transfusion Services, Cape Town, South Africa) to PBMCs from HIV-infected women (Table 3.4). While a similar hierarchy of performance was observed for the 7 expansion methods in HIV- compared with HIV+ individuals, the magnitude of expansion was found to be 2-3-fold higher in HIV- individuals than those infected with HIV (Table 3.4; $p<0.01$). This confirms that HIV infection significantly reduces the polyclonal expansion efficiency of T cells and that the performance of expansion methods would be better in uninfected individuals.

Table 3.4. Comparison of expansion kinetics of PBMC from HIV-infected and uninfected women

Stimulus	HIV-infected Median (IQR) N=5 ($x10^6$)	Uninfected Median (IQR) N=10 ($x10^6$)	Fold difference	p-value
Anti-CD3+IL-2	0.42 (0.41-0.46)	1.27 (1.23-1.28)	3.02	<0.01
Anti-CD3/CD28+IL-2	0.46 (0.40-0.46)	1.28 (1.22-1.32)	2.78	<0.01
Anti CD3+IL-2+IL-7+IL-15	0.53 (0.51-0.55)	1.32 (1.28-1.46)	2.49	<0.01
Dynal Beads(3:1)+IL-2	0.59 (0.58-0.59)	1.39 (1.33-1.50)	2.36	<0.01
Dynal Beads(1:5)+IL-2	0.67 (0.64-0.67)	1.44 (1.28-1.58)	2.15	<0.01
Dynal Beads(1:1)+IL-2	0.67 (0.66-0.68)	1.56 (1.22-1.76)	2.33	<0.01
Dynal Beads (1:1)+IL-2+IL-7+IL-15	0.71 (0.70-0.74)	1.90 (1.83-2.17)	2.68	<0.01

Expansion was carried out for 7 days

3.3.3 Impact of polyclonal expansion of PBMCs on T cell memory phenotype

The impact of each expansion method was next investigated on blood memory T cell phenotypes based on differential expression of CD45RO, CD27 and CCR7 (Figure 3.2 and Figure 3.3). Figure 3.2A shows a representative plot of the gating strategy used to define naive and total memory based on CD45RO and CD27 expression, while Figure 3.2B shows the strategy used to define distinct memory subsets. The following phenotypes were defined: naïve T cells (CD45RO-CCR7+CD27+), intermediate memory T cells (CD45RO-CCR7-CD27+), effectors (CD45RO-CCR7-CD27-), transitional memory T cells (CD45RO+CCR7-CD27+), effector memory (CD45RO+CCR7-CD27-), and central memory T cells (CD45RO+CCR7+CD27+) (Burgers et al., 2009). In order to simplify the analysis, only the proportions of effector memory and central memory are represented on figure 3.2, the rest of the proportions of memory subsets are summarised on figure 3.7.

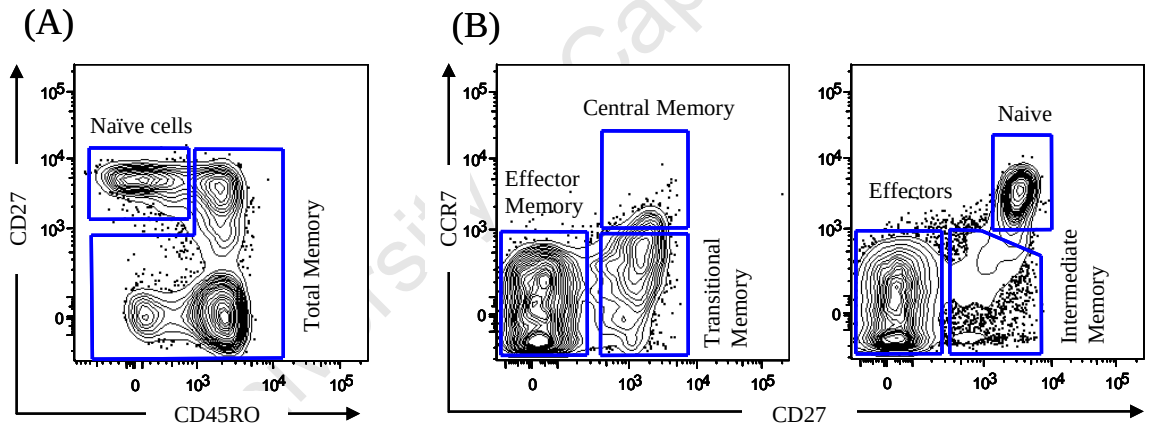


Figure 3.2. Impact of polyclonal expansion on T cell memory phenotype in blood. Cells were first gated on singlets, live cells and then lymphocytes. From this gate, CD3+ cytokine-producing T cells were gated, and then further gated on CD4+ and CD8+ T cells. Gates for each of the memory subsets were defined based on CCR7 and CD27 expression. Representative plots showing **(A)** naïve and antigen-experienced cells (total memory) based on differential staining with CD27 and CD45RO; and **(B)** differentiation markers expression (CD45RO, CD27 and CCR7) on CD8+ T cells from blood. Eight distinct memory subsets were defined from these markers: Central memory cells, ($CD45RO^+CD27^+CCR7^+$), transitional memory cells ($CD45RO^+CD27^+CCR7^-$), effector memory cells ($CD45RO^+CD27^+CCR7^+$), $CD45RO^+CD27^-CCR7^+$, naïve T cells ($CD45RO^-CD27^+CCR7^+$), intermediate memory cells ($CD45RO^-CD27^+CCR7^-$), effector ($CD45RO^-CD27^-CCR7^-$) and $CD45RO^-CD27^-CCR7^-$ cells.

Before expansion, a median of 80% (range 65-91%) of CD8+ T cells and 64% (range 46-81%) of CD4+ T cells were antigen experienced (including CD45RO+ and CD45RO-non-naïve effector cells; Figure 3.3 top panel). Following expansion, significantly increased frequencies (>95%) of both CD4+ and CD8+ T cells expressed CD45RO ($p<0.0001$).

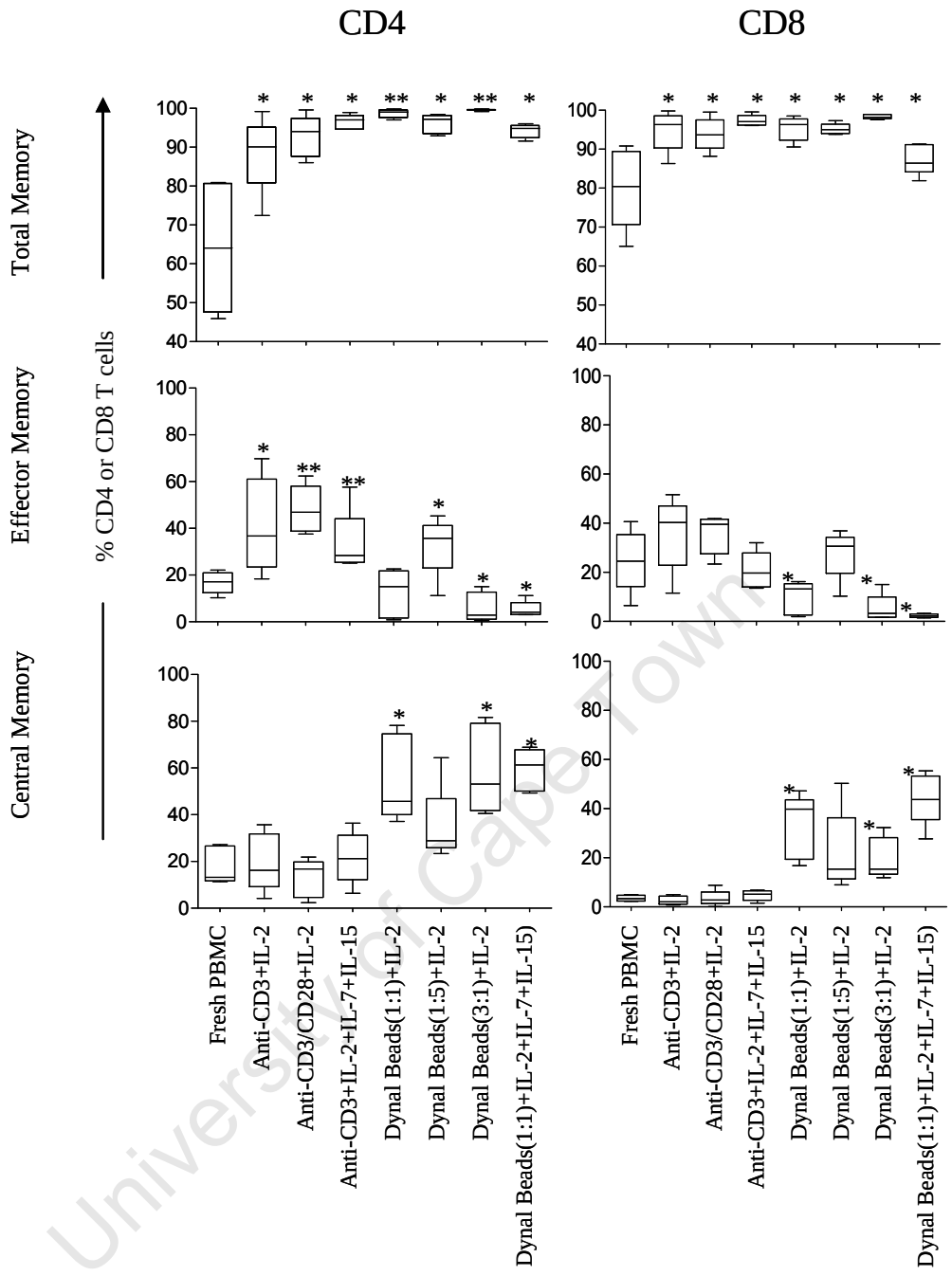


Figure 3.3. Impact of polyclonal expansion on T cell memory phenotype in blood. Comparison of the frequency of total (top panel), effector memory (middle panel) and central memory (bottom panel) subsets expressed as percentages of total $CD4^+$ and $CD8^+$ T cells in chronically HIV-infected individuals (n = 5) before and after expansion using seven different protocols. Each box and whisker plot shows the median (central line), IQR (outer lines of box) and 5-95% range (error bars) of 5 HIV-infected individuals. * indicates $p<0.05$ while ** indicates $p<0.01$ using Wilcoxon ranks test.

Since CD45RO+ memory T cells predominated in blood following expansion, the impact of expansion on distinct memory subsets (effector memory and central memory, Figure 3.3 middle and bottom panel) was further evaluated. It was noted that anti-CD3 and IL-2 stimulation (in the presence or absence of anti-CD28 and/or IL-7 and IL-15) resulted in the accumulation of cells with an effector memory phenotype (CD45RO+CCR7-CD27-) compared with unexpanded PBMC and cells expanded with Dynal beads (Figure 3.3). In contrast to anti-CD3, Dynabeads expansions (with or without IL-7 and IL-15) resulted in the accumulation of with a central memory phenotype. It was noted that expansion of PBMC with Dynal beads at a higher ratio of bead:PBMC (3:1 and 1:1) resulted also in a significantly higher proportion of cells with a central memory phenotype, compared with unexpanded PBMC ($p=0.01$ and $p=0.03$, respectively). Similar results were observed for both CD4+ and CD8+ cells.

3.3.4 Comparison of methods for expanding cervical T cells

A median of 112800 cervical cells (range 91,320-156,000) was obtained per cytobrush *ex vivo* from the 18 HIV-infected women included in this study (Table 3.2). From the results of the PBMC expansions, the three best performing methods [(1) anti-CD3/IL-2, (2) Dynal beads (1:1)/IL-2, and (3) Dynal beads (1:1)/IL-2/IL-7/IL-15] were selected to expand cervical cytobrush-derived cells (Figure 3.4). Fifteen of 18 cytobrushes (83.3%) expanded successfully while 3/18 (16.7%) became contaminated during culture (Table 3.2). This resulted in five independent cytobrushes were successfully expanded for each of the three expansion methods tested (Table 3.2). Despite a wide variation in HIV load (<50–170,000 RNA copies/ml), no significant difference in the distribution of viral loads between expansion groups was noted (data not shown).

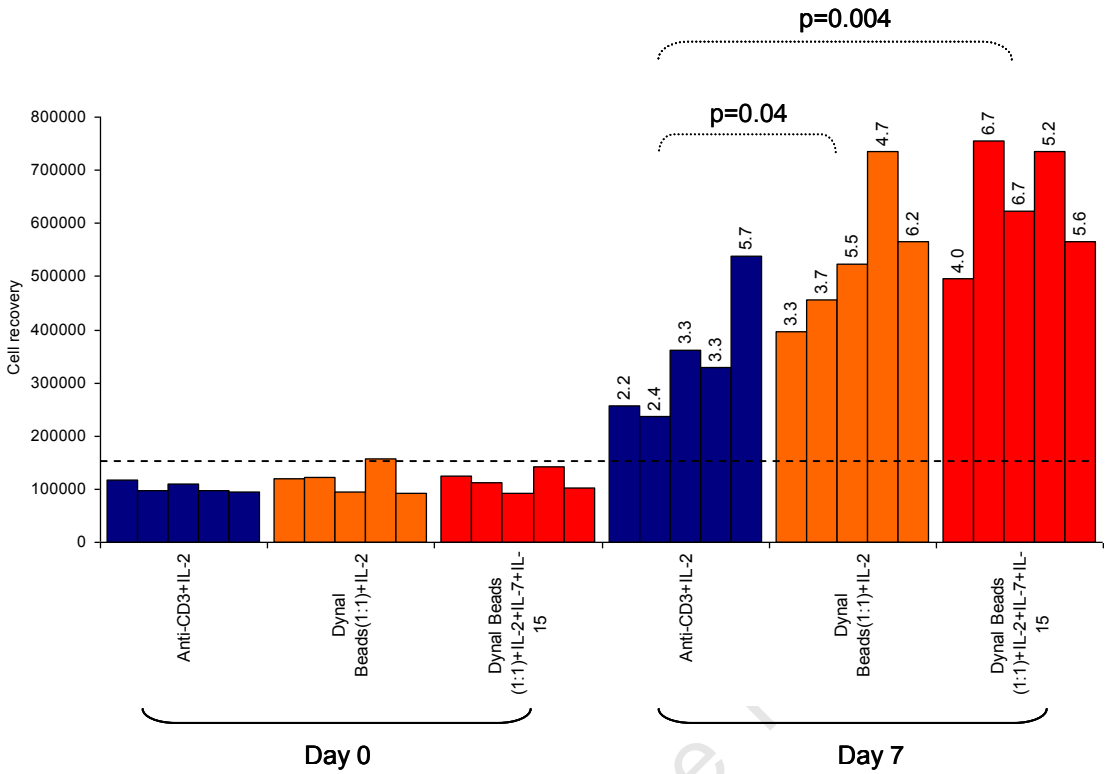


Figure 3.4. Comparison of polyclonal expansion methods for expanding cervical cytobrush-derived T cells. Cervical cells obtained from HIV-infected women were cultured with either anti-CD3/IL-2 (n=5; blue bars), Dynal anti-CD3/CD28 beads (1:1)/IL-2 (n=5; orange) and Dynal anti-CD3/CD28 beads (1:1)/IL-2/IL-7/IL-15 (n=5; red). Bars represent the yield of cells obtained from each donor pre- and post-expansion. Wilcoxon Rank Test was used to compare day 0 and day 7 yields. Figures on top of the bars represent fold expansion relative to day 0.

Expansion with anti-CD3 and IL-2 resulted in a median of 3.3-fold (range 2.2-5.7) increase in cervical CD3+ cells following 7 days expansion with a median yield of 328,000 cells [range 236,000-538,600] and median viability of 87% (range 84.5-89.5; Figure 3.4; Table 3.5). Compared with anti-CD3 and IL-2 expansion, Dynal beads (1:1) and IL-2 resulted in a significant improvement in cervical cell yields with and without additional cytokines IL-7 and IL-15 (p=0.004 and p=0.04, respectively; Figure 3.4). Dynal beads (1:1) and IL-2 alone yielded a median of 524,000 cells (range 396000-736000) with a median of 4.7-fold increase and a median cellular viability of 91.5% (range 90.25-93.5%). Dynal beads (1:1) and IL-2, IL-7 and IL-15 resulted in a median of 624000 (range 496000-756000) with a median 5.6-fold increase. Compared to expansion with anti-CD3 and IL-2, expansion with Dynal beads (1:1) and IL-2 both in the presence and absence of additional IL-7 and IL-15 resulted in improved cervical T cell viability [p=0.09 for Dynal+IL-2 alone and

p=0.02 for Dynal+IL-2+IL-7+IL-15]. From these experiments, it was concluded that Dynal beads (1:1) in the presence of IL-2, IL-7 and IL-15 generated the most significant increase in CD3+ T cell yields and viability in 7 days from cervical cytobrush specimens compared to *ex vivo* yields ($p<0.001$).

Table 3.5. Impact of *in vitro* expansion methods on viability of cervical cells

Stimulus	Cytokines Cocktail	Percentage viability following expansion				P-value
		Day 0		Day 7		
		Median	IQR	Median	IQR	
Anti-CD3 immobilized	IL-2	98.0	(96.0-100)	87.0	(84.5-89.5)	-
Dynal anti-CD3/CD28 beads (1:1)*	IL-2	94.0	(90.0-98.0)	91.5	(90.3-93.5)	0.05
Dynal anti-CD3/CD28 beads (1:1)	IL-2, IL-7, IL-15	96.0	(95.0-96.0)	96.0	(94.0-98.0)	0.02

* Anti CD3/CD28 bead-to-T cell ratios

3.3.5 Impact of expansion on memory T cell phenotype in the female genital tract

The impact of expansion on the memory phenotype of T cells derived from the cervix was next investigated (Figure 3.5). Figure 3.5A shows a representative plot of the gating strategy used to define cervical naive and total memory based on CD45RO and CD27 expression, while Figure 3.5B shows the strategy used to define distinct memory subsets at the cervix. Before expansion, a median of 97.8% (range 66.7-100.0) of CD4+ T cells and 95.3% (range 80-100.0) of CD8+ T cells from the cervix were found to be antigen experienced CD45RO+ T cells (Figure 3.6, top panel). Significantly more cells from the cervix expressed CD45RO compared to blood for both CD4+ ($p=0.0005$) and CD8+ T cells ($p<0.0001$) reflecting the trafficking of antigen-specific cells to mucosal sites. There were no significant changes observed in the frequency of total memory T cells before and after expansion from cervical cytobrush-derived cells (Figure 3.6 upper panel), since memory T cells made up the majority of the population before and after expansion. The impact of expansion on distinct cervical T cell memory subsets (effector memory, and central memory was evaluated; Figure 3.6; middle and lower panels respectively). Expansion of cervical cells for 7 days with anti-CD3 and IL-2 resulted in significantly increased frequencies of effector memory CD8+ T cells compared to fresh cytobrush samples (Figure 3.6). In contrast, Dynal bead (1:1) expansion (with and without IL-7 and IL-15) resulted in

significantly reduced frequencies of effector memory T cells (p=0.02 and p=0.0005, respectively) for CD4+ T cells. While central memory T cells comprised only 2.8% (range 0.0-11.1) of CD4+ and 0.3% (range 0.0-3.3) of CD8+ T cells before expansion, all expansion methods resulted in enrichment for this memory subset. In particular, expansion with Dynal beads (1:1) with and without IL-7 and IL-15 resulted in a 4.2-fold (p=0.0.03) to 11.2-fold increase (p<0.0001) in the frequency of CD4+ T cells and 81.1-fold (p=0.0002) to 154.3-fold increase (p<0.0001) in the frequency of CD8+ T cells that were of a central memory phenotype, respectively.

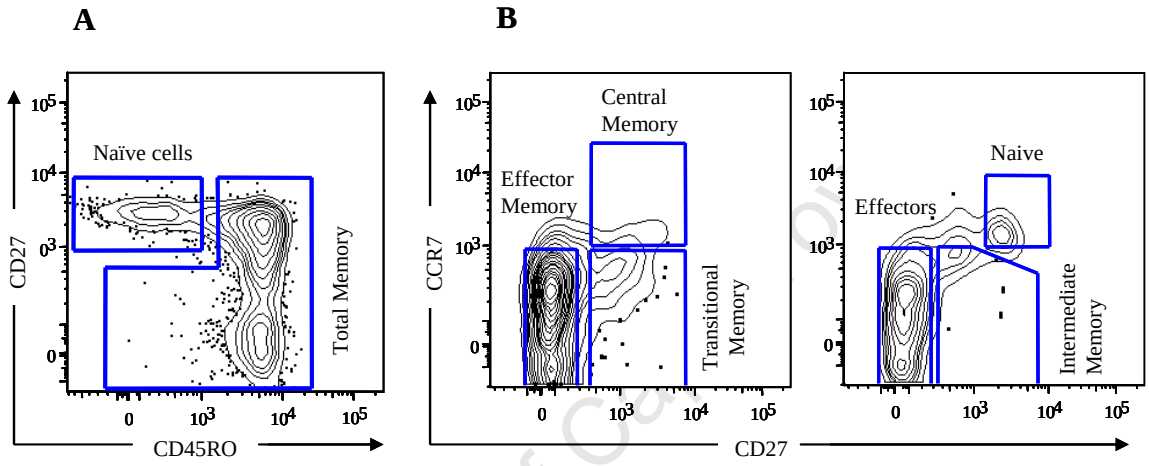


Figure 3.5. Impact of polyclonal expansion on T cell memory phenotype at the cervix. Cells were first gated on singlets, live cells and then lymphocytes. From this gate, CD3+ cytokine-producing T cells were gated, and then further gated on CD4+ and CD8+ T cells. Gates for each of the memory subsets were defined based on CCR7 and CD27 expression. **(A)** Representative plots showing naïve and antigen-experienced T cells (total memory) based on differential expression of CD27 and CD45RO. **(B)** Gating strategy used to define central memory ($CD45RO^+CD27^+CCR7^+$), transitional memory cells ($CD45RO^+CD27^+CCR7^-$), effector memory cells ($CD45RO^+CD27^+CCR7^+$), $CD45RO^+CD27^-CCR7^+$, naïve T cells ($CD45RO^-CD27^+CCR7^+$), intermediate memory cells ($CD45RO^-CD27^+CCR7^-$), effector ($CD45RO^-CD27^-CCR7^-$) and $CD45RO^-CD27^-CCR7^-$ cells. A total of 200, 000 events were acquired on an LSRII flow cytometer (BD Biosciences; San Jose, CA, USA).

