THE MEASUREMENTS OF APOPTOSIS IN HIV-1 INFECTION

By

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DECLARATION

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

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SUMMARY

Acquired immunodeficiency syndrome (AIDS) was first reported in 5 homosexual men in Unite States of America in 1981 as a series of opportunistic infections which occasionally occurred in adults. Subsequently, it has been achieved that human immunodeficiency virus type 1 (HIV-1) is the cause of AIDS and this aetiological agent has spread all over the world. The virus primarily attacks CD4+ T cells and gradually leads to progressive depletion of CD4 T lymphocytes from peripheral blood and lymphoid organs. Since CD4+ T cells are vital immune cells in induction and regulation of both cell-mediated and humoral immune responses, depletion of these cells ultimately results in a profound immunodeficiency characterized by susceptibility to variety of opportunistic infection.

Apoptosis have been commonly proposed as the mechanism of CD4 depletion because elevated levels of apoptosis were observed in HIV-1 infected individuals (Ameisen et al., 1991; Groux et al., 1992 & Oyaizu et al., 1993). Nevertheless, there was evidence showing that HIV-1 infected cells died not from apoptosis (Bolton et al., 2002) and another study reported that inhibition of apoptosis resulted in high viral production (Antoni et al., 1995). These controversial views indicated that the mechanism of CD4 depletion and the immuno-pathogenesis of apoptosis should be considered.

As a pilot sub-study, eight HIV-1 infected subjects were enrolled to determine the methods in measuring apoptosis. Three different cell separations: (1) whole blood cells, (2) buffy coat cells and (3) isolated PBMCs were prepared to determine whether different cell preparations result in different measurements of apoptosis. In addition, FITC-labelled Annexin V, an early marker of apoptosis, and flow-cytometer based scatter methods based on characteristics of apoptotic cells were used to investigate the difference in analytical methods in determining the levels of apoptosis. Firstly, it was found that whole blood samples yielded more precise measurements in measuring

apoptosis, followed by Buffy coat and then PBMC samples. Secondly, this sub-study also indicated that the scatter method as well as fluorenscent labelled Annexin V could be useful markers for apoptosis.

Secondly, different surface markers of apoptosis were used to investigate apoptosis in HIV-1 infected adults. Fifty-eight HIV-1 infected adults were involved in this sub-study. They were classified into three categories based on CDC CD4 category classification (CDC, 1993). According to the data, the level of apoptotic CD4+ T cells measured by the scatter method was high in CD4 category 1, decreased in category 2 and finally increased again in category 3. This tendency was in parallel with CD95 (Fas) expression on CD4+ T cells. The curve formed a "V" shape according to the three CD4 categories. Together with the gradually increased plasma viral load, these data reflect an activated immune response at early stage of infection and under controlled viraemia. This possibly represents the immune response trying to eliminate infected cells as a means of survival. The high level of apoptosis in category 3 could indicate a disordered immune system accounting for the rapid loss of CD4+ T cells and progression to AIDS.
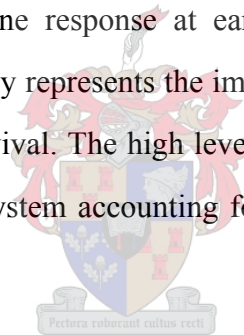
A novel finding of this study was the presence of two CD4+ populations in 10 HIV-1 infected subjects, which were CD4^{dim} and CD4^{bright}. These 10 subjects had relatively high CD4 count and low viral replication. Statistical analysis showed they had significantly higher levels of apoptosis in CD4 and CD8 T lymphocytes, measured by the scatter method, than those subjects presenting single CD4 population. In addition, when comparing the two CD4 subpopulations, it was found that CD4^{dim} cells had significant higher level of apoptosis and CD95 expression than the CD4^{bright} cells.

Finally, the virological and immunological effects of antiretroviral therapy (ART) were investigated in two cohorts of HIV-1 infected children. Fourteen HIV-1 infected children were involved in investigation of 12-month long-term effect, while another five children were involved in a short-term 1-month follow-up study. In addition, a

different assay of detecting apoptosis: terminal deoxynucleotidyltransferase deoxyuridine triphosphates nick end labeling (TUNEL) was conducted to measure the level of apoptotic PBMCs. According to the findings from 12-month and 1-month sub-studies, it appeared that ART could be effective in suppression of viral replication at an early stage. However, the immunological effect, such as CD4 reconstitution, could only be seen as a long-term effect, since immune recovery would take a long time. In addition, different regimens containing protease inhibitors (PIs) might be more effective in inhibiting apoptosis than non-nucleoside reverse transcriptase inhibitors (NNRTIs).
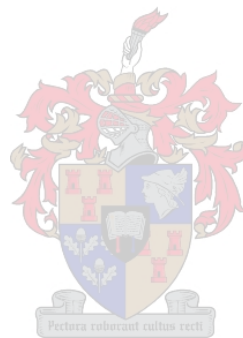
OPSOMMING

Verworwe immuun gebrek sindroom (VIGS) was vir die eerste keer waargeneem in 1981 waar dit voorgekom het in 5 homoseksuele mans in die Verenigde State van Amerika as 'n reeks oppertunistiese infeksies wat by wyle voorgekom het in volwassenes. Die menslike immuungebrek virus tipe 1 (MIV-1) is die oorsaak van VIGS en het vêrreikende gevolge reg oor die wêreld. Die virus val primêr CD4+ T selle aan en veroorsaak geleidelike vermindering van CD4 T limfosiete in perifere bloed en limfoïede organe. Vermindering van CD4+ T selle lei tot grootskaalse immuun gebrek wat gekaraktiseer word deur 'n verskeidenheid oppertunistiese infeksies, aangesien die selle 'n essensiële rol speel in die induksie en regulering van die sellulêre- en humorale immuun sisteme.

Apoptose word algemeen voorgestel as die meganisme van CD4 T sel vermindering, aangesien verhoogde vlakke van apoptose waargeneem was in MIV-1 geïnfekteerde individue (Ameisen et al., 1991; Groux et al., 1992 & Oyaizu et al., 1993). Daar bestaan wel teenstrydige resultate wat aandui dat MIV-1 geïnfekteerde selle nie as gevolg van apoptose sterf nie (Bolton *et al.*, 2002), asook 'n studie wat getoon het dat inhibisie van apoptose verhoogde virale produksie veroorsaak het (Antoni *et al.*, 1995). Hierdie kontroversiële bevindings dui aan dat die meganisme van CD4 T sel vermindering en die immuun-patogenese van apoptose sterk nagesien moet word.

Ag MIV-1 geïnfekteerde pasiënte is betrek by 'n voorloper subproef om die verskillende metodes van apoptose meting te bepaal. Drie verskillende selskeidings, naamlik volbloed selle, "buffy" laag selle en geïsoleerde perifere bloed mononuklêre selle (PBMS), was gebruik om te bepaal of verskillende selvoorbereidings 'n verskil in apoptose bepaling tot gevolg sou gehad het. Addisioneel is FITC gemerkte Annexin V, 'n vroeë merker van apoptose, en vloei sitometrie gebaseerde verstrooings metodes gebruik om die verskil te bepaal in apoptose bepaling tussen die verskillende analitiese metodes. Eerstens was daar vasgestel dat volbloed monsters

beter resultate gelewer het in die bepaling van apoptose, gevolg deur die "buffy" laag metode en dan PBMS monsters. Tweedens het die substudie aangedui dat die verstrooiings metode asook fluoresensie gemerkte Annexin V 'n bruikbare merker van apoptose mag wees.

Verskillende oppervlak merkers van apoptose was gebruik in die studie om apoptose te bestudeer in MIV-1 geïnfekteerde volwassenes. Ag en vyftig MIV-1 geïnfekteerde volwassenes was betrokke by die substudie. Hulle was geklassifiseer in drie kategorieë op grond van CDC CD4 kategorie klassifikasie (CDC, 1993). Die resultate van die studie het aangedui dat die vlakke van apoptotiese CD4+ T selle gemeet deur die verstrooiings metode was hoog in CD4 kategorie 1, laer in kategorie 2 en weer hoog in kategorie 3. Die tendens was in parallel met CD95 (Fas) uitdrukking op CD4+ T selle. Die kurwe van die drie kategorieë het die vorm van 'n 'V' aangeneem. Die resultate van die studie, te same met die die geleidelike verhoging in plasma virale lading, reflekteer 'n geaktiveerde immuun reaksie gedurende vroeë infeksie en onder die beheer van virumie. Dit dui moontlik 'n immuun reaksie aan wat poog om geïnfekteerde selle te verwyder ter wille van oorlewing. Die verhoogde vlakke van apoptose in kategorie 3 mag 'n verwarde immuun sisteem aandui wat probeer opmaak vir die vinnige verlies aan CD4+ T selle en die progressie na VIGS.

Die teenwoordigheid van dubbel CD4+ populasies in 10 van die MIV geïnfekteerde pasiënte was 'n nuwe bevinding van die studie en die populasies staan bekend as CD4^{dof} en CD4^{helder}. Hierdie 10 pasiënte het 'n relatiewe hoë CD4 telling gehad en lae virale replikasie. Statistiese analiese van die verstrooiings metode het gewys dat daar hoër vlakke van apoptose was in CD4 en CD8 T limfosiete van die groep, as wat daar was in pasiënte met 'n enkele CD4 populasie. Daar is verder bevind dat daar beduidende hoër vlakke van apoptose en CD95 uitdrukking teenwoordig was in die CD4^{dof} populasie as in die CD4^{helder} populasie.

Die virologiese en immunologiese uitwerking van antiretrovirale terapie (ART) was ondersoek in twee kohorte van MIV geïnfekteerde kinders. Veertien MIV geïnfekteerde kinders was betrokke by die ondersoek wat gestrek het oor 12 maande en gekyk het na langtermyn effekte, terwyl nog 5 kinders betrokke was in 'n kort termyn studie met opvolging daarvan oor 1 maand. 'n Ander toets vir apoptose, naamlik terminale deoksienukleotidieltransferase deoksiëuridien trifosfaat keep-einde klassifikasie (TUKEK), was gebruik om die vlakke van apoptotiese PBMSe te meet. Die bevindings van die 12 maande en 1 maand substudies het aangedui dat ART vrale replikasie effektief kan onderdruk op 'n vroeë stadium. Die immunologiese effek, soos CD4 herkonstitusie, kan egter net gesien word as 'n lang termyn effek, aangesien immuun herstelling 'n lang tyd sal neem. Verder kan daar gesê word dat verskillende behandelings wat protease inhibitore (PIe) bevat moontlik meer effektief sal wees om apoptose te onderdruk as nie-nukleosied omgekeerde transkriptase inhibitore (NNOTIe).
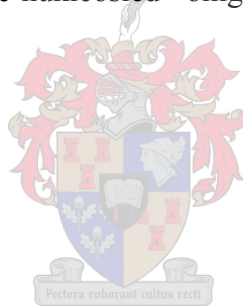
ABBREVIATIONS

$3^{'}$-OH: $3^{'}$-hydrocyl

AIDS: acquired immunodeficiency syndrome

ANOVA: analysis of variance

APCs: antigen presenting cells

ART: antiretroviral therapy

ARV: antiretroviral

CCR5: CC-chemokine receptor 5

CXCR4: CXC-chemokine receptor 4

CDC: Centres for Disease Control and Prevention

CSFs: colony stimulating factors

CTL: Cytototic T Lymphocyte

DNA: deoxyribonucleic acid

dUTP: deoxyuridine triphosphates

EBV: Epstein – Barr virus

EDTA: ethylene diamine tetraacetic acid

FasL: Fas ligand

FDCs: follicular dendritic cells

FSC: Forward Scatter

FITC: fluorescein isothiocyanate

HAART: highly active anti-retroviral therapy

HIV: Human immunodeficiency virus

  HIV-1: Human immunodeficiency virus type 1

  HIV-2: Human immunodeficiency virus type 2

HIV protein

  env: envelope protein

  gag: viral core protein

  nef : negative factor

  rev: the regulator of virion protein

tat: transactivator

vif: virion infectivity factor

vpr: viral protein R

vpu: viral protein U

HSC: hemopoietic stem cell

HTLV: Human T-lymphotropic retrovirus

IDCs: interdigitating cells

IFN: interferon

  IFN-γ: interferon gamma

Ig: immunoglobulin

  IgA: immunoglobulin A

  IgD: immunoglobulin D

  IgE: immunoglobulin E

  IgG: immunoglobulin G

  IgM: immunoglobulin M

IL: interleukin

  IL-2: interleukin 2

  IL-4: interleukin 4

  IL-10: interleukin 10

  IL-12: interleukin 12

LLD: lower limit of detection

mAb: monoclonal antibody

MHC: major histocompatibility complex

  MHC-I: major histocompatiblity complex class I

  MHC-II: major histocompatiblity complex class II

MIP-1α: macrophage inflammatory protein 1α

NK cells: Natural killer cells

NNRTIs: Non-nucleoside reverse transcriptase inhibitors

NRTIs: nucleoside reverse transcriptase inhibitors

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PCD: programmed cell death

PCR: ploymerase chain reaction

PE: phycoerythrin

PerCP: peridinin chlorophyll protein

PI: propidium iodide

PIs: Protease inhibitors

PMNs: polymorphonuclear neutrophils

PS: phosphatidylserine

RANTES: regulation on activation, normal T cell expressed and secreted

RNA: ribonucleic acid

RT: reverse transcription

SIV: simian immunodeficiency virus

SF: syncytium formation

SLE: systemic lupus erythematosus

SSC: side Scatter

TdT: terminal deoxynucleotidyl transferase

TH cells: T helper cells

  TH$_1$ cells: T helper cells type 1

  TH$_2$ cells: T helper cells type 2

TNF: tumour necrosis factor

  TNF-α: tumour necrosis factor alpha

TUNEL: terminal deoxynucleotidyltransferase dUTP nick end labeling

UNAIDS: joint united nations programme on HIV/AIDS

ULQ: upper limit of quantitation

VL: viral load

WHO: World health organisation

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ACKNOWLEDGEMENTS

To my parents and my husband for their endless love and support.
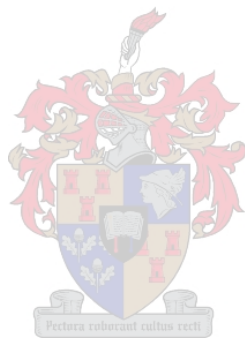
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Basic Anatomy of the Immune System

1.1.1 Lymphoid organs

The immune system is composed of a number of different tissues, organs, and cells. The lymphoid organs are divided into the primary and secondary lymphoid organs. The former includes the thymus and the bone marrow, both are sites where the lymphocytes are generated, differentiate and subsequently mature. On the other hand, the latter (secondary lymphoid organs) such as the spleen and the lymph nodes are where the immune responses of lymphocytes take place.

1.1.1.1 Primary lymphoid organs

The bone marrow becomes the site of haematopoiesis in the last months of foetal development. It gives rise to all the blood elements including the lymphoid cells that migrate as pre-T cells to the thymus for T cell maturation. It is also the site for B cell maturation: pre-B cells are selected and allowed to undergo further differentiation before emerging from the bone marrow as functional B lymphocytes. The selection is based on the phenomenon of tolerance whereby only non-self recognizing cells are selected (In: Instant Notes in Immunology, Ed. Lydyard et al., 2000).

The thymus is active during foetal life and early childhood and undergoes atrophy at puberty. It is full of lymphocytes and accessory cells that are essential for maturation of T cells. A similar process of selection and maturation/differentiation as that to the bone marrow is active in the thymus: all self-reactive T cells are deleted allowing only those recognizing non-self to develop further.

1.1.1.2 Secondary lymphoid organs

The spleen is a highly organized lymphoid tissue with different cell types. The red pulp contains red cells and the periarteriolar lymphatic sheath contains mainly T cells and interdigitating cells (IDCs), while the primary lymphoid follicles are composed predominantly of follicular dendritic cells (FDCs) and B cells. The main immunological function of the spleen is to filter the blood, remove damaged red blood cells and immune complexes from the circulation. Any blood borne antigenic stimulus that arrives in the spleen is processed and subsequently leads to an immune response. This is evident by a splenomegaly upon palpation.

The lymph nodes are spread throughout the body and are found at varying points along the lymphatic system as this drains regions or specific organs (e.g. lungs, digestive system, etc). The lymph nodes are similar in structure to the spleen in that different cell types are located within different regions in the nodes. They are the sites where the immune responses take place against invading microbes originating in the surrounding tissues or drained to the node via the lymph. The clinical indication of an immune response within the regional lymph node is lymphadenopathy, easily palpable by the clinician.

1.1.2 Cells of Immunity

The immune system is usually divided into two systems for ease of study: the innate immune system and adaptive immune system. The innate immune system is present at birth and changes little throughout life. It is characterised by its response time (rapid) but its non-specificity. No immunological memory is generated during an innate response and any subsequent contact with the identical stimulus will require the same time to initiate. The acquired/adaptive immune system on the other hand, is obtained after birth while dealing with the microbial

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infection. It takes longer to develop, but shows specificity and memory. Different cells are involved in the two immune systems.

1.1.2.1 Cells involved in the innate immune system

Phagocytes

Phagocytes are those cells which bind to microorganisms, internalize them and kill them. They are the first line cells of immune system to combat infection. Phagocytes have two major groups of cells - mononuclear phagocytes and polymorphonuclear granulocytes (In: Immunology, Ed. Roitt et al., 2001). Mononuclear phagocytes are long-lived (months or years) phagocytic cells derived from bone marrow stem cells. They are placed where they will encounter pathogens. In blood, they are called monocytes, while in tissue they are macrophages. Their functions are to remove particulate antigen and to present antigenic peptides to T cells. Polymorphonuclear granulocytes play an important role in acute inflammation by phagocytosis and destruction of pathogens. They consist mainly of neutrophils, also known as polymorphonuclear neutrophils (PMNs), which are short-lived (2-3days) phagocytic cells (In: Immunology, Ed. Roitt et al., 2001). Neutrophils have lots of enzymes and antibiotic proteins, such as lysosomes and defensins, which are released during phagocytosis.

Natural killer cells

Natural killer (NK) cells account for up to 15% of blood lymphocytes. They are large granular lymphocytes and contain more cytoplasm than classical lymphocytes. They are produced in the bone marrow and are found throughout the tissues of the body but mainly in the circulation. The absence of CD3, but presence of CD56 and /or CD 16 is currently the most reliable marker of NK cells (In: Immunology, Ed. Roitt et al., 2001). The function of NK cells is to recognize and kill infected cells or transformed cells by

releasing the contents of their granules (perforins and granzymes) upon contact with the cell recognised as transformed or infected. They also can produce certain cytokines, such as interferon gamma (IFN-γ), which is required in the development of adaptive immunity.

Basophils and mast cells

Basophils are found in very small numbers in the circulation, while mast cells locate in connective tissues. The granules in both basophils and mast cells contain heparin, leukotrienes, histamine and eosinophil chemotactic factor of anaphylaxis. When they are activated, they release their granules which increase vascular permeability and develop inflammatory response.

Antigen-presenting cells

Antigen-presenting cells (APC) have a pivotal role in the induction of functional activity of T helper (TH) cells and are seen as the interface between innate and adaptive immune systems.

APCs are found primarily in the skin, lymph nodes, spleen and thymus. IDCs are rich in MHC class II molecules, which are important for presenting antigen to TH cells. Whereas FDCs lack MHC II molecules but bind antigen via complement receptors and these cells present antigen to B cells.

Other cells such as eosinophils, platelets and erythrocytes are also part of the blood elements and may act in the immune system indirectly by the secretion of cytokines, etc. These cells types will not be discussed within this dissertation.

1.1.2.2 Cells involved in the adaptive immune response

T lymphocytes and B lymphocytes are the two types of lymphocytes that provide specificity and memory in the adaptive immune system. They are responsible for the cell-mediated immunity and humoral immunity

respectively. NK cells are another type of lymphocyte, but due to their apparent lack of specificity, function within the innate immune system.

T cells are responsible for cell-mediated immunity and their precursors are derived from hemopoietic stem cells (HSC) in the bone marrow. They differentiate into mature T cells expressing functional receptors within the thymus whereupon, they migrate to secondary lymphoid organs or tissues where they respond to microbial antigens. T lymphocytes consist of two distinct subsets: T helper cells (TH) and Cytotoxic T cells (CTL). In normal peripheral blood of adults, about 55% of total lymphocytes are T helper cells, while 25% are cytotoxic T cells (In: Instant Notes in Immunology, Ed. Lydyard et al., 2000). TH cells are also known as CD4+ T cells because of their expression CD4 on their surfaces. They interact with MHC class II molecules on antigen presenting cells, such as macrophages and dendritic cells. TH cells are divided into two main types: T helper cell type 1 (TH_1) and T helper cell type 2 (TH_2): TH_1 cells are involved in mediating inflammatory immune responses through the activation of macrophages, while TH_2 cells are primarily involved in humoral immunity via activation of B cells. TH_1 cells produce IFN-γ and tumour necrosis factor alpha (TNF-α), whilst TH_2 cells produce interleukin 4 (IL-4) and interleukin 5 (IL-5).

Cytotoxic T cells express CD8 on their surfaces, and are therefore referred to as CD8+ T cells. CD8 binds to MHC class I molecules which present peptides derived from an intracellular microbe, such as a virus. This interaction activates CD8+ T cells and induces killing of the infected cells. This cell killing can be mediated by two pathways. Firstly, the activated CTLs release lytic granules containing perforin and granzymes, such as granzyme B, which is a protease capable of inducing apoptosis of target cells (Heusel et al., 1994). The other pathway is through expression of Fas ligand

(FasL/CD95L) on surface of CTL to interact with Fas (CD95) on target cells, which trigger the cells to undergo apoptosis (Suda et al., 1993 & Lowin et al., 1994).

In human peripheral blood, about 10% of lymphocytes are B cells. They are developed from HSC and mature in bone marrow. B cells produce both cell surface and secreted antibodies, which are coded by multiple genes, against extracellular microbes. Rearrangements of genes generate different B cells with different specificity. Immunoglobulin M (IgM) and immunoglobulin D (IgD) with the same antigen specificity are expressed on mature B cell surface. When they migrate to the secondary lymphoid organs, they respond to foreign antigens by proliferation and development into memory and plasma B cells. However, other classes of immunoglobulins such as immunoglobulin A, G and E (IgA, IgG and IgE) require activation by antigen and involvement of T helper cells (In: Instant Notes in Immunology, Ed. Lydyard et al., 2000).
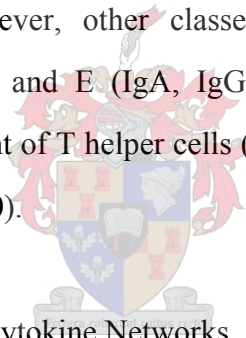
1.1.2.3 Immune regulation: Cytokine Networks

Cytokines are variety of small molecules secreted by different cells. To some extent, they can be classified by the cell populations that produce them, such as interleukins (ILs) are produced primarily by leukocytes, while lymphokines are produced by lymphocytes (In: Cytokines and cytokines receptors, Ed. Hamblin 1993).

Cytokine families include interferon, lymphokines, monokines chemokines and other cytokines, such as colony stimulating factors (CSFs) and tumor necrosis factor. They can induce cell growth, differentiation, activation or enhance cytotoxicity. However, some of them have similar activities, whilst others have antagonistic activities. Both interleukin 4 and 10 (IL-4 and IL-10) produced primarily by TH_2 cells can induce TH_2 and inhibit TH_1 response.

In contrast, interleukin 12 (IL-12) produced by B cells and IFN-γ produced by TH$_1$ and NK cells induce TH$_1$ and inhibit TH$_2$ response (In: Instant Notes in Immunology, Ed. Lydyard et al., 2000). The resulting biological effect is the equilibrium between both positive and negative effects.

1.2 HIV/AIDS pandemic

1.2.1 AIDS recognition

AIDS, in terms of acquired immunodeficiency syndrome, was first recognized in 1981 because of an unusual clustering of diseases which were occasionally observed in young adults, such as *Pneumocystis carinii* pneumonia (Gottlieb et al., 1981 & Masur et al., 1981). As most of the first cases of AIDS involved homosexual men, it was at first suspected that the cause of the disease related to this population. However, as reports showed that AIDS also happened to intravenous drug users, blood transfusion recipients and infants born to AIDS mothers (Curran et al., 1984 & Scott et al., 1984), it is clear that AIDS can be transmitted through sexual contact, exposure to infected blood or from mother to child (In: AIDS: etiology, diagnosis, treatment and prevention, Ed. deVita et al., 1992).

AIDS is defined as the appearance of opportunistic infections, which is life-threatening, at the end of infection with human immunodeficiency virus. It takes about 10-11 years from initial infection to the onset of AIDS (Lemp et al., 1990 & Rutherford et al., 1990). As AIDS is a progressive disease, a classification system for HIV/AIDS has been established by the Centers for Disease Control and Prevention (CDC). It is beneficial for early diagnosis and treatment of the disease. Due to the different spectrum of disease manifestations between adults and children, there are two classification systems, one is for adults (CDC. 1993) and the other is for children younger than 13 years age (CDC. 1994). In addition, as our knowledge of the disease increases and improves, the classification system has

been revised and expanded. In the latest revision for both adults and children, CD4+ T lymphocyte counts or percentages have been integrated with clinical conditions to categorize the immune stages (CDC. 1993 & CDC. 1994). As illustrated in Table1-1, age-specific CD4 count was used in children.

Table 1-1: Paediatric and Adults HIV classification of immune categories.

| Immunologic definitions | children younger than 13 years age | | | | | | Adults | |
| | <12 month | | 1-5 years | | 6-12 years | | | |
	CD4 count(μl)	CD4 %	CD4 count(μl)	CD4 %	CD4 count(μl)	CD4 %	CD4 categories	CD4 count(μl)
No suppression	≥1500	≥25	≥1000	≥25	≥500	≥25	category 1	≥500
Moderate suppression	750-1499	15-24	500-999	15-24	200-499	15-24	category 2	200-499
Severe suppression	<750	<15	<500	<15	<200	<15	category 3	<200
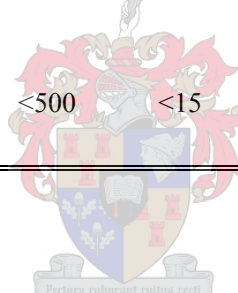
1.2.2 HIV/AIDS worldwide

AIDS has become a great threat and challenge to people all over the world. More than 60 million people have been infected and 20 million have died in less than 25 years. However, the virus has not been fully controlled during the last two decades. Millions of people are suffering from HIV/AIDS, and among them, thousands and thousands of people including children are dying.

In the AIDS epidemic update report of December 2005 by UNAIDS/WHO, the estimated number of people living with HIV/AIDS in 2005 was over 40 million, of which nearly 5 million were newly infected (Table 1-2, UNAIDS/WHO, 2005).

Table 1-2: World estimates of HIV/AIDS epidemics at the end of 2005.

		million
Number of people living with HIV in 2005		
	Total	**40.3**
	Adults	38.0
	Children under 15 years	2.3
People newly infected with HIV in 2005		
	Total	**4.9**
	Adults	4.2
	Children under 15 years	0.7
AIDS death in 2005		
	Total	**3.1**
	Adults	2.6
	Children under 15 years	0.5

The following figure (Fig 1-1) reported by UNAIDS/WHO showed the estimated number of infection in all regions of the world (UNAIDS/WHO, 2005). According to the report, the number of people infected with HIV has increased in all regions except the Caribbean, where no change was recorded over the past two years. The sharpest increase of HIV infection is in Eastern Europe and Central Asia, which is 25% increase from 2003 to 2005 and brings to 1.6 million of people living with HIV. Despite the relatively low prevalence (0.4%) in the large populations of Asian nations, the total number of people infected with HIV is astonishing, such as in India, where over 5 million are infected. Sub-Saharan Africa remains the hardest-affected area in the world by the AIDS epidemic. Approximately two thirds of all people living with HIV are in this region. An estimated 25.8 million people were infected with HIV and 2.4 million people died of HIV-related illnesses by the end of 2005 in this region (UNAIDS/WHO, 2005).

Fig 1-1: Statistics of HIV infection in all regions of the world. (Source: AIDS epidemic update: December 2005. UNAIDS/WHO 2005 Website: www.unaids.org)
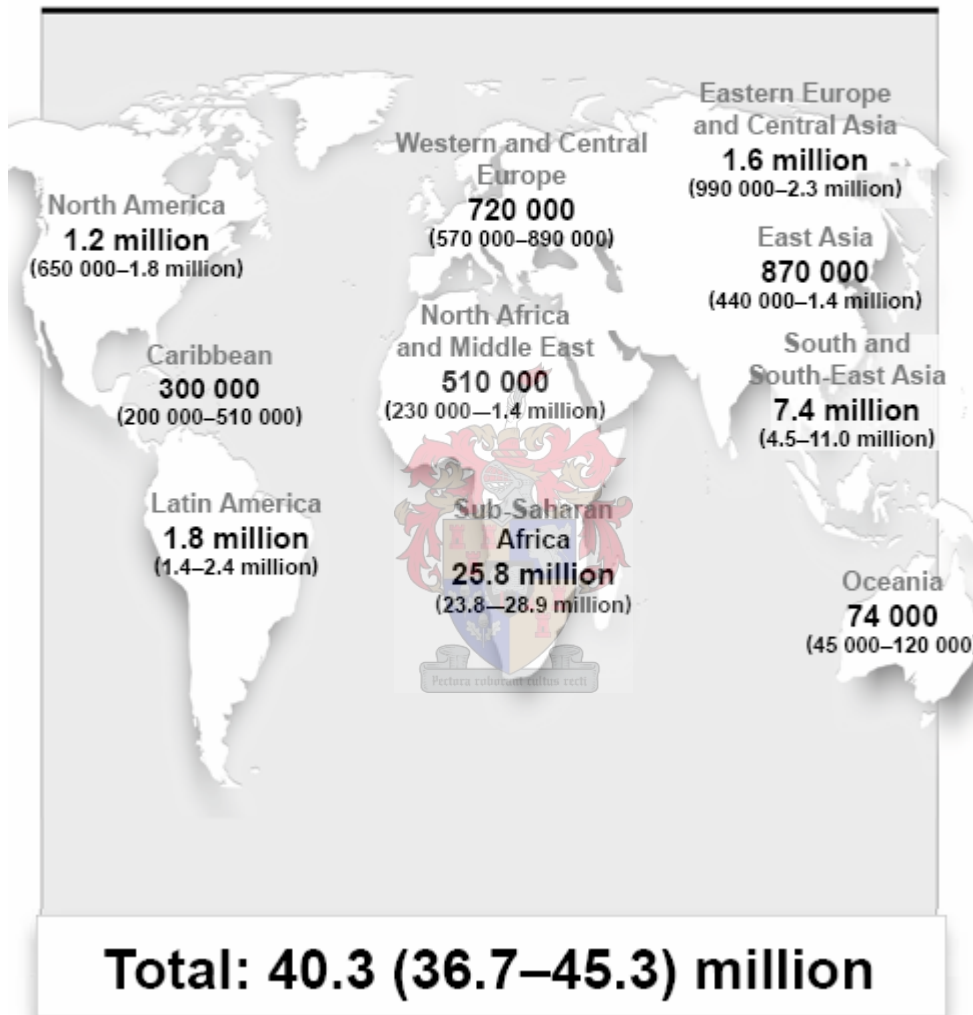
1.2.3 HIV/AIDS in South Africa

AIDS epidemic in South Africa is challenging. According to National HIV and syphilis antenatal seroprevalence survey 2004, which included 16,064 women attending antenatal care, HIV prevalence among pregnant women has reached 29.5% in 2004, which is significantly increased over that of 27.9% observed in 2003 (Department of Health, 2005, Fig 1-2).

Fig 1-2: HIV prevalence among antenatal care attendees in South Africa 1990-2004. (Source: National HIV and syphilis antenatal seroprevalence survey 2004. Department of Health, South Africa 2005)
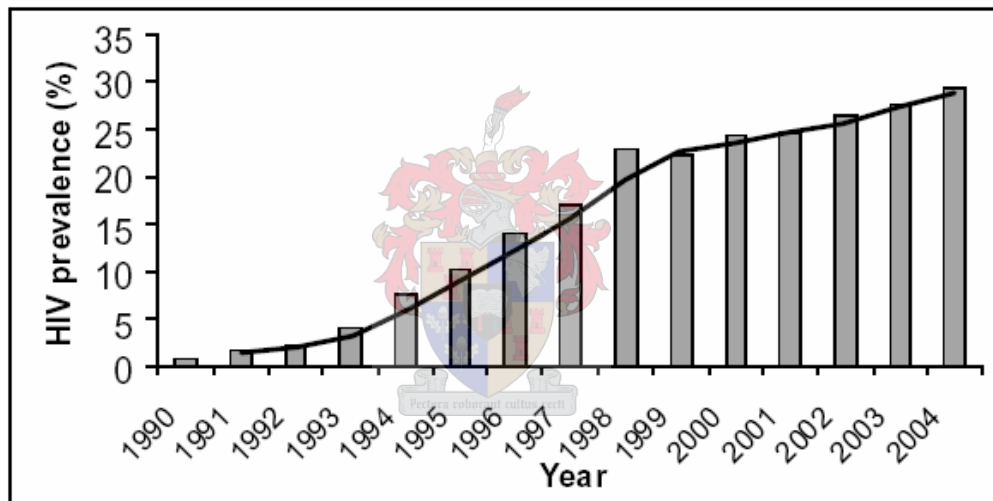
The survey also indicates that the worst-affected province is KwaZulu-Natal, where the prevalence has reached 40% among antenatal clinic attendees, while it has remained between 27% and 31% in the Eastern Cape, Free State, Gauteng, Mpumalanga and North West provinces. Another finding of the survey is that HIV prevalence differs between age groups. About 38.5% of women aged between 25 and 29 years old are infected. Among women younger that 25 or older than 30 years old the prevalence is relatively lower (16.1% to 34.4%). Estimates extrapolated from the prevalence of HIV among antenatal attendees indicate that there were between 5.7million to 6.2 million people are living with HIV in 2004.

Based on mid-year population estimates released by Statistics South Africa, the HIV prevalence rate will reach 9.8% among the total population of adults in 2005 and the highest prevalence is in women aged from 15 to 49 years old, which is 18.1% (Statistics South Africa, 2005a). However, another finding from death notification showed that a shift in the age distribution of mortality has occurred with an increase in the number of deaths among young adults in 1997- 2002 (Statistics South Africa, 2005b, Fig 1-3). The study also indicated that 3.8% of deaths were caused by HIV disease in the 15-49 years aged group in 2001. These finding provide indirect evidences of HIV epidemic in South Africa.

