T lymphocyte inhibitory/exhaustion marker expression in chronic HIV-1 infection and the impact of TB co-infection

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March 2016

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Abstract

Introduction

Chronic HIV-1 infection is driven by inflammation and immune activation which ultimately induces T lymphocyte exhaustion. Co-infection with TB is problematic as the HIV-impaired immune system is unable to effectively contain the TB, and in turn the TB disease promotes further immune activation and exhaustion through additional antigenic burden.

In the current two component study, a total of 101 HIV-1 infected, 25 HIV-1-TB co-infected and 52 uninfected control individuals were included from the Cape Town peri-urban region. A cross sectional investigation of expression of markers of immune activation (CD38 and HLA-DR), exhaustion/inhibition (PD-1, Tim-3, LAG-3, 2B4) and apoptosis (CD95) was investigated on both $CD4^+$ and $CD8^+$ T cells. Functional proliferative responsiveness of T cells was also assessed.

Materials and Methods

Flow cytometry (both 4–colour and 10-colour) was used to determine expression of phenotypic markers using both fresh whole blood (pilot study) and PBMC (main study). $CD4^+$ and $CD8^+$ T lymphocyte populations of all study groups were stained for all the markers and evaluated for positive expression or co-expression.

A functional proliferation assay (αCD3 and αCD28 stimulation) was conducted using CFSE-staining on the $HIV\text{-}1^+$ samples. Impact of blocking Tim-3 and PD-1 pathways was evaluated.

Results

Chronic HIV-1 infection was accompanied by a significant increase in CD38 ($p<0.0001$), CD95 ($p<0.01$), PD-1 ($p<0.01$) and Tim-3 ($p<0.01$) expression on $CD8^+$ T cells (pilot study). TB co-infection led to significantly elevated expression of CD38, CD95 and Tim-3, but not PD-1 (all $p<0.05$). $CD4^+$ T cells displayed decreased expression of all of these markers in the infected groups, except for Tim-3, which was consistently <5%.

In the main study $CD8^+$ T cell-associated CD38, CD95, and PD-1 displayed a similar trend, with significant higher expression in the infected groups ($p<0.0001$, $p<0.05$, and $p<0.0001$, respectively), In contrast to the pilot study, Tim-3 expression was consistently <10%, with no difference between the groups. The novel marker 2B4 showed high level baseline expression (median 59.2%) which was significantly increased in the HIV and HIV/TB groups (69.6% and 75.1% respectively, $p=0.025$). LAG-3 was poorly expressed. Co-expression of

PD-1 and 2B4 as well as CD95 and CD38 was also significantly increased ($p<0.0001$ for both).

Discussion

Increased immune activation and exhaustion was evident in the infected groups in both studies. PD-1 and 2B4 were both strongly expressed on $CD8^+$ T cells and were significantly up-regulated in infected groups. PD-1 correlated positively with Tim-3 and LAG-3, and 2B4 with LAG-3, (both $p<0.01$) indicating that they are useful as biomarkers of exhaustion.

Although significantly elevated exhaustion markers were observed in the TB co-infected setting in the pilot study, this did not reflect in the main study (except for CD95, 2B4). This suggests that immune dysfunction is mainly driven by HIV-1 infection alone. Short-term anti-TB therapy may also have had a restorative impact on the exhaustive marker expression. Differences in expression patterns using fresh whole blood vs. PBMC (especially Tim-3) warrant further investigation. Blocking Tim-3 and PD-1 expression on $CD4^+$ and $CD8^+$ T cells did not appear to have beneficial effects on T cell proliferation.

500 WORDS

Opsomming

Inleiding

Kroniese MIV-1 infeksie word gedryf deur inflammasie en immuun-aktivering wat lei tot T limfosiet uitputting. Mede-infeksie met TB is veral problematies omdat die immuunstelsel nie die infeksie kan oorkom nie, wat lei tot die verdere aftakeling van die immuun stelsel.

Tydens die tweeledige studie ‘n totaal van 178 individue was ondersoek wat bestaan het uit die volgende: 101 MIV-1 positiewe individue, 25 MIV-1-TB mede-geïnfekteerde individue en 52 kontrole individue. Individue wat deel gevorm het van die studie groep was afkomstig van Kaapstad se buitestedelike areas. Die studie het die uitdrukking van merkers van immuun-aktivering (CD38 en HLA-DR), onderdrukking/uitputting (PD-1, Tim-3, LAG-3, 2B4) en seldood (CD95) ondersoek op CD 4^+ en $CD8^+$ selle. Die proliferatiewe reaksie van T selle was ook geassesseer.

Materiale en Metodes

Vloeisitometrie (4-kleur en 10-kleur) was gebruik om die uitdrukking van fenotipiese merkers te ondersoek in beide vars heelbloed en PBMS. $CD4^+$ en $CD8^+$ T limfosiet populasies was ondersoek deur immuun merkers te kleur en daarna te evalueer vir positiewe uitdrukking of ko-uitdrukking.

‘n Funksionele proliferasie toets (αCD3 en αCD28 stimulasie) was uitgevoer op die HIV-1^+ monsters deur middel van CFSE-kleuring. Die impak van Tim-3 en PD-1 paaie was geëvalueer.

Resultate

Kroniese MIV-1 infeksie het gepaard gegaan met ‘n verhoogde- CD38 ($p<0.0001$), CD95 ($p<0.01$), PD-1 ($p<0.01$) en Tim-3 ($p<0.01$) uitdrukking op $CD8^+$ T selle. TB mede-infeksie het gelei tot ‘n verhoogde uitdrukking van CD38, CD95 en Tim-3, maar nie PD-1 ($p<0.05$) nie. $CD4^+$ T selle in geïnfekteerde groepe het ‘n verlaagde uitdrukking van alle immuun-merkers getoon, behalwe vir Tim-3 (<5%).

Die hoof studie het bevind at $CD8^+$ T sel-geassosieerde CD38, CD95, en PD-1 ‘n soortgelyke patroon vertoon het, met verhoogde uitdrukking in geïnfekteerde groepe ($p<0.0001$, $p<0.05$, en $p<0.0001$, onderskeidelik). In kontras met die loodsstudie was uitdrukking van Tim-3 konstant <10%, met geen verskille tussen studie groepe.

Die nuwe merker 2B4 het ‘n verhoogde basislyn uitdrukking getoon (mediaan 59.2%) wat beduidend verhoog was in die HIV-1^+ en HIV-TB groepe (69.6% en 75.1% onderskeidelik, $p=0.025$). LAG-3 was swak uitgedruk. Ko-uitdrukking van PD-1 en 2B4 sowel as CD95 en CD38 was beduidend verhoog ($p<0.0001$).

Bespreking

Verhoogde immuun-aktivering en uitputting was opmerklik in geïnfekteerde groepe in beide studies. PD-1 en 2B4 was albei sterk uitgedruk op $CD8^+$ T selle en was beduidend opgereguleer in geïnfekteerde groepe. PD-1 is positief gekorreleer met Tim-3 en LAG-3, en 2B4 met LAG-3, (beide $p<0.01$), wat toon dat hul gebruik kan word as biomerkers van uitputting.

Alhoewel uitputting merkers beduidend verhoog was in die TB ko-geinfekteerde individue in die loodsstudie, was dit nie gereflekteer in die hoof studie nie (behalwe vir CD95 en 2B4). Dit kan ‘n indikasie wees dat immuunstelsel aftakeling gedryf word deur MIV-1 infeksie. Korttermyn anti-TB medikasie mag ook ‘n restoratiewe impak tot gevolg gehad het. Verskille in die uitdrukking van merkers tussen vars bloed en PBMS dui daarop dat verdere navorsing benodig word. Onderdrukking van Tim-3 en PD-1 uitdrukking op $CD4^+$ en $CD8^+$ T selle het geen voordelige effek gehad op T sel proliferering nie.

497 Woorde

Acknowledgements

I would like to thank Dr. Glashoff for taking the time to explain the most complex concepts in the simplest ways possible. Your patience and knowledge are to be admired.

I would like to thank Dr. de Beer for accepting me into honours and changing my life. Thank you for your kindness and guidance.

I would like to thank the research nurse; Ronell Taylor for collecting my samples, without you this whole project would not have been possible.

Thank you to my funding from the Poliomyelitis Research Foundation. I appreciate it greatly as this money has gone to making me into a better scientist.

I would like to thank all my friends in the department who were so supportive during this thesis and thank you for your input. A great big thank you to Karlien Barnard for translating my abstract from English to Afrikaans, you are always willing to help a friend.

I would like to thank Storm Eckstein who was always willing to “lend an arm” and still smile.

I would like to thank Tim Reid for training me on the Navios. I looked forward to our random chats. Thank you for the jam, you are a multifaceted man.

I would like to thank Vanda O’ Keefe, my dearest friend for helping the words in my head speak clearly on paper.

I would like to thank Nafiisah Chotun and Shahieda Isaacs for helping me with the fine detail. Thank you for stressing along with me. I owe you each a kidney. No words could describe my gratitude.

I would like to thank Ndapewa Ithete and Graeme Jacobs for agreeing to proof-read my thesis at the last minute. Stressful times. Greatly appreciated.

I would like to thank my wonderful parents, Linda and David Golden, I miss you every day, may you both look down from heaven and smile. “You did good.”

Finally, I would like to thank God for all my blessings and for all the wonderful people in this world who have helped me along my journey. Amen.

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Abbreviations:

3TC	Lamivudine
AICD	Activated induced cell death
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
APC	Allophycocyanin
ART	Antiretroviral therapy
BCG	*M. bovis* Bacille Calmette Guerin
Bcl	B cell lymphoma
BD	Becton Dickinson
BKV	Polyomavirus hominis 1
BLIMP-1	B lymphocyte-induced maturation protein 1
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CFSE	5(6)-carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte-associated protein
CXCR	C-X-C motif chemokine receptor
d4T	Stavudine
DC	Dendritic cell
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Treatment, short course
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
ESA	Eastern and Southern Africa
FACS	Fluorescent activated cell sorter
FasL	Fas ligand
FH	Ficoll Histopaque
FITC	Fluorescein isothiocyante
FMO	Fluorescence minus one

G	gravity
GALT	gut associated lymphoid tissue
GIT	Gastrointestinal tract
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV	Human herpes virus
HIC	HIV controllers
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
HTLV	Human T-cell lymphotropic virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INH	Isoniazid
IV	Intravenous
IQR	Interquartile range
JCV	John Cunningham virus
KZN	Kwazulu-Natal
LAG	Lymphocyte-activation gene
LAM	Lipoarabinomannan
LAV	Lymphadenophy-associated virus
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MALT	Mucosal associated lymphoid tissue
MDR TB	Multidrug-resistant tuberculosis
MHC	Major histocompatibility complex
MO	Monocyte
MØ	Macrophage
MSM	Men who have sex with men
MTCT	Mother to child transmission
M.tb	*Mycobacterium tuberculosis*
NFAT	Nuclear factor of activated T cells
NGF	Nerve growth factor

NK	Natural killer cell
NLR	Nod like receptor
NNRTI	Non-nucleoside reverse-transcriptase inhibitor
NOD	Nucleotide-binding oligomerization domain receptor
NRTI	Nucleoside reverse-transcriptase inhibitors
NVP	Nevirapine
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI	protease inhibitor
PrEP	Pre-exposure prophylaxis
PS	Phosphatidyl serine
PZA	Pyrazinamide
RIF	Rifampicin
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Streptomycin
SD	Standard deviation
SIV	Simian immunodeficiency virus
SLAM	Signalling lymphocyte activation molecule
SSA	Sub Saharan Africa
SSC	Side scatter
STI	Sexually transmitted infections
TB	Tuberculosis
TDF	Tenofovir
TCR	T cell receptor
Th	T helper
Tim-3	T cell immunoglobulin mucin 3
TLR	Toll-like receptor
TNF	Tumor necrosis factor

UNAIDS	Joint United Nations programme on HIV/AIDS
VZV	Varicella Zoster Virus
VL	Viral load
WHO	World Health Organization
WNV	West Nile virus
XDR TB	Extensively drug-resistant tuberculosis
ZDV	Zidovudine

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CHAPTER 1: INTRODUCTION

The human immunodeficiency virus (HIV) / acquired immune deficiency syndrome (AIDS) epidemic has had a global impact on millions of lives worldwide since the early 1980s when the first official reports appeared. Over 30 years later, the disease has spread to all countries in the world and has had devastating implications on human health, social wellbeing and economic resources (Mayosi and Benatar, 2014). The virus targets $CD4^+$ T lymphocytes (or T cells) weakening the human immune system against infection. Currently there is neither a vaccine nor a cure (Sharp and Hahn, 2011; Shapiro 2013; Walker *et al*., 2013; Levy *et al*., 2014; Liu *et al*., 2015). It is estimated that 36.9 million people globally are living with HIV-1 and that 25.3 million people have died due to AIDS related diseases since 2000. This number of AIDS related deaths has decreased by 42% with 1.2 million recorded deaths in 2014 compared to 2 million deaths in 2005 (UNAIDS, 2015). It is encouraging that the number of new infections globally has decreased by 35% since 2000. Furthermore, the number of children newly infected worldwide has also decreased by 58% between 2000 and 2014. These numbers equate to 220 000 children newly infected in 2014, compared to 520 000 children newly infected in 2000 (UNAIDS, 2015).

The burden of HIV-1 is amplified by co-infection with tuberculosis (TB). TB is the number one cause of death in people with HIV-1/AIDS. At present, one in three people worldwide are infected with the bacillus, which remains dormant (latent), and kept under control primarily by the $CD4^+$ T helper (Th) lymphocytes cells (Valadas and Antunes, 2005). Once the immune system is compromised (such as in HIV-1), it often fails to contain TB, and the disease transforms into a state of active disease. The target cell for HIV-1 is also the primary effector cell for control of TB.

Together, HIV-1 and TB drive the immune system to exhaustion and collapse by the on-going increased levels of immune activation and immune cell dysfunction. Simultaneously, these two diseases constitute the main burden of infectious diseases in the developing world. Estimates by the World Health Organization (WHO) indicate that there are more than nine million new cases of TB every year, which leads to over two million deaths per year. Consequently, HIV-1/TB co-infection presents particular diagnostic and therapeutic challenges, which burden healthcare systems in developing countries within Africa and elsewhere, due to the large number of individuals in need of basic treatment.

As T cells are the target cells for the virus, and also the effector cells for control, the main focus of this study will be the analysis of T cell phenotype in three main groups, namely: an HIV-1 positive group; an HIV-1/TB co-infected group, and a healthy HIV-1 uninfected control group. T cell markers of activation (CD38 and HLA-DR), exhaustion (PD-1, Tim-3, 2B4 and

LAG-3) and apoptosis (Fas) were analysed. An initial pilot study focused on Tim-3, PD-1, CD38 and Fas expression and was analysed by 4-colour flow cytometry (Becton Dicksinson FACS Calibur, USA). The subsequent main study expanded the marker repertoire to include 2B4, LAG-3 and HLA-DR, analysis was performed using a 10-colour Beckman Coulter Navios instrument.

The literature includes extensive reports that markers of T cell activation, such as HLA-DR and CD38 on $CD8^+$ T cells, are reliable markers of disease progression in chronic HIV infection. More recently, it has been shown that T cell exhaustion occurs in chronic infection, most clearly demonstrated by Programmed Death-1 (PD-1) expression on $CD8^+$ T cells.

According to the literature, blocking of PD-1 has shown encouraging results in improving T cell effector function in exhausted T cell phenotypes in several disease settings, including chronic HIV. In the current study, (αCD3 and αCD28 induced proliferation assays) T cells were monitored by CFSE staining and binary dilution. The PD-1 and Tim-3 inhibitory pathways were blocked using functional monoclonal antibodies, in an attempt to ascertain whether blocking the expression of these markers did in fact restore proliferation ability in both $CD4^+$ and $CD8^+$ T cells.

The primary aim of this current study was to CD4+ and CD9+ T cell phenotypes with respect to activation and inhibition marker expressed in chronic HIV-1 infection with or without co-infection. In particular, the relative expression of the well-described PD-1 and Tim-3 markers compared to the newer less well described 2B4 and LAG-3 was undertaken. The study evaluated the relationship of these markers to standard clinical markers (CD4 count, viral load), and to T cell markers of activation and cell death (CD38, HLA-DR and Fas). The final component of the study was to ascertain whether function could be restored to “exhausted T cells” by blocking PD-1 and Tim-3. This approach could prove beneficial in new treatment strategies, as excessive immune activation is known to drive T cells to an exhaustive state which accelerates disease progression (Kuchroo *et al.,* 2014).

CHAPTER 2 LITERATURE REVIEW

2.1 HIV epidemic: Eastern and Sub Saharan Africa

Approximately 1% of the global population is living in Sub Saharan Africa (SSA)[1], which is home to about 17-20% of the HIV/AIDS burden (Delva and Karim 2014; Mayosi and Benatar, 2014). When looking at the possibility of an AIDS-free SSA, the transmission rate between sexually active persons is of concern; in 2012 UNAIDS found that a staggering 74% of men and 58% of women had never been tested for HIV (UNIADS, 2015). When comparing HIV-1 incidence rates in Swaziland, individuals aged 18-19 years were most affected. Girls in this age group had an overall prevalence of 14.3% when compared to 0.8% for boys (Delva and Karim, 2014). Currently 13 000 Kenyan children become infected with HIV-1 every year, and a further 100 000 children die before the age of five due to the virus (UNAIDS, 2015). According to the 2015 UNAIDS report for Eastern and Southern Africa (ESA[2]), there was a remarkable increase in the number of HIV-1 positive pregnant women on antiretroviral therapy (ART) with approximately 78% in ESA. This resulted in a 50% decrease in the number of babies born with HIV-1 by the end of 2013. Furthermore, in Ethiopia, mother to child transmission (MTCT) has decreased significantly by 57% between 2009 and 2013 (UNAIDS, 2015). Of those children who were infected at birth, Botswana appeared to have the highest number of children on treatment with 80% ART coverage, followed by South Africa, Namibia, Rwanda and Swaziland with 46% in 2013 collectively. It is estimated that approximately 2 million children in ESA are currently HIV-1 positive (UNAIDS, 2015).

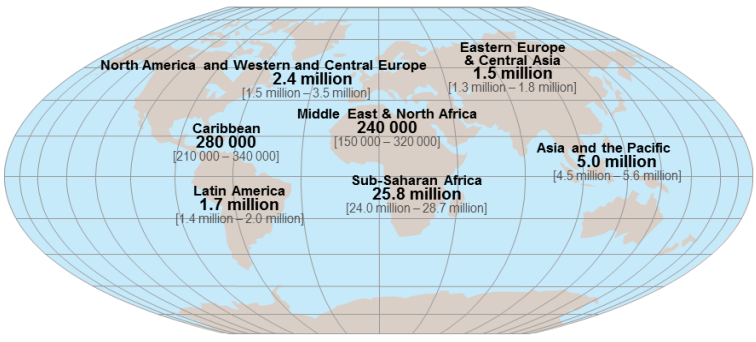


Figure 2. 1 Adults and children estimated to be living with HIV-1 globally in 2014. Adults and children estimated to be living with HIV-1 globally in 2014. SSA has the highest HIV burden in the world, with approximately 25.8 million people currently infected (UNAIDS, 2015).

[1] Swaziland, Lesotho, Botswana, South Africa, Zimbabwe, Namibia, Zambia, Mozambique, Angola and Malawi.
[2] Angola, Botswana, Ethiopia, Kenya, Lesotho, Malawi, Mozambique, Namibia, South Africa, Swaziland, Uganda, Tanzania, Zambia and Zambabwe.

South Africa alone accounts for more than 6.1 million people living with HIV/AIDS and only 2.1 million of these people have access to ART (Delva and Karim, 2014; Mayosi and Benatar, 2014). There has been great improvement in life expectancy and morbidity in people who do have access to ART. UNAIDS (2015) global statistics indicate that 35 million people worldwide are infected with HIV-1. SSA is the worst affected with approximately 25.8 million people infected with the virus (Figure 2.1).

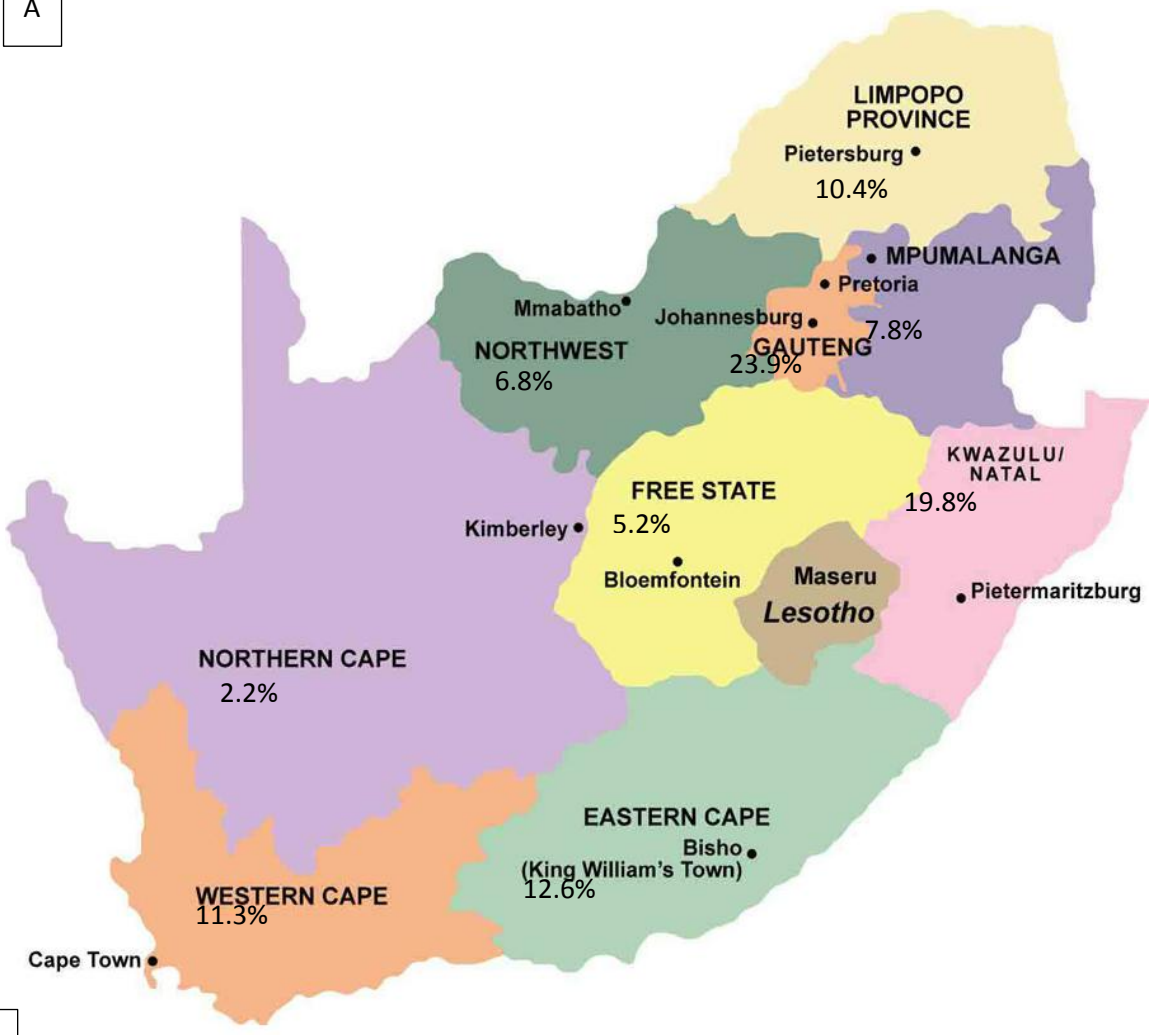
2.1.1 HIV-1 epidemic: South Africa

South Africa has a population of 54.96 million according to the mid-year population estimates for 2015. It has the highest prevalence of HIV-1 compared to the rest of the world, with approximately 6.19 million people living with HIV-1 and a prevalence rate of 16.6% reported in the 15-49 years age group in 2015. The overall HIV-1 prevalence rate is currently 11.2%. Although deaths, as a result of AIDS have decreased from 44.6% in 2002 to 30.5% in 2015 (Statistics South Africa, 2015), the UNAIDS reported the number of AIDS orphans to be 230 0000 with an additional 340 000 children (0-14 aged) living with HIV-1 in 2014 (UNAIDS, 2015).

The South African HIV-1 prevalence is higher than other countries which could be a result of many factors including: poverty, the high prevalence of sexually transmitted infections (STI), sexual abuse of women, limited access to primary health care and education (Mayosi and Benatar, 2014; UNAIDS, 2015; Statistics South Africa, 2015). Women between the ages of 15-19 years have a three times higher risk of HIV-1 infection compared to men (Fatti *et al*., 2014; UNAIDS, 2015). Approximately 1 in 5 young South African female adults will contract HIV-1 during their reproductive years. Young adults aged between 15-24 years had a prevalence rate of 5.59% in 2015, which shows a 1.16% decrease since 2002. However, the 2015 prevalence rate of 16.59% in the age group 15-49 years, has increased by 2.09% since 2002 (Statistics South Africa, 2015).

The province with the highest prevalence of HIV-1 is KwaZulu-Natal (KZN) where 27.6% of individuals aged 15-49 years are currently infected (Figure 2.2). The Western Cape with a prevalence of only 9.2%, which is three times lower than KZN, is the province with the overall lowest prevalence (Delva and Karim, 2014). Globally, South Africa has the highest incidence of childhood infections via MTCT with approximately 280 000 HIV-1 positive pregnant women (Fatti *et al*., 2014).

A



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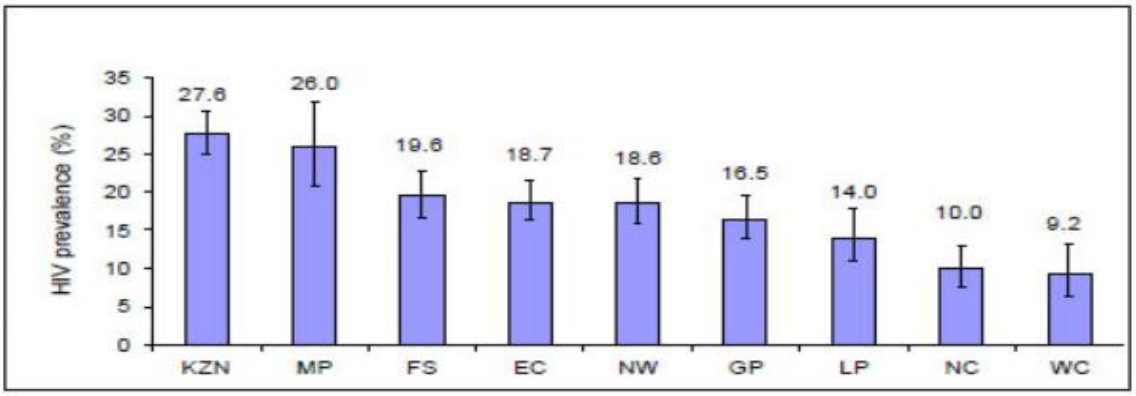

Figure 2. 2 The percentage of people residing in each South African province 2015. (B) South African 2012 HIV prevalence per province for the age group 15-49 years. (A) The percentage of people residing in each South African province 2015. (B) South African 2012 HIV prevalence per province for the age group 15-49 years. Gauteng province is an urban area representing 13 million (23.9%) of the total South African population with an HIV-1 prevalence of 16.5% (HSRC, 2012). KwaZulu Natal province is a rural area representing 10 million (19.8%) of the total South African population (Statistics South Africa, 2015) with an HIV prevalence of 27.6% (HSRC, 2012). The Western Cape has a population of 6 million (Statistics South Africa, 2015) and an HIV prevalence of 9.2% (HSRC, 2012 HIV Report). Overall prevalence for South Africa is 11.2% (Statistics South Africa, 2015).

KZN= Kwazulu Natal; MP= Mpumalanga; FS= Free State; EC= Eastern Cape; NW= North West; GP= Gauteng; LP= Limpopo; NC= Northern Cape; WC= Western Cape

2.1.2 Antiretroviral Therapy globally and in South Africa

Access to ART by those living with HIV-1 has increased from 13.6 million in 2014, to 15 million in 2015 globally (UNAIDS, 2015). While SSA is receiving a high portion of the global ART supplies, many more individuals are still waiting for treatment as shown in Figure 2.3. The number of adults living with HIV-1 who had access to treatment was 41% in 2014 compared to 23% in 2010. Similarly, the number of children who had access to treatment in 2014 was 32% compared to 14% in 2010. Finally in 2014, approximately 73% of all HIV-1 positive pregnant women had access to treatment globally (UNAIDS, 2015).

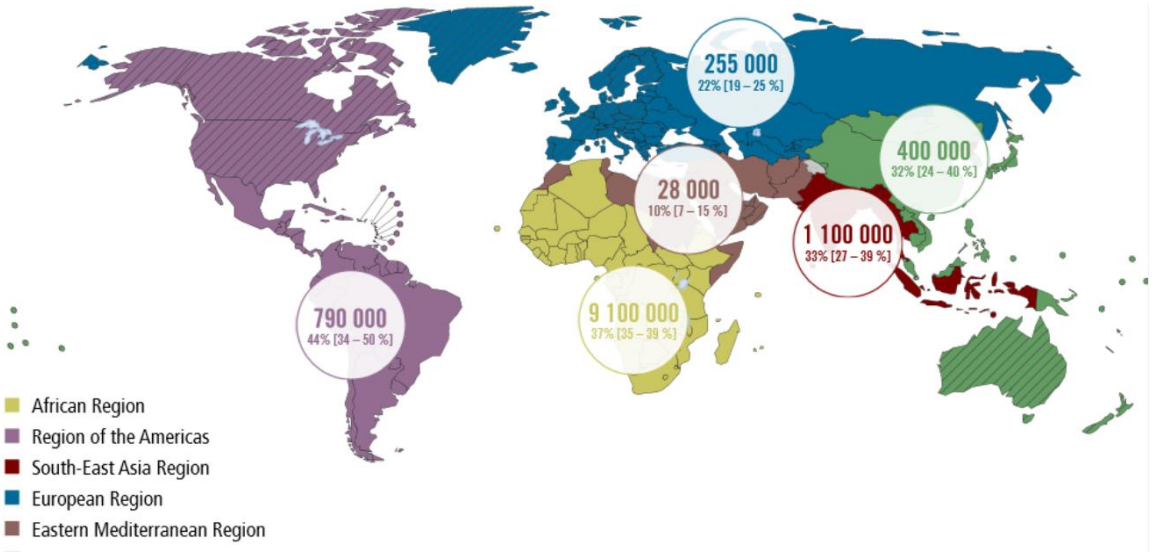

Figure 2.3 The number of people receiving ART globally in 2013. SSA had the highest number of people receiving ART. However, these only accounts for approximately 37% of all individuals infected with HIV/AIDS on the African region (WHO, 2015).

The South African government introduced the ART Rollout Programme in 2003 with the help of international agencies for funding and support (HIV/AIDS Care & Treatment, 2015). Since then, more than two million people have been receiving ART, and as a result there has been a drastic decrease in the morbidity and mortality figures (Delva and Karim, 2014). However, mortality is higher in the first three months of treatment due to: drug toxicity, added disease burden complications, such as pre-existing diseases, malnutrition, low CD4 count, severely low haemoglobin and advanced HIV-1 disease (WHO, 2015). Furthermore, according to a study by Boulle *et al.,* (2014), mortality while on ART is higher in areas with limited resources. This would include areas with limited access to housing and sanitation, limited access to primary health care and areas with severe poverty.

According to the World Health Organization (WHO) 2013 ART drug guidelines for adults, the first line drug treatment should consist of two nucleoside reverse-transcriptase inhibitors (NRTIs) as well as a non-nucleoside reverse-transcriptase inhibitor (NNRTI) for effective viral repression (Table 2.1). Second line drug treatments should include a ritonavir-boosted

protease inhibitor (PI) as well as a combination of the previous three drugs from the first line drug treatment.

Table 2. 1 South African Antiretroviral Therapy Guidelines Standardised National ART Regimens for Adults and Adolescents 2013

First line ART regimens			Second line ART regimens		Third line ART regimens
TDF*	3TC (or FTC)*	EFV*	AZT	3TC (or FTC)	RAL
AZT	3TC	EVP (or NVP)	TDF	3TC (or FTC)	DRV
D4t	3TC	EVP (or NVP)			ETV
TDF	3TC (or FTC)	NVP			

*Preferred first line ART regimen
TDF= Tenofovir (NRTI), AZT= Zidovudine (NRTI), NVP= nevirapine (NNRTI), ETV = Etravirine (NNRTI), DRV= Darunavir (PI), RAL= Raltegravir (InSTI), 3TC= Lamivudine (NRTI), EFV=Efavirenz (NNRTI), D4T= Stavudine (NRTI), FTC=Emtricitabine (NRTI), EVP= Emtricitabine^{+}rilpivirine^{+}tenofovir (fixed dose combination) (WHO, 2015).

In South Africa, when a person is eligible to start ART, the initial regimen from 2003 until 2004 was zidovudine (ZDV) and lamivudine (3TC), together with either nevirapine (NVP) or efavirenz (EFV). Stavudine (d4T) replaced ZDV due to high toxicity in the standard first-line regimen in 2004, as stated in the South African national guidelines (Department of Health SA, 2014). This regimen was further changed in 2013 to the current preferred first-line therapy of tenofovir (TDF), 3TC or emtricitabine (FTC) in combination with EFV. If first line therapy fails due to the inability of the drugs to repress viral replication, second line therapy would be implemented, which consists of a revised combination of drugs depending on the type of resistance to a given first line drug (WHO, 2015). Furthermore, an earlier study by Boulle *et al*., (2010), reported that many people who had been moved to the second-line regimen might not have been completely adherent to their first-line regimen. In order to move onto second-line ART, the patient's plasma HIV-1 RNA load must be greater than 1 000 copies/ml (WHO, 2015). When individuals move onto second-line regimens prematurely, it becomes problematic as more drugs are required to be administered, which is expensive, complex dosing schedules are required, and the issue of further drug resistance becomes apparent.

While ART prolongs life, it does not fully restore the immune system, in turn leading to immune exhaustion as the virus is still able to replicate at a decreased rate. Immune activation can continue, leading to immune exhaustion and resulting in the development of certain chronic conditions, such as cardiovascular disease. Furthermore, long-term therapy

is also associated with drug toxicities, resulting in renal impairment and other complications when compared to the general population (Cockerham *et al*., 2014).

The current study examined the impact of TB co-infection on immune function in chronic HIV-1 infection. For this reason a brief background to the TB epidemic is presented below.

2.2 TB epidemic world statistics

According to the WHO, one third of the world's population is infected with TB and it is the second leading cause of death by an infectious disease worldwide (WHO, 2015). The report emphasizes the profound impact of TB worldwide with 9 million new active TB cases being reported in 2013 and between 1.4 million and 1.5 million TB deaths. Of the 9 million new TB cases, 1.1 million (12%) were HIV-1 positive, while 1.1 million of the reported TB deaths (73%) were among HIV-1 negative patients and 0.4 million (27%) HIV-TB co-infected patients. In 2013, women infected with TB accounted for 3.3 million new and current infections with 510 000 deaths, respectively. Similarly, children in the same year accounted for approximately 550 000 current infections and 80 000 TB deaths worldwide. While TB is still a major global health concern more so in the developing worlds, the total deaths due to TB infection have declined greatly (by 45%) since 1990. Furthermore, the number of newly infected people worldwide has also declined by 1.5% per year. It has also been estimated that the number of lives saved between 2000 and 2013 is in the area of 37 million, which can be attributed to medical advances in timely TB detection and adherence to TB treatment programs (Claassens *et al*., 2012; WHO, 2015). However, despite progress, TB still remains the leading cause of death for those persons co-infected with HIV-1 (Sullivan *et al*., 2015).

It appears that Asia is most highly burdened by the worldwide TB epidemic with 56% of all cases recorded globally in 2013. The SSA/African region accounts for 29%, of all TB cases followed by the Eastern Mediterranean with 8%, Europe with 4% and the American region with 3% (WHO, 2015). The WHO listed the top 10 counties in order of TB burden as: India, China, Nigeria, Pakistan, Indonesia, South Africa, Bangladesh, Philippines, Democratic Republic of the Congo and finally Ethiopia.

2.2.1 TB epidemic in South Africa

According to 2014 global statistics, South Africa has one of the highest TB burdens in the world. This can be causally linked to weakened immunity in those with HIV-1, predisposing individuals to reactivation of latent TB, followed by a more rapid course with more severe disease manifestations. There has been a significant increase in the number of multidrug-resistant-TB (MDR TB) and extensively drug-resistant TB (XDR TB) cases in recent years in the region (Mayosi and Benatar, 2014). In South Africa, TB is responsible for 11% of all

recorded deaths (Osman *et al*., 2015). Osman *et* al. (2015) used the ETR.net TB database in a retrospective study and found that HIV-1 positive individuals between the ages 15-24 years old were almost 5 times more likely to die from TB than those without HIV-1 due to failing immune systems. Cape Town has a TB treatment rate of 83% and the number of TB cases has greatly decreased from 8.7% in 2009 to 6.6% in 2013. In high burden areas of TB and HIV-1 infection, the risk of death while on treatment was most often due to malnutrition and advanced immune suppression; whereas in areas with low TB and HIV-1 disease, the risk factors included increased age, excessive alcohol use, IV drug use and homelessness.

A brief historical background to both diseases will be presented prior to discussing HIV-1 pathogenesis and immune response in more detail.

2.3 History of HIV

HIV-1 was initially called lymphadenopathy-associated virus (LAV) or human T-cell lymphotropic virus III (HTLV-3) (Knipe and Howley, 2013). Despite only first being recognized in the early 1980s, subsequent studies date the HIV-1 epidemic to a much earlier time point. The origin of HIV-1 is via cross species spread from African primates infected with simian immunodeficiency virus (SIV). These primates were naturally infected with SIV across multiple species where the virus had limited negative effects on the immune system and impact on health, while in other species, such as the chimpanzees; the disease appeared to display similar immune dysfunction as that of HIV-1 in humans. HIV-1 and HIV-2 are both lentiviruses and result from the zoonotic transfer between multiple primate species to humans (Sharp and Hahn, 2011). It is believed that transmission of SIVcpz from chimpanzees in south eastern Cameroon to humans gave rise to HIV-1 group M (major), which is regarded as the principal cause of the HIV-1 pandemic (Sharp and Hahn, 2011). The time when humans first became infected with HIV-1 is unclear; however, records from Bioko Island dates monkeys infected with SIV back to at least 30 000 years ago (Worobey *et al*., 2010). HIV-1, group M is the lineage of the virus which is responsible for the AIDS epidemic. Besides group M, groups N, O and P have also been described, but only infect a small percentage of the world's population (Mauclere *et al*., 1997).

