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THE CHARACTERISTICS AND FUNCTIONAL NATURE OF T CELLS UPON HIV-1 INFECTION AND EXPOSURE

Nati Stephina Nyoka

A dissertation submitted to the faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa in fulfilment of the requirements for Masters Degree

2008

DECLARATION

I, Nati Stephina Nyoka, declare that this dissertation is my own unassisted work. It has been submitted for a Masters degree in the University of Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other university.

……..
Stephina Nyoka

…….day of ……., …….

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This project was supervised by Prof Clive Gray, head of the immunology section of the AIDS Unit-National Institute for Communicable Diseases. I would like to thank him for his guidance, motivation, encouragement and his whole support to completion of this dissertation.

Blood samples used for phenotyping of T cells were obtained from a mine setting in Welkom, facilitated by the Aurum Health Research Center. I would like to thank Dr Gavin Churchyard, Edwin Magcuntsu, Alison Grant, Richard Chaisson, Surita Roux and the nursing staff for their efforts in collection of these samples.

Cord blood samples for T cell receptor-spectratyping were collected from Coronation Women and Children's hospital in Johannesburg. I thank Gayle Sherman for co-ordination of sample collection and testing as well as Dr Zinhle Makatini and the nursing staff for sample collection. I am also thankful for the help I got from Dr Marcella Sarzotti-Kelsoe, Dan Ozaki, Scott Langdon and Min He for immnunoscope (spectratyping) training, as well as Dr Guido Ferrari for the use of their facilities at Duke University. I also thank Dr Janet Ottinger and Dr Mike Betts for offering training on intracellular cytokine staining.

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ABSTRACT

Cases of HIV-1 infection are growing in large numbers, and deaths as a result of AIDS are escalating in South Africa. Understanding cellular immune responses to HIV-1 by exploring general effects and changes occurring at a cellular level, including direct engagement of T cells with the virus during exposure or infection will provide information on possible correlates of viral control. This dissertation focuses on three characteristics of T cells during HIV infection, dual HIV/TB co-infection and exposure to HIV. The characteristics examined are 1) memory and activation status of CD4+ and CD8+ T cell subsets; 2) T cell receptor repertoire and 3) HIV-1– specific T cell responses.

There are two hypotheses in this dissertation. Firstly, that co-infection with TB leads to elevated T cell activation, disruption of the T cell receptor repertoire and altered patterns of immunodominance in HIV-1 subtype C-specific T cell responses in infected adults. Secondly, that T cell priming occurs in utero in HIV uninfected babies born to HIV infected mothers.

Four cohorts were examined in this dissertation. Three were recruited from a clinic around the Welkom area and analysed in a cross-sectional and longitudinal manner. The cohorts consisted of HIV-1 infected adults, individuals dually infected with HIV and TB and healthy controls. Whole blood samples from the HIV and HIV/TB infected groups were analysed at baseline (before TB chemotherapy), at 2, 8 and 24 weeks. A

further cohort consisted of babies born to HIV-1 infected mothers, with some being followed up at three months after birth.

This dissertation consisted of five different methods: 1) the use of four colour flow cytometry to measure the frequency of naïve T cells (CD45RA+/CD62L+), memory (CD45RA-CD62L-) and activated (CD38) T cell populations in individuals singly infected with TB and dually with HIV and TB. This investigation was aimed at obtaining the overall representation of T cells involved in HIV-1 and TB co-infection. 2) the use of flow cytometry staining with monoclonal antibodies recognizing different T cell receptor (TCR) Vβ specificities for quantitation of the percentage of particular TCR families in pools of T cells. The aim was to provide an indication of TCR usage in different disease states. 3) the immunoscope assay was used to measure the different CDR3 lengths of the TCR and assess Vβ family repertoires in newborn babies. The aim was to show evidence of T cell maturity at birth and whether there was TCR engagement in utero by analysing cord blood cells. 4) the use of the IFN-γ ELISPOT to measure HIV-specific T cell responses in a cohort of HIV and HIV/TB co-infected individuals. The aim was to identify targeted immunodominant regions and to determine whether TB infection resulted in differing patterns of HIV-1 specific T cell immunity. 5) intracellular cytokine staining (ICS) was used to confirm the responses obtained after initial screening with the IFN-γ ELISPOT and was used to delineate CD4+ and CD8+ T cell responsiveness.

Whole blood was stained with an array of monoclonal antibodies to measure various T cell subsets in HIV-1 and HIV/TB co-infected adults. The CD4+ T cells in HIV-1 infected individuals ranged between 245 - 436, those of HIV/TB co-infected patients were 157-840 and the TB group had CD4+ absolute counts ranging between 583-1757 cells/mm^3. CD4+ T cells were reduced as a result of HIV-1 infection and HIV/TB co-infection, and no loss of these cells was seen as a result of single infection with TB. There was a loss of naïve T cells, with increased memory phenotypes in the presence of TB and HIV single infection, which was more pronounced in the presence of HIV and TB co-infection. The loss of naïve CD4+ and CD8+ T cells was associated with a high HIV-1 plasma RNA load in patients co-infected with HIV and TB. CD8+ T cells in HIV singly infected and HIV/TB co-infected individuals were highly activated when compared to those infected with TB only, which was likely due to the high HIV plasma RNA load. The standard course of six months of TB therapy in HIV/TB co-infected adults did not lead to recovery of absolute CD4 cells, nor did it stem the loss of naïve CD4+ and CD8+ T cells, which remained in a highly activated state: possibly due to unchanged HIV-1 RNA loads.

The fine specific nature of T cell activation was investigated by examining TCR Vβ expansions in HIV and TB single and co-infected individuals. Whole blood was stained with CD3+, CD4+, CD8+, CD38+ and an array of Vβ-specific antibodies. Polyclonal skewing of the TCR Vβ repertoire, showing expansion of various Vβ–CD4+ and -CD8+ families was observed in TB and HIV-1 single and dual infection. A more restricted usage of the T cell repertoire was observed in both HIV and HIV/TB co-infected patients, where major and oligoclonal expansions of Vβ11, Vβ16, Vβ20 and Vβ22 were observed.

No major expansions were observed in TB single infection. Overall, significantly greater use of TCR Vβ families were found in the CD8+ T cell compartment rather than by CD4+ T cells in both HIV-1 and HIV/TB co-infected adults.

A cohort of neonates born to HIV-infected mothers was used to assess TCR usage using immunoscope analysis to support the hypothesis that the TCR repertoire skewing in cord blood cells can be a marker of T cell priming *in-utero.* The repertoire measured in HIV uninfected neonates born to HIV-1 infected mothers displayed a polyclonal skewing of various TCR families and oligoclonal distribution of Vβ5, Vβ6a, Vβ7, Vβ18, and Vβ23 families as compared to the Gaussian distributions seen in healthy controls. This study readily detected perturbations in the TCR repertoire in presumed HIV exposed babies and that newborns possess an intact TCR repertoire.

Measurement of HIV-1-specific CD8+ T cells was made to identify which regions of the expressed HIV genome were immunodominant and what impact of co-infection with TB may have. PBMC samples were thawed and cultured in vitro using CD3+/CD4+ bi-specific antibody to preferentially expand CD8+ T cells and measure IFN-γ producing cells in the ELISPOT and confirmed with the ICS. The ELISPOT results were interpreted in SFU/million PBMC and as percentages of T cell subsets in ICS assays. HIV-1 subtype C-specific CD8+ responses were readily detected in both HIV-1 and HIV/TB co-infected patients, however, patterns of peptide targeting were different between the two groups. Gag was targeted by 85% of HIV-1 infected patients, whereas only 27% of HIV/TB co-infected patients targeted Gag. Pol was targeted by 73% in the HIV/TB group. Gag and

Nef responses observed in some (n =7) of the patients were confirmed using ICS. These data infer that TB co-infection may change patterns of targeting and in how CD8+ T cells recognize HIV antigens.

Collectively, this dissertation demonstrated the existence of highly activated CD8+ T cells, most probably driven by high HIV-1 plasma RNA loads; restricted TCR usage by CD8+ T cells, predominantly in individuals dually infected with HIV and TB; possible shifting of immunodominant HIV-specific CD8+ T cell responses as a result of co-infection with TB. Despite successful treatment of TB with chemotherapy, these immunological observations remained unchanged.

PRESENTATIONS

1. Skewing of the T cell receptor (TCR) repertoire in HIV-1 uninfected neonates born to HIV-1 infected mothers. **2nd South African AIDS conference (7-10 June 2005), a national conference held in Durban, South Africa.** Stephina Nyoka, Gayle Sherman, Marcella Sarzotti-Kelsoe, Dan Ozaki, Zinhle Makatini, Clive Gray.

2. Effect of Mycobacterium tuberculosis on HIV-specific cytotoxic T lymphocyte responses. **3rd South African AIDS conference (5-8 June 2007), a national conference held in Durban, South Africa.** Stephina Nyoka, Gavin Churchyard, Edwin Magcuntsu, Alison Grant, Richard Chaisson, Surita Roux, Clive Gray AIDS Unit Immunology-National Institute for Communicable Diseases, Aurum Health Research Centre.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
AZT	Azidothymidine
BCG	Bacillus Calmette-Guerin
BD	Becton Dickinson
BSA	Bovine serum albumin
BSMAB	Bispecific monoclonal antibody
CBMC	Cord blood mononuclear cells
CD	Cluster of differentiation
CDC	Centre for disease control
CDR3	Complementarity- determining region
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonuclec acid
ELISPOT	Enzyme linked immunospot
EBV	Epstein - Barr virus
EDTA	Ethylenediaminetetracetic acid
FACS	Fluorescent antibody cytometry systems
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
Gp120	Glycoprotein
HCl	Hydrochloric acid
HIV-1	Human Immunodeficiency virus type 1
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HLA-DR	Human leucocyte antigen

HSV	Herpes simple virus
ICS	Intracellular cytokine staining
IFN-γ	Interferon
IL-1β	Interleukin-1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-15	Interleukin 15
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
LiCl	Lithium Chloride
LTR	Long term region
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex II
mRNA	Messenger ribonucleic acid
MTCT	Mother to child transmission
NaCl	Sodium Chloride
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridin chlorophyll
PHA	Phytohaemagglutinin
RNA	Ribonucleic acid
RT	Reverse transcriptase
TB	Mycobacterium tuberculosis
SEB	Staphylococal enterotoxin B
SFU	Spot forming unit
SSC	Side scatter
TCR	T cell receptor
TNF-α	Tumor Necrosis Factor –alpha
UNAIDS	United Nations Programme on HIV/AIDS

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The human immunodeficiency virus (HIV) infection epidemic is growing in developing countries, such as those in Sub-Saharan Africa. Current statistics indicate that 5.5 million persons in South Africa are infected with HIV-1 (UNAIDS 2006, AIDS Epidemic Update). HIV infection initiates a slow progressive degeneration of the immune system. It infects cells bearing the CD4 antigen, along with co-receptors CCR5 and CXCR4 on their surface (Lusso, 2006; Rubbert *et al.,* 1998), mainly macrophages and CD4+ T helper cells (Shacklett *et al.,* 2003). CD4+ T helper cells play an important role in facilitating and enhancing the functions of other immune cells that protect the host from invading pathogens. The hallmark of HIV-1 infection is the gradual decline in the numbers of CD4+ T cells and as a result, the immune system becomes suppressed and allows the development of various opportunistic infections which contribute to disease progression and ultimately Acquired Immunodeficiency Syndrome (AIDS). One of the most common of these opportunistic infections in South Africa is Mycobacterium tuberculosis (TB) (Wilkinson *et a*l., 1997). Understanding the characteristics of T cells in people infected with HIV-1 and during TB infection may provide valuable information on HIV pathogenesis.

One of the most common forms of HIV-1 transmission in South Africa is through mother to child transmission (Brody *et al.,* 2003). This can occur through several routes: foetal exposure to maternal body fluids during gestation, delivery, and breastfeeding (Gordon *et al.,* 1992). It is generally acknowledged that the increase in susceptibility of infants and neonates to viral infections, including HIV-1, is related to the immaturity of their immune system (Krampera *et al*., 2000; Siegrist *et al* 2001) but the correlates of protective immunity to neonatal HIV-1 infection is poorly understood. Understanding the characteristics of T cells in neonates infected or exposed to HIV-1 may hopefully shed light on possible protective mechanisms.

Understanding cellular immune responses to HIV-1 by exploring general effects and changes occurring at a cellular level, including direct engagement of T cells with the virus during exposure or infection will provide information on possible correlates of viral control. This dissertation focuses on three characteristics of T cells during HIV infection, dual HIV/TB co-infection and exposure to HIV. The characteristics examined are 1) memory and activation status of CD4+ and CD8+ T cell subsets; 2) T cell receptor repertoire and 3) HIV-1 antigen – specific T cell responses.

Various cohorts of HIV-1 infected and HIV-1 exposed individuals have been included in this dissertation, including those individuals who are co-infected with mycobacterium tuberculosis (TB). Also included are HIV-1 uninfected children born to HIV-1 infected mothers, aiming at determining the level of antigen exposure.

1.2 LITERATURE REVIEW

1.2.1 HUMAN IMMUNODEFICIENCY VIRUS

Human immunodeficiency virus (HIV) is a retrovirus belonging to the family of Lentiviruses, which was initially described in 1983 (Montagneur, 2002). Infection with HIV shows a chronic course of disease, persistent viral replication in monocytes/macrophages (Zhu *et al*., 2002) and gut-associated lymphoid tissue (Guadalupe *et al*., 2003). HIV is the causative agent of Acquired Immunodeficiency Syndrome (AIDS), a disease associated with severe immunosuppression as a result of infection and loss of CD4+ bearing T lymphocytes (Guadalupe *et al*., 2003).

1.2.1.1 Virus composition and structure

HIV is a genetically complex, 100nm RNA virus consisting of three structural genes; *env*, *pol*, *gag* and six regulatory genes, *tat, rev, vif, nef, vpr and vpu*. A schematic diagram of the structure of HIV is shown in Figure.1.1 and gene arrangement is shown in Figure 1.2. Each viral particle contains 72 glycoprotein complexes, which are integrated into the lipoprotein membrane surrounding it. The external glycoprotein gp120 may be easily shed into the external environment within the host, and can therefore be detected in serum, as well as within the lymphatic tissue in HIV infected individuals. The transactivator gene (*Tat*) stimulates the transcription of HIV-1 DNA into RNA, promote RNA elongation, enhance the transportation of HIV RNA from the nucleus to the cytoplasm and it is also essential for translation. The virion protein expression (*rev*) is a gene that positively regulates the expression of virion proteins. *Nef* has been shown to cause down-regulation of CD4 and HLA class I (A and B alleles) molecules from the

surface of infected cells, therefore facilitating viral escape from immune T cell attack (Aiken *et al.,* 1996), as well as interfering with T cell activation and function by binding to proteins that are involved in intracellular signal transduction pathways. The potential importance of *nef* in disease progression has been shown where deletions of portions of the *nef* gene have been associated with slow disease progression and low viral replication (Collins *et al*., 1999). *Vpr* is essential for viral replication in non-dividing cells such as macrophages. It stimulates the HIV-LTR (long-term region) and it is also important for transport of the pro viral DNA to the host cell nucleus.

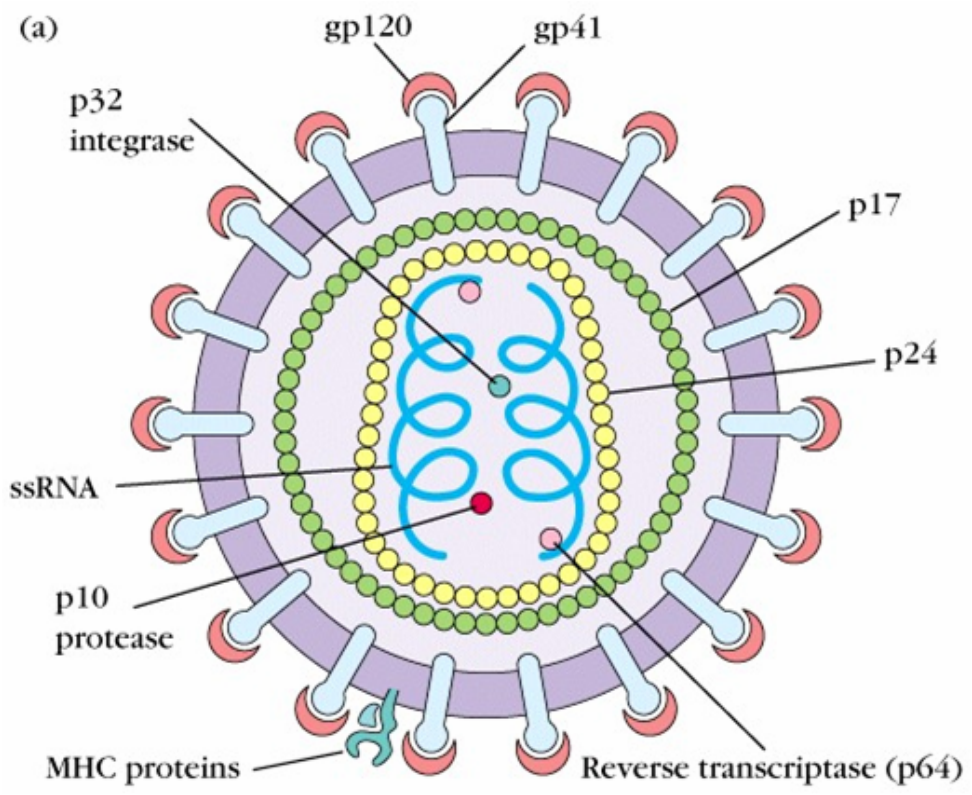


Figure 1.1 Structure of an HIV virion particle (D.W Sears, 2007. http://tutor.lscf.ucsb.edu)

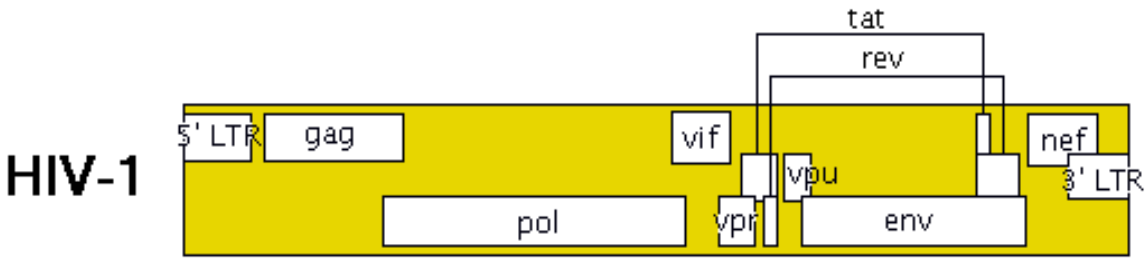


Figure 1.2: Schematic diagram of the genes of HIV-1, showing the three structural genes (*gag, pol, and env*) and the regulatory gene (*nef, tat, rev, vpu,vpr and vif* (Dan Stowell, 2006. http://www.mcld.co.uk/hiv).

The *vif* protein enhances viral infectivity and also enhances cell to cell transmission of HIV-1 (Gabudza *et al*., 1992). *Vpu* is essential for the budding process, because mutations in *vpu* have been shown to dis-able release of viral particles from infected cells (Schubert *et al.,* 1996).

1.2.1.2 Portal of entry and replication

HIV enters target cells (e.g. CD4+ T lymphocytes and monocytes/macrophages, dendritic cells and glial cells) by attaching its gp120 molecule (the envelope glycoprotein) to the CD4+ molecule on the surface of these cells (Randall, 1999; Rubbert *et al*., 1998; Blanco *et al.,* 2002). This attachment requires interaction of gp120 with two host cell receptors, the CD4 molecule and a chemokine receptor, either CCR5 (R5 using) or CXCR4 (R4 using). Gp120 primarily binds to certain epitopes of CD4 and induces conformational changes in gp120 that promote a more efficient interaction of the V3 loop of gp120 with

its respective co-receptor. Binding to the co-receptor in turn triggers a rearrangement of gp41 to extend the α-helical fusion domain, which then interact with the cell membrane. Penetration of the viral particle into the host cell is then followed by uncoating, i.e. when the viral core enters the cytoplasm of the target cell. Viral RNA is then converted to proviral DNA in the cytoplasm of the target cell. This conversion is mediated by the viral enzyme reverse transcriptase (RT) and it is the most important step in viral replication. RT inhibitors are currently widely used to inhibit viral replication (nucleoside and non-nucleoside analogues) and are utilized for effective treatment in HIV-1 infected individuals. The provirus then migrates to the nucleus and integrates into the host chromosome with the help of the viral enzyme, integrase. The pro-viral DNA remains in the cell for as long as the cell survives, and is the continuous source of new viral progeny. The RNA transcript is formed from pro-viral DNA, and it gets processed to yield a complex pattern of subfragments of the initial transcript that serve as messenger RNAs for generation of viral proteins. Viral proteins are then assembled and packaged into a mature virus particle that buds from the cell as a free virion released into the extracellular space (Figure 1.3).

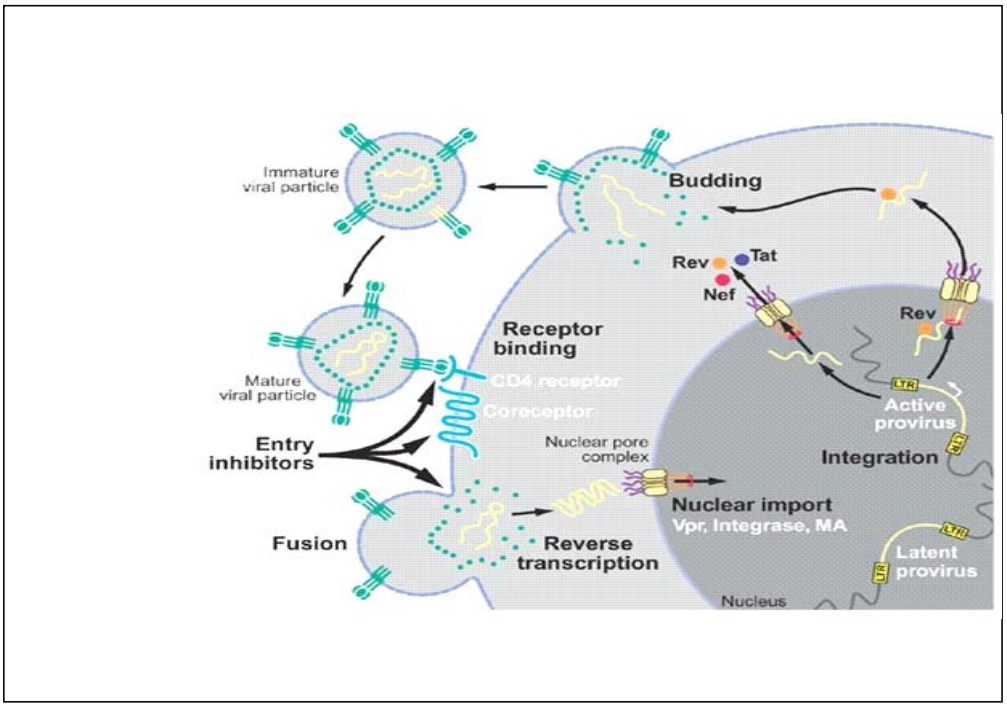


Figure 1.3: The life cycle of HIV virus showing viral entry and replication. (http://www.gladstone.ucsf.edu/gladstone/files/publicaffairs/HIVlifeCycle.gif).

1.2.1.3 Mechanisms of HIV transmission

HIV is transmitted when infected blood, semen, vaginal fluids, or breastmilk enter another person's body. HIV can be transmitted by any infected person or injection of infected blood, or from mother to infant during pregnancy or at the time of birth. Viral transmission can occur in a number of ways (Douglas *et al.,* 1992; Puren *et al.,* 2002; Schmid *et al* 2004).

1.2.1.3.1 Sexual intercourse

The virus can enter the body during sexual intercourse through the mucosal membranes of the anus, vagina, penis, or mouth, as well as through cuts, sores and abrasions on the

skin. Oral sex is risky as well, but not as risky as vaginal and anal intercourse. Infection takes place when the virus crosses the mucosal surface and binds to cells that express CD4 and CCR5 and/or CXCR4. The risks of acquiring infection are increased by the presence of sexually transmitted diseases (Korenromp *et al*., 2005) and lowered by male circumcision (Auvert *et al*., 2001; Auvert *et al*., 2005). Plasma levels of virus broadly correspond to viral loads in the genital secretions, which are associated with the risk of transmission (Hart *et al*., 1999). Heterosexual transmission is the most common route of HIV-1 acquisition in South Africa (Schmid *et al.,* 2004; Hunter *et al*., 1993; Abdool Karim *et al*., 1992).

1.2.1.3.2 Occupational Exposure and infection

Health care workers are at risk of being infected in the case of a needle stick injury. From a study conducted in Durban, South Africa, thirteen percent of hospital staff reported injuries with HIV positive patients (Gouden *et al.*, 2000). Trainee registrars were the highest risk group (60%), with 94% percutaneous and 65% occurred during emergency surgery injuries (Gouden *et al.,* 2000). Needle stick injuries and eye splashing of infected material can also be a source, especially in laboratories where high concentrations of virus is being used for research experiments. Healthcare workers mostly prone to HIV infection are nurses, laboratory technicians, surgeons, housekeepers, morgue technicians, and non-nursing attendants. Based on multicenter trials of more than 3000 healthcare workers, the risk of HIV infection is very high after needle stick injury or parenteral injury, which results in direct inoculation of infected material (Gouden *et al*., 2000). Meanwhile, the risk is very low following mucous membrane exposure (Kandla *et al.,*

1997). Other factors, which affect the risk of HIV infection in health care workers, include the volume of inoculum, the quantity of virus, depth of penetration, type and size of needle, and actual infection of blood (Gouden *et al*., 2000; Veeken *et al*., 1991)

1.2.1.3.3 Blood transfusion

Extensive testing of the blood by donor banks is performed before the blood can be distributed for donation. However, there is still a risk of HIV-infected donated blood because of donors who are in the 'window period' during acute infection (Sitas *et al.,* 1994). The increased use of Nucleic Acid Testing (NAT) (303) (Scuracchio *et al*., 2007) would minimize the use of HIV infected donor blood, as this would detect early infection during acutely infected potential donors. There have also been new policies put in place in South Africa to reduce the prevalence of HIV in blood donors, which included closing donor clinics in areas where HIV prevalence is high (Heyns *et al*., 2006). Following new policies, prevalence of HIV-1 in blood donations declined from 0.17% in 2000 to 0.08% in 2002. The prevalence of HIV in first time donors decreased by 45% (Heyns *et al*., 2006).

1.2.1.3.4 Injection drug use

Small amounts of blood can remain in needles and syringes, which poses a high risk of transmission when the same tools are used without being sterilized. This happens predominantly among drug users. Intravenous abuse of Wellconal was reported in a study conducted in Johannesburg from 86 patients who were admitted to Johannesburg hospital

(Williams *et al*., 1997). Studies on treatment demand pointed to substantial use of heroine, although most of the users tend to smoke rather than inject (Parry *et al.*, 2005).

1.2.1.4 Mother to child transmission (MTCT)

MTCT is referred to as vertical transmission, when the mother is infected with HIV and then transmits the virus to the unborn baby *in-utero* or to the newborn during birth and also through breastfeeding. Intrauterine infection and infection during delivery has been extensively reduced by the use of AZT or single dose Nevirapine during the last trimester of pregnancy or to the baby immediately after birth (Coetzee *et al.,* 2005; Sherman *et al.,* 2004). One of the most common routes of perinatal transmission is via breast milk and the issues around whether mothers should breastfeed or not is currently controversial (Doherty *et al.,* 2006, Chopra *et al*., 2002). Maternal viral load has a direct effect on all three modes of transmission (Thea *et al*., 2006, Rigopoulos *et al.,* 2007), where the higher the viral load, the more likely is transmission (Hayes *et al*., 2006). Caesarean section has been shown to reduce perinatal transmission by more than 50% (European collaborative study, 2005).

1.2.1.4.1 Infection at fertilization and post fertilization stages

It has been shown that free virus or HIV infected leukocytes present in the ejaculate may reach the site of fertilization in the upper oviduct. The embryo may also be susceptible to infection as a result of the presence of virus in the female genital tract (Spinillo *et al*., 2006). Virus has been isolated from the vaginal and cervical fluid and from the cells of the cervix (Maher *et al*., 2005). There is also evidence of a leucocytic infiltration of the

cervix and the uterus following insemination. If CD4-bearing cells are infected, the chances of intrauterine infection are increased. Infection of the zygote is also likely to happen through the motile sperm, carrying free virus from the vaginal and cervical secretions, which has been shown in vitro by expression of HIV proteins from human egg infected at the time of fertilization (Kiessling, 1998; Whitmer *et al*., 1992).

1.2.1.4.2 Infection at implantation

Trophoblasts (cells forming the placental barrier) invade maternal endometrial capillaries within a few days of implantation (Lunghi *et al.,* 2007). The invading trophoblasts encounter infected cells during this time and therefore, contact of the trophoblasts with maternal blood poses hazards of HIV infection since placental trophoblasts will be bathed in maternal blood (Vidricaire *et al.,* 2004; Al-Harthi *et al*., 2002). Direct infection of trophoblasts may cause transmission of the virus to the foetus. Infection was found to be independent of gp120/CD4 interaction but requires heparin sulfate proteoglycans for uptake of the virus (Vidricaire *et al*., 2007).

1.2.1.4.3 Transmission across placental villi and the chorioamnion

Most maternal-foetal exchanges are mediated by the placenta. The placental syncyciotrophoblast plays a major role in these interactions since it is in direct contact with maternal blood (Menu *et al.,* 1999). Some studies have suggested that monocytes/macrophages and epithelial cells lining the foetal capillary may function as a reservoir for the HIV (Maher *et al*., 2005). DC–SIGN may facilitate the transplacental

transmission of HIV, where DC-SIGN is a C-type lectin that is able to bind gp120 with high affinity and has been found to be expressed in the placenta (Soilleux *et al*., 2001).

Another potential route is through the chorioamnion (Menu *et al*., 1999), where the cellular trophoblast and the maternal decidua are joined together. This happens in late gestation. The deciduas contain maternal blood vessels, macrophages, lymphocytes and thus may serve to expose the adjacent trophoblast to HIV. From here the virus or infected cells may cross the foetal connective tissue and amnion and enter the amniotic fluid. It has been demonstrated that women showing positive granulocyte elastase at delivery should be suspected of having had chorioamnionitis during pregnancy, which increases the risk of intrauterine transmission of HIV (Kaseba-Sata *et al.,* 2006; Bhoopat *et al.,* 2005).

1.2.1.4.4 Infection during delivery and postpartum

There are reported risks of transmission even if the baby escapes intra-uterine HIV infection. HIV has been isolated from vaginal and cervical secretions from about one-half of infected women studied (Nunnari *et al.,* 2005; Saracino *et al*., 2000), making it possible that the foetus could be infected. Distinguishing intrapartum and postpartum infection has been difficult to prove. Current methods involve performing a PCR at birth and from specimens obtained within 72 hours of birth to indicate in-utero infection (Mock *et al.,* 1999; Fawzi *et al.,* 2001; Sherman *et al.,* 2005).

1.2.1.4.5 Infection through breastfeeding

Breastfeeding is a potential mode of vertical transmission, especially in those countries where HIV prevalence is high. HIV-1 transmission via breast milk ranges from 8%-16% (Nduati *et al*., 200; Wiktor *et al*., 1999). There is no clear evidence of the timing of transmission during breastfeeding. Studies have demonstrated that the number of infected breast milk cells per million cells was associated with levels of cell-free viral RNA in breast milk (Koulinska *et al.,* 2006; Rousseau *et al.,* 2004). This suggests that infected breast milk cells may play a role in transmission of HIV via breast-feeding than cell free virus.

1.2.2 NEONATAL T CELL IMMUNITY AT BIRTH

Neonatal immune responses are generally considered to be immature and therefore lead to susceptibility of infants to both viral and bacterial pathogens. Neonates depend on maternal antibodies since neonatal humoral immunity is entirely underdeveloped. However, it has been determined that the foetal immune system can be activated to produce antigen specific responses as shown during parasitic infections with Trypanosoma cruzi (Hermann *et al*., 2002) and human cytomegalovirus infection (Marchant *et al*., 2003). Clonal T cell expansions have been previously identified in peripheral blood of HIV-1 infected children (McFarland *et al*., 2002), which further supports the existence of a matured T cell immunity during infancy. It has been demonstrated that human foetal CD8+ T lymphocytes can expand, differentiate, and acquire effector functions during a CMV infection and that this response shares similar characteristics to that found in adults (Marchant *et al*., 2003*)*. It has therefore been

concluded that human CD8+ T cells can be primed in utero and could provide immunity to newborns against viral infections. A better understanding of the immune system in early life is required to develop vaccines that could protect infants from viral infections.

1.2.3 THE EPIDEMIOLOGY OF HIV-1 INFECTION IN SOUTH AFRICA

Worldwide, the number of HIV-1 infected individuals exceeds 40 million. The majority of these live in developing countries of Asia, South America and Sub-Saharan Africa. The profile of the epidemic to date at a national level, as produced by UNAIDS stated that the total number of people living with HIV in South Africa was estimated to be 5.5 million. It is estimated that 240 000 children between the age of 0-14 are infected with HIV-1 and 320 000 AIDS deaths have been reported (UNAIDS update, 2006).

There were around 530,000 new infections between the middle of 2004 and the middle of 2005, and around 340,000 AIDS deaths over the same period (Groenewald *et al*., 2005). The HIV prevalence is still growing, and this has resulted in an estimated 520,000 untreated South Africans who are sick with AIDS and in need of antiretroviral treatment. It is also estimated that 1.5 million South Africans have died of AIDS-related illnesses since the beginning of the epidemic (Groenewald *et al*., 2005). The ASSA2003 (Actuarial Society of South African AIDS and Demographic model) predicts that the total number of HIV infections in South Africa will increase slightly, from 5.2 to 5.8 million by 2010 (Groenewald *et al*., 2005). It is predicted that the annual number of new HIV infections is likely to remain at approximately half a million over the next few years, despite the significant interventions that have already been introduced to limit the spread of HIV.

Amongst other interventions is the implementation of an Operational Plan for Comprehensive HIV and AIDS Care, Management and Treatment. By the end of December 2005, 111 827 people were estimated to be accessing free antiretroviral drugs. Significant progress has also been made in prevention through campaigns such as the Government Mass Media Campaign (Khomanani-http://www.aidsinfo.co.za/) Soul City (http://www.soulcity.org.za/); Love life (http://www.lovelife.org.za/) and others. The Government is also continuously distributing free condoms (UNAIDS update, 2006). According to the South African National HIV Prevalence communication Survey-2005, the highest rate was found to be among South African females at 33.3% (between 25-29 years old) followed by males at 26% (between 30 and 34 years old) (Shisana *et al.*, 2005). There appears to be a high HIV prevalence among South African children which are estimated at 129,621 children aged 2-4 years and 214,102 children aged 5-9 (Shisana *et al*., 2005). The most predominant HIV-1 subtype in South Africa is Clade-C, with only a few of A, B, D, G, and U (Bredell *et al*., 1998; Puren, 2002). These other subtypes represent a very small fraction of the predominant circulating subtypes.

1.2.4 INTERACTION BETWEEN HIV-1 AND MYCOBATERIUM TUBERCULOSIS

Globally, Mycobacterium tuberculosis (TB) is one of the common HIV-associated opportunistic infections and the leading causes of AIDS related deaths. An estimated 1.7 million people (27/100) died from TB in 2004, including those co-infected with HIV (248 000) (WHO report, 2005). The incidence of TB in South Africa is currently 718 cases/100 000 annually, with the prevalence rate of 670 cases per 100 000 population. TB

mortality has been reported at 135 per 100 000 population per annum. 60% of these cases are HIV infected (WHO report, 2005), which are adults between the ages of 15-49 years. Approximately 10% of infected individuals develop active disease, which if left untreated, will kill more than 50% of its victims (Bonfioli *et al*., 2005).

1.2.4.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis is a human-type aerobic acid fast tubercle bacillus, which is classified as a Gram-positive (Fisher *et al*., 1990). The bacterium is an intracellular, growing in mononuclear phagocytes e.g. macrophages (Zhang *et al.,* 1999). It is a slow-growing pathogen with the generation time of 12 to 18 hours. It has a hydrophobic cell wall with a high lipid content, which makes it impermeable to the usual gram stains. Once stained, the cells resist decolorization with acidified organic solvents and are therefore called "acid fast". One commonly used acid-fast staining method for TB is the Ziehl-Neelson stain (Prasanthi *et al*., 2005). The TB smear is fixed, stained with carbol fuchsin (pink dye), and decolorized with acid-alcohol. The smear is then counterstained with methylene blue or certain other dyes. Acid-fast bacilli appear pink in a contrasting background (see Figure.1.4). Laboratory diagnosis of tuberculosis is made by a positive tuberculin skin test, which can be confirmed by X rays of the chest and microscopic examination of the sputum or tissue samples using Ziehl Neelsen stain. This may however, be confirmed by laboratory culture of the bacterium (Toure *et al*., 2006).



Figure 1.4: Mycobacterium tuberculosis appearing as pink rods/bacilli (pointed by the arrow) on a Ziehl Neelsen stain (http://www.search.com/reference/Tuberculosis).

1.2.4.2 Host response to tuberculosis

TB infection commences with the acquisition of bacilli by the susceptible person via infectious droplets from an infectious case (Fennely *et al*., 2004). These droplet nuclei are transmitted from one individual to another via coughing, sneezing, talking and singing. Following inhalation, most of the larger droplets become lodged in the upper respiratory tract (the nose and throat), where infection is unlikely to develop. The smaller droplets pass through to the small air sacs of the lung (the alveoli) where infection begins after 7-21 days post exposure (Johnson *et al.*, 2006). Transmission can only occur from people with active TB disease (see 1.2.3.4). Individuals at risk include immunocompromised patients such as those with HIV/AIDS (Cahn *et al*., 2003), health care workers who serve

high-risk clients (Corbett *et al*., 2007; Naidoo *et al*., 2006), and children exposed to adults in high-risk categories (Soeters *et al*., 2005).

In the alveoli, the mycobacteria are taken up by macrophages and dendritic cells, to the lung parenchyma and eventually to the lymph nodes (Johnson *et al*., 2006; Abdel-Dayem *et al.,* 1997). At this stage lymphocytes begin to infiltrate as they are presented with microbial antigens. TB-antigen specific T cells induce the formation of a granuloma around infected macrophages primarily composed of monocyte-derived macrophages, $CD4^+$ T cells and an outer ring of $CD8^+$ T cells (Tsai *et al*., 2006). In the granulomatous lesion, macrophages are activated by T lymphocytes through production of type 1 cytokines (IFN-γ and TNF-α) (Roach *et al*., 2002). IFN-γ activates macrophages which are now capable of destroying the microbe. It is at this stage that the individual becomes tuberculin-positive, which is a result of the host developing a vigorous cell-mediated immune response. It is also at this stage that the tubercle is formed, which does not allow the microbe to multiply but to persist for extended periods. Many macrophages can be found surrounding this tubercle, but remain in an inactive form, and used by the microbes to replicate. The granuloma persists for years and efficiently contains tubercle bacilli as long as an individual remains immunocompetent (Chan *et al*., 2004).

1.2.4.3 Mycobacterium tuberculosis disease progression

It is thought that progression from TB exposure and infection to TB disease occurs in individuals whose immune system fails to control bacilli and they begin to multiply. This may occur soon after infection (primary TB disease in 1-5% of cases) or many years after infection (post primary TB, secondary TB, reactivation of dormant bacilli in 5-9% of

cases). About 5% of infected persons will develop TB disease in the first two years, and another 5% will develop the disease later in life (McShane *et al.*, 2005). The risk of reactivation increases with immune suppression, especially that caused by HIV (Milburn *et al*., 2001). Apart from HIV infection, there are other conditions that increase the risk of progression to TB disease, which includes silicosis, end stage renal disease, poorly controlled diabetes, chronic malnutrition and those who smoke tobacco (Johnson *et al*., 2006; Ariyothai *et al*., 2004).

TB disease most commonly affects the lungs, where it is referred to as pulmonary TB. Extrapulmonary sites include the pleura, central nervous system (meningitis), lymphatic system, genitourinary system and bones and joints, and the hematogenous spread known as miliary tuberculosis (Farid *et al*., 1999; Maltezou *et al*., 2000).

1.2.4.4 Tuberculosis treatment

TB is currently treated by means of combination therapy, using cocktails of 3-4 drugs with different properties: Isoniazid, rifampicin, streptomycin which is a combination that has antibacterial activity; as well as isoniazid, rifampicin, ethambutol which is a combination used to inhibit the development of resistance. Vaccination against TB is routinely practiced in many countries as a prevention strategy. The Bacillus Calmette-Guerin (BCG) vaccine is a live, attenuated strain of mycobacterium bovis which was introduced in 1922. However, the true efficacy of BCG is unknown. Some studies suggest 60-80% effective rate in children. BCG is still used in South Africa, however the use of BCG in the United States of America (USA) has been stopped because its

effectiveness in preventing infectious forms of TB is uncertain and the reactivity to tuberculin that occurs after vaccination interferes with management of persons who are possibly infected with TB. The primary strategy in the USA is to minimize the risk of transmission by the early identification of persons who have latent TB infection and the use of Isoniazid or Rifampicin for preventing progression of latent TB to active TB disease (CDC-MMWR, 1996).

