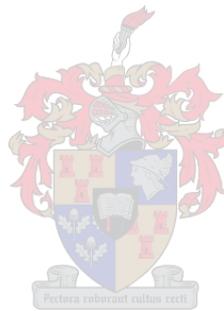


**CORECEPTOR EXPRESSION AND T LYMPHOCYTE SUBSET  
DISTRIBUTION IN HIV-INFECTED AND TB CO-INFECTED SOUTH  
AFRICAN PATIENTS ON ANTI-RETROVIRAL THERAPY**

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Dissertation presented for the degree of Master of Science (Medical Virology)  
at Stellenbosch University

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December 2009

## **DECLARATION**

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2009

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# DEDICATION

I dedicate this work to:

- 1 My wife and dearly, Elysee Tulubukayi
- 2 My children, Emmanuel, Joseph, Ruth and Daniel

## ABSTRACT

In 2007, AIDS caused an estimated 2.1 millions deaths worldwide; about 70% in sub-Saharan Africa. HIV preferentially targets activated CD4 T cells, expressing the major HIV receptor CD4, as well as the major chemokine coreceptors CCR5 and CXCR4. These coreceptors play a prominent role during HIV cell entrance phase, HIV transmission and also disease progression. They have been found to be differentially expressed by CD4 T cell subsets. Tuberculosis coinfection may enhance immune activation *in vivo* thus accelerating HIV disease progression and has become a major challenge in the control of TB in Africa. Introduction of HAART has reduced disease progression to AIDS, as well as risk of further morbidity and mortality. HAART results in a rapid decline of viral load and an initial increase of peripheral CD4 count, however little is known on the effect of HAART in regulation of coreceptor expression, immune activation status and CD4 T cell subset distribution in HIV infection and HIV/TB coinfection.

This study is a cross-sectional analysis of coreceptor expression, immune activation status and CD4 T cell subpopulation distribution in South African HIV and HIV/TB coinfecting patients before and after ARV. A total of 137 South African individuals were investigated, comprising 15 healthy normal donors (healthy subgroup), 10 patients with active pulmonary tuberculosis (PTB subgroup), 33 HIV-1 positive patients without active PTB (HIV subgroup), 23 positive patients with active PTB (HIV/PTB subgroup), 36 HIV-1 positive patients on ARV (HIV on ARV subgroup) and 20 HIV-1 positive patients with active PTB on ARV (HIV/PTB on ARV subgroup).

CD4 absolute count and plasma viral load were determined for all donors. Freshly isolated PBMC were classified by flow cytometry into the following CD4<sup>+</sup> T lymphocyte subsets: naïve (CD45<sup>+</sup>, CD27<sup>+</sup>), effector memory (CD45<sup>-</sup>, CD27<sup>-</sup>), central memory (CD45<sup>-</sup>, CD27<sup>+</sup>), and effector (CD45<sup>+</sup>, CD27<sup>-</sup>). Coreceptor expression and activation status was assessed by CCR5, CXCR4 and CD38 expression on CD4 T cell subsets.

HIV, TB and HIV/TB coinfection was associated with a decrease in percentage CCR5<sup>+</sup> T cells as compared to healthy controls, with the HIV/TB group showing the most extensive decrease. In treatment naïve patients, CD4 T cells showed elevated surface expression of CCR5 and CD38 as determined by mean fluorescence intensity in HIV/TB co-infection compared to HIV infection alone. The percentage of antigen-experienced cells was higher in

the HIV/TB co-infected group compared to the HIV group. The percentage of naïve T cells was decreased in both the HIV infected and the HIV/TB co-infected groups compared to healthy controls. HIV patients with more than 6 months of ARV showed decreased CCR5 and CD38 surface level expression in the HIV and the HIV/ TB co-infected subgroups. An increased percentage of naïve T cells was observed in the HIV infected subgroup, but not in the HIV/TB subgroup, similarly, a decreased percentage of antigen-experienced cells was observed in the HIV subgroup, but not in the HIV/TB co-infected subgroup. A positive correlation was found between CCR5 and CD38 expression, and CXCR4 and CD38 expression (Spearman coefficient of correlation respectively:  $r=0.59$ ,  $p<0.001$  and  $r=0.55$ ,  $p<0.001$ ). Furthermore we found plasma viral load positively associated with CD38 expression ( $r=0.31$ ,  $p<0.001$ ) and percentage activated CCR5+ expressing CD4 T cells positively related to viral load ( $r=0.31$ ,  $p<0.001$ ). Percentage naïve CD4 T cells was positively associated with CD4 count ( $r=0.60$ ,  $p<0.001$ ) and negatively correlated to viral load ( $r=-0.42$ ,  $p<0.001$ ).

These results indicate that TB coinfection exacerbates certain aspects of dysregulation of CD4 T cell homeostasis and activation caused by HIV infection. In addition, ARV-associated decrease in coreceptor expression, immune activation status and a normalisation of CD4 T cell subset distribution was observed in HIV infected individuals, but not in HIV/TB co-infection. Despite viral suppression after ARV treatment, the decline in the immune activation marker CD38 and coreceptor CCR5 expression, increase in percentage naïve CD4 T cells and decrease of antigen-experienced cells did not reach the levels displayed in the healthy control group. This may indicate that ongoing (albeit reduced) T cell immune activation may occur in the presence of ARV. Further longitudinal studies are needed to closely monitor immune activation during ARV treatment.

This study highlighted an association of TB disease with immune activation in HIV infection, the importance of T-cell activation in HIV pathogenesis and its impact on ARV treatment. Further studies are needed to identify causative factors that may lead to a persistent immune activation status during ARV treatment, and how TB coinfection confounds normal responses to ARV.

## OPSOMMING

In 2007 was ongeveer 2.1 miljoen sterftes wêreldwyd veroorsaak deur VIGS; ongeveer 70% in Sub-Sahara Afrika. CD4 T selle is die hoof teiken van MIV, aangesien dit die primêre CD4 reseptor, sowel as een of beide van die vernaamste chemokien koreseptore CCR5 en CXCR4 vrystel. Hierdie koreseptore speel 'n prominente rol wanneer die MIV die sel binnedring, asook tydens MIV oordrag en verloop van die siekte. Dit word ook deur verskillende fraksies van CD4 T selle vrygestel. Gelyktydige TB infeksie mag immunaktivering *in vivo* verhoog en dus die siekeproses versnel. MIV het 'n groot uitdaging geword in die beheer van TB in Afrika. Bekendstelling van HAART het die ontwikkeling van VIGS vertraag, asook die risiko van verdere morbiditeit en mortaliteit. HAART veroorsaak 'n vinnige afname in virale lading 'n toename in CD4 telling, hoewel die spesifieke invloed van HAART op die regulering van koreseptor vrystelling, immunaktivering en verspreiding van CD4 fraksies in MIV en MIV/TB infeksies nog onduidelik is.

Hierdie studie het gepoog om koreseptor vrystelling, immunaktiveringstatus en die verspreiding van CD4 subpopulasies in pasiënte met MIV en MIV/TB voor en na ARV behandeling te ondersoek. 'n Totaal van 137 Suid-Afrikaanse individue is ondersoek en die studiegroep het bestaan uit 15 normale persone (gesonde subgroep), 10 pasiënte met aktiewe pulmonale TB (PTB subgroep), 33 MIV positiewe pasiënte sonder PTB (MIV subgroep), 23 MIV positiewe pasiënte met aktiewe PTB (MIV/PTB subgroep), 36 MIV positiewe pasiënte op ARV (MIV op ARV subgroep) en 20 MIV positiewe pasiënte met aktiewe PTB op ARV (MIV/PTB op ARV subgroep).

Absolute CD4 telling en virale ladings was bepaal vir alle deelnemers. Vars geïsoleerde perifere bloed mononukleêre selle is geklassifiseer deur middel van vloesitometrie as die volgende CD4 T limfosiet subgroepe: naïewe selle (CD45+, CD27+), effektor geheueselle (CD45-, CD27-), sentrale geheueselle (CD45-, CD27+), en effektor selle (CD45+, CD27-). Koreseptor vrystelling en aktivering was beoordeel volgens CCR5, CXCR4 en CD38 vrystelling op CD4 T sel subgroepe.

HIV, TB en MIV/TB ko-infeksie is geassosieer met 'n afname in die persentasie CCR5+ T selle, vergeleke met gesonde kontroles, waar die MIV/TB subgroep die grootste afname getoon het. In onbehandelde pasiënte het die CD4 T selle verhoogde vrystelling van CCR5 en CD38 op die oppervlakte getoon en dit is bevestig deur die gemiddelde fluoësserende

intensiteit in die MIV/TB subgroep vergeleke met die subgroep met slegs MIV. Die MIV/TB subgroep het verder ook 'n verhoogde persentasie totale geheue T selle getoon vergeleke met die MIV subgroep. Die persentasie naïewe T selle was egter verlaag in beide die MIV en MIV/TB subgroepe vergeleke met normale kontroles. MIV pasiënte wat langer as 6 maande op ARV behandeling was in beide die MIV en MIV/TB subgroepe, het 'n verlaagde vrystelling van CCR5 en CD38 op die oppervlakte van die CD4 selle getoon. 'n Verhoogde persentasie naïewe T selle het in die MIV subgroep voorgekom, maar nie in die MIV/TB subgroep nie. 'n Soortgelyke tendens is gevind waar die persentasie totale geheueselle verlaag was in die MIV subgroep, maar nie in die MIV/TB subgroep nie. 'n Positiewe korrelasie is gevind tussen CCR5 en CD38 vrystelling, asook CXCR4 en CD38 vrystelling (Spearman korrelasie koëffisiënt:  $r=0.59$ ,  $p<0.001$  en  $r=0.55$ ,  $p<0.001$  onderskeidelik). Verder het die plasma virale lading 'n positiewe assosiasie getoon met CD38 vrystelling ( $r=0.31$ ,  $p<0.001$ ) en die persentasie geaktiveerde CCR5+ vrystellende CD4 T selle met virale lading ( $r=0.31$ ,  $p<0.001$ ). Die persentasie naïewe CD4 T selle het 'n positiewe assosiasie getoon met CD4 telling ( $r=0.60$ ,  $p<0.001$ ) en 'n negatiewe korrelasie met virale lading ( $r=-0.42$ ,  $p<0.001$ ).

Volgens hierdie resultate vererger TB ko-infeksie sekere aspekte van die disregulasie van CD4 T selhomeostase en aktivering as gevolg van MIV infeksie. Verder kon 'n ARV-geassosieerde afname in koreseptor vrystelling, immuunaktivering en normalisering van CD4 T sel fraksies bespeur word in die MIV subgroep, maar nie in die MIV/TB subgroep nie. Ten spyte van virale onderdrukking veroorsaak deur ARV behandeling, het die afname in die immuunmerker CD38 en koreseptor CCR5, toename in die persentasie naïewe CD4 selle en afname in totale geheue CD4 T selle nie die vlakke van die normale kontrolegroep bereik nie. Dit is moontlik dat volgehoue verlaagde T sel immuunaktivering nog steeds mag plaasvind in die teenwoordigheid van ARV. Verdere longitudinale studies is nodig om immuunaktivering tydens ARV behandeling te monitor.

Hierdie studie het die belangrikheid van T sel aktivering in MIV patogene en dit impak daarvan op ARV behandeling beklemtoon. Verdere studies is nodig om moontlike oorsake of bydraende faktore te identifiseer wat tot volgehoue immuunaktivering tydens ARV behandeling kan lei, asook tot mate waartoe TB ko-infeksie kan inmeng met die normale werking van ARV behandeling.

## ACKNOWLEDGEMENTS

- 1 I wish to express my sincere gratitude to you, my supervisor, Dr Corena de Beer, for your guidance of this work and financial support during this study.
- 2 I thank my copromoter, Dr Richard H Glashoff, for the time spent on supervising me and carefully examining this thesis.
- 3 I thank Professor Wolfgang Preiser for his support. In fact it is through his GHRC project we were able to perform this study.
- 4 I would like to express my sincere gratitude to my other teachers in Medical virology: Dr Gert van Zyl, Prof Susan Engelbrecht and Dr Walter Liebrich for sharing their knowledge of Medical Sciences to me during this study.
- 5 My acknowledgments to the following colleagues for their practical support to this study: Sam Pillay, Jan de Wit, Ronell Taylor, NHLS medical laboratory technologists, Tygerberg Campus.
- 6 Thank you to all friends, colleagues and Medical Virology Staff members for enjoyable times in the laboratory.
- 7 Many thanks to my love and spouse, Elysee Tulubukayi, for her support and encouragement.
- 8 Also I would like to thank Pastor Gerald Johannes Redelinghuis (Lighthouse Ministries), Mr Noel Bekkers, Mr Beya and Mr Guy Olembe for the assistance and encouragement.
- 9 Finally, I wish to thank NHLS Research Trust for financial support.

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## LIST OF ABBREVIATIONS

3 TC	Lamivudine
Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
AR10	AIM-V, RPMI plus 10% serum
ART	Antiretroviral therapy
ARV	Antiretroviral
ATV	Atazanavir
AZT	Zidovudine
BD	Becton Dickinson
CA,USA	California, United States of America
CCR5	Chemokine receptor in the CC chemokine group
CD	Cluster of differentiation
CDC	Centers for Disease Control and prevention
CM	Central memory (cells)
CTL	Cytotoxic T lymphocyte
CXCR4	Chemokine receptor in the CXC family
CYP	Cytochrome protein
d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
DNA	Deoxyribonucleic acid
E	Effector (cells)
EDTA	Ethylene diamine tetra acetic acid
EFV	Efavirenz
EM	Effector memory (cells)
env	Envelope gene
FACS	Fluorescence-activated cell sorter / sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

gag	group antigen gene
GI	Gastro-intestinal
gp	Glycoprotein
GPR15/Bob	G-protein-coupled receptor
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA-DR	Human leukocyte antigen-DR
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRIS	Immune reconstitution inflammatory syndrome
LSD	Least significant difference
LPS	Lipopolysaccharide
LPV/r	Lopinavir
M	Memory (cells)
MDR	Multiple drug resistance
MEIA	Microparticle enzyme immunoassay
MFI	Mean fluorescence intensity
MIP	Macrophage inflammatory proteins
mRNA	messenger Ribonucleic acid
MTB	Mycobacterium tuberculosis
MVC	Maraviroc
NASBA	Nucleic acid sequence based amplification
N	Naïve (cells)
NF-Kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NNRTIs	Non-nucleoside RT inhibitors
NRTIs	Nucleoside RT inhibitors
NSI	Non-syncytium inducing
NtRTIs	Non-nucleoside RT inhibitors
NVP	Nevirapine
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PI	Protease inhibitor
PMTCT	Preventing Mother-to-Child Transmission
pol	polymerase gene
PTB	Pulmonary tuberculosis
QC	Quality control
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RPMI	Tissue culture medium Roswell Park Memorial Institute
RT	Reverse transcriptase
SAAVI	South African AIDS Vaccine Initiative
SI	Syncytium inducing
SIV	Simian Immunodeficiency Virus
SQV	Saquinavir
SSC	Side scatter
T-20	Enfuvirtide
TB	Tuberculosis
TDF	Tenofovir
Th1	T helper cell type 1
Th2	T helper cell type 2
UK	United Kingdom
UNAIDS	Joint United Nations programme on HIV/AIDS
VEIs	Viral entry inhibitors
VIGS	Verworwe Immunitetsgebrek Sindroom
WHO	World Health Organization
XDR	Extensively drug-resistant

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## CHAPTER 1: INTRODUCTION

The first reports of what became known as Acquired Immune Deficiency Syndrome (AIDS) were published in May 1981. They described unusual cases of Pneumocystis pneumonia and Kaposi's sarcoma among injecting drug users and homosexual men in the USA (CDC, 1981). Two years later, HIV-1 (Human immunodeficiency virus type 1) was defined as the primary cause of AIDS (Barre-Sinoussi *et al.*, 1983). A second similar, but antigenically distinct retrovirus named HIV-2 (Human immunodeficiency virus type 2), was isolated from patients with AIDS in West Africa in 1986 (Clavel *et al.*, 1986). HIV-1 is now distributed worldwide, while HIV-2 remains predominantly localised in West Africa.

The earliest known HIV positive serum sample was collected in Leopoldville in 1959 (now Kinshasa, Democratic Republic of Congo) (Zhu *et al.*, 1998; Nahmias *et al.*, 1986; Yusim *et al.*, 2001). Korber *et al.* (2000) have analysed envelope gene sequences of HIV-1 isolates from more than 150 individuals and estimated the common ancestor of the M group at 1931, with a confidence interval of 1915-1941.

In 2007, 26 years after initial description of HIV/AIDS, an estimated 33.2 million people were living with HIV-1 worldwide (Figure 1.1), 2.5 million new HIV infections were reported and 2.1 millions deaths were attributed to AIDS. Every day over 6 800 persons become infected with HIV and over 5 700 persons die from AIDS (UNAIDS/WHO, 2008). There is no region of the world untouched by this pandemic (Incardi and Williams, 2005).

Sub-Saharan Africa remains the most affected region in the global AIDS epidemic. More than two thirds (68%) of all people who are HIV-positive live in this region where more than three quarters (76%) of all AIDS deaths in 2007 also occurred (UNAIDS/WHO, 2008). South African antenatal clinic surveillance data have indicated prevalence rates among pregnant women of 30.2% in 2005, 29.1% in 2006 and 28% in 2007 (National Department of Health, South Africa, 2008). There is as yet no effective AIDS vaccine. Development of a safe, effective, easily administered and affordable HIV vaccine is urgently needed, but remains a major challenge.

Introduction of HAART (highly active antiretroviral [ARV] therapy) has reduced disease progression to AIDS and transformed HIV infection from a fatal condition to a manageable, chronic illness (Incardi and Williams, 2005; Girard *et al.*, 2006; Berrey *et al.*, 2001). Use of HAART results in a rapid decline of viral load, an initial immune reconstitution, as well as a

decrease in risk of further morbidity and mortality (Giovannetti *et al.*, 1999; Palella *et al.*, 1998; Murphy *et al.*, 2001). The most common reasons for ARV failure are variability of HIV strains, latency during the replication cycle, emergence of drug resistance, as well as non-adherence to HIV treatment (Del Rio, 2006).

The CD4 molecule is the primary cellular receptor for HIV (Dalglish *et al.*, 1984). The chemokine receptors CCR5 (also termed CD195) and CXCR4 (also termed CD184 or Fusin) are the major coreceptors involved in HIV infection and have also been implicated in disease progression (Princen and Schols, 2005). That is why in this study, the two major coreceptors, CCR5 and CXCR4, critical in HIV infection have been investigated. The coreceptors are differentially expressed on T cell subsets (Zhang *et al.*, 1998). While reports on North American and European cohorts demonstrated increasing Subtype B usage of CXCR4 as disease becomes more severe, studies from India, Ethiopia, Malawi and South Africa have however reported that Subtype C almost exclusively uses CCR5, with CXCR4 usage being rarely observed (Cilliers *et al.*, 2003). Fraziano *et al.* (1999) reported increased CCR5 expression in active tuberculosis (TB) infection. Similarly, Morris *et al.* (2001) found CCR5 to be the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. More studies of normal CCR5 expression on CD4+ T cells in the South African population where HIV-1 subtype C predominates is needed to understand the role of coreceptor expression in HIV pathogenesis in this region (Morris *et al.*, 2001).

T cell activation is known to be critical for productive viral infection, as activated T cells are the main targets for HIV (Siliciano and Siliciano, 2000). However, little is known on the effective role of HAART in regulation of coreceptor expression, immune activation status and T cell subset distribution in HIV infection and HIV/TB coinfection in South Africa. Concurrent infections, such as tuberculosis, particularly in Africa, may lead to various degrees of immune activation *in vivo*, thus enhancing HIV infection and accelerating disease progression (Bentwich *et al.*, 2000; Morris *et al.*, 2003), eventually resulting in failed ARV therapy (Burman and Jones, 2001). HIV infection has become a major challenge in the control of TB, mainly due to complications involved in optimal management of concurrent treatment.

Previous studies have found that HAART lead to CCR5 normalization, whereas CXCR4 expression did not change significantly (Pierdominici *et al.*, 2002; Giovannetti *et al.*, 2001; Nicholson *et al.*, 2001). Such modification in the expression of host determinants of viral tropism may play a role in the emergence of virus variants when HAART failure occurs

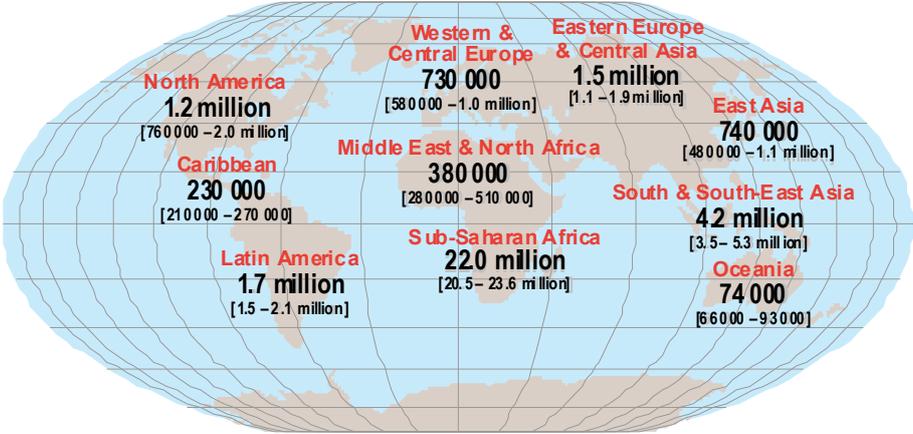
(Giovannetti *et al.*, 1999; Miller *et al.*, 2002; Johnston *et al.*, 2003; Brito *et al.*, 2007). Differences in target cells, tissue distribution and replication characteristics between R5- and X4-tropic viruses may affect the impact of HAART on HIV coreceptor expression (Skrabal *et al.*, 2003; Zhang *et al.*, 2006). R5-tropic viruses are viruses using HIV coreceptor CCR5 and X4-tropic viruses using HIV coreceptor CXCR4 (Berger *et al.*, 1999; Princen and Schols, 2005).

The continued upregulated CXCR4 expression after HAART (Manetti *et al.*, 2000) may also reflect the relative change at the T-cell subset level (Naïve versus memory T cells).

The current research project was a cross-sectional study performed to assess the effect of ARV on coreceptor expression, activation status and CD4 T cell subset distribution in peripheral blood of adult South African HIV and HIV/ TB co-infected patients.



**Adults and children estimated to be living with HIV, 2007**



**Total: 33 million (30 – 36 million)**

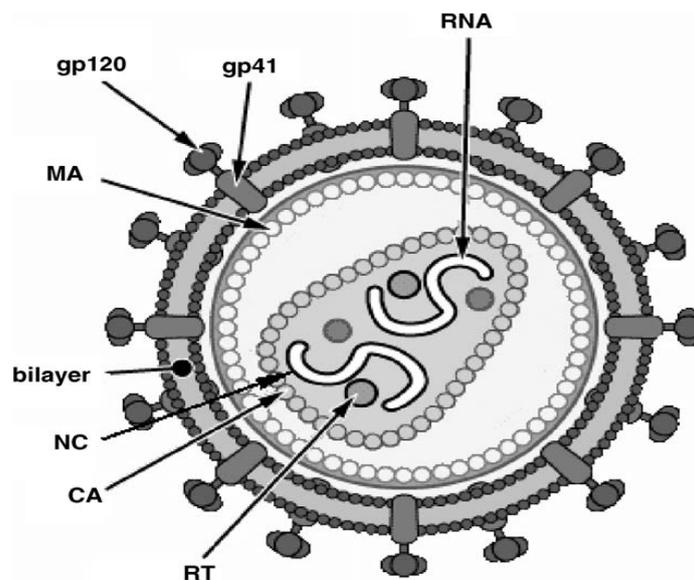
**Figure 1.1.** Estimated number of people living with HIV worldwide in 2007 (from <http://www.unaids.org/2007>). This map shows HIV infection predominantly affecting the Sub-Saharan region of Africa (UNAIDS/WHO, 2008).

## CHAPTER 2: LITERATURE REVIEW

### 2.1. HIV: Structure and Replication

HIV-1 belongs to the lentivirinae subfamily of Retroviridae. They are enveloped RNA viruses producing slow, progressive infection. All lentivirinae, including HIV-1, have a latency period before the manifestation of clinical illness (Luciw, 1996).

The HIV-1 virion, which measures 100 nm in diameter, comprises a core composed of nucleoproteins complexed to two genomic ribonucleic (RNA) molecules, a capsid which encapsulates the ribonucleoprotein particle, a matrix, which surrounds this capsid, and an envelope that in turn surrounds the matrix. HIV-1 has nine genes in its 9 kB RNA, including 3 structural genes (*gag*, *pol* and *env*) (Figure 2.1) and 6 regulatory genes (*tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*).



**Figure 2.1.** Structure of an HIV virion particle. This figure depicts the genomic RNA and viral components coded by the 3 structural genes (*pol*, *gag* and *env*): Env (with gp120 and gp41); Gag (with MA, matrix protein or p17, CA, capsid protein or p24 and NC, nucleocapsid protein or p7); Pol (with RT, reverse transcriptase) (Sierra, 2005)

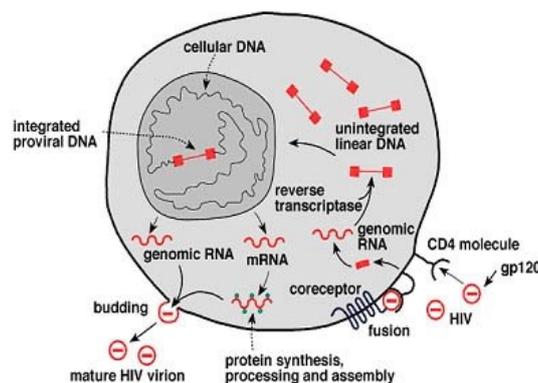
The HIV replication cycle (Figure 2.2) consists of 3 main steps: (i) HIV entry into the cell, (ii) replication and transcription, and (iii) assembly and release. HIV entry into the host cell begins through interaction of the envelope glycoprotein complex, gp120, with both CD4 and a chemokine receptor (CCR5 or CXCR4) (Dalglish *et al.*, 1984; Dimitrov *et al.*, 1998; Feng *et al.*, 1996). This interaction allows the N-terminal fusion peptide, gp41, to penetrate the cell

membrane (Chan and Kim, 1998; Liu, 2007). This process sees the membranes of the virus and the host cell fusing, which allows for subsequent entry of the capsid. Once the viral capsid enters the cell, viral RNA is released from the capsid, a process involving proper uncoating of the core that is critical for the virus to undergo reverse transcription (Dismuke and Aiken, 2006; Nisole and Saib, 2004). Virus-associated reverse transcriptase then converts HIV single-stranded RNA into complementary DNA. The nascent complementary viral DNA is then transported into the cell nucleus and integrated into the host DNA via the action of viral integrase (Zheng, 2005; Fouchier and Malim, 1999).

