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ANALYSIS OF THE EFFECT OF *Mycobacterium tuberculosis* *(M.tb)* ON HIV INFECTION IN THE PRESENCE OF IRON OVERLOAD

by

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THESIS
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I dedicate this work to my mother-in-law, Anastasie Ngandu, a woman of character and integrity.

Anastasie Ngandu Kilambe (1951 - 2004)
However long the night, the dawn will break.

African proverb
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Preface

Introduction

Human immunodeficiency virus / Acquired immunodeficiency syndrome (HIV/AIDS) has brought about enormous public health concerns in sub-Saharan Africa where HIV infection is taking a disproportionate toll on human life. AIDS is characterized by a number of opportunistic infections (in addition to immune system depletion) and the replication of HIV is being influenced by host factors and coexisting infections (Mancino et al., 1997). Tuberculosis (TB) is the most common of the opportunistic infections associated with AIDS in Africa (Moyo et al., 1997; Hopewell, 1989; Johnson and Chaisson, 1991). The spread of HIV in Sub-Saharan Africa has contributed to an increase in the incidences and mortality rates of tuberculosis. Indeed, Mycobacterium tuberculosis (M.t.b) and HIV seem to have a synergistic relationship when simultaneously infecting the same host (co-infection). It is reported that TB disease accelerates the course of HIV disease progression (Ranjbar et al., 2003) and inversely, HIV co-infection stimulates TB in people with reduced numbers of CD4+ cells (Gold et al., 2004).

Iron overload has been shown to play a role in host susceptibility to numerous infections (including those by HIV or M.t.b) and previous in vitro studies in this laboratory (Traoré and Meyer, 2004) and others showed its stimulatory effect on viral infection. The irony of fate is that iron overload and tuberculosis are both very common to sub-Saharan Africa, and now these common ailments are believed to augment AIDS. Thus to control/reduce HIV infection in Africa, it would be advisable to consider treating the two common conditions in parallel.

In this study, the effect of iron overload on co-infection was analyzed by looking at the effect of iron overload on (1) chronically and (2) acutely HIV-infected mammalian cells secondarily infected in vitro with M.t.b. Experimental analysis included evaluating cellular viability/death using viability dyes (Alamar Blue and 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide (MTT)) and flow-cytometry. Specialized ELISAs were used to measure viral replication, cytokine production and nitric oxide secretion. Finally, the effect of iron on bacterial viability during co-infection was assessed using a modified
fluorometric microplate-based Alamar Blue assay. The techniques mentioned allow evaluation of both pathogens and excess iron on selected host cell defence systems and conversely on pathogen survival.

The objectives of this study were to:

i. Investigate the effect of iron-overload on host cell defences during co-infection with the two pathogens

ii. Determine the effect of iron-overload on the replication of both pathogens

iii. Assess differences in both host and pathogen responses during chronic and acute infection

The hypotheses of this study were that:

i. Excess iron promotes pathogen replication while killing host cells

ii. The detrimental effects of excess iron on host cell viability can counteracted through the use of iron chelators

**Layout of thesis**

The literature overview (Chapter 1) highlights HIV disease and opportunistic infections, the detrimental effects of iron loading, and host defence responses during co-infection with HIV and/or *M.tb*. The findings of the experimental section are described, demonstrated and discussed in Chapters 2, 3 and 4 respectively.

**Outcomes of the study**

Data collected and techniques employed during this study are currently being prepared for publication in international, peer-reviewed journals:

**Article submitted**

The influence of excess iron on *Mycobacterium tuberculosis* viability in chronically HIV-infected monocytes (submitted to the Journal of Clinical Microbiology and Infection for publication in October 2004).
1. Variability in assessing the effect of Fe-overload on HIV-infected cells co-infected in vitro with *Mycobacterium tuberculosis*

2. Discrepancies observed with the viability dye Alamar Blue: Fluorescence Vs. absorbance
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I would like to express my sincere gratitude to many people who have helped me in one form or another.

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...Without whom this thesis would never have been possible.
Summary

Analysis of the effect of *M. tb* on HIV infection in the presence of iron overload

By

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Co-Promoter: Prof. Liza Bornman

Department: Department of Chemistry and Biochemistry

Degree: Ph.D. Biochemistry

Background: AIDS is characterized by a number of opportunistic infections and the immune depletion caused by HIV infection is the strongest risk factor for both reactivation of tuberculosis (TB) and progression of *Mycobacterium tuberculosis* (*M. tb*) infection to disease. Numerous studies have shown that concurrent infection of the same host cell by HIV and *M. tb* stimulates replication of both pathogens. The interaction between the two is lethal. A synergistic relationship exists between *M. tb* and HIV. While HIV spurs the spread of TB, mycobacterial infection results in acceleration of HIV disease progression. The requirement for iron as a crucial factor for cellular processes has long been demonstrated. Excess iron leads to infections with harmful consequences such as cell death and function impairment. During infection, iron is required by both the host cell and the pathogens. Iron chelation is believed to modulate some of these effects.

Objectives: *M. tb*, HIV and Fe-overload are common in sub-Saharan Africa and iron plays a major role in determining the outcome of several infections. In view of this, we wanted to (1) investigate the effect of excess iron on host cell defences during co-infection with the mentioned microorganisms, (2) evaluate the differences in both host and pathogen responses during acute and chronic infection in the presence of iron overload and (3) Determine the efficacy of iron chelation (with DFO) as a means of counteracting conditions associated with iron overload.

Hypotheses: The combination of Fe-overload and co-infection of host cells with HIV and *M. tb* in an *in vitro* model should stimulate replication of the pathogens, which would ultimately result in host cell stress manifesting as lower viability or cell death and impaired immune defence functions. Also the detrimental effects of excess iron on host cell viability could be counteracted through the use of iron chelators.

Methods: We analyzed the *in vitro* effect of *M. tb* in both chronically and acutely HIV-infected cells (PBMC's and monocytes), exposed to 500 μM FeSO₄ and/or DFO for 4 days. Host cell viability, survival and death were assessed through viability assays (MTT and Alamar Blue) and flow cytometric analyses of apoptosis/necrosis (using Annexin V and propidium iodide). Secretion of IL-6 and TNF-α and production of total nitrate were monitored as host immune/defence responses using specialized ELISAs. HIV replication was investigated by looking at core protein (p24) contents and reverse transcriptase (RT) activity. *M. tb* replication and growth was monitored using the microplate Alamar Blue assay (MABA) and quantitative culturing.
Results: Co-infection caused a reduction of host cell viability (± 20% and 45% inhibition during chronic and acute infection respectively; as measured by MTT), increases in the numbers of viral particles (2.3 times and 20% increases for chronic and acute infections respectively) and stimulation of both bacterial viability (36%) and host defence responses (30% increase in TNF-α secretion). Excess iron further decreased viability with a marked increase in necrosis of cells and was found to enhance pathogen replication and growth (26% for HIV and 47% for M.tb). Chelation of iron with DFO abrogated the enhanced replication of the pathogens with a marginal restoration of host viability.

Conclusion: The results obtained demonstrate the deleterious effect of excess iron during concurrent infection with both pathogens as well as its stimulating/enhancing properties on pathogens. On the other hand, DFO inhibited pathogen replication and host viability.
Analyse de l'effet du Mycobacterium tuberculoæ (M. tb) sur l'infection par le VIH en présence de la surcharge de fer

Par

Hafsatou Ndama Traoré

Directrice de thèse: Dr. Debra Meyer
Directrice adjointe de thèse: Prof. Liza Bornman
Département: De Chimie et Biochimie
Niveau: Doctorat en Biochimie

Fond: Le SIDA est caractérisé par un certain nombre d'infections opportunistes et l'épuisement immunitaire provoqué par l'infection au VIH est l'un des risques facteurs tant pour la réactivation de la tuberculose (TB) que pour la progression de l'infection avec M. tb à la tuberculose. Plusieurs études ont montrées que l'infection simultanée de la même cellule hôte par le VIH et M. tb stimule la réplication des deux pathogens. En effet, l'action réciproque entre les deux est mortelle. Une influence réciproque existe entre M. tb et VIH. Pendant que le VIH encourage la propagation de la tuberculose, M. tb pour sa part accélère la progression de l'infection au VIH au SIDA. L'exigence pour le fer comme facteur capital aux processus cellulaires a longtemps été démontrée. L'excès de fer cause des infections avec les conséquences malfaisantes comme la mort cellulaire et l'affaiblissement des fonctions cellulaires. Pendant l'infection, le fer est exigé tant par la cellule hôte que par les pathogens. On croit que la chelation du fer module ces effets.

Objectifs: M. tb, VIH et la surcharge en fer sont communs en Afrique sub-Saharienne et le fer determine la finalité de plusieurs infections. En vue de cela, nous avons voulu (1) enquêter l'effet de l'excès de fer sur les défenses de la cellule hôte pendant la co-infection avec les micro-organismes mentionnés, (2) évaluer les différences de réponses tant pour l'hôte que pour les pathogènes pendant l'infection aiguë et chronique en présence de la surcharge en fer et (3) déterminer l'effet de la chelation du fer (avec DFO) comme un moyen de contrer les conditions associées à la surcharge en fer.

Hypothèse: la combinaison d'une surcharge en fer et la co-infection des cellules hôtères avec le VIH et le M. tb dans un modèle in vitro devrait stimuler la réplication des pathogens, qui provoqueront finalement la tension (stress) de la cellule hôte avec une baisse de sa viabilité ou sa mort et un système immunitaire affaibli. Aussi les effets préjudiciables d'excès de fer sur la viabilité de la cellule d'hôte pourraient être contrés à l'aide d'un agent chelant.

Méthodes: Nous avons analysés l'effet in vitro de M. tb sur les cellules infectées avec le VIH (PBMC et monocytes), exposées à 500 mM de FeSO₄ et/ou DFO pendant 4 jours. La viabilité des cellules hôtères, la survie ou mort ont été évaluées grâce aux essais de viabilité (MTT et Alamar Blue) et l'analyse flow cytométrique d'apoptose/nécrose (utilisant Annexin V et propidium iodide). La sécrétion d'IL-6 et TNF-α et la production de nitrate ont été mesurées comme réponses/ défenses immunitaires de l'hôte en utilisant ELISAs. La réplication du VIH a été enquêtée en regardant le contenu de la protéine de base (p24) et l'activité enzymatique du Reverse transcriptase (RT). La réplication de M. tb et sa croissance ont été mesurées en utilisant la microplaquette Alamar Blue essai (MABA) et la culture quantitative.

Résultats: La co-infection a provoqué une réduction de viabilité de la cellule hôte (± inhibition de 20% et de 45% pour l'infection chronique et aiguë respectivement; mesuré par MTT), augmentations dans les nombres de particules virales (2.3 fois et augmentations de 20% pour les infections chroniques et aiguës respectivement) et une stimulation tant de la viabilité bactérienne (36%) que des réponses/défenses immunitaires de l'hôte (augmentation de 30% pour la sécrétion de TNF-α). L'excès en fer diminue davantage
la viabilité de l’hôte avec une augmentation marquée en necrosis des cellules et augmente la replication/croissance des pathogens (26% pour VIH et 47% pour M.tb). La chelation du fer avec DFO a annulé la réplication des pathogens avec une restauration marginale de la viabilité des cellules hôtes.