1.2.4.5 HIV/TB co-infection

Immunosupression, resulting from HIV infection hinders the ability of macrophages to clear TB infection, therefore enabling the bacilli to spread easily throughout the body. The risk of progression to TB disease is between 5 to 10% per year for co-infected patients, compared to a 7-10% risk for the rest of their life for patients with TB only. In areas like Sub-Saharan Africa where TB is endemic, many individuals harbor latent TB infection and reactivation occurs consequently due to the immunosuppressive effect of HIV infection. In this regard, it has been widely noted that TB is a high grade pathogen that is the most common cause of death in HIV infected patients (Cahn *et al*., 2003). As a result, HIV is likely to have significant implications for the management and control of TB infection.

HIV-1 infected patients with tuberculosis were reported to have lower CD4+ and CD8+ T lymphocyte counts than patients with single TB or HIV infection, respectively (Villacian *et al*., 2005; Rodrigues *et al*., 2006). These findings are an indication of disease progression in HIV infected individuals presenting with an opportunistic infection. High

activation of CD8+ T cells has been demonstrated in patients co-infected with HIV-1 and TB (Rodrigues *et al*., 2006). Virological and immunological impact of tuberculosis on HIV disease has been well documented. Among other studies, it was reported that TB provides a milieu of continuous cellular activation and irregularities in cytokine and chemokine circuits that are permissive of viral replication and expansion in vivo (Toosi, 2003). An increase in HIV plasma RNA load was observed soon after onset of TB (Goletti *et al*., 1996) with increased viraemia in bronchoalveolar lavage fluid. In HIV-1 infected persons with pleural tuberculosis, increased HIV-1 activity was demonstrable in both pleural-acellular and pleural mononuclear cells (Richter *et al*., 1994); increased HIV-1 systemic heterogeneity was found in dually infected patients. Distinct quasispecies were found to be more frequent in patients with both HIV-1 and TB, as opposed to HIV-1 infected patients without TB (Richter *et al*., 1994). The overall conclusion from these findings presents a clear indication that sites for active TB infection in subjects co-infected with HIV and TB play a critical role in HIV replication and evolution. As long as these sites harbor TB infection, they contribute to systemic viral activity. Additionally, TNF-α is produced and circulated in abundance in TB infected individuals (Toossi *et al*., 2001; Bal *et al*., 2005, Raja *et al.,* 2004) and has been shown that TNF-α enhances HIV replication in vitro (Kitaura *et al*., 2004).

1.2.5 HIV IMMUNOPATHOGENESIS

Infection with HIV initiates a slow progressive degeneration of the immune system, infecting predominantly cells bearing the CD4+ marker on their surface, mainly macrophages and CD4+ T helper lymphocytes (Klatzmann *et al*., 1984; Dalgleigh *et al*.,

1985). The hallmark of HIV infection is a gradual decline in the numbers of CD4+ T helper cells (Clerici *et al.*, 1997; Alimonti *et al*., 2003; Holm *et al*., 2005). Depletion of CD4+ T cells result in progressive suppression of the immune system which gives rise to introduction of various opportunistic infections that leads to AIDS. CD4+ lymphocyte depletion has been shown to be the result of a combination of specific virus-induced cell death, activation-induced loss of the memory (CCR5+CD45RO+) cell pool, occurring predominantly in the gut associated lymphoid tissues (Veazey et al 2000) and impaired renewal of the naïve (CD45RA+) cell pool (Lawn *et al.,* 2001).

1.2.5.1 CDC classification of HIV infection and disease stages

The CDC classification system for HIV infected adolescents and adults, categorizes persons on the basis of clinical conditions and CD4+ T lymphocyte counts (CDC-MMWR, 1992). The three CD4+ T lymphocyte categories are: 1) Greater than or equal to 500 cells/µl, 2) 200 – 499 cells/µl, 3) less than 200 cells/µl. The clinical categories/stages are: Stage A) Asymptomatic HIV infection B) Symptomatic conditions including candidiasis, Herpes zoster, e.t.c C) AIDS defining illness including Pneumocystis carinni pneumonia, cytomegalovirus disease, wasting syndrome e.t.c. CD4+ T cell counts are higher in infants and young children than in adults. The immunological status of children is therefore based on age-specific CD4+ levels. The three CDC-CD4+ T lymphocyte immunologic categories applied for children are: 1) No evidence of suppression with >= 1500 cells/ml for less than 12 months; >= 1000 cells/ml for 1-5 years; and >= 500 cells/ml for 6-12 months, 2) moderate suppression with 750-1499 cell/ml for < 12

months; 500-999 for 1-5 years; and 200-499 for 6-12 years. 3) Severe suppression with < 750 for < 12 months; <500 for 1-5 years; and < 200 for 6-12 years (CDC-MMWR, 1994).

1.2.5.2 Mechanisms of CD4+ T cell depletion

There are various considered mechanisms of CD4 depletion in HIV-1 infected individuals. It is unclear whether one mechanism predominates or that all operate. The possible reasons for CD4 depletion include:

- Direct cytopathocity: HIV virions are synthesized in an infected cell, which then buds out of the cell in such a way that they create punctures on the cell membrane resulting in cell death (Morita *et al*., 2004 ; Cadd *et al*., 1997).
- Syncytia formation: In in-vitro studies performed using established cell lines, the HIV-envelope protein (gp120) expressed on the surface of infected cells could fuse with the CD4+ molecule of uninfected cells and form large multinucleated cells or syncytia. Syncytia get large, are unwieldy, and ultimately die prematurely (LaBonte *et al*., 2000). Syncytia formation may be an in vitro effect as this has not been observed in vivo
- Apoptosis: CD4+ T lymphocytes have been shown to be killed by autologous HIV-infected cells, without syncytium formation. This cytolytic mechanism depends on gp120–CD4 binding, which triggers apoptotic death (Nardelli *et al.,* 1995; Heinekelein *et al*., 1995; Herbein *et al*., 1998).
- Autoimmunity: HIV–specific CD8+ cytotoxic T cells (CTL) become stimulated by HIV antigens to kill infected CD4+ cells, through secretion of granules

(perforin and granzyme) that mediates the killing process (Shankar *et al.*, 1999; Zarling *et al*, 1999; Liu *et al*., 2007).

1.2.5.3 CD4+ and CD8+ T cell function

CD4+ T helper cells provide critical help to B cells in the production of antibodies against HIV antigens, including stimulatory signals (e.g. IL-2) for B cell proliferation, differentiation into immunoglobulin–producing or memory B cells, and isotype switching from IgM to IgG (Barlett *et al*., 1990; Fogelman *et al.,* 2000). Studies done in mice demonstrated the role of CD4 help in the development of functional CD8 memory (Shedlock *et al*., 2003). Both these immune mechanisms play an important role in the control of viral replication (Xiang *et al*., 2005) cytotoxic T cells (CTL) which differentiates and mediates killing of infected CD4+ T cells via production of perforin and granzyme (Yanai *et al*., 2003; Pardo *et al.,* 2004), as well as cytokine (e.g. TNF- and IFN-γ) pathways (Harty *et al*., 2000) .

1.2.5.4 HIV RNA load

The plasma HIV load is a major indicator of HIV-1 prognosis associated with increased risk of death (Vlahov *et al*., 1998; Mellors *et al.,* 1997). It is influenced by co-existence of other chronic viral infections (e.g. CMV, EBV and HSV), (Grando *et al*., 2005); opportunistic infections such as P. carinii pneumonia, M. avium complex disease, Candida esophagitis, toxoplasmosis, cryptosporidiosis, and mycobacterium tuberculosis (Goletti *et al*., 1996; Cahn *et al*., 2003). A representative diagram of HIV/AIDS disease progression is shown on Figure 1.5. The dissemination of HIV during primary infection

is co-incident with reduction of plasma viral load and detection of strong cellular immunity. It is likely that the strong CTL response contributes to the initial fall in plasma viral load (Koup *et al.,* 1994; Borrow *et al.,* 1994). The deterioration in T cell immunity and the increase in viraemia is accompanied by a gradual depletion of CD4+ T helper cells and the onset of AIDS and increased susceptibility to opportunistic infections (Pantaleo *et al*., 1993). This may take a range of times, from within months to several years.

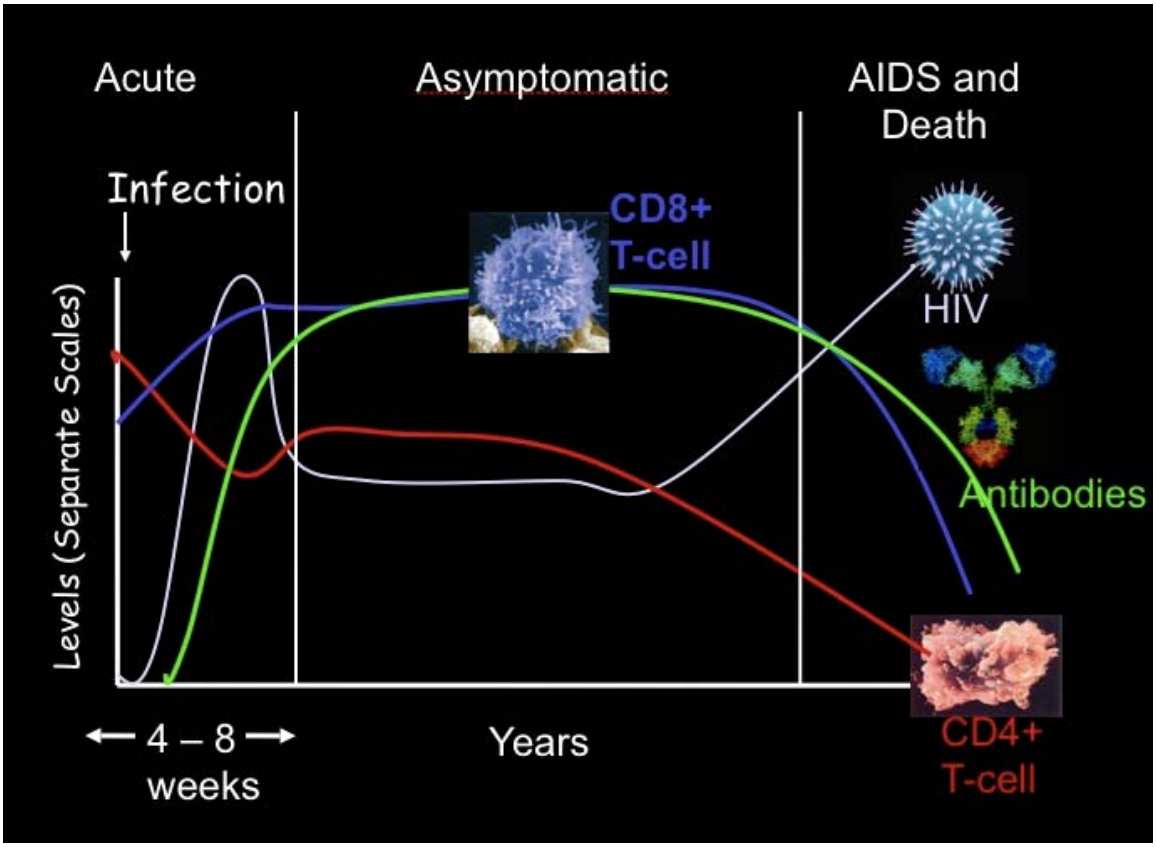


Figure 1.5: Representation of a typical HIV /AIDS disease progression in the absence of antiretroviral therapy, showing a gradual decline in CD4 count, a decline in viral load reaching setpoint and then increasing overtime thereafter. An increase in HIV CTL and neutralizing antibodies which declines gradually as the disease progresses to AIDS (www.studentreader.com, 23rd March 2007).

1.2.6 HIV-1 EXPOSURE AND INFECTION

There are several routes of exposure by HIV-1 which could either lead to HIV infection or no infection if precautionary measures are taken. The most common exposure happens either in an occupational setting, through drug use, sexually, as well as mother-to-child. Vertical/mother-to-child transmission has however, been dramatically reduced with the introduction of antiretroviral treatment and other precautionary measures such as avoidance of breastfeeding and opting for caesarean section (Coetzee *et al*., 2005; Sherman *et al*., 2004, European collaborative study, 2005).

There is a high possibility that infants born to HIV infected mothers may have been exposed to HIV proteins and particles in–utero. This has been shown through several studies as indicated by immune activation (Clerici *et al*., 2000; Kuhn et al., 2002), HIV–positive PCR (Peretz *et al*., 2006; De Andreis *et al*., 1996) and the presence of HIV-specific CD4+ and CD8+ T cell responses masured by flow cytometry (Legrand *et al*., 2006; Kuhn *et al*., 2002).

Impaired progenitor cell function has been observed in HIV-negative infants of HIV-positive mothers as evidenced by lower numbers of naïve CD4+ T cells and reduced thymic output (Nielson *et al*., 2001). HIV envelope peptide specific IL-2 responses associated with Beta-chemokine production were detectable at birth in the majority of uninfected infants of HIV-positive mothers (Wasik *et al*., 1999). It has been reported that children, even during the first year of life, are able to mount functional immune responses as indicated by the IFN-γ ELISPOT (Feeney *et al*, 2005; Legrand *et al.,* 2006). Major expansions of Vβ-restricted T cells have also been described in infants born to HIV-

positive mothers, which were similar to those observed in acutely infected adults. The expansions have been considered to be associated with HIV-specific cytotoxic T lymphocyte activity (Soudeyns *et al*., 2000; McCloskey *et al*., 2002).

1.2.7 T CELL RESPONSES DURING HIV INFECTION

Cellular immunity is made up of CD3+CD4+ T helper lymphocytes, CD3+CD8+ T cytotoxic lymphocytes, and natural killer cells (Steward *et al*., 1985; Parkin *et al*., 2001). The induction of cellular immune response to HIV-1 exposure and infection depends upon the presentation of viral antigens on the surface of infected cells or antigen presenting cells to the CD4+ or CD8+ T lymphocytes (Parkin *et al*., 2001).

Upon encounter and recognition of antigen, CD4+ T helper cells produce cytokines e.g. IL-2, IL-1, IL-6, IL-15, TNF-α, IFN-γ) which, amongst other functions facilitates proliferation and activation of CD8+ cytotoxic T lymphocytes. CD8+ T cells expand and differentiate into effector cells upon HIV infection (Fujiwara *et al.,* 2005; Jordan *et al*., 2006; Jassoy *et al*., 1993). CD8+ cytotoxic T lymphocytes have been shown to be predominantly involved in the partial containment of HIV replication (Benito *et al*., 2004; Musey *et al*., 1997). This has been shown in studies of long term non-progressors, who sustained HIV-specific activity and were able to control viremia (Propato *et al*., 2001; Greenough *et al*., 1999; Bailey et al 2006).

T cells recognize HIV proteins by interaction of the T cell receptor (TCR) with small linear HIV peptide fragments (known as epitopes) on the surface of virally infected cells and antigen presenting cells which may be macrophages and/or dendritic cells (Parkin *et*

al., 2001; Knights *et al*., 1991; Macatonia *et al*., 1992). There are two ways in which MHC molecules loading ofantigen onto MHC molecules occur. 1) During viral replication and protein synthesis in the infected cell, peptide fragments bind and fold in a specific host cell class I MHC molecules and are transported to the cell surface for presentation to CD8+ T cells. 2) For presentation to CD4 T cells, proteins are degraded to peptide fragments in intracellular endosomal compartments or exogenous antigen may be taken up by endocytosis and then loaded onto class II MHC proteins for subsequent presentation. The MHC class I pathway presents endogenous antigen and MHC class II pathway presents exogenous antigens. Figure 1.6 illustrates interaction of the TCR on CD4+ and CD8+ T cells and MHC class-1- and class II epitope complexes. The TCR on the surface of CD4+ T cells recognize HIV antigens presented through MHC class II complex and CD8+ T cells recognize antigens presented through MHC class I (Parkin *et al*., 2001).

MHC-class I molecules consist of two polypeptide chains, an α or heavy chain which has three domains (α1, α2, and α3) and a smaller, non covalently associated chain, the β_2-microglobulin which has one domain (Cossarizza, 1997). The α3 domain and that of the β_2-microglobulin have a folded structure, whereas the α1 and α2 domains pair to generate a long cleft, or groove that is the site at which an epitope binds to the MHC molecule. As discussed, antigens degraded in the cytoplasm, mainly those of endogenous origin, are bound to MHC class I molecules and presented to CD8+ T lymphocytes. MHC class II molecules are formed by two chains, α and β, which forms a structure very similar to that of class I. The two domains forming the peptide-binding cleft (α1 and β1) are given by

different chains. One important difference between MHC class I and II molecules is that the ends of the peptide-binding cleft are open in MHC class II and closed in MHC class I. As a result, the ends of an epitope bound to MHC class I are constrained, whereas the ends of epitopes bound to MHC class II hang over the open binding cleft. Antigens processed in acidified vesicles, mainly exogenous, are bound to class II molecules and are recognized by CD4+ T lymphocytes.

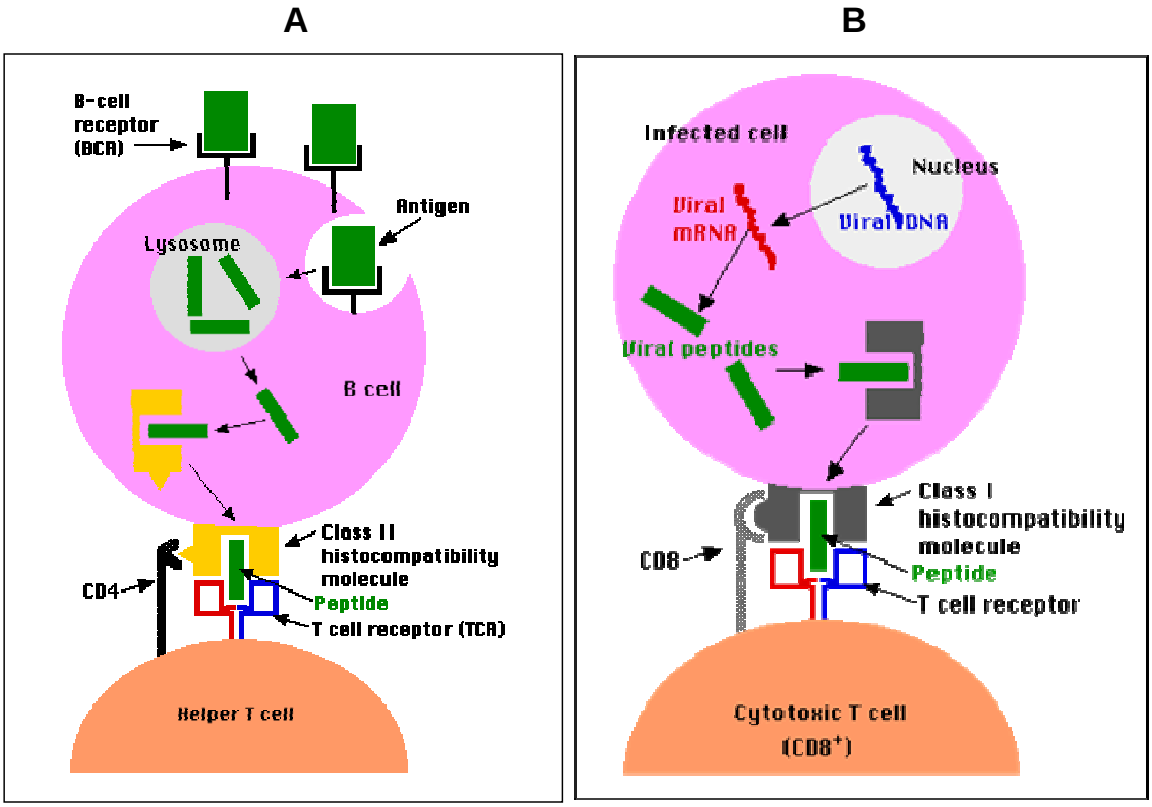


Figure 1.6: TCR and MHC interaction. Viral peptides are presented to the CD4+ T cell via MHC class II (A) and to the CD8+ cytotoxic T cell through MHC-class (B). (users.rcn.com/.../BiologyPages/H/HLA).

In both cases, epitopes are stably bound to MHC molecules so that the upper surface of the molecule is kinked in such a way that residues on the bound epitope are recognized

by the TCR. A complete recognition by the TCR is achieved through a composite of residues of the MHC molecule ($\alpha 1$ and $\alpha_{2)}$ and the epitope. CD8+ T cells increase in numbers in the peripheral blood after HIV infection, mirroring the decline in CD4+ T cells. A large proportion of CD8+ cytotoxic T cells (CTL) are HIV-specific, recognizing different epitopes across the HIV proteome. Many of these cells are activated to varying degrees and co-express markers that reflect activation events, such as CD38 and HLA-DR (Kestens *et al.,* 1994; Benito *et al*., 2004). CD8+ cytotoxic T lymphocytes directly kill HIV infected cells through secretion of cytoplasmic granules (perforin and granzyme A and B). Perforin is a monometric pore-forming protein containing serine esterase that may be involved in the assembly of the lytic complex. In the presence of calcium, the perforin monomers bind to the target cell membrane and polymerize to form transmembrane channels, leading to cell death and removal from the circulation (Yanai *et al*., 2003). Granzyme enters the cell through the pores that are created by perforin. Granzymes are a collection of serine esterases (enzymes) which interact with intracellular pathways in the target cell to activate mechanisms which trigger apoptosis and DNA degradation (Pardo *et al*., 2004).

Another mechanism used by CTL to kill infected cells is the Fas-FasL interaction. Ligation of Fas induces trimerization of the Fas molecule onto the target cell surface, associating with a transducing molecule which recruits and activates caspases 8 and 10, mediating cell killing by apoptosis (Pardo *et al*., 2004; Pinkoski *et al*., 2002).

1.2.8 THE SPECIFICITY OF T CELLS IN RECOGNIZING ANTIGENS

Specificity of T cells is generated during T cell formation in the thymus. Lymphocytes mature in the thymus and recognize cell surface presented MHC-I and MHC-II complexed-HIV epitopes by a specific clonotype T cell receptor (TCR). During intrathymic maturation, T cells undergo a series of events in a positive or negative manner, provoking their expansion and or deletion, respectively, and ultimately encounter a variety of antigens in the periphery resulting in clearance of infected cells. T cell maturation consists of three closely related processes (Pathak *et al*., 2005; Delves *et al*., 2000).

1.2.8.1 Migration and proliferation: Immature T cells (haematopoetic stem cell precursors) arise from the bone marrow, leave the bone marrow, circulate in the blood and enter the thymic cortex, where a high level of proliferation occurs. Selective processes result in death of most of the newly formed cells so that only MHC-restricted, self-tolerant cells survive. Surviving cells then migrate to the medulla and are finally discharged into the periphery (Pathak *et al*., 2005; Delves *et al*., 2000).

1.2.8.2 Differentiation*:* This is development of the mature phenotype of T cells. This involves formation of TCR complexes following formation of functional TCR genes by somatic rearrangement of different gene segments. Some of the accessory molecules acquired by T cells include CD4 and CD8 molecules. Functional differentiation occurs at this stage so that the potential of a thymocyte to become either a helper or a cytolytic T

lymphocyte is already developed before the cell enters the circulation (Pathak *et al*., 2005; Delves *et al*., 2000).

1.2.8.3 Selection: Two selection processes are applied so that T cells are modified or shaped for specificity in recognition of epitopes in association with the MHC molecule. These two processes are referred to as positive and negative selection. Positive selection is the process by which the T cell repertoire becomes self MHC-restricted. This ensures that only those cells expressing TCR capable of recognizing self MHC will be permitted to mature. Negative selection then eliminates potential autoreactive clones, and survival of T cells that do not recognize self antigens (Pathak *et al*., 2005; Delves *et al*., 2000). These two selection processes then result in the self-restricted, self antigen-tolerant mature T cell repertoire (Figure 1.7) as shown in adult mice studies.

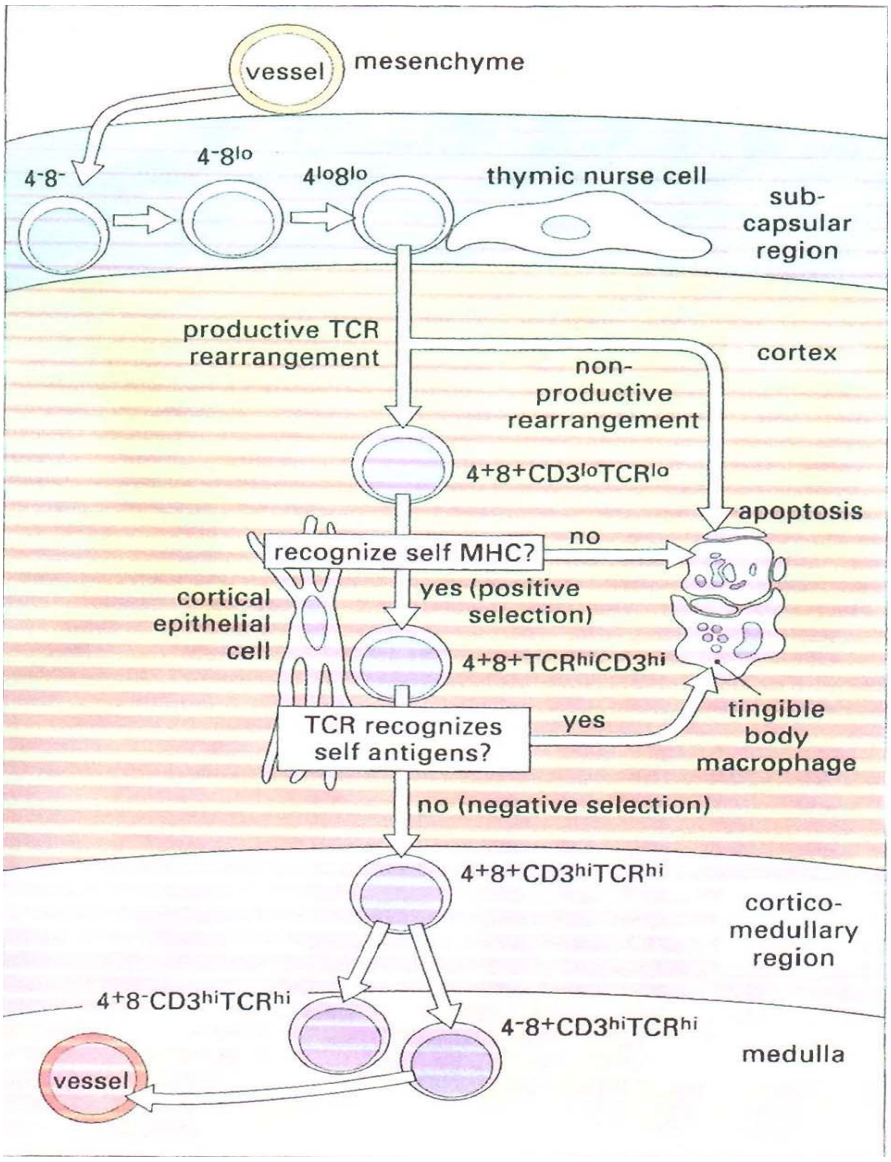


Figure 1.7: Positive and negative selection of thymocytes in the thymus. Immature thymocytes from the bone marrow are subjected to positive or negative selection, where T cells are tuned to recognize host MHC first. During positive selection Double-Positive T cells (CD3+CD8+ and CD3+CD4+) that can recognize self MHC's are selected for proliferation. Those T cells that have high affinity for self MHC die via apoptosis (negative selection). If they escape this elimination, they may subsequently react against self antigens, and cause autoimmune disease. The selection process and apoptosis occur in the thymic cortex and T cell maturity occurs in the thymic medulla. Matured T cells are then transported to the circulating blood (Roitt *et al.*, 2001).

1.2.9 THE TCR GENE: ORGANIZATION, REARRANGEMENT AND GENERATION OF DIVERSITY.

T cell differentiation in the thymus results in cells expressing randomly generated TCR molecules with diverse specificities. This process happens prior to the selection process. T cells of the T lymphocyte lineage possess functional TCR-α and β chain genes, which are capable of being expressed as polypeptides. The antigen-specific TCR on 90% of the T cells in peripheral blood is a di-sulphide-linked, heterodimeric, transmembrane glycoprotein composed of α and β chain (Cossarizza, 1997). Functional α and β genes are formed by DNA rearrangements that generate V-(D)-J genes which are then linked to a C-region segment by RNA splicing, following transcription. Diversity in the TCR is generated by random utilization of a large number of germline V, D, J segments and random insertion of P- and N- nucleotides junctions between the V, D, J segments of the β chain and the V and J gene segments of the α chains. The most variable loops of the TCR namely the CDR3 regions of the α and β chains lie centrally and have the most contact with the presented peptide side chains.

Three key structural features of CDR3 predominantly contribute to this interaction: the amino acid sequence of CDR3, which is critical for physical recognition of the epitope; CDR3 length, which influences how deep the loop can reach into the epitope-binding groove of the MHC molecule and represents the first structural feature to be fixed during maturation of antigen specific immune responses; and the identity of the J segment used in TCR rearrangement, as well as the germ-line J region contributes at least four polymorphic residues to the C-terminal portion of CDR3. A schematic diagram of the

TCR gene is shown on Figure 1.8. The combined sum of all the different TCRs and specificities in an individual forms a repertoire. Both α and β chains play a crucial role in shaping the peripheral T cell repertoire responding to different antigens. At least 57 V gene segments are used to form beta chains, and they can be grouped into 24 families of Vβ1, Vβ2, Vβ3.1, Vβ4, Vβ5.1, Vβ5.2/3, Vβ6a, Vβ6β, Vβ7, Vβ8, Vβ9, Vβ11, Vβ12.1, Vβ13.1/3, Vβ13.6, Vβ14, Vβ15, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21, Vβ22, Vβ23, based upon having 75% or greater sequence homology (Cossarizza 1997).

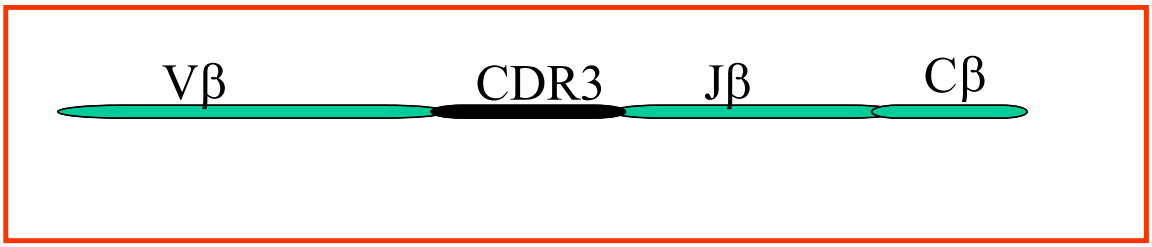


Figure 1.8: The Vβ TCR gene.

1.2.10 THE T CELL RESPONSE TO HIV AND CLONAL EXPANSION

Antigens are presented to naïve CD4+ or CD8+ T cells either by presentation with class II or class I MHC on dendritic cells. This results in immunoactivation and priming of T cells bearing specific T cell receptors (e.g. Vα and Vβ), as shown on Figure 1.9. Dendritic cells prime T cells and then on encounter with antigen on other antigen presenting cells, expansion of T cells occurs. The specific TCR interacts with the specific MHC plus epitope complex, and this determines which Vβ family is expanded. In the absence of infection, the TCR repertoire is fairly stable through time within individual subjects (Garderet *et al*., 1998; Even *et al*., 1995). An indication of TCR engagement

with antigen can be identified by investigation of the T cell repertoire. Perturbations in the repertoire have been previously observed as a result of T cell antigen encounter, i.e. during infection with various viruses and bacteria, including HIV-1 (Mc Farland *et al.,* 2002; McCoskey *et al.,* 2002; Pantaleo *et al*., 1994). The diversity of the T cell repertoire plays a critical role in recognition of antigen. A more diverse repertoire has been shown to give rise to multiple HIV-1 epitope targeting (Douek *et al*., 2002; Gamberg *et al*., 1999). Previous studies have demonstrated changes in the TCR Vβ repertoire during HIV-1 infection (Wilson *et al*., 1998; Kharbanda et al 2003). Gene expression analysis or spectratyping/immunoscope (Pannetier *et al*., 1995) and/or protein expression analysis by flow cytometry provides quantitation of the percentage of particular TCR Vβ families in a pool of T cells.

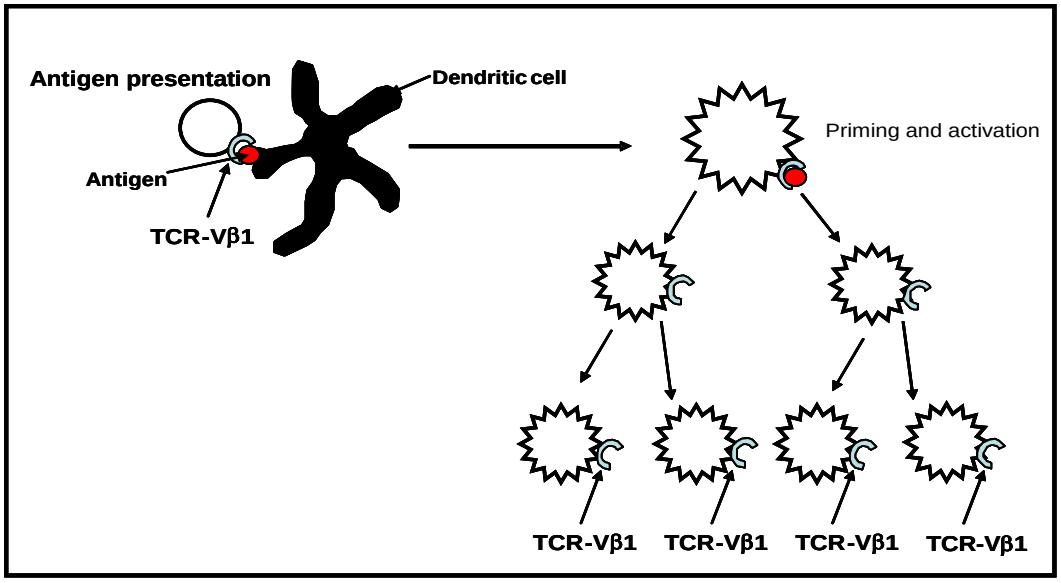


Figure 1.9: Cartoon of the clonal expansion of CD8+ T cells after encounter with presented antigen by a dendritic cell. Priming and activation of a T cell bearing TCR-Vβ1 occurs, resulting in generation of T cell clones bearing the same TCR-Vβ1 (courtesy of Gray CM, 2007).

1.2.11 MISSING GAPS IN KNOWLEDGE

The main objective of this dissertation is to examine the overall characteristics and functional nature of T cells in response to infection and exposure to HIV-1, as well as in the presence of mycobacterium tuberculosis (TB) co-infection. T cell-phenotypic changes have been associated with HIV-1 infection when compared to normal individuals (Vanham *et al*., 1991; Ho *et al.,* 1993). These changes have been observed in various cohorts of HIV-1-infected as well as in HIV/TB co-infected cohorts, mainly indicative of T cell activation. It has been shown that activated T cells are higher in individuals co-infected with HIV-1 and TB (Rodrigues *et al*., 2003; Morris *et al*., 2003; Hertoghe *et al*., 2000). More knowledge of the pathogenesis of HIV-1 in the presence of opportunistic infections needs to be explored.

This dissertation examines different characteristics of T cell responses to HIV-1 and TB in infected, exposed and co-infected individuals; ranging from phenotypes to HIV-specific function. Effective immune reponses to HIV-1 have been associated with both CD4+ and CD8+ T lymphocyte functional responses. HIV-1 proteins have been shown to elicit HIV-1-specific CD8+ cytotoxic T lymphocyte (CTL) responses (Novitsky *et al*., 2003; Masemola *et al*., 2004; Ramduth *et al*., 2005,) as measured by secretion of IFN-γ. The emergence of CTL during the acute stage of infection coincides with a rapid decline in viraemia (Wilson *et al*., 2000; Koup *et al*., 1994). This dissertation explores: a) the nature of T cell reponses to HIV-1 infection in the absence and presence of co-infection with TB; b) the nature of T cell reponses in exposed uninfected individuals and exploring the neonate TCR as a model for exposure.

The immune system of neonates is generally known to be weaker than that of adults (Krampera *et al*., 2000; Qian *et al*., 1997; D'Arena *et al*., 1998), as demonstrated by low expression of Th1 cytokines. However, other studies have shown it to be mature in the context of cellular immunity to pathogens (Regner *et al*., 2004; Hermann *et al*., 2002). The interruption of HIV transmission from mother to child is important, and it involves research studies that focus on investigating the ability of infants to respond to HIV vaccines and whether any immunogenicity would be elicited by those vaccines. The immune response of neonates to HIV-1 has not been extensively defined, therefore hindering possible approaches to vaccine design. A number of vaccine trials have been conducted (Johnson *et al*., 2005; McFarland *et al.*, 2001) which elicited poor immunogenicity compared to adults, or no immunogenicity at all.

1.2.12 AIMS AND HYPOTHESIS

1.2.12.1 AIM 1

Examine changes in T cell phenotypes as a result of HIV-1 infection and co-infection with mycobacterium tuberculosis. This investigation is aimed at obtaining an overall representation of T cells involved in the defense against HIV-1 exposure and infection. This analysis has included the influence of TB disease on T cell immunity in HIV-1 infection.

1.2.12.2 AIM 2

To examine the T cell receptor repertoire by measuring the frequency of TCR-T cell families and the diversity of the T cell populations by investigating CDR3 length

variations. This analysis provides a more refined nature of direct T cell engagement with antigen exposure in vivo using two techniques to measure TCR-usage, flow cytometry and CDR3 length variation to identify TCR Vβ family skewing.

1.2.12.3 AIM 3

To examine HIV-1 antigen-specific CD8+ cytotoxic T lymphocyte responses and profiles in HIV-1 single infection and co-infection with TB.

This analysis provides a further refined approach of investigating antigen-specific T cell immunity at the peptide level in both HIV-infected and dually HIV/TB infected individuals.

1.2.12.4 HYPOTHESIS 1

That the immune response in HIV/TB co-infected individuals leads to elevated T cell activation and disruption of the TCR.

1.2.12.5 HYPOTHESIS 2

That T cell priming can occur in utero as a result of HIV-1-exposure.

1.2.12.6 HYPOTHESIS 3

That the patterns of immunodominance in HIV-1 subtype C–specific T cell responses are altered in the presence of TB disease.

CHAPTER 2

T - CELL PHENOTYPIC ANALYSIS TO MEASURE NAÏVE AND ACTIVATED CELLS

2.1 INTRODUCTION

The pathogenesis of HIV is associated with several immunological dysfunctions, mainly the loss of CD4+ T helper cells as well as phenotypic changes to other T cells. CD4+ and CD8+ have been shown to be activated in response to various pathogens including HIV-1 (Benito *et al.,* 1997; Kerstens *et al*., 1994) and mycobacterium tuberculosis (TB) (Hertoghe *et al*., 2000; S Rodrigues Ddo S. *et al.,* 2003; Morris *et al*., 2003). A major change that occurs during HIV infection is the reduction/depletion of naïve CD4+ and phenotypic alteration of naïve CD8+ T cells (Rodrigues *et al*., 2003; Brinchmann *et al*., 2000). The phenotype and hence the functional quality of these T cells have been shown to change upon encounter with HIV and during the course of HIV infection (Barry *et al*., 2003; Eggena *et al*., 2005).

Activation of the immune system by HIV-1 infection enhances the maturation of naïve T cells into effector memory cells and central memory T cells (Hazenberg *et al*., 2000; Resino S *et al*., 2001). Along with depletion of CD4+ T cells, HIV infection leads to an increase in CD8+ T lymphocyte populations, both in percentage and absolute numbers (Barry *et al*., 2003). Upon infection, T cells express activation markers such as CD38+ and HLA-DR+ (Chun *et al*., 2004, Barry *et al*., 2003; Ho *et al*., 1993). Expression of

CD38+ on CD8+ T cells has been shown to be a strong predictor of HIV disease progression (Liu *et al*., 1997; Lubaki *et al*., 1999; Chun *et al*., 2004; Resino *et al*., 2004).

Measurements of the frequency of naïve, memory and activated T cells from a total leucocyte population have been widely achieved by the use of four colour flow cytometry (Brenchley *et al*., 2002; Landay *et al*., 1990), although this is rapidly changing beyond four colour (Walker *et al*., 2004; de Rosa, 2004). Four colour flow cytometry was used in this dissertation to measure the frequency of naïve T cells (CD45RA+/CD62L+), memory (CD45RA-/CD62L-) and activated (CD38+) T cell populations in individuals infected with HIV-1 and mycobacterium tuberculosis (TB), to examine changes in T cell phenotypes as a result of HIV-1 infection and co-infection with TB. This investigation was aimed at obtaining an overall representation of T cells involved in HIV and HIV and TB co-infection.