Host cellular transcription factors (e.g. NF- $\kappa$ B) are required for transcription of the integrated viral genome. These transcription factors are functional when the host cell is in an activated state (Hiscott, 2001). The integrated provirus is copied to mRNA, which is then spliced into smaller pieces to produce different regulatory proteins (Pollard and Malim, 1998). Structural proteins are also produced from full-length mRNA.

Assembly of new HIV-1 virions starts at the plasma membrane of the host cell. The env polyprotein, gp160, is processed into gp41 and gp120. Those glycoproteins, together with Gag, Pol polyproteins and the genomic RNA, associate to form new virions, which begin to bud from the host cell. Further maturation of virions occurs after budding and with the formation of active proteases, cleaves Gag and Pol polyproteins into functional subunits (Nguyen and Hildreth, 2000).

### Replication Cycle of HIV



**Figure 2.2.** Schematic illustration of the HIV replication cycle. Depicted are - the entrance phase (attachment of virus, fusion and penetration in cell); replication and transcription phase inside of cell; and budding and maturation of HIV virion. Sourced online from [http://www.web-books.com/elibrary/Medicine/Infections/Images/HIV\\_cycle.jpg](http://www.web-books.com/elibrary/Medicine/Infections/Images/HIV_cycle.jpg)

A number of factors have given rise to the diversity of HIV-1 observed today, including the high replication rate and error-prone nature of reverse transcriptase, recombination between virus subtypes or virus groups, as well as cross-species transmission (Mansky, 1998; McCutchan, 2006). The rapid mutation of the virus is primarily related to error-prone reverse transcription at a rate of 1 substitution per genome per replication cycle and the absence of any transcriptional safety checks (Sharp *et al.*, 2001; Korber *et al.*, 1998; McCutchan, 2006).

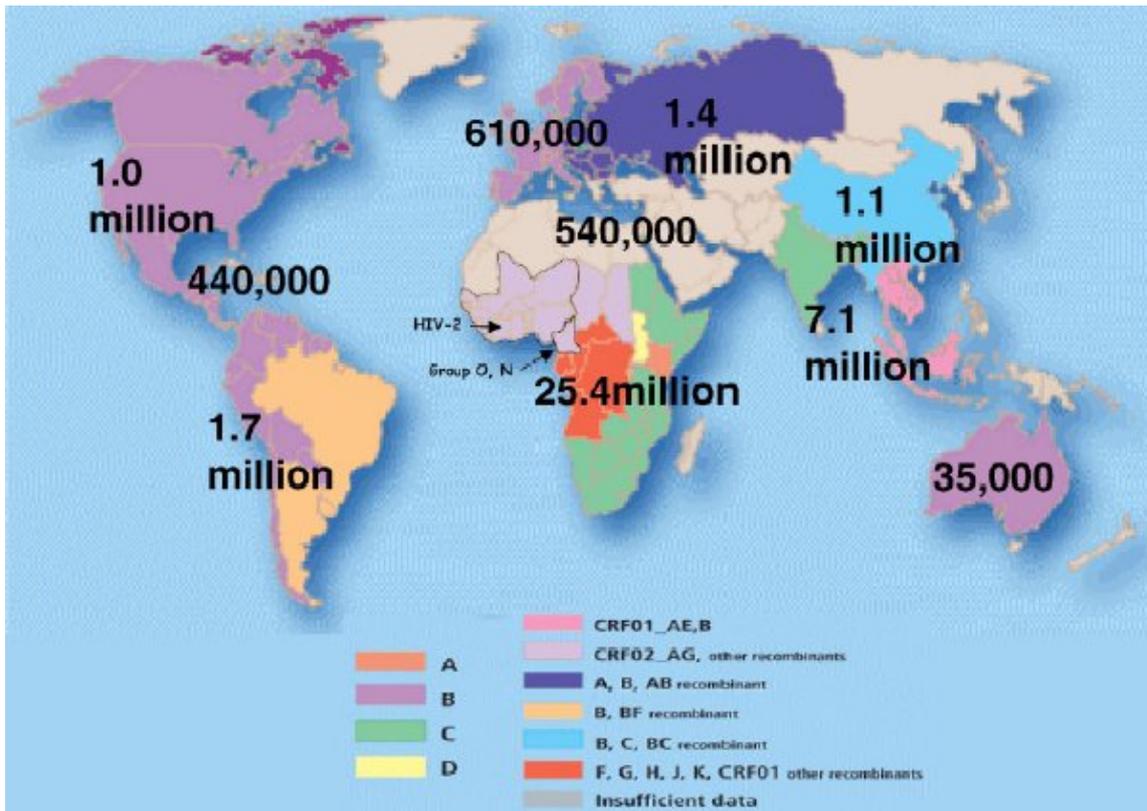
## **2.2. HIV: Natural Host, Origin and Diversity**

Numerous studies have shown that HIV strains have arisen due to cross-species transmission from primates to human beings in Africa (Sharp *et al.*, 2001). The SIVcpz (Simian Immunodeficiency Virus [SIV] from chimpanzees), the virus most closely related to HIV-1, has been isolated from chimpanzees, *Pan troglodytes troglodytes* (Gao *et al.*, 1999; Corbet *et al.*, 2000; Simon *et al.*, 1998). These viruses are non-pathogenic for chimpanzees. Gao *et al.* (1999) observed that the natural range of *Pan troglodytes troglodytes*, in Western Equatorial Africa, coincides uniquely with areas of endemic HIV-1, suggesting that this chimpanzee species was the primary reservoir for HIV-1 strains.

The Sooty Mangabey (*Cercocebus atys*) and the African Green Monkey (*Cercopithecus aethiops*) (AGM) are naturally infected with SIV, but do not develop AIDS like disease (Hirsch *et al.*, 1995; Sharp *et al.*, 2001). Phylogenetic analyses indicate that the only species naturally infected with viruses closely related to HIV-2 is the Sooty Mangabey from Western Africa (Chen *et al.*, 1996).

Three groups of HIV-1 have evolved and spread across the globe: M (major), O (outlier) and N (new). The M group, which accounts for over 90% of reported HIV/AIDS cases, has been further subdivided into 11 subtypes, including A-K, as well as several circulating recombinant forms or RCFs (Wainberg, 2004; Requejo, 2006). The viral subtypes show a distinct geographical distribution (Figure 2.3; McCutchan, 2006). Subtype C viruses continue to dominate worldwide and account for 60% of all HIV-1 infections (Requejo, 2006). In South Africa subtype C accounts for more than 90% of all HIV-1 infection.

The principal means of HIV transmission are through blood, sexual contact or mother-to-child transmission (MTCT) (Levy, 2007).



**Figure 2.3.** Global HIV prevalence and distribution. The estimated numbers of HIV-infected individuals worldwide are indicated. The colors depict regional patterns of HIV variation with different subtypes and recombinant forms distribution (McCutchan, 2006).

### 2.3. Pathogenesis of HIV-1 Infection

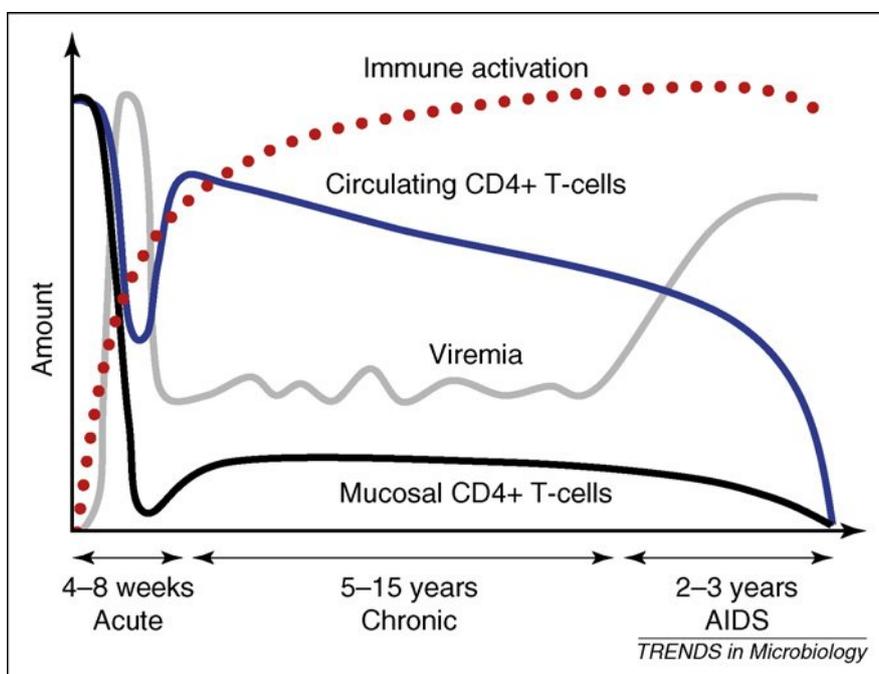
In absence of treatment, the natural history of HIV infection is divided into 3 major phases (Figure 2.4; Kamps and Hoffman, 2007; Levy, 2007):

- 1 Acute phase (also termed acute viral syndrome or primary HIV infection)
  - 2 Chronic phase (also termed persistent or latency period);
  - 3 AIDS (also termed symptomatic period).
- a. After viral entry into the host, the first stage of primary infection is characterized by localized viral replication at the site of entry, usually in the genital tract or rectum. Viruses can infect localized CD4 T cells, macrophages or dendritic cells prior to transportation to localised draining lymph nodes. Once virus moves from the initial site of entry to the local lymph nodes (within 2 days), the infection has become established (Haase, 2005).

Following an infection becoming established, there is a dramatic increase in plasma viremia and a simultaneous depletion of CD4 T cells. Within 10 to 14 days, up to 200 billion CD4+ T cells become infected (Embretson *et al.*, 1993). CD4+ T cell numbers rapidly decrease during this phase and then return to a level below normal, signifying the transition into chronic or persistent infection. CD8+ T cell numbers rise during the viremic phase, as is commonly seen in viral infections, and then return to baseline. In acute infection cellular immune responses appear to be the first antiviral activity produced (Koup *et al.*, 1989), followed later by neutralizing antibodies, which can be detected within days to weeks after exposure (Mackewicz *et al.*, 1994; Willey and Aasa-Chapman, 2008). The acute phase is also marked by the massive depletion in memory CD4 T cells from gut-associated lymphoid tissue, which ultimately leads to damage of the gastrointestinal tract (GIT) (discussed below and in more detail in Section 2.6).

- b. The persistent period or chronic phase begins at resolution of the acute phase at 3 to 6 months after infection and is characterized by an asymptomatic period with virus persistence at low levels in lymph nodes (Siliciano and Siliciano, 2000; Stebbing *et al.*, 2004). Suppression of HIV replication during this period seems to be mediated by antiviral CD8+ cells (Rowland-Jones *et al.*, 1993; Levy *et al.*, 1996) and equilibrium between viral replication and host immune response is reached. Even in the absence of treatment, this period of clinical latency may last 8-10 years or more (Cohen and Fauci, 2001; Forsman and Weiss, 2008). Although chronic infection is asymptomatic, it is a period of chronic immune activation (Hazenbergh *et al.*, 2003; Asther and Sheppard, 1988). Plasma viral loads are generally lower, but rise slowly over time. This is accompanied by a gradual decline in CD4+ T cells throughout the latency period (Grossman *et al.*, 2006). A heightened state of chronic, systemic immune activation before the onset of AIDS has been described by several authors. Chronic immune activation is also associated with damage of the GIT, resulting in leakage of the GIT and chronic innate activation due to lipopolysaccharides (LPS) and other innate immune stimuli entering the body and/or blood stream (Brenchley *et al.*, 2008). The continual presence of antigen is also a driving force in the activation process. This prominent feature distinguishes pathogenic infection of lentiviruses in humans and macaques from non-pathogenic infection in Chimpanzees, AGMs and Sooty Mangabeys (Chakrabarti, 2004; Forsman and Weiss, 2008; Benito, 2008).
- c. Symptomatic period: This phase is usually characterized by CD4+ T cell numbers

dropping below 350 cells/ $\mu$ l, increasing viral load and a general reduction in antiviral CD8+ T cell responses (Mackewicz *et al.*, 1991; Cao and Walker, 2000). These events precede development of AIDS-associated defining clinical illnesses and opportunistic infections. Concurrent infections may provoke an increase of immune activation *in vivo* and thus accelerate disease progression (Bentwich *et al.*, 2000; Wahl *et al.*, 1999; Sodora and Silvestri, 2008). Tuberculosis is the most common major opportunistic infection affecting HIV-infected individuals worldwide (Dolin *et al.*, 1994) and cause of death in patients at late stage of disease (Mukadi *et al.*, 2001; UNAIDS, 2008). HIV/TB coinfecting patients display increased expression of cellular activation markers and higher viral loads (Goletti *et al.*, 1996).



**Figure 2.4.** Schematic representation of a typical course of pathogenic HIV/SIV infection. The figure shows a persistent increase of immune activation throughout the chronic phase of HIV infection.

From Forsman A and Weiss RA, 2008. doi: 10.1016/j.tim.2008.09.004

#### 2.4. HIV-1 Infection and AIDS in South Africa

Subtype C is the predominant circulating HIV-1 strain in South Africa (van Harmelen *et al.*, 1999; Jacobs *et al.*, 2006), however non-subtype C and recombinant HIV-1 strains are also emerging (Jacobs *et al.*, 2007). The majority of HIV transmission in South Africa occurs via sexual contact (Jacobs *et al.*, 2007; Rehle *et al.*, 2007).

In HIV/AIDS epidemiology the term incidence rate is defined as the number of new cases per unit of person-time at risk, whereas prevalence rate is a measure of the total number of cases of disease in a population (Coggon *et al.*, 1997). Throughout South Africa, geographic distribution of HIV infection differs, with the highest antenatal prevalence in 2007 being in KwaZulu-Natal (37.4%) and the lowest in the Western Cape (12.6%) (National Department of Health, 2008). According to the recent Department of Health report on HIV and AIDS in South Africa, HIV prevalence trends suggest a tendency towards stabilization among pregnant women since 2004, as seen in the shift from 29.5% in 2004, to 30.2% in 2005, 29.1% in 2006 and 28% in 2007 (National Department of Health, 2008). Rehle *et al.* (2007) have reported HIV incidence rates of 2.4% in South Africa for the age group 15-49 years with a peak of HIV infection among females in the 20-29-year-old age-group at 5.6%, six times greater than the incidence found in the male population of the same age group (0.9%). Almost 5.7 million people have been reported living with HIV in South Africa at the end of 2007 and 1 000 AIDS-related deaths occur every day (Pembrey, 2008; UNAIDS, 2008). Since the beginning of the HIV epidemic, an estimated 1.8 million people have died of AIDS-related disease in South Africa (UNAIDS, 2008).

The explosive spread of HIV in South Africa is thought to be due to multiple factors, including the predominance of circulating HIV-1 subtype C, poverty, higher TB prevalence rates, sexually transmitted infections (STIs), other infections, and the limits of government action (Pembrey, 2008).

HIV has increased the burden of TB in developing countries (Maher *et al.*, 2005; Lalloo and Pillay, 2008). Corbett *et al.* (2003) reported that South Africa has the largest numbers of co-infected adults in the world, 2.0 million out of 11.4 million (17.5%) of HIV/TB co-infected cases worldwide. In a retrospective study among South African gold miners, the risk of TB infection was found to be increased within the first year of HIV infection (Sonnenberg *et al.*, 2005). On the other hand, HIV/TB coinfecting patients respond differently to TB treatment as seen with TB drug malabsorption and treatment failure with standard regimens (Gurumurthy *et al.*, 2004) This can potentially increase the risk of acquiring or amplifying TB drug resistance, including MDR (multidrug-resistant) TB and XDR (extensively drug-resistant) TB in South Africa (Andrews *et al.*, 2007).

Although the introduction of a structured ARV therapy programme in South Africa started in 2004, its coverage was still only 28% of people in need of treatment at the end of 2007

(Pembrey, 2008; WHO, 2008). Prevention campaigns through different media have also been launched since 1994 to educate people about HIV infection in South Africa (Noble, 2008). A programme to prevent mother-to-child transmission (PMTCT) has been in place countrywide since 2003. PMTCT involves prevention of HIV transmission from HIV positive mothers to their infants during pregnancy, labour, delivery and breastfeeding by the use of ARVs and safer infant feeding practices (Noble, 2008).

More commitment and improved care is required from the government in order to control the HIV epidemic in South Africa. A change of government policy in 2008, as stated by the new health minister, with an enhanced commitment to ARV distribution, will undoubtedly help to address some of the problems faced in the battle against HIV/AIDS in the country.

## **2.5. HIV-1 Coreceptors**

### **2.5.1. Discovery of HIV Coreceptors**

HIV coreceptors are members of the 7-transmembrane G protein-coupled receptor family of chemokine receptors whose physiologic role is to transmit cellular signals following interaction with chemoattractant cytokines (See Figure 2.5).

The primary cellular receptor for HIV entry is CD4 (Dalglish *et al.*, 1984). However, expression of CD4 on a target cell is required, but not sufficient for HIV entry and infection. Several chemokine receptors are known to allow HIV entry when co-expressed with CD4 on the cell surface (Dimitrov *et al.*, 1998). In 1996, chemokine receptors CXCR4 and CCR5 were identified as the major coreceptors for HIV-1 entry (Feng *et al.*, 1996). CXCR4 (also referred to as CD184 or Fusin) is the natural receptor for SDF-1 (or CXCL12). CCR5 (also referred to as CD195) is the natural receptor for RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  (or CCL5, CCL3 and CCL4, respectively).

Prior to identification, the first indication that chemokine receptors might function as coreceptors for HIV-1 entry came from observations that the chemokines RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  suppressed infection of susceptible cells *in vitro* by macrophage-tropic primary HIV-1 isolates (Cocchi *et al.*, 1995).

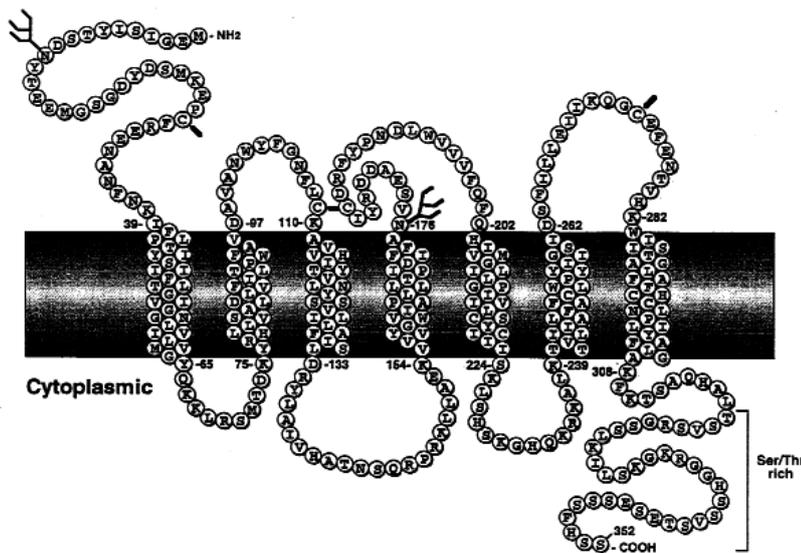
Initial work on HIV-1 coreceptor activity indicated that:

- The chemokine receptor CXCR4 was found to support infection and cell fusion of CD4+

cells by laboratory-adapted T-tropic HIV-1 strains.

- CCR5 was subsequently identified as the principal coreceptor for primary macrophage-tropic strains.
- CCR3 and CCR2b were also identified as coreceptors that supported infection by some strains of HIV-1.

A. CXCR4



B. CCR5

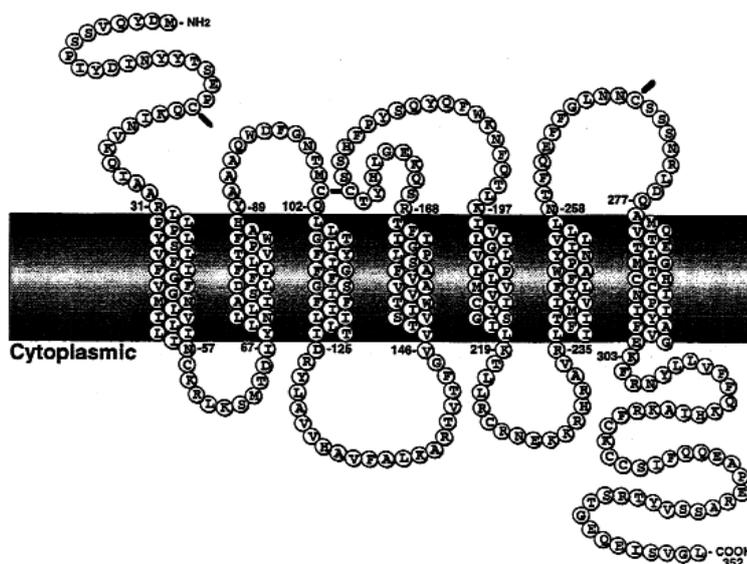


Fig. 1. Primary sequences and predicted membrane topology of the HIV-1 coreceptors CXCR4 (A) and CCR5 (B). Courtesy of R. Doms.

Figure 2.5. Primary sequences and predicted membrane topology of the HIV-1 coreceptors CXCR4 (A) and CCR5 (B) (Dimitrov *et al.*, 1998).

The major coreceptors, CCR5 and CXCR4 (Princen and Schols, 2005; Dimitrov *et al.*, 1998; Moore *et al.*, 1997), play a prominent role in the transmission of HIV and during disease progression towards AIDS. CCR5 is predominantly expressed on dendritic cells, macrophages and CD4 T cells, whereas CXCR4 is expressed on activated T cells (Zhang *et al.*, 1998; Bleul *et al.*, 1997). Additionally, T-cell lines express CXCR4, monocytes/macrophages express CCR5 and primary T-cells express either or both chemokine receptors.

The distinct tropisms of different HIV-1 isolates for various CD4 positive human target cell types were observed *in vitro*. T-tropic virus strains are those adapted for growth in transformed T-cells and which can also replicate in transformed T-cell lines. M-tropic virus strains are those viruses adapted in peripheral blood mononuclear cells (PBMCs) and which can replicate in cells from the macrophage/monocyte lineage. Both T- and M-tropic viruses replicate in activated T-cells. T-tropic strains preferentially use CXCR4 and are syncytium inducing (SI), M-tropic strains use CCR5 and are non-syncytium inducing (NSI). Dual tropic virus strains use both coreceptors (Princen and Schols, 2005; Moore *et al.*, 2004).

Viral isolates obtained from HIV-1 infected persons in the early stages of infection are predominantly M-tropic, while those found at a later stage of disease progression towards AIDS are mostly T-tropic (Berger *et al.*, 1999; Princen and Schols, 2005).

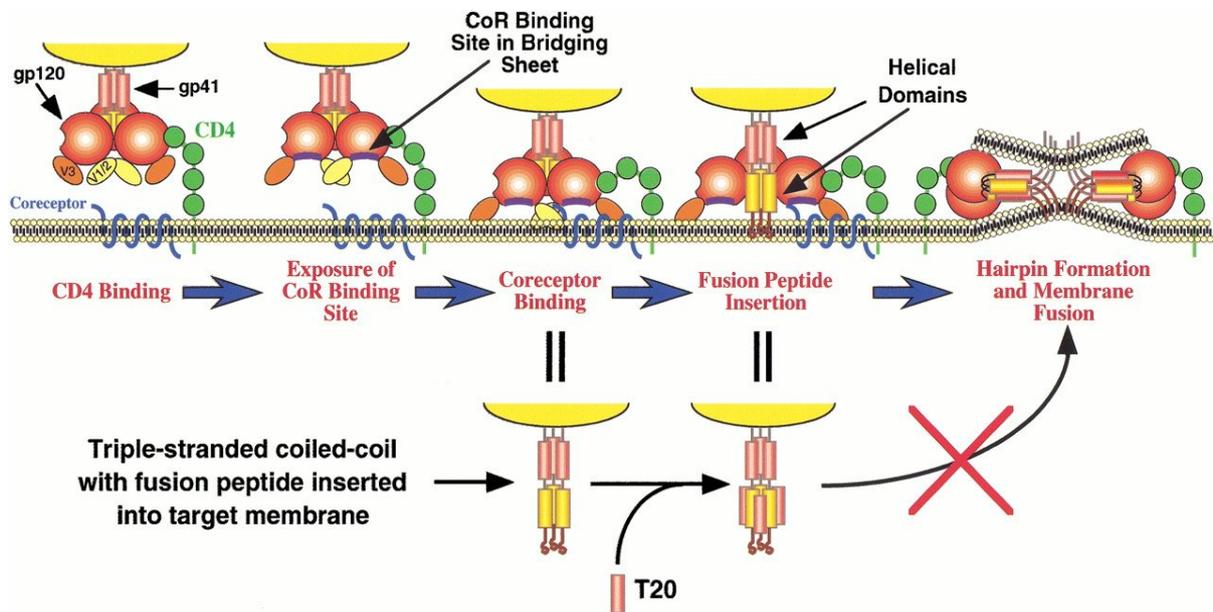
The minor coreceptors of HIV-1 were also found to mediate the entry of HIV-1 strains *in vitro* and include CCR3 (CD195), CCR8, CCR9, CCR2b (CD192), CX3CR9, CXCR6 (STRRL33/Bonzo), APJ, and GPR15/Bob. These coreceptors do not play a critical role in HIV infection (Princen and Schols, 2005).

### **2.5.2. Coreceptor Mediation of HIV Entry**

HIV binds to CD4 antigen on cells, such as T helper lymphocytes or macrophages, via the HIV surface glycoprotein, gp120. The interaction of gp120 and CD4 antigen causes a conformational change in gp120, which is stabilized by the chemokine co-receptor (Dimitrov *et al.*, 1998). This causes gp41 to undergo a conformational change exposing hydrophobic regions that are then embed in the membrane of the host cell. The viral membrane of the virus can fuse with the host membrane and allow the nucleocapsid (containing the RNA genome) to enter the cell cytoplasm.

In brief, the HIV entry process includes the following 3 steps (Figure 2.6):

- Binding of the viral envelope glycoprotein with CD4 receptor.
- Binding of the envelope-CD4 complex to chemokine receptor, and
- Fusion of the viral and cell membranes.



**Figure 2.6.** Schematic presentation of the HIV-1 entry process. This figure displays 3 prominent steps of HIV entrance: Binding of Viral envelope glycoprotein with CD4 followed by the envelope-CD4 complex binding to chemokine receptors and fusion of the viral and cell membranes. (Princen and Schols, 2005).

### 2.5.3. Relationship between HIV Coreceptors and HIV Pathogenesis

The most significant variables influencing the efficiency of viral entry are both CD4 and coreceptor cell surface expression levels (Liu *et al.*, 1996; Doms and Peiper, 1997; Reynes *et al.*, 2003). The activity of HIV-1 coreceptors seems to be a critical determinant of disease progression (Berkowitz *et al.*, 1998). CCR5 appears to be important for NSI strains of HIV (strains most common in early disease), while CXCR4 appears to be more important for SI strains (a more aggressive strain sometimes seen in patients with more aggressive disease).