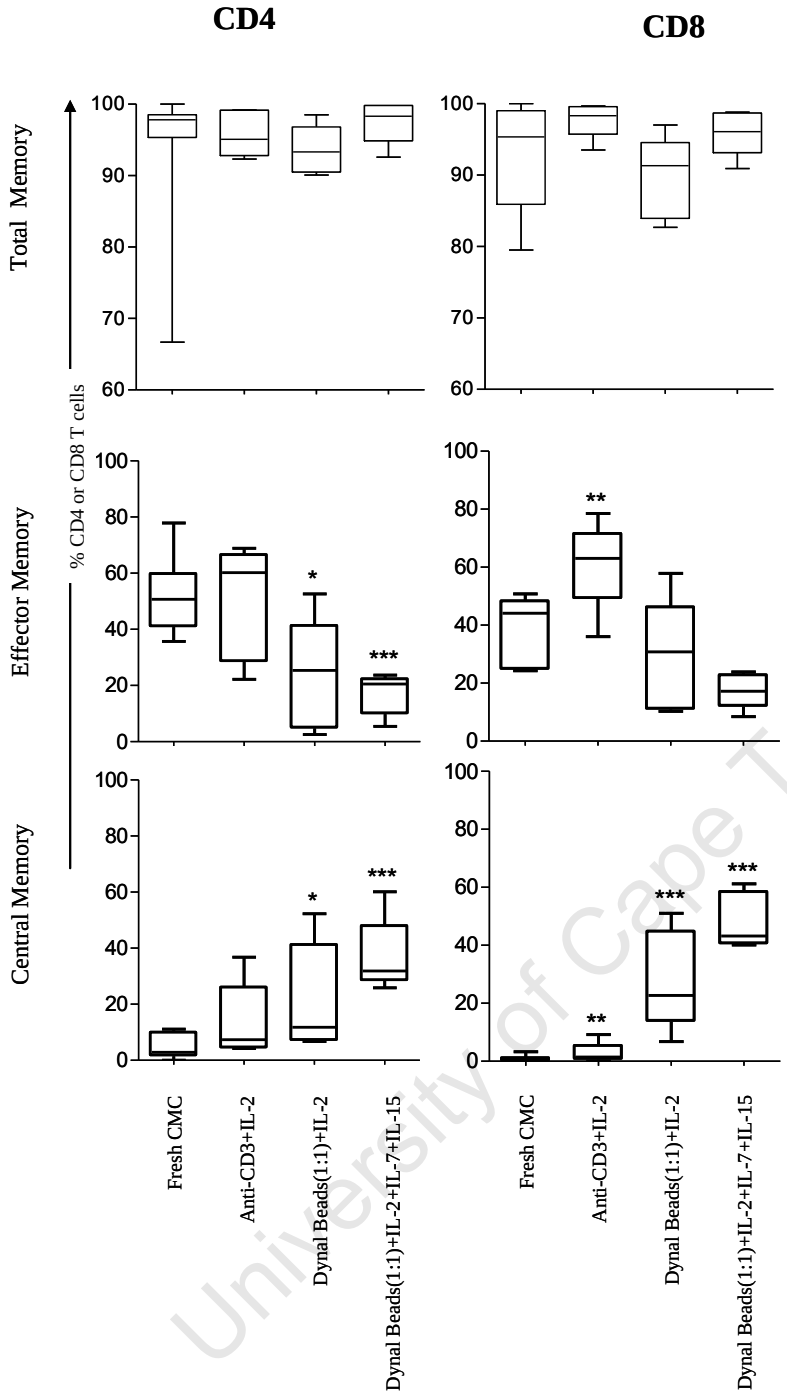


Figure 3.6. Impact of polyclonal expansion on T cell memory phenotype at the cervix. Comparison of the frequency of total (top panel), effector memory (middle panel) and central memory (bottom panel) subsets expressed as percentages of total $CD4^+$ and $CD8^+$ T cells at the cervix of chronically HIV-infected individuals (n = 5) before and after expansion for 7 days using three different protocols. Cervical cells were expanded using the methods that yielded best expansion in PBMC experiments [Dynal Beads (1:1)/IL-2 and IL-2/IL-7/IL-15] and compared with anti-CD3/IL-2 alone. Each box and whisker plot shows the median (central line), IQR (outer lines of box) and 5-95% range (error bars) of 5 HIV-infected individuals. * indicates $p<0.05$ while ** indicates $p<0.01$ using Wilcoxon ranks test.

Figure 3.7 provides a summary of phenotypic changes following expansion of PBMC and cervical cytobrush cells, including all combinations of phenotypes observed with the markers we used. While it is clear that effector memory T cells predominate at the cervix *ex vivo*, effector and naïve cells predominate in fresh blood. Anti-CD3 expansion in the presence of IL-2 maintains this dominance of effector memory T cells in cervical samples while it enriches for this population in expanded PBMCs. In contrast, Dynal beads (particularly in the presence of IL-7 and IL-15) favour the accumulation of central memory subsets in both cervical and PBMC samples. The transitional memory T cell frequencies were generally unchanged irrespective of the expansion method used with the exception of a significant reduction in the frequency of this subset in CD4+ T cells only following expansion with Dynal beads (1:1) and IL-2, IL-7 and IL-15 ($p=0.04$; Figure 3.7).

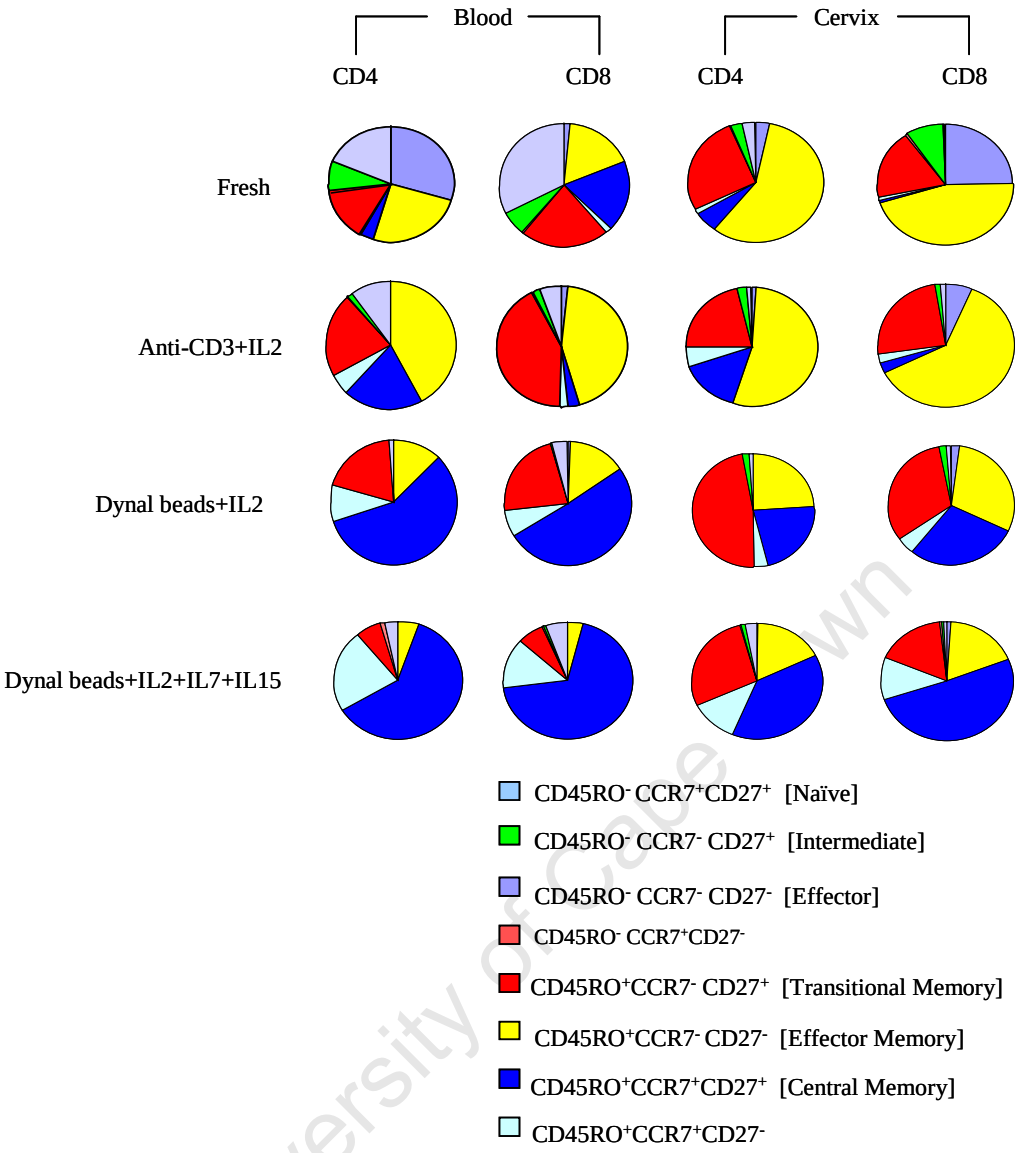


Figure 3.7. Summary of the phenotypic changes observed in distinct T cell subsets at the cervix and blood of HIV-infected women after 7 days of expansion. Eight memory subsets were defined from these markers: Central memory cells, ($CD45RO^+CD27^+CCR7^+$), transitional memory cells ($CD45RO^+CD27^+CCR7^-$), effector memory cells ($CD45RO^+CD27^+CCR7^+$), $CD45RO^+CD27^-CCR7^+$, naïve T cells ($CD45RO^-CD27^+CCR7^+$), intermediate memory cells ($CD45RO^-CD27^+CCR7^-$), effector ($CD45RO^-CD27^-CCR7^-$) and $CD45RO^-CD27^-CCR7^-$ cells. Correlation between fresh and expanded cells memory phenotypes was performed using Spearman Rank test and Spearman Rho and p-values are for each correlation are shown above each pie chart.

Based on significantly improved cell yields and viability, it was concluded that the combination of Dynal beads (1:1) and IL-2, IL-7 and IL-15 was superior to the other methods for expanding both cervical and blood-derived T cells. This method, however, selects for enrichment of central memory over effector memory T cells in cervical samples, resulting in expanded cervical cells differing from those present before expansion. Although anti-CD3 and IL-2 offered the poorest improvement in yield for both PBMC and cervical cells, this method best conserved the memory profile of cervical CD4+ cells following expansion (Figure 3.7; Spearman Rho=0.76; p=0.04).

3.4. Discussion

Understanding T cell responses to HIV in the female genital tract is important since the majority of new HIV infections and transmissions are via this route. Isolation of immune cells from the female genital tract using non-invasive methods is characterized by low cell yields that hamper thorough evaluation of immune function. Although phenotypic and functional qualities of genital immunity have previously been investigated *ex vivo* (Gumbi et al., 2008; Kaul et al., 2000; Shacklett et al., 2000), there is a need to develop methods to improve yields or expand immune subsets *in vitro* (Ibarrondo et al., 2005; Iqbal et al., 2005; Shacklett et al., 2003a). Few studies have directly compared expansion methods (Robinson et al., 2002; Kalamasz et al., 2004) or compared their ability to expand cytobrush-derived T cells from the female genital tract.

This study compared the efficiency of immobilized anti-CD3 alone or in combination with anti-CD28 and Dynal bead-bound anti-CD3/CD28 to expand cervical cytobrush-derived cells and PBMCs in the presence of various combinations of γ-chain cytokines; IL-2, IL-7 and IL-15. Dynal beads in the presence of IL-2, IL-7 and IL-15 were found to be superior to other methods tested in terms of their ability to expand both cervical and blood-derived T cells, but this method enriched for central memory T cells. In contrast, anti-CD3 and IL-2 offered the poorest improvement in yield for both PBMC and cervical cells but best conserved the effector memory profile of cervical cells following expansion.

The strength and duration of signalling that T cells receive, determines their fate (Kalamasz et al., 2004). Kalamasz et al. (2004) reported that optimal expansion of T cells depends on engagement of both the CD3/T cell receptor (TCR) complex and co-stimulation with anti-CD28. In this study, the benefit of co-stimulation by addition of anti-CD28 in the presence of anti-CD3 and IL-2 did not significantly improve expansion yield of PBMCs nor alter the memory profile of expanded cells compared to anti-CD3 and IL-2 alone. Since expansion in general selected for enrichment of the memory T cell pool and memory T cells are known to have differing requirements for co-stimulation than naïve T cells, the influence of CD28 co-stimulation in these

experiments is likely to be dampened by the reduced need of memory cells for this secondary signal (Kalamasz et al., 2004; Sallusto et al., 2004).

This study shows that there are significant changes that occur in individual memory subsets depending on the expansion method applied. Generally, anti-CD3 expansion in the presence of IL-2 alone resulted in the accumulation of effector memory cells, while Dynal beads resulted in selective accumulation of central memory T cells from both cervix and blood. Previous studies have shown that varying the concentration of bead to target cells when using Dynal beads impacts significantly on expansion of naïve versus antigen-experienced cells with high bead:cell ratios favouring naïve cell expansion and low bead:cell ratios favour antigen-specific cell expansion (Kalamasz et al., 2004). The highest yield of T cells was obtained at beads:cell ratios of 1:1 followed by 1:5 and then 3:1, indicating that bead-to-cell ratio impacted on expansion kinetics. This finding supports previous observations that TCR signalling strength is important in driving T cell proliferation (Kalamasz et al., 2004). Further, it was found that reducing the signal achieved by reducing the beads-to-cell ratio resulted in accumulation of effector memory T cells while increasing the signal by increasing the beads-to-cell ratio favoured the accumulation of central memory T cells. It was speculated that the differing phenotypes of T cells that result from the various expansion protocols tested represent a change in the phenotype of the cells present initially (activation induced maturation and cycling) rather than a selective expansion of an existing T cell population.

T cells derived from the female genital tract were predominantly antigen-experienced and more highly-differentiated, with effector memory T cells being the most predominant subset at the cervix. Effector memory cells constitute the major population of CD4+ T cells in extra-lymphoid effector sites such as the intestinal lamina propria and the lung (Sallusto et al., 2004). The difference in the memory status of T cells derived from cervical cytobrush samples compared to blood may also explain the difference in expansion potential of these cells. The female genital tract is colonized by abundant commensal and sometimes pathogenic microflora (Zhou et al., 2004). It was speculated that continuous exposure of immune cells residing in the genital tract to bacterial products might result in increased T cell activation, differentiation and exhaustion.

In this study, supplementation with IL-7 and IL-15 following stimulation with Dynal beads resulted in accumulation of central memory T cells. Previous studies have shown that these growth promoting cytokines override the increased tendency of effector memory T cells to undergo apoptosis (Sallusto et al., 2004), by inducing telomerase activity (Son et al., 2000) and upregulating anti-apoptotic molecules (Boise et al., 1995). Addition of IL-7 and IL-15 in combination with immobilized anti-CD3 resulted in selective accumulation of effector memory CD4+ T cells, which is more consistent with the reported tendency of these cytokines to "protect" effector memory cells from apoptosis.

A limitation of this study is that it focused only on expansion of cervical and blood-derived T cells from HIV-infected women. It was shown in blood that HIV-infection impacts on the extent of expansion of T cells and is therefore also likely to influence *in vitro* expansion of T cells from the genital tract. It has been previously shown that the memory profiles of T cells isolated from the cervix of HIV-infected and uninfected women are similar (Nkwanyana et al., 2009). However, since (1) HIV infection may impact on the baseline characteristics of cervical cells and their ability to expand *in vitro* and (2) the value of this approach would be best applied to uninfected HIV vaccine clinical trial participants, it is likely that this data may under estimate the expansion potential of T cells from the genital tract. It is therefore important to expand these studies to include cervical T cell expansion from HIV negative women.

Conditions that improve the rate of expansion and viability of T cells derived from the female genital tract will not only reduce the time required to improve yields but also reduce the risk of expansion bias and contamination. This study demonstrates here that the extent of expansion of cervical T cells might be impacted by the predominance of effector memory T cells *ex vivo.* The relative expansion of cervical T cells expanded with either anti-CD3/IL-2 or Dynal/IL-2 was 1.4-fold lower than the extent of expansion observed for PBMCs. It was concluded that cervical T cell yields can be best improved by expansion with Dynal beads (1:1) in the presence of IL-2, IL-7 and IL-15, while memory T cell profiles can best be maintained by expansion with anti-CD3 in the presence of IL-2 alone.

CHAPTER 4

Comparison of the functional complexity of HIV-specific T cell responses detected in the female genital tract and blood of HIV-infected women following polyclonal expansion

Table of Contents

4.1 Introduction

Although phenotypic and functional qualities of genital immunity have previously been investigated *ex vivo* (Gumbi et al., 2008; Kaul et al., 2000a; Shacklett et al., 2000), these studies were hampered by low yields making the development of methods to expand immune subsets *in vitro* valuable for more indepth studies of mucosal HIV immune responses (Ibarrondo et al., 2005; Shacklett et al., 2003).

HIV-specific T cell populations at the rectal and genital mucosa have largely been shown to overlap in specificity and clonality with those detected in blood (Shacklett et al., 2000; Kaul et al., 2000a; Musey et al., 2003a; Ibarrondo et al., 2005). In Chapter 2, it was demonstrated that the magnitude of IFN-γ responses to HIV Gag and specific Gag regions targeted by cervical T cell lines from chronically HIV-infected women overlapped with those detected in blood, suggesting that the compartments were connected. Recent studies comparing the functional complexity of HIV-specific T cells at mucosal sites and blood have demonstrated the existence of polyfunctional HIV-specific T cells at mucosal sites such as brochiavilar (Brenchley et al., 2008) and gastrointestinal tract (Critchfield et al., 2007; Macal et al., 2008; Ferre et al., 2009). In these studies, HIV-infected individuals naïve to ART had enhanced HIV Gag responsiveness by mucosal CD8+ T-cells compared to PBMC (Critchfield et al., 2007; Macal et al., 2008; Ferre et al., 2009). Despite recent suggestions that the magnitude of multifunctional cells do correlate with HIV clinical status (Critchfield et al., 2007), the cause and effect relationship between these responses and HIV clinical parameters is unclear (Brenchley et al., 2008; Critchfield et al., 2007; Macal et al., 2008; Ferre et al., 2009).

It has previously been documented that HIV Gag-specific CD8+ T cells responses detected by intracellular staining for IFN-γ production or with MHC class I tetramers by flow cytometry (Ramduth et al., 2005), or by IFN-γ Elispot (Ogg et al., 1998; Edwards et al., 2002; Masemola et al., 2004) were associated with better control of HIV. Studies investigating the relationship between the quality or magnitude of HIV-specific CTL responses and HIV clinical status have, however, generated conflicting results. Some have reported an inverse relationship between the magnitude and breadth of HIV Gag-specific CTL responses (using either MHC class I tetramers or

IFN-γ Elispot) and HIV viral load (Ogg et al., 1998; Edwards et al., 2002). Conversely, others have reported a positive relationship between plasma viral load and the total HIV-specific, Env-, and Nef-specific CD8+ T-cell frequency (Betts et al., 2001) while others observed no correlations between the magnitude (Addo et al., 2003; Kaufmann et al., 2004, Ramduth et al., 2005) or breadth (Addo et al., 2003; Kaufmann et al., 2004; Masemola et al., 2004) of total HIV-specific T cell responses in both CD8+ and CD4+ T cell compartments and viral load, when the entire HIV genome was analysed. Recently, Critchfield et al. (2007) showed that the percentage of Gag-specific CD8+ T-cells in the rectal mucosa which were capable of >3 simultaneous effector functions (polyfunctional) were significantly associated with blood CD4 counts and inversely associated with plasma viral load. In the female genital tract, however, Gumbi et al. (2008) showed that IFN-γ+ T cell responses to HIV Gag were not associated with protection from HIV genital tract shedding.

