Phenotypic and functional characterization of cytotoxic T lymphocytes in HIV-1 infected South African adults

by
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December 2010
“Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any university for a degree.

Signature: 

Date: 16 November 2010
# Table of Contents

Declaration ....................................................................................................................... ii
List of Abbreviations ........................................................................................................ vi
List of Figures .................................................................................................................. x
List of Tables ................................................................................................................. xiii
Abstract ......................................................................................................................... xiv
Opsomming ................................................................................................................... xvi
Acknowledgements ..................................................................................................... xviii

## CHAPTER 1: INTRODUCTION ..................................................................................... 1

## CHAPTER 2: LITERATURE REVIEW ........................................................................... 2

2.1. Overview of the HIV/AIDS Epidemic .............................................................. 2
2.2. The HIV-1 Virus .............................................................................................. 5
2.3.1. The HIV-1 Replication Cycle .......................................................................... 7
2.3.1.1. Entry ............................................................................................................... 7
2.3.1.2. Reverse Transcription and integration ............................................................ 8
2.3.1.3. Transcription and translation ................................................................. 8
2.3.1.4. Assembly and budding ................................................................................... 8
2.4. HIV Pathogenesis .......................................................................................... 10
2.4.1. The Acute Phase .......................................................................................... 10
2.4.2. Clinical Latency/Chronic Phase .................................................................... 11
2.4.3. Progression to AIDS ..................................................................................... 12
2.5. The Immune Response to HIV ..................................................................... 12
2.5.1. The Cellular Immune Response and Importance of CTLs ....... 12
2.5.2. Humoral Immune Response ......................................................................... 13
2.5.3. HIV-associated immune activation and apoptosis ........................................ 14
2.5.4. Importance of CD8+ CTL in host response to HIV infection ......................... 16
2.6. HIV and TB Coinfection .................................................................................. 19
2.7. Cytotoxic mechanisms of CD8+ T cells ........................................................ 21
2.7.1. Granule-dependent exocytosis pathway ....................................................... 22
2.7.1.1. Perforin ......................................................................................................... 22
2.7.1.2. Granzymes ................................................................................................... 23
2.7.1.3. Granulysin .................................................................................................... 26
2.7.2. Receptor-ligand pathways ............................................................................ 27
2.7.2.1. Fas – FasL and TRAIL-DR mediated pathway ............................................. 27
2.7.2.2. The TNF and TNFR type I dependent pathway ............................................. 30
2.8. Characterization of CTL in HIV ...................................................................... 31
2.8.1. Phenotypic markers vs. functional markers .................................................. 31
2.8.2. Techniques for measuring T-cell activity ...................................................... 32
2.8.2.1. Analysis of T cell proliferation ...................................................................... 32
2.8.2.2. Measurement of CTL Surface Marker Changes ............................................ 33
2.8.2.2.1. Flow cytometric quantification of CD107 surface expression ....................... 33
2.8.2.3. Detection of cytokine production ............................................................... 34
2.8.2.4. Measurement of Immune Activation ........................................................... 34
2.8.2.4.1. Markers of Activation ................................................................................. 34
2.9. Importance of detailed characterization of CTL in HIV for evaluating vaccine or therapy efficacy ........................................................................................................... 36
2.10. Aims of this Study ......................................................................................... 36

CHAPTER 3: METHODOLOGY ................................................................................... 38
3.1. Donors .......................................................................................................... 38
3.2. Venipuncture ................................................................................................ 38
3.3. CD4 Count .................................................................................................... 38
3.4. Viral Load ..................................................................................................... 39
3.5. Peripheral Blood Mononuclear Cell (PBMC) preparation ............................. 39
3.6. Freezing of PBMCs ...................................................................................... 40
3.7. Thawing of frozen PBMCs ............................................................................ 41
3.8. HIV gag pool peptides .................................................................................. 41
3.9. In vitro stimulation ........................................................................................ 41
3.10. Degranulation Assay ................................................................................... 43
3.11. Antibody Staining Mix Preparation ............................................................. 43
3.12. Staining Protocol .......................................................................................... 44
3.13. Acquisition of Flow Cytometry data ............................................................ 45
3.15. Gating Strategy for HIV+TB- Cohorts........................................................... 51
3.17. Statistical Analysis........................................................................................ 57

CHAPTER 4: RESULTS ............................................................................................. 58
4.1. Patient Demographic.................................................................................... 58
4.2. CD4 counts and viral loads......................................................................... 58
4.3. Activation status ...................................................................................... 61
4.4. Phenotypic and Functional status.............................................................. 65
4.4.1. Perforin.................................................................................................. 65
4.4.2. Granzyme A ......................................................................................... 67
4.4.3. CD107a/b (LAMP-1/-2) ....................................................................... 70
4.4.4. Fas (CD95) .......................................................................................... 72
4.4.5. FasL (CD95L) ...................................................................................... 74
4.4.6. Cytokines (IFN-γ and TNF-α)................................................................ 78
4.5. Immune Dysfunction – PD-1.................................................................... 81
4.6. CD137 and PD-1 Dual Expression .............................................................. 83
4.7. Correlation between CTL marker expression, CD4 count and Viral Load .... 85

CHAPTER 5: DISCUSSION ..................................................................................... 87
5.1. Expression of the novel activation marker CD137 (4-1BB).......................... 88
5.2. Expression of Fas (CD95) and FasL ........................................................... 90
5.3. Expression of Programmed Death-1 (PD-1; CD279) .................................. 91
5.4. Perforin and Granzyme A............................................................................ 93
5.5. Measurement of Degranulation (CD107a/b)............................................. 95
5.6. Cytokine production - Interferon-gamma (IFN-γ) and Tumour necrosis factor –
alpha (TNF-α) Production............................................................................. 95
5.7. Limitations of this study ......................................................................... 97
5.8. Summary of findings and future questions ............................................. 98

CHAPTER 6: CONCLUSION ............................................................................... 99
REFERENCES.................................................................................................... 101
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-AAD</td>
<td>7-amino actinomycin D</td>
</tr>
<tr>
<td>7-TM</td>
<td>Seven-transmembrane</td>
</tr>
<tr>
<td>51Cr</td>
<td>Radiolabeled Chromium</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>Ape1</td>
<td>Apurinic endonuclease 1</td>
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<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
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<tr>
<td>ARV</td>
<td>Antiretroviral</td>
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<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>BD</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>BLCL</td>
<td>B lymphoblastoid cell lines</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>5(6)-carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CRF</td>
<td>Circulating Recombinant Forms</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signaling-complex</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ELISPOT</td>
<td>Single cell Enzyme-linked immunosorbent assay</td>
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<tr>
<td>Env</td>
<td>Envelope glycoprotein</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FACS</td>
<td>Fluorescent Activated Cell Sorter</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FasL</td>
<td>Fas Ligand</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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<td>------</td>
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<tr>
<td>FATAL</td>
<td>Fluorometric assessment of T lymphocyte antigen-specific lysis</td>
</tr>
<tr>
<td>FCC assay</td>
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</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>gravity</td>
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<tr>
<td>Gag</td>
<td>Group antigen</td>
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<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>gp</td>
<td>Glycoprotein</td>
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<tr>
<td>Group M</td>
<td>Group Major</td>
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<td>Group Outlier'</td>
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<td>Group non M/non O'</td>
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<td>Grzs</td>
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<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
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<td>Human immunodeficiency virus</td>
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<td>HIV-1</td>
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<tr>
<td>HIV+TB-</td>
<td>Human immunodeficiency virus positive Tuberculosis negative</td>
</tr>
<tr>
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</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen-DR</td>
</tr>
<tr>
<td>HMG-2</td>
<td>High mobility group 2</td>
</tr>
<tr>
<td>ICC</td>
<td>Intracellular Cytokine</td>
</tr>
<tr>
<td>IFN</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KZN</td>
<td>Kwazulu-Natal</td>
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<tr>
<td>LAMP</td>
<td>Lysosomal Associated Membrane glycoprotein</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
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<tr>
<td>LTNP</td>
<td>Long-term non progressors</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inhibitory Protein</td>
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</table>
mRNA  messenger RNA
MSM  Men who have Sex with Men
Mtb  *Mycobacterium tuberculosis*
MTPAs  Microtubule-targeted tubulin-polymerizing agents
n  number of subjects
NASBA  Nucleic Acid Sequence-Based Amplification
NK  Natural killer
NM23-H1  Nonmetastatic protein 23 homologue 1
NSI  Non-syncytium-inducing
PBMCs  Peripheral blood mononuclear cells
PBS  Phosphate buffered saline
PCR  Polymerase Chain Reaction
PD-1  Programmed death 1
PE  Phycoerythrin
PerCP  Peridinin chlorophyll protein
PHA  Phytohemagglutinin
PMA  Phorbol myristate acetate
pp32  Phospho-protein 32
RNA  Ribonucleic Acid
ROS  Reactive oxygen species
rpm  revolutions per minute
RT  Reverse Transcriptase
SAS ANCOVA  SAS Software Analysis of Covariance
SEB  Staphylococcal enterotoxin B
SEM  Standard error of the Mean
SI  Syncytium-Inducing
SIV  Simian immunodeficiency virus
SIVcpz  Simian immunodeficiency virus chimpanzees
SIVsm  Simian immunodeficiency virus sooty mangabeys
SIVmac  Simian immunodeficiency virus macaques
Tat  Transactivator protein
<table>
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<tr>
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</thead>
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<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFSF</td>
<td>TNFR superfamily</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor Receptor</td>
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<td>TRAFs</td>
<td>TNF receptor associated factors</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRAIL-R</td>
<td>TNF-related apoptosis-inducing ligand receptor</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>Joint United Nations programme on HIV/AIDS</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Global depiction of adult HIV infection, 2007</td>
<td>3</td>
</tr>
<tr>
<td>2.2</td>
<td>Structure of the HIV-1 virion</td>
<td>6</td>
</tr>
<tr>
<td>2.3</td>
<td>Diagram of the HIV genome</td>
<td>7</td>
</tr>
<tr>
<td>2.4</td>
<td>The life cycle of HIV</td>
<td>9</td>
</tr>
<tr>
<td>2.5</td>
<td>The course of HIV-1 infection</td>
<td>12</td>
</tr>
<tr>
<td>2.6</td>
<td>HIV-specific CD8+ T cell response in HIV controllers</td>
<td>19</td>
</tr>
<tr>
<td>2.7</td>
<td>CD8+ T cell and NK cell interacting with target cell</td>
<td>21</td>
</tr>
<tr>
<td>2.8</td>
<td>Granzyme A and Granzyme B induction of apoptosis</td>
<td>25</td>
</tr>
<tr>
<td>2.9</td>
<td>The internalization of lytic molecules</td>
<td>26</td>
</tr>
<tr>
<td>2.10</td>
<td>The Fas-FasL pathway</td>
<td>30</td>
</tr>
<tr>
<td>3.1</td>
<td>Example of Acquisition template for Healthy Cohorts</td>
<td>47-50</td>
</tr>
<tr>
<td>3.2</td>
<td>Example of Acquisition template for HIV+TB- Cohorts</td>
<td>51-54</td>
</tr>
<tr>
<td>3.1</td>
<td>Example of Acquisition template for HIV+TB+ Cohorts</td>
<td>55-56</td>
</tr>
<tr>
<td>4.1</td>
<td>Box-and-whisker plot representation of CD4 counts data across study groups</td>
<td>59</td>
</tr>
<tr>
<td>4.2</td>
<td>Box-and-whisker plot representation of viral loads across HIV+ groups</td>
<td>60</td>
</tr>
<tr>
<td>4.3</td>
<td>Graph showing the Linear regression of CD137 vs. CD25 expression on CD8+ T cells following 18 hours of SEB stimulation</td>
<td>62</td>
</tr>
<tr>
<td>4.4</td>
<td>Graph showing the Linear regression of CD137 vs. CD69 expression on CD8+ T cells following 18 hours of SEB stimulation</td>
<td>62</td>
</tr>
<tr>
<td>4.5</td>
<td>Comparison of CD137 expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation</td>
<td>64</td>
</tr>
<tr>
<td>4.6</td>
<td>Comparison of Perforin expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation</td>
<td>66</td>
</tr>
<tr>
<td>4.7</td>
<td>Comparison of Granzyme A expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation</td>
<td>68</td>
</tr>
</tbody>
</table>
Figure 4.8. Comparison of Perforin and Granzyme A dual expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation ................................................................. 69

Figure 4.9. Comparison of CD107a/b expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation ................................................................. 71-72

Figure 4.10. Comparison of Fas expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. .... 73

Figure 4.11. Comparison of FasL expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. .... 75

Figure 4.12. Comparison of Fas and FasL dual expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation ................................................................. 76-77

Figure 4.13. Comparison of IFN-γ expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. .... 79

Figure 4.14. Comparison of TNF-α expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation ................................................................. 80-81

Figure 4.15. Comparison of PD-1 expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation .82-83

Figure 4.16. Comparison of CD137 and PD-1 dual expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation ................................................................. 84
<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Markers and associated Patient Numbers</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.</td>
<td>Antibodies used in the study</td>
<td>44</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Patient demographic of the three study groups</td>
<td>61</td>
</tr>
<tr>
<td>Table 4.</td>
<td>CD4 Count Correlation showing r- and p- values Across all Groups in Response to Stimuli</td>
<td>85</td>
</tr>
<tr>
<td>Table 5.</td>
<td>Viral Load Correlation showing r- and p- values Across all Groups in Response to Stimuli</td>
<td>86</td>
</tr>
</tbody>
</table>
Abstract

In just 25 years since the first reported cases in 1981, the number of Human Immunodeficiency virus (HIV) infected people has risen to 65 million, and over 25 million have died of acquired immunodeficiency syndrome (AIDS). Sub-Saharan Africa accounts for 67% of all people living with HIV and 72% of deaths in this region were AIDS related. Tuberculosis (TB) is one of the most common opportunistic infections in AIDS patients, particularly in developing countries, where 60 – 70% of TB cases occur in HIV-1-infected persons. HIV-1 is a high risk factor for the development of TB, the reactivation of a latent *Mycobacterium tuberculosis* infection and also progressive TB.

CD8+ Cytotoxic T Lymphocytes (CTL) are pivotal in the host immune response to HIV infection. CTL are associated with resolution of acute infection and with reduction in viral load. Studies in macaques and humans indicate the importance of CTL in the control of HIV infection, where reduction in CD8+ T cell number has been correlated with progression to AIDS.

The current study was a cross-sectional descriptive study of CD8+ T cells of HIV+ adult South Africans with and without TB co-infection (TB disease). The cohort consisted of anti-retroviral therapy (ART) naïve patients and all CTL analyses were carried out on peripheral blood mononuclear cells (PBMCs). A total of 60 South African adults from the Western Cape were utilized in this study, including 15 healthy controls; 30 HIV+TB- individuals and 15 HIV+TB+ individuals. Expression of phenotypic, activation and functional markers were investigated by flow cytometry with the use of fluorochrome-conjugated antibodies. The markers examined included the novel activation marker CD137, the CTL associated markers Perforin, Granzyme A, CD107a/b, Fas (CD95), and FasL (CD95L), intracellular cytokines IFN-γ and TNF-α and the chronic HIV CTL dysfunction marker PD-1.
HIV infection alone was associated with increased baseline expression of TNF-α, Perforin, Granzyme A, PD-1, Fas (CD95), and FasL (CD95L), but not CD137(4-1BB) or IFN-γ as compared to uninfected controls. TB co-infection resulted in further increased baseline expression of TNF-α, perforin, PD-1, FasL (CD95L), as well as increased IFN-γ. HIV-1 antigen (gag)-specific stimulation \textit{in vitro} indicated that in HIV infection was associated with antigen-specific upregulation of activation and cytotoxicity markers CD137, IFN-γ, TNF-α, Fas, FasL and CD107a/b. In TB co-infection a reduction in antigen-specific degranulation (CD107a/b up-regulation) and also Fas and FasL expression was observed.

TB co-infection (in the form of active pulmonary TB) reduced antigen-specific CTL functional activity, but simultaneously there was an association with increased baseline PD-1 expression and also cytolytic marker expression (Fas, FasL, TNF-α). These cytolytic markers could be involved in non-antigen-specific bystander target cell death. The expression of the co-stimulatory molecule CD137 appeared to correlate with interferon-γ production and levels of degranulation, confirming its usefulness as a putative surrogate marker of functional responsiveness. These data indicate that in addition to impacting on CD4 T cell function, TB co-infection leads to higher baseline expression of CTL-associated markers, but to dysfunctional antigen-specific CTL responses.
OPSOMMING
Slegs vyf en twintig jaar na die eerste berigte van die menslike immuniteitsgebrekivirus (MIV) in 1981, het die getal MIV-geinfekteerde individue gestyg tot 65 miljoen en het meer as 25 miljoen mense alreeds gesterf aan die verworwe immuniteitsgebrek sindroom (VIGS). Sub Sahara Afrika maak 67% uit van alle HIV gevalle en het `n MIV-verwante doodsyfer van 72%. Een van die algemeenste opportunistiese infeksies in VIGS pasiënte is Tuberkulose (TB). In ontwikkelende lande, veral, kom 60-70% van TB gevalle voor in MIV-1 geinfekteerde individue. MIV-1 is `n hoë risiko faktor vir die ontwikkeling van TB, die heraktivering van latente *Mycobacterium tuberculosis* infeksie en progressiewe TB.

Die CD8+ sitotoksiese T Limfosiete (STL) se immuun reaksie teen `n MIV infeksie is noodsaaklik en word geassosieer met `n resolusie van die akute infeksie en `n afname in viruslading. Studies in die mens en macaque het getoon dat sitotoksiese T limfosiete belangrik is vir die beheer van MIV infeksies aangesien die afname in CD8+ sel getalle korrelear met die verloop tot VIGS.