2.2 MATERIALS AND METHODS

2.2.1 Study cohort

Four groups of individuals were recruited from a gold mine in Welkom, and analyzed in a cross-sectional manner. The cohorts consisted of HIV-1 infected, singly TB infected individuals, HIV/TB co-infected individuals and healthy controls. The three infected cohorts with CD4 counts and HIV plasma RNA copies are shown on Table 2.1. The medians and interquartile ranges for both CD4+ counts and plasma RNA copies are shown below the table. The TB and HIV/TB co-infected groups were followed longitudinally over a period of 6 months while they were obtaining TB therapy and

samples were obtained at baseline (before treatment), 2 weeks, 6 months and 12 months. The TB status was determined by sputum-culture.

Table 2.1: Cohorts of HIV infected, HIV/TB co-infected and TB infected

HIV+			HIV/TB			TB+	
Patient ID	CD4 (cells/µl)	Viral load RNA copies /ml	Patient ID	CD4 (cells/µl)	Viral load RNA copies /ml	Patient ID	CD4 (cells/µl)
IM67	270	6861	IM1	140	284612	IM3	1254
IM68	420	9460	IM5	111	29861	IM4	1606
IM70	379	10887	IM6	*	6475	IM7	559
IM71	1225	15828	IM9	*	19522	IM10	957
IM72	260	21588	IM11	833	113	IM12	1073
IM73	287	3192	IM13	437	3763	IM22	585
IM74	539	5106	IM14	1395	36096	IM23	580
IM75	391	225	IM15	*	29401	IM26	627
IM76	*	14556	IM16	583	65976	IM32	1908
IM77	*	*	IM19	510	24658	IM35	464
IM78	*	6093	IM21	177	35413	IM40	537
IM79	537	54822	IM29	945	6378	IM41	2624
IM80	393	*	IM30	66	49172	IM45	1353
IM81	36	67574	IM33	353	82638	IM46	2681
IM82	393	1980	IM34	70	128271	IM57	*
IM83	452	19756	IM36	158	48403	IM59	2285
IM84	643	20599	IM42	873	9334		
IM85	300	1980	IM47	1828	12283		
IM86	717	19756	IM51	155	57230		
IM87	208	26216	IM56	*	12460		
IM88	350	2520	IM58	117	245634		
IM89	304	121	IM60	939	67166		
IM90	222	82150	IM61	189	92239		
IM91	371	219917	IM62	536	5217		
IM92	229	6576	IM63	847	9488		
IM93	56	59463	IM65	469	41398		
			IM66	324	3645		

CD4 Count

Cohort	Median	IQR
HIV+	371	245 - 436
HIV/TB	437	157 - 840
TB+	1073	583 - 1757

HIV RNA LOAD

Cohort	Median	IQR
HIV+	12722	4628 - 22745
HIV/TB	29861	9411 - 61603

IQR: Interquartile range
*No results

The HIV-1 results were obtained by a serological assay (ELISA) at baseline- The GENSCREEN HIV1/2 Ab EIA was used, which is an enzyme immunoassay based on the principle of a two-step sandwich technique. It consists of a solid phase coated purified recombinant gp160 and gp25 proteins. Briefly, plasma samples were added to the wells on the antigen coated plate and incubated at 37^{o}C. Peroxidase conjugate was then added, followed by the substrate solution. The reaction was stopped using sulphuric acid. Samples with ODs above the cutoff were considered to be positive. The Quantiplex HIV-1 RNA 3.0 Assay (bDNA) was used to measure plasma viral load. The method was based on amplification of a signal nucleic acid probe for direct quantification of HIV-1 RNA in human plasma. It is a nucleic acid hybridization procedure. The RNA in plasma is captured to a microwell plate by a set of specific, synthetic oligonucleotide capture probes. A set of target probes hybridizes to both the viral RNA and the pre-amplifier probes. The capture probes (comprised of 17 individual capture extenders) and the target probes (comprised of 81 individual target extenders) bind to the different regions of the pol gene of the viral RNA. The amplifier probe hybridizes to the pre-amplifier forming a branched DNA (bDNA) complex. A chemiluminescent substrate was added and the emitted light was measured and calibrated by means of a standard curve for determination of RNA load. Viral load data for HIV and HIV/TB infected individuals are shown on Table 2.1.

Absolute CD4+ counts were determined using percentages of CD4+ T cells obtained using Becton Dickinson FACSort flow cytometer-CellQuest software version 1.1, which were calculated from total lymphocyte counts obtained from the Coulter Onyx (Coulter)

blood cell counter. Whole blood was stained with APC labeled-CD3 and PerCP labeled–CD4, as described in section 2.2.2. Percentage of CD3+CD4+ T helper cells was then obtained from gated lymphocytes. This percentage CD3+CD4+ was then used to calculate the absolute value of CD3+CD4+, as a proportion of total lymphocytes obtained from Coulter Onyx. Absolute CD4 counts for HIV and HIV/TB co-infected patients are shown on Table 2.1.

2.2.2 Whole blood staining and flow cytometric analysis

Whole blood cells were labeled with FITC (Fluorescein isothiocyanate); PE (Phycoerythrin); PerCP (Peridin Chlorophyll) and APC (Allophycocyanin)–conjugated monoclonal antibodies (Table 2.2). The table describes monoclonal antibodies used to identify cell types/cell populations described by various markers. Whole blood (100µl) and monoclonal antibodies (0.5µl to 10µl) were added to 5ml FACS polypropylene tubes and incubated in the dark at room temperature for 30 minutes. Red blood cells were lysed with 2ml BD 1x FACS lysing solution. Cells were then washed by centrifugation at 1200rpm with 3ml of sheath fluid/haematology diluent (see Appendix 1) and fixed with 300µl of cell fixer (see Appendix 1) for acquisition and analysis on the FACSort.

Table 2.2: Monoclonal antibodies (conjugated to different fluorochromes) and cell types measured

Tube	Measured cell type	FITC	PE	PerCP	APC
1	Naïve CD4+ T lymphocytes	CD45RA	CD62L	CD4	CD3
2	Naïve CD8+ T lymphocytes	CD45RA	CD62L	CD8	CD3
3	Acutely activated CD4+ T lymphocytes	CD69	CD25	CD4	CD3
4	Acutely activated CD8+ T lymphocytes	CD69	CD25	CD8	CD3
5	Chronically activated CD4+ T lymphocytes	HLA-DR	CD38	CD4	CD3
6	Chronically activated CD8+ T lymphocytes	HLA-DR	CD38	CD8	CD3

2.2.3 The gating strategy

A total of 50,000 cells were acquired on the Becton Dickinson FACSort using the CellQuest software-version 1.1. List mode data was analyzed using the same software by gating live-lymphocytes on the FSC/SSC parameter followed by gating of specific T cell populations. An example of the gating strategy is shown on Figure 2.1. Lymphocytes were selected by region 1 (R1, Figure 2.1A), and sequentially gated by region 2 (R2) selecting CD3+ T cells (Figure 2.1B), and then sequentially gated by region 3 (R3) selecting CD8+ T cells (Figure 2.1C). Using this sequential (R1+R2+R3) gating strategy, the percentage of CD3+CD8+CD62L+CD45RA+ (naïve CD8+ T cells) is shown on the upper-right quadrant of Figure 2.1D (4.14%) and memory CD8+ T cells (34%) labelled as CD3+CD8+CD62L-CD45RA- is shown on the bottom left quadrant. Using a sequential gating strategy of R1 + R2, a percentage of activated CD8+CD38+ T cells was determined (Figure 2.2). Activated CD8+ T cells are shown on the upper right quadrant of Figure 2.2.C, which is 65.15% of T cells expressing the CD38 marker.

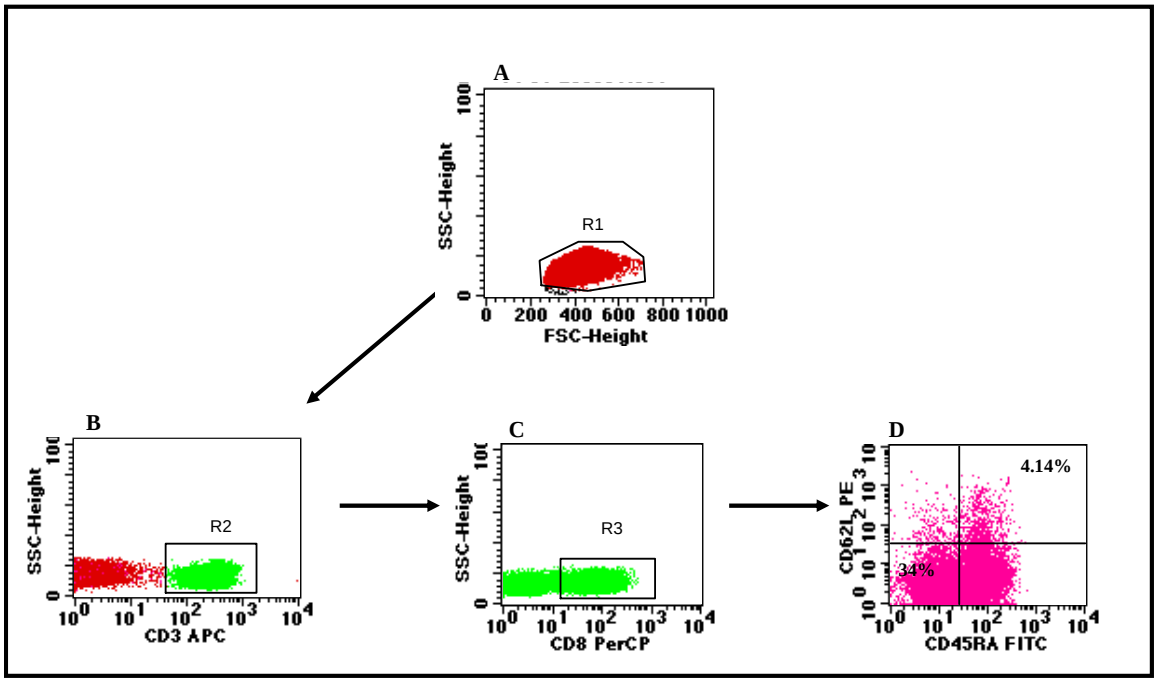


Figure 2.1: Flow cytometry dot plots showing the phenotype analysis of naïve and memory T cell populations. Events (50,000) were acquired on FACSort and the frequency of activated CD8+ T cells was measured. The gating strategy for CD8+CD45RA+CD62L+ (4.14%) and CD8+CD45RA-CD62L- (34%) T cells are illustrated. The percentages of both naïve CD8+ T cells and memory T cells were measured using a sequential gating strategy (R1+R2+R3) as shown on plot -A, B, and C.

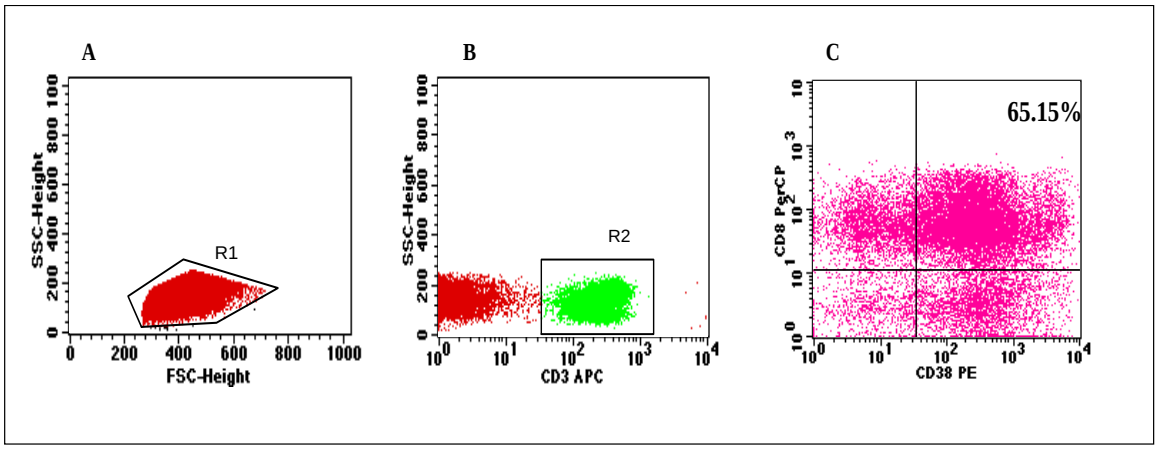


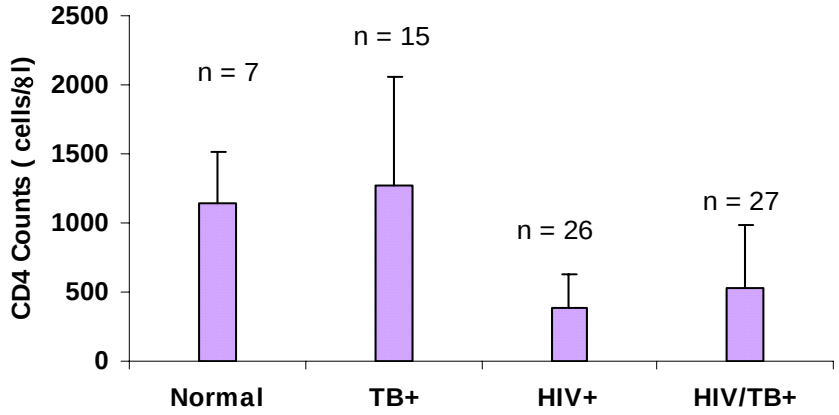
Figure 2.2: Flow cytometry plots showing gating strategy to measure activated T cells. The gating strategy for CD3+CD8+CD38+ (65.5%) is illustrated. The percentage of activated CD8+ T cells was measured using a sequential gating strategy (R1+R2) as shown on plot -A, B, and C.

2.3 RESULTS

2.3.1 Comparison of CD4 + T lymphocyte counts between TB infected, HIV-1 infected and HIV/TB co-infected individuals

To understand the clinical picture of these groups, CD4 absolute counts were compared. Both HIV-1 infected and HIV/TB co-infected groups had lower CD4+ T cell counts when compared to healthy controls ($p = 0.004$ and $p = 0.029$ respectively) as shown in Figure 2.3. No differences were observed between the normal and the TB infected group ($p = 1.000$) as well as between the HIV-1 infected group and the HIV/TB co-infected group. Both HIV-1 infected and HIV/TB co-infected groups had lower CD4+ T cell counts when compared to the TB infected group ($p = 0.0001$). Statistical data (P values) is shown in Figure 2.3. The significant difference by group was calculated using Bonferroni, (Statacorp, College station, Texas), which is a pairwise test that measures

differences between groups. Similar results were obtained when percent CD4 counts were compared between the four groups.



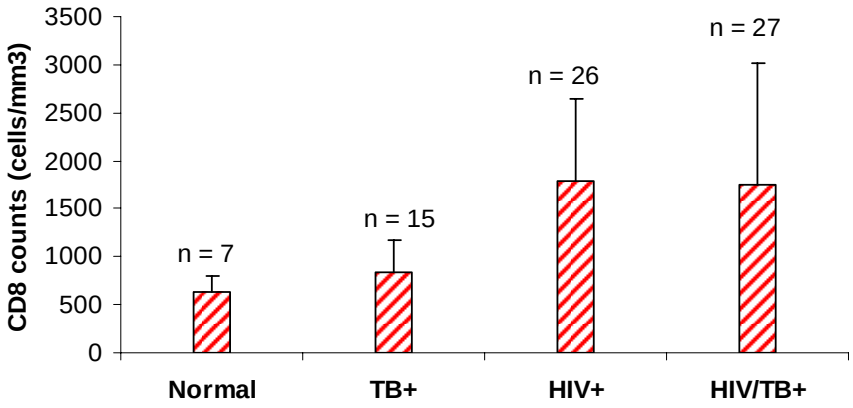
	Normal	HIV+	TB+
HIV+	0.004		
TB+	1.000	0.0001	
HIV/TB+	0.029	1.000	0.0001

Figure 2.3: Differences in absolute CD4 counts between healthy controls and the three cohort groups. Statistical P values are shown in the table below the graph.

2.3.2 Comparison of CD8+ T lymphocyte counts between HIV-1 infected, TB infected and HIV/TB co-infected individuals

Both HIV-1 infected and HIV/TB co-infected groups had significantly higher CD8+ T cell counts when compared to the normal controls ($p = 0.027$ and $p = 0.034$ consecutively) as shown in Figure 2.4. No differences were observed between the normal and the TB infected group ($p = 1.000$) as well as between the HIV-1 infected group and

the HIV/TB co-infected group. Both HIV-1 infected and HIV/TB co-infected groups had higher CD8+ T cell counts when compared to the TB infected group (p = 0.016 and 0.022 respectively). Statistical data is shown in Figure 2.4.



	Normal	HIV+	TB+
HIV+	0.027		
TB+	1.000	0.016	
HIV/TB+	0.034	1.000	0.022

Figure 2.4: Differences in absolute CD8 counts between healthy controls and the three cohort groups. Statistical P values are shown in the table below the graph.

The CD4:CD8 ratios were the lowest in the HIV-1 (0.02 – 0.86) and the HIV/TB co-infected groups (0.01 – 0.97) compared to the healthy controls donors (0.94 - 2.02) and the TB infected group (0.48 – 2.95) (Table 2.3).

Table 2.3: The CD4:CD8 ratios from the three study cohorts and the normal controls

CD4/CD8 ratio	Normal	TB+	HIV+	HIV/TB+
Median	1.68	1.60	0.25	0.24
Range	0.94 - 2.02	0.48 - 2.95	0.02 - 0.86	0.01- 0.97

These data show that CD4 counts are suppressed in HIV infected individuals, with or without TB and that TB infection alone did not significantly impair either the CD4+ or CD8+ compartment.

2.3.3 Comparisons of HIV-1 RNA load between HIV and HIV/TB co-infected individuals

HIV-1 RNA load was compared between 24 HIV-1 infected and 27 of the HIV/TB co-infected individuals. Although there was no statistically significant differences observed between the two groups ($p = 0.075$), the HIV/TB patients had a higher trend of viraemia than the individuals with HIV-1 only (Figure 2.5). These data may suggest that the elevated levels of viraemia in co-infected patients may result in different pathologies between the two groups.

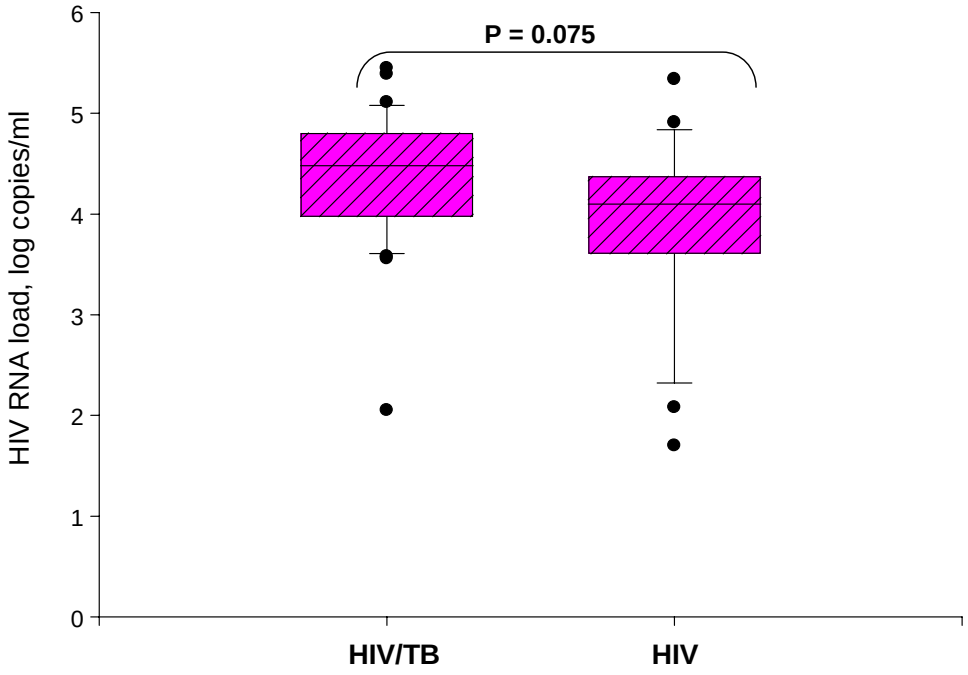


Figure 2.5: Comparison of HIV-1 plasma RNA load between 24 HIV/TB and 27 HIV-1 infected individuals.

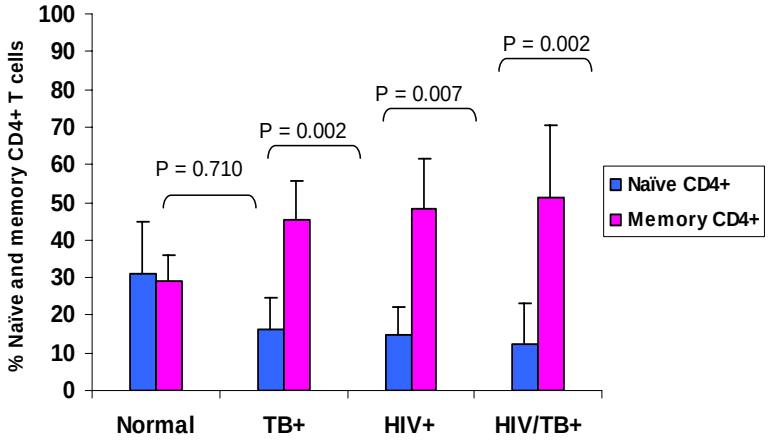
2.3.4 Phenotypic analysis of naïve and memory T cell populations in the TB, HIV-1 and HIV/TB co-infected individuals

The first T cell subsets to be determined were the frequencies of naïve and memory T cells. Flow cytometry data for this phenotypic analysis was expressed as percentages of total T lymphocytes as shown in the gating strategy example in Figure 2.1A-C. The mean percentages of naïve and memory T cells between healthy individuals (n = 7), TB infected (n = 16), HIV-1 infected (n = 26) and HIV/TB (n = 27) co-infected individuals were compared.

2.3.4.1 CD4+ T cells

Differences in the mean frequencies of CD3+CD4+CD62L+CD45RA+ T cells were observed amongst the four groups. The healthy controls had a significantly higher

frequency of naïve CD4+ T cells to that of TB, HIV-1 and HIV/TB co-infected individuals (Figure 2.6). There was a statistically higher frequency of memory CD4+ T cells (CD3+CD4+CD62L-CD45RA-) in the three disease cohorts when compared to healthy donors and conversely lower frequencies of naïve CD4+ T cells. It was interesting to note, that although the gross CD4 count in TB infected individuals (without HIV infection) did not differ from healthy controls (see Figure 2.3), there were as few naïve CD4+ T cells in the CD4 compartment as in those infected with HIV-1 (Figure 2.6). The lower proportion of naïve CD4+ T cells probably reflects the transition from naïve to memory T helper cells.



Naïve CD4

	Normal	HIV+	TB+
HIV+	0.001		
TB+	0.005	1.000	
HIV/TB+	0.0001	1.000	1.000

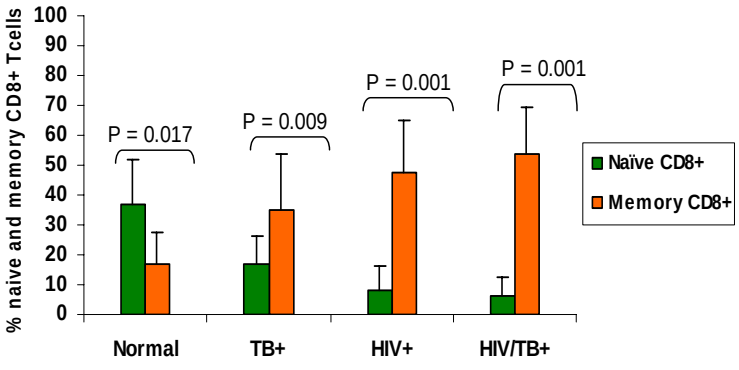
Memory CD4

	Normal	HIV+	TB+
HIV+	0.017		
TB+	0.093	1.000	
HIV/TB+	0.004	1.000	1.000

Figure 2.6: Differences in naïve CD4+ T cells (CD3+CD4+CD62L+CD45RA+) and memory CD4+ T cells (CD3+CD4+CD62L-CD45RA-) between healthy controls and the three cohort groups. Statistical P values are shown in the tables. Statistical P values were determined by using ANOVA and Bonferroni pairwise analysis for these three cohorts consecutively when compared with the healthy controls.

2.3.4.2 CD8+ T cells

The control group had the highest frequency of naïve CD8+ T cells when compared with all the groups (p = 0.0001). The plot and statistical tables are shown on Figure 2.7. Lower frequencies of naïve CD8+ T cells were observed in the HIV+ (p = 0.012) and the HIV/TB co-infected group (p = 0.001) when compared with the TB infected group. Higher frequencies of memory CD8+ T cells were observed in the HIV+ (p = 0.0001) and the HIV/TB group (p = 0.0001) as compared to the normal donors. The CD8+ memory pool in the TB+ group was significantly lower than that observed in the HIV/TB co-infected group (p = 0.004). The frequencies of both memory CD4+ T cell and memory CD8+ T cell populations in all three infected groups were higher than the naïve T cells populations (Figure 2.6 and 2.7), and the over-representation of circulating memory T cells, coupled with a preferential loss of the naïve T cell population, is likely due to persistent antigen and maturation of naïve to memory T cells upon interaction with both HIV-1 and TB antigens.



Naïve CD8

	Normal	HIV+	TB+
HIV+	0.0001		
TB+	0.0001	0.0120	
HIV/TB+	0.0001	1.0000	0.0010

Memory CD8

	Normal	HIV+	TB+
HIV+	0.0001		
TB+	0.1120	0.1220	
HIV/TB+	0.0001	1.0000	0.0040

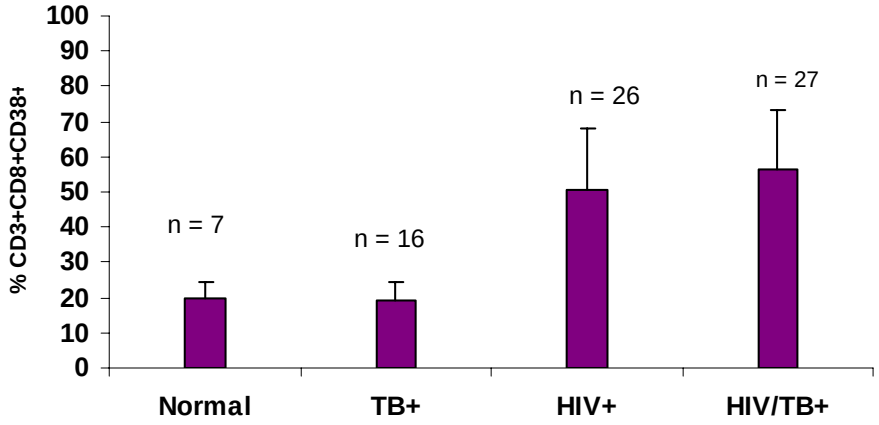
Figure 2.7: Differences in naïve CD8+ T cells (CD3+CD8+CD62L+CD45RA+) and memory CD8+ T cells (CD3+CD8+CD62L-CD45RA - between healthy controls three infected groups. Statistical P values are shown in the tables.

2.3.5 Phenotypic analysis of activated CD8+ T cell populations in the TB, HIV-1 and HIV/TB+ co-infected individuals

The increased frequencies of memory CD8+ T cells, demonstrated in the infected cohort groups, were also accompanied by increased T cell activation. The level of activation was achieved by measuring the percentages of CD3+CD8+ T cells expressing CD38. The phenotypic patterns of activated CD8+ T cells between the 7 healthy controls, 16 TB infected, 26 HIV-1 infected and 27 HIV/TB co-infected individuals were compared (Figure 2.8)

A larger proportion of CD3+CD8+ T cells expressing CD38+ were observed in the HIV/TB group when compared with healthy controls and TB infected individuals ($p = 0.0001$) followed by the HIV-1 infected group which showed higher levels when compared to TB and healthy control groups ($p = 0.0001$); as shown in Figure 2.8, no significant differences were observed between the normal controls and the TB group ($p = 1.0000$). The presentation of the opportunistic infection (TB) in the co-infected individuals was associated with higher frequencies of T cells expressing CD38 and is perhaps in agreement with accelerated progression to AIDS (Kerstens *et al*., 1994). The increased activation status observed in the HIV/TB co-infected group is associated with the high levels of CD8+ memory T lymphocytes as described in section 2.3.4, as well as the high absolute CD8+ T cell counts. HLA-DR was expressed at very low percentages in the CD8+ T cells of all cohort groups and no significant differences were observed. No differences were observed in the frequency of acutely activated T cells:

CD3+CD8+CD69+ and CD3+CD8+CD25+ T cells between the four cohort groups - (p = 1.000).



	Normal	HIV+	TB+
HIV+	0.0001		
TB+	1.0000	0.0001	
HIV/TB+	0.0001	1.0000	0.0001

Figure 2.8: T cell phenotype results showing the percentage (mean ±SD) of activated CD8+ T cells (CD3+CD8+38+). The percentages of CD8+ T cell activation was compared amongst the normal controls and the three infected cohort groups. Statistical P values are shown in the table.

2.3.6 Associations between naïve/memory T cells and HIV plasma RNA copies in individuals singly infected with HIV-1

This dissertation has demonstrated enhanced maturation of naïve CD4+ and CD8+ T cells to memory T cells in the presence of HIV-1 and with increased frequencies of T cell activation markers. It is hypothesized that viral load causes enhanced maturation of naïve to memory phenotypes with increased T cell activation. The association between naive/memory T cells and HIV-1 plasma RNA copies was performed in 24 HIV-1

infected individuals. There was an inverse trend between naïve CD4+ T cells (R = -0.354, p = 0.0877) and viral load, although not reaching significance (Figure 2.9A). A similar association was observed between naïve CD8+ and viral load (R = -0.475, p = 0.0192, Figure 2.9B). These data may suggest that a high burden of viraemia enhances the maturation of naïve T cells to memory. Although not significant, the trends towards positive correlations were observed between memory CD4+ (R= 0.171, p = 0.419) and CD8+ T cells (R = 0.328, p = 0.116) and HIV-1 RNA load (Figure 2.9C and D), suggesting that levels of viraemia may drive maturation of T cells from naïve to memory.

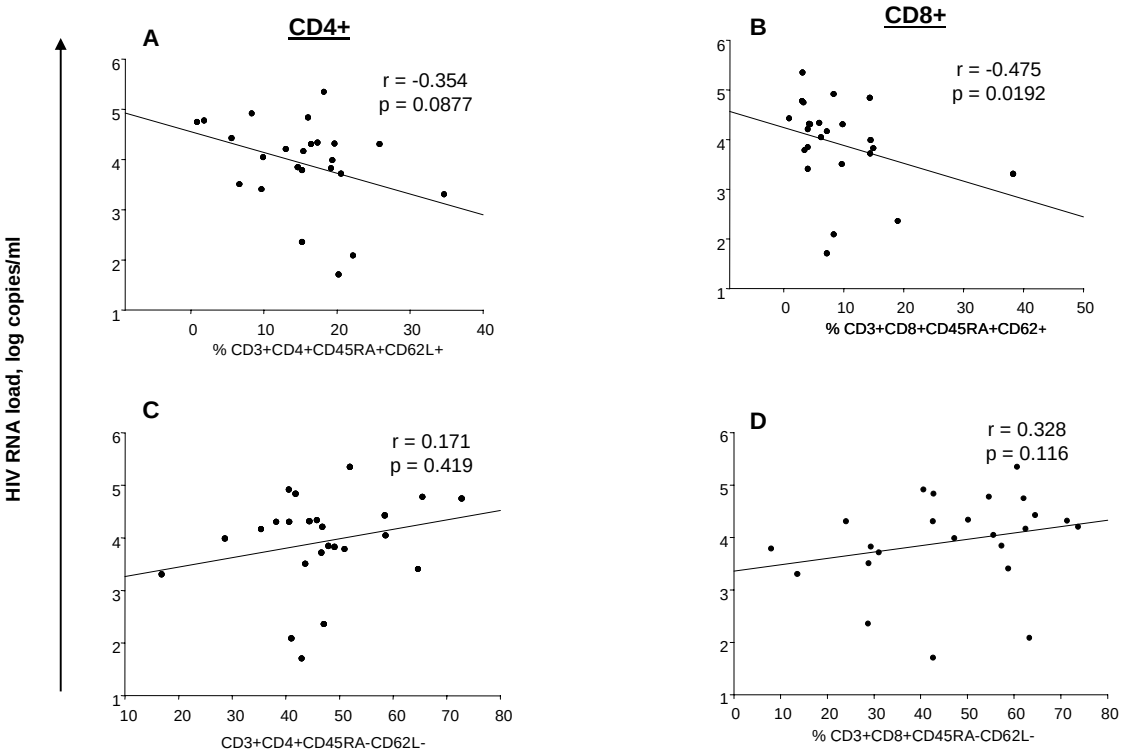


Figure 2.9: The association between naïve CD4+ (A), naïve CD8 (B), memory CD4+ (C) and memory CD8+ (D) in patients infected with HIV-1, using Spearman Rank Order Correlation tests.

2.3.7 Associations between naïve /memory T cells and HIV plasma RNA copies in individuals dually infected with HIV-1 and TB.

The relationship between viral load and the percentage of naïve CD4+ and CD8+ T cells was determined from a total of 27 HIV/TB co-infected individuals. A statistically significant negative association was observed between naïve CD4+ T cells and viral load (R = -0.476 and p = 0.0124, Figure 2.10A), with a similar association observed between naïve CD8+ T cells and viral load in the same group (R = -0.503 and p = 0.031, Figure 2.10B). No significant association was observed between memory CD4+ T cells (Figure 2.10C) or memory CD8+ T cells (Figure 2.10D) and viral load in this group of individuals.

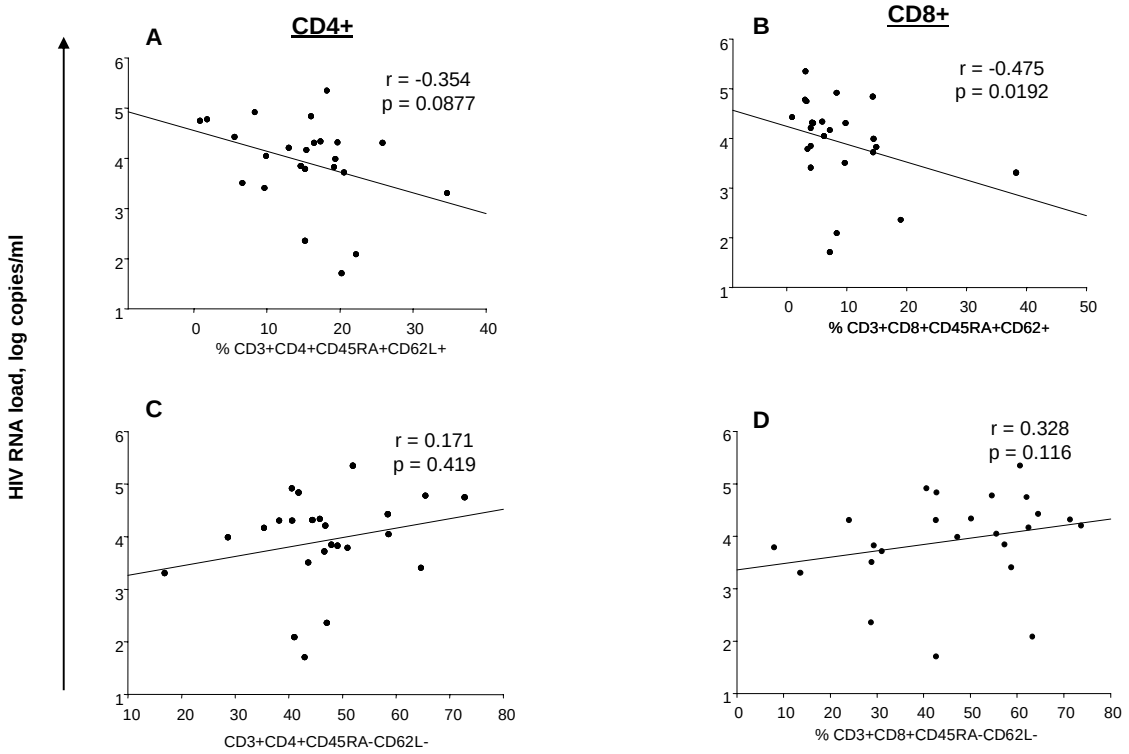


Figure 2.10: The association between naïve CD4+ (A), naïve CD8 (B), memory CD4+ (C) and memory CD8+ (D) in patient dually infected with HIV-1 and TB.

2.3.8 The association between activated T cells and HIV-1 plasma RNA load

Activation of the immune system in HIV infection can be measured by investigating the proportions of CD8+ T cells co-expressing CD38, which has been associated with disease progression, coinciding with high levels of viraemia (Barry *et al*., 2003). This dissertation examined the association between HIV-1 plasma RNA copies and the level of T cell activation, where it is hypothesized that immune activation positively associates with viraemia. Associations were made in 27 HIV/TB co-infected and 24 HIV infected individuals. A significant positive association was indeed observed between activated CD8+ T cells and HIV–plasma viral load (R = 0.411, p = 0.0455) in HIV-1 infected individuals (Figure 2.11A). Although not reaching significance there was a similar association seen in the HIV/TB co-infected group (R = 0.338, p = 0.0836) shown on Figure 2.11B.

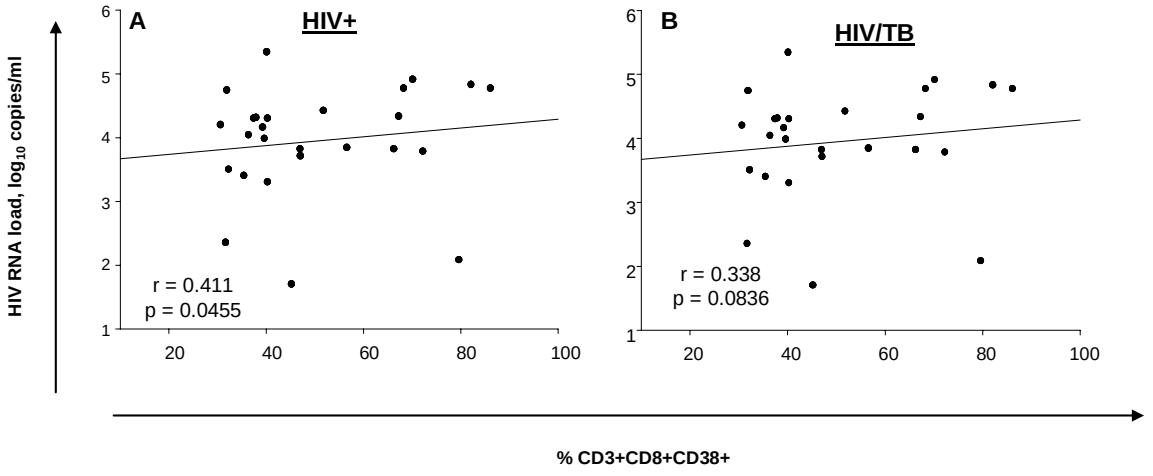


Figure 2.11: The relationship between activated CD8+ T cells and HIV-1 RNA load for 24 HIV-1 infected individuals (A) and 27 HIV/TB co-infected individuals (B) using Spearman Rank Order Correlation tests.

These data supports the notion that HIV replication drives T cell activation, as measured by the CD38 marker.

2.3.9 The effect of TB treatment on CD4+ counts and HIV-1 RNA load in HIV/TB co- infected individuals

Successful treatment of TB in HIV/TB co-infected individuals may represent an effective strategy in elimination of some of the immunopathology associated with dual HIV and TB infection. With this in mind, one of the aims of this dissertation was to explore whether anti-TB therapy could alleviate or reverse markers of immune activation and restore levels of naïve T cells.