Conclusion: Les résultats obtenus démontrent l’effet nuisible de l’excès de fer pendant l’infection simultanée avec les deux pathogens aussi bien que son effet stimulant/augmenteur pour les pathogens. D’autre part, DFO a inhibé la réplication des pathogens et la viabilité de l’hôte.
List of Abbreviations

°C  degree celsius
µg  microgram
µl  microliter
µM  micromolar
%  Percentage
A  absorbency
Ab  antibody
ACK  Ammonium chloride potassium
Ag  antigen
AIDS  Acquired immune deficiency syndrome
Anti-DIG-POD  Antibody to digoxigenin conjugated to peroxidase
APC  Antigen presenting cell
ATCC  American type culture collection
ATP  Adenosine triphosphate
AZT  Zidovudine
BrdUTP  Bromo deoxyuridine triphosphate
BSA  Bovine Serum Albumin
C282Y  mutation of a cysteine to a leucine at position 282
CAT  Catalase
CC  Chemokine co-receptor
CCR5  Chemokine co-receptor for HIV
CD  Cluster of differentiation
CFU  Colony forming unit
CO₂  Carbon dioxide
CMI  Cell-mediated immunity
CSR  Coulter stopping reagent
CTL  Cytotoxic lymphocytes
CV  Coefficient of variation
ddC  dideoxycytidine
ddl  dideoxyinosine
D-Exo Desferriexochelin 772 SM
DFO Desferrioxamine
dH2O distilled water
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
dT Deoxothymidime
EIA Enzyme immuno assay
ELISA Enzyme-linked immunoadsorbent assay
env Envelope gene
FACS Fluorescence-activated cell sorter
FBS Foetal bovine serum
Fe Iron
Fe2+/Fe3+ Ferric and ferrous states
FeSO4 Ferrous sulfate
FITC Fluorescein isothiocyanate
gag group specific antigen
gp glycoprotein
GS Gentamycin sulfate
HFE Hereditary hemochromatosis protein
HH Hereditary hemochromatosis
HAART Highly active anti-retroviral
HIV Human immunodeficiency virus
HLA Human leukocyte antigen
H2O2 Hydrogen peroxide
h hour
HRP Horseradish peroxidase
HTLV Human T cell leukemia virus
ICL- 670A 4-(3,5-bis (hydroxyl-phenyl)-[1,2,4] triazol-1-yl)-benzoic acid
ICP-AES Inductively coupled plasma atomic emission spectroscopy
ICTV International Committee on the Taxonomy of viruses
IdeR  Iron dependent regulator
IFN-γ  Interferon gamma
IL  Interleukin or Illinois
IN  Integrase
INH  Isoniazid
kDa  kilo Dalton
L1  Deferiprone
LAV  Lymphadenopathy associated virus
LPS  Lipopolysaccharide
LTR  Long terminal repeat
MA  matrix
MABA  Microplate Alamar Blue assay
MDR-TB  Multi-drug resistant TB
MHC  Major histocompatibility complex
MIC  Minimal inhibitory concentration
min  minute
ml  milliliter
mm  millimeter
mM  millimolar
MRC  Medical research council
mRNA  messenger Ribonucleic acid
M.tb  Mycobacterium tuberculosis
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide
nef  negative effector
NCI  National Cancer Institute
NF-κB  Nuclear factor κB
NIAID  National institute of allergy and infectious diseases
NIH  National institute of health
NICD  National institute for communicable diseases
NK  Natural killer
NO  Nitric oxide
<table>
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<tr>
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<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NR</td>
<td>Non-reduced</td>
</tr>
<tr>
<td>NRF</td>
<td>National research foundation</td>
</tr>
<tr>
<td>NS</td>
<td>Non-specified/Non-syncytium inducing</td>
</tr>
<tr>
<td>OH*</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OIs</td>
<td>Opportunistic infections</td>
</tr>
<tr>
<td>OONO-</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>O2•-</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>p</td>
<td>Protein</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCD</td>
<td>Programmed cell death</td>
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<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide or protease inhibitor</td>
</tr>
<tr>
<td>pol</td>
<td>polymerase</td>
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<td>PS</td>
<td>Phosphatidyl serine</td>
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<td>Rifampicin</td>
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<td>Reactive oxygen species</td>
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<tr>
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<td>Ribonucleic acid</td>
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<td>Rosewell park memorial institute</td>
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<tr>
<td>RR</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RTI</td>
<td>Reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>SI</td>
<td>Syncytium inducing</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
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<td>SIV_sm</td>
<td>SIV sooty mangabey</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>tat</td>
<td>transactivation</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TC</td>
<td>T cytotoxic</td>
</tr>
<tr>
<td>TCID50</td>
<td>Tissue culture infectious dose 50</td>
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<td>TH</td>
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TMB  Tetramethyl benzendine
TNF-α  Tumor necrosis factor alpha
Ts cell  T suppressor cell
t-RNA  transfer RNA
UNAIDS  United nation AIDS program
URL  Uniform resource locator
UV  Ultraviolet
WHO  World health organization
XTT  2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
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Literature Review

1. HIV and opportunistic infections

1.1. HIV/AIDS

1.1.1 The global epidemic
For more than 20 years, HIV/AIDS has brought devastating effects throughout the world. According to 2004 estimates from the Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), more than 40 million people worldwide have been infected with HIV and AIDS has caused almost 22 million deaths. These numbers are still increasing globally despite antiretroviral therapy and prevention programs implemented to reduce AIDS and to cut down the number of new infections respectively. The majority of HIV-infected people live in the developing world where poverty, poor health care systems and limited resources for prevention and care, are some of the causes contributing to the spread of the virus (UNAIDS/WHO, 2004).

Although Africa is home to just over a tenth of the world’s population, yet the region has 70% of all HIV infection. Southeast Asia has 16%, while the rest of the world accounts for the remaining 14%. The epidemic is spread mainly through heterosexual contact in Africa and the Caribbean, it is concentrated among injecting drug users and prostitutes in Asia, Latin America and Eastern Europe. In high-income countries, transmissions are concentrated among marginalized groups of people such as injecting drug users, gay men, immigrants and refugees (Avert Org, 2004).
1.1.2 The Southern African plague

Sub-Saharan Africa has some of the best HIV surveillance systems in the world, showing the area being the most affected by the epidemic with an estimated 30 million people living with HIV/AIDS (Avert Org, 2004). However, large variations exist among different countries with the HIV prevalence ranging from less than 1% in Mauritania to 40% in Botswana and Swaziland.

There are many reasons as to why AIDS is spreading at its current rate throughout the region. Some of them were well documented by Ateka (2001). Briefly, these include high rates in both migrant labour and prevalence of sexually transmitted diseases, the coalescence of multiple strains and the sense of denial (Cohen and Makgoba, 2000). Also, the cost of drugs and the fact that AIDS is seen as the "invisible disease" in regard to the period HIV takes to develop to full blown AIDS.

Additionally, the African pandemic is worsened by a myriad of co-factors such as (1) various infectious diseases present in the region including tuberculosis or malaria and spurred on by conditions such as (2) iron overload commonly found in the region.

1.1.3 The causative agent: HIV

**Origin of the virus**

Researchers, from the Pasteur institute in Paris, isolated a previously unrecognized virus (containing reverse transcriptase) from a patient who had swollen lymph glands, and called the virus lymphadenopathy associated virus (LAV, Barre-Sinoussi et al., 1983). They subsequently reported that LAV was tropic for T-helper cells. In 1984, Robert Gallo, then head of one of the laboratory of the National Cancer Institute (NCI), described the isolation of a cytopathic T-lymphotropic virus from AIDS patients which was designated the Human T-leukaemia virus III (HTLV-III, Gallo et al., 1984). By 1985; it became clear that the two viruses represented different isolates of the same virus based on the analyses of their nucleotide sequences (Wain-Hobson et al., 1985a and b). The virus was later renamed, by the International Committee on the Taxonomy of viruses (ICTV), "Human Immunodeficiency Virus" (HIV, Coffin et al., 1986).
Chapter 1: Literature Review

Structure of the virus

HIV-1 is a lentivirus, which is believed to evolve from simian immunodeficiency virus (SIV), crossing from chimpanzees to human beings during the second half of the 20th century (Sleasman and Goodenow, 2003). There are two recognized strains of HIV, namely HIV-1 and HIV-2. HIV-2 is more related to SIVsm, but it is less common and less pathogenic than HIV-1. HIV-1 is divided into three groups and many subtypes with differences in their geographic distributions according to their origins (Sleasman and Goodenow, 2003). These three groups are, M (and its subtypes A to J), N and O. The most common subtypes of group M are: B; found in North America, Europe, parts of South America and India, C; predominant form in sub-Saharan Africa and also in India, and E; found in south East Asia. These subtypes differ both epidemiologically and antigenically causing major implications for vaccine initiatives.

The basic morphology of HIV-1 is shown in Figure 1.1 and is similar to that of other retroviruses. The HIV-1 virion has 2 copies of the viral genome. It is coated with viral glycoproteins (gp160 precursor of gp120 and the transmembrane protein gp41) protruding from the host-derived lipid envelope. Within the virus, a matrix protein called p17 forms the inner surface of the viral lipid envelope where an icosahedral shell of proteins (capsid or p24) is enclosed (Ennifar et al., 1999).

Organization of the viral genome

The "classical" structural diagram of a retroviral genome (Fig. 1.2) is: 5'LTR-gag-pol-env-LTR3'. The long terminal repeat (LTR) region represents the two end parts of the viral genome, which are connected to the host cell DNA after integration and do not encode for any viral proteins (Davies et al., 1999). The gag (group-antigen) gene encodes structural proteins, including the matrix p17, the capsid p24 and the nucleocapsid p6/p7. Then there is the polymerase (pol) gene, which encodes all the enzymes; reverse transcriptase, protease and integrase. The next gene is the envelope (env) gene encoding for the envelope glycoproteins, which include the CD4-binding region and envelope fusion protein.
The LTR 3' end of the genome enables the viral genome to form a circular plasmid-like structure by linking with the 5' LTR. In addition to the three major reading frames, HIV-1 contains in its RNA six accessory genes, some of that are essential for replication and contribute to its complexity. The transcription of transactivation (tat) gene encodes a protein essential for HIV replication. The product of the rev gene is a regulator of structural gene expression and is also essential for viral replication. Other viral genes include vpr that encodes a weak transcriptional factor, vif, which encodes a viral infectivity factor, vpu, that is required for efficient virion budding and nef which is a negative effector (Davies et al., 1999).
HIV life cycle

To get into the host cell, the proteins on the outside of the viral lipid envelope bind to receptors on the host cell. This lipid envelope will fuse with that of the target cell and release the nucleocapsid into the cell (Fig 1.3) where uncoating occurs, followed by the reverse transcription in the cytoplasm. The resulting cDNA travels into the nucleus where it is sliced, by integrase into the host chromosome randomly. The cDNA has a promoter that can respond to host cell transcription factors and codes for the proteins and nucleic acids necessary to form progeny virions. The newly formed progeny virions travel to the cell surface and bud out with a host-derived lipid envelope (Lewis et al., 1992).
The use of receptor by HIV

Besides being the primary receptor for HIV, CD4 was found not to be sufficient to allow entry of the virus. Therefore the existence of additional co-receptors were suggested and proven (Cocchi et al., 1995). These secondary receptors were later found to be members of the chemokine family of receptors (Davies et al., 1999). Chemokines are a large group of cytokines that act as chemical attractants for cells. These are classified into α- and β-chemokines according to their function and sequence homology: the α-chemokines have a characteristic cys-x-cys motif (CXC) and attract neutrophils, whereas β-chemokines have a characteristic cys-cys motif (CC) and attract monocytes and lymphocytes. The secondary receptors consist of CXCR4, CCR5 and C2. CXCR4 is the major co-receptor for T-cell line adapted isolates and CCR5 for non-T-cell line adapted isolates (Tscherning et al., 1998). CXCR4 is known as Lestr or Fusin and is expressed in dendritic cells, monocytes and B-cells (Bleul et al., 1996). The HIV-1 isolates from individuals with early asymptomatic infections are non-syncytium inducing (NSI) and utilize CCR5 as major co-receptor and infect cells of the monocyte-macrophage lineage as well as primary T-cells. In contrast, the
syncytium inducing (SI) viruses are found in individuals with more advanced disease. These isolates use CXCR4 as co-receptors. In South Africa, where the epidemic is largely due to subtype C HIV-1 viruses, CCR5 is used as the main co-receptor (Morris et al., 2001). However, recent reports have demonstrated that subtype C can use CXCR4 or both CXCR4 and CCR5 for entry (Cilliers et al., 2003; Treurnicht et al., 2002). These observations are of extreme importance to the designing of an effective vaccine against this subtype.