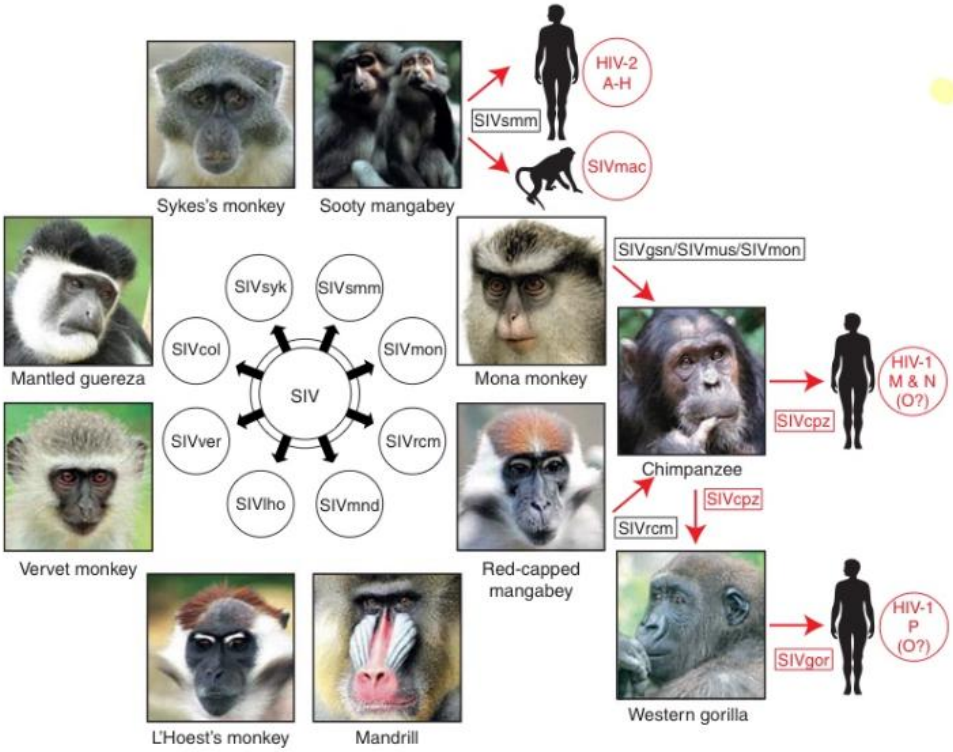

Figure 2.4 Transmission of HIV from African monkeys to humans. HIV-1 is believed to have been transferred to humans by the Chimpanzee and the Western Gorilla. HIV-2 is believed to be transmitted to humans from the Sooty mangabey (Sharp and Hahn, 2011).

Phylogenetic analysis and statistical modelling has estimated that HIV-1 group M entered the human domain between 1910 and 1930 (Korber *et al*., 2000; Worobey *et al*., 2008). Molecular virologists believe that the virus went unnoticed in West-Central Africa for approximately 50-70 years spreading among the local people and travellers in the area of Kinshasa (Vidal *et al*., 2000).

HIV-1 infection was first officially recorded in Los Angeles and San Francisco, USA, in 1981. A small group of young homosexual men displayed *Pneumocystis carinii pneumonia*, Kaposi's's sarcoma and failing immune systems followed by rapid death (Sharp and Hahn, 2011; Masia *et al*., 2014). HIV-1 was initially believed to be a disease of the gay community. A year after the first description in 1982 the CDC named the disease AIDS, a universally accepted nomenclature which superseded the numerous earlier designations (AIDS, 2011).

In 1984, Gallo, Montaigner and Levy were independently the first to confirm that the causal agent of AIDS was a virus (HIV-1) (Barre-Sinoussi *et al.,* 1983; Gallo *et al*., 1984; Levy *et al*., 1985). In the same year, reports from the CDC confirmed that the virus is transmitted via sharing needles among the intravenous (IV) drug users, unprotected sex among all sexual orientations and via blood transfusions in the hospital setting (Sharp and Hahn, 2011). One year later in 1985, the virus had been confirmed to have spread to all continents of the world.

In 1986, the official name HIV-1 was given to the causative virus (A Timeline of HIV/AIDS, 2015).

2.4 History of TB

As mentioned above, TB is the second leading cause of death from an infectious disease worldwide (Yang *et al*., 2013; Jiang *et al*., 2014; Yang *et al*., 2014). Evidence collected from Egyptian mummies suggested that TB has been plaguing humans for around 4 000 years. Once referred to as “white plague” or “consumption”, TB has caused one billion human deaths in the time spanning 1700 to 1900. Fortunately, in 1882, Robert Koch, a German scientist and physician, identified *Mycobacterium tuberculosis* (M.tb), which is the bacterium responsible for TB (Morris *et al*., 2013; Nunes-Alves *et al*., 2014; Stanley *et al*., 2014; Stylianou *et al*., 2014). Koch won the Nobel Prize in 1905 as a result of his research and discovery of tuberculosis. Since Koch’s discovery, three other scientists, Paul Ehrlich, Gerhard Domagk and Selman Walksman, have been awarded the Nobel Prize for their contribution towards compounds that are able to slow down or stop the growth of M.tb. The combination of these compounds, which include Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), Ethambutol (EMB) and Streptomycin (STR), still forms the majority of the TB medication currently in use today (Department of Health SA, 2014).

2.4.1 Diagnosing TB in South Africa

The diagnosis of TB involves clinical examination and radiographic and/or mycobacteriological confirmation. A biological specimen (sputum) is obtained and analysed using a smear microscopy. Alternatively, M.tb is cultured (in broth or plates) or confirmed using molecular techniques (see below) (WHO, 2015). The smear methodology refers to two staining procedures for acid-fast bacilli, known as either Ziehl-Neelsen or fluorescent auramine staining. These tests depend on the ability of the bacterium to retain the dyes when treated with acid and alcohol solutions. This is a well-developed method of testing for TB in patients with pulmonary TB, and can be performed within 48 hours. However, this method is less sensitive in detecting TB in patients who have low bacillary load in sputum, such as those with HIV-1 co-infection (Department of Health SA, 2014). A more sensitive test would be the culture method of detecting TB, which is also able to detect TB in children and those infected with HIV-1 with smear negative pulmonary TB. However, this test is more expensive, prone to contamination, as the bacteria are grown on a plate or tube, and time consuming, as results take up to 6 weeks to complete (Department of Health SA, 2014). Two of the most commonly used molecular techniques in the detection of TB, and the resistance to TB drugs in South Africa, is the Xpert MTB/RIF (Cepheid, CA, USA) and the line probe assay called Genotype MTBDRplus (Hain Lifesciences, Nehen, Germany). Both

these tests are polymerase chain reaction (PCR) techniques with the Xpert MTB/RIF testing for M.tb, as well as resistance to RIF, generating a result within two hours. The Xpert MTB/RIF is a preferred technique as each sample is tested in a closed cartridge system which minimises the risk of contamination and human error. Similarly, the line probe assay detects the presence of MDR-TB and TB drug resistance to RIF and INH which are two of the most powerful first-line TB drugs currently available in the world. While the line probe assay is effective in detecting TB and MDR-TB in seven days, it is labour intensive and is prone to human error, as this test requires three different rooms to carry out the entire experiment (Department of Health SA, 2014). The Quantiferon TB Goldtube test is a blood test, which is used to distinguish between latent and active disease. This test is an immune diagnostic test, which involves T cell stimulation and measures the amount of interferon (IFN)-γ production (Quest Diagnostics, NJ USA). Besides laboratory diagnostics; a clinical diagnosis of TB can be given via X-ray abnormalities and physical symptoms such as coughing for an extended period of time (longer than three weeks), past history of TB, sudden weight loss and night sweats (WHO, 2015).

2.4.2 TB treatment

Most healthy individuals who come in contact with M.tb effectively recover and control the bacteria for a lifetime. This is classified as latent TB (Stanley *et al*., 2014). In the event that a person has latent TB, the bacteria remain dormant and contained within granulomas. The granuloma consists of a collection of T cells, macrophages (MØ) and fibroblasts, which slowly replace themselves over time to maintain the entire structure (Walker *et al*., 2013; Nunes-Alves *et al*., 2014). These cells act as a wall-like structure in which the bacterium is unable to multiply. However, in 10% of cases, latent TB will go through a process of reactivation (Morris *et al*., 2013; Yang *et al*., 2013; Jiang *et al*., 2014; Yang *et al*., 2014). This occurs when the immune cells have become compromised due to autoimmune disease, organ transplant medications, or immuno-suppression due to HIV/AIDS. The T cells are unable to maintain the wall-like structure or to keep bacterial replication in check and the bacteria are able to break through the granuloma to actively replicate. Active TB disease is classified when the immune cells are unable to control M.tb. At this point, first-line chemotherapy will be administered. First-line defence against TB is conducted by the use of a combination of chemotherapy agents, typically RIF, INH, EMB and PZA, which are international standard treatments recommended by the WHO. These approved TB drugs have either bactericidal or bacteriostatic properties. Combination therapy reduces risk of drug resistance developing (UNAIDS, 2015). INH, RIF and PZA are bactericidal agents which destroy the bacteria in a matter of hours of administering the drugs. For example, between INH and RIF, 90% of the active bacilli are destroyed and become incapable of

actively causing disease after 24 hours of administration. Furthermore, EMB has bacteriostatic properties and minimises the risk of drug resistance (Department of Health SA, 2014). This treatment lasts six months or longer in order to clear the active disease to a controlled latent form. In the first two months of treatment, INH, RIF, PZA and EMB are prescribed in order to rapidly destroy the tubercle bacilli. This is- then followed by a final four month treatment of INH with a target of rapid to intermediate growing bacilli, and RIF with a target of all degrees of growing bacilli, including dormant bacilli to ensure that active M.tb infection is cleared to minimize risk of future active M.tb infection (Department of Health SA, 2014).

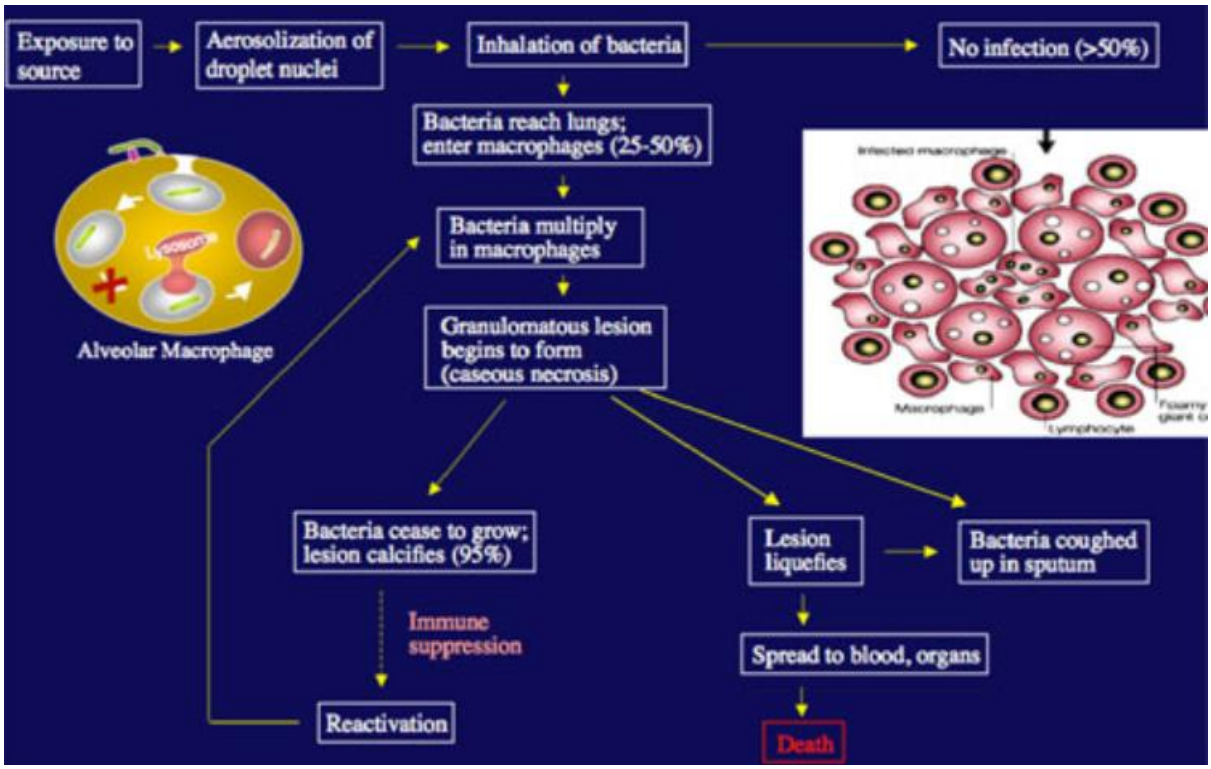

Figure 2.5: Steps in the pathogenesis of tuberculosis. The bacterium travels from one infected person to another through the air and into the lungs. Half of the newly infected individuals are able to clear the infection. The other half of the newly infected individuals will form bacterial containing structures called granulomas. Granulomas consist of a collection of immune cells which are able to stop the spread of the infection. However, for a few individuals with a compromised immune system, granuloma formation is hindered and the bacteria are able to spread (Tuberculosis, 2013).

2.4.3 Directly Observed Treatment, short course in South Africa

According to Loveday *et al*., (2012), KZN is the province in South Africa with the highest recorded incidence of TB, at 1163 cases per 100 000 population. Fortunately, the WHO developed the Directly Observed Treatment short course (DOTS) programme, which was designed to one day be able to eradicate TB entirely. In 1972, the WHO implemented this programme to help control the spread of TB infection in the world through a course of chemotherapy. The DOTS programme is based on directly observed treatment surveillance

where people in the community volunteer to ensure that patients infected with TB adhere to taking all their TB medication for the recommended time period (Pasipanodya and Gumbo, 2013). While there are many individual tablets that are administered during the course of treatment, The World Bank states that the DOTS programme is one of the most cost-effective of all health interventions (Frieden and Sbarbaro, 2007). Although this treatment is between six and nine months in length, financial support from various international organizations has made it possible for these people to have access to medication. However, some of the patients, now estimated at 500 thousand per year, will be reported to have MDR-TB, requiring second-line drugs that are more expensive, toxic, and may require up to two years of supervised adherence (Henao-Tamayo *et al*., 2011).

As chronic HIV-1 infection is the core component of the current study, a more detailed introduction to the causative agent, its replication, pathology and the immune responses are covered in the sections below.

2.5 HIV causal agent

There are two strains of HIV that have been found to cause disease in humans. Both HIV-1 and HIV-2 are RNA viruses of the Retroviridae family and lentivirus group. The virus is classified as a retrovirus because of the ability to utilise a reverse transcriptase enzyme to produce DNA from RNA. Furthermore, this virus is then able to insert the cDNA copy of the full viral genome directly into the human genome using the viral enzyme intergrase (Sierra *et al*., 2005). Once the viral genome is integrated into the human genome, the virus is free to use host proteins and machinery (particularly polymerase enzymes for transcription and translation) to manufacture new virus particles. HIV-2 is less virulent than HIV-1 and found mostly in West African regions. HIV-1 is the predominant strain infecting humans and is responsible for the AIDS epidemic worldwide (Abram *et al*., 2014; Holmes *et al*., 2015). The virus is grouped into M, N, O, P and further subdivided into subtypes A-K (Girard *et al.,* 2011). In the South African population, Group M (major), subtype C is the predominant circulating strain of HIV-1 found among infected individuals (Jacobs *et al.,* 2014).

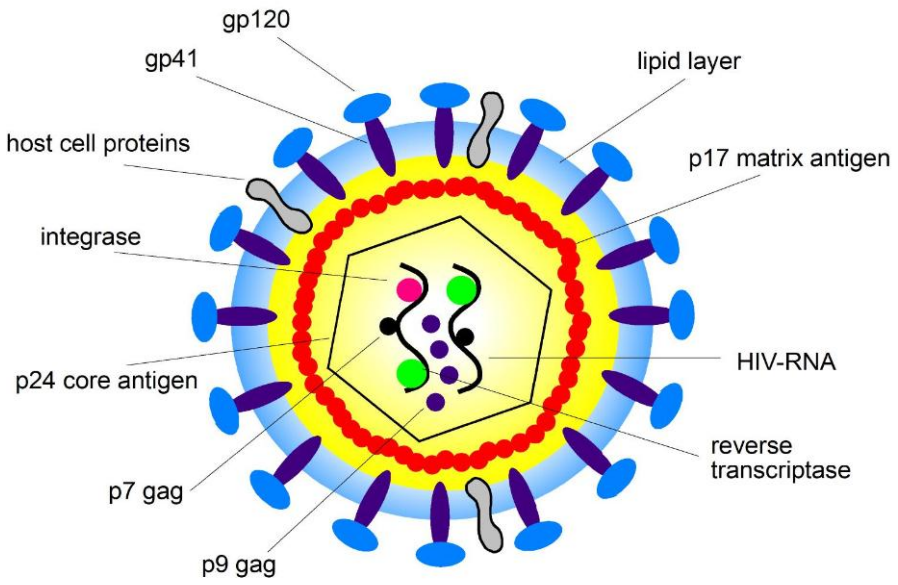

Figure 2.6: HIV viral structure. The virus is composed of a lipid membrane, gp120 and gp41 envelope proteins, followed by an outer P17 matrix protein matrix with a p24 capsule core, which contains two single stranded RNA molecules, reverse transcriptase enzyme, integrase, P7/P9 Gag, regulatory proteins (Tat and Rev) and accessory proteins (Vif, Vpr and Vpu) (Rubbert *et al.*, 2011).

The mature HIV-1 virion is approximately 120 nm in diameter and is composed of an external bi-lipid layer membrane called the envelope, followed by an internal p24 capsid protein layer (Figure 2.6). Inside the capsid, the intergrase, reverse transcriptase, two identical single strain positive sense RNA molecules and viral proteins can be found (Girard *et al*., 2011). The genome is 9.7kbp in length and codes for nine genes within many open reading frames (ORF). These ORFs encode three structural (Gag, Pol and Env), three accessory (Vif, Vpr and Vpu) and three regulatory proteins (Tat, Rev and Nef). The HIV-1 genes, their products, functions and localization are indicated in Table 2.2.

Table 2. 2 List of HIV RNA gene and functions

Name	Size	Function	Localization
Gag	P17, p24, p7, p9	Membrane anchoring	Virion
Protease	P11	Gag/pol cleavage	Virion
Reverse Transcriptase	P66	Reverse transcriptase activity	Virion
Env	Gp120/gp41	External viral proteins	Plasma membrane, virion envelope
Tat	P16/p14	Viral transcriptional transactivator	Primary in nucleus
Rev	P19	RNA transport	Primary in nucleus
Vif	P23	Promotes viral maturation	Cytoplasm, Virion
Vpr	P15	Inhibits cell division	Virion, nucleus
Vpu	P16	Promotes extracellular release of viral proteins	Integral membrane protein
Nef	P27	Down regulation of CD4 proteins	Plasma membrane, cytoplasm

P=protein, GP=glycoprotein, number e.g. 19 = kd size, 19kD (Knipe and Howley, 2013).

Flanking the structural and functional genes are the long terminal repeat (LTR) sequences, functioning as transcription promoters. Expression of the full genome requires multiple frame shifts to synthesize mRNA from overlapping genes (Holmes *et al.*, 2015). Figure 2.7 displays the genomic organisation of the afore-mentioned viral genes of HIV-1.

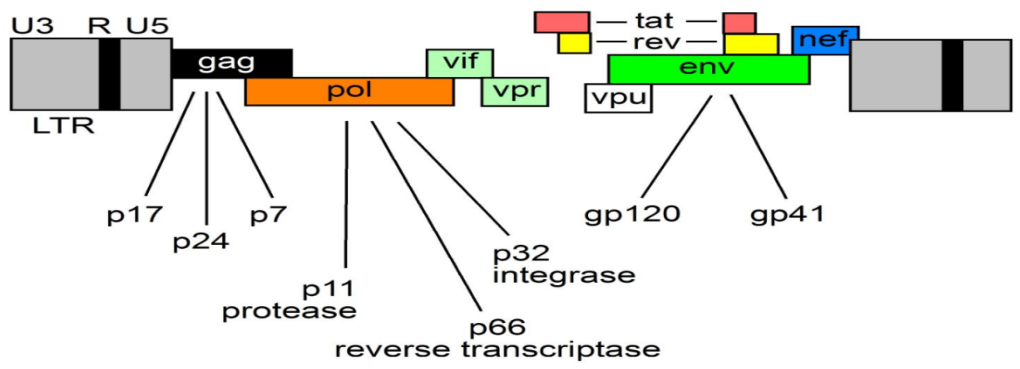

Figure 2.7: Genome of HIV. HIV-1 genome is organized into 9 genes which include *gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu* coding for various proteins and enzymes. The intergration of proviral DNA from RNA into the host cell's chromosome is carried out by reverse transcriptase. Diagram illustrates gene overlaps and LTR flanking regions (Rubbert *et al.,* 2011).

2.5.1 HIV replication cycle

HIV-1 replication is initiated once a replication competent virus successfully crosses the epithelial layer/mucosal lining and attaches to a susceptible host cell containing surface host proteins CD4 as the primary receptor, together with CCR5 or CXCR4 as the co-receptors (Girard *et al*., 2011; Arrildt *et al*., 2012). CD4 is expressed on T cells, dendritic cells (DCs) and MO/MØ, thymocytes (immature thymus T cells) and microglial cells (Shankar *et al*., 2015). CCR5 is expressed on activated and memory $CD4^+$ T cells and MØ, with CXCR4 most often expressed on naïve $CD4^+$ T cells (Arrildt *et al*., 2012). The HIV-1 replication cycle can be divided into an early phase which consists of the virus attaching to the surface of the cell until when the virus has integrated into the human genome; and a late phase which consists of viral gene expression until budding and maturation of the new virion (Freed, 2015). Upon entering the host, the virus will attached to the host receptors, a conformational change occurs which allows the virus to empty the viral capsid into the host cytoplasm. The viral envelope fuses with the host cell membrane; the capsid enters the cell cytoplasm, and is then uncoated, releasing viral RNA and enzymes into the cytoplasm (Figure 2.8). The reverse transcriptase enzyme reverse transcribes the RNA into double-stranded molecule viral DNA. This is termed complimentary or cDNA. Integration occurs next, and is when viral cDNA enters the nucleus and is integrated into the host's DNA via the viral integrase enzyme. At this point the viral DNA is called a provirus (Sierra *et al.,* 2005). Once integrated, the cDNA can lay latent (integrated provirus constitutes the so-called "latent reservoir" in treated patients) until the host cell is activated and host cell gene transcription occurs. Integrated viral DNA is used as a template to make viral RNA in a process called transcription. Transcription produces multiple RNA copies of the cDNA which translocate from the nucleus to the cytoplasm. During the late phase, translation of the viral RNA leads

to formation of new viral proteins, both structural proteins and enzymes. Assembly involves viral proteins, viral RNA and enzymes moving towards the host cell's membrane to form a bud in preparation to exit the host cell (Dinkins *et al*., 2014). Release and maturation are the final stage in the cycle, where the virus buds from the host cell membrane and the viral enzyme protease cuts the long polypeptide protein chain to form a functional core structure. The entire replication cycle can be completed in approximately 20 hours, at which time the newly formed virus leaves the first host cell and will continues to infect adjacent cells (Holmes *et al.,* 2015).

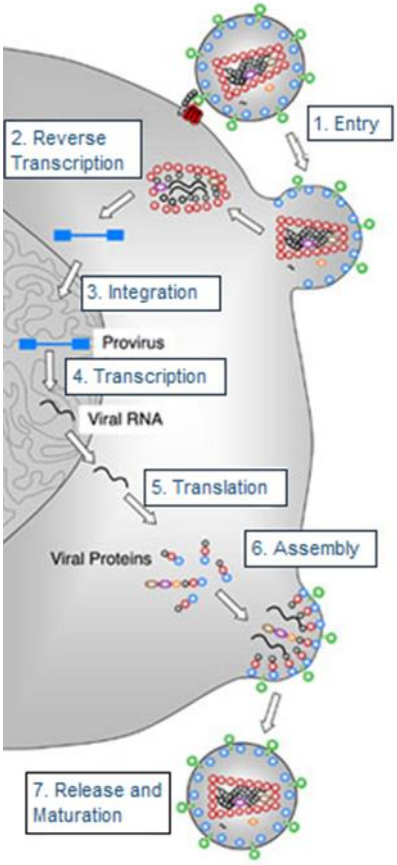

Figure 2.8: HIV replication cycle. HIV-1 replication cycle divided into seven main points of viral formation in the host cell. The cycle begins with the virus attaching to the host cell and ends with the release of a mature virus particle able to infect the next $CD4^+$ T cell (AIDS research centre, 2012).

2.5.2 Mode of HIV transmission

HIV-1 enters the human body in a number of different ways and at different sites, namely: 1) through unprotected sexual intercourse (female genital tract or rectal mucosa); 2) by vertical infection, between an infected mother to her child *in utero*, during childbirth, post-partum or via breast feeding; 3) parenteral transmission by means of a) sharing or reusing

contaminated IV needles; b) work related needle stick injuries and c) blood transfusions in a hospital setting (Girard *et al*., 2011; Sharp and Hahn, 2011; Walker *et al*., 2013).

According to Delva and Karim (2014), transmission of the virus is most often seen in heterosexual populations with multiple sex partners or in monogamous relationships where HIV-1 status of a partner is not known at the start of the relationship. The number of new infections is also high for sex workers and their clients.

WHO summarised (in the 2015 guidelines) that the five groups bearing the highest risk for new HIV-1 infection globally to include: men who have sex with men (MSM), sex workers, people who inject drugs, transgendered people and people in prison with the strong recommendation to administer pre-exposure prophylaxis (PrEP) in order to minimise future infection. Importantly, in South Africa, heterosexual people who engage in multiple sexual partners are at an equal, if not higher risk of HIV-1 infection as the above mentioned groups (WHO, 2015).

2.6 TB and HIV pathogenesis

TB and HIV-1 both have pathogenesis pathways involving multiple cell types and immune mediators. The co-occurrence of both pathogens in a single host naturally further complicates the process. It is well known that having either disease predisposes the individual to worse outcome if acquiring the other disease. In the following section, the pathogenic processes of both diseases are presented, highlighting pathways that may be most important when co-infections occur.

2.6.1 TB pathogenesis

TB is transferred between people via aerosol droplets from coughing (Nunes-Alves *et al*., 2014). Once the bacteria have been inhaled into the lungs, they settle in the distal lung alveoli, which initiate the recruitment of inflammatory cells to the site of infection (Russell, *et al*., 2010). The first point of contact is the alveolar resident MØ which engulf the M.tb. MØ have the ability to secrete proteolytic enzymes and cytokines that exhibit anti-microbial effects, including interleukin (IL)-1 which is associated with inflammation, IL-6 which enhances proliferation of B cells, IL-10 and tumour necrosis factor (TNF)-α which signals the production of nitric oxide metabolites and granuloma formation (Van Crevel *et al*., 2002). This is subsequently followed by recruitment of DCs, which also engulf the M.tb. Initial innate recognition of the bacteria by MØ and DC is possible by pathogen-associated molecular patterns (PAMPs) on the surface of the bacteria via receptors on host cell surface, namely toll-like receptors (TLR), nucleotide-binding oligomerization domain receptor (NOD) and nod like receptors (NLRS) (Mesman and Geijtenbeek, 2012; Rustagi and Gale, 2013; Nunes-

Alves *et al*., 2014). Recognition of M.tb occurs primarily through lipoarabinomannan (LAM), which is the major cell wall component of M.tb. The main receptor responsible for recognising LAM is TLR-2 on the phagocytic cells (Quesniaux *et al*., 2004). The receptor on the phagocytic cell that is responsible for inducing the uptake of the bacteria could be either complement receptor (CR) 1, CR3, mannose receptor or a type A scavenger receptor (Van Crevel *et al*., 2002). M.tb, which escapes or survives this initial innate immune response, will multiply. Increased antigen will be detected by resident phagocytes, which in turn, signals recruitment of blood MO to the site of infection. The MO differentiates into MØ, which engulf the M.tb but do not destroy the bacterium, as the MØ requires further activation from the T cells (Pawlowski *et al.,* 2012).

DCs engulf the antigen and migrate to the closest lymph node (Morris *et al*., 2013; Lai *et al*., 2014; Nunes-Alves *et al*., 2014). It is here, in the lymph node, where the DCs display a peptide fragment of the antigen to the naïve T cells via MHC (Larsson *et al*., 2013; Morris *et al*., 2013). Once the T cell has recognised the peptide fragment, it is able to mount a powerful immune response to control the infection (Pawlowski *et al*., 2012; Lai *et al*., 2014). In order for a T cell to become activated it requires two signals (Figure 2.9). The first signal is between the major histocompatibility complex (MHC) receptor of the DC and the T cell receptor (TCR) of the T cell (Larsson *et al*., 2013; Martinez *et al*., 2015). The second signal is between the co-stimulating molecules CD80/CD86 with CD28, which results in clonal expansion and differentiation into effector T cells (Larsson *et al*., 2013; Littman, 2015).

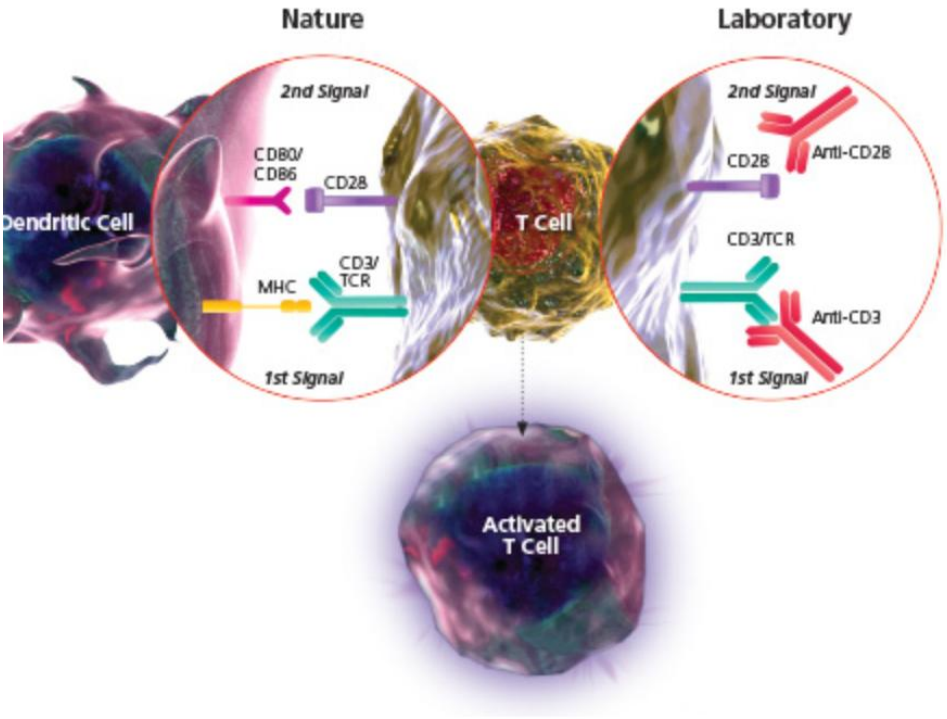

Figure 2.9: Nature and laboratory co-stimulation of T cells. T cell activation requires at least two signals to become fully activated. The first occurs after engagement of the TCR by MHC, and the second by subsequent engagement of co-stimulatory molecules. The most potent T cell co-stimulator is CD28. These signals are transmitted to the nucleus and result in 1) Clonal expansion of T cells; 2) Up-regulation of activation markers on the cell surface; 3) Differentiation into effector cells; 4) Induction of cytotoxicity or cytokine secretion; 5) Induction of apoptosis (T cell activation, 2015).

The effector T cell then migrates to the lung in the form of T helper (Th) 1 cells, which are able to activate the MØ to destroy the engulfed M.tb via the production of IFN-γ (Van Crevel *et al*., 2002). Interestingly, IL-12 which is expressed by activated MØ and DCs plays a role in the activation of Th1 cells which in turn stimulate the activation of the MØ to destroy the bacterium and control infection (Lai *et al*., 2015). While TB can spread to other organs in the human body, pulmonary TB infection will be the main focus of this study as it is the most commonly reported co-infection found in the South African HIV-1 population.

2.6.2 $CD4^+$ T cells as a link between TB and HIV

The role of $CD4^+$ T cells in the human immune system is numerous; in particular, Th1 cells are responsible for the activation of other immune cells to effectively ward off infections. Firstly, Th1 cells are responsible for the activation of MØ, which aid in enhancing elimination of M.tb (Yang *et al*., 2013; Bandaru *et al*., 2014; Boer *et al*., 2014; Lai *et al*., 2014; Stylianou *et al*., 2014;). Th1 cells are recruited to the site of M.tb infection, where they secrete the cytokines IFN-γ and TNF-α, which activates MØ to control bacterial growth (Hertoghe *et al*., 2000; Tousif *et al*., 2011; Qiu *et al*., 2012; Day *et al*., 2014; Lai *et al*., 2014; Stanley *et al*., 2014). CD4 knockout mice were more prone to TB infection when compared to wild type

mice (this holds true for HIV-1 patients with a very low CD4 count as they too are at a greater risk of TB infection) (Tousif *et al*., 2011; Yang *et al*., 2013). In a similar study by Bandaru *et al*., (2014), IL-7, a pro-inflammatory cytokine, also had an important role in immunity against M.tb. IL-7 has been recorded as having a positive effect in vaccine-induced protective immune responses against M.tb infection. After vaccination with *M. bovis* Bacille Calmette Guerin (BCG), Th17 cells are shown to activate chemokine production, which recruits IFN-γ producing T cells. Secondly, Th1 cells are responsible for the activation of the killing action of $CD8^+$ cytotoxic T lymphocytes (CTL). Thirdly, Th1 cells generally have a central role in the activation and maturation of B cells, which aid in antibody production against HIV-1 and TB. Fourthly, Th1 cells secrete IL-2, IFN-γ and lymphotoxin, which are important for autoimmunity and for the development and maintenance of secondary lymphoid organ structure, as well as in the formation of memory T cells (Munier *et al*., 2013; Kahan *et al*., 2014; Levy *et al*., 2014). And finally, Th1 cells delay hypersensitivity reactions.

Due to the vast number of activities performed by Th1 cells, when a person is infected with HIV-1, all these functions are impaired due to $CD4^+$ T cells being the target cell of HIV-1 and the cell type selectively deleted (Shankar *et al*., 2015).

2.6.3 HIV pathogenesis

The pathogenesis of HIV-1 can be broadly divided into three distinct phases based on CD4 count, viral load (VL) and AIDS defining opportunistic infections resulting in death. These three stages are **acute phase (primary infection)**, **chronic phase (clinical latency**) and **finally AIDS (progression to death)** (Figure 2.10). The acute phase is characterised by primary infection, followed by plasma VL increasing drastically to around 10^6 -10^7 viral copies per ml and accompanied by flu-like symptoms (Veazey *et al.,* 1998; Veazey *et al.,* 2000). The rate of viral replication will gradually decrease to a set point, which will continue throughout the chronic phase or clinical latency. It is possible for the chronic phase to last up to 10 years without ART or even longer with ART. During the chronic phase, untreated HIV-1, $CD4^+$ T cells are continually depleted and the susceptibility to opportunistic infections increases. The final AIDS stage is marked by extremely low CD4 count (<250 cells/mm^3), high VL and stage III or stage IV WHO disease classification. This phase results in death (UNAIDS, 2015).

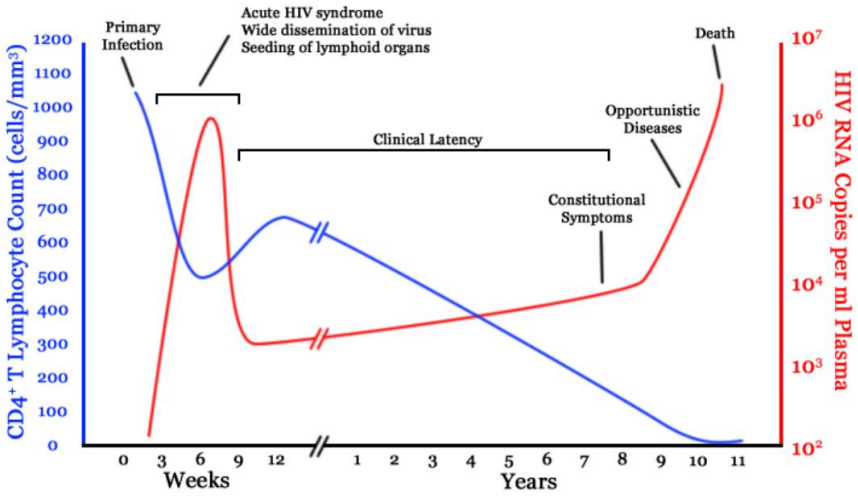

Figure 2.10: HIV disease stages according to CD4 count and VL. The acute stage is characterized by a drastic peak in viral load and depletion of $CD4^+$ T cells; the clinical latency stage is characterized by a gradual decrease of $CD4^+$ T cells and gradual increase in VL; and the final AIDS stage results in high VL and low CD4 count, opportunistic infections, wasting and death (HIV and Disability, 2010).

2.6.4 Depletion of $CD4^+$ T cells

In the acute phase of HIV-1 infection, a rapid depletion of $CD4^+$T cells in peripheral and mucosal lymphoid tissue accompanied by high levels of virus are common hallmarks (Ghiglione *et al*., 2014). Arnoczy *et al*., (2012), explained that during the acute phase of HIV-1 infection, the high level of virus is due to the limitation of the T cell repertoire against this newly encountered virus. It is during the acute phase, when HIV-1 is able to directly and indirectly destroy the normal functioning of $CD4^+$ T cells, which in turn, leads to lifelong immunodeficiency (Sauce *et al*., 2011; Kulpa *et al*., 2013; Munier *et al*., 2013; Shankar *et al*., 2015). The virus directly affects the production of T cells by infecting the thymus, inhibiting thymopoiesis and affecting thymic output, decreasing the total number of new naïve T cells entering into circulation (Sauce *et al*., 2011).

There are various mechanisms by which HIV-1 decreases the total number of $CD4^+$ T cells. Firstly, viral replication inside the $CD4^+$ T cell causes cell damage resulting in apoptosis. It is believed that the budding off of the new viral particle causes an influx of calcium into the cell, which activates apoptosis. This process also increases the amount of water moving into the cell, which also leads to osmotic lysis. Secondly, $CD4^+$ T cells are depleted by the virus hijacking the host cell machinery to assemble viral protein, instead of the essential proteins needed for host cell survival. A less common action of HIV-1 on the host cell is giant cell formation (syncytium) between an HIV-1 infected $CD4^+$ T cell and an uninfected $CD4^+$ T cell. Both $CD4^+$ T cells are joined by the gp120-CD4 interaction on the HIV-1 infected $CD4^+$ T cell. $CD4^+$ T cells are unable to function as a multinucleated giant $CD4^+$ T cell and as a

result, the host cell dies. DCs and MØs also display the CD4 marker and are also susceptible to HIV-1 infection. However they are more resistant to the disease, but can transfer the viral infection to $CD4^+$ T cells and therefore assist in the depletion of the overall $CD4^+$ T cell number. And finally, the DCs attach and transport the virus and directly display it to the naïve T cell, increasing HIV-1 infection of the $CD4^+$ T cells (Larsson *et al*., 2013; Dinkins *et al.,* 2015). A final pathway of T cell death is inflammation/immune activation-associated up-regulation of death receptors and AICD, which will be discussed in subsequent paragraphs.

2.6.5 $CD8^+$ T cell response to HIV

$CD8^+$ T cells play a crucial role in the killing of virus-infected cells, as well as defending against cancer (Zajac *et al*., 1998; Wherry, 2011; Legat *et al*., 2013; Rahim *et al*., 2013; Buzon *et al*., 2014; Ghiglione *et al*., 2014). In a study by Yamamoto *et al*., (2011), it was found that as soon as the $CD8^+$ T cells were unable to effectively launch a cytotoxic response in HIV-1 infected individuals, the progression to AIDS and death was significantly increased due to the inability to control the virus. Furthermore, in the absence of $CD4^+$ T cells, the $CD8^+$ T cells lack the overall ability to effectively produce cytokines and proliferation was decreased after *in vitro* stimulation. Moreover, $CD8^+$ T cells were more sensitive to cell death in populations infected with HIV-1 when compared to $CD8^+$ T cells in healthy controls. As a result, targeting the effector function and survival of $CD8^+$ T cells could enhance immune control of HIV-1 infection and therefore increase survival of the individual (Zajac *et al*., 1998; Munier *et al*., 2013). As mentioned earlier, CTLs recognise antigen on the surface of cells that display the MHC I molecule. The activation of CTLs is reliant on the help of $CD4^+$ T cells via cytokine release. However, the full activation of CTLs is initiated upon activation of $CD4^+$ T cells by the antigen presenting cells. Once the $CD4^+$ T cell is activated, it secretes cytokines, such as IL-2, IL-6, IL-12 and IL-15, which trigger the differentiation of the $CD8^+$ T cell into an activated MHC class I restricted CTL. These CTLs are able to combat a virus-infected cell through several mechanisms, such as lytic granules, which would include perforins, granzymes and the cytokine TNF-α. The granules are able to induce pore formation in the membrane of the virus-infected cell within minutes (Sakhardi *et al*., 2012; Day *et al*., 2014). Furthermore, CTLs are also able to induce apoptosis by means of the Fas pathway (Ju *et al*., 1994). Importantly, $CD8^+$ T cells also play a functional role in the management of M.tb by means of killing the bacteria by granzyme independent mechanisms (Qiu *et al*., 2012; Day *et al*., 2014; Lindenstrom *et al*., 2014).