Fig 1-3: Distribution of deaths by age and year of death, 1997-2002. (Source: Mortality and causes of death in South Africa, 1997-2003: Finding from death notification. Statistics South Africa, 2005)
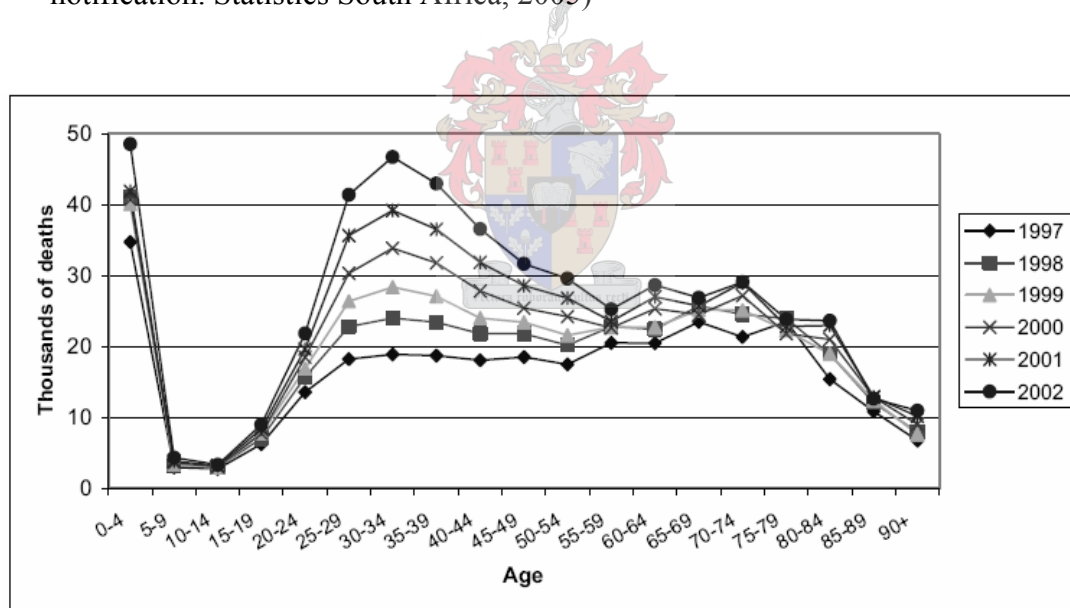
1.3 Introduction of human immunodeficiency virus

1.3.1 Human immunodeficiency virus type 1

One of the earliest known characteristics of AIDS was that T helper lymphocytes (CD4+ cells) decreased in numbers and became functionally impaired in AIDS patients (Ammann et al., 1983). Human T-lymphotropic retrovirus (HTLV) was

the first human retrovirus known to infect T-helper lymphocytes at that time and it was proposed to be the etiological agent of AIDS (Gallo et al., 1983 & Barre-Sinoussi et al., 1983). Soon after, further characterization of the virus revealed that HTLV-III, now termed human immunodeficiency virus type 1 (HIV-1) was the cause of AIDS (Popovic et al., 1984).

The structure of the HIV-1 has been well characterized. The HIV-1 virion is about 100 nm in diameter. The structural proteins of the virus particle are encoded by *gag* (viral core protein, contain p24) and *env* (envelope protein) genes. *Env* protein is translated on the rough endoplasmic reticulum(ER) as a precursor, is cleaved to gp41 and gp120 (Robey et al., 1985; Allan et al., 1985 & Willey et al., 1986).The protein gp120 is critical to the infection, it can be released from the surface of virions to infect target cells, such as CD4 T cells (Moore et al., 1990). It has also been reported that gp120 is responsible for binding to the CD4 receptor (McDougal et al., 1986), whereas gp41 is involved in the fusion process (Kowalski et al., 1991), which is essential for syncytium formation (SF).

Unlike other retroviruses, the HIV-1 genome contains additional genes---*tat* (transactivator), *rev* (the regulator of virion protein) and *nef* (negative factor), which are functional in the regulation of virus replication (Fisher et al., 1986; Chang et al., 1989 & Kestler 1991). In addition, the virus has a few accessory proteins including *Vif* (virion infectivity factor), *Vpr* (viral protein R), *Vpu* (viral protein U): they also play a variety of roles during the infection (Guy et al., 1991; Cohen et al., 1990; Strebel et al., 1988 & Rucker et al., 2004).

The spread of HIV-infection is primarily determined by the life cycle of the virus, which is essential for itself to escape clearance by the immune system. Infection of a target cell begins via an interaction between viral envelope protein (gp120) and cellular CD4 molecule (CD4 receptor) on cell surface. The binding of gp120 and CD4 receptor induces the exposure of a previously hidden domain on viral

envelope protein and results in a second binding to the chemokine co-receptor CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4) (Moore et al., 2004).

After the binding, fusion of the virus and cell membranes occurs to allow virus entry into the cytoplasm, and then the viral RNA is converted to cDNA (complementary DNA) by the reverse transcriptase for which the virus itself encodes. Once the viral DNA integrates into the nucleus, infection is established. Expression starts when viral DNA is transcribed into RNA by the host polymerase II. The viral RNA is processed by splicing and exported to the cytoplasm and translated into viral protein. The virus capsid combines two copies of viral RNA into the newly formed virus while budding through the plasma membrane (In: AIDS: etiology, diagnosis, treatment and prevention, Ed. de Vita et al., 1992).

1.3.2 Human immunodeficiency virus type 2

Human immunodeficiency virus type 2 (HIV-2) was found predominantly in West Africa (Clavel et al., 1986 & 1987). A further study revealed that simian immunodeficiency virus (SIV) in Green monkey was closer to HIV-2 than HIV-1 (Hirsch et al., 1989). HIV-2 appears to be less virulent than HIV-1, but it can also be associated with AIDS or dual infection with HIV-1 (Marlink et al., 1988 & Rayfield et al., 1988).

1.3.3 The immune response to HIV

When the immune system encounters HIV, both innate and adaptive immune systems are activated to defend against the virus. However, as most innate immune responses, such as phagocytosis, are not sufficient, adaptive immune responses including T cell (cell mediated) response and B cell (humoral) response play central roles in controlling the infection. Firstly, CD8+ T cells, referred to cytotoxic T cells are responsible for recognizing and eliminating the virus infected cells. Consequently, it has been reported that HIV-1-specific CTL activity present

in HIV-1 infected patients following primary HIV-1 infection (Borrow et al., 1994). Furthermore, HIV-suppression factors are released by CD8+ T cells as evidence of the immune response to the virus (Cocchi et al., 1995). Secondly, the humoral immune response mediated by B cells is potentially important in controlling viral replication because B cells can produce specific antibodies binding to HIV antigen. It has been reported that in Long-term non-progressors, neutralizing antibodies are detected and the titers were significantly higher than in infected individuals showing progression (Pantaleo et al., 1995).

This body of evidence clarified that the host immune responses, both cell-mediated and humoral, were present during HIV-1 infection. Nevertheless, due to the impaired function of immune cells such as CD8+ T cell and B cells (reviewed later), the immune responses ultimately fail to control HIV-1 replication and lead to disease progression.

1.4 Immunopathology of T cells in HIV/AIDS

The biggest threat for AIDS is the collapse of the host immune system caused by depletion and dysfunction of T lymphocytes. Since HIV-1 envelope protein gp120 has a high affinity with CD4 receptor, T helper cells are involved early in the infection because of expression of CD4 molecules on their surface. Cytotoxic T cells are responsible for killing virus-infected cells. Expansion and activation of CD8+ T cells occur during primary infection, which represents immune responses to suppress the virus replication. However, as the disease progresses, these HIV-1 specific CTLs become exhausted and lose their functions.

1.4.1 Immunopathology of CD4+ T cells

The hallmarker of HIV infection is the progressive depletion of CD4+ T cell. During the acute phase of the infection, a rapid drop of CD4+ T cells occurs, which lasts for a few weeks. Thereafter, the CD4 cell numbers recover but never

to their original level.   Following this phase, during an asymptomatic period of 10 to 11years, the CD4 counts maintain at a relatively stable level or gradually decrease to 500 cells per microliter. In a study of HIV infected Long-term nonprogressors, CD4+ cells counts remain stable for many years with a low viral load and remarkable antiviral responses (Pantaleo et al., 1995). However, as the disease moves to the symptomatic stage, CD4 counts accelerate their decline to less than 200 cells per microliter, and eventually when moving to AIDS, CD4+ cell count drops below 50 cells per microliter (In: AIDS: etiology, diagnosis, treatment and prevention, Ed. de Vita et al., 1992).

Together with the decline in absolute count of CD4+ T lymphocytes, the dysfunction of the cells also occurs throughout the disease. A series of early reports showed that CD4+ T cells lose their proliferative response to antigen, alloantigen and mitogen during HIV infection (Lane et al., 1985 & Clerici et al., 1989). However, Chou et al. suggested that the low proliferative responses to antigens could only be partly due to a decrease of memory CD4+ T cells because they found that memory CD4+ T cells were not selectively decreased in HIV-seropositive subjects (Chou et al., 1994). Fan and his colleagues found altered levels of different cytokines in HIV seropositive individuals, such as IFN-γ and IL-2 (Fan et al., 1993). Moreover, a later study reported that expressions of activation markers HLA-DR and CD38 on CD4+ T cells were significantly higher in HIV infected subjects than in HIV negative controls (Kestens et al., 1994).

1.4.2 Immunopathology of CD8+ T cells

Despite the decline of CD4 + T cells, numbers of CD8+ T cells increase during the primary infection. Correlations between the expansion of CD8+ T cells and decreased viral load support the notion that CTLs play an important role in combating HIV at early infection (Koup et al., 1994; Borrow et al., 1994 & Ogg et al., 1998). Furthermore, CD8+ T cells have been shown to release a variety of chemokines, such as macrophage inflammatory protein 1 alpha (MIP-1α) and

regulation on activation, normal T cell expressed and secreted (RANTES), which suppress virus replication (Cocchi et al., 1995). However, as the disease develops, proliferative capacity of CD8+ T cells is diminished and HIV-specific activity is ultimately lost (Carmichael et al., 1993, & Effros et al., 1996). Similarly in another study, an expansion of CD8+CD28- subpopulation, which has poor proliferative response and no cell division on stimulation, was found (Brinchmann et al., 1994). Another finding in advanced HIV disease is the decrease in naïve CD8+ cell subset (Roederer et al., 1995). The same laboratory also found that naïve CD8+ T cells correlated with total CD4+ T cell counts (Rabin et al., 1995). They suggested that the loss of naïve CD8 cells may contribute to the defects in cell mediated immunity.

1.4.3 Immunopathology of B cell

B cells are responsible for generating specific antibodies against the virus. However, during HIV-infection, this cell population is also indirectly affected by the virus because the maturation and activation of B cells require CD4+ T cells, particularly TH_2 cells, which are main target cells of HIV-1. An early study indicated an increase of immature B cells in the circulation (Rogers et al., 1989). In addition, a new finding suggested that B cells were directly infected by virus by expression chemokine receptor-CXCR4 (de Silva et al., 2001). Furthermore, it has been reported that B cells from HIV-1 infected individuals have significantly higher levels of apoptosis than seronegative controls (Samuelsson et al., 1997). Moreover, in another laboratory, it was found that B cells from HIV-1 patients displayed increased annexin V binding and increased Fas Ligand (FasL) after overnight incubation and they suggested that apoptosis in B cells might be triggered via constimulatory signals delivered by T cells (Lewis et al., 1999). Washmuth et al. also reported than HIV-infected patients had significantly higher numbers of CD95+ cells and Annexin V binding cells in B cells than healthy controls. However, they did not gradually increase with disease progression (Washmuth et al., 2000).

1.4.4 Immunopathology of NK cells

As previously mentioned, NK cells are predominantly functional in innate immunity. They kill infected virus with non-specificity. However, an early study showed that NK activity was significantly reduced in AIDS patients (Ortona et al., 1988). Elevated levels of apoptosis in NK cells as well as in CD4+ and CD8+ T cells were also observed in HIV-infected patients (Washmuth et al., 2000). Recently, it was found that NK cells suppressed HIV replication by secreting CC-chemokines (Kottilil et al., 2003). Furthermore, Mavilio et al. also suggested that expansion of CD56- NK cell subset in HIV viremic individuals contributed to the dysfunction of total NK cells (Mavilio et al., 2005).

1.5 Treatment of HIV/AIDS

1.5.1 Antiretroviral therapy

Although we have not yet found efficient strategies to eliminate HIV-infection or AIDS, antiretroviral therapy (ART) was developed to block or suppress the replication of HIV. There are 3 drug categories which are currently available on the market: the nucleoside reverse transcriptase inhibitors (NRTIs), Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and Protease inhibitors (PIs). NRTIs and NNRTIs can disturb the life cycle of the virus by interfering with the reverse transcriptase enzyme in the replication process of the virus, while PIs interfere with the formation of viable daughter virions in the life cycle of the virus. As viral resistance is an ongoing concern, it is recommended to use a triple-combination of the drugs. Highly active antiretroviral therapy (HAART) is the most widely used regimen in HIV-infected individuals. HAART contains 2 nucleoside analogues and 1 or 2 protease inhibitors. A study from European countries from 1994-1998 showed that HAART could significantly reduce the morbidity and mortality of HIV-1 infection (Mocroft et al., 1998). Another report from the United States also confirmed the benefit of intensive antiretroviral therapy (Palella et al., 1998).

Since HIV infection leads to a depletion of CD4+ T lymphocytes and ultimately results in a collapse of the immune system, it is critical to monitor the immunological parameters such as apoptosis during antiretroviral therapy. A lot of investigations have been done to assess the role of ART on apoptosis in HIV-infection. A report from Johnson and Parkin showed that apoptosis was reduced after 4-6 weeks of therapy with an initial rise of CD4+ T cells (Johnson et al., 1998). Moreover, a decrease in anti-CD95 induced apoptosis of CD4 and CD8 T cells was found during ART in another study conducted by Bohler and his co-worker (Bohler et al., 1999). In a later study, Benito and his co-workers found that HAART reduced apoptosis of CD4+ and CD8+ lymphocytes to normal levels without significant differences when comparing subjects receiving PI or NNRTI triple combinations (Benito et al., 2002). However, more recently, it was reported that CD95 expression and annexin V binding were elevated during phase I of treatment without PI and began to decline only after the addition of a PI in phase II of HAART (Wasmuth et al., 2003).

1.5.2 HIV vaccine

HIV vaccines represent the ultimate prevention tool, which complement the existing antiretroviral therapy in place. An ideal HIV vaccine should induce both neutralizing antibodies and cell-mediated responses. It could prevent either HIV infection or disease progression. However, HIV-1 has a complex structure and its high mutation rate provides obstacles for the development of a safe and effective vaccine (Preston et al., 1997 & McCutchan et al., 2000).

The early vaccine studies were conducted in Rhesus monkeys or Macaques by means of the Simian model for AIDS. The results suggested that inactivated whole simian immunodeficiency virus (SIV) vaccine could be effective in inducing immune response and protect against low challenge dose of SIV (Descrosiers et al., 1989; Murphey-Corb et al., 1989 & Carlson et al., 1990).

Presently, more evidences for the critical role of CTL in controlling of HIV-infection (Schmitz et al., 1999 & Goulder et al., 1999), accumulate a number of novel vaccine strategies, such as live recombinant vectors and plasmid DNA immunogens are currently under development. These have been shown to elicit specific cytotoxic response to the virus and infected cells (Shen et al., 1991 & Frankel et al., 1995). Another line of research concerns the natural route of transmission of HIV-infection, namely the mucosal surfaces. Use of vaccines for mucosal immunization has been considered as an effective approach for prevention of transmission (Stevceva et al., 2004 & Belyakov et al., 2004).

CHAPTER 2

LITERATURE REVIEW OF APOPTOSIS

2.1 Characteristics of apoptosis

Apoptosis was originally described by Kerr et al. as one of the forms of cell death which occurs in the absence of pathological manifestations (Kerr et al., 1972). The term "apoptosis" is from the Greek word for "falling off" of leaves from a tree. It is a normal physiologic process in T cell maturation as well as in maintenance of tissue homeostasis.

The apoptotic program is characterized by morphological and biochemical features, such as cell shrinkage, membrane blebbing, chromatin condensation. The end of apoptosis is that the cell's DNA is broken down to 180-200 base pair fragments. These DNA fragments are recognized and eliminated from circulation by phagocytes without stimulating inflammation (Wyllie et al., 1984 & 1992). By contrast, necrosis is an inadvertent death of cells, accompanied by cell swelling and lysis, loss of cell membrane integrity. Necrosis does not result in DNA fragmentation, but released cytoplasmic contents can trigger an inflammatory reaction in surrounding cells or tissue.

Apoptosis is an important physiological process. During embryonic life, for example, it is involved in the remodeling of tissues, such as the developing vascular system. In addition, during lymphocytes development, most of the thymocytes generated each day die by apoptosis with less than 5% surviving. In the thymus, those T cells binding too strongly to MHC molecules (negative selection) are eliminated by apoptosis to prevent autoimmune responses (self reactive T cells). Hence, apoptosis plays a crucial role in shaping the adaptive immune repertoire. However, it also accounts for some pathological processes. For example, anti-DNA antibody can cause systemic lupus erythematosus (SLE) and cross reactive antibodies generated can account for the

leucopaenia observed in this condition. In an *in vitro* study, it was found apoptosis was increased in SLE patient (Bijil et al., 2001).

2.2 Measurements of apoptosis

Early methods to detect apoptotic cells include detecting morphologic changes under light microscopy, electron microscopy using nuclear stains or fluorescent dyes. However, most of the methods used to monitor apoptosis are cumbersome, non-specific and difficult to quantify or require the destruction of cell integrity. In addition, as apoptotic bodies can be cleared rapidly from peripheral blood, the detection of cells undergoing apoptosis must be determined at an early stage (Facchinetti et al., 1991 & Schmid et al., 1994). After flow cytometer was developed, new approaches have been established to identify apoptotic events (Darzynkiewicz et al., 2000). Apoptotic cells characterized by cell shrinkage and condensation of chromatin are detectable by flow cytometry based scatter method, which show diminished forward scatter (cell size) and increased side scatter (cell granularity) on a dot plot (Cotton et al., 1997).
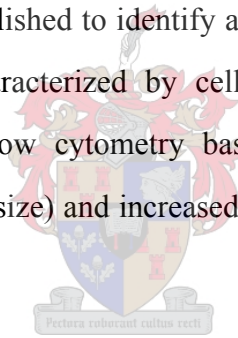
Changes on the membrane of apoptotic cells happen in the early stage of apoptosis. One of these changes is the loss of phospholipids asymmetry with exposure of phosphatidylserine (PS), which is translocated from the inner plasma membrane to the outer leaflet while the cells are undergoing apoptosis (Fadok et al., 1992). Annexin V, a calcium dependent phospholipid-binding protein possesses high affinity for PS (Andree et al., 1990) and thus this method has been developed to quantify early apoptotic cells by flow cytometry by conjugating the protein with a fluorescent dye (Koopman et al., 1994). As Annexin V binds to cells early in apoptosis and continues to be bound through cell death, Propidium Iodide (PI) is used to distinguish early apoptotic cells from necrotic cells in Annexin V apoptosis assays (Vermes et al., 1995 & Zhang et al., 1997). Since PI stains DNA, but cannot get through the plasma membrane, the early apoptotic cells which maintain membrane integrity cannot be

stained by PI, they show Annexin V positivity and PI negativity. However, necrototic cells, which have lost membrane integrity and expose PS to Annexin V, show both Annexin V positive and PI positive events (Vermes et al., 1995).

At the final stage of apoptosis, DNA is broken down to fragments. Therefore, another method to measure apoptotic cells is called terminal deoxynucleotidyltransferase deoxyuridine triphosphate nick end-labeling (TUNEL), which is a method for detecting fragmented DNA utilizing a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT) (Darzynkiewicz et al., 1997). TdT enzyme catalyzes a template independent addition of FITC labeled deoxyuridine triphosphates (dUTP) to $3^{'}$-hydrocyl ($3^{'}$-OH) ends of double and single-stranded DNA (Li et al., 1995a). Early studies showed the advantages in the TUNEL assay's sensitivity and specificity in apoptosis (Whiteside et al., 1998 & Negoescu et al., 1998). However, by using a flow cytometer, it has been reported that the TUNEL method is both sensitive and specific for the measurement of apoptosis (Kylarova et al., 2002 & Olano et al., 1996). Recently TUNEL method has been widely used to investigate apoptosis in HIV-infected disease (Niehues et al., 2001& Liegler et al., 1998).

2.3 Apoptosis involved in HIV infection

We have acquired an extensive knowledge of the molecular characteristics of the human immunodeficiency virus since its first isolation. However, the mechanisms of the HIV-related disease progression are not fully understood. A number of hypotheses have been proposed and among those, accelerated apoptosis is the main reason to account for the depletion of CD4 T- lymphocytes, which leads to the collapse of host's immune system. Early studies have shown that apoptosis was involved in the HIV-infection and elevated levels of apoptosis were observed in HIV-infected patients (Ameisen et al., 1991; Laurent-Crawford et al., 1991; Meyaard et al., 1992 & Oyaizu et al., 1993). However, there was controversial *in vitro* evidence, which

showed that E1B 19K protein inhibited HIV-induced apoptosis while it enhanced virus production (Antoni et al., 1995). Blton and his laboratory suggested that death of CD4+ T cells is not via apoptosis (Blton et al., 2002). Moreover, some authors have made observations that indicated apoptosis to be correlated with disease progression (Pandolfi et al., 1995; Gougeon et al., 1996; Prati et al., 1997; Cotton et al., 1997 & Samuelsson et al., 1997), while others have reported that no correlations between apoptosis and disease progression was found (Meyaard et al., 1994b & Muro-Cacho et al., 1995).

As apoptosis was proposed to account for the progressive depletion in CD4+ T cells, whether it occurs in virus-infected and /or in uninfected lymphocytes remains a question needing confirmation. There has been *in vivo* evidence showing that apoptosis occurs predominantly in bystander lymphocytes but not in the infected cells (Finkel et al., 1995), while Herbein et al. observed that 90% of CD4+ T cells undergoing apoptosis were HIV infected cells ,while 10% of cells were uninfected bystander cells or cells early in the infectious cycles. Furthermore, the authors found that in the presence of monocyte-derived macrophages, HIV-specific T cell apoptosis occurred at a significantly higher rate in bystander cells than in infected cells (Herbein et al., 1998).

2.4 Immunological mechanisms contribute to apoptosis in HIV-1 infection

2.4.1 HIV proteins involvement in apoptosis

Firstly, it was proposed that when the HIV envelope protein gp120 cross-links the CD4 receptor, this mechanism induces apoptosis in the CD4+ T cells (Banda et al., 1992). It was also proposed that cross linking of CD4 upregulated Fas antigen expression due to IFN-γ and TNF-α (Oyaizu et al., 1994). Secondly, the role of *tat* in apoptosis during HIV infection remains controversial. There is evidence that *tat* protein enhances apoptosis (Westendorp et al., 1995 & Li et al., 1995b). Furthermore, it was reported that *tat*-induced apoptosis is associated with

increased expression of caspase-8, a signaling molecule in apoptotic pathways (Bartz et al., 1999). In contrast, some investigators found that *tat* played an anti-apoptotic role in HIV infection (Gibellini et al., 1995 & Zauli et al., 1993). It might be reasonable to hypothesize that *tat* can regulate apoptosis to the benefit of the virus in HIV-infection (McCloskey et al., 1997). In addition, other HIV proteins such as *nef* and *vpr* are also involved in apoptosis (Azad, 2000).

2.4.2 Fas/ Fas Ligand system mediates T cell apoptosis in HIV infection

Fas also known as (CD95/Apo-1), which is a 45kDa cell receptor, belongs to the tumour necrosis factor family. It has cytoplasmic tails (called death domains) which can activate a series of caspase enzymes initiating an apoptotic signal. Fas was first described as a molecule expressed on the surface of certain cell lines that could mediated programmed cell death when ligated by a specific monoclonal antibody (mAb) (Yonehara et al., 1989). It was reported that peripheral blood mononuclear cells (PBMC) from HIV infected individuals had elevated levels of Fas (Gehri et al., 1996 & Silvestris et al., 1996). Mueller and co-workers also showed that CD8+ cells exhibited increased sensitivity to Fas induced apoptosis (Mueller et al., 2001). Moreover, there is evidence suggesting that the amount of Fas expression correlated with disease progression in HIV infected adults (McCloskey et al., 1995) and children (McCloskey et al., 1998).

2.4.3 Antigen-presenting cells mediated killing

According to Badley et al. they found that HIV-infected macrophages mediate apoptosis of T cells from HIV-infected individuals.Moreover this macrophage-dependent killing targets CD4+, but not CD8+ T lymphocytes (Badley et al., 1997).

Although the literature contains various opinions and hypothesis which seem to be controversial, they are all contributing to the understanding of the mechanisms of HIV-infection and the underlying cell loss during the infection.

2.5 Apoptosis in Memory /Naïve T cells in HIV infection

Peripheral blood T cells can be divided into two functionally different subsets based on the reciprocal expression of different surface markers. In particular, the subset that expresses CD45RO refers to memory cell. These cells respond rapidly to recall antigen and secrete a wide variety cytokines in the response to the stimulation. The other subset confined as naïve cell expressing CD45RA, has a relatively slow response or non-response to antigen and a poor or no cytokine secretion in the response (Morimoto et al., 1985 & de Jong et al., 1991). In addition, memory /naïve cells are at different sites in the body. Naïve lymphocytes home preferentially through lymphoid tissues, whereas memory cells have a much wider tissue distribution such as gut and the skin (Mackay, 1993).

HIV-infection is accompanied by the depletion of T lymphocytes and progressive loss of immune function. Since memory/naïve cells contribute differently to the immune response, investigations have been centred on whether naïve or memory T cells are preferentially depleted in HIV-infection. According to the observation of Meyaard et al. despite a selective loss of memory cells in early HIV-infection, both naïve and memory T cells lose their functions in later stages of infection (Meyaard et al., 1994a).

However, Roederer et al. presented different results from 266 HIV-infected adults showing that naïve CD8+ T cells were progressively lost and fell in parallel with overall CD4+ T cell counts (Roederer et al., 1995). They also implied that the increase in total CD8 counts in most HIV-infected individuals was primarily due to an expansion of memory cells. In addition, similar results were found in HIV-infected children (Rabin et al., 1995). In another later observation, using magnetic beads to select naïve and memory subsets of isolated CD4+ and CD8+ lymphocytes and polymerase chain reaction (PCR) to quantitate HIV sequences, it was shown that the frequency of infection and proportion of virus load contributed by naïve and memory subsets of CD4 lymphocytes varied from absolute CD4 count: CD4 naïve cells

contributed the majority of infected cells in those with high CD4 count, while memory cells were predominantly infected in those with low CD4 counts. However, this did not occur in CD8 T lymphocytes (Mcbreen et al., 2001). Moreover, in a more recent report, it was shown that memory CD8+ T cells are more resistant to apoptosis than naïve cells (Grayson et al., 2002). In an *in vitro* study, it was suggested that although both memory and naïve CD4 subsets can bind the HIV envelope protein gp120, HIV-infection of memory cells was enhanced in SF and reverse transcription (RT) (Helbert et al., 1997). There is another hypothesis indicating that the two subsets have different susceptibility to *in vitro* HIV-infection, that is naïve cells were more susceptible to CCR5 infection while memory cells remained resistant to infection and viral replication (Riley et al., 1998).
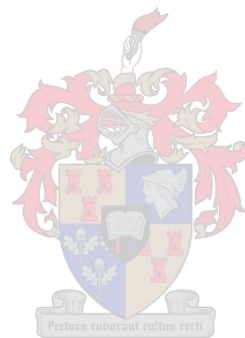
CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design of Study

The study was designed to investigate apoptosis in adults and children and to correlate this measurement to disease stage and other surrogate markers of disease activity. Three sub-studies contributed to the study. Firstly, different methods were compared to determine the approach of measuring apoptosis: did the cell preparation (i.e. whole blood or isolated cell suspensions) make a difference? Secondly, once the method of choice had been determined, different surface markers of apoptosis were used to investigate apoptosis in HIV-1 infected adults as well as in HIV-1 infected children. Lastly, a different assay to detect apoptosis (TUNEL) was conducted in the blood samples from HIV-infected children to study the effect of antiretroviral therapy.

For comparing the different cell preparations for measuring apoptosis, each subject had six FACS tubes labeled as follows:

    i. whole blood control

    ii. whole blood Annexin V

    iii. Buffy coat control

    iv. Buffy coat Annexin V

    v. PBMC control

    vi. PBMC Annexin V.

The degree of Annexin V binding (delta positive) was determined by subtracting the percentage in the control tubes from Annexin V tubes. The Scatter based analysis of apoptosis (described below) was performed in these "control" tubes.

For measuring different cell surface markers of apoptosis both in HIV infected adults and children, every subject had four FACS tubes labeled as follows:

<ol type="i">
Control
Annexin V direct
CD95 direct
CD45RA direct.

The "control" tubes were fluorescence free tubes while "direct" labeled tubes received fluorescent Annexin V or other antibodies respectively. The degree of fluorescence binding (delta positivity) was determined by subtracting the percentage in the "control" tubes from that of "direct" tubes. As to subsets of memory and naïve CD4+/CD8+ T cells, the percentage of apoptotic cells were determined by scatter analysis.

For TUNEL analysis in the sub-study in HIV infected children, one FACS tube was involved, which was labeled as APO-TUNEL. The fixed cells isolated from sodium heparin anti-coagulated blood were transferred to this tube and processed for DNA staining and analysed on the flow cytometer according to the manufacturer's instructions.
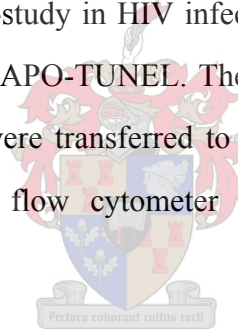
3.2 Study subjects and materials

3.2.1 Study subjects

The subjects involved in this study consisted of three different groups of HIV-1 infected individuals seen at Tygerberg Hospital. Ethical permission was obtained from the Research Council committee and signed consent was obtained from the adult volunteers or from the parents of pediatric patients.

The first group of 8 adult HIV-1 infected individuals was randomly selected and one tube of ethylene diamine tetraacetic acid (EDTA) anti-coagulated blood was drawn from each patient. All 8 patients were attending the Infectious Diseases Outpatient clinic at Tygerberg Hospital as part of their clinical management:

routine CD4 T cell counts and viral loads were determined as part of this visit. The mean CD4 absolute count of them was 620 cells per microliter blood (range 234-1022 cells per microliter blood). However, their clinical information was blinded in this sub-study. The aim of this sub-study was to determine whether the cell preparation (whole blood versus isolated cell suspensions) had any impact on the measurement of apoptosis. The ideal would be to have a quick, reproducible and sensitive assay that could be applied routinely to samples received in the laboratory.

The second group of 58 HIV-1 infected adults was randomly involved in studying different markers of apoptosis. They were enrolled when visiting the Infectious Diseases Clinic at Tygerberg Hospital during the period of February to July 2005. CD4 T cell counts and viral loads were determined as part of routine of their visits. The mean age of this group was 37.2 years (range from 23 to 53 years old). The gender breakdown of this group was as follows: 47 females and 11 males. All subjects were regrouped according to different characteristics in order to study the differences between groups (shown in Table 3-1):
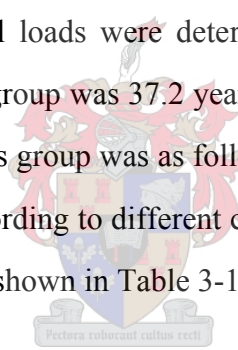
Table 3-1: Characteristics of grouping study subjects.

Characterisation of the groups	Number of individuals (n)
Total HIV-1 infected individuals	58
CDC classification of CD4 category	
Category 1: CD4 count $\geq$ 500 cells/μl	12
Category 2: CD4 count 200-499 cells/μl	27
Category 3: CD4 count<200 cells/μl	19
On antiretroviral therapy	29
Co-infected with tuberculosis	10

The third group of 19 HIV-1 infected children was enrolled for studying apoptosis in pediatric samples. They were roll out patients from KID-CRU (Children's Infectious Diseases Clinical Research Unit) at Tygerberg Hospital. This group consisted of two sub-groups.

The first sub-group included 14 HIV-1 infected children, who were first enrolled in another clinical trial in 2002. The mean age of this sub-group at that enrolment was 3.9 years (range from 1 to 7 years old). They were enrolled in this present study at the time of their routine visits at clinics during February to May in 2004. Afterwards, all of them were followed up for 12 months.

The second sub-group contained 5 HIV-1 infected children. They enrolled in this study when they started antiretroviral therapy during March to May in 2005. The mean age of this sub-group was 3.4 years (range from 2 to 6 years old). All of the subjects were followed up from baseline to 1 month, 3 month and 6 month after initiation of antiretroviral therapy. However, the cut-off time for the analysis of this sub-study was 1 month.

3.2.2 Reagents and buffer

Reagents

Monoclonal antibodies: CD4-PerCP, CD8-PE, CD45RA-FITC (BD Biosciences, Scientific Group, South Africa)

Multiset Antibody: CD3-FITC/CD4-APC/CD8-PE/CD45-PerCP (BD Biosciences, Scientific Group, South Africa)

TruCounts beads tube (BD Biosciences, Scientific Group, South Africa)

Apoptosis markers: Annexin V-FITC (Sigma, South Africa), CD95-FITC (BD Biosciences, Scientific Group, South Africa)

DNA staining Kit: APO-DIRECT™ Kit (BD Biosciences, Scientific Group, South Africa)

Buffers or solutions

    a) 10% FACS lysing solution: the working solution was made up by diluting the 10X concentrate purchased from the supplier (BD Biosciences, Scientific Group, South Africa) in MilliQ water. This solution was kept at room temperature

and used to lyse red blood cells and fix the resulting white blood cell suspension.

b) 5% fixative: cells were fixed for later analysis using this fixative. Five milliliters (5ml) of formaldehyde (Merck, South Africa) was diluted with 95ml phosphate buffered saline (PBS) and stored at 4°C until use.

c) 6% dextran solution: this solution was used to prepare buffy coat cells from whole blood. Six grams of dextran was weighed out and mixed with PBS. This solution was sterilized by autoclaving prior to use. The stock solution was stored at 4°C.

d) 70% ethanol: 70 mls of absolute ethanol was diluted with 30 ml of MilliQ water in order to generate a 70% solution. This was used as a post-fixative in the TUNEL assay. It was stored at -20°C and used cold.

e) HEPES Buffer: This buffer was used as the binding buffer in all Annexin-V assays. The HEPES powder was weighed out together with the following salts to yield the following concentrations: 10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$, (PH=7.2).

f) His-1077 density gradient solution: the density gradient solution was used to prepare mononuclear cells from whole blood. This was purchased from Sigma-Aldrich and used without any dilution.

g) Phosphate buffered saline (PBS): this buffered salt solution was used for all cell washing steps. The sachets of pre-weighed powder was added to a liter of MilliQ water and sterilized by autoclaving. It was generally stored at 4°C and used without further dilution. The final solution had a final pH of 7.2.

3.3 Staining Protocols

3.3.1 Cell preparations

Different cell preparations were employed dependent on the sub-study in question. Fresh whole blood, buffy coat cells and peripheral blood mononuclear cells (PBMCs) from EDTA blood were prepared when comparing the different methods of measuring apoptosis in sub-study 1. The aim of this study was to determine whether the cell preparation had any impact on the measurement of apoptosis.

For sub-study 2 (the measurements of different markers of apoptosis in HIV-infected adults), only fresh whole EDTA blood was used. This was due to the fact that sub-study 1 had shown that the EDTA whole blood assay was sensitive and reproducible.