The aim of this Chapter was to compare the magnitude of polyfunctional HIV Gag-specific responses from *ex vivo* and *in vitro* expanded cervical and blood-derived T cell lines from HIV-infected women. In this study, Cells that simultaneously expressed three or four cytokines in response to HIV peptide stimulation were considered "polyfunctional." Polyclonal cervical and blood T cell lines were generated using anti-CD3 or Dynal anti-CD3/CD28 expansion in the presence of IL-2, IL-7 and IL-15 (optimized in Chapter 3). Polychromatic flow cytometry was used to simultaneously assess the frequency of expression of CD107a, IFN-γ, TNF-α, and MIP-1β by CD8+ and CD4+ T cells in response to HIV Gag stimulation.

Finally, the relationship between the magnitude and complexity of T cell responses to HIV in each compartment were compared with markers of HIV disease progression (CD4 T cell counts in blood and viral load).

4.2 Materials and Methods

4.2.1 Study participants and sample collection

Twenty eight chronically HIV-infected women were recruited from the Nyanga Day Hospital in Nyanga, Cape Town, South Africa for this study. All HIV-infected women were naive to anti-retroviral (ARV) therapy at the time of study. Women, who were menstruating, post-menopausal, had undergone a hysterectomy, or had visible evidence of genital tract infections or discharges were excluded from the study. All women gave written informed consent, and the Research Ethics Committee of the University of Cape Town approved all aspects of the study (UCT REC REF 206/2002).

4.2.2 PBMC isolation

Forty millilitres of whole anti-coagulated blood was collected in ACD vacutainer tubes (BD Biosciences, Plymouth, UK) from each woman. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich, Egham, Runnymede, UK) and LeucoSep® centrifuge tubes (Greiner Bio-one, Frickenhausen, Germany) as previously described in Chapter 2 section 2.2.2 and fresh samples were used.

4.2.3 Collection and processing of cervical cytobrush specimens

Cervical cytobrush samples were collected from the female genital tract of all women under speculum examination using a Digene cervical sampler as previously described (Chapter 2, section 2.2.2). Cervical cytobrush-derived T cells were either investigated fresh or following polyclonal expansion. The absolute number of CD3+ T cells in each cytobrush sample was counted using a Guava automated cell counter (Guava Technologies) according to the method described by Nkwanyana et al. (2009). Viability of cervical mononuclear cells was determined by Trypan staining (Sigma-Aldrich, Irvine, UK) using a Fast Read Haemocytometer.

4.2.4 Polyclonal expansion of cervical and blood-derived T cell lines

Cervical cytobrush and blood-derived T cells were expanded using either Dynal anti-CD3/CD28 beads (16/28 women enrolled); anti-CD3 (7/28 women enrolled); or investigated directly *ex vivo* (7/28 women enrolled). Dynal anti-CD3/CD28 beads were used in the presence of IL-2, IL7 and IL-15 while immobilized anti-CD3 was used in the presence of IL-2 alone (to mimic expansion reported in Chapter 2) according to the method described by Chapter 3, section 3.2.2. Table 4.1 summarizes the expansion methods and cytokine combinations used in this study. Cervical and blood cells were cultured in a 5% CO_2 at 37^{o}C for a total of 14 days and fresh R10 medium with cytokines was replenished every 2 days. T cell yields and viability was determined by Guava automated cell counting (Guava Technologies, Hayward, CA, USA) and Trypan staining using a Fast Read Haemocytometer by microscopy (Sigma-Aldrich, Irvine, UK), respectively.

Table 4.1: Summary of the polyclonal expansion methods used

Stimulus	Cytokine cocktail			References
	IL-2 (IU/ml)	IL-7 (ng/ml)	IL-15 (ng/ml)	
Anti-CD3 immobilized	200	-	-	Yang *et al*. (1996)
Dynal anti-CD3/CD28 beads (1:1)	200	20	20	Onlamoon *et al*. (2006)

* Anti CD3/CD28 bead-to-T cell ratios

4.2.5 Antibodies used for flow cytometry

The following antibodies were used to investigate the phenotype and functionality of cervical and blood HIV Gag-specific T cell responses: CD3-Allophycocyanin-H7 (CD3-APC-H7), CD4-PerCp-Cy5.5, CD107-FITC, TNF-α-Cy7-PE, MIP1-β-PE, IFN-γ-Alexa fluor 700 (all BD Biosciences, San Diego, CA, USA), CD8-quantum dot (QD) 605 (Invitrogen, Carlsbad, CA, USA), CD14-PacBlue (BD Biosciences San Diego, CA, USA), and CD19-PacBlue (Invitrogen, Carlsbad, CA, USA). All antibodies were pre-titered to optimal concentrations.

4.2.6 Intracellular cytokine staining

Intracellular cytokines staining (ICS) assays and phenotypic staining were carried out on *ex vivo* "primary" cervical cells and PBMC, and *in vitro* expanded cervical and

blood T cell lines. *Ex vivo* cervical and blood-derived T cell lines were rested overnight at 37^{o}C, 5% CO_2 while expanded T cell lines were deprived of IL-2, IL-7 and IL-15 for 24hrs prior to stimulation with HIV Gag peptides. HIV-1 subtype C Du422 Gag overlapping peptides spanning the entire Gag sequence were kindly provided by the NIH AIDS Research and Reference Reagent Program (Bethesda, Maryland, USA) and consisted of 121 peptides (15-mer overlapping by 11 amino acids). The Du HIV strains used are close to a South African consensus sequence (Williamson et al., 2003).

For these experiments, cervical and blood-derived T cells were stimulated with (1) pooled HIV Gag peptides (1 μg/ml); (2) Staphylococcus enterotoxin B (SEB, 5 mg/ml; positive control); (3) PMA (0.1 μg/ml)/ionomycin (0.1 μg/ml) [positive control]; and (4) media alone (negative control). To each stimulation, 10μl of R10 stimulation mix (4.5 ml R10, 500 μl DNase I, anti-CD49d (1 μg/ml), anti-CD28 (1 μg/ml), BFA (0.5 μg/ml) and monensin (10 μg/ml) (Sigma Aldrich) was added. In addition, anti-CD107a-FITC antibody (5μl) was included in each stimulation tube**.** With the exception of PMA, cells were stimulated with antigen for 16 h at 37°C, 5 % CO_2 (Thermo Electron Corporation). For PMA/ionomycin stimulation, cells were incubated for only 4 h.

Following stimulation, cells were washed with FACS wash buffer (1% FBS and 0.01% NaN_3, GIBCO® PBS, Invitogen™, Carlsbad, CA, USA) and centrifuged at 838 x g for 3 (2300rpm) min at 4 ºC. Cells were re-suspended in 50 μl PBS containing violet viability reactive dye ("Vivid"; Invitrogen) and incubated for 20 min at room temperature. For surface staining, a staining mix, consisting of anti-CD19-Pacific Blue, anti-CD14-Pacific Blue, anti-CD8-Qdot 605, anti-CD4-PerCP-Cy5.5 and PBS, was prepared and cells were stained for 20 min at room temperature. Cells were fixed and permeabilized at room temperature for 20 min using Cytofix/Cytoperm buffer (BD Biosciences) and stained intracellularly with anti-CD3-APC-H7, anti-IFN-γ-Alexa fluor 700, anti-TNF-α-PE-Cy7, and anti-PE-MIP-1β. All staining were prepared in PBS and a final volume of 50 μl was added to each sample. Cells were washed, and finally resuspended in 1% paraformaldeyde (Cell fix BD Biosciences) and stored at 4^{o}C until acquisition (within 24 hours).

Samples were acquired on the LSRII (BD Biosciences) with FACSDiva software version 6.0 (BD Biosciences). The number of events collected ranged between 200,000 and 1,000,000 for both blood and cervix. Data analysis was performed using FlowJo v8.8.6 (Tree Star). Dead cells (ViVid), monocytes (CD14+), and B cells (CD19+) were excluded from the analysis. For experiments measuring four functional responses (CD107a, IFN-γ, MIP-1β, and TNF-α), individual cytokine gates were evaluated alone and also processed through Boolean combinations. Flow cytometry data were biexponentially transformed in order to include all events. Fluorescence minus one (FMO) was used to set gates. SPICE software (kindly provided by Dr Mario Roederer, Vaccine Research Center, NIAID/NIH, Bethesda, MD) was used to analyze functional complexity data.

4.2.7 Gating strategy used to detect HIV-specific T cell responses to Gag

The gating strategy used to detect CD8+ and CD4+ T cell responses using the optimized polychromatic panel are illustrated in Figure 4.2. A singlet gate based on forward scatter was followed by a viability gate (ViVid negative) and then selection of lymphocytes based on forward/side scatter. CD3+ T cells were then selected from a lymphocyte gate and gated against a cytokine to cater for cytokine-producing T cells that may down-regulate CD3 and CD8 expression, thereby becoming dim cells. Gating on CD4- and CD8- (after the CD3+ gate) and then CD8+ and CD4+ respectively ensured that these two subsets were discriminated and double positive cells were excluded from analysis. These cells were further subdivided into individual populations consisting of cells positive for IFN-γ, TNF-α, CD107a and MIP-1β. Values from these populations were either analyzed directly or the populations were further subdivided into all possible combinations of functions as previously described (Betts et al., 2006; Critchfield et al., 2008; Ferre et al., 2009). In all cases, values were background-corrected using values from cells that were not stimulated.

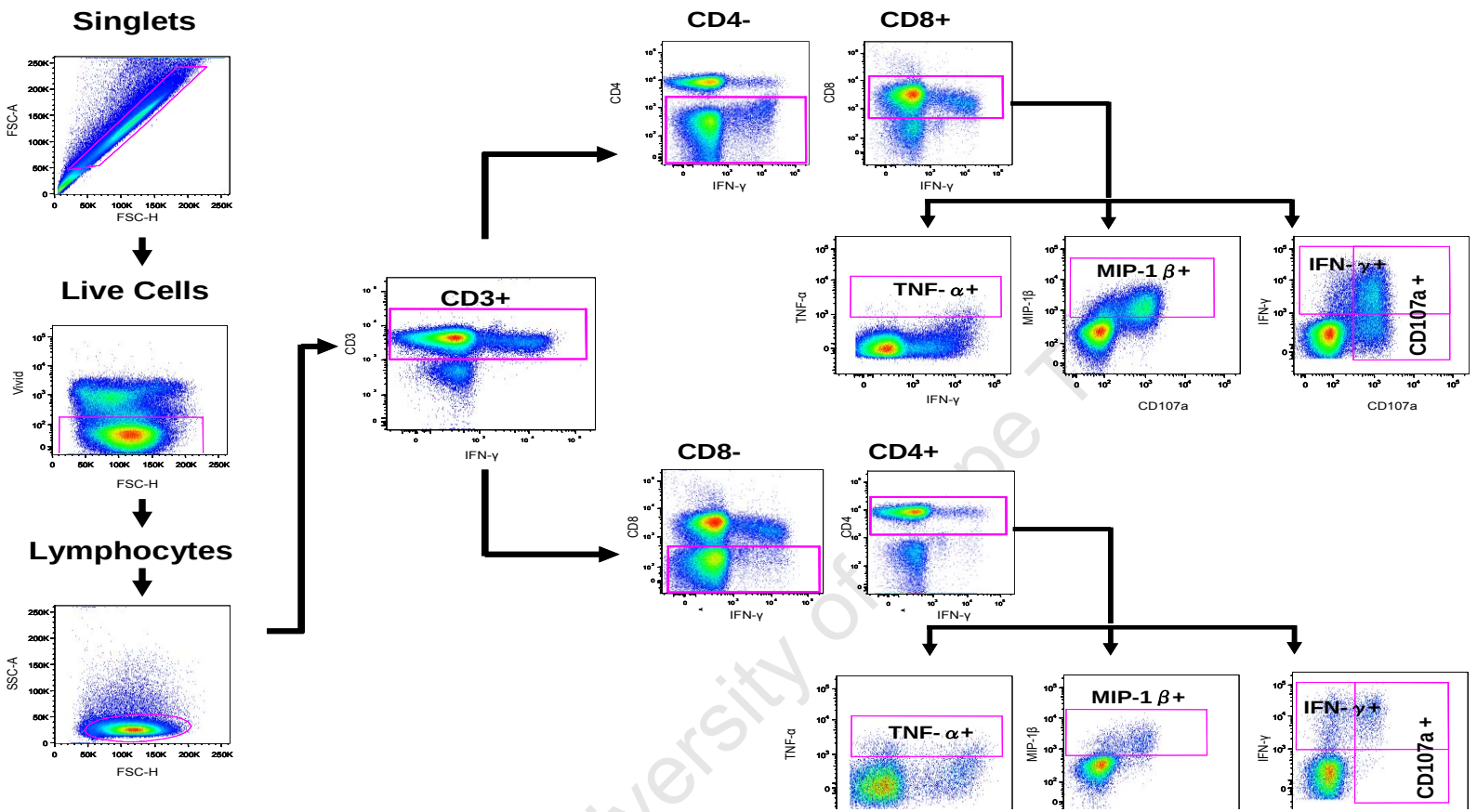


Figure 4.1. Gating scheme used for polychromatic flow cytometry of HIV Gag-specific T cell responses in the female genital tract and blood. Cells were first gated on singlets, live cells and then lymphocytes. From this gate, CD3+ cytokine-producing T cells were gated, and then further gated on CD4+ and CD8+ T cells. Gates for each of the four functions were set based on FMOs and negative control and were kept constant for all samples analysed. Data analysis was performed using FlowJo v8.8.6.

4.2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5® (GraphPad Software, San Diego California USA), Pestle version 1.6.2 and SPICE version 5.1 (both kindly provided by Dr Mario Roederer, Vaccine Research center, NIAID, NIH). Mann-Whiteny U test was applied for independent sample comparison, the Wilcoxon Ranks Test was used for matched non-parametric comparisons and Spearman Ranks correlation was applied for correlation comparisons. The false discovery rate (FDR) step-down procedure was used to reduce false-positive results when performing multiple comparisons (Columb and Sagadai, 2006). P-values ≤ 0.05 were considered significant. As stated in Chapters 2 and 3, due to the rarity of the mucosal samples used throughout this study and because the analyses were mainly exploratory, multiple comparison adjustment was not widely performed. In addition, guidelines for statistical correction of multiparameter flow cytometry data has not been standardized in the field.

4.3 Results

Twenty eight chronically HIV-infected women were included in this study to compare the functional complexity of HIV Gag-specific T cell responses in the female genital tract with those detected in blood. Of these 28 women, cervical and blood T cell responses were measured immediately *ex vivo* from 5/28 while the remaining 23/28 cervical and blood samples were expanded into T cell lines using short term *in vitro* expansion with either Dynal beads (16/23) or anti-CD3 antibodies (7/23). The clinical details of each of these groups of women are included in Table 4.2. All women were naïve to anti-retroviral therapy at the time of study. The median age of all of the HIV-infected women was 35 years (ranging from 33 to 37 years). Their median CD4 cell counts were 295 cells/μl (IQR 260-431 cells/μl) and their median plasma viral load was 7500 HIV RNA copies/ml (IQR 2,600-15,000 copies/ml). Twenty five of 28 (89.3%) of HIV-infected women had detectable HIV RNA in plasma. In the genital tract, 22/28 (78.6%) of these women had detectable HIV RNA [4950 copies/ml in cervical secretions (IQR 753-9,850)]. There was no significant difference between the three groups with respect to age, CD4 count, plasma and cervical viral load. None of the women had visible vaginal discharge or infections at the time the cytobrush was taken.

Table 4.2: Clinical status of the HIV-infected women included in this Chapter

Characteristic	Dynal beads expansion	Anti-CD3 expansion	Direct *Ex vivo*
N	16	7	5
Age [years; median (IQR)]	35 (33-37)	38 (33-37)	35 (34-37)
CD4 count [cell/μl; median (IQR)]	311(263-442)	275 (246-342)	328 (288-365)
Plasma viral load [RNA copies/ml; median (IQR)]	10,900 (5325-81,750)	59,50(3,425-7,200)	6,800 (510-7,500)
Number of women with detectable HIV RNA in plasma [N/Total (%)]	14/16 (87.5%)	6/7 (85.7%)	5/5 (100%)
Genital tract viral load [RNA copies/ml; median (IQR)]	9,400 (2,300-14,000)	625 (450-800)	600 (460-725)
Number of women with detectable HIV RNA at cervix [N/Total (%)]	13/16 (81.3%)	6/7 (85.7%)	2/5 (40.0%)

4.3.1 Feasibility of ex vivo polychromatic assessment of the magnitude and complexity of cervical cytobrush-derived HIV-specific T cell responses

Cervical cytobrush sampling of the female genital tract classically yields relatively few cells (Nkwanyana et al., 2009), making thorough immunological analysis difficult. This dissertation has previously explored *in vitro* polyclonal expansion to improve genital tract T cell yields (Chapter 2 and Chapter 3) although direct comparison with *ex vivo* genital tract T cell responses was not possible because splitting the sample would have compromised the success of *in vitro* expansion. To illustrate the impact of low cervical cytobrush yield on the quality of immunological analyses (particularly when multiple functions are being assessed simultaneously), the magnitude and complexity of *ex vivo* cervical T cell responses (compared to blood) to HIV Gag were initially investigated using intracellular cytokine staining of CD8+ and CD4+ T cells for IFN-γ, CD107a, TNF-α, and MIP-1β. The representative figures in Figure 4.2 illustrate the differences in quality of the multiparameter flow cytometry data collected from direct *ex vivo* cervical mucosal and blood T cells.

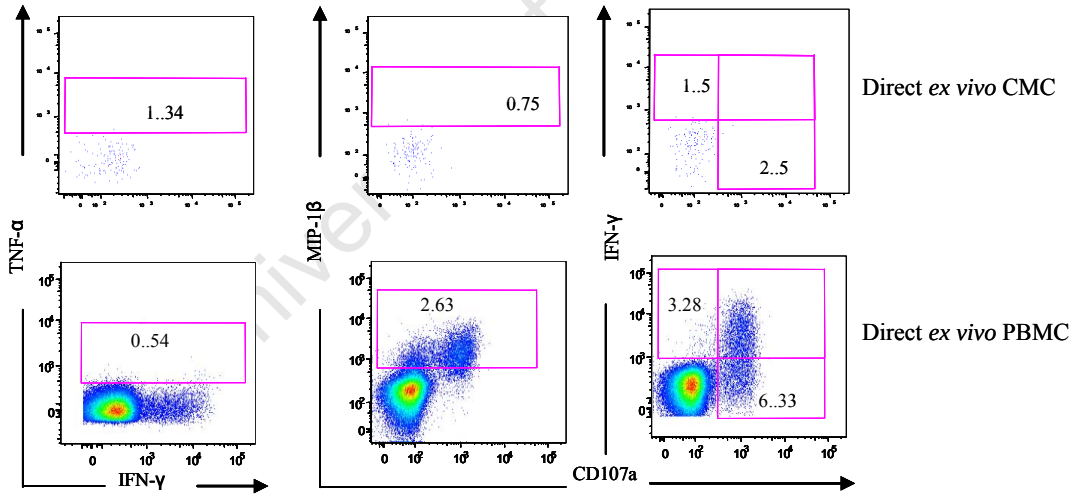


Figure 4.2. Representative plots of Gag-specific CD8 T cell responses as measured by polychromatic flow cytometry *ex vivo* at the cervix or in blood. Gates for each of the four functions were set based on FMOs and the unstimulated (negative) control. The percentages shown in each gate represent the frequency of CD8+CD3+ live T cells producing each of the respective cytokines.

A median of 110,235 cervical mononuclear cells (range 105,890-113450 cells; n=5) were obtained *ex vivo* per cytobrush. These *ex vivo* derived cervical cells were split

into three tubes and either (1) not stimulated, (2) stimulated with HIV Gag or (3) stimulated with PMA/ionomycin. From *ex vivo* cervical specimens, total CD3+ T cell event counts captured per stimulation condition for analysis ranged from 185-1008 events (with a median of 525 events; with all cells per condition acquired). This was significantly lower than *ex vivo* events acquired from matching blood samples where cell numbers are not a limiting factor (p=0.001; Table 4.3). Of these CD3+ events, CD4+ T cell captured events made up the minority and ranged from 20–140 (with a median of 96 events) while CD8+ events made up the majority and these ranged from 145–5594 events (with a median of 379 events). When event counts for individual cytokine responses to HIV Gag were evaluated, the number of positive CD107a, IFN-γ, MIP1-β and TNF-α events captured by flow cytometry ranged from 0 to 7 events (Table 4.3). Such low event counts are obviously not conducive to accurate statistical analysis using Boolean conditions and this data strongly supports the need for improved yields of cells acquired from the female genital tract. *In vitro* expansion of T cells from cytobrushes offered a feasible method to overcome these limitations (Chapter 2 and 3).

There are a number of ways of determining what constitutes a positive response. In this study, a positive response was taken as net percentage response (subtraction of the background unstimulated sample value). All medians were below 0.05 % for both CD4+ and CD8+ T cell subsets in blood. The level of non-specific cytokine production for *ex vivo* cervical samples was higher than in blood, with medians that were just below 0.50 % for both CD4+ and CD8+ T cell subsets.