2.7 Chronic viral infection

Chronic HIV-1 infection leads to compromises in cell-mediated immunity, which hinders the ability to ward off opportunistic infections (Camargo *et al*., 2014). Furthermore, diseases that were once kept under control by the immune system are no longer controlled (Masia *et al*., 2014). Diseases such as Epstein-Barr virus (EBV), which infect 90% of the general population, are associated with a higher risk of lymphomas in HIV-1 positive individuals (Sachithanandham *et al*., 2014). Similarly, the reactivation of varicella-zoster virus (VZV) in HIV-1 positive individuals is associated with ophthalmic and neurological complications. Another reactivated virus that is associated with many lifelong conditions is cytomegalovirus (CMV) (Masia *et al*., 2014). In HIV-1 positive individuals, CMV is directly linked to CD4 count and the most common disease is retinitis, which affects 15 to 40% of patients with a low CD4 count (Griffiths *et al*., 2012). Bacterial infections are also problematic, Abimbola and Marston (2012), showed that TB, cryptococcal meningitis, pneumonia and diarrheal illness are the most common diseases responsible for early mortality of HIV-1 positive patients living on the African continent.

There are also a number of STIs that are associated with increased risk of acquisition and with accelerating HIV-1 disease progression and death. One of the best researched is human papilloma virus (HPV), which has a direct role in the development of cervical cancer. In healthy young women, the immune system is able to control and even eliminate the virus. However, in HIV-1 positive women there is a rise in mild dysplasia by 80% when compared to 27% found in healthy, HIV-1 negative women. Furthermore, in HIV-1 positive women there is a drastic increase in the development of aggressive lesions and a lower response to treatment when compared to healthy HIV-1 negative women (Camargo *et al*., 2014).

2.8 Immune activation and immune exhaustion in chronic HIV-1 infection

When a cell exhibits immune activation or exhaustion due to chronic viral infection, nuclear transcription factors induce production of host proteins, which are presented on the cell surface as receptors or ligands (cell biomarkers). These receptor proteins can either induce non-responsiveness or down-regulate cell defences (immune exhaustion) or activate the cell (immune activation) on binding with its respective ligand. The up-regulation of immune exhaustion markers on the surface of the cell is protective as the immune system attempts to minimise cellular and tissue damage due to long term inflammation and cytokine production. Conversely, this up-regulation of inhibitory surface markers is also detrimental to the immune system as the down-regulation of the immune cell function enables the virus to gain an advantage (Zajac *et al.,* 1998).

In HIV-1 negative persons, acute viral infections stimulate inflammation and immune activation, which are quickly resolved in the clearance of the antigen (Haas *et al*., 2013). The regular course of action entails the naïve T cells becoming activated and differentiating into effector T cells. These effector cells proliferate to form a collection of specialised cells, which are able to clear the infection. Once the infection has been cleared, inflammation and immune activation is decreased and the activated T cells are cleared due to apoptosis or transition to long-lived memory cells. Only a small number of effector T cells are retained as memory T cells, which have the ability to transform into effector T cells on secondary infection of the same pathogen.

Conversely, in several chronic diseases such as HIV-1, lifelong inflammation and immune activation is seen, resulting in immune and tissue damage. This is due to the constant filtration of the immune cells and the immune response via pro-inflammatory cytokines (Haas *et al*., 2013). Furthermore, chronic immune activation is associated with immune aging and weakening of the immune cells referred to as immuno-senescence (Chalan *et al*., 2015; Shankar *et al*., 2015). Over time, functional impairment of the immune cells increases vulnerability to opportunistic infections and cancers, which are not normally found in healthy populations (Camargo *et al*., 2014; Mutlu *et al*., 2014).

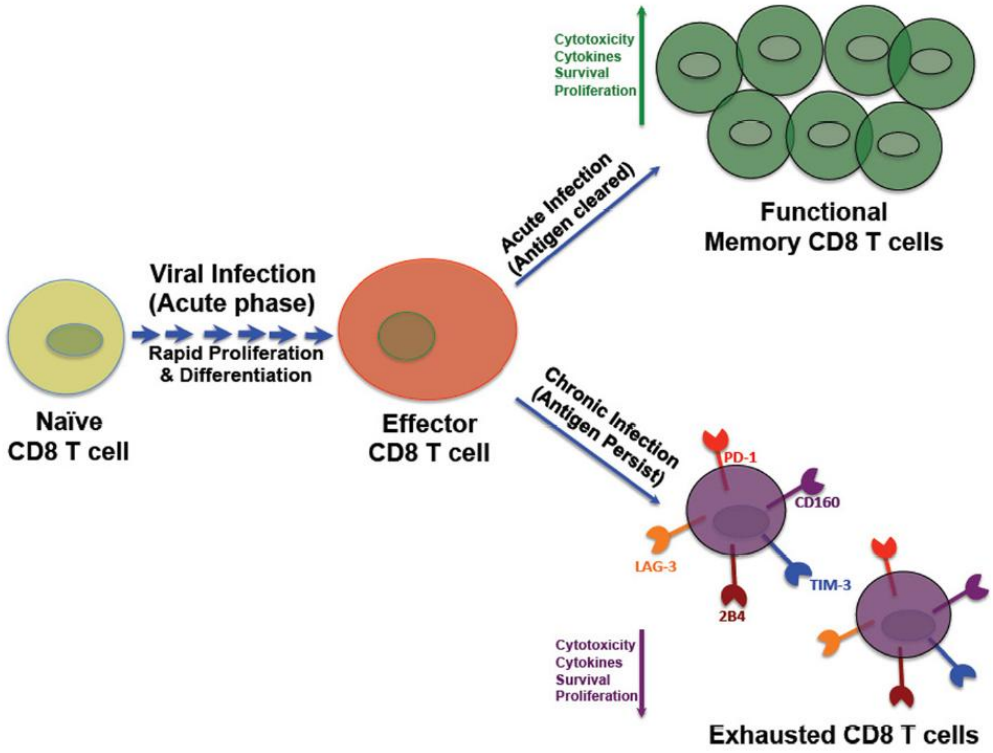

Figure 2.11: Chronic viral infection leading to immune exhaustion. Acute viral infections result in T cell effector function, and clearance of the antigen results in memory T cells. Chronic viral infection results in T cell effector function, increase in T cell activation and inflammation due to prolonged exposure to the antigen, leading to the activation of exhaustion surface markers, decrease in T cell function and lack of memory T cell formation (Velu *et al.,* 2015).

Studies of lymphocytic choriomeningitis virus (LCMV) in mice have contributed greatly to the current knowledge about immune exhaustion (Moskophidis *et al*., 1993; Zajac *et al*., 1998). In chronic viral infection, T cells are constantly exposed to the virus and this is the primary cause of immune exhaustion (Zajac *et al*., 1998; Sakhdari *et al*., 2012; Ezinne *et al*., 2014; Wherry and Kurachi, 2015). Immune exhaustion results from a process of unresolved and prolonged inflammation of the T cells due to immune activation and the inability of these cells to eliminate the pathogen burden over an extended time period (Wittkop *et al*., 2013; Lu *et al*., 2015; Martinez *et al*., 2015). PD-1 and Tim-3, among others in this study, are two examples of markers of T cell exhaustion up-regulated on T cells in patients with a viral or bacterial infection that is associated with prolonged unresolved immune activation (Martinez *et al*., 2015). Both exhaustion markers are involved in the down-regulation of T cell function, and both are categorised as T cell inhibitory markers. T cell exhaustion is damaging to the overall functioning of the immune system by means of multiple factors including the loss of a robust T cell effector function, up-regulation of inhibitory receptors on the cell surface, errors in the expression of transcription factors and limited cytokine production (Sakhdari *et al*., 2012; Smith *et al*., 2013; Wherry and Kurachi, 2015). With a combination of all these disabling factors, T cells are unable to transition into a resting state and form memory T cells

(Wherry and Kurachi, 2015). Similarly, Wang *et al*., (2011), found that inhibitory marker Tim-3 was up-regulated on virus-specific $CD8^+$ T cells in patients with chronic progressive HIV-1 infection, and also on antigen-specific $CD8^+$ T cells in patients with active TB.

In a recent KZN-based study, Sullivan *et al*., (2015), found immune activation and inflammation in active TB patients with or without HIV-1 infection. It has been well documented in the relevant literature that HIV-TB co-infected individuals display increased levels of inflammation, as well as an increased up-regulation of surface markers due to the added disease burden (Shankar *et al*., 2015). Furthermore, co-infection results in a significant increase in the amount of immune activation as evidenced by increased human leukocyte antigen (HLA)-DR and CD38 expression on T cells (Wittkop *et al*., 2013; Tenorio *et al*., 2014; Shankar *et al*., 2015). HIV-1 positive patients with latent TB also expressed high levels of immune activation when compared to those HIV-1 positive, but TB negative patients. It has been found that immune activation in HIV-1 positive patients is linked to immune exhaustion and progression to AIDS and is a better marker of survival than $CD4^+$ T cell count and VL (Sauce *et al*., 2011; Dunham *et al*., 2014).

HIV-related inflammation is well described in the literature resulting from mechanisms such as: microbial translocation, co-infections such as CMV reactivation, persistent HIV antigenemia and the direct effect of the virus on $CD4^+$ T cell depletion (Kolter *et al.*, 1984; Hunt *et al.*, 2011; Burgener *et al*., 2015). Doitsh *et al*., (2014), found that when $CD4^+$ T cells were depleted, IL-1β cytokine was released which is known to have potent pro-inflammatory properties. Chronic immune activation and inflammation can remain high throughout the lifetime of patients, despite ART. Non AIDS related illnesses, such as cardiovascular disease, renal disease and type II diabetes are believed to be related to this chronic activation/inflammation and, as a result, add strain to the already compromised immune system (Deeks and Phillips, 2009).

Microbial translocation is associated with physical damage of the mucosal associated lymphoid tissue (MALT) of the gastrointestinal tract (GIT) primarily due to destruction of tight junctions in the epithelium. The damage and associated translocation is a driver of on-going inflammation and immune activation, which adds to immune burden and equates to the accelerated HIV-1 disease progression to death (Guadalupe *et al*., 2003). In the majority of healthy HIV-1 negative individuals, the integrity of the gut lining is preserved due to the immunological and anatomical barriers which stop microbes and microbial products from entering across into the internal environment. The point of contact is the single layer of cells lining the gut which is covered by a protective mucus layer containing immunoglobulin (Ig) A and glycocalyx. Directly below this single layer of cells, in the lamina propria, DC, MØ,

granulocytes, mast cells, B and T cells can be found. The majority of the $CD4^+$ T cells found in the gut associated lymphoid tissue (GALT) are activated, displaying the CCR5 co-receptor, which is one of the core receptors needed for HIV-1 to bind and enter the host cell. During acute phase HIV-1 infection, memory $CD4^+$ T cells (which are $CCR5^+$) found in the lamina propria is most severely depleted. The GALT is the storage location of the majority of memory T cells, and is thus the favoured site for viral replication (Novati *et al*., 2015). This sudden depletion of $CD4^+$ T cells is accompanied by an increase in immune activation and inflammation, which continues for many years throughout the chronic phase of the disease (Guadalupe *et al*., 2003). While ART has shown to significantly decrease VL in peripheral blood, the GALT serves as a viral reservoir for the virus as it continuously replicates and infects $CD4^+$ T cells in this region (Anton *et al*., 2003; Chun *et al.,* 2008).

As early as 1984, Kotler *et al.,* (1984), reported a distinct link between damage of the gut, microbial movement into the blood and progression to AIDS. A few years later it was found that high levels of microbial lipoplysaccarides (LPS) in the blood correlated with high levels of immune activation and inflammation of the gut lining. The movement of LPS across the gut lining elicits both innate and adaptive responses in efforts to preserve the integrity of the internal environment (Brenchley *et al.,* 2006). Reactivation of latent diseases, such as CMV and VZV, further burden the immune system through eliciting high proliferation responses in mucosal lining (Guadalupe *et al.,* 2003). Microbial translocation also worsens with the addition of antibiotic therapy (Berg, 1981), burns (Deitch and Berg, 1987) and haemorrhagic shock (Rush *et al.,* 1988).

2.9 Characterization of T cell-associated negative regulatory and exhaustion markers

Yamamoto *et al*., (2011), reported that the $CD8^+$ T cell response during chronic viral infection in mice is controlled by a pattern of inhibitory, exhaustion and activation receptors. These receptors include Tim-3, PD-1, 2B4, LAG-3 and many others (Wherry, 2011; Legat *et al*., 2013). Furthermore, many studies have found that the greater the degree of exhaustion exhibited by the virus-specific $CD8^+$ T cells in mice and humans, the greater the co-expression of inhibitory receptors (Yamamoto *et al*., 2011; Legat *et al*., 2013; Lanteri *et al*., 2014).

In the current study, we have examined a number of markers associated with immune activation, exhaustion and death. These together with others (such as CTLA-4) are discussed below.

2.9.1 CTLA-4

One of the first T cell markers to be characterised in the early 1980's was Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) which is believed to have a role in T cell activation (Littman, 2015). This marker is part of the immunoglobulin family similar to that of CD28. CTLA-4 (also known as CD152) is responsible for regulating the magnitude of the T cell response and as such was first described as a down-regulator of T cell stimulation. CTLA-4 surface marker expression depends on the strength of the TCR signal. CTLA-4 is able to reduce this TCR signalling process and in turn down-regulate cell function. Furthermore, CTLA-4 is also able to down-regulate expression of the co-stimulatory ligand CD80 and CD86 on the antigen presenting cells, thereby reducing T cell CD28 engagement by means of the second signal of T cell activation (Wang *et al.,* 2015). CTLA-4 acts as an antagonist to CD28 by means of blocking the PI3K pathway, which in turn has a negative effect on protein synthesis and cell survival (Wang *et al.,* 2015). Mice deficient in CTLA-4 produce a lethal condition of hyper proliferation of T cells, which reinforces the regulatory importance of CTLA-4 is a negative regulator of T cell activation (Callaham and Wolchok, 2015).

Most of the current advances in medical research concerning immune check point therapies such as; cancer, autoimmune disease and chronic viral infections have focused on CTLA-4 and PD-1 (Littman, 2015; Ye *et al.,* 2015). CTLA-4 is expressed on antigen presenting cells, B cells and most importantly, T cells. In advanced HIV-1 diseased T cells, it was found that CTLA-4 was highly expressed on exhausted $CD4^+$ T cells but not expressed on CTL's. Additionally, blocking of CTLA-4 on exhausted $CD4^+$ T cells was found to improve proliferation and effector function (Kaufmann and Walker, 2009).

2.9.2 PD-1 inhibitory marker

PD-1 is a member of the CD28/cytotoxic T lymphocyte-associated protein-4 (CTLA-4)/B7 Ig family and is classified as a marker of immune exhaustion by inhibiting or down-regulating gene expression when interacting with its ligand PD-1L (Tousif *et al*., 2011; Dietze *et al*., 2013; Kulpa *et al*., 2013; Larsson *et al*., 2013; Ezinne *et al*., 2014). In 1992, PD-1, also referred to as CD279, and has subsequently been shown to have beneficial properties in controlling inflammation in many disease models by decreasing T cell activity (Littman, 2015). PD-1 is found on the surface of many immune cells, including T cells, B cells, natural killer (NK) cells and MØ cells (Tousif *et al*., 2011; Larsson *et al*., 2013; Seung *et al*., 2013; McBerry *et al*., 2014). In bacterial infections such as TB, Jiang *et al*., (2014), found that by blocking the PD-1 pathway, there was significant improvement in the production of IFN-γ, which is central to M.tb control in MØ.

PD-1 expression is controlled at the transcriptional level and the exact mechanism is currently still unknown. However, the PD-1 pathway is believed to be regulated by mechanisms involving T-bet, a cell-associated transcription factor, and B lymphocyte-induced maturation protein 1 (BLIMP-1) on $CD8^+$ and $CD4^+$ T cells (Shin *et al*., 2009; Kao *et al*., 2011; Lu *et al*., 2014). In the presence of antigen, TCRs signal PD-1 gene expression via the transcriptional factor NFATc1 pathway. It was once thought that T-bet had a role in the suppression of the transcription pathway, but this was discredited as T-bet could not express a strong enough response to decrease PD-1 expression in large quantities. Lu *et al*., (2014), found that during acute viral infections, transcription repressor BLIMP-1 was able to suppress the expression of PD-1 on effector T cells. BLIMP-1 knockout mice were used to further support this observation and it was confirmed that these mice displayed higher levels of PD-1 and reduced effectiveness in clearing acute infections (Lu *et al*., 2014). In chronic viral infection, PD-1 was also found to have a role in the TCR signalling pathway of T cells decreasing IL-2, IFN-γ and TNF-α production which are crucial for T cell survival (Wherry, 2011; Kahan *et al*., 2014; Martinez *et al*., 2015). Similarly, in a review by Balkhi *et al*., (2015), it was discussed that exhausted T cell which displayed PD-1 had a distinct gene transcription profile that is very different from that of healthy T cells; notably these unique transcription factors including BLIMP-1, Eomes, T-bet, IKZF and FoxO1 were up-regulated. Balkhi *et al*., (2015), noted that the action of IL-2 was believed to be two fold, one for cell survival and the other in a feedback loop to PI3K in NFATc1 pathway (Figure 2.12). While this signalling pathway of IL-2 during exhaustion is not fully understood, it is proposed that the binding of PD-1/PDL-1 could negatively influence the production of IL-2 resulting in the progression of T cell exhaustion (Balkhi *et al*., 2015).

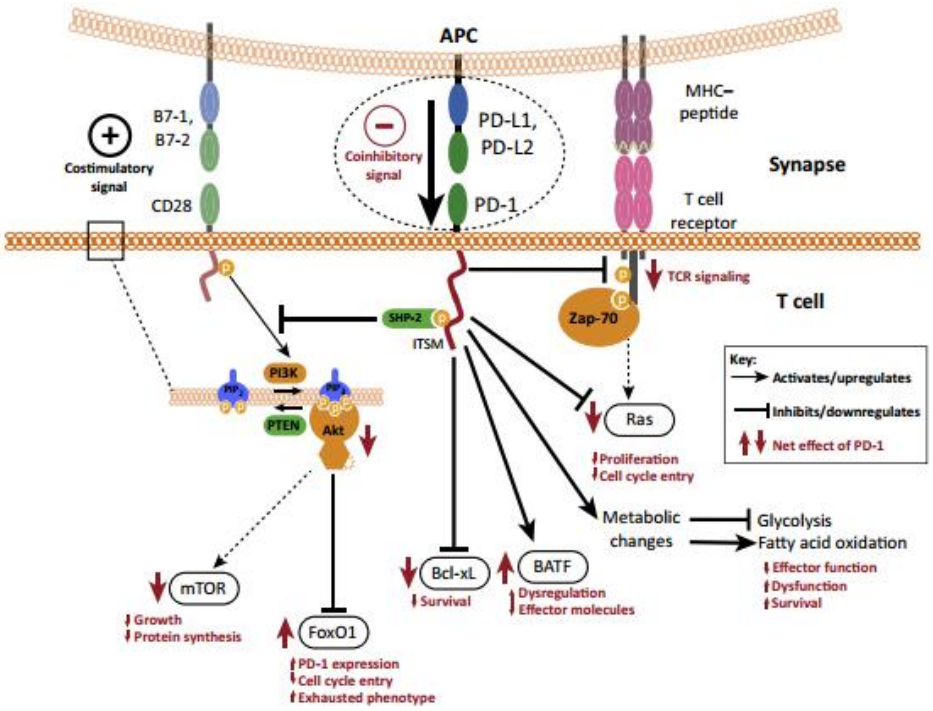

Figure 2.12: PD-1 pathway. A schematic of the proposed PD-1/PD-L1 pathway. PD-1/PD-L1 binding activates SHP-2 in binding with the ITSM tail of PD-1. This decreases the TCR signalling by means of blocking CD3 and inhibits PI3K/Akt pathway. PD-1/PD-L1 binding leads to a down-regulation of survival transcription factor mTOR, Bcl-xl and Ras while promoting dysregulation and increased expression of PD-1 via BATF and Fox01 (Chinai *et al.,* 2015).

One of the first studies that noted the importance of PD-1 with respect to $CD8^+$ T cell exhaustion was characterised using the LCMV model (Moskophidis *et al*., 1993; Larsson *et al*., 2013; Legat *et al*., 2013; Seung *et al*., 2013). This model showed that by blocking certain pathways, there could be a reverse effect in the dysfunction of the cell and this, in turn, could promote an improved ability to control viral replication (Larsson *et al*., 2013; Munier *et al*., 2013; Ezinne *et al*., 2014; Kloverpris *et al*., 2014). Furthermore, Smith *et al*., (2013), found that PD-1 was upregulated on $CD8^+$ T cells with respect to infection with the Hepatitis C virus (HCV), West Nile virus (WNV), Hepatitis B virus (HBV) and HIV-1 (Seung *et al*., 2013; Lanteri *et al*., 2014). It was also found that the level of PD-1 expression positively correlated with the extent of disease progression (Tousif *et al*., 2011; Rahim *et al*., 2013; Lanteri *et al*., 2014). In a similar study with a group of SIV positive macaques, PD-1 blocking antibodies was administered, resulting in effective $CD8^+$ T cell immunity and survival conserved (Kloverpris *et al*., 2014).

Smith *et al*., (2013), found that PD-1 expression on $CD4^+$ T cells was elevated on those cells that also express CD38 and HLA-DR in both HIV-1 positive and negative groups. In the same study, Smith *et al*., (2013), showed higher levels of activation on $CD4^+$ T cells in samples from patients infected with HIV-1, followed by EBV, CMV, HSV and VZV infections.

This hierarchy of immune activation may be related to the level of persistence or the likelihood of reactivation of the virus.

Many studies have highlighted the link between PD-1 expression on the surface of HIV-1 specific $CD8^+$ T cells, VL and disease progression to AIDS (Yamamoto *et al*., 2011; Rahim *et al*., 2013; Smith *et al*., 2013). PD-1 expressing $CD8^+$ T cells are more susceptible to both activation induced cell death (AICD) and Fas-mediated apoptosis. This was illustrated in a study by Yamamoto *et al*., (2011), who observed a reduction in viral load in patients initiating ART. In addition greater T cell survival, which was believed to be due to a decrease in PD-1 levels on $CD8^+$ T cells were also observed.

2.9.3 Tim-3 inhibitory marker

The T cell marker, Tim-3, is a membrane protein that was first documented in mouse models and functions as a negative regulator on T cells (Qiu *et al*., 2012; Dietze *et al*., 2013; Yang *et al*., 2013). Galectin-9 is the ligand for Tim-3 in T cells, however, this is debated by some, as phosphatidyl serine (PS) has also been able to bind to Tim-3 on the surface of phagocytes (Dietze *et al*., 2013; Leitner *et al*., 2013; Zhang and Shan, 2014). Nevertheless, when Tim-3 binds to its ligand, the interaction is either T cell death or T cell tolerance (Khaitan and Unutmaz, 2011; Sakhdari *et al*., 2012; Larsson *et al*., 2013; Leitner *et al*., 2013; Zhang and Shan, 2013). The proposed pathways is similar to that of PD-1, with Galactin-9 binding to Tim-3 activating the phosphorylation of SHP-2 to block the PI3K pathways which intern blocks NF-AT and limits IL-2 and other pro survival cytokines. Moreover, the binding of Tim-3 with Galactin-9 also inhibits the T cell receptor and down regulates TCR signalling (Tomkowicz *et al.,* 2015).

Additionally, Tim-3 is expressed on MO, MØ and DCs, but especially severely exhausted T cells, which secrete low levels of cytokines and have poor proliferative abilities (Gautron *et al*., 2014; Kuchroo *et al*., 2014). In a study by Wang *et al*., (2011), Tim-3 was found to have immunological suppressive effects on active TB-infected individuals. Furthermore, the regulation of $Th17^+$ cells, which are known for their role in arthritis with respect to inflammatory properties, is also under a form of control by Tim-3 (Khaitan and Unutmaz, 2011; Gautron *et al*., 2014). Increased levels of Tim-3 on activated Th1 cells are linked to the autoimmune mediated bleeding disease called immune thrombocytopenia. This disorder is characterised by a decreased production of platelets (Zhang and Shan, 2013). Several other reports also confirmed that CTLs in individuals infected with HIV-1, have higher levels of Tim-3 and displayed poor proliferation ability, as well as a dysfunctional cytokine response (Larsson *et al*., 2013; Leitner *et al*., 2013; Gautron *et al*., 2014). Likewise, studies have found that patients with active TB infection demonstrated increased expression of Tim-3 on $CD8^+$ T

cells when compared to healthy controls, but no significant difference could be found on $CD4^+$ T cells. Higher levels of Tim-3 expression were found on $CD8^+$ T cells which also expressed lower levels of IFN-γ. This is an important finding, as IFN-γ is one of the main cytokines in MØ defence against M.tb and also a key cytokine in the immune response against HIV-1 (Wang *et al*., 2011 Leitner *et al*., 2013; Yang *et al*., 2013).

2.9.4 HLA-DR and CD38 as markers of immune activation

HLA-DR, a MHC class II molecule, was characterised as an activation marker of T cells as early as 1995, and studies have shown that levels of HLA-DR and CD38 on $CD8^+$ T cells are significantly increased in HIV-1 positive patients. CD38 is a transmembrane glycoprotein, which is expressed on numerous activated cells, especially activated T cells in the presence of chronic viral infection (Deeks *et al*, 2004). Expression of CD38 is linked to processes such as cell-to-cell adhesion, production of cytokines, and increased proliferation of $CD4^+$ T cells. CD38 upregulation is also linked to exposure of oxidative stress and reactive oxygen species (Reyes *et al.,* 2015). High levels of CD38 expression on $CD8^+$ T cells are used as an instrument to determine immune activation in HIV-1 positive individuals (Lederman *et al*., 2000).

Xiao *et al*., (2011), found an increase in HLA-DR and CD38 expression on $CD8^+$ T cells from 8% in healthy individuals, and 49% in asymptomatic HIV-1 positive individuals. HLA-DR and CD38 expression on $CD8^+$ T cells is believed to be a strong predictor of HIV-1 progression to AIDS (Xiao *et al*., 2011; Tenorio *et al*., 2014; Sullivan *et al*., 2015).

Musyoki *et al*., (2014), measured the expression of HLA-DR and CD38 in pregnant women with HIV-1, versus non-pregnant women with HIV-1. The results of the study showed that pregnant women expressed lower levels of HLA-DR and CD38 on $CD8^+$ T cells when compared to the non-pregnant women. The lower levels of activation on $CD8^+$ T cells inversely correlated with an increased survival of $CD4^+$ T cells. Multiple other studies have found that the expression of CD38 and HLA-DR on $CD8^+$ T cells is correlated with its cytotoxic effector function and therefore increased clearance of viral infected cells (Hertoghe *et al*., 2000; Cockerham *et al*., 2014; Musyoki *et al*., 2014; Tenorio *et al*., 2014).

Hua *et al*., (2014), analysed the phenotype of HIV-1 controllers (HIC) in relation to CD38 and HLA-DR expression. It was established that the HIC group expressed low levels of CD38 with high levels of HLA-DR on their T cells. These findings are not in line with previous studies, as low levels of CD38 are associated with an inactive state and high levels of HLA-DR are associated with an activated state. While these findings are contradicting, B cell lymphoma (Bcl)-2 expression was also found at significantly higher levels, which is well known for its apoptotic protective properties. With the phenotype $CD38^{low}HLA\text{-}DR^{high}$,

together with an increased expression of Bcl-2, T cells were shown to limit the degree of activation and overall effector function (Hua *et al*., 2014).

Finally, in a drug study conducted by Tenorio *et al*., (2014), Rifaximin (which is a common ART drug used in first-line therapy in South Africa) was tested in relation to HLA-DR and CD38 expression. It was found that, even after starting ART, 25% of individuals did not increase their $CD4^+$ count to more than 350 cells/mm^3 after 3 years, and that both activation markers continued to be highly expressed. Those individuals expressing high levels of HLA-DR and CD38 seemed to be less responsive to drug therapy. Therefore, high levels of HLA-DR and CD38 expression are strong predictors of disease progression.

2.9.5 2B4 and LAG-3 as putative markers of activation and inhibition

CD244 (2B4) is classified as a signalling lymphocyte activation molecule (SLAM) (or CD150) and controls immune reactions (Aldy *et al*., 2011; Yang *et al*., 2013; Ezinne *et al*., 2014). This receptor is expressed on NK cells, CD4 T cells, CD8 T cells, MOs, eosinophils and basophils and is upregulated in viral infections (Ezinne *et al*., 2014; Liu *et al*., 2014). 2B4 interacts with ligand CD48 (Kahan *et al*., 2014), which upon binding, leads to cell proliferation, IL-2 production and cell survival by means of activating the SHP-2 pathways which in turn activates the PI3K/AKT signalling pathway (Aldy *et al*., 2011; Ezinne *et al*., 2014). Furthermore, Elishmereni and Levi-Schaffer (2011), found that decreasing the binding of ligand to the receptor led to a decrease in CTL activity. Similarly, Aldy *et al*., (2011), found that the levels of $2B4^+$ $CD8^+$ T cells were increased in HIV-1 positive individuals when compared to the control group, and that the 2B4 pathway may be implicated in the cytotoxic action of $CD8^+$ T cells. Furthermore, it was found that, by blocking the 2B4 pathway, the killing action of the CTLs was impaired. Other groups have also reported 2B4's dual activating and inhibitory functions, with activation manifesting at low level expression and inhibition at high 2B4 expression (Yang *et al*., 2011; Larsson *et al*., 2013; Kuchroo *et al*., 2014; Liu *et al.*, 2014). Furthermore, in relation to chronic viral infections such as HBV, HCV, LCMV and HIV-1, the expression of 2B4 on $CD8^+$ T cells was increased which resulted in decreased virus-specific proliferation and cytotoxic function and is then classified as a marker of T cell dysfunction (Yang *et al*., 2013).

LAG-3 is a transmembrane protein found on T cells, B cells, NK cells and DCs (Sega *et al*., 2014). The ligand to LAG-3 is MHC class II which, according to Juno *et al*., (2015), binds with greater affinity to MHC class II than CD4. The signalling pathway for LAG-3 has not been confirmed, however it is known that the intracellular domain KIEELE is involved (Nirsch and Drake, 2013).

Mouse models demonstrated that LAG-3 was inducible on T cells, and this was linked to the functional exhaustion of $CD8^+$ T cells in persistent infections (Larsson *et al*. 2013; Sega *et al*., 2014). Furthermore, according to Khaitan (2011), during HIV infection LAG-3 has shown to correlate with HIV viral load in patients unresponsive to antiretroviral therapy. Similar to PD-1, LAG-3 has been linked to the down regulation of T cell proliferation, activation and suppression of Treg function. Furthermore, Kahan *et al*., (2014), found that LAG-3 was able to decrease calcium levels which influence the TCR signalling pathway. LAG-3 is also associated with a decrease of cytokine production and T cell proliferation when co expressed with PD-1 (Kahan *et al*., 2014; Sega *et al*., 2014). Blocking PD-1 and LAG-3 on exhausted HIV infected T cells resulted in an increase in viral control and T cell functioning was significantly improved (Pena *et al.,* 2014).

2.9.6 Fas activation-associated and death pathway

Fas (CD95) is a death receptor on the surface of cells that leads to programmed cell death or apoptosis (extrinsic pathway) (Hertoghe *et al*., 2000). Another pathway leading to apoptotic cell death is the intrinsic pathway. The intrinsic pathway refers to the apoptosis pathway process inside the cell, in the mitochondrial membrane. When the mitochondrial membrane is disrupted, bcl-2 proteins activate the release of cytochrome c resulting in activation of caspase 3, which in turn initiates apoptosis of the cell (Figure 2.13). Conversely, the extrinsic pathway is associated with the binding of ligand to receptor via the Fas/FasL apoptosis pathway on the outside of the cell (Nardacci *et al.,* 2015). The extrinsic pathway is also associated with TNRF1 and TRAILR, which together activate apoptosis. For the purpose of this study, Fas/FasL (CD95/CD95L) will be the main focus. Together these apoptotic surface receptors bind to their respective ligands and activate the release of caspases 8 and 10, which further activates caspases 3 and 7, leading to death of the cell (Alimonti *et al.,* 2003). Fas/FasL is part of the TNF family (Baier-Bitterlich *et al*., 1997) and the nerve growth factor (NGF) family of receptors and is highly expressed on activated T cells (Waring and Mullbacher, 1999).

Fas have a central role in the regulation of the immune response after the elimination of antigen. The process is important in limiting damage and inflammation to the surrounding tissue by restricting clonal expansion of T cells (Waring and Mullbacher, 1999; Cencioni *et al*., 2015). Mice deficient in the gene, which codes for Fas/FasL, displayed lymphoproliferative autoimmune disorders, such as systematic lupus erythematosus as a result of $CD4^+$ and $CD8^+$ T cell build-up (Takahashi *et al.,* 1994; Watanabe-Fukunaga *et al.,* 1999).

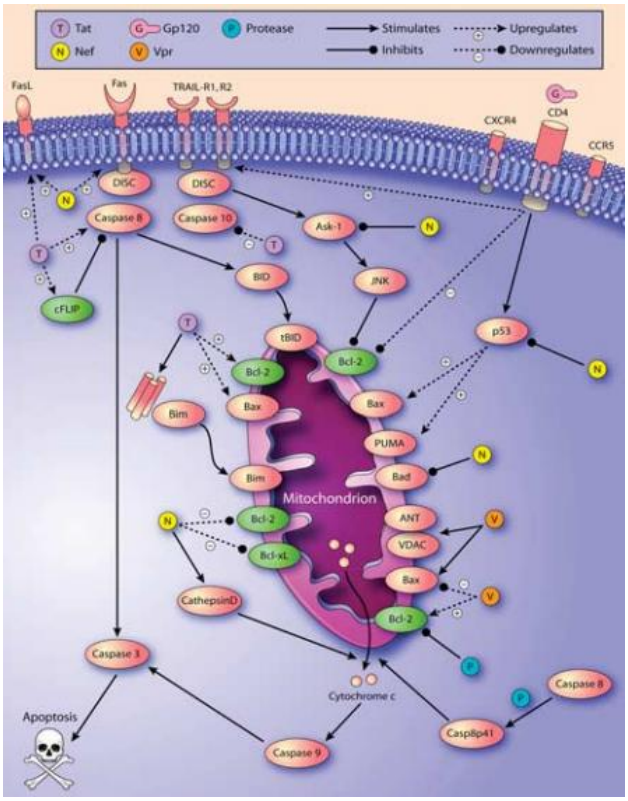

Figure 2.13: Fas death pathway. A schematic depicting viral proteins activating the intrinsic pathway by means of disrupting the mitochondrial membrane resulting in cell death. The Fas and FasL pathways are also depicted which activate caspase 8 leading to the activation of caspase 3 and/or disruption of the mitochondrial membrane resulting in cell death (Cummins and Badley, 2010).

In HIV-1 positive individuals, the largest depletion of $CD4^+$ T cells is as a result of AICD induced by the Fas/FasL pathway. This pathway is significantly up-regulated in individuals with persistent viral infections (Barathan *et al*., 2015). Fas/FasL pathway is activated by the interaction of gp120 viral proteins, CXCR4, CCR5 with the $CD4^+$ T cell membrane resulting in cell death (Oyaizu *et al*., 1994). Uninfected, bystander $CD4^+$ T cells are most affected by this process, which results in drastic $CD4^+$ T cell depletion increasing progression of morbidity and mortality. Furthermore, Nardacci *et al.,* (2015), concluded that with a decrease of bystander $CD4^+$ T cells in HIV positive individuals, a marked decrease in immune protection can be found against intracellular microbes, including helminthes and fungal infections which further weaken the immune system.

Problem statement

South Africa has one of the highest rates of HIV-1/TB co-infection and mortality in the world. While it is known how these two diseases increase the burden on the immune cells, little is known on how this impacts specific markers of the immune system in South African individuals. This problem was addressed by using 3 distinct patient groups namely a healthy control group, HIV positive group and a dually HIV/TB positive group. HIV-1 is associated with exhaustion (PD-1 and Tim-3), activation (HLA-DR and CD38), apoptosis (Fas) and inhibitory or negative regulatory markers (LAG-3 and 2B4). TB has similarly been associated with differential marker upregulation. This study addressed co-infection and how these markers link immune activation and exhaustion in such a setting.

Hypothesis

Chronic HIV-1 infection together with active TB disease amplifies the expression of all activation and exhaustion/inhibitory markers (CD38, HLA-DR, Fas PD-1, TIM-3, LAG-3 and 2B4) on $CD4^+$ and $CD8^+$ T cells.

Aims

The primary aim of this current study was to investigate the impact of TB (active disease) on exhaustion marker expression in chronic untreated HIV.

Objectives

1) To examine expression of the inhibitory markers PD-1 and Tim-3 on $CD8^+$ T cell subsets in 3 patient groups – uninfected, HIV-1 infected and HIV-1/TB co-infected individuals.

2) To compare PD-1, Tim-3, $CD38^+$ and HLA-DR expression to other less well-characterized inhibitory markers (LAG-3 and 2B4) and their association with clinical markers of disease progression (CD4 count and viral load).

3) To assess functional responsiveness of αCD3 stimulation T cells in the 3 study groups, and the impact of inhibitory pathway blockade.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design

Ethics approval

The Health Ethics Committee of Stellenbosch University approved the study. Registration numbers N07/06/133 (2007 to 2011) and N12/02/008 (2012 to 2015)(Addendum A).

Three participant study groups were included in the current study: HIV-1 infected, HIV-1/TB co-infected and an uninfected control group. The study comprised two components: a pilot study, which examined two exhaustion/negative regulatory markers, PD-1 and Tim-3, in fresh whole blood. A follow-up main study assessed these two markers in addition to 2B4 and LAG-3 in frozen PBMC. The main study also included a functional component in which αCD3 and αCD28 stimulated $CD4^+$ and $CD8^+$ T cells were monitored for proliferation by CFSE dilution, in the presence or absence of blocking of the PD-1 and Tim-3 pathways.