The effect of TB therapy on CD4+ T lymphocyte count and HIV-1 viraemia were evaluated from 10 HIV/TB infected individuals, which were selected because of availability of longitudinal samples, although some of the patients were lost to follow up. Blood samples were collected at baseline, 2 weeks, 8 weeks and 6 months of TB therapy. There was no change in CD4 counts or viraemia over time of treatment (Figure 2.12)

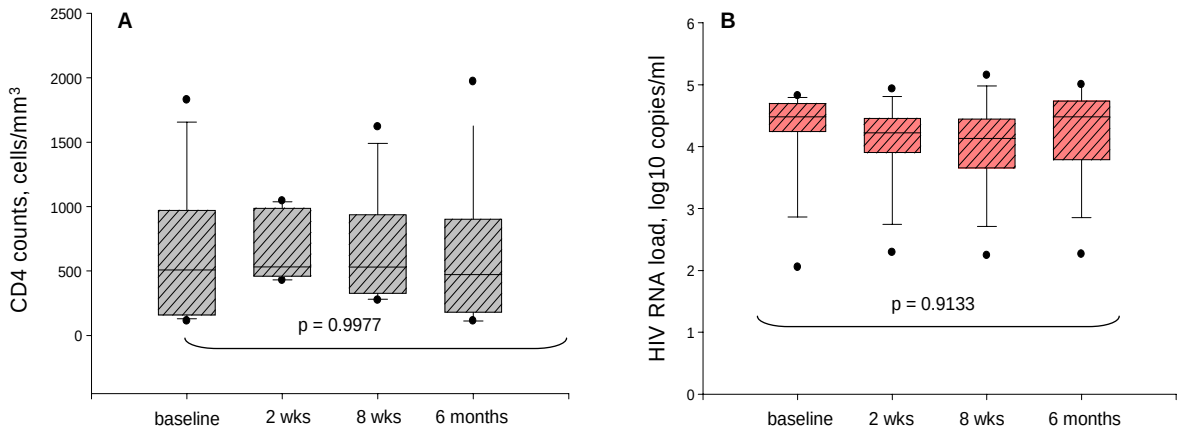


Figure 2.12: The effect of TB treatment on CD4+ T lymphocyte count (A) and HIV-1 RNA load (B) in HIV/TB infected patients over six months of anti-TB treatment.

2.3.10 The effect of TB treatment on T cell phenotypes in TB singly infected individuals

Naïve CD4+ T cells increased significantly by 2 weeks of anti-TB therapy ($p = 0.000$), and then declined to pre-treatment levels at 8 weeks and were maintained at the same level for 6 months (Figure 2.13A). As shown on Figure 2.13B, levels of memory CD4+ T cells were significantly reduced at 6 months ($p = 0.008$). Anti-TB treatment in this cohort group did not improve restoration of naïve CD4+ T cells; however, the memory T cell pool was significantly reduced. Naïve CD8+ T cells increased significantly at 8 weeks ($p = 0.019$) and declined to baseline levels at 6 months (Figure 2.13C). There were no differences in levels of memory CD8+ T cells (Figure 2.13D). Interpretation of these data suggests that anti-TB treatment had variable effects and there was probably too much inter-patient variation to identify conclusive effects of treatment. Activated CD8+ T cells

(CD3+CD8+CD38+) were shown to be at the same level as those of healthy controls in this study group, and this did not change during anti-TB treatment (Figure 2.13E). As these results showed no meaningful or long term effect of anti-TB treatment on the phenotypes of T cells in TB infected individuals, these data infer that reduced naïve CD4+ T cells in this study group may not be associated with TB infection per se. One of the limitations of this study was the absence of longitudinal follow-up of healthy control donors, to establish the variation of these phenotypes without disease or pathology.

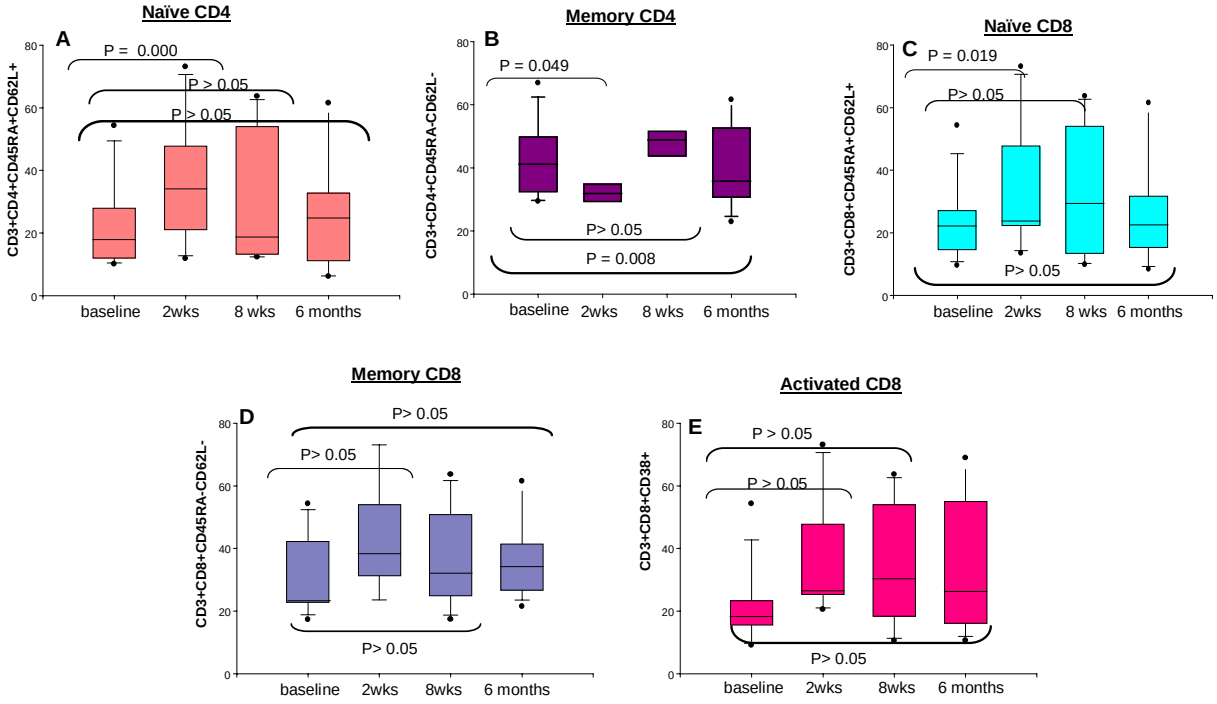


Figure 2.13: The effect of TB treatment on naïve CD4+ (A) memory CD4+ (B), naïve CD8+ (C) memory CD8+ (D) and activated CD8+ T cells (E) in TB single infected individuals.

2.3.11 The effect of TB treatment on T cell phenotypes in HIV/TB co-infected individuals

This dissertation demonstrated elevated levels of CD38+-expressing CD8+ T cells in dual infection. A decrease in T cell activation as a result of TB therapy has been previously demonstrated in individuals with HIV/TB co-infection, and most of these studies described changes in the levels of cytokines (IFN-γ, IL-6 and TNF-α) and expression of HLA-DR as a marker of activation (Lawn *et al*., 1999, Wallis *et al*., 1993). This dissertation describes the effect of anti-TB therapy on phenotypic changes including CD3+CD4+ and CD3+CD8+ T cells expressing CD45RA+CD62+ (naïve), CD45RA-CD62L-(memory), and CD38+ (activated) phenotypes.

Frequencies of naïve CD4+ T cells in this study group were significantly reduced although when adjusting for CD4+ T cell count, the frequencies of naïve CD4+ T cells were sustained throughout the duration of anti-TB treatment ($p > 0.05$)(Figure 2.14A). The elevated frequencies of memory CD4+ T cells were also sustained throughout anti-TB therapy ($p > 0.05$) (Figure 2.14B), suggesting that presumed removal of TB bacillus load made no impact on the naïve to memory imbalance. Adjusting for CD8+ T cell counts at each time point of anti-TB treatment in individuals co-infected with HIV-1 and TB, there was a marginal significant increase in naïve CD8+ T cells at 6 months ($p =0.049$) (Figure 2.14C), coinciding with a decrease in memory CD8+ T cells ($p = 0.000$) (Figure 2.14D). Elevated frequencies of CD8+ T cells expressing CD38+ were observed in HIV-1 infected and HIV/TB co-infected individuals, whereas those individuals infected with only TB had the same frequency of CD38–expressing CD8+ T cells as

healthy controls. No significant difference in CD8+ T cell activation occurred at all time points during TB therapy in this cohort group (Figure 14E), suggesting that TB infection probably had a minor role to play in T cell activation observed in HIV/TB dual infection.

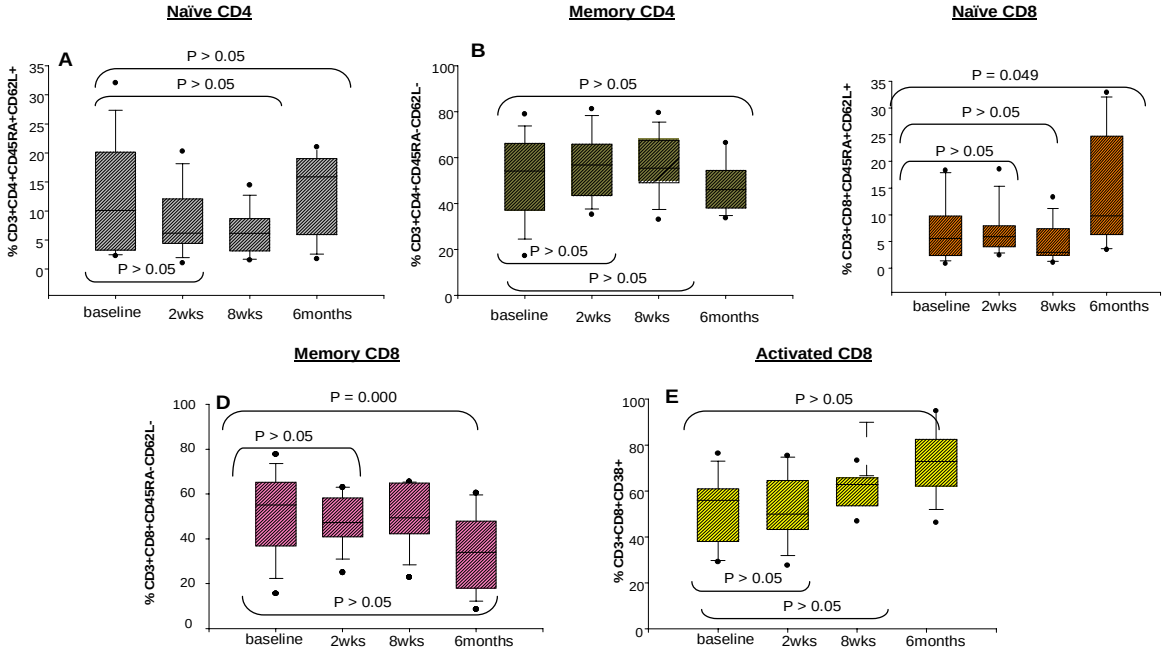


Figure 2.14: The effect of TB treatment on naïve CD4+ (A) memory CD4+ (B), naive CD8+ (C), memory CD8+ (D) and activated CD8+ T cells (E) in HIV/TB co-infected patients.

2.4 DISCUSSION

Phenotypic analysis of T cells in this dissertation has demonstrated that changes occur in T cells, as a result of either single HIV-1, TB infection or co-infection (HIV/TB). Loss of CD4+ T cells is commonly associated with HIV-1 disease progression to AIDS (Rodrigues *et al.*, 2003; Brinchmann *et al.*, 2000). In this study, CD4+ T cells were observed to be reduced as a result of HIV-1 infection and HIV/TB co-infection, which was concurrent with high CD8+ T cell counts. Although infection with TB alone has

been previously reported to result in apoptosis and therefore loss of CD4+ T cells (Hirsch *et al*., 2001), no loss of CD4+ T cells was observed as a result of TB infection in the cohort studied. CD4+ absolute cell counts in patients infected with TB in this study were similar to those observed in healthy controls.

The loss of CD4+ T cells in HIV-1 infected patients was not significantly affected by co-infection with TB, suggesting that loss of these cells is predominantly due to HIV-1. This was further confirmed by approximately similar values of the CD4:CD8 ratio observed in patients who were singly infected with HIV-1 and those co-infected with HIV-1 and TB which were both lower than the ones observed from single infection with both HIV-1 and TB. The CD4:CD8 ratio is used as a marker of HIV/AIDS disease progression (Taylor *et al*., 1989) where lower ratios reflect the more advanced stage of HIV-1 infection.

The results from this chapter have demonstrated that the loss of CD4+ T cells is caused by HIV-1, and was probably not due to TB infection in these cohorts. Since anti-TB treatment did not restore this T cell population, it may be necessary to consider the use of both antiretroviral therapies concurrently with anti-TB treatment to increase survival in individuals co-infected with both pathogens. Previous studies have shown no significant reduction in HIV-1 plasma viraemia during anti-TB treatment (Lawn *et al*.,1999; Toossi *et al*., 2001) however, contrasting report showed a decrease in viral load after 3 months of treatment and which was maintained at 6 months (Morris *et al*., 2003). The findings in this dissertation demonstrate maintenance of high viraemia in HIV-1 infected individuals who are co-infected with TB, despite anti-TB treatment. A significant loss of

CD3+CD4+CD45RA+CD62L+ (naïve CD4+) T cells in all three infected groups, concurrent with an increase in the memory phenotype was demonstrated in this dissertation, which was maintained throughout a six months period of anti-TB therapy in HIV/TB co-infected individuals. This reduction of naïve CD4+ T cells was concurrent with a reduction in absolute CD4+ T lymphocyte counts and an increase in HIV-1 plasma load, which were not affected by anti-TB therapy. Maturation of naïve CD4+ T cells to memory phenotype may pose a detrimental effect since it has been reported that memory CD4+ T cells are more susceptible to HIV-1 infection (Veazey *et al*., 2000), consequently leading to an increased loss of the CD4+ T lymphocyte pool. HIV-1 infection has also been associated with impaired renewal of naïve CD4+ T cell pool (Lawn *et al*., 2001), which may also be a factor in the reduction of these T cells. The influence of high HIV-1 RNA copies in the loss of naïve CD4+ T cells is also demonstrated. These results demonstrate that loss of naïve CD4+ T cells occurs, largely in the presence of co-infection with HIV-1 and TB, which is driven by high HIV-1 plasma load. Anti-TB treatment did not improve the loss of this T cell population.

Similarly, HIV/TB co-infected individuals had the highest levels of memory CD8+ T cells, and the lowest levels of naive CD8+ T cells, which may imply that the high HIV-1 RNA load as well as co-existence of TB may be the two factors driving maturation of naïve CD8+ T lymphocytes to memory phenotype. It was further demonstrated that naïve CD8+ were elevated and memory CD8+ T cells were reduced at 6 months during anti-TB therapy, which was not observed with CD4+ T cells, suggesting that removal of TB antigens with treatment allowed the restoration of CD8+ T cells probably because they

are not directly infected by HIV-1. This study did not identify any reduction of absolute CD8+ T lymphocytes in HIV/TB co-infected individuals, but contrasting reports demonstrated loss of absolute CD8+ T cells in individuals co-infected with HIV and TB, whereas these cells were found in large numbers during the natural progression of HIV-1 infection (Rodrigues *et al*., 2005). These results demonstrate that despite severe loss of CD4+ T cells in advanced HIV-1 disease, anti-TB therapy could restore a pool of naïve CD8+ T lymphocytes which could possibly play a role in the potential control of HIV-1 disease progression.

Elevated frequencies of CD38-expressing T cells are a strong marker of HIV-1 disease progression to AIDS (Liu *et al*., 1997). CD38 reflected high levels of activation which appeared in large numbers in patients who were singly infected with HIV-1 as well as those co-infected with TB in this study. There was no difference between activation levels in single HIV-1 infection and HIV/TB co-infection suggesting that activation of CD8+ T lymphocytes was predominantly elicited by HIV-1 rather than TB. In this respect, anti-TB therapy had no impact on the level of CD8+ T lymphocyte activation, suggesting that activation of CD8+ T lymphocytes is driven by high HIV-1 RNA load, despite anti-TB therapy. The influence of HIV-1 plasma RNA copies may be implicated in the persistence of T cell activation since there was a significant association between viral load and the level of activation which did not change during TB therapy. Sustained high levels of activation and HIV-1 plasma RNA load could therefore be related to advancing disease.

In summary, alterations in T cell phenotypes in this chapter appeared to be more influenced by HIV-1 than TB infection, as anti-TB treatment and removal of bacterial burden did not significantly alter T cell activation or uniformly affect the pool of circulating naïve and memory T cell populations.

CHAPTER 3

T-CELL RECEPTOR USAGE IN HIV-1 INFECTED ADULTS CO-INFECTED WITH MYCOBACTERIUM TUBERCULOSIS

3.1 INTRODUCTION

T cell priming and activation occurs when antigens are presented to naïve CD4+ or CD8+ T cells by dendritic cells. The T cell receptor allows this process to occur through binding to the MHC and the restricted epitope (see chapter 1, section 1.2.7). This process results in expansion of T cells bearing specific T cell receptors and in the absence of infection or persistent infection, the TCR repertoire is fairly stable throughout time within an individual (Garderet *et al*., 1998; Even *et al*., 1995). An indication of TCR engagement with antigen can be investigated by measuring the T cell repertoire. Perturbations in the repertoire have been previously observed as a result of T cell antigen encounter during infection with various bacterial and viral infections, including HIV-1 (Mc Farland *et al*., 2002; McCoskey *et al*., 2002; Pantaleo *et al*., 1994). The diversity of the T cell repertoire plays a critical role in recognition of antigen and the more diverse repertoire has been shown to give rise to multiple HIV-1 epitope targeting (Douek, 2002; Gamberg *et al*., 1999).

Previous studies have demonstrated changes in the TCR Vβ repertoire during HIV-1 infection (Wilson et al., 1998; Kharbanda et al., 2003). Gene expression analysis or spectratyping/immunoscope (Pannetier *et al*., 1995) and/or protein expression analysis by flow

cytometry (Wilson *et al*., 1998) are among the most frequently used assays for analysis of the TCR repertoire in HIV-1 infected individuals.

Flow cytometry staining with monoclonal antibodies recognizing different TCR Vβ specificities provides quantitation of the percentage of particular TCR Vβ families in a pool of T cells, and can give an indication of T cell receptor (TCR) usage in response to antigens. Flow cytometry was applied in this dissertation to determine TCR usage in individuals infected with HIV-1 and the impact of TB co-infcetion. Approximately 80% of the T cell repertoire can be assessed using monoclonal antibodies specific for the variable beta TCR and so provides a measure for surface expression of different TCR families on T cells. Thus, this approach, using flow cytometry, allows simultaneous measurements of Vβ-expressing CD4+ and CD8+ T cells.

3.2 MATERIALS AND METHODS

3.2.1 Study cohort

TCR repertoire changes were investigated in three patient cohorts: 26 HIV-1 infected, 27 HIV-1 and TB co-infected, 16 TB infected patients and 7 healthy controls. The TB and HIV/TB co-infected groups were followed longitudinally while on anti-TB therapy for a period of 6 months and samples were obtained at baseline (pre-treatment), 2 weeks, 8 weeks and 6 months (as described in section 2.2.1).

3.2.2 Whole blood staining and flow cytometric analysis

Whole blood (100µl) and different volumes of monoclonal antibodies (Table 3.1) for CD3+CD4/8+CD38+Vβ+ T cells ranging from 0.5 to 10µl were added to 5ml FACS polypropylene tubes and incubated in the dark at room temperature for 30 minutes. Red blood cells were lysed with 2ml BD 1x FACS Lysing solution. Cells were then washed by centrifugation at 1200rpm with 3ml of wash solution (see description on Appendix 1A) and fixed with 300µl of cell fixer (see description on appendix 1A). A total of 50,000 cells were acquired on a Becton Dickinson FACSort using CellQuest software-version 1.1. List mode data was analysed using the same software by gating live-lymphocytes on the FSC/SSC parameter followed by gating of specific T cell populations. The gating strategy is shown on Figure 3.1. Gating of CD3+CD8+ T cells (R1) and CD4+ T cells (CD3+CD8-) as R2 finally resulted in measurements of 1.99% of CD8-CD38+ (CD4+) T cells –expressing Vβ5.1 and 2.73% of CD8+CD38+)-expressing Vβ5.1. As it was shown in Chapter 2, CD38 surface expression was used to assess which Vβ TCR were used by activated T cells. Panels for analysis of Vβ1, Vβ2, Vβ6.7, Vβ9, Vβ18 were excluded for analysis of activated T cells for all cohort groups due to unavailability of relevant monoclonal antibodies/fluorochrome combinations.

3.2.3 Interpretation of the TCR results

T cell receptor usage was classified using arbitrary definitions of minor and major T cell expansions, using the ratio of Vβ TCR expression in infected cohorts divided by those found in healthy controls. Minor expansions were defined as those Vβ-expressing T cells with greater than two–fold but less than ten-fold higher than frequencies observed in healthy

controls. Major expansions were those that were ten-fold and higher than frequencies found in healthy controls. Thus, major expansions were considered those where Vβ TCR+ T cells had a ratio of ≥10. Oligoclonal expansion of T cells, reflecting a restricted use of the T cell repertoire and polyclonal expansion of T cells, reflecting diverse Vβ TCR usage, were defined as multiple minor and major expansions respectively. Representative examples of polyclonal and oligoclonal expansions are shown on Figure 3.2.

Table 3.1: List of Monoclonal antibodies used

Tube	APC	PerCP	PE	FITC
1	CD3	CD8	Vβ1	CD45RA*
2	CD3	CD8	Vβ2	CD45RA*
3	CD3	CD8	CD38	Vβ3.1
4	CD3	CD8	CD38	Vβ5.1
5	CD3	CD8	CD38	Vb5.2
6	CD3	CD8	CD38	Vβ5.2/3
7	CD3	CD8	CD38	Vβ6.7
8	CD3	CD8	CD38	Vβ7
9	CD3	CD8	CD38	Vβ8
10	CD3	CD8	Vβ9	CD45RA*
11	CD3	CD8	CD38	Vβ11
12	CD3	CD8	CD38	Vβ 12.1
13	CD3	CD8	CD38	Vβ13.1/3
14	CD3	CD8	CD38	Vβ13.6
15	CD3	CD8	CD38	Vβ14
16	CD3	CD8	CD38	Vβ16
17	CD3	CD8	CD38	Vβ17
18	CD3	CD8	Vβ18	CD45RA*
19	CD3	CD8	CD38	Vβ20
20	CD3	CD8	CD38	Vβ21.3
21	CD3	CD8	CD38	Vβ22
22	CD3	CD8	Vβ23	CD45RA*

**CD45RA was used in some four colour staining due to lack of availability of the relevant antibody*

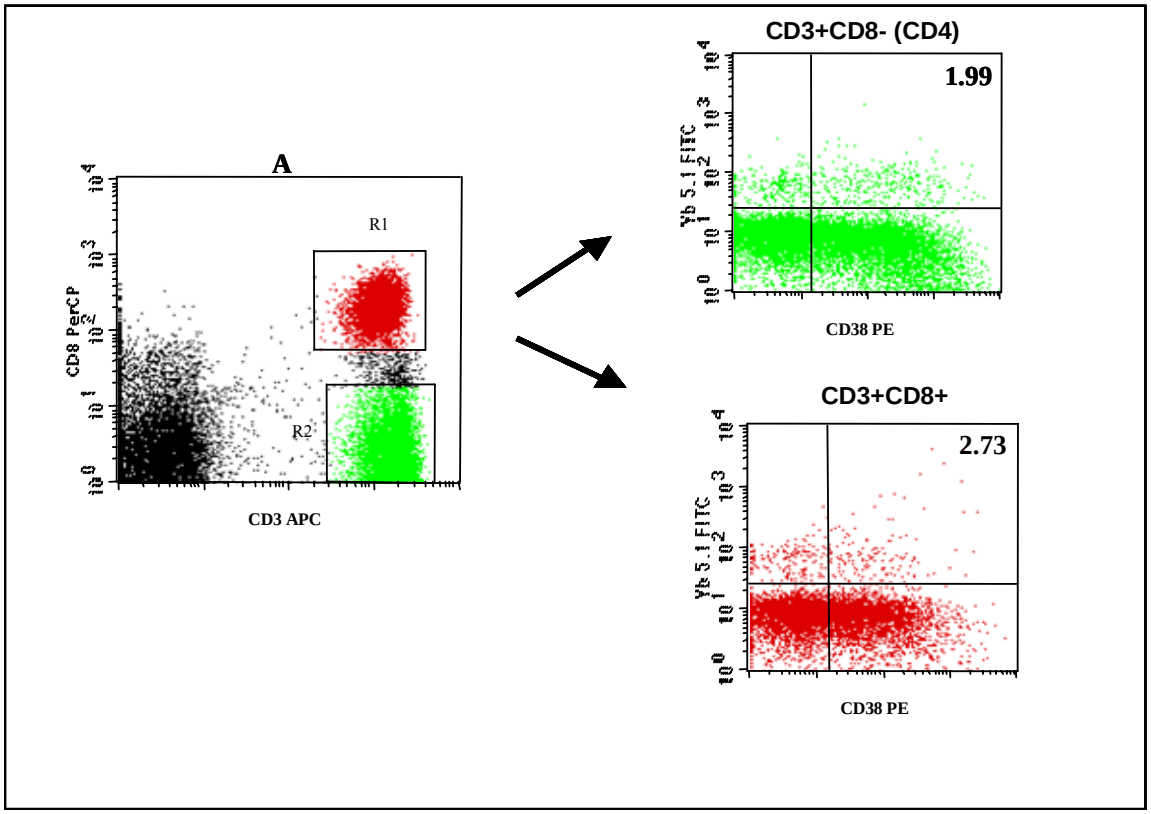


Figure 3.1: The gating strategy for Vbeta analysis by flow cytometry. Events (50,000) were acquired on the flow cytometer and the frequency of activated CD3+CD8-(CD4) and CD3+CD8+ T cells expressing Vbeta TCR were measured as illustrated, using a sequential gating strategy (R1+R2) as shown in this figure.

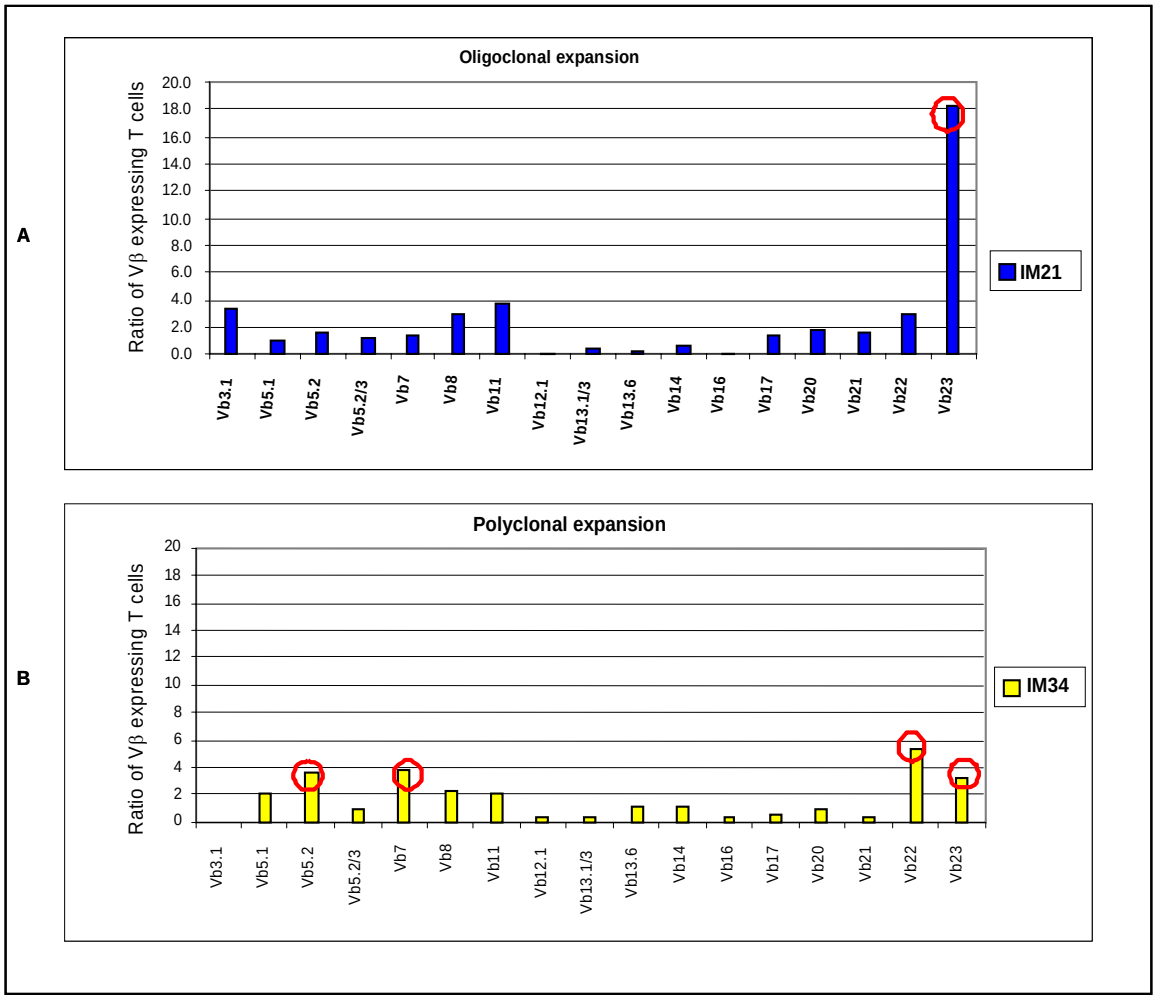


Figure 3.2: Representative examples of oligoclonal (major) and polyclonal (minor) expansions of activated CD8+ T cells for two HIV/TB co-infected individuals. (A) Major expansion of CD8+ T cells expressing Vβ23 (shown by red circles) (B) polyclonal minor expansions of cells expressing Vβ5.2, Vβ7, Vβ22 and Vβ23 (shown by red circles).

3.3 RESULTS

3.3.1 T cell receptor usage in HIV/TB dual infected individuals

It has been demonstrated in previous chapters of this dissertation that CD8+ T cells are persistently activated during HIV-1 and TB co-infection, and predominantly due to HIV. In this chapter, the fine specific nature of T cell activation was investigated by examining TCR Vβ expansions in HIV and TB single and co-infected individuals. It was hypothesized that different Vβ TCR families are expanded in single and dually infected patients. The total T cells expressing 22 Vβ –TCR from 27 HIV/TB co-infected individuals were measured by flow cytometry as described in section 3.2.2. The ratio of Vβ expressing cells in HIV/TB dual infected patients versus healthy controls was calculated as described in section 3.2.3, and the results are shown in Tables 3.2 and 3.3 for CD4+ and CD8+ T cell subsets, respectively. The data shows for activated CD4+ T cells that there was polyclonal skewing (minor expansions) of activated CD4+ T cells in 14 of the 27 co-infected patients (52%), where there was major expansion of Vβ16 TCR using cells in two patients as shown on Figure 3.3. For CD8+ T cells, there was polyclonal skewing (minor expansions) of activated CD8+ T cells (Table 3.3) in 22 of the 27 individuals analysed (82%), with oligoclonal expansions of Vβ11 and Vβ20 observed in three individuals (Figure 3.4). Using Fisher's Exact test, there was a significant difference ($p=0.0418$) in the frequency of polyclonal skewing, with a greater number of TCR being used by CD8+ than CD4+ T cells in dually infected individuals. In summary, multiple T cell receptors were used by CD8+ T cells rather than CD4+ T cells in individuals co-infected with HIV-1 and TB.

Table 3.2: Ratios of frequencies of Vβ-expressing CD4+ T cells

TCR	IM1	IM5	IM6	IM9	IM11	IM13	IM14	IM15	IM16	IM19	IM21	IM29	IM30	IM33	IM34	IM36	IM42	IM47	IM51	IM56	IM58	IM60	IM61	IM62	IM63	IM65	IM66
Vb3.1	1.3	0.3	1.1	0.4	0.9	0.3	2.3	1.4	2.0	0.8	2.4	0.2	0.4	0.3	0.2	0.3	0.5	0.2	0.5	1.5	0.4	0.1	0.2	0.7	0.6	0.2	1.2
Vb5.1	2.2	0.8	2.3	0.8	1.2	0.9	0.9	1.7	2.5	0.5	5.5	1.9	0.6	0.7	0.6	0.7	1.3	0.5	0.7	0.9	0.5	0.9	1.2	1.0	0.9	0.3	0.1
Vb5.2	0.0	2.0	1.0	1.1	1.0	1.9	2.6	2.1	1.6	0.8	1.9	0.8	0.5	1.0	0.0	0.4	1.0	0.7	0.5	0.5	0.7	1.1	0.7	0.7	0.3	0.0	7.2
Vb5.2/3	2.0	1.3	0.5	0.8	1.1	1.1	1.1	5.0	2.7	2.4	1.2	0.7	0.6	1.1	0.1	0.8	1.1	2.7	0.5	0.7	0.6	1.3	0.5	0.5	0.7	0.5	0.6
Vb7	2.7	1.0	0.5	0.9	1.2	1.1	0.6	0.7	0.7	0.9	0.8	0.5	0.6	0.6	0.0	0.9	0.3	0.6	0.3	0.7	0.8	0.8	1.0	0.6	0.9	0.4	1.0
Vb8	0.6	0.8	0.8	0.5	0.6	0.9	0.7	3.7	0.2	1.2	0.2	0.4	0.3	0.9	1.2	0.4	0.5	0.4	0.2	0.7	0.8	0.9	0.5	0.3	0.3	0.5	0.3
Vb11	2.0	1.2	1.0	0.4	0.2	1.1	0.3	1.5	1.4	0.4	3.5	0.2	0.0	0.3	0.0	0.9	0.4	0.3	0.4	0.3	0.4	0.7	0.1	0.7	0.3	0.0	0.5
Vb12.1	2.5	2.5	2.0	1.4	0.4	0.9	0.5	1.4	4.0	4.7	0.8	1.8	2.4	1.2	2.9	0.8	0.0	0.6	0.8	1.0	3.4	3.5	0.7	1.4	0.4	1.9	0.9
Vb13.1/3	0.9	1.1	1.2	0.7	0.7	1.0	0.4	1.0	1.1	1.4	0.8	0.3	0.2	0.7	0.0	0.7	0.8	0.8	0.9	0.8	0.9	1.1	0.6	1.1	0.8	0.2	0.2
Vb13.6	2.1	0.8	1.6	1.4	0.7	1.0	0.4	1.5	1.4	1.0	1.4	0.4	0.4	0.8	0.0	0.8	1.3	0.4	1.3	0.8	1.0	1.0	0.4	1.0	0.5	0.4	0.6
Vb14	1.2	0.8	1.2	1.3	0.1	0.9	0.1	0.8	0.9	0.5	1.9	0.6	0.0	0.2	3.4	0.4	0.3	0.1	0.5	0.6	0.0	0.4	0.5	1.4	0.0	0.5	0.5
Vb16	3.7	2.2	1.5	1.0	0.5	1.9	0.8	2.4	27.8	1.1	26.5	0.5	0.2	1.5	0.5	1.3	2.0	0.8	1.3	0.5	0.2	1.6	0.8	0.7	0.8	0.5	7.6
Vb17	1.4	0.8	0.3	0.8	0.7	0.8	0.4	1.2	0.9	0.4	2.4	0.4	0.2	0.8	0.5	0.7	0.9	0.5	1.0	0.9	0.5	1.0	0.8	0.8	0.6	0.6	0.1
Vb20	2.4	0.8	1.4	1.6	1.3	1.2	0.8	1.7	2.1	0.7	3.0	1.7	0.2	0.9	1.3	0.6	1.2	0.5	1.1	1.3	0.5	1.6	1.9	2.0	0.6	0.6	1.1
Vb21	2.6	0.7	0.9	1.4	0.8	0.9	0.7	1.6	2.3	1.0	2.3	2.5	0.4	0.9	0.0	1.1	1.1	0.4	0.5	0.7	0.6	1.1	0.6	0.9	0.8	0.4	0.0
Vb22	2.2	0.9	1.0	1.1	1.5	1.2	0.7	2.0	2.2	0.9	2.3	0.7	0.1	0.9	0.4	1.0	1.1	0.4	1.1	1.1	1.0	1.3	0.8	1.2	1.2	0.2	1.3

TCR usage as shown by ratios of Vβ-expressing CD4+ T cell populations between HIV/TB co-infected individuals and healthy controls. The blue shaded squares are minor expansions and the yellow shaded ones are major expansions

Table 3.3: Ratios of frequencies of Vβ-expressing CD8+ T cells.

TCR	IM1	IM5	IM6	IM9	IM11	IM13	IM14	IM15	IM16	IM19	IM21	IM29	IM30	IM33	IM34	IM36	IM42	IM47	IM51	IM56	IM58	IM60	IM61	IM62	IM63	IM65	IM66
Vβ3.1	0.6	0.4	1.5	0.3	1.0	0.3	1.6	2.0	3.3	1.0	3.5	0.3	0.4	1.9	0.2	0.2	0.7	0.4	1.1	1.1	3.5	0.1	0.1	0.8	4.0	0.5	0.9
Vβ5.1	2.4	3.8	3.7	2.0	0.9	2.7	1.8	3.6	1.8	0.6	0.9	0.7	1.7	1.0	2.3	1.7	0.4	2.3	1.2	2.3	1.3	0.5	1.0	1.3	0.9	1.1	0.7
Vβ5.2	0.0	1.5	0.4	0.3	2.7	4.7	4.3	1.0	1.1	0.2	1.7	0.6	0.2	1.1	0.6	1.2	0.4	4.3	2.6	0.6	2.8	0.3	3.0	2.4	0.8	0.2	3.3
Vβ5.2/3	3.2	2.8	3.9	1.8	1.5	3.3	1.0	3.8	1.8	0.8	1.2	1.1	0.4	0.8	1.2	1.5	0.4	2.9	3.0	0.9	4.6	2.5	1.7	1.9	0.7	1.4	1.0
Vβ7	2.8	2.1	0.7	4.8	2.5	1.5	1.5	2.4	0.5	1.4	1.4	3.0	0.4	0.7	2.8	1.2	0.0	1.5	1.0	1.5	2.8	2.8	3.1	1.1	1.1	0.5	1.8
Vβ8	1.2	0.7	1.8	0.8	0.5	0.8	0.8	2.2	0.1	1.2	3.1	1.0	0.2	1.5	1.2	7.7	0.2	0.9	1.3	0.9	0.5	2.2	1.1	1.5	1.2	1.9	0.0
Vβ11	22.7	0.4	19.4	0.3	0.4	0.9	0.8	0.3	0.8	0.5	3.8	0.2	0.0	0.7	1.2	0.4	0.1	15.0	0.5	0.2	0.5	0.5	0.7	0.6	1.9	0.2	1.5
Vβ12.1	1.3	1.4	0.6	0.3	0.4	0.1	0.3	0.3	0.2	0.1	0.2	0.6	1.1	0.1	0.1	0.2	0.2	0.2	1.4	0.1	0.1	3.0	0.3	0.7	0.2	2.0	1.1
Vβ13.1/3	1.5	0.8	1.9	0.7	0.4	1.3	0.4	1.1	0.6	2.7	0.5	0.1	0.2	1.1	2.2	0.3	0.0	0.4	0.8	1.1	0.7	2.1	1.9	1.1	0.5	0.3	0.5
Vβ13.6	0.5	0.1	1.2	0.7	0.2	0.7	0.4	1.2	0.3	2.2	0.3	0.4	0.2	0.5	0.7	0.2	0.1	0.3	0.3	0.4	1.1	0.3	1.5	0.3	0.2	0.3	0.4
Vβ14	0.2	0.3	1.1	1.2	0.2	1.1	0.1	0.3	0.4	0.2	0.6	0.4	0.0	0.2	2.1	1.0	0.3	0.0	0.1	0.2	0.2	0.2	1.4	1.2	0.1	0.2	0.2
Vβ16	1.9	0.2	0.2	0.9	0.1	0.3	0.2	0.5	1.1	3.2	0.1	0.1	0.0	0.2	0.3	0.4	3.4	0.3	0.1	0.2	1.0	0.4	0.9	0.1	0.1	0.1	0.8
Vβ17	2.2	0.6	2.1	0.6	0.4	1.1	1.0	0.8	0.4	0.4	1.4	0.3	0.3	1.8	2.2	0.6	1.6	1.4	0.7	2.6	0.5	0.9	0.9	3.4	0.6	0.4	0.1
Vβ20	13.2	1.2	2.3	1.2	1.0	3.2	1.0	0.4	1.6	0.4	1.9	1.8	0.3	0.6	3.1	0.8	0.3	1.6	0.8	1.8	0.2	0.3	2.8	0.9	0.4	0.5	1.4
Vβ21	3.1	0.4	1.0	1.3	1.8	3.3	2.4	5.4	1.1	0.8	1.6	2.3	0.4	2.5	3.5	0.9	0.5	1.3	0.4	4.4	0.7	4.4	1.5	3.3	0.6	0.9	0.0
Vβ22	0.8	1.9	4.0	0.9	1.3	2.5	0.9	3.2	3.8	0.7	3.1	0.6	0.1	1.3	1.2	3.7	0.6	1.4	1.0	1.6	1.2	1.1	2.5	0.4	0.8	0.2	1.8

TCR usage as shown by ratios of Vβ-expressing CD8+ T cell populations between HIV/TB co-infected individuals and healthy controls. The blue shaded squares are minor expansions and the yellow shaded ones are major expansions.