Table 4.3: *Ex vivo* blood and cervix event counts for HIV Gag positive responses

Parameters		Positive Event counts	
		Cervix (N=5)	Blood (N=23)
CD3	–	525 (86 - 7240)	71,041 (28,699 – 156,222
CD4	–	96 (9 - 837)	9,011 (6,279 – 26,176)
CD8	–	379 (63 - 5594)	35,682 (8,797 – 85,494)
CD4:CD8	–	0.25	0.25
CD107a	CD4	0 (0 - 8)	2 (0 - 822)
	CD8	6 (1 - 244)	1,064 (0 - 4486)
IFN-γ	CD4	0 (0 - 2)	7 (0 - 567)
	CD8	3 (0 - 11)	304 (0 – 2,684)
MIP1-β	CD4	0 (0 - 10)	32 (0 - 971)
	CD8	7 (0 - 334)	276 (5 – 3,532)
TNF-α	CD4	0 (0 - 13)	2 (0 - 590)
	CD8	0 (0 - 46)	1 (0 - 609)

4.3.2 Polyclonal expansion of HIV-specific T cells from the female genital

Cervical-cytobrush and blood-derived T cells were expanded with either Dynal beads in the presence of IL-2, IL-7 and IL-15 (n=16) or anti-CD3 in the presence of IL-2 (n=7) from chronically HIV-infected women. For these expansion experiments, a median of 113450 cervical mononuclear cells (range 104,595-126,470 cells; n=23) were obtained *ex vivo* per cytobrush (Table 4.4). Following *in vitro* expansion with Dynal beads, this number was increased to a median of 1.8 x 10^6 cells (range 1.3-2.2 x 10^6 cells) in 14 days of culture in the presence of IL-2, IL-7 and IL-15. This represents a 15-fold increase in cervical T cell numbers compared to *ex vivo* cell yields. In comparison, anti-CD3 expansion yielded a median of 1.1 x 10^6 cells (range 1.1-1.3 x 10^6 cells; 10-fold) after 14 days and this was significantly lower than obtained from Dynal bead expansion (p=0.004 for yield; Table 4.4). Of the 23 cervical T cells lines initiated, 1/23 (4.3%) became contamination during the first few days of culture and were excluded from further analysis. T cell lines derived from PBMCs showed a significant fold increase in T cell numbers in comparison to matched cervical lines after 14 days of expansion with either anti-CD3 (19 vs.10-fold; p=0.02) or Dynal beads (29 vs. 15-fold; p=0.0006) [Table 4.5].

Ex vivo cervical mononuclear cell viability ranged from 88-92% (with a median of 90%) while PBMCs viability ranged from 98-100% (median of 99%). Following

Dynal beads expansion, the median viability of cervical lymphocytes was 85% (ranging from 80-90%) while expanded blood median viability was 92% (ranging from 88-97%). After anti-CD3 expansion, the viability of cervical and blood lymphocyte were a median of 80% (ranging from 80-86%) and 88% (ranging from 87-93%), respectively and these were significantly different between the two compartments (p=0.03). However, comparison between the two methods showed a significant difference in blood T cell median viabilities (p=0.04) but not for cervical lymphocytes (p=0.63) [Table 4.5].

Table 4.4: Comparison of T cell expansion kinetics and viability for cervical cytobrush and blood-derived T cells using Anti-CD3 alone or Dynal anti-CD3/28 T cell expander beads

Characteristic	Dynal Beads expansion CMC Median (IQR) N=16	Anti-CD3mAb expansion CMC Median (IQR) N=7	P-value	Dynal Beads PBMC Median (IQR) N=16	Anti-CD3 expansion PBMC Median (IQR) N=7	P-value
Ex vivo T cell recovery	0.11x 10^6 (0.1-0.13 x 10^6)	0.11x 10^6 (0.1-0.12 x 10^6)	0.1	100,000	100,000	–
Percentage ex vivo T cell viability	90 (88-93)	88 (89-91)	0.89	99 (98-100)	98 (97-98)	0.1
Expanded T cell recovery	1.8 x 10^6 (1.3-2.2 x 10^6)	1.1 x 10^6 (1.1-1.3 x 10^6)	**0.004**	3.6 x 10^6(3.3-3.9x 10^6)	1.9 x 10^6(1.6-2.2x 10^6)	**0.0002**
Percentage expanded T cell viability	84.5 (80-90)	80 (80-86)	0.63	92 (88-96.5)	88 (87-92.5)	**0.04**
Fold T cell expansion [Day 14:Day 0]	15.4 (13.2-17.3)	10.1 (9.3-11.9)	**0.001**	29 (25-34.3)	18.6 (16.4 -21.9)	**0.002**

Figure 4.3 provides a representative figure to illustrate the improvement in the quality of plots for multiparameter flow cytometry data collected from cervical mucosal T cells after they were expanded using Dynal beads. Expanded cervical samples showed increased levels of background cytokine production, with a median of approximately 1.5% for both CD4+ and CD8+ T cell compared to ex vivo cervical samples (median below 0.50 %).

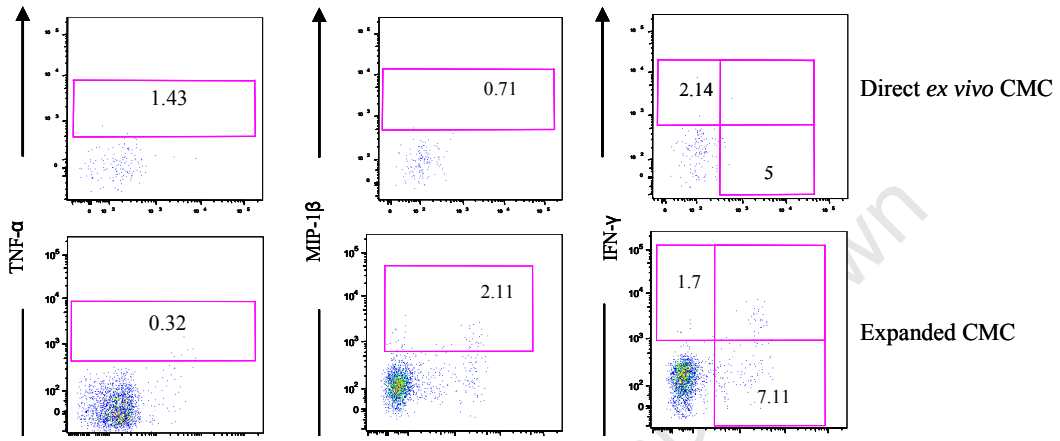


Figure 4.3. Representative plots of Gag-specific cervical CD8 T cell responses as measured by polychromatic flow cytometry *ex vivo* or following *in vitro* expansion with Dynal beads in the presence of IL-2, IL-7 and IL-15 for a period of 2 weeks. Gates for each of the four functions were set based on FMOs and the unstimulated (negative) control. The percentages shown in each gate represent the frequency of CD8+CD3+ live T cells producing each of the respective cytokines.

Following polyclonal expansion of cervical specimens, expanded cervical CD3+ T flow cytometry event counts resulted in a median of 6,546 CD3+ events (ranging from 4,225-10,395 events) after Dynal beads expansion, which was significantly higher than obtained *ex vivo* (p=0.01). Similarly, anti-CD3 expansion of cervical T cells resulted in a median of 6,661 CD3+ events (ranging from 3,443-9,543). This was significantly higher than events acquired from *ex vivo* cervical samples (p=0.01; Table 4.5). Dynal beads expansion resulted in a median of 2,200 CD4+ T cell events (ranging from 1,014–4,182 events) compared to a median of 2,847 CD8+ events (ranging from 1022–3433 events). It was interesting to note that Dynal beads expansion of CD4+ T cells were 2.6-fold higher (although not significantly different) than anti-CD3 expansion while anti-CD3 expansion of CD8+ T cells was 1.4-fold

higher than Dynal beads. When event counts for individual cytokine responses were evaluated, the number of positive CD107a, IFN-γ, MIP1-β and TNF-α events captured by flow cytometry ranged from 3-46 events and 1-38 following Dynal beads and anti-CD3 expansion respectively (Table 4.5).

Table 4.5: *Ex vivo* and expanded cervix event counts for HIV Gag positive responses

Parameters		CMC Positive Event counts:		
		Direct Ex vivo (IQR) N=5	Dynal Beads expansion (IQR) N=16	Anti-CD3 expansion (IQR) (N=7)
CD3		525 (185 – 1,008)	6,546 (4,225 – 10,395)	6,661 (3,443 – 9,543)
CD4		96 (20 - 140)	2,200 (1,014 – 4,182)	858 (283 – 1,564)
CD8		379 (145 - 576)	2,847 (1,022 – 3,433)	4,073 (1,231 – 6,188)
CD4:CD8 ratio		0.15	1.1	0.18
CD107	CD4	0 (0 - 1)	6 (4 - 25)	7 (4 - 21)
	CD8	6 (3 - 44)	46 (30 - 129)	38 (15 - 73)
IFN-γ+	CD4	0 (0 - 1)	13 (2 - 33)	3 (2 - 6)
	CD8	3 (0 - 8)	20 (8 - 83)	5 (1 - 34)
MIP-1β+	CD4	0 (0 - 1)	9 (4 - 14)	4 (1 - 24)
	CD8	7 (0 – 159)	37 (16 - 66)	21 (19 - 181)
TNF-α+	CD4	0 (0 - 1)	3 (1 - 16)	1 (1 - 3)
	CD8	0 (0 - 1)	3 (1 - 5)	5 (3 - 25)

4.3.3 Comparison between ex vivo versus in vitro expanded cervical T cell responses to HIV Gag

Although fresh *ex vivo* and expanded cervical responses were not evaluated from the same women due to cell number constrants, the differences in magnitude or functional profile of HIV-specific responses from fresh *ex vivo* or *in vitro* expanded cervical T cell lines were investigated (Figure 4.4). The individual and cumulative frequencies of cytokines produced by both CD8+ (Figure 4.4A and 4.4B) and CD4+ (Figure 4.4C and 4.4D) T cells were determined. *Ex vivo* cervical CD8+ T cells responding to HIV Gag predominantly produced MIP1-β and CD107a. Cervical T cell lines expanded with Dynal beads had significantly higher frequencies of cumulative functional responses (sum of the 4 functions) by CD8+ T cells to HIV Gag than fresh or anti-CD3 expanded T cell lines (Figure 4.4 A; $p \leq 0.05$). The frequency of CD8+ T cells expressing CD107a in response to Gag stimulation following anti-CD3 expansion was significantly lower than those detected following Dynal beads expansion ($p=0.007$). Frequencies of CD8+ T cells producing IFN-γ and MIP1-β following Dynal bead expansion were significantly higher than either fresh or anti-CD3 expanded lines (Figure 4.4 A; $p \leq 0.05$). In addition to these two cytokines; MIP1-β and CD107a, Gag-specific responses also included IFN-γ production, following Dynal bead expansion (Figure 4.4B). After anti-CD3 expansion, CD107a expression was lost by cervical CD8+ T cells in all individuals while TNF-α production was gained (Figure 4.4B).

Cervical CD4+ T cells responding to HIV Gag were present at low frequencies (Figure 4.4C and D) compared to CD8+ T cells responses. The total magnitude of HIV Gag-specific responses by CD4+ T cells was significantly higher in Dynal bead expanded T cell lines compared to fresh samples (Figure 4.4C; $p<0.05$). At the individual cytokine level, significantly higher frequencies of expanded CD4+ T cells produced IFN-γ in response to Gag stimulation compared to fresh cells ($p=0.03$; Figure 4.4C). IFN-γ responses predominated in Dynal beads expanded CD4+ T cells while CD107a expression predominated anti-CD3 expanded cells (Figure 4.4D).

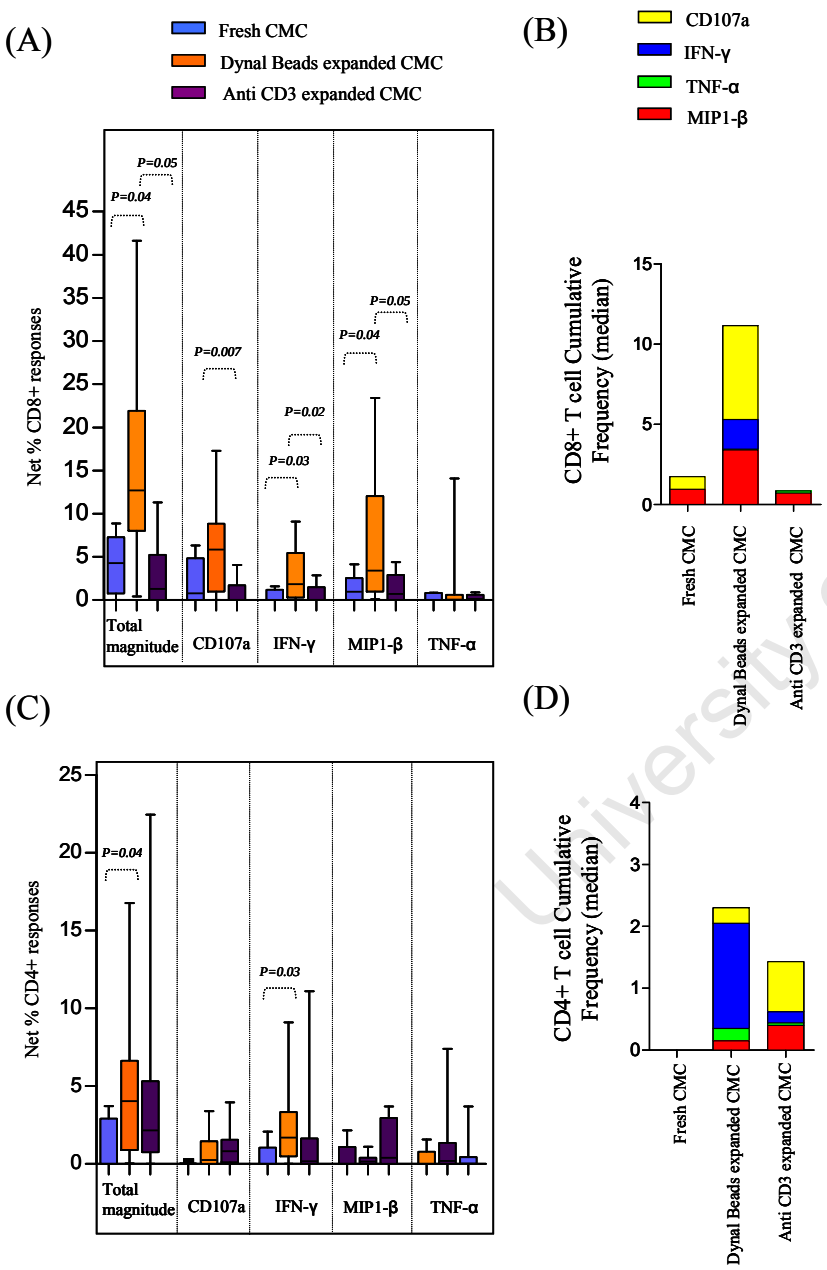


Figure 4.4. Comparison between fresh and expanded cervical T cell responses to HIV Gag. Comparison of the net magnitude of cumulative and individual cytokine responses expressed as percentages of (A) total CD8+ and (C) CD4+ T cells in chronically HIV-infected individuals before (blue boxes), or after Dynal bead (orange boxes) or anti-CD3 expansion (purple boxes). (B and D) The cumulative frequency for four T cell functions investigated; CD107a (yellow), IFN-γ (blue), TNF-α (green) and MIP1-β (red) was investigated. Each box and whisker plot shows the median (central line), IQR (outer lines of box) and 10-90% range (error bars) of HIV-infected individuals. Responses are plotted after background subtraction and the middle lines on the plots represent median response after background subtraction. Mann Whitney Test was used to compare fresh and expanded responses.

4.3.4 Comparison between fresh versus expanded cervical polyfunctional T cell responses to HIV Gag

Polyfunctionality in HIV-specific responses was next compared in fresh versus expanded cervical T cell lines (Figure 4.5). HIV Gag–specific CD8+ and CD4+ T cells responses at the cervix before and after expansion showed only low to undetectable magnitude frequencies of four-functional cells. Compared to direct *ex vivo* samples, there was a higher frequency of Gag-specific tri-functional CD8+ T cells detected in expanded cervical samples (0% vs. 0.2%) and they made up only a small proportion of Gag responsive cells (2%) (Figure 4.5A and B). The net frequency of dual-functional cells was highest in Dynal beads expanded cells (2.5%); followed by fresh cervical samples (1.5%) and lowest for anti-CD3 expanded cells (1%). The proportion of HIV Gag–specific dual functional cells was lower in fresh samples (10%) than in expanded CMCs (15%). In the CD8+ T cell compartment, mono-functional responses were the predominant responses detected (Figure 4.5A) making up ~80% of HIV-specific responses, irrespective of whether the cells were expanded or analysed fresh (Figure 4.5B). Due to the low frequencies of HIV-specific CD4+ T cell events detected in fresh samples, polyfunctionality could only reliably be assessed in expanded samples (Figure 4.5 C and D). Analogous to CD8+ T cells, monofunctional cells predominated in CD4+ T cell compartment, making up >90% of the responses to Gag. Following Dynal beads expansion, both the magnitude (0.25 % vs. 0.1%) and proportion (5% vs 2.5%) of dual-functional CD4+ T cells was higher than that detected after anti-CD3 expanded cervical cells (Figure 4.5C and D). There were no significant differences within the magnitude or proportion of any combinations of functions in both the CD8+ and CD4+ T cell compartment between the methods investigated.

Expansion did not result in reduced polyfunctional T cell responses at the cervix and cells tended to retain the same proportion of polyfunctionality pre- and post-expansion. Because pre- and post-expansion in the same cervical samples could not be compared, these types of experiments were performed in blood to validate the impact of expansion on T cell functions.

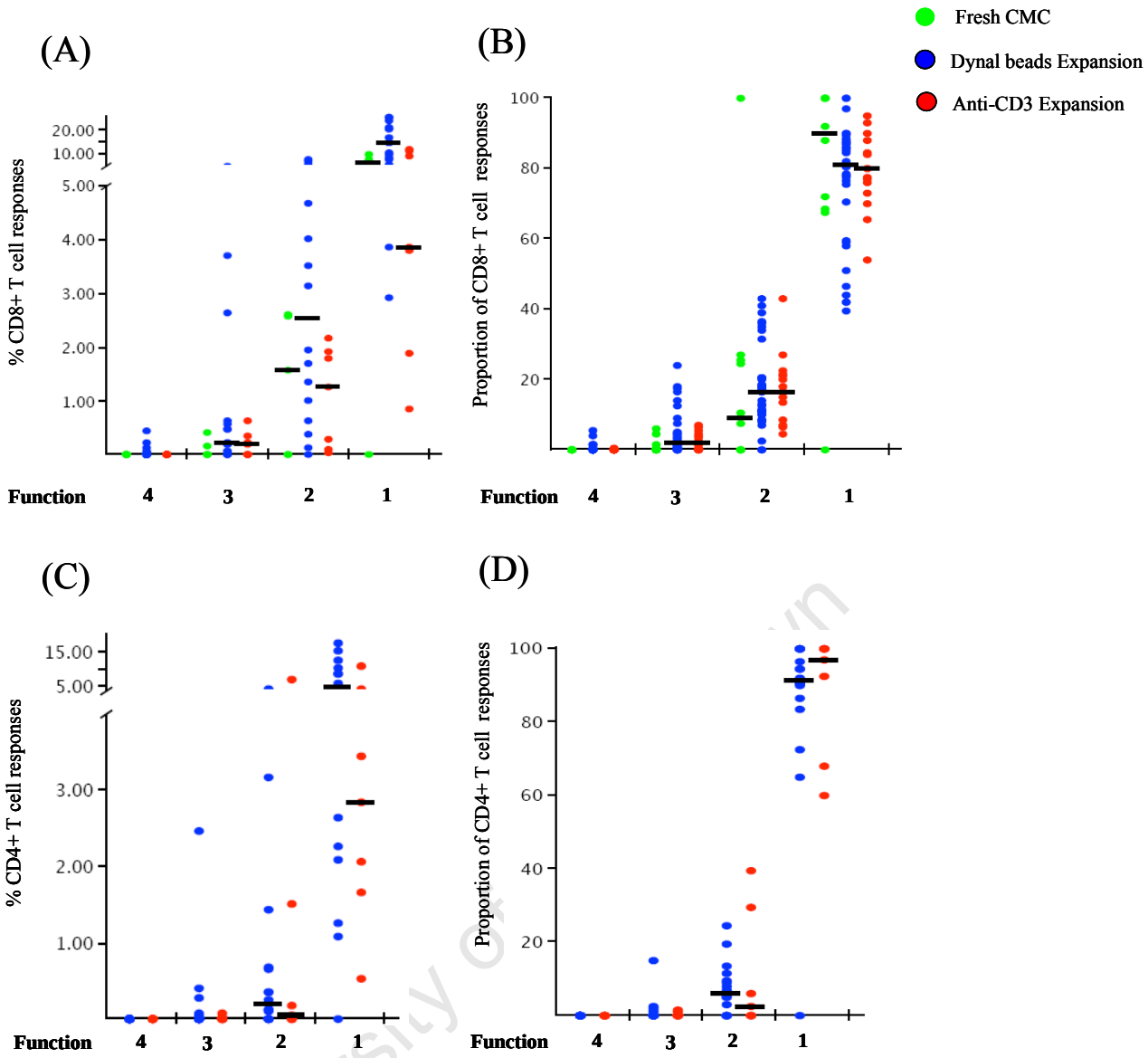


Figure 4.5. Comparison of polyfunctional HIV-specific T cell responses in fresh cervical (green dots), or Dynal bead (Blue dots) and anti-CD3 (red dots) expanded cervical T cell lines from HIV-infected women. **(A)** Net frequency of CD8+ T cell responses. (**B**) Relative proportion of the combinations of functions (4+, 3+, 2+ and 1+ functional cells). **(C)** Net frequency and **(D)** relative proportion of Gag-specific responses for each polyfunctional CD4+ T cells subset. For the CD4+ cervical T cell compartment, only expanded T cell line functionality is shown. Responses were considered 4-, 3-, 2- or 1-functional if any combination of 4, 3, 2 or 1 of the 4 functions investigated were detected, respectively. The middle bars on the plots represent median response after background subtraction. Mann-Whitney U test was used to compare blood and cervical responses.