For sub-study 3 however (when measuring apoptosis in HIV infected children), fresh whole EDTA blood was used for the Annexin-V assay while PBMCs from sodium heparin anticoagulated blood was prepared for the TUNEL assay.

3.3.1.1 Fresh whole EDTA blood

One hundred microliters (100μl) of the fresh blood was removed from the EDTA vacuum tube and used without any further manipulation. All antibodies

were added to the blood and the red blood cells were finally lysed by the addition of the Lysis buffer described above.

3.3.1.2 Buffy coat cells

Two milliliters (2.0 ml) of the blood was removed from the EDTA tube and diluted to 10.0 ml with PBS in a 15ml conical tube. Thereafter, 4.0 ml of a 6% dextran solution was added and mixed well. This was allowed to stand for 20 minutes at room temperature during which time most of the red blood cells sedimented to the bottom of the tube. The supernatant containing the white blood cells was transferred to another tube and centrifuged at 1500rpm for 15 minutes at 4°C. After discarding the supernatant, the cell pellet was suspended and 100µl was used for further processing (staining, etc.).

3.3.1.3 PBMCs in EDTA tube

Two milliliters (2.0 ml) of whole blood was removed from the EDTA tube and double diluted using cold PBS. Then the blood was carefully overlaid onto 3.0 ml of His-1077 density gradient solution (Sigma, South Africa) in a conical tube. After centrifugation at 1800rpm for 25 minutes at 4°C, the mononuclear cells at the interface between the plasma and HistoPaque were carefully transferred to another clean conical tube by a transfer pipette. The cells were washed once with 10.0 ml cold PBS by centrifugation (1500rpm, 15 minutes, 4°C). The supernatant was discarded, the cell pellet was suspended and 100µl was used for further processing.

3.3.1.4 Preparation of PBMCs in Sodium heparin blood for TUNEL assay

Two milliliters (2.0 ml) of Sodium Heparin anticoagulated blood was used. The blood was double diluted with cold PBS, and carefully overlaid on 3 ml His-1077 density gradient solution in a conical tube. After centrifugation at 1800rpm for 25 minutes, the mononuclear cell layer between plasma and HistoPaque was aspirated using a transfer pipette and transferred to another

clean 15 ml conical tube. Then 10.0 ml PBS were added to the conical tube and cells were pelleted by centrifuging at 1500rpm for 15 minutes. The cell pellet was washed again in 5.0 ml PBS. After washing, the cell pellet was resuspended in 0.5 ml PBS and fixed by adding 5.0 ml 5% fixative for 15 minutes at 4^0C. The cells were washed twice to discard the fixative by centrifuging at 1500 rpm for 5 minutes. Afterwards, the cell pellet was resuspended by gently vortexing in 200µl PBS and transferred to a FASC tube labeled as APO-TUNEL and stored in 2.0 ml 70% cold ethanol at -20^oC before DNA staining as per APO-DIRECT kit instructions.

3.3.2 CD4 counts determination

The routine CD4 absolute counts were done by the Division of Immunology, Department of Medical Microbiology, Tygerberg Hospital as part of the routine clinical management of the patients. Briefly, 50 µL of whole EDTA blood was incubated with an aliquot of 20µl MultiTest[TM] monoclonal antibody mixture: CD3-FITC/CD4-APC/CD8-PE/CD45-PerCP in a TruCount beads tube for 15 minutes in the dark at room temperature. Afterwards, 450 µL of lysing solution (FACSLyse, BD Bioscience, Scientific Group, South Africa) were added and the cells were incubated for a further 15 minutes. Thereafter, the samples were analysed immediately on a flow cytometer (FACS Calibur, Becton Dickinson) using the automated software MultiSet[TM]. The results of each sample analysed were expressed as both percentages of each subset as well as absolute counts determined using the beads in each tube. The ratio of CD4:CD8 was determined by the computer.

3.3.3 Lymphocyte and its subsets definition

For the analysis of lymphocytes (total) or subsets of lymphocytes (e.g CD4+, CD8+, or memory/naïve cells), the flow cytometric analysis was conducted using the CellQuest[TM] software. Basically, a dot plot of forward scatter (FSC, indication of cell size) versus side scatter (SSC, indication of cell granularity/complexity)

was drawn for every sample. On the dot plot, one could identify three different cell populations: cells at the top with the highest FSC and SSC were neutrophils, whereas cells with similar FSC and intermediate SSC were monocytes. Lymphocytes could be identified at the bottom of the plot: these cells had the lowest SSC (least complex and granular) and lowest FSC (smaller cell size) compared to the monocytes and neutrophils. This is illustrated in Figure 3-1A below. It can be clearly seen that an electronic gate could be set around the cells of interest namely the lymphocytes.

In order to analyse the subsets of lymphocytes, CD4+ and/or CD8+ T lymphocytes were defined by using CD4-PerCP/ CD8-PE fluorescent antibodies. Cells expressing CD4-PerCP positive were considered as CD4+ T lymphocytes, while those expressing CD8-PE positive were considered as CD8+ T lymphocytes. These subsets were analysed within the total lymphocyte gate set as described above (example Figure 3-1B1 and Figure 3-1B2 below).

Similarly, naïve /memory cells were defined by using the CD45RA-FITC fluorescent antibody. Cells showing CD45RA-FITC positive were considered as naïve cells, whereas CD45RA-FITC negative were considered as memory cells (Figure 3-1C below).
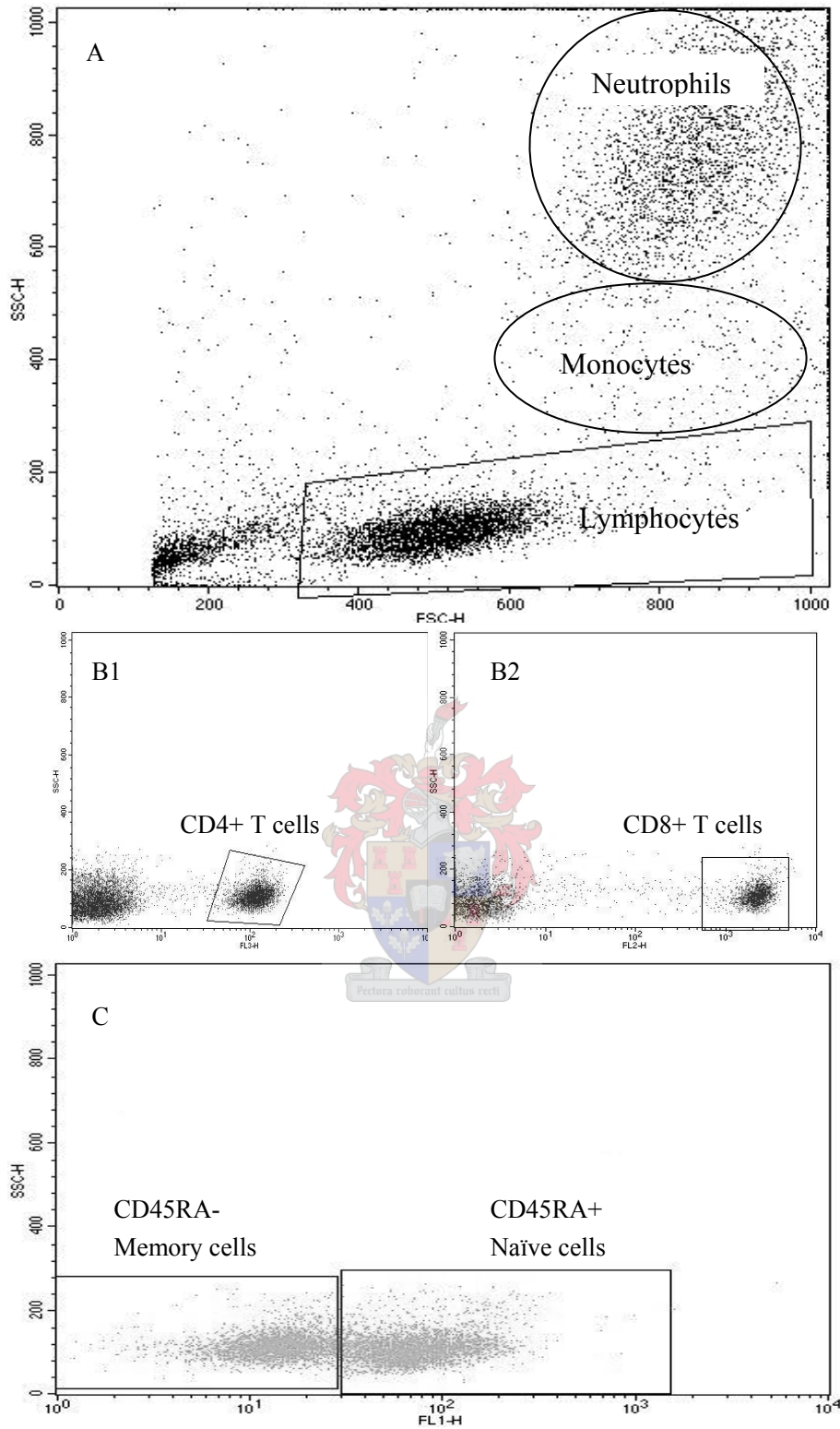
Fig 3-1: Definition of lymphocyte and its subsets on flow cytometer parameters.

(A) Lymphocytes, monocytes, and neutrophils were gated according to their different characterization of cell size and granularity. (B1, B2) CD4+ and CD8+ T lymphocytes were gated within lymphocytes gate by fluorescent antibodies: CD4-PerCP (FL3-H) and CD8-PE (FL2-H). (C) Within CD4+ T cell gate, two cell subsets were distinguished by CD45RA-FITC (FL1-H): CD45RA- (memory) and CD45RA+ (naïve) cells.

3.3.4 Measurements of apoptosis

3.3.4.1 Comparing different cell preparations of measuring apoptosis using Annexin V assay

The various cell preparations as described above (namely, whole blood versus Buffy coat cells or PMBCs) were labeled with 20µl of anti-CD4-PerCP conjugated monoclonal antibody (BD Biosciences, Scientific group, South Africa). Following 20 minutes incubation in the dark at room temperature, 1.0 ml BD FACS ^{TM} Lysing solution (BD Biosciences, Scientific Group) was added to all samples and incubated for a further 20 minutes. Once the red blood cells had been lysed, the tubes were centrifuged at 1500rpm for 10minutes and washed twice with 2.0 ml cold PBS. To the control tubes, 500µl 5% fixative was added and the tubes were stored at 4 ^{o}C until analysis on the flow cytometer. To the Annexin V tubes, 100µl HEPES Buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl$_2$, PH=7.4) was added and incubated for 10 minutes. Thereafter, 2.0µl Annexin V-FITC (Sigma, South Africa) was added and incubated for another 15 minutes. The cells were washed once with 1.0 ml HEPES buffer and finally suspended in 500µl fixative and stored at 4°C. The Annexin V tubes together with control tubes were analysed on a four-color flow cytometer (FACScan Becton Dickinson).

3.3.4.2 Measuring different apoptotic surface markers

One hundred microliters (100µl) whole blood was incubated with either the anti-CD4 or anti-CD8 fluorescent labeled antibodies (each of 20µl) for 20 minutes in the dark at room temperature. The red blood cells were lysed by incubation another 20 minutes with 1.0 ml lysing solution. The White Blood Cells were pelleted by centrifuging at 1500rpm for 10 minutes, then the cells were washed twice with 2.0 ml cold PBS. As described above, the cells in the "control" tubes were fixed by adding 500µl of 5% fixative and kept in the fridge at 4°C until acquisition on the flow cytometer. For "direct" tubes of apoptosis measurement, the cells were suspended in 100µl HEPES buffer

and incubated for 15 minutes with 2.0 µl of Annexin V-FITC and anti-CD95-FITC or anti-CD45RA-FITC monoclonal antibodies respectively. After binding thoroughly, the cells were washed with 1.0 ml HEPES buffer by centrifugation. The cell pellet was then resuspended in 500µl fixative, and stored at 4°C until flow cytometric analysis was performed.

3.3.4.3 Staining of DNA fragmentation

APO-DIRECT™ Kit (BD Bioscience, Scientific Group, South Africa) was used for the staining protocol. Negative and positive controls were available within the Kit and each batch run included both a sample of the negative and positive controls. One milliliter (1.0 ml) of Negative control and 1.0 ml of Positive control cells were transferred to the FACS tubes and centrifuged at 1500 rpm for 5 minutes together with the PBMCs that had been stored in 70% ethanol at -20°C. We discarded the ethanol supernatant from all tubes and washed the cells twice with 1.0 ml Wash Buffer provided in the Kit. Afterwards, the cell pellet was suspended in 50µl of staining solution. This solution was prepared according to the manufacturer's instruction: briefly, it contained 10µl Reaction Buffer, 0.75µl TdT enzyme, 8µl FITC dUTP and 32µl distilled H_2O. All reagents were supplied in the kit and were ready for use.   The cells were then incubated in the staining solution for 60 minutes at 37°C in a CO_2 incubator. At the end of the incubation, the cell pellet was rinsed twice by 1.0 ml Rinse Buffer (provided in the Kit). After last wash the cell pellet was resuspended in 0.5 ml PI/ RNase A solution (provided in the Kit) and incubated another 30 minutes in the dark at room temperature. Analysis of the cells in PI/ RNase solution was performed on the flow cytometry within 1 hour after staining.

3.3.5 HIV viral load

Plasma HIV-1 viral loads were done by the Department of Virology, Tygerberg Hospital/University of Stellenbosch. Basically, the viral loads were detected by

LCx HIV RNA Quantitative assay which is an *in vitro* reverse transcription-polymerase chain reaction assay. It measures ribonucleic acid (RNA) of HIV-1 in human plasma. The lower limit of detection (LLD) is 50 copies of HIV-1 RNA per ml for 1.0 ml sample volume procedure and 178 copies per ml for 0.2 ml sample. The upper limit of Quantitation (ULQ) is 1,000,000 copies of HIV-1 RNA per ml for 1.0 ml sample and 5,011,872 copies of HIV-1 RNA per ml for 0.2 ml sample.

In this present study, limited detection level of 50 copies/ml was for HIV-1 infected adult sample (1.0 ml), while 178 copies/ml for paediatric sample (0.2 ml). An arbitrary value of 25 or 89 copies was assigned respectively to samples with undetectable levels. The transformation of logarithm ($\log_{10}$) of viral load was used in this thesis for analysis.

3.4 Flow cytometry analysis of apoptosis

3.4.1 Scatter-based assay

Flow cytometry based scatter analysis was developed based on the light scatter characteristics of apoptotic cells. As described by Cotton and his colleagues, sorting experiment using γ-irradiated PBMCs confirmed the validity of the scatter assay (Cotton et al., 1997).

The scatter characteristics of CD4+ T lymphocytes, CD8+ T lymphocytes, CD4 memory/naïve cells, CD8 memory/naïve cells were analysed on the flow cytometer using the CellQuest^{TM} software. Within the total lymphocytes population, CD4 and CD8 cells subpopulation were electronically gated using the CD4-PerCP and CD8-PE fluorescent antibodies. Those cells exhibiting diminished forward scatter and increased side scatter were considered as apoptotic CD4 and CD8 cells respectively. An example is shown in Figure 3-2 A, B and D below. The degree of apoptosis in CD4+ and CD8+ T cells populations were

defined as the number of apoptotic CD4 or CD8 cells divided by number of total CD4+ or CD8+ T cells (expressed as a percentage).

Similarly, memory/naïve cells were defined by the CD45RA-FITC fluorescent antibodies. Within the CD4 positive or CD8 positive cell subpopulation, cells displaying CD45RA+ were considered as naïve CD4 or CD8 cells, while CD45RA- cells were considered as memory CD4 or CD8 cells. Within the memory and naïve cell gates, a scatter dot plot was displayed as described above. Those cells exhibiting diminished FSC and slightly increased SSC characteristics area were considered as apoptotic cells (example Figure 3-2 C, E and F below). The degree of apoptosis was calculated in the memory/naïve cell subpopulation as a percentage of the total memory/naïve cells.
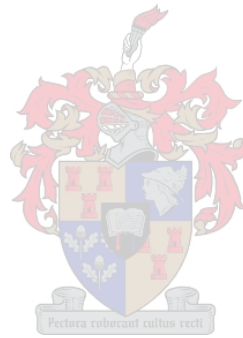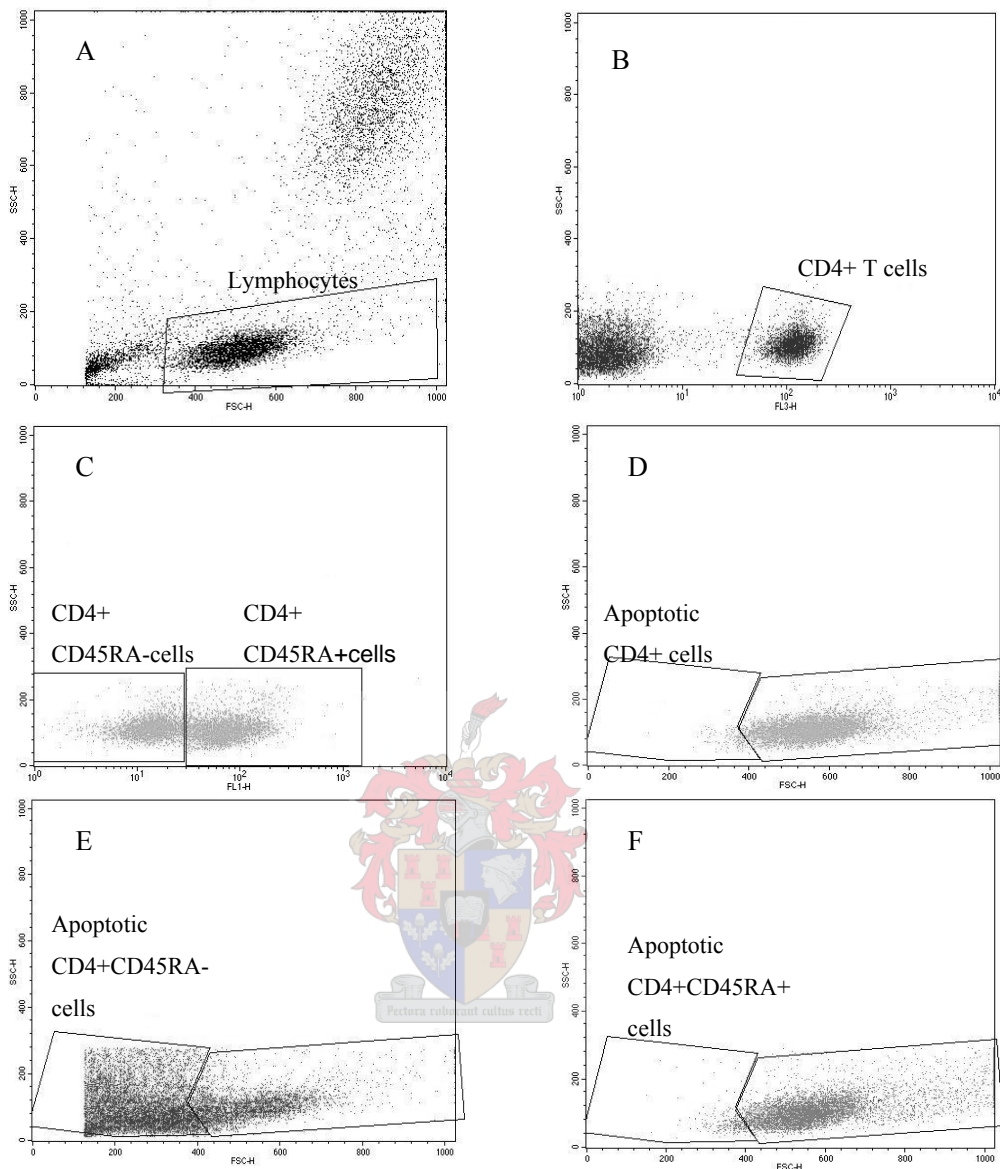
Fig 3-2: Scatter analysis of apoptotic cells in CD4+T cells and its subset of memory and naïve cells.

(A) Lymphocytes gate was set on a FSC/SSC dot plot. (B) Within the lymphocytes gate, CD4+ T cells were determined by CD4-PerCP fluorescent antibody (FL3-H). (C) Memory and naïve cells were defined as CD45RA- and CD45RA+ (FL1-H) in CD4+ T cells gate. (D) Within the CD4+ T cell, cells exhibiting decreased forward scatter were gated and considered as apoptotic cells. (E, F) Two FSC/SSC dot plots were generated from gates of CD4+CD45RA- and CD4+CD45RA+ cells, respectively. The same gate of apoptotic CD4+ T cells was copied to the plots and the apoptotic memory and naïve cells were shown.

3.4.2 Annexin V binding assay and CD95 expression assays

The determinations of Annexin V binding and/or CD95 expression were performed on the FACSCalibur™ flow cytometer and 50,000 total events were acquired using CellQuest™ software. The CD4+/CD8+ T lymphocytes were gated using the anti-CD4-PerCP and anti-CD8-PE fluorescent antibody as described above. The Annexin V binding within these gates was determined by brightly expressing Annexin V-FITC indicated within the M1 region (example Figure 3-3 below).   Similarly, CD95-FITC positivity was determined within the CD4+ or CD8+ gates (data not shown). The percentage of Annexin V binding or CD95 expression was defined as number of CD4+/CD8+ Annexin V+ or CD95+ cells divided by number of total CD4+/CD8+ T cells.
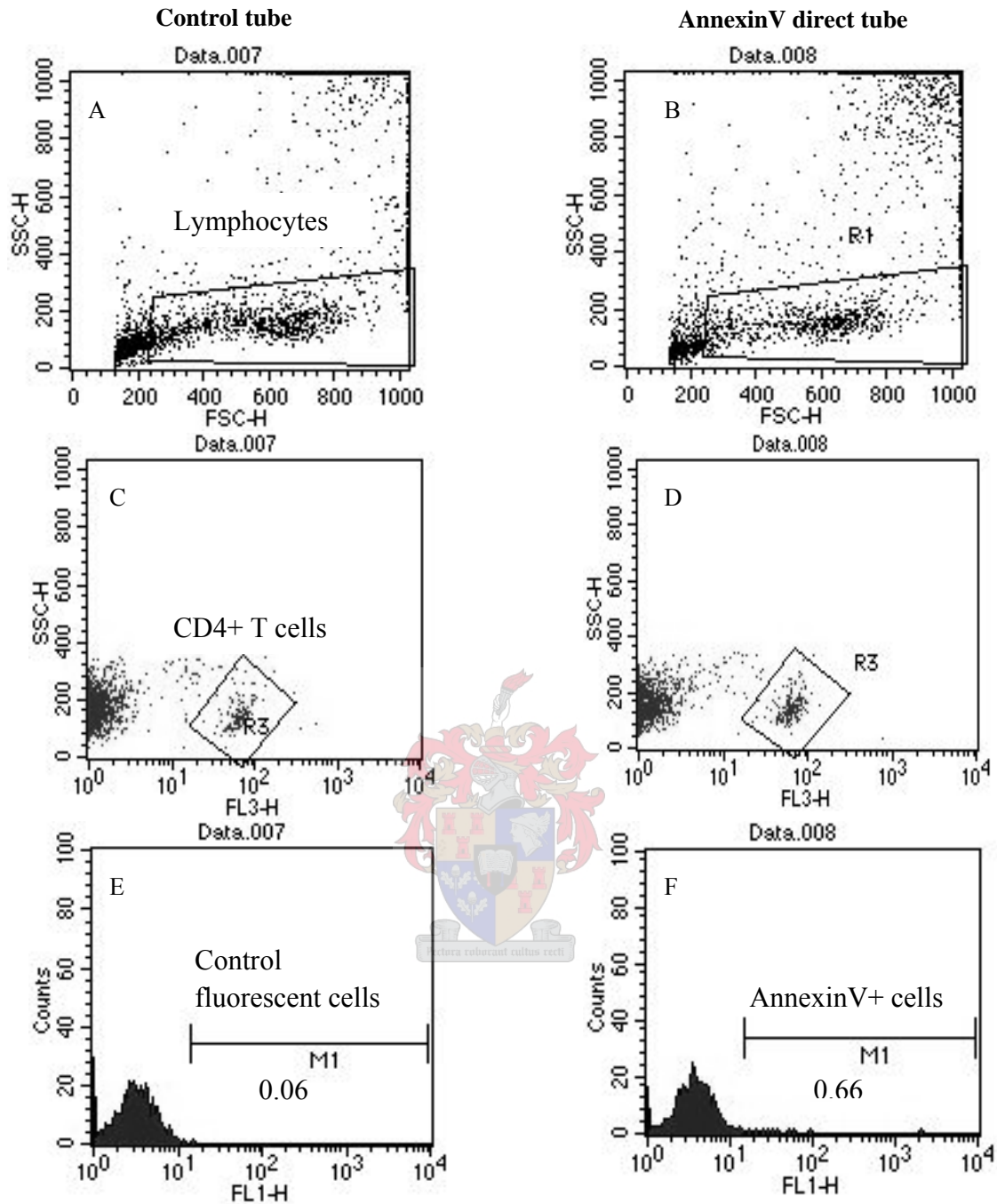
Fig 3-3: Determination of percentage of apoptotic CD4+ T cells by Annexin V binding analysis on flow cytometer.

The histograms on the left (A, C, E) were control tubes, which were fluorescence free, while on the right (B, D, F) were Annexin V direct tubes which received Annexin V-FITC. (A, B) Lymphocyte gate was defined as low FSC and low SSC (R1). (C, D) CD4+ T cells (R3) were gated by cells expressing high CD4-PerCP (FL3-H) within lymphocyte gate. (F) Annexin V+ cells (M1) were defined by cells expressing high Annexin V-FITC (FL1-H). The percentage of apoptotic CD4+ T cells was determined by subtracting the percentage of control fluorescent cells (E, 0.06) in control tubes from that of AnnexinV+ cells (F, 0.66) in direct tubes.

3.4.3 TUNEL assay

The TUNEL analysis was run on the flow cytometer using the CellQuest[TM] software. A dot plot of FL2-A versus FL2-W was created indicative of DNA area (Y-axis) versus DNA width (X-axis). This display allowed one to exclude cells which were clumped (these would have increased FL2-W features). Such events are referred to as "doublets". An electronic gate was drawn around the non-clumped cells (refer to Figure 3-4A below). Within this gate, the second dot plot was generated: the cells that stained positive with FITC labeled deoxyuridine triphosphate (FITC-dUTP) were defined as apoptotic cells, while the cells that were negative were considered as non-apoptotic cells (Fig 3-4B below). The level of apoptosis was determined as the percentage of apoptotic cells in total cells (apoptotic and non-apoptotic cells).
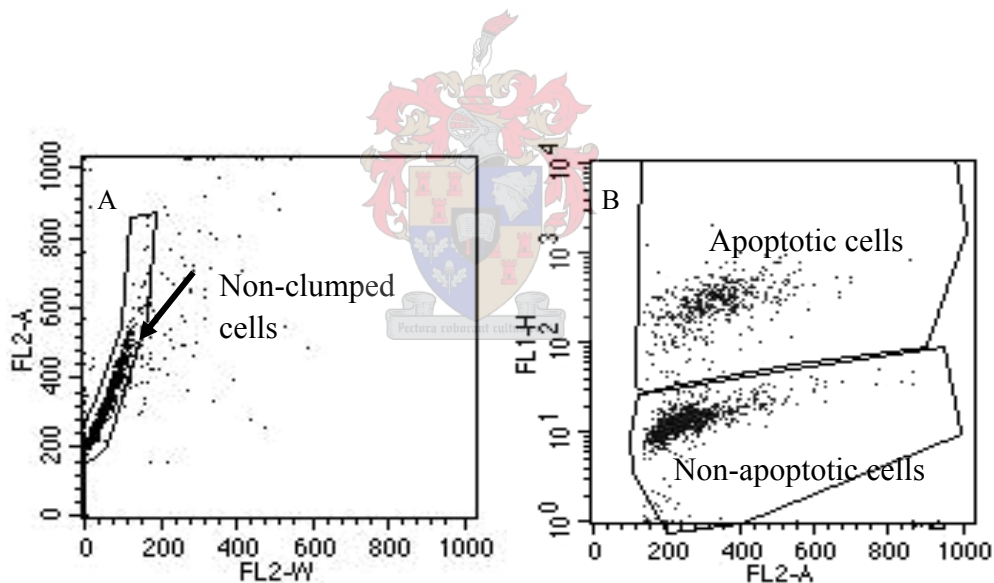
Fig 3-4: TUNEL analysis of apoptosis on flow cytometer

(A) Non-clumped cells (non-doublets) were gated in a dot plot of DNA area (FL2-A) versus DNA width (FL2-W). (B) Within the Non-clumped cells, cells stained positive dUTP-FITC (FL1-H) were considered as apoptotic cells, while the negative events were non-apoptotic cells.

3.5 Statistical analyses

Statistical analyses were done using the Statistica 7.0 software (Statsoft, Inc.1984-2005). Advice was sought from an expert available to post-graduate students within the faculty (Prof. DG Nel). The t-tests for independent groups and one-way ANOVA were used to evaluate the differences in means between two or more groups. The t-test for dependent sample was use to compare the differences in means between two dependent variables. ANOVA repeated measures was use to compare the differences at different time point measured on same subject.

General linear model was used to compare multiple dependent variables, such as cell preparations and measuring methods. Person product-moment correlation coefficient was applied to determine the correlation between different variables. Significance was set at p≤0.05 for all parameters analysed.
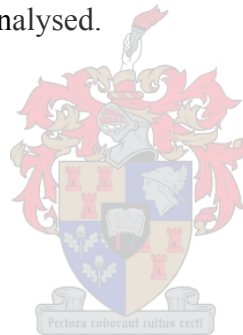
CHAPTER 4

EVALUATION OF DIFFERENT CELL PREPARATIONS
FOR MEASURING APOPTOSIS

4.1 Background

Apoptosis is proposed as one of the mechanisms of CD4 depletion in HIV-1 infection. A number of studies have been done or are being done to measure apoptotic events in HIV-1 infected individuals. However, different levels of apoptosis are reported at different laboratories. Apparently, the differences in methodologies could account for the different conclusions, such as the cohort of study subjects or the methods used to detect apoptosis. Different methodologies were applied due to different objectives of the studies. It would be the best to find a method measuring *in vivo* apoptotic events, which would be beneficial to understand the pathogenesis or mechanisms of CD4 depletion in HIV-1 infection. In addition, it is also crucial to find a simple and specific method which would be reproducible, easily applied to clinical samples and one that detects apoptosis rapidly and exclusively.
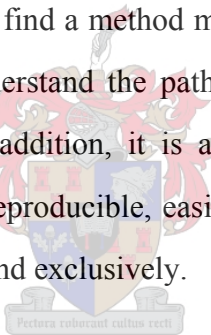
When one considers the different studies and cell preparations used, most of the apoptosis studies in HIV-1 infection have used isolated PBMCs, stimulated or unstimulated, cultured or uncultured to determine *in vitro* or *ex vivo* apoptosis, while few refer to the use of whole blood sample (unfractionnated cells). Although there are no reports that state that the levels of apoptotic events would be influenced by manipulating the blood, one study showed that whole blood samples had greater level of apoptosis than PBMC samples under identical experimental condition (Fowke et al., 2000).

With respect to the method of detecting apoptosis, fluorescent labelled Annexin V is well established and widely used as an early marker of apoptotic activity because of its specificity and high affinity to PS which is exposed on the surface of apoptotic

cells (Andree et al., 1990 & Darzynkiewicz et al., 2000). Another method – flow cytometry based scatter analysis is founded on the characteristics of apoptotic cells, such as cells shrinkage and increased granularity. As a consequence of cell shrinkage, chromatin condensation and nuclear fragmentation, a decrease in cell size and increase in cell granularity (which is indicated by diminished forward scatter and increased side scatter) on a histogram of flow cytometer is observed when cells undergo apoptosis (Scherer et al., 1999 & Darzynkiewicz et al., 2000). However, Studzinski in his book suggested that increased side scatter only occurs at a relatively early stage of apoptosis. When apoptosis is more advanced and the cells become small, the side scatter diminishes as well as the forward scatter (In: Apoptosis---A practical approach. Ed. Studzinski, 1999).

In this present study, three different cell preparations – Whole blood, Buffy coat cells and isolated PBMCs were used to determine whether different cell preparations made any differences in the measurement of apoptosis. In addition, FITC-labelled Annexin V as well as the flow cytometry based scatter method was applied to investigate the differences in analytical methods for measuring apoptotic cells. The levels of apoptosis are determined as the percentage of Annexin V-FITC positive cells or the percentage of cells displaying decreased forward scatter and slightly increased or decreased side scatter on the flow cytometer FSC /SSC dot plot.

4.2 Results

4.2.1 Analysis of apoptosis by flow cytometry

As described in the Materials and Method section (chapter 3), every study subject had 6 FACS tubes to measure percentages of apoptotic CD4+ T cells in three different cell preparations. Each analytical method was applied in Whole blood samples, Buffy coat samples as well as PBMC samples. The flow cytometric analysis of Annexin V was also illustrated in the methods section. The scatter based assay was done in the fluorescent free tubes of different cell preparations. An example of the results obtained is shown in Figure 4-1. The first row of figures

(Figure 4-1 A, B & C) represents the results obtained using a whole blood sample; The second row of figures (Figure 4-1 D, E & F) represents the results obtained using the Buffy coat cell sample and the last row of figures (Figure 4-1 G, H & I) represents the results obtained using the PBMC sample. All these data were generated using the same donor but the blood was processed differently as described in the Methods section.

It can be seen that the highest percentages of apoptotic cells was yielded in whole blood sample (5.04%) with respect to the same study individual, while the lowest result was exhibited by the PBMC sample (2.04%).

4.2.2 Results of comparing apoptosis in three cell preparations by Annexin V and scatter method assays

4.2.2.1 By FITC-labeled Annexin V binding method

Five out of 8 patients showed greater levels of apoptotic (Annexin V +) cells in fresh whole blood samples than that in their Buffy coat sample. When whole blood samples were compared to isolated PBMC sample, 6 of 8 HIV-infected subjects had lower percentage of Annexin V binding in PBMC samples. The mean percentage of apoptotic cells of the 8 patients is illustrated in Figure 4-2 A.