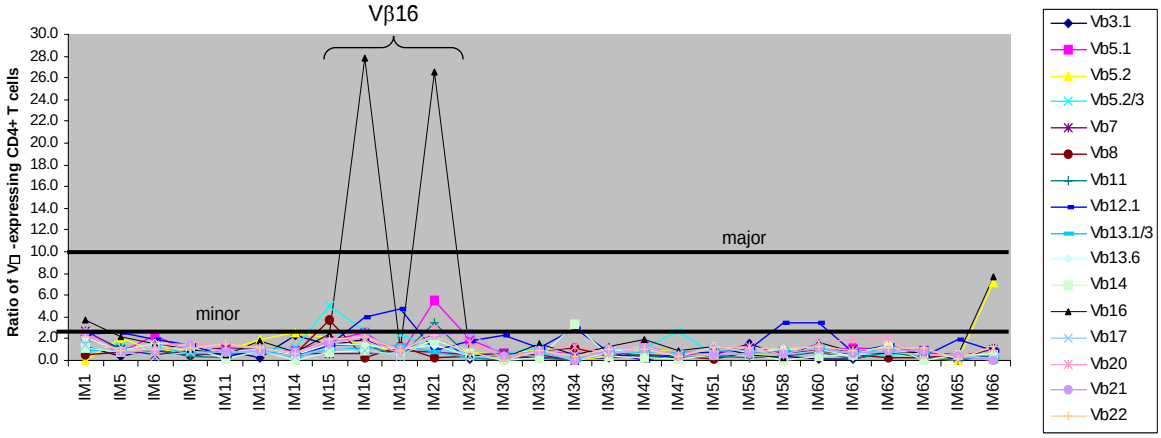


Figure 3.3: TCR usage of CD4+ T cells in 27 HIV/TB dually infected individuals. Major oligoclonal expansion of CD4+ T cells expressing Vβ16 in two HIV/TB infected individuals (IM16 and M21) is observed, as well as minor expansions in fourteen individuals. The horizontal blue line at the ratio of 2 indicates the cutoff for minor expansions and the red line at 10.0 indicates the cutoff for major expansions.

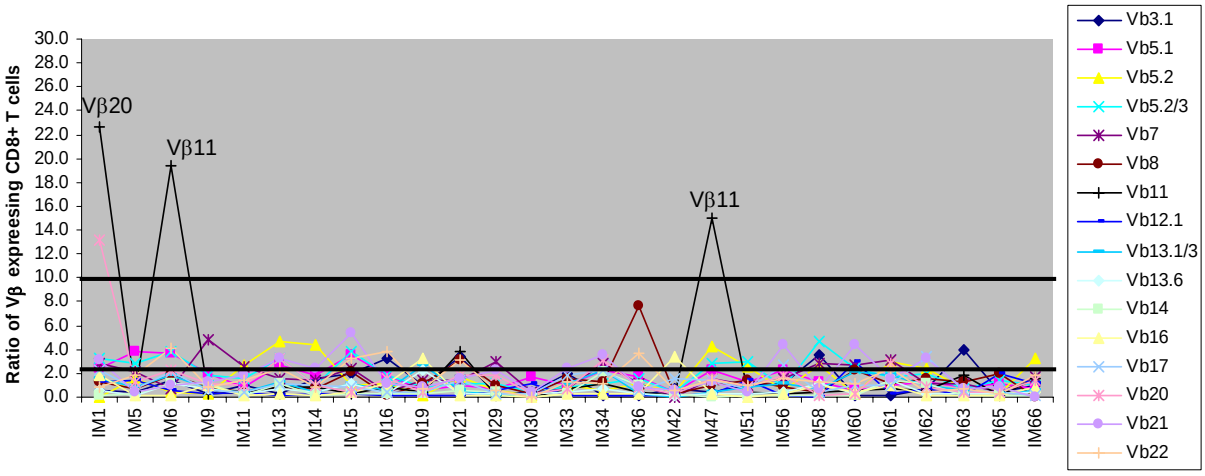


Figure 3.4: TCR usage of CD8+ T cells in 27 HIV/TB dually infected individuals. Major oligoclonal expansion of CD8+ T cells expressing Vβ11and Vβ20 in three HIV/TB infected individuals (IM1, IM6 and IM47), as well as minor expansions in twenty-two individuals. The horizontal blue line at the ratio of 2 indicates the cutoff for minor expansions and the red line at 10.0 indicates the cutoff for major expansions.

3.3.2 T cell receptor usage in HIV-1 single infected individuals

Minor expansions of activated CD4+ T cells were observed in sixteen of the 26 individuals (62%), as shown in Table 3.4. None of the patients showed oligoclonal expansion of activated CD4+ T cells (Figure 3.5). As shown in Table 3.5, minor expansions of activated CD8+ T cells were observed in 20 of the 26 individuals (77%). There was oligoclonal skewing of CD8+ T cells expressing Vβ11 and Vβ22 in 3 patients (Figure 3.6). In summary, as observed in HIV/TB infected individuals, usage of multiple TCRs (polyclonal skewing) by CD8+ T cells rather than CD4+ T cells was observed in HIV-single infection, although this was not significant (p=0.37).

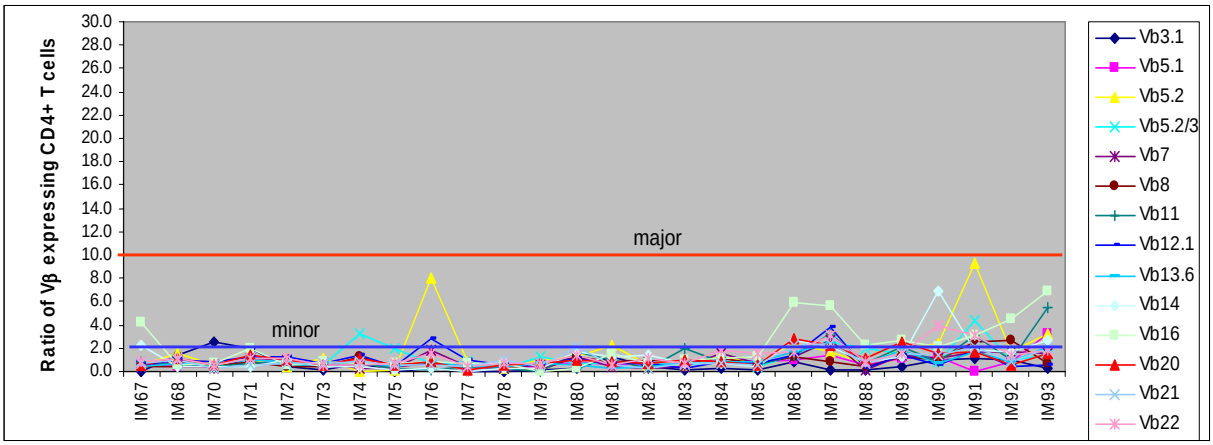


Figure 3.5: TCR usage of CD4+ T cells in 26 HIV-1 singly infected individuals. Expansions of CD4+ T cells-expressing Vβ3.1, Vβ5.1, Vβ5.2, Vβ5.2/3, Vβ8, Vβ11, Vβ12.1, Vβ14, Vβ14, Vβ21 and Vβ22 were observed in sixteen individuals. The horizontal blue line at the ratio of 2 indicates the cutoff for minor expansions and the red line at 10.0 indicates the cutoff for major expansions. The horizontal line at the ratio of 2.0 indicates the cutoff for minor expansions.

Table 3.4: Ratios of frequencies of Vβ-expressing CD4+ T cells.

TCR	67	68	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93
Vb3.1	0.1	1.4	2.5	2.0	0.4	0.1	0.7	0.1	0.3	0.0	0.1	0.1	0.4	1.3	0.4	0.1	0.3	0.2	0.8	0.1	0.2	0.4	1.0	1.1	0.9	0.2
Vb5.1	0.6	0.5	0.5	0.9	0.6	0.4	0.8	0.4	0.7	0.5	0.3	0.4	0.9	0.6	0.7	0.6	1.2	0.8	1.0	1.4	0.6	1.1	1.2	0.0	0.9	3.2
Vb5.2	0.6	1.5	0.5	0.9	0.4	1.1	0.0	0.2	8.1	0.9	0.3	1.0	1.3	2.3	0.5	0.5	1.3	0.7	2.4	1.6	0.6	2.0	2.3	9.4	1.6	3.2
Vb5.2/3	0.4	0.7	0.4	0.9	0.6	0.6	3.3	2.0	0.7	0.9	0.3	1.3	0.6	0.7	0.7	0.5	0.9	0.4	1.5	2.5	2.0	1.5	0.9	4.4	1.0	1.4
Vb7	0.5	0.8	0.2	1.1	0.9	0.7	0.3	0.3	1.8	0.4	0.3	0.6	1.3	0.4	0.3	0.4	1.6	0.8	1.3	2.7	0.1	1.4	1.1	1.7	1.7	1.5
Vb8	0.5	0.4	0.4	0.8	0.4	0.4	1.2	0.4	0.7	0.4	0.4	0.2	1.2	0.8	1.0	0.9	1.1	0.7	1.2	0.8	0.4	1.2	1.0	2.6	2.7	0.7
Vb11	0.8	0.5	0.4	0.7	0.8	0.5	0.6	0.3	0.2	0.5	0.3	0.0	1.7	1.0	0.3	2.0	0.9	0.5	1.5	3.2	0.7	2.1	0.8	3.1	1.0	5.5
Vb12.1	0.6	1.0	0.9	1.3	1.2	0.8	1.4	0.4	2.9	1.0	0.5	0.6	1.1	0.8	0.7	0.3	0.9	0.7	1.4	3.9	0.3	1.4	0.6	1.8	0.5	0.6
Vb13.1/3	0.3	0.3	0.3	0.5	0.4	0.3	0.3	0.3	0.9	0.3	0.4	0.3	0.5	0.4	0.4	0.5	0.8	0.6	0.6	1.1	0.8	0.7	0.5	1.3	0.4	0.3
Vb13.6	0.5	0.7	0.7	1.0	0.8	0.7	0.8	0.5	0.8	0.4	0.4	0.7	0.6	0.3	0.4	0.7	0.8	0.8	1.7	2.5	0.7	1.8	0.7	1.7	0.8	2.1
Vb14	2.2	0.4	0.4	0.4	0.9	0.4	0.4	0.2	0.3	0.1	0.3	0.3	1.7	1.2	1.3	0.6	0.7	0.5	2.7	2.1	0.9	1.3	6.9	1.8	1.7	2.7
Vb16	4.2	0.5	0.7	2.0	0.6	0.8	0.7	0.6	0.8	0.7	0.8	0.0	0.2	1.5	0.7	1.1	1.1	1.3	5.9	5.7	2.3	2.7	2.2	3.2	4.5	6.9
Vb17	0.4	0.6	1.4	0.9	0.0	0.7	0.7	0.3	0.7	0.5	0.7	0.7	0.7	0.6	0.8	0.8	0.8	0.1	1.1	1.2	0.4	1.0	1.8	1.0	0.6	1.1
Vb20	0.6	1.1	0.5	1.5	1.0	0.6	1.1	0.6	0.9	0.1	0.5	0.7	1.0	0.8	0.7	1.0	0.8	0.5	2.7	2.2	1.2	2.6	1.5	1.7	0.6	1.5
Vb21	0.8	0.9	0.3	0.5	0.6	0.7	0.9	0.6	0.6	0.4	0.9	0.4	2.2	0.5	0.3	0.7	0.7	0.6	2.2	2.4	0.8	1.5	1.4	2.1	0.9	2.0
Vb22	0.8	1.1	0.6	1.1	1.1	0.5	0.6	0.7	1.6	0.4	0.6	0.7	1.5	0.6	1.1	0.8	1.8	1.5	1.7	3.2	0.8	1.5	4.0	3.0	1.4	1.8

TCR usage as shown by ratios of Vβ-expressing CD4+ T cell populations between 26 HIV infected individuals and healthy controls. The blue shaded squares are minor expansions.

Table 3.5: Ratios of frequencies of Vβ-expressing CD8+ T cells.

TCR	IM67	IM68	IM70	IM71	IM72	IM73	IM74	IM75	IM76	IM77	IM78	IM79	IM80	IM81	IM82	IM83	IM84	IM85	IM86	IM87	IM88	IM89	IM90	IM91	IM92	IM93
Vb3.1	0.0	1.5	0.8	1.1	0.3	0.0	1.3	0.0	0.3	0.1	0.0	0.2	0.2	1.2	0.2	0.0	0.2	0.0	0.3	0.0	0.2	0.2	1.6	0.6	1.1	0.1
Vb5.1	1.7	2.0	0.7	1.1	1.2	0.5	3.5	1.5	0.6	2.6	1.3	3.7	1.6	1.2	1.4	1.4	1.8	1.1	0.6	2.0	0.6	1.2	2.3	3.5	1.6	2.1
Vb5.2	0.3	1.2	0.7	0.5	0.6	0.1		1.7	1.9	1.1	0.4	0.8	0.4	1.0	3.3	0.1	0.4	0.3	1.4	4.3	0.6	0.4	0.9	7.4	0.4	1.0
Vb5.2/3	0.8	0.8	5.5	1.1	1.1	0.4	1.8	1.0	1.8	1.9	1.2	2.2	2.0	1.0	1.4	0.7	2.1	1.4	0.5	1.3	1.6	1.0	1.1	3.0	1.1	2.5
Vb7	1.5	1.3	0.1	1.1	4.3	0.6	0.4	0.6	9.2	1.0	0.2	1.3	1.9	0.5	1.4	1.5	1.2	0.9	2.2	2.2	0.0	0.9	1.2	6.6	0.9	6.4
Vb8	0.7	0.8	0.5	0.5	3.8	0.1	0.9	1.0	1.0	0.7	0.2	0.3	0.9	0.7	0.7	0.2	0.3	0.1	0.2	0.6	0.3	0.6	1.3	1.2	1.4	1.7
Vb11	1.3	0.7	0.3	0.3	0.4	0.3	1.1	0.2	0.2	0.6	0.1	0.1	1.2	1.6	0.6	3.4	2.4	1.7	0.7	3.3	0.7	2.1	11.7	2.1	0.6	2.2
Vb12.1	0.4	0.3	0.6	0.8	0.6	0.2	0.8	0.1	0.9	4.3	0.0	0.1	0.1	0.3	0.3	0.0	0.1	0.1	0.1	0.7	0.0	0.3	0.4	0.4	0.2	0.1
Vb13.1/3	0.4	0.3	0.1	0.2	0.4	0.1	0.0	0.1	0.8	0.1	0.2	0.7	0.3	0.4	0.3	0.4	0.8	0.3	0.1	0.8	0.1	0.4	0.2	0.3	0.2	0.1
Vb13.6	0.4	1.0	0.1	0.3	1.1	0.1	0.7	0.1	1.0	0.1	0.1	1.8	0.8	0.6	0.3	0.3	0.4	0.9	0.7	2.2	0.4	0.6	0.5	0.4	0.4	0.4
Vb14	0.7	0.3	4.3	0.1	0.1	0.0	0.6	0.1	0.2	0.2	0.1	0.1	1.9	3.5	1.5	0.2	0.3	0.8	1.0	1.6	1.7	1.1	8.1	2.0	0.8	1.7
Vb16	0.1	0.2	0.3	0.1	0.2	0.3	2.1	0.2	0.5	0.3	0.3	2.2	0.7	0.3	0.2	0.1	0.1	0.4	0.1	0.9	0.7	0.4	0.2	1.3	0.7	0.3
Vb17	1.2	0.8	1.3	0.5	0.0	0.1	3.5	0.9	0.9	0.8	0.2	1.4	0.8	1.4	1.5	0.2	0.5	0.9	1.8	0.8	0.5	0.6	1.6	0.9	0.9	1.4
Vb20	3.6	1.0	1.0	1.4	0.5	0.4	3.9	1.3	0.4	0.2	0.4	0.1	1.6	1.0	0.9	0.6	1.3	1.0	0.4	1.3	0.7	1.2	5.6	1.1	0.7	1.2
Vb21	1.3	1.0	0.6	0.4	1.6	1.0	1.8	0.5	0.3	0.7	2.0	1.0	1.2	1.7	1.1	0.8	0.9	2.2	0.5	1.5	0.7	1.1	3.0	2.1	0.6	1.4
Vb22	1.8	0.6	0.6	0.9	0.7	0.1	0.5	0.3	2.5	1.0	1.4	0.7	2.8	1.6	2.2	0.5	2.5	3.1	1.0	7.9	1.8	10.1	2.7	2.8	1.5	16.5

TCR usage as shown by ratios of Vβ-expressing CD8+ T cell populations between 26 HIV infected individuals and healthy controls. The blue shaded squares are minor expansions and the yellow shaded ones are major expansions.

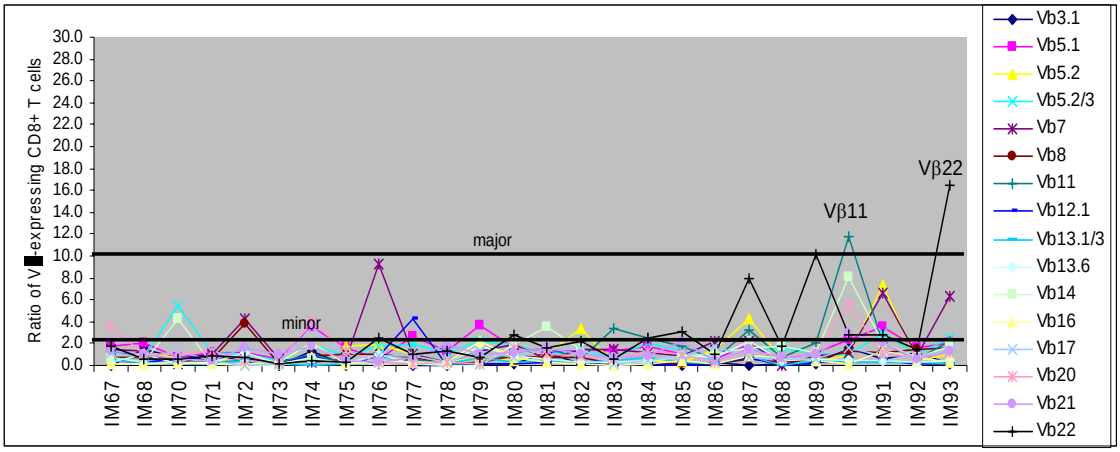


Figure 3.6: TCR usage of CD8+ T cells in 26 HIV-1 singly infected individuals. Major expansions of CD4+ T cells expressing Vβ11 and Vβ22 in two HIV/TB infected individuals (IM90 and IM91) were observed, as well as minor expansions in fourteen individuals. The horizontal blue line at the ratio of 2 indicates the cutoff for minor expansions and the red line at 10.0 indicates the cutoff for major expansions.

3.3.3 T cell receptor usage in TB singly infected individuals

Minor expansions of Vβ3.1, Vβ5.2, Vβ7, Vβ11, Vβ13.1/3 and Vβ22-expressing CD4+ T cells were observed in 6 of the 16 TB infected individuals (Table 3.6 and Figure 3.7). As shown in Table 3.7, minor expansions of Vβ3.1, Vβ5.1, Vβ5.2, Vβ5.2/3, Vβ7, Vβ8, Vβ11, Vβ13.1/3, Vβ16, Vβ17, Vβ20, Vβ21 and Vβ22 were observed in activated CD8+ T cells in 15 of the 16 individuals (94%), with no oligoclonal expansions (Figure 3.8). The frequencies of individuals showing polyclonal skewing in the CD8+ T cell compartment was significantly ($p=0.0021$) different from skewing found in the CD4+ T cell population.

Table 3.6: Ratios of frequencies of Vβ-expressing CD4+ T cells.

TCR	IM3	IM4	IM7	IM10	IM12	IM22	IM23	IM26	IM32	IM35	IM40	IM41	IM45	IM46	IM57	IM59
Vb3.1	0.2	0.1	0.2	0.2	0.7	0.4	0.7	2.5	0.3	0.3	0.4	0.1	0.3	0.2	0.7	0.1
Vb5.1	0.5	0.8	0.6	0.9	0.8	0.7	0.6	0.5	1.0	1.0	0.9	0.9	0.7	0.7	0.6	0.6
Vb5.2	0.0	0.4	0.6	1.8	0.7	0.8	4.9	1.4	0.4	0.3	0.6	0.4	0.4	0.5	0.6	0.6
Vb5.2/3	0.3	0.5	0.2	1.3	0.8	0.9	1.2	1.7	0.2	0.5	0.6	0.6	0.4	0.5	0.7	0.5
Vb7	0.5	1.1	0.5	1.4	0.8	0.6	0.4	2.3	0.4	0.3	0.6	1.2	0.8	0.3	0.5	0.2
Vb8	0.2	0.2	0.3	0.7	0.8	0.7	0.8	0.7	0.6	0.2	0.3	0.2	0.3	0.4	0.9	0.4
Vb11	0.4	0.5	0.4	0.7	0.9	0.3	0.3	0.2	0.5	0.3	0.6	0.4	0.3	2.1	0.5	0.6
Vb12.1	0.5	1.2	0.7	0.6	1.1	0.5	1.7	0.8	0.4	0.7	0.8	0.8	1.0	1.6	0.5	0.6
Vb13.1/3	0.5	1.2	0.6	0.8	0.9	3.3	1.1	0.8	0.6	0.5	0.7	1.0	0.8	0.7	0.4	3.8
Vb13.6	0.6	1.0	0.5	0.6	0.6	0.5	0.8	1.0	0.7	0.7	0.9	0.9	0.8	0.7	0.6	0.5
Vb14	0.4	0.3	0.2	0.4	0.9	0.4	0.4	0.5	0.6	0.3	0.3	0.3	0.3	0.3	0.4	0.4
Vb16	0.8	0.6	0.5	1.3	0.7	0.9	0.8	0.9	1.3	1.0	0.9	1.0	0.9	0.6	0.8	0.9
Vb17	0.6	0.8	0.4	1.0	0.8	0.6	0.7	1.1	0.9	0.5	0.6	0.8	0.9	0.7	0.7	0.6
Vb20	0.5	1.1	0.5	1.3	1.0	0.7	1.3	0.1	0.6	0.3	0.8	0.9	1.3	0.5	0.7	0.7
Vb21	0.7	1.1	0.6	0.8	0.9	0.6	1.0	1.5	0.7	0.5	1.8	0.7	0.7	0.8	0.7	0.4
Vb22	1.2	2.4	1.1	1.1	1.1	1.1	1.2	1.3	0.8	1.4	1.4	1.5	1.5	1.1	0.7	0.6

TCR usage as shown by ratios of Vβ-expressing CD8+ T cell populations between 16 TB infected individuals and healthy controls. The blue shaded squares are minor expansions.

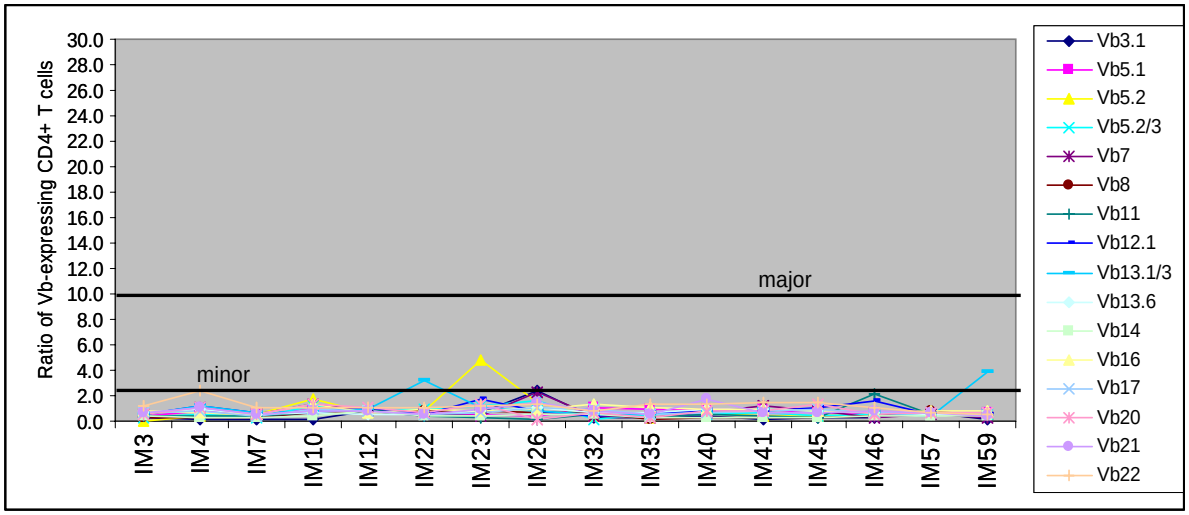


Figure 3.7: TCR usage of CD4+ T cells in 16 TB singly infected individuals. Expansions of CD4+ T cells-expressing Vβ3.1, Vβ5.2, Vβ7, Vβ11, Vβ13.1/3 and Vβ22 were observed in sixteen individuals. The horizontal line at the ratio of 2 indicates the cutoff for minor expansions and the red line at 10.0 indicates the cutoff for major expansions.

Table 3.7: Ratios of frequencies of Vβ-expressing CD8+ T cells.

TCR	IM3	IM4	IM7	IM10	IM12	IM22	IM23	IM26	IM32	IM35	IM40	IM41	IM45	IM46	IM57	IM59
Vb3.1	0.6	0.3	2.0	0.1	1.8	1.4	0.5	0.6	0.1	0.2	0.6	0.0	0.2	0.2	1.0	0.2
Vb5.1	1.6	1.8	1.2	0.4	1.4	0.4	0.3	2.0	0.8	0.7	2.0	1.1	0.6	0.9	0.9	2.3
Vb5.2	0.0	1.3	2.7	0.4	0.5	0.5	2.3	0.9	0.8	0.5	0.0	1.1	0.3	2.0	0.7	1.1
Vb5.2/3	1.0	0.9	1.8	3.2	0.4	1.7	0.4	0.7	2.1	1.3	1.0	0.8	1.6	0.6	2.5	0.8
Vb7	4.4	0.5	0.8	0.7	0.5	0.6	0.2	1.0	0.2	0.8	1.8	1.9	1.7	0.1	0.3	0.5
Vb8	1.2	2.6	1.9	0.4	0.9	0.3	0.6	0.7	0.5	0.7	1.4	0.9	1.1	1.0	0.9	1.2
Vb11	0.6	1.5	0.9	0.9	1.6	0.2	0.5	0.5	0.3	0.2	1.7	0.5	0.1	8.5	1.1	1.4
Vb12.1	0.2	0.3	0.2	0.2	0.1	0.7	0.2	1.4	0.2	0.0	1.8	0.0	0.0	0.0	0.2	0.4
Vb13.1/3	0.6	1.1	1.8	5.3	0.7	0.6	0.5	0.7	0.2	0.5	1.2	0.9	2.0	0.4	0.3	2.3
Vb13.6	0.4	0.5	0.6	0.1	0.3	0.1	0.1	0.4	0.1	0.7	0.9	0.3	0.2	0.2	0.2	0.3
Vb14	0.1	0.5	0.5	0.5	0.1	0.1	0.1	0.2	0.2	0.6	0.9	0.3	0.2	0.1	0.3	1.0
Vb16	0.2	0.2	0.2	0.2	0.3	0.3	0.1	0.2	3.2	2.0	0.3	0.7	1.9	0.2	0.3	0.2
Vb17	0.5	1.0	0.6	2.0	0.6	0.8	0.8	0.8	1.3	2.1	1.9	4.5	0.9	0.4	0.5	1.2
Vb20	0.5	2.7	7.5	0.5	1.2	0.4	1.4	0.6	0.7	1.6	1.5	0.9	1.1	0.6	0.9	0.9
Vb21	0.8	1.8	1.0	0.5	1.5	0.6	1.6	0.7	0.5	0.8	3.7	0.7	2.6	0.7	0.5	0.6
Vb22	1.1	3.8	1.7	0.6	2.2	0.5	1.6	1.3	0.4	2.7	1.9	0.9	0.5	1.3	0.6	0.9

TCR usage as shown by ratios of Vβ-expressing CD8+ T cell populations between 16 TB infected individuals and healthy controls. The blue shaded squares are minor expansions.

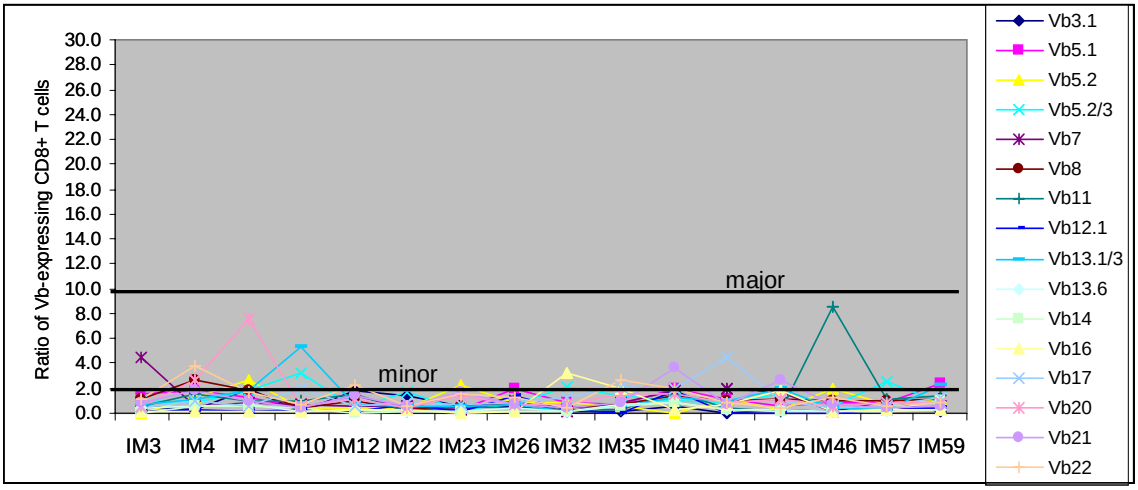


Figure 3.8: TCR usage of CD8+ T cells in 16 TB singly infected individuals. Minor expansions of CD8+ T cells-expressing Vb3.1, Vβ5.1, Vβ5.2, Vβ5.2/3, Vβ7, Vβ8, Vβ11, Vβ13.1/3, Vβ16, Vβ17, Vβ21 and Vβ22 were observed in sixteen individuals. The horizontal line at the ratio of 2 indicates the cutoff for minor expansions and the line at 10.0 indicates the cutoff for major expansions.

3.4 DISCUSSION

Viral and bacterial antigens evoke T cell immunity which results in activation, proliferation and expansion of T cells bearing specific TCR (Fujiwara *et al*., 2005; Jordan *et al*., 2006; Jassoy *et al*., 1993). Findings in this dissertation have demonstrated elevated absolute CD8+ T cell counts and enhanced activation of CD8+ T cells in HIV-1 and HIV/TB co-infected patients, which were associated with expansions of CD8+ T cells expressing various Vβ T cell receptors as seen in this chapter. It has also been shown that multiple TCR, as measured by polyclonal skewing, were significantly used by CD8+ T cells rather than CD4+ T cells in two groups: HIV/TB co-infected and HIV-1 single infected. No significant skewing in either CD4+ or CD8+ TCR usage was observed in TB single infection. These data would suggest that a) either CD8+ T cells play an active role in the immune response to HIV with or without TB co-infection or b) that expanded CD4+ T cells reside elsewhere rather than in the peripheral circulation. It has been previously reported in HIV infection that expanded circulating CD8+Vβ+ T cells mediates HIV-specific cytotoxicity (Pantaleo *et al*., 1994).

Previous reports have determined the existence of skewing/perturbations of the T cell repertoire in HIV infected children and adults using flow cytometry (McFarland *et al*., 2002;, Halapi *et al*., 1996; Piltch *et al*., 2000; Soudeyns *et al*., 2002; Dalgleish *et al*., 2002), and considerable oligoclonality of CD8+ T cells have been shown to occur and that they persist frequently due to antigen stimulation by persistent viruses (Wilson *et al*., 1998). In agreement with these findings, oligoclonal expansions of Vβ11, Vβ20, and Vβ22 were observed in HIV-1 infected individuals as well as in dual infection with HIV

and TB. Although a more quantitative approach to this analysis was applied, limited TCR usage observed in HIV-1 single infection in this study may indicate a more focused recognition of HIV-1 epitopes. A more refined analysis of limited TCR repertoire in HIV-1 infection has been shown (Kalams *et al*., 1994), where these authors observed that a high degree of HIV-1 specific CTL activity may be due to oligoclonal expansion of specific effector cells and that this limited TCR diversity against immunodominant epitopes may limit recognition of mutated viral variants in regions interacting with the TCR and therefore allow productive viral infection.

Although there were significant minor expansions of various TCR usage on CD8+ T cells within groups, there was no evidence of a common pattern of TCR usage between individuals in the same cohort group in this study. This may not be surprising as multiple epitopes would be restricted by the various HLA types present in these cohorts, although these possibilities were not measured. However, oligoclonal expansion of Vβ11 TCR families was observed in 3 HIV/TB infected patients and Vβ22 TCR families in 2 HIV-1 infected individuals..

Analysis of the T cell repertoire has been performed extensively in HIV-1 infected individuals, but there is little data on the same type of analysis in patients singly or dually infected with TB. This dissertation has determined that CD8+ TCR Vβ+ T cell perturbations in the presence of single and dual infection with TB and HIV-1 are significantly more frequent than CD4+ TCR Vβ+ usage and that TB likely result in expanded peripheral blood circulating CD8+ T cell clones.

CHAPTER 4

THE T CELL RECEPTOR (TCR) REPERTOIRE IN HIV-1 UNINFECTED NEONATES BORN TO HIV-1 INFECTED MOTHERS

4.1 INTRODUCTION

In the previous chapter this dissertation explored TCR usage by CD8+ T cells using flow cytometry in adults infected with HIV and TB. This chapter investigates the TCR repertoire in another cohort of HIV-1 exposed uninfected infants to assess TCR usage without infection. Paediatric HIV-1 infection is largely acquired via transmission from the HIV-1 infected mother and there is a high probability that infants born to HIV infected mothers have been exposed to HIV and antigens in-utero. This has been shown through several studies as indicated by immune activation (Kuhn *et al.*, 2002; Clerici *et al*. 2000), HIV–positive PCR (Vazquez *et al*., 2006; De Andreis *et al*., 1996) and the presence of HIV-specific CD4+ and CD8+ T cell responses (Legrand *et al*., 2006; Kuhn *et al*., 2001).

It has been reported that children, even during the first year of life, are able to mount functional immune responses as indicated by flow cytometry (ICS) and the IFN-γ ELISPOT assay (Feeney *et al*., 2005; Legrand *et al.,* 2006; Kuhn *et al.,* 2001). Previous studies have demonstrated transient TCRVβ –expansions of CD8+ T cells in HIV-1 infected children and infants (Halapi *et al.*, 1996; Silvestri *et al*., 1996) and at lower

levels in uninfected children born to HIV-1 infected mothers (Soudeyns *et al*., 2000). Apart from measuring the TCR using a repertoire of monoclonal antibodies, the repertoire can also be analyzed based on CDR3 length variations within Vβ gene families, a method referred to as the TCR immunoscope (Pannetier *et al*., 1995, Pilch *et al*., 2000). The immunoscope technique was used in this dissertation to a) assess the maturity of the TCR repertoire in newborn infants and b) show evidence of TCR engagement in-utero by analysing cord blood mononuclear cells (CBMC) isolated at birth from babies born to HIV-1 infected mothers. It was hypothesized that TCR repertoire skewing is a marker of T cell priming in-utero as a result of exposure to HIV-1-specific antigens from the mother.

4.2 MATERIALS AND METHODS

4.2.1 Study cohort

The cohort used in this dissertation was recruited from Coronation Women and Children's Hospital in Johannesburg. Peripheral blood and cord blood samples were obtained from 20 HIV-1 infected mothers. The mother and cord/baby samples were analyzed as pairs. The mothers received a single dose of Nevirapine at birth. Twenty-five HIV-1 uninfected babies and 10 uninfected mothers were included in this cohort as negative controls. The HIV status of the mothers was determined by ELISA. For the babies, heel prick blood samples were deposited onto Whatman filter paper as dried blood spots and used for the detection of HIV-1 by polymerase chain reaction (PCR). In order to establish existence of other common congenital infections, ELISA assays were

performed for detection of Syphilis, Toxoplasma, Rubella and Cytomegalovirus on both mothers and babies.

4.2.2 Immunoscope experimental procedure

RNA was extracted from cryopreserved cord blood mononuclear cells (CBMC) (see 4.2.3). cDNA was synthesized (see 4.2.4) and amplified by PCR (see 4.2.5) using primers for each of the 23 Vβ families (list is shown in Appendix 3). Amplification was enabled by labelling the 3' end of the TCR gene with a Cβ (constant region) primer and the 5' end was labelled with a specific Vβ primer (Inqaba Biotechnical Industries, Hatfield, South Africa). A schematic diagram of a TCR gene with three different regions is shown on Figure 1.8 (chapter 1), which is amplified and analysed to obtain fragments of various Vβ genes. The PCR products were processed in a run-off PCR reaction using a fluorochrome labelled (blue 6-FAM) primer, purified using DyeEx resin columns and acquired on the ABI sequencer. Fragment sizes and peak heights were then analysed using Applied Biosystems Genemapper software. Figure 4.1 shows the experimental design of the immunoscope procedure.

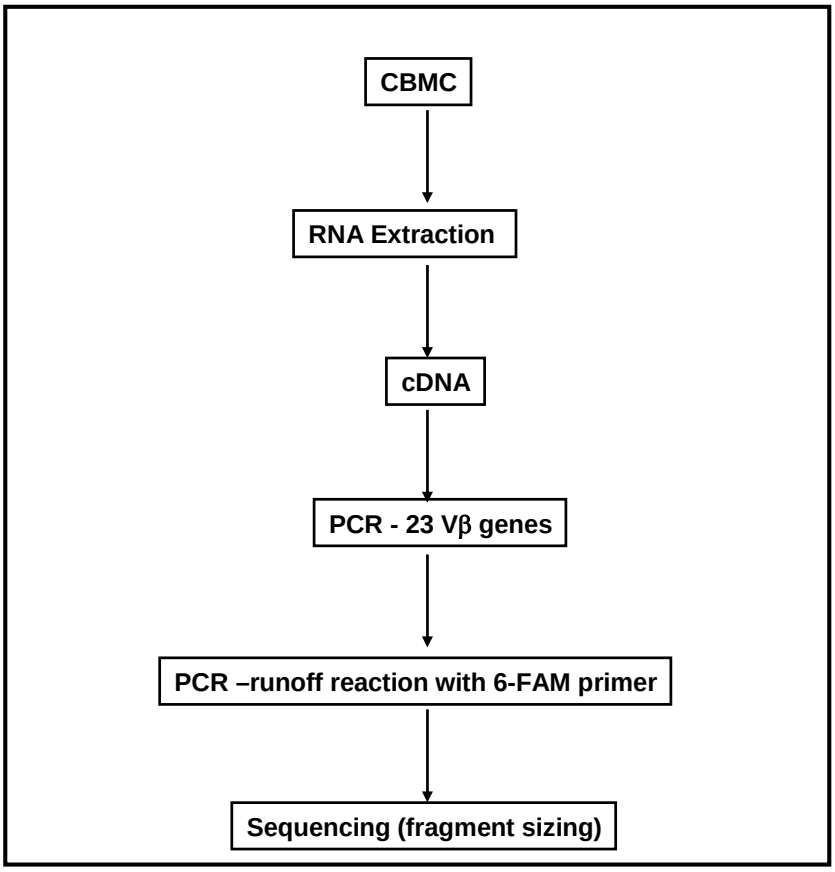


Figure 4.1: The immunoscope experimental design

4.2.3 RNA extraction

Cord and peripheral blood was collected in ACD-anticoagulated tubes and mononuclear cells were isolated using the standard Ficoll Hypaque method (AEC Amersham) and stored in liquid nitrogen. When required, cells were quick-thawed in a waterbath at 37^{o}C and using a sterile 1ml pipette, the cells were transferred to a sterile 5ml snap-cap tube. An aliquot of 20µl of the cell suspension was removed and counted using trypan blue exclusion using the haemocytometer. The cells were then centrifuged at 1400 rpm for 10

minutes. The supernatant was decanted and the cells were suspended in 1ml of Trizol (Invitrogen Life Technologies) lysis reagent which lysed the cells and enabled mRNA to be isolated. The RNA suspension was then transferred into 1.5 microfuge tubes. RNA was either extracted directly or Trizol suspensions were stored at –80^{o}C for batch isolation. Chloroform isoamyl alcohol (200µl)(Sigma-Aldrich) was added to the RNA suspension, incubated for 15 minutes at room temperature after vigorous shaking for 10 seconds and centrifuged at 12000 rpm for 15 minutes. Isopropanol (500µl) was added, followed by gentle vortexing and incubated for 10 minutes at room temperature. The tubes were centrifuged at 12000 rpm for 10 minutes in the cold room (4^{o}C). Ethanol (1ml) was added and the tubes were centrifuged at 15000 rpm for 3 minutes. The RNA pellet was suspended in 20µl of sterile diethylpyrocarbonate (DEPC)-treated water and incubated at 56^{o}C for 10 minutes. RNA (1µl) was diluted 1/100 in DEPC water and optical density (OD) readings were performed on a spectrophotometer (WPA Lightwave, Labotec) and the RNA concentrations were calculated to make a final concentration of 1µg/µl using the following formula:

RNA concentration = A260 x dilution (100) x 40 x volume

The RNA was then stored at –80^{o}C until ready to synthesize cDNA. The purity of RNA was visualized by loading a fraction of RNA on a 1% agarose gel, and examined for sharp 28S and 18S bands (Preparation of RNA gel is shown in Appendix 2).