A homozygous genetic defect resulting in a 32 base pair deletion ( $\Delta 32$ ) in CCR5 correlates strongly with protection against HIV-1 infection *in vivo* and *in vitro* (Liu *et al.*, 1996). Individuals who are heterozygous for a defective CCR5 allele are at best weakly protected against infection and have only a moderately slowed disease progression.

NSI strains of HIV are the most common sexually transmitted form of the virus. It has been argued that R5 viruses are preferentially transmitted because of the patterns of expression of coreceptors and their ligands in memory T cells at mucosal sites after virus deposition during sexual intercourse (Moore, 1997; Moore *et al.*, 2004; Philpott, 2003).

Expression of the two major coreceptors (CCR5 and CXCR4), as well as their respective chemokines, appears to be critical in determining T-cell susceptibility to HIV-1 infection (Giovannetti *et al.*, 1999; Taylor *et al.*, 2001; Schmitt *et al.*, 2003). Chemokines, as natural chemokine receptor ligands, block HIV-1 binding to the chemokine receptors and thereby impede viral entry into cells (Bleul *et al.*, 1996; Cocchi *et al.*, 1995).

There is a switch in coreceptor usage during the course of infection in 50% of infected individuals from predominance of CCR5-using strains at the early stage of infection, to CXCR4-using HIV variants at the late stage of disease (Berger, 1998; Philpott, 2003; Moore *et al.*, 2004). The depletion of CCR5 expressing memory cells in acute infection may drive viral evolution towards CXCR4 (Moore *et al.*, 2004). This pattern is not a universal phenomenon, but rather a tendency in non-subtype C (predominantly subtype B) infection (Cilliers *et al.*, 2003).

T cell-associated expression of CCR5 was found to be upregulated in HIV-1 infected individuals, while CXCR4 appears downregulated on both CD4 and CD8 T cells when compared to normal controls (Giovannetti *et al.*, 1999; Bleul *et al.*, 1997; Ostrowski *et al.*, 1998). Analysis of chemokine receptor expression patterns shows that CCR5 and CXCR4 are differentially expressed on naïve and memory T cells (Blaak *et al.*, 2000). R5 virus tropism for memory cells and X4 virus for naïve cells may drive the evolution of phenotypes with disease progression (Giovannetti *et al.*, 1999; De Roda Husman *et al.*, 1999; van Rij *et al.*, 2000; Gorry *et al.*, 2004).

#### **2.5.4. Implications for HIV Therapy**

The discovery of HIV-1 coreceptors has stimulated new efforts for identification of entry inhibitors, which would prevent the coreceptor interactions with the env-CD4 complex, and thus prevent membrane fusion and viral entry (Dalglish *et al.*, 1984). The best CXCR4 antagonists described are Bicyclam derivatives, which consistently block X4, but also R5/X4 viral replication in PBMCs (Princen and Schols, 2005; Schols *et al.*, 1997). Maraviroc is one of the small-molecule CCR5 inhibitors currently in ongoing clinical development (Dorr *et al.*,

2005).

Both CXCR4 and CCR5 chemokine coreceptor inhibitors will have to be administered simultaneously and possibly even in combination with other ARV drugs that target other aspects of the HIV replication cycle to obtain optimal antiviral therapeutic effects (Princen and Schols, 2005).

A concern with regard to coreceptor inhibitors is whether the inhibition of the normal physiological functions of CCR5 will be tolerated or whether blocking this receptor will have special adverse consequences in individuals with HIV-related immune impairment (Lederman *et al.*, 2006; Kuhmann and Hartley, 2008). It is also unclear if blocking CCR5 *in vivo* will lead to the emergence of resistance to this class of coreceptor inhibitor or in a shift toward CXCR4-using strains (Blanpain C, 2002; Princen and Schols, 2005).

## **2.6. Dynamics of CD4 T Cell Distribution, Activation and Proliferation in HIV-1 Infection**

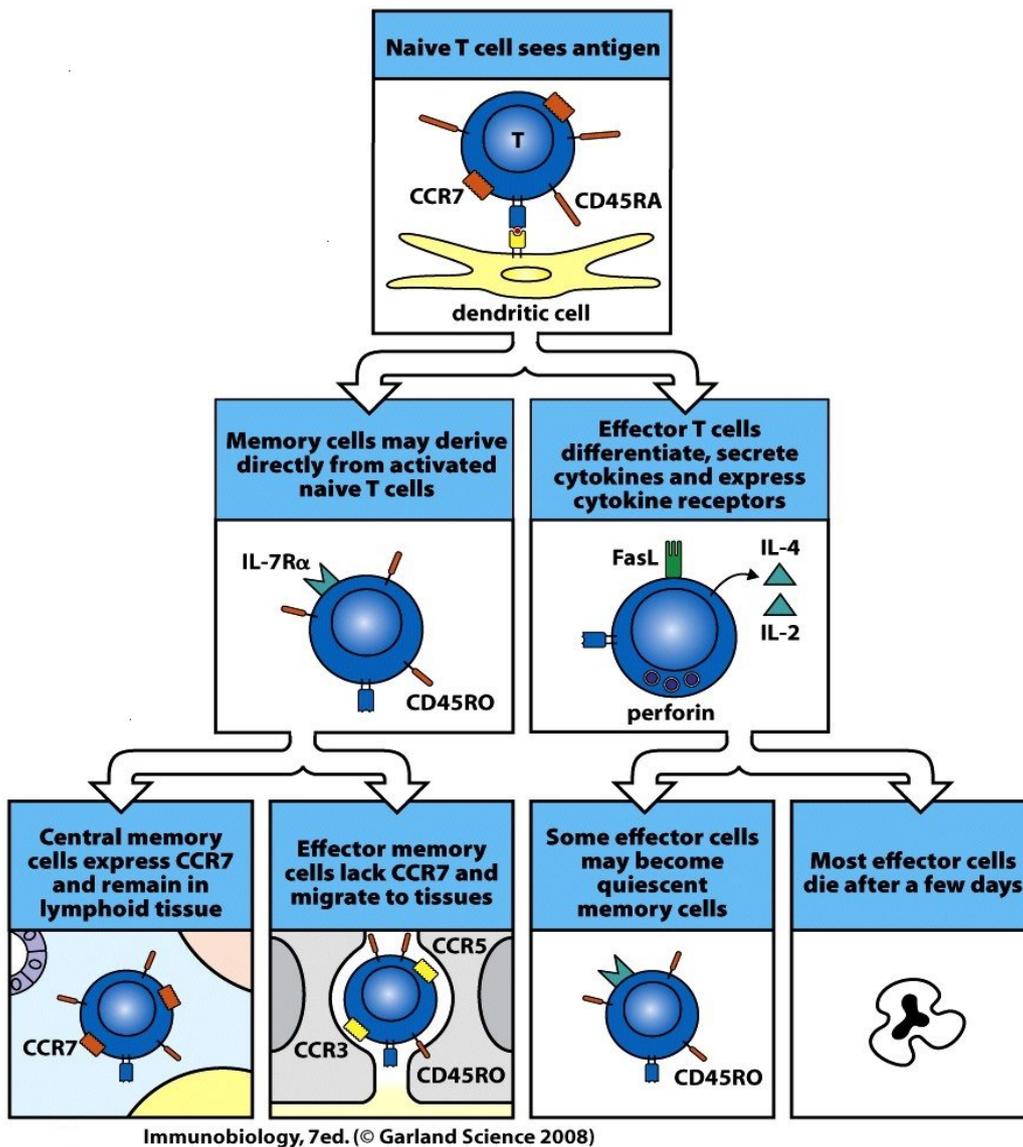
T lymphocytes can be classified according to their maturation status or antigen experience into naïve (N) cells (those that have not yet encountered cognate antigen), effector (E) cells (those that have met their antigen, have become activated and differentiated further into fully functional lymphocytes) and memory (M) cells (those that have been activated by antigen and differentiated for long-lasting immunity). In primary response to new viral infection, naïve T cells become activated in lymphoid tissue and differentiate into effector T cells, which then migrate to peripheral sites to orchestrate viral clearance. Memory cells respond rapidly on re-exposure to the antigen that originally induced them (Verhoeven *et al.*, 2008). Memory cells are divided into two subsets - effector memory (EM) cells, which are located primarily in mucosal tissues, and central memory (CM) cells, which are located in lymphoid tissue (Figure 2.7). During viral infection, EM cells present an immediate, but not sustained, defence at pathogen sites of entry, whereas CM T cells maintain the response by proliferating in the secondary lymphoid organs and producing a supply of new effectors (Halwani *et al.*, 2006). Central memory T cells are thought to be responsible for the long-term maintenance of immune memory. A preserved CD4<sup>+</sup> T Central memory cells and activated EM CD4<sup>+</sup> T-cell subsets have been associated with HIV disease progression (Potter *et al.*, 2007). Previous reports have shown that loss of central memory CD4<sup>+</sup> T cells during primary SIV mac 251 infection associated with plasma viral load ( Karlsson *et al.*, 2007; Sun *et al.*, 2007).

Furthermore the survival in vaccinated SIV-challenged monkeys was associated with preserved central memory CD4<sup>+</sup> T lymphocytes and could be predicted by the magnitude of vaccine-induced cellular immune response (Letvin *et al.*, 2006). Mason *et al.* (2008) also found a significant association between preservation of CM CD4<sup>+</sup> T cells and control of viremia in SIV-infected pigtail macaques.

Markers such as CD45RA, CD45RO, CD27, CCR7 and CD62L have been used to define memory cells phenotypically (Figure 2.8). Various researchers have proposed schemes in which T cell subsets are defined based on the presence of various combinations of these receptors. Most work on memory cells has been on CD8 T cell memory rather than CD4 T cells, but schemes used for CD8 T cells have generally been applied to CD4 T cells as well. The most common marker combinations include:

- CD45RA<sup>+</sup>/CD62L<sup>+</sup> (N); CD45RO<sup>+</sup>/CD62<sup>+</sup> (M) (Giovannetti *et al.*, 1999; Seder and Ahmed, 2003)
- CD45RA<sup>+</sup>/CD62L<sup>+</sup>/CCR7<sup>+</sup> (N); CD45RO<sup>+</sup>/CD62<sup>+</sup>/CCR7<sup>+</sup> (CM) (Seder and Ahmed, 2003; Sallusto *et al.*, 1999)
- CD45RA<sup>-</sup>/CD62L<sup>+</sup> or CD45RA<sup>-</sup>/CD62<sup>-</sup> (M) (Giovannetti *et al.*, 1999; Bell *et al.*, 1998)
- CD45RA<sup>+</sup>/CD45RO<sup>-</sup>(N); CD45RA<sup>+</sup>/CD45RO<sup>+</sup> (M) (Tortajada *et al.*, 2000)
- CD45RA<sup>-</sup>/CD62<sup>+</sup>/CCR7<sup>+</sup> (CM); CD45RA<sup>-</sup>/CD62<sup>+</sup>/CCR7<sup>-</sup> (EM) (Seder and Ahmed, 2003; Sallusto *et al.*, 1999);
- CD45RO<sup>-</sup>/CD27<sup>+</sup> (N); CD45RO<sup>+</sup>/CD27<sup>+</sup> (CM); CD45RO<sup>+</sup>/CD27<sup>-</sup> (EM) (Di Mascio *et al.*, 2006).

These markers have natural functional activities, for example CD45 RA/RO is involved in modulation of T cell receptor signalling; CD62 is associated with homing to lymphoid tissue; and CCR7 is a chemokine receptor for lymphoid homing. Newer classification schemes include use of markers such as CD127 (part of receptor for IL-7) and Bcl-2 (cell survival).



**Figure 2.7.** T cell differentiation into CM and EM subsets. This figure displays a differentiation of naïve T cell after exposure to an antigen into memory T cells and effector T cells. Memory T cells in turn differentiate into central memory and effector memory. Memory T cells can also arise from activated effector cells. (Murphy *et al.*, 2008).

Protein	Naive	Effector	Memory	Comments
CD44	+	+++	+++	Cell-adhesion molecule
CD45RO	+	+++	+++	Modulates T-cell receptor signaling
CD45RA	+++	+	+++	Modulates T-cell receptor signaling
CD62L	+++	-	Some +++	Receptor for homing to lymph node
CCR7	+++	+/-	Some +++	Chemokine receptor for homing to lymph node
CC69	-	+++	-	Early activation antigen
Bcl-2	++	+/-	+++	Promotes cell survival
Interferon- $\gamma$	-	+++	+++	Effector cytokine; mRNA present and protein made on activation
Granzyme B	-	+++	+/-	Effector molecule in cell killing
FasL	-	+++	+	Effector molecule in cell killing
CD122	+/-	++	++	Part of receptor for IL-15 and IL-2
CD25	-	++	-	Part of receptor for IL-2
CD127	++	-	+++	Part of receptor for IL-7
Ly6C	+	+++	+++	GPI-linked protein
CXCR4	+	+	++	Receptor for chemokine CXCL12; controls tissue migration
CCR5	+/-	++	Some +++	Receptor for chemokines CCL3 and CCL4; tissue migration

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**Figure 2.8.** Changes in marker expression when naive T cells become memory T cells. This figure lists a selection of different markers that can be used to define memory T cells. (Murphy *et al.*, 2008).

In natural HIV infection, the major feature of the acute phase is a massive loss of CD4 T cells (particularly of the mucosal-associated EM subset) residing in the lamina propria of the GIT (Paiardini *et al.*, 2008). These cells are highly susceptible to viral infection, as they express CCR5 co-receptor molecules and are readily activated (Douek *et al.*, 2002; Douek *et al.*, 2003).

The GIT CD4 T cell depletion requires some viral spread, as this is distal to the genital mucosae where initial HIV-1 infection usually occurs. The massive depletion of these cells is accompanied by a simultaneous loss of peripheral CD4 T cells and a massive increase in plasma viral load. Although the peripheral CD4 count recovers after acute infection, the EM population is not replenished and antigen is never totally eliminated due to viral integration into host cells (Douek *et al.*, 2003; Munier and Kellerher, 2007; Brenchley *et al.*, 2004; Veasy *et al.*, 1998; Paiardini *et al.*, 2008).

Chronic infection is characterized by persistent T cell activation – a feature linked to continual antigen presence and also more recently linked to chronic innate immune activity due to GIT damage. Continual onslaught by various pathogens is also believed to occur as the protective EM cells are no longer present and cannot control infections or antigen level. The massive depletion of memory cells from the GIT leads to damage and loss of GIT integrity. This may lead to leakage of gut antigen (e.g. LPS seen in HIV) – in turn leading to innate immune activation and a chronic stimulation of the whole immune system (Brenchley *et al.*, 2008; Brenchley *et al.*, 2006). This continual activation ultimately leads to T cell dysfunction and impaired functional activity (proliferation, cytokine production, CTL activity) (Appay and Sauce, 2008). Chronic HIV is also accompanied by enhanced levels of apoptosis of T cells, most likely due to increased Fas and FasL expression in activated T cells, i.e. bystander cell death as opposed to direct viral-associated cytotoxic effects (Appay and Sauce, 2008; Gougeon, 2005).

Different subsets of T cells are thus affected in different ways in natural HIV infection. This is related to susceptibility to viral infection (related to CD4, co-receptor expression and activation status) and also responses generated in order to restore T cell balance after the acute phase damage.

CD4 EM cells are the most susceptible subset for transmission of HIV from dendritic cells (Groot *et al.*, 2006). CD4 EM cells are also more susceptible to CTL killing than CM, N or E subsets (Liu and Roederer, 2007). Even if new EM cells are generated, they remain the primary target for destruction; hence the inability to restore the population and normal immune status in HIV patients. Destruction of a large proportion of memory T cells is now acknowledged to place a huge immunological burden on the host from which it never recovers (Guadalupe *et al.*, 2003; Hazenberg *et al.*, 2000). Interestingly, primate species naturally infected with SIV and not displaying any disease pathology, tend to have a much lower number of CCR5 expressing cells (Veazy *et al.*, 2000; Chase *et al.*, 2006).

The mere entry of virus into a T cell is not sufficient for viral replication. The cell must also divide. Without cell division, viral products are broken down and thus no productive infection is possible (Davenport *et al.*, 2002; Stevenson *et al.*, 1990). The viral production from naïve cells is much lower than viral production from memory cells. It is possible that high division rate of memory T cells compared to that of naïve T cells in an infected host provide an advantage for a memory cell-tropic (R5) virus at the early stage of infection (Davenport *et al.*,

2002; McCune *et al.*, 2000).

CCR5 expression on CD4 T cells has been associated with other measures of disease progression, such as viral load and CD4 count (Shang Hong *et al.*, 2005; Lin *et al.*, 2002), and markers of cellular activation, such as CD38 (Giovannetti *et al.*, 1999; Nicholson *et al.*, 2001; Shang Hong *et al.*, 2005). Cellular activation is also known to be critical for productive HIV-1 infection (Stevenson *et al.*, 1990; Oswald-Richter *et al.*, 2004). Different cellular markers of T-cell activation include CD38, CD69, CD95, Ki67, HLA-DR, and loss of CD127 (Appay and Rowland-Jones, 2002; Savarino *et al.*, 2000; Kestens *et al.*, 1992; Ziegler *et al.*, 1994; Kiazky and Fowke, 2008; Shepard *et al.*, 2008).

Previous studies have shown consistent increase of viral load when the immune system of HIV-1 infected individuals is activated by exogenous stimuli, such as opportunistic pathogens (Zhang Zi-ning *et al.*, 2006; Shang Hong *et al.*, 2005; Cohen Stuart *et al.*, 2000). A decline of CD4+ T cells is also strongly associated with an increased level of activation markers on CD4 populations (Savarino *et al.*, 2000; Sousa *et al.*, 2002). T cell activation is one of the important factors determining survival of HIV-1 infected patients, with lower activation being protective (Hazenberg *et al.*, 2003; Mahalingan *et al.*, 1993). There is a strong interaction between HIV replication and T-cell activation, because productive HIV infection is largely restricted to activated CD4+ T cells (Stevenson *et al.*, 1990; Cohen Stuart *et al.*, 2000; Zack *et al.*, 1990). Persistently increased expression of chemokine receptors and their ligands in HIV-1 coinfection with active TB may further provide a potential mechanism for increased HIV replication, and may contribute to the persistence of immune activation and HIV viremia observed in African cohorts (Morris *et al.*, 2001; Sodora and Silvestri, 2008; Wolday *et al.*, 2005; Rosas-Taraco *et al.*, 2006). Several groups have demonstrated that immune activation is central to CD4 cell depletion in HIV infection and immune reconstitution during HAART treatment (Anthony *et al.*, 2003; Hazenberg *et al.*, 2000; Benito *et al.*, 2005; Aiuti and Mezzaroma, 2006).

## **2.7. Effect of Antiretroviral Therapy on HIV Coreceptor Expression, Activation Status and T Cell Subset Distribution**

The function of ARV treatment is to suppress or stop retroviral replication and since the most important human retrovirus infection is HIV, the term usually refers to anti-HIV drug treatments. In practice, approved ARV agents refer to anti-HIV drugs in clinical use (De

Clercq, 2004). The first ARV drugs were introduced between 1987 and 1990, but showed modest successes since approaches were focused on monotherapy (use of a single drug). AZT, the first ARV introduced early in 1987, did not provide durable efficacy (Hoffman and Mulcahy, 2007; Concorde, 1994). The same scenario emerged from the other nucleoside analogues (ddC, ddI and d4T) introduced somewhat later as monotherapies. Then in June 1996, at the World AIDS Conference in Vancouver, the new concepts of “AIDS drug cocktails” and HAART emerged, which refers to the combination of 3-antiretroviral drugs.

Approved ARV drugs currently in use (Hoffman and Mulcahy, 2007; De Clercq, 2004) are:

1. ARV drugs targeting the reverse transcriptase (RT) enzyme at the transcription step of HIV replication. These include Nucleoside analogue reverse transcriptase inhibitors (NRTIs) e.g. Zidovudine (AZT), Didanosine (ddI), Stavudine (d4T), Lamivudine (3TC); Nucleotide analogue inhibitors (NtRTIs) e.g. Tenofovir (TDF); Non-nucleoside RT inhibitors (NNRTIs) e.g. Nevirapine (NVP) and Efavirenz (EFV).
2. ARV drugs that target the virion packaging step of HIV replication, including Protease inhibitors (PIs) e.g. Saquinavir (SQV), Atazanavir (ATV), Lopinavir (LPV/r) and Ritonavir (Norvir).
3. Viral entry inhibitors (VEIs) including Enfuvirtide (T-20) and Maraviroc (MVC).

Current first line approaches (initial regimens) consist of two NRTIs combined with a boosted PI, an NNRTI or a third NRTI (Hoffman and Mulcahy, 2007).

Although there has been an increase in the availability of ARV agents over the last few years, the selection of optimal combination regimens that could eliminate HIV-1 replication continues to be challenging, because of the development of HIV-1 drug resistance (Hanna and D’Aquila, 2001; van Vaerenbergh, 2001; Rodes *et al.*, 2005) and also the continued presence of low-level viral antigen even when “undetectable” in standard diagnostic tests.

In South Africa, the ARV treatment programme in the public sector which started in 2004 use the following recommended treatment guidelines for Adult HIV positive patients (National Department of Health South Africa, 2004):

- 1 Regimen 1a (for all men and for women on contraception): d4T / 3TC / Efavirenz
- 2 Regimen 1b (for women who are unable to guarantee reliable contraception while on

ARV): d4T / 3TC / NVP

- 3 Regimen 2 (patients with treatment failure despite demonstrated adherence): AZT / ddI / Lopinavir.

HAART treatment of HIV-infected individuals result in a decrease in plasma viral load, an increase in peripheral CD4 count and a decrease in general T cell activation (Collier *et al.*, 1996; Autran *et al.*, 1997; Autran *et al.*, 1999). The level of T cell apoptosis is also markedly reduced and proliferative capacity increased (Autran *et al.*, 1997). Rebound or persistent viremia in patients on HAART is usually linked to development of drug resistant mutations. In some cases, however, increased viral load may be due to non-compliance (treatment interruption), adverse drug interactions or drug compartmentalization (Bezemer *et al.*, 2006). Rebound or persistent viremia is generally accompanied by a decreasing CD4 count and an increase in T cell activation; however in certain cases a discordant CD4 increase or maintenance of existing CD4 count accompanies viremia (Price *et al.*, 2003; D'Ettorre *et al.*, 2002).

Since HAART results in a broad inhibition of immune activation, normalization of CCR5 and CXCR4 expression after prolonged suppression therapy appears to be linked to reduced levels of immune activation (Anderson *et al.*, 1998; Nicholson *et al.*, 2001; Pierdominici *et al.*, 2002; Brito *et al.*, 2007). Peripheral redistribution of naïve/memory T cell compartment and decrease in the level of T cell activation have initially been suggested to be responsible for the change observed in coreceptor expression after HAART (Giovannetti *et al.*, 2001; Brito *et al.*, 2007; Smith, 2002). In contrast, Briz *et al.* (2008) recently working on a longitudinal cohort for 2 years did not find a significant change in coreceptor expression after HAART treatment.

Previous studies have found T cell turnover in HIV infection related to immune activation (Hazenberg *et al.*, 2000; Galati and Bocchino, 2007). Anthony *et al.* (2003) demonstrated a direct relationship between activation and proliferation of T cells. Increased turnover of T cells in HIV infection is associated mainly with immune activation even after long-term HAART, suggesting that T cell activation and turnover play a prominent role in CD4 depletion in HIV infection and influence the potential for T cell normalization after treatment. A study on factors influencing T-cell turnover in HIV-1 patients by McCune *et al.* (2000), showed normalization of circulating T cell turnover as a function of time after therapy. After 3 months of ARV, this turnover still remained high, but normalized at 12-36 months.

Tortajada *et al.* (2000) have confirmed that immune reconstitution of T cell subsets after 12 months of HAART was more likely to occur when treatment was initiated in early stages of HIV infection rather than at late stage of disease. Delobel *et al.* (2006) compared the virological and immunological features of 2 groups of patients, one having poor CD4+ T cell restoration despite sustained virological responses, and the second having good immunological and virological responses. They found X4 viruses playing a role in the pathogenesis of poor immune reconstitution on HAART by enhancing T-cell activation and bystander apoptosis via gp120-CXCR4 interactions, leading to naïve T-cell depletion. This possibility must be considered in future when therapeutic strategies using CCR5 entry inhibitors reach clinical application.

Development of drug resistance is important, as mutated viruses do not respond to existing treatment regimens. It appears as if different viral subtypes have different levels of mutation; possibly due to viral fitness, replication potency and other factors. Transmission of mutated viruses is problematic, as newly infected individuals will be non-responsive to treatment. Transmitted mutated viruses appear less fit, but appear to maintain cytopathogenicity (Solomon *et al.*, 2005). Although transmitted mutated viruses are associated with exacerbated CD4 depletion in early acute infection (due to drug unresponsiveness), in later infection this does not appear detrimental. In fact, mutated viruses are associated with reduced immune activation, which may confer some long-term protection (Hunt *et al.*, 2006; Shet and Markowitz, 2006).

The levels of T cell activation and apoptosis in rebound or persistent drug resistant viremia may be lower than in acute infection or chronic untreated (no HAART) viremia (Hunt *et al.*, 2006). This may be due to reduced viral fitness and replication efficiency. Interestingly, it has also been documented that drug resistant viruses may be more replication competent and pathogenic than wild type viruses (Solomon *et al.*, 2005). Despite all those findings, it is still unclear why discordant immunologic and virologic responses occur during HIV ARV therapy.

## **2.8. Impact of Tuberculosis on the HIV-1 Epidemic in South Africa**

*Mycobacterium tuberculosis* (MTB) infection is the most common opportunistic coinfection and cause of mortality in people living with HIV/AIDS. An estimated 13% of AIDS deaths worldwide are related to TB disease (UNAIDS, 2008). HIV infection also predisposes an

individual to reactivation and progression of active TB (Corbett *et al.*, 2003; Djoba Siawaya *et al.*, 2007; Toossi, 2003; Badri *et al.*, 2001). It primarily involves macrophages (the target cell for TB) and T<sub>H1</sub> CD4 T cells. The bacteria reside in macrophages and drive a CD4 T<sub>H1</sub> response as the host attempts to kill the pathogen via T cell-induced activation and/or destruction of infected macrophages (Boom *et al.*, 2003; Schluger and Rom, 1998). Thus, in TB the presence of high numbers of activated CD4 T cells is ideal for the replication of HIV.

TB provides a milieu of continuous cellular activation that is accommodating to HIV replication (Toossi, 2003), consequently enhancing HIV disease progression (Badri *et al.*, 2001; Collins *et al.*, 2002; Whalen *et al.*, 1995). On the other hand, HIV fuels the TB epidemic due to the overlap of these two populations. In Sub-Saharan Africa, the spread of HIV has undoubtedly increased the incidence of TB (Lawn *et al.*, 2006; Badri *et al.*, 2001).

Previous studies have confirmed the interaction between the HIV and TB epidemics by showing an increased prevalence of HIV infection among people with TB disease and an amplification of TB infection rate in people living with HIV/AIDS (Corbett *et al.*, 2003; Badri *et al.*, 2001; Wood *et al.*, 2000). Recently, MacPherson *et al.* (2009) have reported TB as the main cause of deaths (44.3%) in public-sector patients, initiating HAART in a South African rural under-resourced region.