Hierdie deursnit-beskrywende studie het die CD8+ T selle van MIV+ volwasse Suid-Afrikaners, met of sonder`n TB mede-infeksie, ondersoek. STL analise is gedoen op die perifere bloed mono-nuklêre selle (PBMS) van pasiënte wat geen teen-retrovirale terapie (TRT) ontvang het nie. `n Totaal van sestig Suid-Afrikaanse volwassenes van die Wes-Kaap het deelgeneem aan die studie wat 15 gesonde kontroles; 30 MIV+TB-en 15 MIV+TB+ individue ingesluit het. Die uitdrukking van fenotipiese, aktivering en funksionele merkers is ondersoek deur middel van vloeisitometrie en fluorochroomgekonjugeerde teenliggaampies. Laasgenoemde het ingesluit die nuwe aktiversingsmerker CD 137, die STL geassosieerde merkers Perforien en Gransiem A, CD 107a/b, Fas (CD95) en FasL (CD95L), intrasellulêre sitokiene IFN-γ en TNF-α en PD-1, die merker vir chroniese MIV CTL disfunksie.
Daar is gevind dat `n TB mede-infeksie (in die vorm van aktiewe pulmonêre TB) die antigeen-spesifieke STL funksie verlaag en terselfdertyd `n verhoging in die uitdrukking van PD-1 en sitolitiese merkers (Fas, FasL, TNF-α) bewerkstellig. Hierdie sitolitiese basislyn merkers is moontlik betrokke by die dood van nie-antigeen-spesifieke omstander teiken selle. Die uitdrukking van die mede-stimulatoriese molekule CD 137 blyk om te korreleer met die produksie van STL IFN-γ en die vlakke van degranulasie. Dit bevestig die merker se bruikbaarheid as `n gewaande surrogaat merker vir funksionele reaksies. Die data toon verder dat `n TB mede-infeksie nie net `n effek het op die CD4 T sel funksie nie, dit lei ook tot `n verhoogde basislyn uitdrukking van STL-gearrelateerde merkers, maar met disfunksionele antigeen-spesifieke STL reaksies.

Hierdie studie het bepaal dat `n MIV infeksie verbind word met `n toename in die basislyn uitdrukking van TNF-α, Perforien, Gransiem A, PD-1, Fas (CD95) en FasL (CD95L). Dit is egter nie die geval wanneer die uitdrukking van CD 137 (4-1BB) of IFN-γ vergelyk word met nie-geinfekteerde kontroles. `n TB mede-infeksie het `n verdere toename in die uitdrukking van TNF-α, Perforien, PD-1, FasL (CD95L) getoon, asook `n verhoring in IFN-γ vanaf die basislyn. In vitro MIV-1 antigeen (gag)-spesifieke stimulasies het aangedui dat `n MIV infeksie met die antigeen-spesifieke op-regulasie van aktiverings en sitotoksiese merkers CD137, IFN-γ, TNF-α, Fas, FasL en CD107a/b geassosieer word. In `n TB mede-infeksie, is `n verlaging van antigeen-spesifieke degranulasie (CD 107a/b op-regulasie) asook die uitdrukking van Fas en FasL waargeneem.
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CHAPTER 1: INTRODUCTION

The cellular immune response is extremely important in the control of HIV-1 infection. In particular, CD8+ cytotoxic T lymphocytes (CTL) have been shown to play a role in the control of viraemia after acute infection and for the maintenance of low viral load during chronic infection [1]. A decline in CTL numbers and function at the later stages of chronic infection is coupled with rebound viraemia and immune system collapse [1]. Although numbers of CTL remain fairly consistent throughout chronic HIV infection, a gradual diminishing of function has been reported – due to continued antigen stimulation and “immune exhaustion” [2, 3, 4].

Tuberculosis and HIV-1 have become a lethal duet of infectious diseases in sub-Saharan Africa. TB is the most common cause of death in HIV-infected individuals in this region. HIV-1 appears to worsen the TB prognosis in a co-infection setting, and in turn TB seems exacerbate pathologic phenomena associated with later stage chronic HIV infection [5]. How these two diseases interact and cause a generally worse outcome is not fully understood. Immunologically, TB is a Th1 CD4+ T cell driven disease, as CD4 T cells are the primary activators of macrophages, the preferred host cell of *M. tuberculosis*. HIV-1 is also a disease strongly linked to CD4+ T cells, as these cells are the primary target for HIV-1 infection. It can be seen that continued stimulation of CD4+ T cells by TB antigen would provide HIV with activated host cells for replication. Conversely, depletion of CD4+ T cells in HIV could lead to worse outcome in TB as the primary effector cells are reduced in number (and function).

Due to the pivotal role of CD8+ CTL in controlling viral replication in HIV infection, it is of interest to understand how TB co-infection may impact on this crucial subset of cells. The objective of the research undertaken for this study was to gain some additional understanding of the phenotype and function of CTL in HIV-infected individuals with and without TB co-infection (active TB disease). The study includes the novel activation marker CD137 (4-1BB), which is a member of the Tumour Necrosis Factor Receptor (TNFR) family and which has been shown to be restricted to recently activated T cells.
The thesis is structured as follows – an Introduction to the study is described in Chapter 1, and then a Review of the Literature follows in Chapter 2, ending with a presentation of the Aims of the study and a working Hypothesis. Chapter 3 presents the Material and Methods of the study. Chapter 4 presents the Results attained, and finally Chapter 5 presents a Discussion of the Results and a general Conclusion in Chapter 6.

CHAPTER 2: LITERATURE REVIEW

2.1. Overview of the HIV/AIDS Epidemic

The first known cases of acquired immunodeficiency syndrome (AIDS) were reported on June 5, 1981 by the Center for Disease Control involving five cases of a hitherto rare disease, *Pneumocystis carinii* pneumonia (more recently defined as *Pneumocystis jiroveci* pneumonia) in young homosexual men in Los Angeles [6]. Two years later a retrovirus was isolated from an AIDS patient, this virus was later named human immunodeficiency virus type 1 (HIV-1) [7]. In 1986, a second type of human immunodeficiency virus was isolated from an AIDS patient in West Africa [8]. This retrovirus was similar but antigenically different, and was subsequently named human immunodeficiency virus type 2 (HIV-2) [9]. In just 25 years since the first reported cases, the number of HIV infected people had risen to 65 million, and over 25 million had died of AIDS [7]. In 2007, there were between 30 - 36 million individuals living with HIV. Sub-Saharan Africa accounts for 67% of all people living with HIV and in 2007, 72% of deaths were AIDS related (figure 2.1).
In almost all regions outside of sub-Saharan Africa HIV predominantly affects injecting drug users, men who have sex with men (MSM), and sex workers. Recent data (2006) shows that in developed parts of the world like Europe and North America, the United Kingdom had the highest number of newly infected HIV MSM cases (between 2500 – 3000) and Canada had the highest number of new cases in the injecting drug user group (between 200 – 300). Interestingly, more up-to-date studies in sub-Saharan Africa indicate high infection levels among constituents of these developed world risk groups in this region as well. HIV infections among men of the MSM group are also increasing rapidly in parts of Asia [10]. A number of early studies (case series, case-controlled and seroprevalence) reported that African patients with HIV/AIDS, in most instances, did not report being MSM or injection drug users. These two factors were and are still considered major risk behavior criteria in the USA and Europe [11]. Adult HIV risk in Africa was and is still mainly due to the number and frequency of change in heterosexual partnerships [12].
Sub-Saharan Africa contains less than ten percent of the world’s population, but includes 90% of all paediatric cases of HIV and 68% of adult HIV cases [13]. In 2007, this region accounted for 76% of AIDS-related deaths worldwide. Women in sub-Saharan Africa comprise 57% of the HIV infected adults and women aged 15-24 years make up three quarters of young people on the continent living with HIV. South Africa contains the highest number of individuals infected with HIV at an estimated 5.5 million people [14]. The number of people living with AIDS in South Africa is higher than the total population of the neighbouring countries of Botswana, Swaziland and Lesotho, combined [14]. In 2006, HIV/AIDS related deaths accounted for 71% of all deaths in adults aged 15-49 years and there were an estimated 1 400 new HIV infections a day, averaging almost one infection a minute [15]. By the year 2015, it is projected that 5.4 million South Africans will suffer AIDS related deaths; 6 million people will be infected with HIV and approximately 797 000 people will develop clinical AIDS [15].

Over 95% of HIV infected people reside in resource-limited settings where access to antiretroviral therapy (ART) was generally unavailable in South Africa before 2003 [16]. Due to AIDS activist and advocacy group efforts and the influx of bilateral and multilateral funding, access to ART has increased from 2003 in regions that are highly affected by HIV. In South Africa, approximately 300 000 HIV infected patients had access to ART by March 2007 [17]. Studies in 2007; showed that over 70% of patients in need of ART were still living without it. This delay of ARV access has come too late for the 2.1 million people who suffered AIDS-related deaths in 2006. In South Africa, the UNAIDS/WHO 2008 report showed that at the end of 2007 there were between 22–36% of people in need of ARTs receiving treatment.

Tuberculosis (TB) is one of the most common opportunistic infections in AIDS patients. HIV-1 is the most frequent risk factor for the development of TB, the reactivation of a latent *Mycobacterium tuberculosis* infection and progressive TB. Sixty to seventy percent of TB cases in developing countries occur in HIV-1-infected persons [17]. Studies have indicated that HIV-associated immunodeficiency is aggravated by TB [18-23]. HIV patients with tuberculosis present severe clinical manifestations, with diffuse
pulmonary involvement and extra pulmonary dissemination [24]. It is therefore thought that several different immunological parameters may be altered in AIDS patients co-infected with TB, as compared to each individual disease [5].

2.2. The HIV-1 Virus

HIV belongs to the genus Lentivirus within the family Retroviridae. Viruses belonging to this family are enveloped ribonucleic acid (RNA) viruses that cause slow, progressive infections. Replication depends on an active reverse transcriptase (RT) that transforms the viral RNA genome into a proviral DNA copy. The proviral DNA integrates itself into the host cell chromosome. The provirus is then transcribed into mRNAs that encode viral proteins and progeny genomic RNA [24]. The Lentviruses are generally “slow viruses”, i.e. disease manifested by slow onset and progression. These viruses can infect a wide range of mammalian host cells. HIV-1 is phylogenetically related to a Simian immunodeficiency virus (SIV) in chimpanzees (SIVcpz), and is thought to have arisen from a single transmission event from chimpanzees to humans. HIV-2 is phylogenetically related to SIVmac (macaques), the viral agent of simian AIDS as well as SIVsm a commensal virus in sooty mangabeys) [24]. Of the two types of HIV, HIV-2 is the less virulent, less transmissible and prevalent only in West Africa. HIV-1, unlike its counterpart, is prevalent worldwide and causes most human HIV infections [8].

HIV-1 is subdivided into three groups, viz. M (‘major’), O (‘outlier’) and N (‘non M/non O’). HIV-1 Group M strains are responsible for the greater part of the worldwide AIDS pandemic. The M group is then divided into at least ten genetic subtypes or clades, designated by letters A to K [25]. Dual and superinfections have produced recombinant forms of these subtypes which are called Circulating Recombinant Forms (CRF). In the Americas and Western Europe, subtype B is most prevalent. Asia has a mixture of subtypes B and C. Subtype C is responsible for most of infections in southern Africa, Eastern Africa and India [16]. In South Africa >90% of cases are subtype C [26].
The HIV-1 virion itself is 120nm in diameter and consists of an outer envelope, a core protein shell and a cone-shaped inner core. The envelope is composed of a lipid bilayer which is traversed by 72 glycoprotein spikes surrounding the core (figure 2.2).

**Figure 2.2.** Structure of the HIV-1 virion. The figure illustrates the lipid membrane consisting of glycoproteins gp120 and gp41, as well as host cell-derived proteins. The p17 matrix protein and the p24 core antigen are also shown housing two copies of HIV RNA, components of gag (p9 and p7), integrase and reverse transcriptase enzymes [27].

The core consists of nucleoproteins complexed to two genomic RNA molecules [28]. The HIV genome is a single stranded positive sense RNA molecule, approximately 9.5 kb in length, which encodes the typical retrovirus proteins - Gag, that gets cleaved into M (matrix), C (capsid) and N (nucleocapsid) components; Pol that gets cleaved into protease, reverse transcriptase and integrase; and Env a 160 kD glycoprotein eventually cleaved into an external gp120 subunit and a transmembrane gp41 subunit that when combined form the trimeric spikes on the surface of the virion. The genome of HIV-1 encodes for various non-structural proteins, viz. the transactivator protein Tat [29], a splice regulator protein Rev [30] and accessory proteins Nef [31,32], Vif [33], Vpr (in certain retroviruses other than HIV-1, Vpx) [34, 35] and Vpu (figure 2.3).
Figure 2.3. Diagram of the HIV genome. This figure illustrates the genes gag, pol and env, and the proteins, glycoproteins or enzymes they code for as well as accessory genes vif, vpu, vpr, tat, rev and nef [27].

2.3.1. The HIV-1 Replication Cycle
2.3.1.1. Entry

HIV-1 enters target cells by fusing the virus envelope with the target cell membrane (figure 2.4). This entails high affinity binding between the surface envelope glycoprotein gp120 and cellular receptor, CD4 which is a surface marker present on T lymphocytes and monocytes [36, 37]. After this initial attachment, conformational changes occur in gp120 and it binds with a chemokine coreceptor. Next there are conformational changes within gp41, insertion of the terminal fusion peptide of gp41 into the target cell membrane, and finally fusion of the viral envelope with the target cell membrane [36, 37]. After these fusion events the viral nucleocapsid is released into the cytoplasm of the target cell [38].

Apart from the CD4 major HIV-1 receptor, a number of seven-transmembrane (7-TM) chemokine receptors have been defined as entry coreceptors for HIV-1. The α-chemokine receptor CXCR4, is a key coreceptor for so-called T-cell line tropic (T-tropic) group of HIV-1 syncytium-inducing (SI) viruses, or X4 viruses. The β-chemokine
receptor CCR5, is another important coreceptor for the so-called macrophage-tropic (M-tropic) HIV-1 non-syncytium-inducing (NSI) viruses [39] or R5 viruses.

2.3.1.2. Reverse Transcription and integration

After entry into the target cell and uncoating, virion-associated RT copies the genomic viral RNA into double-stranded proviral DNA [40]. This proviral DNA migrates into the cell nucleus and integrates into the cellular DNA through the activity of viral integrase. The host cell can be latently or actively infected. Latent infection entails no viral RNA being produced and a progressive infection may not occur. An active infection involves the host cell producing mature virions by transcription of proviral DNA [41].

2.3.1.3. Transcription and translation

Tat is a major controller of the HIV-1 transcription process [41]. The proviral DNA is transcribed by the cellular RNA polymerase II. This results in the production of new viral messenger RNA (mRNA). Cellular poly-ribosomes then translate the mRNA. In the cytoplasm, the mRNA is translated into HIV-specific structural proteins that integrate with viral core particle components.

2.3.1.4. Assembly and budding

Viral proteins along with genomic RNA are then transported to the cellular membrane and the new virion is assembled there. Assembly of viral structural proteins requires the core and envelope proteins be produced first. The Env proteins migrate to and then anchor within the plasma membrane. Genomic RNAs bound by Gag then move to the plasma membrane at the site of the Env insertion [41].

The virus goes through a final maturation by a process of reverse endocytosis (budding) at the plasma membrane. During this process the virus core acquires a portion of the host cell plasma membrane that contains gp41 and gp120. Propagation of the virus
thereafter occurs either through free infectious particles that are released from the budding process or via cell to cell transfer [42].

Figure 2.4. The life cycle of HIV. This figure shows the different stages of the HIV life cycle from entry (top) to budding (bottom) [43].
2.4. HIV Pathogenesis

HIV pathogenesis can be divided into three stages, the acute phase, the latent/chronic phase and progression to AIDS.

2.4.1. The Acute Phase

The most frequent route of infection is across mucosal barriers and since heterosexual transmission is the predominant mode of transmission, the genital tract is the major mucosal site. Within a week after infection, HIV can be detected in mucosal-tissue associated lymph nodes. HIV also interacts with mucosa-associated dendritic cells (DCs), which then carry the virus on their surface to lymph nodes. There is an increase in viral production within the draining lymph nodes, where DCs and monocytes/macrophages interact with viral antigen-specific CD4+ T cells. This results in amplification of infection through these CD4+ T cells [44, 45]. During this time of infection the virus also spreads to the gut-associated lymphoid tissue (GALT) resulting in further amplification of infection of CD4+ T effector memory cells, which are located in GALT, a process which leads to their rapid depletion [46-49]. The earliest events in the lymphoid tissues, HIV-1 targeting of memory CD4+CCR5+ T cells [50, 51] can be demonstrated 3 to 4 days after infection.

The rapid and severe loss of CD4+ T cells (figure 2.5) in the gut is believed to impact on the integrity of the gut itself, which in turn leads to translocation of bacteria across this barrier. This process activates innate immune responses (particularly DCs and monocytes/macrophages) via Toll-like receptors [52, 53]. This secondary or “bystander” activation of the immune system appears to worsen the already excessive activation associated with primary HIV infection. The abundant loss of CD4+ effector memory T cells in the gut is synchronous with the changes in total CD4+ T cells in the blood. No major changes in the effector memory T cell subset in the blood are however observed
The quick depletion of CD4+ effector T cells in the gut corresponds to peak plasma viral production (viraemia) at around 21 days post-infection.

2.4.2. Clinical Latency/Chronic Phase

This stage of HIV infection generally occurs after 12 weeks and may last for up to 7 to 10 years in typical progressors. Chronic infection is characterized by the virus getting trapped in lymphoid tissues by follicular dendritic cells, declining CD4+ T cell count, low levels of HIV plasma viraemia and stable but early evidence of declining CD8+ T cell levels. Viral set point is now established in patients that are not on anti-retroviral therapy (ART). HIV-1 infection of resting memory or naïve CD4+ T cells, macrophages or monocytes results in a non-productive latent infection [40, 54]. Two forms of latency can be defined viz. a labile pre-integration form and a stable post-integration form. Latency may occur during highly active ART (HAART), reducing plasma viraemia. Even though there are low numbers of latently infected cells containing replication-competent virus, they show minimal decay and are sufficient enough to impart a lifelong persistence of HIV in a HIV infected patients on ART regimens [42].