4.2.4 cDNA synthesis

RNA (10µl) at an optimal concentration of 1µg/µl was added to 0.6 ml microfuge tubes (Eppendorf) after thawing on ice. 10mM dNTP (2µl 10mM) (Roche Diagnostics, South Africa) and Oligo-dT (2µl) was added and incubated at 70^{o}C for 10minutes and placed on ice for > 1 minute and centrifuged for 5-10 seconds. RT buffer (4µl) (Roche), RNAse inhibitor (1µl) and AMV-RT (1µl) were added and incubated for 1 hour at 42^{o}C. The mixture was then placed on ice for > 1 minute and spun for 5-10 seconds. cDNA samples were subsequently stored at –20^{o}C until they were used in the PCR.

4.2.5 Polymerase Chain Reaction (PCR)

A set of primers (5µl, 2.5mM) corresponding to each Vβ family were added to each of 24 tubes per sample. Details of the PCR mix used are given in Appendix 4 and 20µl was distributed, along with each primer, into each of the tubes plus a positive cell control (Jurkat cell line known to express VB8, supplied by Highveld Biological, South Africa) and a positive cDNA control (GAP-DH, Roche). The tubes were then placed on a thermocycler (GeneAmp PCR System 9700, Applied Biosystems) and the mixture was subjected to 40 cycles of denaturation for 25 seconds at 94^{o}C, annealing for 45 seconds at 60^{o}C and elongation for 45 seconds at 72^{o}C with a extended final elongation step of 5 minutes at 72^{o}C. Products were visualized on a 2% agarose gel (Sigma-Aldrich) in 10X TBE using Ethidium Bromide (Sigma-Aldrich) staining before using 2µl of the amplified product for a run-off reaction with the 6 Fam labeled HuβC2 primer. Preparations of 10X TBE, agarose gel and gel loading buffer are shown on Appendix 5. The gel was then

run at 230 volts using the power pack and photographed on a UVP-White/UV transilluminator (Vacutec, South Africa). Figure 4.2 shows a photograph of a gel for 23 Vβ products, including the Jurkat cell line as a positive control. Final DNA products appear as single white bands.

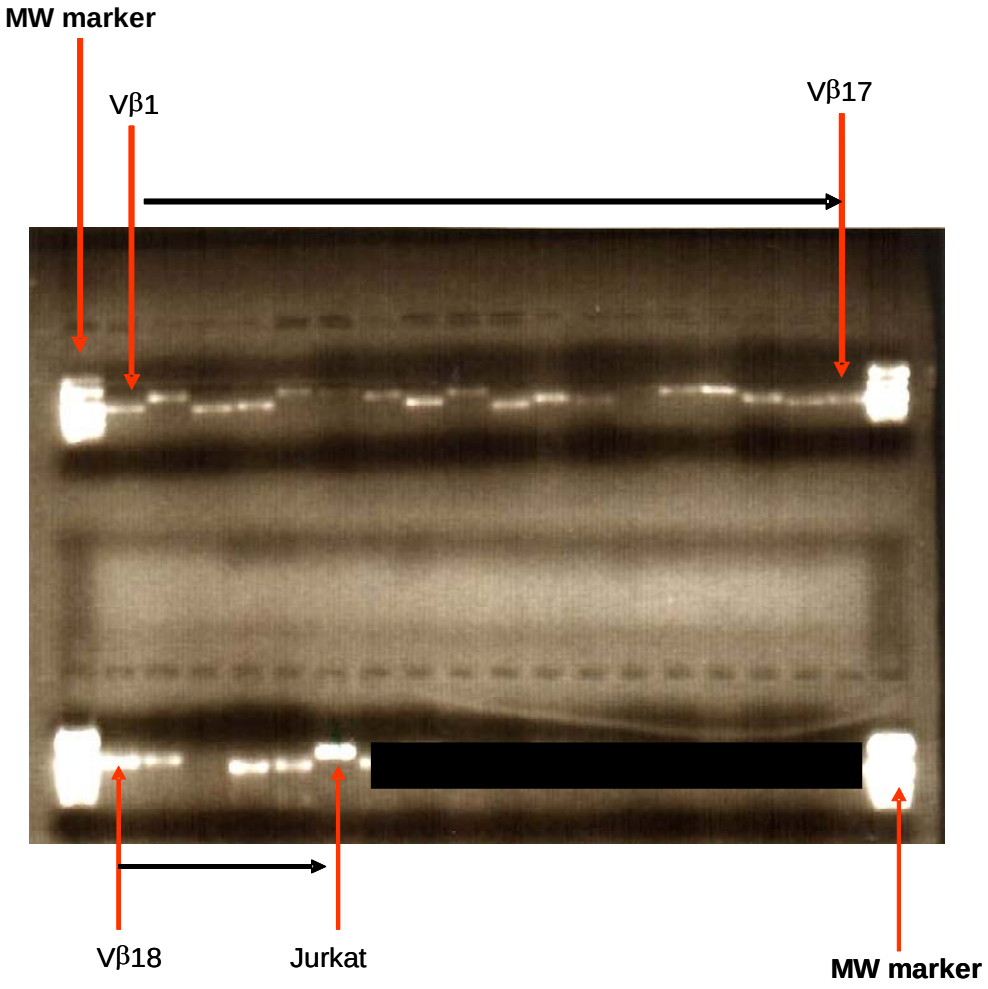


Figure 4.2: DNA gel showing the molecular weight (MW) control marker, 23 Vβ genes and a Jurkat cell line as a positive control. cDNA for each of the 23 Vβ T cell receptor genes was amplified and the PCR products were run on 2% agarose gel. The bands appearing on top are showing PCR products for Vβ1, Vβ2, Vβ3, Vβ4, Vβ5, Vβ6a,Vβ6B, Vβ7, Vβ8, Vβ9, Vβ10, Vβ11,Vβ12,Vβ13a,Vβ14,Vβ15,Vβ16 to Vβ17 as shown by the arrow. The bottom row consists of PCR products for Vβ18, Vβ20, Vβ21, Vβ22, Vβ23 and the Jurkat cell line.

4.2.6 Polymerase chain run-off reaction for immunoscope analysis

A PCR run-off reaction was performed using 24 PCR products. Reactions were prepared in 0.6µl microfuge tubes. The PCR mixture details are shown in Appendix 6. Each PCR product (2µl) and 8µl of PCR mixture were added into each tube and placed on the Applied Biosystems thermocycler and subjected to 94^{o}C for 2 minutes, 5 cycles of 94 ^{o}C for 25 seconds; 60 ^{o}C for 45 seconds; 72 ^{o}C for 45 seconds and to a cycle of 72 ^{o}C for 3 minutes, followed by holding at 4 ^{o}C. Samples were stored at 4^{o}C until they were purified for fragment analysis.

4.2.7 Purification of run-off PCR products

Products were purified using Performa DTR 96 well standard plate resin columns (Southern Cross Biotechnology, SA). The resins are gel filtration plates consisting of 0.8µl volume columns in a standardized array, packed with gel matrix optimized to effectively remove dye terminator dNTPs, salts and other low molecular weight materials from the sequencing reactions. Reactions were performed in 0.2µl microfuge tubes on strips (Roche Diagnostics). Product volumes were increased by addition of 10µl of sterile water to 20µl in each of the 24 Vβ product tubes. The top and bottom adhesive tapes from the Performa plates were removed. The plates were covered with a lid, stacked on top of an empty plate and centrifuged at 850xg for 3 minutes. The eluate was discarded and the complete volume of each product was transferred to the purification plate using a multichannel pipette. The plates were stacked on top of a sterile 96 well receiver plate and centrifuged at 850xg for 3 minutes. The eluates (products) in the receiving plates were retained for sequencing. The remaining product was stored at –20^{o}C.

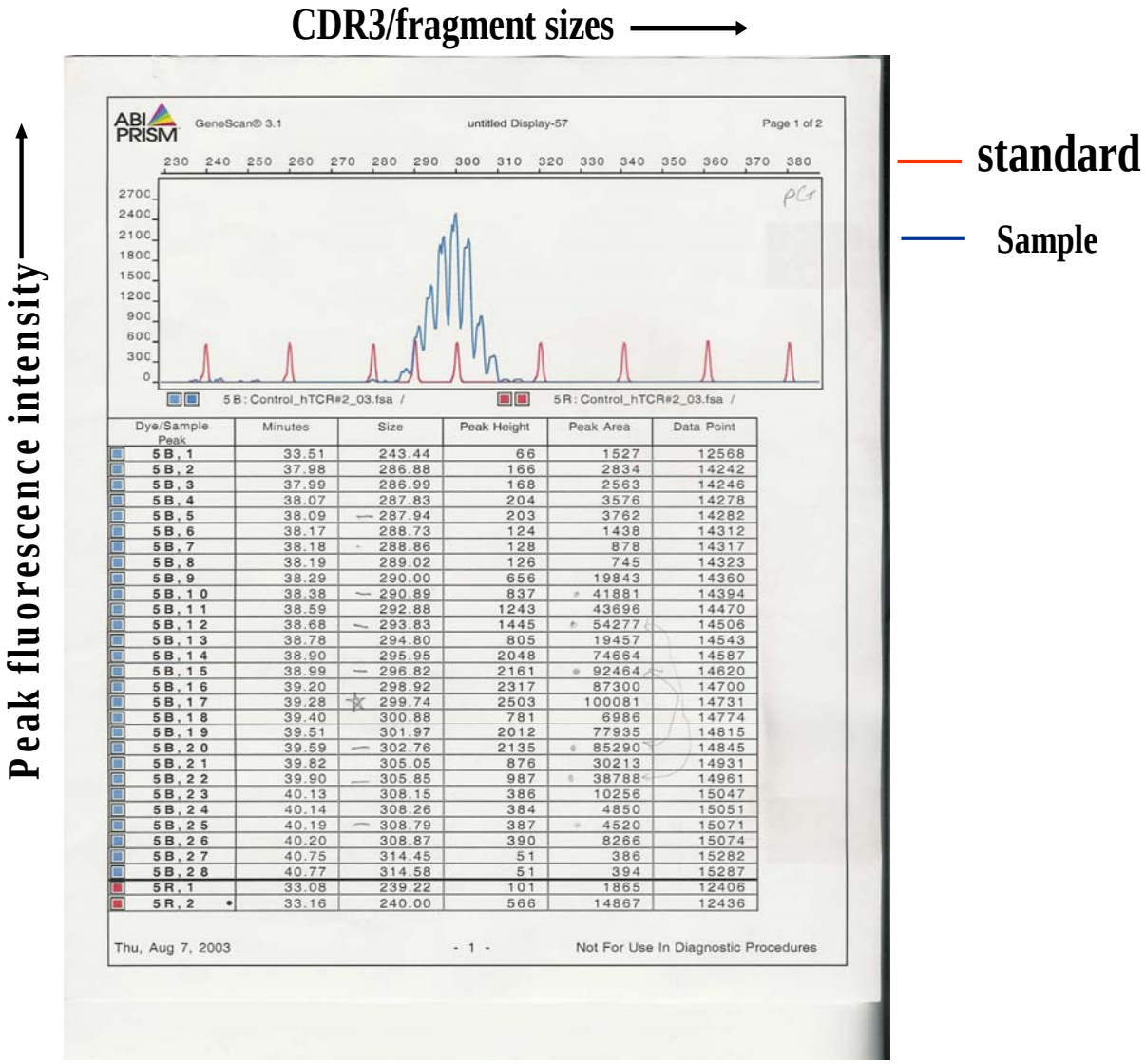
4.2.8 Preparation of product and sequencing acquisition

Dye–Ex formamide (1ml) (Applied Biosystems) was mixed with 5µl of ROX 400 Standard (Applied Biosytems). This mixture (5µl) and 5µl of purified product was added to the 96 well sequencer plate (Applied Biosystems). The mixture was heated up at 90^{o}C for 2 minutes and allowed to cool on ice. The samples were acquired on a 3100 ABI Genetic analyzer (Applied Biosytems) using a 3100 Data Acquisition Software with the running module described on Appendix 7, using a 50cm array length and POP6. The instrument equipped with a laser provides the peak fluorescence intensity (height), peak size and peak area. The data was automatically stored in the relevant folder for analysis.

4.2.9 Gene fragment analysis using Genemapper software

Data was analyzed using Genemapper software (Applied Biosystems). Standard peaks appeared red and the sample peaks appeared blue as per fluorescent dye used. The sample peak sizes, lengths and areas were determined by comparison with the ROX 400 standard. A representative snapshot of peaks and data table is shown on Figure 4.3. The expected highest peak size were determined by measurements of the size of the Vβ primer length to residue 95 of the gene (i.e. the beginning of the CDR3-which lies between residue 95 and 106), plus the Cβ2 constant gene distance to residue 106 (73), plus the CDR3 length of 30 nucleotides (10 amino acids)**.** The highest peak was expected to be 10 amino acids (aa) in length and subsequent peaks to be 3aa apart with the CDR3 length of 9aa and other peaks following in descending order of variable CDR-3 lengths indicated by various fragments. Figure 4.3 shows a representative example of the immunoscope graphs for Vβ2 with sample peaks (blue) measured against the standard

(red). The peak sizes, heights and areas are shown in the table below the graph and the size of the expected highest peak is 299.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
5B,1	33.51	243.44	66	1527	12568
5B,2	37.98	286.88	166	2834	14242
5B,3	37.99	286.99	168	2563	14246
5B,4	38.07	287.83	204	3576	14278
5B,5	38.09	287.94	203	3762	14282
5B,6	38.17	288.73	124	1438	14312
5B,7	38.18	288.86	128	878	14317
5B,8	38.19	289.02	126	745	14323
5B,9	38.29	290.00	656	19843	14360
5B,10	38.38	290.89	837	41881	14394
5B,11	38.59	292.88	1243	43696	14470
5B,12	38.68	293.83	1445	54277	14506
5B,13	38.78	294.80	805	19457	14543
5B,14	38.90	295.95	2048	74664	14587
5B,15	38.99	296.82	2161	92464	14620
5B,16	39.20	298.92	2317	87300	14700
5B,17	39.28	299.74	2503	100081	14731
5B,18	39.40	300.88	781	6986	14774
5B,19	39.51	301.97	2012	77935	14815
5B,20	39.59	302.76	2135	85290	14845
5B,21	39.82	305.05	876	30213	14931
5B,22	39.90	305.85	987	38788	14961
5B,23	40.13	308.15	386	10256	15047
5B,24	40.14	308.26	384	4850	15051
5B,25	40.19	308.79	387	4520	15071
5B,26	40.20	308.87	390	8266	15074
5B,27	40.75	314.45	51	386	15282
5B,28	40.77	314.58	51	394	15287
5R,1	33.08	239.22	101	1865	12406
5R,2	33.16	240.00	566	14867	12436

Figure 4.3: A representative immunoscope analysis graph of Vβ2-TCR showing the peaks for the standard (red) and the peaks for the sample (blue). The table is showing the peak lengths, areas and sizes. Each peak represents a Vβ2-T cell clone with a specific CDR3 length. The highest peak has a CDR3 length of 10aa.

4.2.10 Interpretation of the immunoscope results

To quantitate Vβ-CDR3 length variability, and hence a measure of TCR skewing, the following formulas were used for *Polyclonal Gaussian (PG)*, *Polyclonal Skewed (PS)* and *Oligoclonal Skewed (OS)* distributions (representative examples):

$$\%\ PG = \frac{\text{number of PG V}\beta\text{ families} \times 100}{\text{Total V}\beta\text{ analyzed (23)}}$$

and

$$\%\ PS = \frac{\text{number of PS V}\beta\text{ families} \times 100}{\text{Total V}\beta\text{ analyzed (23)}}$$

and

$$\%\ OS = \frac{\text{number of OS V}\beta\text{ families} \times 100}{\text{Total V}\beta\text{ analyzed (23)}}$$

"No skewing" of the T cell repertoire is measured as a ***Polyclonal Gaussian (PG)*** **distribution**, which is observed in naïve T cells prior to antigen engagement; a ***Polyclonal Skewed (PS)*** distribution which is observed after a T cell response resulting in multiple antigen-specific T cell clones; ***Oligoclonal Skewed (OS)*** distribution which is observed when there has been a clonal expansion of a T cell recognizing antigen using a specific TCR. Examples of these peak interpretations and CDR3 distributions are shown on Figure 4.4, where each of the PG, PS and OS are shown:

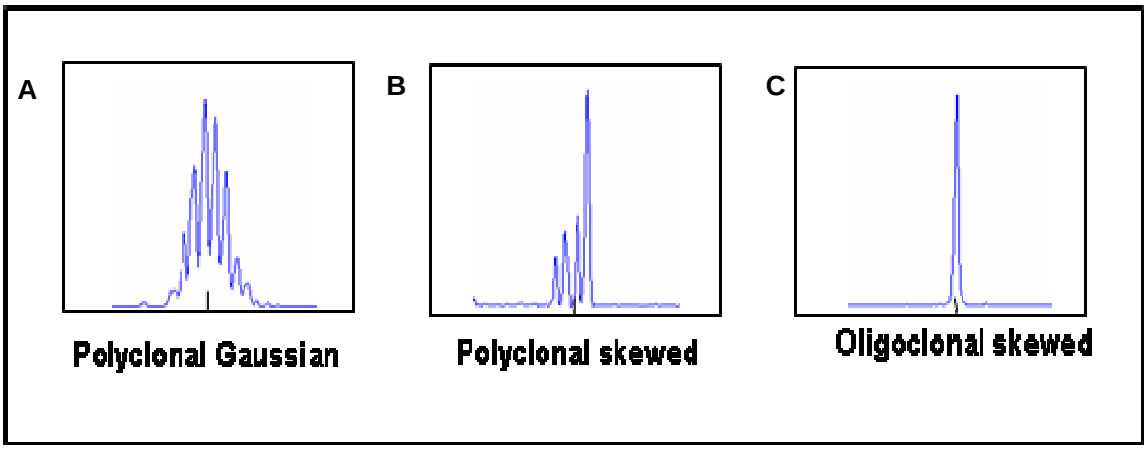


Figure 4.4: Immunoscope analysis showing polyclonal Gaussian (A), polyclonal skewed (B) and oligoclonal skewed (C) distribution observed in peripheral blood mononuclear cells.

4.3 RESULTS

To ensure that optimal peaks were detected, several optimization experiments were completed prior to applying the technique to the cohort samples.

4.3.1 Optimization of the PCR run-off reaction

In order to obtain optimal conditions for the run off-PCR reaction, various conditions and reagent titrations were performed. The Cβ2 primer in the PCR reaction mixture was used at three different volumes which were identified as ***Mix 1*** (0.12µl), ***Mix 2*** (0.24µl) and ***Mix 3*** (0.48µl). Volumes of reagent used for different PCR reaction mixtures are shown on table 4.1. In addition to these, each PCR mixture was combined with 2µl ***(Condition 1)***, 4µl ***(Condition 2)*** and 6µl ***(Condition 3)*** of the PCR product for amplification used at 8µl, 6µl and 4µl consecutively. Conditions 1, 2 and 3 were applied as indicated in Figure 4.5, where condition 1 consists of 2µl of PCR product combined with PCR mixture 1, 2

and 3; condition 2 consists of 4µl of PCR product combined with mix 1A, 2A and 3A and condition 3 consists of 6µl of PCR product mixed with PCR mixtures mix 1B, 2B and 3B. Three representative Vβ-TCRs were analysed under these conditions and the graphs are shown on Figure 4.5 (Vβ1,Vβ3 and Vβ 5). Graphs A, B and C are showing data for various conditions and the immunoscope peak heights/signals from mix 1, mix 2 and mix 3 were the highest, when 4µl of the PCR product was used (Condition1). The Cβ2 primer was finally used in all reaction mixtures at 0.12µl **(*MIX 1)*** and the final volume of PCR product used was 4µl ***(condition 2).***

Table 4.1: PCR reaction mixtures used for optimization of the PCR-run off reation

Reagent	**Mix 1**	**Mix 2**	**Mix 3**
Buffer	1.1µl	1.1µl	1.1µl
dNTPs	1.1µl	1.1µl	1.1µl
$MgCl_2$	1.32µl	1.32µl	1.32µl
Cβ2 primer	0.12µl	0.24µl	0.48µl
H_2O	5.12µl	5.0µl	4.76µl
Taq polymerase	0.04µl	0.04µl	0.04µl

Volume of mix = 8.8µl

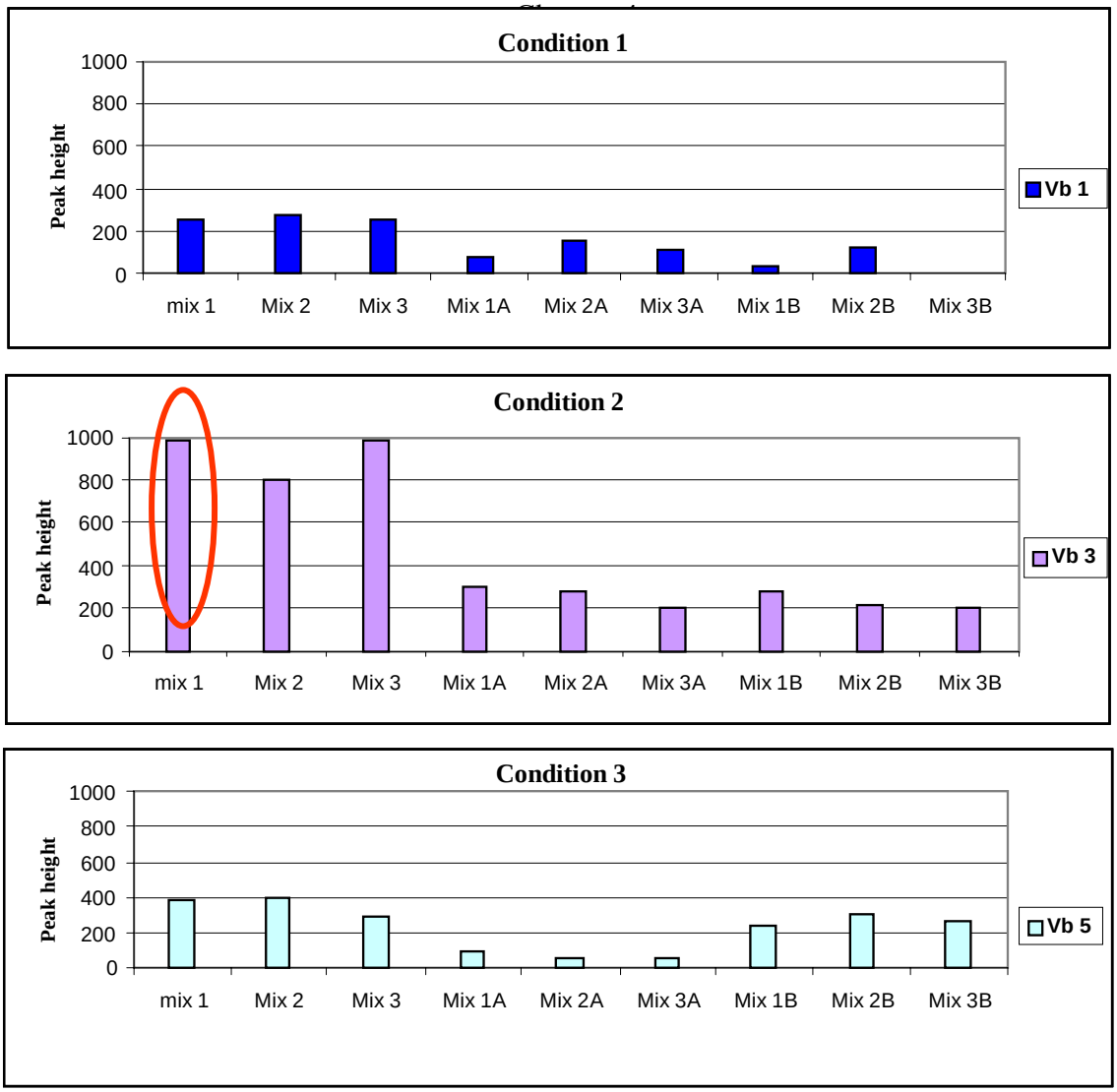


Figure 4.5: Optimization results for titration of the 6-Fam labeled Cβ2 primer and different product volumes used in a PCR run-off reaction. The CB2 primer was titrated and used in three different concentrations in the PCR reaction mixtures. The Cβ2 primer in the PCR reaction mixture was used at three different volumes which were identified as Mix 1 (0.12µl), Mix 2 (0.24µl) and Mix 3 (0.48µl). In addition to these, each PCR mixture was combined with 2µl (Condition 1), 4µl (Condition 2) and 6µl (Condition 3) of the PCR product at 8µl, 6µl and 4µl of consecutively. Condition 1 consists of 2µl of PCR product combined with PCR mixture 1, 2 and 3; condition 2 consists of 4µl of PCR product combined with mix 1A, 2A and 3A and condition 3 consists of 6µl of PCR product mixed with PCR mixtures mix 1B, 2B and 3B. the final reaction mixture used in all PCR reaction mixtures is highlighted in 'Red'.

4.3.2 Vβ TCR usage in cord blood samples of neonates born to HIV-1 infected mothers– A measure of exposure to HIV-1 in-utero.

Having optimized the method, the immunoscope technique was used to obtain a measure of the diversity of the T cell repertoire in a scenario of possible antigen exposure. The CDR3 length distributions were analyzed by assessing the proportions of polyclonal Gaussian (PG), polyclonal skewed (PS) and oligoclonal skewed (OS) profiles from the 20 HIV-1 babies born to HIV-1 infected mothers; 25 healthy control babies; 12 HIV-1 infected mothers, and 10 HIV healthy control mothers.

A representative Vβ TCR distribution from a healthy control baby showed a polyclonal Gaussian distribution for each family (Figure 4.6). A further representative Vβ distribution from an HIV-1 exposed (HIV uninfected) baby showed evidence for a polyclonal skewed distribution of almost every Vβ family (Figure 4.7). Each panel represents the results of the amplified PCR products from individual TCR Vβ families (Vβ1- Vβ23) and the 24th panel represents the Vβ8 PCR–amplified product of the Jurkat cell line (cDNA control). Overall, the Vβ TCR distribution profile observed in healthy control babies were 46% polyclonal Gaussian and 54% polyclonal skewed, with zero oligoclonal skewing (see table in Appendix 8). The polyclonal skewing observed in healthy control babies may be expected due to in utero exposure of multiple antigens derived from the mother. However, these data may differ from what was reported previously in other studies, where no clonal dominance was observed in healthy cord blood (147) (Than *et al.*, 1999). Conversely to that found in the healthy controls, the TCR distribution measured in babies born to HIV infected mothers (and presumed

exposed in utero) showed 1% polyclonal Gaussian distribution, 72% polyclonal skewed and 27% oligoclonal skewed (see Appendix 9), as shown in Figure 4.8. The TCR repertoire of babies born to HIV-1 infected mothers was highly skewed as shown by a statistically higher proportion of oligoclonal skewing ($p < 0.05$) and a lower proportions of polyclonal Gaussian ($p<0.001$) when compared to healthy control babies (Figure 4.8). There was no difference between the distribution of polyclonal skewed TCR between healthy and "exposed" babies and the high degree of oligoclonality in the latter group suggests engagement of the TCR and clonal expansion. It is tempting to suggest that this may be due to engagement with antigens derived from HIV in these babies. The oligoclonal expansion of T cells in babies born to HIV-1 infected mothers were mainly Vβ18, Vb23, Vβ5, Vβ6a, Vβ7, Vb 6b and Vβ23-families (Figure 4.9), which had five to seven out of of twenty babies showing oligoclonal skewing of the repertoire.

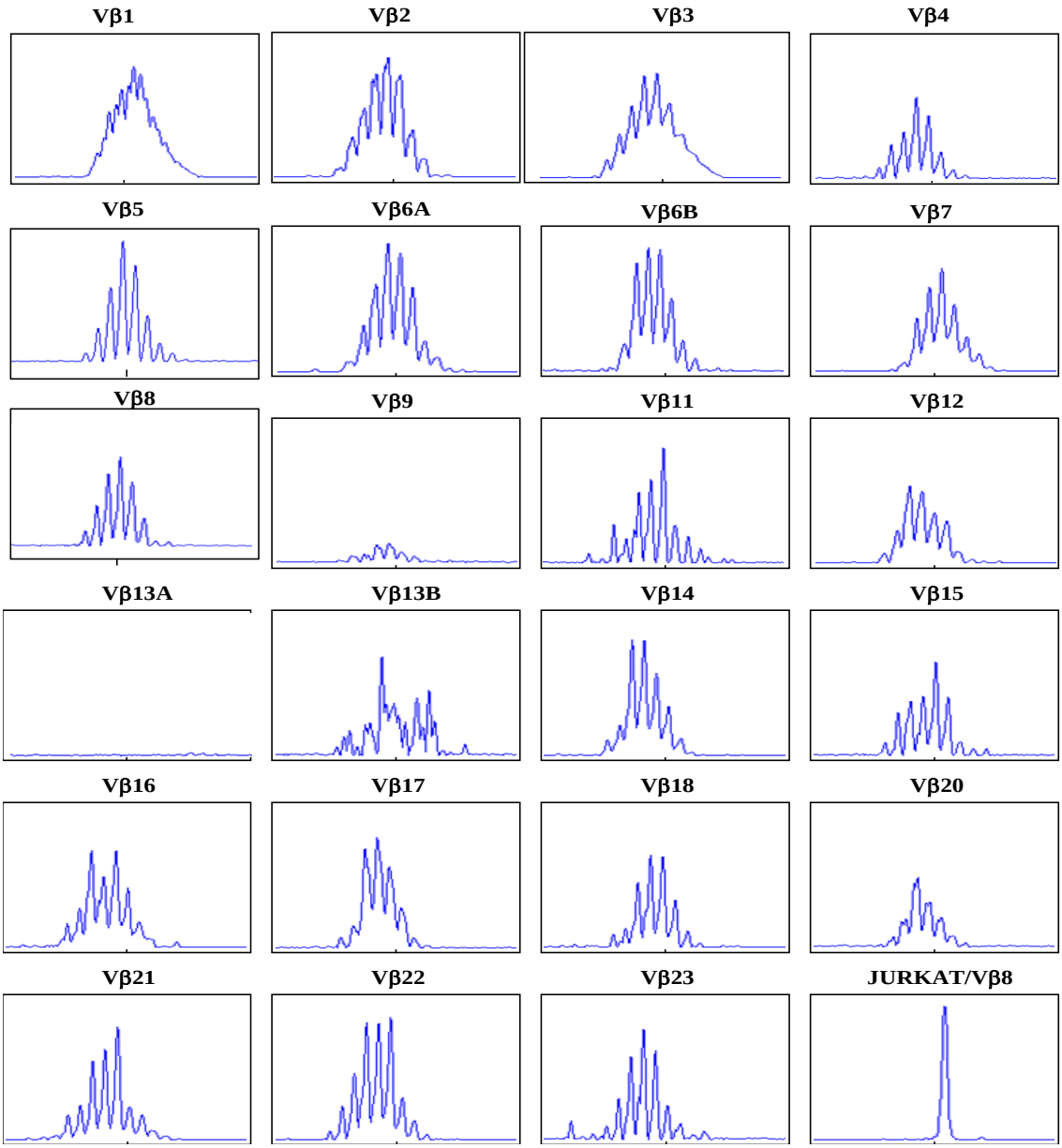


Figure 4.6: Immunoscope profile of a normal control baby, appearing as 100% Gaussian distribution. Each TCR result is shown as a density histogram with the CDR3 sizes shown on the x-axis and the peak fluorescence intensity shown on the y-axis. 23 Vβ families were analysed, including the Jurkat cell line as a positive control.

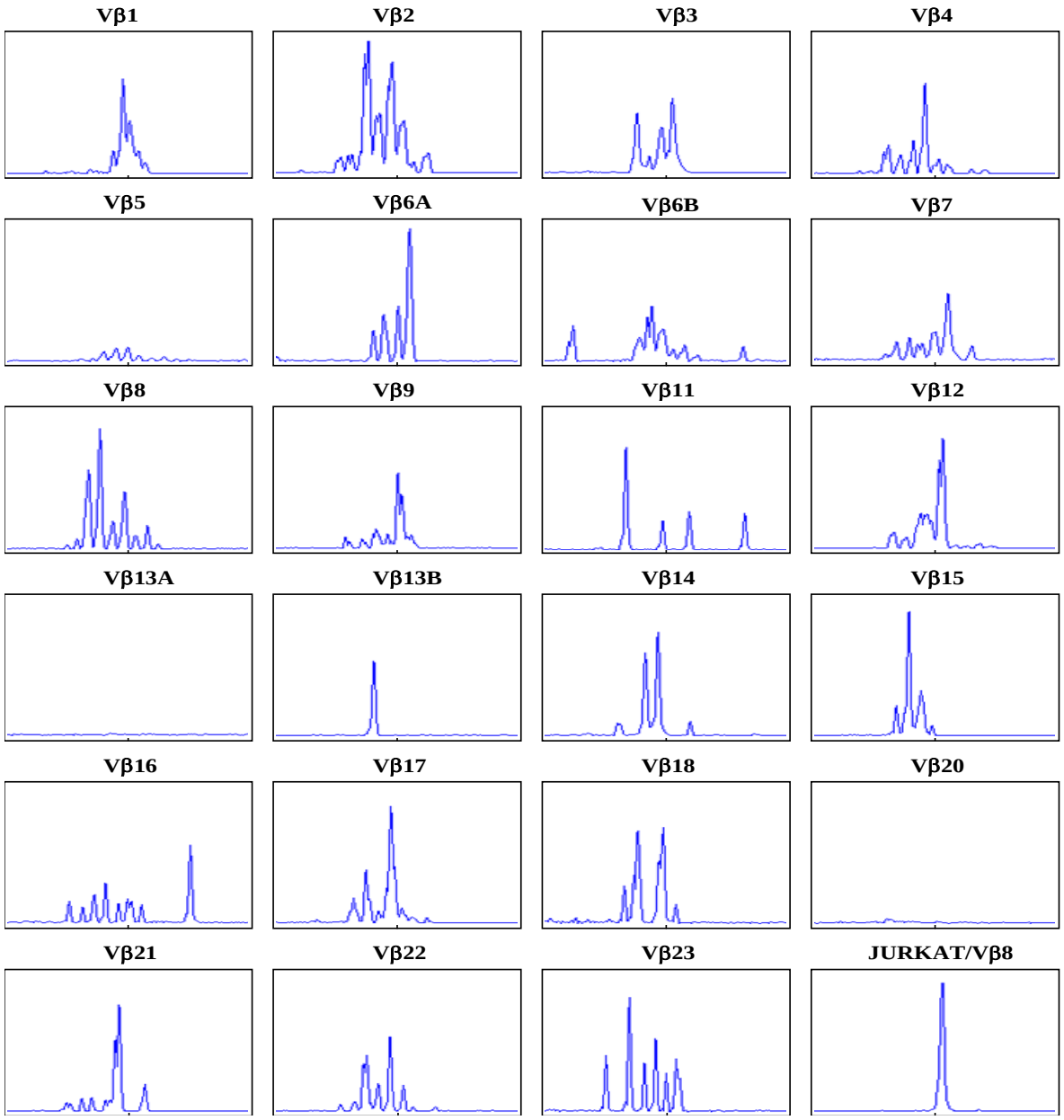


Figure 4.7: Immunoscope profile of a baby born to an HIV-1 infected mother, appearing as polyclonal skewed and oligoclonal skewed (e.g. Vβ9, Vb13B, Vβ17, Vβ21) distributions. Each TCR result is shown as a density histogram with CDR3 sizes shown on the x-axis and the peak fluorescence intensity shown on the y-axis. 23 Vβ families were analysed, including the Jurkat cell line as a positive control.

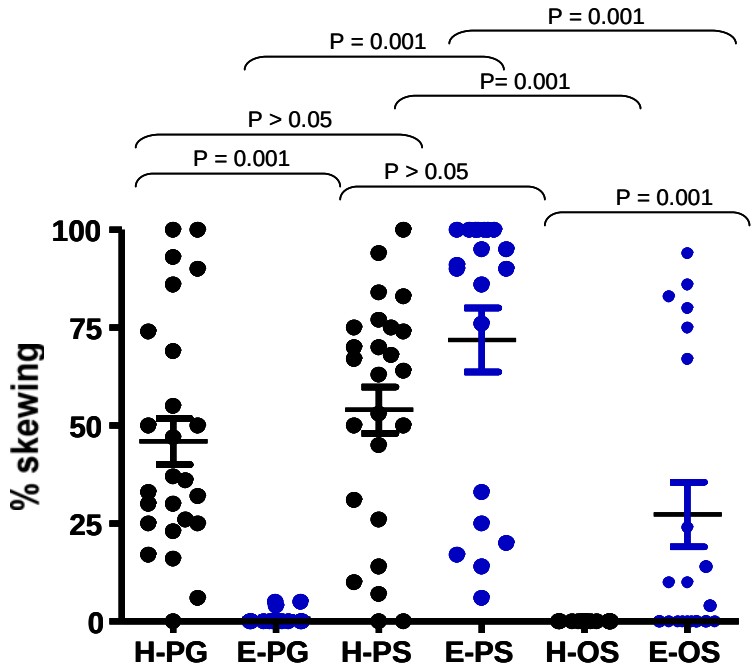


Figure 4.8: The T cell receptor profiles for 20 HIV-negative babies born to HIV-1 infected mothers (identified as E) compared with those of 25 healthy control babies (identified as H). Proportions of polyclonal Gaussian (PG), polyclonal skewed (PS) and oligoclonal skewed (OS) TCR are shown on the Y-axis as percentage of skewing within the total repertoire. Statistical p values were obtained using Mann-Whitney Rank Sum test.

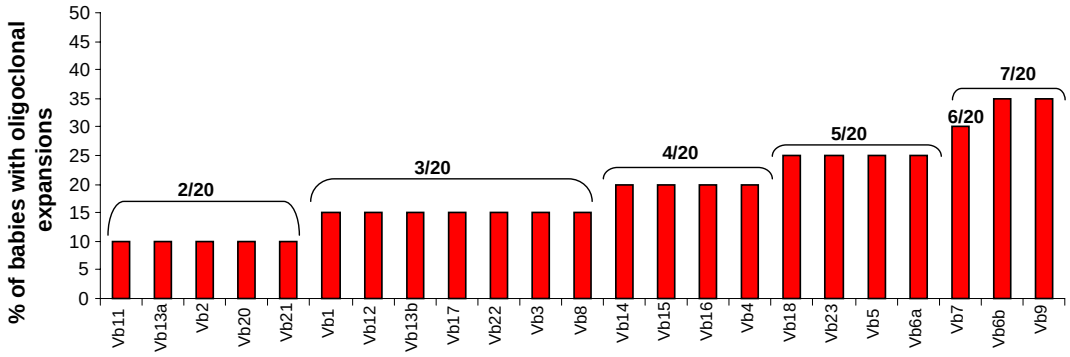


Figure 4.9: The frequency of oligoclonal skewing from 20 babies born to HIV-1-infected mothers. Proportions of babies with oligoclonal skewing of the repertoire are shown on top of the bars.

In order to discount the possibilities that the TCR patterns observed from cord blood T cells isolated from babies born to HIV-1 infected mothers were due to contaminating T cells from the mother, the TCR patterns in 11 mothers paired with their babies were compared. The TCR profiles of HIV-1 infected mothers and paired babies are shown in Table 4.2, where it can be seen that oligoclonal skewing of T cells was higher in the mothers compared to their corresponding babies. Conversely, high polyclonal skewing was observed in babies, but not mothers. These data suggest that because the Vβ family profiles were different between mother and baby, it is unlikely that there was contamination of maternal cells in the baby cord blood T cell population.

Table 4.2: The TCR profiles for 12 HIV uninfected babies born to HIV-1 infected mothers compared with their paired mothers

Patient ID	Oligoclonal Skewed (%)	Polyclonal Skewed (%)	Polyclonal Gaussian (%)
M1	88	12	0
B1	0	95	5
M2	88	12	0
B2	0	100	0
M3	75	25	0
B3	80	20	0
M4	36	64	0
B4	10	90	0
M5	79	21	0
B5	10	90	0
M6	33	67	0
B6	4	91	4
M7	62	38	0
B7	75	25	0
M8	5	95	0
B8	24	76	0
M9	71	29	0
B9	0	100	0
M10	94	6	0
B10	83	17	0
M11	100	0	0
B11	94	6	0
M12	86	14	0
B12	0	100	0

4.3.3 Vβ TCR usage at 3 months in babies born to HIV-1 infected mothers

It was possible to perform a 3-month follow-up in four babies by obtaining a peripheral blood sample. Three babies (B1, B2 and B3) had 70-90% polyclonal skewed TCR distribution at birth, which was maintained at 3 months after birth (Figure 4.11). However, baby B4 had 94% oligoclonal distribution at birth, which was altered to a polyclonal skew distribution at 3 months. This would suggest that babies born with polyclonal populations of T cells remain polyclonal and that perhaps heavily perturbed oligoclonal populations become more diverse in potential recognition of antigens after birth.