Although ARV therapy leads to reduction of AIDS-related opportunistic infections and mortality (Palella *et al.*, 1998; Hung *et al.*, 2003; Dheda *et al.*, 2004; Breen *et al.*, 2006; Badri *et al.*, 2002), HIV/TB coinfecting patients experience adverse effects that can result in poor adherence and failure of therapy (Dean *et al.*, 2002; Burman and Jones, 2001). Also ARV may amplify the presentation of active TB due to TB inflammatory reaction of immune reconstitution syndrome (IRIS) (Breen *et al.*, 2006; Dhasmana *et al.*, 2008). TB IRIS can be observed in two different situations: either unrecognized active TB may be unmasked early after initiation of ARV or a known TB patient on anti-TB treatment may develop a deteriorating clinical picture after the introduction of ARV (Djoba Siawaya *et al.*, 2007; Lawn *et al.*, 2007).

TB treatment involves 6 months of treatment with a 2-month initial phase (with daily or 3 times weekly isoniazid, rifampicin, pyrazinamide and ethambutol) and a continuation phase of 4 months (with daily or three times weekly isoniazid and rifampicin) or 6 months (with daily isoniazid and ethambutol) (Maher *et al.*, 2005; WHO, 2003b). Rifampicin is a potent inducer

of CYP enzymes, producing marked reductions in plasma concentrations of PIs and NNRTI, and resulting in drug-drug interactions in concurrent therapy (Niemi M, 2003; Breen *et al.*, 2006). The implication for HIV/TB coinfecting patients treated with a regimen with rifampicin throughout is that ARV therapy should be delayed or co-administered using ARV drugs compatible with rifampicin (e.g. Efavirenz) (Maher *et al.*, 2005; Niemi *et al.*, 2003).

Patients on TB medication and ARVs are taking a large number of drugs, which may lead to a poor adherence of treatment. There is a challenge in concurrent management of HIV/TB coinfection with regard to the adverse effects, complex drug interactions, overlapping toxicities and IRIS (Beck *et al.*, 2001; Idemoyor, 2007; Breen *et al.*, 2006; National Department of Health South Africa, 2004). These problems have promoted the view that anti-TB therapy should be given initially without HAART (Pozniak *et al.*, 1999; Breen *et al.*, 2006). Unfortunately in this context, the delay of HAART increases the risk of AIDS events or death in advanced HIV/TB coinfecting individuals (Dean *et al.*, 2002; Dheda *et al.*, 2004).

In South Africa, treatment guidelines for adult HIV infected patients with concomitant TB is as follows (National Department of Health South Africa, 2004):

1. If TB develops while on ARV: continue ARV therapy throughout TB treatment in patients on first-line therapy, as previously described. Regimen 1b containing Nevirapine should generally be swapped to Efavirenz (because of increased hepatotoxicity).
2. If TB infection is present before starting ARV: in patients with CD4 count >200 cells/ $\mu$ l, start TB treatment and assess the need for ARV after completing TB therapy; in patients with CD4 count <200 cells/ $\mu$ l, delay ARV until after 2-months intensive phase of TB therapy; in patients with CD4 count <50 cells/ $\mu$ l, introduce ARV after no less than 2 weeks of TB therapy.

Balance of logic needs to be established between the risk of progressing AIDS and possible death if ARV is deferred, against the risk of adverse events in concurrent therapy. The practical therapeutic approach could be to vary the ARV regimen at the time when anti-TB treatment is started, in view of the potential drug-drug interactions without interruption of antiretroviral therapy. Further studies are required to establish the optimal regimens and optimal time at which to start HAART in the context of HIV/TB coinfection. (Breen *et al.*, 2006; National Department of Health South Africa, 2004).

In summary, HIV infection is now a major challenge in the control of TB, especially in the Western Cape region of South Africa, which has a very high background incidence of TB as previously described by Yach, (1988). More studies are needed in such areas to better understand the impact of concomitant infections in order to set up strategies for optimal management of HIV/TB disease.

## **2.9. Aim and Objectives of this Study**

The aim of this study was to investigate differences in coreceptor expression, immune activation status and T cell subpopulation distribution in South African HIV and HIV/TB coinfecting patients, pre- and post-ARV and also in ARV-responders vs. ARV-non-responders.

The major objectives of this study were:

1. To determine the expression of HIV co-receptors CCR5 and CXCR4 on CD4+ T cells of South African HIV positive individuals pre- and post-ARV treatment
2. To determine the distribution of naïve, effector memory, central memory and effector memory CD4 T cell subpopulations in South African HIV positive individuals pre- and post-ARV treatment
3. To investigate HIV co-receptor expression on each of the 4 T cell subpopulations in South African HIV positive individuals pre- and post-ARV treatment
4. To assess activation status of T cells expressing either CCR5, CXCR4 or both in South African HIV positive individuals pre- and post-ARV treatment
5. To determine the effect of TB co-infection on coreceptor expression, T cell subset distribution and activation.

## **CHAPTER 3: MATERIALS AND METHODS**

### **3.1. Patient Demographics**

Approval for this study was obtained from the Committee for Human Research of the University of Stellenbosch (study number N07/08/179). Blood samples were collected from Karl Bremer Hospital, TC Newman Hospital and Tygerberg Hospital between April and October 2008 by a qualified research nurse. Samples were only collected if patients agreed to complete and sign the relevant consent forms. The control (HIV negative) samples were obtained from volunteers among Stellenbosch University staff at Tygerberg campus. Their status was confirmed by HIV-1 serology and normal CD4 count profiles.

Venous blood (40 ml) was collected by venipuncture into EDTA vacutainer tubes (BD Vacutainer<sup>®</sup>, Plymouth, UK). Bloods were transported to the laboratory at Medical Virology, Tygerberg Campus, Stellenbosch University on the day of collection and processed.

The only inclusion criterion for the study subgroups was HIV positive status, as confirmed through routine voluntary counselling and testing (VCT) at the different hospitals. However, Healthy controls and TB positive subgroup (newly diagnosed individuals) with HIV negative were also included. No specific exclusion criteria were defined for this study. Patients with secondary or co-infections, e.g. TB, were not excluded from the study, but formed part of unique subgroups. Based on clinical information contained in patient folders as confirmed by the clinician, active TB was considered in cases of a clinical diagnosis with appropriate radiological evidence of pulmonary TB (PTB) and/or with a positive Ziehl-Neelsen smear result for acid-fast bacilli. CD4 count at the baseline was considered as an objective indication of the stage of disease.

Cross-sectional analysis was performed on a total of 137 South African individuals, comprising 6 groups: 15 healthy normal donors (healthy subgroup), 10 patients with active TB (TB subgroup), 33 HIV-1 positive patients without active PTB and treatment naive (HIV subgroup), 23 HIV-1 positive patients with active PTB (HIV/PTB subgroup), 36 HIV-1 positive patients on ARV (HIV on ARV subgroup) and 20 HIV-1 positive patients with active PTB on ARV (HIV/PTB on ARV subgroup).

## **3.2. Methods**

### **3.2.1. PBMC Isolation**

PBMCs were isolated by density gradient centrifugation using Ficoll-Histopaque as first described by Boyum (1968). EDTA anticoagulated blood samples were centrifuged at 500 x g for 10 minutes to extract plasma (for viral load and HIV serology, see below) and to concentrate the cellular fraction. An equal volume of PBS (phosphate buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup>, Gibco, Invitrogen Corporation, UK) was added to the rest of sample and mixed well to dilute the blood. Three milliliters (3 ml) Histopaque<sup>®</sup>-1077 was added to a 15 ml conical centrifuge tube (CELLSTAR, Greiner Bio-one, Frickenhausen, Germany). Carefully and slowly, 3-6 ml of diluted blood was overlaid onto the Histopaque<sup>®</sup>-1077. Centrifugation was performed at room temperature at 400 x g for 30 minutes (Eppendorf centrifuge, Merck, Hamburg, Germany). After centrifugation, the mononuclear cell layer was collected at a volume of approximately 1-3 ml per centrifuge tube and washed in 10 ml of sterile PBS at 250 x g for 10 minutes. The washing step was repeated, the supernatant fluid aspirated and discarded and the cells resuspended in 1 ml of AR10 medium (50% RPMI, 50% AIM-V plus 10% heat inactivated FBS) (Gibco, Invitrogen Corporation, UK).

For counting, 15 µl of cells were mixed with an equal volume of Turks solution (0.02% crystal violet, 7% glacial acetic acid in water) and cells were counted using a Neubauer hemocytometer and microscope (Wild Heerbrugg M20, Switzerland). Cell concentrations were adjusted to 5 x 10<sup>6</sup>/ml by centrifugation of the cells and resuspension in the appropriate volume of AR10 medium.

Plasma samples were sent to the diagnostic laboratory at Medical Virology, Tygerberg Campus, Stellenbosch University on the same day and determination of HIV-1 viral load performed on all samples collected from infected individuals.

### **3.2.2. HIV-1 Viral Load Determination**

EasyQ HIV-1 v1.2 is a nucleic acid amplification protocol for the quantitative determination of HIV-1 RNA in human EDTA plasma. It consists of nucleic acid sequence-based amplification (NASBA) combined with a simultaneous real-time detection step using molecular beacons on a NucliSens EasyQ analyzer (Yao *et al.*, 2005; de Mendoza *et al.*, 2005; Leone *et al.*, 1998).

Nucleic acid isolation, amplification and detection of plasma viral loads were performed according to the manufacturer's instructions provided with each assay kit. In brief, an automated procedure was used for nucleic acid isolation using the NucliSens easyMAG magnetic extraction method. Amplification and detection were performed as previously described by Yao J *et al.* (2005). Firstly, 10 µl of primer solution (mixture of synthetic primers, synthetic molecular beacon probes, nucleotides, dithiothreitol, KCl and MgCl<sub>2</sub>) was added to a tube containing 5 µl purified nucleic acid extract of each sample. The tubes were incubated at 65°C for 2 minutes in a NucliSensQ incubator (Biomerieux, Marcy l'Etoile, France) and cooled to 41°C for 2 minutes. Thereafter, 5 µl of enzyme solution was added to each tube. Eight-well strip caps (Biomerieux, Marcy l'Etoile, France) were used to seal the tubes. All tubes were then transferred to a 41°C temperature controlled NucliSens EasyQ analyzer (Biomerieux, Marcy l'Etoile, France) and read 120 times for fluorophores. The NucliSens EasyQ Director Software (Biomerieux, Marcy l'Etoile, France) was used to quantify viral load in each sample automatically (Yao J *et al.*, 2005; de Mendoza *et al.*, 2005).

### **3.2.3. HIV-1 Serology**

Using AxSYM HIV Ag/Ab Combo (Abbott, Wiesbaden, Germany), a microparticle enzyme immunoassay (MEIA), qualitative detection of antibodies to HIV type 1 and/or type 2 and HIV p24 antigen in human plasma was performed. AXSYM HIV Ag/Ab Combo is based on MEIA technology utilizing recombinant HIV (*E. coli*) antigens and HIV p24 monoclonal (mouse) antibodies coated on microparticles to capture antibodies against HIV-1/HIV-2 and HIV p24 antigen. Biotin-labelled recombinant antigens react with captured antibodies/antigen. An anti-Biotin alkaline phosphatase conjugate was used to detect the complexes (Kwon *et al.*, 2006; Sickinger *et al.*, 2004).

A reaction mixture was set up, comprising specimen diluent, sample, and microparticles coated with recombinant antigens and monoclonal antibodies in the sample well of the reaction vessel (Abbott, Wiesbaden, Germany). In the presence of antibodies or p24 antigen in the sample, a complex antigen-antibody is formed on the microparticles. Washing steps were performed to remove material not bound to microparticles. Biolabelled recombinant antigens were added to form an immune complex revealed with an anti-Biotin alkaline phosphatase conjugate, which acts on the substrate to give a fluorescent product measured by MEIA optical assembly (Kwon *et al.*, 2006; Sickinger *et al.*, 2004).

### 3.2.4. PBMC Surface Staining:

Freshly extracted PBMCs were labelled with fluorochrome-conjugated monoclonal antibodies prior to flow cytometric analysis. The following fluorochrome-conjugated antibodies were used in the current study: CD4-PerCP (clone SK3, mouse IgG, K; BD Biosciences, San Jose, CA, USA), CD45RA-FITC (Clone JS-83, mouse IgG1, K; eBioscience, San Diego, CA, USA), CD27-APC (clone 0323, mouse IgG1, K; eBioscience, San Diego, SA, USA), CD38 (clone H1T2, mouse IgG1, K; eBioscience, San Diego, SA, USA), CCR5-PE (clone R22 / 7, mouse IgG1, K; BD Biosciences, San Jose, CA, USA), CXCR4-PE (12 G5, mouse IgG2a, K; BD Biosciences, San Jose, CA, USA), CCR5-APC (clone 3A9, mouse IgG2a, K; eBioscience, San Diego, CA, USA).

The staining mixes used in the study allowed characterization of CD4 T cells into Naïve (N, CD45<sup>+</sup> and CD27<sup>+</sup>), effector memory (EM, CD45 RA<sup>-</sup> and CD27<sup>-</sup>), central memory (CM, CD45 AR<sup>-</sup>, CD27<sup>+</sup>) and effector (E, CD45 RA<sup>+</sup>, and CD27<sup>-</sup>) populations and also allowed determination of chemokine receptor expression (CCR5, CXCR4) on CD4 T cells. Activation status was determined using CD38.

Antibodies were used at concentrations previously found to be optimal for lymphocyte staining in our laboratory. The first step was to set up different staining mix tubes. Three different tubes were prepared to define CCR5 and CD4 subsets (Tube 1), CXCR4 and CD4 subsets (Tube 2) and CCR5 / CXCR4 and CD4 activation (Tube 3). A total of  $5 \times 10^5$  cells/well were labelled with 25  $\mu$ l of each staining mix. Three sets of staining mixes were set up per sample as indicated below.

Tube 1		Tube 2		Tube 3	
CD4-PerCP:	3.5 $\mu$ l	CD4-PerCP:	3.5 $\mu$ l	CD4-PerCP:	3.5 $\mu$ l
CD45RA- FITC:	2.5 $\mu$ l	CD45RA-FITC:	2.5 $\mu$ l	CCR5-FITC:	2.5 $\mu$ l
CD27-APC:	5.0 $\mu$ l	CD27-APC:	5.0 $\mu$ l	CD38-APC:	5.0 $\mu$ l
CCR5-PE:	1.5 $\mu$ l	CXCR4-PE:	1.5 $\mu$ l	<u>CXCR4-PE:</u>	<u>1.5 <math>\mu</math>l</u>
<u>Staining buffer:</u>	<u>12.5 <math>\mu</math>l</u>	<u>Staining buffer:</u>	<u>12.5 <math>\mu</math>l</u>	<u>Staining buffer:</u>	<u>12.5 <math>\mu</math>l</u>
<b>Total:</b>	<b>25.0 <math>\mu</math>l</b>	<b>Total:</b>	<b>25.0 <math>\mu</math>l</b>	<b>Total:</b>	<b>25.0 <math>\mu</math>l</b>

Antibodies were mixed before their use as described. Staining buffer was made using PBS without Ca<sup>2+</sup>/Mg<sup>2+</sup> + 0.1% FBS (Foetal Bovine Serum, Gibco, Invitrogen corporation, UK).

The staining mix volume of each tube had to be multiplied by the number of wells to be stained (plus additional to ensure sufficient volume). For example: for 6 wells to be stained the staining mix was multiplied by 8. Staining mixes and antibodies were kept on ice during the preparation of the cells and staining procedure.

Once staining mixes were prepared, the PBMC pellet of sample was resuspended, concentration of cells adjusted to  $5 \times 10^6$ /ml with AR10 medium and plated in 96-well U bottomed microtitre plates (CELLSTAR, Greiner BIO-one, Frickenhausen, Germany), at 100  $\mu$ l per well, such that a total of  $5 \times 10^5$  cells were present in each well. One well was prepared unstained as a control and three wells for the different staining mixes. Centrifugation (Jouan centrifuge GR4.12, Jouan SA, France) of PBMC microtitre plate was performed at 250 x g (at 8°C) for 5 minutes.

Supernatant was carefully removed using a Gilson pipette (approximately 75  $\mu$ l) ensuring that the pellet remained undisturbed. The pellet was then resuspended by holding microplate on vortex. Staining mixes (25  $\mu$ l) were then added to each well and the microplate incubated at 4°C for 30 minutes. Initial experiments included isotype control antibody staining, isotype controls being used at same volumes as standard antibodies. After staining, 175  $\mu$ l of staining buffer were added to each well and the microplate was centrifuged at 250 x g for 5 minutes at 8°C. After centrifugation, supernatants were removed, and pellets resuspended on a vortex as before.

Two hundred microliters (200  $\mu$ l) of staining buffer were added for the second washing step. The microplate was centrifuged at 250 x g for 5 minutes, supernatants discarded and pellets resuspended on a vortex. Finally, 200  $\mu$ l of staining buffer was added to each well, and contents mixed. Labelled FACS tubes (Falcon, BD Sciences, San Jose, CA, USA) were separately filled with 300  $\mu$ l of staining buffer per tube, wherafter 200  $\mu$ l of stained samples from each well were transferred to the corresponding FACS tube, mixed to make the total sample volume to 500  $\mu$ l per tube and kept at 4°C in the dark until analysed on the Flow cytometer.

### **3.2.5. Whole Blood Staining for CD4 Count**

Using BD Multitest reagent CD3-FITC / CD8-PE / CD45-PerCP / CD4-APC, whole blood was stained in a TruCount tube containing microbeads to enumerate human T lymphocytes.

BD-TruCount tubes were labelled with patient numbers and 20 µl of multitest reagent was pipetted and deposited with a Gilson pipette above the steel retainer at the bottom of the tube. Well-mixed whole blood (50 µl) was added carefully into the bottom of the tube, avoiding smearing blood on the side of the tube. The tube was then gently vortexed and incubated for 15 minutes in the dark at room temperature. 450 µl of 1X FACS lysing solution (BD Biosciences, San Jose, CA, USA) was added to the tube and vortexed to mix. Incubation for 15 minutes in dark at room temperature was performed and the sample was directly analyzed on flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA).

### **3.2.6. Assessment of Coreceptor Expression, Activation Status and CD4 T Cell Subset Distribution**

#### **3.2.6.1. Acquisition of Data**

Using a BD FACSCalibur flow cytometer with CellQuest™ software (BD Biosciences, San Jose, CA, USA), acquisition and analysis were performed on stained PBMC samples. Before acquiring data, events to be acquired, data storage files and acquisition conditions were defined and saved.

Instrument settings and colour compensation values were based on those defined using matched isotype control antibodies and the antibody panels defined for the study. Manually adjusted (fine tuned) colour compensation settings from these optimization steps were also saved. Once settings were optimized, the same settings were used throughout the study. Minor adjustments were made to ensure that cells consistently gave the same scatter patterns.

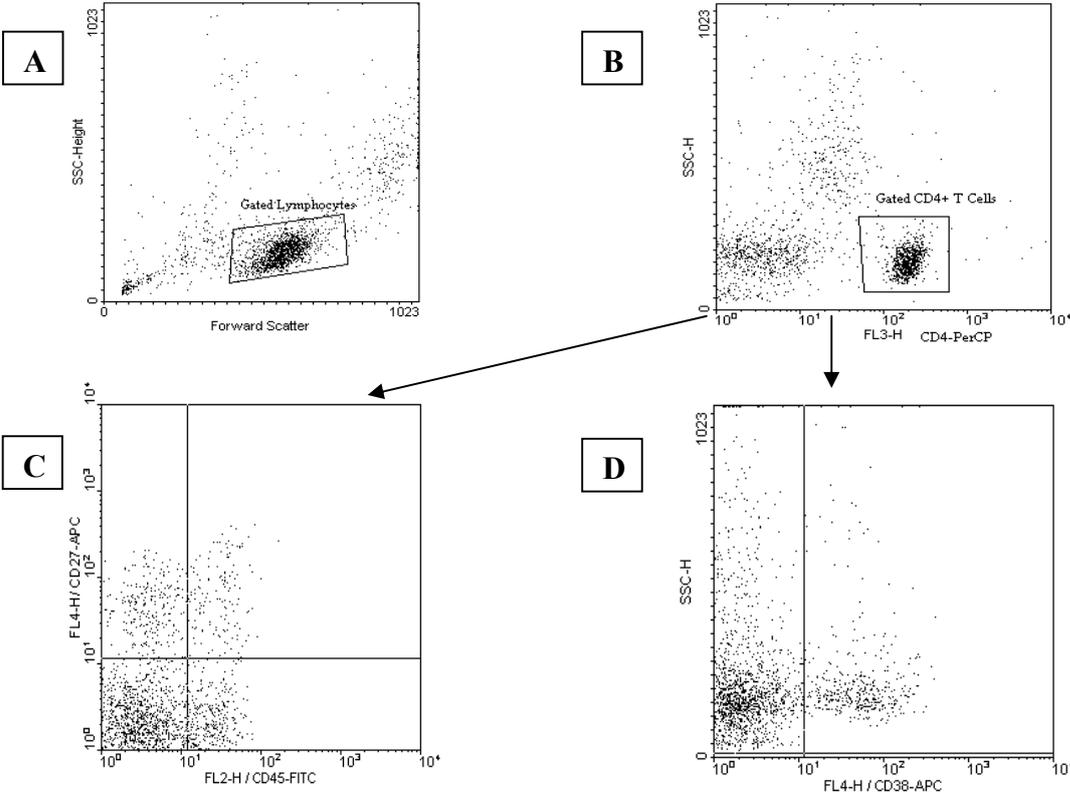
The acquisition template for the study had 8 dot plots created for each of the 4 tubes per sample as described previously. Plot parameters were defined as following: FSC versus SSC (FSC / SSC), FL3 / SSC, FL4 / SSC, FL1 / SSC, FL1 / FL4, FL2 / FL4, FL2 / SSC and FL1 / FL2 (or FL1 / FL4). Five gated regions were created respectively: G1=R1 (lymphocytes based on scatter); G2=R2 (CD4 positive events); G5=R1+R2; G3=R3 (CD4 positive + expressing CCR5 or CXCR4); G4=R4 (CD4 positive expressing CD38). Quadrant markers were also created on different plots to generate statistics.

Data were acquired as follows: Lymphocytes were identified by gating on forward scatter versus side scatter then CD4+ T cells (by gating around CD4-PerCP stained cells). CD38 expression, CCR5 and CXCR4 expression on total CD4 T cells and different CD4 T cell

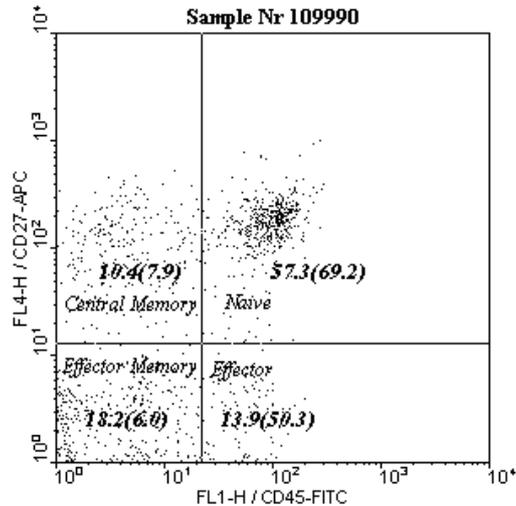
subsets were determined from different created quadrants. Fifty thousand (50000) of total events were acquired per sample.

### 3.2.6.2. Analysis of Data

Data was analyzed after acquisition by defining regions and gates, creating quadrant markers and generating statistics. For analysis plots, templates of both mean fluorescence intensity (MFI) data and percentage positive data (% of cells expressing a particular antigen) were used. An example of data analysis for defining cell subsets is given in Figure 3.2.



**Figure 3.1.** Example of gated regions. A: lymphocytes gated (G1); B: CD4 T cells gated (G2) and created quadrants; C: CD4 T cells staining (FL4 vs FL2); and D: CD4 T cells staining (FL4 vs SSC).



**Figure 3.2.** Example of Characterization of CD4 T cell subset. This plot is based on gating of Lymphocytes (FS vs SS) plus CD4 staining (G1 plus G2). Data are expressed as percentage (%) of each subset. MFI values for expression of CD45 and CD27 are indicated for each subset in parentheses.  
 Naïve CD45RA+, CD27+; Effector CD45RA+, CD27-; Central Memory CD45RA-, CD27+; and Effector Memory CD45RA-, CD27-

### 3.2.7. Determination of CD4 Count

After defining parameters and instrument settings for BD Multiset™ (BD Biosciences, San Jose, CA, USA) data was acquired and absolute cell counts (number of positive cells per  $\mu\text{l}$ ) were determined automatically by the software. Standard procedure was followed for CD4 count as per accredited SOP (CD4 SOP - SANAS).

### 3.2.8. Data Collection and Statistic Analysis

All flow cytometric data were collected into spreadsheet format (Excel). Statistical analysis was performed in consultation with a statistician at the Centre for Statistical Consultation, Stellenbosch University.

For comparison of measurements between different subgroups, one-way ANOVA was performed. Post-hoc comparisons were done using Fisher least significant difference (LSD). To determine relationships between different measurements, Spearman correlations were calculated. A 5% significance level ( $p < 0.05$ ) was used as guideline for determining significant relationships.

Where p values are indicated, comparison with uninfected controls is implied (unless stated otherwise). P values are scored as follows:

\*\*\*,  $p < 0.001$     \*\*,  $p = 0.001$  to  $0.01$     \*,  $p = 0.01$  to  $0.05$     NS,  $p > 0.05$

Graphs: Box and Whiskers graphs show mean, 25<sup>th</sup> and 75<sup>th</sup> percentile values (boxes) and the error bars represent the 10<sup>th</sup> and 90<sup>th</sup> percentiles.

## **CHAPTER 4: RESULTS**

### **4.1. Demographic, Virological and Immunological Characteristics of Individual Subgroups**

In this study, a cross-sectional analysis was performed on 137 blood samples collected from individual donors comprising different subgroups as described in Chapter 3. Demographic, virological and immunological characteristics of the sample collection are shown in Table 4.1.

The median age of individual donors was 35 years (range between 19-65 years), and female sex was predominant across all subgroups (Table 4.1). The median duration of treatment was 6 months in the HIV infected on ARV subgroup, and 2 months and 2 weeks in the HIV/TB coinfecting subgroup on ARV (data not shown). The median duration of TB treatment was 1 month in the HIV/TB coinfecting subgroup, and 3 months and 2 weeks in the HIV/TB subgroup on ARV (Table 4.2).

CD4 count differences were not significant between the HIV subgroup and the HIV/TB subgroup without ARV. Median CD4 count in individuals with active TB (in absence of HIV infection) was strangely found low when compared to the healthy subgroup (544 Vs 1408), however the number of participants with CD4 count data in TB disease subgroup was smaller (Table 4.1) than in the healthy subgroup (7 Vs 15). After initiation of treatment, however, the CD4 count was found to be higher in the HIV on ARV subgroup (median 235.5) compared to the HIV/TB on ARV subgroup (median 141). The increase of 62 cells/ $\mu$ l in the HIV subgroup represents a 53.7% enhancement following ARV. In the HIV/TB subgroup, the increase following initiation of ARV was 25.5 cells/ $\mu$ l, only a 22% enhancement.