Treatment available for HIV

Eradicating HIV is currently not a realistic target of antiretroviral therapy; however, the use of highly active antiretroviral therapy (HAART) drugs has the potential to dramatically improve the health and extend the lives of people with HIV/AIDS (Palella et al., 1998). Yet the high cost and demanding clinical requirements of these drugs make them out of reach for most infected people, especially in developing countries, where HIV infection is high and associated to poor public resources (Morley and Power, 2000). Also in industrialized countries, the cost of HAART is substantial, even if there is a positive change in both health and life expectancy. Furthermore, these drugs and HIV healthcare are not available to many in some developed countries (Morley and Power, 2000). In addition, the complexity of therapy and long-term treatment with HAART do not work for everyone and some viral strains are becoming drug-resistant (Smit et al., 2004). The main disadvantage of HAART is its inability to completely suppress HIV replication (Turpin, 2003). Therefore, a constant search for new/improved antiretrovirals and targets becomes critical. HAART consists of reverse transcriptase inhibitors (RTI's), protease inhibitors (PI's) and emerging classes of HIV inhibitors, including entry (T-20; Ketas et al., 2003), coreceptor, integrase (SCH-C, beta-Diketos; Turpin, 2003) and viral enzymes (Agenerase; King et al., 2003) inhibitors. A list of these drugs is indicated in Table 1.1 and has been approved by the National Institute of Allergy and Infectious Diseases (NIAID). Moreover, the drugs shown here are not a comprehensive list of all the drugs that are available and several additional drugs are still in developments. In general, these drugs reduce viral load and increase CD4+ cell counts in patients in all stages of HIV infection and are used in combination to lower the possibility of viral drug-resistance (Feinberg et al., 1998).
Although these drugs suppress the virus, immune reconstitution is not achieved. Thus, restoring the immune system is an important factor when managing the disease (Angel, 2001). Immune-based therapies may help to restore host immune responses against pathogens without any side effects. Some studies have evaluated the safety and immunologic effects of these therapies such as, granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin (IL)-2 and inactivated HIV-1 immunogen (Angel, 2001) and shown both a durable viral suppression and a decrease in toxicity.

Over the past decades, researchers in the antiretroviral field have also turned to many traditional medicines that includes a “cocktail of natural products”, in order to determine the scientific basis of their remedial effects (Bedoya et al., 2001). More than 50% of the drugs available on the market today have their origin in nature (Jung et al., 2000). Natural products are good sources for the search for effective anti-HIV agents with decreased toxicity. Some of the plants deliver a single chemical entity or harbour multicomponent bioactive ingredients that enhance the immune system while others contain antiviral chemotherapeutic agents (Houghton, 1996).

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Generic name</th>
<th>Trade name</th>
<th>Manufacturers</th>
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<tr>
<td>Nucleoside analogs</td>
<td>AZT</td>
<td>Retrovir</td>
<td>Glaxo-Wellcome</td>
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<td></td>
<td>DDI</td>
<td>Videx</td>
<td>Bristol-Meyers Squibb</td>
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<tr>
<td></td>
<td>DDC</td>
<td>HIVID</td>
<td>Hoffman-LaRoche</td>
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<tr>
<td></td>
<td>3TC</td>
<td>Epivir</td>
<td>Glaxo-Wellcome</td>
</tr>
<tr>
<td></td>
<td>D4T</td>
<td>Zerit</td>
<td>Glaxo-Wellcome</td>
</tr>
<tr>
<td>Non-nucleoside RT</td>
<td>Nevirapine</td>
<td>Viramune</td>
<td>Boeringer Ingelheim</td>
</tr>
<tr>
<td>inhibitors</td>
<td>Delavirdine</td>
<td>Rescriptor</td>
<td>Pharmacia &amp; Upjohn</td>
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<tr>
<td></td>
<td>Abacavir</td>
<td>Ziagen</td>
<td>Glaxo-Wellcome</td>
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<td></td>
<td>Efavirenz</td>
<td>Sustiva</td>
<td>DuPont-Merck</td>
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<td>Pro tease inhibitors</td>
<td>Saquinovir</td>
<td>Fortavase</td>
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<td>Indinavir</td>
<td>Crixivan</td>
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<td>Ritonavir</td>
<td>Norvir</td>
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<td>GlaxoSmithKline</td>
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<tr>
<td>Fusion inhibitors</td>
<td>Pentafuside</td>
<td>T-20</td>
<td>Trimeris/Roche</td>
</tr>
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<td></td>
<td>R724</td>
<td>T-1249</td>
<td>Trimeris/Roche</td>
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1.2 Opportunistic infections associated with HIV infection

The impairment of immune function induced by HIV infection exposes individuals to a variety of infections, called opportunistic infections. These concurrent infections have been reported to stimulate viral infection (Juffermans et al., 2000) and are associated with enhanced HIV replication. However, the exact mechanism of action of these opportunistic infections on HIV-1 is unclear (Toossi et al., 2001). These include *Mycobacterium avium* (Denis and Ghadirian, 1994), *Pneumocystis carinii* (Israel-Biet et al., 1993), herpes simplex virus-1 (Heng et al., 1994) and *Mycobacterium tuberculosis* (*M.tb*, the most common HIV-related opportunistic pathogen; Shattock et al., 1993). The growing epidemic of HIV has given new life into an old enemy - tuberculosis (TB). TB is one of the initial manifestations in people dying from AIDS (Scharer and McAdam, 1995). Most of the cases of dual HIV/TB infection have been reported in sub-Saharan Africa (Toossi et al., 2001).

1.2.1 TB/*M.tb*

Pathophysiology of TB

TB is a communicable disease caused by the tubercle bacilli *M.tb*. It is spread by airborne droplet nuclei that are infectious aerosols of 1-5 μm in diameter (Frieden et al., 2003). After inoculation, the droplets lodge in the alveoli in the distal airways where the bacilli are phagocytosed by alveolar macrophages (Scharer and McAdam, 1995; Frieden et al., 2003). This will result in either successful containment of the infection or progression to active disease (primary progressive disease). Normally, the initial phase of tuberculosis infection is without symptom but in few cases, *M.tb* can overcome control by macrophages and TB develops. The signs of tuberculous disease include malaise, fatigue, weakness, weight loss, fever (Murray et al., 1998) and night sweats (Scharer and McAdam, 1995).

After HIV/AIDS, TB is the second most common cause of death from infectious diseases throughout the world (Frieden et al., 2003). TB, a disease long associated with overcrowding, malnutrition, race (ethnic minority groups) and poverty, was thought to be almost eradicated worldwide until recently (Piddock et al., 2000). The number of tuberculosis cases has increased in many developing and developed countries (Davies and Grange, 2001). In 2000, an estimated 9 million new cases of tuberculosis were reported...
with the highest rate found in sub-Saharan Africa (Frieden et al., 2003). However the most populated countries in Asia have the largest numbers of cases. The resurgence of TB can be attributed to the HIV/AIDS pandemic, the emergence of multidrug-resistant strains of \textit{M.\textit{tb}} (MRD-TB) and to the economic and health services decline in some countries (Piddock et al., 2000; Frieden et al., 2003). While TB is still prevalent in areas where malnutrition and poor living conditions occur, in America and Europe, tuberculosis increase is mainly associated with HIV infection. Nevertheless, others factors such as immigration, poverty and genetic factors contribute to the increase of tuberculosis in developed countries (Davies and Grange, 1998).

\textit{Structure of \textit{M.\textit{tb}}}

\textit{Mycobacteria} are aerobe prokaryotic organisms, members of the genus \textit{Mycobacterium}, and called acid-fast organisms because they do not stain easily with stains such Kinyoun, but once stained, they resist decolourization by acidified organic solvents (Jawetz et al, 1982). The group includes pathogenic organisms that cause chronic diseases. Two main species are responsible for tuberculosis in humans, \textit{Mycobacterium tuberculosis} and \textit{Mycobacterium bovis}.

\textit{M.\textit{tb}}, the primary etiologic agent of tuberculosis, is a facultative intracellular pathogen (Gobin and Horwitz, 1996), which grows within macrophages (Ehlers and Daffe, 1998). It is a fairly large thin rod-shaped bacterium measuring $0.4 \times 3 \ \mu m$ and resistant to chemical agents because of the hydrophobic nature of the cell surface (Fig. 1.4). It possesses a complex cell wall formed by a peptidoglycan skeleton (Murray et al, 1998). The cell wall is made of lipids, largely bound to proteins and polysaccharides. Up to 60% of the cell wall is formed by lipids (located on the outer layers) which confer to them the acid-fast properties. These lipids include complex lipids, fatty acids and waxes (Jawetz et al, 1982; Murray et al, 1998). A characteristic of virulent strains of \textit{M.\textit{tb}} is the “cord” formation (Jawetz et al, 1982) via association of cord factor (6, 6”-dimycolate of trehalose) resulting in a parallel alignment of bacilli rows. The proteins bound to a wax fraction are biologically important because they can stimulate the host to produce antibodies (Murray et al, 1998).
Polypeptides (e.g., PPD)

Free lipids (e.g., waxes, mycosides, cord factor)

Arabinogalacton mycolate layer

Peptidoglycan layer

Cytoplasmic membranes

Cytoplasm

Mycolic acids

D-arabinose and D-galactose

N-acetyl glucosamine and
N-acetyl muramic acid

D-glutamic acid, m-diaminopimelic
acid, L- and D-alanine

Figure 1.4. *M.tbc* surface structure. Beneath the protective coat, a plasma membrane encloses a cytoplasmic compartment (taken from Murray et al, 1998).

Treatment for *M.tbc*

The goal of TB treatment include cure without relapse, preventing death, stopping transmission and preventing the emergence of drug resistance (Frieden et al., 2003). The need for long-term treatment is necessary because of (1) the bacilli can remain dormant for long periods and (2) the existence of naturally resistant mutants. All recommended treatment regimens have two phases including an initial intensive phase and a continuation phase. The initial phase is designated to kill actively growing and semi dormant bacilli while the second phase is when all residual bacilli are eliminated (Frieden et al., 2003). The common anti-tuberculosis drugs are isoniazid (INH), ethambutol, rifampicin (RIF) and streptomycin (Jawetz et al, 1982). But, most mycobacteria become resistant to these drugs.
Effective therapy for infection with \textit{M.\textit{tb}} requires concomitant use of these drugs in order to avoid the selection of resistant organisms. The most commonly used is isoniazid combined with rifampicin (Murray \textit{et al}, 1998). Since Mycobacteria multiply slowly, treatment has to be taken for 6 months. The emergence of multi drug-resistant tuberculosis (MDR-TB) has added a huge burden to TB therapy. Currently, immunotherapeutic investigation are being done to reduce the duration of therapy and to find an alternative treatment for MDR-TB (Prabha \textit{et al}, 2003). In the case of TB in HIV-infected people, the goal of co-infection management is to provide potent and safe antiretroviral therapy and anti-tuberculosis treatment enough to cure and prevent recurrence and resistance (Pedral-Sampaio \textit{et al}, 2004). However, severe adverse reactions are reported in TB-HIV co-infected people during simultaneous treatment (Munsiff and Fujiwara, 2000). A recent study has found Efavirenz to be sufficient and safe to treat HIV/TB patients who are on antiretrovirals (Pedral-Sampaio \textit{et al}, 2004).

1.2.2 HIV and \textit{M.\textit{tb}} interactions

Eleven percent of all AIDS-related deaths are due to tuberculosis (Pedral-Sampaio \textit{et al}, 2004) and the role of \textit{M.\textit{tb}} in enhancing HIV-1 morbidity and mortality has clearly been demonstrated (Toossi \textit{et al}, 2001). The two infections fuel each other and together they form the leading infectious causes of mortality worldwide (Aliyu and Salihu, 2003). Whether patients contract HIV or \textit{M.\textit{tb}} first, the synergy between the two organisms is deadly. Tuberculosis-HIV infection presents particular diagnostic and therapeutic challenges and constitutes a big burden on the health care systems of heavily infected countries. A synergistic relationship exists between mycobacteria and HIV, in which each enhances the replication/pathogenicity of the other (Shafer \textit{et al}, 1996). HIV-infected people are more susceptible to mycobacterial infections, a result of an impairment of immune response to the bacteria. Conversely, mycobacterial infection stimulates (through its immunostimulant effect) the replication of HIV and therefore accelerates disease progression (Biswa et al., 2003; Goletti \textit{et al}, 1996; Meylan \textit{et al}, 1992). Several mechanisms leading to viral replication during co-infection have been suggested and revolve all around the host cell immune activation (Mancino \textit{et al}, 1997; Goletti \textit{et al}, 1996; Ho, 1996). One such mechanism would be via cytokines that are triggered during
phagocytosis of M.tb (Nakata et al., 1997; Zhang et al., 1995). Also, the changes in surface markers associated with M.tb-induced differentiation of cells could facilitate viral replication (Mancino et al., 1997; Juffermans et al., 2000). In addition, reactive oxygen intermediates (produced by M.tb-infected macrophages) could initiate viral replication (Mancino et al., 1997). Finally, the induction of antigen-specific T cell proliferation by M.tb-infected cells could also induce viral replication (Mancino et al., 1997).