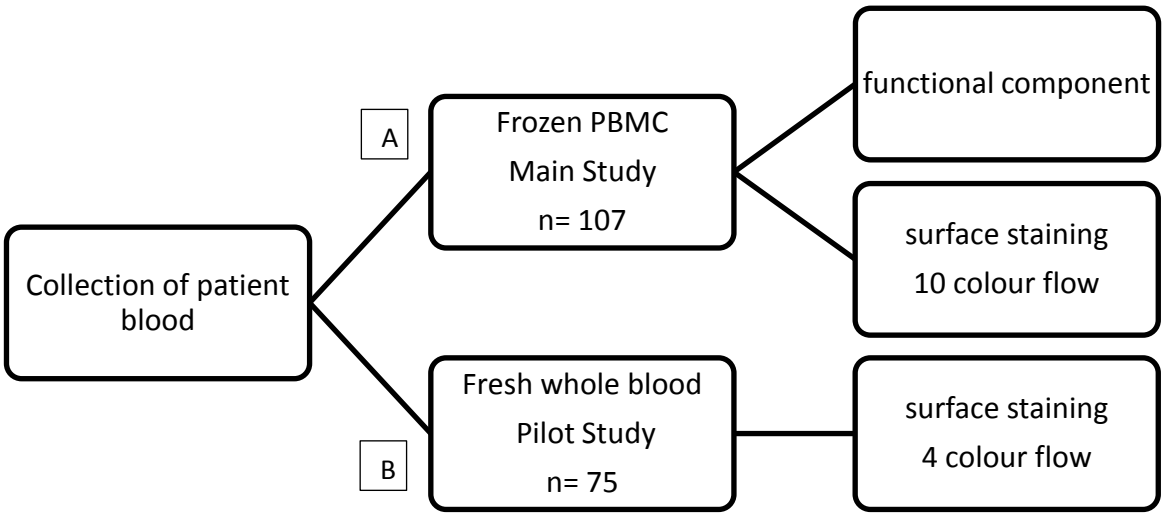

Figure 3.1: Overview of work flow. (A) Main study using frozen Peripheral blood mononuclear cells (PBMC) on a ten colour flow cytometer, markers included DAPI viability,CD45, CD3, CD4, CD8, PD-1, Tim-3, Fas, CD38, 2B4, LAG-3, HLA-DR (B) Pilot study using fresh whole blood on a four colour flow cytometer, markers included CD3, CD4, CD8, CD38, Fas, PD-1 and Tim-3.

Both studies were cross-sectional in design with a functional experimental component in the main study. The main objective of these studies was to determine the phenotype of $CD4^+$ and $CD8^+$ T lymphocytes which have been exposed to chronic viral infection with/without active TB disease by means of evaluation of a range of T cell surface markers. Markers of T cell inhibition included the well-defined PD-1 and Tim-3 markers together with the less well-defined LAG-3 (CD223) and 2B4 (CD244) markers. Fas (CD95) is associated with T cell

death, but its expression is also related to immune activation. Other markers of T cell activation include CD38 and HLA-DR. To try to obtain a clearer understanding of the relationship between these processes, a range of exhaustion/negative regulatory markers with markers of immune activation and death was combined. PBMC and fresh whole blood was used in phenotypic assessment by flow cytometry techniques. Flow cytometry panels (Tables 3.1 and 3.3) were constructed to evaluate expression of inhibitory markers together in relation to the other markers. The functional capacity of the T cells (following αCD28 and αCD3 stimulation) in chronic HIV-1 infection was measured by means of one flow panel (Table 3.7).

3.2 Study participants

Study participants were recruited from various HIV-1 and Wellness Clinics in the Western Cape area which include, Kayamandi Clinic, Durbanville Clinic, Idas Valley Clinic, Klapmuts Clinic, Kraaifontein Community Health Centre, Eerste River Hospital, Stellenbosch Provincial Hospital, Tygerberg Hospital, and Karl Bremer Hospital. Study participants were recruited by a research nurse and written consent was given by participants before inclusion into this study. Routine VL and CD4 counts were done on arrival of the blood at the Division of Medical Virology, NHLS laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus.

3.3 Inclusion criteria

The pilot study utilized fresh whole blood and the main study utilized cryopreserved PBMCs. The control group for both studies consisted of 52 patients who had never been infected with HIV-1 and showed no clinical signs of active TB infection. Clinical records were referenced to confirm either infection. The HIV-1 positive group consisted of 100 HIV-1 positive patients. HIV-1 status of all 100 patients was confirmed with the use of two rapid HIV-1 testing strips which was performed at the clinics. Patients were regarded as TB negative if there were no clinical symptoms of active infection and lack of record of any such disease in the medical records. Finally, for the 25 patients dually infected with HIV-1 and active TB, confirmation of HIV-1 was confirmed by rapid HIV testing strips and active TB infection was diagnosed when medical records confirmed culture positivity or affirmative chest x-ray (CXR). ART was an exclusion criterion for this group (i.e. all participants were treatment naïve). All patients in the TB group were on early phase TB treatment (less than or equal to 3 months maximum).

3.4 Blood collection by venipuncture

Whole blood was collected from the clinics mentioned in section 3.2 by the research nurse and transported to the NHLS Medical Virology laboratory, Faculty of Medicine and Health

Sciences, Tygerberg Campus within 2 hours of collection. A total of 15ml was collected from each patient: 10ml in sodium Heparin tubes and 5ml in ethylenediamine tetraacetic acid (EDTA) tubes. HIV-1 VL testing and CD4 counts were done on the EDTA blood (from plasma and whole blood, respectively), while p24 enzyme-linked immunosorbent assay (ELISA) was done on plasma from sodium heparin tubes.

3.5 Data collection methods and instruments

The HIV-1 positive group underwent two rapid HIV-1 tests for confirmatory purposes at the clinic. The TB positive group underwent sputum culture tests, pulmonary chest x-ray and clinical comprehensive examination to confirm their status at the clinic before enrolling in this study. The HIV-1 negative group first underwent a rapid HIV-1 test (First response HIV test) at the clinic. A confirmationatory p24 ELISA test (Vironostika, BioMerieux, The Netherlands) was done using their plasma from their blood at the in the NHLS Medical Virology laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus.

3.5.1 Rapid *HIV test*

For both the pilot and the main study, rapid HIV-1 tests were administrated by a registered nurse at the clinics before the blood was collected and brought to NHLS Medical Virology laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus for processing. Once the patient had agreed to take part in this study, an informed consent form was signed. The First Response HIV test (Premier Medical Corporation Limited, Daman, India) was the rapid test used to check for antibodies against HIV-1 and HIV-2. In the event that the rapid test was positive for HIV-1 or HIV-2, a secondary confirmatory test (Abon Biopharm, Hangzhou, China) was preformed to verify the result. In the event that one test was positive and one test was negative, a repeat of the first test was performed using the First Response HIV test.

The sensitivity and specificity of the First Response HIV test is 94% and 91% respectively and it can detect both HIV-1 and HIV-2. The device contains membrane coated recombinant HIV-1 antigens on a specific test area of the strip, which binds to HIV-1 and HIV-2 antibodies in the patient full blood sample.

3.5.2 p24 ELISA

This assay was conducted in order to ensure that all samples collected from HIV-1 negative patients were true negatives. The Vironostika HIV-1 Antigen Microelisa system (BioMerieux, The Netherlands) is a quantitative enzyme-linked immunosorbent assay (ELISA) specific for the HIV-1 p24 core antigen and was done on HIV negative patient samples only.

Briefly, the Vironostika p24 ELISA was performed as follows. Murine monoclonal antibodies to the HIV-1 p24 antigen are attached to the microwells of the plate, which was provided with the assay kit. Disruption buffer was added to all the microwells, whereafter standards, controls and plasma were added to all respective microwells, the plate was incubated at 37°C for 1 hour. In the event that there was HIV-1 present in the sample being tested, viral antibodies in the sample would bind to the antigen attached to the wells. Unbound protein was then removed by washing the plate on a Multiwash II Tri-continental plate washer (Healthcare Technologies, Cape Town, South Africa). Monoclonal mouse anti-human HIV-1 conjugate labelled with horse radish peroxide (HRP) was then added to all microwells and the plate incubated again at 37°C for 1 hour. During this incubation step, the labelled antibody could bind to the solid phase antibody/antigen complex. Another wash step followed to remove all unbound complexes before adding the tetramethylbenzidine (TMB) substrate. During a 30 minute incubation period at 30°C, a colour change developed, which was stopped with 1 M sulphuric acid. This changed the blue colour to yellow and the plate is then read on a spectrophotometer at 450 nm. The intensity of the colour was directly proportional to the concentration in mg/ml, which was calculated from the standard curve generated by an Elx800 ELISA reader according to SOP.BSL3.00026.2, NHLS Medical Virology laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus.

3.5.3 CD4 count

CD4 counts were conducted for all patients in the three study groups. CD4 tests are regarded as a clinical marker of HIV-1 disease progression. Flow cytometry was used with the BD Multi Test True Count kit (BD Biosciences, San Jose, CA, USA), which includes the associated antibodies and tubes needed for this study. The flow cytometer utilised a four-colour technique for CD4 quantification (BD FACS Calibur, San Jose, CA, USA). All CD4 counts were determined in the in the Medical Virology Laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus.

Fresh blood collected in EDTA tubes was stained with an antibody cocktail (CD3/8/45/4) that enables quantification of total $CD8^+$ and $CD3^+$ cells. The results from this test show total T cells ($CD3^+$), Th cells ($CD3^+CD4^+$) and CTL cells ($CD3^+CD8^+$). Both absolute counts and percentages are determined when using the BD MultiSET® staining kit with Tru-count tubes (BD Biosciences, San Jose, CA, USA) in accordance with SOP.MOL.00016.5, NHLS Medical Virology laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus.

3.5.4 Viral load tests

HIV-1 VL was measured on all HIV-1 positive and HIV-1/TB co-infected patient samples. This test was done according to the SANAS accredited Abbott RealTime HIV-1 (Abbott

Laboratories, Germany) method in the routine NHLS Medical Virology laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus (SOP: HAE1455 NHLS version 5). It quantifies and reports the number of viral copies of HIV-1 that are present in 1 ml of whole blood in EDTA tubes (Abbott Laboratories, Germany). All VL tests were determined in the in the NHLS Medical Virology laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus.

3.5.5 PBMC isolation

Processing of whole blood for PBMC isolation was performed as outlined in the standard protocol used in the NHLS Medical Virology laboratory, Faculty of Medicine and Health Sciences, Tygerberg Campus (SOP: VIRO.BSL3.SOP.00042.0). Briefly, on arrival, the sample was centrifuged at 2000 rpm (~ 500 x g) for 10 minutes to separate the cells into their respective layers as shown (Figure 3.2). The plasma layer of the sample was removed and replaced with an equal volume of phosphate buffered saline (PBS) (LONZA, BioWhittaker, Belgium). Plasma was stored at -80°C in 1 ml cryogenic vials for subsequent p24 ELISA testing of HIV-1negative samples.

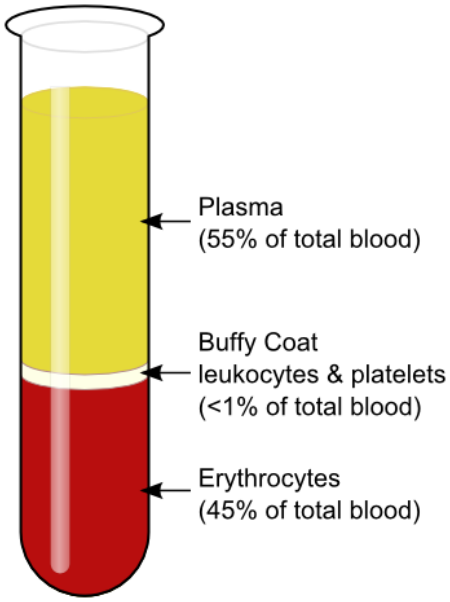

Figure 3.2: Blood components. A visual representation of the 3 main layers of blood including plasma (containing cytokines), buffy coat (containing leukocytes and platelets) and erythrocytes (the red blood cell) (Blood and Immunity Test, 2015).

Ficoll Histopaque (FH) (Sigma Life Sciences, St. Louis, MO, USA) was used in the process of separating PBMC based on density gradient. The concept behind FH is that once fresh whole blood has been layered on top of FH and centrifuged, the cells with the highest

density (red blood cells) move below while less dense cells (PBMC) remain above the FH. FH was used in a 1:2 ratio with the blood-PBS mixture. In a separate tube, the whole blood was carefully layered on top of the FH with special care not to mix the two layers together. Once layered, the sample was centrifuged again at 2000 rpm (~500 x g) for 30 minutes at room temperature (22ºC – 26ºC).

On removing the sample tube from the centrifuge, PBMC layer was clearly visible (Figure 3.3). The PBMC layer and was removed in a circular motion using a Pasteur pipette 3 ml (Lasec, SA) and transferred to a 15 ml BD falcon tube (BD Biosciences, San Jose, CA, USA). PBS was then added to the 15ml mark to perform the first wash step. The tube was centrifuged again at 1200 rpm (~300 x g) for 17 minutes at room temperature. The supernatant fluid was poured off and the pellet was re-suspended. PBS was again added to the sample and a second wash step was done by another centrifugation step at 1200 rpm (~300 x g) for 17 minutes at room temperature. The supernatant fluid was discarded again and the pellet re-suspended in 1ml of PBS.

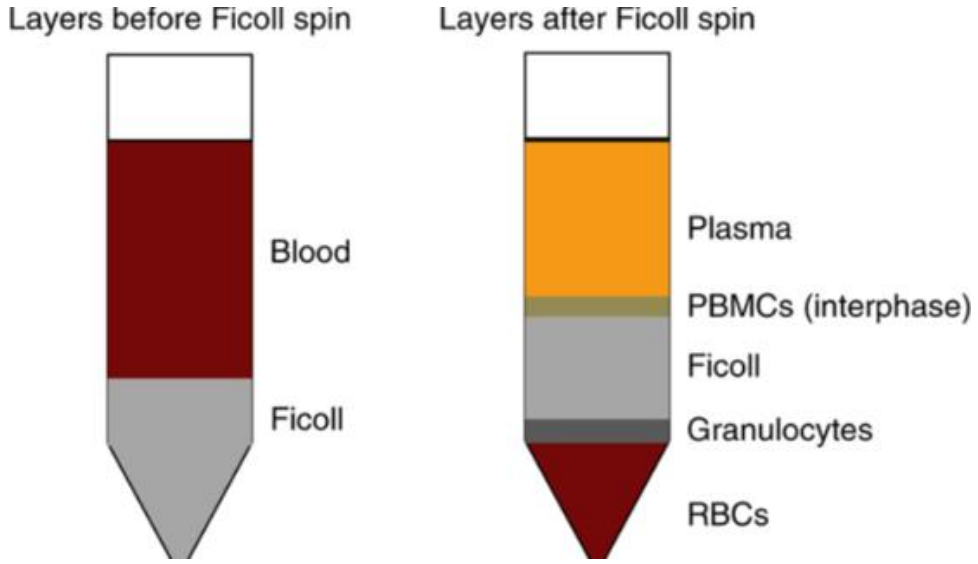

Figure 3.3: Separating blood into individual components using Ficoll Histopaque. A visual representation of the layering of blood on top of Ficoll Histopaque as a method of separating cells according to size (Lin *et al.,* 2014).

3.5.6 Cell viability

Viable cell counting was performed by adding 10 µl Trypan blue which stains dead cells based on the principle that viable/live cells do not take up this dye (LONZA, BioWhittaker, USA) to 10 µl of PBMC sample and mixed thoroughly. The mixture was then placed on a BIO-RAD dual chamber cell counter slide (BIORAD, USA) and inserted into the automated cell counter (BIORAD, USA). Cell counts are performed to determine both number of cells and their viability prior to preparation a total of one million cells for freezing in 2 ml cryogenic vials (Corning Incorporated, New York) before cryopreservation.

3.5.7 PBMC freezing procedure/ Cryopreservation

Freezing medium was prepared, consisting of 10% dimethyl sulfoxide (DMSO) in foetal bovine serum (FBS) (Capricorn Scientific, South America) and DMSO (Sigma Life Sciences, Mo, USA). It was added to the cells in a drop-wise manner in order to minimize cell damage. One million cells were frozen per ml. PBMCs in cryogenic vials were placed in a Mr Frosty™ freezing container (Nalgene, USA) and storedovernight in a -80°C ultra-low temperature freezer. The cryogenic vials were then individually stored in appropriate cryogenic boxes for long term storage in the liquid nitrogen tank at -127°C until further processing and analysis.

3.5.8 Thawing of frozen PBMC

A water bath was heated to 37°C prior to thawing the cells for 1-2 minutes. Once thawed fully, the content was quickly poured into a 15 ml BD falcon tube. Growth medium was prepared prior to defrosting the sample consisting of 10% FBS and 90% RPMI 1640 (Life Technologies, UK). The growth medium was added to the defrosted sample to the 15 ml mark and centrifuged for 10 minutes at 1000 rpm (~250 x g). The pellet was gently re-suspended and centrifuged at 1000 rpm (~250 x g) for 10 minutes. The supernatant was discarded and the pellet was gently re-suspended in 1 ml of growth medium prior to cell count. Cells were counted and adjusted to the correct concentration based on the Trypan blue staining results. Once the cell count was performed, the tube was centrifuged again at 1000 rpm (~250 x g) for 10 minutes to separate cells from the medium in preparation for staining for flow cytometry or for functional experiments (Refer to sections 3.6.1, 3.6.3 and 3.7.1). For staining purposes, the supernatant was discarded and 1 ml of staining solution (BioWhittaker, Walkersville, MD, USA) was added to the cells.

3.6 Surface marker antibody staining

3.6.1 Fresh blood processing for pilot study

The fresh whole blood sample was divided into 100 µl aliquots, which were each added to the individual panels using four 5 ml FACS tubes (BD Falcon, BD Biosciences, MA, USA) respectively. Blood was added to the antibody cocktail and the sample was incubated for 30 minutes in the dark at room temperature in order for the antibodies to bind to the blood. The sample was kept in the dark in order to preserve the fluorochromes of the antibodies (Table 3.1). After the first incubation period, 2 ml of lysing solution (BD San Jose, CA, USA) was added to lyse the red blood cells.

The sample was incubated for a further 15 minutes in the dark at room temperature. The sample centrifuged for five minutes at 1500 rpm (~375 x g). Supernatant and 2 ml of staining buffer were added. Staining buffer (BioWhittaker Walkersville, MD, USA) was used to stabilise the cells, and as a medium to wash the cells of leftover cell debris.

The FACS tube was lightly tapped in order to gently mix the remaining pellet into solution. The sample was again centrifuged at 1500 rpm (~375 x g). The resulting supernatant was discarded and 350 µl of staining buffer was added to ensure the integrity of the cells until the sample could be processed later on the same day using a BD FACS CALIBUR flow cytometer. BD Stabilising Fixative (BD San Jose, CA, USA) was used to preserve the cells for up to a week to stop degradation of the markers and antibodies until ready to process on the BD FACS CALIBUR flow cytometer (San Jose, CA, USA). These samples were processed within a 2-day time frame using the BD FACS CALIBUR flow cytometer. All sample data were collected using BD CellQuest Pro Software (BD Biosciences, San Jose, CA, USA).

Table 3.1: Pilot study antibody cocktails for assigned panels. Four antibodies were added per tube to make up the cocktail mix in panel. Then whole blood was added to each tube. Both panels 1 and 2 are focused on CD38 and PD-1 expression on $CD8^+$ and $CD4^+$ T cell activity. Panels 3 and 4 are focused on Tim -3 and CD95 expression on $CD4^+$ and $CD8^+$ T cell activity.

Panel	FITC	PE	PerCP	APC	Total volume (µl)
1	CD3 (10 µl)	PD-1 (2.5 µl)	CD8 (10 µl)	CD38 (2.5 µl)	25 µl
2	CD3 (10 µl)	TIM-3 (2.5 µl)	CD8 (10 µl)	CD38 (2.5 µl)	25 µl
3	CD3 (10 µl)	TIM-3 (2.5 µl)	CD4 (10 µl)	CD95 (2.5 µl)	25 µl
4	CD3(10 µl)	PD-1 (2.5 µl)	CD4 (10 µl)	CD95 (2.5 µl)	25 µl

PerCp = Peridinin chlorophyll protein; APC = Allophycocyanin; PE = Phycoerythrin; FITC = Fluorescein isothocyanate

3.6.2 Sources of monoclonal antibodies for pilot study

Below is a comprehensive tabulated list of all antibodies used in this study. Volumes were based on manufacturer's recommendations and subsequently confirmed by titration (Table 3.2).

Table 3.2: Pilot study: List of antibodies, manufacturers, isotypes and clones

Antibodies	Panel	Manufacturer	Cat #	Volume (µl)	Clone	Isotype
CD3 - FITC	BB	BD Biosciences, CA, USA	555339	10	HIT3a	IgG2,k
Tim-3 - PE	P2,P3	Biolegend, CA, USA,	345006	2.5	F38-2E2	IgG1,k
CD95 - APC	P3,P4	Biolegend, CA, USA	305612	2.5	DX2	IgG1,k
PD-1 - PE	P1,P4	Ebioscience, CA, USA	737659	2.5	J105	IgG1, k
CD4 - PerCP	P3,P4	BD Biosciences, CA, USA	345770	10	sk3	IgG1,k
CD8 - PerCP	P1,P2	BD Biosciences, CA, USA	34774	10	HB-7	IgG1,k
CD38 - APC	P1,P2	Biolegend, CA, USA	345807	2.5	HIT2	IgG1,k

PerCp = Peridinin chlorophyll protein; APC = Allophycocyanin; PE = Phycoerythrin; FITC = Fluorescein isothocyanate; P1 = Panel 1; P2 =Panel 2; P3= Panel 3; P4= Panel 4; BB = Back bone.

3.6.3 Preparation of panels for main study

Antibodies were combined to form two antibody cocktails as shown in Table 3.3. The sample was divided into 0.5 million cells which were used for the individual panels in two 5 ml FACS tubes (BD Biosciences, MA, USA). Sample was added to the antibody cocktail based on manufacturer recommendations and titration experiments and the sample was incubated for 30 minutes in the dark at room temperature in order for the antibodies to bind to the cells.

After the incubation period, the sample was centrifuged for ten minutes at 1000 rpm (~250 x g). The FACS tube was lightly tapped in order to gently mix the remaining pellet into solution. Sample was again centrifuged at 1000 rpm (~250 x g) for 10 minutes. The resulting supernatant was discarded and 500 µl of staining buffer was added to ensure the integrity of the cells until the sample could be processed later on the same day using the Navios, Beckman Coulter flow cytometer (Beckman Coulter, CA, USA). Five hundred microliters of BD Stabilising Fixative (BD Biosciences, San Jose, CA, USA) was used to preserve the cells for up to a week to stop degradation of the markers and antibodies until ready to process on the Beckman Coulter flow cytometer. However, samples were usually processed within a day.

Table 3.3: Main study: Antibody cocktails for assigned panels. Nine antibodies were added per tube to make up the cocktail mix in panel. Panel 1 includes novel inhibitory/exhaustion markers. Panel 2 includes markers associated with inhibitory/exhaustion and apoptosis.

Panel	FITC	PE	ECD	PE5	PE7	A647	A700	APC	PB	KO	TV
1	CD244 (2.5µl)	TIM-3 (2.5µl)	CD8 (10µl)	X	CD223 (2.5µl)	PD-1 (2.5µl)	CD3 (2.5µl)	CD4 (5µl)	DV (2.5µl)	CD45 (10µl)	40µl
2	CD95 (10µl)	CD38 (10µl)	CD8 (10µl)	DR (10µl)	X	PD-1 (2.5µl)	CD3 (2.5µl)	CD4 (5µl)	DV (2.5µl)	CD45 (10µl)	62.5µl

PE5=PE-CY5; PE7=PE-CY7; A647=ALEXA647; A700=ALEXA700; PB=PACIFIC BLUE; KO=KROME ORANGE; DV=DAPI VIABILITY; DR= HLA-DR; TV=Total volume in µl.

3.6.4 Sources of monoclonal antibodies for main study

Below is a comprehensive tabulated list of all antibodies used in this study. Volumes were based on manufacturer's recommendations and subsequently confirmed by titration (Table 3.4).

Table 3.4: Main study: List of antibodies, manufacturers, isotypes and clones

Antibodies	Panel	Manufacturer	Cat #	Volume (µl)	Clone	Isotype
CD95 – FITC	P2	Beckman Coulter, France	IM1506	10	UB2	IgG1
CD38 – RPE	P2	Beckman Coulter, France	A07779	10	LS198-4-3	IgG1
CD4 – APC	BB	Beckman Coulter, France	A94682	5	13B8.2	IgG1
CD8 – ECD	BB	Beckman Coulter, CA, USA	737659	10	SFCI12Thy2 D3[5,6,22,23]	IgG1[23]
DAPI Viability	BB	Beckman Coulter, CA, USA	B30437	2.5	REF: B30437	PN: B37432-AB
CD45 – KO	BB	Beckman Coulter, France	A96416	10	J.33	IgG1,k
CD223 – PE-Cy7	P1	eBioscience, CA, USA	252239	2.5	3DS223H	IgG1, k
CD244 – FITC	P1	BioLegend, CA, USA	329506	2.5	C1.7	IgG1.k
TIM-3 – PE	P1	BioLegend, CA, USA	345006	2.5	F38-2E2	IgG1, k
PD-1 – AF647	BB	BioLegend, CA, USA	329910	2.5	EH12.2H7	IgG1, k
CD3 – AF700	BB	BioLegend, CA, USA	317340	2.5	OKT3	IgG2a, k
HLA-DR–PE-CY5	P2	BD Biosciences, CA, USA	551375	10	TU36	IgG2b, k
αCD3	**F**	**eBiosciecnes, CA, USA**	**160037**	**50**	**OKT3**	**IgG2a, k**
αPD-1	**F**	**BioLegend, CA, USA**	**329912**	**50**	**EH12.2H7**	**IgG1, K**
αCD28	**F**	**eBiosciences, CA, USA**	**160289**	**50**	**CD28.2**	**IgG1, k**
αTim-3	**F**	**BioLegend, CA, USA**	**345004**	**50**	**F38-2E2**	**IgG1, k**

PerCp = Peridinin chlorophyll protein; APC = Allophycocyanin; PE = Phycoerythrin; FITC = Fluorescein isothocyanate; F= Functional; P1 = Panel 1; P2 =Panel 2; BB = Back bone

Last four antibodies were used in the proliferation assay as blocking agents.

3.7 Functional Component for main study

3.7.1 *In vitro* stimulation

A total of ten patient samples were used for this component. Selection was based on sufficient stored material being available (>4 vials per patient remaining after completion of the previous staining procedures). This work included stimulation of PBMC with αCD3/αCD28 in the presence/absence of αPD-1 and/or αTim-3. The control included an unstimulated sample. In the pathway component five conditions were analysed in order to understand if blocking certain inhibitory pathways results in improved cell effector function.

3.7.2 Coating plates for stimulation assay

Round bottom 96 well plates (Greiner Bio-One, Germany) were used with corresponding plate lids (Greiner Bio-One, Germany). A final concentration of 0.25 µg/µl of functional αCD3 (eBioscience, USA) was used with 10% RPMI working stock as recommended by the manufacturer for T cell stimulation. Fifty microliters was then added to four wells in preparation for cell stimulation. Fifty microliters of PBS was added to one well which was used as the unstimulated control well (Table 3.5). The plate was covered with the plate lid and left overnight at room temperature as recommended by the manufacturer to obtain plate-bound αCD3 (eBioscience, USA).

Table 3.5: Experimental plate setup. Each well was coated with functional αCD3 (except for unstimulated)

	Well 1	**Well 2**	**Well 3**	**Well 4**	**Well 5**
	US	SO	STim-3	SPD-1	S^+T^+P
PBS	50µl	X	X	X	X
Functional grade αCD3	X	50µl	50µl	50µl	50µl

US= unstimulated; SO= stimulated only; STim-3= stimulated Tim-3; SPD-1; S^+T^+P= stimulated and Tim-3 and PD-1

3.7.3 Cell staining with CFSE for proliferation determination

A 5 mM 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) stock solution was prepared by adding 1 vial of CFSE 100 µg (BioLegend, CA, USA) into a 5 ml BD falcon tube together with 36 µl of DMSO. This was then further diluted by adding 1 ml of PBS. The final CFSE concentration which was added to the cells was 5 µM. The amount of CFSE (BioLegend, CA, USA) added to the cells for *in vitro* proliferation was dependent on the cell count per ml as indicated on the product package insert.

Cells were incubated for 10 minutes at 37°C. The reaction was stopped by adding 44 ml of ice cold 10% RPMI stock solution. Cells were centrifuged for 10 minutes at 1000 rpm (~250 x g). Supernatant was discarded and pellet re-suspended in 500 µl of growth medium.

3.7.4 Cell stimulation and pathway blocking

Fifty microliters of CFSE-labelled cells were transferred to each of the five wells as shown in Table 3.6. In the unstimulated well: 150 µl of growth medium (RPMI+ 10% FBS) was added to the cells as shown in Table 3.6.

In the stimulated only well (i.e. no blocking): 100 µl of growth medium was added to the cells, diluted with growth medium for a final concentration of 10 µl of functional αCD28.

In the stimulated and αTim-3 well, 50 µl of growth medium was added to the cells diluted with growth medium for a final concentration of 10 µl of functional αCD28. Fifty microlitres of functional αTim-3 at a final concentration of 2.0 µg/µl was added to the cells with growth medium. An additional 50 µl of growth medium was added.

In the stimulated and αPD-1, 50 µl of growth medium was added to the cells diluted with growth medium for a final concentration of 10 µl of functional αCD28. Fifty µl of functional αPD-1 at a final concentration of 0.5 µg/µl was added to the cells with growth medium. An additional 50µl of growth medium was added.

Finally, in the stimulated well with αPD-1 and αTim-3, 50µl of growth medium was added to the cells diluted with growth medium for a final concentration of 10 µl of functional αCD28. 50 µl of functional αPD-1 at a final concentration of 0.5 µg/µl was added to the cells with growth medium. And 50 µl of functional αTim-3 at a final concentration of 2.0 µg/µl was added to the cells with growth medium.

The plates were covered and placed in the incubator at 37°C for 24-48 hours as recommended by the manufacturer (eBiosciences, USA).

Table 3.6: Antibody blocking setup. Reagents add to each round bottom well per patient sample

	Well 1	**Well 2**	**Well 3**	**Well 4**	**Well 5**
	US	SO	STim-3	SPD-1	$S^{+}T^{+}P$
Functional grade αCD28 (µl)	X	50µl	50µl	50µl	50µl
Functional grade αTim-3 (µl)	X	X	50µl	X	50µl
Functional grade αPD-1 (µl)	X	X	X	50µl	50µl
Cells (µl)	50µl	50µl	50µl	50µl	50µl
10% RPMI working stock (µl)	150µl	100µl	50µl	50µl	X
Total in each well (µl)	200µl	200µl	200µl	200µl	200µl

US= unstimulated; SO= stimulated only; STim-3= stimulated Tim-3; SPD-1; $S^{+}T^{+}P$= stimulated and Tim-3 and PD-1

3.7.5 Functional staining panel

The 96 well plates were centrifuged for ten minutes at 1000 rpm (~250 x g). The supernatant was discarded and the content of each well was pipetted into five 5 ml BD Falcon tubes in preparation for cell surface staining (Table 3.7). Sample was added to the antibody cocktail as recommended by manufacturer or as per titration and incubated for 30 minutes in the dark at room temperature. After the incubation period, the sample was centrifuged for ten minutes at 1000 rpm (~250 x g). The supernatant was discarded, the pellet re-suspended in 1 ml of BD fixative and processed on Beckman Coulter flow cytometer on the same day.

Table 3.7: Functional panel 3. Using CFSE staining to track the change in proliferation events

Panel	**FITC**	**PE**	**ECD**	**PE5**	**PE7**	**A647**	**A700**	**APC**	**PB**	**KO**
3	CFSE	CD38	CD8	X	X	PD-1	CD3	CD4	DV	CD45

PE5=PE-CY5; PE7=PE-CY7; A647=ALEXA647; A700=ALEXA700; PB=PACIFIC BLUE; KO=KROME ORANGE; DV=DAPI VIABILITY

3.8 Flow cytometry data acquisition for pilot study

Flow cytometry was performed using BD CellQuest Pro software (BD Biosciences, CA, USA) for the BD FACS CALIBUR flow cytometer. Cells were gated based on FSC verse SSC (G1 –R1) and SSC versus CD3 (G2 – R2). $CD8^+$ on $CD4^+$ associated expression of Fas, PD-1, Tim-3 and $CD38^+$ was then possible on a two colour dot plots.

With respect to data acquisition, events acquired were 20 000 $CD3^+$ lymphocyte gated events.

3.9 Flow cytometry data acquisition for main study

Samples were acquired and analysed on a Beckman Coulter, Navios flow cytometer with Navios software (Beckman Coulter, CA, USA). Additional post-acquisition analysis was performed using Kaluza version 1.3 software (Beckman Coulter, FL, USA).

A total of 100 000 events was acquired although in some patients with low overall CD4 count of <250 µl less than 100 000 events were acquired. Cells were gated according to singlet discrimination (Population 1, P1) followed by DAPI viability dye which stains dead cells (Population 2, P1) followed by CD45 vs. SS for lymphocytes (Population 3, P1), CD3 for T lymphocytes (population 4, P1) CD4 and CD8 (Population 5, P1). Gating on these first five populations was common to all three panels conducted in this study. Additionally, panel 1 included markers of exhaustion while panel 2 included markers of activation and apoptosis. All surface markers of interest were plotted against each other on both $CD4^+$ T cells and $CD8^+$ T cells. Forward scatter and side scatter were used to define the lymphocyte population (Figure 3.4).

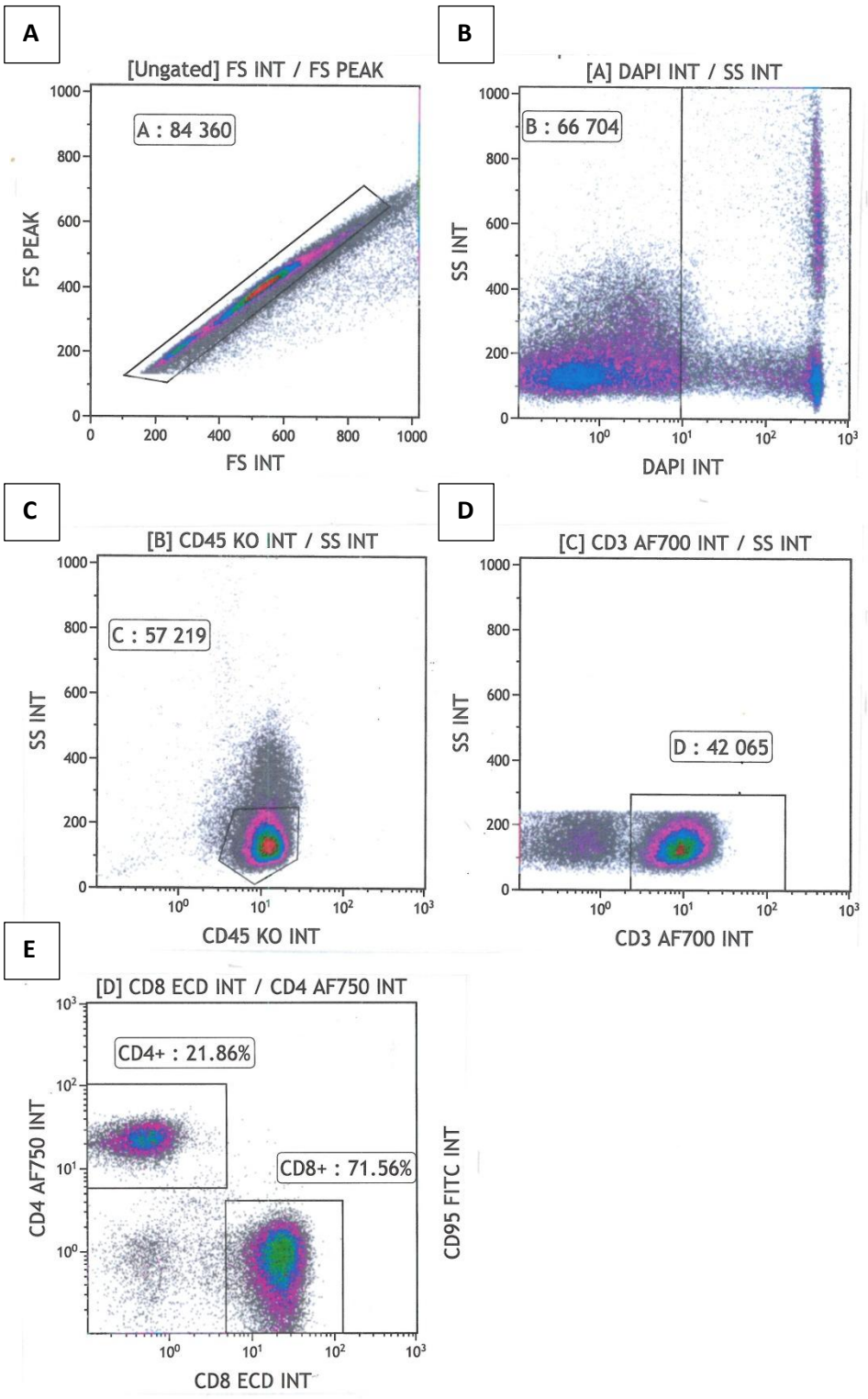

Figure 3.4 Gating strategy common to all panels in the main study. (A) single cells, (B) cell viability, (C) $CD45^+$ for leukocytes, (D) $CD3^+$ lymphocytes, (E) $CD4^+$ T cells and $CD8^+$ T cells

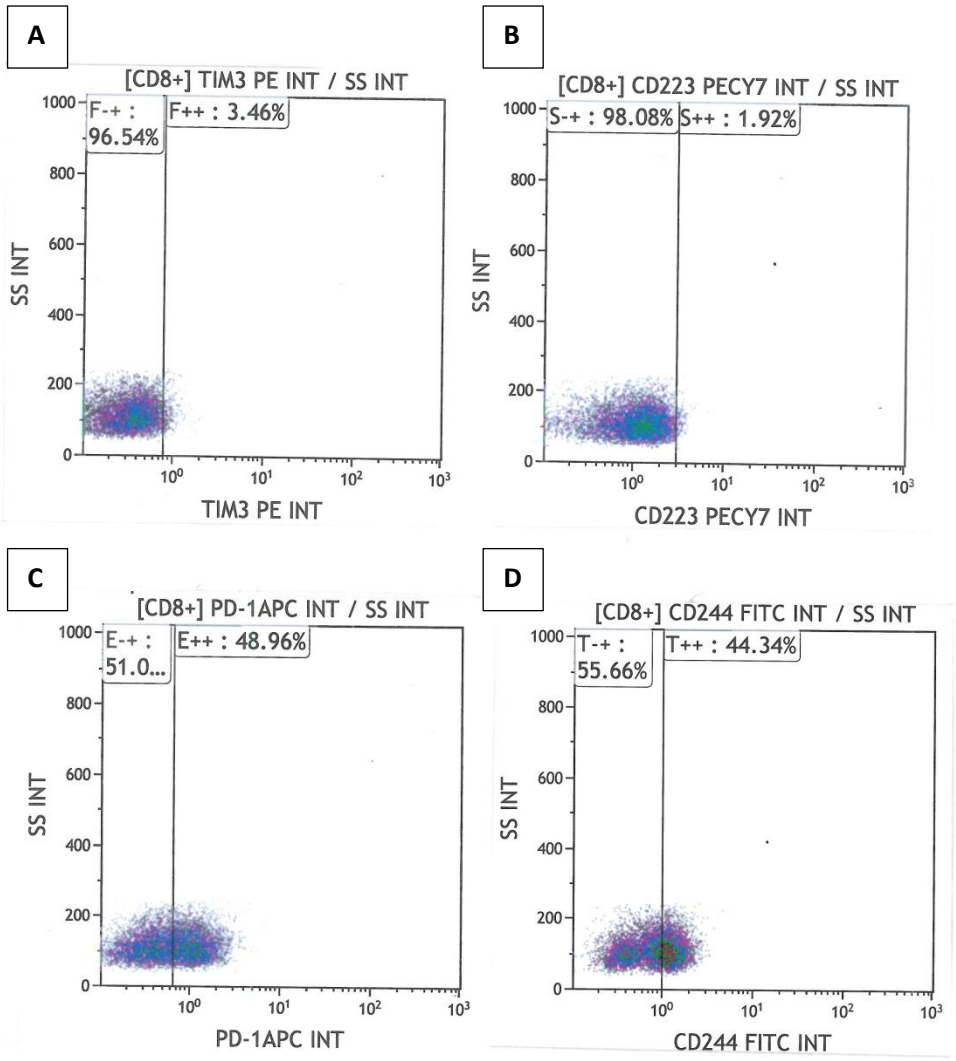

Figure 3.5 Panel 1. Highlighting marker expression on $CD8^+$ T cells of LAG-3, 2B4, Tim-3 and PD-1 which was the common expression marker between the two panels. The same gating strategy was used all markers for $CD4^+$ T cells (not shown).