4.2.2.2 By flow cytometer based scatter method

When the scatter method was applied to the same blood samples to determine the levels of apoptotic cells, 6 of 8 patients had higher percentage of apoptotic cells in whole blood compared to the Buffy coat samples or the PBMC samples. The mean percentage of apoptotic cells by the scatter assay is shown in Fig 4-2 B.
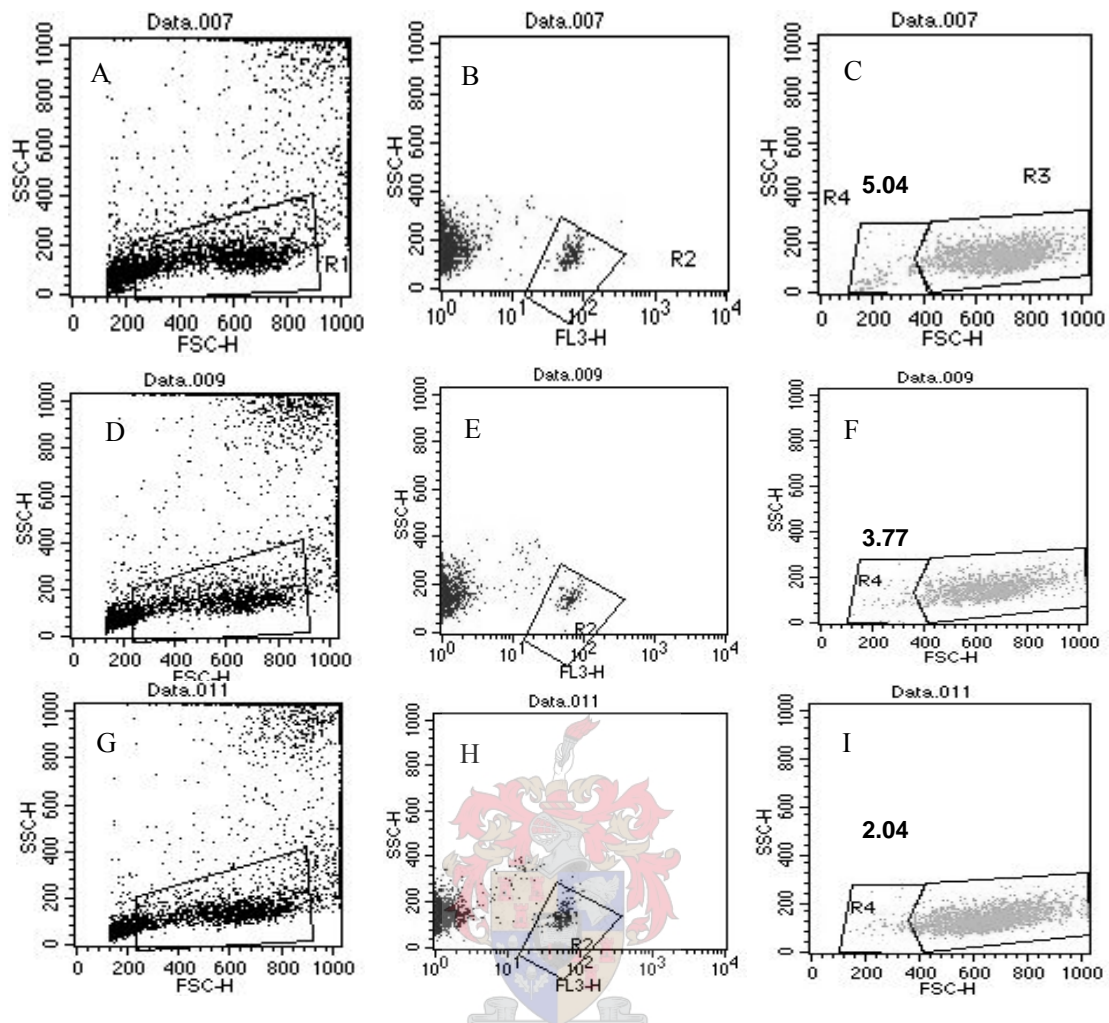
Fig 4-1: Flow cytometric analysis of scatter method in three different cell preparations.

Dot plots show levels of apoptosis measured by the scatter method in whole blood (A, B, C), Buffy Coat (D, E, F) and PBMC (G, H, I). A lymphocyte gates (R1) was drawn by low side scatter and low forward scatter in three different cell preparation samples (A, D, G). CD4+ T cells (R2) were gated as highly expressing CD4-PerCP monoclonal antibody (FL3-H) within lymphocytes population (B, E, H)). Apoptotic CD4+ T cells (R4) were defined as those cells showing decreased forward scatter and increased or decreased side scatter within CD4 population (C, F, I). The percentages of apoptosis in the three samples are indicated in the plots respectively. It appeared that the highest level of apoptosis was in whole blood sample (C, 5.04%) followed by Buffy coat (F, 3.77%) and PBMC (I, 2.04%) samples.

Fig 4-2: Mean percentages of apoptotic cells from 8 HIV-infected individuals by measuring Annexin V binding and scatter.

Whole blood sample showed the highest percentage of apoptotic cells in the three cell preparations both in Annexin V binding (A) and scatter (B) measurements, while PBMC had the lowest level. It indicates that the whole blood samples gave the highest mean level of apoptosis compared to Buffy coat or PBMC samples.

A Annexin V	Whole Blood	Buffy coat	PBMC
AnnexinV	0.71	0.46	0.32

B scatter	Whole Blood	Buffy coat	PBMC
scatter	6.00	4.04	3.01
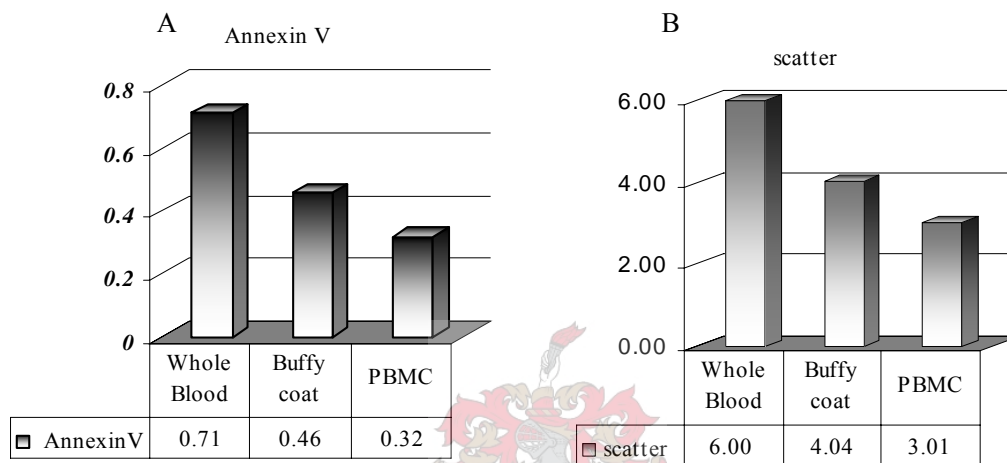
4.3 Statistical analyses

4.3.1 Comparing differences in cell preparations and analytical methods

4.3.1.1 Cell preparations: Whole blood & Buffy coat & PBMC

The *t*-test for dependent samples was performed to determine the statistical difference between the three cell preparations. It appeared that whole blood samples had significantly greater levels of apoptosis than the PBMC samples either by scatter method (p=0.008, Fig 4-3) or by Annexin V binding method. However, significant differences were not apparent when one compared the results of the whole blood versus the Buffy coat samples (data not shown). Differences between buffy coat and PBMC samples were not significant.

Fig 4-3: Boxplot representation of the scatter assay using the three cell preparations.
The *t*-test for dependent samples was used. The percentage of apoptotic cells (Y-axis) while the three cell preparations are presented on the X-axis. The plot showed that the highest level of apoptotic cells was in whole blood cell sample, followed by Buffy coat and PBMC samples. In addition, whole blood samples had significantly high levels than PBMC samples (p=0.008), however, when compared to Buffy coat samples, the difference was not significant (NS).
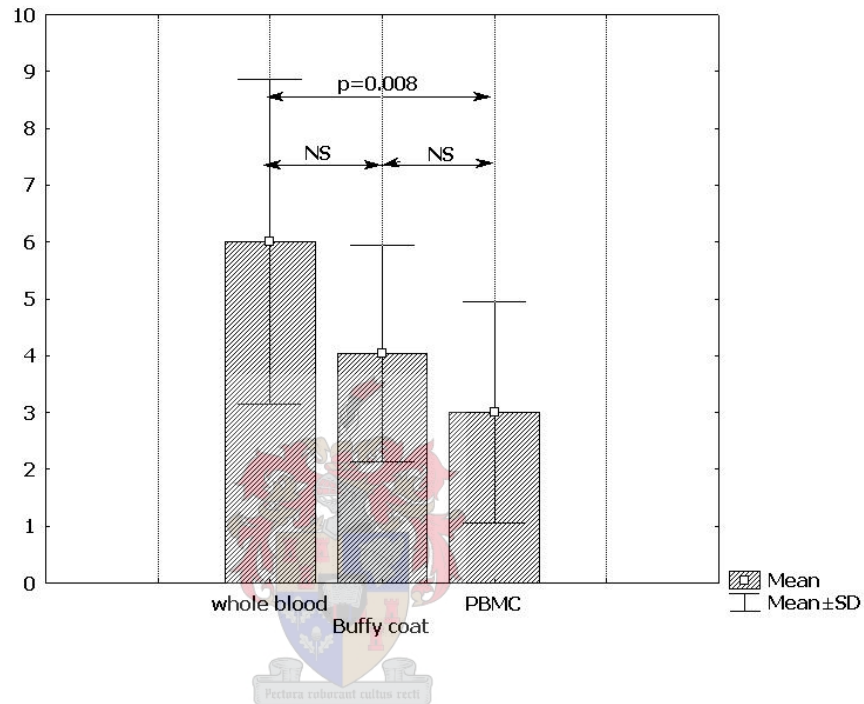
4.3.1.2 Analytic methods: Annexin V binding versus Scatter assay

When comparing Annexin V binding with the flow cytometry based scatter method, Wilcoxon matched pairs test showed that the scatter method provided significantly higher levels of apoptosis than the Annexin V in all three cell preparations (p=0.012).

According to the General linear model test, both the cell preparations and methods of measurements had significant effects on the levels of apoptosis: the whole blood preparation yielded the greater levels of apoptosis when compared to the Buffy coat or PBMC preparations and the scatter method gave higher results than the Annexin V method. However, although the interactions of the two factors

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(method versus cell preparations) were close, they were not significantly different (Figure 4-4).

Fig 4-4: General linear model analysis of the different parameters in determination of levels of apoptosis.

The mean percentage of apoptotic cells were calculated as least squares means (LS Means).   (A) The difference between three cell preparations in measuring apoptosis was statistically significant (p=0.020). The figure indicates that Whole blood preparation provided highest levels of apoptosis followed by Buffy coat and PBMC samples. (B) The difference between methods of measuring apoptosis was highly significant (p<0.001). The scatter method had higher levels of apoptosis when compared to the Annexin V binding method. (C, D) The interaction of cell preparations and measuring methods did not reach statistical significance (p=0.087).

Cell preparations; LS Means
Current effect: F(2, 14)=5.2403, p=.02001

Measuring methods; LS Means
Current effect: F(1, 7)=64.849, p=.00009

Cell preparation*Measuring methods; LS Means
Current effect: F(2, 14)=2.9198, p=.08713

C

Annexin V binding
Scatter method

Cell preparation*Measuring methods; LS Means
Current effect: F(2, 14)=2.9198, p=.08713

D

Whole blood
Buffy coat
PBMC
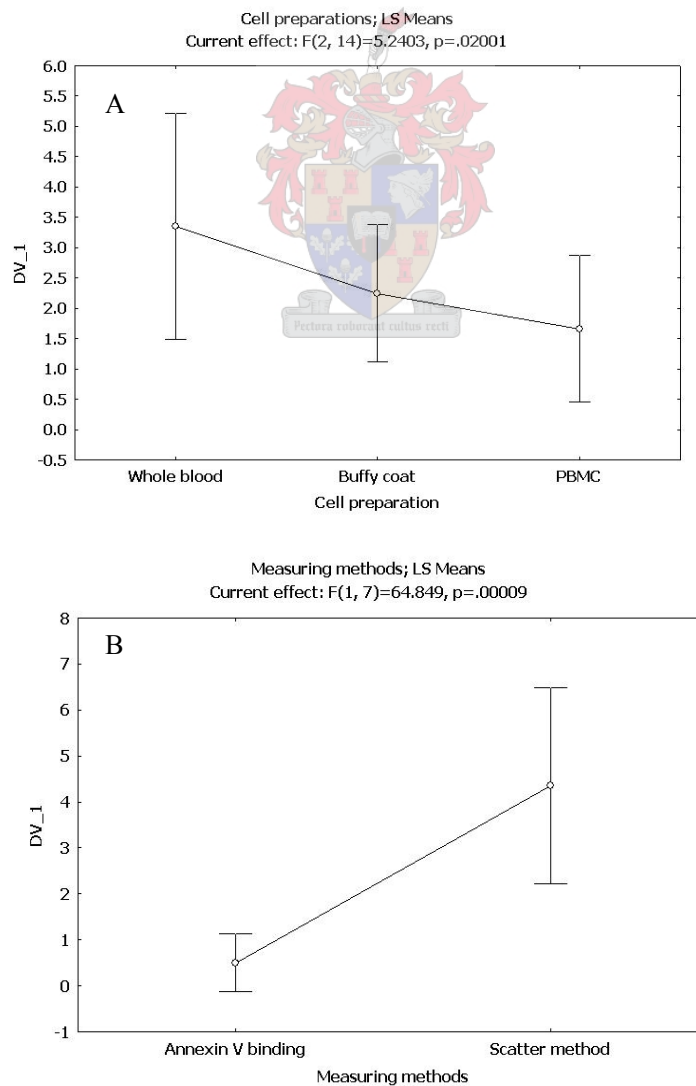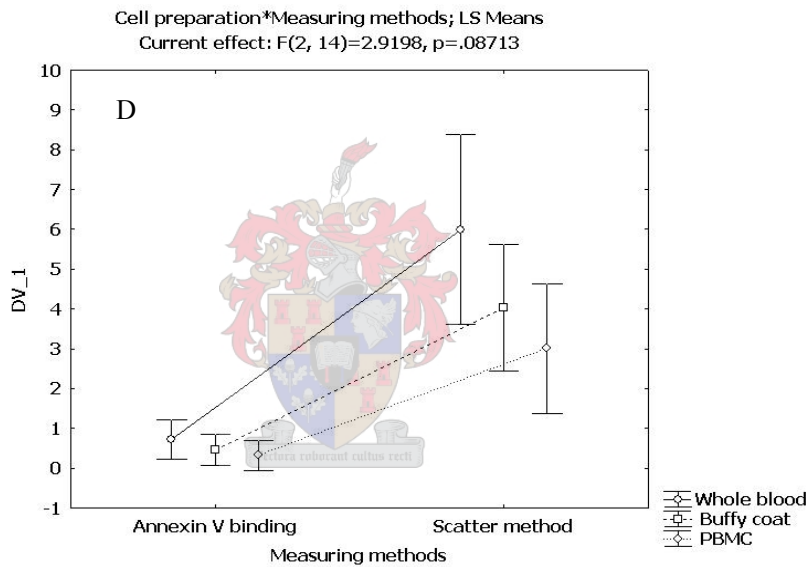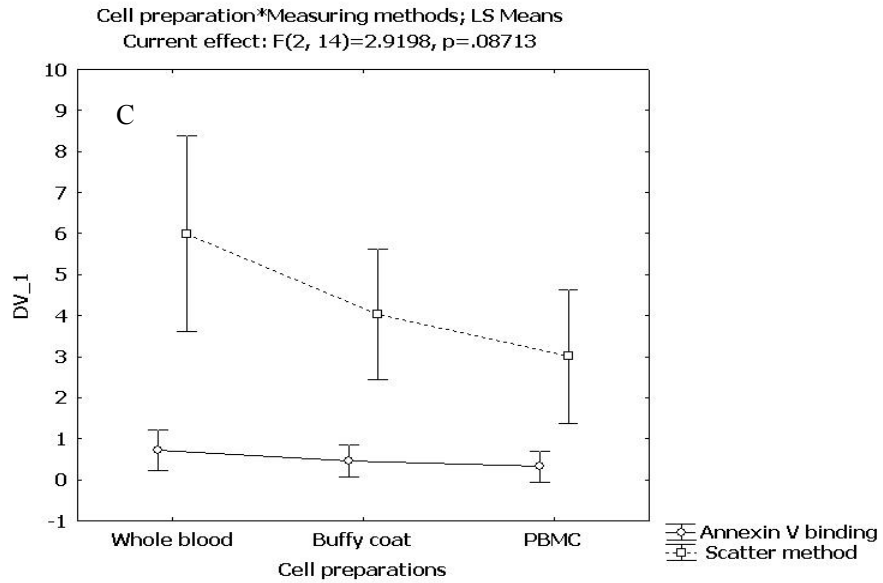
4.3.2 Correlations between CD4 counts and measurements of apoptosis

According to the correlation analyses, the CD4 counts were inversely (negatively) and significantly correlated to the level of apoptosis in the CD4+ T cells as measured by the scatter method only in whole blood samples (p = 0.014, r = -0.948, Figure 4-5). However, the Annexin V binding assay did not significantly correlate to CD4 counts (data not shown). This may be due to the small sample size used (n = 8). No correlation between Annexin V binding

assay and flow cytometry based scatter method was found in the three cell preparations.

Fig 4-5: Scatterplot of correlation between levels of apoptosis and CD4 count.

The percentage of apoptotic cells measured by scatter method in whole blood samples (Whole blood scatter, Y-axis) versus the CD4 absolute counts (CD4 count, X-axis). In 5 of the HIV-infected individuals, whose CD4 counts were presented, the scatterplot indicated that in whole blood samples, apoptosis was inversely correlated to the CD4 count (r= -0.948,p=0.014). The CD4 counts were unfortunately not available for the remaining 3 samples.
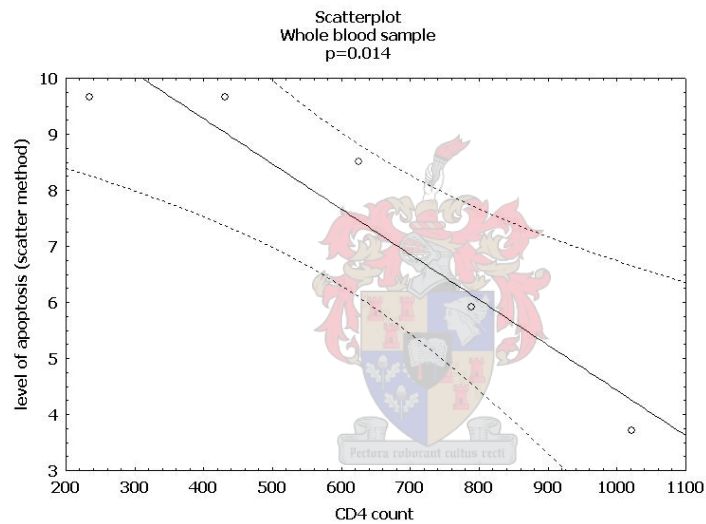
4.4 Summary and discussion

The aim of this sub-study was to investigate whether different cell preparations and measuring methods could affect detection of apoptosis. Eight HIV-1 infected individuals were involved in this sub-study. Three cell preparations: whole blood, Buffy coat cells and isolated PBMCs and two kinds of measuring methods: FITC-labeled AnnexinV binding and flow cytometer based scatter method were applied for the investigation.

According to the results, different cell preparations could affect the level of apoptosis. Fresh whole blood sample yielded greater levels of apoptotic cells when compared to the Buffy coat and/or the PBMC preparations when measured by either Annexin V binding or scatter analysis. Our results are in agreement with those published by Fowke and his colleagues (Fowke et al., 2000). We feel that this might due to *in vitro* manipulation of the samples leading to a decrease in cell viability or even cell loss during the Buffy coat and PBMC processing: whole blood sample does not change the cellular composition of the blood and resembles the *in vivo* condition.

With respect to the methods of measuring apoptosis, Annexin V binding and the flow cytometric based scatter assay were significantly different in all cell preparations. The level of apoptosis was much greater when measured by scatter method compared to the Annexin V binding assay. Furthermore, the inversely significant correlation between CD4 count and level of apoptosis indicated that the CD4 depletion might due to increased apoptosis. However, this significant correlation was only observed in whole blood sample by scatter analysis. This further confirms the fact that whole blood sample provides better result and the scatter analytical method could be another useful method for measuring apoptosis.

This methodological sub-study acted as a pilot study for the further study of measuring apoptosis in HIV-1 infected adults and children. The aim of this sub-study was not to correlate apoptosis to viral loads or disease stage: it provided us with the data that indicated that the whole blood method as a cell preparation could be used as a rapid and reproducible cell source. Furthermore, this sub-study also indicated that the scatter method as well as fluorescent labelled Annexin V could be useful markers for apoptosis. Both methods were used throughout the remainder of this study and it was decided that the whole blood sample was the ideal sample to be processed: this did not require labour intensive separation methods which could ultimately change the profile of the sample.

It must also be stated however that our results might somehow be different to other published studies. Firstly, the levels of apoptosis in HIV-infected subjects as measured by Annexin V were relatively low compared to other studies. This may be due to the fact we measured apoptosis in unstimulated and/or uncultured cells. Most studies either culture the blood cells prior to the measurement of apoptosis. In addition, the flow cytometric based scatter method detects not only early apoptotic events but also late apoptotic cells (or dead cells). It is a non-specific method for determination of apoptosis. We are aware of the limitation of the current sub-study: the sample size was small (n = 8) and we cannot differentiate between live apoptotic cells and dead (necrotic) cells. We do feel confident however that the use of whole blood and the use of various methods for detecting apoptotic events is applicable to the larger studies and that it would yield important results.
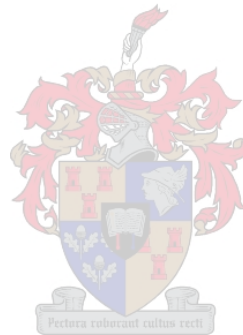
CHAPTER 5

MEASUREMENTS OF APOPTOSIS IN
HIV-1 INFECTED ADULTS

5.1 Background

Enormous progress has been made in the understanding of HIV-1 infection and AIDS over the last two decades. The virus has been well characterized, antiretroviral therapies have been developed and vaccines are under investigation. However, despite this expanse of knowledge, we have not conquered the disease. A lot of questions remain unsolved and the immunopathological mechanisms of HIV infection are unclear. For example, the progressive loss of CD4 T lymphocytes, which leads to the destruction of the immune system, remains controversial and unclear. The better we understand the mechanisms, the better the strategies we can develop to eliminate the virus.

Apoptosis, which is a cell's program to die (programmed cell death), is a normal biological phenomenon to eliminate immature cells or infected cells. As elevated levels of apoptotic CD4 T lymphocytes are found in HIV-1 positive individuals, it has been proposed as a contributory factor of the depletion of CD4 T lymphocyte during HIV-1 infection (Laurent-Crawford et al., 1991 & Meyaard et al., 1992). The mechanism of the CD4 apoptosis is not fully understood. One of the assumptions is that the process involves the Fas/FasL mediated T lymphocytes apoptosis (Silvestris et al., 1996 & Mueller et al., 2001).

The present study was set up to measure apoptosis in both CD4+ and CD8+ T lymphocytes and their memory/naïve subsets. Fluorescent labelled Annexin V and flow cytometric scatter-based methods were used to determine the levels of apoptotic events in HIV-1 infected adults. In addition, to study the correlation between

Fas/FasL pathway and apoptosis, fluorescent labelled anti-CD95 (Fas/FasL) antibody was used to measure CD95 expression on CD4+ and CD8+ T lymphocytes.

A series of questions were addressed when starting the study. First of all, what's the difference between apoptosis in CD4 T lymphocytes and in other lymphocyte subsets? Secondly, does the level of apoptosis in HIV-1 infection gradually increase while the disease progresses? In addition, does antiretroviral therapy affect apoptosis? That is, is there any difference in levels of apoptosis between antiretroviral treated and non-treated HIV-1 infected individuals? Moreover, if HIV-1 infected individuals are co-infected with tuberculosis (TB), does the level of apoptosis in these individuals differ significantly from individuals not having the co-infection?

The aim of this chapter is to present to the reader our findings in a cohort of HIV-infected adults where these questions have been posed.

5.2 General review of the study subjects

As described under the Materials and Methods section, 58 HIV-1 infected adults were prospectively enrolled in the study. All patients were attending the outpatient clinic at the hospital and informed consent obtained from all participants. These patients were enrolled between February to July 2005 and a single time point of study was applied to all: this was a cross-sectional study and we did not require follow up samples from the patients. Detailed clinical notes were available for all the patients.

The following table illustrates the characteristics of these study subjects (Table 5-1). The mean age of all the subjects was 37.2 years (ranged from 23 to 53 years old). More than 80% of the subjects were female and they were predominantly black or of mixed ancestry. All of the subjects had been tested HIV-1 seropositive for at least 1 month. Twenty-nine of 58 subjects were on antiretroviral treatment for at least 1 month: most of them were on Nucleoside reverse transcriptase inhibitors (NRTIs) and Non-nucleoside reverse transcriptase inhibitors (NNRTIs) combination regimens.

Only one subject was receiving 2 NRTIs plus 1 Protease inhibitor (PI) combined therapy.

Table 5-1: Description of 58 HIV-1 infected study subjects

Total HIV-1 infected adults	n=58		
		mean age (yr)	37.2
Gender			
		Male (n)	11
		Female (n)	47
Race			
		Black (n)	26
		Mixed (n)	23
		Caucasian (n)	3
		Others (n)	6
Time since first HIV-1 positive test			
		Shortest	1 month
		Longest	14 years
Time since antiretroviral treatment			
		Shortest	1 month
		Longest	6 years

According to CD4+ T lymphocyte category of CDC classification system for HIV infection among Adults (CDC 1994), all 58 subjects were grouped into one of the three disease stages, defined as follows:

   i.   CD4 category 1: CD4 count greater than or equal to 500 cells/µl blood

   ii. CD4 category 2: CD4 count 200-499 cells/µl blood

   iii. CD4 category 3: CD4 count less than 200 cells/µl blood.

Furthermore, with respect to antiretroviral treatment and co-infection with tuberculosis, subgroups of treated/not-treated, co-infected/not co-infected were formed accordingly and analysed appropriately (refer to Table 5-2). A new surprising finding of the study, some patients exhibited CD4 double populations when cells were stained with the monoclonal antibodies: we could therefore form a new grouping of

patients exhibiting single CD4+ cell population versus those exhibiting double CD4+ cells. As shown in Fig 5-1, subjects showing a single population of CD4+ T cells were grouped in CD4 single population group, whereas subjects having two sub-populations of CD4+ T cells were grouped in CD4 double population group (Table 5-2).

Fig 5-1: Example of the flow cytometric characteristic of CD4 double/single population patients.

Using the same staining protocol and analytic method, two kinds of CD4-PerCP antibody expressions were observed. (A2) CD4+ T cells were gated as cells expressing CD4-PerCP (FL3-H) positive within lymphocytes gate. These cells were defined as CD4 single population. (B2) CD4+ T cells gate consisted of two separate sub-populations, both of which contained cells were CD4-PerCP positive.
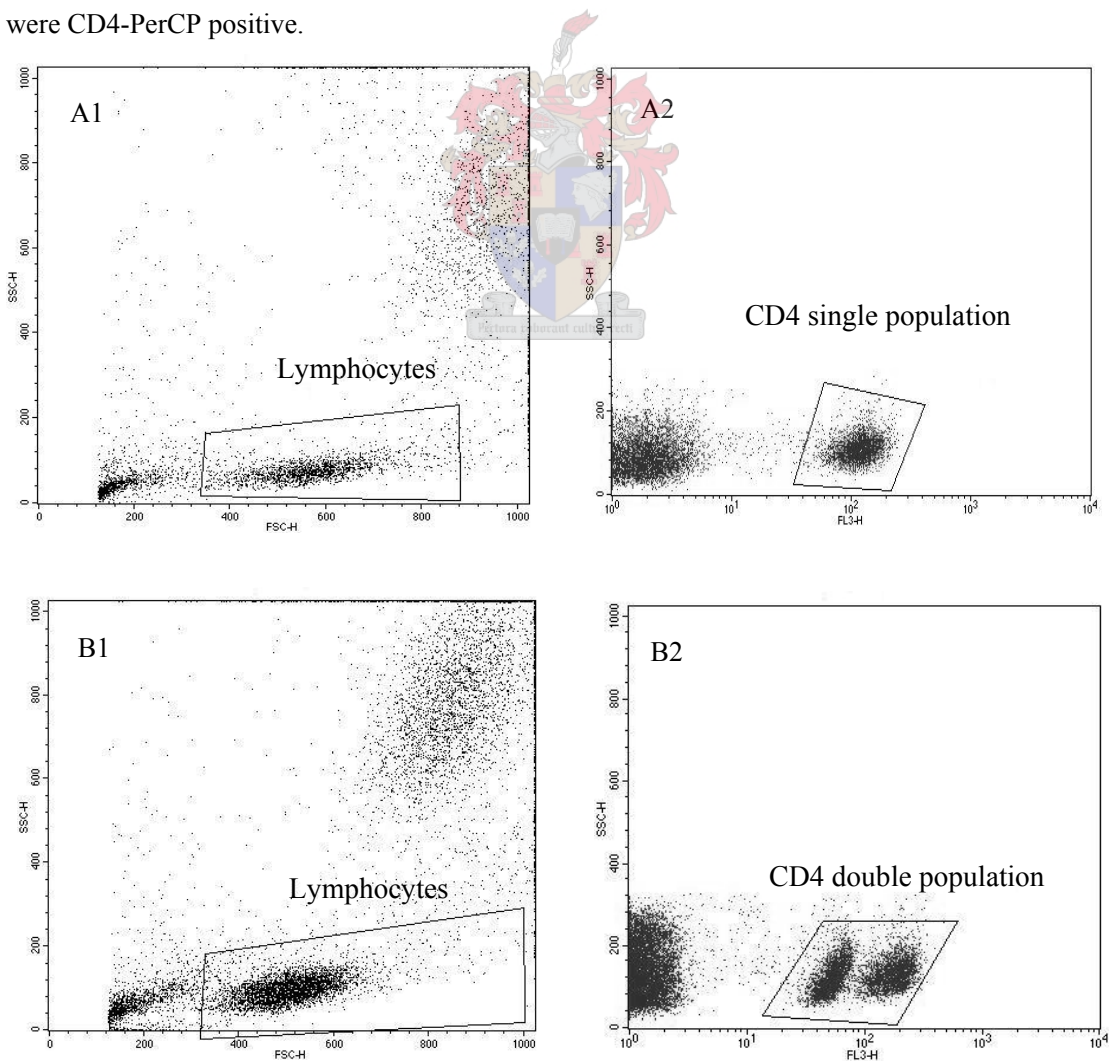
Table 5-2: Characteristic of sub-groupings of study subjects

| | Total (n) | CD4 Categories[*] | | |
		1	2	3
All subjects	58	12	27	19
ARV Treated	29	9	12	8
ARV Not treated	29	3	15	11
Co-infected with TB	10	0	4	6
Not co-infected with TB	48	12	23	13
CD4 double population	10	7	3	0
CD4 single population	48	5	24	19

*CD4 category was defined according to the CD4 count.

CD4 category 1: CD4 count greater than or equal to 500 cells/µl blood;

CD4 category 2: CD4 count 200-499 cells/µl blood;

CD4 category 3: CD4 count less than 200 cells/µl blood.

As summarized in Table 5-2, the number of antiretroviral treated and not treated subjects were same in the whole cohort (50% in either group) spread across the CD4 category. However, the percentage of treated subjects in CD4 category 1 group was greater than that of not treated, which indicated that the high CD4 counts (greater than or equal to 500 cells/µl blood) were artificially raised by antiretroviral drugs and accordingly, these individuals were falsely placed in Category 1 at the time of study. We did not attempt to correct for this by grouping these individuals according to their original baseline CD4 counts.

Co-infection with TB occurred predominantly in CD4 category 3 subjects, while there was no TB co-infection in category 1 patients. This suggested that the lower CD4 count indicative of disease progression, the higher the risk of the patient developing tuberculosis. Conversely, CD4 double population group were mostly in CD4 category 1, when CD4 counts were relatively high (mean CD4 count was 761 cells/µl blood). Further observation of this subgroup showed that all 10 subjects were not co-infected with TB, and all of them had low viral load (less than 50 copies/ml plasma or

undetectable) except one subject, whose plasma sample failed in the HIV viral load test (data not shown). These findings are interesting in that it implies that the presence of the double CD4 cell population in these patients would represent a beneficial apoptotic activity due to the immune cells increasing their self destruction in order to control viral replication. There was prior *in vitro* evidence showed that the inhibition of apoptosis (by anti-apoptotic agents) leads to increased viral replication (Antoni et al., 1995).

5.3 Comparison of apoptosis at different disease stages

5.3.1 Differences in levels of apoptosis at different disease stages

The 58 study subjects were classified into three groups according to the CDC's classification system. This classification system refers to the disease stages of HIV-1 infection, with those individuals with the lowest CD4 count having the most advanced disease. The results of Means ± SD are summarized in Table 5-3 below. These results do not take into consideration whether the patients are treated/non-treated, co-infected with tuberculosis or whether they exhibit the double CD4+ phenomenon.

The mean CD4 count in the CD4 category 1 (858 cells/μl) was the highest in the three categories, whereas the Log Viral Load (LOG VL) at this stage was the lowest in the same category. Statistical analysis (t-test for independent groups) confirmed that the differences in LOG VL between CD4 category stage 1 and 2, category stage 1 and 3 were statistically significant whereas this was no longer significant when categories 2 and 3 were compared.

The levels of apoptosis in CD8+ T cells were significantly higher at category 1 than category 2 ($p < 0.02$) when scatter method was used (scatter of CD8). However, when Annexin V binding assay was used to determine the level of apoptotic CD4+ T cells (CD4+AnnexinV+), it was significantly lower at CD4

category 1 than category 3 (p<0.02). These two parameters were mirror images at this category of disease stage (Table 5-3).

The levels of apoptosis in the memory subsets of CD4+ T cells as well as in the same subset of CD8 T cells (scatter of CD4 CD45RA- and scatter of CD8CD45RA- respectively) were increased significantly from category 1 to category 2 groups (p<0.02). On the other hand, CD95 expressions on CD8+ T cells (CD8+CD95+) were significantly highest at category 3 when compared to category 1 (p<0.02) and 2 (p<0.03) (Table 5-3).

Table 5-3: Comparing levels of apoptosis by measuring different apoptotic markers of different T lymphocytes at different CD4 categories.