Numerous factors have been speculated on as being co-factors contributing to the spread and elevated incidence of HIV-M.tb co-infection. One such factor is hereditary or dietary iron overload commonly found in Africa (Moyo et al., 1997). This condition is described in detail in section 3 and the implications associated with it will be discussed in Chapter 4.

2. Immune responses to infections and nitric oxide production

2.1 Overview of the immune system

The different types of mammalian immune responses resort into two groups, namely innate (or non-adaptive) and adaptive (or acquired/specific) immune responses. These two responses differ on the basis that an adaptive immune response displays a specific reaction to a particular pathogen. Also, it shows four major characteristics: antigenic specificity, diversity, immunologic memory and self/non-self recognition (Goldsby et al., 2000; Roitt et al., 2001). Although the innate immune response does not change after repeated exposure to a given pathogen, the adaptive response improves with each encounter with the same infectious agent by inducing a heightened state of immune reactivity. Finally, the adaptive immune response responds only to foreign agents, showing its ability to distinguish self from non-self (Goldsby et al., 2000).

Immune responses are produced primarily by leukocytes of which there are phagocytes and lymphocytes. The phagocytic cells, which include monocytes, macrophages and polymorphonuclear neutrophils, eliminate microorganisms in primitive non-specific recognition systems: by binding, internalizing and killing them. These cells initiate the first line of defence against infection (Fig. 1.5). Moreover, the adaptive immune response comprises of two branches: humoral and cellular immunity. These two branches are formed by B and T lymphocytes. While B cells eliminate extracellular pathogens by releasing
antibody (humoral immunity) that specifically binds to the target molecule (antigen), T cells have a wider range of activities. Some (T_H) are involved in both the control of B cell development and antibody production and others (T_C) help destroy pathogens taken up by phagocytic cells (Roitt et al., 2001). Once a pathogen is internalized by antigen specific phagocytic cells called antigen-presenting cells (APC); it is degraded and shown to T_H cells. These T_H cells recognize only antigens presented on the surface of an APC by major histocompatibility complex (MHC) class I molecules (Roitt et al., 2001). This will lead to activation of T_H cells and secretion of various cytokines. When a T_C is presented with an antigen by MHC class I and is stimulated by T_H induced cytokines, it proliferates and differentiates into functional cytotoxic T-lymphocytes (CTL’s), and this constitutes the cellular immunity part (Ward et al., 1998; Roitt et al., 2001). The humoral immune response involves the production of antibodies and is mediated by B cells, which displays both immunoglobulins and MHC class II proteins on their surfaces. When B cells encounter antigens that bind to their particular immunoglobulins, engulf bound antigen molecules; digest them into fragments that are then displayed at the cell surfaces nestled inside a class II MHC. Helper T cells specific for this structure will bind the B cells and secrete lymphokines, which stimulate B cells to proliferate and differentiate. (Goldsby et al., 2000; Roitt et al., 2001).

Figure 1.5. The humoral and cell-mediated branches of the immune system. Taken from www.people.virginia.edu (URL active on 21 October 2004).
2.2 Immune responses induced by HIV and *M. tb*

2.2.1 Immune response induced by HIV

A major problem encountered when designing effective immunotherapies and vaccines is the fact that HIV induces an inexorable decline in the immune system, resulting in continuous viral replication despite the presence of HIV-specific responses (Cohen and Fauci, 2001). Some evidence suggests that particular immune responses to HIV may actually be harmful to the host (Cohen and Fauci, 2001). However, the slow progression to disease and strong HIV-specific immune responses show a beneficial role for both humoral and cell-mediated immune responses.

Neutralizing antibodies, responsible for partial control of HIV replication in vivo, are of two forms, type specific (specific to one viral isolate) and group specific (specific to a board range of viral isolates). Both forms of neutralizing antibodies appear to be prognostically relevant in the course of HIV infection (Cohen and Fauci, 2001).

A role for CD8$^+$ cytotoxic T lymphocyte (CTL) in suppressing HIV replication has been shown after the detection of high levels of HIV-specific CTL’s in HIV-infected patients (Cohen and Fauci, 2001). It was also shown that CTL levels correlate inversely with viral load. CD8$^+$ cells recognize and kill HIV-infected cells, which displays MHC class I proteins. These cells produce chemokines that bind to their seven-transmembrane G protein-coupled receptors thus blocking HIV entry into macrophages or T-cells (Copeland, 2002). While CTLs can directly interfere with the HIV life cycle, CD4$^+$ T$_H$ cells exert their function through the secretion of cytokines (reviewed in detail in section 2.3).

2.2.2 Immunity to *M. tb* and hypersensitivity

During primary infection, the host acquires a certain resistance and hypersensitivity to the tubercle bacilli (Jawetz *et al.*, 1982) and cell-mediated immunity develops after 2-8 weeks (Frieden *et al.*, 2003). Hypersensitivity and cell-mediated immunity, two different immune host responses, can stop the tubercle bacilli multiplication and spread. Indeed, the role of hypersensitivity is to kill inactivated macrophages in which tubercle bacilli are multiplying. Cell-mediated immunity (CMI) by T-lymphocytes activates macrophages so they can kill and digest bacilli they have ingested (Fig. 1.6). If there is no defect in CMI, the infection
remains contained and the disease is resolved. The response is mediated via the secretion of complex cytokine pattern by antigen-specific T-cells. The cytokine pattern in TB is suggestive of TH1 type (Prabha et al., 2003). These cytokines including gamma interferon (IFN-γ), Tumor Necrosis Factor (TNF)-α, IL-2, IL-12 and IL-18 are the ones that bring about granulomas formation, intramacrophage elimination of the bacillary antigens and fibrosis leading to the resolving of the disease (Prabha et al., 2003; Mariani et al., 2001).

**Invading bacilli**

1. Invading bacilli
2. Phagocytosis

**Sensitize lymphocytes**

**Excretion of lymphokines**

**Activation of Macrophages**

**Arrest multiplication**

**Destroy Bacilli**

**Figure 1.6. Cell-mediated immunity during M.tb infection.** Cell-mediated immunity is well established when granuloma occurs: Lymphocytes have released lymphokines and macrophages are activated (Drawn according to information from Murray et al., 1998 and Scharer and Mc Adam, 1995).
2.3 Cytokines and HIV/M.tb infections

Cytokines play a major role in controlling the balance of the immune system and determine the outcome of any infectious processes (Kedzierska and Crowe, 2001; Cohen and Fauci, 2001). All cytokines are proteins that sometimes contain carbohydrate moieties. Cytokines with activity against or produced in response to HIV or M.tb fall into a number of categories as shown in table 1.2.

Cytokines have a large variety of names (Roitt et al., 2001) and the confusion in their names is based on the fact that cytokines can have multiple functions (Tizard, 1995). The majority of cytokines normally act locally by having a direct effect on the target cell that releases them (autocrine) or over very short distances on target cells in their immediate vicinity (paracrine). Cytokines are highly potent and elicit their biological effects at very low concentrations due to their specificity and affinity towards binding to their receptors on target cells (Tizard, 1995). In simple words, they function in a complex network where production of one will influence the production of several others (Roitt et al., 2001).

Cytokines that are produced by the host in response to an infection will determine the outcome of the infectious process (Cohen and Fauci, 2001). HIV infection results in the deregulation of the cytokine profile in vivo and in vitro. Several of the resulting alterations in cytokine secretion contribute to HIV infectivity by inducing viral replication and suppressing any efficient antiviral response. Moreover, HIV infection leads to a decrease in secretion of T-helper cell type 1 (TH1) cytokines such as interleukin (IL)-2 and antiviral interferon (IFN)-γ whereas the production of T-helper cell type 2 (TH2) cytokines, IL-4, IL-10, proinflammatory cytokines (IL-1, IL-6, IL-8) and tumor necrosis factor (TNF)-α is increased (Kedzierska and Crowe, 2001; Cohen and Fauci, 2001). In addition IFN-γ, a TH1-type cytokine reported to be essential in TB immunity, is the single most important factor for macrophage activation and TNF-α induction (Prabha et al., 2003). However in the case of dual infections with M.tb and HIV, signals released during phagocytosis of M.tb to kill mycobacteria are potent inducers of HIV replication (Collins et al., 2002; Imperiali et al., 2001). Their mechanism of action is also well documented (Toossi et al., 1999). TNF-α activates the cellular transcription factor NF-κB, which is an inducer of HIV LTR-mediated transcription. Although IL-6 stimulates HIV replication by a posttranscriptional
mechanism, it can react with NFκB-inducing cytokines to enhance HIV transcription (Cohen and Fauci, 2001).

Conversely, the production of HIV by cells can be inhibited by addition of antiproinflammatory cytokines or receptor antagonists (ra) such as IL-1ra. In cultures, the viral-suppressive activity of some cytokines such as IL-10 is mainly due to their ability to inhibit the secretion and activity of HIV-inducing proinflammatory cytokines (Cohen and Fauci, 2001). Thus, such cytokines can easily influence HIV production by infected cells due to their antiproinflammatory and antiproliferative activities. The use of these cytokines as therapeutic agents and elements to reconstitute immune response in HIV disease has increased over the years. Accordingly, administration of IL-2 to HIV-infected patients receiving antiretroviral therapy, significantly increased CD4+ cells with no increases in viral load (Cohen and Fauci, 2001). Further studies are investigating the role of cytokines in the immune system and their role on AIDS progression and include an exotic list of cytokines such as IL-12 and IL-16 (Cohen and Fauci, 2001).

Table 1.2. The effects of different types of cytokines on HIV and M.tb (compiled from Kedzierska et al., 2003; Frieden et al., 2003).

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Examples</th>
<th>Effects on HIV</th>
<th>Effects on M.tb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukins</td>
<td>IL</td>
<td>IL-2, IL-3, etc</td>
<td>IL-10, 13, 16 inhibitory, IL-1, 6 stimulatory, IL-4 bifunctional</td>
<td>IL-12 indirect inhibition</td>
</tr>
<tr>
<td>Interferons</td>
<td>IFN</td>
<td>IFN-α, -β, -γ</td>
<td>Inhibitory effects</td>
<td>IFN-γ indirect inhibition</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>TNF</td>
<td>TNF-α, -β</td>
<td>Stimulatory effects</td>
<td>TNF-α inhibition</td>
</tr>
<tr>
<td>Growth factors</td>
<td>GF</td>
<td>NGF, EGF</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Colony stimulating</td>
<td>CSF</td>
<td>M-CSF, G-CSF, GM-CSF</td>
<td>M-CSF stimulates G-CSF inhibits</td>
<td>NS</td>
</tr>
<tr>
<td>factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemokines</td>
<td>RANTES, MCP-1, MIP-1-α</td>
<td>β-chemokines Inhibitory</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS: not specified
2.4 Nitric Oxide production

Nitric Oxide (NO) is a small molecule produced by many mammalian cell types and plays an important role in many biological processes (Bertholet et al., 1999; Ciani et al., 2002). NO, known as a mediator of several physiological functions depending on the place of its release, is implicated in various immunological disorders (Blond et al., 1998). It can be a neurotransmitter, regulate blood pressure or inhibit blood coagulation. In addition, NO can act as a cytostatic agent and its presence may halt the proliferation of cancer and pathogens. Moreover, NO is produced abundantly during host defence reactions (Torre and Ferrario, 1996) and has been involved in microbicidal activity of cells (Blond et al., 1998). Its implication with HIV infectivity was demonstrated by high levels of nitrite (NO$_2^-$) and nitrate (NO$_3^-$) found in the serum of infected individuals (Blond et al., 1998), especially in those co-infected with M.tuberculosis (Tone et al., 2002). However, excessive production of NO may result in tissue damage and various disorders (Torre and Ferrario, 1996) suppress both T-cell proliferation and production of antibodies (Torre and Ferrario, 1996; Blond et al., 1998) and therefore down-regulates immune response against pathogens (Torre and Ferrario, 1996).