Before ARV treatment, the median viral load was 3.95 fold increased in the HIV/TB subgroup as compared to the HIV subgroup (71 000 copies/ml vs. 18 000 copies/ml). The median viral load in both the HIV and HIV/TB subgroups on ARV was decreased to undetectable limit ( $\leq$  357 copies/ml).

### **4.2. Coreceptor Expression in Individual Subgroups**

Due to the important role that CCR5 and CXCR4 coreceptors play in HIV entry and disease progression, assessment of their expression on CD4 T cells within the defined subgroups was performed. CCR5 and CXCR4 expression on CD4 T cells was determined by immunostaining

and flow cytometric analysis of fresh PBMCs isolated from individual blood samples as described in Chapter 3.

**Table 4.1. Demographic, virological and immunological characteristics of individuals within donor subgroups**

	Healthy	PTB	HIV	HIV/TB	HIV on ARV	HIV/TB on ARV
No of Subjects	15	10	33	23	36	20
Age (median years)	43	42	35	33	37	34
% of Males	37.7	40	27.3	43.5	25	45
% of Females	64.3	60	72.7	56.5	75	55
CD4 count (median)	1408	544*	<b>173.5**</b>	<b>115.5**</b>	235.5	141
Viral Load copies/ml (median)			<b>18000</b>	<b>71000</b>	LDL***	LDL
Log10 copies/ml (median)			<b>4.3</b>	<b>4.9</b>	2.5	2.5

Subgroups: Healthy = normal donors; HIV = HIV infected patients; PTB = active pulmonary tuberculosis patients; HIV/TB =- HIV/TB coinfecting patients; on ARV = on antiretroviral therapy.

\* 3 CD4 count data not included, median based on n=7. \*\* 1 CD4 count data not included, median based on n=32 (HIV) and n=22 (HIV/TB). \*\*\*LDL = lower than detectable limit ( $\leq 357$  copies/ml).

Note: Of total CD4 count data (136) 54 (40%) were performed on fresh blood. Other CD4 data were collected from file within last 3 months.

**Table 4.2: TB treatment of the individuals with pulmonary TB\***

	HIV/TB	HIV/TB on ARV
<b><u>Duration</u></b>		
Pre-treatment	6	6
0-2 months	10	4
More than 2 months	5	7

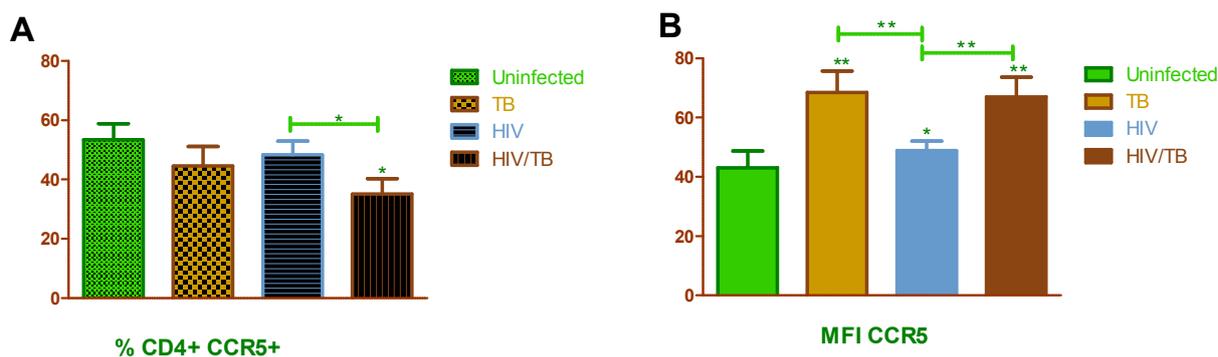
\*Data are expressed as number of patients with documented TB treatment.

The percentage of CD4 T cells expressing CCR5+ and CXCR4+ are shown in Figures 4.1 and 4.2 and Table 4.3. The percentage CCR5 positive CD4 T cells in the HIV/TB subgroup (28.6%) was significantly reduced compared to the HIV subgroup (52.5%,  $p < 0.05$ ), and the healthy control group (55.9%,  $p < 0.05$ ). The HIV/TB subgroup also showed reduced expression when compared to the PTB subgroup (53%), however this was not statistically significant.

The MFI of CCR5 expression on CD4 T cells (Figure 4.1) in the HIV/TB coinfecting subgroup (median MFI = 64.6) was significantly higher than the HIV infected subgroup (median MFI = 46.4;  $p < 0.05$ ) and healthy controls (median MFI = 40.2;  $p < 0.005$ ). As with percentage data, the difference of CCR5 level expression in the HIV/TB subgroup was found to be not significant compared to the PTB control subgroup. These figures indicate that HIV/TB disease leads to a significant additional reduction in the percentage of CCR5 expressing CD4+ T lymphocytes compared to HIV alone. The reduced number of CCR5 positive cells is accompanied by a significant increase in CCR5 receptor expression per cell.

The percentage of CXCR4+ CD4 T cells (Figure 4.2; Table 4.3) was higher in healthy controls (57.9%) compared to all the infected subgroups: PTB (48.9%,  $p = 0.057$ ), HIV (40%,  $p < 0.005$ ) and HIV/TB (27.4%,  $p < 0.001$ ). As with CCR5, TB co-infection lead to an exacerbated reduction in CXCR4 expressing CD4 T cells beyond that observed in HIV infection alone.

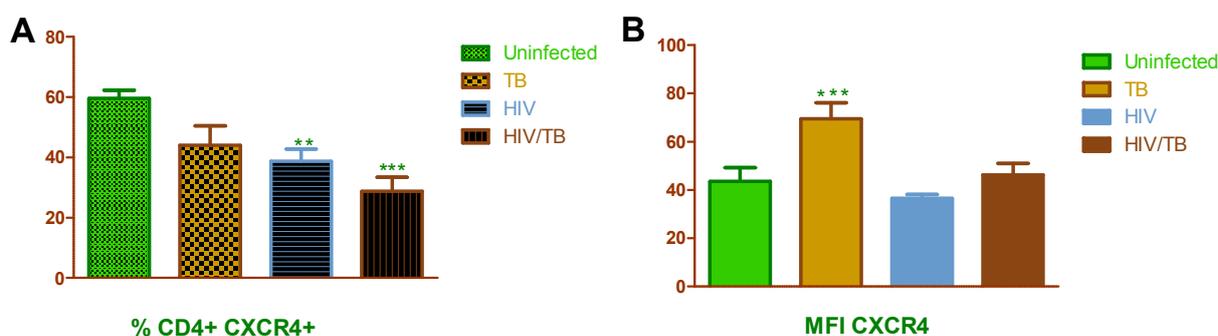
The MFI of CXCR4 expression on CD4 T cells (Figure 4. 2) in the PTB subgroup (median MFI = 73.3) was significantly higher compared to the HIV/TB subgroup (median MFI = 41.6;  $p < 0.005$ ), the HIV subgroup (median MFI = 35.3;  $p < 0.0001$ ) and healthy controls (median MFI = 34.3;  $p < 0.001$ ). However, unlike CCR5 expression, CXCR4 expression was not significantly increased in the HIV/TB subgroup compared to the HIV infected subgroup ( $p = 0.058$ ) and healthy controls ( $p > 0.05$ ). These figures illustrate that HIV/ TB disease was associated with exacerbated reduction in CXCR4 expressing cells.



**Figure 4.1.** Comparison of CCR5 expression on CD4+ T cells in individuals within donor subgroups. PBMCs were stained with CD4-PerCP, CD45-FITC, CD27-APC and CCR5-PE. Data are expressed as Means with SEM (standard error of the mean) of percentage cells positive for CCR5 and CCR5 MFI (indicating the fluorescence intensity of CD4 T cell expressing CCR5+).

A: Reduced percentage of CCR5 positive cells is shown in all infected subgroups, with HIV/TB disease the most severely reduced. There was also a significant difference between the HIV subgroup ( $p < 0.05$ ) compared to the HIV/TB subgroup

B: CCR5 MFI was increased in all infected groups compared to the healthy control group, the PTB subgroup ( $p < 0.05$ ); the HIV subgroup ( $p < 0.01$ ) and the HIV/TB ( $p < 0.005$ ) subgroup



**Figure 4.2.** Comparison of CXCR4 expression on CD4+ T cells in individuals within donor subgroups. Four-colour flow cytometry analysis was performed on PBMC using CD4-PerCP, CD45-FITC, CD27-APC and CXCR4-PE mAbs. Data expressed as Means with SEM.

A: A significant decrease in percentage CXCR4 + in the HIV/TB subgroup ( $p < 0.001$ ) and the HIV subgroup ( $p < 0.005$ ) compared to healthy controls

B: A significantly increased CXCR4 expression in the PTB subgroup ( $p < 0.0001$ ) compared to the HIV subgroup

### 4.3. Activation Marker Expression in Individual Subgroups

T cell immune activation is crucial in productive HIV infection as HIV preferentially targets activated CD4 T cells. To determine the immune activation status of CD4 T cells in the different donor subgroups, CD38 expression was measured as a marker of activation.

The median percentage CD38+ CD4 T cells (Figure 4.3 and Table 4.3) was higher in the HIV/TB and the HIV subgroups (33.4% and 30.4% respectively) compared to healthy controls (23.4%). The immune activation status of CD4 T cells in the PTB subgroup (median MFI = 77.5) was significantly higher compared to the HIV subgroup (median MFI = 50.5;  $p < 0.05$ ) and healthy controls (median MFI = 43.2;  $p < 0.05$ ). CD38 expression in the HIV/TB subgroup (median MFI = 64.2) was significantly higher compared to the HIV subgroup (median MFI = 50.1;  $p < 0.005$ ) and healthy controls (median MFI = 43.2;  $p < 0.005$ ).

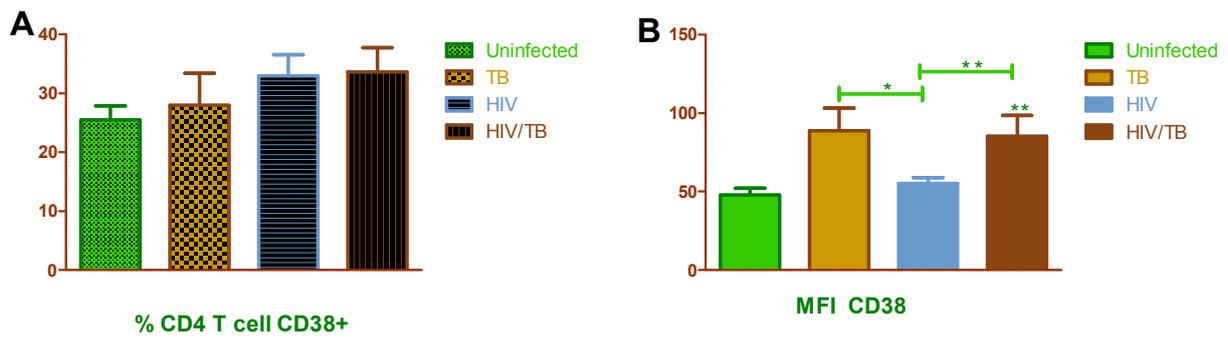
Expression of CD38 on CCR5- and CXCR4-expressing CD4 T cell subsets was also determined. Percentage activated CD4 T cells expressing CCR5+ and CXCR4+ are shown in Figure 4.4. Activated CD4 T cells expressing CCR5+ were found to be significantly increased in the HIV/TB (72.9%) and HIV subgroups (64.5%) compared to healthy controls (46.5%;  $p < 0.005$ ).

Conversely, activated CD4 T cells expressing CXCR4+ did not differ significantly within the donor subgroups. In the PTB subgroup, activated CD4 T cells expressing CCR5+, but not CXCR4+, were higher compared to healthy controls (79.3% vs. 46.2% CD4+CD38+CCR5+ and 50% vs. 56.9% CD4+CD38+CXCR4+).

**Table 4.3. CCR5, CXCR4 and CD38 positive percentage of CD4 T cell in Individuals within the donor subgroups\***

	Healthy	PTB	HIV	HIV/TB	HIV on ARV	HIV/TB on ARV
CCR5+	55.9	53.0	52.5	28.6	69.3	36.8
CXCR4+	57.9	48.9	40.0	27.4	55.0	32.8
CD38+	23.4	24.3	30.4	33.4	34.2	31.3

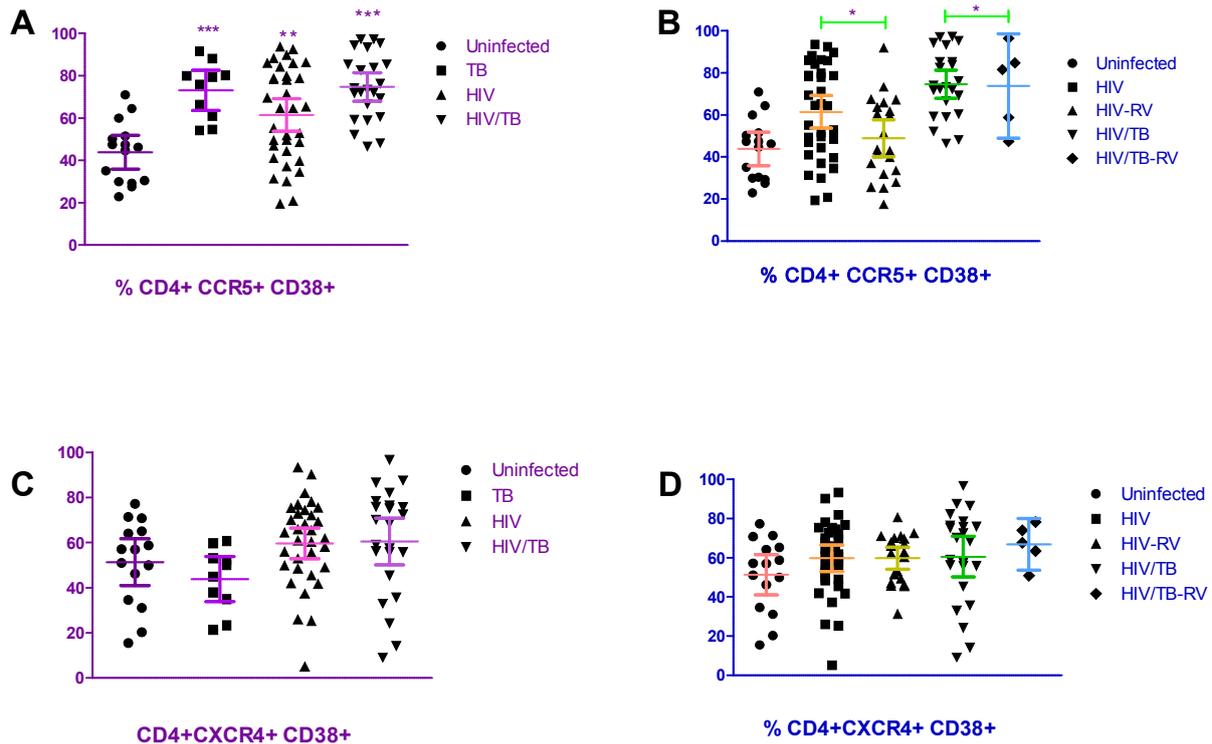
\* Data are expressed as Median percentage positive CD4 T cells. Subgroups: Healthy, normal donors; HIV, HIV infected patients; PTB, active tuberculosis patients; HIV/TB, HIV coinfecting patients; on ARV, on antiretroviral therapy



**Figure 4.3.** Comparison of CD38 expression on CD4<sup>+</sup> T cells in individuals within donor subgroups. PBMCs were stained with CD4-PerCP, CCR5-FITC, CXCR4-PE and CD38-APC mAbs. Data is expressed as Means with SEM.

A: Increased CD4 T cell CD38 expression in the HIV/TB and HIV subgroups compared to healthy controls

B: Increased CD38 expression in the PTB ( $p < 0.05$ ) and HIV/TB subgroups ( $p < 0.005$ ) compared to the HIV subgroup



**Figure 4.4.** Comparison of mean percentage positive activated CD4 T cells expressing CCR5+ and CXCR4+. Data expressed in scatter dot plot as means with SEM.

- A: Before ARV treatment, a significant increase of activated CCR5+ CD4 T cells in the HIV/TB ( $p < 0.005$ ), HIV ( $p < 0.05$ ) and PTB subgroups ( $p < 0.05$ ) compared to healthy controls
- B: After ARV treatment, a significant decrease of CCR5+ CD4 T cell activation in the HIV/TB ( $p < 0.05$ ) and HIV on ARV subgroups ( $p < 0.05$ ) as compared to untreated patients
- C: Activated CXCR4+ CD4 T cells within different subgroups before ARV treatment did not show a significant difference within the subgroups
- D: Activated CXCR4+ CD4 T cells within different subgroups after ARV treatment did not show a significant difference within the subgroups

#### 4.4. CD4 T Cell Subset Distribution in Individual Patient Subgroups

CD4 T cell susceptibility to HIV infection is related to the mode of HIV transmission and activation status of the T cells. Infection with HIV also impacts on T cell subset distribution, since certain subsets of cells are more readily infected than others. To phenotypically define different CD4 T cell subsets in this study, CD27 and CD45 were used as markers. After immunostaining of fresh PBMCs from individual donors, different dot plots were constructed and quadrants created to allow characterization of CD4 T cells into naïve, EM, CM and

effector cells as described in Chapter 3.

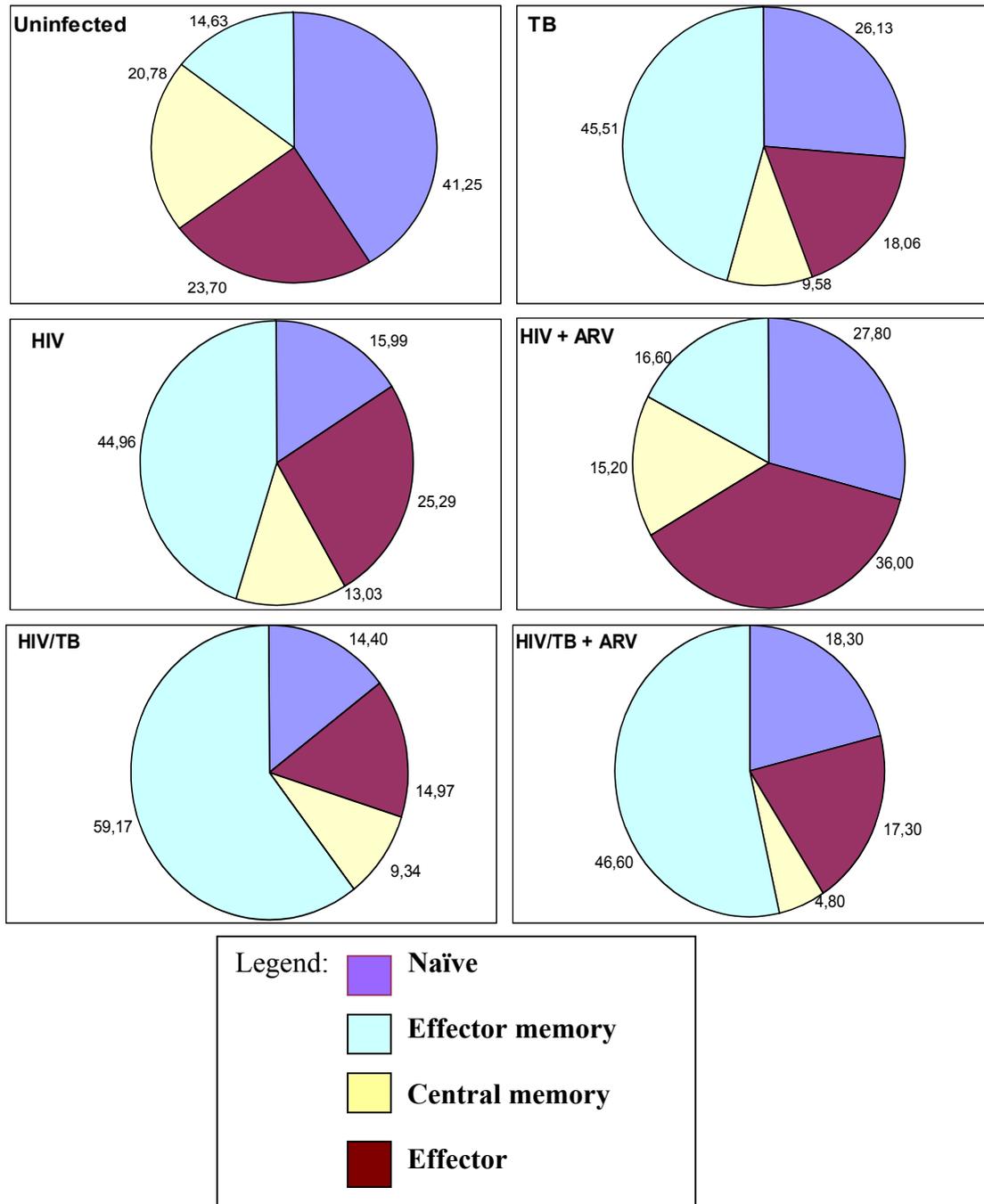
Based on co-expression of two markers (CD27/ CD45), data were depicted as the percentage positive-defining CD4 T cell subset populations. Distribution of the naïve CD4 T cell, effector CD4 T cell, CM and EM CD4 T cell subsets is reported in Figures 4.5 to 4.10 respectively.

Antigen-experienced cells including non-naïve cells were also calculated by summing proportions of effectors, central memory and effector memory CD4 T cells (Figure 4.10).

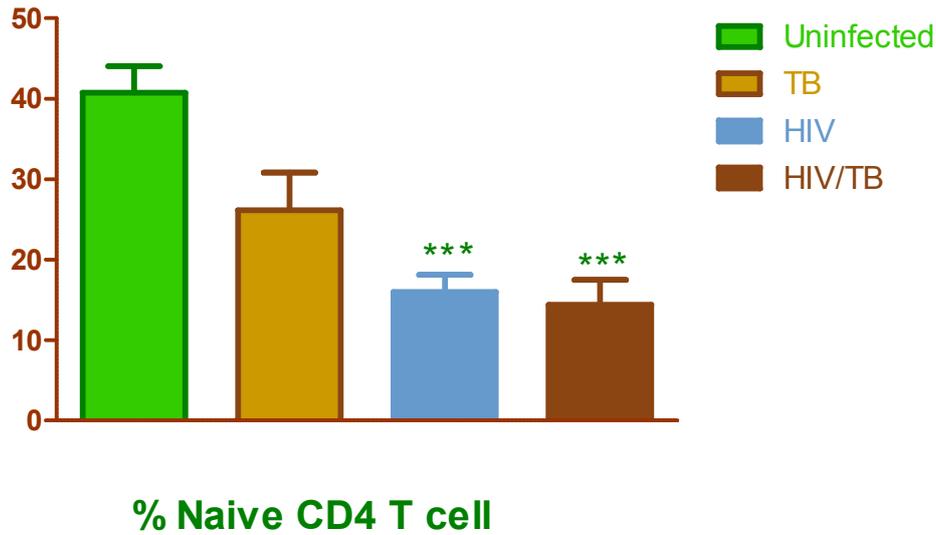
The percentage of naïve CD4 T cells in the HIV (13.7%) and HIV/TB coinfecting subgroups (11%) (Figures 4.5 and 4.6) was substantially decreased compared to healthy controls (41.1%;  $p < 0.0001$ ). The percentage of effector CD4 T cells was higher in the HIV subgroup (24.9%) compared to the HIV/TB (13%,  $p < 0.005$ ), PTB (20.1%) and healthy subgroups (22.1%) (Figures 4.5 and 4.7). The percentage of central memory CD4 T cells (Figures 4.5 and 4.8) was considerably decreased in the HIV/TB (6.8%,  $p < 0.0001$ ) and HIV subgroups (12.6%,  $p < 0.005$ ) compared to healthy controls (20%). The percentage of effector memory CD4 T cells were higher in the HIV/TB subgroup (58.9%) compared to the HIV infected subgroup (37.7%,  $p < 0.05$ ) and healthy controls (14.6%,  $p < 0.0001$ ). The percentage of antigen-experienced cells was increased in the HIV/TB subgroup (78.7%) compared to the HIV infected subgroup (75.1%) and healthy controls (56.7%,  $p < 0.005$ ).

In addition to subset distribution in peripheral blood, we examined coreceptor expression in different CD4 T cell subsets. The effect of ARV on CD4 T cell subsets expressing CCR5+ and CXCR4+ was also determined. Data is shown in Figures 4.11 and 4.12, and Table 4.4.

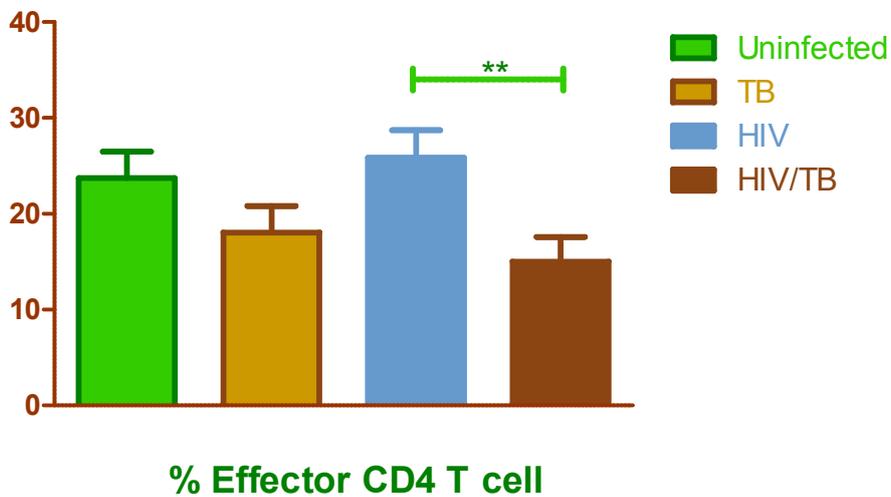
Within the patient subgroups, as expected, naïve CD4 T cell subsets expressed more CXCR4+ than CCR5+, for example 47.7% CXCR4+ vs. 42.6% CCR5+ in healthy controls. The CCR5 expressing CM subsets were increased in the HIV (16.9%) and HIV/TB subgroups (14.8%) compared to healthy controls (9.2%). CXCR4 expression of the naïve T cell subsets were decreased in the HIV/TB (24.7%) and HIV subgroups (22.8%) compared to healthy controls (47.7%). Expression of CCR5 and CXCR4 on the effector CD4 T cell subset was higher in the HIV subgroup (36.8% and 43.7%) compared to the HIV/TB subgroup (29.0% and 29%) and healthy controls (28.8% and 26.3%).