Using the SIV-infected monkey model, the virus can be detected in all lymphoid tissues; the evidence of productive infection is first located in the paracortex of the lymph nodes, the periarteriolar sheaths in the spleen, and the thymic medulla which underlies the thymic cortex, which contains mature lymphocytes. At this stage of HIV infection, the virus evolves its coreceptor usage and can switch from CCR5-tropic to CXCR4-tropic. This coreceptor switching is seen in up to 50% of HIV-1 subtype B and D[55-58] infections at the very late stage of disease, this event is however rarely seen in HIV-1 subtype C infections [59, 60]. Viral RNA and proteins can be detected over germinal centers due to antibody trapping the virus and their interaction with follicular DCs [61]. Thymic infection occurs during a period of CD4 T-cell regeneration, resulting in thymic involution. T cell progenitors are released from the thymus at a higher rate than the rate of T-cell death in the periphery [62].
2.4.3. Progression to AIDS

AIDS progression in HIV-1 infected patients is largely due to the decline in CD4+ T lymphocytes. These lymphocytes are crucial for the induction of specific humoral and cell mediated immune response. The consequence of damage to the immune defense is the susceptibility of the infected organism to opportunistic infections that ultimately lead to death [38].

![Diagram of HIV infection](image)

**Figure 2.5.** The course of HIV-1 infection. This diagram shows the relationship between CD4+ T cell count and HIV-1 viral load over time in a typical untreated HIV-1 infection [63].

2.5. The Immune Response to HIV

2.5.1. The Cellular Immune Response and importance of CTLs

The cellular immune response is extremely important in controlling HIV replication and affording protection from progression to AIDS. Both CD4+ and CD8+ T cells contribute to the response to HIV, but it is CD8+ CTL have been most closely associated with resolution of viraemia and asymptomatic presentation during chronic infection. Although CD8+ CTL are protective, they are ultimately unable to eradicate the virus, and at later stages of chronic infection their function becomes impaired.
During acute infection, prior to seroconversion activation of HIV-1-specific cytotoxic CD8+ T lymphocytes occurs. This CTL response is more successful in controlling viral replication than HIV-1-specific antibodies. Previous studies indicated that the number of HIV-1-specific CTLs is inversely associated with viral load [1]. CTLs recognize HIV-1-infected antigen-presenting cells via the T cell receptor (TCR). The CTL TCR binds to viral peptides combined with Major Histocompatibility Complex (MHC) Class I molecules at the surface of infected cells and induces protease (granzyme) and perforin release, resulting in lysis of the infected target cells [64].

Another killing pathway is the interaction of Fas ligand (FasL) expressed on the CTL surface with Fas molecules on the surface of target cells. This interaction leads to induction of apoptotic lysis of the infected cell [19]. Other than cell lysis, binding of CTLs to HIV-1 infected cells promotes the release of non-cytotoxic antiviral compounds such as the cytokine interferon-gamma (IFN-γ) and the chemokines macrophage inhibitory protein (MIP)-1α, MIP-1β and RANTES [65]. These chemokines are the natural ligand for one of HIV-1’s coreceptors, CCR5, and have been shown to have an inhibitory effect on R5 HIV-1 strains. The important role of specific CTL for the control of viral replication has been observed in long-term non progressors (LTNP) [66]. These patients have shown strong qualitative and quantitative HIV-1-specific CTL response. Studies have shown that highly exposed yet seronegative individuals had detectable amounts of HIV-1-specific CTLs which may indicate that the CTL can prevent seroconversion [66].

2.5.2. Humoral Immune Response

The humoral response appears to play a minor role in minimizing the impact of HIV-1 infection. Binding of neutralizing antibodies to virus particles induces the incorporation and destruction of virions by phagocytes. In vitro studies involving sera form HIV-1 infected patients indicate that they aren’t capable of considerably reducing viral infectivity [67]. The antiviral activity of host neutralizing antibodies against HIV-1 glycoprotein gp120 is often overcome by complete replacement of neutralization-
sensitive virus via immune escaping HIV-1 variants. This is possible either by mutation-induced conformational gp120 changes and/or by altered glycosylation patterns of gp120. These antiviral mechanisms are ineffective in most patients in preventing chronic phase immune decline in HIV-1 infection or clearing HIV-1 infected cells [66].

2.5.3. HIV-associated immune activation and apoptosis

*In vivo* studies on HIV-1 induced immune activation have shown that viral-associated chronic immune activation has an impact not only on the biology of the virus but also the host cell. Viral components may induce apoptosis, as well as immune activation and bystander cell death (explained further in section 2.6.2.1). Immune activation may result in increased HIV-1 replication as well as changes in HIV-1 phenotype and genotype, increased apoptosis of the host immune cells and the suppression of hematopoietic regeneration. Chronic activation of the Tumour Necrosis Factor (TNF)-α-signaling pathway plays an important role in HIV-1 pathogenesis [68], leading to increased viral transcription, initiation on mononuclear cell apoptosis and suppressing hematopoiesis. The HIV-1 envelope glycoprotein gp120 activates TNF-α secretion by peripheral blood mononuclear cells (PBMCs) [69-71] and upregulates viral replication in an autocrine and paracrine mode in chronically infected cell lines [72-74] as well as PBMCs [75].

Depletion of CD4+ and C8+ T cells in chronically infected individuals may be due to apoptosis resulting from unsuitable induction of activation-induced cell death. During the chronic phase, ongoing immune activation associated with HIV-1 infection increases signaling through the proapoptotic pathways of T lymphocytes. A large percentage of cells undergoing apoptosis are activated memory cells expressing Human Leukocyte Antigen (HLA)-DR, CD38, CD45RO+ (memory CD4+ T cells) and Fas [76]. Induction of apoptotic cell death does not only occur in virus infected cells or HIV antigen-specific (responder) cells but also in surrounding bystander cells which account for the majority of cells lost in this disease [77]. Studies indicate that virus-encoded Tat protein is released by the infected cells and is taken up by surrounding, uninfected cells via an endocytic pathway [78, 79] to accentuate this bystander apoptotic effect exerted by HIV.
Susceptibility of PBMCs to apoptosis is dependant upon lymphocyte activation in peripheral blood [72] and in lymphoid tissue [80]. Modeling analysis shows the degree of systemic immune activation correlates to CD4+ T-cell loss [81]. CD4+ T cell depletion is not due only to apoptosis but a combination factors including specific virus-induced cell death, widespread activation-induced loss of the memory cell pool (CD45RO+) and impaired renewal of the naïve cell pool (CD45RA+). Immune activation may lead to clonal deletion of CD4+ T cells during the process of major histocompatibility complex class II-restricted antigen presentation [82-84]. This phenomenon may contribute to the progressive loss of T-cell responses to frequent recall antigens observed during HIV-1 disease progression [85].

Two major apoptotic pathways exist – the extrinsic and intrinsic. The classical (intrinsic) apoptotic pathway, involving the cell’s mitochondria, is regulated by the Bcl-2 family of proteins. This includes anti-apoptotic (Bcl-2, Bcl-XL) and pro-apoptotic (Bax, Bid, Bim) members that carry out their function primarily at the mitochondrion by either thwarting or inducing mitochondrial dysfunction. Once the intrinsic death signal is received, pro-apoptotic proteins translocate from the cytoplasm to the outer mitochondrial membrane, where they intermingle with their pro-apoptotic partners. This event is followed by mitochondrial dysfunction, release of pro-apoptotic proteins out of the mitochondrion (of which a major role can be attributed to cytochrome C) and consequent caspase activation [86]. One of the triggers for the mitochondrial pathway of apoptosis is the disturbance of dynamic formation/interaction of microtubules in the cell. This disturbance may be triggered by a variety of microtubule-targeted tubulin-polymerizing agents (MTPAs). Once MTPAs have been taken up intracellularly, they bind to β-tubulin and promote tubulin polymerization that impedes the function of the mitotic spindle resulting in mitotic arrest at the metaphase-anaphase transition and subsequent activation of the mitochondrial pathway of apoptosis [86]. The extrinsic (receptor-mediated) pathway is discussed in more detail below.
2.5.4. Importance of CD8+ CTL in host response to HIV infection

The importance of CD8+ CTL in controlling infection has been demonstrated in non-human primate studies, where depletion or blocking of CTL leads to rapid disease progression. In addition, adoptive transfer of CTL confers protection [87, 88]. Depletion of CTLs in SIV-infected macaques results in the inability to lower viraemia during the acute phase of infection, or during an increase of viraemia in elite controller animals [89-92]. The outcomes of these studies indicate CD8+ T cells are the primary mediator of viral control.

There are small populations of infected individuals who have low to non-detectable plasma viraemia and do not progress to AIDS. Such individuals can be divided into two groups, one termed long-term non-progressor (LTNP) that have low viral loads and the second group is termed elite controllers (or HIV controllers) with levels of < 50 RNA copies/ml [93]. Studies on these individuals indicate that they have CD8 T cell responses that target Gag over other proteins with diverse HLA class I alleles. Elite controllers show more CD4 and CD8 T cells that secrete IFN-γ and interleukin-2 (IL-2), and lower levels or less protection conferred by HIV neutralizing antibodies [94]. The action of virus specific T cells is dependant upon the interaction between TCRs and their cognate antigenic peptides. TCR utilization is determined by peptide-HLA complexes [95]. A sizeable amount of the elite controllers have specific protective MHC-I alleles, viz. HLA-B57, HLA-B27 [94], with certain conserved alleles such as HLA-B*5701 being highly protective [96]. MHC-1 genes encode proteins that determine the viral peptides that CD8+ T cells target and they are very polymorphic.

HLA-B27 presents peptides with an arginine residue that attaches the peptide to the MHC-1 molecule [102]. Studies between humans and animal models indicate MHC-I and peptide complexes on professional antigen-presenting cells prime CD8+ T cell responses. Studies on HLA-B27 and HLA-B57 responses established that viruses with escape mutations within the Gag epitopes had in vitro fitness deficiencies [107-109].
In controllers there are a high number of IFN-γ-secreting HIV-specific CD8+T cells even though there are low detectable viral antigens. Researchers have found that there is a high frequency of HIV-specific CD8+IFN-γ+ cells in progressors and low in HAART responders; this is dependant on detectable viral antigens as there is less detectable viral antigens in the latter group [66, 110, 111]. HIV replication in AIDS progressors have been associated with distorted maturation of HIV-specific CD8+ T cells [112-114]. HIV-specific CTLs are more differentiated in controllers than in progressors but the expression of their markers and specificities vary from patient to patient [115, 116]. This suggests that differentiation and maturation may not be the determining factor for the effectiveness of HIV-specific CTLs. The activation status of these CD8+ T cells in controllers is also of interest, they express low CD38 but high HLA-DR [116]. Both markers are highly expressed in viraemic patients or aviraemic patients on HAART [117]. Low CD38 expression indicates these cells aren’t activated but the high HLA-DR expression shows a high capacity for expansion, as HLA-DR is used as an indicator of proliferative potential [116]. Progressors exhibit high CD38 expression which may be due to high viral load and induced by cytokines (figure 2.6) [2, 118, 119].

This activation status would drive the cell to have higher programmed death 1 (PD-1) expression, functional inefficiency (non-responsiveness), senescence and eventually apoptosis [2, 118, 119]. The relationship of PD-1 in chronic viral infections was elucidated in studies with mice. The results showed an upregulation of this marker in acute infections and downregulation once the antigen has been cleared. Antigen persistence is associated with continued PD-1 expression. Blocking PD-1’s interaction with its cognate ligand restores proliferative and cytokine secreting capabilities in ‘exhausted’ CD8+ T cells [120]. In HIV-infected patients, virus-specific T cells exhibit high levels of PD-1. This elevated expression of PD-1 in vitro leads to a high vulnerability to apoptosis [2, 4]. Studies by Petrovas et al. [121] on SIV-infected macaques, showed the reliance of PD-1 expression on antigen exposure by investigating its expression CD8+ T cells that select for viral escape mutations upon acute infection. Escape resulted in a decrease in PD-1 expression as it was no longer possible for these cells to establish their MHC + peptide complex and couldn’t recognize
high amounts of antigen. HIV-specific CD8+ T cells in HIV controllers display low PD-1 expression [122].

In contrast to progressors, HIV-specific CTLs in controllers, after antigen stimulation, can produce multifunctional responses like degranulation, chemokine and cytokine secretion [123]. These proliferating CTLs exhibit expression of perforin [65] and increased degranulation capacity [123]. These factors may explain the ability of HIV-specific CD8+ T cells in HIV controllers in eliminating infected CD4+ T cells [116]. The functional capacity of the TCRs of these cells may aid in producing an effective CD8+ T-cell response by supporting proliferation of these cells through providing important survival signals [124]. These signals might be determined by the TCR avidity for its cognate antigenic peptide. High avidity clonotypes may react when limited amounts of antigen are present. High-avidity HIV-1-specific CD8+ T cell clonotypes are abundantly preserved in HIV controlling patients [125] and lost in progressive chronic HIV-1 infection. Researchers suggest that low-avidity T cells would have better protection than high-avidity T cells and would be spared with respect to low antigen burden [126]. It can be seen that the TCR of HIV-specific CD8+ T cells from HLA-B57 patients exhibit increased structural flexibility that could allow it to accommodate epitope variants [96, 127].

In HLA-B27+ HIV-infected humans, a single CD8+ T cell response directed towards an epitope in Gag may be sufficient enough to control viral loads to low levels. In one LNTP patient, an escape mutation in the epitope resulted in a sudden increase in HIV viraemia [128]. In another study where Indian rhesus macaques were challenged with a DNA/Ad5 Gag vaccine, the temporary lowering of set-point viraemia was seen after infection, but the increases in viral load couldn’t be associated with escape in defined Gag epitopes [129]. Research of escape during chronic infection indicates that some CD8+ lymphocytes are actively suppressing viral replication during this time. Other evidence for the importance of CD8+ T cells is that their depletion during chronic infection in macaques results in a spike in viraemia [90].
Figure 2.6. HIV-specific CD8+ T cell response in HIV controllers. This figure shows the expression of HLA-DR and lack of CD38 on HIV controller CD8+ T cells in contrast to CD8+ T cells of progressors. It also illustrates that the HIV controller CD8+ T cells are polyfunctional producers of cytokines (e.g. IFN-γ, TNF-α, ad IL-2) in response to an HIV infected CD+ T cell [130].

2.6. HIV and TB Coinfection

*Mycobacterium tuberculosis* (Mtb) is the causative agent of TB. Although primarily targeting macrophages, both HIV and TB are intracellular pathogens which establish infection in immune cells and which are characterized by latency.

HIV infected individuals have a 40-80 times higher susceptibility to TB infection [97]. In 2007, there was an estimated 9.27 million tuberculosis cases globally, of which 1.37 million (15%) were HIV+. Africa accounted for 79% of those reported cases and Southeast Asia made up 11% [98]. In that year, there were 456 000 deaths within HIV+
and TB coinfected individuals. That figure accounts for 23% of the estimated 2 million HIV-associated deaths in 2007. Southern Africa is the hardest hit by HIV+ and TB coinfection, nine countries in particular (viz. South Africa, Swaziland, Lesotho, Namibia, Botswana, Mozambique, Zambia, Zimbabwe and Malawi) make up 50% of global cases [99].

TB and HIV coinfection elicits both innate and adaptive immune antimicrobial effector pathways. The cell mediated arm of immunity that is most important for protection against TB i.e. CD4+ T cell mediated activation of infected macrophages is gradually destroyed by HIV infection. HIV infection leads to depletion of CD4+ T cells which in turn leads to reduced ability to activate macrophages and destroy Mtb. Both TB and HIV drive immune activation, and immune activation is a major factor in the progression of HIV disease ultimately leading to immune exhaustion.

Studies have shown that polyfunctional T cells viz. those secreting Th1 cytokines and cytolytic effector molecules ensure long term host defence. In terms of Th1 cytokines, IFN-γ [100] and TNF-α [101] are the most important in mounting a protective response to TB. They act together to activate macrophages and T cell subsets and other immune cell responses in TB [103].

Another way of eliminating intracellular Mtb infection as well as HIV infected cells [104] is via the granule-mediated exocytosis pathway (see below for explanation of this pathway) of target cell lysis. This method is carried out by cytolytic T cells and NK cells through the release of perforin and granulysin (see below) [97, 105]. A last example of Mtb manipulating the immune system is when the organism infects macrophages and induces the Fas/FasL dependant apoptosis of Th1 cells which leads to a downregulation of T cell killing activity towards Mtb-infected cells [106].
2.7. Cytotoxic mechanisms of CD8^+ T cells

Cytotoxicity is a major functional response of the cell-mediated arm of the immune system. The process entails target cell death via cytotoxic effector cells. The two main populations of cells involved in cytotoxicity are natural killer (NK) cells and CD8^+ T cells (figure 2.7). These cells remove abnormal or infected cells to prevent the development or spread of malignant cells and the elimination of intracellular pathogens [131-134].

![Figure 2.7. CD8+ T cell and NK cell interacting with target cell. This diagram shows the different mechanisms of target cell recognition by CD8+ T cells and NK cells. CD8+ T cells recognize HLA class I restricted peptide antigens on the target cell and destroy the target cell. Shown here is the Fas-FasL method of inducing cell death, where Fas molecules expressed on the target cell interacts with the FasL present on CTLs. TCR: T cell receptor; ILT: immunoglobulin-like transcripts; NKG2: NK cell group 2 transmembrane receptors, KIR: killer immunoglobulin-like receptor [135].](image)

CD8^+ T cells kill target cells by inducing apoptosis. Cells in the process of apoptosis undergo morphological changes that are carried out in a programmed and controlled manner. Firstly, there is restricted chemical alteration on the apoptotic cell membrane, followed by chromatin condensation, cytoplasmic shrinking, dilation of endoplasmic
reticulum and lastly a packaging of intracellular contents into cell bladders called “apoptotic bodies”. These apoptotic bodies are phagocytozed by the surrounding phagocytes. This prevents the release of cytoplasmic contents to the outside that would otherwise cause an inflammatory response and surrounding tissue damage [136, 137]. CD8+ T cells induce apoptosis by at least three different pathways: i) granule-dependent exocytosis pathway, ii) receptor-ligand pathways (Fas – FasL and TRAIL-DR), and iii) cytokine pathway (TNF and TNFR type I).

2.7.1. Granule-dependent exocytosis pathway

This pathway is activated through intracellular signaling after target cell recognition by a cytotoxic lymphocyte. In exocytosis or degranulation, microtubules are mobilized, leading to preformed cytolytic “granules” or lysosomes of the cytotoxic cell moving towards the point of contact with the target cell, then releasing the stored lytic molecules [138, 139]. Degranulation can be distinguished by the exposure of the lysosomal-membrane-associated glycoproteins, CD107a, CD107b and CD63, on the lymphocyte surface [140]. These glycoproteins are located in the granule-membrane inner surface and are exposed onto the lymphocyte surface through degranulation [140-143]. The lytic granule contains a proteoglycan matrix that preserves protease enzymes in an inactive stage [143]. Lytic granules mature through a hMunc 13-4-dependent maturation process, which is required before efficient release of lytic molecules onto the target cell can occur [144]. The lytic molecules stored in granules that are responsible for inducing apoptosis are perforin, granzymes (Grzs) and granulysin.