NO is produced by nitric oxide synthase (NOS), which has three distinct isoforms and three genes encoding NOS. Two are believed to be constitutively expressed (eNOS and nNOS) and are characterized in endothelial and neuronal cells respectively. By contrast, the third one is inducible (iNOS) and modulated by several stimuli including lipopolysaccharide (LPS) and INF$_\gamma$ (Blond et al., 1998). NO is formed by the conversion of L-arginine and O$_2$ into the co-products NO and L-citrulline (Torre et al., 2002). In the cell, NO can react with some molecules and is scavenged by superoxide to form peroxynitrite (OONO$^-$) which in turn rearranges to nitrate (Wanchu et al., 2002). OONO$^-$ is a free radical that can initiate inflammation-mediated nucleic acid (via guanine residues) and protein damage (via tyrosine residues) leading to tissue injury (Zhang et al., 2002).
3. Iron and infection

3.1 Iron overload

3.1.1 Classification of iron overload

In clinical practice, the most common condition is iron overload as illustrated in Table 1.3. Fe overload is either primary (as a result of an inherited disorder) or secondary (acquired or as a result from diseases) to a problem resulting in an increase in iron absorption (Harrison and Bacon, 2003). By far, hereditary hemochromatosis (HH, about 10% - 12% of Caucasians are heterozygous for the condition) is the most common genetic disorder with the HFE-related HH being the prevalent form. The HFE protein is normally associated with the transferrin receptor and prevents internalization of iron-transferrin complex in the cells (Gross et al., 1998), thus acting as a brake on cellular iron uptake. Most cases of HFE-related HH are caused by the C282Y (mutation of a cysteine to a leucine at position 282) mutation. This mutation disrupts the folding of the HFE protein, which can no longer associate with the transferrin receptor and therefore does not dampen iron uptake by cells (Harrison and Bacon, 2003; Lebron et al., 1998). Increasing evidences are emerging regarding the existence of other inherited forms of iron overload that are not caused by HFE mutations (Harrison and Bacon, 2003). These include mutations in the genes coding for ferroportin 1 and transferrin receptor-2, whereas Juvenile hemochromatosis is not considered as an inherited disorder.

Secondary iron overload results from the increased absorption of iron from erythrocytes, independently from HH. Some of the examples are all iron-loading anaemia, chronic liver diseases and parental iron overload.

3.1.2 African iron overload

African iron overload is a result of increased absorption of iron occurring in individuals from sub-Saharan Africa and in African-Americans. Its causes are not completely understood (Barton et al., 2003). For many years it was believed that African iron overload occurred in only beer drinkers (Gordeuk, 2002) where enormous amounts of Fe were found in traditional beers brewed (from local grains) in steel drums. Recent studies show that in addition to dietary iron content, a genetic mutation may also be involved in iron overload in
Africans (Gordeuk, 2002; Barton et al., 2003; Beutler et al., 2003). However, the mutation is different from the ones observed in the HFE gene found in Caucasians because it is not HLA-linked (Beutler et al., 2003). Studies, on an autosomal dominant form of iron overload, suggested that mutations of the ferroportin gene may be responsible for the iron overload found in both African-Americans and the South African Bantu population (Beutler et al., 2003; Barton et al., 2003).

Table 1.3. Classification of iron overload (taken from Harrison and Bacon, 2003)

<table>
<thead>
<tr>
<th>Categories</th>
<th>Types</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hereditary hemochromatosis</strong></td>
<td></td>
</tr>
<tr>
<td><em>HFE</em>-related</td>
<td>C282Y/C282Y</td>
</tr>
<tr>
<td></td>
<td>C282Y/H63D</td>
</tr>
<tr>
<td></td>
<td>Other HFE mutations</td>
</tr>
<tr>
<td><strong>Non-HFE-related</strong></td>
<td>Juvenile hemochromatosis (<em>HFE</em> 2)</td>
</tr>
<tr>
<td></td>
<td>Transferrin receptor-2 mutations (<em>HFE</em> 3)</td>
</tr>
<tr>
<td></td>
<td>Ferroportin 1 mutations (<em>HFE</em> 4)</td>
</tr>
<tr>
<td></td>
<td>African overload*</td>
</tr>
<tr>
<td><strong>Secondary iron overload</strong></td>
<td></td>
</tr>
<tr>
<td>Acquired iron overload</td>
<td>Iron-loading anaemia’s</td>
</tr>
<tr>
<td></td>
<td>Parental iron overload</td>
</tr>
<tr>
<td></td>
<td>Chronic liver disease</td>
</tr>
<tr>
<td></td>
<td>Dysmetabolic iron overload syndrome</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neonatal iron overload</td>
</tr>
<tr>
<td></td>
<td>Acerulopasminemia</td>
</tr>
<tr>
<td></td>
<td>Congenital atransferrinemia</td>
</tr>
</tbody>
</table>

* African overload is a Non-HFE-related inherited condition.
3.1.3 Effect of iron overload on host defence mechanisms
Through its participation in the Fenton reaction, iron overload becomes harmful with adverse effect on immunity. Iron overload seems to affect the immune system by altering the proliferation of both T- and B-lymphocytes (Means and Krantz, 1992; Weiss et al., 1992), which results in host defence mechanisms being hampered. Increased iron will lead to inhibition of IFN-γ mediated defence mechanisms and NO-dependent antibacterial activity of macrophages (Cronjé and Bornman, 2004). This is seen as a reduction of the metabolic pathways and a reduction in cytotoxic activity toward intracellular pathogens. In addition, a reduction of MHC class II expression occurs in increased intracellular iron levels (Gordeuk et al., 2001) resulting in a shift in the ratio CD4⁺ (T-helper) and CD8⁺ (T-suppressor) cells with expansion of CD8⁺ cells (Marx, 2002). Programmed cell death (PCD) is another host defence system affected by iron (Jacob et al., 1997). However, the role played by iron is still not clearly defined. Therefore, iron appears to be a two-edged sword. On one hand it is essential for growth and proliferation but on the other, in excess it contributes to increase in ROS production resulting in the induction and acceleration of PCD (Cronjé and Bornman, 2004).

3.2 Competition between host and pathogens for iron

3.2.1 General aspects of the competition
Most microorganisms live in cavities inside and on the surface of the body thus providing a kind of protection for the host against other pathogenic species (Marx, 2002). During infection, iron is important for both host and pathogen and will lead to a competition between host and parasite that ultimately becomes critical to the outcome of many infections (Marx, 2002; Jurado, 1997). Inside a potential host, iron is mostly stored in red cell haemoglobin and in biological fluids attached to high affinity proteins such as transferrin, ferritin or lactoferrin (Marx, 2002; Jurado, 1997), and is therefore not directly available to microorganisms. In contrast during infection, iron is released from storage by inflammatory mediators, such as TNF-α and NF-KB. Then, the “free” iron becomes part of the host phagocytic function where it has a role in the killing of microorganisms by producing the antimicrobial defence molecules superoxide anion radical (O₂⁻; Marx, 2002).
In addition, this iron is involved in host macrophage-mediated cytotoxicity by catalyzing the production of reactive oxygen species (ROS) within the phago-lysosome as a mean for host cell defence against microorganisms (Gordeuk et al., 2001). However, the “free” iron would also be easily available to the pathogens that have developed sophisticated strategies to extract iron from the various ligands and receptors.

In their host, microorganisms acquire iron from several systems including the siderophore system, the enzymatic breakdown of iron-binding proteins, the reduction of the Fe$^{3+}$ complex to Fe$^{2+}$ complex (resulting in iron release) and the interaction of microbial receptors with the Fe-glycoprotein complex (Jurado, 1997). Microbial uptake of iron is possible with help from receptors or channels (localized in the membranes of the microbes) and siderophores that are released in the environment to catch iron (Marx, 2002). This acquisition is a complicated system caused by the difficulties associated with iron transportation across cellular membranes. Based on strategies used by pathogens to gain access to iron from hosts, iron can then be classified as a co-factor for microbial virulence.

3.2.2 Iron and HIV/Mtb

The high levels of iron seen in sub-Saharan Africa due to dietary intake (Gordeuk, 2002), genetic factors (Barton et al., 2003; Beutler et al., 2003) and HIV (where an alteration in iron metabolism occurs because of viral infection; Savarino et al., 1999) could be a contributing factor to increased rates of several infections including HIV and M.tb. Excess iron assists in the replication of both pathogens independently. There are numerous mechanisms reported as to how iron is involved in HIV replication (Georgiou et al., 2000; Weinberg, 1996). First, iron activates NF-κB (contained in the viral LTR) via increase of reactive oxygen species (ROS; van Asbeck et al., 2001), which in turn will result in the up-regulation of viral transcription. The second mechanism implicates the presence of an iron dependent host enzyme, ribonucleotide reductase (RR) involved in viral DNA synthesis (Lori et al., 1994). Over the years, the involvement of iron in M.tb virulence has been demonstrated in several studies summarized by Lounis et al. (2001). Indeed, high levels of iron has been shown to result in M.tb infection and replication (Cronjé and Bornman, 2004; Lounis et al., 2001). A large number of enzymes encoded in the M.tb genome, need iron as obligate cofactor (Cole et al., 1998). This includes all the enzymes that are controlled by
the iron-dependent regulator IdeR and those involved in oxygen metabolism and DNA synthesis, also iron-containing superoxide dismutase (SOD) and catalase (CAT).

3.3 Iron toxicity and host iron withholding strategy

3.3.1 Iron toxicity
The amount of iron within the cell is carefully regulated in order to provide an adequate level of nutrient while preventing its accumulation and toxicity. The property of iron to easily donate or accept an electron turns it into a potential biohazard, because the metal is able to catalyze the generation of noxious radicals (Papanikolaou and Pantopoulos; 2005). Iron's toxicity resides on the Fenton and Haber-Weiss chemistry where catalytic amounts of iron yield reactive oxygen intermediates (ROIs) such as, hydroxyl radicals (OH) from superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). These oxygen intermediates can also be produced by the NADPH oxidase complex, which is an important tool for the antimicrobial defence of host cell (Papanikolaou and Pantopoulos; 2005). During infection, the enzyme complex assembles thus generating high levels of superoxide. The resultant products including peroxynitrite (ONOO$^-$) and hypochlorite (OCI$^-$) will amplify the bactericidal (and cytotoxic) capacity of phagocytic cells. Moreover, redox iron catalyzes the generation of not only hydroxyl radicals, but also of organic reactive species.

Free radicals, being highly reactive species, may promote oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. The increased generation of reactive oxygen and/or nitrogen species is referred to as "oxidative stress". Increase of redox active iron induces an aggravation of oxidative stress and results in accelerated tissue degeneration. This is seen in disorders associated with hereditary or secondary iron overload (Tam et al., 2003).

Under normal conditions, iron is trapped by transferrin where it becomes unavailable for reactions with ROIs. However, if iron imbalance leads to the mobilization of iron from transferrin, the released iron can participate in redox reactions generating ROIs. In principle, the antioxidant system (SOD, catalase, GPX) prevents the damage induced by free radicals, but oxidative damage will occur when ROIs production exceeds cellular
antioxidant defences. A fundamental mediator of tissue injury in human diseases is the result of free radical injury (Papanikolaou and Pantopoulos, 2005; Tam et al., 2003).

3.3.2 The iron withholding system

Host iron withholding mechanisms that forms part of innate immunity (Weinberg, 2000a), involve (1) iron being integrated in essential proteins such as haemoglobin, myoglobin and cytochromes, and (2) iron bound to iron-binding-proteins (transferrin, lactoferrin and ferritin; Jurado, 1997). This defence system functions to sequester hazardous amounts of iron, protect host defence cells, and deprive pathogens of the metal (Weinberg, 1999) therefore controlling replication of pathogens (Ryu et al., 2000). The iron-withholding mechanism’s constituents can be separated in 3 classes (Table 1.4) according to their nature, the type of response induced and the type of pathogens added on (Weinberg, 2000b).