	CD4 category 1 (n=12)	CD4 category 2 (n=27)	CD4 category 3 (n=19)
CD4 count (cells/μl) [a, b, c]	858±235	339±87	121±58
LOG VL [a, b]	1.62±0.36	2.88±1.71	3.87±1.96
CD4+AnnexinV+ (%) [b]	0.05±0.07	0.11±0.11	0.13±0.08
CD8+AnnexinV+ (%)	0.07±0.07	0.06±0.05	0.05±0.05
Scatter of CD4 (%)	6.85±3.77	4.75±2.58	5.47±4.20
Scatter of CD8 (%) [a]	8.96±5.20	5.65±2.96	5.36±4.73
CD4+ CD95+ (%)	4.18±6.49	3.02±5.11	4.61±7.17
CD8+ CD95+ (%) [b, c]	2.46±2.27	3.24±4.35	7.21±6.80
Scatter of CD4+ CD45RA- (%) [a]	58.40±15.09	71.84±15.42	69.81±16.84
Scatter of CD8+ CD45RA- (%) [a]	56.96±16.73	69.62±14.22	67.67±15.81
Scatter of CD4+ CD45RA+ (%)	8.46±3.66	7.95±10.58	8.11±4.73
Scatter of CD8+ CD45RA+ (%)	11.35±5.34	9.48±6.46	9.61±6.11

[a] significant between CD4 category 1 and 2, p<0.03

[b] significant between CD4 category 1 and 3, p<0.03

[c] significant between CD4 category 2 and 3, p<0.02

One way ANOVA analysis was used to compare the differences in apoptosis and CD95 expression between three categories. As shown in Fig 5-2 A, levels of apoptosis in CD4+ T cells (scatter of CD4) paralleled that of CD95 expression on cells surfaces (CD4+CD95+). Both of them had a "V" shape tendency throughout the disease which was high at category 1, then dropped at category 2 and finally at category 3 increased again. This was not statistically significant. However, the trend was different when the CD8 subset was examined. CD95 expression of CD8+ T cells were different with those of CD4+ T cells (Fig 5-2 B): this increased gradually from category 1 through to category 3 while, in parallel, the level of apoptotic CD8+ cells (as measured by scatter of CD8) gradually decreased in the same category. As indicated in Figure 5-2B, this was statistically significant.
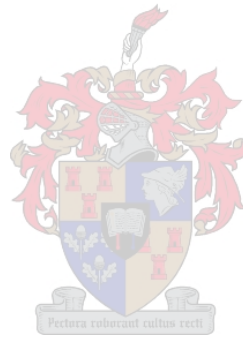
Fig 5-2: ANOVA analysis of Apoptosis (scatter assay) and CD95 expression on the CD4 and CD8 T lymphocytes at the different CD4/disease category.

(A) Scatter of CD4 cells and CD95 expression of CD4 T lymphocytes. The tendency was for the levels of apoptosis in CD4+ T cells (scatter of CD4, dashed line) and CD95 expression on CD4+ T cells (CD4+CD95+, solid line) was to exhibit a similar "V" shape, which was highest at category 1 and declined at category 2, but increased at category 3. (B) Scatter of CD8 cells and CD95 expression on CD8 T lymphocytes. Contrary to the above, the tendency was for the level of CD8+ apoptosis (scatter of CD8, dashed line) to decrease from one category to the next while the CD95 expression (CD8+CD95+, solid line) increased.

CD4 Category; LS Means
Wilks lambda=.93532, F(4, 106)=.90095, p=.46626

A

CD4 Category

CD4+ CD95+
scatter of CD4

CD4 Category; LS Means
Wilks lambda=.74076, F(4, 108)=4.3708, p=.00258

B

CD4 Category

CD8+ CD95+
scatter of CD8
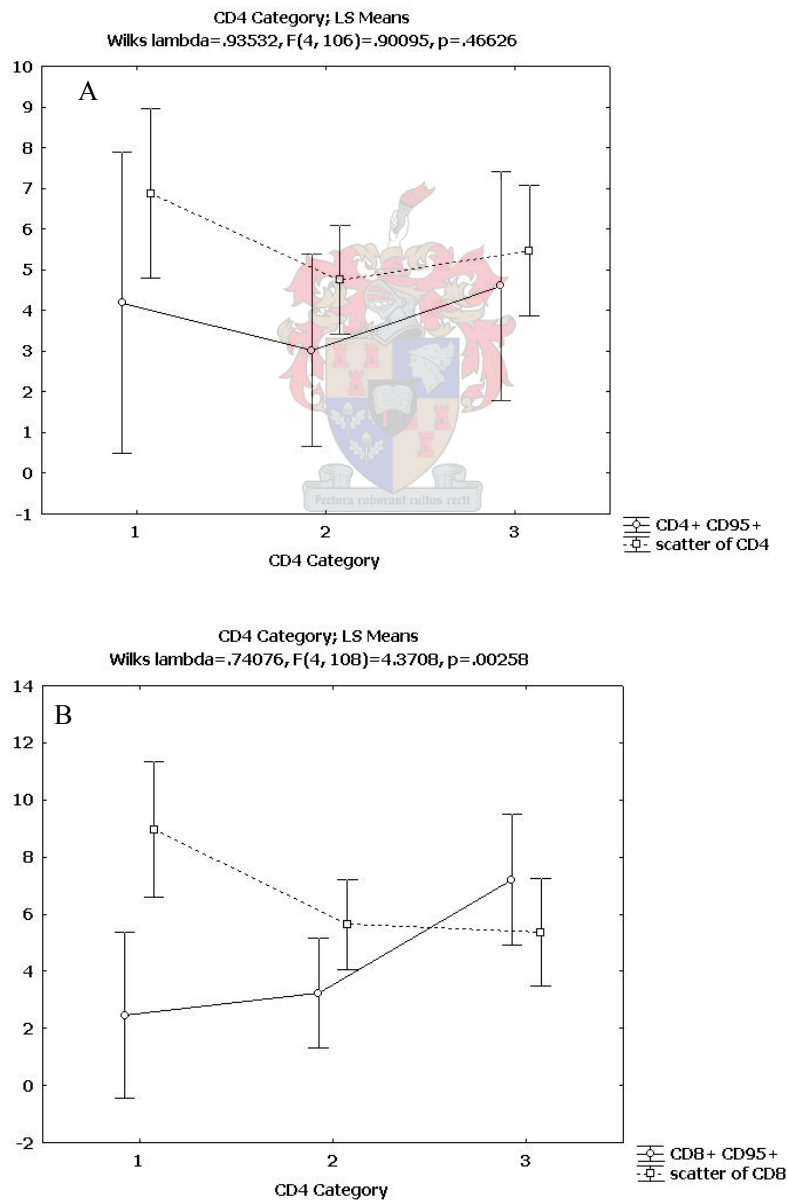
5.3.2 Differences in levels of apoptosis between CD4 and CD8 T lymphocytes

The differences in levels of apoptosis between CD4+ and CD8+ T cells varied depending on the CD4/disease category. Levels of Annexin V positivity in CD4 T lymphocytes (CD4+Annexin V+) were significantly higher than that in CD8 T lymphocytes (CD8+Annexin V+) at CD4 category 3 (p<0.005). Levels of apoptosis in memory CD4+ cells were higher than that in CD8+ cells at all stages, while naïve CD4+ cells were lower than that in CD8+ cells, although these differences did not reach statistical significance. However, levels of apoptosis in memory cells were significantly higher than that in naïve cells both in CD4 and CD8 T lymphocytes regardless of CD4 category (p<0.001).

5.3.3 Correlations between different markers of apoptosis at different disease stages

Correlations between different apoptosis markers were done respectively according to different CD4 categories. The scatterplots are presented in Figure 5-3. Each correlation is discussed individually:

    i. The CD4 counts were significantly correlated to levels of apoptotic CD4+ cells (CD4+ Annexin V+) within the CD4 category/disease stage 1 (p=0.001; Figure 5-3A). This is an interesting observation implying that those patients with the highest CD4 absolute counts would exhibit high levels of apoptosis (as measured by Annexin V binding). This will be further discussed at a later stage.

    ii. Secondly, the correlation between the levels of apoptotic activity (determined by the scatter-based method) within both subsets of T cells (CD4+ and CD8+) was strongly correlated within all disease categories (p<0.001). The Figure 5-3B shows the correlation within category 2 patient group.

    iii. Lastly, correlation between the CD95 expression on CD4 T

lymphocytes (CD4+CD95+) were found to be statistically significant when correlated to the level of apoptotic CD8 T lymphocytes as measured by AnnexinV binding (CD8+Annexin V+) within the disease/CD4 category 2 patient population (Figure 5-3C; p=0.015). Once again, this could represent an immune-induced mechanism of protection whereby the immune cells could show enhanced apoptosis in order to survive the viral replication. This will be discussed in depth later.
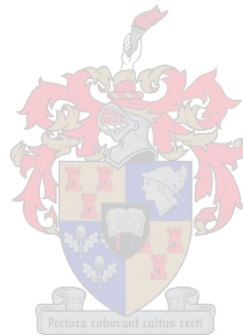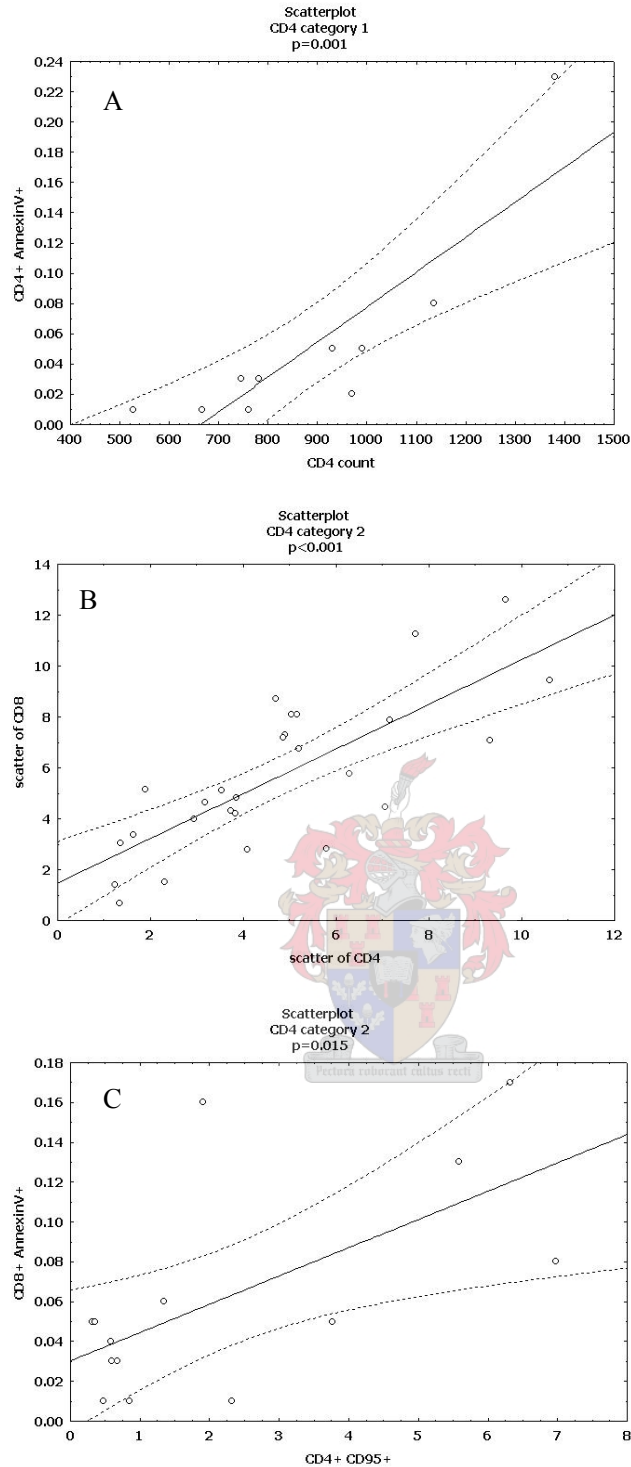
Fig 5-3: Scatterplots of correlation between different apoptosis markers.

(A) Level of apoptotic CD4+ T cells (Y-axis, CD4+AnnexinV+) was positively correlated to CD4 count (X-axis) at CD4 category 1 (p=0.001). (B) Scatterplot of correlation of CD4 apoptosis and CD8 apoptosis. Level of apoptotic CD8+ T cells (Y-axis, scatter of CD8) and CD4+ T cells (X-axis, scatter of CD4) were significantly correlated at CD4 category 2 (p<0.001). (C) Scatterplot of correlation between CD8 apoptosis and CD95 expression on CD4+ T cells. Level of CD8 apoptosis (Y-axis, CD8+AnnexinV+) correlated to level of CD95 expression (X-axis, CD4+CD95+) at category 2 (p=0.015).

5.4 Comparisons of apoptosis in antiretroviral treated and non-treated HIV-1 infected individuals

5.4.1 Differences between two groups irrespective of CD4 count (irrespective of disease category)

The means of LOG VL were lower in antiretroviral treated group than that in non-treated group, while the means of CD4 count were higher in treated group than that non-treated group, but the differences did not reach statistical significance (Table 5-4).

Table 5-4: Comparison means of Log viral load and CD4 count between treated and non-treated by *t*-test for independent groups.

	Treated (n=29)	Non-treated (n=29)	p value
LOG VL	2.50	3.37	p=0.076
CD4 count (cells/μl blood)	423	327	p=0.213

Similarly the mean levels of apoptotic CD4+, CD8+ T cells and their memory/naïve subsets determined by scatter method were slightly lower in treated group than that in the non-treated group, which again, this was not statistically significant.

5.4.2 Differences between treated and non-treated in CD4 category 2 group subjects

Since no significant differences were found between antiretroviral treated and non-treated groups in the whole group (5.4.1 above), the analyses were repeated in the CDC sub-groups. As shown in Table 5-2 above, the number of patients in the category 2 sub-groups were evenly balanced (12 antiretroviral treated subjects versus 15 non-treated subjects), which justified the analysis as two subgroups. The means of LOG VL and CD4 count were similar between the treated and non-treated sub-groups and this was not statistically significant (LOG VL: 2.57 versus 3.12 log, p>0.4, CD4 count: 333 versus 344 cells/μl blood, p>0.7). The

levels of apoptotic CD4+, CD8+ T cells and their naïve subsets determined by scatter method were lower in the treated group. However, it was different in their memory subsets, which was higher in treated group than in non-treated group. In addition, different with scatter method, Annexin V binding method to determine levels of apoptosis showed that levels of apoptotic CD4+ and CD8+ were higher in treated group than that in non-treated group, especially level of apoptotic CD8+ T cells, which was significant higher in treated group (p=0.021).

5.4.3 Differences between CD4 and CD8 T lymphocytes irrespective of disease category

The comparison of apoptosis (Annexin V assay) between the CD4+ and CD8+ T cells was analyzed in the treated and non-treated groups irrespective of CD4 counts and disease categories. As shown in Figure 5-4, the levels of apoptotic CD4+ T cells (CD4+ AnnexinV+) were significantly higher than that of CD8+ T cells (CD8+AnnexinV+) in non-treated group (p=0.012).
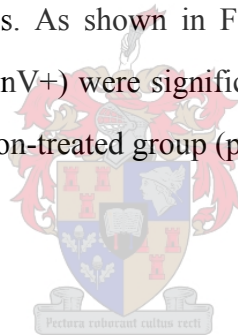
Fig 5-4: Boxplot representation of levels of apoptosis (Annexin V assay) of CD4 and CD8 T cells in non-treated group.

The mean level of apoptotic CD4+ cells were significantly higher than that of CD8+ cells in the non-treated group (p=0.012).
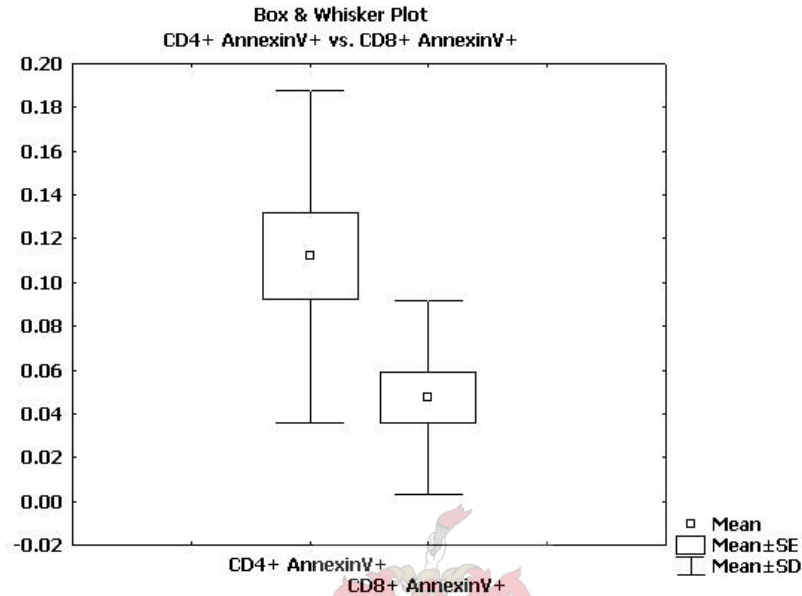
5.4.4 Correlations of markers of apoptosis in the treatment groups (treated versus non-treated)

Correlations between the variables were done between the treated (n=29) and non-treated (n=29) sub-groups. Each correlation will be discussed individually hereunder:

i. The LOG VL was inversely correlated to the level of apoptotic CD8+ T cells (scatter of CD8) both in treated (r = -0.505, p= 0.023) as well as in the non-treated group (r = -0.609, p=0.021).

ii. Levels of apoptotic CD4+ and CD8+ cells (scatter of CD4 and CD8) was strongly correlated regardless of whether the patients were being treated or not (r = 0.804, p<0.001).

iii. Furthermore, a positive correlation was found between CD95 expression on CD8+ T cells (CD8+ CD95+) and the level of apoptosis in CD4+ T cells (scatter of CD4) in the treated subgroup

within the CD4 category 2 (p=0.023, Figure 5-5). Once again, this could imply that the CD4 apoptotic activity could be linked to the CTL activity and this could represent a protective phenomenon while the immune system of the infected patient is relatively intact.

Fig 5-5: Scatterplot of correlation between the levels of CD4 apoptosis and CD95 expressions on CD8+ T cells in treated group.

Level of apoptotic CD4+ T cells measured by scatter method (Y-axis, scatter of CD4) and level of CD95 expression on CD8+ cells (X-axis, CD8+ CD95+) were significantly correlated in antiretroviral treated group when CD4 count lies between 200 and 499 cells/μl blood.

Scatterplot ,treated
CD4 category 2
p=0.023
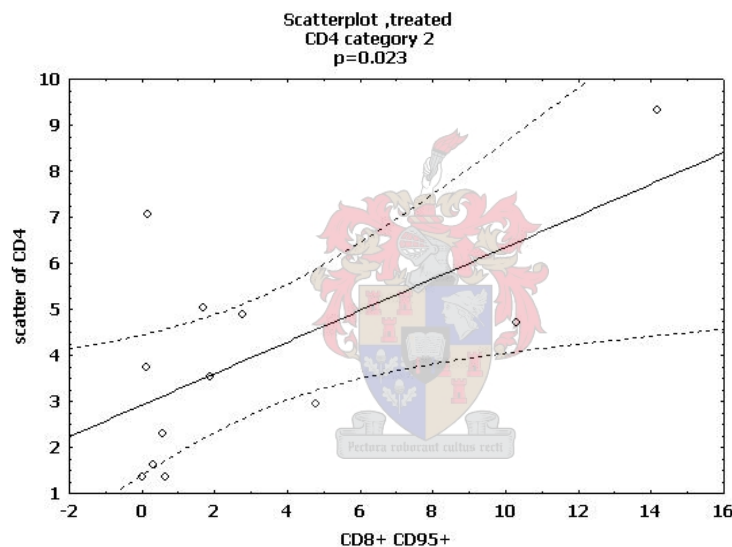
5.5 Comparisons of apoptosis in HIV-1 infected patients co-infected with TB

5.5.1 Differences between the two groups (irrespective of disease category)

As already shown in Table 5-2 above, there were 10 HIV-1 infected individuals co-infected with tuberculosis. When they were compared to the remaining 48 HIV-1 patients without TB, a significantly higher level of LOG VL was found in co-infected with TB group than in those non-co-infection group (mean LOG VL: 4.31 versus. 2.65 log, p=0.007) was observed. Moreover, a significant difference in the CD4 count was also observed: HIV-1 co-infected with TB group had lower CD4 counts than those patients without TB group (p=0.041, Fig 5-6). Again, this

re-enforces the observation that HIV-infected patients with the lowest CD4 counts run the highest risk of developing one of the AIDS-defining opportunistic infection.

The t-test analysis also showed that the levels of apoptotic CD4+, CD8+ T lymphocytes and their memory/naïve cells were lower in co-infected patients than in those patients without TB. However, these differences did not reach statistical significance (data not shown).

Fig 5-6: Difference in CD4 count between HIV-1 with TB and without TB co-infection.

The mean CD4 count in HIV without TB infection group was 411 ±306 cell/µl, while it was 203 ±103 cells/µl in HIV co-infection with TB group. ANOVA analysis showed a significant lower CD4 count in HIV-1 co-infection with TB (p=0.041).
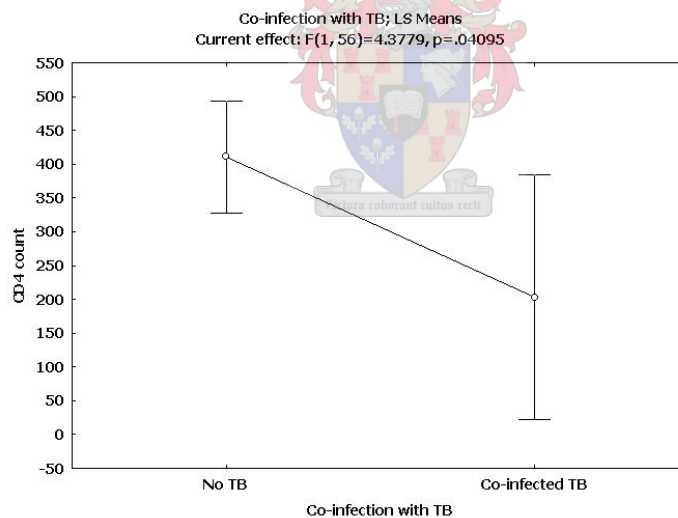
As Table 5-2 indicated, the sample sizes of subgroups of co-infection with TB in CD4 category 2 and 3 were too small; no further analyses could be conducted in these subgroups. This is unfortunate since this would have yielded interesting observations.

5.5.2 Correlations of the markers of apoptosis in the two groups (co-infected with TB versus no co-infection)

According to the correlation analyses, the LOG viral load was inversely correlated to the levels of apoptotic CD4+ and CD8+ T lymphocytes in HIV-1 without TB co-infected group. Both of these correlations were highly significant ($p<0.001$, $p=0.002$, respectively). This was not found in the co-infection with TB group. Again, this implies the viral replication is controlled by a high apoptotic activity. Secondly, the correlation analysis found that in the HIV-1 TB-co-infected patients, the levels of CD8+ CD95 expressing cells correlated to levels of apoptotic memory CD4+T cells ($p=0.014$) and CD8+ T cells ($p=0.027$). This is represented in Figure 5-7 hereunder. Once again, this would implicate the role of CD8+ cells in the apoptotic process of CD4+ cells.

Fig 5-7: Scatterplot of correlation between CD95 expressions on CD8+ T cells and levels of apoptotic memory subset of CD4+ cells in co-infected with TB patients. Level of apoptotic memory cells of CD4 T lymphocytes (Y-axis, scatter of CD4 CD45RA-) was correlated to level of CD95 expression on CD8+ T cells (X-axis, CD8+CD95+) of in HIV-1 co-infection with TB group ($p = 0.014$).
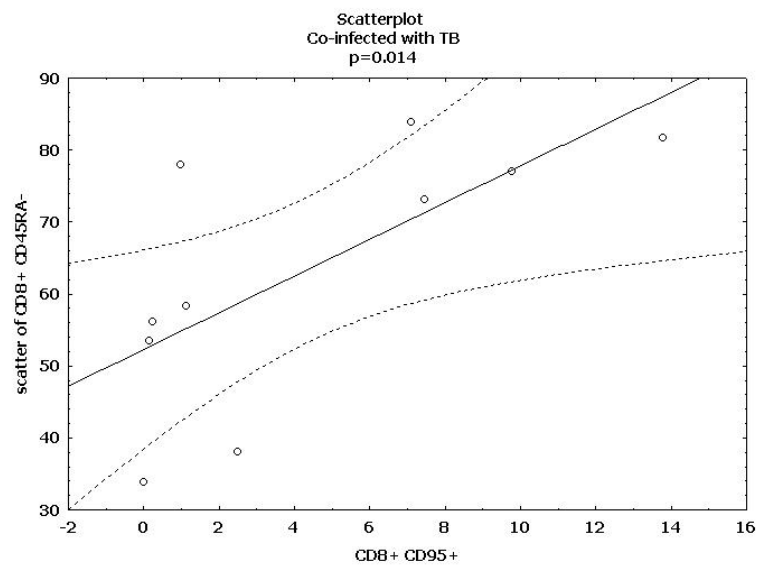
5.6 Comparisons of apoptosis in individuals with CD4 single population and those with CD4 double population

5.6.1 Differences between the two groups (irrespective of disease category)

As described earlier in the description of the study subjects, the group showing the CD4 double population had higher CD4 count and lower viral loads than those with a single CD4 cell population. These differences were confirmed as statistically significant by ANOVA ($p<0.001$, $p=0.006$, CD4 count and Log VL respectively; Figure 5-8). This is a most interesting observation: it would imply that the presence of double CD4 positive cells is an indication of a physiological response to the virus: CD4 cells are actively undergoing apoptosis in order to control the viral replication. In other words, apoptosis may in fact be a protective phenomenon but only at certain stages of the infection. This is discussed in depth at a later stage.

Furthermore, when comparing the levels of apoptosis between the two groups, the CD4 double population sub-group had higher percentages of apoptotic cells both in CD4 and CD8 T lymphocytes ($p<0.006$ and $p<0.004$ respectively). This was observed when the degree of apoptosis was determined by the scatter method.

Fig 5-8: Differences in CD4 count and Log Viral load between CD4 double and single population groups.

(A) CD4 double population group had a significantly higher CD4 count than those with a single population group (mean ±SD: 761±363 versus 295±203 cells/μl, p<0.001). (B) LOG VL was significantly lower in the CD4 double population group than in those patients with a single CD4+ population group (1.46±0.13 versus 3.20±1.80 log, p=0.006).
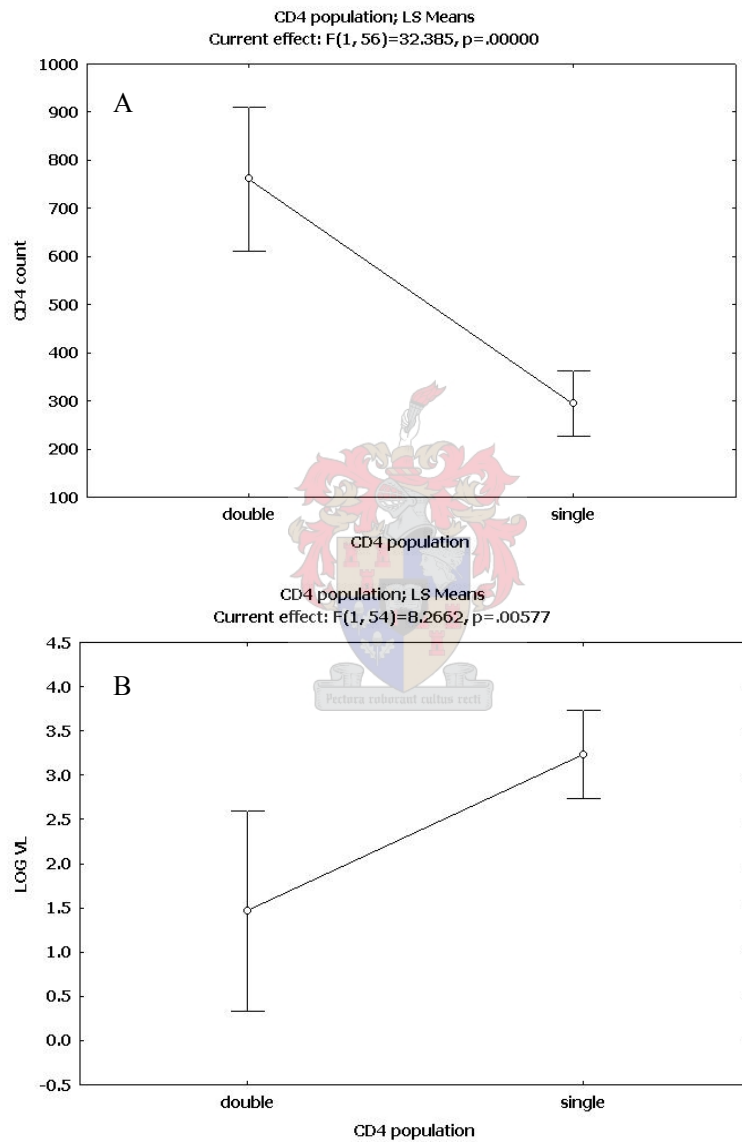
5.6.2 Correlations between the two groups (single CD4 population versus double positive population)

When the CD4 cell counts were taken into consideration, the correlations in the two groups (double CD4 cell population versus single population) were quite different.

i.   Firstly, in the CD4 single population group, it was found that LOG VL inversely correlated to CD4 count (p=0.004, Figure 5-9).

ii.   Secondly, the levels of apoptotic CD4+ and CD8+T cells determined by the scatter method was also correlated to the presence of single positive CD4 cells (scatter of CD4 and scatter of CD8, p=0.013 and p=0.001, respectively). Furthermore, levels of CD95 expression on CD4+ T cells significantly correlated to that on CD8+ T cells (p<0.001) in this sub-group of patients. Although similar correlations were found in CD4 double population group, this was not statistically significant.

iii.   Thirdly, in those patients exhibiting the double CD4 population, levels of apoptotic CD4+ and CD8+ T cells determined by Annexin V binding (CD4+AnnexinV+ and CD8+AnnexinV+) were significantly and inversely correlated (p=0.009, Figure 5-10). Levels of CD95 expression on CD4+ T cells were positively correlated with levels of apoptotic CD8+ T cells (p<0.001).

Fig 5-9: Scatterplot of correlation between CD4 count and LOG VL in CD4 single population group.

LOG VL (Y-axis) inversely correlated to CD4 count (X-axis) in the sub-group exhibiting the single CD4 cell population (p=0.004).
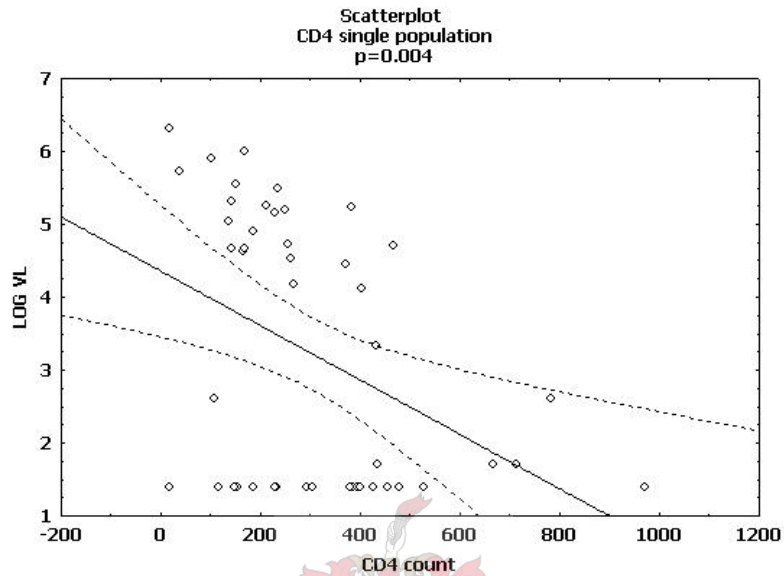
Fig 5-10: Scatterplot of correlation between levels of apoptosis in CD4+ and CD8+ cells in CD4 double population group.

Level of apoptotic CD8+ T cells (Y-axis, CD8+AnnexinV+) was inversely correlated to that of apoptotic CD4+ T cells (X axis, CD4+AnnexinV+) in CD4 double population group (p=0.009).
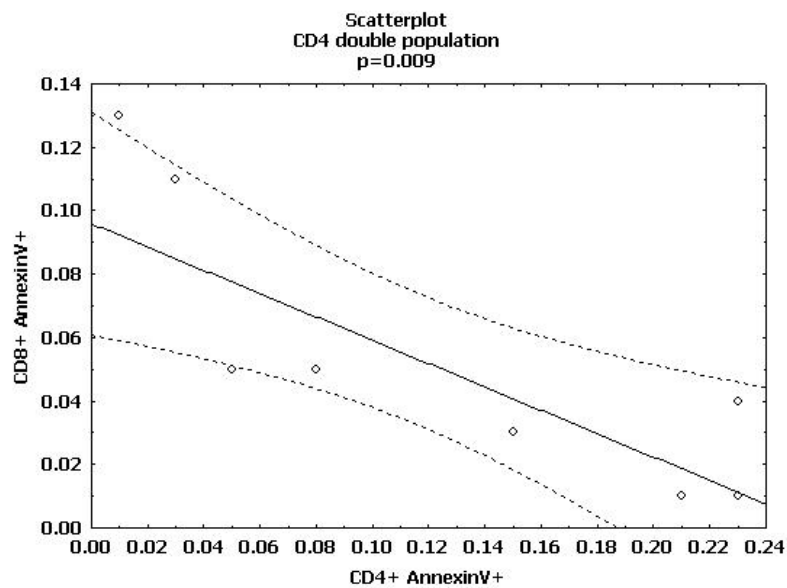
5.6.3 Focusing on CD4 double population.

The presence of certain patients exhibiting the double CD4 cell population made it imperative to examine this sub-group of patients in depth. The following flow cytometric graphs (Figure 5-11) clarified the different expression of CD4-PerCP and the difference in Annexin V binding between the two subpopulations of CD4 T cells, which were CD4^{dim} and CD4^{bright}. It appeared that the apoptotic activity, whether measured by Annexin V binding or CD95 expression was significantly higher in the CD4^{dim} population compared to those expressing higher levels of CD4 marker. The levels of apoptosis measured within the total CD4 gate was contributed predominantly by the apoptotic activity in the CD4^{dim} gate.

Similarly, further analysis of the data made it clear that levels of apoptosis in CD4^{dim} were higher than those in CD4^{bright} sub-population when the alternative assays of apoptosis were conducted in parallel. The CD4^{dim} T cells expressed more CD95 than the CD4^{bright} T cells. Analysis by *t*-test also confirmed that the differences between CD4^{dim} and CD4^{bright} were significant (Fig 5-12).
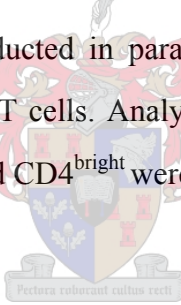
Fig 5-11: Analysis of apoptosis in CD4 and its sub-populations.