2.7.1.1. Perforin

Perforin is a soluble monomer when it is located within granules. After the cytotoxic-cell/target-cell connection is made, perforin is released by exocytosis [139]. Once anchored into the target cell, perforin begins polymerization in the presence of calcium (Ca$^{2+}$) to create cylindrical pores with a diameter of 5 to 20 nm [145-147]. These pores serve as passive conductors of granzymes and granulysin through the target cell
membrane and may also allow an ionic exchange, causing osmotic unbalance leading to cell death [147]. Once inside the target cell, granzymes and granulysin induce apoptosis [148]. Studies have now also shown that Grzs can adhere to the cell surface via electrostatic linkage; in contrast, perforin pores induce Ca\(^{2+}\) flow from the extracellular towards the intracellular environment [149]. The influx of Ca\(^{2+}\) causes activation in the target cell, which tries to amend the pore in the cell membrane to prevent necrosis [149]. The Grzs are then internalized with perforin and are released in the cytoplasm of the target cell. The presence of perforin is necessary for the induction of apoptosis [150]. Perforin is an unstable molecule and the baseline concentrations vary according to the cytotoxic cell population [149, 151].

### 2.7.1.2. Granzymes

These molecules are soluble proteins of globular structure, belonging to the serine-protease family. The most abundant granzymes in lytic granules are Grz-A and Grz-B [152]. Granzymes are released as multi-molecular complexes which induce apoptosis by caspase-independent or-dependent pathways [151]. Caspases are located in the cell cytoplasm as inactive precursor molecules that must be hydrolyzed to begin their activity. Grzs are grouped according to their function viz. i) inflammatory, ii) initiator of stress signals, and iii) effector of apoptosis [151, 152].

Grz-A activates caspase-independent apoptosis, inducing a slow process of cell death. Grz-A cleaves single-stranded DNA, and hydrolyzes proteins consisting of basic amino acids such as arginine or lysine [153, 154]. Grz-A triggers an endoplasmic reticulum associated complex (the SET complex), which is formed by two tumour-suppressor proteins, phospho-protein 32 (pp32) and nonmetastatic protein 23 homologue 1 (NM23-H1), and 3 Grz-A substrates: oncoprotein SET, high mobility group 2 (HMG-2) protein and apurinic endonuclease 1 (Ape1) [153, 155, 156]. One of the features of apoptosis is an increase of reactive oxygen species (ROS) and decrease of the mitochondrial membrane potential, this process plays a pivotal role in the SET translocation into the cell nucleus [153, 154, 157]. Inside the cell nucleus, Grz-A cleaves SET (specific
inhibitor for NM23-H1); this cleavage releases NM23-H1, which degrades chromosomal DNA [153, 155]. There is also evidence to suggest that Grz-A cleaves histone 1, changing the nucleosomal center, so chromatin is relaxed and DNA is fragmented by endonucleases [158]. Studies have shown that Grz-A may fragment the IL-1β pro-peptide at the Asp116 site that gives rise to an active form of IL-1β for activating cells (e.g. fibroblasts) to secrete cytokines, such as IL-6, IL-8 and IFN-γ [153, 155, 159, 160].

Grz-B cleaves protein substrates in the carboxyl side of acidic amino acids, mainly aspartic acids [151]. Grz-B induces apoptosis in two ways (see figure 2.8). In the first, Grz-B activates caspase 3, promoting fragmentation of DNA, or of nuclear membrane vital components or of the cytoskeleton [161, 162]. In the second pathway, Grz-B induces the permeability of the mitochondrial outer membrane and cleaves Bid, a molecule from the Bcl-2 family [161]. Bid then promotes cytochrome-C release from mitochondria and other apoptogenic intermembrane molecules, like HtrA2/Omi, endoG and AIF into the cytoplasm [162]. Cytochrome-C triggers the formation of apoptosomes and activates caspase 9, which in turn enhances caspase 3 activation, decreasing mitochondrial function resulting in cell death [163].
Figure 2.8. Granzyme A and Granzyme B induction of apoptosis. After entering the target cell, Grz-A activates the endoplasmic reticulum (ER) associated complex which is conformed by phosphoprotein 32 (pp32), nonmetastatic protein 23 homologue 1 (NM23-H1), oncoprotein SET, high mobility group 2 (HMG-2) protein, and apurinic endonuclease 1 (Ape1). There is an increase of reactive oxygen species (ROS) and a decrease in mitochondrial membrane potential inducing the translocation of the SET/NM23-H1 complex into cell nucleus. Grz-A then cleaves SET, which activates NM23-H1, an endonuclease that fragments chromosomal DNA. Grz-B cleaves Bid or activates caspase 3 directly, which degrades DNA. Truncated-Bid causes permeability of the mitochondrial outer membrane and cytochrome-C release with a decrease of mitochondrial function. Cytochrome-C induces activation of caspase 9 that increases the apoptosis process by downstream activation of caspase3. TCR: T cell receptor [135].

Other granzymes present in humans are Grz-K, -H and –M. Grz-K is a protease like trypsin, that triggers apoptosis by Bid-dependent mitochondrial outer membrane damage [164]. Grz-H has a synergistic function with Grz-B and is a chymotrypsin-like protease (chymase). Grz-M is a serine protease that is highly expressed in NK cells and induces cell death by cleaving the actin-plasma membrane linker, ezrin and the microtubule component α-tubulin, disrupting the microtubule network [165].
2.7.1.3. Granulysin

This is a cytolytic molecule and a member of the saposin-like protein family. It is stored in granules from NK cells, cytotoxic T cells, helper T cells and NKT cells. It is proposed that when granulysins interact with the negative charge of the target-cell mitochondrial membrane lipids, it induces cell membrane damage [166]. This releases cytochrome-C and lowers mitochondrial function, which is related with the perforin-pore (figure 2.9) [167]. Granulysin may also induce apoptosis via caspase-3 activation [167]. At nanomolar concentrations it can act as a leukocyte chemoattractant, at micromolar concentrations it causes cell lysis [168, 169].

![Figure 2.9](image.png)

**Figure 2.9.** The internalization of lytic molecules. This diagram shows Perforin polymerizing on the target cell surface creating a pore, through which Grz-A, Grz-B and granulysin enter to induce apoptosis [135].
2.7.2. Receptor-ligand pathways
2.7.2.1. Fas – FasL and TRAIL-DR mediated pathway

This cytolytic pathway is critical in the control of T cell overstimulation and is involved in induction of tolerance to self-antigens, as well as being a homeostatic mechanism of cytotoxic T cell activity and target cell killing. In HIV infected patients, there is an overexpression of Fas on CD4+ T cells which has been linked to increased bystander cell death which can be induced by activated CD8+ or CD4+ T cells expressing FasL [170]. Effector T cells express FasL (CD178), whereas target cells express Fas (CD95 or Apo-1), and these target cells are susceptible to apoptosis induced by receptor-ligand interaction (figure 2.10) [171, 172]. The Fas molecule is a cell surface protein and belongs to the TNF receptor (TNFR)-I type family. Fas has one extracellular domain rich in cysteines that binds FasL, and another cytoplasmic domain involved in initiation of death signal transduction [173]. FasL is an inductive molecule that is expressed on T cells and is homologous to the cytokine TNF. It is a member of the TNFR-II type family [174]. FasL is constitutively expressed on cells in immune privileged organs and protects from the action of immune cells by acting as an additional regulatory mechanism of self-tolerance. Such organs include the brain, anterior chamber of the eyes and testes [175]. FasL expression is regulated at the transcriptional level. The positive regulators of FasL expression are NFaT, Egr2/Egr3, NFκB, AP-1, c-myc SP1, and B1/Cdk1, while the negative regulators are c-Fos and CIITA [176-178]. Some of these regulatory factors function by binding directly to FasL-encoding DNA, whereas others indirectly regulate transcription factors [178].

FasL is expressed in three ways: the first as highly arranged trimers anchored on the cell surface membrane, this is the primary mediator of apoptosis. The second is when FasL is anchored to intracellular membrane microvesicles; here it is stored until expressed on the cell surface in response to physiological stimuli. The third way is in the form of soluble FasL which is produced by degradation of the membranous form (during the first few minutes of expression) due to the activity of a metalloprotease matrix whose function is to catalyze the degradation of extracellular matrix proteins [179–182].
Soluble FasL may have either pro-apoptotic or anti-apoptotic properties since soluble FasL is an ineffective homotrimer binding to Fas. When these molecules act together the result is null signaling with no apoptosis. However soluble FasL can promote apoptosis after its association or aggregation with extracellular matrix proteins. Apoptosis can also be induced when soluble FasL forms tetramers or other highly arranged structures [182]. The Fas/FasL pathway has a critical role in graft rejection [183], where soluble FasL has a chemotactic effect on neutrophils, during the acute rejection of a graft transplant [184].

When Fas and FasL bind it causes trimerization and recruitment of Fas-associated death domain (FADD) proteins through homotypic death domain interactions (see figure 2.10). The trimerized FADD then recruits either procaspase 8 or 10, which undergo a process of autoproteolysis to become an activated caspase [185]. Once these components assemble they form a death-inducing signaling-complex (DISC), which is important in receptor-dependent apoptosis [185]. Caspase 8 interacts with procaspases 3, 6 or 7 and becomes activated through a process of transproteolysis. It is in this activated form, that effector caspases cleave DNA. Caspase 8 can also hydrolyze Bid, which results in damage to the mitochondrial outer membrane and triggers cytochrome-C release [186, 187].

Other than FasL, another member of the TNFR family is the TNF-related apoptosis-inducing ligand (TRAIL), a.k.a. Apo-2L [188]. TRAIL has multiple receptors of which two are pro-apoptotic. The receptors also belong to the TNFR family. These receptors are TRAIL-R1 (death receptor 4, DR4) and TRAIL-R2 (DR5) [189, 190]. Once linked to TRAIL, these receptors recruit FADD proteins in their cytoplasm portion. FADD proteins then employ procaspase 8 that is activated within DISC [191, 192]. Caspase 8 is able to induce the apoptosis either through interaction with procaspases 3, 6, 7 or Bid cleavage as mentioned previously.

Induction of apoptosis by death receptors occurs in two ways, depending on the cell type. Cells that die via apoptosis accompanied by large amounts of activated caspase-8
originated at the DISC are termed type I, and cells where receptor-mediated death relies primarily on the release of pro-apoptotic factors from the mitochondria are termed type II [193]. Type I cells quickly internalize Fas into an endosomal compartment in a clathrin-actin dependent manner [194], which is necessary to assemble DISC components. Therefore type I cells need Fas internalization to improve signaling events toward the apoptosis process [195]. Type II cells require the magnification of the apoptosis signal through a contribution from the mitochondria [194]. Studies show the FasL-induced endocytic vesicles reach the mitochondrial compartment resulting in type II cell death [196].

The same process occurs with TRAIL receptor; however TRAIL receptor internalization is not required for DISC formation and apoptosis induction [197]. TRAIL-induced apoptosis requires a loss of mitochondrial membrane potential, which induces the release of cytochrome-C [162, 193]. Cytochrome-C is critical to start the apoptosis process in the target cell by downstream activation of caspase 3 [162, 198].

One of the main differences between the Fas-FasL intercellular linkage-mediated pathway and the granule-dependent exocytosis pathway is the constant induction of apoptosis by FasL in cytotoxic T cells. The elimination of FasL (internalization) from the cell surface requires 2 to 3 hours; this period allows for the exertion of cytotoxicity in the absence of stimulus via TCR [199]. In this way, Fas expressing neighbor cells could be eliminated, even though they do not express the specific antigen recognized by cytotoxic cell (the so called “bystander cell death” phenomenon).
Figure 2.10. The Fas-FasL pathway. This figure illustrates the interaction of the FasL trimer with Fas. The interaction induces the trimerization of Fas-associated death domain (FADD) molecules that employ and activate procaspase 8 or 10. Increase of intracellular signaling boosts the recruitment of FADD molecules. Caspase 8 activates procaspases 3, 6 or 7, which activates Bid promoting cytochrome-C release. Caspase 8 also cleaves and activates procaspase 9. In the final step, activated caspases 3, 6, and 7, degrade chromosomal DNA leading to target cell death [135].

2.7.2.2. The TNF and TNFR type I dependent pathway

TNF-α is a cytokine made by activated cells that initiates cell apoptosis, inflammatory processes, cell activation and differentiation [199, 200]. The TNF molecule can induce receptor oligomerization increasing the binding affinity of the ligand [201]. The receptors of TNF (TNFR) can be assembled into three classes: i) having cytoplasmic death domains, ii) linked to adaptor molecules termed TNF receptor associated factors (TRAFs), and iii) soluble receptors [202, 203].
In TNF-induced apoptosis through the contribution of FADD molecules, after the preliminary interactions with TNF, the TNFR undergoes multimerization to form the DISC signaling downstream through the caspase activation cascade and mitochondrial changes [204]. Furthermore, the TNF-TNFR complex enlists TRAF molecules that signal downstream, activating the transcription factors NF-κB and JNK [205, 206]. Studies show the TRAFs have an important role in regulating the increase of intracellular reactive oxygen species (ROS), as well as regulating the cellular redox status [207]. The TNF-TNFR complex also enhances NADPH oxidase activity promoting a burst of oxidative stress and leading to necrotic cell death (hence the term "necrosis factor") [208, 209].

2.8. Characterization of CTL in HIV

2.8.1. Phenotypic markers vs. functional markers

T-cell responses can be divided into early, intermediate and late functional phases. Early functions consist of intracellular calcium flux and phosphorylation of key signaling proteins on serine, threonine or tyrosine residues [233]. Intermediate functions consist of degranulation, cytotoxicity and cytokine production [233]. The range of cytokines produced by T cells is a function of their differentiation and can include IL-2 as well as Th1 cytokines (e.g. IFN-γ, TNF-α), Th2 cytokines (e.g. IL-4, IL-5, IL-13) or regulatory cytokines (e.g. TGF-β, IL-10) [233]. Late functions consist of proliferation, as well as apoptosis or activation-induced cell death. Phenotypic markers of CTL are all markers associated with CTL function but not indicative of function [233]. Functional markers (such as degranulation, induced cytokine production) are important as they demonstrate integrity of CTL function, which is not always retained despite phenotypic marker expression [233].
2.8.2. Techniques for measuring T-cell activity

There are many assays available for the ex vivo examination of T-cell responses. These assays measure proliferation, define phenotype or changes in phenotype or measure function. Phenotypic changes include activation marker expression as well as alteration in other markers being expressed (e.g. memory). Functional changes in CD8+ T cells are measured by detecting the cytolytic mechanisms of these cells, e.g. intracellular cytokine production and degranulation in response to antigen or target cell killing. Flow cytometry can be used to measure degranulation by detecting the cell-surface expression of CD107 from cytotoxic granules [140]. The traditional assay for cytotoxicity is to measure cell lysis via release of $^{51}$Cr from labeled target cells. This process has been modified to measure the loss of dye-labeled target cells by flow cytometry [161]. Quantifying cytokines can be done with a wide range of techniques. There are bulk culture assays (ELISA for cytokine protein, and PCR or RNase protection for cytokine mRNA) and single-cell assays (ELISPOT and cytokine flow cytometry).

Functional T-cell responses can be measured following stimulation or polyclonal activation using mitogens and other stimulators. These include phorbol myristate acetate (PMA) plus ionomycin, phytohaemagglutinin (PHA), anti-CD3 and Staphylococcal enterotoxin B (SEB). One of the major applications of functional assays is to examine antigen-specific cell responses.

2.8.2.1. Analysis of T cell proliferation

Proliferation can be detected by the uptake of $^3$H-thymidine or bromodeoxyuridine (BrdU), or by dilution of a dye such as 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE). Techniques utilizing membrane-associated fluorescent dye CFSE have been used to detect dividing cells by flow cytometry through the serial reduction in CFSE intensity [162, 210]. This method allows the visualization of six or more distinct cycles of cell division by flow cytometry in vitro. It is compatible with identification of multiple cell-surface and intracellular markers on the dividing cells [211, 212], in addition
to live-cell sorting of proliferating cell populations. This method has been used in monitoring immune responses with respect to vaccine potency [212] and in examining immune competence during disease progression [211, 213 – 215].

2.8.2.2. Measurement of CTL Surface Marker Changes

2.8.2.2.1. Flow cytometric quantification of CTL activity by CD107 expression

Lytic granules contain perforin and a set of serine proteases called granzymes [141, 216, 217]. The granule core is enclosed by a lipid bilayer containing lysosomal associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1), CD107b (LAMP-2) and CD63 (LAMP-3) [140, 141, 218]. These proteins become temporarily expressed on the T cell surface after granule exocytosis [141, 218]. Betts et al. [140, 219] investigated the recruitment of CD107 to the surface of activated T cells in peripheral blood mononuclear cell (PBMC) cultures stimulated in the presence of the secretion inhibitor monensin. Because of the transient surface expression and fast internalization of CD107 via the endocytotic pathway [218], staining for CD107 is maximized by the addition of the corresponding antibody during cell stimulation and by adding monensin. CD107 and intracellular cytokines can be evaluated at the same time utilizing 4-6 hours of stimulation.

The CD107 mobilization assay can be used as a substitute to $^{51}$Cr release assays, even though effector cell degranulation is not the same measurement as target cell death. Researchers have however shown a good correlation between degranulation and cytotoxic activity of CTLs as measured in a flow-cytometry-based killing assay [140]. Rubio et al. [220] have also shown an accurate correlation between the cytotoxic activity of tumour-specific CD8+T-cell clones, as measures by $^{51}$Cr release, and the level of CD107 induction on those cells. Degranulation and cytokine production are diversely dependent on the concentration of stimulating peptide [221]. The level of effector response might be cytolytic and cytokine-producing or only cytolytic.
Other advantages of flow cytometric assays over the $^{51}$Cr-release assay is that they’re faster, safer, more sensitive, a single cell based assay (allowing for other cell surface markers to be studied), allows for the study of the outcome of effector cells and sorting experiments done with flow cytometry can be performed to analyze the profile of gene expression in CTL-susceptible and CTL-resistant target cells [222].