Table 1.4. The host iron withholding system (Compiled from Kontogiorges and Weinberg, 1995 and Weinberg, 1999).

<table>
<thead>
<tr>
<th>Therapeutic agents</th>
<th>Constitutive components</th>
<th>Processes induced during infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agents that alter iron metabolism</td>
<td>Siderophilins (Transferrin and Lactoferrin)</td>
<td>Suppression of dietary iron assimilation (up to 80%)</td>
</tr>
<tr>
<td>Vaccines containing microbial iron acquisition antigens</td>
<td>Ferritin</td>
<td>Increase of ferritin synthesis</td>
</tr>
<tr>
<td>Iron chelation by desferrioxamine</td>
<td></td>
<td>Synthesis of nitric oxide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decrease in TfR expression</td>
</tr>
</tbody>
</table>
3.4 Iron chelation therapy

Because human beings do not have effective physiologic means of eliminating excess body iron, treatment of iron overload is usually achieved with iron chelating agents (Brittenham, 2003). The aim of chelation therapy is (1) to decrease excess iron in tissue to concentrations where there is no iron-induced toxicity and (2) iron detoxification by chelating agents able of complexing with iron (Kushner et al., 2001). In order to achieve iron balance, chelators have to access the two major iron pools (Kushner et al., 2001). The first is the intracellular labile iron derived from both the catabolism of ferritin and iron uptake of transferrin or non-transferrin bound to cells. The second source, its derivation from the catabolism of red blood cells. Iron chelation is beneficial because it leads to both the inhibition of pathogen replication and host immune responses’ restoration (Thuma et al., 1998).

3.4.1 Different types of iron chelators

Iron chelators are small molecules, developed to eradicate any excess iron. These molecules are classified according to their origin (synthetic or biological; Kontoghiorghes and Weinberg, 1995) and are structurally different. Only a few of the iron chelators available are used in clinical trials (Galanello, 2001) because of their toxic side effects. Example of chelators are desferrioxamine (DFO), desferriexochelin 772SM, ICL 670A, deferiprone (L1) etc. The therapeutic applications for these chelators include (1) acting on oxidative stress, and (2) both antiviral and antimicrobial activities (D-Exo; Amersi et al., 2001; Galanello, 2001; Boelaert et al., 1996).

DFO, the most commonly used chelator in disorders associated with iron overload, has a high affinity to iron in a 1:1 ratio. Its use has demonstrated the effectiveness of using iron chelators to treat and prevent iron overload (Brittenham, 2003). Unfortunately, treatment with DFO is cumbersome and unpleasant. Thus many efforts have been made to develop alternative and more effective agents for the removal of excess iron leading to new iron chelators becoming available for clinical trials (Fig. 1.7; Brittenham, 2003).
Hexadentate iron chelators

A. DFO
Deferoxamine B mesylate

B. HBED
$N,N'$-bis(2-hydroxybenzyl)ethylenediamine-$N,N'$-diacetic acid

Tridentate iron chelators

C. 4'-OH-dadmDFT
$(S)$-4,5-dihydro-2-(2,4-dihydroxyphenyl)-4-thiazolecarboxylic acid

D. ICL670A
4-[3,5-bis-(hydroxyphényl)-1,2,4-triazol-1-yl]-benznic acid

Bidentate iron chelator

E. Desferiprone (L1, DMHP, CP20)
1,2-dimethyl-3-hydroxypyridin-4-one

Figure 1.7. Selected iron-chelating agents (taken from Britenham, 2003).
3.4.2 Iron chelation and infections

Iron is required for the growth and metabolism of both pathogens and hosts. However, excess iron results in malfunctioning of host defence mechanisms against invading pathogens (Taramelli et al., 2000). Hosts have evolved strategies to restrain availability of iron to pathogens while retaining enough for their own use. Therefore, the use of chelators to regulate iron availability can be looked on as therapeutic (Weinberg, 1994) and when reduction of excess iron occurs, host defence systems should improve (Legssyer et al., 2003). Several chelators (Table 1.5) display anti-pathogen/microorganism activities to (1) malaria (Weinberg, 1994), (2) HIV (Georgiou et al., 2000), (3) fungus (Taramelli et al., 2000), and (4) bacteria (Gomes et al., 2001). Iron chelation as a therapeutic approach has been carried out in vitro for several infections including M. avium (Gomes et al., 1999), hepatitis C (Sartori et al., 2001) and HIV (Georgiou et al., 2000; Sappey et al., 1995).

Table 1.5. Effects of some iron-chelating agents on pathogens (Adapted from Boelaert et al., 1996).

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Chelators</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus fumigatus</td>
<td>DFO</td>
<td>Stimulation</td>
<td>Stimulation</td>
</tr>
<tr>
<td></td>
<td>L1</td>
<td>None</td>
<td>NS</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>DFO</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>L1</td>
<td>None</td>
<td>NS</td>
</tr>
<tr>
<td>Cryptococcus</td>
<td>DFO</td>
<td>None</td>
<td>Stimulation</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>DFO</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>DFO</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td>Histoplasma capsulatum</td>
<td>DFO</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td>Leishmania</td>
<td>DFO</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>L1</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td>Mycobacterium avium</td>
<td>DFO</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>DFO</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td>Neoforans</td>
<td>L1</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CP94</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td>Pneumocystis carinii</td>
<td>DFO</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td>L1, CP94</td>
<td>Inhibition</td>
<td>None</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>DFO</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CP94</td>
<td>Inhibition</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: non-specified
4. Direct and indirect techniques used as investigation tools

*In vitro* techniques are used as alternative means to animal testing for anti-bacterial, antiviral, vaccine production, etc. *In vitro* experimentation is also lower in cost and provides results quicker. Issues evaluated this way include cell viability or growth rates, the integrity of cellular compartmentalization, the oxidation-reduction pathways, the cell signal transduction pathways, induction of apoptosis and necrosis, interfering with normal cellular metabolism, and DNA metabolism. A major disadvantage of *in vitro* tests is the difficulty in extrapolating the results obtained to the real *in vivo* situation.

4.1 Methods to enumerate viability

4.1.1 Host cell viability

Various assays have been developed to investigate cellular viability. Some of these assays rely on the integrity of the plasmalemma of the cell and others reflect not only cell membrane integrity but also cellular metabolic state (Gomes *et al.*, 1997). Such assays include the reduction of tetrazolium salts (Fig. 1.8) into formazan pigments (Kairo *et al.*, 1999) that is caused by enzymes of the mitochondria. The most frequently used of these dyes are 3-(4, 5-dimethylthiazol-2-y1)2, 5-diphenyltetrazolium bromide (MTT) and sodium 3’-[1-phenylamino-carbonyl-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT). MTT is a non soluble single reagent system where the yellow dye is reduced by mitochondrial dehydrogenase (succinate-tetrazolium reductase) in living cells to produce an insoluble purple formazan crystal (Mshana *et al.*, 1998; Kairo *et al.*, 1999). The produced crystals are solubilized with DMSO or acidified alcohol which destroys the cells allowing only a single measurement. XTT has an advantage over MTT in that it is soluble in culture media therefore continuous monitoring can be performed. However, XTT is less sensitive than MTT (Traoré and Meyer, 2002).

Another non-radioactive colorimetric dye, Alamar blue can also be used in the current study. This dye contains a redox indicator that exists in more than one form (Zhi-Jun *et al.*, 1997). The oxidized form is non-fluorescent and of blue colour while the reduced form is pink in colour and highly fluorescent. It is soluble, non-toxic to cells and used to measure
cell viability (Ahmed et al., 1994; Voytik-Harbin et al., 1998). This dye can be evaluated visually, fluorometrically or spectrophotometrically.

\[ \text{Figure 1.8. Reduction of tetrazolium salts} \]

4.1.2 Bacterial viability
Numerous methods have been developed to assess mycobacterial viability including counting the colony forming units (CFU) and a microplate Alamar blue assay (MABA). Alamar blue can be used as an indicator of host cell and/or mycobacterial growth. Collins and Franzblau (1997) and Yajko et al. (1995) have applied the MABA technique to screen compound active against \textit{M. tb}. This technique agrees with the agar proportion method (the proportion of resistant organisms in a given inoculum) when determining the minimal inhibitory concentration (MIC) and shows high correlation with the radiometric BACTEC 460 system (Franzblau et al., 1998). These assays look at bacterial susceptibility to compound by comparing the number of colonies growing on the drug-free medium with the number growing on drug-containing medium.

The CFU or quantitative culture is one of the most common methods for the enumeration of mycobacteria and is used to determine viable \textit{M. tb} populations in a culture. This method requires serial dilutions of a suspension of bacteria that are plated on a solid growth medium. The replication of the mycobacteria results in the formation of visible colonies (Nolte and Metchock, 1995). This technique has been adapted for the investigation of antimicrobial susceptibility testing. The major limitation to this method is the length of time required for colonies to grow.
4.2 Measurement of host defence responses in vitro

Measuring immune responses during infections is critical for understanding the interactions between pathogens and host cells and how the latter defend themselves. Such measurements have important diagnostic and prognostic relevance. Because of the complexity of the immune response, it can be measured using several methods, e.g. by detecting cytokine secretion, nitric oxide. The interaction between host cell and pathogens results in activation of potentially anti-pathogen mechanisms, including nitric oxide and the production of cytokines (such as IL-6 and TNF-α), which influence subsequent induced innate- and specific immune response (Message and Johnston, 2004). In vitro studies have shown that during concurrent HIV and M.tb infections, host immune responses directed to the killing of one of the pathogens is being used by the other to replicate (Imperiali et al., 2001). Our interest in measuring IL-6, TNF-α and NO arises from the ability of HIV to utilize both cytokines for its replication through a mechanism involving NO. In addition, cytokines determine the outcome of any infectious processes (Kedzierska and Crowe, 2001; Cohen and Fauci, 2001). Infection with pathogens such as HIV will result in the dysregulation of the normal cytokine profile in vivo and in vitro, contributing in turn to the stimulation of viral replication by suppressing any efficient immune response.

Interleukin 6 (IL-6) is a multifunctional protein that plays important roles in host defence, and immune responses. TNF-α, also known as cachectin is a pleiotropic cytokine that can induce disease through its toxicity (tissue injury) and improve host defence mechanisms (stimulation of inflammation and increase of immune cell function). Both IL-6 and TNF-α assays are “sandwich type” of enzyme immunoassays in which a monoclonal anti-human IL-6 or TNF-α antibody is bound onto polyester microplate wells. Human IL-6 or TNF-α present in a given sample is captured by the antibody and non-bound material is removed by washing. Subsequently, a biotinylated polyclonal antibody to human IL-6 or TNF-α is added. This antibody binds to the IL-6 or TNF-α-antibody complex present in the microplate well. Excess biotinylated antibody is removed by washing, followed by addition of Horseradish Peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the IL-6 or TNF-α sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A colored product is
formed in proportion to the amount of IL-6 or TNF-α present in the sample. The enzyme-substrate reaction is stopped by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at 450 nm. From the absorbance of samples and those of a standard curve, the concentration of the desired cytokine can be determined by interpretation with the standard curve.

NO is produced in trace amounts by neurons and endothelial cells in response to homeostatic and it is a small molecule produced by many mammalian cell types. It plays an important role in many biological processes (Bertholet et al., 1999; Ciani et al., 2002). The assay used is based on the conversion of nitrate to nitrite utilizing nitrate reductase followed by the addition of reagents that convert nitrite into a deep purple azo compound. Photometrically measurement of the absorbance due to this azo chromophore accurately determines NO$_2^-$ concentration.