Flow cytometric graphs of gating total CD4 (A), CD4 ^{dim} (C) and CD4^{bright} (E) populations. From graph A, the total CD4 population consisted of two separate sub-populations. Both of them were stained positive with CD4-PerCP (FL3-H), however, the sub-population on the left expressed CD4 dimly, while the one on the right expressed CD4 brightly. When FITC-labelled Annexin V was applied to analyse the levels of apoptosis in these subpopulations (B, D, F), the CD4 ^{dim} cell population had the higher percentage of AnnexinV+ cells than that of CD4^{bright} cell population.
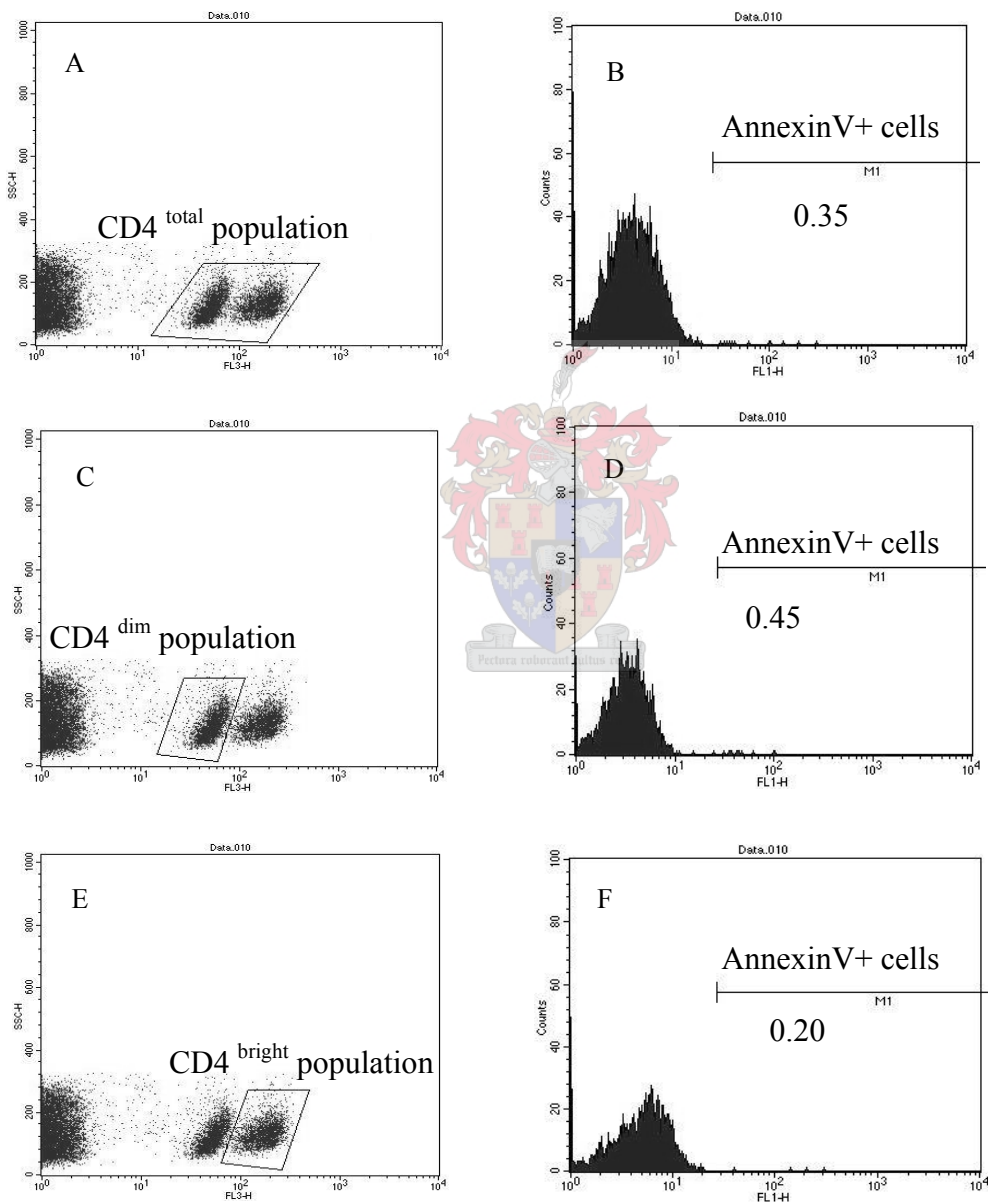
Fig 5-12: Comparison of differences in levels of apoptosis and CD95 expression between the two CD4 subpopulations (n=10).

(A) CD4^{dim} cells had significant higher level of apoptosis than CD4^{bright} cells (p=0.002). (B) CD4^{dim} cells had significant higher level of CD95 expression than CD4^{bright} cells (p=0.038).
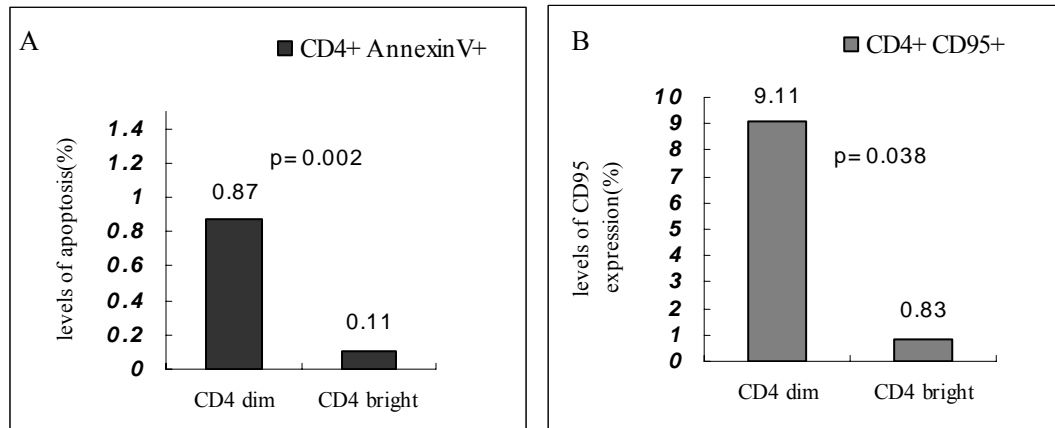
5.7 Summary

The study was undertaken with the idea that different methods of measuring apoptosis (such as fluorescent labelled Annexin V binding, flow cytometer scatter-based method or fluorescent labelled CD95 antibody) could yield different information concerning differences between different cell populations and different sub-cohorts of HIV-1 infected subjects. The data generated can be summarised as follows:

a) The CDC defined CD4 cell categories, which reflects the immune status of the host's immune system (hence the disease stages), had an interesting effect on apoptosis. The level of apoptotic CD4+ T cells measured by the scatter method was high at CD4 category 1, decreased during category 2 disease stage and finally increased again at category 3. This tendency was paralleled by the data generated using the CD95 (Fas) expression on CD4+ T cells. The curve formed a "V" shape according to the three CD4 categories. On the other hand, the marker of viral replication (LOG viral load) gradually increased from CD4 category 1 to category 3. The V-shape of

apoptosis measurement would imply that this cellular activity was high during the initial stage of infection, possibly representing the immune response trying to eliminate infected cells as a means of survival (beneficial effects). The high levels of apoptosis at stage 3 of the infection could represent a chaotic phenomenon accounting for the rapid loss of CD4 cells as the disease progresses to full blown AIDS. At this late stage of infection it is known that many mechanisms (viral replication, auto-immune process, innocent bystander cell loss, etc) may account for the total cell loss.

b)   A novel finding of this study was the presence of a double CD4+ population seen in 10 HIV-1 infected subjects. Among them, 7 were in CD4 category 1 and 3 were in CD4 category 2. All of them had low viral load, which was undetectable or less than 50 copies/ml plasma. These subjects had two distinct and separated CD4 populations, both of which were expressing CD4: one was dimly positive while the other was brightly expressing the membrane marker. These cells, based on their SSC characteristics and the fact that they were gated within the total lymphocyte population could not be any other blood cell known to express the CD4 marker (for instance monocytes). On the other hand, because we used CD4-PerCP antibody to gate CD4 population within lymphocytes, which was more specific than using CD45-antibody and made it possible to display this CD4^{dim} subpopulation. This can explain why these two CD4 populations have never been described before. Furthermore, five of these 10 study subjects were on HAART while the other 5 were not. We could therefore not conclude that the presence of two CD4 populations associated with treatment. Follow-up studies should be conducted in order to determine if this is a marker for Long Term Non-progressors.

Statistical analysis showed that the subjects who exhibited the double population had significantly higher levels of apoptosis in CD4 and CD8 T lymphocytes as well as their naïve subsets as measured by the scatter method than those subjects having a single CD4 population. In addition, when comparing the two CD4 subpopulations, it was found that CD4^{dim} cells had significant higher level of apoptosis and CD95 expression than the CD4^{bright} cells. Interestingly, in an *in vitro* study, which used cultured lymphocytes to detect apoptotic cells suggested that apoptotic lymphocytes displaying a CD45^{dim} phenotype (Carbonari et al., 1994). As the cell preparation and flow cytometer analysis (cell population gated) were different, these CD4^{dim} cells are apoptotic cells or other phenotype are not certain, further studies should be prospected.

c) Co-infection of HIV-1 positive patients with tuberculosis occurred predominantly in the CD4 category 2 and 3 group. The mean CD4 count of this co-infection group was 203 cells/µl blood, which was significantly lower, while LOG VL was higher than non-co-infection group. There was no significant difference in the levels of apoptosis and CD95 expression on CD4 and CD8 T lymphocytes between the two groups. This higher LOG VL in co-infected patients implies that the mycobacterial infection could enhance viral replication.

d) When antiretroviral treatment was used to classify the study subjects, no significant differences were found between treated and non-treated group. When applying the disease categories to the data, it was found that the subgroups of treated and non-treated in CD4 category 2 patients yielded significant correlations: although lower levels of apoptosis (as measured by the scatter method as well as

CD95 expression) in the CD4 and CD8 T subsets were found in the treated group when compared to the non-treated group, these findings still did not reach statistical significance. One puzzling finding was that the level of apoptotic CD8+ cells measured by FITC-labelled Annexin V was significantly higher in treated group. Whether this represents the recovery of CTL activity post-initiation of therapy, we cannot conclude at present.

Correlations between the various apoptosis markers analysed in the different sub-cohorts of subjects also showed interesting findings: there was an inverse correlation between the plasma viral load (LOG VL) and the CD4 count, implying that CD4 depletion associates with viral replication. This was even more evident in the cohort of patients exhibiting the CD4 single population: the worse off the patient was immunologically speaking (lower CD4 cell counts), the higher the viral replication. Moreover, the level of apoptotic CD4+ T cells (measured by Annexin V positivity) correlated to the absolute CD4 count in CD4 category 1 group, when the CD4 count was relatively high. This would lead us to believe that the high apoptotic activity is possibly protective and that it is the immune system's way of controlling the viral replication. The protective characteristic of apoptosis is indirectly indicated by other viruses, such as Epstein – Barr virus (EBV), which can protect infected cell from apoptosis (Henderson et al., 1991). Consequently, in our study, the LOG VL was inversely correlated to the level of apoptotic CD4+ and CD8+ T cells as measured by the scatter method in most groups. Lastly, another interesting significant correlation was found between the levels of CD4+CD95+ and the levels of apoptotic CD8+ T cells (measured by Annexin V binding) in CD4 category 2 group individuals, as well as the non-treatment group and those patients exhibiting the double CD4 cell population. Once again, we are tempted to speculate that those patients having the two CD4 cell populations maintain the integrity of their immune system by allowing apoptosis to take place (CD95 expression of the cell surface). The introduction of therapy may inhibit this process. We do not have sufficient data to be able to

conclusively state whether this is clinical relevant as far as disease prognosis is concerned. However, the data generated would seem to suggest that apoptosis may in fact be a protective activity especially during the early stage of the infection: at this stage it may be a controlled process. The high levels of apoptosis observed during the late stage of infection, may be a chaotic and non-controlled process leading to the total destruction of the host's immunity.

The presence of two CD4 populations in individuals with the higher apoptotic activity and lower viral loads implies that the immune cells are able to regulate the viraemia by self-destroying; they effectively remove the biochemical pathways necessary for on-going viral replication. The question whether this could be a marker of Long-Term Non-Progressors is impossible to answer at present. Further long term follow-up studies are required but it is nevertheless tempting to speculate that this could indeed be a marker of non-progression.

We may conclude that in this cohort of adult HIV-1 infected patients, the levels of apoptotic activity were different with respect to different sub-cohorts of HIV-1 infected subjects, especially disease stages. The novel finding of the double CD4 cell population may also indicate disease stages and needs further in depth study in larger groups of patients.

CHAPTER 6

MEASUREMENT OF APOPTOSIS IN
HIV-INFECTED CHILDREN

6.1 Study design

According to the UNAIDS/WHO report, an estimated 700,000 children became infected with HIV-1 and 570,000 died of AIDS in 2005 (UNAIDS/WHO report 2005). HIV-1 infection in children is becoming a crisis to the whole world because the disease progresses more rapidly and children develop opportunistic infections at higher CD4 count than in adults (Leibovitz et al., 1990).

Since potential antiretroviral treatment is also widely used in HIV-1 infected children, this present study sought to investigate the long-term as well as short-term effects of antiretroviral treatment in HIV-1 infected children. In addition, long-term treatment, using different regimens were also compared. The effects included virological and immunological changes after treatment. The virological change was determined in the plasma viral load, while the immunological changes were studied in apoptosis of lymphocytes. Two different cohorts of HIV-1 infected children as well as different observations were involved in this study.

The first cohort consisted of a sub-study of a cohort of 14 HIV-1 infected children followed up over a period of 12-months. The 14 study subjects had been enrolled in another clinical trial which was initiated in 2002. The mean age at the time of enrolment was 3.9 years (range from 1 to 7 years old). They were enrolled in this present study at the time of their routine visits at clinics, which were considered as "visit 1" for this study, during February to May in 2004. The second visit (visit 2) in this study was 12 months after first the visit. The long-term effects of antiretroviral treatment were determined by measurement of their viral load, CD4 count as well as in the apoptotic activity in different cell populations, such as CD4+ T cells, total

lymphocytes and total PBMCs. As to the measuring methods of apoptosis, both FITC-labeled AnnexinV and scatter methods were employed in the study. In addition, the TUNEL method, which was to detect DNA fragmentation (late apoptosis marker), was also applied in the study.

The second sub-study included a cohort of 5 HIV-1 infected children over a follow-up period of 1-month in order to determine the early effects of antiretroviral treatment. The mean age of this group was 3.4 years (range 2 to 6 years). They were enrolled in this sub-study when they started antiretroviral treatment, which were considered as "baseline" visits, during March to May in 2005. The second visit was one month after treatment considered as "visit 1". Similar to the 12-months follow-up sub-study, viral load and CD4 count were also studied in this 1-month sub-study. In addition to the measurement of apoptosis in the CD4+ T cells and PBMCs, apoptosis was also measured in the CD8+ T cell subset as well as in their memory/naive subsets. Moreover, CD95 expression on CD4+ and CD8+ T cells were used to study the Fas/FasL pathway of apoptosis. However, in this 1-month study, FITC-labeled AnnexinV was not used due to modification of the concentration of reagents between "baseline" and "visit 1". Flow cytometer based scatter and TUNEL method were applied in the determination of the levels of apoptosis in the different cell subsets.

6.2 Long-term effects of antiretroviral treatment (Cohort 1)

6.2.1 The characteristics of study subjects

All of the 14 12-months follow-up HIV-1 infected children were on triple combination of antiretroviral treatment. Six of them were receiving regimens containing 2 NRTIs plus 1 NNRTI, the other 8 had combination of 2 NRTIs plus 1 PI. When they enrolled in this present study, 12 of them had been on treatment for 18 months, 1 of them for 12 months, and 2 of them for 6 months. Both the virological and immunological status was determined at enrolment in our study. Table 6-1 summarizes of the characteristics of the study subjects at enrolment. Six

of the 14 children had undetectable viral load levels (less than 50 copies/ml plasma), while 3 still had higher than 100,000 copies/ml plasma VL's after 18 months of anti-retroviral treatment. The immunological categories were based on age-specific CD4+ T lymphocytes count and percentage of total lymphocytes, which was according to CDC's classification system for children less than 13 years age (CDC, 1994). As described in Table 6-1, no subjects had severe immunological suppression, which indicates that the CD4 count and percentages of total lymphocytes were relatively high after 18 months of treatment.

Table 6-1: Virological and immunological status of the cohort of 14 children enrolled in the 12-months follow-up study.

The data represented their virological and immunological status subjects at enrolment.

		Visit 1
Viral load (copies/ml plasma)[&]	Undetectable	n=6
	<10,000	n=3
	10,000-100,000	n=2
	>100,000	n=3
Immunological categories*	No suppression	n=8
	Moderate suppression	n=6
	severe suppression	/

[&]Viral load were defined as HIV-1 RNA in plasma.
*Immunological categories were defined as age-specific CD4+ T lymphocytes count and percentage of total lymphocytes (CDC, 1994).

6.2.2 The long-term effects of antiretroviral treatment

The cut-off time of the study was 12 months after visit 1, considered as "visit 2". During visit 1 and visit 2, all subjects were continuously receiving antiretroviral treatment.

6.2.2.1 Virological effect

After 12 months of antiretroviral treatment, the viral loads of the three subjects who had greater than 100,000 copies/ml plasma at the study start,

dropped at different rates. All the subjects (except one) with undetectable viral loads at the start remained at undetectable levels: the one patient increased to higher than 10,000 copies/ml plasma at visit 2. Two viral loads, which were less than 10,000 copies/ml plasma, decreased to undetectable levels. Nevertheless, repeated measures ANOVA indicated there was no significant differences in two means of LOG VL between visit 1 and visit 2 (p=0.406). Wilcoxon matched pairs test (Non parametric test) also showed a non-significant difference (p=0.314).

6.2.2.2 Immunological effects

Number of lymphocytes

The mean CD4 count of the group as a whole at visit 1 was 1301 cells/μl blood while it was 1930 cells/μl blood at visit 2. The mean CD4 percentages between visit 1 and visit 2 did not differ from each other (26% and 27% for visit 1 and 2 respectively). However, as shown in Figure 6-1A, the absolute CD4 count was significantly higher after 12 months of treatment (p<0.001). Also, in parallel, the mean CD8 count significantly increased from 2532 to 3172 cells/μl blood between visit 1 and visit 2 (p=0.037). However, CD8 percentage significantly decreased from 48% at visit 1 to 40% at visit 2 (p<0.001, Fig 6-1B). These data indicated that antiretroviral treatment was beneficial to immune reconstitution and that at visit 2, the patients were less immune suppressed.

Fig 6-1: ANOVA analysis of difference in number of lymphocytes between two visits.

(A) The mean CD4 count was significantly increased from visit 1 to visit 2 (mean±SD: 1302±601 versus 1930±610 cells/µl, p<0.001). (B) The mean CD8 percentage was significantly decreased from visit 1 to visit 2 (mean±SD: 48±10 versus 40±11%, p<0.001).
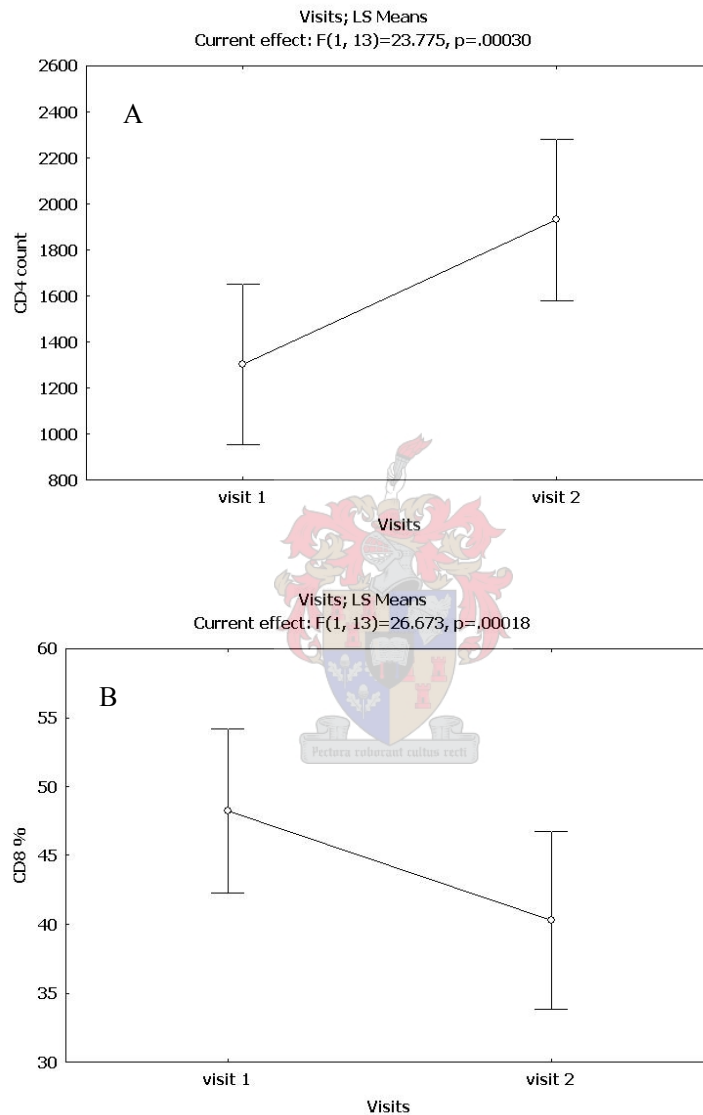
Levels of apoptosis in CD4+ T cells

The levels of apoptosis in the CD4+ T cells were measured by FITC-labelled AnnexinV and flow cytometric based scatter methods. ANOVA analysis showed a difference in the two measurements (Figure 6-2). Although not statistically significant (p>0.1), the level of apoptosis in CD4+ T cells

measured by the AnnexinV binding assay (CD4+AnnexinV+, Figure 6-2 A) decreased after 12 months of treatment. However, in the same cell populations, the scatter method indicated that the level of apoptotic CD4+ T cells (scatter of CD4) was significantly increased after treatment ($p<0.004$, Figure 6-2 B).

Fig 6-2: ANOVA analysis of differences in levels of apoptosis between two visits.
(A) The level of apoptotic CD4+ T cells measured by FITC-labelled AnnexinV (CD4+AnnexinV+) decreased from visit 1 to visit 2 (mean±SD: 0.47±0.71 versus 0.17±0.29%). However, the difference between them was not statistically significant ($p=0.140$). (B) The level of apoptotic CD4+ T cells measured by scatter method (scatter of CD4) was significantly increased from visit 1 to visit 2 (mean±SD: 6.94±3.10 versus 12.17±4.08%, $p=0.004$).

Visits; LS Means
Current effect: $F_{(1, 13)}=2.4741$, $p=.13975$

Visits; LS Means
Current effect: $F_{(1, 13)}=11.933$, $p=.00427$
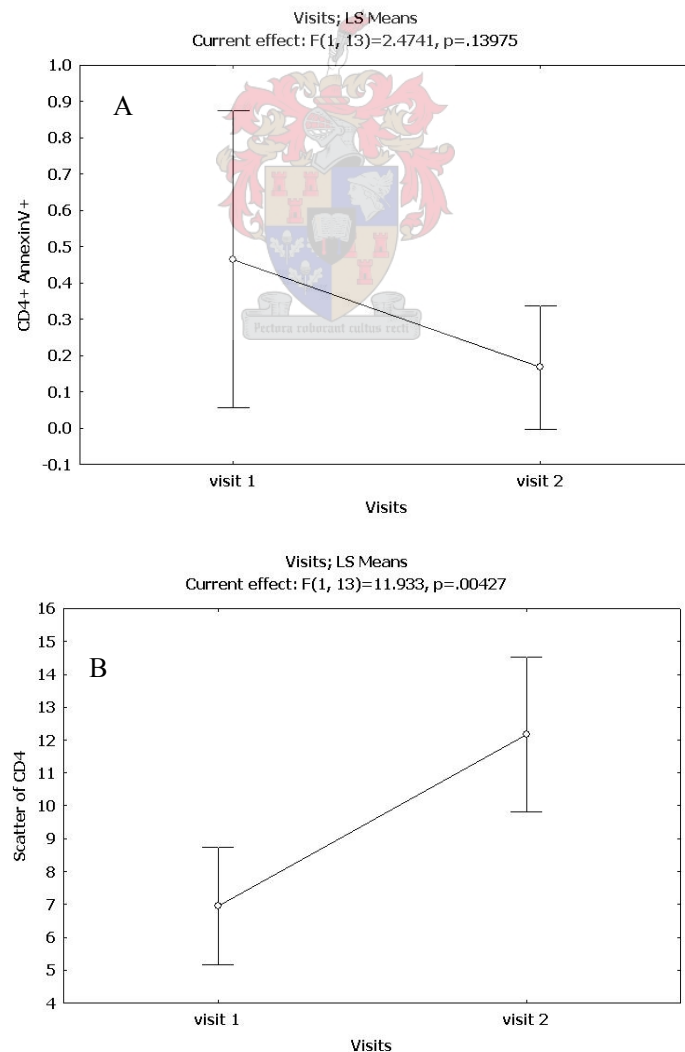
Levels of apoptosis in PBMCs

The levels of apoptotic PBMCs were measured by the TUNEL method, which was to detect DNA fragmentations during cell apoptosis (late event apoptosis). The following dot plots (Figure 6-3) represents the levels of apoptotic PBMCs measured by TUNEL method at two time point in the same subject: Figure 6-3A measured at visit 1 and Figure 6-3B, measured at visit 2. The mean level of apoptotic PBMCs of all the subjects was 4.84% at visit 2, which was slightly higher when compared to the value at visit 1 (4.00%). However, this change did not reach statistical significance (p=0.492, data not shown).

Fig 6-3:   A representative example of the levels of apoptosis at visit 1 and visit 2 in the same individual.

The percentages of apoptotic cells were calculated as events in R3 divided by total events (R2+R3). According to this case, apoptotic level dropped only slightly (from 4.28% at visit 1 to 4.09% at visit 2) after treatment.
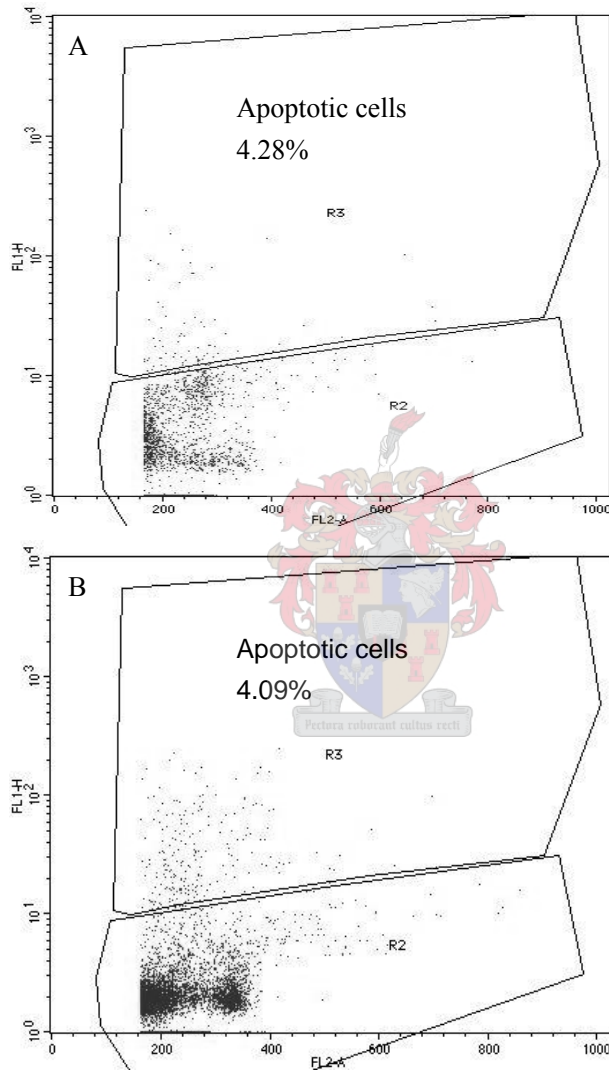
Levels of apoptosis in total lymphocytes

The total lymphocytes include CD4+ and CD8+ T cells, B cells as well as NK cells. All of them might be undergoing apoptosis during HIV-1 infection. Apoptotic total lymphocytes were measured by the scatter method. ANOVA repeated measures showed that the level of apoptosis in the total

lymphocyte gate significantly increased from 11.55% to 16.76% (p=0.025, Fig 6-4).

Fig 6-4: ANOVA analysis of difference in level of apoptotic total lymphocytes between two visits.

The level of apoptosis in total lymphocytes population was significantly increased from visit 1 to visit 2 (mean±SD: 6.94±3.10 versus 12.17±4.08, p=0.025).
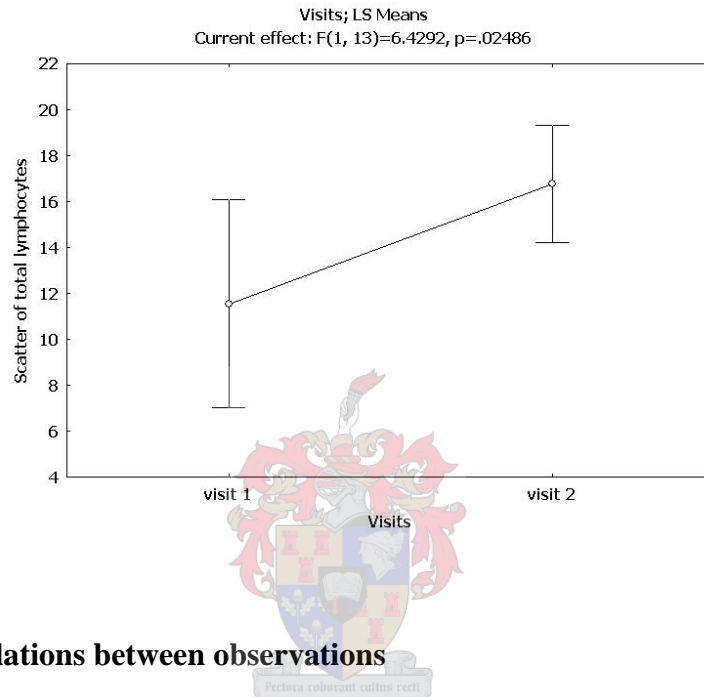
6.2.3 Correlations between observations

With respect to the possible influences of antiretroviral treatment on various parameters, the correlations between the measurements were investigated at different time points (visit 1 and visit 2, respectively). At the time of visit 1, a significant correlation was found (p=0.003, r = 0.738, Figure 6-5 A) between CD4 count and levels of apoptotic CD4+ T cells measured by Annexin V (CD4+AnnexinV+). This correlation seems to indicate that the levels of apoptosis are directly related to the absolute CD4 counts implying that the higher apoptotic activity was responsible for viral replication control hence leading to the higher CD4 counts. In addition, an inverse correlation between CD8 percentage and level of CD4+ AnnexinV+ was also found (p=0.021, r = -0.609, Figure 6-5B). This again reinforces our hypothesis that the level of apoptosis is CD8 mediated and indirectly leads to viral control (hence increased CD4 counts). However, both

of these significant correlations were not observed at the time of visit 2. It was found at visit 2 that both CD8 count and percentage were inversely correlated to level of apoptotic total lymphocytes (p=0.026 and p=0.036, respectively).

Fig 6-5: Scatterplot of correlation between lymphocytes and level of apoptotic CD4+ T cell at visit 1.

(A) The level of apoptosis in CD4+ T cells (Y-axis, CD4+AnnexinV+) positively correlated to its absolute number (X-axis, CD4 count) at visit 1 (p=0.003, r = 0.738). (B) The level of apoptosis in CD4+ T cells (Y-axis, CD4+AnnexinV+) inversely correlated to CD8 percentage (X-axis, CD8%) at visit 1 (p=0.021, r = -0.609).
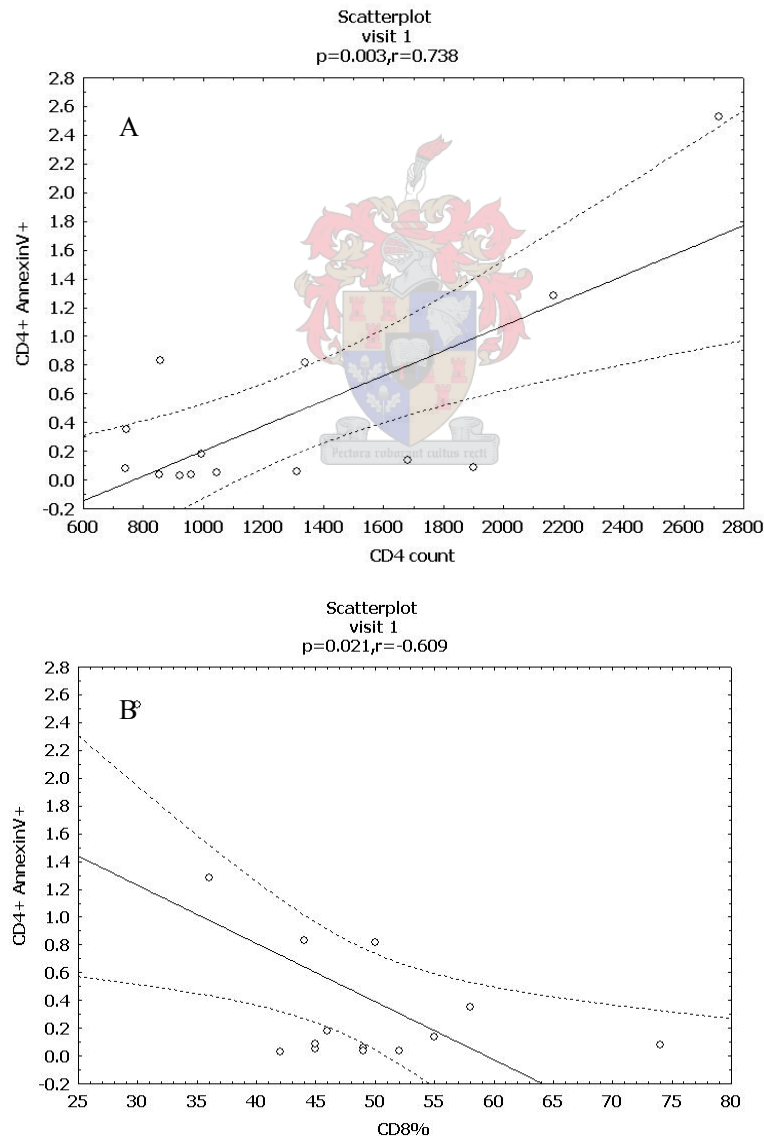
6.2.4 Different effects when NNRTI and PI were compared

Firstly, all 14 HIV-1 infected subjects were grouped with respect to the regimens they received. The subjects on triple combination therapy containing one NNRTI were in NNRTI group (n=6), while the others receiving PI were regrouped in the so-called PI group (n=8). The mean levels of apoptosis of two groups were analyzed by t-test. As displayed in Figure 6-6, after treatment for 12 month, the level of apoptotic PBMCs (as determined by the TUNEL assay method) were significantly lower in PI treated group than that in NNRTI treated group (mean percentages: 3.76% versus 6.28% respectively, p=0.017). However, no significant difference was found in apoptosis of CD4+ T cells.

Fig 6-6: Boxplot representation of levels of apoptotic PBMCs in NNRTI and PI treated groups.