2.8.2.3. Detection of cytokine production

Most CD107$^+$ cells that respond to viruses produce IFN-$\gamma$ [140]. To measure IFN-$\gamma$ production, berfeldin A is added in addition to monensin to optimize detection of this cytokine as well CD107. The measurement of IFN-$\gamma$ production is a universal standard for expressing a cellular immune response to vaccination. In HIV-infected patients, using ELISPOT that detects IL-2 in combination with IFN-$\gamma$ by antigen-specific CD4+ T cells correlates to control of viraemia and non-progression [211, 223]. Researchers have used polychromatic flow cytometry (up to 12 colours) to measure antigen-specific immune responses [224]. Investigators using four anti-cytokine antibodies (MIP-1$\beta$, TNF-$\alpha$, IFN-$\gamma$ and IL-2) together with anti-CD107 demonstrated a loss of polyfunctional CD8+ T cells in patients with progressive HIV infection [225]. Long term non-progressors were found to have a consistent presence of CD8+ T cells that were polyfunctional and produced all four of the aforementioned cytokines and also degranulate in response to antigen.

2.8.2.4. Measurement of Immune Activation

2.8.2.4.1. Markers of Activation

Activated T cells can be characterized by the expression of newly synthesized surface proteins called activation markers. These activation markers can be identified through flow cytometry by utilizing fluorochrome conjugated monoclonal antibodies that are reactive to the surface marker in question. Examples of activation markers that are readily examined include: CD25 (the $\alpha$ chain of the IL-2 receptor), T cells expressing this marker are ready to proliferate and differentiate in response to antigen recognition;
CD38 (which is not only expressed on activated T cells but also activated B cells and plasma cells); CD69 (also known as an activation inducer molecule or AIM), is expressed on a number of activated leukocytes; HLA-DR (major histocompatibility complex class II antigen expressed on primed lymphocytes upon activation) is a good predictor of disease progression in HIV-infected individuals [228-230]; and CD137 which is a costimulator of T cells that induces cell division and IL-2 secretion [226,227]. CD137 (4-1BB) is a member of the TNFR superfamily (TNFSF9) and a type I transmembrane protein [243]. CD137 expression has been observed on activated CD4 and CD8 T cells [244, 245] as well as certain subsets of Dendritic cells, mast cells, Natural Killer cells, human monocytes and eosinophils [248]. Upon activation, T cells exhibit short-lived expression of this marker, the peak being at approximately 48 hours post activation and declining by 4 to 5 days [244,249]. CD137 activation brings about pro-survival signals during the peak of the immune response effector phase. This is critical in avoiding activation induced cell death of the effector cell population giving rise to larger memory pools. Researchers have shown the importance of the CD137 – CD137 ligand interaction in effector CTL induction as well as the establishment of long-lasting memory CTLs [250, 275, 300, 301]. Other methods of measurement of immune activation include measuring Calcium flux in signal transduction [231, 232] or the increased phosphorylation of signal transduction proteins [234-242].

Cytotoxicity can also be quantified in the following ways: measuring the activation of caspases within target cells [222, 246-247]; detecting lysed target cell that were stained with CFSE and 7-Amino actinomycin D (7-AAD) [251,252]; measuring the release of a radioactive compound, $^{51}$Cr from lysed target cells that were previously loaded with this compound [253] and the visual detection of biotinylated refolded HLA-peptide complexes that are attached to a streptavidin molecule, which binds to T cells expressing an appropriate TCR [1, 254-262].
2.9. Importance of detailed characterization of CTL in HIV for evaluating vaccine or therapy efficacy

The importance of CD8+ T cells has been shown in SIV-infected macaques and also HIV-infected humans, where their role in determining the rapidity of disease progression has been defined. It is clear that CD8+ T cell-mediated immunity will most likely be a key component of a successful AIDS vaccine. Studies involving SIV-infected rhesus macaques mimic, to an extent, CD8+ T cell responses in HIV-infected humans. Three important areas of research involving SIV-infected macaques are (i) investigating the immunological mechanisms underlying MHC-I connections with elite controllers, which is mediated during acute infection; (ii) establishing the immune responses generated by live-attenuated vaccines that contribute to their effectiveness, and endeavour to duplicate using alternative vaccination strategies and finally (iii) to create viruses that closely mimic the diversity selected for in HIV, so as to provide accurate assessments of vaccines in non-human primates.

Based on the discussion above, it can be seen that the HIV-specific CTL response is of great importance, and measuring CTL activity in normal progressors, elite controllers as well as natural simian host, offers valuable insight. The CD8+ T cell activity in TB and HIV coinfected individuals has not been well documented thus far, especially in South Africa where this coinfection is readily seen.

2.10. Aims of this Study

The purpose of the current study was to evaluate CTL phenotypically and functionally in chronic HIV and to assess the impact of TB co-infection on CTL. HIV and TB co-infection is very prevalent in South Africa and the knowledge of how Mtb infection impacts on the immune system of HIV+ individuals still needs further investigation. CTLs were characterized in this study as they known to be important in mounting an anti-HIV protective response. CTL dysfunction and loss have been shown to precede the onset of AIDS. There have been reports on the influence of TB on HIV CTLs but this
study is the first to combine a range of CTL killing/apoptosis associated markers, immune exhaustion markers, cytokine markers and an activation marker in the SA setting.

CD4 count and viral load was determined and baseline expression levels of various phenotypic markers of CTL were assessed in uninfected, HIV-infected and HIV-TB co-infected subgroups. The novel marker of immune activation CD137 was also included to determine whether ex vivo expression was enhanced in HIV infection, and whether expression was related to other markers of CTL function. Antigen-specific (pooled gag peptide) stimulation of CTL was then performed, and the impact of stimulation on marker expression and also on degranulation was determined.

A working hypothesis for this study was that TB co-infection promotes generalized immune activation and that this is a driving force in progression to AIDS. This study was aimed at linking this concept to CTL function.
CHAPTER 3: METHODOLOGY

3.1. Donors

All donors were sourced from clinics in the Tygerberg area including infectious disease clinics of Tygerberg Hospital and Karl Bremer Hospital. Ethical approval was attained for this study from Stellenbosch University Ethics Committee (N07/03/057). All donors were adults (> 17 years of age) who had not undergone any antiretroviral (ARV) treatment (i.e. treatment naïve). All donor volunteers were briefed on the study and signed consent forms in their preferred language. Donors were divided into three groups: Healthy HIV negative (negative control); HIV+ but tuberculosis negative (HIV+TB-) and HIV+TB+. There were 15 healthy volunteers, 30 HIV+TB- volunteers and 15 HIV+TB+ volunteers. HIV status was confirmed by standard serological screening. The HIV+ volunteers were recruited at the clinics before initiation of ARV treatment. The TB co-infected group consisted of individuals displaying active TB disease. TB disease status was confirmed by positive chest X-ray or clinical symptoms (persistent cough, night sweats, fever, and weight loss). All but two of the TB+ volunteers had started TB treatment a month prior to their enrolment into this study.

3.2. Venipuncture

A research nursing sister employed by the Division of Medical Virology, carried out blood extraction (venipuncture) on the volunteers. Two 10ml EDTA Vacutainer tubes (BD Biosciences, San Jose, California) were used for this purpose. Approximately 20ml was obtained from each donor.

3.3. CD4 Count

The BD MultiTEST™ CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC Reagent (BD Biosciences, San Jose, California) and TruCOUNT tubes (BD Biosciences, San Jose, California) were used for CD4 counts. TruCOUNT tubes were appropriately labeled with
patient information. 20µl of MultiTEST reagent (antibody mix) was then pipeted into the bottom of the tube above the steel retainer above the bead pellet. Then, 50µl EDTA blood was then added to the tube and the tube was then vortexed. The tube was then incubated for 15 minutes in the dark at room temperature. After incubation, 450µl of 1X Fluorescent Activated Cell Sorter (FACS) lysing solution (BD Biosciences) was then added and vortexed. The tubes were then incubated for 15 minutes at room temperature and then analyzed on the flow cytometer (Becton Dickinson FACSCalibur with MultiSET™ software). This method is the standard CD4 SOP used in the Division of Medical Virology, which has been accredited by SANAS.

### 3.4. Viral Load

After the 50µl of blood was aliquoted for the CD4 count, the EDTA tubes were centrifuged at 20°C at 1200 revolutions per minute (rpm) or 300 X gravity (g) for 12 minutes. Approximately 1ml of plasma was decanted into a Greiner Bio-one cryotube (Greiner Bio-One GmbH, Frickenhausen, Germany) and the sample was sent for routine viral load testing in the Division of Medical Virology. The viral load assay performed was a NucliSens EasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands) which has a detection range from 350 to 10^6 copies/ml. This is an HIV-1 RNA quantitative assay that monitors viral load by applying nucleic acid sequence-based amplification (NASBA) technology combined with detection of real-time molecular beacons. This method is also SANAS approved.

### 3.5. Peripheral Blood Mononuclear Cell (PBMC) preparation

All centrifugation procedures were carried at approximately 20°C (room temperature). Vacutainer tubes of fresh blood were centrifuged (Jouan CR412 centrifuge, M4 swing out rotors) at 1200 rpm (300g) for 12 minutes (at room temperature) to obtain a buffy layer containing leukocytes. The buffy layer was then collected (5ml per vacutainer tube). 4ml D-PBS (phosphate buffered saline without Ca^{2+} and Mg^{2+}, Gibco, Invitrogen Corporation, UK) was then added to the 5ml buffy layer. The 9ml of diluted buffy layers
were carefully added to centrifuge tubes containing 4ml Histopaque-Ficoll® (Sigma-Aldrich). After centrifugation at 1600rpm (400g) for 30 minutes (at room temperature), 5ml of interface monolayer was pipetted into tubes and washed again with 12ml D-PBS buffer (Gibco, Invitrogen Corporation, UK). These tubes underwent another round of centrifugation at 1000rpm (250g) for 10 minutes (at room temperature) and the supernatants were removed and pellets resuspended. If the donor had two tubes of PBMCs, they were combined at this point. After the final stage of centrifugation at 1000rpm (250g) for 10 minutes (at room temperature), the supernatant was removed from each tube and the cells were resuspended in AR-10 medium (50% AIM V + 2mM L-glutamine; 50% RPMI 1640 + 2mM L-glutamine + 1mM Sodium pyruvate supplemented with 10% heat inactivated foetal bovine serum; 50mg/ml Gentamycin, Gibco). Cells were then counted using a haemocytometer following staining with Turks staining solution (0.02% crystal violet, 7% glacial acetic acid in water).

3.6. Freezing of PBMCs

Samples were frozen away and thawed later for bulk batch analysis. After cell counting, the extracted PBMCs were first centrifuged at 1300rpm (325g) for 10 minutes (at room temperature) and the cell concentration was adjusted to $3 \times 10^6$ cells/ml using AR-10 media. The cells were then pelleted by re-centrifugation at 1300rpm (325g), and the resuspended pellet mixed with 1ml of freezing medium (DMSO in Foetal Bovine Serum,Gibco, Invitrogen Corporation, UK). Aliquots (1ml) of these cell suspensions were then transferred to cryovials (Corning, Greiner Bio-one, Frickenhausen, Germany). They were then wrapped in absorbent tissue paper (Carlton paper). The tubes were then stored at –20°C overnight. The next day the tubes were transferred to boxes and stored in the designated place in a –80°C freezer. If storage was for a period longer than one month, the cell vials were transferred to liquid nitrogen storage.
3.7. Thawing of frozen PBMCs

Cryovials were removed from the –80°C freezer or liquid nitrogen, and immediately were placed in a beaker with water from a 37°C water bath for transport and then moved into a 37°C water bath. Once thawed, AR-10 medium was added to the vial in a drop-wise style up to 2ml. The contents of the vial was transferred to 15ml BD Falcon tubes (BD Biosciences, San Jose, CA, USA), to which AR-10 media was pipeted to make up a total volume of 12ml. The tubes then underwent centrifugation at 1000rpm (250g) for 10 minutes (at room temperature) and had their supernatant removed. Pellets were resuspended and tubes were again filled with AR-10 media to the 12ml mark. This centrifugation step was repeated. After the final centrifugation step at 1000rpm (250g) for 10 minutes (at room temperature), the supernatant was removed, AR-10 media was then added up to the 5ml mark and the cells were counted with a haemocytometer after mixing 1:1 with Trypan blue (Sigma-Aldrich). Cell concentrations were calculated and altered to meet the assay protocol requirement. Generally cells were adjusted to 5 x 10^5 cells/ml.

3.8. HIV gag pool peptides

Peptides were used for restimulation of PBMC for intracellular cytokine (ICC) staining. Gag peptides were derived from an HIV-1 subtype C consensus sequence covering the full gene (NICD, Johannesburg). A total of 66 peptides (15-20mer overlapping by 10) were included. For ICC a superpool of all peptides was used for restimulation. Peptides were used at a final concentration of 0.45 µg/ml.

3.9. *In vitro* stimulation

96-well round-bottomed Microtitre plates (Greiner) with cells suspended in AR-10 media at a concentration of 5 x 10^5 cells/well were used for stimulation. Unstimulated cells were exposed to AR-10 medium alone. The stimulated wells included 1µg/ml staphylococcal-enterotoxin B (SEB, Sigma-Aldrich) or 0.45µg/ml HIV gag peptides. All
wells were co-stimulated with 10µg/ml anti-CD28 antibody (BD Biosciences, San Jose, CA, USA). The plates were incubated for an hour in a 37°C 5% CO₂ incubator; after which Monensin (1:250 dilution of Golgistop, Pharmingen, San Diego, Calif.) was then added to all the wells. Brefeldin A (1:250 dilution of Golgiplug, Pharmingen, San Diego, Calif.) was added to wells in which IFN-γ was to be investigated as previous experiments (data not shown) in the laboratory had shown that Brefeldin A was better for the detection of this cytokine. The samples were incubated a further 17 hours in a 37°C 5% CO₂ incubator. After this step, the plate was stored in a 4°C fridge prior to surface or intracellular staining. Only samples that showed a positive SEB response were evaluated for gag-specific responses.

Due to inadequate cell numbers not all the markers were investigated on all the patients. This was due to low lymphocyte counts in certain patients, and also as a result of cell loss during the freezing-thawing process. The table below shows the markers and numbers of individuals in each experimental group that were evaluated for marker expression.

**Table 1. Markers and associated Patient Numbers.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Healthy</th>
<th>HIV+TB-</th>
<th>HIV+TB+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD137</td>
<td>15</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Perforin</td>
<td>15</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Granzyme A</td>
<td>15</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>CD107a/b</td>
<td>15</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>12</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>Fas*</td>
<td>15</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>FasL*</td>
<td>15</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>12</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>PD-1</td>
<td>15</td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>

Healthy = uninfected donors; HIV+TB- = HIV infected and TB uninfected; HIV+TB+ = HIV and TB coinfected donors. Note: Fas and FasL expression following SEB stimulation was performed in 14/15 Healthy donors.
3.10. Degranulation Assay

PBMCs in round-bottomed 96-well microtitre plates (Greiner), at a cell concentration of 5 x 10^5 cells/ml per well were used. The plate was placed in a 37°C 5% CO₂ incubator overnight. The next day, media was removed from wells, and stimuli were added to them. Some PBMCs were plated in the presence of stimulants (1µg/ml SEB, 30µg/ml HIV Gag peptides) and others were unstimulated. FITC-labeled anti-CD107 was added to each well. The plate was then incubated for an hour at 37°C. Monensin (1:250 dilution of Golgistop, Pharmingen, San Diego, Calif.) was then added to all the wells. The plate was incubated in a 37°C CO₂ incubator (17 hours), after which it was stored in a 4°C fridge overnight and stained with antibodies the following day using the intracellular staining protocol outlined below.

3.11. Antibody Staining Mix Preparation

All reagents were kept on ice and centrifugation was carried out at approximately 8°C. Monoclonal antibodies were taken out of the 4°C storage, and were placed on ice. A Becton Dickinson FACScalibur flow cytometer was used for detection of these antibodies. A total of 5 surface stains were used in this study (CD8, CD137, Fas, FasL, PD-1). CD137 expression was evaluated on normal donor PBMC following SEB stimulation for 18 hours, as discussed in section 3.9. After stimulation, cells were stained with monoclonal antibodies to CD8 (PerCP), CD25 (FITC), CD69 (PE) and CD137 (APC). Staining procedures were as outlined in section 3.12. In addition, 4 intracellular stains (perforin, granzyme A, TNF-α and IFN-γ) were used. Finally, CD107a/b was used for degranulation staining. Volumes of individual antibodies were determined from antibody titrations performed in the laboratory for related studies. Details of all the antibodies, the volumes used and their manufacturers are presented in table 2. The surface stain cocktails were adjusted to a final volume of 25µl/well in staining buffer, whereas intracellular stain cocktails were adjusted to a volume of 50µl/well in permwash buffer. These volumes have are routinely used in the laboratory, and have been found to yield consistent staining patterns.
Table 2. Antibodies used in the study.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Manufacturer</th>
<th>Volume (µl) per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8 PerCP</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>5</td>
</tr>
<tr>
<td>CD137APC</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>3.5</td>
</tr>
<tr>
<td>Perforin FITC</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>2.5</td>
</tr>
<tr>
<td>Granzyme PE</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>1.5</td>
</tr>
<tr>
<td>PD-1 PE</td>
<td>eBioscience, Inc., San Diego, Calif.</td>
<td>1.5</td>
</tr>
<tr>
<td>FasL PE</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>1.5</td>
</tr>
<tr>
<td>Fas FITC</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>2.5</td>
</tr>
<tr>
<td>IFN-γ FITC</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>2.5</td>
</tr>
<tr>
<td>TNF-α PE</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>1.5</td>
</tr>
<tr>
<td>IFN-γ PE</td>
<td>BD Pharmingen™, San Diego, Calif.</td>
<td>1.5</td>
</tr>
</tbody>
</table>

PerCP = Peridinin chlorophyll protein; APC = Allophycocyanin; PE = Phycoerythrin; FITC = Fluorescein isothiocyanate; H = Height; IFN-γ = Interferon-gamma; TNF-α = Tumor necrosis factor-alpha.