4.3 Direct assessment of cell death using flow-cytometry

Cytometry refers to the measurement of the physical properties of cells. In flow cytometry, single particles or cells pass in a fluid stream through a measurement point surrounded by an array of detectors (Dean, 1997). This technique allows measurement to be made on cells at high speed. Measurements are based on light scatter and fluorescence, making it possible to measure simultaneously many cellular parameters at once. In the flow cytometer, labelled cells are pumped through the flow chamber and pass the measuring point where they intersect a beam of light from a laser producing light of a single wavelength of high intensity. This light interacts with natural fluorescent pigments present in cells or with fluorescent stains added to cells prior to analysis. The excited fluorochromes emit light that is collected over a range of angles by different detectors, amplified and digitally converted. Forward scatter is detected in a narrow angle from the direction of the laser beam while the right one detects the opposite angle of the beam. The intensity of both forward and right scatters is proportional to the size, shape and optical homogeneity of cells or particles. The light intensity is measured by photodiodes and then the computer processes the amplified signals. The data collected are displayed as frequency histograms, dot plots, contour plots or isometric plots (Ormerod, 1999; Dean, 1997). Flow cytometry is a rapid, sensitive and
reproducible technique with a wide variety of applications such as the measurement of apoptosis/necrosis, cell sorting, and measurement of cell surface antigens and analysis of DNA.

Cell death forms an integral part of normal development, maturation and disease (Kanduc et al., 2002; Guimarães and Linden, 2004). However, the mechanisms underlying these processes are poorly understood. There are two distinct and opposite modes of cell death events: apoptosis (or programmed cell death) and necrosis (or accidental cell death). The latter represents a state of "no return" in cell life. Apoptosis is a physiological form of cell death that is involved in the development and maintenance of multicellular organisms (Loo and Rillema, 1998). It is characterized on morphological and biochemical criteria. Phosphatidylserine (PS) is a negatively charged phospholipid found in the inner layer of the plasma membrane which flips through translocation to the outer layer and becomes exposed on the outer surface during apoptosis. Annexin V-FITC a calcium and phospholipid-binding protein binds to the exposed PS and can thus be used to detect early apoptotic cells. Since Annexin V also detects necrotic cells, apoptotic cells have to be differentiated from necrotic cells by the use of propidium iodide (PI). PI is a dye that stains nucleic acids and can not cross the plasma membrane of viable cells or cells which maintain plasma membrane integrity. PI selectively labels late stage apoptotic or necrotic cells. The surface exposure of PS is monitored using Annexin V and membrane disruption is monitored by PI. Early apoptotic cells are defined as positive for Annexin V-FITC (green fluorescence) and negative for PI (red fluorescence). PI positive cells are necrotic and/or late apoptotic cells (Vermes et al., 1995; Koopman et al., 1994).

Biosafety was a concern when using the flow cytometer for monitoring cell death of co-infected cells (due to the presence of M. tb). Aerolization of droplets containing M. tb can occur during analysis. The problem was overcome by treating samples with paraformaldehyde prior to analysis in order to inactivate all biohazardious agents.
Enzyme-linked immunosorbent assays (ELISA's)

ELISA is the most commonly used test when detecting antibodies to HIV. There are also ELISAs in existence for detecting virion-associated proteins like HIV's p24 or HIV enzymes such as reverse transcriptase. A large number of these available commercial kits are classified as indirect, competitive and antigen capture or sandwich assays (Chiengsong-Popov et al., 1995). Most of them are made of HIV antigens attached to a solid phase and incorporate a conjugate and substrate detection system. Each of these different types of ELISAs functions in a similar manner.

4.4.1 RT ELISA

RT is a RNA-directed DNA polymerase of immense importance in the life cycle of retroviruses such as HIV. Assays for reverse transcriptase activity have been widely used to monitor retroviral propagation. The Roche colorimetric RT assay (Roche Diagnostics; Germany) is based on the fact that if RT is present in the sample, PolyA that is bound to the surface of the plate will serve as template and oligo-dT as primer for the synthesis of a complementary DNA strand. A base analogue to dTTP, Bromo-deoxyuridine triphosphate (BrdUTP) will be incorporated in the growing DNA strand. This ELISA allows the use of non-radiolabelled nucleotides in contrast to classical RT assays. In place of radiolabelled nucleotides, digoxigenin- and biotin-labelled nucleotides are incorporated into the same DNA molecule.

4.4.2 p24 ELISA

The HIV-1 p24 Antigen assay is an enzyme immunoassay (EIA) or ELISA, developed for the detection and quantification of the HIV-1 p24 core protein. In this ELISA, the viral capsid protein p24 is captured by a monoclonal antibody bound to the microwell and this bound antigen is detected with a biotinylated secondary antibody and conjugated streptavidin-horse-radish peroxidase. The addition of a substrate reagent containing tetramethylbenzidine (TMB) and hydrogen peroxidase forms a blue colour. The reaction is terminated by addition of acid, and the absorbance is measured spectrophotometrically. The intensity of colour is directly proportional to the amount of p24 in the sample.
Chapter 2: Materials and Methods

Materials and Methods

1. Study population

The first study (for chronic infection) was composed of HIV-infected patients attending the IC2 clinic at Helen Joseph hospital with no history of tuberculosis (TB). The individuals were from Johannesburg (Gauteng; South Africa) and its surroundings. The diagnosis of TB was made on the basis of medical history. They were recruited after ethical approval by the WITS Ethics Committee (M01-04-03; University of the Witwatersrand, Johannesburg) and informed consent was obtained from them with complete confidentiality and anonymity insured. Only information regarding sex, average age and race were documented and the patient assigned a number. The sample consisted of 45 females and 18 males with ages ranging from 24 to 63 years. Blood (5 ml) was collected in EDTA-containing tubes (Beckman Coulter; Florida). The second study involving acute infection included healthy donors (students from Rand Afrikaans University) with ages ranging from 20 to 34 and consisted of 8 females and 3 males.

2. Materials

2.1 Reagents
FeSO₄·7H₂O was purchased from Merck, Germany. MTT, DFO and Paraformaldehyde were obtained from Sigma-Aldrich (St Louis, MO), while Alamar Blue was obtained from Serotec Ltd. (Oxford, UK). MTT and Alamar Blue were added in an amount equal to 10% of the culture media. MTT was prepared at a concentration of 5 mg/ml in phosphate buffered saline (PBS) solution, filtered through a 0.2 μm pore membrane and stored at 4°C. Alamar blue came as a ready to use solution and was stored in the dark as specified by the manufacturer.
Tissue culture reagents (RPMI 1640, foetal bovine serum (FBS), gentamycin) were obtained from Highveld Biological (Sandrigham, South Africa) unless otherwise stated. PHA and IL-2 were purchased from Sigma-Aldrich. RPMI 1640 medium containing 2 mM L-glutamine and supplemented with 10% heat-inactivated (56°C, 30 min) FBS and 1% gentamycin sulphate (GS) is referred to as complete media hereafter. Gentamycin was used because of its inability to affect viability of *M. tb* (Mancino et al., 1997).

All the Middlebrook media (7H9) and agar (7H10) for bacterial culture were obtained from Difco laboratories (Detroit; MI).

A 5 mM stock solution of both iron and desferrioxamine were prepared in distilled water (dH2O), filtered through a 0.2 µm pore membrane and stored at 4°C and -20°C respectively. Prior to the addition to the test plate, appropriate dilutions of chemicals were made in complete RPMI media.

2.2 Viral and bacterial cultures

2.2.1 Virus

HIV-1 subtype C isolates were obtained from the National Institute for Communicable Diseases (NICD; Sandrigham, South Africa) who kindly donated DU-151a2-P2 isolates.

2.2.2 Bacterial cultures

A virulent *M. tuberculosis* strain, H37Rv (ATCC no. 25618) was obtained from the American Type Culture collection (ATCC, Rockville, MO) and grown by the National Tuberculosis Research Program, Medical Research Council (MRC, Pretoria, South Africa). Bacterial cultures were diluted to $1 \times 10^8$ bacilli/ml in Middlebrook 7H9 broth media (Difco Laboratories, Detroit, MI) supplemented with 0.2% glycerol (Sigma Chemical, St Louis, MO) and 10% Middlebrook OADC enrichment (Merek, Darmstadt, Germany), hereafter referred to as Middlebrook 7H9 media, aliquoted and stored at -80°C. For each experiment, 200 µl of bacteria was thawed overnight at 37°C in a shaking water bath and
sonified (Bandelin Electronic UW 2070, Berlin, Germany) for 3 cycles of 10 seconds at 50% power.

3. Methods

3.1 Confirmation of HIV status with Rapid HIV test
Rapid tests are screening tests that produce very quick results between 5 to 30 minutes. Most rapid tests display similar or identical sensitivity and accuracy as ELISA and western blot assays (Branson, 2000; Phillips et al., 2000) and are confirmed by the latter assays. There are a variety of rapid tests that differ based on different principles. In this project, these tests were used to confirm HIV status of the patients. Although the blood used in the course of the study was obtained from an HIV clinic, a verification of HIV status was decided on. One of the tests used was the Abbott® Determine HIV-1/2 test (Abbott Lab, Abbott Park; IL). This test can detect antibodies to HIV-1 and HIV-2 in human sera, plasma or whole blood and provide results within 10 min. Briefly; it is a flow-through device and employs solid-phase capture technology, which involves the immobilization of HIV antigens on a porous membrane. The specimen flows through the membrane and is observed into an absorbent pad. A dot or a line visibly forms on the membrane when developed with a signal reagent (usually a colloidal gold or selenium conjugate). The appearance of a coloured dot or line at the patient window confirms the presence of HIV antibodies. The other test used was the Efoora Rapid HIV test (Efoora Inc., Buffalo Grove, IL) that detects the presence of antibodies to HIV in samples within 20 min. Here the sample (followed by a buffer) is applied to an absorbent pad, migrates through the strip and combines with a signal reagent. A positive reaction results in a visual line on the membrane where HIV antigen has been applied. A procedural control line is applied to the strip beyond the HIV-antigen line.

3.2 Choice of cells for in vitro studies
The methods for studying HIV infections in patients mainly focused on analysing viral growth in Peripheral blood mononuclear cells (PBMCs) and T cell lines. However, subsequent studies using different cell types indicated that HIV-1 isolates were unable to
grow in T cell lines (Asjo et al., 1986). The cellular tropism of HIV is based on the ability of viral isolates to grow in macrophages and transformed T-cell lines in vitro. Isolates that grow well in macrophages and PBMCs are called macrophage-tropic or non-syncytium inducing (NSI) viruses, whereas isolates that grow well in PBMCs and T-cell lines are designated as T-tropic or syncytium inducing (SI) viruses (Cohen and Fauci, 2001). In addition, T-cells and macrophages are key targets of HIV infection and have a dual role in the pathogenesis of tuberculosis and HIV-1 infection (Cohen and Fauci, 2001; Gobin and Horwitz, 1996). Furthermore, cells of the monocytes/macrophage lineage serve as reservoirs of viral infection and dysfunction of these cells contributes to CD4+ T-cell dysfunction and as well as to impaired host defence against intracellular pathogens such as mycobacterium tuberculosis (Weiss, 2002). Because of this knowledge and the need to achieve an effective infection, PBMCs and monocytes were therefore the cells of choice for in vitro studies.

3.3 Preparation of cells

PBMC were isolated by density gradient centrifugation using Ficoll-hypaque (Sigma, St. Louis, MO). Anti-coagulant treated blood was diluted with RPMI media (1:1). Diluted blood was slowly layered on top of the Ficoll-hypaque with a ration of 2:1 blood-Ficoll. Centrifugation at 1028 x g for 20 min followed. The cloudy layer of cells was then carefully removed, washed twice with plain RPMI media and incubated for 10 min with Ammonium chloride potassium (ACK) buffer to lyse any red blood cells present. The cells were then washed in RPMI media and resuspended at 10^6 cells/ml in complete media containing 2 µg/ml of purified PHA and 1 ng/ml purified human IL-2.

Monocytes were isolated by culturing PBMC on plastic Petri dishes (Nunc, Kamstrupvej; Denmark) at 37°C for 1 h and non-adherent cells removed by washing with PBS. Finally, monocytes were also adjusted to 10^6 cells/ml in complete media containing 2 µg/ml of purified PHA and 1 ng/ml purified human IL-2.
3.4 General study design

3.4.1 Acute and chronic infections of cells
In this study, chronically infected cells referred to cells isolated from blood collected from HIV-infected patients (those infected with HIV for longer than 12 months).