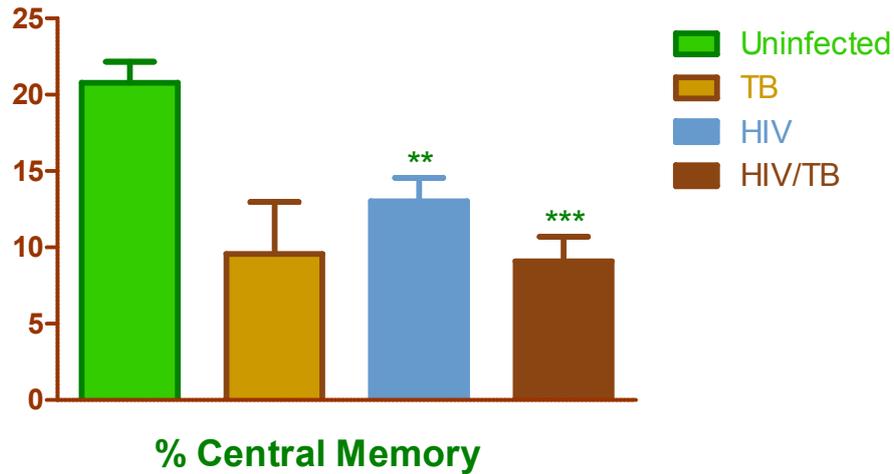
**Figure 4.5.** Representation of CD4 T cell subset distribution in pie graph format. This figure shows different CD4 T cell subset distribution within patient subgroups. Data expressed as positive % of Naïve, Effector, Central Memory and Effector Memory CD4 T cells.



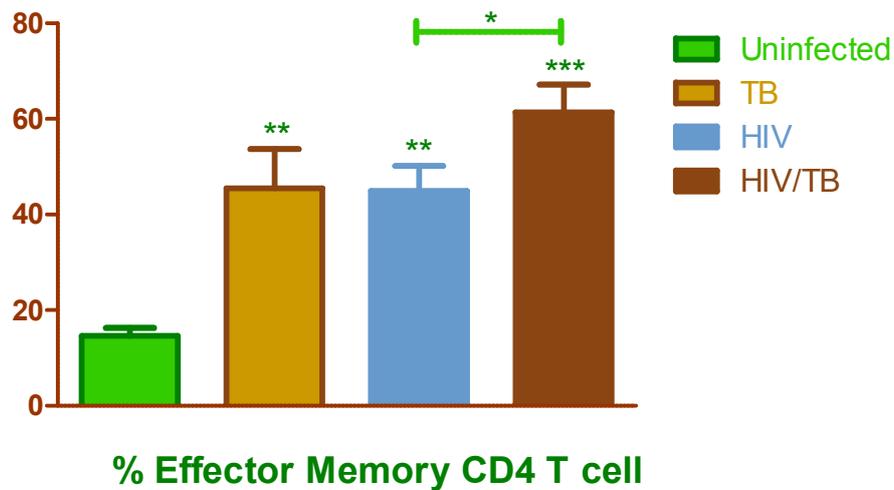
**Figure 4.6.** Distribution of Naïve CD4 T cell subsets in individual subgroups. Phenotypic analysis of PBMCs following staining with mAbs was performed. Data expressed as means with SEM of percentage positive indicated the number of Naive CD4 T cell subset is significantly decreased in the HIV subgroup and HIV/TB subgroups compared to healthy controls ( $p < 0.0001$ ).



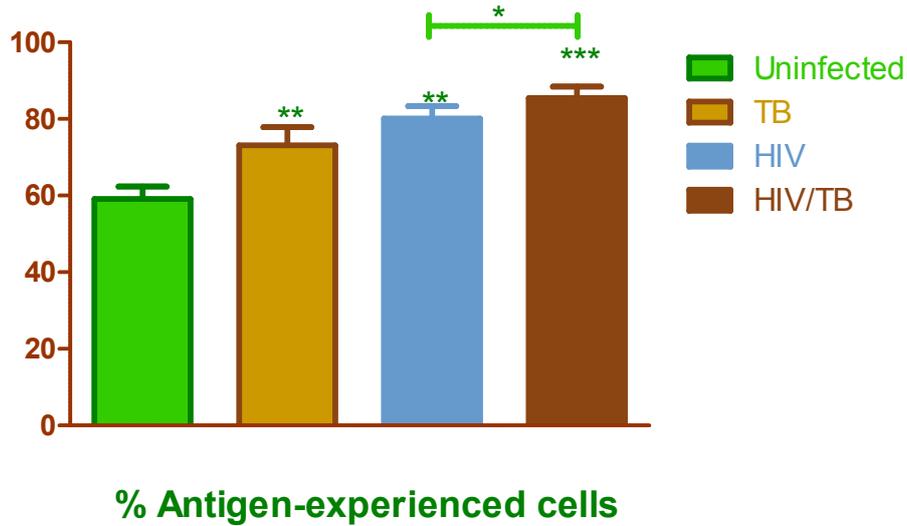
**Figure 4.7.** Distribution of Effector CD4 T cell subsets. After four-colours staining of PBMCs, phenotypic analysis was done by FACS. Data expressed as means with SEM of percentage positive present the numbers of Effector CD4 T cell subset indicating a significant increase in the HIV subgroup ( $p < 0.005$ ) compared to the HIV/TB subgroup.



**Figure 4.8.** Distribution of Central Memory CD4 T cell subsets. Data are expressed as means with SEM. This figure indicates a significant decrease of central memory CD4 T cells in the HIV/TB ( $p < 0.0001$ ) and HIV subgroups ( $p < 0.005$ ) compared to healthy controls.



**Figure 4.9.** Distribution of Effector memory CD4 T cell subsets. Data are expressed as means with SEM. The percentage of effector memory CD4 T cells is significantly higher in the HIV/TB subgroup compared to the HIV subgroup ( $p < 0.05$ ) and healthy controls ( $p < 0.0001$ ).



**Figure 4.10.** Distribution of antigen-experienced CD4 T cell subsets. Data expressed as means with SEM. The figure shows a significant increase of antigen-experienced (effector + central memory + effector memory) CD4 T cells in the HIV/TB subgroup compared to the HIV subgroup ( $p < 0.05$ ) and healthy controls ( $p < 0.0001$ ).

After ARV treatment, CCR5 expression of antigen-experienced subsets were significantly decreased in the HIV subgroup (75.3% untreated vs. 67.3% treated,  $p < 0.05$ ), but not in the HIV/TB on ARV subgroup (69.2% vs. 78.6%) (Figure 4.11 and Table 4.4).

**Table 4.4****Coreceptor expression of CD4 T cell subsets in Individuals within the donor subgroups<sup>♦</sup>**

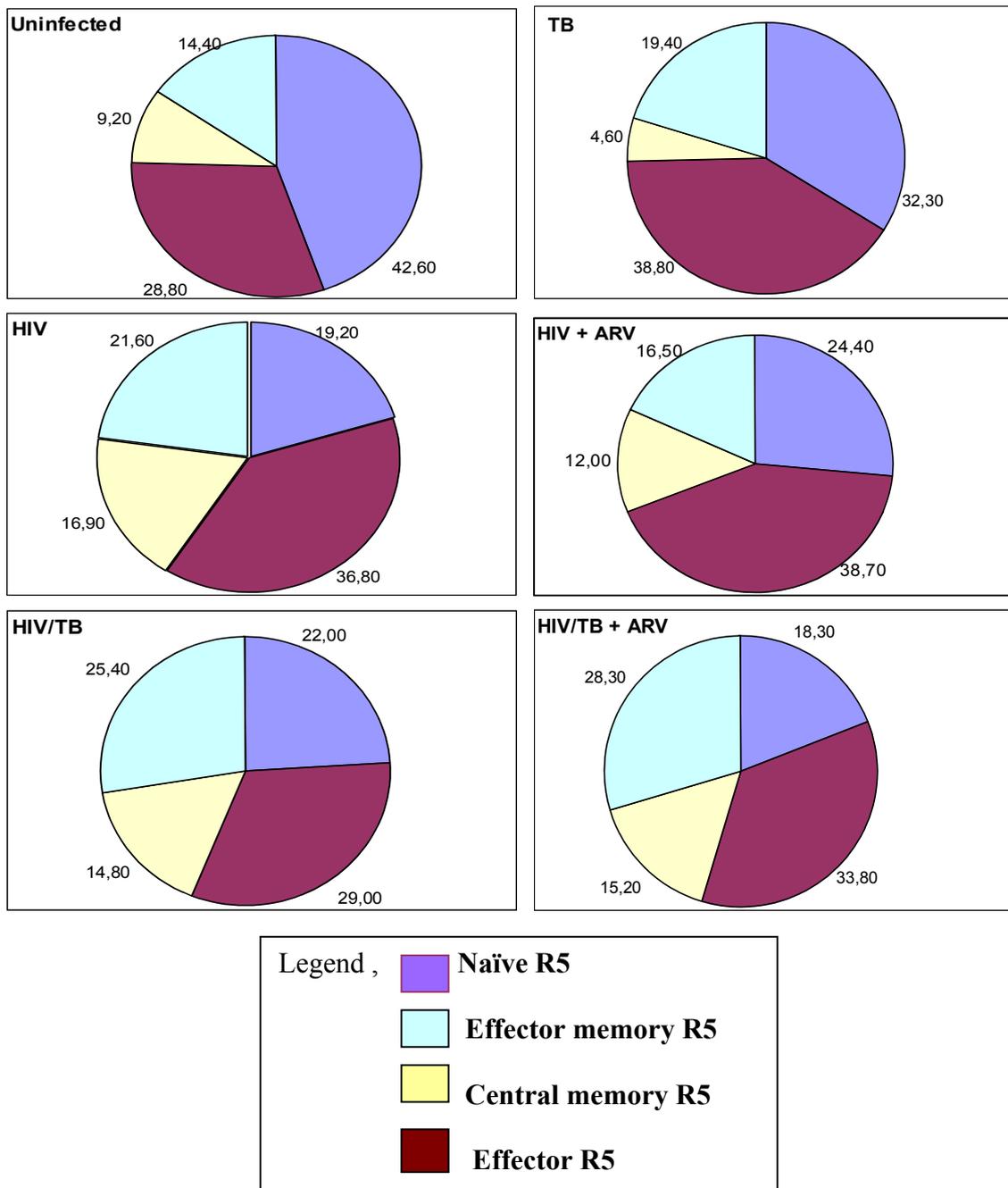
		Healthy	PTB	HIV	HIV/TB	HIV on ARV	HIV/TB on ARV
<b><u>CD4 T cell subset</u></b>	<b><u>Cell marker</u></b>						
Naïve	CCR5+	42.6	32.3	19.2	22.0	26.9	16.5
Effector	CCR5+	28.8	38.8	36.8	29.0	39.1	38.4
Central Memory	CCR5+	9.2	4.6	16.9	14.8	12.1	14.4
Effector Memory	CCR5+	14.4	19.4	21.6	25.4	16.1	25.8
Antigen-experienced- cells	CCR5+	52.4	62.8	75.3	69.2	67.3 <sup>**</sup>	78.6 <sup>***</sup>
Naïve	CXCR4+	47.7	33.6	22.8	24.7	30.3	17.2
Effector	CXCR4+	26.3	36.9	43.7	29.0	45.6	42.8
Central Memory	CXCR4+	5.6	5.5	6.1	7.4	4.2	6.7
Effector Memory	CXCR4+	18.0	66.5	22.8	31.6	15.6	28
Antigen-experienced- cells	CXCR4+	49.9	29.6	72.6	68.0	65.4	77.5

<sup>♦</sup> Data are expressed as Median of positive percentage. Subgroups: Healthy, normal donors; HIV, HIV infected patients; PTB, active tuberculosis patients; HIV/TB, HIV coinfecting patients; on ARV; on antiretroviral therapy.

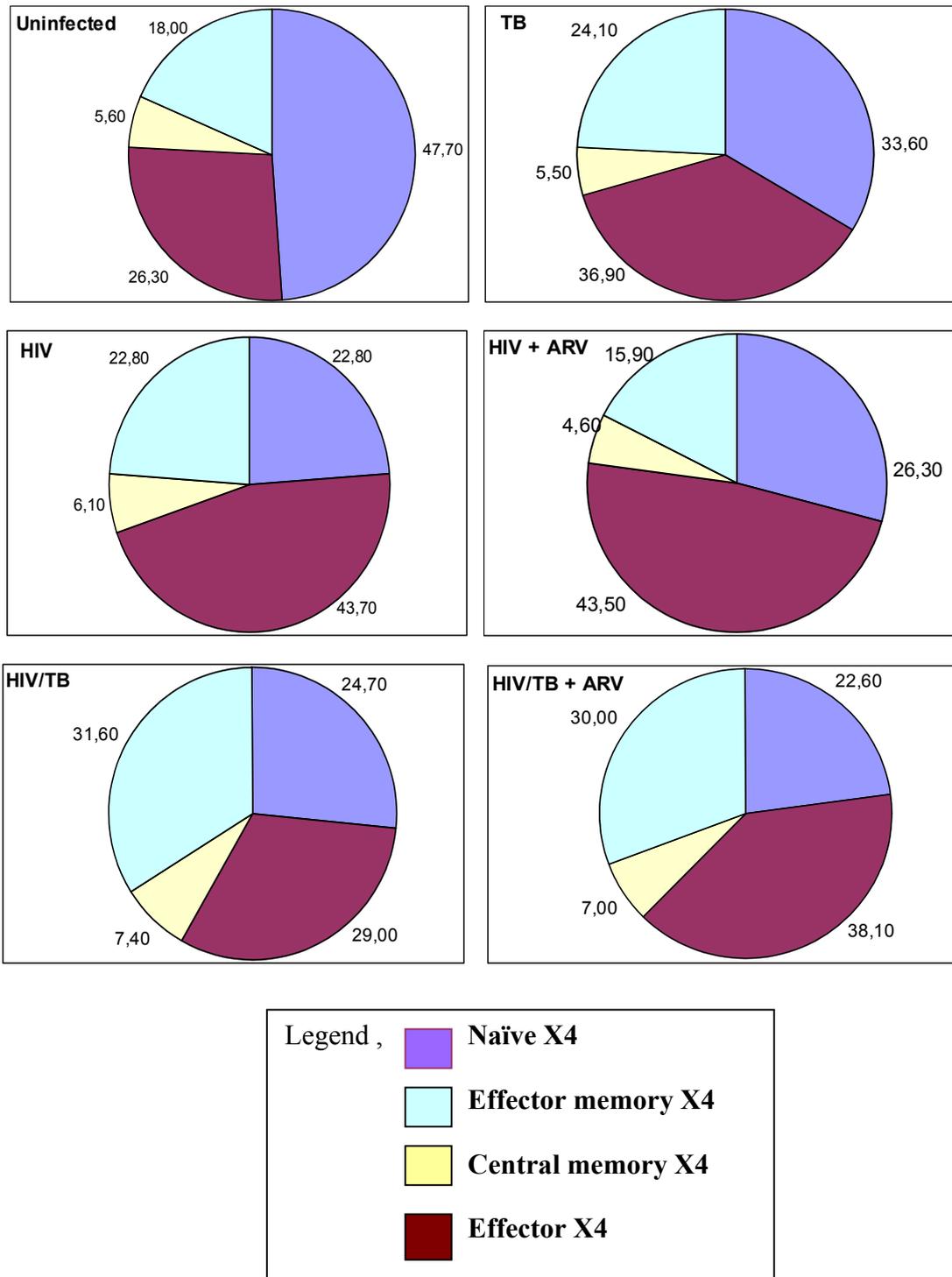
Note. Percentage of CD4 T cell subset expressing CCR5+ or CXCR4+ was calculated then Median of % determined.

<sup>\*\*</sup> p<0.05 as compared to HIV subgroup.

<sup>\*\*\*</sup> p>0.05. Not significant as compared to HIV/TB subgroup.



**Figure 4.11.** Representation of T cells subset distribution of CD4 T expressing CCR5+ within subgroups. Data expressed in % positive of CD4 T subset expressing CCR5+. Naïve, Effector, Central memory and Effector memory subsets are indicated.



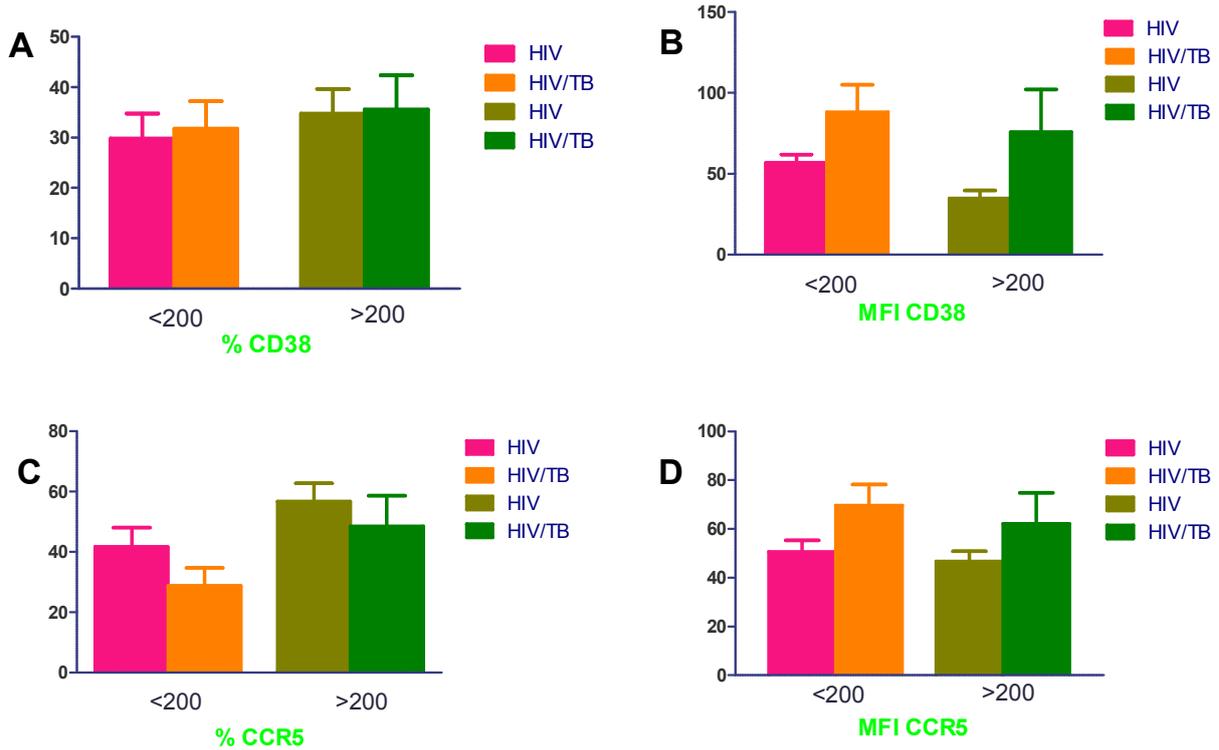
**Figure 4.12.** Representation of T cells subset distribution of CD4 T expressing CXCR4+ within the subgroups. Data expressed in % positive of different subset CD4 T cells expressing CXCR4+. Naïve, Effector, Central memory and Effector memory subsets are indicated.

#### **4.5. Comparison of Coreceptor expression, activation marker expression and CD4 T cell distribution in the HIV infected and HIV/TB co-infected subgroups according to CD4 count level**

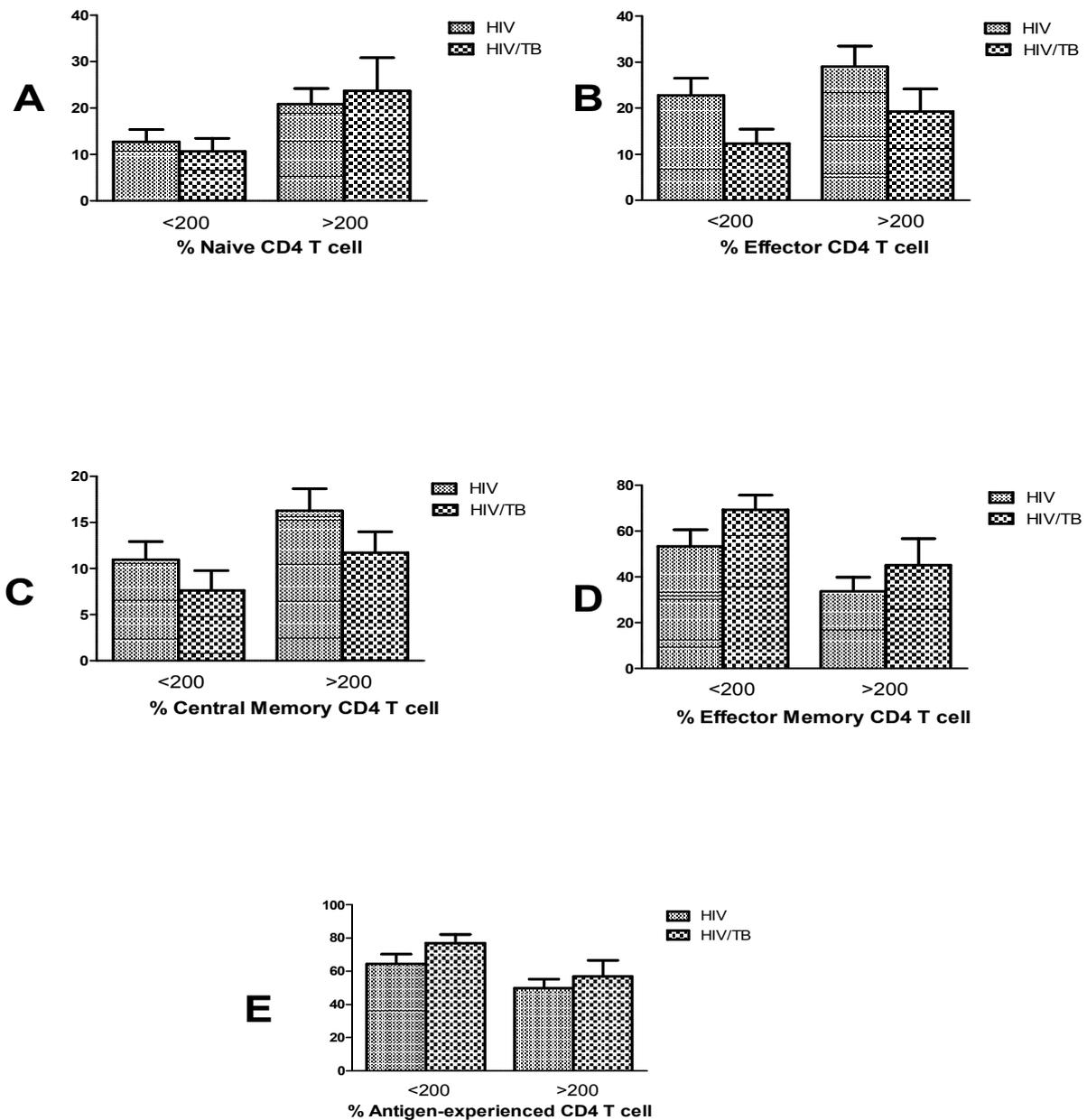
CD4 count is one of the classic markers used to define HIV disease progression and to monitor ARV treatment. Cut-off for High vs. Low CD4 count comparison in this study was 200 cells/ $\mu$ l, as this is the CD4 count used as a guide for ARV initiation in South Africa.

Based on the CD4 count cut-off of 200 cells/ $\mu$ l, the HIV infected and HIV/TB coinfecting subgroups were compared to assess differences in coreceptor expression, activation markers and CD4 T cell distribution with regard to the degree of immunosuppression. As information on clinical stage of disease within the subgroups was incomplete, this comparison was used to help to correlate CD4 level with the severity of immunosuppression with different markers expressed on CD4 T cells. The <200 cells/ $\mu$ l CD4 count HIV subgroup including 20 patients while the >200 cells/ $\mu$ l CD4 count HIV subgroup was composed by 12 patients. The <200 cells/ $\mu$ l CD4 count HIV/TB subgroup including 15 patients whereas the >200 cells/ $\mu$ l CD4 count HIV subgroup was with 7 patients.

The data illustrated in Figures 4.13 and 4.14 indicate that overall numbers of activated CD4+ T cells (as measured by CD38 %) was reduced in the <200 cells/ $\mu$ l CD4 count HIV group, but the MFI was increased. This seems to point towards a loss of activated T cells, but generally more activation marker expression per cell. HIV/TB disease also tended to increase both % CD38 positive cells and MFI of CD38 expression. These data were not statistically significant, due to the small sample size when stratifying groups based on CD4 count. CCR5 expression seems to follow a similar pattern to CD38.



**Figure 4.13:** Comparison of expression in patients with CD4 >200 cells/ $\mu$ l and CD4 <200 cells/ $\mu$ l  
 Panel A and B. Comparison of activation marker expression in patients with CD4 >200 cells/ $\mu$ l and CD4 <200 cells/ $\mu$ l. Data expressed as means with SEM. Higher expression of CD38 in the HIV/TB subgroup compared to the HIV subgroup was observed in both CD4 subsets.  
 Panel C. Comparison of CCR5 expression in patients with CD4 <200 cells/ $\mu$ l and CD4 >200 cells/ $\mu$ l. Data expressed in means with SEM. Increased CD4 T cell CCR5 expression in the HIV subgroup compared to the HIV/TB subgroup.  
 Panel D. Comparison of CCR5 expression in patients with CD4 <200 cells/ $\mu$ l and CD4 >200 cells/ $\mu$ l. Data expressed in means with SEM. Increased CCR5 expression in the HIV/TB subgroup compared to the HIV subgroup.



**Figure 4.14:** Comparison of T cell subset distribution in patients with CD4 count <200 cells/ $\mu$ l. Data expressed as means with SEM. Increased effector and central memory CD4 T cells indicated in the HIV subgroup compared to the HIV/TB subgroup in graphs A, B and C.

Increased effector memory and antigen-experienced subsets in the HIV/TB subgroup compared to the HIV subgroup illustrated in graphs D and E.

Comparison of T cell subset distribution in patients with CD4 count >200 cells/ $\mu$ l. Percentage of effector CD4 T cells and central memory CD4 T cells in graphs B and C are higher in the HIV subgroup compared to the HIV/TB subgroup, whereas proportions of effector memory subset, antigen-experienced cells and naïve CD4 T cells in graphs A, D and E are higher in the HIV/TB subgroup.