4.3.5 Investigation in blood of the impact of in vitro expansion on the magnitude of HIV-specific T cells (matched pre- versus post-expansion)

Because polyclonal expansion of T cells may introduce expansion bias with certain T cell clonotypes potentially out competing others (refer to Chapter 2, section 2.3), the effect of *in vitro* expansion with Dynal anti-CD3/28 T cell expander beads or anti-

CD3 on HIV-specificity of PBMC-derived T cells was evaluated pre- and post-expansion in matched samples. The magnitude and the cumulative frequency of HIV Gag-specific T cell responses from 28 chronically infected individuals are shown in Figure 4.6. No significant differences between the total magnitudes (sum of the 4 functions) of responses in blood for both CD8+ and CD4+ T cells was observed when comparing fresh to expanded HIV Gag-specific responses (Figure 4.6 A and C, respectively).

Fresh and Dynal bead-expanded HIV Gag-specific CD8+ T cell responses detected in blood were predominantly CD107a+ (Figure 4.6A), although the net frequency of CD107a+ events in fresh blood was significantly higher than frequencies detected following Dynal bead ($p=0.009$) and anti-CD3 ($p=0.01$) expansion. Generally, low magnitudes (<0.02%) of Gag-specific CD8+ T cells producing TNF-α were observed before and after expansion. Both fresh and Dynal bead expanded blood-derived T cell lines produced CD107a, IFN-γ and MIP-1β in response to Gag while anti-CD3 expanded lines only produced IFN-γ and MIP-1β but failed to express CD107a (Figure 4.6B). Strikingly, this dropping away of CD107a expression in expanded blood samples was not previously observed in expanded cervical samples (Figure 4.4). The background levels of cytokine production in media control wells for both CD4+ and CD8+ T cell subsets following polyclonal expansion of PBMC was lower (below 1.2%) compared to expanded CMC (below 1.5%), however this was higher than background levels obtained in fresh PBMC (below 0.05%).

Fresh and anti-CD3 expanded HIV-specific CD4+ T cell responses in blood were predominantly MIP1-β+, while Dynal bead expanded CD4 T cell lines produced IFN-γ (Figure 4.6C and D). Compared with fresh and anti-CD3 expansion, Dynal bead expanded T cell lines produced a broader range of cytokines in response to HIV Gag stimulation (Figure 4.6C and D). TNF-α production was only noted in Dynal bead expanded T cell lines and the net frequencies of TNF-α were significantly higher in Dynal expanded compared to fresh PBMCs ($p=0.02$). CD107a expression in response to Gag stimulation was rare in the CD4+ T cell compartment (Figure 4.6D).

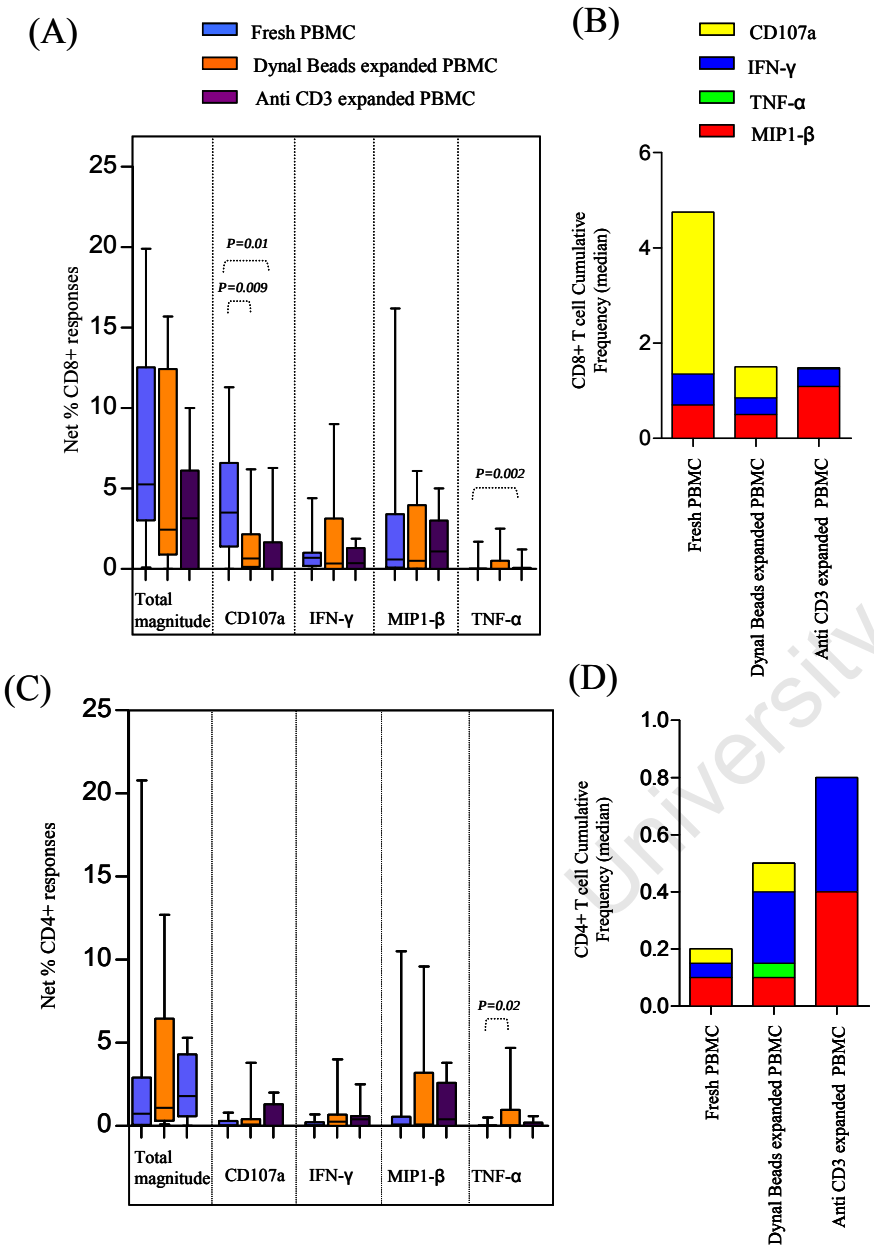


Figure 4.6. Impact of polyclonal expansion on the cumulative and individual net frequencies of functional responses to HIV Gag measured in fresh versus expanded blood T cell lines. (A and C) Comparison of the net magnitude of total or individual cytokine responses expressed as percentages of (A) total CD8+ and (C) CD4+ T cells in chronically HIV-infected individuals before (blue bars) and after Dynal beads (orange bars) or anti-CD3 expansion (purple bars). (B and D) The cumulative frequency of HIV Gag-specific responses by CD8+ (B) and CD4+ (D) T cells producing CD107a (yellow), IFN-γ (blue), TNF-α (green) and MIP1-β (red) was investigated. Each box and whisker plot shows the median (central line), IQR (outer lines of box) and 10-90% range (error bars) of HIV-infected individuals. Responses are plotted after background subtraction. Mann Whitney Test was used to compare fresh and expanded responses.

Since matching analysis was performed on fresh and expanded T cell lines from blood in each of the women included in this study, the relationship between the net magnitude of total and individual cytokine responses in fresh and expanded blood lines was investigated. Despite CD107a frequencies being significantly reduced following both Dynal and anti-CD3 expanded blood T cell lines (Figure 4.6A), the frequency of CD8+ T cells expressing CD107a after expansion was significantly correlated with frequencies of CD107a measured in fresh blood samples in response to Gag stimulation, irrespective of the expansion method used ($p=0.003$ for Dynal and $p=0.009$ for anti-CD3; Spearman Ranks test). Despite very low frequencies of TNF-α being produced in response to Gag, the frequencies of CD8+ ($p=0.04$ for Dynal and $p=0.05$ for anti-CD3) and CD4+ T cells ($p=0.02$) producing TNF-α in response to Gag were significantly associated before and after Dynal bead and anti-CD3 expansion.

4.3.6 Investigation in blood of the impact of polyclonal expansion on polyfunctional T cell responses to HIV Gag (pre- versus post-expansion)

The impact of *in vitro* expansion using Dynal beads or anti-CD3 on the frequency of polyfunctional T cell responses to HIV Gag was investigated (Figure 4.7). T cells positive for CD107a, IFN-γ, MIP-1β and TNF-α were subdivided into 15 possible combinations of functions and these were examined as previously described (Critchfield et al., 2008). Figure 4.7A and C shows net frequencies of Gag-specific CD8+ and CD4+ T cell responses that were 1, 2, 3 or 4 functional (based on combinations of expression of CD107a, IFN-γ, MIP-1β and/or TNF-α). Figure 4.7B and D shows the proportion of these combinations of functions (4+, 3+, 2+ and 1+ producing cells) relative to the total magnitude of Gag-specific responses.

HIV Gag–specific CD8+ T cells responses in blood, irrespective of whether they had been expanded on not, showed very low net frequencies (median of 0%; range, 0-0.5%) of four-functional cells. Few Gag-specific tri-functional CD8+ blood T cells were detected in fresh blood and they made up only a small proportion of Gag responsive cells (2%) and these were higher than those observed in expanded blood (1%) (Figure 4.7A and B, respectively). The frequency of dual-functional cells was comparable between fresh PBMCs (1%), anti-CD3 (0.8%) and Dynal beads (1.2%) expanded cells. Similarly, the proportion of HIV Gag–specific dual functional cells

was ~10-15% in fresh and expanded PBMCs. The median frequency of HIV Gag–specific mono-functional cells in fresh PBMCs was 3.2% and this was lower than observed for Dynal beads (4.3%) and anti-CD3 expanded (5.8%) PBMCs. The frequencies of Gag-specific CD8+ T cell responses in both fresh and expanded blood were dominated by monofunctional cells (~80-90%). HIV Gag–specific CD4+ T cells responses in blood before and after expansion were predominately mono-functional (~90-95%; Figure 4.7C and D). Fresh and expanded HIV Gag–specific CD4+ T cell showed only a low proportion of dual-functional cells (~2-5%) with no tri- and four-functional cells being detected (Figure 4.7A D). These finding suggests that there are low frequencies of polyfunctional cells in blood of chronically HIV-infected individuals and the majority of HIV Gag responses are dominated by mono-functional T cells. These responses are not significantly alerted following polyclonal expansion.

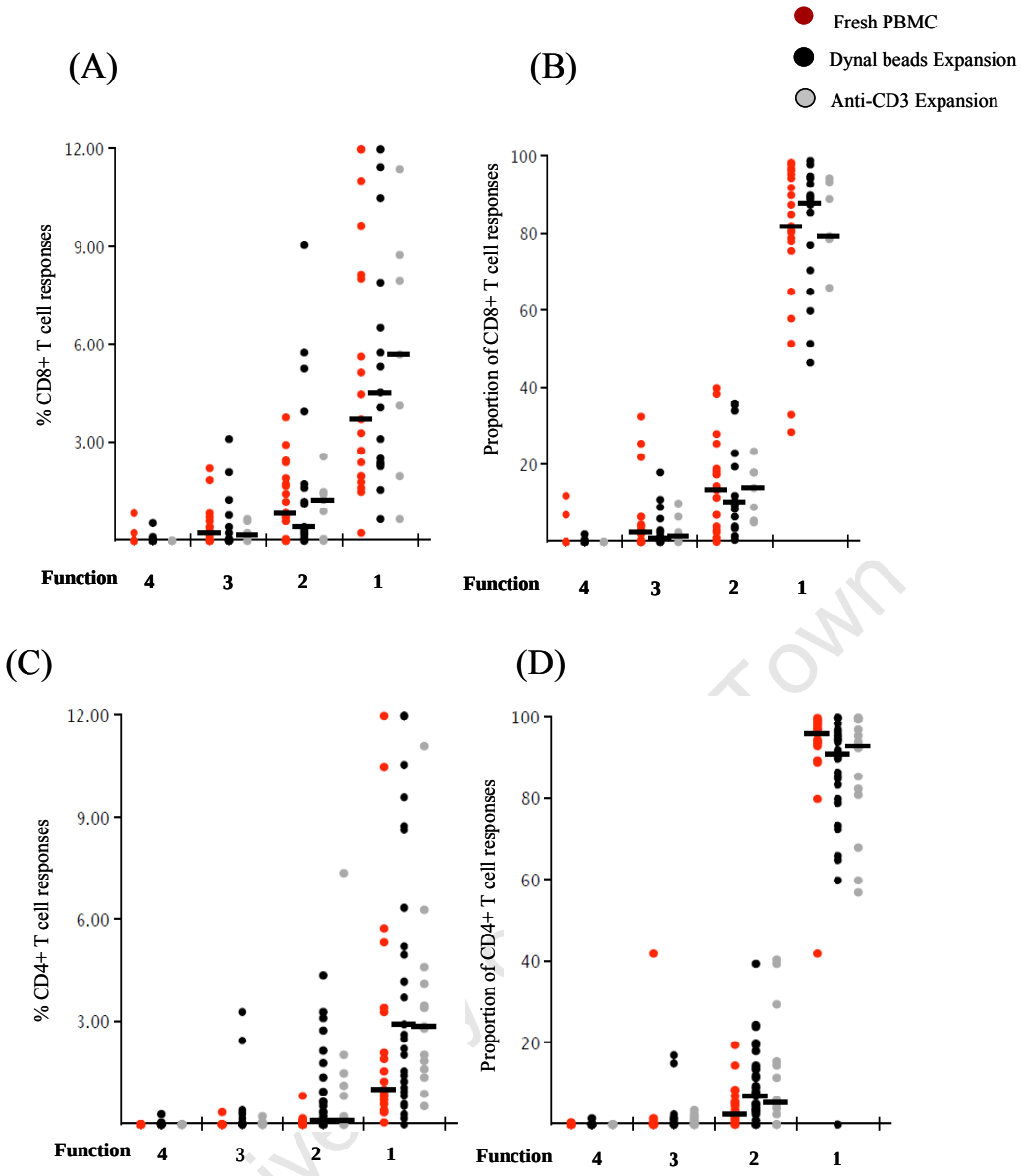


Figure 4.7. Impact of expansion on the polyfunctional profile of HIV-specific T cell responses detected in blood. The (A) net frequency and (B) relative proportion of CD8+ T cell responding to HIV Gag in fresh (grey circles) and expanded (black circles) PBMCs. The **(C)** magnitude and **(D)** proportion of CD4+ T cells responding to HIV Gag in fresh (red dots), Dynal (black dots) or anti-CD3 expanded (grey dots) PBMCs. Responses were considered 4-functional if all 4 of the markers included in the polyfunctional panel were detected (IFN-γ, MIP-β, TNF-α and CD107a). Responses were considered 3-, 2- or 1-functional if any combination of 3, 2 or 1 of the 4 functions investigated were detected, respectively. The middle bars on the plots represent median net responses (after background subtraction). Wilcoxon Rank Test was used to compare matched fresh and expanded responses.

4.3.7 Comparison between the frequency and quality of Gag-specific T cell responses at the cervix and in blood after expansion with Dynal beads

The magnitude and functionality of Gag responses detected in cervical T cell lines were compared with those detected in matched blood T cell lines (Figure 4.8 and 4.9). The total magnitude of CD8+ HIV Gag-specific T cell responses (the sum of all 4 responses measured) detected at the cervix was significantly higher than those detected in matching blood T cell lines derived from the 16 HIV-infected women expanded with Dynal beads (p=0.004; Figure 4.8A). Of the individual functions measured, CD107a expression was the predominant responses detected in HIV-specific CD8+ T cell responses from both compartments although higher frequencies of cervical CD8+ T cells expressed CD107a than blood (p=0.007; Figure 4.8A). Similarly, the frequency of MIP1-β expression by Gag-specific CD8+ T cells at the cervix was significantly higher than detected in blood (p=0.03). Although the cumulative frequency of cervical Gag specific CD8+ T cell responses was higher than blood, both blood and cervix showed similar cytokine response profiles (Figure 4.8B).

HIV-specific CD4+ T cells were found to produce a broader range of cytokines in both blood and cervix (Figure 4.8D) than CD8+ T cells, in that they exhibited all four of the functions measured (CD107a, IFN-γ, MIP-1β and TNF-α) while CD8+ T cells only produced 3 of these functions (CD107a, IFN-γ, and MIP-1β but not TNF-α). A median of 1.7% (range 0-3.2) of CD4+ T cells present at the cervix produced IFN-γ in response to Gag and this was significantly higher than IFN-γ responses detected in blood [median of 0.3 (range 0.0-0.63); p=0.002]. Blood and cervical CD4+ T cells showed a similar functional profile in response to Gag stimulation with all 4 functions being detected (Figure 4.8B).

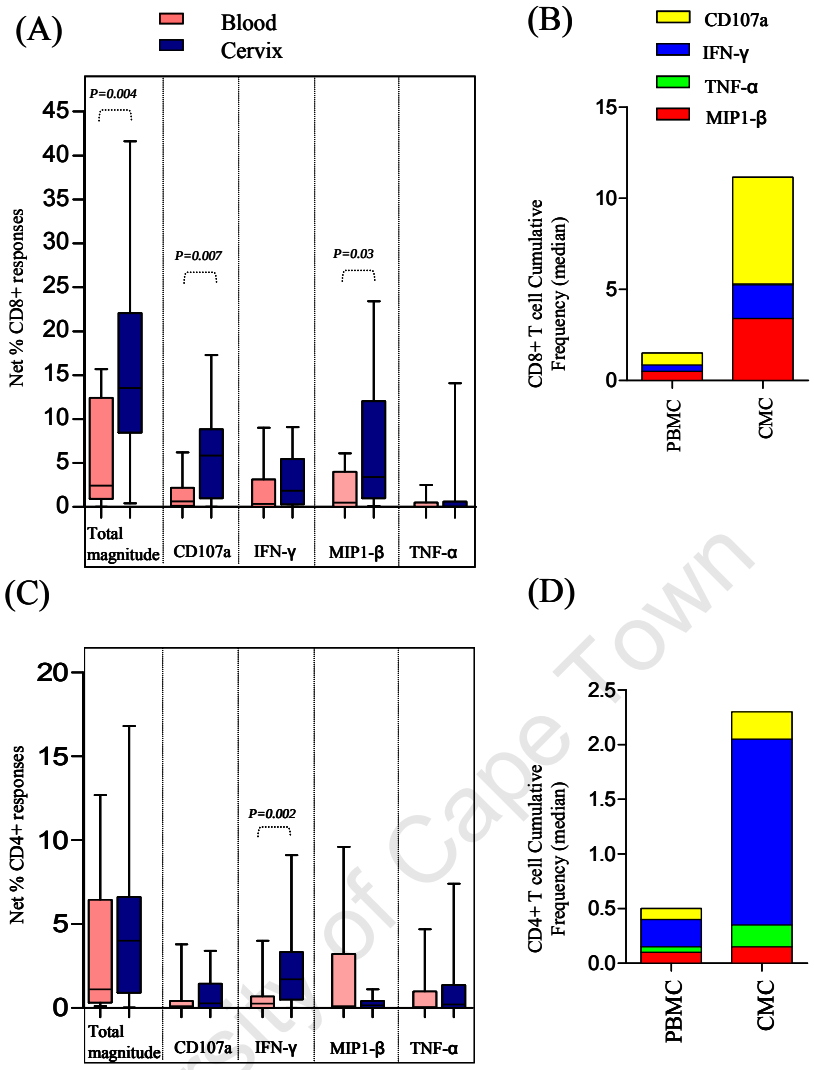


Figure 4.8. Comparison between female genital tract and blood CD8 and CD4 T cell responses to HIV Gag in T cell lines expanded with Dynal beads from HIV-infected women. **(A)** Total and individual frequencies of CD8+ responses detected in blood (pink boxes) and cervix (dark blue boxes) from Dynal bead expanded lines. (B) Cumulative frequencies of CD8+ CD107a (yellow bar), IFN-γ (blue bars), MIP-1β (red bars), and TNF-α (green bars) T cell responses in PBMC and cervix (CMC). **(C)** Total and individual frequencies of CD4+ responses detected in blood (pink boxes) and cervix (dark blue boxes) from Dynal bead expanded lines. (B) Cumulative frequencies of CD4+ CD107a (yellow bar), IFN-γ (blue bars), MIP-1β (red bars), and TNF-α (green bars) T cell responses in PBMC and cervix (CMC). Each box and whisker plot shows the median (central line), IQR (outer lines of box) and 10-90% range (error bars) of 16 HIV-infected individuals. Wilcoxon Rank Test was used to compare fresh and expanded responses and a $p<0.05$ indicates a significant difference.