3.12. Staining Protocol

All reagents were kept on ice and the centrifugation steps were carried out at 8°C; cell concentrations for each well were 5 x 10⁵ cells/well. Microtitre plates were centrifuged at 1300rpm (325g) for 5 minutes and to the resuspended cells, 25µl of the corresponding surface-staining mix was added. The plates were incubated in the dark in a 4°C fridge for 30 minutes. At the end of incubation, 175µl staining buffer (0.25% Heat-inactivated Fetal Calf Serum in PBS) was added to each well. The plates were centrifuged at 1300rpm (325g) for 5 minutes, had their supernatant removed and their pellets resuspended. 100µl of fixative and permeabilizing reagent (Cytofix-cytoperm, BD Biosciences) was then added to the wells. The microtitre plates were incubated in the dark in a 4°C fridge for 20 minutes, after which 100µl of a 1:10 diluted permwash buffer (BD Pharmingen™, San Diego, Calif., this 1:10 diluted solution was used for the rest of the staining procedure) was added. The plates were centrifuged at 1600rpm (400g) for 5 minutes (at room temperature) and supernatant removed. The pellet was
resuspended and 200µl of Permwash buffer was again added to each well; thereafter the plates were centrifuged at 1600rpm (400g) for 5 minutes (at room temperature). The supernatant was removed, pellet resuspended and 50µl of the corresponding intracellular stains added to the corresponding wells. The plates were incubated in a 4°C fridge for 30 minutes. After this 150µl of permwash buffer was added to each well and centrifuged at 1600rpm (400g) for 5 minutes (at room temperature). The supernatant was removed, pellet resuspended and 200µl of permwash buffer was added. The microtitre plate then again underwent centrifugation at 1600rpm (400g) for 5 minutes (at room temperature). The supernatant then was removed and pellets resuspended and a repeat wash step was performed after adding 200µl of staining buffer. The supernatant was removed and after pellets were resuspended, 200µl of staining buffer was again added to each well. The contents of the wells were pipetted to correspondingly labeled FACS tubes. These FACS tubes had 300µl staining buffer added to them (to make a final volume of 500µl). The tubes were stored at 4°C until analysis on by flow cytometry.

3.13. Acquisition of Flow Cytometry data

Samples were acquired and analyzed on a Becton Dickinson (BD) FACSCalibur flow cytometer with CellQuest™ software (BD Biosciences, San Jose, CA, USA). Additional post-acquisition analysis was performed using BD FacsDiva Software (BD Biosciences, San Jose, CA, USA).

Fifty thousand events were collected for each sample. Cells were gated according to CD8 expression (population 1, P1) and forward and side scatter patterns to define the lymphocyte subset. The percentage of CD8+ T cells expressing each marker was then established (Examples of full flow cytometry panel analysis of a representative uninfected and HIV-infected individual is presented in Figures 3.1, 3.2 and 3.3). Instrument settings and colour compensation values were derived from those established using matched isotype control antibody stained samples (BD Biosciences, San Jose, CA, USA). Manual adjustments to the colour compensation settings were
performed initially using single antibody stained cells and antibody mixes. Following establishment of optimal colour compensation and instrument settings, these settings were used unaltered throughout the study.

There were three gating strategies employed, the first was \( P_1 = R_1 \) (this was based on positive CD8+ T cell populations). The FSC-H vs SSC-H density plot was generated from R1 and the second gate \( (P_2 = R_2) \) was based on the lymphocyte population scatter pattern (low side scatter). The remaining density plots were generated using \( P_3 = R_1 + R_2 \). Additional data analysis was performed using FacsDiva Software version 6.0 (BD Biosciences, San Jose, CA, USA). The general gating strategy for each group can be seen in Figures 3.14, 3.15 and 3.16.
**Figure 3.1.** Example of Acquisition template for Healthy Cohorts. This diagram shows the acquisition template based on patient 110095. Density plot A represents CD8+ T cells, from there P1=R1 was created from the CD8 positive population. A second gate P2=R2 was created on the predefined lymphocyte population (plot B). Subsequent density plots of cytokines and markers were derived from R1+R2. Plots A, B, C, E, G, I, K, M, O, Q and S are based on Unstimulated marker expression and plots D, F, H, J, L, N, P, R and T are based on Gag-stimulated marker expression. FSC = Forward Scatter; SSC = Side Scatter; US = Unstimulated, gag = Gag stimulated; FL = Fluorescence; H = Height; PerCP = Peridinin chlorophyll protein; PE = Phycoerythrin; FITC = Fluorescein isothiocyanate; Q = Quadrant; P = Population.
Figure 3.2. Example of Acquisition template for HIV+TB- Cohorts. This diagram shows the acquisition template based on patient 110316. Density plot A represents CD8+ T cells, from there P1=R1 was created from the CD8 positive population. A second gate P2=R2 was created on the predefined lymphocyte population (plot B). Subsequent density plots of cytokines and markers were derived from R1+R2. Plots A, B, C, E, G, I, K, M, O, Q and S are based on Unstimulated marker expression and plots D, F, H, J, L, N, P, R and T are based on Gag-stimulated marker expression. FSC = Forward Scatter; SSC = Side Scatter; US = Unstimulated, gag = Gag stimulated; FL = Fluorescence; H = Height; PerCP = Peridinin chlorophyll protein; PE = Phycoerythrin; FITC = Fluorescein isothiocyanate; Q = Quadrant; P = Population.
**Figure 3.3.** Example of Acquisition template for HIV+TB+ Cohorts. This diagram shows the acquisition template based on patient 110189. Density plot A represents CD8+ T cells, from there P1=R1 was created from the CD8 positive population. A second gate P2=R2 was created on the predefined lymphocyte population (plot B). Subsequent density plots of cytokines and markers were derived from R1+R2. Plots A, B, C, E, G, I and K are based on Unstimulated marker expression and plots D, F, H, J and L are based on Gag-stimulated marker expression. FSC = Forward Scatter; SSC = Side Scatter; US = Unstimulated, gag = Gag stimulated; FL = Fluorescence; H = Height; PerCP = Peridinin chlorophyll protein; PE = Phycoerythrin; FITC = Fluorescin isothiocyanate; Q = Quadrant; P = Population.

### 3.17. Statistical Analysis

Flow cytometric data in the form of percentage positive gated events were collected on a Microsoft Excel spreadsheet. Statistical analysis was performed using the statistical program GraphPad PRISM Version 5.03 (GraphPad Software, Inc., San Diego, CA, USA). Student t test analyses, linear regression determination and 1 way Analysis of Variance (ANOVA) were performed. Significant differences or correlations were defined as having a P value \( \leq 0.05 \) and were performed on Statistica. A statistician was consulted to confirm that the statistical tests used were correct and applicable for the data sets generated in this study.
CHAPTER 4: RESULTS

4.1. Patient Demographic

A total of 60 individuals were recruited for this study. The demographic breakdown of the subgroups is indicated in the materials and methods section, and also summarized in table 4. The mean age in years for the healthy cohort was 38.9 (range of 25-49), for the HIV+TB- group, the mean age was 32.8 (range of 17-46) and for the HIV+TB+ cohort the mean age was 34.9 (range of 23-43). The summary of this can be seen in Table 4.

The reported mean marker expression data indicated in this chapter are all based on percentage positive gated events (CD8+ and lymphocyte subset) acquired from flow cytometric analysis of PBMC. Data is presented using box-and-whisker plots, where the middle line represents the median, the box represents the interquartile range (25-75 percentile), the whiskers represent the 10-90 percentile and the outliers are indicated by dots.

4.2. CD4 counts and viral loads

The mean CD4 counts (cells/µl) were as follows Healthy: 1354; HIV+TB-: 211.2 and HIV+TB+: 191.6. The HIV+TB+ group therefore has a mean CD4 count below 200, which according to WHO staging places most of the patients in this category of severely immunosuppressed. Using student t test analyses, significant differences were found between Healthy cohorts vs. HIV+TB- (p=<0.0001) and Healthy vs. HIV+TB+ (p=<0.0001). A summary of the CD4 data can be seen in Table 4.
**Figure 4.1.** Box-and-whisker plot representation of CD4 counts data across study groups. Healthy: uninfected donors; HIV+TB-: HIV infected and TB uninfected; HIV+TB+: HIV and TB co-infected donors. Statistically significant differences are indicated by bars with P values. A summary of the median values, mean values with standard error of the mean (SEM) and number of subjects (n) are indicated below the graph.

The mean viral load of the HIV+TB- group was 283 687 copies/ml, which was almost twice the mean of the HIV+TB+ group (162 120 copies/ml) (figure 4.2.). (see Table 3).

The low CD4 count and high viral load of the 2 patient cohorts was due to their being recruited at HIV treatment clinics. The majority of patients only approach clinics when they are symptomatic, hence preferential recruitment of patients with low CD4 counts (and high viral loads).
Figure 4.2. Box-and-whisker plot representation of viral loads across HIV+ groups. HIV: HIV infected and TB uninfected; HIV+TB: HIV and TB co-infected donors. A summary of the median values, mean values (with SEM) and sample sizes are indicated below the graph. One outlier from each group was removed.
### Table 3. Patient demographic of the three study groups.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>HIV+TB-</th>
<th>HIV+TB+</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of Donors</td>
<td>15</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Age range</td>
<td>25 - 49</td>
<td>17 - 46</td>
<td>23 - 43</td>
</tr>
<tr>
<td>Age (mean years)</td>
<td>38.9</td>
<td>32.8</td>
<td>34.9</td>
</tr>
<tr>
<td>% of Males</td>
<td>26.7</td>
<td>23.3</td>
<td>26.7</td>
</tr>
<tr>
<td>% of Females</td>
<td>73.3</td>
<td>76.7</td>
<td>73.3</td>
</tr>
<tr>
<td>CD4 Count Range cells/µl</td>
<td>685 - 2343</td>
<td>14 - 569</td>
<td>7 - 801</td>
</tr>
<tr>
<td>CD4 Count cells/µl (mean)</td>
<td>1354*</td>
<td>211.2</td>
<td>191.6</td>
</tr>
<tr>
<td>CD4 Standard Deviation</td>
<td>428.8</td>
<td>149</td>
<td>250</td>
</tr>
<tr>
<td>CD4 Count cells/µl (median)</td>
<td>1336.5*</td>
<td>185</td>
<td>120</td>
</tr>
<tr>
<td>Viral Load Range copies/ml</td>
<td></td>
<td>357 - 6300000</td>
<td>357 - 1100000</td>
</tr>
<tr>
<td>Viral Load copies/ml (mean)</td>
<td></td>
<td>283 687</td>
<td>162 120</td>
</tr>
<tr>
<td>Viral Load Standard Deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral Load copies/ml (median)</td>
<td></td>
<td>23 000</td>
<td>46 000</td>
</tr>
<tr>
<td>Log10(median viral load)copies/ml</td>
<td></td>
<td>4.36</td>
<td>4.66</td>
</tr>
<tr>
<td>Log10(mean viral load)copies/ml</td>
<td></td>
<td>5.45</td>
<td>5.21</td>
</tr>
</tbody>
</table>

Healthy = uninfected donors; HIV+TB- = HIV infected and TB uninfected; HIV+TB+ = HIV and TB coinfected donors.

*One CD4 count not included in the median calculation, therefore median is based on n=14.

Note: CD4 count measurements of 42% of participants in this study (25 out of 60 individuals) were performed on fresh blood on the day of PBMC extraction. The remaining CD4 counts were collected from recent clinic CD4 count data (performed within the previous three months).

### 4.3 Activation status

To test for CD8 activation, the novel early activation marker CD137 was examined. This marker has been associated with early activation by signaling through TCR can be utilized to identify antigen-responsive CD8+ T-cell subset [302]. Preliminary research was carried out on eight Healthy volunteers, to directly compare expression CD137 expression changes on stimulation by SEB vs. other routine markers of activation viz.
CD25 and CD69. Linear regression analysis was done on CD137 vs. CD25 and CD137 vs. CD69.

![Graph showing the Linear regression of CD137 vs. CD25 expression on CD8+ T cells following 18 hours of SEB stimulation.](image)

**Figure 4.3.** Graph showing the Linear regression of CD137 vs. CD25 expression on CD8+ T cells following 18 hours of SEB stimulation.

![Graph showing the Linear regression of CD137 vs. CD69 expression on CD8+ T cells following 18 hours of SEB stimulation.](image)

**Figure 4.4.** Graph showing the Linear regression of CD137 vs. CD69 expression on CD8+ T cells following 18 hours of SEB stimulation.
These results showed that CD137 was statistically positively correlated to comparable markers of activation, the p value for CD137 vs. CD25 was 0.0002 and the p value for CD137 vs. CD69 was 0.0249. The statistical significance was attained with a relatively small sample size, which also confirms the relatedness of CD137 to CD25 and CD69. Based on these findings, we used CD137 as a valid marker of early activation. Ideally a comparison of CD137 expression with a classical activation marker throughout the study should have been included, but limitations due to cell numbers and fluorochrome combinations in 4-colour flow cytometry prevented this (see Discussion).

The HIV+TB- and healthy cohorts shared a similar baseline level of CD137 expression on CD8 T cells (healthy mean baseline: 1.528%; HIV+TB- mean baseline: 1.579%), whereas the HIV+TB+ group had a much higher baseline level (mean: 4.829%). There were significant differences in the baseline levels between the controls vs. HIV+TB+ (p=<0.001) and HIV+TB- vs. HIV+TB+ (p=<0.001).

The low levels of ex vivo expression of CD137 in the HIV-infected cohorts are similar to findings of low CD25 expression [303]. This may indicate that CD137, although a good marker of induced activation, is less beneficial for ex vivo screening than markers such as CD38 and HLA-DR [122, 303].

In response to HIV gag peptide stimulation, healthy control cohorts as expected displayed no effect whereas both HIV+ groups had a positive response, with the HIV+TB+ cohort having the strongest HIV antigen-specific response (healthy gag mean: 1.153%; HIV+TB- mean: 1.860%; HIV+TB+ mean: 5.427%). The gag induced expression of the control group had a significant difference in comparison to the HIV+TB+ (p=0.0023) as well as between HIV+ groups (p=0.001). These data are illustrated in figure 4.5.
Figure 4.5. Comparison of CD137 expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+.
and TB- cohorts; HIV+TB: HIV; TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.

4.4. Phenotypic and Functional CTL status

CD8 phenotype and function was investigated by measuring various markers including those associated directly with cytolytic function such as Perforin, Granzyme A, Fas and FasL and also the CD107a/b degranulation-associated marker. Also included were intracellular cytokine levels of IFN-γ and TNF-α, cytokines associated with Th1 responses, and CTL activity in HIV infection.

4.4.1. Perforin

Perforin is an important component of CTL granules and is plays a vital role in CTL killing. The baseline level of Perforin staining in the HIV+ groups was markedly higher than that of the healthy donors, but the HIV+TB+ co-infected group displayed more than double the baseline perforin of the HIV+TB- group. The mean percentage positive baseline values for the uninfected, HIV+ and HIV+TB+ groups were 0.9380, 2.491 and 6.272 respectively. There were significant differences between the healthy group and the HIV+TB- (p=0.008) as well as the HIV+ cohorts (p=0.0179). Gag peptide stimulation resulted in a non-response in the healthy volunteer group, whereas both the HIV+ cohorts showed a positive response (healthy mean gag stimulated value: 0.93; HIV+TB- stimulated mean: 2.554; HIV+TB+ stimulated mean: 6.711) (figure 4.6). Significant differences were found between the HIV+ cohorts (p=0.0116).
Figure 4.6. Comparison of Perforin expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.
4.4.2. Granzyme A

Granzyme A is a serine protease enzyme that is found in CTL granules, and upon entry into target cells, induces apoptosis. Baseline Granzyme A expression levels in HIV+TB- volunteers was highest (mean: 60.95%), nearly twice that of healthy controls (mean: 33.17%). The co-infected HIV+TB+ group also had high baseline levels (mean: 56.03) but these were somewhat lower than that of HIV+TB- group. There were significant differences between the healthy group and the HIV+TB- group (p=0.0008) as well as the controls and the TB co-infected group (p=0.0111). The mean Gag response in healthy, HIV+TB- and HIV+TB+ groups was 49.18%, 60.70% and 55.87%, respectively (figure 4.7.). This equates to a very slight antigen-specific reduction in GzA content in the two HIV+ groups. This may be attributed to Granzyme A exiting the CD8 T cell following stimulation, a process not matched or overcome by production of new granzyme A within the cells. The increase in granzyme A in the control stimulated group was due to an outlier with unaccountably high granzyme expression within a small sample group.

(a)

![Graph showing CD8+GzA+ levels](a)

<table>
<thead>
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<th>Control</th>
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<th>HIV+TB</th>
</tr>
</thead>
<tbody>
<tr>
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<td>63.65</td>
<td>53.70</td>
</tr>
<tr>
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</tr>
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</table>
Figure 4.7. Comparison of Granzyme A expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.

Dual positive expression of Perforin and Granzyme was also examined in all three groups. The results are as follows: Healthy unstimulated baseline: 0.9960%, Gag stimulated: 1.207%; HIV+TB- unstimulated: 2.418%, Gag stimulated: 2.547%; HIV+TB+ unstimulated: 5.864%, Gag stimulated: 6.217% (see figure 4.8.). The difference in baseline levels between healthy and TB+ cohorts were shown to be significant with a p value equal to 0.0136, as was the difference between the HIV+ groups (p=0.0256). The differences in gag stimulated expression between HIV+ cohorts were significant, p=0.0286. The pattern of both baseline and Gag-stimulated expression for both perforin and Granzyme A was similar to that of perforin alone. This indicates those cells positive for GzA were generally positive for perforin as well, but not the other way round.
Figure 4.8. Comparison of Perforin and Granzyme A dual expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.
The percentage of Granzyme A single producers that were also producing both Granzyme A and Perforin are as follows: Baseline Controls: 3.00%; Gag stimulated Controls: 2.45%; Baseline HIV+TB-: 3.97%; Gag stimulated HIV+TB-: 4.20%; Baseline TB+: 10.47%; Gag stimulated HIV+TB+: 11.13%. The percentages of Perforin single producers that were dual producers are as follows: Baseline Controls: 100%; Gag stimulated Controls: 100%; Baseline HIV+TB-: 97.07%; Gag stimulated HIV+TB-: 99.73%; Baseline TB+: 93.49%; Gag stimulated HIV+TB+: 92.64%. It can be seen that more perforin single producers were also Granzyme A and Perforin dual producers.