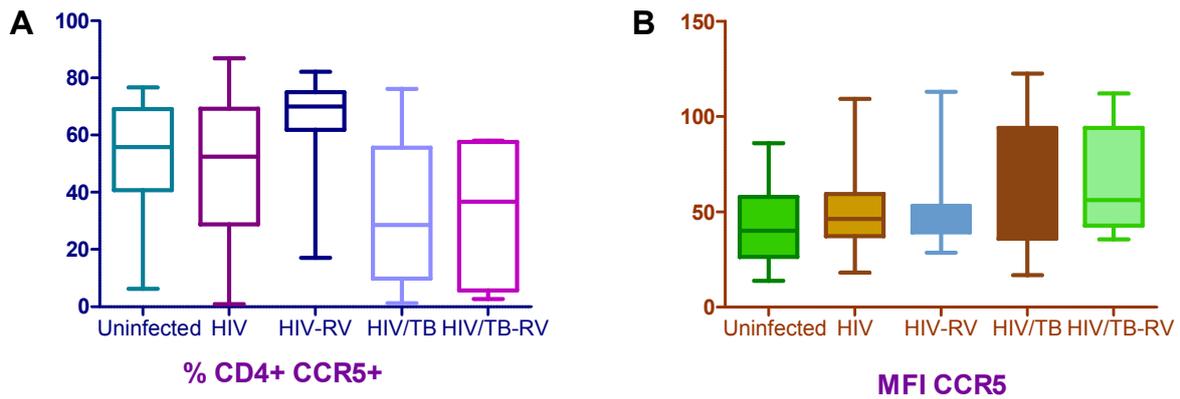
#### **4.6. Effect of ARV on Coreceptor Expression, Activation Marker Expression and CD4 T Cell Subset Distribution in Individual Patient Subgroups**

The impact of ARV is largely evaluated by observing changes in the viral load and peripheral CD4 count. Little, however, is known of the impact of ARV on coreceptor expression, CD4 T cell subset distribution (or redistribution) and CD4 T cell immune activation status, particularly in South African individuals. In the current study, an assessment of CCR5, CXCR4, CD38 expression and CD4 T cell subset distribution was performed on naive patients and patients with treatment to evaluate the effect ARV in HIV infected and HIV/TB coinfecting patients.

##### **4.6.1. Effect of ARV on Coreceptor Expression, Activation Marker and T Cell Subset Distribution**

To assess the effect of ARV on coreceptor expression, the levels of CCR5, CXCR4, CD38 expression and CD4 T cell subset distribution were compared according to the duration of ARV treatment within individual subgroups on ARV and without ARV. Data is shown in Figures 4.15 to 4.22.

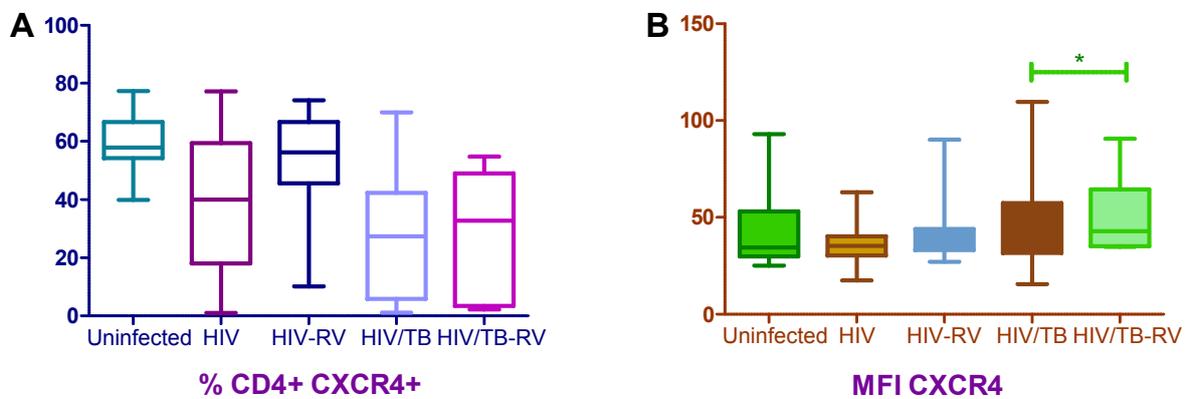
Overall data on the effect of ARV indicates a decrease of CCR5 expression and CD38 expression (data expressed as means of MFI) after 6 months of ARV within all subgroups (not statistically significant); a significant ( $p < 0.05$ ) decrease in CXCR4 expression (data expressed as means of MFI) in the HIV/TB on ARV subgroup; % naïve CD4 T cells were significantly increased ( $p < 0.05$ ) in the HIV subgroup, but not the HIV/PTB on ARV subgroup after  $\geq 6$  months of treatment. Effector T cells showed a significant increase in the HIV on ARV subgroup ( $p < 0.005$ ), but not in the HIV/PTB on ARV subgroup ( $p > 0.05$ ) after  $\geq 6$  months of treatment; central memory T cells significantly increased ( $p < 0.05$ ) in the HIV on ARV subgroup, but not in the HIV/TB subgroup; Effector memory and antigen-experienced CD4 T cells were significantly decreased ( $p < 0.005$  and  $p < 0.0001$  respectively) in the HIV on ARV subgroup, but not in the HIV/TB subgroup.



**Figure 4.15:** Effect of ARV on CCR5 expression in patients after  $\geq 6$  months of treatment.

**A:** Data (expressed as % positive) show CCR5+ CD4 T cells increased (not significantly) after ARV in the HIV and HIV/TB subgroups compared to untreated patients.

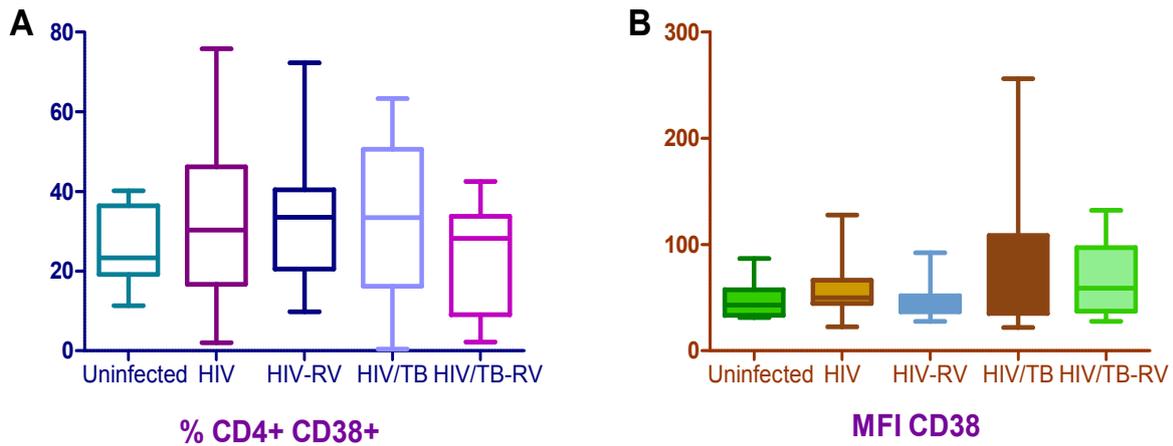
**B:** Data (expressed as means of MFI) show CCR5 expression decreased in the treated HIV/TB and HIV subgroups compared to the untreated subgroups ( $p > 0.05$ ).



**Figure 4.16:** Effect of ARV on CXCR4 expression in patients after  $\geq 6$  months of treatment.

**A:** Mean data indicates increased CXCR4 expression after treatment in the HIV subgroup ( $p > 0.05$ ), whereas in the HIV/TB subgroup there is a significant decrease of CXCR4 expression ( $p < 0.05$ ).

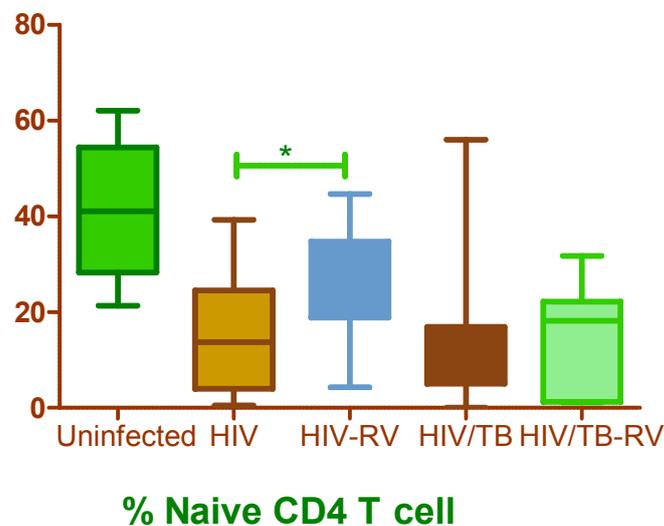
**B:** Mean data indicates weakly increased CXCR4 expression after treatment in the HIV subgroup ( $p > 0.05$ ), whereas in the HIV/TB subgroup there is a significant decrease of CXCR4 expression ( $p < 0.05$ ).



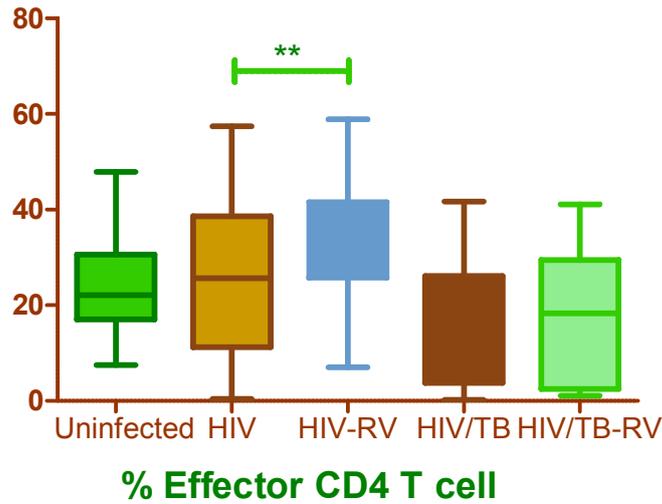
**Figure 4.17:** Effect of ARV on activation marker in patients after  $\geq 6$  months of treatment.

A: Mean data shows a minor increase of CD38 expression after ARV treatment in the HIV, but not HIV/TB subgroups.

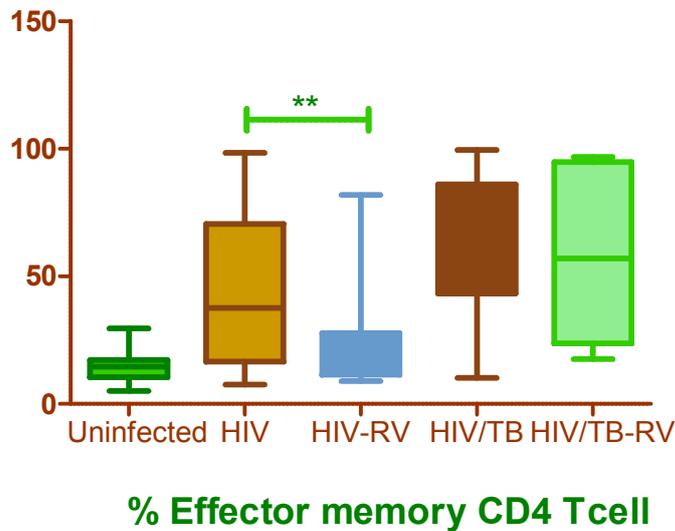
B: Mean data indicates a decrease of CD38 expression in the HIV on ARV and HIV/PTB on ARV subgroups compared to untreated subgroups ( $p > 0.05$ ).



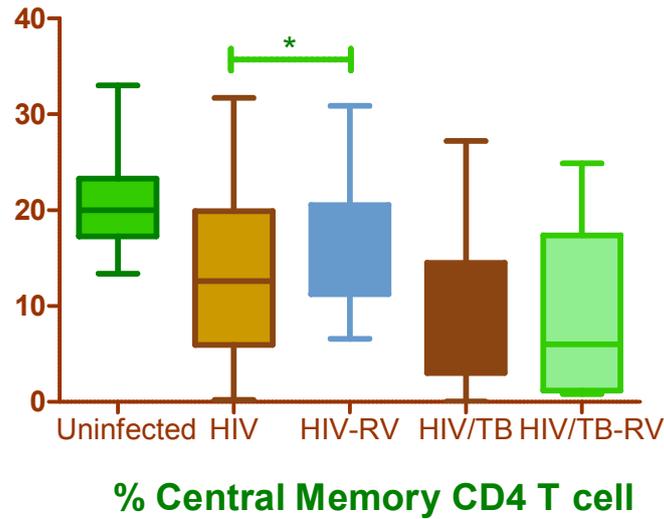
**Figure 4.18:** Effect of ARV on Naive CD4 T cell subsets. Data expressed as % positive indicates a significant increase of Naive T cells in the HIV on ARV subgroup ( $p < 0.05$ ), but not the HIV/PTB on ARV subgroup ( $p > 0.05$ ) after  $\geq 6$  months of treatment.



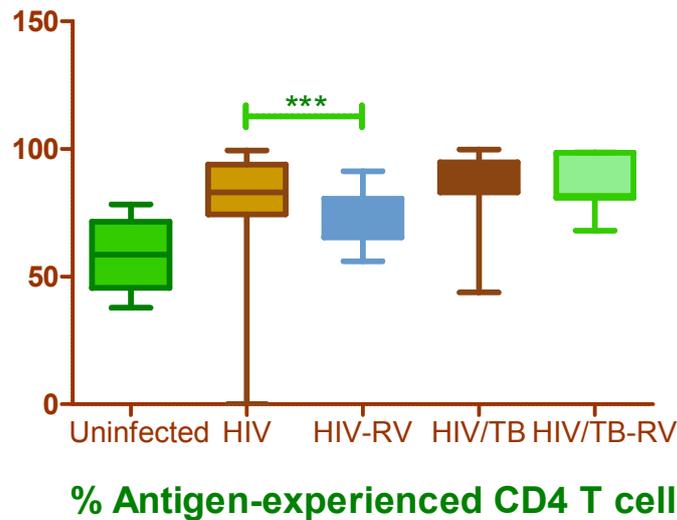
**Figure 4.19:** Effect of ARV on effector CD4 T cell subsets. Data expressed as % positive indicates a significant increase of effector T cell numbers in the HIV on ARV subgroup ( $p < 0.005$ ), but not in the HIV/PTB on ARV subgroup ( $p > 0.05$ ) after  $\geq 6$  months of treatment.



**Figure 4.20:** Effect of ARV on effector memory CD4 T cell subsets. Data are expressed as percentage positive and show a decrease in this subset after  $\geq 6$  months of ARV treatment in the HIV subgroup ( $p < 0.005$ ) and the HIV/PTB subgroup ( $p > 0.05$ ).



**Figure 4.21:** Effect of ARV on central memory CD4 T cell subsets. Data expressed as percentage positive shows the number of central memory T cells increased significantly in the HIV ( $p < 0.05$ ) and HIV/PTB subgroups ( $p > 0.05$ ) after  $\geq 6$  months of treatment.



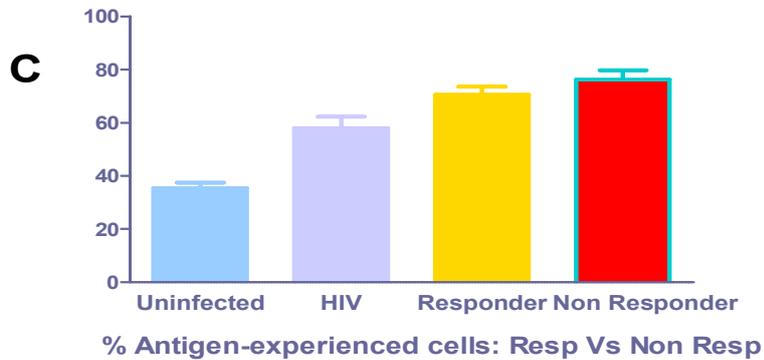
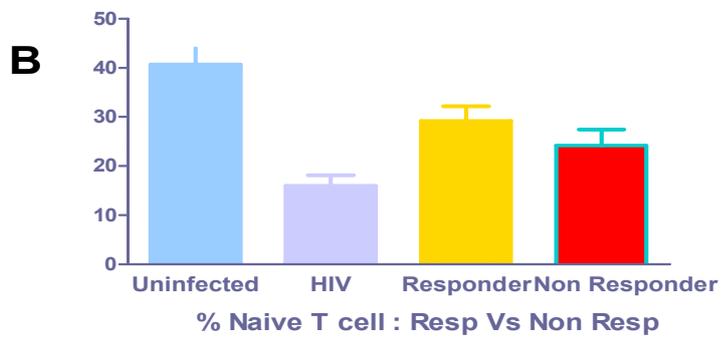
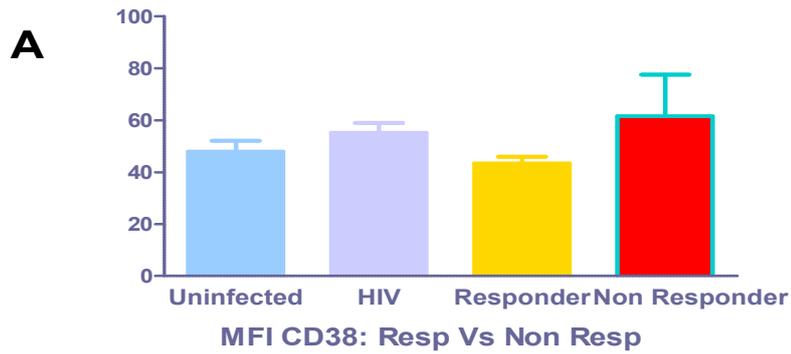
**Figure 4.22:** Effect of ARV on antigen-experienced CD4 T cell subsets. Data expressed as percentage positive of antigen-experienced cells indicates a decrease of this subset in the HIV on ARV subgroup ( $p < 0.0001$ ), but not the HIV/PTB coinfecting on ARV ( $p > 0.05$ ) after  $\geq 6$  months of treatment.

#### **4.6.2. Comparison of activation marker expression, CD4 T cell subset distribution in patients classified as ARV Responders vs. non Responders**

Immunological and virological responses observed during ARV treatment allowed for the subdivision of HIV patients in two groups: ARV responders and ARV non responders.

Given that CCR5, CXCR4 and CD38 expression, as well as CD4 T cell subset distribution, are affected by ARV treatment, the differences in coreceptor expression, immune activation and CD4 T cell subset distribution between ARV responder and ARV non responder subgroups was assessed.

Based on the CD4 count and viral load results, HIV on ARV subgroup (in the absence of TB) after  $\geq 6$  months was subdivided in 2 different subgroups as follows: ARV Responder - patients on ARV with CD4 counts  $\geq 200$  cells/ $\mu$ l and viral load lower than limit of detection; ARV non-Responder - patients on ARV with CD4 counts  $< 200$  cells/ $\mu$ l and detectable viral load (i.e. higher than limit of detection of 357 copies/ml). Data is expressed as median MFI, percentage of activation marker (CD38) and percentage positive of CD4 T cell subsets, and are illustrated in Figure 4.23. Comparison of CD38 expression showed ARV non responders (means MFI = 56.7; means % 42.3) had higher expression compared to ARV responders (means MFI = 43.4; means % 28.1).



**Figure 4.23:**A: Comparison of activation marker expression in different groups. Data expressed as mean with SEM of MFI revealed an elevated CD38 expression in the ARV non responder subgroup

B: Comparison of naïve CD4 T cell subset distribution. Increased naïve T cell subset numbers in the ARV Responder subgroup was observed.

C: Comparison of antigen-experienced CD4 T cell distribution. Increased antigen-experienced CD4 T cell subset numbers in the ARV non Responder subgroup was observed.

The percentage of Naïve CD4 T cells was higher in the ARV responder (29.2%) compared to the ARV non responder (24.2%) subgroups; Antigen-experienced CD4 T cells were higher in ARV non-responder (76.2 %) compared to the ARV responder (70.6 %) subgroups.

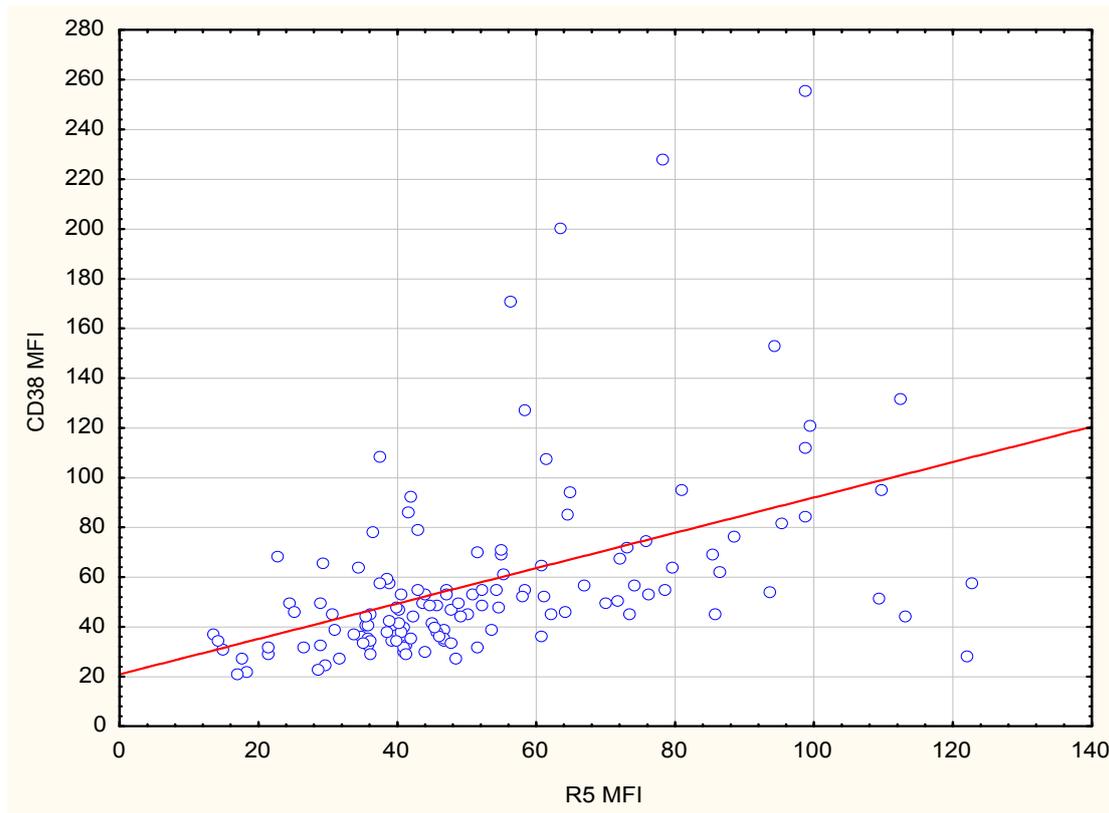
Comparison of coreceptor expression in ARV responders vs. ARV non responders did not reveal statistically significant differences (data not shown), due to small sample size when stratifying groups. ARV responder subgroup including 9 HIV infected patients and ARV non responder subgroup with 5 HIV infected patients after  $\geq 6$  months of therapy.

#### **4.7. Relationship between Coreceptor Expression, Immune Activation and T cell Subset Distribution**

To assess whether the expression of coreceptors was associated with immune activation status, different coefficients of correlation were calculated between CCR5, CXCR4 and CD38 expression, and CD4 T cell subset distribution within the patient subgroups.

##### **4.7.1. Correlation between Coreceptor Expression and Immune Activation**

Significant positive relationships we observed between CCR5 and CXCR4 expression ( $r=0.66$ ,  $p<0.001$ ), CCR5 and CD38 expression ( $r=0.59$ ,  $p<0.001$ ), and CXCR4 and CD38 expression ( $r=0.55$ ,  $p<0.001$ ) on CD4 T cells.



**Figure 4.24:** A significant positive relationship was found between CD38 and CCR5 expression with Spearman coefficient of correlation ( $r$ ) =0.059 and  $p$  value <0.001.

#### 4.7.2. Correlation between Coreceptor Expression, Immune Activation, Viral Load and CD4 Count

The relationship between CD38 expression and viral load was positive ( $r=0.31$ ,  $p<0.001$ ). The percentage of activated CCR5+ expressing CD4 T cells was negatively associated with CD4 count ( $r=-0.43$ ,  $p<0.001$ ) and positively related with viral load ( $r=0.31$ ,  $p<0.001$ ). CCR5 expression was positively correlated to viral load ( $r=0.27$ ,  $p<0.001$ ) although the correlations were found statistically significant, they were weak. We did not find a significant correlation between coreceptor expressions and CD4 count.

#### 4.7.3. Correlation of Coreceptor Expression, Immune Activation and T Cell Subset Distribution

The percentage of naïve CD4 T cells was positively associated with CD4 count ( $r=0.60$ ,  $p<0.001$ ) and negatively correlated with viral load ( $r=-0.42$ ,  $p<0.001$ ). The percentage of naïve CD4 T cells was also found negatively associated with antigen-experienced CD4 T cells ( $r=-0.80$ ,  $p<0.001$ )

The percentage of antigen-experienced CD4 T cells was positively related to viral load ( $r=0.40$ ,  $p<0.001$ ) and negatively associated with CD4 count ( $r=-0.47$ ,  $p<0.001$ ).

The percentage of activated CCR5+ expressing CD4 T cells was positively related to percentage of antigen-experienced CD4 T cells ( $r=0.40$ ,  $p<0.001$ ) and negatively associated with percentage of naïve CD4 T cells ( $r=-0.30$ ,  $p<0.001$ ).

## CHAPTER 5: DISCUSSION

CCR5 and CXCR4 are the major HIV coreceptors, critical for viral entry into host cells (Feng *et al.*, 1996; Wu, 2009). They are differentially expressed by CD4 T cell subsets, and their relative expression has been associated with disease pathogenesis (Bleul *et al.*, 1997; Lee *et al.*, 2008). Immune activation, particularly T cell activation, is critical for productive HIV infection (Siliciano and Siliciano, 2000). Immune activation is now also recognised as a major driving force in AIDS pathogenesis (Hazenberg *et al.*, 2003; Biancotto *et al.*, 2008). Immune activation also directly impacts on T cell coreceptor expression (Shang H *et al.*, 2005). TB and HIV coincide in Sub-Saharan Africa, and as both diseases are marked by immune activation and changes in T cell homeostasis, it is important to assess the impact of one disease on the other. ARV therapy is a potent suppressor of HIV-1 replication, and also of generalised immune activation, related to removal of antigenemia. An assessment of the role of ARV in regulation of coreceptor expression, immune activation (CD38) and CD4 T cell subset distribution, as well as the impact of HIV/TB disease, has been investigated in this study.

### 5.1. HIV Infected and HIV/TB Coinfected Patients without ARV

Before initiation of ARV treatment, HIV/TB coinfecting patients displayed increased levels of CCR5 expression ( $p < 0.05$ ), CD38 expression ( $p < 0.05$ ), percentage of effector memory CD4 T cells ( $p < 0.05$ ) and percentage of total memory CD4 T cells when compared to HIV infected patients and healthy controls. There was also a significant decrease of naïve CD4 T cells in both the HIV and HIV/TB subgroup when compared to healthy controls ( $p < 0.0001$ ).

The level of coreceptor expression per cell (as determined by MFI) and the percentage of cells expressing a particular coreceptor were not necessarily comparable. For example, the increased levels of CCR5 expression per cell in all groups (TB, HIV and TB/HIV) were accompanied by a decreased percentage CCR5 positive cell. This trend was not observed in the expression of CD38 (Figure 4.3 and Table 4.3), where increased marker expression was accompanied by increased percentage positive cells. These findings would appear to indicate the CCR5-expressing cells are preferentially lost from the peripheral blood at the same time that coreceptor expression is increased, probably related to increased activation.

To our knowledge, this is the first South African study to show an upregulated CCR5 expression and immune activation status of CD4 T cells and an elevated percentage of

antigen-experienced CD4 T cells in HIV/TB coinfecting patients.