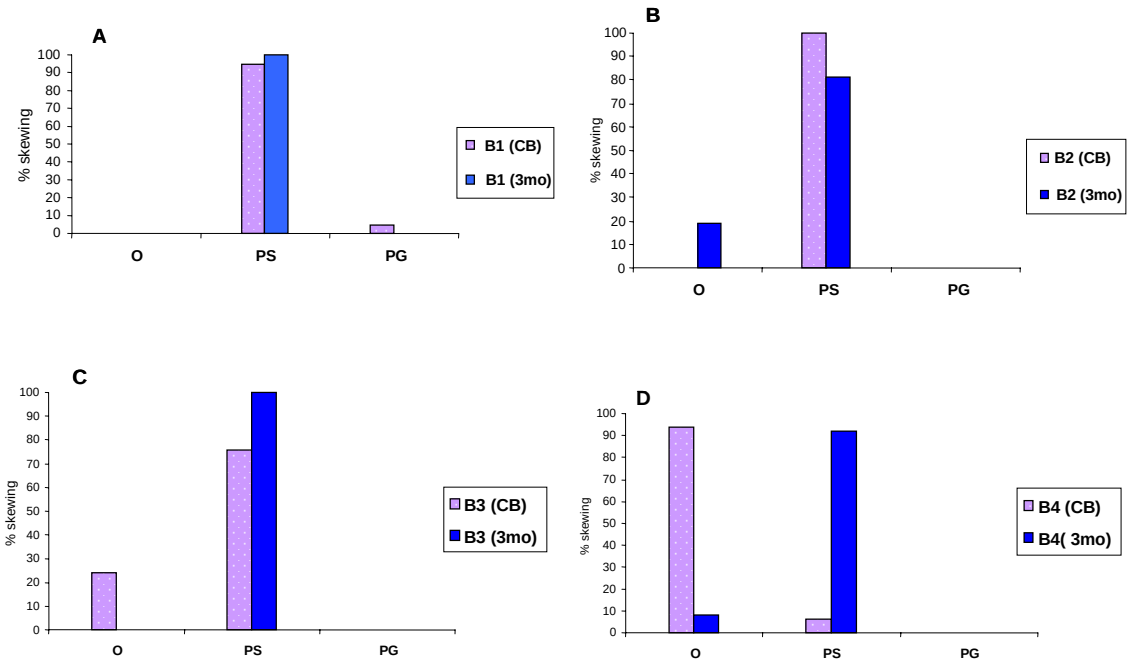


Figure 4.10: TCR profiles of four HIV-1-uninfected babies born to HIV-1 infected mothers, followed at 3 months after birth. The babies are identified by lab numbers e.g. B1 cord blood (CB) and CTL B1-3 months The immunoscope assay was performed from cord blood and from peripheral blood mononuclear cells at 3 months after birth (3mo).

4.4 ELISA results for other congenital infections – A measure of in-utero exposure to antigens

In order to determine some of the source of TCR skewing observed in the babies and that skewing may have been due to exposure to other infections, besides HIV, ELISA assays were performed for antibodies to syphilis (RPR), toxoplasma (IgG and IgM), rubella (IgM) and cytomegalovirus (IgG and IgM) in plasma from 17 babies born to HIV-1 positive mothers and their corresponding mothers, as well as in 19 healthy control babies and their mothers. Measurement of IgM was used to detect the presence of acute infection in both mothers and babies, whereas IgG was used for detection of existing immunity to previous infections in the mother, and the existence of IgG in cord blood was unlikely to be meaningful as this cannot be distinguished from maternal derived immunity. Acute infection in both baby and mother was thus confirmed by the detection of IgM in cord blood and maternal plasma respectively. Detection of IgM in the mother at birth would also result in the possibility that the baby may have been exposed to the pathogen in utero.

Three babies born to HIV-1 infected mothers tested positive for syphilis. Seven babies tested positive for toxoplasma IgG and two babies were seropositive for rubella IgM. The two babies with evidence of the presence of rubella IgM showed more than 90% polyclonal skewing with no evidence of oligoclonal expansion (Table 4.3). Seven of the nine babies that showed evidence of oligoclonal-T cell expansion (B6, B7, B8, 4153B, B14, B4, B17) were not IgM seropositive for any of the pathogens tested. All babies, including healthy control babies and mothers were seropositive for CMV IgG (Table 4.4),

which coincided with polyclonal skewing of the T cell repertoire, suggesting that the babies may have been exposed to CMV antigens at a certain stage in utero. A prior study by Marchant showed that maternal CMV infection induced oligoclonal expansion of T cells in foetal life. It is tempting to suggest that maternal CMV and HIV-I infection was able to induce polyclonal and oligoclonalskewing of the TCR in HIV uninfected babies.

Table 4.3: ELISA results and TCR profiles of HIV-uninfected babies born to HIV-1 - infected mothers (B) and their paired mothers (M)

	ELISA						TCR Profiles		
SAMPLE ID	Syphilis (RPR)	TOXO(IgG)	TOXO(Igm)	RUBELLA(IgM)	CMV(IgG)	CMV(IgM)	O	PS	PG
M2852	neg	neg	neg	neg	pos	neg			
B2852	neg	neg	neg	pos	pos	neg	0	96	4
M4000	neg	neg	neg	neg	pos	neg	88	12	0
B4000	neg	neg	neg	neg	pos	neg	0	95	5
M4004	neg	pos	neg	neg	pos	neg	75	5	0
B4004	neg	pos	neg	neg	pos	neg	80	20	0
M4089	neg	neg	neg	neg	pos	neg			
B4089	neg	neg	neg	neg	pos	neg	10	90	0
M4096	neg	neg	neg	neg	pos	neg			
B4096	neg	neg	neg	neg	pos	neg	0	100	0
M4138	neg	neg	neg	neg	pos	neg	79	21	0
B4138	neg	neg	neg	neg	pos	neg	10	90	0
M4140	pos	neg	neg	pos	pos	neg			
B4140	pos	neg	neg	pos	pos	neg	0	100	0
M4153	neg	neg	neg	neg	pos	neg	33	67	0
B4153	neg	neg	neg	neg	pos	neg	4	91	4
M4211	neg	neg	neg	neg	pos	neg	30	70	0
B4211	neg	pos	neg	neg	pos	neg			
M4214	neg	pos	neg	neg	pos	neg			
B4214	neg	pos	neg	neg	pos	neg	0	100	0
M4215	pos	neg	neg	neg	pos	neg	5	95	0
B4215	pos	pos	neg	neg	pos	neg	24	76	0
M4219	neg	pos	neg	neg	pos	neg	71	29	0
B4219	neg	pos	neg	neg	pos	neg	0	100	0
M4223	neg	pos	neg	neg	pos	neg	94	6	0
B4223	neg	pos	neg	neg	pos	neg	83	17	0
M4227	neg	neg	neg	neg	pos	neg	100	0	0
B4227	neg	neg	neg	neg	pos	neg	94	6	0
M4230	pos	neg	neg	neg	pos	neg			
B4230	pos	neg	neg	neg	pos	neg	14	86	0
M4232	neg	neg	neg	neg	pos	pos	86	14	0
B4232	neg	neg	neg	neg	pos	neg	0	100	0
M4256	neg	neg	neg	neg	pos	neg			
B4256	neg	pos	neg	neg	pos	neg	67	33	0

Table 4.4: ELISA results and TCR profiles of healthy negative control babies (B) and their paired mothers (M).

ELISA							TCR Profiles		
SAMPLE ID	RPR	TOXO(IgG)	TOXO(Igm)	RUBELLA(IgM)	CMV(IgG)	CMV(IgM)	O	PS	PG
M20	neg	neg	neg	neg	pos	neg			
B20	neg	neg	neg	neg	pos	neg	0	74	26
M21	neg	neg	neg	neg	pos	neg	8	92	0
B21	neg	neg	neg	neg	pos	neg	0	50	50
M22	neg	neg	neg	neg	pos	neg			
B22	neg	neg	neg	neg	pos	neg	0	63	37
M23	neg	neg	neg	neg	pos	neg			
B23	neg	neg	neg	neg	pos	neg	0	10	90
M24	neg	neg	neg	neg	pos	neg	0	100	0
B24	neg	neg	neg	neg	pos	neg			
M25	neg	pos	neg	neg	pos	neg			
B25	neg	pos	neg	neg	pos	neg	0	64	36
M26	neg	neg	neg	neg	pos	neg			
B26	neg	neg	neg	neg	pos	neg	0	50	50
M27	neg	neg	neg	neg	pos	neg	0	100	0
B27	neg	neg	neg	pos	pos	neg	0	94	6
M28	neg	neg	neg	neg	pos	neg	0	100	0
B28	neg	neg	neg	neg	pos	neg	0	68	32
M29	neg	neg	neg	neg	pos	neg	18	82	0
B29	neg	neg	neg	neg	pos	neg	0	100	0
M30	neg	neg	neg	neg	pos	neg			
B30	neg	neg	neg	neg	pos	neg	0	75	25
M31	neg	neg	neg	neg	pos	neg			
B31	neg	neg	neg	neg	pos	neg	0	7	93
M32	neg	neg	neg	neg	pos	neg			
B32	neg	neg	neg	neg	pos	neg	0	77	23
M33	neg	neg	neg	neg	pos	neg			
B33	neg	neg	neg	neg	pos	pos	0	45	55
M34	neg	neg	neg	neg	pos	neg			
B34	neg	neg	neg	neg	pos	neg	0	70	30
M35	neg	neg	neg	neg	pos	neg			
B35	neg	neg	neg	neg	pos	neg	0	84	16
M36	neg	neg	neg	neg	pos	neg			
B36	neg	neg	neg	neg	pos	neg	0	75	25
M37	neg	pos	neg	neg	pos	neg	0	93	0
B37	neg	pos	neg	neg	pos	neg			
M38	neg	neg	neg	neg	pos	neg			
B38	neg	neg	neg	neg	pos	neg	0	14	86

4.5 DISCUSSION

Distribution of the TCR repertoire in healthy cord blood has previously been shown to have a Gaussian distribution. (Than *et al*., 1999, Sarzotti *et al*., 2003), and the repertoire in normal cord samples were shown to be diverse, with consistent representation of all 23 Vβ families with no evidence of clonal dominance. In this dissertation, an equal mix of Gaussian and Polyclonal skewed TCR families was shown in cord bloods from healthy babies born from HIV uninfected mothers. Polyclonal skewing was measured when there was a slight shift in TCR peak position and is sensitive to any form of TCR engagement with antigen. It is thus expected that healthy babies may have been exposed to an array of maternal antigens giving rise to polyclonal skewing. Converse to this, oligoclonal skewing was observed in cord blood TCR from HIV-1 uninfected babies born to HIV-1 infected mothers, suggesting that clonal expansion may be linked to exposure to HIV-1 antigens from the mother in-utero. This was further supported from the results looking at syphilis (RPR), toxoplasma, rubella and cytomegalovirus immunity in mother and baby pairs.

Studies performed in HIV-1 infected children have demonstrated evidence of clonal dominance in HIV infected children (Than *et al*., 1999; McFarland *et al*., 2002). Although we identified TCR expansions in total CBMC, clonal expansions have been reported to be common in CD8+ T cells in HIV-1 infected individuals, as shown by others (Wedderburn *et al*., 2001), and also described in the TCR analysis of adults conducted elsewhere in this dissertation (Chapter 3). A limitation of this study was that the functional implication of these clonal expansions in exposed uninfected babies was

not determined. Although other neonatal infections, or antigen exposure, could have given rise to the TCR skewing observed using spectratyping, it is tempting to speculate that this was linked to HIV infection of the mother. Indeed, similar expansions in HIV-1 infected children and adults have been associated with HIV-specific cytotoxic T lymphocyte activity. (Feeney *et al*., 2005, Legrand *et al*., 2006).

To discount the possibility of TCR skewing observed in exposed uninfected neonates due to other possible antigen exposure or congenital infections, IgG and IgM plasma antibodies for four possible pathogens [syphilis (RPR), rubella, toxoplasma and cytomegalovirus) were screened by ELISA. Detection of CMV IgG observed in all babies coincided with polyclonal expansion of T cells in all babies, including healthy control babies, whereas, oligoclonal expansion of T cells was observed only in babies born to HIV-1 infected mothers. Hence, oligoclonal expansion of T cells in these babies is shown to be associated with HIV infection rather than other antigens. Overall, the TCR data collected at enrollment and three months later from exposed–uninfected neonates showed persistent TCR expansions. The finding that an oligoclonal skewed population observed in one baby, which then normalized at three months after birth showed that the oligoclonality detected at birth was a biological characteristic and not a technical issue.

These findings are in agreement with previously reported data showing perturbations of the TCR Vβ repertoire in uninfected children younger than 2 months, whereas in older children less expanded TCR populations were observed (Silvestri *et al.*, 1996). In summary, oligoclonal skewing of the T cell repertoire in HIV-1 uninfected babies born to

HIV-1 infected mothers in this study infers that there was exposure to HIV-1 antigens in-utero.

The oligoclonal dominance of T cells in one neonate was transient, and possibly influenced by the presence of antigen. These findings also suggest that it is only in the absence of persistent antigen exposure that the diversity of the TCR repertoire in neonates broadens, at least as observed in babies born to HIV-1 infected mothers. Future studies are necessary to determine whether there is a link between TCR skewing in HIV uninfected babies with HIV antigen specificity. This association was not made in this dissertation and represents a limitation of the study in terms of attempting to understand whether T cell expansion in newborns is associated with protective immunity or a marker of exposure to antigen. Further studies are required to determine whether TCR clonal populations generated in HIV-1 exposed cord blood and 3 months follow up are HIV-1 specific. Other studies have demonstrated that 25% of exposed but uninfected infants show CTL activity against HIV-1 targets, but this activity is only detectable between 4 to 12 months of life (Halapi *et al*., 1996; Sandberg *et al*., 2003).Overall, this chapter has shown that it is possible to detect perturbations in the TCR repertoire in likely exposed and HIV uninfected babies. This study has also shown that healthy newborn babies are born with an intact TCR repertoire. It remains to be seen whether perturbations of the TCR due to exposure to antigens in utero provides immune competence or induces a weakness in potential T cell responses upon encounter with antigens at some point after birth.

CHAPTER 5

MEASUREMENT OF HIV-1 - SPECIFIC T CELL ACTIVITY

5.1 INTRODUCTION

In this dissertation, several characteristics of T cells have been investigated in different cohorts of HIV infected and HIV exposed, but uninfected individuals. In this chapter, the fine specific nature of CD8+ T cells has been measured by investigating HIV-specificity using the IFNγ ELISPOT assay and intracellular cytokine staining.

The role of antigen-specific T cells, especially that of CD8+ cytotoxic T cells (CTL) in the control of HIV-1 has been well documented (Schmidz *et al*., 1995; Klein *et al*., 1995; Walker *et al*., 1996; Xia *et al*., 1999). It has been suggested that ultimate control of HIV-1 infection and disease will probably rely on an effective CTL-based vaccine. Proof of this concept has been shown in pre-clinical experiments when immune responses in rhesus macaques were elicited using DNA vaccines and then challenged with SHIV (Amara *et al*., 2001; Barouch *et al*., 2001; Egan *et al*., 2000). The immune responses in these vaccinated macaques were able to control viraemia to nearly undetectable levels and prevent immunodeficiency and clinical disease, as opposed to control monkeys, which exhibited high viraemia and significant disease progression. These studies emphasize the requirement for more studies to examine correlates of immune control against HIV in humans. The complexities of finding a CTL-based vaccine that will work

in humans has been recently thwarted by the failed clinical trial of an adenovirus type 5 vector-based immunogen containing the HIV-1 *gag* gene. After enrolling 3000 volunteers into the STEP trial, it was shown that the vaccine arm had no efficacy and possibly may have exacerbated HIV transmission (http://www.hvtn.org/media/pr/step1207.html). Whether this was a failure of the concept of CTL-based vaccines or a failure of the vaccine product remains to be seen. Nevertheless, understanding CD8+ T cell immunity in natural HIV infection remains a valuable foundation for understanding what may be required from a vaccine that stimulates T cell immunity.

CD8+ T cells arise in the peripheral blood after HIV infection resulting in a large proportion of CD8+ cytotoxic T cells (CTL) that are HIV-specific, recognizing different epitopes across the HIV-expressed genome (Edwards *et al*., 2002; Addo *et al*., 2003; Peretz *et al*., 2005). Many of these cells are activated to varying degrees and co-express markers that reflect activation events, such as CD38 and HLA-DR as described in chapter 2 of this dissertation. This activation response has been associated with increased levels of cytokines, including gamma interferon (IFN-γ) in the peripheral blood (Fan *et al*., 1993; Jassoy *et al*., 1993; Jassoy *et al*., 1992), CSF and germinal centers of the lymph node (Hosmalin *et al.,* 2006).

HIV-specific T cells from peripheral blood can be identified and enumerated by detection of antigen induced interferon gamma (IFN-γ) secreted at a single cell level using various methods. 1) The intracellular cytokine staining method (ICS) which allows detection of multiple peptides and whole proteins which can be added to stimulate the cells and then

analyzed by flow cytometry. Multiple T cell types, allowing analysis of the T cell lineage can be measured simultaneously using ICS. 2) Along with the ICS assay, the ELISPOT assay allows the quantitative measurement of the frequency of cytokine secreting cells at the single cell level directly ex vivo without in vitro expansion or manipulation of cell populations. This assay is sensitive and allows for enumeration of low frequencies of T lymphocytes. 3) The use of tetramers and flow cytometry analysis, which requires the HLA type of the analyzed sample to be known and individual MHC-class I preparation contains only a single peptide epitope. These assays can also be used in measurements of other cytokines that are secreted in response to HIV antigens, for example IL-2 and TNFalpha.

HIV-1 –specific T cell activity in this dissertation was measured on cryopreserved PBMC, by using a combination of the ELISPOT assay and intracellular cytokine staining (ICS). This chapter focuses on HIV-1-specific T cell recognition of HIV in a cohort of HIV/TB co-infected individuals. The aim was to determine whether mycobacterium tuberculosis had any effect on HIV-1-specific T cell immunity.

5.2 MATERIALS AND METHODS

5.2.1 Study cohort

Two groups of individuals consisting of HIV-1 infected and HIV/TB co-infected were studied in a cross-sectional and longitudinal manner. The ELISPOT assay was performed on 14 HIV-1 and 11 HIV/TB co-infected individuals for cross-sectional analysis and 10 HIV/TB individuals were analysed for longitudinal analysis (see Appendix 8 for cohort

details). The 10 individuals were followed from baseline to 12 months following a six-monthly course of TB treatment as described in chapter 2.

5.2.2 Thawing of cryopreserved PBMC

PBMC were removed from the liquid nitrogen tank and rapidly thawed by suspending the vial in the waterbath and warmed at 37^{o}C. Using a 1ml pipette, the cells were added drop wise into 50ml tubes with 10ml RPMI containing 10% FCS, 0.5% gentamycin (R10) and 50 units/ml benzonase. The cells were centrifuged at 1200rpm for 10 minutes. The supernatant was decanted and the cells were suspended in 15 ml R10. The cells were centrifuged again at 1200rpm for another 10 minutes and resuspended in 1-5ml warm RPMI + 20% FCS (R20). 20µl of the cell suspension was removed, mixed with 180ml ViaCount solution and counted on the Guava cell counter. An additional volume of R20 was added to the cells to make a concentration of 2 x10^{6} cells/ml. The cells were incubated for 12-18 hours (overnight) at 37^{o}C, 5% CO_2 with the caps loosened. The cells are washed once with warmed R10 and resuspended in 1-5ml R10 for viable and total cell counts. The cells are then ready for the ELISPOT and ICS assays.

5.2.3 The IFN-γ ELISPOT

Measurement of IFN-γ was performed from cryopreserved PBMC, which were either thawed, rested in the incubator overnight and stimulated with HIV-1 subtype C superpools ex vivo, (these cells were then referred to as *"fresh")* or cultured with CD3+CD4+ bispecific monoclonal antibody (BSMAB, kind gift from Dr Guido Ferrari, Duke University) to specifically expand CD8+ T cells. When added to PBMC in the

presence of IL-2, CD3,4B BSMAB there is a selective elimination of CD4+ T cells by promoting complement-mediated lysis of CD4+ T cells (Wong *et al.,* 1987). Briefy, 2 x 10^6 PBMC suspended in RPMI + 10% foetal calf serum and 0.5 % gentamycin were placed into individual wells of a 24 well plate + 25U/ml IL-2 + 0.1 μg/ml CD3+CD4+ BSMAB. After every 2 days, the medium was partially exchanged with fresh medium containing IL-2 and cultures were continued for five days**.** These cells were then referred to as *"expanded"*. The ex vivo IFN-γ ELISPOT was performed on freshly thawed PBMC from 10 HIV/TB individuals and on expanded CD8+ T cells from 14 HIV-1 infected and 11 HIV/TB co-infected individuals.

The ELISPOT assay was performed on polyvinylidene difluoride membrane -96 well plates (MAIP S45, Millipore, Johannesburg, SA), which were coated overnight at 4^{o}C with 50μl of 2μg/ml anti-IFNγ-monoclonal antibody (1-D1K, Mabtech, Stockholm, Sweden). Nine peptide superpools that spanned the complete HIV-1 subtype C whole genome were used and details pertaining to peptide length, sequence strain (or concensus) is shown in Table 5.1. Peptides were synthesized using 9-fluorenylmethoxycarbonyl-based solid phase chemistry (Natural and Medical Sciences Institute, University of Tübingen, Tübingen, Germany). All peptides were checked for the correct molecular weight by Elektrospray QTOF-mass spectrometry. Peptide purities ranged from 70-80%. Gag, Vpu, Vpr and Tat were synthesized from the consensus sequence of HIV-1 subtype C (a generous gift from Marcus Altfeld, Massachusetts General Hospital) and Pol, Nef, Env, Vif and Rev were synthesized from viral sequence strains that were closest to the consensus subtype C sequence and used as a basis for HIV

vaccine manufacture (Williamson *et al.,* 2003). Nef peptides were synthesized as 15-mers overlapping by 11 amino acids (aa), and the remaining peptides varied from 15-18-mers overlapping at 10 residues. Peptides were dissolved in 100% dimethyl sulfoxide at an initial concentration of 10mg/ml and were pooled at 40µg/ml/peptide stock in phosphate-buffered saline (PBS) in which the final concentration of dimethyl sulfoxide was always less than 0.5%.

Table 5.1: Synthetic peptides used in the ELISPOT

Protein	Number of peptides/superpool	Length of peptide	Strain
Gag	14	15-18	Concensus
Pol	24	15-18	Du 151
Nef	10	15	Du 151
Env	24	15-18	Du 179
Vif	12	15-18	Du 151
Rev	14	15-18	Du 151
Vpu	9	15-18	Concensus
Vpr	11	15-18	Concensus
Tat	12	15-18	Concensus

The plate layout is shown in Table 5.2, showing the nine peptide superpools, two wells of media and cells only serving as negative controls and two wells of PHA as a positive control. The wells were then washed 3 times with PBS-Phosphate Buffered Saline (in-house made) and blocked with 100µl of RPMI-10% FCS for at least 2 hours. Peptide (50µl, used at a final concentration of 2µg/ml) was added to the wells, followed by 50µl of 0.5 -2 x 10^5 PBMC. The plate was incubated for 16-18 hours in a 37^{o}C, 5% CO_2 incubator. Any positive reaction resulted in a release of IFN-γ, which was captured by the

anti-IFN-γ monoclonal antibody coating the surface of the membrane. To identify the positive reactions, wells were washed three times with PBS followed by three washes with PBS supplemented with 0.05% Tween 20 (Sigma Aldrich, SA) + 1% FCS. A secondary detection antibody (50 µl biotinylated IFN-γ (clone 7-B6-1; Mabtech, 2µg/ml) was then added and incubated at room temperature for 3 hours. After washing the plate six times with PBS-Tween 20, 50µl of streptavidin–bound horseradish peroxidase (2µg/ml -Pharmingen, Cupertino) was added and incubated for 1 hour. Developed spots were visualized by addition of 100µl of Novared substrate (Vector, Burlingame, California, USA) according to the manufacturer's instructions and incubated at room temperature in the dark. As soon as the spots were visible on the membrane (as shown in Figure 5.1), the wells were rinsed with tap water and air-dried. When dry, developed spots were counted on the ELISPOT reader (CTL, ImmunoSpot, Cleveland, Ohio). The frequency of cells responding to each of the peptide pools was normalized as spots/million PBMC. A positive cutoff was defined as above 100 SFU/10^6 PBMC.

Table 5.2: Plate layout of the ELISPOT assay using HIV-1 subtype C peptide superpools

1	2	3	4	5	6	7	8	9	10	11	12
Gag	Pol	Nef	Env	Vif	Vif	Tat	Rev	Vpu	Vpr	Media	Media
Cells	Cells	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	PHA	PHA

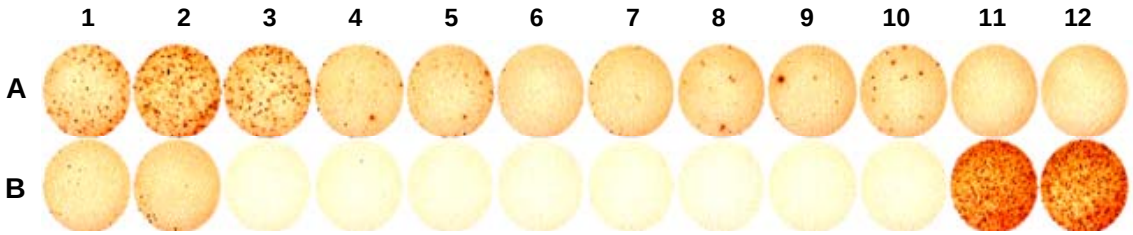


Figure 5.1: The ELISPOT membrane showing HIV-1 antigen responding /IFN-γ+ cells appearing as spots on A1, A2, A3, A4 e.t.c. and PHA (positive control), which is shown on well B11 and B12. The plate layout is shown on table 5.2.

5.2.4 Intracellular cytokine staining (ICS)

The ICS assay was used to confirm the HIV-1 subtype C specific responses obtained by the ELISPOT assay and to identify the phenotype of responding cells e.g. (CD4+ and CD8+ T cells). The main advantage of this method is that more than one cytokine as well as the lineage of T cell subsets can be measured simultaneously. The method involves three steps consisting of PBMC-peptide stimulation; staining of stimulated cells with selected monoclonal antibodies; flow cytometry acquisition and analysis.

5.2.4.1 Cell stimulation

Cryopreserved PBMC were thawed and resuspended in RPMI + 10% FCS at 1 x10^6/ml in 5ml falcon FACS tubes. PBMC suspension (1ml) was stimulated with 1µg each of anti - CD28/CD49d (Becton Dickinson-Pharmingen, San Jose, California) as costimulatory antibodies and 2µg/ml of the peptide superpool to be confirmed. Cells containing CD28/CD49d only were used as a negative control and cells stimulated with positive control (SEB- staphylococcus enterotoxin B which is a superantigen obtained from

Sigma Aldrich) were included in each experiment. The cultures were incubated at 37^{o}C in the presence of 5% CO_2 for 1 hour with addition of the cytokine secretion inhibitor Brefeldin A used at a final concentration of 10μg/ml (Sigma Aldrich, SA) followed by an additional incubation for 5 hours. The cells were then stored at 4^{o}C (for a maximum of 16-18 hours) until ready for permeabilization and staining.

5.2.4.2 Flow cytometry analysis

EDTA (100μl of 20mM, Sigma Aldrich) was added to peptide stimulated and control tubes and incubated at room temperature for 15 minutes. The cells were then suspended in FACS wash solution (PBS + 1% BSA + 0.1% sodium azide) and centrifuged at 1200rpm for 10 minutes. The cells were fixed and lysed in 2ml of 1x FACS lysing solution (BD Immunocytometry systems, San Jose, California) for 10 minutes at room temperature in the dark, and permeabilised with 0.5ml of 1x FACSPerm II (BD) for 15 minutes at RT. Wash solution (1ml) was added and the tubes were centrifuged at 1800rpm for 10 minutes. Permeabilized cells were then stained with directly conjugated CD3-APC, CD8-PerCP, CD69-PE or IL-2 PE and IFN-γ-FITC (all BD) for 30 minutes at room temperature in the dark. After an additional wash the cells were then resuspended in the fixing solution (1% paraformaldehyde, 0.05% sodium azide: in -house). Stained cells were stored at 4^{o}C for a maximum of 24 hours for flow cytometry acquisition and analysis. Four colour flow cytometry was performed on the Becton Dickinson FACSCalibur using CellQuest software (BD, San Jose, California). Live events (100,000-130,000) were acquired, gating small viable lymphocytes and saving 50,000 CD3+ T lymphocytes. List mode data files were analyzed using FlowJo (Tree Star, Inc. Ashland,

Oregon, and USA). The gating strategy is shown on Figure 5.2. Plot A is showing the lymphocyte gate with 21.8% of total cells, plot B is showing gated CD3+ T cells with 43.3% of total lymphocytes which were collected from the lymphocyte gate. Frequencies of CD8+IFNγ+ and CD4+IFNγ+ T cell for unstimulated cells (0.035 and 0.013%) and SEB (3.57% and 0.6%) are shown as percentages of total T cells. HIV-1-specific responses were rated as positive when the frequency of CD8+IFNγ+ or CD4+IFNγ+ was $\geq$0.05% above the percentage of unstimulated cells.

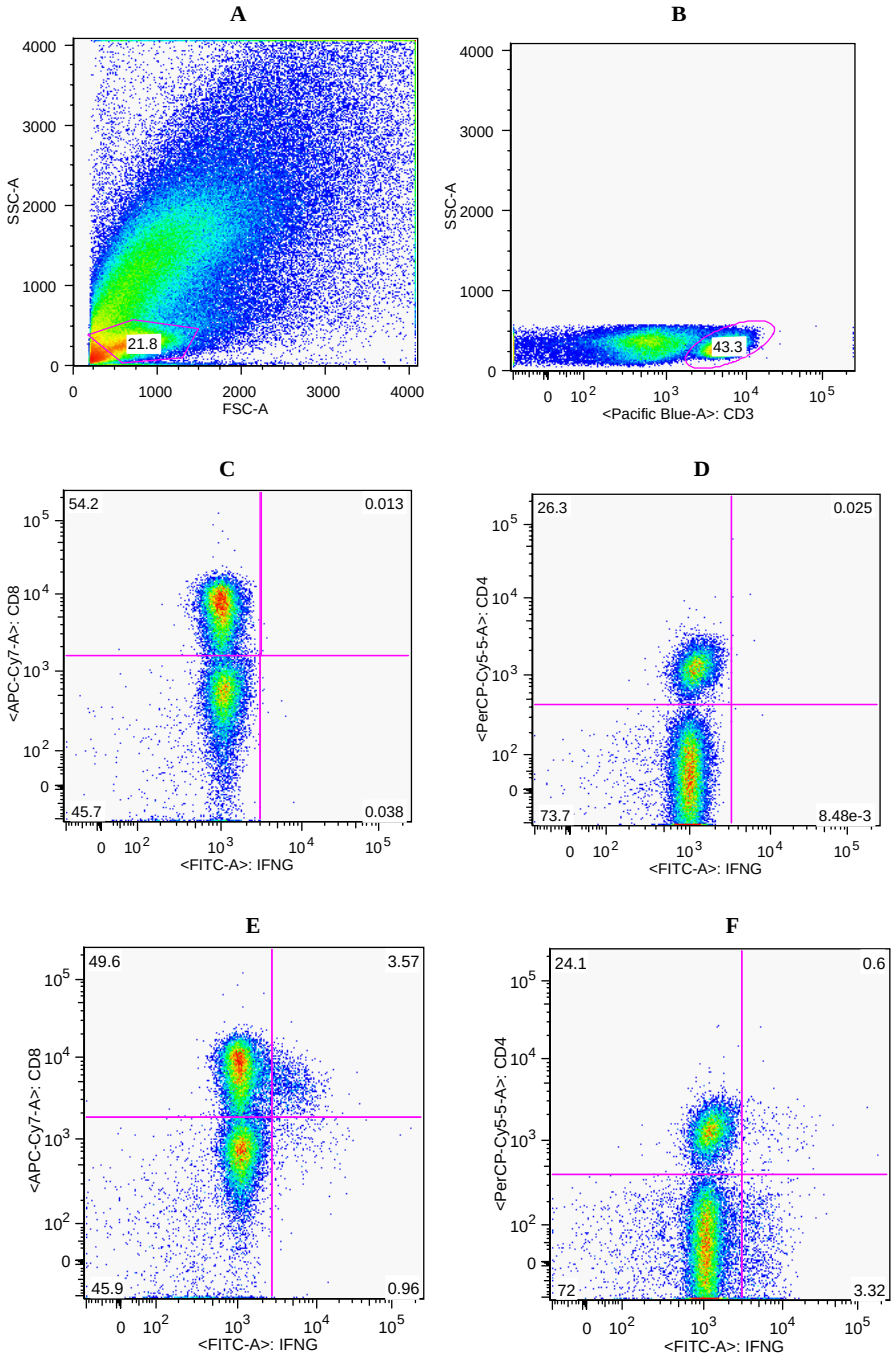


Figure 5.2: Flow cytometry plots showing analysis of IFN-γ producing T cells as analyzed by FlowJo. Plot A is showing the lymphocyte gate with 21.8% of total cells, plot B is showing gated CD3+ T cells with 43.3% of total lymphocytes which were collected from the lymphocyte gate. Frequencies of CD8+IFNγ+ and CD4+IFNγ+ T cell for unstimulated cells (0.035, plot C and 0.013%, plot D) and SEB (3.57%, plot E and 0.6%, plot F) are shown as percentages of either CD3+CD4+ or CD3+CD8+ T cells.

5.3 RESULTS

5.3.1 The frequency of response to each region (i.e how many individuals are responding to each protein region)

It has been shown in this dissertation that CD8+ T cells are activated by HIV-1, and more enhanced by the presence of TB co-infection. It was further noted that clonal dominance of the CD8+ T cell population occurs following activation, indicating usage of selected T cell receptors. Measurement of HIV-1 specific T cells was made to identify which recognised regions were immunodominant. To identify HIV-specific CD8+ T cells, PBMC were expanded for 5 days with bispecific antibody (CD3+CD4+) and stimulated with 2μg/ml each of 9 HIV-1 subtype C peptide superpools in the IFN-γ ELISPOT assay. CD8+ T cells from both HIV-1 and HIV/TB co-infected patients responded to one or more of the nine HIV-1 subtype C peptides.Of the 14 HIV-1 singly infected individuals, 85% recognised Gag; 78% recognized Pol, 64% recognized Nef, 43% recognized Vif, 36% recognized Env, Rev and Vpr; and 21% recognized Tat (Figure 5.3A and Appendix 12). Of the 11 HIV/TB co-infected patients, 73 % targeted Pol, Vif and Nef, 45% recognized Env; 27% recognized Vpr and Gag, 18% recognized Tat and Vpu; and 9 % recognized Rev (and Figure 5.3B and Appendix 13). These data shows that the most frequently targeted HIV-1 genome regions in HIV-1 infected individuals differed from those individuals with TB and HIV co-infection.

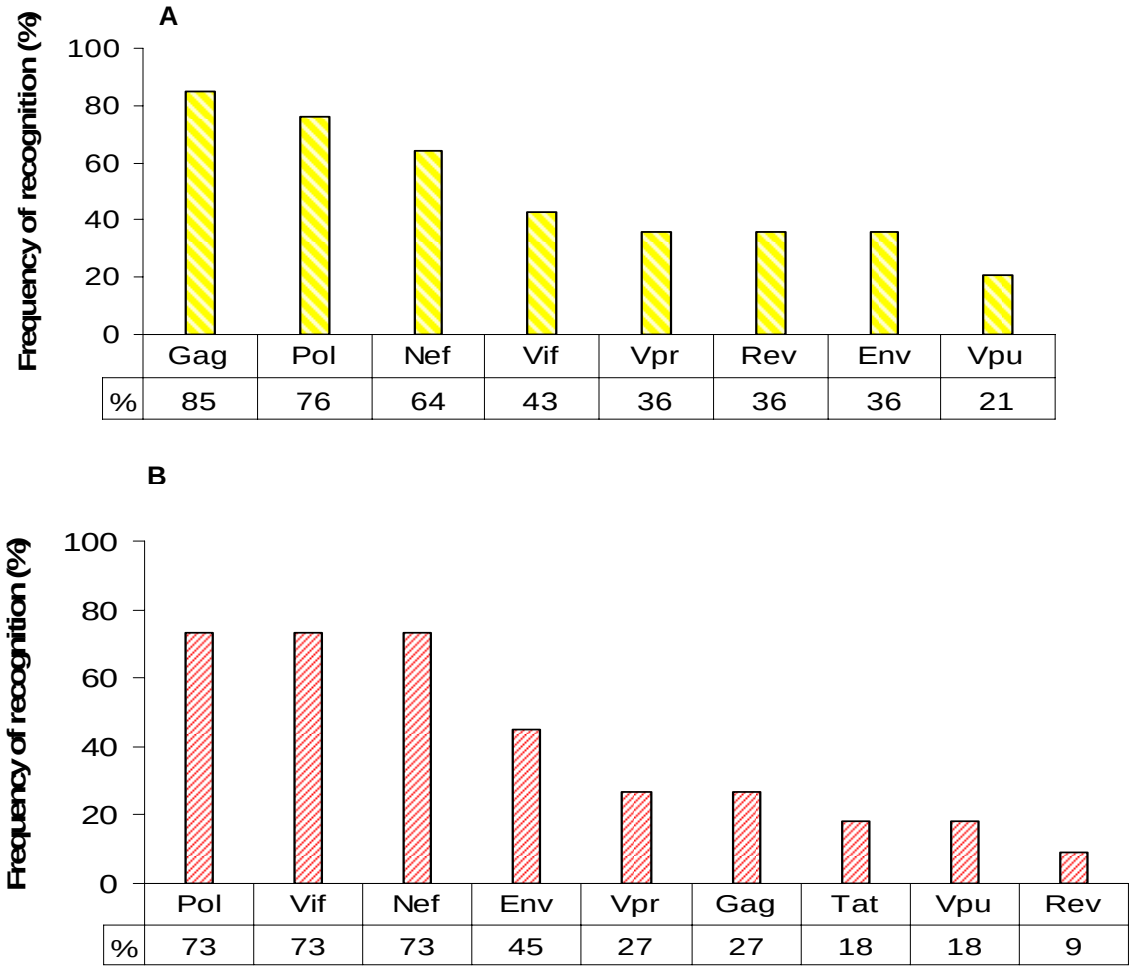


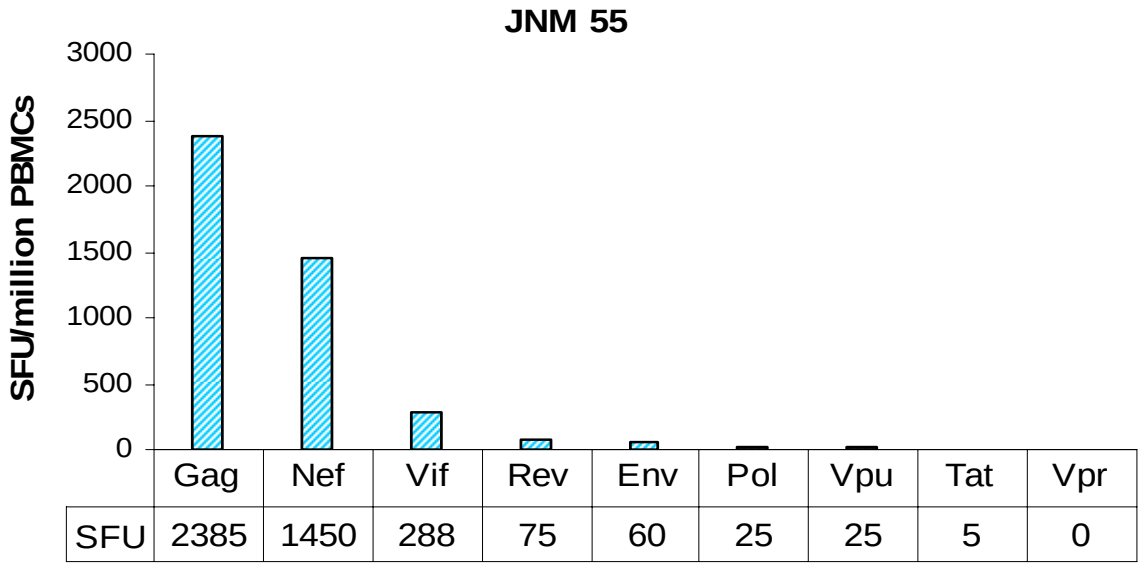
Figure 5.3: Targeting of HIV-1 subtype C-peptide superpools by HIV-1 (A) and HIV/TB (B) co-infected patients. Frequencies of individuals targetting various HIV-1 subtype C superpools within both cohort groups are shown at the bottom of the graph.