4.4.3. CD107a/b (LAMP-1/-2)

CD107a/b or Lysome-associated membrane-protein 1/2 (LAMP-1/-2) is an important functional marker of CTL activity. Once a CD8+ T cell is activated upon antigen stimulation, this lysosomal protein translocates to the cell surface. The degranulation assay (CD107 upregulation) showed that the HIV+ groups had higher baseline levels than the healthy controls (healthy control baseline mean: 1.390%; HIV+TB- mean: 2.230%; HIV+TB+ mean: 7.048%), in other words HIV+TB+ patients displayed a 5-fold higher expression than that of healthy controls and a 3-fold higher expression than the HIV+TB- group. Significant differences were calculated between healthy and TB+ cohorts (p=<0.001) and between HIV+ cohorts (p=<0.001). In response to HIV gag peptides, the HIV+TB+ cohort exhibited the highest induced expression of the three groups (HIV+TB+ mean: 6.894%), as the other two had very low positive enhancement (healthy control gag mean: 1.577%; and HIV+TB- mean: 2.840%) (figure 4.9.). Significant differences in response to gag stimulation were seen between the healthy cohorts and the TB+ groups (p=0.0230) and between the HIV+ groups (p=0.0006). The co-infected group showed an antigen-specific reduction in CD107a/b expression, whereas the HIV cohort showed an increased expression on stimulation. This appears to indicate an inhibition of responsiveness in the co-infected cohort. These data are however not conclusive due to some gag-specific changes in the control group. This may be due to the small control group size for gag-specific stimulation. In addition, the
high baseline CD107a/b expression may be linked to cell fragility, general immune activation status complications and even enhanced NK degranulation, as we only included CD8 as a marker for T cells not CD3.
Figure 4.9. Comparison of CD107a/b expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.

4.4.4. Fas (CD95)

Fas expression is important in defining cell targets which may be receptive to FasL-mediated CTL activity and also bystander cell death induced by any other FasL expressing cell. Fas baseline levels in the HIV+ groups were approximately twice the level observed in the healthy controls (healthy mean baseline: 1.201%; HIV+TB- mean: 2.848%; HIV+TB+ mean: 3.095 %). There was a significant difference between the Healthy and TB+ cohorts (p=0.0012). The expression of Fas on resting (or unstimulated) CD8 T cells indicates that a minority of such cells is marked for
elimination. The loss of CD8 T cells in later stage disease may be linked to Fas expression, and the increased baseline expression in the co-infected group may be indicative of the stage of disease of the patients. Stimulation with Gag peptides showed positive induced expression in the HIV+ groups but at low levels (healthy gag stimulated mean: 1.057%; HIV+TB- gag mean: 3.140%; HIV+TB+ gag mean: 3.129%) (figure 4.10.).
Figure 4.10. Comparison of Fas expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.

4.4.5. FasL (CD95L)

FasL expression, unlike Fas expression, is considered indicative of CTL activity rather than susceptibility to apoptosis. Baseline expression of FasL was higher in HIV+ groups when compared to that of the healthy cohorts (healthy control baseline mean: 0.9127%; HIV+TB- mean: 3.430%; HIV+TB+ mean: 7.281%). The healthy cohort was significantly different in Fas expression as compared to the TB+ group (p=0.0123). As with Fas, the co-infected group had highest FasL expression. The HIV+TB- and HIV+TB+ groups showed a three and five times higher expression, respectively than uninfected controls. The healthy controls didn’t show any change following Gag peptide stimulation. A Low positive antigen-specific increase was seen in the HIV+TB- group however there was a reduction in the TB+ group (healthy mean: 0.7714; HIV+TB- mean: 3.714; HIV+TB+ mean: 6.789), (figure 4.11). The non-responsiveness with regard to FasL expression following stimulation in the co-infected group may indicate immune dysfunction (see PD-1 data below), however the high baseline expression coupled with high baseline Fas expression in this group would seem to indicate that CD8 T cells are at risk for elimination. Without CD8 count data, this cannot however be confirmed.
Figure 4.11. Comparison of FasL expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.
Dual positive expression of Fas and FasL was also examined in all groups, with HIV+TB+ as expected having the highest baseline expression (healthy control baseline mean: 0.2786%; HIV+TB- mean: 0.5704%; HIV+TB+ mean: 1.619%). As observed with FasL, the co-infected group displayed a null gag-specific response (healthy gag mean: 0.3200%; HIV+TB- gag mean: 1.307%; HIV+TB+ gag mean: 1.636%) (see figure 4.12). These data may indicate antigen non-responsiveness, but may also indicate that any increase in either marker on stimulation may lead to cell death and thus elimination of the cells expected to show increased expression.
Figure 4.12. Comparison of Fas and FasL dual expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.

The percentage of Fas positive cells that were dual positive for Fas and FasL were as follows: Baseline Controls: 23.19%; Gag stimulated Controls: 100%; Baseline HIV+TB-: 20.03%; Gag stimulated HIV+TB-: 41.63%; Baseline TB+: 52.30%; Gag stimulated HIV+TB+: 52.30%. The percentages of FasL positive cells that were dual positive were as follows: Baseline Controls: 30.52%; Gag stimulated Controls: 100%; Baseline HIV+TB-: 16.63%; Gag stimulated HIV+TB-: 35.20%; Baseline TB+: 22.23%; Gag stimulated HIV+TB+: 24.10%. The Fas positive cells were therefore more likely to be dual Fas and FasL positive.
4.4.6. Cytokines (IFN-γ and TNF-α)

The two cytokines examined in this study were IFN-γ and TNF-α. IFN-γ is a cytokine produced by activated CTLs to activate macrophages and the cytokine is operative in both the innate and adaptive cell-mediated immune responses. IFN-γ baseline levels in the healthy and HIV+TB- groups were similar (Healthy means: 1.617%; HIV+TB- mean: 1.275%; HIV+TB+ mean: 4.387%), but the TB+ group had a much higher baseline level. This mirrors the higher baseline level compared to HIV+ alone observed in several markers (including TNF-α and PD-1 discussed below, but also Fas, FasL and perforin presented above). The baseline IFN-γ positive events (>1%) were higher than in many reported studies. This may be due to the use of thawed PBMC but may also be related to interference by NK cells. No CD3 marker was included in the current study to exclude CD8+ NK cells. NK cells may have higher baseline IFN-γ than T cells, which could impact on the result. Significant differences were found between the baselines for the healthy cohort vs. TB+ cohort (p=0.0302) and between the two HIV+ groups (p=0.0222). In response to Gag stimulation, the healthy controls had a decreased IFN-γ expression level (healthy control gag mean: 1.040%). There were positive responses from both of the HIV+ groups, the TB- group having the higher of the two (HIV+TB- gag mean: 2.885%; HIV+TB+ gag mean: 4.473%) (figure 4.13.). SEB stimulation was good in all three of the groups (healthy SEB mean: 12.68%; HIV+TB- SEB mean: 14.82%; HIV+TB+ SEB mean: 15.86%).
Figure 4.13. Comparison of IFN-γ expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.
TNF-α was examined because it is a marker associated with CD8+ T cell polyfunctionality and cytotoxicity, and it is also associated with driving HIV-replication. TNF-α baseline levels in HIV+TB- individuals (mean: 6.186%) were more than three times that of the negative controls (healthy control mean: 1.993%), and in the TB+ cohort the expression was over five times that of the healthy controls (HIV+TB+ mean: 10.78%). Significant differences in baseline levels were seen between the Healthy cohorts and the HIV+TB- cohorts (p=0.0146) as well as the healthy controls and the TB+ group (p=0.0176). Gag peptide stimulation brought about low positive expression in HIV+ individuals, and a overall negative response in healthy cohorts (healthy gag mean: 1.480%; HIV+TB- gag mean: 6.558%; HIV+TB+ gag mean: 12.08%) (figure 4.14.). Unlike with the IFN-γ data, the co-infected individuals displayed the highest HIV antigen-specific induction of TNF-α (> 3-fold higher than HIV+ group alone, 1.300%). The elevated TNF-α levels reported are relevant as the HIV+ cohorts generally had low CD4 counts and the cells were subjected to freezing and thawing rendering them fragile and “leaky”.

(a)

<table>
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<tr>
<th>Group</th>
<th>Median %CD8+TNF-α+</th>
<th>Mean ± SEM</th>
<th>n</th>
</tr>
</thead>
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<td>1.993 ± 0.5628</td>
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</tr>
<tr>
<td>HIV</td>
<td>4.37</td>
<td>6.186 ± 1.477</td>
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<tr>
<td>HIV+TB</td>
<td>7.08</td>
<td>10.78 ± 4.706</td>
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Figure 4.14. Comparison of TNF-α expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.

4.5. Immune Dysfunction – PD-1

To assess dysfunction of the CD8+T cells examined for expression of CTL markers, the PD-1 marker was investigated. PD-1 is a receptor present on CD8+ T cells and its cognate ligands (PD-L1 and PD-L2) are present on antigen presenting cells, B cells and on non-lymphocyte tissue like endothelial cells and cardiac myocytes. It is thought that the interaction of PD-1 with its ligands negatively regulates T cells in peripheral tissue.

The TB+ group data was based on a sample size of 6 for this marker (table 1), and this may account for the large range of the expression levels observed. Baseline levels of PD-1 in HIV+TB- individuals (mean: 7.319%) were over seven times that of the healthy group (mean: 0.9760%), whereas TB+ volunteers displayed a seventeen times higher expression of PD-1 (mean: 16.74%; but with a mean range of: 2.82 – 43.57%). Baseline
expression between the healthy and the HIV+TB- (p=<0.0001) as well as the healthy and the TB+ (p=0.0005) groups were significantly different. In response to Gag peptides, healthy controls had a negative induced expression, i.e. decrease (healthy gag mean: 0.9300%); HIV+TB- individuals had a low positive antigen-specific response (HIV+TB- gag mean: 7.521%), but the TB+ cohort had a high positive induced expression in comparison with the other two (HIV+TB+ gag mean: 21.18%; HIV+TB+ mean range: 3.34 – 48.71%) (see figure 4.15). The gag stimulated expression between the HIV+ cohorts was significantly different (p=0.0200). The co-infected group thus expressed both the highest baseline PD-1 and the greatest HIV antigen-specific induced expression.
**Figure 4.15.** Comparison of PD-1 expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.

### 4.6. CD137 and PD-1 Dual Expression

To assess activation as well as immune exhaustion the dual expression of CD137 and PD-1 was examined. Baseline expression for the healthy, HIV+TB- and TB+ cohorts were 0.04833%, 0.2117% and 1.330% respectively. Significant differences were seen between control vs. HIV+ (p=0.0013); control vs. TB+ (p=<0.0001) and between HIV+ cohorts (p=0.0078). Gag stimulated dual expression across the three groups were as follows: Controls: 0.0300%; HIV+TB-: 0.3617% and HIV+TB+: 5.427%. There were significant differences found between the healthy vs. TB+ (p=0.0115) and between the HIV+ cohorts (p=0.0016) (see figure 4.16.). Although the dual expression of these markers was generally low, the dramatic differences between all the groups may warrant further investigation.
Figure 4.16. Comparison of CD137 and PD-1 dual expression on CD8+ T cells across the three different groups (a) ex vivo (baseline) and (b) in response to gag stimulation. The box-and-whisker plots are based on composite percentage positive gated results. Control: healthy cohorts; HIV: HIV+ and TB- cohorts; HIV+TB: HIV and TB co-infected cohorts; Dif: Difference between stimulated and unstimulated means. The significant p-values are indicated on the graph.
4.7. Correlation between CTL marker expression, CD4 count and Viral Load
Correlations were performed to assess the link of CD4 counts and Viral load to CTL function and activation status. The majority of markers did not show significant correlation to CD4 count. Only Fas showed a baseline significant negative correlation, however CD137 and Granzyme A both showed negative correlation approaching significance levels (table 4). There were greater significant correlations between CTL markers and viral load, which may be indicative of CTL responsiveness to elevated antigen levels. Baseline CD137, perforin, Fas, TNF-α and PD-1 were all significantly positively correlated to viral load, with granzyme A approaching baseline significance. These findings are presented in table 5.

Table 4. CD4 Count Correlation showing r- and p- values Across all Groups in Response to Stimuli.

<table>
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<th>r value</th>
<th>p value</th>
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</thead>
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</tr>
</tbody>
</table>

Gag = Gag pool peptide stimulated

**Table 5. Viral Load Correlation showing r- and p- values Across all Groups in Response to Stimuli.**
CHAPTER 5: DISCUSSION

The hypothesis of the current study was that TB co-infection impacts on CTL activation and function in HIV infected individuals. To test this we looked at activation, apoptosis and immune dysfunction/exhaustion marker expression (CD137, Fas, FasL and PD-1) as well as functional CTL marker expression (CD107, Perforin, Granzyme A, IFN-γ and TNF-α production) in healthy volunteers and HIV+ patients with and without TB.

Studies in non-human primates have shown the importance of cytotoxic T lymphocytes in controlling HIV [89-92]. In humans, elite controllers of the virus are noted for possessing polyfunctional CTLs. Daucher et al. concluded that it was the quality of virus-specific CTLs rather than the quantity that elicited effective control of viral replication. The markers used to define polyfunctional CD8+ T cell “quality” include CD107, IFN-γ, MIP-1β, TNF-α and IL-2 [263]. In a large percentage of untreated HIV individuals, the increase in viral load and decline in CD4+ T cell numbers over time results in progression to AIDS. HIV-specific CTLs are detectable in these individuals, but despite this, their progression to AIDS suggests that CTLs become functionally impaired over time (or “quality” diminishes) [112, 118, 264, 265]. This may be due to ongoing aberrant immune activation associated with continual antigen stimulation. Zhang and colleagues have shown evidence that up-regulation of the inhibitory receptor PD-1 in HIV infected patients is linked to HIV-specific CTL functional exhaustion. Exhausted CD8+ T cells are also more susceptible to apoptosis, resulting in inability of CTLs to suppress viral replication [2-4]. Other studies indicate that PD-1+ CTLs have a range of dysfunctions, for example, defective perforin and IFN-γ expression and impaired proliferative potential. Research linking PD-1 expression to increasing viral load suggest that this exhaustion of the CTL immune function or heightened expression of PD-1 may be attributed to over stimulation by viral antigen [2, 3] and the PD-1 upregulation may actually represent the immune system trying to counteract the continued activation (exhaustion). Another possible explanation for CTLs failure to control viral replication during chronic HIV infection is the inability of CD8+ T cells to traffic into infected lymph nodes as HIV disease progresses [266]. This is probably due
to changes in homing receptor expression and also changes in lymph node architecture [267, 268].

_Mycobacterium tuberculosis_ antigen-specific CTLs may not directly play a vitally important part in protective immunity in TB, but they are present and do undergo activation due to antigenic stimulation [269]. TB co-infection in HIV appears to impact on the total CTL population as evidenced in the study by Hertoghe and colleagues where they showed elevated expression of HLA-DR, CD38 and CD95 (Fas) on CD8+ CTL. Higher levels of immune activation have been noted in TB co-infection, especially in the CD4+ T cell subset, a finding confirmed in a parallel study in our laboratory (Kabue _et al._, personal communication). Most of the TB+ patients in the current study were receiving anti-TB treatment while enrolled. Previous studies in West Africa have shown elevated levels of viral load and TNF-α even after 3 months of TB treatment [304] and another study showed that despite successful TB treatment there is still a decline in immune function [305].

In the current study heightened expression of all markers was observed in the co-infected group, and of particular interest was the combination of the activation marker CD137, PD-1 and Fas/FasL. The enhanced expression of markers associated with activation (CD137), apoptosis (Fas/FasL) and dysfunction or non-responsiveness (PD-1) may explain why TB co-infection leads to rapid disease progression, and often death. Activation would drive viral replication, apoptosis would enhance cell loss (both CTL and other) and lack of responsiveness would prevent immune control.

### 5.1. Expression of the novel activation marker CD137 (4-1BB)

Activation status was characterized in this study by the expression of CD137 (4-1BB), which is a CD8-specific costimulatory molecule that is expressed on activated T cells [270]. CD137 is a member of the TNF receptor family, and the addition of anti-CD137 monoclonal antibodies suppressed the antiviral immune response during acute infection of lymphocytic choriomeningitis virus (LCMV) Armstrong – or A/PR8/34 influenza – in
mice [271]. These findings highlight the importance of this marker in antiviral CTL activation and responsiveness. Ligation of CD137 may induce cell division and cytokine secretion and might play a significant role in the survival of activated CTLs late in their response, as shown by Bertram and colleagues assessing primary and secondary influenza infection in mice [272]. This marker has been used to define antigen-responsive cells, in a study comparing it with other markers of activation [273]. In preliminary experiments for the current study we compared CD137 with CD25 and CD69 in normal donors following stimulation, and showed that although absolute levels of induced CD137 were lower than the other markers, there was an antigen-specific increase in expression following stimulation and expression of CD137 was correlated with the other 2 markers.

The expression of CD137 was found to correlate with viral load and not CD4 count in our study (table 4 and 5). No studies to date have used CD137 to characterize HIV-specific antigen-primed CTLs, but a study in Uganda showed that patients with HIV with/out TB that were not on ARTs showed that HIV cohorts with TB co-infection expressed the highest level of the activation markers HLA-DR and CD38 [269]. The results from the current study indicate that baseline CD137 was similar in the uninfected and HIV+ groups, but elevated in the co-infected individuals. On stimulation with gag peptides however the HIV+ group showed stronger increased expression than the co-infected group. This seems to indicate higher in vivo activation in TB co-infection, but less inducible activation in vitro. A statistical significant difference in expression in CD137 between HIV infected, and dual infected groups – indicates that this marker may be useful as a marker of enhanced immune activation in co-infection. A more in depth analysis of expression of CD137 vs. expression of classical markers of activation in patients is still required. It is important to convey that CD137 is not considered a better marker than regular activation markers like CD25 and CD38, but it may be beneficial in screening for activation status as it is an early activation marker. As with the classical activation markers CD25 and CD69 however, ex vivo expression levels appear to be generally low.
5.2. Expression of Fas (CD95) and FasL

Fas is a member of the TNF receptor family and is expressed on the surface of T cells and other cell types. When Fas on target cells bind to Fas ligand (FasL) a signaling cascade is initiated which leads to apoptotic cell death [274]. The Ugandan study mentioned above showed that HIV+ cohorts with and without TB had heightened Fas expression. Their research showed the highest baseline expression of Fas came from patients with HIV+TB+ dual infection. Our study appears to contradict these findings as our HIV+TB- group had the highest background expression of Fas receptor, however this was not statistically significant, and both the infected groups had elevated Fas compared to uninfected controls. There was a moderate increase in Fas expression following Gag stimulation in the two HIV+ groups, and as with CD137 the greater response was in the absence of co-infection, with the HIV+TB- cohorts displaying almost twice as high a response as the TB+ group.