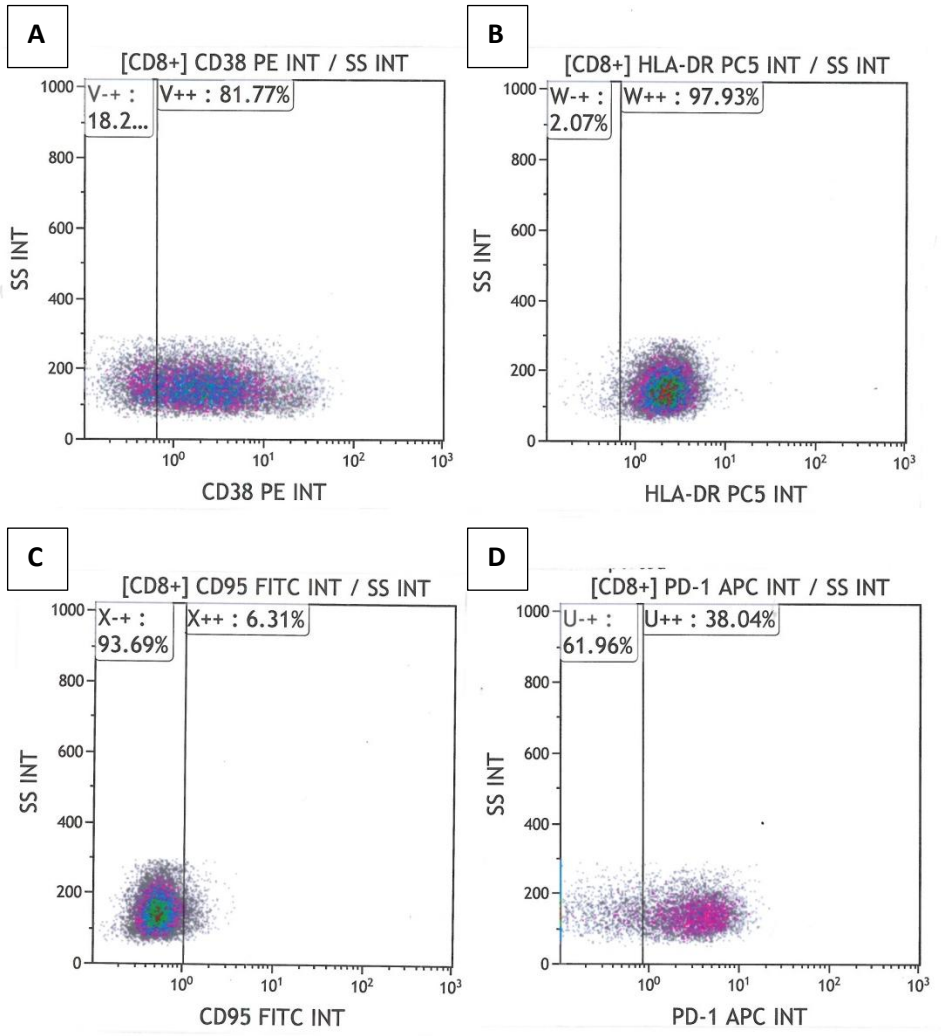

Figure 3.6 Panel 2. Highlighting marker expression of Fas, CD38, HLA-DR and PD-1 which is the common expression marker between the two panels. The same grating strategy was conducted for $CD4^+$ T cells (not shown).

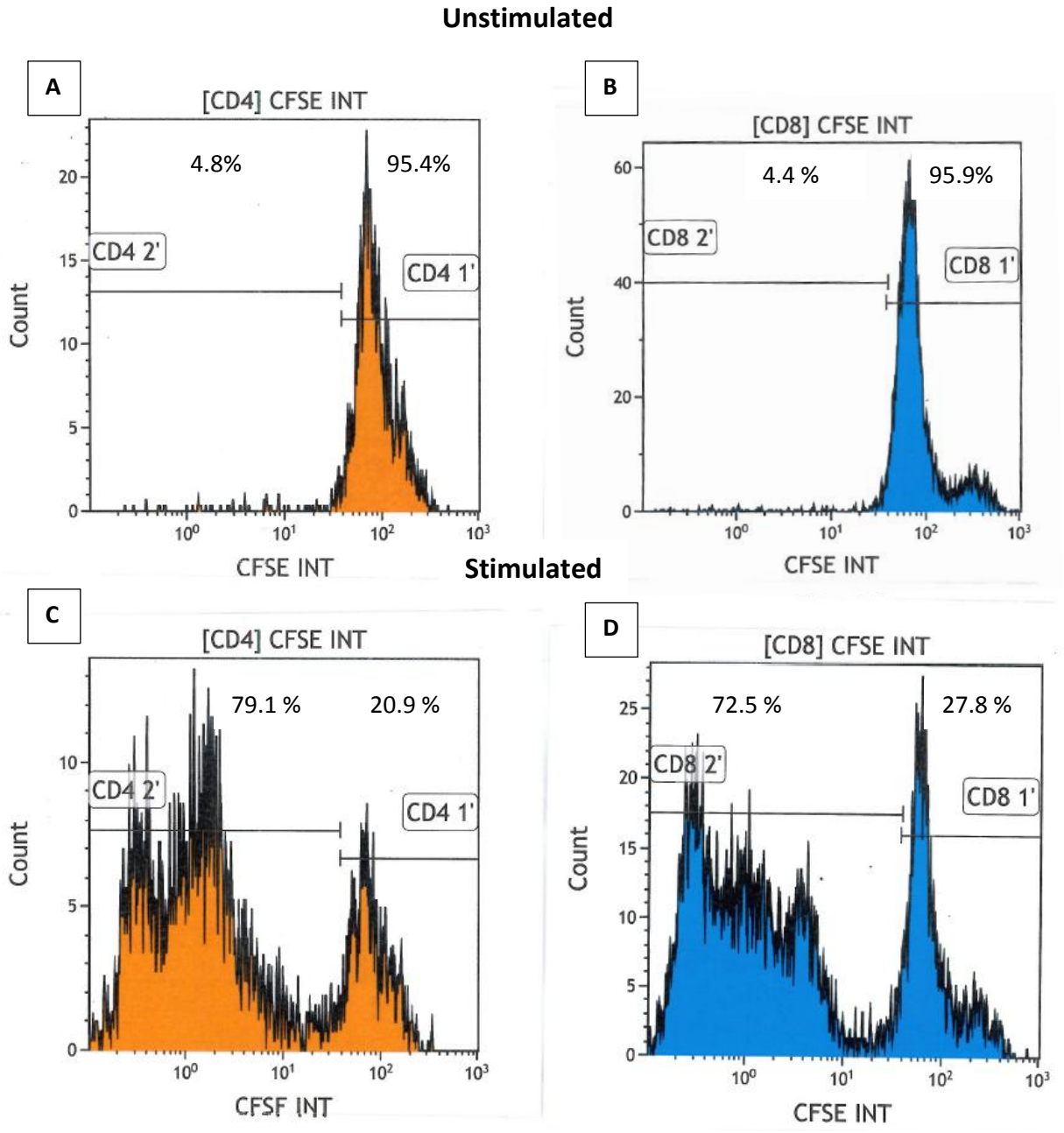

Figure 3.7 Panel 3. Highlighting the five blocking conditions and how well the cells were able to proliferate thereafter. Diagram A and B are resting, unstimulated $CD4^+$ and $CD8^+$ T cells. Diagram C and D illustrate proliferation of the cells with marked populations on the left side of each diagram for both $CD4^+$ and $CD8^+$ T cells.

3.10 Calculations of percentage of proliferating cells

The percentage proliferating cells was calculated by setting a population region discriminator in the unstimulated cells. All events to the left of the CFSE peak were considered proliferating. In the above figure the unstimulated $CD4^+$ T cells displayed 4.80% proliferation, and the stimulated 9.30%. Similarly, in the $CD8^+$ T cells, the unstimulated cells displayed 4.55% proliferating cells, whereas the stimulated 7.57%.

3.11 Optimizing flow cytometry

Standardization/optimization was divided into three sections namely, antibody titrations, Fluorescence Minus One (FMO) and compensation (Maeker and Trotter, 2006).

3.11.1 Titrations

Original manufacturer's recommended volumes were used, which were later re-adapted based on titrations. Titrations are preformed to ensure that the correct amount of antibody is added to the cells in order not to over or under saturate the cells of interest as well as save money by not wasting antibodies. Titrations were conducted for the main study for antibodies of interest. The titrations were conducted on healthy PBMC controls which had been exposed to cryopreservation conditions for three weeks before conducting these experiments. A total of one million PBMCs were added to the antibody cocktails as suggested by the manufacturer except for the antibody of interest. For example, the manufacturer suggests that 10 µl of CD4 antibody be added to each test. The current titration experiment was divided into five tubes with five diverse amounts of CD4 antibody. In the first tube, double the recommended amount of CD4 antibody was added (20 µl). In the second tube, recommended amount of antibody was added (10 µl). In the third tube, the recommended amount minus 2.5 µl was added (7.5 µl). In the fourth tube, the recommended amount minus 5 µl was added (5 µl). In the fifth tube, the recommended minus 7.5 µl was added (2.5 µl). Regular staining protocol was followed as shown in section 3.6.3. All five tubes were analysed on the Beckman Coulter Navios flow cytometer and the results and graphs were produced using Kaluza Analysis Software v1.3 (Beckman Coulter, Miami, FL, USA). Final selected values are indicated in Table 3.3.

3.11.2 Fluorescence Minus One

FMO is a control which is used to identify positive events and eliminate background noise from the sample. When conducting FMO experiments, all the regular antibodies are added to the normal amount of cells except for the one antibody of interest. The full panel is analysed and discriminator set for the missing antibody (minus one). All events in channel of interest

for the antibody were negative. Once FMO has been set a full panel is run including the previously absent one to check that positive vs. negative discrimination is accurate.

3.11.3 Compensation

The principle of compensation is to adjust for spectral overlap between fluorochromes emitting light that can be detected in more than one detector channel. When there are two or more fluorochromes used in a staining panel, whose emission spectra overlap, compensation has to be conducted to subtract the signal which is detected in the adjacent detector channel. For example FITC and PE are often used in routine flow cytometry. Their emission spectra overlap and portions of the PE signal is detected in the FITC channel, and vice versa. By using single and dual/multiple stained samples (often done with beads), the signal spilling into the incorrect channel can be compensated for (Hardung 2014).

3.12 Statistical analysis

Once all the samples were analysed using the flow cytometer, the data was compared and statistical analysis was conducted to confirm whether there was a significant difference between the median levels of inhibitory markers between the groups of interest. Flow cytometric data in the form of percentage positive gated events were collected on a Microsoft Excel spread sheet. All statistics were analysed using GraphPad Prism 6 software 2015 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used for analysing all three groups together (HIV^- group, $HIV\text{-}1^+$ group and the $HIV\text{-}1^+TB^+$ group) based on group wise comparison. A non-parametric t-test was used when comparing two variables such as VL for the $HIV\text{-}1^+$ group compared to the VL of the $HIV\text{-}1^+TB^+$ group. Hypothesis testing was done at 95% confidence intervals and results were regarded as significant if $p<0.05$ and highly significant if $p<0.01$.

CHAPTER 4: RESULTS

In this section there will be a focus on the comparison between the results of the pilot study and the results of the main study. The pilot study examined four markers expressed on both $CD4^+$ and $CD8^+$ T cells namely Tim-3, PD-1, Fas and CD38 together with CD4 count and HIV-1 VL as clinical markers of disease progression. Furthermore, the main study included all the markers of the pilot study as well as LAG-3, 2B4, and HLA-DR in efforts to better understand if there was a connection between the respective markers and immune activation and/or immune exhaustion.

4.1 Pilot study demographics

The total number of participants who enrolled for the pilot study was 75 (Table 4.1). They were between the ages of 17 and 63 (mean age of 33.5 ± 11.6 years). There were 18 (24%) participants from Durbanville Clinic, 38 (51%) participants from Khayamandi Clinic and 19 (25%) participants from Idas Valley Clinic.

Table 4.1 Patient demographics in pilot study

	Total recruited	Controls	HIV$^+$	HIV$^+$/TB$^+$
Recruited	75	30	30	15
Analysed	73	30	28	15
Male : Female	19 : 54	2 : 28	10 : 18	7 : 8
Age (years)	Mean ± SD	43.0 ± 12.4	30.0 ± 10.3	42.0 ± 10.2
CD count	Mean ± SD	1020.0 ± 337.7	336.5 ± 251.6	277.7 ± 258.4
(cells/µl)	Median	1014.0	309.5	234.0
	IQR	824.3-1150	220.3-380.8	48.0-443.0
Viral Load (copies/ml)	Mean ± SD	Not done	124 056.0 ± 186 652.0	385 603.0 ± 455 869.0
	Median		43 688.0	213 650.0
	IQR		14 604.0-158 361.0	119 345.0-550 990.0

IQR = Interquartile range; SD=standard deviation

4.1.1 Viral load

The co-infected group had a significantly higher VL than the HIV-1 positive group ($p<0.01$). Median VL of HIV-1 positive group was 43 688.0 copies/ml, whereas in the co-infected group it was 213 650.0 copies/ml (Figure 4.1A). The co-infected group had a VL that is almost 5 times more than the HIV-1 positive group. Viral load data is illustrated in Figure 4.1.A. All graphic representation of data across groups is by box-and whisker plot. In each plot the horizontal line represents the median, the box encompasses 50% of the data spread (interquartile range or 25-75% confidence limits), the upper and lower whisker-like projections represent the upper 90% and the lower 10% (i.e. data within range from tip to tip of whisker encompasses 80% of data, or 10-90% confidence limits). Outliers are shown as dots, these are values outside of the 80% spread (McKillup, 2008).

4.1.2 CD4 count

The CD4 count for the control group was 1 014.0 cells/µl (IQR 824.3- 1150.0); in the HIV-1 positive group 309.5 cells/µl (IQR 220.3- 380.8) and in the co-infected group 234.0 cells/µl (IQR 48.0- 443.0). The interquartile range denotes the middle 50% of values shown from 25% to 75%. There was a significant difference ($p < 0.01$) between the 3 groups. The HIV-1 VL of the two infected groups show an inverted spread when compared to CD4 count (Figure 4.1B).

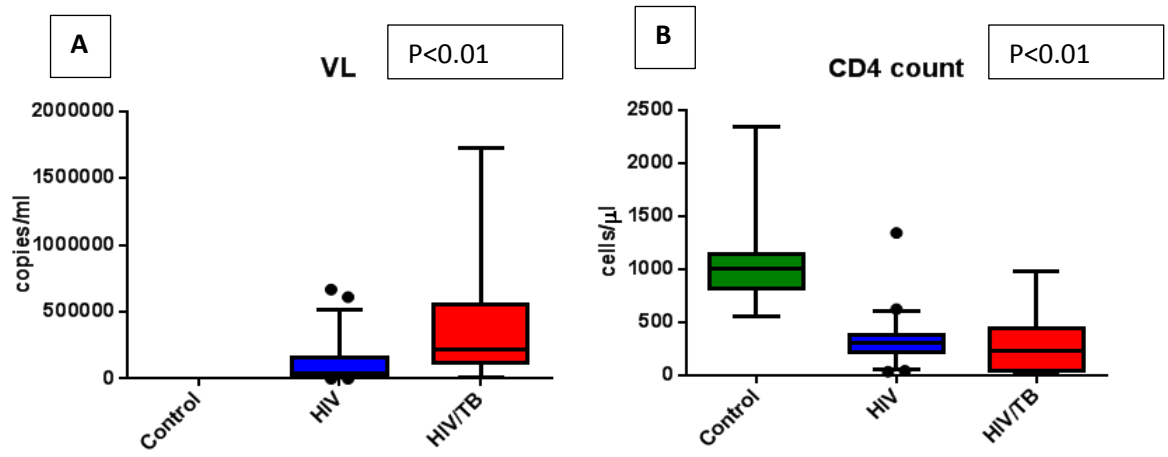

Figure 4.1 (A) Standard clinical markers of HIV and immune activation. (A) Viral load. The VL with respect to the number of copies found per ml of blood was significantly different between the two infected groups. There also appears to be a larger data spread in the HIV-1 positive group compared to the HIV-1/TB co-infected group. (B) CD4 count. The CD4 count was highest among the control group and lowest amount the HIV-1 positive and HIV-1/TB co-infected groups.

4.2 Single marker expression

Recognition of clear patterns of spread of markers of activation, exhaustion and apoptosis expressed on $CD4^+$ T was problematic throughout the pilot and the main study. As noted in the literature review, $CD4^+$ T cells are the target cell for HIV-1 and a large proportion of these cells are lost in the acute infection stage (refer to section 2.6.3 and Figure 2.10) (Shankar *et al.,* 2015). For this reason, the expression patterns displayed on $CD4^+$ T cells may be more variable than on $CD8^+$ counterparts. Consequently, a greater focus will be on $CD8^+$ T cells expression pattern and how this relates to disease progression.

4.2.1 CD38 expression on $CD4^+$ and $CD8^+$ T cells

CD38 is a marker of immune activation on T cells. CD38 is known to be a useful marker of disease progression to AIDS when expressed on $CD8^+$ T cells which is normally positively correlated to VL (Hua *et al.,* 2014). CD38 expression was determined for both $CD4^+$ and

$CD8^+$ T cells. The CD38 expression on $CD4^+$ T cells for the control group was 21.0% of cells (IQR 14.6-26.5), in the HIV-1 positive group 13.4% of cells (IQR 8.2-21.1) and in the co-infected group 8.0% of cells (IQR 3.6-13.9). The three groups were significantly different ($p<0.01$) from each other with respect to CD38 expression (Figure 4.2A).

The CD38 expression on $CD8^+$ T was the inverse of $CD4^+$ cells. The control group had a median value of 10.5% of cells (IQR 7.1-14.5), in the HIV-1 positive group 39.3% of cells (IQR 32.3-52.4) and in the co-infected group 51.7% of cells (IQR 33.9-70.8). There was a significant difference ($p<0.01$) between the three groups (Figure 4.2B). CD38 appears to have a similar expression pattern on $CD8^+$ T cells as VL. CD38 was highly expressed on the co-infected group which also has the highest VL. CD38 expression on $CD8^+$ T cells also appears to display an opposite expression pattern to CD4 count as the control group had the lowest CD38 expression and also the highest CD4 count compared to the other two groups with increased CD38 expression. TB co-infection was associated with the highest level of CD38 expression.

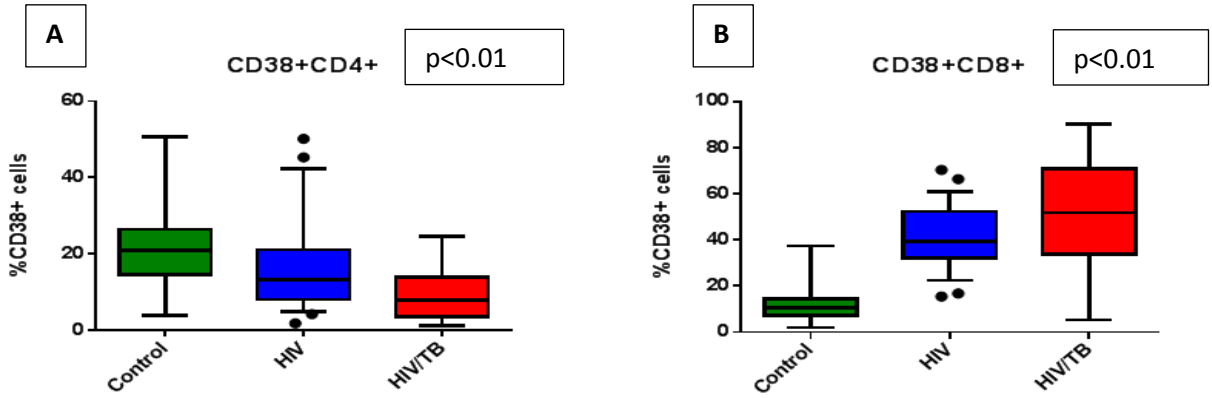

Figure 4.2 (A) Activation marker CD38 on $CD4^+$ T cells. There is a high level of CD38 expression on $CD4^+$ cells in the control group and there is a significant decrease between the HIV-1 positive group and the HIV-1/TB co-infected group; this was not expected as CD38 is a marker of activation and it would not be expected that the control group would have higher expression than the HIV-1/TB co-infected group. A similar pattern of expression to the $CD8^+$ T cells would be expected (B) Activation marker CD38 on $CD8^+$ T cells. CD38 expression was highest in the co-infected group. CD38 expression on $CD8^+$ T cells follows the expected pattern as the data displays greater immune burden and a higher expected activation. The overall CD38 expression was similar between the two infected groups when compared to the control.

4.2.2 Fas expression on $CD4^+$ and $CD8^+$ T cells

Fas is a death marker receptor and its interaction with FasL induces apoptosis. Fas expression has also been associated with T cell activation (Cencioni *et al.,* 2015). The Fas expression on $CD4^+$ T cells in the control group had a median value of 31.1% of cells (IQR 26.4- 52.0), in the HIV-1 positive group 11.9% of cells (IQR 8.7- 16.0) and in the co-infected group 17.3% of cells (IQR 4.2- 17.9). There was a significant difference ($p<0.01$) between

the three groups (Figure 4.3A). As with CD38 data, the Fas expression on $CD8^+$ T cells was significantly higher in the disease groups. The control group had a median value of 14.6% of cells (IQR 10.9- 19.7), in the HIV-1 positive group 48.6% of cells (IQR 37.3- 60.5) and in the co-infected group 55.4% of cells (IQR 49.5- 75.1). There was a significant difference ($p<0.01$) between the three groups (Figure 4.3B). HIV-1/TB co-infection was associated with the highest level of Fas expression.

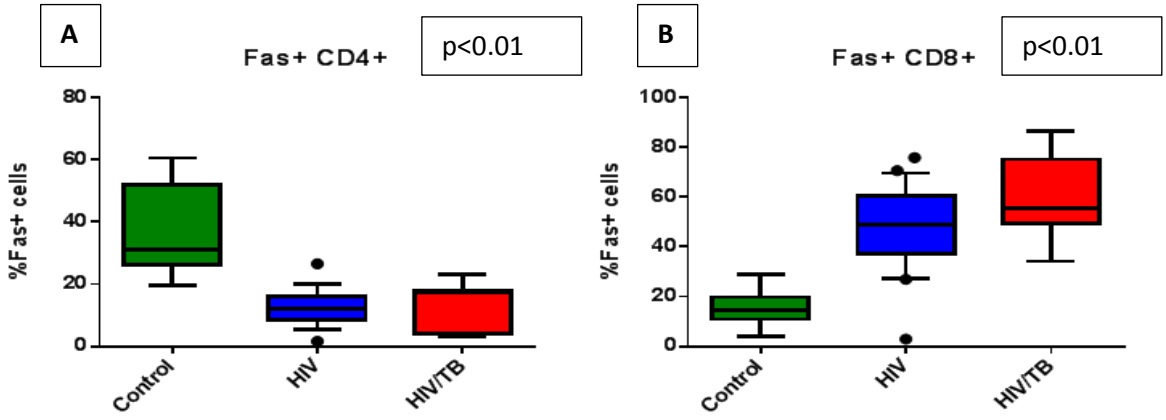

Figure 4.3 Death marker Fas on $CD4^+$ T cells. (A) Similar to the CD38 expression, Fas expression on $CD4^+$ T cells was highest for control group and lowest for the HIV-1/TB co-infected group. By the looks of the figure, $CD4^+$ T cells appear to display higher marker expression, but this may not be a true representation of $CD4^+$ T cell processes. (B) Death marker Fas on $CD8^+$ T cells. Alternatively, Fas expression on $CD8^+$ T cells was highest on the HIV-1/TB co-infected group and lowest on the control group which appears to be more constant with the literature.

4.2.3 PD-1 expression on $CD4^+$ and $CD8^+$ T cells

PD-1 is the best described T cell exhaustion marker and highly expressed on exhausted T cells exposed to chronic viral infections. PD-1 is a member of the CD28/CTLA-4 family and once bound to its ligand, PD-L1 and PD-L2, down-regulates T cell function (Kloverpris *et al.,* 2014). The PD-1 expression on $CD4^+$ T cells in the control group had a median of 8.9% of cells (IQR 5.1- 13.8), in the HIV-1 positive group 5.6% of cells (IQR 3.9- 9.5), and in the HIV-1/TB co-infected group 4.2% of cells (IQR 2.9- 6.9). There was a significant difference ($p<0.05$) between the three groups (Figure 4.4A). The PD-1 expression on $CD8^+$ T cells in the control group had a median value of 2.8% of cells (IQR 1.3- 6.3), in the HIV-1 positive group 15.2% of cells (IQR 9.6- 27.1), and in the HIV-1/TB co-infected group 11.5% of cells (IQR 5.7- 35.2). There was a significant difference ($p<0.01$) between the three groups (Figure 4.4B). Unlike for CD38 and Fas, the HIV-1/TB co-infected did not display the highest level of PD-1.

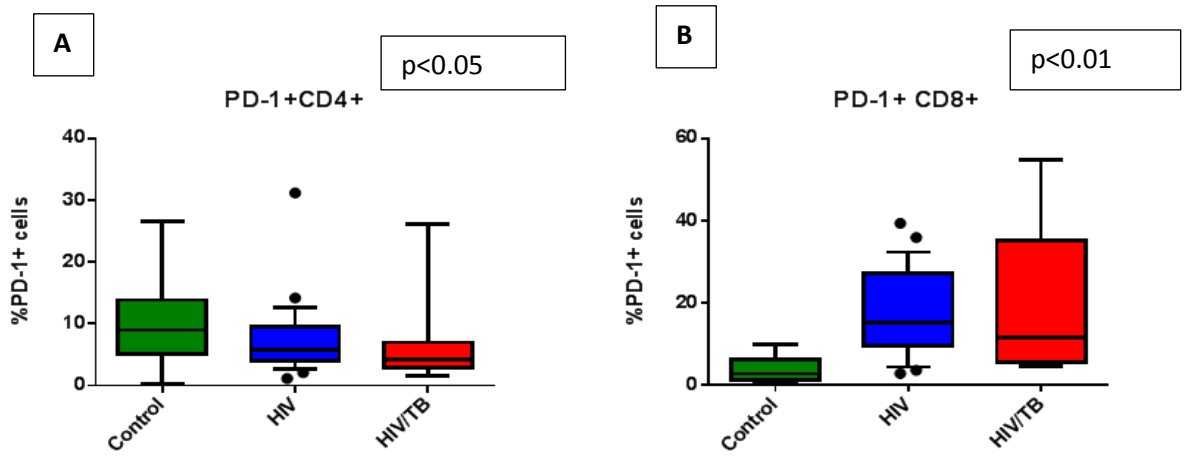

Figure 4.4 Inhibitory marker PD-1 on $CD4^+$ and $CD8^+$ T cells. (A) Inhibitory marker PD-1 on $CD4^+$ T cells. PD-1 expression is higher in the control group and lowest in the HIV-1/TB co-infected group. Similar to the previous mentioned markers, it would not be expected that the healthy cells could display high levels of exhaustion when compared to co-infection with HIV and TB. As mentioned previously, the inconsistencies of the $CD4^+$ T cells could be attributed to the high loss of $CD4^+$ T cells due to viral infection. (B) Inhibitory marker PD-1 on $CD8^+$ T cells. PD-1 expression on $CD8^+$ T cells is highest in the HIV-1/TB co-infected group and lowest on the control group. These findings would be more in line with what was expected as chronic viral infection leads to an increase in PD-1 expression

4.2.4 Tim-3 expression on $CD4^+$ and $CD8^+$ T cells

Tim-3, like PD-1 has been associated with the exhaustion phenotype (Gautron *et al.,* 2014). The Tim-3 expression on $CD4^+$ T cells in the control group had a median value of 0.4% of cells (IQR 0.1- 0.6), in the HIV-1 positive group 2.3% of cells (IQR 0.4- 4.7) and in the HIV-1/TB co-infected group 1.3% of cells (IQR 0.9- 5.7). There was a significant difference ($p<0.01$) between the three groups (Figure 4.5A).

The Tim-3 expression on $CD8^+$ T cells in the control group had a median value of 0.7% of cells (IQR 0.2- 3.3), in the HIV-1 positive group 5.9% of cells (IQR 1.4- 11.9), and in the HIV-1/TB co-infected group 11.7% of cells (IQR 7.0- 23.9). There was a significant difference ($p<0.01$) between the three groups (Figure 4.5B). As with PD-1 expression, the HIV-1/TB co-infected group did not display the highest median Tim-3 expression.

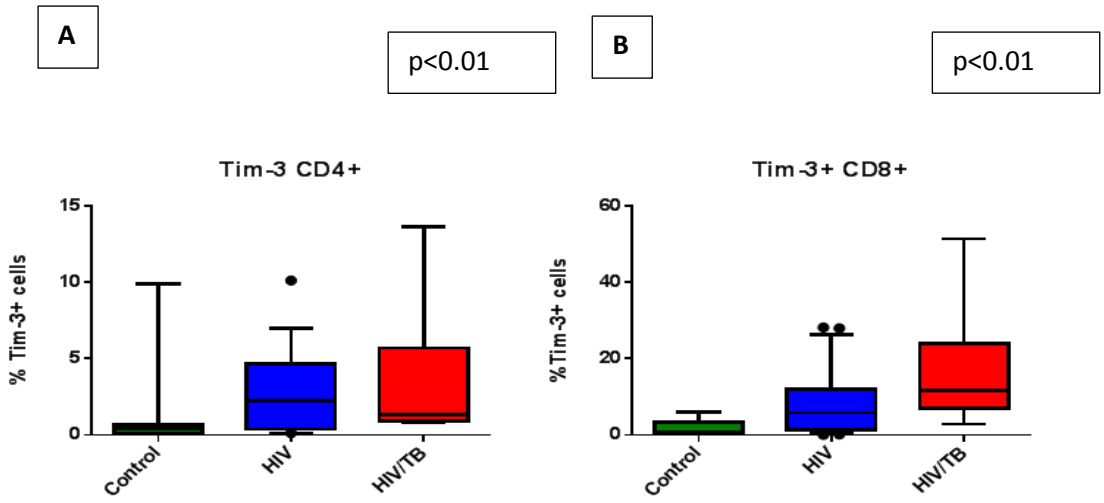

Figure 4.5 Inhibitory marker Tim-3 on $CD4^+$ and $CD8^+$ T cells. (A) Inhibitory marker Tim-3 on $CD4^+$ T cells. The control group appear to be displaying extremely low levels of Tim-3 while the HIV positive group and the HIV-1/TB co-infected group appear to have significantly higher levels. Tim-3 expression on $CD4^+$ T cells appears to follow a similar pattern to the $CD8^+$ T cells (B) Inhibitory marker Tim-3 on $CD8^+$ T cells. Tim-3 is lowest in the control group and highest in the HIV-1/TB co-infected group which would be expected as the infected groups are undergoing a process of immune exhaustion which is displayed by an upregulation of the inhibitory marker.

4.2.5 Summary of the main findings of the pilot study

The main finding of the pilot study was that CD38, Fas and PD-1, all followed similar expression patterns, with high expression of the marker in the control group for $CD4^+$ T cells, while higher expression of the marker was found of the HIV-1/TB co-infected groups for $CD8^+$ T cells. Tim-3 however did not follow this trend as the other markers in the pilot study were less than 5% expression on $CD4^+$ T cells and below 20% expression on $CD8^+$ T cells (however despite low expression on $CD8^+$ cells, expression pattern was similar to PD-1). Based on the findings from the pilot study, a larger study examining activation, exhaustion and negative-regulation in T cell subsets was implemented. For the main study, correlations were calculated for all the markers of interest for both $CD4^+$ and $CD8^+$ T cells, which will be referred to as the expression data.

4.3 Main study demographics

The total number of participants who enrolled for this study was 107 (Table 4.2). They were between the ages of 17 and 62 (mean age of 37.5). There were six (5%) participants from Stellenbosch Day Hospital, 39 (36%) participants from Durbanville Clinic, 41 (38%) participants from Kayamandi Hospital, and 21 (20%) participants from Idas Valley Day Hospital.

Table 4.2 Patient demographics in main study

	Total recruited	Controls	HIV$^+$	HIV$^+$/TB$^+$
Recruited	107	22	73	12
Analysed	105	22	73	10
Male: Female	42:63	7:15	32:41	3:7
Age (years)	Mean±SD	37.4±13.7	35.6±10.6	39.6±8.6
CD4 count	Mean±SD	1021.6±254.1	340.6±225.4	371.8±356.1
(cells/µl)	Median	1042.0	305.0	282.5
	IQR	788.3-1188.0	190.0-456.0	92.0-487.8
Viral Load	Mean±SD		237 515.5 ± 421 055.9	184 888.6 ± 219 069.1
(copies/ml)	Median	Not done	46 000.0	129 347.5
	IQR		15 145.0- 354 910.0	5 635.0- 293 561.0

IQR= interquartile range; SD= standard deviation

4.3.1 Viral load

The VL in the HIV-1 infected group had a median value of 46 000.0 copies/ml, (IQR 15 145.0- 354 910.0) whereas in the HIV-TB co-infected group it was 129 347.5 copies/ml (IQR 5 635.0- 293 561.0). The median VL was higher in the co-infected group compared to the HIV-1/TB co-infected group although this difference was not significant (p=0.9) (Figure 4.6A). When comparing the differences between the VL of the two studies, it appears that the pilot study has a 1.7 times higher VL in the HIV-1/TB co-infected group compared to the main study's HIV-1/TB co-infected group. Conversely, the pilot study and the main study appear to have similar VLs among the HIV-1 positive groups.

4.3.2 CD4 count

The CD4 count in the control group was 1 042.0 cells/µl (IQR 788.3- 1 188.0); in the HIV-1 positive group 306.0 cells/µl (IQR 190.0- 456.0) and in the co-infected group 282.3 cells/µl (IQR 92.0- 487.8). There was a significant difference (p< 0.01) between the three groups.

The VL of the two infected groups did have an impact on the CD4 count, with a negative correlation (r=-0.4630, p<0.01 refer to Addendum D) (Figure 4.7A). The control groups between the two studies appear to be almost equal with a CD4 count of 1 014.0 and 1 042.0 cells/µl. Comparing the CD4 count between the HIV-1 only positive groups, it was interesting to find that even though there was a two-fold difference in HIV-1 VL between the two groups, the data spread rendered this difference statistically insignificant (p=0.90).

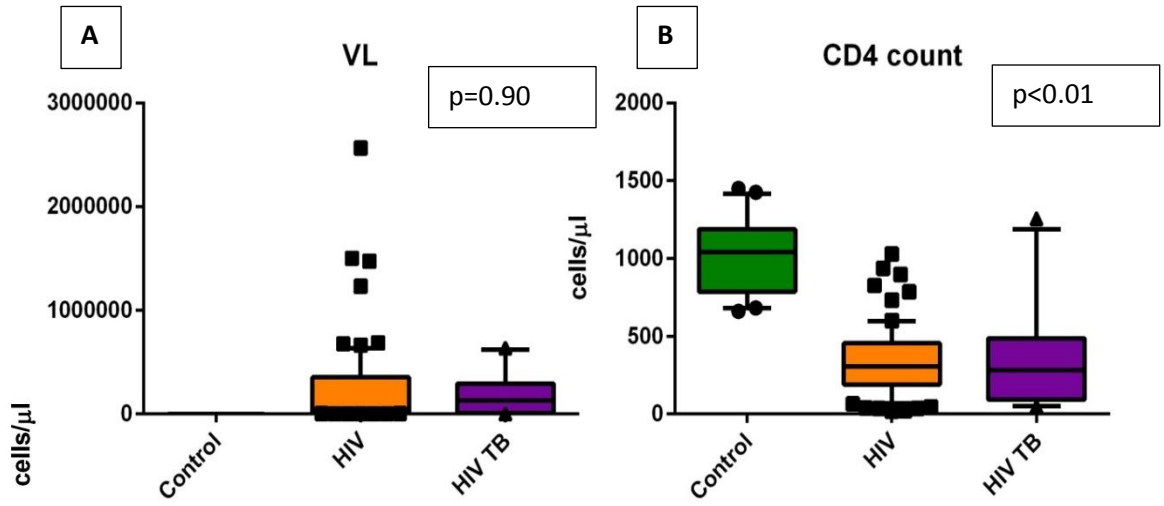

Figure 4.6 Standard clinical markers of HIV and immune activation (A) Viral load. The VL with respect to the number of copies found per ml of blood was not significantly different between the two infected groups. There also appears to be a larger data spread in the HIV-1 positive group compared to the HIV-1/TB co-infected group. (B) CD4 count. The CD4 count was highest among the control group and lowest amount the HIV-1 positive and HIV-1/TB co-infected groups.

4.4 Single marker expression

4.4.1 CD38 expression on $CD4^+$ and $CD8^+$ T cells

When compared to the pilot study, expression of CD38 was much higher in the main study on both $CD4^+$ and $CD8^+$ T cells, and unlike the pilot study the $CD4^+$ T cells expression followed patterns of $CD8^+$ T cells, i.e. increased in diseased groups. For the main study, median CD38 expression on $CD4^+$ T cells for the control group was 16.7% of cells (IQR 9.3- 23.1), in the HIV-1 positive group 42.8% of cells (IQR 26.0- 53.9), and the co-infected group 41.7% of cells (IQR 12.3- 49.3). There was a significant difference (p<0.01) between the three groups (Figure 4.3 A). The CD38 expression on $CD8^+$ T cells for the control group was 49.6% of cells (IQR 35.9- 57.1), in the HIV-1 positive group 88.6% of cells (IQR 71.9- 96.1), and the co-infected group 80.3% of cells (IQR 71.0- 96.7). There was a significant difference (p<0.01) between the three groups (Figure 4.3B). According to the pilot study, with respect to $CD4^+$ T cells, the HIV-1/TB co-infected group has the lowest expression of CD38 which was 1.7 times lower than the HIV-1 positive group and 2.2 times lower than the control

group. Conversely, in the main study, CD38 expression was highly expressed on the HIV-1/TB co-infected group which was 2.2 times higher than the control but 1.1 times lower than the HIV-1 positive group. HIV-1 VL and CD38 expression were positively correlated ($r=0.4104$, $p<0.01$, refer to Addendum E). The median CD38 expression value on $CD4^+$ T cells was 13% for the pilot study and 43% for the main study in the HIV-1 infected group. The discrepancy may be due to a number of factors (see Discussion). Figure 4.8 and 4.9 displays the expression pattern between the three groups.