4.3.8 Comparison between the frequency and quality of Gag-specific T cell responses at the cervix and in blood after expansion with anti-CD3

In contrast to Dynal bead expanded T cell lines, no significant differences between either total or individual frequencies of CD8+ (Figure 4.9A and B) and CD4+ T cell (Figure 4.9C and D) responding to HIV Gag were found when cervical and blood-derived T cell lines expanded with anti-CD3 were compared. In contrast to Dynal bead expanded T cell lines which predominantly expressed CD107a in response to Gag, anti-CD3 expanded CD8+ T cell responses in blood and at the cervix were dominated by MIP1-β (Figure 4.9B). In contrast to Dynal bead expanded CD4+ T cells which exhibited all 4 functions measured (CD107a, IFN-γ, MIP-1β and TNF-α), CD4+ T cells resulting from anti-CD3 expansion were predominantly producing a more restricted subset of these (MIP1-β and IFN-γ; Figure 4.9D). There were no significant differences detected in the frequency of CD4+ T cells at the cervix and in blood responding to Gag in any of the 4-functional categories.

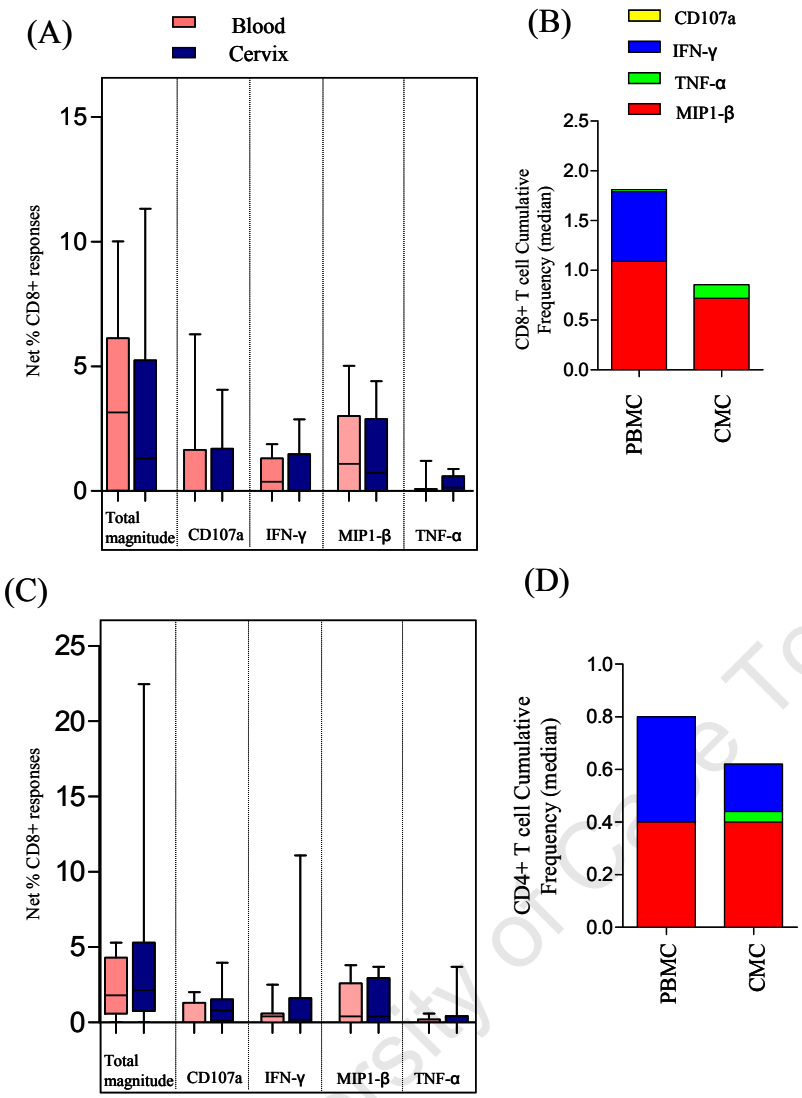


Figure 4.9. Comparison between female genital tract and blood CD8 and CD4 T cell responses to HIV Gag in T cell lines expanded with anti-CD3 from HIV-infected women. **(A)** Total and individual frequencies of CD8+ responses detected in blood (pink boxes) and cervix (dark blue boxes) from anti-CD3 expanded lines. (B) Cumulative frequencies of CD8+ CD107a (yellow bar), IFN-γ (blue bars), MIP-1β (red bars), and TNF-α (green bars) T cell responses in PBMC and cervix (CMC). **(C)** Total and individual frequencies of CD4+ responses detected in blood (pink boxes) and cervix (dark blue boxes) from expanded lines. (B) Cumulative frequencies of CD4+ CD107a (yellow bar), IFN-γ (blue bars), MIP-1β (red bars), and TNF-α (green bars) T cell responses in PBMC and cervix (CMC). Each box and whisker plot shows the median (central line), IQR (outer lines of box) and 10-90% range (error bars) of 7 HIV-infected individuals. Wilcoxon Rank Test was used to compare fresh and expanded responses and a $p<0.05$ indicates a significant difference.

4.3.9 Comparison of polyfunctional HIV-specific T cell responses in Dynal bead-expanded blood and cervical T cell lines

The frequency of polyfunctional HIV-specific responses at the cervix and blood was compared (Figure 4.10 and Figure 4.11, respectively). Following Dynal beads expansion, there were very low frequencies (median of 0%; range, 0-0.5%) of HIV Gag-specific four functional CD8+ T cells in both blood and cervix (Figure 4.11A and B). Although few Gag-specific tri-functional (CD107a, IFN-γ, MIP-1β) CD8+ cervical T cells were detected and they made up only a small magnitude (0.2%) and proportion (~2%) of Gag responsive cells (Figure 4.10A and B, respectively), these were higher than those observed in blood (although not significantly). Similarly, cervical cells had a greater magnitude and proportion of dual-functional (CD107a and MIP1-β) Gag-specific CD8+ T cells than blood (2.5% vs. 0.5%) and (15% vs.10 %) respectively, although this was not significant. The frequency of Gag-specific CD8+ T cell responses in both blood and cervix were dominated by monofunctional cells (>80%). Although the net frequency of HIV Gag-specific monofunctional cells was significantly higher at the cervix than detected in blood (Figure 4.10A; p=0.002) following Dynal beads expansion, the relative proportion of monofunctional cells at the cervix was lower than detected in blood, although this was not significant (Figure 4.10B). This finding suggests that a significantly higher frequencies of cervical T cells responded to Gag compared to blood and these genital cells tended to have a higher proportion of cells with ≥2 functions compared to blood.

Like CD8+ T cells, monofuctional CD4+ T cell responses dominated Gag-specific responses detected in blood and cervix (Figure 4.10C and D). Of the CD4+ mono-functional responses detected at the cervix, IFN-γ responses made up ~30% of total responses to Gag. Blood CD4+ responses, in contrast, were predominantly MIP1-β+ (~30%). Although the frequencies of Gag-specific CD4+ mono-, and dual-functional T cell were higher at the cervix than in blood, these were not significantly different and neither was the proportion of responses (Figure 4.10C and D).

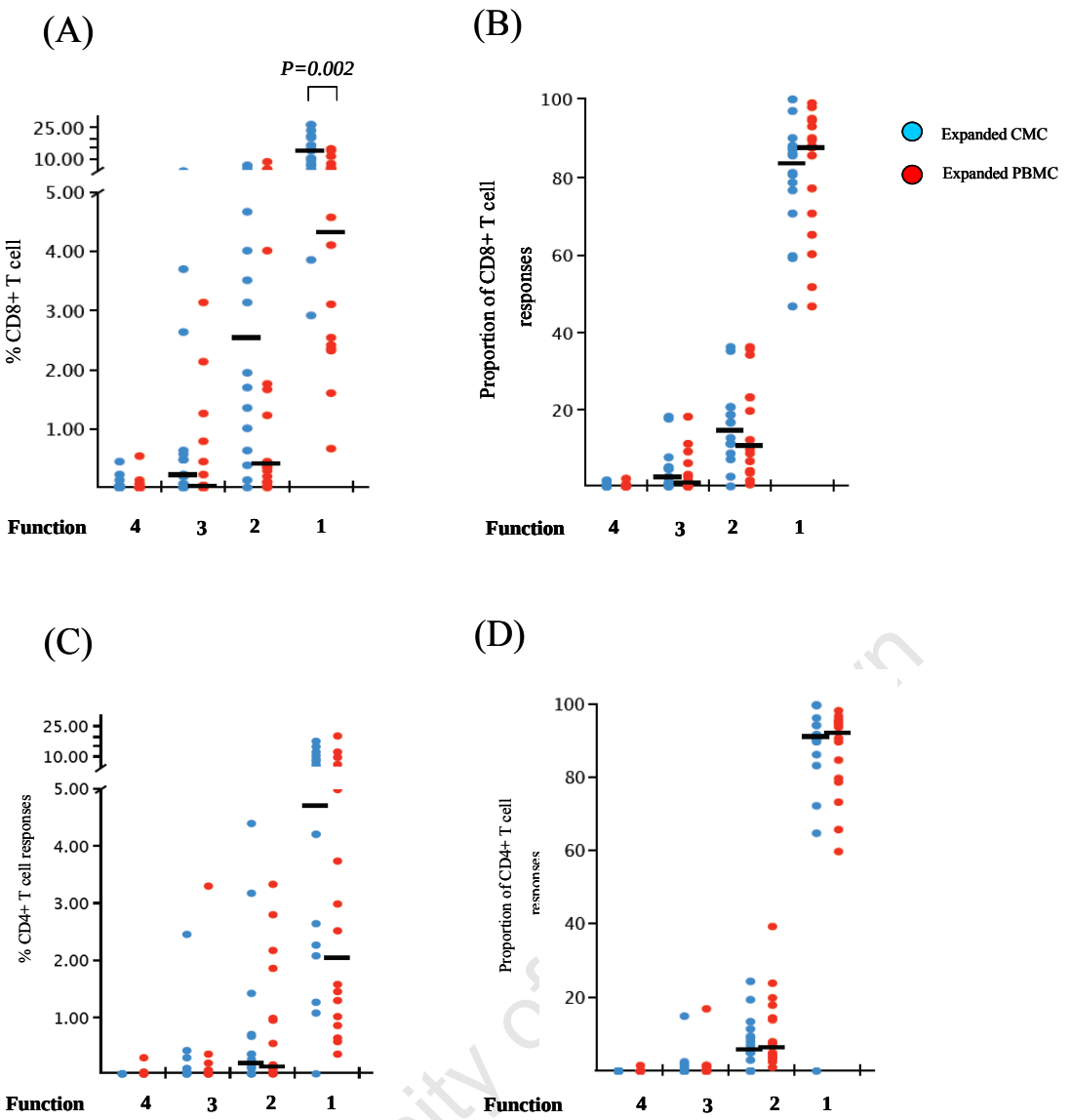


Figure 4.10. Polyfunctional HIV-specific T cell responses between Dynal beads expanded blood (red dots) and cervix (blue dots) from 16 chronically HIV-infected women. **(A)** Frequency of CD8+ responses within each functional category. (**B**) Proportion of CD8+ T cell responses within each functional category. **(C)** Frequency of CD4+ T cell responses for each polyfunctional subset. **(D)** Proportion of CD4+ polyfunctional responses. Responses were considered 4-functional if all 4 of the markers included in the polyfunctional panel were detected (IFN-γ, MIP-1β, TNF-α and CD107a). Responses were considered 3, 2 or 1-functional if any combination of 3, 2 or 1 of the 4 functions investigated were detected, respectively. The middle bars on the plots represent median response after background subtraction. Wilcoxon Rank Test was used to compare blood and cervical responses.

4.3.10 Comparison of polyfunctional HIV-specific T cell responses in anti-CD3-expanded blood and cervical T cell lines

Similar to Dynal beads expansion, very low frequencies of T cell lines generated by anti-CD3 expansion were four-functional (Figure 4.11). Only a small proportion (2%) of Gag-specific tri-functional CD8+ T cells (IFN-γ, MIP-1β and TNF-α) were detected in both blood and cervix (Figure 4.11A and 4.10B, respectively). Similar frequencies (~1%) and proportions (~15%) of Gag-specific dual-functional CD8+ cells were detected in both blood and at the cervix following anti-CD3 expansion. Mono-functional Gag-specific T cell responses dominated both blood and cervical CD8+ T cell responses (Figure 4.11A and B). These mono-functional subsets accounted for approximately 80% of the total Gag specific CD8+ T cell responses in both compartments (Figure 4.11B).

After anti-CD3 expansion, the magnitude of HIV Gag-specific dual-, tri-, and four functional CD4+ T cell was approximately 0.5% while that for mono- functional cells was ~3% in both blood and cervix (Figure 4.11C and 4.10D). However, the proportion of HIV Gag-specific dual-functional cells was higher in blood than at the cervix (10% vs. 2%). Mono-functional responses also dominated the Gag-specific CD4+ T cell responses and these made up close to 100% for cervical cells and 85% in blood of the total CD4+ T cell responses (Figure 4.11 C and D).

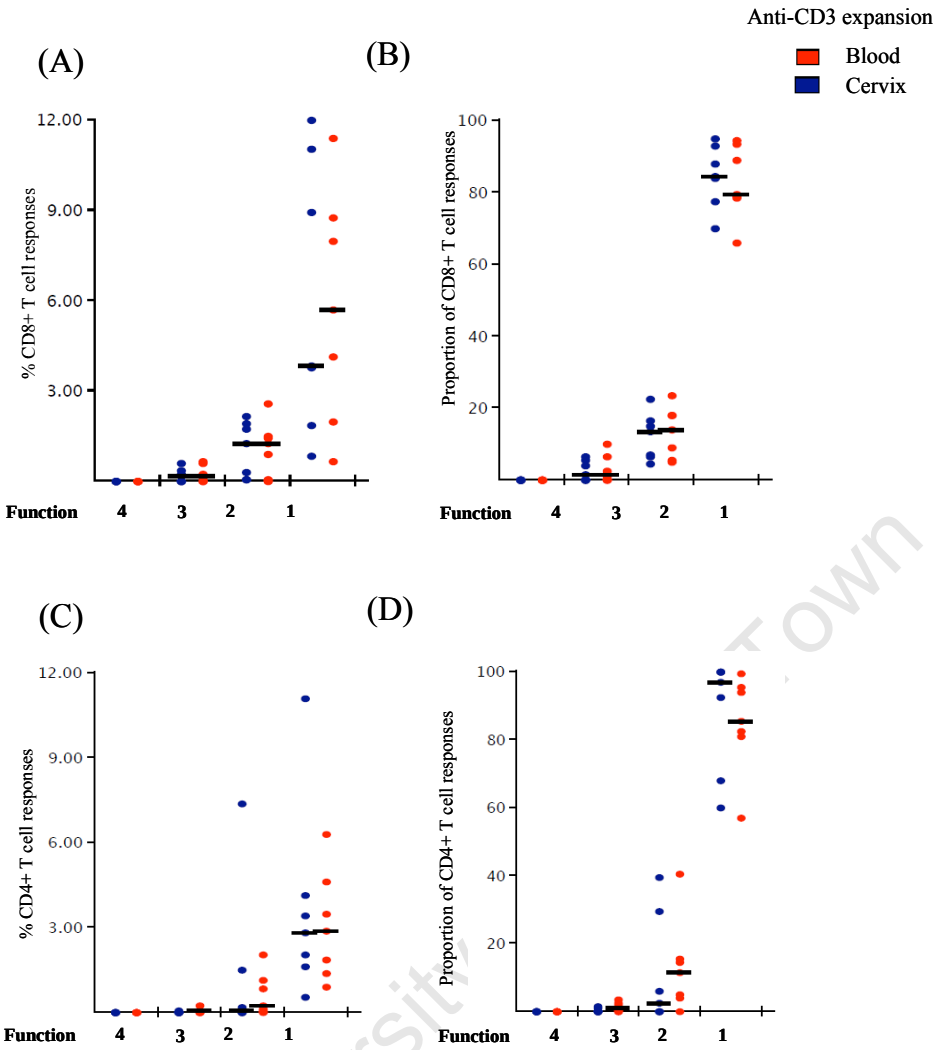


Figure 4.11. Polyfunctional HIV-specific T cell responses between anti-CD3 expanded blood (red) and cervix (blue) from 7 chronically HIV-infected women. **(A)** Frequency of CD8+ responses within each functional category. (**B**) Proportion of CD8+ T cell responses within each functional category. **(C)** Frequency of CD4+ T cell responses for each polyfunctional subset. **(D)** Proportion of CD4+ polyfunctional responses. Responses were considered 4-functional if all 4 of the markers included in the polyfunctional panel were detected (IFN-γ, MIP-1β, TNF-α and CD107a). Responses were considered 3, 2 or 1-functional if any combination of 3, 2 or 1 of the 4 functions investigated were detected, respectively. The middle bars on the plots represent median response after background subtraction. Wilcoxon Rank Test was used to compare blood and cervical responses.

4.3.11 Relationship between the frequency and functional profile of HIV Gag-specific responses at the cervix and in blood

To investigate whether HIV-specific responses detected in blood correlated with those detected in the female genital tract, the relationship between both the magnitude and complexity of HIV Gag-specific T cell responses in blood and cervical T cell lines (expanded with Dynal beads or anti-CD3) was evaluated. A significant correlation between blood and cervix in the total magnitude of CD4+ T cell responses was observed after Dynal beads expansion (p=0.002; Spearman Rank). For CD4+ T cells responding to Gag, there was a significant correlation between cervix and blood for CD107a (Rho=0.8; p=0.003) and TNF-α (Rho=0.7, p=0.004; Spearman Ranks test). For CD8+ T cells, there was a significant correlation between cervix and blood for CD107a (Rho=0.8, p=0.0002), IFN-γ (Rho=0.6, p=0.01) and TNF-α (Rho=0.8, p=0.0006) following Dynal bead expansion (Spearman Ranks test). This data shows that the magnitude of blood and cervical T cell responses to HIV Gag were broadly shared between compartments

Following anti-CD3 expansion, a significant correlation between blood and cervix in the total magnitude of CD8+ T cell responses was observed (Rho=0.89; p=0.01; Spearman Rank). Similar to responses detected after Dynal beads expansion, the frequency of anti-CD3 expanded CD8+ T cells in blood and at the cervix expressing CD107a (Rho=0.87, p=0.01), IFN-γ (Rho=0.8, p=0.03), and TNF-α (Rho=0.88, p=0.01) correlated significantly (Table 4.7; Spearman Ranks test). For CD4+ T cells, both the total magnitude (Rho=0.88, p=0.01) and the frequency of cells in blood and at the cervix expressing CD107a (Rho=0.87, p=0.01) were significantly associated. Similarly to Dynal beads expansions, this data shows that the responses magnitudes of blood and cervical T cells following anti-CD3 expansion are associated between compartments and confirms that immune cells can traffic freely between blood and cervical compartment in search of antigens.

The relationship between polyfunctional T cell responses (2+, 3+ and 4+ functions) detected in blood and at the cervix was next investigated (Figure 4.12). A significant association was observed between blood and cervical polyfunctional CD8+ T cell responses to Gag in T cell lines expanded with Dynal beads but not anti-CD3 (Figure 4.12 A and B, respectively). Because CD4+ T cell polyfunctional T cell responses were detected on comparatively low frequencies compared to CD8+ T cell polyfunction, no significant associations between compartments were found for CD4+ T cells.

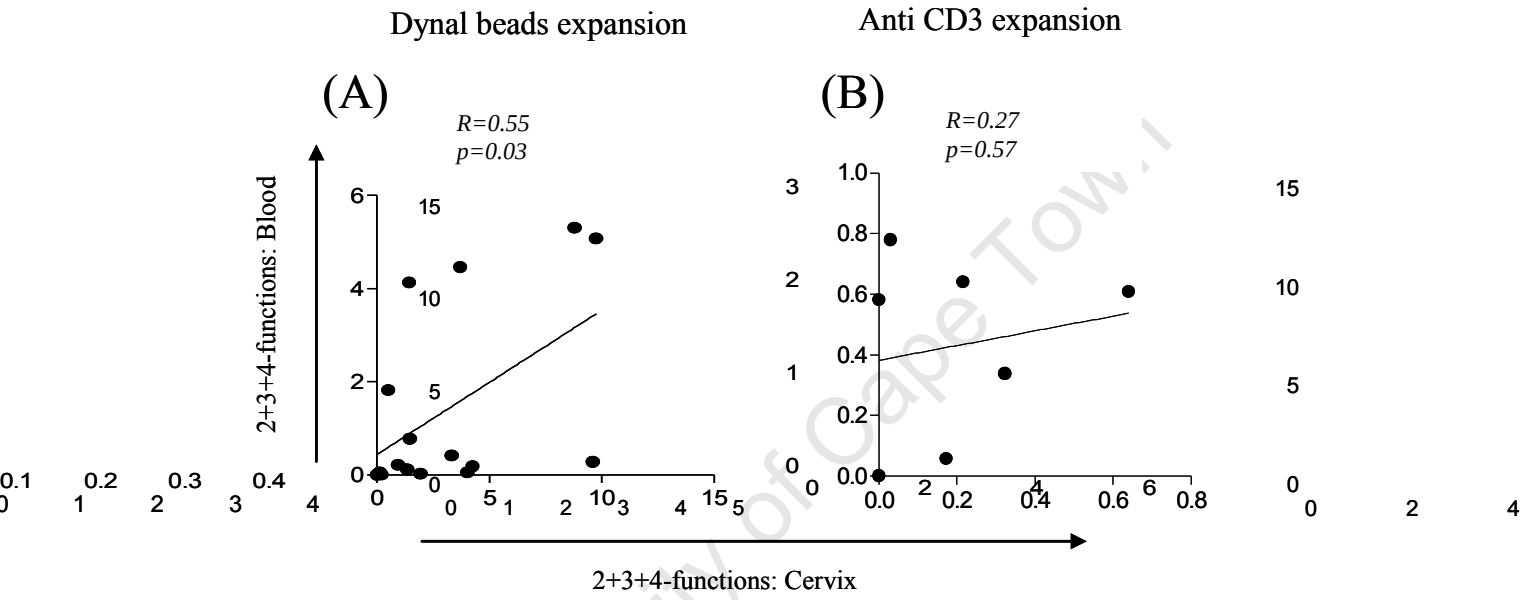


Figure 4.12. Relationship between polyfunctional HIV Gag-specific CD8+ T cell responses detected at the cervix and in blood of HIV-infected women following Dynal bead (A) or anti-CD3 expansion (B). Polyfunctional responses were defined as the total of 2+, 3+ and 4+ functions in blood and at the cervix of CD8+ T cells. Spearman Rank Test was applied to test correlations and p-values <0.05 were considered significant. Spearman Rho score is shown on each plot.