The differences between the Uganda study and the current study may be attributed to different methodologies and cohorts. Hertoghe and colleagues worked on fresh whole blood as opposed to cryo-preserved PBMCs. The cryo-preservation may have lowered the responsiveness of the CD8+ T cells. The markers used by these researchers (viz. CD3+CD8+CD95+) were more specific than the ones in our study (CD8+CD95+). The cohort size of the HIV+TB+ volunteers in Uganda was almost twice that of our TB+ cohorts. The mean CD4 counts for the HIV+ group were almost 200 cells/µl higher than ours, and their TB+ cohorts had over 100 cells/µl than our corresponding cohorts.

Fas ligand (FasL) is a membrane protein that, like Fas is a member of the TNF family of proteins and is expressed on activated T cells. The binding of this ligand with its cognate receptor Fas, results in the initiation of apoptosis within that target cell [274]. Dyrhol-Riise [276] et al. showed that HIV-1 infected patients in Norway had a mean baseline FasL expression of 18.1% ± 10.5 before starting HAART. The baseline FasL levels reported in our study did not mirror the results of the Norwegian study and expression levels of our cohorts were much lower (Healthy: 0.9127; HIV+TB-: 3.430;
HIV+TB+: 7.281). Dyrhol-Riise et al. performed their study on fresh tonsillar mononuclear cells. There were also differences in the cohorts chosen for that study. The number of HIV+ individuals in the Norwegian investigation was less than half of the study described here. In terms of mean CD4 counts, the study presented here had 178.8 cells/µl lower than the Norwegian cohorts. The log viral load value for their study was 0.85 lower than our HIV+ group.

FasL expression is dependent on immune activation, and different levels of expression are probably related to immune activation status. Interestingly baseline FasL expression in our study was higher than Fas expression, and there was a trend of increased FasL from uninfected, to HIV+ to HIV+TB+ groups. As with Fas the Gag stimulation led to a better induction in the HIV+TB- group compared to the TB+: group. These findings may also be linked to expression of PD-1, with the TB co-infected group being least responsive to antigen-stimulation due to highest baseline PD-1 expression (>13% in the co-infected group), and an associated reduced capacity to respond to antigen stimulation. A Spearman nonparametric correlation was done on FasL and PD-1 expression on the TB+ cohorts and the correlation a shown to be significant, p=0.0333, which would appear to confirm this hypothesis.

5.3. Expression of Programmed Death-1 (PD-1; CD279)

The PD-1 cell surface receptor belongs to the CD28 family. It is inducibly expressed on activated T cells, NK T cells, B cells and myeloid cells [276–279]. Its cognate ligands PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273), are expressed on non-/professional antigen presenting cells and they are also present on non-hematopoietic cells [280, 281]. Binding of PD-1 to either of its aforementioned ligands, induces an immunosuppressive pathway [280 – 282]. Researchers have shown that PD-1 ligand expression on APCs may switch off autoreactive T cells and induce peripheral tolerance. Expression of these ligands on virus-infected cells may suppress functional effector T cells and diminish or block the destruction of virus-infected cells [283, 284]. Studies indicate that the PD-1/PD-L pathway can mediate exhaustion of virus-specific CD8+ T cells during chronic infection as shown in the murine model using lymphocytic
choriomeningitis virus (LCMV) as the infectious agent [120] and HIV-infected humans [2, 4, 3]. These studies indicate that PD-1 expression correlates with HIV-specific CD8+ T cell exhaustion and that PD-1 expression may be driven by high HIV antigen levels.

A study in Beijing, China that recruited 26 typical progressors of HIV infection showed that HIV-specific CD8+ T cells expressed mean positive values of 45% ± 16.6% for PD-1 expression [122]. Our values of PD-1 expression were considerably lower and didn’t directly correspond to the values quoted in this study. Zhang et al., utilized fresh PBMCs and used one more marker to define PD-1 expression compared to our study viz. CD3+CD8+PD-1+. The cohorts in their investigation had an almost 100 fold lower viral load than those presented here.

A study conducted in Kwazulu Natal (KZN) showed HIV-infected patients having a median of nearly 40% of CD8+PD-1+ cells [2]. Our HIV+ cohorts showed baseline levels HIV+TB-: 7.319% and HIV+TB+: 16.74%. These values were still markedly higher than baseline healthy levels of expression. The HIV+TB+ group had a markedly higher response to Gag than the HIV+TB- cohorts (HIV+TB-: 7.521%; HIV+TB+: 21.18). Day et al. also performed their experiments on fresh PBMCs and had a sample size of over 500. Their cohorts had almost two times the median viral load as our HIV+ group and a median CD4 count of 178cells/µl higher than our volunteers.

PD-1 expression was negatively correlated to CD4 counts and positively correlated to viral load, indicating that with increase in viral antigen exposure there was an increase in PD-1 expression on CD8 T cells. These correlations between PD-1 and viral load as well as CD4 counts were also found in the KZN study [2]. Differences in PD-1 expression in chronically infected individuals may not only be related to CD4 count and viral load but also immune activation. The fact that antigen-stimulation resulted in even higher PD-1 expression in the TB co-infected group together with findings that antigen-stimulation showed greater reduction in expression of other CTL markers, points to increased dysfunction in this group.
5.4. Perforin and Granzyme A

Perforin plays an important role in the cytolytic function of CTLs. After a CTL recognizes a target cell through the interaction between its TCR and a corresponding antigenic peptide that is bound to the MHC-I complex, lytic granules (one of which being perforin) inside the CTL migrate towards the CTL-target cell contact site and fuse with the plasma membrane. Degranulation then occurs and perforin inserts into the target membrane and polymerizes to form pores. This gives way to the entry of proteases and granzymes from CTLs to the target cell, initiating an apoptotic cascade [285, 146].

Harari and colleagues [286] performed a study in 2009 on HIV-1 infected individuals, which yielded results of baseline perforin expression of 10%, much higher than the baseline expression in our HIV+TB- cohorts of 2.491%. In our study, the HIV+TB+ group had the highest expression (6.272%) suggesting that co-infection is linked to increased perforin expression. This may be due to increased viral load or increased immune activation, or both. Harari et al. utilized more specific markers (i.e. MHC I tetramer CD8+Perforin+) to demonstrate CTL Perforin expression. Their cohorts had a higher mean CD4 count (>550 cells/µl) and a lower mean log viral load (1.06 more than our study).

A study in 2007 based on 13 HIV infected individuals pre-ART in Chandigarh, India showed that upon Gag stimulation, CD8+T cells of their cohorts produced total mean percentage expression values of 41.52 ±7.7 [287]. Our research revealed a much lower mean percentage positive response to Gag peptides. Pallikkuth et al. had utilized fresh PBMCs for their studies but had a lower mean CD4 count (120 cells/µl lower) in comparison to ours. Their stimulation conditions were also different to ours, we had incubated gag pool peptides for 18 hours at a final concentration of 0.45µg/ml whereas the Indian study used gag p24 antigen for 48 hours at a concentration of 4µg/ml.

Antigen-stimulated CTLs release granzymes to induce apoptosis in the target cell. Human granzymes include granzymes A, B, H, K and M. Granzyme A induces
apoptosis in most cell types in approximately 24 hours [288]. A number of studies have examined GzB expression in CTL in HIV [289] and also compared expression patterns of the cytolytic enzymes granzyme A, granzyme B, and perforin. Differences in GzA and GzB expression have been reported by Chattopadhyay and colleagues [289]. Their study was carried out on HIV-infected patients in the USA, what they found was that CD4+ and CD8+ T cells frequently expressed GzA alone and that GzB was never expressed without GzA. Chattopadhyay et al. performed their study on rectal lymphocytes from 16 HIV+ individuals, 4 of whom were on ART. Their cohorts had a higher mean CD4 count (>200 cells/µl) and a lower mean viral load as our HIV+ individuals had >200 000 copies/ml than their volunteers.

Harari et al. also examined GzA production in their study and reported background GzA levels of 80% in HIV-infected patients’ CTLs [286]. The baseline levels reported in our HIV+TB- groups was lower than their patients (GzA mean of 60.95%). Researchers in San Francisco California, USA doing research on 12 HIV-1 positive individuals not on ART observed CD8+ T cells expressed granzyme A levels of 45% in response to a Gag SL9- epitope [290]. Our study indicated that Gag stimulation reduced GzA expression in the HIV+TB- group, but not the co-infected group. This lowering of the functional response may be due to an impairment of Gag peptide recognition brought on by TB coinfection or conversely Gag stimulation brought about increased degranulation in the HIV group alone, thus lowering absolute levels. This latter argument seems to be confirmed by the degranulation data, which shows reduced expression of CD107 in the TB co-infected group following stimulation with gag. Both HIV+ groups had markedly higher baseline levels of granzymes A than the healthy volunteers. Harari et al. also looked at baseline dual expression of perforin and GzA in HIV-infected patients, and reported very low expression. Our study concurred with their findings.
5.5. Measurement of Degranulation (CD107a/b)

When CD8+ T cells are stimulated to produce proteases and granzymes, lytic granules migrate towards the CTL target cell interface and degranulate or release the contents of their lytic granules into the target cell. Glycoproteins CD107a, CD107b and CD63 are located in the granule-membrane inner surface and are exposed onto the lymphocyte surface through degranulation [140 – 142]. Baseline levels of CD107a/b were higher in the HIV groups than in healthy controls. The HIV+TB+ group had over 4 times the baseline level of HIV+TB- group, which appears to indicate increased active immune function in this group (a finding mirrored in the baseline IFN-γ response discussed below). Gag stimulation led to an increase in CD107 expression in the HIV group (0.6100%), but a ten-fold diminished response in TB co-infection. McElroy and colleagues performed a study in Uganda on gag peptide responses on CD8+ T cells in HIV-infected patients and they reported a median antigen-induced expression of 1.10% above baseline [291]. Another study performed in California, reported median values above background in response to the same stimulus of approximately 0.6% [292]. This last study compares well with our data observed in HIV+TB- cohorts. TB co-infection therefore diminishes HIV-specific degranulation. This also confirms that the increase in GzA in the co-infected group may indeed be due to inhibition of degranulation, and also emphasizes the trend of reduced responsiveness linked to increased PD-1 expression.

5.6. Cytokine production - Interferon-gamma (IFN-γ) and Tumour necrosis factor – alpha (TNF-α) Production

IFN-γ has antiviral-associated properties, and is an important activator of macrophages. It has critical roles in the innate and adaptive immune response. Expression of IFN-γ stimulates the expression of MHC class I and II molecules as well as costimulator marker expression on antigen presenting cells [293]. Baseline levels for IFN-γ were high for healthy cohorts (1.617%) compared to other studies. A study performed in Colombia, South America for example showed that healthy donors exhibited mean CD8+IFN-γ+ expression of 0.033% [294] and a study in Japan reported a healthy baseline level of
0.39±0.3% [295]. The 10-fold variance across these studies is indicative of the general problem of standardizing flow cytometric based readouts across laboratories. The current study showed approximately 4 times higher baseline than the Japanese cohort. This may have been a result of the freezing-thawing process as evidenced in a lymphocyte viability of approximately 70%, which could result in membrane damage or loss of membrane integrity. This loss of viability through freeze thawing appears to be related to the late stage of HIV infection in this cohort and the concomitant low CD4 counts. The absence of CD3 marker in the current study panel may also have contributed to a higher baseline, as CD8+ NK cells or other non-T lymphocytes may have inadvertently been included.

The current study did not screen patients for other co-infections besides TB. It is possible that HIV+ patients with low CD4 counts (as were many in this study), had active infections which could prime a baseline immune responsiveness evidenced by high IFN-γ positivity. The HIV+TB- IFN-γ baseline levels were lower than that of the healthy group, but no statistically significant differences were observed. The HIV+TB+ group had a distinctly elevated baseline level of 4.387% but upon HIV Gag peptide stimulation the response was reduced compared to the HIV group. A study carried out by an Italian group showed results of IFN-γ response to gp160 Env peptides in ARV untreated individuals had a CD8+ T cell mean difference between stimulated and unstimulated percentage expression of 1.71 ± 0.06 [296]. Our HIV+TB- study cohorts had a slightly lower yet comparable response to HIV gag peptides (1.610%). This lowering of the IFN-γ response in the presence of TB, once again indicates the dysfunction of the immune response with co-infection, as observed with other markers.

TNF-α functions as part of a network of cytokines that is engaged in the regulation of the immune system [297]. TNF-α is produced by macrophages and monocytes in response to infections and results in an increase in macrophage and CTL potential [298, 299]. Our study revealed high baseline levels of TNF-α but more so in the TB+ group (HIV+TB-: 6.186%; HIV+TB+: 10.78%). The largest mean difference between stimulated and unstimulated Gag peptide-induced response was elicited in the co-infected group.
(1.300% vs. 0.3720%). The Italian study mentioned above showed that their cohorts expressed a mean percentage of 0.75±0.22 in response to HIV peptides [296]. TNF-α is thus different from IFN-γ in that TB co-infection was characterized by higher baseline TNF-α positivity in CTL, and also enhanced production after HIV antigen stimulation. This may not however be indicative of a more protective response, as secreted TNF-α is also implicated in bystander cell death and immune dysfunction [68-71]. In addition, TNF-α promotes HIV replication [68-71], and higher levels of this cytokine in TB co-infection could be a driver of increased viraemia as well as cell death via apoptosis. Both these pathogenic processes have been implicated in worsening prognosis in HIV infection.

5.7. Limitations of this study

There were several hindering factors in this study, the first was sample size. The sample size of our cohorts was small, largely due to low frequency of patients not on HAART and also clearly defined subsets of patients with HIV infection alone or co-infected. The implication of this small sample size is the statistical insignificance of many of the results, which would be alleviated with a greater sample size. This was a cross sectional study and as such we could not measure the patients response over time. A longitudinal study was not possible as patient recruitment and follow up was a problem at the clinics, and also most patients began ARV therapy after their initial visits. Absolute CD8 counts weren’t assessed in his study, so the correlation between CD8+ T cell number and functional markers or between CD8 T cells, viral load and CD4 count could not be determined. A number of HIV studies have utilized more than one antigen to assess HIV-specific CD8+ T cell response, (e.g. Nef, Pol, Env and Gag or combinations of all) whereas financial constraints in our study allowed the use of a single antigen. The problem here is that there may be a broader response to a combination of HIV antigens depending on CTL recognition of HIV determinants. There were concerns over fresh versus frozen samples as preliminary data (data not shown) indicated that fresh samples had lower expression of activation markers in healthy individuals. The flow cytometer used in this study was only a four colour instrument, so
there were many restrictions on staining combinations and analysis of co-expression of certain markers was limited.

5.8. Summary of findings and future questions

In summary, TB co-infection led to higher baseline level expression of numerous markers. TB co-infection also seems to have inhibited antigen-specific responsiveness, which is reflected in the higher PD-1 expression observed in this group as well. Differences in levels of marker expression in both healthy and infected cohorts in this study as compared to other studies may be due to the staging of the disease, with many patients being in the untreated, late stage sub-group.

In this study we have demonstrated the active TB co-infection results in changes to not only standard measures of HIV disease progression (CD4 count and viral load), but also changes in both baseline expression and HIV-antigen induced expression of a range of phenotypic and functional markers in CD8+ CTL. TB co-infection led to elevated baseline expression of all markers examined, which may be associated with lower CD4 count, or heightened immune activation, or both. The trend was not observed following antigen stimulation, as there was diminished CD137, Fas, FasL, IFN-γ and CD107 expression in the TB co-infected group. The increased perforin and GzA expression in the co-infected group which appears to indicate a “better” response may be due to the decreased degranulation, which would confirm inhibition of responsiveness. The reduction in responsiveness to antigen was inversely related to the PD-1 expression patterns. The only marker linked with CTL “quality” that showed enhanced antigen-specific expression in the co-infected group was TNF-α, and this itself may not necessarily be protective.
CHAPTER 6: CONCLUSION

Researchers have shown that as HIV progresses there is associated CTL dysfunction and depletion, and it is these events that precede development of AIDS [266]. This is partly due to overstimulation by viral antigen and appears to be exacerbated by TB co-infection. Our results reinforce this statement as HIV+TB+ cohorts had the highest expressions of activation markers, and the highest expression of a T cell dysfunction marker (PD-1). It could also be seen that the TB+ cohorts had almost three times the viral load of the HIV+TB- group. TNF-α production was high in the HIV+ groups, and this is in agreement with previous studies that suggest TNF-α secretion plays a role in HIV disease progression [68-71]. With regard to IFN-γ and CD107a/b production the lower levels of expression in HIV+TB+ individuals compared to that of HIV+TB- patients is indicative of immune dysfunction, as both IFN-γ and degranulation are associated with functional and beneficial CTL response in HIV. From the IFN-γ and CD107 data, it can be seen that if these two functional responses are impaired in HIV+TB+, enhancing them (or reducing/inhibiting PD-1) may assist in promoting a better disease outcome. Monitoring of the expression of these markers, may be useful in evaluating responsiveness to both HIV therapy and/or TB therapy.

The data gathered from this study has implications on disease outcome for patients with both HIV and TB. Longitudinal studies are needed to assess the impact that ARV treatment has on expression of these markers. A more comprehensive analysis of CTL activation and inclusion of markers defining cell subsets (i.e. effector, memory etc) would aid in clarifying the findings in further studies. A larger scale study is needed to truly assess consensus marker expression in the co-infection setting in response to multiple HIV epitopes, as well as following up these markers in typical progressors, LTNPs and elite controllers to determine which functional components are most important.
The thrust for more extensive basic research prior to vaccine development necessitates further studies into HIV-TB co-infection in South Africa, as the models provided in HIV infection alone in a first-world setting may not be adequate when addressing both diseases together.
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