The level of apoptotic PBMCs was significantly lower in PI group than that in NNRTI group (mean ±SD: 3.76±1.10 versus 6.28±2.30%, p=0.017).
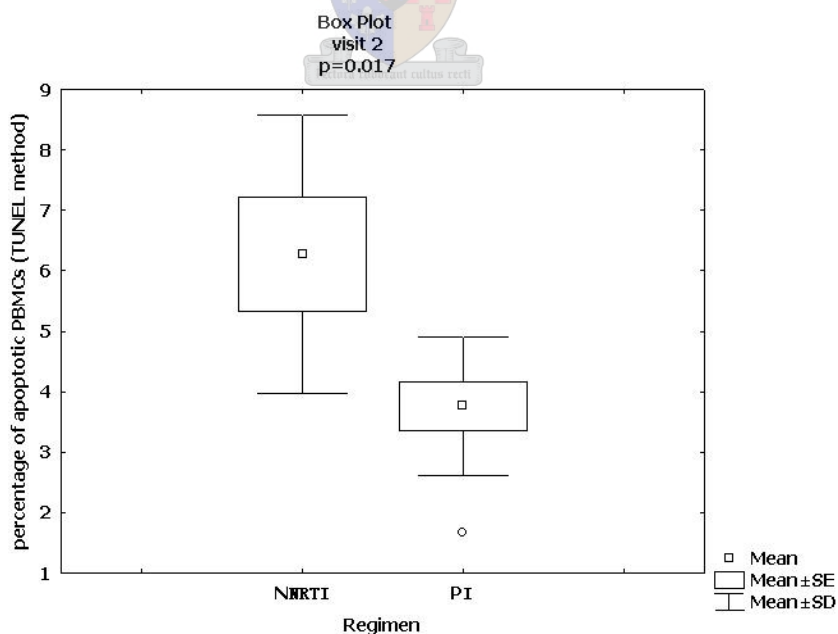
Secondly, when comparing the virological and immunological effects of the 12-months treatment, the two regimens showed similar effects. However, it appeared that PI was more effective than NNRTI in increasing the CD4 count. The mean CD4 counts of visit 1 and visit 2 in PI treated group increased from 1342 to 2083 cells /ul blood (t-test, p=0.004), while in NNRTI treated group, this increased from a mean count of 1248 to a mean count of 1727 cells/μl blood (t-test, p=0.050).

Finally, correlation analyses were performed in NNRTI and PI treated groups, respectively. Both CD4 percentage and CD8 percentage were significantly correlated to the levels of apoptotic total lymphocytes (scatter method) in subjects treated by PI for 12 months (Figure 6-7). However, no such correlation was found in NNRTI treated group. In the case of the CD4 percentages and apoptosis (scatter method), the correlation was positively correlated (Figure 6-7A), once again confirming our hypothesis that the apoptotic activity was possibly protective. In the case of the CD8 correlation (Figure 6-7B), this once again, was inversely correlated: again confirming our hypothesis of CD8 mediated apoptosis. Why the difference between the PI versus the NNRTI? At this stage we cannot provide any clarification since one would have expected similar responses in both groups but this does not seem to be the case. We are not aware of any studies having shown differences in the apoptotic activity when different regimens were compared. This requires further investigation in larger groups of patients.

Fig 6-7: Scatterplots of correlations between lymphocytes and apoptosis.

(A) The level of apoptotic lymphocytes (Scatter of lymphocytes, Y-axis) positively correlated to CD4 percentage (CD4%, X-axis) after treatment with PI for 12 month (p=0.020). (B) The level apoptotic lymphocytes (Scatter of lymphocytes, Y-axis) was inversely correlated to CD8 percentage (CD8%, X-axis) after treatment with PI for 12 month (p=0.024).
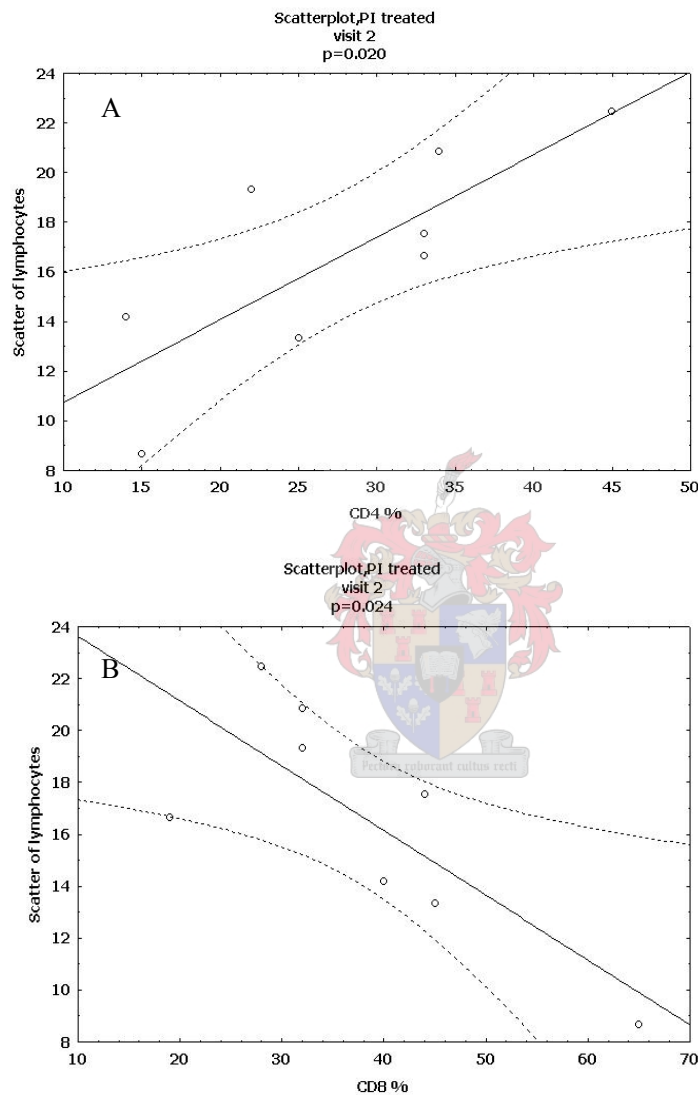
6.3 Early effects of antiretroviral treatment on virological and immunological parameters
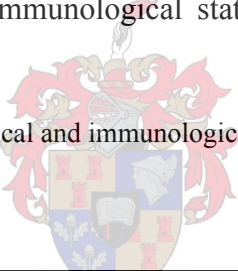
6.3.1 The characteristics of study subjects

The study subjects in this small sub-study included 5 HIV-1 infected children. The mean age was 3.4 years (range 2 to 6 years). In order to study the early effects of antiretroviral treatment, the cut-off follow up time was one month after the

initiation of the treatment although some patients had follow-ups of 3 and 6 months. However, the numbers were too small for meaningful analysis. The regimen of ARV's used were the same as in the long-term study above: combination of 2 NRTIs plus 1 NNRTI or 1 PI (4 received NNRTI and 1 received PI). At baseline visits when the 5 HIV-1 infected children were enrolled in the study, all of them had high viral loads. As summarized in Tabel 6-2, three of them had greater than 100,000 copies/ml plasma, while the lowest one of the 5 was 9584 copies/ml plasma. Following the CDC's immunologic classification by age-specific CD4+ T cell count and percentage of total lymphocytes (CDC, 1994), 3 out of 5 subjects had no evidence of suppression, 1 had moderate suppression and 1 had severe suppression (Table 6-2).

Table 6-2: Virological and immunological status of 5 children enrolled in the 1-month follow-up study.

The data represented their virological and immunological status subjects at baseline visit.

		Baseline
Viral load (copies/ml plasma)	<10,000	n=1
	10,000-100,000	n=1
	>100,000	n=3
Immunological categories	No suppression	n=3
	Moderate suppression	n=1
	severe suppression	n=1

6.3.2 Early effects of antiretroviral treatment

6.3.2.1 Virological effect

After treatment for a month, all subjects had dramatically decreased their viral load. In one subject, the viral load dropped to undetectable level. The mean of LOG viral load remarkably decreased from 5.04 at baseline to 2.87 at 1 month visit. The statistical significance was confirmed by ANOVA repeated measures (p<0.001, Figure 6-8). The decrease in viral load

implicated that the patient had a successful response to antiretroviral treatment and that the desired virological response had been attained.

Fig 6-8: Comparison of LOG viral load between baseline and visit 1.

The graph showed a significant decrease from baseline to visit1 of the two means (mean±SD 5.04±0.69 versus 2.87±0.82 log, p<0.001).

Visits; LS Means
Current effect: F(1, 4)=197.94, p=.00015
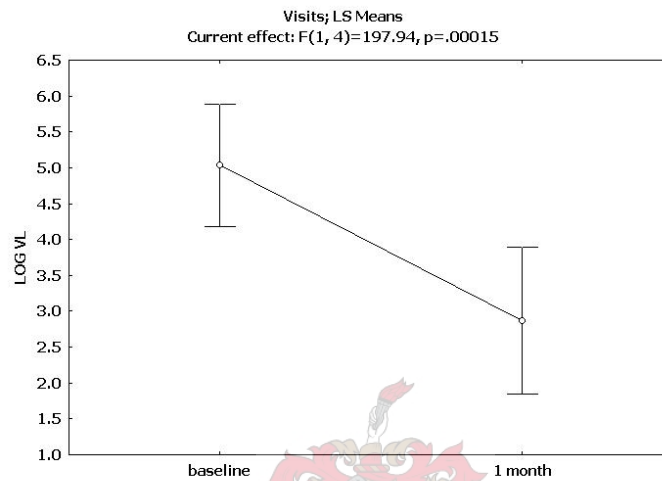
6.3.2.2 Immunological effects

Number of lymphocytes

Most of the children showed an increase in CD4 count after starting the treatment for a month (1 month), except one child who had an extremely high CD4 count (11776 cells/μl) at baseline, and this dropped to 4212 cells/μl after treatment for a month. When comparing the means of CD4 count and percentage between baseline and 1 month visits in the 5 subjects, both were decreased at 1 month visit. (CD4 count decreased from 3223 cells/μl blood to 1844 cells/μl blood, CD4 percentage from 26% to 25%). However, this could be due to the one subject who was considered as an outlier: when this extremely high CD4 count and percentage was excluded, the mean of CD4 count of the remaining 4 subjects increased from 1085 cells/μl at baseline to 1252 cells/μl after treated whereas the percentage of CD4 elevated from 20% to 21.5%. ANOVA repeated measures showed that

the increases in CD4 count and percentage were not statistically significant (p=0.090, p=0.444, respectively).

According to the CD8 count and percentage, this decreased in 2 subjects, while it increased in the other 3 subjects after treatment. The means between baseline and "1 month" visit was not significantly different (data not shown).

Levels of apoptosis in CD4+ and CD8+ T cells

The mean level of apoptosis in CD4+ T cells did not show substantial difference between baselines and "1 month" visit values (4.60% versus 4.00%). However, the mean level of apoptotic CD8+ T cells decreased after one month of antiretroviral treatment (5.96% versus 3.53 %). ANOVA repeated measures showed the difference was not statistically significant (p=0.083).

Levels of apoptosis in memory and naïve subsets

The determinations of apoptotic activity within the memory and naïve subsets of CD4 and CD8 cells were described in the Materials and Methods section. Although none of the difference in the levels of apoptosis were statistically significant between memory and naive cells or between baseline and "1 month" visit, it appeared that the apoptotic activity within the memory cell subset were about 10-fold greater than that within the naïve cells in both the CD4 and CD8+ T cells. Antiretroviral treatment seemed to inhibit apoptosis in all subsets when comparing the levels of apoptotic cells between baseline to that at 1 month visit (Figure 6-9).

Fig 6-9: Mean percentages of apoptotic cells in four cell subsets.

Memory cell subsets (CD4+CD45RA- and CD8+CD45RA-) had greater percentages of apoptotic cells than naive cell subsets (CD4+CD45RA+ and CD8+CD45RA+). Percentages of apoptotic cells in all subsets decreased at 1 month compared to baseline.
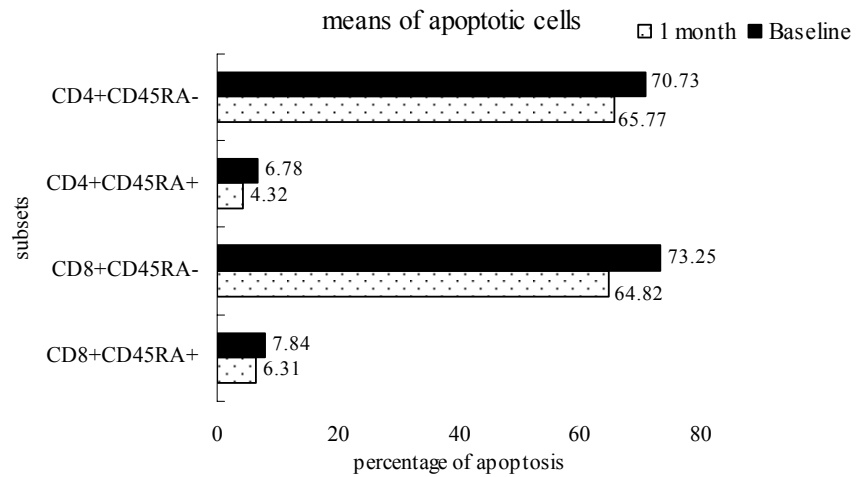
Fig 6-10: ANOVA repeat measures of levels of apoptotic PBMCs (TUNEL assay) at two visits.

Although the level of apoptosis dropped about 2-fold after initial of treatment, the difference between baseline and visit 1 was not significant (mean±SD: 5.14±1.35 versus 3.32±1.71%, p=0.228).
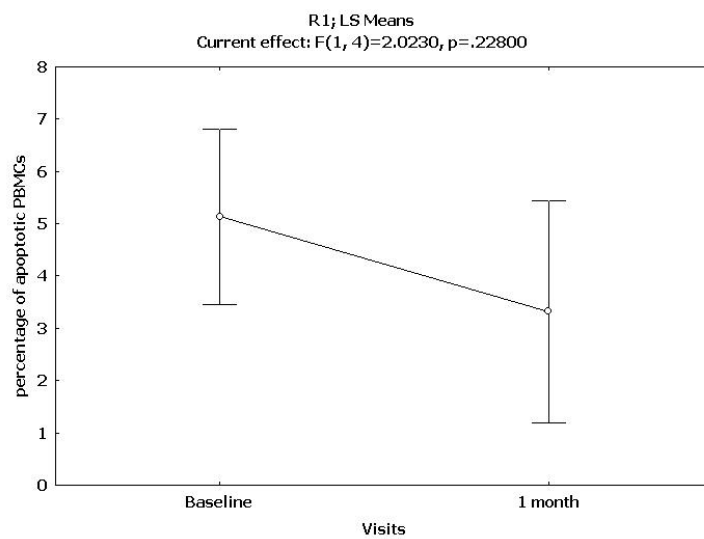
Levels of apoptosis in PBMCs (TUNEL assay)

With respect to the measurement of apoptosis by the TUNEL assay, all subjects except one showed decreased percentage of apoptotic PBMCs after 1 month of treatment. When we compared the mean percentages of the two visits, it was lower at the second visit (1 month after treatment) than that at baseline (5.14% versus 3.32% respectively). However, the decrease did not reach statistical significance (p=0.228, Figure 6-10).

Levels of CD95 expression on CD4 and CD8 T lymphocytes

CD95 expression on CD4 and CD8 cells was determined by using the fluorescent antibody CD95-FITC. As described under the Materials and Methods section, cells expressing CD95-FITC^{bright} were considered as CD95 positive cells. There was an interesting finding in that the range of the percentage of CD95 positive cells was enormous: this ranged from 0.44% (in one subject) up to 24.61% (in another subject) at baseline. The same measurement repeated at the follow-up visit (1 month) showed more homogeneity: range 0.01% - 3.43% depending on the individuals (Table 6-3). However, when comparing the mean levels of CD95 expression, there was no significant difference in levels of CD95 expression on CD4 or the CD8 subset between baseline and "1 month" visit. This was obviously due to the wide range of measurement.

Table 6-3: Descriptions of levels of CD95 expression on CD4+ and CD8+ T cells of all subjects at baseline.

Among the 5 subjects, two had high levels of CD95 expression on CD4+ T cells, while one of them also had similar level on CD8 subset. Taken together, both means and standard deviations (SD) were high at baseline. As to "1 month" visit, most of the levels of CD95 expression were low.

	Baseline		1 month	
	CD4+CD95+ (%)	CD8+ CD95+ (%)	CD4+CD95+ (%)	CD8+ CD95+ (%)
Subject 1	0.44	3.05	3.41	0.75
Subject 2	23.96	24.14	1.54	3.43
Subject 3	24.61	8.44	0.02	0.02
Subject 4	1.33	5.97	0.09	0.05
Subject 5	1.03	2.55	0.15	0.01
Mean	10.27	8.83	1.04	0.85
SD	12.8	8.89	1.47	1.48

6.3.3 Correlations between observations

6.3.3.1 CD4 count/percentage and apoptosis

The CD4 percentage was found to significantly correlate to the level of apoptotic CD4+ and CD8+ T cells (measured by the scatter method, scatter of CD4 and scatter of CD8) at baseline ($p=0.042$, $p=0.043$, respectively, Figure 6-11).

Fig 6-11: Scatterplot of correlation between CD4 percentage and level of apoptosis at baseline in cohort 2.

(A) Level of apoptotic CD4+ T cells (scatter of CD4, Y-axis) correlated to CD4 percentage (CD4%, X-axis) at baseline (p=0.042). (B) Level of apoptotic CD8+ T cells (scatter of CD8, Y-axis) correlated to CD4 percentage (CD4%, X-axis) at baseline (p=0.043).
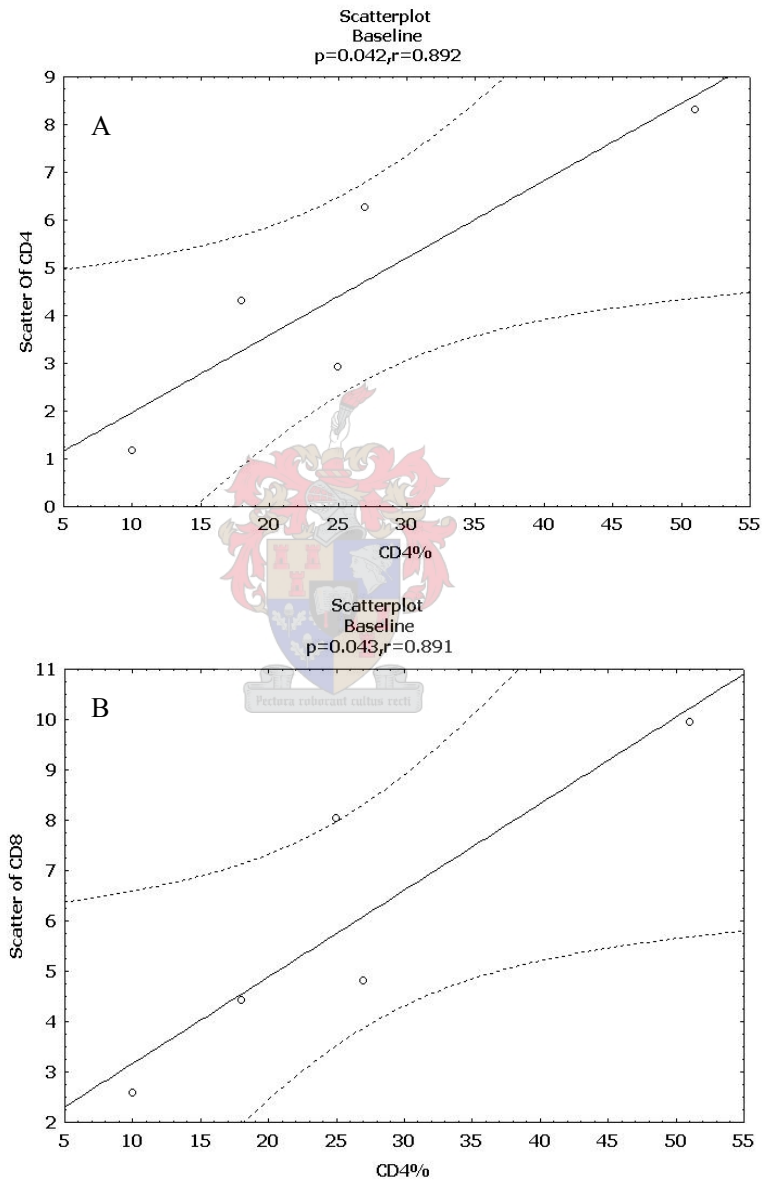
6.3.3.2 CD95 expression with apoptosis

The level of CD95 expression on CD4+ T cells surfaces correlated to level of apoptotic CD4+ T cells (scatter method) at "1 month" visit (p=0.069). This was especially significant in the naïve subset (CD4+CD45RA+, p= 0.029, Figure 6-12). In addition, the level of CD4+ CD95+ cells also correlated to the level of apoptotic CD8+ T cell at "1 month" visit (p=0.031, r = 0.912).

Fig 6-12: Scatterplot of correlation between CD95 expression and level of apoptotic naïve CD4 cells in early responses to ART.

Level of apoptotic naïve CD4 cells (CD4+CD45RA+, Y-axis) positively correlated to CD95expression on CD4+ T cells (CD4+CD95+, X-axis) after 1 month of treatment (p=0.029).
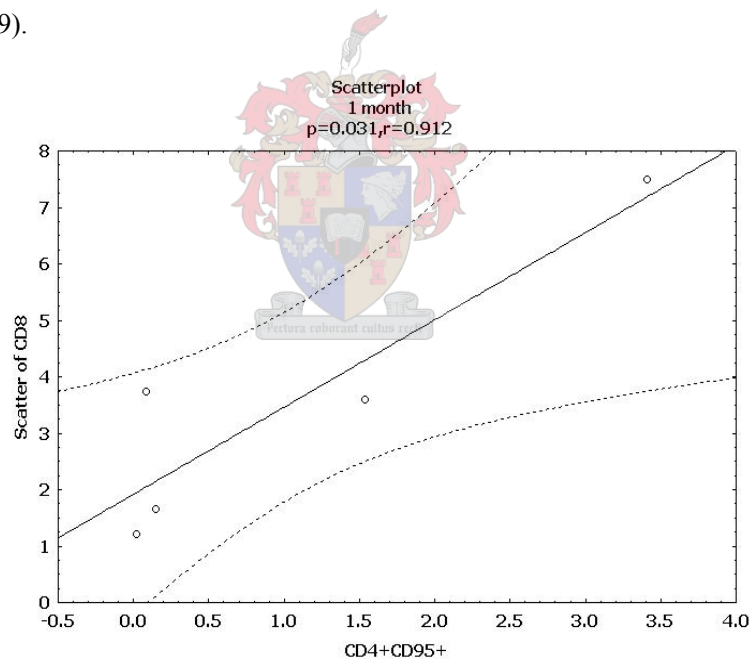
6.4 Summary

The above results showed that antiretroviral treatment significantly decreased plasma viral load after one month. Although the treatment did not make a difference in viral load as a long-term effect, it increased CD4 count as well as CD8 count after 12 month, which was not found in the short-term sub-study. Possibly the short term sub-study was too early to detect these changes.

The long term effects on levels of apoptosis were different depending on the cell populations and analytical methods. It was found that levels of apoptotic CD4 measured by scatter (scatter of CD4) were significantly increased. In addition, the long-term study also showed that CD8 count was positively correlated to level of apoptotic total lymphocytes, which included apoptotic T cells, B cells and NK cells.

There appeared to be differences between the treatments of regimens: the PI treated group had lower levels of apoptotic PBMCs than the NNRTI group. In addition, comparison of the CD4 counts between visit 1 and visit 2, both NNRTI and PI could increase CD4+ T cells, while PI was more effective. These results indicated that PI could suppress apoptosis in HIV-1 infected children. This was also in line with other studies in adults (Phenix et al., 2000 & Matarrese et al., 2002). However, this was contrary to what was found in another study as reviewed previously (Chapter 1.5.1), which suggested that antiretroviral therapy decreased apoptosis of lymphocytes without differences in whether received PIs or NNRTIs (Benito et al., 2002).

The early effect of antiretroviral treatment on apoptosis was uncertain. Firstly, this sub-group was extremely small and, although decreased levels of apoptotic CD4+ and CD8+ T cells and their memory/naive subsets as well as PBMCs were seen after 1 month of treatment, the differences were not significant. In parallel, levels of CD95 expression on CD4+ and CD8+ T cells also decreased but again, this was not significant. However, the level of CD95 expression on CD4+ T cells was found to be significantly correlated to the level of apoptotic CD8+ T cells and naïve CD4+ T

cells. Although the sample size was small, there were remarkable drops in both apoptosis and CD95 expression on treatment, which did not reach significance. However, a much larger study population is required before we can make any comments concerning the early effects of ARV treatment on the apoptotic activity.

We did find CD4 double population in one of the 5 HIV-1 infected children with no immune suppression status (6 years old, CD4 count 1480 cells/µl blood) and a viral load of 73218 copies/ml plasma. However, as the sample size was too small, we could not do statistical analysis for this finding. It needs to be further investigated together with adults in this CD4 double population.

Taken together, the results obtained from the 12-months and 1-month follow-up studies, it appears that the early effect of antiretroviral treatment might be the immediate decrease in viral load (which indicates the successful suppression of viral replication). However, the immunological effect of antiretroviral treatment could be only seen as a long-term effect, since immune recovery would take longer than the period used for the short-term study (1 month).
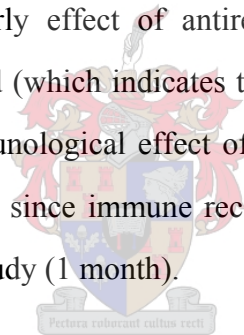
CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS
AND FUTURE PERSPECTIVES

7.1 Discussion of Results

The present study was undertaken in an attempt to investigate the role of apoptosis in HIV-1 infection and to compare this measurement of programmed cell death between adult infected patients to paediatric cases of the pandemic. To date, most literature concerning the measurement of apoptosis relates to the presence of high levels of this activity in the bloods of infected patients (Ameisen et al., 1991; Groux et al., 1992 & Oyaizu et al., 1993), but very little has been demonstrated concerning its correlation to disease stage/clinical surrogate markers of disease activity, and especially, whether this cellular activity may afford the host some sort of protection from the virus.

Moreover, the current literature is fraught with conflicting reports as to whether the apoptotic activity is elevated in this infection: this may be due to the varying methods employed to its measurement, the types of blood samples studied and whether the blood cells were cultured in order to increase the signal of measurement. We have addressed this issue and will conclude hereunder.

The physiological role of apoptosis in normal tissue morphogenesis is well established as well as its immunological role in the differentiation of mature cells within the thymus and the other central lymphoid organ, namely, the bone marrow. The role of apoptosis in chronic viral infections other than HIV-1 has been studied and it is remarkable that such studies have indicated that the immune cells have the capacity to control apoptosis in order to control viral replication. For instance, it has been established that in EBV infection *in vitro*, the immune cells undergo apoptosis in order to decrease the ability of the virus to replicate and to infect other cells in culture (Henderson et al., 1991). As far as HIV-1 infection is concerned, it has also been

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suggested that the inhibition of apoptosis of the immune cells *in vitro* leads to enhanced viral replication (Antoni et al., 1995) suggesting that the physiological response of the immune cells is to limit the damage caused by the virus and this is achieved by enhancing the apoptotic activity in the cells.

Our study has brought to light several interesting findings. The cross-sectional study conducted in infected adults revealed that the cellular response of the immune cells to the infection may be very different to that of children infected with the very same virus. It has been known that the virological and immunological response is so different that different criteria are used to classify the children (CDC, 1994). The current study also reinforces this in that the degree of apoptosis measured by our methods reveals that:

1) In infected adults, the levels of apoptosis are not clearly related to the stage of disease in adults: at the early stage of infection (when the immune cells are still mounting a robust response to the virus), the apoptotic activity of the immune cells is high followed by a nadir during stage 2 and finally, as the patient progresses to full-blown AIDS, the apoptosis is again on the increase. Graphically, this is in the form of a "V". We believe that this phenomenon is due to the fact that early in the infective process, the immune cells are able to control the viral replication by eliminating the very machinery of viral replication. The eventual virological set-point is achieved and followed by the period of latency. As the disease progresses, the apoptosis is activated once again, possibly become uncontrolled and cells are lost by several mechanisms. This is reflected by our findings as measured by both the expression of CD95 as well as the scatter method.

2) The correlation of plasma viral loads and CD4+ apoptosis confirms that the cell death may protect the host: the inverse correlation

implies that in order to control the replicative cycle of the virus, the CD4+ cells undergo extensive apoptosis thereby removing the biochemical machinery required for gene transcription by the virus.

3) A novel finding of our study is the presence of double positive CD4 cells. To our knowledge, this has never been described before. The most interesting observation is the fact the population of CD4 positive lymphocytes exhibit the highest degree of apoptosis: this being measured by both the Annexin V assay as well as the measurement of CD95 expression. This CD4^{dim} population not only showed the higher degree of apoptosis but also exhibited the lower (even undetectable) levels of plasma viral loads. This would imply that the CD4 positive cells which have been programmed to undergo cell death would down-regulate their membrane markers and in so doing would present a challenge to the virus for replication. Interesting is the fact that only one case in the paediatric study showed a similar phenomenon.

4) In adults, co-infection with tuberculosis leads to increased viral replication although this is not reflected in the degree of apoptosis.

5) The introduction of treatment in patients leads not only to viral control but we seem to observe an increase in the degree of apoptosis in CD8+ cells. This is strange in that we would have expected that the levels of CD4+ cell death would have increased, possibly induced by the CD8+ cells (CTL activity). The rationale is that the ARV's would control the viral replication and in so doing, the equilibrium of immune control and immune cell loss would have tipped in favour of control. This is not reflected in our data: on the contrary, it seems that the introduction of treatment may in fact inhibit the levels of apoptosis. Whether this is clinically relevant is difficult to conclude at present.

6) In the paediatric cohort of patients, the effects of anti-retroviral

therapies were examined: in a larger group of children, it was established that those who had been on treatment for at least 12 months showed elevated levels of apoptosis and that certain regimen (including a PI) would promote this activity. Indeed, we found that there were differences between those regimens containing a PI versus those without the use of a PI. Whether this can be translated into a clinical benefit is difficult to determine at present but if our hypothesis concerning the protective role of apoptosis is correct, then the PI containing regimens would be more beneficial.

7.2 Conclusions

7.2.1 Conclusions drawn for the investigation of the methodology employed

The methodological sub-study acted as a pilot investigation for the later studies of measuring apoptosis in HIV-1 infected adults and children. It was found that fresh whole blood samples yielded the greatest level of apoptotic CD4+ T cells, followed by Buffy coat samples and PBMC samples (measured by either FITC-labeled Annexin V binding or scatter analysis). This finding was in line with Fowke and his colleagues (Fowke et al., 2000) who supported the choice of using whole blood as a cell preparation for apoptosis study in HIV-1 infection. Another finding in our methodology investigation was that Annexin V binding and flow cytometer based scatter method were significantly different in all cell preparations. The level of apoptosis measured by scatter method was much greater than that by Annexin V binding method. The CD4 count was inversely correlated to the level of apoptotic CD4+ T cells as measured by scatter method in whole blood sample. All of these findings formed our basic premise that scatter method as well as fluorescent labelled Annexin V could be useful markers for apoptosis. We therefore employed the two methods to measure apoptosis in our cohort of patients and we only used whole blood as source of cells. This facilitated the studies in that

no lengthy cell separation methods needed to be applied and it could therefore be used as a routine laboratory test.

7.2.2 Conclusions concerning the measurement of apoptosis in HIV-1 infected adults

In the last decade, apoptosis is under active investigation by several laboratories. It was mostly elicited as a mechanism of CD4 depletion in HIV-1 infection (Meyaard et al., 1992). Elevated level of apoptosis was found in HIV-1 infected individuals (Groux et al., 1992 & Oyaizu et al., 1993). However, there was also evidence that clearly showed that inhibition of HIV-induced apoptosis enhanced virus production (Antoni et al., 1995). In addition, HIV-1 proteins, such as Tat and Vpr, were found to inhibit apoptosis (Zauli et al., 1993 & Conti et al., 1998). These studies imply that the virus itself codes for some genes which directly down-regulate apoptosis in an attempt to survive the immunological response directed against it. As such, it should be assumed that apoptosis is in fact a protective mechanism activated by the host.

What exactly is the role of apoptosis during the different phases of HIV-1 infection? In this present study, we hypothesized that it acts as a protector early in the disease. In the middle of infection, it is inhibited by the virus. Ultimately, during the last stages of the infection, apoptosis is driven by both an autoimmune process (innocent bystander killing by CTL's) and by the virus itself (cell lysis, etc). Originally, apoptosis was described as a significant physiological process (Wyllie et al., 1980). It is a normal biological process to eliminate immature cells or virus-infected cells. It is therefore plausible that the increased level of apoptosis is due to a protective immune response because the host immune system is trying to eliminate the virus infected cells and suppress viral replication via apoptosis at early infection. Certainly, as the viruses require live and functional cells for their own replication, they inhibit cell death. Later on, the viruses once formed need to be released and certain genes would then make provision for the induction of apoptosis. It is reasonable to suggest that HIV-1 can regulate apoptosis related to

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their life cycles because many viruses, such as the poliovirus have been found to inhibit and induce apoptosis (Tolskaya et al., 1995).

This hypothesis could explain the finding of a "V" shape tendency of apoptosis in CD4+ T cells in this study. At CD4 category 1, the level of apoptotic CD4+ T cells was relatively high whereas viral load was relatively low due to immune response. When the disease progressed to CD4 category 2, the level of apoptosis decreased as a result of suppression by the viruses. In addition, the viral load was increased at that stage. Eventually, at category 3, when CD4 count dropped to less 200 cells/µl blood, the level of apoptosis climbed up because not only was the immunity activated, but also the viruses induced apoptosis (in order to release the virons) and consequently, this leads to an increase in viral load. Furthermore, the inverse correlations between viral loads and the levels of apoptotic CD4+ and CD8+ T cells reinforce this hypothesis.

There was a new finding in this study that CD4 double population (CD4^{dim} and CD4^{bright} cells) was seen in 10 HIV-1 infected subjects. These 10 subjects had relatively high CD4 count and low viral replication. Levels of apoptosis in these subjects were significantly higher than those subjects having a single CD4 population. In addition, when comparing the two CD4+ cell subpopulations, it was found that CD4^{dim} cells had significant higher level of apoptosis and CD95 expression than CD4^{bright} cells had. The immunophenotype of the two CD4+ cell subpopulations were not identified in this study. However, we hypothesized that those CD4^{dim} cells might be a subset of CD4+ T cell, which express CD4+ CD25+ and are known as regulatory T cells. The finding of high level of apoptosis and CD95 expression in these cells was in line with a recent finding by Fritzsching and his colleagues showing that regulatory T cells are highly sensitive to CD95 mediated apoptosis (Fritzsching et al., 2005). However, there was another study which suggested that depletion of regulatory T cells enhances T cell response to HIV antigens (Aandahl et al., 2004). This might indicate that regulatory T cells are present at the early stage of HIV-1 infection to control the immune response,

while later during the disease, the disappearing of these cells leads to auto-immunity of the host. This is purely hypothetical at present but nevertheless deserves further investigation.

The results of HIV-1 infected subjects co-infected with tuberculosis showed significant lower CD4 count and higher viral load in the co-infection group subjects. This confirms the notion and clinically relevant observation that tuberculosis weakens (or deviates) the immune system further leading to active viral replication.

According to the results of apoptosis in the memory and naïve subsets of CD4+ and CD8+ T cells, it appeared that the levels of apoptotic memory cells were much greater than naïve cells. This implicated that memory cells were more susceptible to apoptosis, which contradicted another report (Grayson et al., 2002). Furthermore, it was found that levels of apoptosis in CD4+ and CD8+T cells were strongly correlated to their naïve subsets respectively. The disagreements might be due to different definition of memory cells (CD45RA negative cells were considered as memory cells), and different methods of measurements (scatter method was used to determine apoptotic memory cells) in this present study.