4.3.12 Relationship between HIV clinical status and HIV-specific responses detected in the female genital tract or blood

To investigate whether there was a relationship between HIV clinical status and functional/polyfunctional characteristics of HIV Gag-specific T cells in blood or at the cervix, the relationship between individual response profiles and markers of HIV disease progression (plasma viral load and CD4 counts) were evaluated (Figure 4.13 and 4.14). As previously described (Gumbi et al., 2008), HIV viral loads in plasma were found to be significantly associated with the amount of HIV detected in genital

secretions in this study (Rho=0.75, p=0.0008; Figure 4.13A). A significant inverse relationship was observed between plasma viral loads and the frequency of CD8+ T cells producing CD107a in blood (Rho=-0.53, p=0.04) and at the cervix (Rho=-0.64, p=0.03) in Dynal bead expanded T cell lines (Figure 4.13B and C). Since a significant positive association was previously demonstrated between blood and cervical CD107a expression for both CD8+ and CD4+ T cells in response to Gag (p=0.002 and p=0.003, respectively; Table 4.6), this similar relationship between HIV plasma viral load and CD107a expression in both compartments is likely to reflect this. As with Dynal bead expanded T cell lines, the frequency of fresh blood CD8+ T cells producing CD107a in response to HIV Gag stimulation correlated inversely with plasma and cervical viral loads (Figure 4.13D and E). There were no other significant relationships identified between any other T cell responses in either compartment and blood CD4 counts or plasma viral loads.

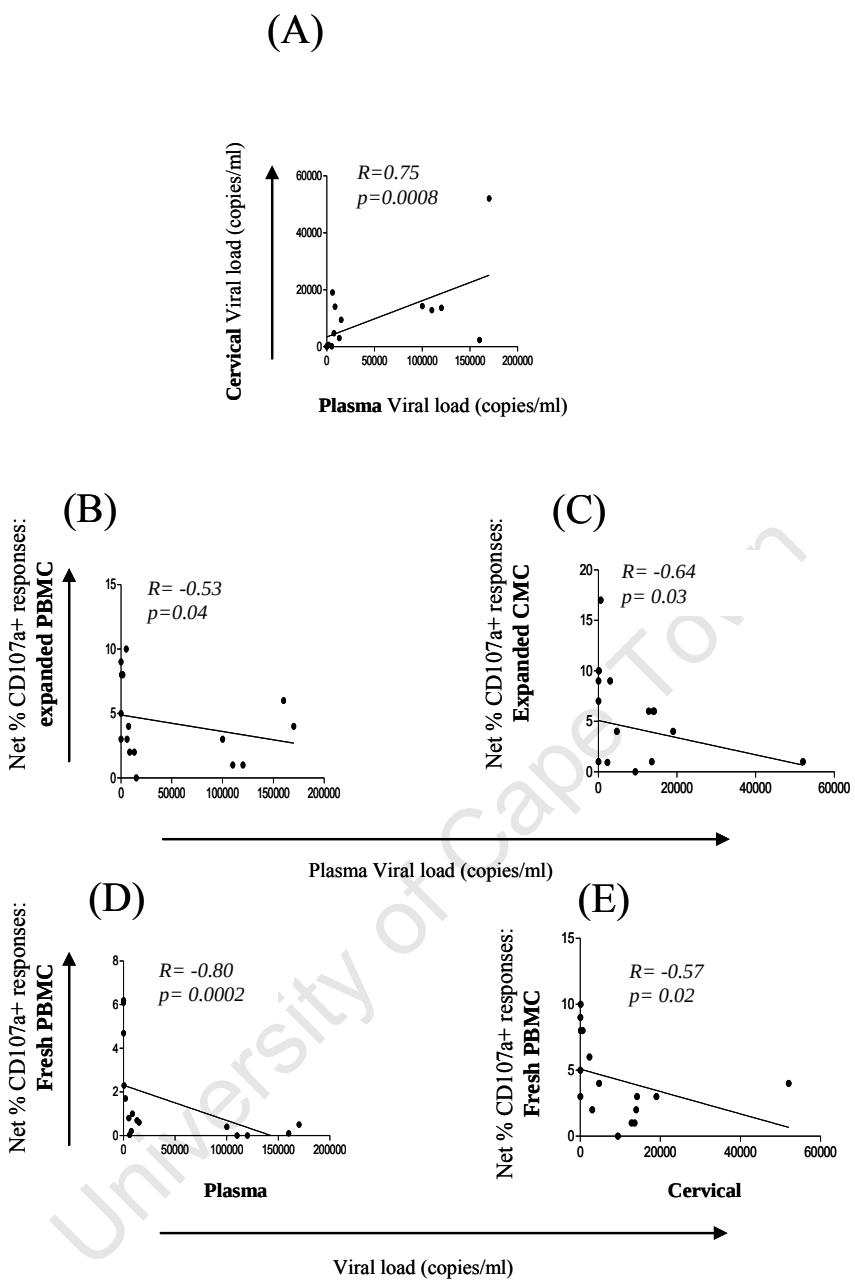


Figure 4.13. Relationship between HIV clinical status and blood or cervical CD8+ T-cell responses to Gag in fresh or Dynal bead expanded T cell lines. (A) Correlation between plasma and cervical viral load. Relationship between Dynal beads expanded blood **(B)** and cervical **(C)** CD8+ T-cell responses (shown as percentages of cells producing CD107a) and plasma viral load. **(E)** Association between CD107a expression in fresh PBMC and plasma **(D)** and cervical **(E)** load. Spearman Rank Test was applied to test correlations and p-values <0.05 were considered significant. Spearman Rho scores are shown on each plot.

The frequency of Gag-specific CD8+ T cell polyfunctional responses detected in blood (sum of 2, 3 and 4-functional responses) was significantly inversely associated with plasma viral loads (Rho=-0.51, p=0.04) and weakly positively associated with blood CD4 counts (Rho=0.33; p=0.21) (Figure 4.14 A and B). Previously, the 2+, 3+, and 4+ functions were shown to correlate between compartment (Figure 4.11) and here, the frequency of Gag-specific polyfunctional CD8+ T cell responses at the cervix were significantly positively associated with blood CD4 counts (Rho=0.56; p=0.03; Figure 4.14 C) and significantly inversely associated with plasma viral loads (Rho=0.52; p=0.04; Figure 4.14 D). In contrast to CD8+ T cell polyfunctionality, the frequency of polyfunctional CD4+ T cell responses in blood and at the cervix were not associated with HIV clinical status (data not shown).

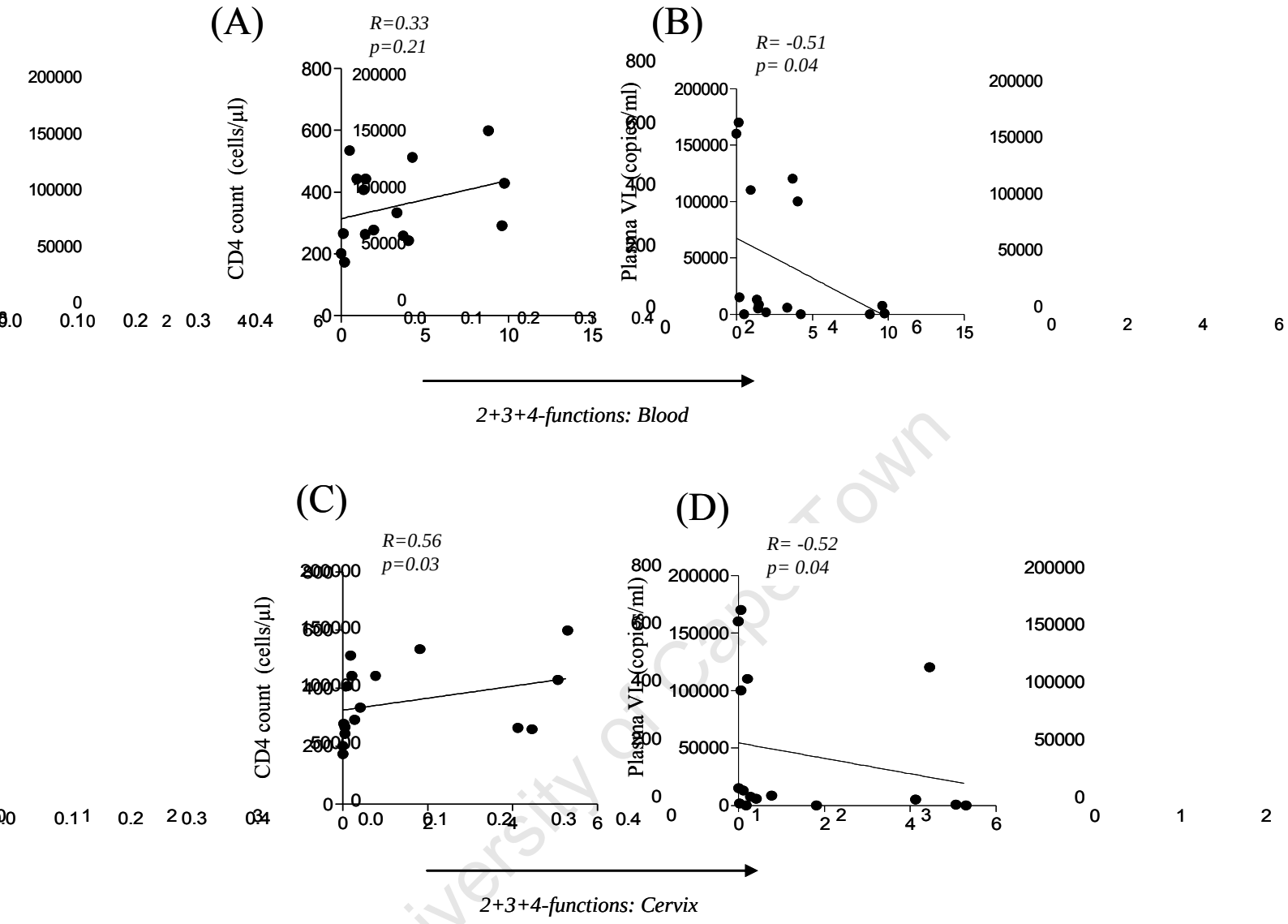


Figure 4.14. Relationship between HIV clinical status and polyfunctional Gag-specific CD8+ T cell responses in blood and at the cervix. (A) Comparison between polyfunctional responses (sum of 2, 3 and 4-functions) in blood and blood CD4 counts. (B) Comparison between blood polyfunctional responses and plasma viral load. (C) Comparison between cervical CD8+ polyfunctional responses and blood CD4 counts. Relationship between cervical polyfunctional responses and plasma viral load. Spearman Rank Test was applied to test correlations and p-values <0.05 were considered significant. Spearman R-value is shown on each plot.

4.4 Discussion

While there is clear evidence that HIV-specific cytotoxic T lymphocytes (CTLs) in blood play an important role in controlling HIV replication systemically (Addo et al., 2003; Betts et al., 2001; Edwards et al., 2002; Kaufmann et al., 2004; Ramduth et al., 2005), their frequency and the complexity of their effector function at the cervix is not well understood. Cervical cytobrushing is relatively non-invasive, but low cervical cell yields associated with this approach have significantly impacted on thorough evaluation of HIV-specific T cell responses (Kaul et al., 2000; Gumbi et al., 2008; Shacklett et al., 2003a). Although *in vitro* expansion of cervical cytobrush-derived T cells would circumvent the problem of low yield, there have been few studies investigating the ability of cervical cytobrush-derived T cells to expand *in vitro* (Chapter 2 and Chapter 3). Furthermore, little is known about how these polyclonal techniques differ in the amount of bias they may introduce into HIV-specific T cells from the cervical mucosa. This study compared the efficiency of immobilized anti-CD3 and Dynal bead-bound anti-CD3/CD28 to expand cervical cytobrush-derived cells and PBMCs in the presence of various combinations of γ-chain cytokines (IL-2, IL-7 and IL-15) using expansion protocols optimized in Chapter 3.

This Chapter showed that HIV-specific cervical and blood T cell responses are largely monofunctional (>80% of responses were either single functional CD107a, IFN-γ, MIP-1β or TNF-α) although significantly higher frequencies of cervical CD8+ T cells were shown to be polyfunctional (≥2 functions simultaneously) than CD8+ T cells from blood (1.96% vs. 1.08%). The functional profile (CD107a, IFN-γ and TNF-α) in response to Gag stimulation by CD8+ T cells in blood and at the cervix correlated significantly. In the CD4+ compartment, a more restricted functional profile (comprising only CD107a, and TNF-α expression) were significantly associated between blood and cervix. Furthermore, this Chapter showed that the frequency of polyfunctional CD8+ T cells responding to Gag (total of 2+, 3+ and 4+ functions) correlated significantly between the blood and cervix, regardless of the expansion technique employed. There were no significant associations in the CD4+ T cell compartment and this may be the result of polyfunctional responses being present in such low overall proportions that associations were not evident or relevant. It was previously established (Chapter 2) that polyclonal expansion of T cells resulted in

increased levels of background cytokine responses by ELISpot, here expanded T cell lines were deprived of IL-2, IL-7 and IL-15 for 24hrs prior to stimulation with HIV Gag peptides. This resulted in comparable background cytokine levels between fresh and expanded cells. Because analysis of multiparameter flow cytometry data has not been standardised in the field, in order to simplify analysis in this study, a positive response was taken as net percentage response (subtraction of the background unstimulated sample value).

The finding that relatively low frequencies of blood T cells were polyfunctional in chronically HIV-infected women confirms the findings of previous studies (Betts et al., 2006; Brenchley et al., 2008; Critchfield et al., 2007; Macal et al., 2008; Ferre et al., 2009). Betts et al. (2006) has shown that polyfunctional HIV-specific CD8 T cells are rarely found in the blood of individuals with progressive HIV infection. In this study, only a small proportion of three and four-functional CD8+ T cells (~1%) were detected in blood, with most Gag-specific T cell responses being mediated by mono-functional T cells. Furthermore, the magnitude and proportion of 3 functional T cells was higher at the cervix compared to blood following Dynal beads expansion, although this was not significant.

Several studies have confirmed the existence of polyfunctional HIV-1 specific T cells at mucosal sites such as rectum and lungs (Brenchley et al., 2008; Critchfield et al., 2007; Macal et al., 2008; Ferre et al., 2009). Brenchley and colleagues (2008) simultaneously measured expression of IFN-γ, TNF-α and IL-2 in HIV-infected individuals after stimulation with HIV Gag, Pol, Nef, and Env peptides and showed that ~ 5% of HIV-specific CD8+ T cells in lungs were polyfunctional compared to (< 1% in blood CD8 T cells. Moreover, HIV-specific CD4+ T cells from lungs were present at significantly higher frequencies and manifested a significantly more polyfunctional response (~ 15%) compared to HIV-specific CD4+ T cells from blood (<1%). In their study, Brenchley et al. (2008) also reported that the majority of responses present in both lungs and blood were monofunctional (~ 80%). At the rectal mucosa, Critchfield et al. (2007) evaluated five distinct effector functions (CD107a, IFN-γ, MIP-1β, IL-2 and TNF-α) of HIV Gag-specific CD8+ T-cells and found that rectal CD8+ responses had a higher frequency of Gag specific responses compared to PBMC. The mean total percentage of rectal CD8+ T-cells responding to HIV Gag

stimulation was 5.9% for therapy-naïve HIV-infected individuals and nearly one-half of the rectal response in these patients was comprised of polyfunctional cells capable of 3, 4, or 5 responses, simultaneously. In blood of HIV-infected individuals not on ART, the mean percentage of HIV Gag-specific CD8+ T-cells was lower than in rectal mucosa (3.8% vs. 5.9%) and approximately 30% of these responses were derived from cells capable of >3 responses. Ferre et al. (2009) measured the production of CD107a, IFN-γ, MIP-1β, IL-2 and TNF-α and reported that HIV Gag-specific CD8+ T-cell responses in rectal mucosa were more strongly associated with controller status than blood responses. The magnitude of Gag-specific mucosal CD8+ T-cell responses was higher in controllers (6.6%) compared to non-controllers (4.1%) and persons on HAART (1.4%). Controllers had higher frequencies of mucosal CD8+ T cells capable of 3 or more simultaneous effector functions than non-controllers. In addition, controllers with protective alleles had a significantly greater frequency of HIV-responsive T cells at the rectal mucosa than measured in blood (7.4% vs 3.0%). Taken together, the finding from this Chapter and from previous studies shows that blood and mucosal compartments (cervix, lungs and rectal) are dominated by mono-functional T cells. The complexity of T cell responses increases in HIV-infected individuals who were controlling HIV better in both blood and mucosa.

Studies investigating the relationship between the magnitude or breadth of HIV-specific CTL and HIV clinical status have reported conflicting findings. Some studies have found no direct relationship (Dalod et al., 1999; Addo et al., 2003). Others have reported an inverse relationship suggesting that high frequencies of HIV-specific CTLs are associated with low plasma viraemia (Ogg et al., 1998; Edwards et al., 2002). In direct contrast, others have found a positive correlation between plasma viral load and the total HIV-specific, Env-, and Nef-specific CD8+ T-cell frequency implying that antigenic load was associated with high frequencies of responses (Betts et al., 2001). Few studies have evaluated the relationship between the frequency or complexity of HIV-specific T cell responses at mucosal surfaces and HIV clinical status. Critchfield et al. (2007) reported that the frequency of polyfunctional rectal mucosal Gag-specific CD8+ T-cells was associated to clinical status (positively with blood CD4 count and inversely with plasma viral loads) suggesting that these responses may play an important role in mucosal immune surveillance. This is further supported by the finding that polyfunctional T cell responses at the rectal mucosa

were enriched among individuals who control HIV in the absence of therapy compare to non-controllers (Critchfield et al. 2007). In this Chapter, a significant inverse relationship between plasma viral loads and CD8+ T cells producing CD107a in fresh blood, expanded blood and expanded cervical lines was observed. This indicates that the ability of CD8+ T cells from both the cervix and in blood to degranulate was highest in women with lowest plasma viral loads. It was also found that blood CD8 T cell producing CD107a correlated with both plasma and cervical viral load. Although one might speculate that cervical polyfunctional responses suppress HIV genital shedding, this study found no relationship between cervical polyfunctional T cell responses and cervical viral loads. Similarly, in a study by Gumbi et al. (2008), monofunctional IFN-γ+ T cell responses to HIV Gag were not associated with protection from HIV genital shedding.

The present study found that the polyfunctionality of cervical Gag-specific CD8+ T-cells was positively related to blood CD4 count and inversely related to plasma viral load. Thus, immunologic features of circulating HIV-specific CTLs may contribute to the apparent control of viral replication. Alternatively, worse clinical disease status may be associated with compromised HIV-specific polyfunctional responses. Since a number of studies have identified deficits in HIV-specific CD8 T cell responses in the absence of CD4 helper functions in individuals with low CD4 T cell counts, the later explanation may be more accurate. Accumulation of polyfunctional HIV-specific cellular responses have been associated with restoration of mucosal CD4 + T cells, despite persistent mucosal CD4+ T-cell proviral reservoirs and immune activation in individuals on long-term HAART (Macal et al., 2008). Taken together, these studies point at the importance of polyfunctional T cells in mucosal compartments. In the present Chapter, HIV-infected women who were controlling viraemia were also more likely to have better responses because they were not so immunocompromised compared to HIV-infected women who were not controlling viraemia.

Despite several studies showing polyfunctional cells associate with control of HIV and other infections, their function is still controversial. HIV studies have shown the importance of polyfunctional T cells in individuals who naturally control virus, have low viral loads and do not progress to disease (Betts *et al*., 2006; Duvall *et al*., 2008; Kannanganat *et al*., 2007b). Thus, due to a limited amount of literature surrounding

this topic, it is still unclear whether polyfunctionality controls viral replication or is the result of low viral loads.