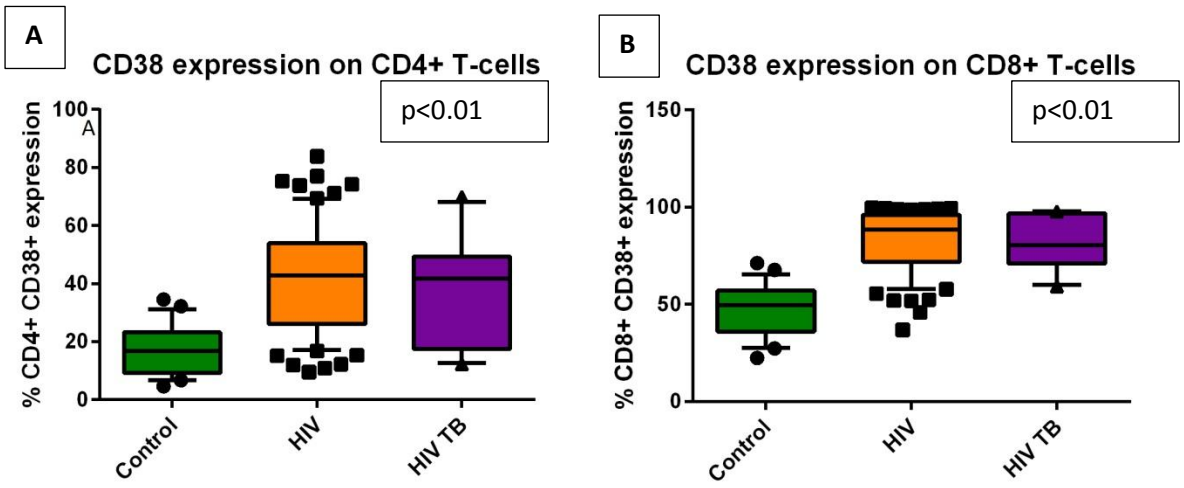

Figure 4.7 Activation marker CD38 on $CD4^+$ and $CD8^+$ T cells (A) Activation marker CD38 on $CD4^+$ T cells. The control group appears to be displaying low levels of CD38 while the HIV-1 positive and HIV-1/TB co-infected group appear to have significantly higher levels. (B) Activation marker CD38 on $CD8^+$ T cells. CD38 expression in the HIV-1 positive group and the HIV-1/TB co-infected group is higher than in the control group. For both studies this marker was significant across the board which was expected as it is a marker of immune activation

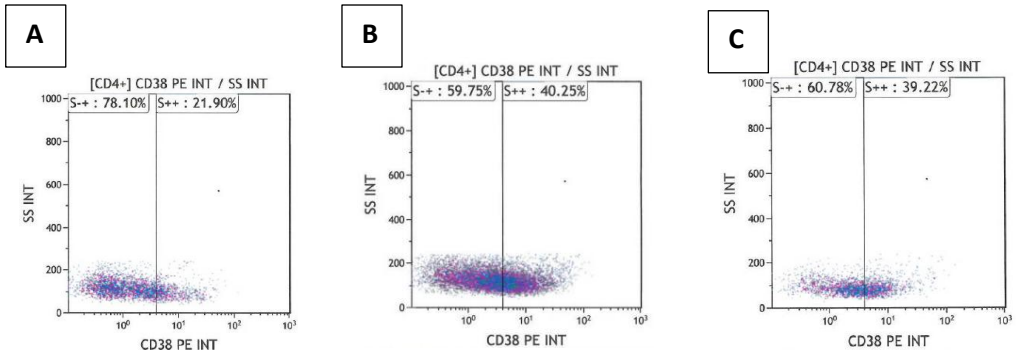

Figure 4.8 Surface marker CD38 expressed on CD4+ T cells as displayed using flow cytometry. Surface marker CD38 expressed on $CD4^+$ T cells as displayed using flow cytometry using Software plots. Each plot is representative of one individual patient sample. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. It can be seen from these diagrams that the expression of CD38 on $CD4^+$ T cells of the control is almost double that of the infected groups. These illustrates that when comparing the amount of activation expressed between the HIV-1/TB positive group and the co-infected group, TB co-infection appear to not influence the amount of activation expressed on $CD4^+$ T cells.

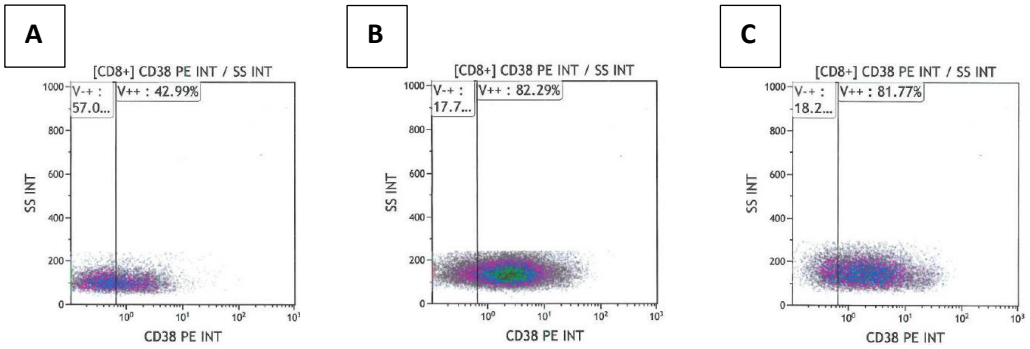

Figure 4.9 Surface marker CD38 expressed on $CD8^+$ T cells as displayed using flow cytometry. Surface marker CD38 expressed on $CD8^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. Similar to the $CD4^+$ group, it appears that co-infection with TB does not have such a big difference between the HIV-1 and HIV-1/TB co-infected groups as shown in the pilot study. The expression of CD38 of the main study was two times the expression of the pilot study in the HIV-1 positive groups. Similar to the $CD4^+$ cells, there was a higher level of granularity in the HIV-1 positive group

4.4.2 Fas expression on $CD4^+$ and $CD8^+$ T cells

Having found changes in Fas expression in the pilot study, further investigation was needed as there was an interest in the negative regulation (apoptosis) of exhausted T cells. In the main study, Fas expression on $CD4^+$ T cells for the control group was 53.5% (IQR 43.0-59.4), in the HIV-1 positive group 52.9% of cells (IQR 35.2- 75.2), and the co-infected group 58.4% of cells (IQR 39.1- 93.1). There was no significant difference between the three groups (p=0.4 refer to Addendum C) (Figure 4.10). The Fas expression on $CD8^+$ T cells displayed similar expression pattern to the pilot study, with the control group expressing 15.9% of cells (IQR 6.7- 26.4), in the HIV-1 positive group expression was 34.0% of cells (IQR 16.1- 52.8), and the HIV-1/TB co-infected group expression was 43.9% of cells (IQR

23.1- 63.8). There was a significant difference ($p<0.05$) between the three groups but there was no significant correlation found between Fas and VL or CD4 count ($r=0.0615$, $p=0.6$); ($r=0.0196$, $p=0.9$ refer to Addendum F) respectively (Figure 4.11).

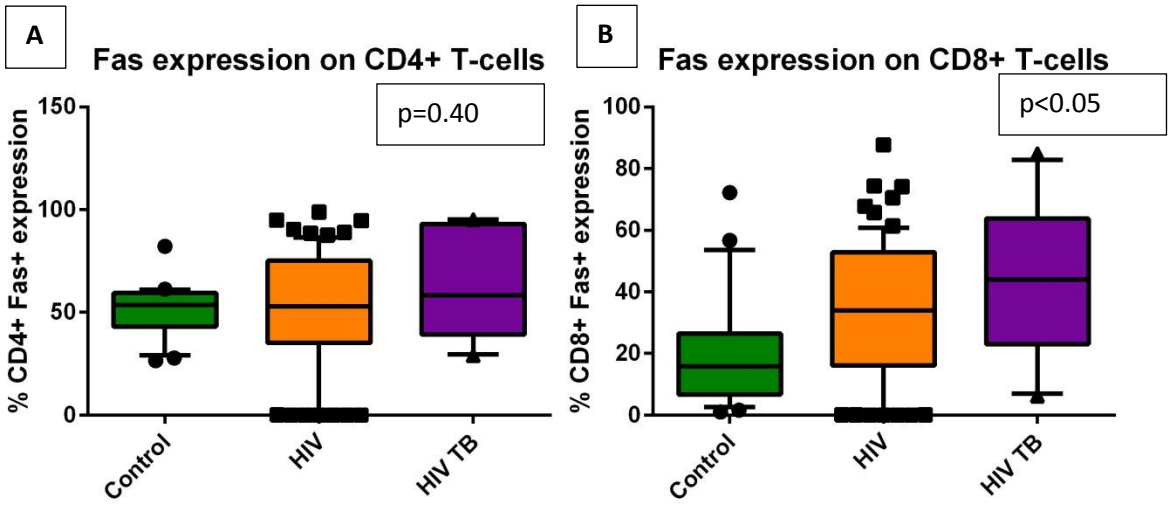

Figure 4.10 Death marker Fas on CD4$^+$ and CD8$^+$ T cells (A) Death marker Fas on CD4$^+$ T cells. The HIV-1/TB co-infected group appears to be displaying higher levels of Fas while the HIV-1 positive and control group appear to have no significantly difference. By observing the above figure, it is unlikely that Fas would be equally expressed between the three groups at such a high expression of 50% (B) Death marker Fas on CD8$^+$ T cells. Fas expression in the HIV-1 positive group and the HIV-1/TB co-infected group is higher than in the control group. This expression pattern of Fas on CD8$^+$ T cells was also found in the pilot study and is also the common trend of the other surface markers in this study.

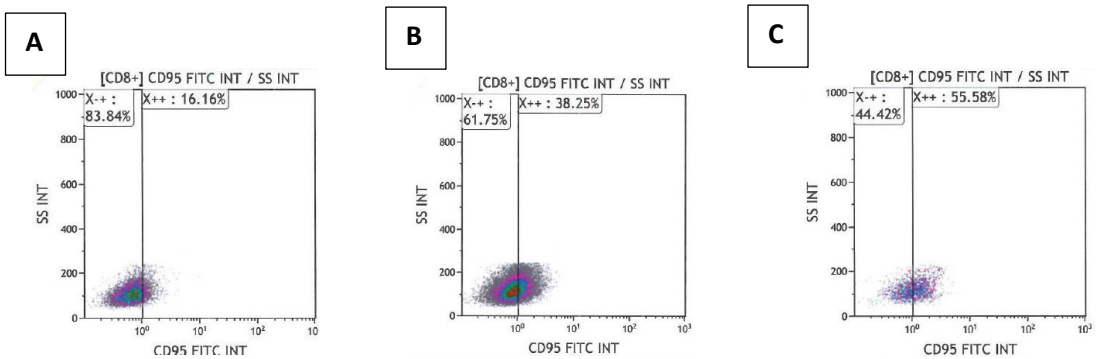

Figure 4.11 Surface marker CD38 expressed on CD8$^+$ T cells as displayed using flow cytometry Surface marker CD95 expressed on CD8$^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. The expression of Fas was over three times higher in the HIV-1/TB co-infected groups when compared to the control group. There was a two fold increase between the control and the HIV-1 positive group.

4.4.3 PD-1 expression on $CD4^+$ and $CD8^+$ T cells

PD-1 expression on $CD4^+$ T cells for the pilot study was lower than the main study. The PD-1 expression on $CD4^+$ T cells for the control group was 53.5% (IQR 43.0- 59.1), for the HIV-1 positive group 53.9% of cells (IQR 35.3- 75.2), and for the HIV-1/TB co-infected group 58.4% of cells (IQR 39.1- 93.1). Similar to Fas, there was high level of expression but no difference across the groups (Figure 4.13A). The PD-1 expression on $CD8^+$ T cells for the control group was 9.5% (IQR 3.9- 15.3), in the HIV-1 positive group 31.6% (IQR 2.9- 43.3), and the HIV-1/TB co-infected group 34.9% (IQR 22.7- 45.5). There was a significant difference ($p<0.01$) between the three groups (Figure 4.12, Figure 4.13 and Figure 4.14). The PD-1 data on $CD8^+$ T cells was comparable across the two studies.

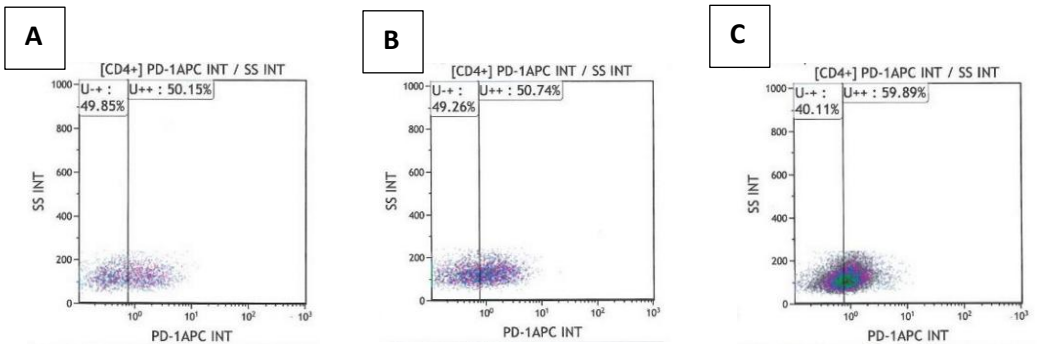

Figure 4.12 Surface marker PD-1 expressed on $CD4^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. Only a small difference was observed between the three groups which are also found on $CD4^+$ T cells expressing Fas.

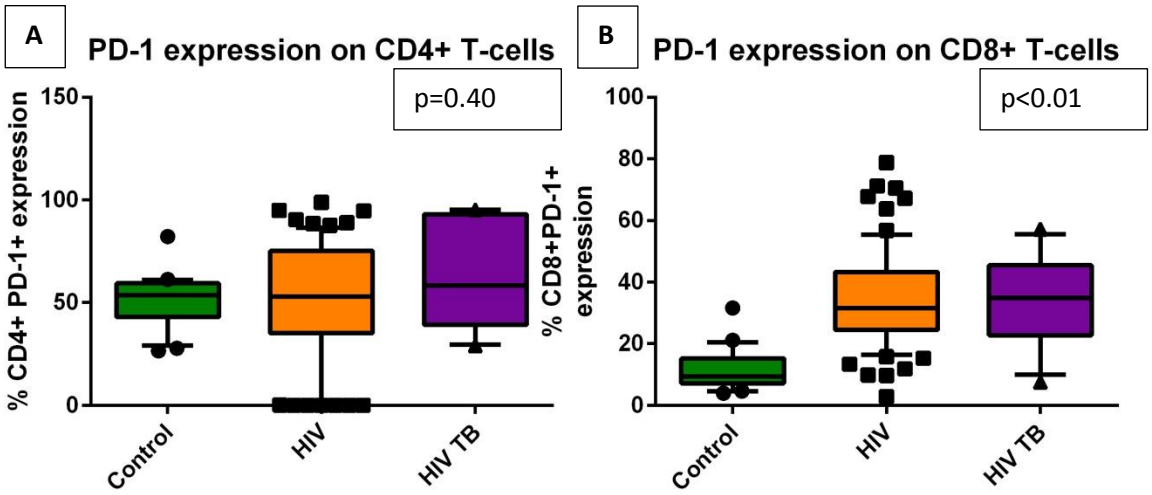

Figure 4.13 Exhaustion marker PD-1 on $CD4^+$ and $CD8^+$ T cells. (A) Exhaustion marker PD-1 on $CD4^+$ T cells. The control group appears to be displaying almost equal levels of PD-1 as the HIV-1 positive group while the HIV-1/TB co-infected group appear to have significantly higher levels. It would not be expected that all three group would express similar amounts of PD-1 suggesting that all three groups express the same levels of exhaustion regardless of HIV infection. Reflecting on the pilot study, lower levels of PD-1 expression on $CD4^+$ T cells was found although higher levels of PD-1 was expressed in the control groups which was unexpected (B) Exhaustion marker PD-1 on $CD8^+$ T cells. PD-1 expression in the HIV-1 positive group and the HIV-1/TB co-infected group is higher than in the control group. Comparing the PD-1 expression between the infected groups, it appears that there was almost no difference, the same was found to be true in the pilot study.

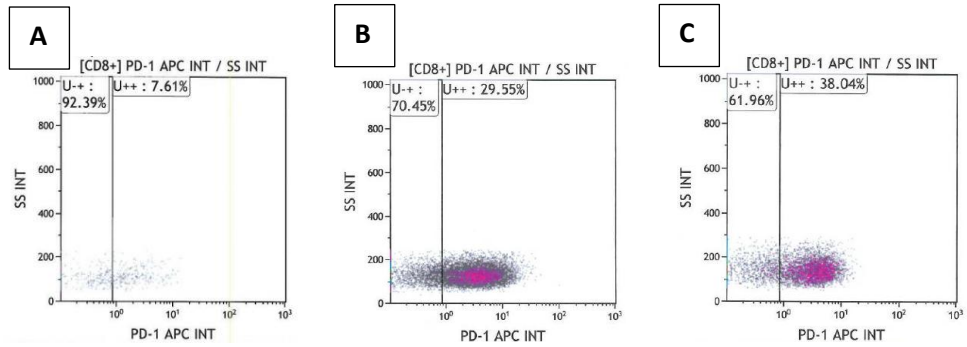

Figure 4.14 Surface marker PD-1 expressed on $CD8^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. It can be seen that PD-1 expression was very low on the control group and significantly higher in the infected groups.

4.4.4 Tim-3 expression on CD4$^+$ and CD8$^+$ T cells

In the pilot study, the freshly isolated cells expressed very low levels of Tim-3 with a median expression of 3% in the HIV-1 group when compared to a median value of almost 20% for frozen PBMC. High level marker expression was also found for Fas, CD38 and PD-1. The Tim-3 expression on CD4$^+$ T cells for the control group was 26.6% (IQR 4.8- 12.7), for the HIV-1 positive group 19.6% of cells (IQR 3.2- 13.2) and for the HIV-1/TB co-infected group 18.8% (IQR 2.7- 17.9). There was no significant difference between the three groups (p=0.6 refer to Addendum C) (Figure 4.15A). The Tim-3 expression on CD8$^+$ T cells for the control group was 6.3% (IQR 4.8- 12.7), for the HIV-1 positive group 5.5% of cells (IQR 3.5- 13.2) and for the HIV-1/TB co-infected group 3.5% (IQR 2.7- 17.9). There was also no significant difference between the three groups (p=0.6, Refer to Addendum D) (Figure 4.15B). When fresh blood was tested, Tim-3 expression on CD8$^+$ T cells was almost two times higher in the HIV-1/TB co-infected group than in the HIV-1 only group. The control group expressed a very low amount of Tim-3 as expressed with a mean value of 1.8 compared to the HIV-1/TB co-infected group with a mean value of 16.0 which is over eight times higher in the HIV-1/TB co-infected group than the control group, and a difference of over 4.5 times more Tim-3 expression in the HIV-1 group than the control group.

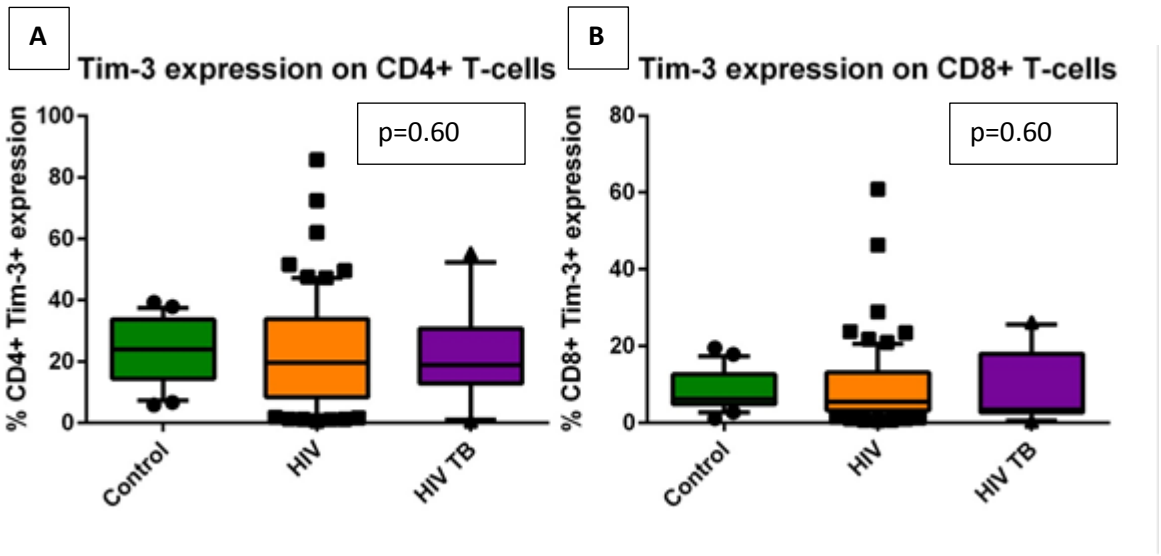

Figure 4.15 Exhaustion marker Tim-3 on CD4$^+$ and CD8$^+$ T cells. (A) Exhaustion marker Tim-3 on CD4$^+$ T cells. The control group appears to be displaying slightly higher levels of Tim-3 while the HIV-1 positive and HIV-1/TB co-infected group appear to display no difference. When compared to the pilot study, Tim-3 expression was 3% in the pilot study compared to 20% expression in the main study (B) Exhaustion marker Tim-3 on CD8$^+$ T cells. Tim-3 expression in the HIV-1 positive group and the HIV-1/TB co-infected group is slightly lower than the control group.

4.4.5 2B4 expression on CD4$^+$ and CD8$^+$ T cells

2B4 is an inhibitory marker which has been documented to increase in expression on exhausted CD8$^+$ T cells with disease progression to AIDS (Aldy *et al.,* 2011) The 2B4

expression on CD4$^+$ T cells in the control group was 21.6% (IQR 9.2- 41.3), in the HIV-1 positive group 10.7% of cells (IQR 3.1- 21.1) and in the HIV-1/TB co-infected group 16.1% (IQR 2.5- 22.3). There was a significant difference ($p<0.05$ refer to Addendum C) between the three groups (Figure 4.17A and Figure 4.16). The 2B4 expression on CD8$^+$ T cells for the control group was 59.2% of cells (IQR 49.7- 69.5), in the HIV-1 positive group 69.6% of cells (IQR 58.91- 80.98) and in the HIV-1/TB co-infected group 75.1% of cells (IQR 44.3- 79.6). There was a significant difference ($p<0.05$) between the three groups (Figure 4.17B and Figure 4.18). Interestingly the 2B4 data showed a trend observed in the pilot study for CD38, Fas and PD-1 – namely decreased expression in CD4$^+$ T cells across groups, but increased expression in CD8$^+$ T cells across the groups.

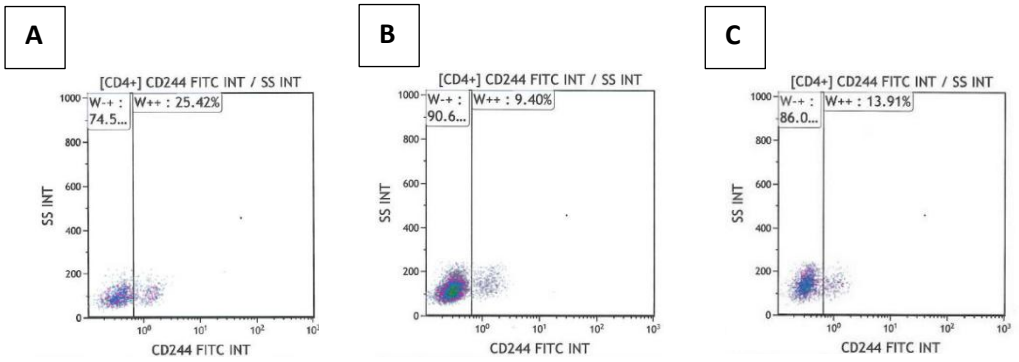

Figure 4.16 Surface marker CD244 expressed on CD4$^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. It appears that this marker is highly expressed on the control and to a lesser extend expressed on the infected groups.

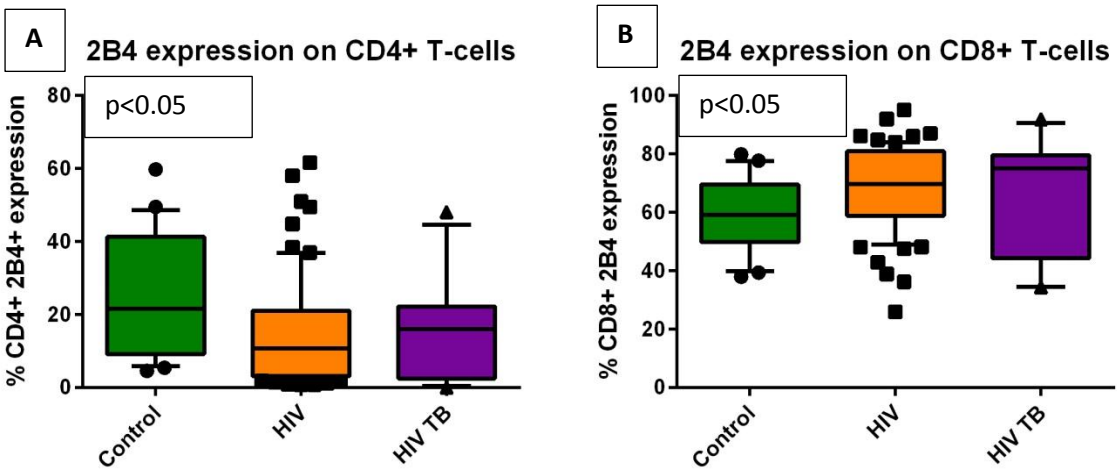

Figure 4.17 Inhibitory marker 2B4 on CD4$^+$ and CD8$^+$ T cells. (A) Inhibitory marker 2B4 on CD4$^+$ T cells. The control group appears to be displaying higher levels of 2B4 while the HIV-1 positive and HIV-1/TB co-infected group appear to have significantly lower levels. (B) Inhibitory marker 2B4 on CD8$^+$ T cells. 2B4 expression in the HIV-1 positive group and the HIV-1/TB co-infected group is higher than in the control group. Novel marker 2B4, displayed an opposite expression pattern between CD4$^+$

and CD8$^+$ T cells which is similar to the expression pattern of Fas in the main study and Fas, CD38 and PD-1 in the pilot study. The expression of 2B4 between the control groups three times higher in CD8$^+$ T cells. The expression of this marker was also seven times higher on the CD8$^+$ T cells compared to the CD4$^+$ T cells.

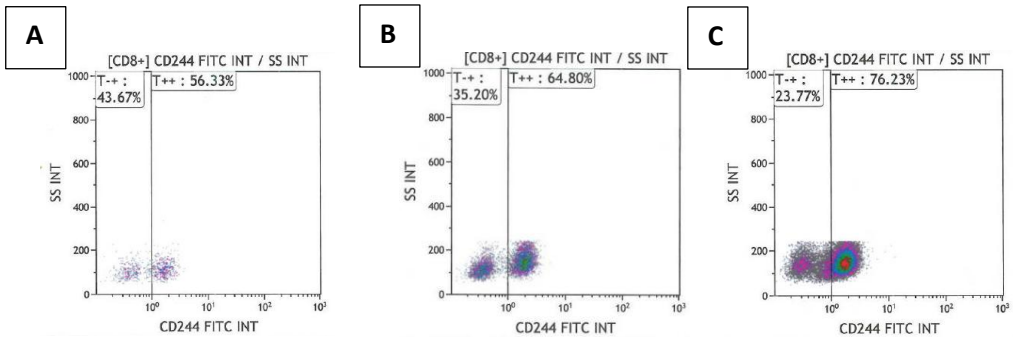

Figure 4.18 Surface marker CD244 expressed on CD8$^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. This gradual increase in 2B4 expression could suggest that this marker is an indicator of immune exhaustion in CD8$^+$ T cells

4.4.6 HLA-DR expression on CD4$^+$ and CD8$^+$ T cells

HLA-DR is a marker of immune activation and normally follows a similar expression pattern to CD38 when expressed on CD8$^+$ T cells (Sullivan *et al.,* 2015). HLA-DR expression on CD4$^+$ T cells did indeed appear to follow a similar expression pattern to CD38 which would be expected as both are markers of immune activation. The HLA-DR expression on CD4$^+$ T cells for the control group was 1.9% of cells (IQR 0.9- 2.5), in the HIV-1 positive group 5.7% of cells (IQR 3.2- 9.8) and the HIV-1/TB co-infected group 5.9% of cells (IQR 3.7- 19.5). There was a significant difference ($p<0.01$ refer to Addendum C) between the three groups (Figure 4.19A and Figure 4.20). The HLA-DR expression on CD8$^+$ T cells for the control group was 100% (IQR 99.9- 100), in the HIV-1 positive group 99.9% of cells (IQR 99.9- 100) and the HIV-1/TB co-infected group 100% (IQR 99.9- 100). There was no significant difference between the three groups ($p=0.5$ refer to Addendum D) (Figure 4.19B). These were unexpected data – and may be indicative of antibody saturation or other factors (see Discussion)

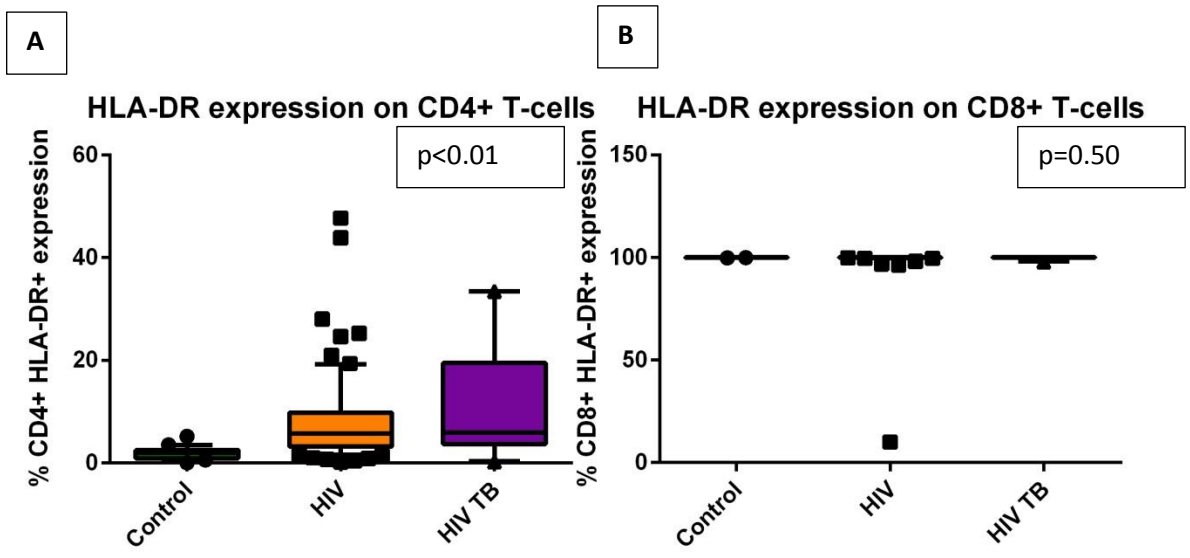

Figure 4.19 Activation marker HLA-DR on $CD4^+$ and $CD8^+$ T cells. (A) Activation marker HLA-DR on $CD4^+$ T cells. The control group appears to be displaying lower levels of HLA-DR while the HIV-1 positive and HIV-1/TB co-infected group appear to have significantly higher levels. Comparable to CD38 as a marker of T cell activation, HLA-DR is following a similar expression pattern. (B) Activation marker HLA-DR on $CD8^+$ T cells. HLA-DR expression in the HIV-1 positive group, HIV-1/TB co-infected group and the control group are out of range and will be discussed in the results section.

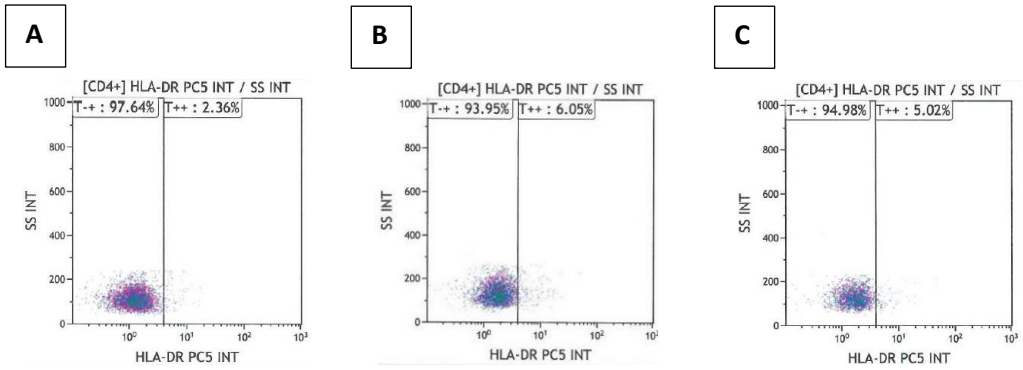

Figure 4.20 Surface marker HLA-DR expressed on $CD4^+$ T cells as displayed using flow cytometry. Surface marker HLA-DRC expressed on $CD4^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. Even though the expression of HLA-DR is quite low, it can be seen that the expression pattern increases as disease burden increases.

4.4.7 LAG-3 expression on $CD4^+$ and $CD8^+$T cells

High expression of LAG-3 on the surface of T cells is associated with advanced HIV-1 disease (Pena *et al.,* 2014). The LAG-3 expression on $CD4^+$ T cells for the control group was 2.9% (IQR 1.2- 5.4), in the HIV-1 positive group 1.3% of cells (IQR 0.2- 4.4) and the HIV-1/TB co-infected group 1.4% of cells (IQR 0.5- 7.0). There was no significant difference between the three groups (p=0.1 refer to Addendum C) (Figure 4.21A). The LAG-3 expression on $CD8^+$ T cells for the control group was 3.3% of cells (IQR 2.0- 4.6), in the HIV-1 positive group 3.2% of cells of cells (IQR 1.1- 5.9) and the HIV-1/TB co-infected group

2.2% of cells (IQR 1.0- 6.9). There was no significant difference between the three groups (p=0.9 refer to Addendum D) (Figure 4.21B). This was thus overall poor expression of this marker in both cell types examined and in both healthy and diseased groups.

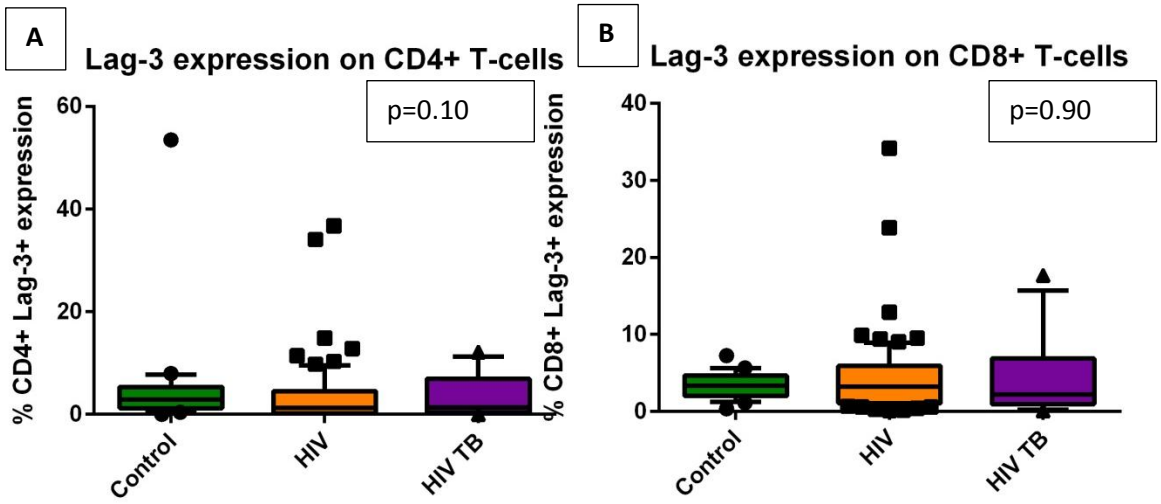

Figure 4.21 Inhibitory marker LAG-3 on $CD4^+$ and $CD8^+$ T cells. (A) Inhibitory marker LAG-3 on $CD4^+$ T cells. The control group appears to be displaying slightly higher levels of LAG-3 while the HIV-1 positive group and HIV-1/TB co-infected group appear to have equal levels of expression. (B) Inhibitory marker LAG-3 on $CD8^+$ T cells. LAG-3 expression in the HIV-1 positive group and the control group is almost equal while the HIV-1/TB co-infected group is slightly lower. It was disappointing to find that LAG-3 did not give significantly difference expression patterns between the groups, however, LAG-3 appears to be expressed mostly in the control groups which is not in accordance with the literature which describes this marker as being elevated on exhausted $CD8^+$ T cells.

4.4.8 Summary of the main findings of the main study in relation to single marker expression

To summarize the above data, it appears that markers 2B4, PD-1, Fas and CD38 were up-regulated on $CD8^+$ T cells in disease groups and share a similar expression pattern. There was not a significant difference in the marker expression between the groups for LAG-3, HLA-DR and Tim-3. Co-expression of PD-1, LAG-3, Tim-3, 2B4, Fas and CD38 were further analysed to understand if co-expression of these marker is higher than single expression and how this relates to disease progression.

4.5 Dual marker expression (co-expression)

Extensive analysis of dual expression was performed. From this several markers gave statistical significant differences across the patient groups, these are indicated below.

4.5.1 PD-1 and LAG-3 expression on CD8$^+$ T cells may be linked

Although only PD-1 showed a significant increase in the infected groups in single expression, co-expression with LAG-3 on CD8$^+$ T cells was significantly different between the groups, despite being very low expression levels. The dual expression of these markers on CD8$^+$ T cells for the control group was 0.4% of cells (IQR 0.2- 0.8), in the HIV-1 positive group 1.2% of cells (IQR 0.3- 2.7) and the HIV-1/TB co-infected group 1.1% of cells (IQR 0.3- 3.5). There was a significant difference ($p<0.05$) between the three groups (Figure 4.22 A). PD-1 and LAG-3 positively correlated ($r=0.3825$, $p=0.01$ refer to Addendum F) on CD8$^+$ T cells which could imply that as PD-1 is co-expressed with LAG-3 on exhausted T cells in low amounts when compared to single expression of 3.2% and 31.6% for LAG-3 and PD-1 respectively. The dual expression of these markers on CD4$^+$ T cells for the control group was 0.7% of cells (IQR 0.2- 1.1), in the HIV-1 positive group 0.5% of cells (IQR 0.1- 2.0) and the HIV-1/TB co-infected group 0.2% of cells (IQR 0.0- 1.1). There was no significant difference between the three groups ($p=0.9$ Refer to Addendum C) (Figure 4.22 and Figure 4.23A).

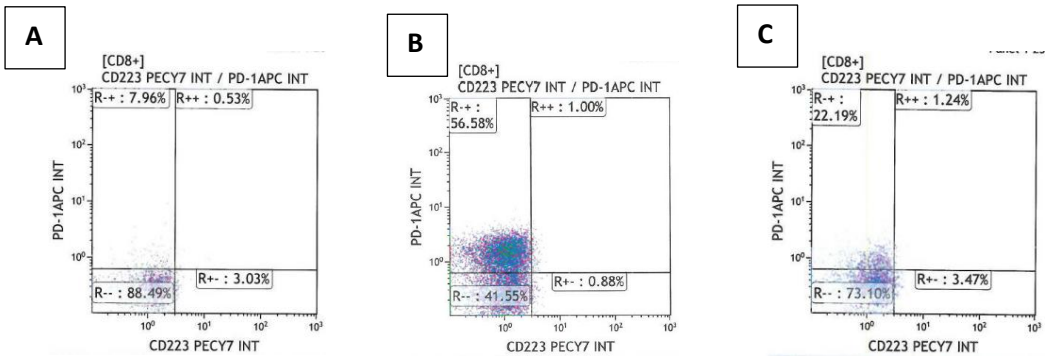

Figure 4.22 Surface markers LAG-3 and PD-1 expressed on CD8$^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. It can be seen from the diagrams above that the overall dual expression of these markers is low compared to the more dominant expression of PD-1 alone.