These data suggest that recognition of epitopic regions differ between individuals singly or dually infected with HIV and TB.

5.3.2 Confirmation of HIV-1–specific responses by intracellular cytokine staining (ICS)

IFN-γ ELISPOT responses from selected peptide pools were confirmed using the ICS assay, which enabled simultaneous analysis of both CD8+ and CD4+ T cells. PBMC samples from 5 HIV/TB patients were stimulated and stained with CD3 Pacific Blue, CD8 APC-Cy7, CD4 PerCP Cy5.5 and IFN-γ FITC and IL-2 PE. The cells were acquired on the LSR II (Becton Dickinson) using FACSDiva software. List mode data was analysed using FlowJo (Tree Star Inc.) software.

A representative example of ELISPOT results for patient JNM 55 (Figure 5.4), showing responses to Gag, Nef and Vif was confirmed using ICS. The ICS results confirming a Gag response are shown on Figure 5.5, as analysed by FlowJo software. Plot A and B are showing CD8+ T cells (y-axis) and CD4+ T cells (y-axis)–expressing IFN-γ (x-axis) for unstimulated (negative control), SEB-stimulated (C and D) and Gag-stimulated (E and F) PBMC. The frequency of CD8+ T cells expressing IFN-γ for this patient is 2.5% after subtracting the background of 0.013%, and the number of CD4+ T cells expressing IFN-γ is 0.12%. Other T cell responses to Gag and Nef, which were confirmed, are shown in Table 5.3, presented as scores of the ELISPOT (SFU x 10^6) and the ICS (% IFN-γ positive cells). HIV-1 –specific responses obtained by the ELISPOT were readily confirmed by the ICS and were predominantly mediated by CD8+ T cells, although there were at least 3 CD4+ responses to Gag and Nef (patients 1, 2 and 5).



	Gag	Nef	Vif	Rev	Env	Pol	Vpu	Tat	Vpr
SFU	2385	1450	288	75	60	25	25	5	0

Figure 5.4: The ELISPOT and ICS results showing Gag, Nef and Vif responses for patient JNM 55.

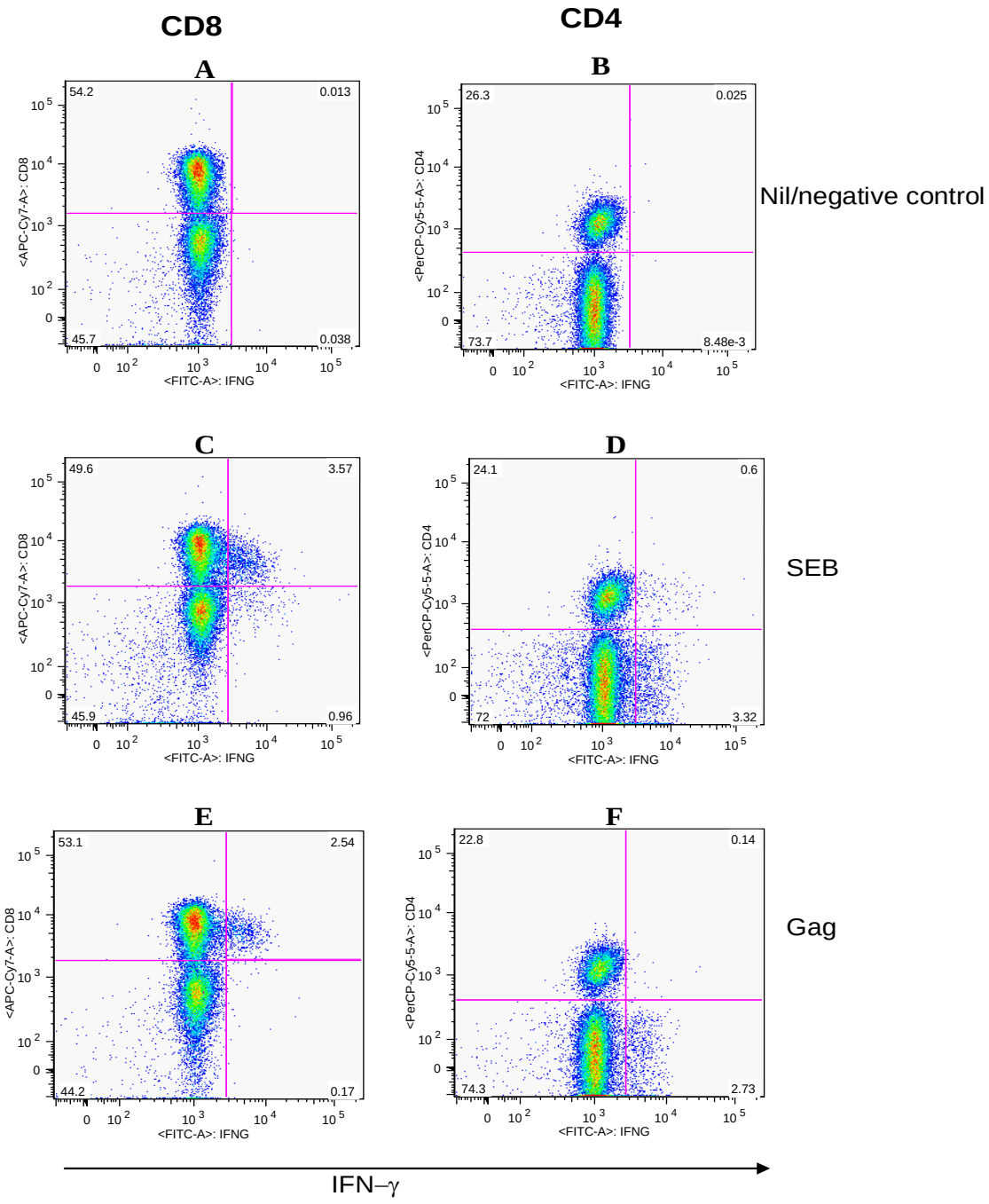


Figure 5.5: The ICS results confirming Gag-specific T cell response from patient JNM 055 identified by ELISPOT. The dot plots show frequencies of CD8+/IFN-γ+ and CD4+/IFN-γ+ for unstimulated (A and B) , SEB (C and D) and Gag (E and F) were gated from the CD3+ T cells.

Table 5.3: ELISPOT and ICS results

PID	HIV-1 superpool	ELISPOT	ICS- CD8	ICS-CD4
1	Gag	++	-	+
2	Nef	+	+	+
3	Gag	++	++	-
4	Nef	+++	+	-
5	Gag	++	+++	+
6	Gag	++	++	-
7	Gag	+	+	-

ELISPOT:

+ ≥ 400 SFU

++ = 401-4000 SFU

+++ ≥ 4000

ICS

+ > 0.05-0.5 %

++= 0.5-2.0 %

+++ >2.0%

5.3.3 Comparison between freshly thawed PBMC and in-vitro expanded CD8+ T cells in the ELISPOT assay.

The use of bispecific monoclonal antibodies for in vitro enrichment of T cells has been shown to allow expansion of CD8+ T cell populations without loss of specificity (Jones *et al*., 2003). The bispecific enriched cells in this study demonstrated comparable repertoire to the original, unexpanded PBMC. In this dissertation, freshly thawed PBMC from 10 HIV/TB individuals were analysed in the ELISPOT assay and compared with in vitro expanded PBMC. PBMC from these individuals were expanded as discussed on section 5.2.3. Low frequencies of CD8+ T cells were readily expanded, and yielded higher levels of responses (p= 0.019) as shown on Figure 5.6. These data supports the in vitro expansion of specific cell populations from low frequencies when multiple

functional studies are required. These findings suggest that there was probably enrichment of memory T cell responses leading to expanded CD8+ T cell specificities.

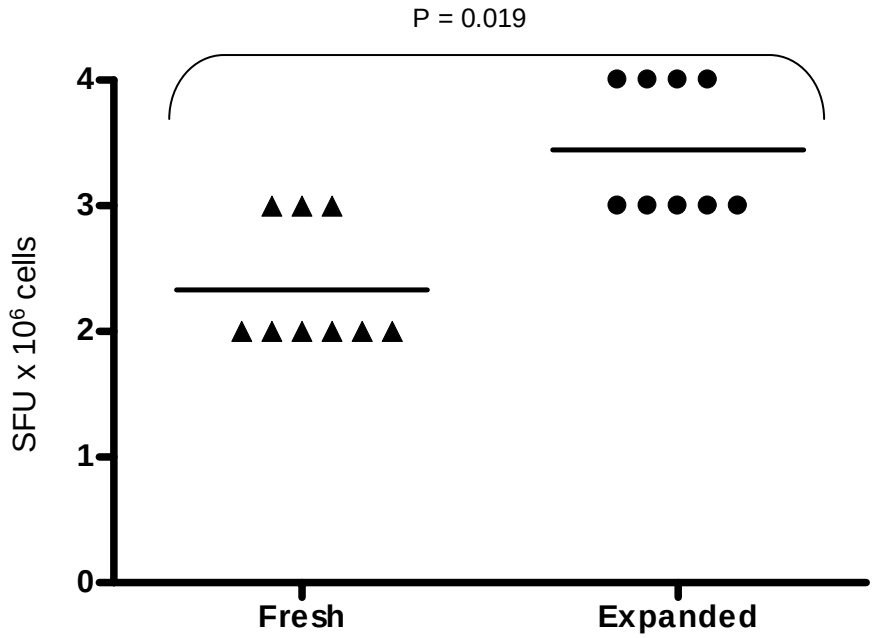


Figure 5.6: HIV-specific T cell responses from freshly thawed and in vitro expanded PBMC. The p-value was calculated by the Mann-Whitney U test.

5.3.4 The relationship between HIV-1 RNA load and HIV-1-specific responses from HIV/TB co-infected individuals

Effective cytotoxic T cell responses have been shown to cause a drop in plasma viral load in acute HIV-1 infection (Klein *et al.,* 1995; Walker *et al*., 1996, Jin *et al*., 1999) and a reduction of viral replication (Koup *et al*., 1994) has been demonstrated. Gag p24-specific immune responses have also been reported in the control of viraemia (Novisky *et al*., 2003) whereas a significant positive association was observed between Nef and viral load (Masemola *et al*., 2004) from HIV-1 subtype C infected individuals. This

dissertation examined the association between HIV-1 subtype C specific responses and viral load in 10 HIV-1 infected patients with mycobacterium tuberculosis co-infection. Freshly thawed PBMC samples were stimulated in a 6 hour ELISPOT assay with nine HIV-1 subtype C proteins (whole genome), as described in section 5.2.3. There was no significant relationship between the viral load and HIV-1-specific T cell responses (cumulative SFU/10^6 cells) for any of the HIV-1 proteins (Figure 5.7). Even though these were not statistically significant, Env, Tat and Vpu showed a trend towards a positive correlation and Gag showed a trend towards negative correlation with viraemia.

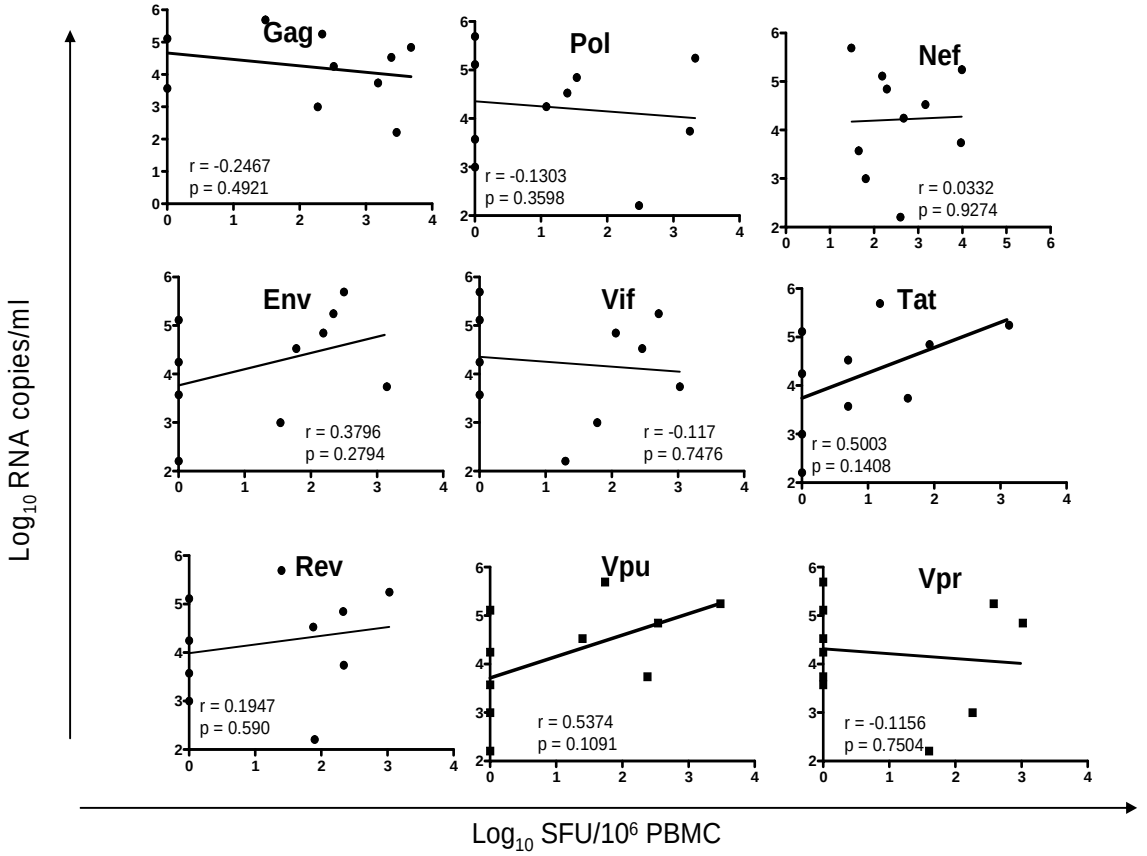


Figure 5.7: The relationship between HIV-1 RNA load and HIV-1-specific responses to the nine HIV-1 subtype C peptide superpools from HIV/TB co-infected individuals. The correlation coefficient and the p values were obtained using GraphPad Prism version 4.

HIV-1 subtype C responses to individual gene regions did not have any significant impact on viraemia in HIV/TB co-infected individuals. In addition, correlation of response to complete protein regions (cumulative SFU/10^6 PBMC) with viral load was investigated to determine whether total responses would have an impact on viraemia. There was no statistically significant association between viraemia and the total SFU/10^6 PBMC in these patients (Figure 5.8A). Similar results were observed when viraemia was correlated with breadth of responses in these individuals (Figure 5.8B), which was defined as the number of peptide pools targeted by each individual.

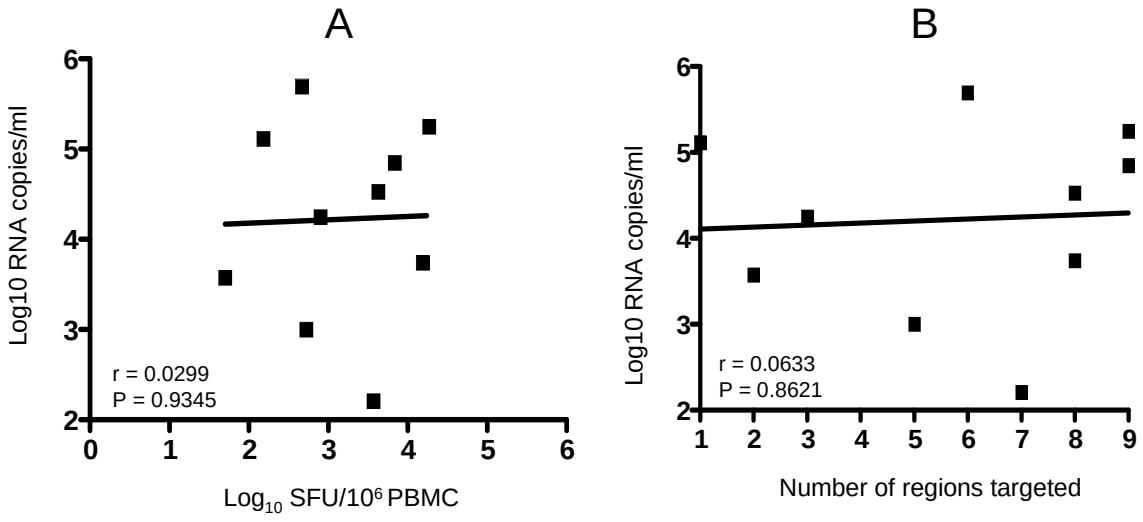


Figure 5.8: Correlation between (A) Cumulative responses and viral load and (B) Number of targeted HIV-1 subtype C regions (breadth) and viral loads.

The association between CD4 count and total T cell response was performed to determine whether a higher magnitude of response or recognition of most of the HIV-1 proteins would correlate with a higher CD4 count; used as a measure of HIV-1 disease progression to AIDS. No statistically significant correlation was observed between both

the cumulative SFU/10^6 PBMC (Figure 5.9A) and the number of targeted gene regions by HIV/TB co-infected patients (Figure. 5.9B).

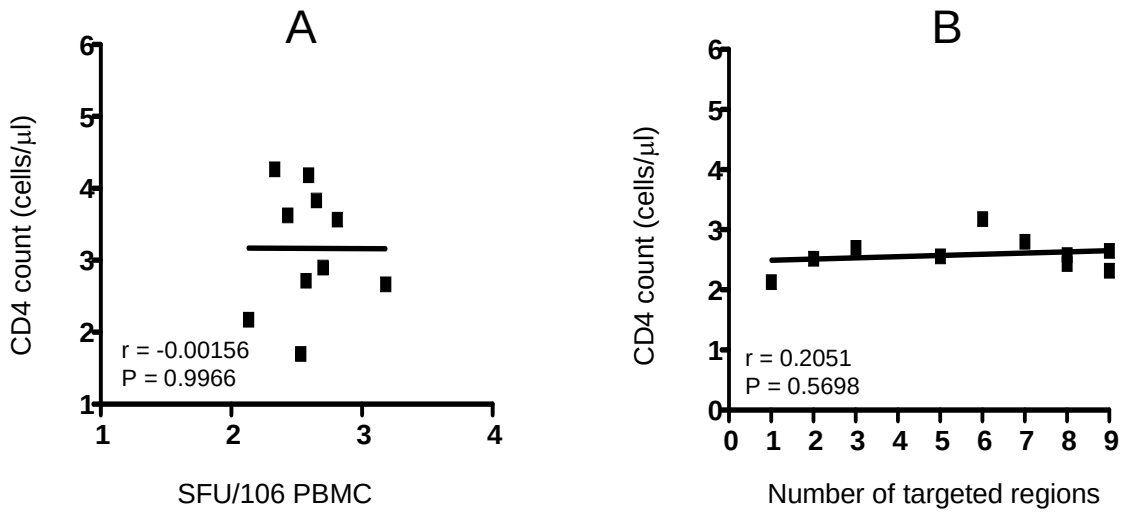
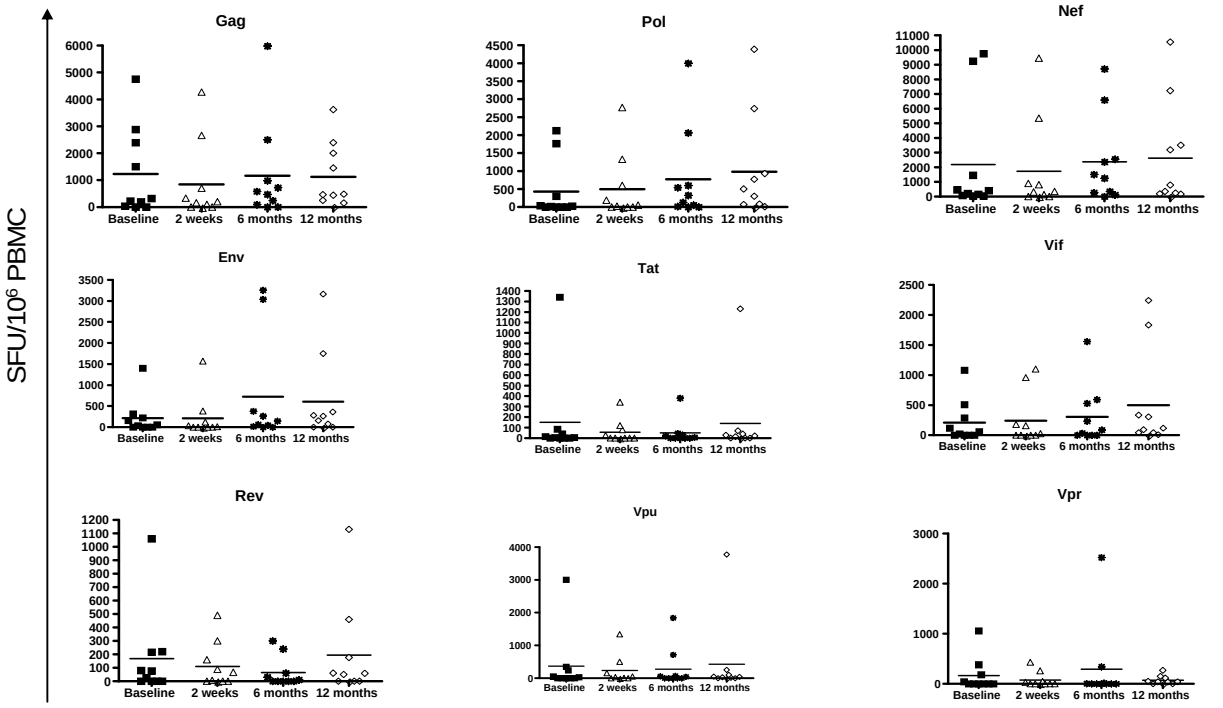


Figure 5.9: Correlation between (A) Cumulative responses and CD4 count and (B) Number of targeted HIV-1 subtype C regions and CD4 count.

5.3.5 Effect of TB treatment on HIV-1 subtype C-antigen specific CD8+ T cell responses.

This dissertation examined the pattern of response to pooled peptides in individuals who were treated for TB disease, mainly to determine the effect of TB on T cell responses to HIV-1 subtype C. The pattern of responses was altered in the presence of co-infection with TB and it is hypothesized that TB treatment would enhance responses to the most dominant proteins in this cohort group. PBMCs from 10 HIV/TB patients were processed in the ELISPOT assay and the total mean SFU/10^6 cells for each of the nine peptide superpools were compared at 4 different time points (baseline, 2 weeks, 6 months and 12

months). The One-way ANOVA, Prism 4 Bonferroni Test established that there were no significant differences in the magnitude of responses at different time points (Figure 5.10) during TB therapy.



	Gag	Pol	Vif	Vpr	Tat	Rev	Vpu	Env	Nef
P-value	0.9483	0.6859	0.8047	0.6579	0.8047	0.6908	0.9572	0.4624	0.9481

Figure 5.10: HIV-1-specific T cell responses to nine HIV-1 subtype C peptides at 4 different time points during TB therapy. The magnitude of responses were compared, and the statistical P values for each peptide superpool are shown in the table.

5.4 DISCUSSION

HIV-1 subtype C-specific T cell responses were readily detected in both HIV-1 infected and HIV/TB co-infected individuals in this dissertation. Previous studies have demonstrated HIV-1–specific responses in HIV-1 subtype C infected individuals (Novitsky *et al.,* 2003, Masemola *et al*., 2004). The effect of TB on T cell responses has not been extensively studied. It has been reported previously that Gag-specific CTL contribute to maintenance of the asymptomatic state by effectively controlling HIV-1 replication (Klein *et al.,* 1995). It was further suggested that the more immune pressure the host's immune system can mount against HIV Gag, and in particular Gag p24, the more the virus can be controlled (Zuniga *et al*., 2006). The fact that recognition of Gag was dominant in HIV-1 singly infected individuals in this dissertation, and this was not the case in HIV-1 infected patients that were co-infected with TB, may have an impact on the control of HIV-1. However, the magnitude of response to Gag was not significantly affected by TB co-infection. These data provide an understanding of factors that may possibly skew the dominance of an effective HIV-1 specific immune response, and co-infection with other pathogens should therefore be considered when peptide based vaccines are designed. The intracellular cytokine staining (ICS) method proved to be effective for confirmation of T cell responses observed from the ELISPOT, and it had an advantage of identifying CD4+ and CD8+ lymphocytes that respond to specific HIV-1 peptides. HIV-1–specific responses were largely mediated by CD8+ cytotoxic T lymphocytes. Even though this method was only used to measure HIV-1 –specific cells in this dissertation, the use of ICS can be extended to analyse the lineage of HIV-1-specific

T cells which provides valuable information on the functionality of various T cell subsets as well as differentiated cells and their quality in HIV-1 control.

Some previously reported studies demonstrated no correlation between HIV-1-specific CTL responses and viraemia (Addo *et al*., 2003, Peretz et al 2005), whereas some have shown that patients who mounted strong gp160-specific responses, as measured by the ELISPOT assay showed rapid reduction of acute plasma viraemia (Borrow *et al*., 1994) and others have reported an important role of HIV-1 Gag p24-immune responses in the control of viraemia and no restraint of viral load from responses to Nef (Novinsky *et al*., 2003, Edwards *et al*., 2002). Preferential targeting of Gag has been previously shown to be associated with low viraemia in HIV-1 subtype C infected individuals (Masemola *et al*., 2004). However, it should be noted that the protective role of HIV-1-specific CTL responses predominantly occurs in primary infection (Koup *et al*., 1994). A progressive loss of HIV-1 specific cytolytic activity in the advanced stages of the disease in the presence of substantial CTL responses has been reported (Klein *et al*., 1995, Pantaleo *et al*., 1990). The results from this dissertation supports the former findings, where all the markers of HIV-1 disease progression (CD4+ T cell counts and viral load) showed no association with measurements of T cell response in HIV-1 infected individuals co-infected with TB, although there were trends and lack of significance, which may be due to small numbers of individuals. Findings from this dissertation showed no effect of HIV-1 –specific T cell response on the control of viraemia and HIV-1 disease progression in the presence of TB. A previously reported longitudinal study revealed that rapid progressors were able to mount substantial transient Gag-specific CTL response which

disappeared with disease progression (Klein *et al*., 1995). In this dissertation, HIV-positive individuals with TB co-infection did not appear to preferentially target Gag. Whether this may influence or be an effect of TB disease (infection) would need to be further studied. Does the lack of Gag targeting in these individuals relate to more rapid disease progression?

CONCLUDING REMARKS

Various aspects of cellular immunity to HIV-1 were examined in this dissertation, using four different cohorts which demonstrated phenotypic changes, including CD8+ T cell activation, restricted T cell receptor usage and altered HIV-1 specific responses due to co-infection with TB. The data have implications for understanding pathogenesis in HIV infected patients co-infected with TB. An array of techniques was applied to measure these properties: a) Flow cytometry, b) ICS, c) Immunoscope, d) ELISPOT. Using these assays, various aspects examining the characteristics and functionality of T cells were explored.

The loss of CD4+ T cells observed in both HIV-1 infected and HIV/TB co-infected patients was shown to be caused by HIV-1, and not by TB. There was a remarkable loss of naïve CD4+ T cells due to HIV infection, which was driven by viral load. Maintenance of high viraemia and low CD4 counts was demonstrated, despite TB co-infection. The level of activation in HIV-1 infected individuals was high, regardless of TB co-infection, which was also probably driven by HIV plasma RNA load (viral load). The activated T cells were sustained, despite TB treatment in HIV/TB co-infected patients, providing evidence for advaced stage HIV-1 infection.

In addition to these changes selected expansions of CD8+ T cells expressing Vβ T cell receptor were demonstrated. Polyclonal and oligoclonal expansions were noted in both HIV-1 and HIV/TB co-infected individuals. Minor polyclonal expansions were also

observed in individuals with TB infection only. However, there was no common pattern of expanded T cell families observed within HIV-1 single infection or in the presence of TB co-infection. Expansion of totally different Vβ families within the same cohort group may suggest usage of unique T cell receptors by each individual in response to HIV-1. Oligoclonal expansions of T cells observed in some of the patients in this dissertation indicate limited and restricted usage of the T cell repertoire, which may impose limited recognition of viral variants, leading to immune escape and enhanced viral replication and disease progression. Further studies are necessary to determine whether these expanded T cell populations can elicit a protective immune response to HIV-1 infection. It may also be necessary to examine the diversity of HIV-1 specific clones in the presence of opportunistic infection such as mycobacterium tuberculosis.

The TCR repertoire in another cohort of HIV-1 exposed uninfected neonates was investigated to assess TCR usage without infection. Oligoclonal and polyclonal expansions observed in HIV uninfected babies born to HIV-infected mothers suggested that these babies were exposed to HIV-1 antigens in-utero, and that they possess a mature T cell repertoire at birth. Future studies are necessary to determine whether T cell expansions noted in these neonates are associated with protective immunity to HIV-1 or they may merely be a symbol of exposure to antigen.

Analysis of HIV-1 –specific responses in both HIV-1 and HIV/TB co-infected patients revealed that CD8+ T cells from both cohorts were able to recognize and respond to one or more of the nine HIV-1 Subtype C proteins. Different patterns of recognition observed

between the two cohort groups suggest that co-infection with TB may possibly change the pattern of recognition across the HIV-1 genome. Shifting of immunodominance was observed in the presence of dual infection with HIV and TB. These data may lead to the hypothesis that TB co-infection alters patterns of T cell targeting and recognition.

Overall, this study demonstrated high activation of T cells driven by high HIV-1 plasma RNA load in response to HIV-1 infection, restricted TCR usage and shifting of immunodominant CD8+ T cell responses as a result of co-infection with TB. TB treatment had no impact on the loss of naïve T cells or reduction of immune activation. This may imply that TB had no role to play in the significant phenotypic alterations observed in HIV-1 infected individuals. Thus, combination of TB treatment with HAART may be considered a better option in patients with dual infection This dissertation further demonstrated that it is possible to detect perturbations in the TCR repertoire in likely exposed and HIV uninfected babies, as a result of exposure to antigens in utero. It remains to be seen whether these perturbed T cell populations provide immune competence upon encounter with antigens at some point after birth.

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APPENDICES

APPENDIX 1

Recipe for Haematology diluent

dH_2O	482.2 g
NaCl	16.8 g
Kcl	15.6 g
KH_2PO_4	141.0 g
LiCl	25.8 g
EDTA	21.6 g
N HCl	75 ml

Recipe for Cell Fixer

PBS + 2% BSA +1.5% Formalin

APPENDIX 2

RNA Verification

The RNA was extracted from the PBMC samples but no product was seen from cDNA amplification in some of the samples. To investigate whether the RNA has not degraded, an RNA gel was run following the procedure outlined below:

RNA Loading Buffer

- 0.5 M EDTA 4 µl
- 1% Bromothemol blue 500µl
- 1% Xylene Cyanol 500µl
- 100% glycerol 1000µl

10X RNA running buffer

- 200mM MOPS 20.95g/500ml
- 50mM NaAc 3M 2.051g/500ml
- 10mM EDTA 0.35M(PH7.00) 1.861g/500ml

1% Gel mix

- Agarose 0.4g
- H2O 27ml
- 10 X RNA running buffer 4ml
- Add 8.5ml formaldehyde

The gel is poured into the gel casting tray and allowed to set at room temperature

RNA mix (use 10-20µg RNA)

- DEPC water 3.3µl
- 10X RNA running buffer 1.5µl
- Formamide 7.5µl
- Formaldehyde 2.7µl
- RNA loading buffer 1.5µl
- RNA 10µl

  Final volume = 26µl

- Heat the mixture at 55^{o}C for 10 minutes
- Place on ice
- RNA was then loaded on a gel and run at 30mA.
- The gel was then stained with Ethidium bromide, rinsed with five changes of water and then photographed (figure 27). The gel showed clearly separated two bands of RNA.

APPENDIX 3

A list of TCR primers used and their sequences.

Primer	Sequence
HuVβ1	ccg cac aac agt tcc ctg act tcg
HuVβ2	cac aac tat gtt ttg gta tcg tc
HuVβ3	cgc ttc tcc ctg att ctg gag tcc
HuVβ4	ttc cca tca gcc gcc caa acc taa
HuVβ5	gat caa aac gag agg aca gc
HuVβ6a	gat cca att tca ggt cat act g
HuVβ6b	cag gg(g/c) cca gag ttt ctg ac
HuVβ7	cct gaa tgc ccc aac agt tct
HuVβ8	Ggt aca gac aga cca tga tgc
HuVβ9	ttc cct gga gct tgg tga ctc tgc
HuVβ11	gtc aac agt ctc cag aat aag g
HuVβ12	tcc (c/t) cc tca ctc tgg agt c
HuVβ13a	ggt atc gac aag acc cag gca
HuVβ13b	agg ctc atc cat tat tca aat ac
HuVβ14	ggg ctg ggc tta agg cag atc ctg
HuVβ15	cag gca cag gct aaa ttc tcc ctg
HuVβ16	gcc tgc aga act gga gga ttc tgg
HuVβ17	tcc tct cac tgt gac atc tct cag cct cca
HuVβ18	ctg ctg aat ttc cca aag agg gcc
HuVβ20	tgc ccc aga atc tct cag cct cca
HuVβ21	gga gta gac tcc act ctc aag
HuVβ22	gat ccg gtc cac aaa gct gg
HuVβ23	att ctg aac tga aca tga gct cct
HuCβ1	ggg tgt ggg aga tct ctg c
HuCβ2	Fam-aca cag cga cct cgg gtg gg

APPENDIX 4

PCR mixture for immunoscope assay

DEPC-H_2O	315µl
25mM Mg+ (Invitrogen Life Tech)	50µl
10X Taq buffer (Invitrogen Life Tech)	62µl
2mM DNTP (Roche Diagnostics)	62µl
BC Primer (Inqaba Biotechnical Industries)	3µl
Taq DNA polymerase (Invitrogen Life Tech)	3µl
CDNA (or DEPC H_2O) for control	5µl
Total mixture	500µl

APPENDIX 5

Preparation of 0.9 M Tris Borate, 20 mM EDTA [10 X TBE] buffer (in-house)

Tris mw 121.1	1080gm
Boric acid	550gm
0.5M EDTA PH 8.0	400ml

Preparation method:

Dissolve the TRIS in + 6 litres of water

Add the Boric acid directly to the solute

Add water up to 9.5 litres (leave space for EDTA) and shake until dissolved

Add the EDTA and make up to ten litres

Filter sterilize with positive pressure - Store at $4^{o}C$

Note: Use a 293 filter for 3 x 10 litres

Preparation of 2% agarose gel and loading of samples:

- 2g of agarose poured onto-
- 100ml of TBE in a sterile glass bottle
- The gel was heated in a microwave until the mixture was clear
- The mixture was allowed to cool and poured onto a gel tank with wells created using a gel comb and allowed to set.
- 5µl of the product was mixed with 3µl of DNA loading buffer:

Preparation of DNA loading buffer:

To make 1ml: v/v 50% glycerol	*500µl*
10 x TBE	*100µl*

200µl 0.5% Bromothemol blue and 200µl 0.5% Xylene Cyanol. (Store at -20^{o}C).

APPENDIX 6

PCR mixture for 24 runoff reactions:

27.5µl	10X AmpliTaq buffer (Applied Biosystems)
27.5µl	2mM each DNTPs (Roche)
33µl	25mM $MgCl_2$ (3mM final- Applied Biosystems)
3µl	Human 6 FAM labeled cβ-2 primer (Inqaba Biotech)
128µl	Sterile water
1µl	AmpliTaq enzyme (Applied Biosystems)
220 µl	Total volume of mixture

APPENDIX 7

Running Module for acquisition of samples on a sequencer for immunoscope analysis

- Select dye set D
- Run temp $50^{o}C$
- Capillary Fill volume 184
- Pre-run Voltage 12.2
- Injection voltage 20
- Run voltage 15
- Data Delay Time 900
- Run Time 3250
- Analysis module is GS400Analysis.gsp

APPENDIX 8

The TCR profile of healthy control babies

Sample ID	Oligoclonal Skewed (%)	Polyclonal Skewed (%)	Polyclonal Gaussian (%)
4863	0	70	30
4867	0	84	16
4857	0	7	93
4835	0	68	32
4829	0	53	47
4923	0	83	17
4855	0	75	25
4925	0	0	100
4861	0	45	55
4827	0	64	36
4791	0	50	50
4795	0	63	37
4831	0	50	50
4859	0	77	23
4908	0	0	100
4797	0	10	90
2163	0	67	33
4817	0	31	69
4833	0	94	6
4893	0	75	25
4837	0	100	0
4897	0	14	86
4935	0	70	30
4789	0	74	26
4933	0	26	74
mean	0	54	46

APPENDIX 9

The TCR profile of HIV uninfected babies born to HIV-1 infected mothers

Sample ID	Oligoclonal Skewed (%)	Polyclonal Skewed (%)	Polyclonal Gaussian (%)
B1	0	95	5
B2	0	100	0
B3	24	76	0
B4	94	6	0
B5	0	95	5
B6	0	100	0
B7	80	20	0
B8	86	14	0
B9	10	90	0
B10	10	90	0
B11	0	100	0
B12	4	91	4
B13	0	100	0
B14	75	25	0
B15	0	100	0
B16	0	100	0
B17	83	17	0
B18	14	86	0
B19	0	100	0
B20	67	33	0
mean	**27**	**72**	**1**

APPENDIX 10

The TCR profile of HIV-1 infected mothers

Sample ID	Oligoclonal Skewed (%)	Polyclonal Skewed (%)	Polyclonal Gaussian (%)
4000	88	12	0
4003	75	25	0
4038	36	64	0
4139	79	21	0
4152	33	67	0
4204	62	38	0
4211	30	70	0
4216	5	95	0
4218	71	29	0
4222	94	6	0
4226	100	0	0
4231	86	14	0
Mean	63	37	0

APPENDIX 11

Cohort groups

HIV/TB (freshly thawed)

SAMPLE ID NUMBER	CD4	VL
JLD 20	136	132001
LMM 072	446	70764
BRG 113	1517	500000
BBS 077	588	164
NTM 119	374	1008
ZKC 084	506	17878
JNM 55	199	33569
NYJ 060	335	3773
TMZ 123	385	5547
MPM 135	216	178465

HIV/TB+ (in vitro expanded)

SAMPLE ID NUMBER	CD4	VL
IM018		
IM021	177	35413
IM029	945	6378
IM030	66	49172
IM060	939	67166
IM062	536	5217
IM066	324	3645
PMM071	117	545898
VSG049	990	9010
NPM100	736	338
SBK121	691	482

HIV+ (in-vitro expanded)*

Dur10
Dur24
Dur27
JHB30
JHB31
JHB32
JHB39
JHB41
IM077
IM080
IM086
IM087
IM089
IM092

*Clinical data was not available for the HIV+ in-vitro expanded cohort.

APPENDIX 12

ELISPOT results of HIV-1 singly infected individuals presented as SFU/10^6 PBMC

	Gag	Pol	Vif	Vpr	Tat	Rev	Vpu	Env	Nef
Dur10	1364	498	20	0	0	50	0	0	2930
Dur24	206	280	10	0	170	20	40	12	7
Dur27	730	125	240	300	400	0	140	180	24
JHB30	200	197	0	70	0	0	10	10	40
JHB31	522	105	230	0	10	3270	0	76	1526
JHB32	4216	1670	230	360	60	100	120	140	192
JHB39	176	390	380	450	200	1560	590	1196	18
JHB41	116	245	75	110	20	130	10	48	408
IM077	9	33	50	0	77	242	33	128	253
IM080	364	335	1690	0	60	460	0	0	218
IM086	250	130	45	40	10	40	20	10	15
IM087	0	0	74	4	0	4	0	0	349
IM089	116	15	20	530	0	210	0	0	112
IM092	80	305	1150	80	0	0	0	128	908

APPENDIX 13

ELISPOT results of HIV/TB co-infected individuals presented as SFU/10^6 PBMC

	Gag	Pol	Vif	Vpr	Tat	Rev	Vpu	Env	Nef
IM018	0	0	0	0	0	300	0	0	0
IM021	14	328	169	0	100	10	20	185	161
IM029	75	103	66.25	40	480	20	50	48	383
IM030	75	422	469	1725	38	62.5	388	729	50
IM060	60	47	120	200	0	0	0	0	113
IM062	60	253	600	0	0	20	0	217	283
IM066	720	1543	2303	0	0	40	0	45	2053
PMM071	50	213	460	1580	0	0	0	260	2555
VSG049	0	1035	5260	0	20	0	160	860	148
NPM100	680	50	0	60	40	0	50	2	0
SBK121	1232	427.5	240	0	20	50	40	80	240

APPENDIX 14: ETHICAL CLEARANCE

This study was approved by the University of the Witwatersrand Ethical Committee, protocol number M080114. Patients were recruited after informed consent was obtained and confidentially of all records ensured.