These results are similar to those previously reported by Juffermans *et al.* (2001) and Wolday *et al.* (2005) when examining expression of CCR5 and CXCR4 on CD4 T cells during active TB in HIV/TB coinfection. Our findings, however, contrast with those of Shalekoff *et al.* (2001) and Hanna *et al.* (2005), who could not demonstrate elevated CCR5 expression on CD4 T cells in HIV/TB infection. Distinct methodologies and differences in study populations may explain the contrast in findings. The two studies mentioned above have been performed using whole blood, which is different from this study performed on PBMCs isolated from fresh blood.

Infection with *Mycobacterium tuberculosis* in immunocompetent individuals does not usually lead to active disease. The immune response mounted to TB infection is usually efficient in containing bacteria. Therefore, in most cases, TB infection leads to an asymptomatic infection known as a clinically latent infection. During such a latent infection, *M. tuberculosis* is in a dormant non-replicating state, located primarily within macrophages contained within a granuloma in infected individual organs. However, in certain cases, due to the lack of an appropriate immune response, some patients may acquire active TB. In case of immunodeficiency (such as HIV infection) reactivation of latent infection may occur (Murphy *et al.*, 2008; Flynn and Chan, 2001).

MHC class II molecules are able to capture peptides from pathogens that have entered the vesicular system of macrophages. Thus TB infection is characterised by an antigen specific CD4 T cell response. The effector CD4 T cell subset primarily involved in combating TB infection is the T<sub>H</sub>1 subset, which can release cytokines (particularly interferon- $\gamma$ ) that activate macrophages to allow them to destroy pathogens (Murphy *et al.*, 2008). Therefore, during active tuberculosis, infected macrophages induce stimulation of T<sub>H</sub>1 cells to produce pro-inflammatory cytokines, which in turn will activate macrophages, but will also lead to increased CCR5 expression on CD4 T cells (Murphy *et al.*, 2008; Stenger and Modlin, 1999; Boom *et al.*, 2003; Schluger and Rom, 1998; Almeida *et al.*, 2009). Consequently, during HIV/TB disease there are more activated CD4 T cells available because of the amplification of immune activation generated by the two infections (Wolday *et al.*, 2005): one from HIV infection itself (antigenic stimulation) and another one produced through T<sub>H</sub>1 stimulation in active tuberculosis.

Active TB in HIV infection may create a microenvironment enhancing the productive infection of T cells by HIV (Bentwich *et al.*, 1995; Valerie Garrait, 1997; Fraziano *et al.*, 1999; Bentwich *et al.*, 2000; Djoba *et al.*, 2007). This is also in line with Morris *et al.* (2003) who have demonstrated that R5 viral variants were preferentially recovered from patients with active TB. This can be related to the increased CCR5 expression on CD4 T cells and activated CCR5 expressing T cells found during HIV/TB disease (Figure 4.1 and Table 4.3). Furthermore, this observation is confirmed by a positive correlation found between CCR5 expression and viral load in this study ( $r=0.27$ ,  $p<0.001$ ).

There is a strong mutual interaction between HIV and TB infection as previously reported by Toossi (2003), who demonstrated continuous cellular activation and irregularities in cytokine and chemokine circuits that are permissive of viral replication. Interaction and interplay between HIV and TB infection was highlighted several years ago in an epidemiological study by Badri *et al.* (2001) in South Africa when assessing the impact of TB on HIV-1 disease progression in an area with high TB prevalence among HIV infected adults in Cape Town.

Our findings support the concept that increased prolonged immune activation induced by HIV/TB disease may lead to increased HIV replication and enhance HIV disease progression (Corbett *et al.*, 2003; Badri *et al.*, 2001; Wood *et al.*, 2000). Although the occurrence of HIV and TB infection simultaneously in a single host leads to increased immune activation and CCR5 expression, this finding is coupled with the observation that the percentage of CCR5 expressing CD4 T cells is reduced. The activation of this subset of T cells is probably associated with increased apoptosis due to upregulation of Fas and FasL. We did not assess these markers or the levels of apoptosis in our study, but similar findings have been reported by others (Gougeon, 2005; Cannavo *et al.*, 2001; Paiardini *et al.*, 2004).

A limitation of this study is that we had insufficient data on the occurrence and impact of other opportunistic coinfections, such as Hepatitis C virus (Tan *et al.*, 2006) in our study groups. As a result we could not determine their impact on immune activation status and their interaction with HIV infection and how this relates to the impact of TB coinfection. Several groups have demonstrated that helminthic infections increase the susceptibility to active TB in HIV dually infected patients (Borkow and Bentwich, 2004; Beyers *et al.*, 1998; Elias *et al.*, 2007; Boraschi *et al.*, 2008; Alvar *et al.*, 2008). However, TB may be the most common opportunistic infection in the Western Cape area (Yach, 1988; Mehtar, 2008) that drives a CD4 T cell-mediated immune response (Boom *et al.*, 2003).

Positive correlation was found in this study between CCR5 and CD38 expression, and CXCR4 and CD38 expression (Spearman coefficient of correlation:  $r=0.59$ ,  $p<0.001$  and  $r=0.55$ ,  $p<0.001$  respectively) (Figure 4.24). Ostrowski *et al.* (1998) previously reported a similar positive relationship when evaluating the levels of CCR5 and CXCR4 expression in HIV infected patients. This finding is also in accordance with Shang Hong *et al.* (2005), who demonstrated a correlation of coreceptor expression and immune activation of CD4 T cells in HIV/AIDS patients of China, although this study did not find a significant correlation between CXCR4 expression and immune activation markers. These findings appear to indicate that the upregulation of coreceptor expression is directly associated with increased expression of immune activation-associated markers. This seems feasible – as control of receptor expression is regulated by transcription factors, which in turn are primed in a generalized immune activation state (Lee *et al.*, 2000; Moriuchi *et al.*, 1997; Camargo *et al.*, 2009).

The median viral load in the untreated HIV/TB coinfecting subgroup was 3.95 fold higher than in the HIV infected subgroup. Furthermore, we found the plasma viral load positively associated with CD38 expression ( $r=0.31$ ,  $p<0.001$ ) and the percentage of activated CCR5+ expressing CD4 T cells positively related to the viral load ( $r=0.31$ ,  $p<0.001$ ) for the HIV infected and HIV/TB coinfecting subgroups. This is consistent with Cohen Stuart *et al.* (2000) who found in subjects without anti-HIV treatment, that plasma HIV-1 RNA level correlated with expression of CD38 in CD4+ and CD8+ T cells. Increased immune activation provides additional target cells for increased HIV replication, since HIV is known to replicate most efficiently in activated CD4 T cells (Cullen and Greene, 1989; Wolday *et al.*, 2005; Sodora and Silvestri, 2008).

In this study, comparison of coreceptor expression, activation marker and CD4 T cell distribution in the HIV subgroup and the HIV/TB coinfecting subgroup in patients with CD4 counts above or below a cut-off of 200 cells/ $\mu$ l revealed consistent comparable trends. In both patients with CD4 < 200 cells/ $\mu$ l, as well as CD4 >200 cells/ $\mu$ l, CCR5 and CD38 expression were increased in the HIV/TB subgroup when compared to the HIV only subgroup (Figure 4.13). Hence level of immunosuppression as indicated by CD4 count drop is not associated with the increased CCR5 and CD38 expression as demonstrated in this study. The overexpression of HIV coreceptors and the enhanced immune activation marker CD38 observed in the HIV/TB subgroup is rather related to TB as an opportunistic infection and to generalized immune activation.

Although we did not collect data on clinical symptoms of patients and their date of HIV infection as required by the CDC classification system for HIV/AIDS patients (CDC, 1992), the level of severe immunosuppression usually associated with the late stage of disease seems clearly not to be the cause of higher CCR5 and CD38 expression found in the HIV/TB subgroup and the HIV subgroup when compared to healthy controls, but rather the increased immune activation found in HIV disease.

Chronic immune activation has been broadly reported as playing a prominent role in AIDS pathogenesis in recent years. HIV-infected patients display increased expression of a range of markers of activation (Forsman and Weiss, 2008; Boasso and Shearer, 2007). Originally, the common cause of T cell immune activation in HIV infection was focused on antigenic stimulation by the virus, and its induction of an adaptive immune response. The inability to clear the virus in untreated individuals leads to persistent or chronic immune responsiveness. There are, however, other causes or drivers of immune activation to be considered during the course of HIV infection. These include production of HIV proteins, reactivation of other viruses (e.g. cytomegalovirus), bacterial translocation and opportunistic coinfections e.g. active TB (Brenchley *et al.*, 2006; Appay and Sauce, 2008). The findings of this study suggested active TB as a potent cause of increased immune activation status. However, we did not investigate TB infection status of healthy controls or infected people without over TB disease in this study to evaluate if this may also influence HIV disease progression.

In this study, CD4 counts in TB-infected individuals were found lower than the healthy individuals (Table 4.1). This may suggest a loss of CD4 cells in TB infection. Activated T cells are predisposed to apoptosis or programmed cell death. It is plausible that elevated susceptibility of MTB-responsive T cells to undergo apoptosis may contribute to the low T cells responses found in individuals with active MTB infection (Hirsch *et al.*, 1999; Aleman *et al.*, 2002). In patients with advanced pulmonary TB, more CD4+ T cells may be trapped in the infected lungs, reflecting a decreased circulating CD4+ T cells (Tsao *et al.*, 2002; Uppal *et al.*, 2004; Swaminathan *et al.*, 2000; Deveci *et al.*, 2006).

T cell subset distribution in HIV/AIDS infection is disrupted (Galati *et al.*, 2007). In the current study, elevated effector memory and total memory CD4 T cells were observed in the HIV/TB coinfecting patients as compared to the HIV infected patients and healthy controls. A significant decrease of naïve CD4 T cells was also observed in both the HIV and HIV/TB subgroups compared to healthy controls. These findings confirm that HIV leads to disrupted T

cell distribution and homeostasis and that TB is a confounding factor in this disruption. These findings support the concept that there is a high turnover of T cells and a disruption in subset distribution (imbalance between production and death of T cells subset) in HIV infection (McCune *et al.*, 2000; McCune, 2001; Mohri *et al.*, 2001). Human TCM are involved in secondary responses and long term protection, whereas human TEM are memory cells that are characterized by rapid effector function and involved in immediate response (Lanzavecchia and Sallusto, 2005). TEM cells are short-lived and enriched for expression of CCR5 compared to other memory CD4 T cells (Oswald-Richter *et al.*, 2007; Blanpain *et al.*, 2002). The normal balance of T cell distribution in peripheral blood of healthy individuals displays a picture of increased naïve CD4 T cells, elevated TCM CD4 cells and decreased TEM CD4 cells (Murphy *et al.*, 2008; Sallusto *et al.*, 2004). The relative distribution of T cell subsets in the peripheral blood is not indicative of the situation in lymph nodes or other lymphoid organs or tissues.

Previous studies have shown HIV preferentially infects memory rather than naïve CD4 T cells *in vivo* (Douek *et al.*, 2002; El-Far *et al.*, 2008). Chronic immune activation may drive the dynamics of T cell homeostasis in HIV infection (Ribeiro *et al.*, 2002) and cause a disruption in T cell subset distribution (McCune, 2001; Leng *et al.*, 2001). This may lead to the expansion of TEM CD4 T cells at the expense of naïve and memory T cell pools. This imbalance will affect the immune system's capacity to generate T cell mediated responses to antigens, e.g. progressive CD4 TCM cell decline resulting in CD4 TEM insufficiency (Picker, 2006; Okoye A, 2007; Sodora and Silvestri, 2008). The decrease in percentage of naïve CD4 T cells found in this study has been similarly described in previous reports suggesting the concept that naïve T cell depletion is one of the hallmarks of HIV infection (Di Mascio *et al.*, 2006; Hazenberg *et al.*, 2000; Hellerstein *et al.*, 2003).

In this study, we also found increased CD4 T cell memory positively associated with viral load, and negatively correlated with naïve CD4 T cells and CD4 count. This finding is in accordance with the concept of AIDS as a disease characterized by a prematurely ageing immune system (enhanced immune senescence). Through a process of chronic immune activation, HIV infection leads to an acceleration of the adaptive immune system ageing process, resulting in early disintegration of T cells (Sodora and Silvestri, 2008; Appay and Rowland-Jones, 2002).

## **5.2. HIV Infected and HIV/TB Coinfected Patients after more than 6 months of ARV**

ARV therapy is an effective treatment strategy that leads to reduced viral load and increased CD4 count. In this study, the untreated HIV subgroup displayed a median CD4 count of  $173.5 \pm 135.1$  and a median viral load of 18 000 (4.3 log<sub>10</sub> copies/ml). The median CD4 absolute count and viral load in the HIV on ARV subgroup were  $235.5 \pm 162.2$  and LDL (< 357 copies/ml), respectively. This finding is comparable to the recent report made by Hammond and Harry (2008) who suggested that ARV can be used effectively within the continent of Africa.

The results from the current study indicated a decline in immune activation status and expression of HIV coreceptors after  $\geq 6$  months of ARV and normalization of CD4 T cell subset distribution in HIV infection, but not HIV/TB disease.

CXCR4 expression (both percentage positive CD4<sup>+</sup> T cells and MFI) in the HIV on ARV subgroup tends to increase (normalize) compared to untreated HIV infected patients (Figure 4.12 and Figure 4.16). Redistribution of CD4 T cells after ARV treatment may explain this increase in CXCR4 expression (Smith *et al.*, 2002; Pierdominici *et al.*, 2002), as it may be related to the increase of percentage of naïve CD4 T cell, typically found to express more CXCR4<sup>+</sup> (Table 4.4).

CCR5 and the immune activation marker CD38 display the same trend i.e. decreased expression after initiation of ARV, which is in accordance with the positive correlation found between these two parameters in this study. CCR5 expression decline may be considered as the consequence of decreased immune activation status as previously demonstrated by several groups (Giovannetti *et al.*, 2001; Zhang *et al.*, 2006; Smith *et al.*, 2002). Interestingly those authors also found that this phenomenon does not occur with regard to CXCR4 expression. The relative importance of CD4<sup>+</sup> T cell subset redistribution and/or immune activation in explaining the increased CXCR4 expression still needs to be addressed in further longitudinal studies.

The change of CCR5 and CD38 expression in this study is not statistically significant ( $p > 0.05$ ) in treated compared to untreated patients. We speculate that this could be a result of the limited sample size and difference in standard deviation of means in our study group. To better understand and confirm the change in expression of CD4 T cells surface markers after

initiation of ARV, we suggest a larger longitudinal study.

Interestingly, similar trends to those in the current study have recently been reported by Briz *et al.* (2008) when longitudinally examining HIV infected patients on ARV. They found that the long-term suppression of plasma HIV-RNA did not significantly influence CCR5 expression on T lymphocytes. However, they did not find a difference of CCR5 level expression and percentage of naive CD4 T cells in HIV infected patients and healthy controls, which is in contrast with the findings of the present study. Zhang Zi-ning *et al.* (2006) have also found in a longitudinal study, that the decreased activation of CD4+ CCR5+ T cells was not significant after 6 months HAART compared to the level after 3 months of therapy.

Despite the suppression of plasma viral load to undetectable limit after  $\geq 6$  months of ARV, a reduced percentage of activated CD4 T cells expressing CCR5+ (Table 4.3) and decreased CCR5 coreceptor expression (Figure 4.15), ARV surprisingly still did not induce changes equivalent to levels observed in healthy controls. Previous studies have demonstrated the identical phenomenon (Smith *et al.*, 2002; Al-Harhi *et al.*, 2004; Benito *et al.*, 2005; Mohri H, 2001; Cohen Stuart *et al.*, 2000; Almeida *et al.*, 2007; Aiuti and Mezzaroma, 2006; Valdez *et al.*, 2002). This indicates that there is a loss of CCR5 expressing CD4 T cells that is never fully restored and also that immune activation continues despite an undetectable viral load. The gastro-intestinal tract damage model implies continued leakage of bacterial components from the blood and chronic innate immune stimulation. This model suggests that activation would continue even when viral load is under control (Dandekar, 2007; Brenchley and Douek, 2008). Brenchley *et al.* (2006) have demonstrated that microbial translocation plays a central role causing HIV-related immune activation throughout the chronic phase of disease. They showed that increased LPS, used as an indicator of microbial translocation, correlated with elevated immune activation. Interestingly, they also found that HAART did not reduce microbial translocation completely. In this study, we did not look at LPS plasma levels in HIV infection to assess the effect of HAART on this parameter, however future studies should include such a component. TB-IRIS was not investigated in the HIV/TB group on ARVs to see if this may be associated with increased activation. However, the increased activation has been associated with active TB in this study even in the context of HIV/TB disease without ARV treatment.

In line with previous observations, this finding may possibly be due to the persistence of HIV antigens from the reservoir (i.e. virus from the latent pool) (Pierson *et al.*, 2000; Belmonte *et*

*al.*, 2003; Delobel *et al.*, 2006), supplying a consistent antigen stimulation that may explain why T cell activation fails to revert to the levels found in healthy individuals (Cohen Stuart *et al.*, 2000; Benito *et al.*, 2005; Anthony *et al.*, 2003). This may render eradication of HIV replication almost impossible being given that the latently infected cells form a drug-insensitive reservoir that importantly contributes to the life-long persistence of HIV despite effective ARV therapy (Williams and Greene, 2007; Shen and Siliciano, 2008).

Pierson *et al.* (2000) have demonstrated that several lines of evidence suggest that new cells may become infected in patients on HAART whose plasma viral loads are below the limit of detection of current assays. Grossman *et al.* (2006) also suggested that the frequent activation of the spared cells in the lymph nodes during the chronic phase of HIV infection may facilitate continued viral replication. This may raise another question of what is a better or more suitable indicator to be used for monitoring ARV response, given this discordance between viral suppression and immune activation in patients on ARV treatment. Interestingly, previous studies have demonstrated for example that SIV-infected Sooty Mangabeys and African Green Monkeys which do not develop immunodeficiency, display reduced T cell activation despite high level of viremia (Appay and Sauce, 2008; Silvestri *et al.*, 2003; Chakrabarti, 2004). Additional monitoring of ARV responses using immune activation biomarkers added to the classic indicators currently used may help to better assess the outcome of ARV.

In this study, we did not assess the lymphoid tissue for a possible relationship between cells infected from the reservoir, immune activation and surface markers during HAART. Further studies on the role of latent HIV and resting CD4 T cells from lymphoid tissue during HAART are needed, however such studies are difficult due to the invasive nature of lymph node biopsy or aspiration.

In this study, the percentage of naïve CD4 T cells was positively associated with CD4 count and negatively correlated to viral load in all subgroups. Furthermore restoration of naïve T cells in patients on ARV was reflected in the CD4 count. A significant increase of naïve CD4 T cells and decrease of antigen-experienced CD4 T cells in HIV treated patients, suggestive of a normalization of T cell distribution after initiation of ARV, has been demonstrated in this study. Previous studies have reported similar findings (Carcelain *et al.*, 1999; Landay *et al.*, 2002; Di Mascio *et al.*, 2006).

The effect of ARV on immune activation status shown by a decrease of CD38 expression may lead to viral suppression and logically explain the normalization of CD4 T cell distribution, which was disrupted before treatment. Normalization of CD4 T cell distribution after ARV treatment was moreover confirmed by the increased percentage of naïve CD4 T cells found in the HIV ARV responder subgroup when compared to ARV non-responder subgroup and elevated percentage of antigen-experienced cells in HIV ARV non-responder when compared to HIV ARV responder subgroup.

Surprisingly, increased naïve CD4 T cells after ARV in HIV infection was still far from the normal percentage of naïve CD4 T cells found in healthy controls ( $p < 0.005$ ). This is in accordance with previous reports (Al-Harathi *et al.*, 2004; Anthony *et al.*, 2003) which demonstrated that T cell turnover is decreased after 12 months of ARV, but does not normalize despite suppression of viral load. This finding supports the hypothesis that increased turnover in HIV infection is mainly associated with ongoing immune system activation and not with T cell homeostasis (Anthony *et al.*, 2003; Benito *et al.*, 2005; Aiuti *et al.*, 2006; Blankson *et al.*, 2002).

In this study, we assessed the effect of ARV on CD4 T cells from PBMC. This process may limit the interpretation of our findings as previous studies have shown that blood is not representative of lymphoid tissues that are critical highly active immunological sites (Fleury and Pantaleo, 1999; Guadalupe *et al.*, 2003). Peripheral blood may not always give a correct reflection of what is occurring within the body. The number of CD4 T cells circulating in the blood where only 15% may be memory cells, may not reflect what is present in lymph nodes (Levy, 2007). Approximately 98% of total CD4 T cell pool in the body is found in secondary lymphoid tissue. However peripheral blood is the most commonly studied immune compartment because of its accessibility (Schacker, 2008).

The effect of ARV on immune activation-associated surface marker expression and coreceptor expression has been reported by several groups since the introduction of ARV for the treatment of HIV infection (Evans *et al.*, 1998; Lempicki *et al.*, 2000; Hunt *et al.*, 2003; Hazenberg *et al.*, 2000). There has however been minimal reporting in the context of HIV/TB coinfection. To our knowledge, this study is the first to assess the effect of ARV on coreceptor expression, *in vivo* immune activation-associated surface marker expression and CD4 T cell subset distribution in HIV/TB coinfection. The present study has shown that there is an increased immune activation of CD4 T cells, which leads to an over expression of

coreceptors and consistent alteration in turnover of CD4 T cells leading to changes in subset distribution in HIV/TB disease.

In HIV/TB coinfecting patients after  $\geq 6$  months of ARV there is a partially reduced immune activation marker and HIV coreceptor expression. Naïve CD4 T cells were found not to be significantly increased and antigen-experienced CD4 T cells as well, were not decreased compared to untreated HIV/TB coinfecting patients. This feature is comparable to what is found in the HIV ARV non responder subgroup (Figure 4.23) as shown above in this study.

This observation may indicate a clear negative influence of HIV/TB disease on ARV treatment outcome and explain the reason why treatment of HIV/TB coinfecting individuals does not seem to display expected changes as observed in HIV infection alone. This is most likely due to the exacerbated immune activation status found in active TB. This finding must be correlated with clinical outcome to confirm our observation. However, failure of therapy or insufficient viral suppression may also possibly lead to such negative outcome. On the other hand, we did not collect information on patient adherence to ARV and/or TB treatment, as well as clinical outcome in this study, especially possible drug resistance, which does not allow us to discuss the outcome of treatment with ARV in the context of TB in depth. Due to the limited sample collection, we were not able to study the effect of concurrent ARV and TB treatment. However two-thirds of our HIV/TB coinfecting patients (Table 4.2) were on TB treatment. Further longitudinal studies are needed to investigate how this may influence the treatment outcome.

An inherent weakness of this study (and any cross-sectional immune-based study) is the fact that we did not repeat assessment of HIV coreceptor and immune activation marker expression at baseline and different point times during the ARV treatment to see if the trends were consistent. Nevertheless, previous prospective studies on HIV and HIV/TB coinfecting patients as discussed above have been reported with similar observations to our findings.

Increased immune activation is a feature of HIV/TB disease determining to what extent HIV coreceptor over expression, as well as CD4 T cell turnover, has an influence on the negative outcome of ARV treatment (as shown in this study). However, its aetiology is multifactorial (Appay and Sauce, 2008; Sodora and Silvestri, 2008). Antigen stimulation itself is not sufficient as the cause of increased immune activation in HIV/TB disease, because the suppression of plasma viral load to undetectable limit after  $\geq 6$  months of ARV found in the

HIV/TB on ARV subgroup in this study (Table 4.1) was not automatically followed by the expected change in immune activation status, coreceptor expression and CD4 T cell distribution after ARV treatment. However, in the HIV on ARV subgroup, a considerable change could be observed after ARV treatment.

Because of limited participant numbers, we did not investigate the concomitant effect of TB treatment and ARV treatment. Further longitudinal studies with large cohorts of HIV/TB coinfecting patients on both treatments will help to elucidate the cause of negative ARV response.

One could speculate on the role of microbial translocation in HIV infection during ARV treatment, as Brenchley *et al.* (2006) found LPS not reduced after treatment, which is comparable to the finding of this study when assessing the effect of ARV on immune activation marker, coreceptor expression and CD4 T cell subset distribution in HIV/TB disease. Alternatively, persistent TB antigen may also impact on continued immune activation. Perhaps HIV/TB disease leads to an increased LPS plasma level, which would be partially reduced by ARV treatment. This may occur through damage to the lung in addition to the GIT.

## CHAPTER 6: CONCLUSION

This study was performed to assess the role of ARV on regulation of coreceptors expression, immune activation status and CD4 T cell subset distribution in HIV infected and HIV/TB coinfecting adult patients.

The findings of this study revealed:

1. In the absence of ARV therapy, CD4 T cell immune activation is increased in HIV infection and further enhanced in HIV/TB infection and may consequently cause over expression of HIV coreceptors, as well as a high turnover of CD4 T cell subsets distribution;
2. After more than 6 months of ARV treatment, a decrease of immune activation may lead to a decrease of CCR5 expression and normalization of CD4 T cell subset distribution in HIV infected patients, but not in HIV/TB coinfecting patients.

Enhanced increase of immune activation status found in HIV/TB disease may explain the reason why treatment of TB coinfection seems not to display a change as expected and show a clear negative influence of HIV/TB disease on ARV treatment outcome. However, failure of therapy or insufficient viral suppression may possibly lead to such negative outcome.

Despite viral suppression after ARV treatment, the decrease of CD4 T cell immune activation and CCR5 expression, increase of percentage naïve CD4 T cells and decrease of antigen-experienced CD4 T cells did not reach normal levels compared to healthy controls. This finding may indicate an ongoing T cell immune activation, which is partially reduced, and suggest further longitudinal studies to closely monitor immune activation during ARV treatment. It would be beneficial even in the classic routine monitoring of ARV treatment to include the assessment of CD4 T cell immune activation biomarker for a better evaluation of treatment outcome.

This study, which was a cross sectional analysis with limited sample collection, could not totally help our understanding on the interactions of other coinfections during ARV treatment, different factors involved in HIV immune activation during ARV treatment and effect of concomitant treatments (ARV and TB treatment). In addition, reports on clinical symptoms and adherence to ARV and TB treatment are required in future to confirm the findings of this

study. Hence, more studies are needed to extend the list of different factors besides the antigen stimulation that may lead to a persistent immune activation status during ARV treatment.

These results suggest a different or modified approach to ARV treatment in HIV positive patients with TB disease. The current findings may point to the fact that the bottom line in management of HIV infection, as well as HIV/TB disease treatment should evaluate if such a reduction in immune activation is indeed followed by improved outcome. The goal of ARV therapy (or other therapies) should be to reduce as much as possible the increased immune activation status. Treatment that could contain the renewal of HIV replication from latent reservoirs in the lymphoid tissue and also suppress the effects of LPS (or other GIT stimuli) in plasma, consequently reducing immune activation status in HIV infection, as well HIV/TB disease should be highly beneficial.

This study has highlighted an association of TB disease with immune activation in HIV infection and its impact on ARV treatment. In future, successful ARV treatment of HIV infection and HIV/TB disease would suggest treatment, which efficiently reduces immune activation status.

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