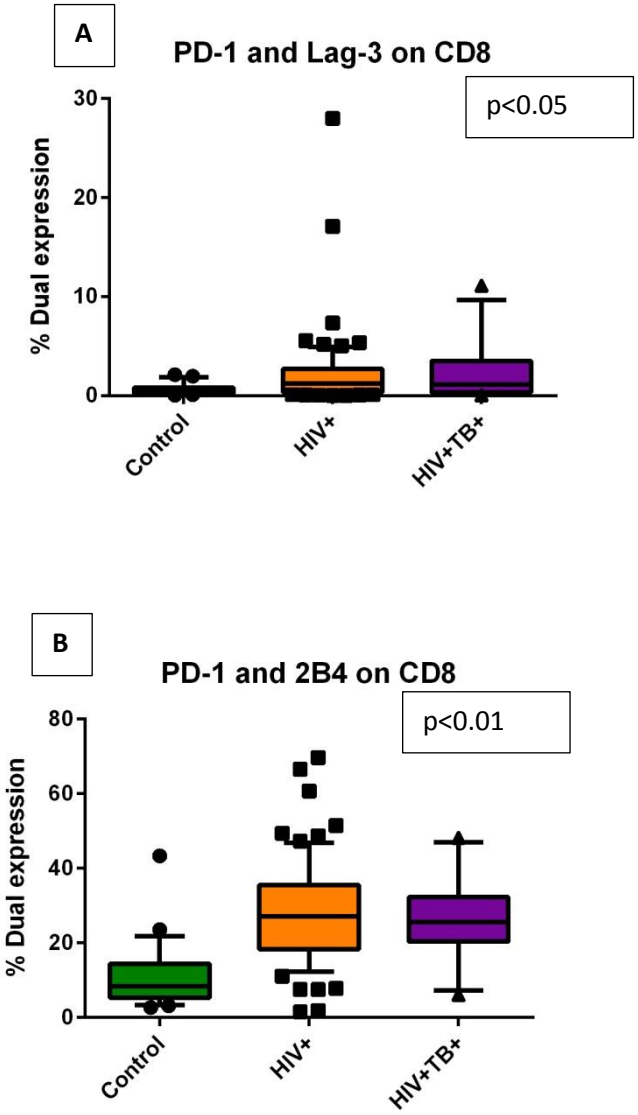

Figure 4.23 Exhaustion marker PD-1 and inhibitory marker LAG-3 on CD8$^+$ T cells (A) Exhaustion marker PD-1 and inhibitory marker LAG-3 on CD8$^+$ T cells. The control group appears to be displaying lower levels of both markers while the HIV-1 positive and HIV-1/TB co-infected group appear to have significantly higher levels. (B) Exhaustion marker PD-1 and inhibitory marker 2B4 on CD8$^+$ T cells. Both markers are expressed at significantly higher levels in the HIV-1 positive group and the HIV-1/TB co-infected group when compared to the control group.

4.5.2 PD-1 and 2B4 expression on CD4$^+$ and CD8$^+$ T cells

Both PD-1 and 2B4 displayed significant differences as single markers across the study groups. The dual expression of these markers on CD4$^+$ T cells for the control group was 3.9% of cells (IQR 1.7- 11.2), in the HIV-1 positive group 5.4% of cells (IQR 1.8- 48.6) and the HIV-1/TB co-infected group 3.8% of cells (IQR 0.8- 15.8). There was no significant difference between the three groups (p=0.7 refer to Addendum C). The dual expression of these markers on CD8$^+$ T cells for the control group was 8.3% of cells (IQR 5.4- 14.4), in the HIV-1 positive group 27.2% of cells (IQR 18.3- 35.5) and the HIV-1/TB co-infected group

25.6% of cells (IQR 20.4- 32.2). While there was a significant difference ($p<0.01$) between the three groups (Figure 4.23B and Figure 24), there was no correlation between the two markers ($r=0.2065$, $p=0.1$ refer to Addendum F).

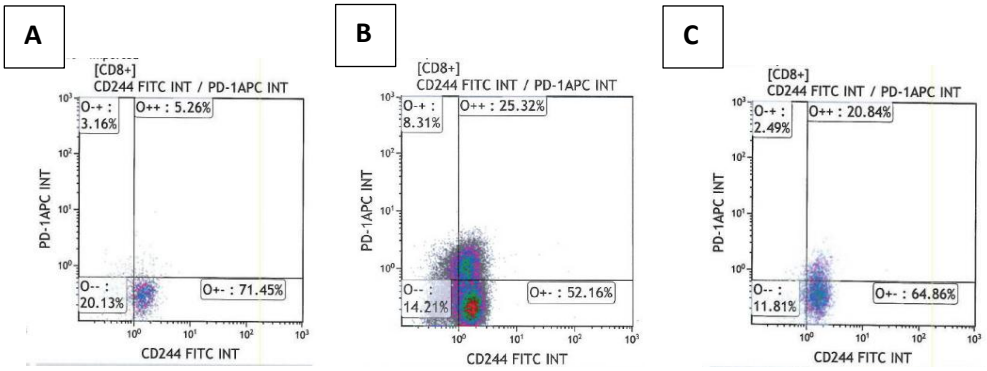

Figure 4.24 Exhaustion marker PD-1 and inhibitory marker 2B4 expressed on $CD8^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. On first glance, it appears that PD-1 and 2B4 expression is highest when expressed together on $CD8^+$ T cells; however, single expression of these markers has shown to be even higher in expression.

4.5.3 Tim-3 and 2B4 expression on $CD4^+$ and $CD8^+$ T cells

The dual expression of these markers on $CD4^+$ T cells for the control group was 7.3% (IQR 3.2- 15.2) in the HIV-1 positive group 3.5% of cells (IQR 1.1- 7.4) and the HIV-1/TB co-infected group 2.4% of cells (IQR 1.1- 6.8). There was a significant difference ($p<0.05$) between the three groups (Figure 4.25 and Figure 4.26) as well as displaying a positive correlation ($r=0.4918$, $p<0.05$ refer to Addendum E). The dual expression of these two markers is lower than the single expression of these markers, which could suggest that these markers, while positively correlated, are better markers of exhaustion when examined individually on $CD4^+$ T cell. The dual expression of these markers on $CD8^+$ T cells for the control group was 5.6% of cells (IQR 2.8- 8.4), in the HIV-1 positive group 3.8% of cells (IQR 2.1- 8.8) and the HIV-1/TB co-infected group 3.1% (IQR 1.2- 11.3). There was no significant difference between the three groups ($p=0.6$ refer to Addendum D).

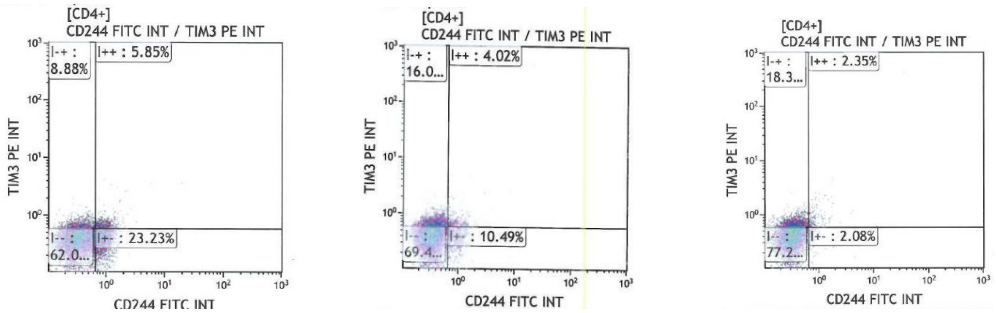

Figure 4.25 Surface markers 2B4 and Tim-3 expressed on CD4$^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. The overall single expression of Tim-3 is almost twice that of 2B4. While compared to the single expression of these markers, Tim-3 expression was over 5.5 times higher than dual expression of these markers.

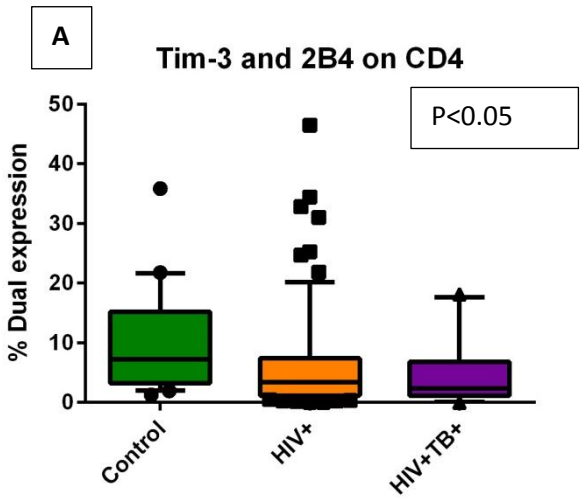

Figure 4.26 Exhaustion marker Tim-3 and inhibitory marker 2B4 on CD4$^+$ T cells. The control group appears to be displaying higher levels of both markers while the HIV-1 positive and co-infected group appear to have significantly lower levels.

4.5.4 LAG-3 and 2B4 expression on CD4$^+$ and CD8$^+$ T cells

The dual expression of these markers on CD4$^+$ T cells for the control group was 1.0% of cells (IQR 0.4- 2.4), in the HIV-1 positive group 0.2% of cells (IQR 0.1- 1.2) and the HIV-1/TB co-infected group 0.2% of cells (IQR 0.0- 0.8). Even though the expression was low on CD4$^+$ T cells, there was a significant difference ($p<0.05$) between the three groups (Figure 4.27 and Figure 4.28) and a positive correlation ($r=0.0113$, $p=0.01$ refer to Addendum E) between the two markers. Similar to the dual expresion of Tim-3 and 2B4, these two inhibitory markers are expressed higher individually than mutrually. The dual expression of these markers on CD8$^+$ T cells for the control group was 1.9% of cells (IQR 1.2- 3.2), in the

HIV-1 positive group 2.6% of cells (IQR 0.9- 4.6) and the HIV-1/TB co-infected group 1.8% of cells (IQR 0.8- 3.6). There was no significant difference between the three groups (p=0.6 refer to Addendum D).

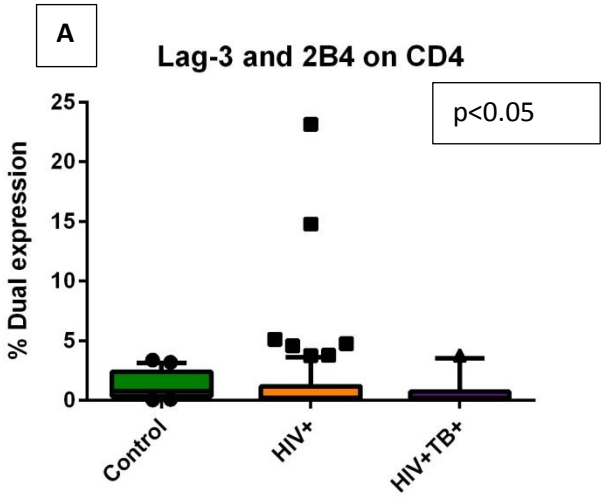

Figure 4.27 Inhibitory markers LAG-3 and 2B4 on CD4$^+$ T cells. Both markers are expressed at significantly lower levels in the HIV group and the HIV-1/TB co-infected group when compared to the control group. Both these sets of surface markers expressed on CD4$^+$ T cells correlated positively however, that dual expression was lower than single expression of these markers.

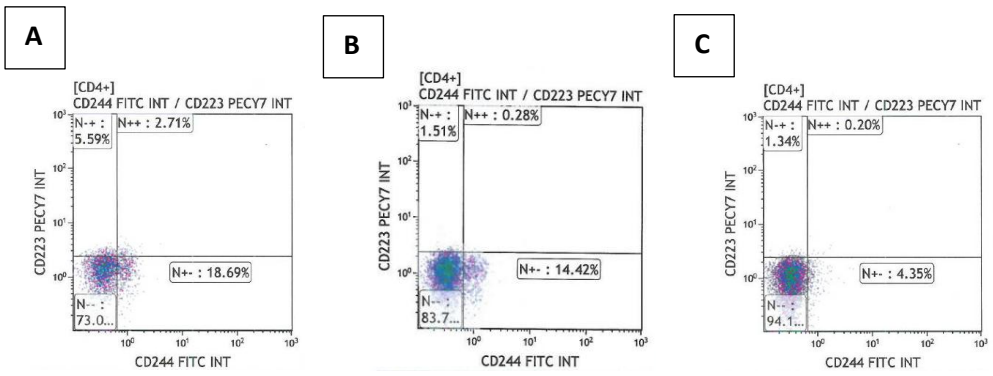

Figure 4.28 Surface markers CD244 (2B4) and CD223 (LAG-3) expressed on CD4$^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. Overall the expression of 1.3% for single expression of LAG-3 was over six times higher than the co-expression of both markers of interest.

4.5.5 Fas and CD38 expression on CD4$^+$ and CD8$^+$ T cells

The dual expression of these markers on CD4$^+$ T cells for the control group was 0.4% of cells (IQR 0.2- 0.7), in the HIV-1 positive group 2.2% of cells (IQR 0.8- 3.4) and the HIV-1/TB co-infected group 2.4% of cells (IQR 0.6- 16.7). There was a significant difference (p<0.01) between the three groups (Figure 4.29 and Figure 4.30) but there was no correlation of these markers on either CD4$^+$ or CD8$^+$ T cells. The dual expression of these

markers on $CD8^+$ T cells for the control group was 1.8% of cells (IQR 0.3- 4.1), in the HIV-1 positive group 14.6% of cells (IQR 0.0- 22.4) and the HIV-1/TB co-infected group 18.4% of cells (IQR 3.2- 36.4). There was a significant difference ($p<0.01$) between the three groups (Figure 4.30 and Figure 4.31). While there was no correlation between these two markers on $CD4^+$ T cells there was six times higher expression on the co-infected group when compared to the control group. Between the infected groups, the HIV-1/TB co-infected group expressed over two times more Fas and CD38 when compared to the HIV-1 only group. Dual expression of these two markers on $CD8^+$ T cells was highest on the HIV-1/TB co-infected group and lowest on the control group which is in line with the current literature. Dual expression of these markers was 1.3 times higher in the HIV-1/TB co-infected group than the HIV group and over nine times higher in the HIV-1/TB co-infected group compared to the control group.

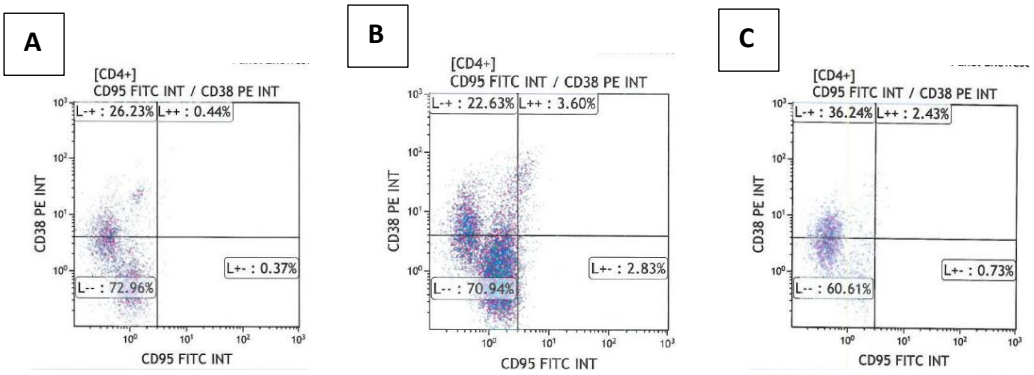

Figure 4.29 Surface markers CD38 and CD95 (Fas) expressed on $CD4^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. These two markers were not correlated even though the co-expression was significantly difference between the groups. The low levels of co-expression compared to the high single expression of CD38 could mean that these markers are better analysed separately.

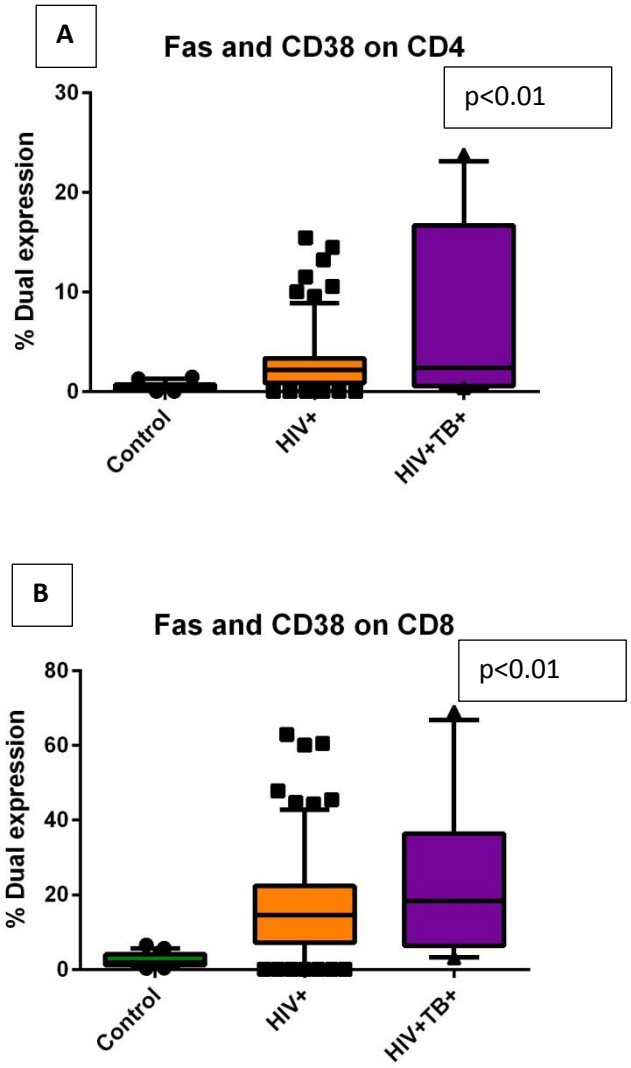

Figure 4.30 Death marker Fas and activation marker CD38 on $CD4^+$ and $CD8^+$ T cells. (A) Death marker Fas and activation marker CD38 on $CD4^+$ T cells. The control group appears to be displaying low levels of both markers while the HIV-1 positive and HIV-1/TB co-infected group appear to have significantly higher levels. (B) Death marker Fas and activation marker CD38 on $CD8^+$ T cells. Dual expression in the HIV-1 positive group and the HIV-1/TB co-infected group is higher than in the control group. These are two important markers of T cell activation and apoptosis. However, dually expressed, these markers did not correlate as it would be expected. It would be expected that a large number of $CD4^+$ cells would express Fas as well as CD38 as these cells are the target cell for this virus.

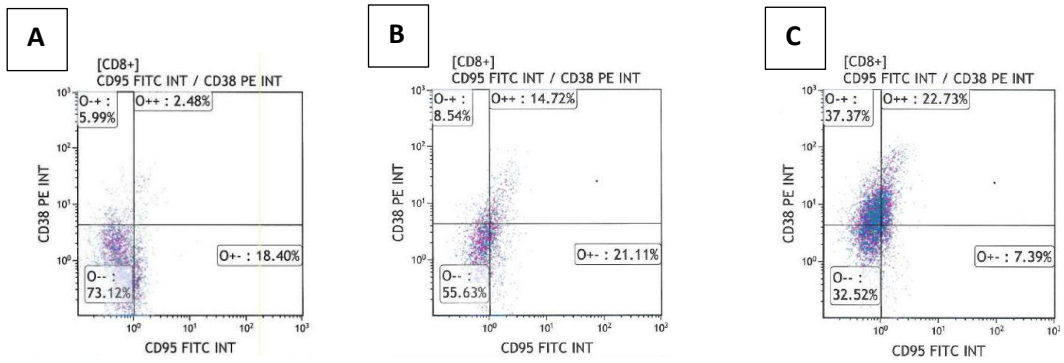

Figure 4.31 Surface markers CD38 and CD95 (Fas) expressed on CD8$^+$ T cells as displayed using flow cytometry. (A) Control group; (B) HIV-1 positive group; (C) HIV-1/TB co-infected group. Dual expression of CD38 and Fas appears to be following a pattern of increased express as disease burden increases among the groups. However, it was also found that single expression of both these markers was higher than dual expression.

4.6 T cell stimulation (Proliferation)

Having established in both the pilot study and the main study that diseased groups display elevated levels of activation, exhaustion and death markers (especially the CD8$^+$ T cell subset), the next step was to investigate whether these cells *in vitro* were functionally impaired and whether blocking exhaustion markers could restore function. Functional responses were monitored in the HIV-1 positive group alone, following αCD3 and αCD28 stimulation of CFSE labelled cells. To assess the role of PD-1 and Tim-3 exhaustion pathways, these markers were blocked independently and together.

The stimulation procedure resulted in increased median proliferation in both CD4 and CD8 T cells. The median unstimulated proliferation in CD4$^+$ T cells was 4.8%, and following stimulation 9.3% of cells. In CD8$^+$ T cells unstimulated proliferation was 4.6%, and following stimulation 7.8% of cells. In both CD4$^+$ and CD8$^+$ T cells, the change in proliferating cells was statistically significant (Refer to Table 4.3). The blocking of the Tim-3 and PD-1 pathways was not effective in enhancing responsiveness overall. Only Tim-3 blocking in the CD8$^+$ group showed an increased in median proliferating cell percentage (10.1%), however this was not statistically significant.

When comparing the CD8$^+$ T cells it appeared that blocking Tim-3 and PD-1 was not as beneficial as blocking Tim-3 alone with a mean value of 12.28 (Figure 4.32B). One of the main findings of the current study was the negative correlation between CD4 count together, Tim-3 (inhibition marker), CD38 (activation marker) and VL on CD8$^+$ T cells. It appears that CD8$^+$ T cells were more susceptible to Tim-3 blocking than PD-1 blocking which is unexpected as the expression of Tim-3 on CD8$^+$ T cells was on 6% compared to PD-1 of 31.6%.

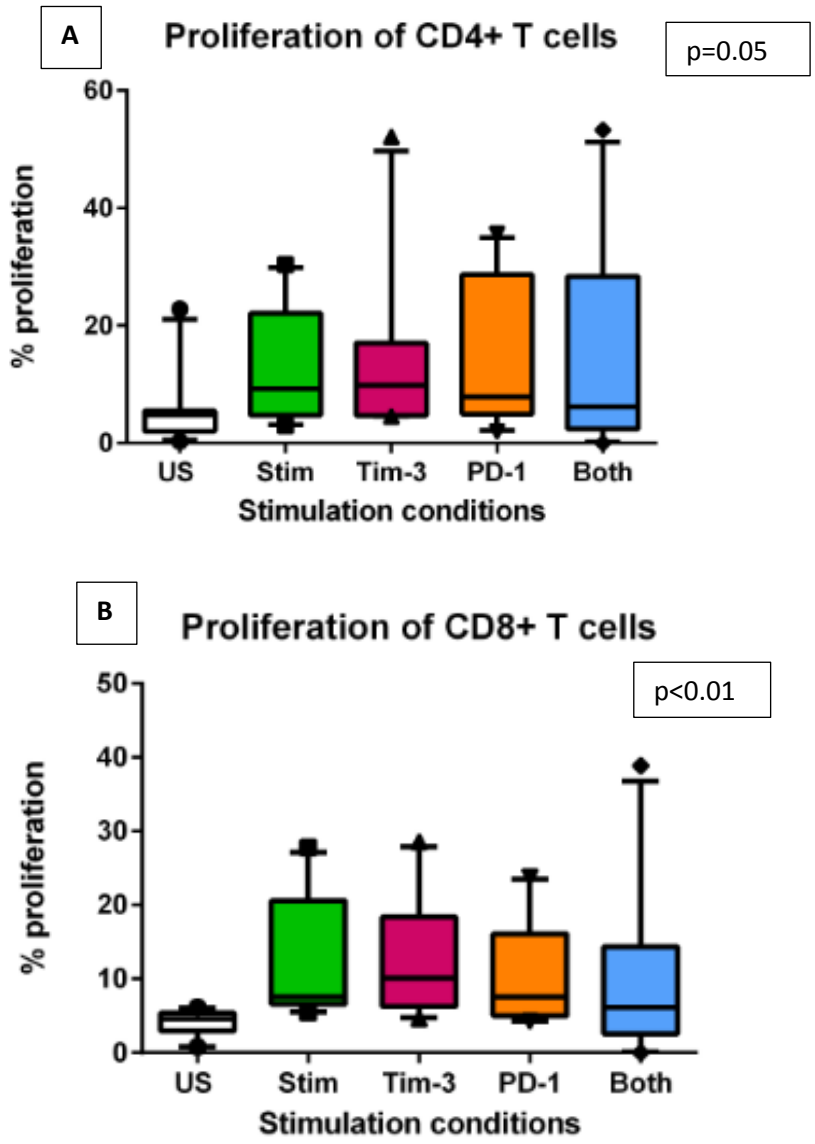

Figure 4.32 Proliferation assay. (A) Proliferations assay on $CD4^+$ T cells displaying the five cell conditions. (B) Proliferation assay on $CD8^+$ T cells displaying the five cell conditions.

Table 4.3 Functional responses to αCD3 and αCD28 proliferation assay. This blocking and proliferation assay was conducted using five conditions.

	Unstimulated	Stimulated	Block Tim-3	Block PD-1	Block both
$CD4^+$ T cells					
Median	4.8	9.3 (4.5% change)	9.85 (0.5% change)	7.88 (-1.97% change)	6.52 (-1.36% change)
IQR	2.0-5.5	4.8-22.1	4.75-17.0	5.0-28.7	2.4-28.4
$CD8^+$ T cells					
Median	4.55	7.57 (3.02% change)	10.11 (2.54% change)	7.58 (-2.53% change)	7.63 (0.05% change)
IQR	2.9-5.4	6.6-20.2	6.3-18.4	5.0-16.1	2.6-14.4

CHAPTER 5: DISCUSSION

In this study the expression of a range of co-inhibitory/exhaustion markers (PD-1, Tim-3, LAG-3 and 2B4) together with activation markers (HLA-DR and CD38) and death marker CD95/Fas was investigated in uninfected, HIV-1 infected and HIV/TB co-infected patients. The primary aim was to relate these markers to clinical indicators (CD4 count and VL) of disease progression to AIDS.

Inhibitory/exhaustion markers are a topic of intense research as they offer potential targets for therapeutic intervention in chronic disease. Human check-point therapy in cancer has proven potentially highly efficacious and the application of this approach in chronic infectious diseases is envisaged in the future. Another primary aim of this study was to ascertain if TB co-infection (active disease) exacerbates the expression of these markers and also whether any of the novel markers (LAG-3 and 2B4) are better biomarkers/targets based on good relevance (correlation) to standard clinical markers. While the two studies (pilot study on fresh whole blood and the main study on frozen PBMC) were comparable with respect to sampling criteria, we did however find differences in the marker expression which was unexpected.

5.1 Clinical markers of disease progression to AIDS

As described in many studies, we observed that the VL for the HIV-1 positive groups (mono- and co-infected) was highest among these individuals with the lowest CD4 count (Wanchu *et al.,* 2010; Hunt *et al.,* 2011; Sauce *et al*, 2011). As reported by Pillay *et al.,* (2009) and Hunt *et al.,* (2011), TB co-infection with HIV-1 leads to dysfunctional CTL responses, which could then lead to an inability of the CTL to control viral replication. With high viral replication in the blood and tissues, this leaves the individual exposed to opportunistic infections, which could result in death.

$CD4^+$ T cells (as reviewed by Siawaya *et al.,* 2007), play a major role in the protection against TB infection and reactivation. When a patient is infected with HIV-1, the $CD4^+$ T cells are depleted and cannot control the TB either. The CD4 count was significantly different between all three study groups as expected with the infected groups displaying the lowest CD4 count. This appears to be in line with the current literature which states that there is an inverse relationship between VL and CD4 count (Hertoghe *et al*., 2000; Smith *et al*., 2013). This has been attributed to dysfunctional CTL responses that lead to the inability to control viral replication, resulting in an increase in the number of circulating viral particles. With an increase in the number of circulating virus particles, there is depletion in the total number of infected $CD4^+$ T cells and uninfected bystander $CD4^+$ T cells. Nardacci *et al.,* (2015), state

that with a decrease in uninfected bystander $CD4^+$ T cells there is a greater likelihood of opportunistic diseases due to weakening of the immune system.

The control of TB in HIV uninfected individuals is well documented to be linked to functional $CD4^+$ T cells producing cytokines, such as IFN-γ and TNF-α. These cytokines lead to an increased recruitment of cells, such as monocytes and granulocytes to the granuloma. Increased cytokine production in turn, would lead to enhanced and more effective phagocytosis of TB bacteria and containment of disease (Li *et al.,* 2007; Siawaya *et al.,* 2007). CTLs only become activated during active TB infection and not during latent TB infection. Furthermore, in line with previous studies, Rozot *et al.,* (2013), highlighted the essential role of $CD4^+$ T cells in protection against TB, since $CD4^+$ T cell depletion is also associated with TB reactivation in HIV-1 infected patients and those with uncontrolled mycobacterial growth.

5.2 Clinical marker of immune activation and disease progression

CD38 is a well characterised marker of immune activation and is regularly used as an indicator of accelerated disease progression to death (Cockerham *et al.*, 2014). In the main study it was found that there was a significant negative correlation between CD38 expression on $CD8^+$ T cells and CD4 count in the HIV-1/TB co-infected group. The theory behind pairing immune activation and disease progression is due to highly activated $CD8^+$ T cells leading to T cell exhaustion and the inability of the $CD8^+$ T cell to perform its cytotoxic function in controlling viral replication (Musyoki *et al.,* 2014). While the expression pattern was found to be true for both the pilot and the main study, no significant correlation was found to support the findings by Musyoki and colleagues.

Furthermore, in the main study it was found that CD4 count negatively correlated with Tim-3 expression, CD38 expression on $CD8^+$ T cells and viral load. As stated earlier, This could then suggest that with high activation (CD38) over an extended period of time, the $CD8^+$ T cells tire, effective cytotoxic function decreases leading to an increase in VL and a decrease in CD4 count (Dietze *et al.,* 2013; Ezinne *et al.,* 2014). To support our current findings, Gornalusse *et al.*, (2015), found that there was a link between immune activation and the number of CCR5 receptors available for HIV binding to $CD4^+$ T cells resulting in a decrease in CD4 count. Furthermore, this is in line with the literature that explains the high levels of CD38 expression on $CD8^+$ T cells is a good clinical marker of disease progression (Holm *et al*., 2008; Sullivan *et al*., 2015). It was found in this study that both infected groups showed higher expression of CD38 on $CD8^+$ T cells when compared to the control group. Siawaya *et*

al., (2007), found that a 10% increased expression of $CD38^+$ on $CD8^+$T cells is associated with an 88% added risk that AIDS could develop. Similarly, as reviewed by Hua *et al.,* (2014), CD38 expression on $CD8^+$ T cells is one of the strongest predictors of disease progression towards AIDS. A low $CD38^+$ expression was observed in the control group (as expected) in both studies.

Similarly in the current study, CD4 count negatively correlated with CD38 and VL as well as HLA-DR on $CD4^+$ T cells for the HIV-1 positive group. As mentioned earlier, VL increases the expression of CD38 and HLA-DR on activated $CD4^+$ T cells which also express more CCR5 receptors leading to an increase in infection of these cells (Oyaizu *et al.,* 1994). Smith *et al*., (2013) also found that CD38 and HLA-DR were up-regulated on $CD4^+$ T cells in HIV-1 positive groups with a high VL. Our findings are in line with previous studies that have found an inverse relationship between VL and CD4 count together with an increase in immune activation on both $CD4^+$ and $CD8^+$ T cells as the disease progresses to AIDS (Cockerham *et al*., 2014). Bastidas *et al*., (2013), states that continuous loss of $CD4^+$ T cells and an increase in $CD8^+$ T cell activation is expected in individuals with HIV infection with as high as 60% dual expression of CD38 and HLA-DR. In line with the current study, , CD38 and HLA-DR dual expression (of almost 40%) on $CD8^+$ T cells was the two highest dual expressing markers in this study ($p<0.01$).

In comparison, studies which examined the dual expression on CD38 and HLA-DR in elite HIV controllers, have found that these individuals have a genetic mutation which allows them to maintain viral replication at a very low level and in turn decrease the adverse effects of immune activation (Hua *et al*., 2014). These individuals are able to maintain a healthy CD4 count (> 500 cells/µl) for more than 10 years without ART (Munier *et al.*, 2013). Therefore, by limiting the amount of circulating virus in the blood for many years, they are able to slow down the progression to AIDS (Sauce *et al.*, 2011; Smith *et al.*, 2013). This also limits the amount of immune activation of the T cells

The expression of HLA-DR on $CD8^+$ T cells in the main study was problematic as all three groups appear to express almost 100% of this marker. This would incorrectly imply that the control group was expressing the same degree of immune activation as the HIV-1 positive and co-infected groups, which is not in line with the current literature. We surmised that the result may have been due to antibody saturation, non-specific binding, incorrect compensation or voltage setting. The absence of a similar finding in the $CD4^+$ group seems to exclude saturation as a potential cause.

5.3 The effects of cryopreservation on T cell marker expression

It was interesting to note that an overall expression of CD38 on $CD8^+$ T cells for the HIV-1 positive group of 40% for fresh whole blood and 88% for frozen PBMCs, which could be attributed to the process of long term cryopreservation. While the topic of long term cell preservation and whether it causes irreversible damage to the cells is still in debate, Holm *et al*., (2008), found that the expression of CD38 tended to decrease on frozen T cell when compared to the fresh sample which is the opposite of what was found in the current study. They also found that cryopreserved $CD4^+$ T cells from HIV positive individuals experienced a high level of apoptosis at a rate loss of approximately -45.7 cells per year. The rate loss denotes that for every year which a HIV-1 positive $CD4^+$ T cells are kept under cryopreservation conditions, on average 45.7 $CD4^+$ T cells are lost due to apoptosis. Germann *et al.*, (2013), also found that cells exposed to the harsh conditions of cryopreservation were more prone to apoptosis than those cells which had not undergone the process. Their study concluded that cells expressing high levels of PD-1 and CD38 on $CD8^+$ T cells experienced the greatest $CD4^+$ T cell loss. In relation to the poor overall performance of the $CD4^+$ T cells in this study, Holm *et al.,* (2008), supports the notion that frozen cells performed less than optimally as a result of extremely high levels of $CD38^+$ on $CD8^+$ T cells together with PD-1. It has been well documented that the $CD8^+$ T cells which express CD38 and PD-1 are less able to control viral replication leading to a decrease in $CD4^+$ T cells (Cockerham *et al.,* 2014). It should be noted that in the current study, cryopreservation was not the only variable between the pilot and the main study; whole blood versus PBMC, 4 colour verse 9 colour flow cytometry and different gating strategies (with or without CD45 and viability) were additional variables.

On the other hand, Weinberg *et al,* (2009), found no significant difference between fresh and frozen T cells with regard to the markers expressed, viability and proliferation of the cells due to long term storage. Similarly, Riccio *et al.*, (2002), also found that PBMCs which were exposed to cryopreservation did not increase the likelihood of apoptosis. It must be noted that Weinberg *et al.,* (2009), examined cells which were cryopreserved for a period not longer than 15 months, which does not address the issue of longer term damage caused by cryopreservation for samples stored for a number of years. In the current study, cells were kept under cryopreservation conditions for almost 36 months.

5.4 PD-1 down regulates T cell effector function

PD-1 is a well characterised exhaustion marker of T cells expressed in chronic HIV infection. PD-1 has been found to down-regulate T cell function during inflammation (Wherry, 2011).

While down-regulation can be viewed as a protective mechanism, decreasing $CD8^+$ T cell function has a negative effect on the overall immune survival by decreased effector function in the control of viral replication. Rozot *et al.* (2013), showed that CTLs responded to high TB antigen burden, as the protective role of CTLs in TB is based on the ability to lyse infected cells.

In the pilot study, PD-1 expression on $CD8^+$ T cells was 3 times as high in the HIV-1/TB group as the control group ($p<0.01$). According to Kulpa *et al.,* (2013), chronic viral infection leads to increase levels of PD-1 and a reduced ability to clear viral infection. The PD-1 expression on $CD8^+$ T cells in the pilot study was almost double the amount on the co-infected cells when compared to the HIV-1 only group ($p<0.01$). This holds true to the literature that states that an increased immune burden in a co-infected setting is associated with an increase in exhaustion markers.

5.5 Novel marker Tim-3 expression was inconsistent

The purpose of the pilot study was to investigate if there was a significant difference expressed of the marker Tim-3. On completion of the pilot study it was found that Tim-3 displayed a significant difference in levels of expression across the three groups on $CD4^+$ and $CD8^+$ T cells. Tim-3 was expressed in lower levels on $CD4^+$ T cells (less than 5%) when compared to $CD8^+$ T cells. Further research was needed to understand if Tim-3 acts independently of PD-1 expression or if Tim-3 was up-regulated simultaneously with PD-1. It was concluded in the pilot study that Tim-3 was most likely unrelated to PD-1 because Tim-3 was expressed at much lower levels than PD-1 and with a very different expression pattern on $CD4^+$ and $CD8^+$ T cells.

Tim-3 was added to the main study to investigate if other related surface markers of activation and exhaustion would have an effect on the Tim-3 expression pattern. The pilot study showed that Tim-3 expression on $CD8^+$ T cells was almost twice as high in the HIV-1/TB group as in the HIV-1 only group. The expression of Tim-3 in the HIV-1/TB group was almost 9 times higher than in the control group ($p<0.01$). This would be expected as Tim-3 has been documented as a marker of immune exhaustion, which is not expected to be present in healthy participants. Similarly, a study by Sakhdari *et al.,* (2012), found that there was an increase in expression of Tim-3 on $CD8^+$ T cells, which were infected with HIV-1. In the main study it was found that Tim-3 expression on $CD4^+$ T cells while low was slightly higher in the control group when compared to the HIV and co-infected groups. As Tim-3 is a marker of T cell exhaustion, it would be expected that both infected groups would elicit greater expression. The Tim-3 expression pattern on $CD8^+$ T cells did not follow that of the pilot study and is not in line with the literature. Perhaps the low level of Tim-3 expression on

$CD8^+$ T cells could be attributed to the fact that this group had a lower VL when compared to the pilot study suggesting that these cells were not as exhausted as those cells found in the pilot study. Alternatively, as noted above in section 5.3, cryopreservation may have led to the loss of exhausted Tim-3 cells. Also, a similar trend was observed for Fas/CD95 expression.

Wang *et al.,* (2011), found that Tim-3 was up-regulated on virus-specific $CD8^+$ T cells in patients with chronic progressive HIV-1 infection and that Tim-3 was up-regulated on antigen-specific $CD8^+$ T cells in patients with active TB. This illustrates that the inhibitory receptor and the ligand interaction have a role in host immunity to HIV-1 and TB. In a normal immune response, there would be a confirmatory interaction of the antigen T cell receptor in addition to secondary co-stimulation. At the end of an immune response there is a switch to co-inhibition to restore the immune system to a homeostatic set point. However, in chronic infection, co-stimulatory interactions (B7 and CD28) are up-regulated even if the antigen is cleared, which over extended periods of time may lead to immune exhaustion.