The levels of CD95 expression on CD8+ T cells were positively correlated to the levels of apoptotic CD4+ T cells in antiretroviral treated group (CD4 count between 200 and 499 cells/µl blood) while in the co-infection with TB group, it was found that CD95 expression on CD8+ T cells were correlated to level of apoptotic memory CD4 and CD8 subsets. Somehow, it might suggest that CD8+ T cells express FasL to interact with Fas on CD4+ T cells so as to induce apoptosis of CD4+ T cells.

7.2.3 Conclusions concerning the effects of antiretroviral treatment in HIV-1 infected children

The potent combination antiretroviral therapy has been reported to have enormous impact on HIV-1 infection (Mocroft et al., 1998). It was shown that antiretroviral therapy inhibited apoptosis of CD4+ and CD8+ T cells with increase in CD4 count (Johnson et al., 1998). Moreover, Navarro and his colleagues indicated a positive correlation between activated CD8+ T cells and viral load in their study of HIV-1 infected children (Navarro et al., 2001). In another long-term study of HIV-1 infected adults undergoing therapy, it was suggested that the increase of CD4+ T cells might be due to decreased level of apoptotic CD8+ cells (Grelli et al., 2004).

In the present study, both long-term and short-term effects of ART were addressed in HIV-1 infected children. The results showed that ART dramatically suppressed viral replication after one month of treatment. Although the long-term treatment did not make difference in plasma viraemia, it boosted the immune restoration through increasing the number of CD4+ and CD8+ T cells, which was not found in the short-term treatment study. In addition, under long-term treatment, it was also found that CD8 count was positively correlated to level of apoptotic total lymphocytes, which included apoptotic T cells, B cells and NK cells. This might indicate that restoration of CD8+ T cells activated the cytotoxic killing, which contributed to the hypothesis that apoptosis is a protective immune response when the immune system exists or has recovered.

The differences in NNRTI and PI showed that PI might be more effective in inhibiting apoptosis of PBMCs than NNRTI. Subjects undergoing combination therapy could achieve similar immunological benefits, nevertheless, since PI induced a greater CD4 + T cell increase.

Despite the certainty of effective viral suppression under short-term treatment, the immunological effects were vague. Although decreased levels of apoptosis in all T

cell subsets were observed after 1 month of treatment, neither of the differences was significant. This may be due to the short time line between treatment initiation and the immunological investigation. The long term immunological benefit may only be evident after the 1 month treatment period.

7.3 Future perspective

With respect to all the data reported in this study and the conclusions drawn, there are many questions that remain unanswered. Some of our results were in disagreement with other reports. We therefore propose that further studies should address the following:

(a) Investigation of CD4 subsets such as the regulatory T cells (T_{reg}) to explore their role in HIV-1 infection and possible viral replication and apoptosis;

(b) The use of well defined sub-cohorts of HIV-1 infected subjects, such as rapid progressors or non-progressors in order to investigate the different immuno-pathogeneses in different subjects.

(c) A study to investigate the reason of the variation of levels of CD95 expression in different HIV-1 infected subjects especially among children.

(d) Further studies of the cytokine responses (TH_1 versus TH_2) during HIV-1 infection and the correlation of these cellular responses in relation to apoptosis and cell loss.

(e) Identification of more patients exhibiting the double CD4 positive phenomenon: this is novel and deserves further in depth research.

(f) A further study with increased cohort size in order to examine the early effects of ART on apoptosis.

REFERENCES

Allan JS, Coligan JE, Barin F, McLane MF, Sodroski JG, Rosen CA, Haseltine WA, Lee TH, Essex M. *Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III*. Science 1985; 228(4703):1091-1094

Ameisen JC, Capron A. *Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis*. Immunol Today 1991; 12(4):102–105

Ammann AJ, Abrams D, Conant M, Chudwin D, Cowan M, Volberding P, Lewis B, Casavant C. *Acquired immune dysfunction in homosexual men: Immunological profiles*. Clin Immunol Immunopathol 1983; 27(3):315-325

Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM. *Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers*. J Biol Chem 1990; 265(9):4923-4928

Antoni BA, Sabbratini P, Rabson AB and White E. *Inhibition of apoptosis in human immunodeficiency virus-infected cells enhances virus production and facilitates persistent infection*. J virol 1995; 69(4): 2384-2392

Azad AA. *Could Nef and Vpr proteins contribute to disease progression by promoting depletion of bystander cells and prolonged survival of HIV-infected cells?* Biochem Biophys Res Commun 2000; 267(3):677-685

Badley AD, Dockrell D, Simpson M, Schut R, Lynch DH, Leibson P, Paya CV. *Macrophage-dependent apoptosis of CD4+ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor*. J Exp Med 1997; 185(1):55-64

Banda NK, Bernier J, Kurahara DK, Kurrle R, Haigwood N, Sekaly RP, Finkel TH. *Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis*. J Exp Med 1992; 176(4):1099-1106

Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L. *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)*. Science 1983; 220(4599): 868-871

Bartz SR, Emerman M. *Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8*. J Virol 1999; 73(3):1956-1963

Belyakov IM, Ahlers JD, Berzofsky JA *Mucosal AIDS vaccines: current status and future directions*. Expert Rev Vaccines 2004; 3(4 Suppl):S65-S73

Benito JM, Lopez M, Martin JC, Lozano S, Martinez P, Gonzalez-Lahoz J, Soriano V. *Differences in cellular activation and apoptosis in HIV-infected patients receiving protease inhibitors or nonnucleoside reverse transcriotase inhibitors*. AIDS Res Hum Ret 2002; 18(18):1379-1388

Bijl M, Horst G, Limburg PC, Kallenberg CG. *Anti-CD3-induced and anti-Fas-induced apoptosis in systemic lupus erythematosus (SLE)*. Clin Exp Immunol 2001; 123(1): 127-132

Bohler T, Walcher J, Holzl Wenig G, Geiss M, Buchholz B, Linde R, Debatin KM. *Early effects of antiretroviral combination therapy on activation apoptosis and regeneration of T cells in HIV-1-infected children and adolescents*. AIDS 1999; 13(7):779-789

Bolton DL, Hahn BK, Park EA, Lehnhoff LL, Hornung F and Lenardo MJ. *Death of CD4 T-Cell lines caused by human immunodeficiency virus type 1 does not depend on caspases or apoptosis*. J virol 2002; 76(10): 5094-5107

Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection*. J Virol 1994; 68(9):6103-6110

Brinchmann JE, Dobloug JH, Heger BH, Haaheim LL, Sannes M, Egeland T. *Expression of costimulatory molecule CD28 on T cells in human immunodeficiency virus type 1 infection: functional and clinical correlations*. J Infect Dis 1994; 169(4):730-738

Carbonari M, Cibati M, Cherchi M, Sbarigia D, Pesce AM, Anna LD, Modica A, Fiorilli M. *Detection and characterization of apoptotic peripheral blood lymphocytes in human immunodeficiency virus infection and cancer chemotherapy by a novel flow immunocytometric method*. Blood 1994; 83(5):1268-1277

Carlson JR, McGraw TP, Keddie E, Yee JL, Rosenthal A, Langlois AJ, Dickover R, Donovan R, Luciw PA, Jennings MB, et al. *Vaccine protection of rhesus macaques against simian immunodeficiency virus infection*. AIDS Res Hum Retroviruses 1990; 6(11):1239-1246

Carmichael A, Jin X, Sissons P, Borysiewicz L. *Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease*. J Exp Med 1993; 177(2):249-256

CDC *1993 Revised classification system for HIV infection and expanded surveillance case Definition for AIDS among adolescents and adults*. MMWR 1992; 41 (RR17):1-19. Website: www.cdc.gov

CDC *1994 Revised Classification System for Human Immunodeficiency Virus Infection in Children Less Than 13 Years of Age. MMWR 1994; 43(RR 12):1-10*. Website: www.cdc.gov

Chang DD, Sharp PA. *Regulation by HIV Rev depends upon recognition of splice sites*. Cell 1989; 59(5):789-795

Chou CC, Gudeman V, O'Rourke S, Isacescu V, Detels R, Willams GJ, Mitsuyasu RT, Giorgi JV. *Phenotypically defined memory CD4+ cells are not selectively decreased in chronic HIV disease*. J Acquir Immune Defic Syndr 1994; 7(7):665-675

Clavel F, Guetard D, Brun-Vezinet F, Chamaret S, Rey MA, Santos-Ferreira MO, Laurent AG, Dauguet C, Katlama C, Rouzioux C, et al. *Isolation of a new human retrovirus from West African patients with AIDS*. Science 1986; 233(4761):343-346

Clavel F, Mansinho K, Chamaret S, Guetard D, Favier V, Nina J, Santos-Ferreira MO, Champalimaud JL, Montagnier L. *Human immunodeficiency virus type 2 infection associated with AIDS in West Africa*. N Engl J Med 1987; 316(19):1180-1185

Clerici M, Stocks NI, Zajac RA, Boswell RN, Lucey DR, Via CS, Shearer GM. *Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging*. J Clin Invest 1989; 84(6):1892-1899

Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. *Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells*. Science 1995; 270(5243):1811-1815

Cohen EA, Terwilliger EF, Jalinoos Y, Proulx J, Sodroski JG, Haseltine WA. *Identification of HIV-1 vpr product and function*. J Acquir Immune Defic Syndr. 1990; 3(1):11-18

Cohen JJ, Duke RC, Fadok VA, Sellins KS. *Apoptosis and programmed cell death in immunity*. Annu Rev Immunol. 1992; 10: 267-93

Conti L, Rainaldi G, Matarrese P, Varano B, Rivabene R, Columba S, Sato A, Belardelli F, Malorni W and Gessani S. *The HIV-1 vpr Protein Acts as a Negative Regulator of Apoptosis in a Human Lymphoblastoid T Cell Line: Possible Implications for the Pathogenesis of AIDS*. J Exp Med 1998; 187(3): 403-413

Cotton MF, Ikle DN, Rapaport EL, Marschner S, Kurrle R, Finkel TH. *Apoptosis of CD4 and CD8+ T cells isolated immediately ex vivo correlates with disease severity in Human immunodeficiency virus infection*. Pediatr Res 1997; 42(5) 656-654

Curran JW, Lawrence DN, Jaffe H, Kaplan JE, Zyla LD, Chamberland M, Weinstein R, Lui KJ, Schonberger LB, Spira TJ, et al. *Acquired immunodeficiency syndrome (AIDS) associated with transfusions*. N Engl J Med 1984; 310(2):69-75

Darzynkiewicz Z, Bedner E. *Analysis of apoptotic cells by flow and laser scanning cytometry*. Methods Enzymol 2000; 322: 18-39

Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. *Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis)*. Cytometry 1997; 27(1):1-20

de Jong R, Brouwer M, Miedema F, van Lier RA. *Human CD8+ T lymphocytes can be divided into CD45RA+ and CD45RO+ cells with different requirements for activation and differentiation*. J Immunol 1991; 146(7):2088-2094

de Silva FS, Venturini DS, Wagner E, Shank PS, Sharma S. *CD4-independent infection of human B cells with HIV type 1: Detection of unintergrated viral DNA*. AIDS Res Hum Retroviruses 2001; 17(17):1585-1598

de Vita VT, Hellman S, Rosenberg SA. *AIDS: etiology, diagnosis, treatment and prevention---third edition*. J. B. Lippincott Company, 1992

Department of Health. *National HIV and syphilis antenatal sero-prevalence survey in South Africa 2004*. Department of Health 2005, Pretoria. Website: www.health.gov.za

Desrosiers RC, Wyand MS, Kodama T, Ringler DJ, Arthur LO, Sehgal PK, Letvin NL, King NW, Daniel MD. *Vaccine protection against simian immunodeficiency virus infection*. Proc Natl Acad Sci USA 1989; 86(16):6353-6357

Effros RB, Allsopp R, Chiu CP, Hausner MA, Hirji K, Wang L, Harley CB, Villeponteau B, West MD, Giorgi JV. *Shortened telomeres in the expanded CD28-CD8+ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis*. AIDS 1996; 10(8):F17-F22

Facchinetti A, Tessarollo L, Mazzocchi M, Kingston R, Collavo D, Biasi G. *An improved method for the detection of DNA fragmentation*. J Immunol Methods 1991; 136(1):125-131

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. *Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages*. J Immunol 1992; 148(7):2207-2216

Fan J, Bass HZ, Fahey JL. *Elevated IFN-gamma and decreased IL-2 gene expression are associated with HIV infection*. J Immunol 1993; 151(9):5031-5040

Finkel Th, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, Baba TW, Ruprecht RM, Kupfer A. *Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV-and SIV-infected lymph nodes*. Nat Med 1995; 1(2): 129-134

Fisher AG, Feinberg MB, Josephs SF, Harper ME, Marselle LM, Reyes G, Gonda MA, Aldovini A, Debouk C, Gallo RC, et al. *The trans-activator gene of HTLV-III is essential for virus replication*. Nature 1986; 320(6060):367-371

Fowke KR, Behnke J, Hanson C, Shea K, Cosentino LM. *Apoptosis: a method for evaluating the cryopreservation of whole blood and peripheral blood mononuclear cells*. J Immunolo Methods 2000; 244(1-2):139-144

Frankel FR, Hegde S, Lieberman J, Paterson Y. *Induction of cell-mediated immune responses to human immunodeficiency virus type 1 Gag protein by using Listeria monocytogenes as a live vaccine vector*. J Immunol 1995; 155(10):4775-4782

Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J, Popovic M. *Isolation of human T cell leukemia virus in acquired immune deficiency syndrome (AIDS)*. Science 1983; 220(4599); 865-867

Gehri R, Hahn S, Rothen M, Steuerwald M, Nuesch R, Erb P. *The Fas receptor in HIV infection: expression on peripheral blood lymphocytes and role in the depletion of T cells*. AIDS 1996; 10(1):9-16

Gibellini D, Caputo A, Celeghini C, Bassini A, La Placa M, Capitani S, Zauli G. *Tat-expressing Jurkat cells show an increased resistance to different apoptotic stimuli, including acute human immunodeficiency virus-type 1 (HIV-1) infection*. Br J Haematol 1995; 89(1): 24-33

Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A. *Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: Evidence of a new acquired cellular immunodeficiency*. N Engl J Med 1981; 305(24): 1425-1431

Gougeon ML, Lecoeur H, dulioust A, Enouf MG, Crouvoiser M,Goujard C, Debord T, Montagnier L. *Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression*. J Immunol 1996; 156(9): 3509-3520

Grayson JM, Harringyton LE, Lanier JG, Wherry EJ, Ahmed R. *Differential sensitivity of naïve and memory CD8+ T cells to apoptosis in vivo*. J Immunol 2002; 167(7):3760-3770

Groux H, Torpier G, Monte D, Mouton Y, Capron A, Ameisen JC. *Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals*. J Exp Med 1992; 175(2):331-340

Guy B, Geist M, Dott K, Spehner D, Kieny MP, Lecocq JP. *A specific inhibitor of cysteine proteases impairs a Vif-dependent modification of human immunodeficiency virus type 1 Env protein*. J Virol 1991; 65(3):1325-1331

Hamblin AS. *Cytokines and cytokine receptors*. Oxford University Press, 1993

Helbert MR, Walter J, L'Age J, Beverley PCL. *HIV infection of CD45RA+ and CD45RO+ CD4+ T cells*. Clin Exp Immunol 1997; 107(2):300-305

Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E, Rickinson A. I*nduction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death*. Cell 1991; 65(7): 1107-1115

Herbein G, Van Lint C, Lovett JL, Verdin E. *Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes*. J virol 1998; 72(1):660-670
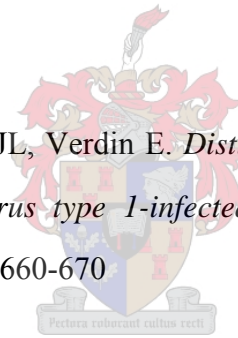
Heusel JW, Wesselschmidt RL, Shresta S, Russell JH, Ley TJ. *Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells*. Cell 1994; 76(6):977-987

Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR. *An African primate lentivirus (SIVsm) closely related to HIV-2*. Nature 1989; 339(6223): 389-392

Johnson N, Parkin JM. *Anti-retroviral therapy reverses HIV-associated abnormalities in lymphocyte apoptosis*. Clin Exp Immunol 1998; 113(2):229-234

Kerr JF, Wyllie AH, Currie AR. *Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics*. Br J Cancer 1972; 26(4):239-257

Kestens L, Vanham G, Vereecken C, Vandenbruaene M, Vercauteren G, Colebunders RL, Gigase PL. *Selective increase of activation antigens HLA-DR and CD38 on CD4+ CD45RO+ T lymphocytes during HIV-1 infection*. Clin Exp Immunol 1994; 95(3):436-441

Kestler HW 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, Desrosiers RC. *Importance of the nef gene for maintenance of high virus loads and for development of AIDS*. Cell 1991; 65(4):651-662

Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. *Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis*. Blood 1994; 84(5):1415-1420

Kottilil S, Chun TW, Moir S, Liu S, McLaughlin M, Hallahan CW, Maldarelli F, Corey L, Fauci AS. *Innate immunity in human immunodeficiency virus infection: Effect of viremia on natural killer cell function*. J Infect Dis 2003; 187:1038–1045

Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome*. J Virol 1994; 68(7):4650-4655

Kowalski M, Bergeron L, Dorfman T, Haseltine W, Sodroski J. *Attenuation of human immunodeficiency virus type 1 cytopathic effect by a mutation affecting the transmembrane envelope glycoprotein*. J Virol 1991; 65(1):281-291

Kylarova D, Prochazkova J, Mad'arova J, Bartos J, Lichnovsky V. *Comparison of the TUNEL, lamin B and annexin V methods for the detection of apoptosis by flow cytometry*. Acta Histochem 2002; 104(4):367-70

Lane HC, Depper JM, Greene WC, Whalen G, Waldmann TA Fauci AS. *Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome. Evidence for a selective defect in soluble antigen recognition.* N Eng J Med 1985; 313(2): 79-84

Laurent-Crawford AG, Krust B, Muller S, Rivière Y, Rey-Cuillé MA, Béchet JM, Luc Montagnier L and Hovanessian AG. *The cytopathic effect of HIV is associated with apoptosis.* Virology 1991; 185(2): 829-839

Leibovitz E, Rigaud M, Pollack H, Lawrence R, Chandwani S, Krasinski K, Borkowsky W. *Pneumocystis carinii pneumonia in infants infected with the human immunodeficiency virus with more than 450 CD4 T lymphocytes per cubic millimeter.* N Engl J Med 1990; 323(8): 531-533

Lemp GF, Payne SF, Rutherford GW, Hessol NA, Winkelstein W Jr, Wiley JA, Moss AR, Chaisson RE, Chen RT, Feigal DW Jr, et al. *Projections of AIDS morbidity and mortality in San Francisco.* JAMA 1990; 263(11):1497-1501
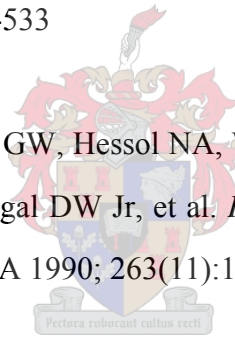
Lewis DE, Ng Tang DS, Wang X and Kozinetz C. *Costimulatory pathways mediated monocyto-dependent lymohocyes apoptosis in HIV.* Clin Immunol1999; 90(3):302-312

Li CJ, Friedman DJ, Wang C, Metelev V, Pardee AB. *Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein.* Science 1995a; 268(5209):429-431

Li X, Darzynkiewicz Z. Labelling DNA strand breaks with BrdUTP. *Detection of apoptosis and cell proliferation.* Cell Prolif 1995b; 28(11):571-579

Liegler TJ, Yonemoto W, Elbeik T, Vittinghoff E, Buchbinder SP, Greene WC. *Diminished spontaneous apoptosis in lymphocytes from human immunodeficiency virus infected long-term nonprogressors.* J Infect Dis 1998; 178(3):669-679

Lowin B, Hahne M, Mattmann C, Tschopp J. *Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways*. Nature 1994; 370(6491):650-652

Lydyard PM, Whelan A and Fabger MW. *Instant Notes in Immunology*. BIOS Scientific Publishers Limited, 2000

Mackay CR. *Homing of naive, memory and effector lymphocytes*. Curr Opin Immunol 1993; 5(3):423-427

Marlink RG, Ricard D, M'Boup S, Kanki PJ, Romet-Lemonne JL, N'Doye I, Diop K, Simpson MA, Greco F, Chou MJ, et al. *Clinical, hematological and immunologic cross-sectional evaluation of individuals exposed to human immunodeficiency virus type 2(HIV-2)*. AIDS Res Hum Retroviruses 1988; 4(2):137-148
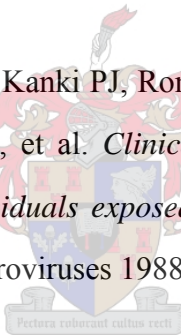
Masur H, Michelis MA, Greene JB, Onorato I, Stouwe RA, Holzman RS, Wormser G, Brettman L, Lange M, Murray HW, Cunningham-Rundles S. *An outbreak of community acquired Pneumocystis carinii pneumonia: Initial manifestation of cellular immune dysfunction*. N Engl J Med 1981; 305(24): 1431-1438

Matarrese P, Giammarioli AM, Cauda R, Malorni W. *Antiapoptotic activity by HIV protease inhibitors either alone or boostered*. JAIDS 2002; 31(5):545-546

Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, O'Shea MA, Kinter A, Kovacs C, Moretta A, Fauci AS. *Characterization of CD56-CD16+ natural killer (NK) cells: A highly dysfunctional NK subset expanded in HIV-infected viremic individuals*. Proc Natl Acad Sci USA 2005; 102(8): 2886-2891

Mcbreen S, Imlach S, Shirafujki T, Scott GR, Leen C, Bell JE, Simmonds P. *Infection of the CD45RA+ (naïve) subset of peripheral CD8+ lymohocytes by human immunodeficiency virus type 1 in vivo*. J virol 2001;75(9): 4091-4102

McCloskey TW, Ott M, Tribble E, Khan SA, Teichberg S, Paul MO, Pahwa S, Verdin E, Chirmule N. *Dual role of HIV Tat in regulation of apoptosis in T cells*. J Immunol 1997; 158(2):1014-1019

McCloskey TW, Oyaizu N, Bakshi S, Kowalski R, Kohn N, Pahwa S. *CD95 expression and apoptosis during pediatric HIV infection: early upregulation of CD95 expression*. Clin Immunol Immunopathol. 1998; 87(1):33-41

McCloskey TW, Oyaizu N, Kaplan M, Pahwa S. *Expression of the Fas antigen in patients infected with human immunodeficiency virus*. Cytometry 1995; 22(2):111-114

McCutchan FE. *Understanding the genetic diversity of HIV-1*. AIDS 2000; 14(suppl3): S31-S44

McDougal JS, Kennedy MS, Sligh JM, Cort SP, Mawle A, Nicholson JK. *Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110K viral protein and the T4 molecule*. Science 1986; 231(4736):382-385

Meyaard L, Otto SA, Hooibrink B, Miedema F. *Quantitative analysis of CD4+ T cell function in the course of human immunodeficiency virus infection*. J Clin Invest 1994a; 94(5); 1947-1952

Meyaard L, Otto SA, Jonker RR, Mijnster MJ, Keet R PM, Miedema F. *Programmed death of T cells in HIV-1 infection*. Science 1992; 257(5067): 217-219

Meyaard L, Otto SA, Keet IP, Roos MT, and Miedema F. *Programmed cell death of T cells in HIV infection: no correlation with progression to disease*. J Cli Invest 1994b; 93(3):982-988

Mocroft A, Vella S, Benfield TL, Chiesi A, Miller V, Gargalianos P, d'Arminio Monforte A, Yust I, Bruun JN, Phillips AN, Lundgren JD. *Changing patterns of mortality across Europe in patients infected with HIV-1*. Lancet 1998; 352(9142):1725-1730

Moore JP, Kitchen SG, Pugach P, Zack JA. *The CCR5 and CXR4 coreceptors---Central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection*. AIDS Res Hum Retroviruses 2004(1): 111-126.

Moore JP, McKeating JA, Weiss RA, Sattentau QJ. *Dissociation of gp120 from HIV-1 virions induced by soluble CD4*. Science 1990; 250(4984):1139-1142

Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. *The isolation and characterization of the human helper inducer T cell subset*. J Immunol 1985; 134(6):3762-3769

Mueller YM, De Rosa SC, Hutton JA, Witek J, Roederer M, Altman JD, Katsikis PD. *Increased CD95/Fas-induced apoptosis of HIV-specific CD8+ T cells*. Immunity 2001; 15(6):867-870

Muro-Cacho CA, Pantaleo G, Fauci AS. Analysis of apoptosis in lymph nodes of HIV-infected persons: *Intensity of apoptosis correlates with the general state of activation of the lymphoid tissue and not with stage of disease or viral burden*. J Immunol 1995; 154(10): 5555-5566

Murphey-Corb M, Martin LN, Davison-Fairburn B, Montelaro RC, Miller M, West M, Ohkawa S, Baskin GB, Zhang JY, Putney SD, et al. *A formalin-inactivated whole SIV vaccine confers protection in macaques*. Science 1989; 246(4935):1293-1297

Navarro J, Resino S, Bellon JM, Abad ML, Gurbindo D, Fernandez-Cruz E and Munoz-Fernandez A. *Association of CD8+ T lymphocytes subsets with the most commonly used markers to monitor HIV type 1 infection in children treated with highly active antiretroviral therapy*. AIDS Res Hum Retroviruses 2001; 17(6): 525-532

Negoescu A, Guillermet C, Lorimier P, Brambilla E, Labat-Moleur F. *Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity*. Biomed Pharmacother 1998 52(6): 252-258

Niehues T, McCloskey TW, Ndagijimana J, Horneff G, Wahn V, Pahwa S. *Apoptosis in T-lymphocyte subsets in human immunodeficiency virus-infected children measure immediately ex vivo and following in vitro activation*. Clin Diagn Lab Immunol 2001; 8(1):74-78
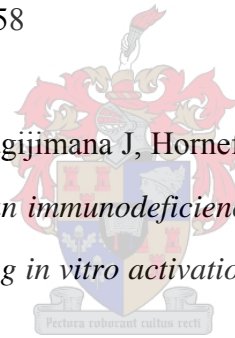
Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ. *Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA*. Science 1998; 279(5359):2103-2106

Olano JP, Wolf D, Keherly M, Gelman BB. *Quantifying apoptosis in banked human brains using flow cytometry*. J Neuropathol Exp Neurol 1996; 55(11):1164-72

Ortona L, Tamburrini E, Tumbarello M, Ventura G, Cauda R. *Natural killer (NK) activity in patients with HIV infection*. Boll Ist Sieroter Milan. 1988; 67(2):135-141

Oyaizu N, McCloskey TW, Coronesi M, Chirmule N, Kalyanaraman VS, Pahwa S. *Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals*. Blood 1993; 82(11): 3392-3400

Oyaizu N, McCloskey TW, Than S, Hu R, Kalyanaraman VS, Pahwa S. *Cross-linking of CD4 molecules upregulates Fas antigen expression in lymphocytes by inducing interferon-gamma and tumor necrosis factor-alpha secretion*. Blood 1994; 84(8):2622-2631

Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD. *Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection*. N Engl J Med 1998; 338(13):853-860

Pandolfi F, Pierdominici M, Oliva A, D'Offizi G, Mezzaroma I, Mollicone B, Giovannetti A, Rainaldi L, Quinti I, Aiuti F. *Apoptosis-related mortality in vitro of mononuclear cells from patients with HIV infection correlates with disease severity and progression*. J Acquir Immune Defic Syndr Hum Retrovirol 1995; 9(5):450-458

Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, Montefiori D, Orenstein JM, Fox C, Schrager LK, et al. *Studies in subjects with long-term nonprogressive human immunodeficiency virus infection*. N Engl J Med 1995; 332(4):209-216

Phenix BN, Angel JB, Mandy F, Kravcik S, Parato K, Chambers KA, Gallicano K, Hawley-Foss N, Cassol S, Cameron DW, Badley AD. *Decreased HIV-associated T cell apoptosis by HIV protease inhibitors*. AIDS Res Hum Retroviruses 2000;16(6):559-67

Popovic M, Sarngadharan MG, Read E, Gallo RC. *Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and preAIDS*. Science 1984; 224(4648):497-500

Prati E, Gorla R, Malacarne F, Airo P, Brugnoni D, Gargiulo F, Tebaldi A, Castelli F, Carosi G, Cattaneo R. *Study of spontaneous apoptosis in HIV+ patients: correlation with clinical progression and T cell loss AIDS Res Hum Retroviruses*. 1997; 13(17):1501-1508

Rabin RL, Roederer M Maldonado Y, Petru A, Herzenberg LA, Herzenberg LA. *Altered representation of naïve and memory CD8+ cell subsets in HIV-infected children*. J Clin Invest 1995: 95(5): 2054-2060

Rayfield M, De Cock K, Heyward W, Goldstein L, Krebs J, Kwok S, Lee S, McCormick J, Moreau JM, Odehouri K, et al. *Mixed human immunodeficiency virus (HIV) infection in an individual: Demonstration of both HIV type 1 and type 2 proviral sequences by using polymerase chain reaction*. J Infect Dis 1988; 158(6):1170-1176

Riley JL, Levine BL, Craighead N, Francomano F, Kim D, Carroll RG, June CH. *Naïve and memory CD4 T cell differ in their susceptibilities to human immunodeficiency virus type 1 infection following CD28 costimulation: implications for transmission and pathogenesis*. J virol 1998; 72(10): 8273-8280

Robey WG, Safai B, Oroszlan S, Arthur LO, Gonda MA, Gallo RC, *Fischinger PJ. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients*. Science 1985; 228(4699):593-595

Roederer M, Dubs JG, Anderson MT, Raju PA, Herzenberg LA,Herzenberg LA. *CD8 naïve T cell counts decrease progressively in HIV-infected adults*. J clin Invest 1995; 95(5): 2061-2066

Rogers LA, Forster SM, Pinching AJ. *IgD production and other lymphocyte functions in HIV infection: immaturity and activation of B cells at different clinical stages*. Clin Exp Immunol. 1989; 75(1):7-11

Roitt I, Brostoff J, Male D, *Immunology---- Sixth Edition*. Harciyrt Publishers Limited 2001

Rucker E, Grivel JC, Munch J, Kirchhoff F, Margolis L. *Vpr and Vpu are important for efficient human immunodeficiency virus type 1 replication and CD4+ T-cell depletion in human lymphoid tissue ex vivo*. J Virol 2004; 78(22): 12689-12693

Samuelsson A, Brostrom C, van Dijk N, Sonnerborg A, Chiodi F. *Apoptosis of CD4+ and CD19+ cells during human immunodeficiency virus type-1 infection correlation with clinical progression, viral load ,and loss of humoral immunity*. Virology 1997; 238(2):180-188

Scherer JM, Stillwell W, Jenski LJ. *Anomalous changes in forward scatter of lymphocytes with loosely packed membranes*. Cytometry 1999; 37(3):184-190

Schmid I, Uittenbogaart CH, Giorgi JV. *Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry*. Cytometry 1994; 15(1):12-20

Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC,

Rieber EP, Letvin NL, Reimann KA. *Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes*. Science 1999; 283(5403):857-860

Scott GB, Buck BE, Leterman JG, Bloom FL, Parks WP. *Acquired immunodeficiency syndrome in infants*. N Engl J Med 1984; 310(2):76-81

Shen L, Chen ZW, Miller MD, Stallard V, Mazzara GP, Panicali DL, Letvin NL. *Recombinant virus vaccine-induced SIV-specific CD8+ cytotoxic T lymphocytes*. Science 1991; 252(5004): 440-443

Silvestris F, Cafforio P, Frassanito MA, Tucci M, Romito A, Nagata S, Dammacco F *Overexpression of Fas antigen on T cells in advanced HIV-1 infection: differential ligation constantly induces apoptosis*. AIDS 1996; 10(2):131-141

Statistics South Africa. *Mid-year population estimates 2005*. Statistics South Africa, 2005a, Pretoria. Website: www.statssa.gov.za

Statistics South Africa. *Mortality and causes of death in South Africa, 1997-2003: Finding from death notification*. Statistics South Africa, 2005b, Pretoria. Website: www.statssa.gov.za

Stevceva L, Strober W. *Mucosal HIV vaccines: where are we now?* Curr HIV Res 2004; 2(1):1-10

Strebel K, Klimkait T, Martin MA. *A novel gene of HIV-1, vpu, and its 16-kilodalton product*. Science 1988; 241(4870): 1221-1223

Studzinski GP. *Apoptosis--- A practical approach*. Oxford University Press, 1999

Suda T, Takahashi T, Golstein P, Nagata S. *Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family*. Cell 1993; 75(6):1169-1178

Tolskaya E, Romanova L, Kolesnikova M, Ivannikova T, Smirnova E, Raikhlin N and Agol V. *Apoptosis-inducing and apoptosis-preventing functions of poliovirus*. 1995; 69(2): 1181-1189

UNAIDS/WHO AIDS epidemic update: December 2005. Website: www.unaids.org

Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. *A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V*. J Immunol Methods 1995; 184(1):39-51

Wasmuth JC, Hackbarth F, Rockstroh JK, Sauerbruch T, Spengler U. *Changes of lymphocyte apoptosis associated with sequential introduction of highly active antiretroviral therapy*. HIV Med 2003; 4(2):111-119

Wasmuth JC, Klein KH, Hackbarth F, Rockstroh JK, Sauerbruch T, Spengler U. *Prediction of imminent complication in HIV-1-infected patients by markers of lymphocyte apoptosis*. J Acquir Immune Defic Syndr 2000; 23(1): 44-50

Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH. *Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120*. Nature 1995; 375(6531):497-500

Whiteside G, Cougnon N, Hunt SP, Munglani R. *An improved method for detection of apoptosis in tissue sections and cell culture, using the TUNEL technique combined with Hoechst stain*. Bra Res Pro1998 2(2):160-164

Willey RL, Rutledge RA, Dias S, Folks T, Theodore T, Buckler CE, Martin MA. *Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus*. Proc Natl Acad Sci USA 1986; 83(14):5038-5042

Wyllie AH, Kerr JF, Currie AR. *Cell death: the significance of apoptosis*. Int Rev Cytol 1980; 68: 251-306

Wyllie AH, Morris RG, Smith AL, Dunlop D. *Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis*. J Pathol 1984; 142(1): 67-77

Yonehara S, Ishii A, Yonehara M. *A cell killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumour necrosis factor*. J Exp Med 1989; 169(5):1747-1756

Zauli G, Gibellini D, Milani D, Mazzoni M, Borgatti P, La Placa M, Capitani S. *Human immunodeficiency virus type 1 Tat protein protects lymphoid, epithelial, and neuronal cell lines from death by apoptosis*. Cancer Res 1993; 53(19):4481-4485

Zhang G, Gurtu V, Kain SR, Yan G. *Early detection of apoptosis using a fluorescent conjugate of annexin V*. Biotechniques 1997; 23(3):525-531