Comparison between expanded blood and cervical T cell responses to Gag showed that the magnitude of responses correlated significantly between compartments [CD107a, TNF-α, and IFN-γ in CD8+ T cells; CD107a and TNF-α in CD4+ T cells]. Ibarrondo *et al*. (2005) found that both the magnitude and breadth of HIV-specific CTL responses in blood mirrored those seen in the rectal mucosal compartment during chronic HIV infection (using IFN-γ ELISpot). Ibarrondo et al. (2005) found that discordant CTL responses were only found in responses that were of a low frequency in either compartment. Although a subset of functions were significantly altered following expansion in this study (CD107a for CD8+ T cells and TNF-α for CD4+ T cell), I found that half of the CD8+ functional responses (CD107a and TNF-α) were significantly associated following expansion while only a quarter of CD4+ functional responses (TNF-α) were associated pre- and post-expansion. Minor fluctuations in Gag-specific responses following expansion might have been as a result of a change in memory phenotype observed following expansion. Results from Chapter 3 have shown that polyclonal expansion of cervical cytobrush-derived cells and PBMCs using Dynal beads in the presence IL-2, IL-7 and IL-15 enriched for central memory T cells while anti-CD3 expansion maintained the effector memory dominant phenotype of cervical cytobrush cells. Effector memory cells are associated with rapid effector functions (Sallusto et al., 2004). Romero et al. (2007) showed in humans that significantly higher frequencies of effector memory CD8 T cells expressed cytolytic perforin and granzyme B and display greater cytotoxic abilities than central memory cells. In contrast, Barber et al. (2003) showed in mice that *in vivo* re-activated central memory CD8 T cells are equally efficient at cytotoxic killing as effector memory cells. Romero et al. (2007) suggested that central memory may be more effective at mediating protective immunity by virtue of their ability to proliferate, which may be particularly important for persistent infections. Since the HIV-infected women included in this study were not taking ARVs and T cell culture did not include ARVs, it was likely that the ongoing viral replication that is likely to have occurred during polyclonal expansion of T cells might have been responsible for driving central memory to acquire these effector memory-like functions.

Besides the measurement of four T cell functions mentioned in this study, there are other cytokines that could have been evaluated. IL-2 is one of the primary cytokines that $CD4^+$ T cells secrete upon antigen recognition (reviewed in Malek et al., 2004). The ability of blood $CD4^+$ T cells to secrete IL-2 has been shown to inversely correlate with viral control in chronic HIV infection (Harari et al., 2004). It would have been interesting to investigate whether similar correlations are observed in cervical CD4+ T cells.

In summary, this Chapter shows that *ex vivo* evaluation of cervical cytobrush T cell responses to Gag are hampered by low cell yields making polychromatic evaluation of the complexity of these responses problematic. This Chapter has highlighted the need to improved yields of cells acquired from the female genital tract and *in vitro* expansion of T cells from cytobrushes was found to be a feasible method to overcome these limitations. The data presented in this Chapter suggests that expanded T cell from both the genital tract and blood generally maintain their cytokine response profiles following *in vitro* expansion. Furthermore, this study shows that HIV Gag-specific polyfunctional T cell responses were detectable in the female genital tract of HIV-infected women. These polyfunctional cells were largely present in women with high CD4 T cell counts in blood and low plasma viral loads. HIV-specific profiles (polyfunctional and cytokines secreted) correlated between compartments. Because of the differing microenvironments in blood and at the cervix, it is important to understand the differences in immune pressures imposed on HIV in these compartments.

CHAPTER 5

Discussion

5.1 Discussion

More than 90% of new HIV infections occur in developing countries, with the majority of infections occurring in Sub-Saharan Africa (UNAIDS, 2008). HIV is spread mainly through heterosexual contact among adults in Africa. Amongst young adults in South Africa, the prevalence of HIV is highest in women (UNAIDS, 2008) and several factors have been proposed to account for this apparent increased susceptibility of young women to HIV infection. Ray et al. (2000) suggested that this may be due to an increased mucosal area of exposure in the female genital tract, increased susceptibility through greater mucosal trauma, the presence of undiagnosed sexually transmitted infections, prolonged exposure to infected male secretions and other immunological differences between men and women. HIV transmission is dependent on the concentration of virions in the genital secretions, with increased heterosexual transmission of HIV being associated with higher viral loads in blood (Lee et al., 1996; Quinn et al., 2000).

Mucosal T cell responses and early events

HIV targets the mucosal immune system early during infection and depletes most of the CD4 T cells at this site (Koup et al., 1994; Reynolds et al., 2005; Miller et al., 2005). Mucosal surfaces also represent the major route of HIV transmission. Studies in macaques infected with SIV have suggested that mucosal T cells may be actively involved in the early defense against HIV-1 infection (Lohman et al., 1995). Most studies in humans have focused on analysis of HIV-specific immunity in blood (reviewed in Pope et al., 2004). Despite the recognized importance of mucosal tissues during transmission and disease progression, comparatively few studies have described immunity at these sites. In humans, mucosal tissues are more difficult to sample with only small numbers of cells being recovered non-invasively from these tissues. Because mucosal immunity to HIV will not only be a desirable but also a necessary component of an effective HIV vaccine, there is a clear need to establish reliable and sensitive methods for investigating mucosal immune responses in future vaccine studies.

Aims and major findings

This dissertation describes the optimisation and validation of methods that can be applied to bulk up cervical cytobrush-derived T cells from the female genital tract. This study describes an approach to overcome some of the limitations of low cell yields through the use of polyclonal *in vitro* expansion of cervical T cells from cytobrush samples using either anti-CD3 monoclonal antibody or Dynal bead stimulation in the presence of various combinations of γ-chain cytokines (IL-2, IL-7 and IL-15). This was achieved by comparing the specificity and breadth of cervical and peripheral blood T cell IFN-γ, CD107a, TNF-α and MIP-1β responses to HIV-1 sub-type C overlapping Gag peptides. Furthermore, this dissertation investigated the role of multifunctional cervical mucosal T-cell in chronically HIV-infected individuals in determining clinical status. This study is one of the most comprehensive to date in comparing and optimizing mucosal mononuclear cell expansion protocols. Evaluation of HIV-specific immune responses, particularly at the genital tract, will provide new information for the better understanding of virus–host interaction during HIV infection and subsequent loss of viral control and disease progression.

T cell recovery and phenotyping

Anti-CD3 in the presence of IL-2 was shown to successfully expand cervical T cells and yield sufficient cells to map individual HIV-specific peptide response by ELISpot (Chapter 2). The finding that cervical cytobrushing and anti-CD3-mediated polyclonal expansion only enabled detection of Gag-specific T cell responses in the genital tracts of women with correspondingly high systemic Gag-specific responses set a standard against which future efforts in this field can be measured and demonstrated the need for more efficient expansion methodologies. Conditions that improve the rate of expansion and viability of T cells derived from the female genital tract will not only reduce the time required to improve yields but also reduce the risk of expansion bias and contamination. The efficiency of immobilized anti-CD3 alone (described in Chapter 2) was compared to alternative expansion modalities including anti-CD3 in combination with anti-CD28; or Dynal bead-bound anti-CD3/CD28 to expand cervical cytobrush-derived cells and PBMCs in the presence of IL-2, IL-7 and IL-15 (Chapter 3). The relative expansion of cervical T cells expanded with either anti-

CD3/IL-2 or Dynal/IL-2 was 1.4-fold lower than the extent of expansion observed for PBMCs. This demonstrates that the extent of expansion of cervical T cells may be lower than blood T cell expansion and may be affected by the predominance of effector memory T cells sampled by cytobrush *ex vivo*. Previous studies have reported that effector memory T cells have a reduced proliferative capacity which has been shown to correlate with reduced telomere length (Sallusto et al., 1999). Despite this, it was confirmed in Chapters 3 and 4, using several alternative expansion methods, that cervical cytobrush-derived T cells can be expanded sufficiently *in vitro* to significantly improve yields of T cells available and subsequently the type of analysis that can be conducted. In Chapter 3, it was concluded that cervical T cell yields can be best improved by expansion with Dynal beads (1:1) in the presence of IL-2, IL-7 and IL-15 while memory T cell profiles were best maintained by expansion with anti-CD3 in the presence of IL-2 alone.

Although previous studies have shown that responses were better after in vitro stim with HIV antigen (Goonetilleke et al., 2006., Hanke et al., 2007) where *in vitro* peptide-stimulated culture expanded both CD4+ and CD8+ T-cell responses, which were often undetectable in *ex vivo* assays, and showed more than 24-fold increases in the frequency of specific T cells measured by ex vivo IFN-γ ELISPOT, this study used polyclonal expansion which did not selectively expand HIV-specific T cells and therefore some of the response after expansion declined. Our results, however, are supported by previous reports which highlighted the idea that TCR signalling strength coupled with the co-stimulation with anti-CD28 are important factors in driving T cell proliferation as well as expansion of antigen specific cells (Kalamasz et al., 2004., Onlamoon et al., 2006).

Correlation between plasma and cervical viral load

There have been a number of studies showing a significant association between plasma viraemia and genital viral loads and that plasma viral load was one of the best predictors of HIV shedding in genital secretions (Uvin et al., 1997; Iversen et al., 1998; Goulston et al., 1998; Clyde et al., 1999). Similarly, in this study, there was a significant association between plasma viral load and HIV genital shedding. Higher plasma viral load, lower CD4 T cell counts and the presence of sexually transmitted infections has previously been associated with the presence of HIV in the genital

secretions (Garcia-Bujalance et al., 2004). Understanding changes in HIV species between these two compartments is crucial for studying the effects of systemic or antiviral therapy and the epidemiology of viral transmission (Coombs et al., 2001).

Viral and T cell compartmentalisation between compartments

In depth comparison of the phylogenetic relatedness between genital and plasma-derived HIV during chronic infection has demonstrated distinct phenotype and genetic differences, suggesting some degree of compartmentalization between these distinct sites (Ellerbrook et al., 2001; Overbaugh et al., 1996; Philpott et al., 2005). It was suggested that there is a continual process of migration of a restricted population of HIV-infected cells to mucosal sites from systemic circulation and that this is followed by localized HIV-1 expansion and independent evolution of the new quasispecies (Poss et al., 2005). Because of these difference is viral genotype and phenotype that have been identified; it has led many researchers to speculate that HIV-specific immune responses by T lymphocyte subsets at the mucosal compartments are also likely to differ from those in blood. This is likely to be partially due to the presence of detectable compartment-specific diversity in HIV genotypes present in each compartment (Musey et al., 2003) and also because the mucosa represents a distinct milieu with higher antigen exposure. Studies comparing the specificity of HIV targeted responses at the genital mucosa and blood during the chronic phase of HIV-infection have yielded conflicting data. Some have demonstrated that there are some differences in HIV targeting between cervical and peripheral blood CTL (Shacklett et al., 2000; Ibarrondo et al., 2005), while others have shown that the blood and mucosal compartments have largely overlapping HIV-1 epitope specificities (Musey *et al*., 1997; 2003a; Kaul et al., 2000a; Musey et al., 2003a; Ibarrondo et al., 2005). Based on these conflicting findings, there is a clear need to further investigate the issue of compartmental T cell diversity to HIV. In this dissertation, it was found that HIV Gag-specific blood T cell responses were detectable in 14/16 (88%) women with chronic HIV-infection while 8/16 (50%) of these had matched cervical responses to Gag (Chapter 2). It has previously been shown that identical HIV-specific CTL clones were present in both blood and mucosal compartments of the same HIV-infected individual, demonstrating that a subset of blood and mucosal HIV-specific CTL can have a common origin and can traffic freely between these distinct compartments (Musey et al., 2003a). In this study, response magnitudes were found to correlate

between compartments and largely target the same regions of Gag (Chapter 2) and with overlapping response profiles (Chapter 4). Similarly, Kaul et al. (2003) showed concordance between HIV-specific responses in blood and at the cervix *ex vivo* during chronic infection with 8/10 women studied having matching responses at the cervix as they had in blood. Shacklett et al. (2000) reported only 3/8 chronically HIV-infected women with detectable HIV-specific responses in blood had a corresponding response at the cervix.

The existence of polyfunctional T cells at the cervix and other mucosal sites

This study used polychromatic flow cytometry to simultaneously assess whether HIV specific T-cell response quality is an important factor in HIV disease progression at the female genital tract. This study detected only a small proportion of three and four-functional CD8+ T cells (~1%) at the cervix and the frequency of these polyfunctional responses were not significantly different from those detected in blood (<0.5%). Most of the Gag-specific T cell responses in both compartments were mediated by mono-functional T cells. Previously, Betts et al. (2006) showed that polyfunctional HIV-specific CD8 T cells are rarely found in the blood of individuals with progressive HIV infection. However, several studies of functional capacity and the magnitude of HIV-specific T cells in other mucosal sites and have confirmed the existence of polyfunctional HIV-1 specific T cells at mucosal sites (Brenchley et al., 2007; Critchfield et al., 2007; Macal et al., 2008; Ferre et al., 2009). Brenchley and colleagues (2007) simultaneously measured expression of IFN-γ, TNF-α and IL-2 in 26 therapy naïve individuals and showed that HIV-specific CD8+ T cells in lungs tended towards a more polyfunctional phenotype (~5%) compared to blood CD8 T cells (<1%). Moreover, HIV-specific CD4+ T cells from lungs were present at significantly higher frequencies and manifested a significantly more polyfunctional response (~15%) compared to HIV-specific CD4+ T cells from blood (<1%). The majority of responses present in both lungs and blood were monofunctional (~80%). At the rectal mucosa, Critchfield et al. (2007) evaluated five distinct effector functions (CD107a, IFN-γ, MIP-1β, IL-2 and TNF-α) of HIV Gag-specific CD8+ T-cells in rectal mucosa and blood and found that rectal CD8+ responses are stronger (all 31 non-overlapping categories combined) compared to PBMC (5.9% vs. 3.8%). Their data showed that blood and mucosal compartments (cervix, lungs and rectal) are predominated by mono-functional T cells, however, the complexity of T cell responses seems to increases in individuals who were controlling HIV better in both blood and cervix.

Correlation between T cell responses and HIV clinical status

A significant inverse relationship was observed between plasma viral load and CD8+ T cells producing CD107a in fresh blood, expanded blood and expanded cervical lines. This indicates that the ability of CD8+ T cells from both the cervix and in blood to degranulate was highest in women with lowest plasma viral load. It was also

found that blood CD8 T cell producing CD107a correlated with both plasma and cervical viral load. Although one might speculate that cervical polyfunctional responses suppresses HIV genital shedding, this study found no relationship between cervical polyfunctional T cell responses (or any subset of individual T cells responses) and cervical viral load. In a study from our laboratory by Gumbi et al. (2008), monofunctional IFN-γ+ T cell responses to HIV Gag also were not associated with protection from HIV genital shedding. The present study found that the polyfunctionality of cervical Gag-specific CD8+ T-cells was positively related to blood CD4 count and inversely related to plasma viral load. While it could be argued that the measurement of four functions will not necessarily provide more relevant information than the measurement of a single function, recent findings have in fact demonstrated the added value of this approach. Recently, Critchfield et al. (2007) reported that the frequency of rectal mucosal Gag-specific CD8+ T-cells capable of 3, 4 or 5 simultaneous effector functions was significantly associated to blood CD4 count and inversely related to plasma viral load suggesting that these responses may play an important role in mucosal immune surveillance, as suggested by their relative enrichment among persons who control HIV in the absence of therapy (Critchfield et al. 2007). Thus, immunologic features of circulating HIV-specific CTLs may contribute to the apparent control of viral replication. Alternatively, worse clinical disease status may be associated with compromised HIV-specific polyfunctional responses. Since a number of studies have identified deficits in HIV-specific CD8 T cell responses in the absence of CD4 helper functions in individuals with low CD4 T cell counts. Accumulation of polyfunctional HIV-specific cellular responses have been associated with restoration of mucosal CD4 + T cells, despite persistent mucosal CD4+ T-cell proviral reservoirs and immune activation in individuals on long-term HAART (Macal et al., 2008). Taken together, these studies point at the importance of polyfunctional T cells in mucosal compartments. In the present study, women controlling viraemia are also more likely to have better responses because they are not so immunocompromised. Viral load correlate between compartments as do magnitude of responses (Chapter 2) and functional profile of responding cells (Chapter 4). Together this implies that both virus and T cells in blood traffic to genital tissue during chronic infection and blood serves as the major reservoir for virus and T cell immunity at mucosal sites.

Summary

This dissertation presents evidence that cervical T cells can be isolated by cytobrushing and *in vitro* polyclonal expansion of T cells is a useful approach to increase the number of cells available from inaccessible mucosal sites. There were significant changes that occurred in individual memory subsets depending on the expansion method applied which is expected since the very act of expansion of T cells would drive differentiation. It is important, however, to note that the ability to obtain cultured T cells that retain a similar functional phenotype as their unexpanded counterparts is important to the usefulness of expansion methods. Generally, anti-CD3 expansion in the presence of IL-2 alone resulted in the accumulation of effector memory cells, while Dynal beads resulted in selective accumulation of central memory T cells from both cervix and blood. Different bead-to-cell concentrations led to different proportions of effector and central memory and this was attributed to TCR signalling strength. This data suggests that the optimal expansion technique depends on what the cells are to be used for following expansion.

Recommendations

Despite great advances in flow cytometry, allowing multiple functional markers to be investigated, thus enhancing the potential to discover correlates of control, there are still unanswered questions regarding the issue of which parameters are to be measured, and which immunological method should be used to provide the most relevant information. Although HIV-specific T cells are capable of producing a wide range of cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, IL-8, IL-13, these are rarely measured in the majority of HIV studies (Goonetilleke et al., 2006). In addition, the most important cytokines or functions to measure are still not well known (Betts et al., 2006; 2008; Hunt et al., 2008; Deeks et al., 2004). One of the limitations in this present study lies in the use of a surrogate markers such as CD107a which might not necessarily or accurately reflect direct T cell cytotoxicity. CD107a is a surface-expressed marker so it does not prove that degranulating cells can actually kill target cells, although CD107a expression has been shown to directly correlate with cytotoxic activity (Betts et al., 2003). However, perforin expression by *ex vivo* HIV-specific CD8+ T cells was recently shown to be significantly higher in elite controllers compared to patients with uncontrolled viral replication (Hersperger et al., 2010). Thus, the ability of HIV-

specific CD8+ T cells to rapidly express perforin might define a novel correlate of control in HIV infection. It might be more reliable to include markers such as perforin, granzyme A or B, or even granulysin. It may be useful in future studies to explore a wider range of surrogate markers for cytotoxicity as well as proliferation. In addition, it might be meaningful to consider viral suppression assays as these are more biologically relevant to T cell functioning.

Another weakness of this study and many other studies is the use of HIV Gag peptides derived from consensus subtype C sequences in determining T cell responses. The problems with these consensus sequences are that they may not match the actual circulating virus.

Although this study clearly identified the presence of low frequencies of polyfunctional T cells at the cervical mucosa, it was not clear what the nature of the causal relationship was between these responses and tissue viral loads. In addition, it may have been useful to include additional cytokines such as IL-2 in the functional evaluations conducted. Because of the differences observed in expansion of HIV- and HIV+ PBMCs, it would also be important in future studies to evaluate the effect of including ARVs in culture on expansion kinetics and yield. This is likely to result in increased cellular yields after expansion of HIV+ cells.

Another limitation of this study was that asymptomatic STIs such as BV and HSV-2 were not measured or controlled for. These have an effect on inflammation and hence might affect cellular recruitment to the female genital tract, which in turn has an effect on cytobrush composition and hence affect polyclonal expansion. However, this study set a platform to identify key questions for future studies.

Finally, due to the rarity of the mucosal samples used in this study, this thesis was mainly a hypothesis generating work and analyses were mainly explanatory and therefore not corrected for multiple comparisons.

Conclusions

In summary, this study shows that e*x vivo* evaluation of cervical cytobrush T cell responses to Gag were hampered by low cell yields making polychromatic evaluation

of the complexity of these responses problematic under Boolean conditions. Direct *ex vivo* data strongly supported the need for improved yields of cells acquired from the female genital tract and *in vitro* expansion of T cells from cytobrushes offered a feasible method to overcome these limitations. HIV Gag-specific cervical T cell responses can be detected in HIV-infected women and correlate with responses detected in blood. T cells derived from the female genital tract were predominantly effector memory in phenotype. Furthermore, HIV Gag-specific cervical T cell were shown to be mostly monofunctional and these did not differ markedly across the two methods tested. These polyfunctional cells were largely present in women with high blood CD4 count and low plasma viral load and cervical viral load. It is likely that polyfunctional responses are a consequence of an intact immune system in individuals with low viral replication, rather than the cause of low virus replication. HIV-specific functional profiles investigated in Chapter 4 were quite broadly shared between compartments. Because of the differing microenvironments in peripheral blood and cervix, it is important to understand the differences in immune pressures imposed on HIV in these compartments. Since we do not fully understand what would constitute protective immunity against HIV, and immunity at the genital mucosa is likely to play an important role in preventing HIV acquisition, the incorporation of mucosal sampling at the female genital tract and understanding HIV-specific events at this site should be a priority during HIV vaccine trials.

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