5.6 Fas was well expressed on $CD8^+$ T cells

CD95/Fas expression on $CD8^+$ T cells was highest in the HIV-1/TB co-infected group for both studies. This was in accordance with a review article by Alimonti *et al.,* (2003), who also found that HIV-1 infection resulted in both $CD4^+$ and $CD8^+$ T cells expressing higher levels of Fas and undergoing apoptosis more readily than non-infected individuals. Our $CD8^+$ T cell data confirms up-regulated expression in the HIV positive and co-infected groups, which is related to T cell activation. CD4 data was problematic for both the pilot and the main study as it was assumed that $CD4^+$ T cell expression of Fas would follow the same expression pattern as $CD8^+$ T cells. In the pilot study, Fas expression on $CD4^+$ T cells was highest in the control group; this may be due to the $CD4^+$ T cells undergoing apoptosis during the ex *vivo* manipulation, therefore, apparent high Fas expression may be lost and the decrease may not be a true reflection for the pilot study. While in the main study, Fas on $CD4^+$ T cells was equally expressed across all three groups suggesting that HIV-uninfected individuals are experiencing equal levels of cell apoptosis as individuals who are co-infected with HIV and TB. This does not follow the findings of the literature as Hertoghe *et al.*, (2000), states that the expression of Fas is increased on both $CD4^+$ and $CD8^+$ T cells in both HIV-1 and co-infection groups with the highest level of Fas expression found on $CD8^+$ T cells with up to 75% expression when compared to the current main study of approximately 62% expression on the co-infected group ($p<0.05$). Similarly, in the pilot study, $CD8^+$ T cells display approximate 75% expression ($p<0.01$) of Fas in the co-infected groups which appears to be

in line with the literature. The high level of Fas expression combined with exhaustion implies that cells are both functionally susceptible to death in chronic infection.

5.7 Expression of PD-1 was higher on frozen PBMCs on both $CD4^+$ and $CD8^+$ T cells

PD-1 on $CD8^+$ T cells was highly expressed on both fresh and frozen samples in our study. On fresh whole blood PD-1 expression was over 4 times higher in the HIV-1 positive group than the control group. On frozen PBMCs, PD-1 was expressed on 3 times as many cells in the HIV-1 positive and co-infected groups than the control group. The expression in the HIV-1 positive and co-infected groups appeared to be similar.

On fresh whole blood, PD-1 expression on $CD4^+$ T cells was significantly different between the different groups with a 1.2 times higher expression in the HIV-1 positive group than the co-infected group. PD-1 expression appeared to be highest in the control group which was 1.4 times higher than in the HIV-1 positive group. In comparison, PD-1 expression on frozen PBMCs did not differ significantly across the groups as expected. The overall expression of PD-1 in the HIV positive group was 5% for fresh whole blood and 53% for frozen PBMCs. Campbell *et al*., (2009), investigated the expression of PD-1 using fresh whole blood compared to frozen PBMCs and their findings suggested that cryopreservation does have a negative effect on this surface marker expression. Furthermore, Campbell *et al.,* (2009), found that cryopreservation has little effect on the expression of PD-1 on $CD4^+$ T cells when compared to fresh whole blood, however, on $CD8^+$ T cells there was a 2.8 times decrease in PD-1 which underwent cryopreservation when compared to fresh samples.

5.8 Expression of PD-1 and LAG-3 correlate on CD8+ T cells; whereas PD-1 expression does not correlate with 2B4

LAG-3 is described in the literature as a marker which down regulates T cell effector function when the cell is exposed to chronic viral infection (Sega *et al*., 2014). In the main study LAG-3 displayed low levels of expression on the infected groups (approximately 2.7%) and highest levels of expression on the control group (3.3%) for both $CD4^+$ and $CD8^+$ T cells which is not in accordance with most of the literature. In favour of our current findings, Bertram *et al.,* (2010), found that LAG-3 expression on $CD8^+$ T cells exposed to chronic viral infection to be lower than 5%. Alternatively, Juno *et al.,* (2015), reports that LAG-3 is highly expressed on the surface of exhausted $CD8^+$ T cells and in healthy controls the expression is also negligible. Grosso *et al.,* (2007), found that LAG-3 was expressed in low amounts on antigen naïve $CD8^+$ T cells, and upon exposure to the antigen, LAG-3 was up-regulated. As

discussed earlier, cryopreservation and apoptosis of the most exhausted cells may be playing a role.

In the current study, the co-expression of PD-1 and LAG-3 was significantly different on $CD8^+$ T cells between the three group ($p<0.05$) with a positive correlation ($r=0.3668$, $p= 0.01$) implying that as PD-1 expression increases, so does the expression of LAG-3. The expression of these markers together was 3.8 times higher in the infected groups than in the control group which is in line with the findings of the current literature which also found that co-expression of these two markers was up-regulated on exhausted $CD8^+$ T cells (Grosso *et al.*, 2007; Grosso *et al.*, 2009; Wherry 2011). Hurst *et al.,* (2015), found that co expression of PD-1 and LAG-3 to be 5% while in our study co-expression was 1.2%. In the main study, PD-1 single expression was 31.6% which is 26 times higher than co-expression with LAG-3. This could suggest that PD-1 is a marker that is best for characterising exhausted T cells. Similar to the main study, Hurst *et al.,* (2015), found that PD-1 expression was highest when expressed singly with 15% on $CD8^+$ T cells. Dual expression of these markers on $CD4^+$ T cells did not differ significantly which could possibly be due to the initial questionable PD-1 expression on these cells. Similar to our study, Khaitian and Unutmaz, (2011), found that while PD-1 was also highly expressed on exhausted $CD4^+$ and $CD8^+$ T, and both cell types were unable to elicit the correct effector function in the presence of chronic viral infection.

2B4 is an inhibitory marker expressed on the cell surface of T cells which are experiencing stress due to chronic viral infection (Larsson *et al.,* 2013). In the current study, 2B4 and PD-1 were significantly co-expressed (27%) on $CD8^+$ T cells in the infected groups ($p<0.01$). In support of our findings, Pombo *et al.,* (2015), examined the co-expression pattern of PD-1 and 2B4 and found that together the expression was almost 50% on $CD8^+$ T cells of the HIV-1 positive group. While there was no correlation between these two markers, 2B4 has been reported to co-express with PD-1, Tim-3 and LAG-3 on exhausted T cells (Wherry, 2011; Yamamoto *et al.,* 2011). 2B4 single expression on $CD8^+$ T cells was even higher than co-expression with 75% expression on the co-infected group. Low expression of 2B4 on $CD8^+$ T cells were found to be beneficial to the cell and increase proliferation while high expression of 2B4 was associated with a decrease in T cell effector function of HIV-1 positive individuals (Aldy *et al.,* 2011). With 2B4 being so highly co-expressed with PD-1 on $CD8^+$ T cells; it suggests that perhaps 2B4 is a strong marker for disease progression to AIDS.

5.9 Co-expression of LAG-3 and 2B4 as well as Tim-3 and 2B4 displayed unusual expression patterns on $CD4^+$ T cells.

In the literature, $CD8^+$ T cells are well studied in relation to immune exhaustion. Conversely, $CD4^+$ T cells are less well described and display unusual patterns of expression of all the exhaustion markers as also seen in the current study. Furthermore, marker expression is difficult to generalise due to low end stage CD4 count which is a hallmark of this disease and of many individuals in the current study. Nevertheless, expression of Tim-3 resulted in a positive correlation with 2B4 and there was dual expression of 3.5%. Similarly, LAG-3 and 2B4 also resulted in a positive correlation and co-expression was found to be the highest on the control group. While it seems problematic to find high expression of a marker on the control groups, Costantini *et al.,* (2003), also found that cryopreservation may significantly increase effector function related markers on T cells. Hurst *et al.,* (2015), found that LAG-3 alone was highly expressed on $CD4^+$ T cells with almost 24% expression when compared to the current study of 1.3% in HIV-1 positive group. Additionally single marker expression of Tim-3 was found to be 50.2% which is drastically higher than the almost 20% found in the present study. Moreover, the main study showed that Tim-3 and LAG-3 displayed higher levels of expression when expressed independently than collectively, which was also found to be true for Hurst *et al.,* (2015).

5.10 Proliferation/ Functional responsiveness

The proliferation assays were performed in an attempt to block Tim-3 and PD-1 independently and simultaneously in order to improve T cell function. According to the literature, blocking PD-1 alone proved to be beneficial in improving T cell effector function by restoring proliferative and survival capabilities (Kulpa *et al.,* 2013). Likewise, T cells which express high levels of PD-1 on the cell surface are also more likely to be associated with apoptosis (Nilsson *et al.,* 2006). In the current study, it was found that blocking PD-1 alone did not significantly restore T cell proliferation ability with a low median proliferation value. Campbell *et al.,* (2009), found that PD-1 and PD-L1 binding was significantly decreased on $CD8^+$T cells which had been cryopreserved. In the current study, blocking Tim-3 alone appeared to have the greatest impact on proliferative ability for both $CD4^+$ and $CD8^+$ T cells groups, however this was statistically insignificant. Similar to our study, Kuchroo *et al.,* (2014) found that by blocking Tim-3 alone there was some improvement in T cell capabilities.

Conversely, blocking of both PD-1 and Tim-3 did not improve the proliferative capabilities of the $CD4^+$ or $CD8^+$T cells which was unexpected as blocking Tim-3 alone had a favourable

outcome. According to the literature Tim-3 and PD-1 co-expression on T cells is associated with an exhausted phenotype and blocking these surface markers has resulted in an increase in T cell function (Pauken and Wherry, 2015). Blocking antibodies may not have been optimally titrated in the current study or the cells were not preforming optimally due to cryopreservation procedures. Owen *et al.,* (2007), found that T cells which have been exposed to long term cryopreservation have a loss in T cell responsiveness in both $CD4^+$ and to a worse extent, $CD8^+$ T cells. Furthermore, Owen *et al.,* (2007), found that the T cells which had been stored for one year or longer displayed the worst T cell responses compared to T cells which had only been stored for a few months. According to the current study, the cells were cryopreserved for 36 months before these experiments were conducted which could perhaps explain why the cells preformed so poorly. However, there was slight change in T cell proliferation events which means that the assay worked to some extent and the proof of concept was achieved. In the future, fresh whole blood would be the preferred working sample to use in this type of proliferation assay.

5.11 Limitations and strengths of the study

To the best of our knowledge, this study was the first to compare a broad and extensive range of activation and inhibitory surface markers simultaneously in chronically HIV-1 and HIV/TB co-infected South Africans. By conducting this study, we were able to observe marker expression patterns in the South African HIV-1 and HIV-1/TB infected populations. This could prove beneficial as a focus for further research into new therapeutic and treatment strategies to alleviate exhaustion phenotype and restore immune cell function.

Despite the interesting findings, this study did have limitations. The co-infected group was small, and as such yielded some inconclusive data on the significant impact of TB co-infection. In addition, in order to ascertain the contribution of TB alone to T cell activation and exhaustion, ideally a TB mono-infected group should have been included.

The study involved two sub-studies – one utilizing fresh whole blood and the other cryopreserved PBMCs. The reason for this is that the earlier sub-study was a general survey study with insufficient material to cryopreserve the PBMC. The latter study was performed on material stored from another unrelated study. Were this study to be repeated - in order to fully understand the effects of cryopreservation on the cells a pre-experiment could have been included to ascertain marker variation on the cells found between the two sub-studies. This pre-experiment could consist of a small amount of blood collected from the HIV positive individuals (n=15) from the first pilot study as well as the main study (n=15) and exposed the cells to both fresh and frozen methodologies. By conducting this pre-experiment, a clearer understanding of the effects of cryopreservation on cell activation and cell death would be possible.

In certain instances, frozen PBMCs expressed unusual marker patterns which could be as a result of induced activation and/or cell death from exposing the cells to DMSO during the freezing and thawing processes.

The functional assay did not perform as well as expected, which could be due to the initial binding of the αCD3 antibody to CD3 receptor of the T cell while loading the cells into the 96 well plates. While there were wash steps conducted, the CD3 receptors may have been saturated with αCD3 which could have resulted in the inability of the staining CD3 antibodies to bind to the respective receptors. Gating on CD45 instead of CD3 may have proven to be more beneficial surface marker in the monitoring of T cell proliferation ability.

In summary, the findings from this study also point to the importance of using fresh blood where possible for both phenotyping and functional profiling, and also standardized flow cytometry protocols throughout.

CHAPTER 6: CONCLUSION

$CD4^+$ T cells are responsible for defending the body against tuberculosis by means of activating infected macrophages and enhancing their bactericidal abilities. On infection with HIV-1, $CD4^+$ cells are drastically diminished, as evidenced by falling CD4 count. In a TB situation, the impairment of the $CD4^+$ T cell compartment, results in impaired macrophage function which in turn manifests in uncontrolled TB replication. This in turn would increase the antigenic stimulation of T-cells – promoting exhaustion and/or death. The two diseases thus amplify the overall morbidity – with dire consequences for the host.

In this study we showed that chronic HIV-1 infection was accompanied by a significant increase in CD38 ($p<0.0001$), CD95 ($p<0.01$), PD-1 ($p<0.01$) and Tim-3 ($p<0.1$) expression on $CD8^+$ T cells in fresh whole blood. TB co-infection led to significantly elevated expression of CD38, CD95 and Tim-3, but not PD-1 (all $p<0.05$).

$CD8^+$ T cell-associated CD38, CD95, and PD-1 displayed a similar trend in cryopreserved PBMCs in a separate cohort, with significant higher expression in the infected groups ($p<0.0001$, $p<0.05$, and $p<0.0001$, respectively), In contrast to the whole blood finding, Tim-3 expression was consistently <10%, with no difference between the groups. The novel marker 2B4 showed high level baseline expression (median 59.2%) which was significantly increased in the HIV and HIV/TB groups (69.6% and 75.1% respectively, $p=0.025$). LAG-3 was however poorly expressed. Co-expression of PD-1 and 2B4 as well as CD95 and CD38 was also significantly increased ($p<0.0001$ for both).

These findings suggest that immune dysfunction is apparent in both HIV-1 infection and HIV-1/TB co-infection. Nevertheless, PD-1 and 2B4 single marker expression (on both $CD4^+$ and $CD8^+$ T cells) as well as co-expression on $CD8^+$ T cells, suggests a potentially co-ordinated up-regulation pattern between these two markers. 2B4 is an exciting biomarker as its high level expression patterns, its correlation to LAG-3 expression and its significant PD-1 co-expression, suggest it plays a major role in regulating T cell function. Although blocking Tim-3 and PD-1 on $CD4^+$ and $CD8^+$ T cells did not appear to have beneficial effects on T cell proliferation in this study, the functional implications of manipulating these exhaustion pathways needs to be elucidated. Additional work is needed to ascertain conclusively whether blocking these markers plus 2B4 or LAG-3 could restore functional integrity.

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Addendum A

Ethics approval letters

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
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17 July 2007

Prof W Preiser
Division of Medical Virology
Dept of Pathology

Dear Prof Preiser

RESEARCH PROJECT : "VIROLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION OF CRYOPRESERVED BLOOD AND VIRUS SAMPLES"
PROJECT NUMBER : N07/06/133

It is my pleasure to inform you that the abovementioned project has been provisionally approved on 16 July 2007 **for a period of one year from this date.** You may start with the project, but this approval will however be submitted at the next meeting of the Committee for Human Research for ratification, after which we will contact you again.

Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary to make their final decision.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

In future correspondence, kindly refer to the above project number.

I wish to remind you that patients participating in a research project at Tygerberg Hospital will not receive their treatment free, as the PGWC does not support research financially.

The nursing staff of Tygerberg Hospital can also not provide extensive nursing aid for research projects, due to the heavy workload that is already being placed upon them. In such instances a researcher might be expected to make use of private nurses instead.

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: cjvt@sun.ac.za

CJVT/pm

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Afdeling Navorsingsontwikkeling en -steun • Research Development and Support Division
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20 August 2008

Dr C de Beer
Dept of Pathology
Division of Medical Virology

Dear Dr C de Beer

RESEARCH PROJECT : "VIROLOGICAL AND IMMUNOLOGICAL CHARACTERISATION OF CRYOPRESERVED BLOOD AND VIRUS SAMPLES"

PROJECT NUMBER : N07/06/133

Your letter dated 5 August 2008 refers.

The Chairperson of the Committee for Human Research approved the subject data form in accordance with the authority given to him by the Committee.

This approval will be submitted to the Committee for ratification.

However, a small change to the participant informed consent form is recommended: On page 2, after line 1 (starts with "General information…"), please insert the line: ***In some participants, questions of a more personal nature may be asked, including sexual history.***

Then please send a copy of the amended form to us for record keeping.

Kind regards

pp
Prof PJJ de Villiers
Chairperson: Committee for Human Research
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: mertrude@sun.ac.za

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10 September 2008

Dr C de Beer
Dept of Pathology
Division of Medical Virology

Dear Dr de Beer

RESEARCH PROJECT : "VIROLOGICAL AND IMMUNOLOGICAL CHARACTERISATION OF CRYOPRESERVED BLOOD AND VIRUS SAMPLES"

PROJECT NUMBER : N07/06/133

My letter dated 20 August 2008 refers.

At a meeting that was held on 3 September 2008 the Committee for Human Research ratified the Chairperson's approval of the amendment.

Kind regards

pp
Prof PJT de Villiers
Chairperson: Committee for Human Research
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: mertrude@sun.ac.za

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12 February 2009

Dr C de Beer
Dept of Pathology
Division of Medical Virology

Dear Dr de Beer

RESEARCH PROJECT : "VIROLOGICAL AND IMMUNOLOGICAL CHARACTERISATION OF CRYOPRESERVED BLOOD AND VIRUS SAMPLES"

PROJECT NUMBER : N07/06/133

At a meeting of the Committee for Human Research that was held on 4 February 2009 the progress report for the abovementioned project has been approved and the study has been granted an extension **for a period of one year from this date.**

Please remember to submit progress reports in good time for annual renewal in the standard CHR format.

For standard CHR forms and documents please visit:
www.sun.ac.za/knowledgepartner/committees_CHR.htm

Kind regards

pp
Prof PJT de Villiers
Chairperson: Committee for Human Research
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: mertrude@sun.ac.za

Approval Date: 4 February 2009 **Expiry Date**: 4 February 2010

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23 June 2010 **MAILED**

Dr C de Beer
Department of Pathology
6th Floor,clinical building
Stellenbosch University
Tygerberg Campus
7505

Dear Dr de Beer

"Virological and immunological characterisation of cryopreserved blood and virus samples."

ETHICS REFERENCE NO: N07/06/133

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 14 June 2010, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 14 June 2010 Expiry Date:14 June 2011

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

23 June 2010 10:45 Page 1 of 1

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Approval Notice
New Application

08-Mar-2012
De Beer, Corena C

Protocol #: N12/02/008
Title: Virological and immunological characterisation of cryopreserved blood and virus samples

Dear Doctor Corena De Beer,

The **New Application** received on **24-Feb-2012**, was reviewed by staff members of the REC office on **06-Mar-2012** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **06-Mar-2012 -06-Mar-2013**

Please remember to use your **protocol number** (**N12/02/008**) on any documents or correspondence with the REC concerning your research protocol.

Please note that the REC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired.
The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number projects may be selected randomly for an external audit.
Translation of the consent document in the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Hlne Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard REC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further help, please contact the REC office at 0219389207.

Included Documents:
Consent
Checklist
Synopsis
CV
Declaration
Application
Protocol

Sincerely,

Mertrude Davids
REC Coordinator
Health Research Ethics Committee 2

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Ethics Letter

02-Apr-2013

Ethics Reference #: N12/02/008
Title: Virological and immunological characterisation of cryopreserved blood and virus samples

Dear Doctor Corena De Beer,

At a review panel meeting of the Health Research Ethics Committee that was held on 4 March 2013, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date:4 March 2013 Expiry Date: 4 March 2014

If you have any queries or need further help, please contact the REC Office 0219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

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Ethics Letter

25-Jun-2014

Ethics Reference #: N12/02/008
Title: Virological and immunological characterisation of cryopreserved blood and virus samples

Dear Doctor Corena De Beer,

At a review panel meeting of the Health Research Ethics Committee that was held on 16 April 2014, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 16 April 2014 Expiry Date: 16 April 2015

If you have any queries or need further help, please contact the REC Office 0219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

Investigator Responsibilities

Protection of Human Research Participants

Some of the responsibilities investigators have when conducting research involving human participants are listed below:

1.Conducting the Research. You are responsible for making sure that the research is conducted according to the REC approved research protocol. You are also responsible for the actions of all your co-investigators and research staff involved with this research.

2.Participant Enrollment. You may not recruit or enroll participants prior to the REC approval date or after the expiration date of REC approval. All recruitment materials for any form of media must be approved by the REC prior to their use. If you need to recruit more participants than was noted in yourREC approval letter, you must submit an amendment requesting an increase in the number of participants.

3.Informed Consent. You are responsible for obtaining and documenting effective informed consent using **only** the REC-approved consent documents, and for ensuring that no human participants are involved in research prior to obtaining their informed consent. Please give all participants copies of the signed informed consent documents. Keep the originals in your secured research files for at least five (15) years.

4.Continuing Review. The REC must review and approve all REC-approved research protocols at intervals appropriate to the degree of risk but not less than once per year. There is **no grace period.** Prior to the date on which the REC approval of the research expires, **it is your responsibility to submit the continuing review report in a timely fashion to ensure a lapse in REC approval does not occur.** If REC approval of your research lapses, you must stop new participant enrollment, and contact the REC office immediately.

5.Amendments and Changes. If you wish to amend or change any aspect of your research (such as research design, interventions or procedures, number of participants, participant population, informed consent document, instruments, surveys or recruiting material), you must submit the amendment to the REC for review using the current Amendment Form. You **may not initiate**any amendments or changes to your research without first obtaining written REC review and approval. The **only exception** is when it is necessary to eliminate apparent immediate hazards to participants and the REC should be immediately informed of this necessity.

6.Adverse or Unanticipated Events. Any serious adverse events, participant complaints, and all unanticipated problems that involve risks to participants or others, as well as any research related injuries, occurring at this institution or at other performance sites must be reported to the REC within **five (5) days** of discovery of the incident. You must also report any instances of serious or continuing problems, or non-compliance with the RECs requirements for protecting human research participants. The only exception to this policy is that the death of a research participant must be reported in accordance with the Stellenbosch Universtiy Health Ethics Committee Standard Operating Procedures www.sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Centres%20and%20Institutions/Research_Development_Support/Ethics/Application_package All reportable events should be submitted to the REC using the SAE Report Form.

7.Research Record Keeping. You must keep the following research related records, at a minimum, in a secure location for a minimum of fifteen years: the REC approved research protocol and all amendments; all informed consent documents; recruiting materials; continuing review reports; adverse or unanticipated events; and all correspondence from the REC

8.Reports to MCC and Sponsor. When you submit the required annual report to the MCC or you submit required reports to your sponsor, you **must** provide a copy of that report to the REC. You may submit the report at the time of continuing REC review.

9.Provision of Emergency Medical Care. When a physician provides emergency medical care to a participant without prior REC review and approval, to the extent permitted by law, such activities will not be recognized as research nor the data used in support of research.

10.Final reports. When you have completed (no further participant enrollment, interactions, interventions or data analysis) or stopped work on your research, you must submit a Final Report to the REC.

11.On-Site Evaluations, MCC Inspections, or Audits. If you are notified that your research will be reviewed or audited by the MCC, the sponsor, any other external agency or any internal group, you must inform the REC immediately of the impending audit/evaluation.

Addendum B

Beckman Coulter Flow- check pro fluorosphere

Flow-Check Pro Fluorospheres

REF A69183 - 3 x 10 mL

PN A70448-AE

BECKMAN COULTER

Flow Cytometer Alignment Verification

For Research Use Only.
Not for use in diagnostic procedures.

PRODUCT DESCRIPTION

Flow-Check Pro Fluorospheres is a suspension of fluorescent microspheres, which may be used for daily verification of a flow cytometer's optical alignment and fluidics system.

SUMMARY AND EXPLANATION

In flow cytometric analysis, optical and fluidics systems are aligned to maximize the detection of fluorescence and scatter signals. The use of uniform fluorospheres to verify optical alignment has been well established.[1-3]
Flow-Check Pro is a mixture of three different types of fluorospheres, each with a uniform and stable size and fluorescence intensity. The uniformity of these product parameters allows for adjustment and/or verification of the alignment of the optical and fluidics systems of flow cytometers.

REAGENTS

Flow-Check Pro Fluorospheres
REF A69183 - 3 x 10 mL

REAGENT CONTENTS

Flow-Check Pro Fluorospheres consists of a mixture of 10 µm fluorospheres with a fluorescence emission of 515 nm to 800 nm when excited at 488 nm, 6 µm fluorospheres with a fluorescence emission of 640 nm to 800 nm when excited at 635 nm, and 3 µm fluorospheres with a fluorescence emission of 400 nm to 500 nm when excited at 405 nm, respectively. The mixture is suspended in an aqueous medium containing surfactants and preservatives at a total concentration of 2×10^6 fluorospheres/mL (nominal concentration).

STATEMENT OF WARNINGS

WARNING
reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC# 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC# 220-239-6](3:1) <0.05%
May cause an allergic skin reaction.
Wear protective gloves, protective clothing and eye/face protection.
If skin irritation or rash occurs: Get medical advice/attention.
Take off contaminated clothing and wash it before use.
SDS Safety Data Sheet is available at *techdocs.beckmancoulter.com.*

1. This product should only be used in its suspension medium. Addition of organic solvents or high ionic strength solutions may irreversibly swell or aggregate fluorospheres.
2. Fluorospheres will settle over extended periods. Ensure the fluorospheres are completely resuspended before use.
3. Do not use fluorospheres beyond the expiration date on the vial label.
4. Use Good Laboratory Practice (GLP) when handling this reagent.
5. Do not aspirate fluorospheres directly from the vial. Aspirate fluorospheres from a test tube.

STORAGE CONDITIONS AND STABILITY

This reagent is stable to the expiration date on the vial label when stored at 2-8°C. Do not freeze. Minimize exposure to light. Open vial stability is 65 days. Open vials must be refrigerated after use.

EVIDENCE OF DETERIORATION

Inability to obtain expected results may indicate product instability or deterioration. The presence of a bimodal peak is not necessarily indicative of product deterioration. Refer to the appropriate Instrument Manuals for performance specifications and determining expected results.

REAGENT PREPARATION

No preparation is necessary. Flow-Check Pro Fluorospheres is used directly from the vial with no dilution. Proper mixing is required prior to use.

PROCEDURE

MATERIAL SUPPLIED

Flow-Check Pro Fluorospheres
REF A69183 - 3 x 10 mL

MATERIALS REQUIRED BUT NOT SUPPLIED

Appropriately sized test tubes
Flow cytometer
Vortex Mixer

Procedure for Daily Verification of Alignment and Fluidics

1. Use the filter set recommended by the manufacturer for detecting the appropriate fluorescence parameters (refer to Instrument Manuals).
2. Use a Flow-Check Pro Fluorospheres test protocol containing single parameter histograms for FS and each desired linear fluorescence parameter. Create a gate region FCblue that encompasses the 10 µm beads. Assign that gate to the linear fluorescence single parameter histograms which correspond to the blue (488 nm) laser channels. Create a gate region FCred that encompasses the 6 µm beads. Assign that gate to the linear fluorescence single parameter histograms which correspond to the red (635 nm) laser channels. Create a gate region FCviolet that encompasses the 3 µm beads. Assign that gate to the linear fluorescence single parameter histograms which correspond to the violet (405 nm) laser channels. Create a linear region within each single parameter histogram. Set color compensation to zero (0) percent for all fluorescence compensations. Set a stop on 5,000 gated events (see Figure 1).
3. Vigorously mix the Flow-Check Pro Fluorospheres vial.
4. Dispense 15-20 drops (about 0.5 mL) of Flow-Check Pro Fluorospheres into a test tube.

IMPORTANT: TO AVOID CONTAMINATION AND DEGRADATION, DO NOT ASPIRATE DIRECTLY FROM THE VIAL.

5. Vortex and aspirate the fluorospheres sample from the test tube.
6. Adjust the appropriate detector settings as needed to place each peak within the peak position (X-Mean) range established.
7. Record the HPCV and peak position for each desired parameter.

   NOTE: For forward scatter channel record only FCblue (10 µm) HPCV values.

8. Record the daily HPCV values for each desired parameter on its respective Levey-Jennings graph.

   NOTE: When running BCI Auto Setup Flow-Check Pro protocols, the PASS/FAIL criteria observed at the end of acquisition represents HPCVs exceeding the upper limit defined in the protocol's region name. Review the QC Levey Jennings data.

9. Repeat steps 1-8 following instrument start up.
10. Refer to the troubleshooting section when results fall outside expected limits.

LIMITATIONS

1. Flow-Check Pro Fluorospheres analyzed at increased flow rates may exhibit wider population distributions and a higher HPCV.
2. Day to day analysis of Flow-Check Pro Fluorospheres should be conducted using the same peak positions determined in Procedure for Daily Verification of Alignment and Fluidics.

TROUBLESHOOTING

1. Ensure that the fluorospheres sample has not been diluted or contaminated. Dilution of Flow-Check Pro Fluorospheres may increase the HPCV values recovered.
2. Ensure the Flow-Check Pro Fluorospheres has been adequately mixed.
3. Check for bubbles in the sheath filter. Review histogram patterns to check for a clog. If a clog or bubble is suspected, flush or prime the sample line.
4. Refer to Instrument Manuals for additional troubleshooting steps.

EXPECTED RESULTS

Refer to appropriate Instrument Manuals for performance specifications.

Expected results may vary slightly due to instrument differences such as color filters, laser power, laser emission wavelength, laser mode, flow cell type, sample delivery rate, and statistical analysis package.

REFERENCES

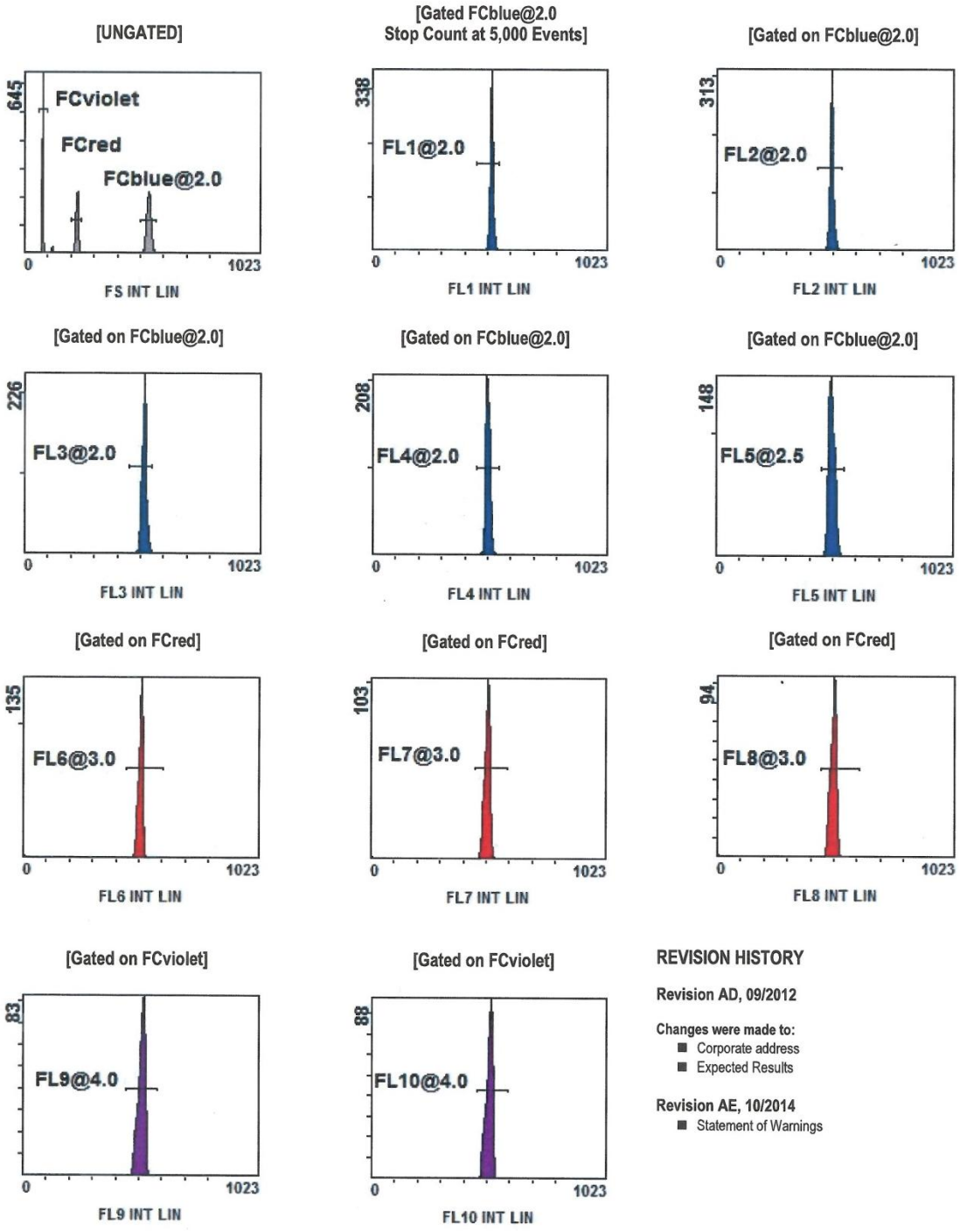
1. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline-Second Edition. 2007. CLSI document H42-A2.
2. Guideline for Flow Cytometric Immunophenotyping: A Report From the National Institute of Allergy and Infectious Diseases, Division of AIDS. 1993. Cytometry 14:702-715.
3. 1994 Revised Guidelines for the Performance of CD4+ T-Cell Determinations in Persons with Human Immunodeficiency Virus (HIV) Infection.1994. Mortality and Morbidity Weekly Report (MMWR). 43:7-8.

PRODUCT AVAILABILITY

Flow-Check Pro Fluorospheres
REF A69183 - 3 x 10 mL vials

TRADEMARKS

Figure 1. Flow-Check Pro Fluorospheres sample histograms for a ten-color three laser system.



REVISION HISTORY

Revision AD, 09/2012

Changes were made to:

- Corporate address
- Expected Results

Revision AE, 10/2014

- Statement of Warnings

Addendum C:

Single and dual expression tables for $CD4^+$ T cells

Addendum C Table 1 Significance of Single Expression on CD4+ T cells (median values)

	p-value	Control	HIV^+	HIV^+/TB^+
PD-1	0.4	53.5	53.9	58.4
Tim-3	0.6	26.6	19.6	18.8
LAG-3	0.1	2.9	1.3	1.4
2B4*	0.02	21.6	10.7	16.1
CD38*	0.0001	16.7	42.8	41.7
HLA-DR*	0.0001	1.9	5.7	5.9
Fas*	0.4	53.5	52.9	58.4

Surface markers indicated with an * denote significance

Addendum C Table 2 Significance of Dual Expression on $CD4^+$ T cells (median values)

	p-value	**Control**	**HIV^+**	**HIV^+/TB^+**
PD-1 and Tim-3	**0.6**	**5.1**	**7.2**	**8.0**
LAG-3 and PD-1	**0.9**	**0.7**	**0.5**	**0.2**
PD-1 and 2B4	**0.7**	**3.9**	**5.4**	**3.8**
Tim-3 and LAG-3	**0.2**	**1.6**	**0.5**	**0.3**
Tim-3 and 2B4*	**0.01**	**7.3**	**3.5**	**2.4**
LAG-3 and 2B4*	**0.01**	**1.0**	**0.2**	**0.2**

Surface markers indicated with an * denote significance

Addendum C Table 3 Significance of Dual Expression on $CD4^+$ T cells (median values)

	p-value	Control	HIV^+	HIV^+/TB^+
PD-1 and Fas*	0.01	7.2	16.7	22.8
PD-1 and CD38*	0.0001	0.5	7.5	4.9
PD-1 and HLA-DR*	0.0001	0.2	1.6	1.2
HLA-DR and CD38*	0.0001	1.4	4.4	4.9
Fas and CD38*	0.0001	0.4	2.2	2.4
Fas and HLA-DR*	0.0001	0.2	1.0	1.4

Surface markers indicated with an * denote significance

Addendum D:

Single and dual expression tables for $CD8^+$ T cells

Addendum D Table 1 Significance of Dual Expression on $CD8^+$ T cells (median values)

	p-value	Control	HIV^+	HIV^+/TB^+
PD-1 and Tim-3	0.2	1.5	2.6	1.9
LAG-3 and PD-1*	0.01	0.4	1.2	1.1
PD-1 and 2B4*	0.0001	8.3	27.2	25.6
Tim-3 and LAG-3	0.9	0.5	0.4	0.1
Tim-3 and 2B4	0.6	5.6	3.8	3.1
LAG-3 and 2B4	0.6	1.9	2.6	1.8

Surface markers indicated with an * denote significance

Addendum D Table 2 Significance of Single Expression on $CD8^+$ T cells (median values)

	p-value	Control	HIV$^+$	HIV$^+$/TB$^+$
PD-1*	0.0001	9.5	31.6	34.9
Tim-3	0.6	6.3	5.5	3.5
LAG-3	0.9	3.3	3.2	2.2
2B4*	0.01	59.2	69.6	75.1
CD38*	0.0001	49.6	88.6	80.3
HLA-DR	0.5	100.	99.98	100.00
Fas*	0.01	15.9	34.0	43.9

Surface markers indicated with an * denote significance

Addendum D Table 3 Significance of Dual Expression on CD8$^+$ T cells (median values)

	p-value	Control	HIV$^+$	HIV$^+$/TB$^+$
PD-1 and Fas*	0.01	6.0	14.9	22.0
PD-1 and CD38*	0.0001	1.9	17.1	18.6
PD-1 and HLA-DR*	0.01	32.7	37.8	43.9
HLA-DR and CD38*	0.0001	5.9	38.6	25.4
Fas and CD38*	0.0001	1.8	14.6	18.4
Fas and HLA-DR*	0.01	14.9	31.9	43.5

Surface markers indicated with an * denote significance

Addendum E:

Correlation tables for CD4$^+$ T cells

Addendum E Table 1 Co-expression on CD4$^+$ T cells correlations

		r value	star rating	direction	p-value
PD-1	Fas	0.5791	****	+	0.01
Tim-3	PD-1	0.4428	****	+	0.01
	2B4	0.4918	****	+	0.05
	LAG-3	0.9195	****	+	0.01
	HLA-DR	0.3224	**	+	0.01
	Fas	0.6437	****	+	0.01
HLA-DR	VL	0.2547	*	+	0.05
	PD-1	0.4743	****	+	0.01
	Fas	0.3945	***	+	0.01
	CD38 ON 4	0.6013	****	+	0.01
2B4	PD-1	0.5977	****	+	0.01
	LAG-3	0.5579	****	+	0.01
	Fas	0.3990	***	+	0.01
	CD38 ON 4	0.3269	**	-	0.01

LAG-3	PD-1	0.4110	***	+	0.01
	Fas	0.5327	****	+	0.01
CD38	2B4	0.4130	***	-	0.01
	LAG-3	0.2913	*	-	0.05
	HLA-DR	0.3871	***	+	0.01
	CD4 COUNT	0.3588	**	-	0.01
	VL	0.4104	***	+	0.01
	Fas	0.1464	x	x	0.21
CD4 COUNT	HLA-DR	0.3264	**	-	0.05
VL	HLA-DR	0.2547	*	+	0.05
	CD4 COUNT	0.4630	****	-	0.01

Addendum F:

Correlation tables for CD8$^+$ T cells

Addendum F Table 1 Co-expression on CD8$^+$ T cells correlations

Fas	PD-1	0.2808	*	+	0.05
	CD4	0.0196	X	X	0.9
	Tim-3	0.4386	***	+	0.01
	2B4	0.2842	*	+	0.05
	LAG-3	0.5752	****	+	0.01
	HLA-DR	0.4528	****	+	0.01
	VL	0.0615	X	X	0.6
PD-1	Tim-3	0.3825	***	+	0.01
	LAG-3	0.3668	**	+	0.01
	2B4	0.2065	X	X	0.1
Tim-3	2B4	0.2722	*	+	0.05
	LAG-3	0.8799	****	+	0.01
	CD4 COUNT	0.2366	*	-	0.05
HLA-DR	LAG-3	0.2577	*	+	0.05
2B4	LAG-3	0.3695	**	+	0.01
CD4 COUNT	CD38	0.3588	**	-	0.01
	VL	0.4630	****	-	0.01