Acutely infected cells are cells isolated from blood collected from healthy donors and infected in vitro. HIV-1 infection of cells was accomplished by incubating cells ($10^6$/ml), with 1 ml of virus diluted 300 times (final TCID$_{50}$ of 500), overnight in a humidified atmosphere (37°C, 5% CO$_2$) and then washing 3 times with warm medium. Cells were then cultured in 24-well plates. These cells pre-infected with HIV-1 were then co-infected with M.tb H37Rv as described below. Cells infected with HIV-1 after 4 days of culture in media only were used as control.

The difference between the two types of infections will be the amount of detectable viral particles.

3.4.2 Infection of cells with Mtb
Mycobacteria were opsonized in 10% autologous human plasma under agitation in 37°C for 30 min. and subsequently washed twice with PBS (centrifugation at 18 894 $\times$ g for 5 min). The pellet resuspended in plain RPMI media and used to infect HIV-infected cells (acutely and chronically) at a cell:bacteria ratio of 1:10. Cells were allowed to ingest the bacteria for 3 h in a humidified atmosphere (37°C, 5% CO$_2$). Extracellular bacteria were removed by washing twice with PBS and co-infected cells resuspended in complete media containing PHA at a concentration of $1 \times 10^6$ cell/ml for further experiments.

3.4.3 Experimental plates for assays
Both 96-well and 24-well plates were used for the assays. The outer wells of the plates were filled with complete RPMI media. Co-infected cells were plated at $10^6$/ml in all wells except the second column in a final volume of either 200 µl (96-well) or 2ml (24-well) in
the absence or presence of 500 μM Fe and/or DFO for 4 days. Non-treated HIV-infected cells were plated in the second column and served as controls.

4. Preliminary studies

4.1 CCR5 receptor expression on PBMCs
The viral determinant of cellular tropism is one of the major determinants for coreceptor use. Indeed, M-tropic isolates use CCR5 as a coreceptor, whereas most T-tropic isolates use CXCR4 (Cohen and Fauci, 2001). Therefore prior to initiating our study, the presence of CCR5 receptors on the surface of PBMCs was confirmed by flow cytometry in conjunction with the use of a Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody against CCR5 (BD Pharmingen, Ontario; Canada). Briefly, duplicate aliquots of cells (1 x 10^6/ml) were washed in PBS, the pellets resuspended in 1 ml of PBS containing 20 μl of FITC-conjugated antibody was added and samples were incubated for 20 min in the dark. Cells were subsequently washed again and resuspended in 1 ml of PBS for analysis. Cells with no antibodies (untreated) were included as control.

4.2 Screening reagents for toxicity: Concentration study
A concentration study was done to determine at which concentrations the test reagents were not toxic. HIV-infected PBMC were plated in multi-well plates containing a range of Fe, DFO or Fe+DFO concentrations. The final volume in all wells was 200 μl. The plates were incubated in a humidified atmosphere at 37°C for 4 days. The activity of the compounds was assessed by MTT and Alamar blue. Each assay included triplicate samples for each concentration.

4.3 Assays on pathogens in a host cell-free environment
Iron and DFO were screened for pro- or anti-activities on both HIV and M.tb.
4.3.1 Mycobacterial viability after treatment with iron and/or DFO (in the absence of host cells)

*Mtb* Rv were diluted to a concentration of $0.1 \times 10^7$ or $1 \times 10^7$ CFU/ml in either Middlebrook 7H9 or RPMI media, seeded in 96-well plates and treated with 500 µM Fe and/or DFO. The reason for using these two media types was to test all necessary controls including the different culture milieus for bacteria when grown on their own (in Middlebrook media) or in the presence of host cells which require RPMI. Controls included Middlebrook or RPMI media only, non-reduced Alamar Blue and 100% reduced Alamar Blue (autoclaved 10% Alamar Blue; 120°C for 20 min). Heat-killed *M.tb* was prepared by autoclaving mycobacteria (120°C, 20 min). Bacterial cultures were incubated at 37°C for 4 days and following incubation the MABA assay was applied where 10% Alamar Blue and 10% Tween 80 (Merck, Darmstadt, Germany) were added to each the cultures. Fluorescence readings were recorded 24 h after addition of Alamar Blue at an excitation wavelength of 544 nm and an emission wavelength of 590 nm (Fluoroskan Ascent, Labsystems, Helsinki, Finland).

4.3.2 Effect of iron overload and its chelation on reverse transcriptase directly

This assay was done using a colorimetric Reverse transcriptase assay (Roche Diagnostics, Basel; Switzerland) in conjunction with the recombinant HIV-1 Reverse Transcriptase (AEC Amersham, Kelvin; South Africa). The enzyme was diluted 200-300 ng/ml in lysis buffer. A stock solution of Fe and DFO were prepared in DMSO (Highveld biological) after which a ten times dilution was prepared in lysis buffer. In each test well, 20 µl of diluted enzyme (4-6 ng); 20 µl of diluted test reagents and 20 µl of reaction mixture (template/primer hybrid poly (A) × oligo (dT015)) were dispensed. The final concentration of reagents being 500 µM. Negative control wells contained 40 µl of lysis buffer and 20 µl of reaction mixture. Positive control wells contained 20 µl of diluted enzyme, 20 µl of lysis buffer and 20 µl of reaction mixture. The plate was covered and incubated in the dark at 37°C for 1 h. After incubation, the plate was washed 5 times to remove any unbound particles and 200 µl of antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POG) were added to the wells and the plate re-incubated at 37°C for another hour. After another
washing step, 200 µl of peroxidase substrate (ABTS) was added to all wells and the colour was left to develop at room temperature (15-30 min). The absorbance was measured at 405 nm and the percentage inhibitory activity of test reagents were calculated using the formula:

\[
\text{Percentage inhibition} = 100 - \left( \frac{\text{Test reagent}}{\text{Positive Control}} \times 100 \right)
\]

4.4 Evaluating the ability of Alamar Blue and MTT dyes to assess viability of \textit{M.\textit{tb}}-infected cells

4.4.1 Showing increase in Alamar Blue reduction during co-infection

Cells (HIV-infected only and co-infected PBMC) were seeded at 1×10^6/ml in 96-well plates. Controls included RPMI media only, 100% reduced Alamar Blue and media containing Alamar Blue. Mycobacteria in media and heat-killed bacteria were also prepared. Fluorescence was measured on days 0, 4 and 6 of incubation where Alamar Blue is added for 4 hr and the absorbance read at 540 nm.

4.4.2 Reduction of dyes after lysis of cells

Plates (24-well) containing experimental cultures (HIV-infected only, Co-infected only and treated co-infected cells) in RPMI media were incubated at 37°C for 4 days. After incubation, cells were harvested, centrifuged (850 × g, 10 min) and lysed by means of freeze thawing several times. Briefly, 200 µl of sterile water was added to the pellet and the sample was frozen (liquid nitrogen) and thawed (56°C water-bath) three times. The lysates were washed twice with PBS, centrifuged once again (850 × g, 10 min) and the final pellet resuspended in 1ml complete RPMI. The suspensions were plated in 96-well plates, dyes added in a final volume of 10% (v/v) and absorbance read after 4 hr incubation.
5. Bio-assays to assess host cell and pathogen responses

5.1 Host cell defence responses: Biochemical and immunological assays

5.1.1 Assessing host cell viability

Viability was assessed using MTT and Alamar Blue.

**MTT:** To each well of the plate, 20 µl of MTT solution (5 mg/ml) was added. The cells were mixed and MTT evenly distributed. The plate was incubated at 37°C in a CO₂ incubator. After a suitable incubation period, solubilization of the formazan crystals was achieved by adding 100 µl of 0.1 N HCl in isopropanol. Complete dissolution of crystals was achieved by placing the plate on a shaker for 10 min. Finally the optical density was measured on a 96-well plate reader (Labsystems Multiskan MS, Helsinki, Finland) with a filter setting at 540 nm (reference filter setting was 690 nm).

**Alamar Blue:** To the 200 µl of cells and test material, 20 µl of Alamar Blue solution was added. The plate was re-incubated at 37°C, 5% CO₂ for 4 h. Colorimetric analysis was done by measuring absorbance at 540 nm (reduced state) and 620 nm (oxidized state). The fluorometric analysis was achieved by reading absorbance at excitation wavelength of 544 nm (emission wavelength was set at 590 nm).

5.1.2 Monitoring cell death by flow-cytometry

Treated or non-treated cells and appropriate controls were washed in PBS and resuspended in binding buffer. To 490 µl of cells, 5 µl each of PI and Annexin V were added simultaneously and cells were incubated on ice in the dark for 10 min. After another washing step, cells were fixed for 10 min with 3% paraformaldehyde in PBS at room temperature and washed once again. Analysis of samples was performed using a Beckman Coulter ALTRA (Coulter, Miami; FL) from the Flow cytometry unit at the department of Immunology (University of Pretoria; South Africa). Ten thousand cells of each sample were analyzed.
5.1.3 *In vitro* Cytokine assays

The cytokines, IL-6 and TNF-α were quantified in culture supernatants using ELISA kits (OptEIA, Biosciences PharMingen, San Diego, CA). Standard curves were prepared from 0 to 200 pg/ml using recombinant cytokine provided in each kit. And the concentrations of corresponding cytokines in the supernatant estimated, based on the absorbance read by an ELISA reader (Labsystems Multiskan MS, Helsinki, Finland) at 450 nm.

5.1.4 Nitric oxide colorimetric assay

The NO accumulation in 4-day was assessed using the Nitrate/Nitrite colorimetric assay kit based on the Griess reaction (Cayman chemical, Ann Arbor; MI). Briefly the nitrate present in the culture supernatants (80 μl) was reduced to nitrite by reduced cofactor (10 μl) in the presence of the nitrate reductase mixture (10 μl). Total nitrite (reduced nitrate plus nitrite) was quantified by the addition of 100 μl of Griess reagent. Reactions were performed in duplicate at room temperature for 10 min. Chromophore absorbance was then measured at 540 nm in a microplate reader (Labsystems Multiskan MS, Helsinki, Finland). Total NO concentrations were evaluated by comparison with sodium nitrite standard curve (supplied with the kit).

5.2 Pathogen behaviour: Microbiological analyses

5.2.1 HIV quantification

*p24 expression of cells*

As described by the manufacturer (Beckman Coulter), 200 μl of diluted sample or standard were transferred to the murine monoclonal-coated 96-well plate and 20 μl of lyse buffer (Triton X-100, dipotassium EDTA, Tween-20, thimerosal 0.05%) was added. After incubation and following a wash step, 200 μl of biotinylated human anti-HIV-1 IgG was added and the plate incubated for another 1 h. In a final step, a substrate reagent containing tetramethylbenzendine (TMB, 200 μl) was added. The reaction was stopped by the addition of 50 μl of Coulter stopping reagent (CSR, 4N H₂SO₄) and the absorbance was measured at 450 nm.
Reverse Transcriptase (RT) ELISA

After experimental incubation (4 days), samples were transferred to 15 ml conical tubes and centrifuged at 30 \( \times \) g for 10 min. Supernatants were transferred to clean tubes and 1 ml of 30% polyethylene glycol was added and the resulting mixture was thoroughly mixed. Following an overnight incubation on ice, samples were centrifuged at 800 \( \times \) g for 1 h in a refrigerated centrifuge at 4°C. The pellets were resuspended in 40 µl of lysis buffer, transferred to fresh tubes and incubated at room temperature for 30 min. Twenty µl of a reaction mixture (template/primer hybrid poly(A) \( \times \) oligo (dT015) was added to tubes containing samples and incubated at 37°C. After 4 h incubation, samples were transferred to appropriate wells of a plate pre-coated with streptavidin followed by 1 h incubation at 37°C. The plate was then washed 5-times to remove any unbound particles and 200 µl of antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POG) were added to the wells and the plate re-incubated at 37°C for 1 h. After additional washings, 200 µl of peroxidase substrate (ABTS) was added to all wells. Following colour development (15-30 min) at room temperature, absorbance was measured at 405 nm with reference wavelength at 492 nm.

5.2.2 Evaluation of bacterial survival

To determine viability and replication of intracellular mycobacteria post-infection, host cells were lysed, bacteria recovered and allowed to grow and viability/replication assessed using MABA and agar cultures.

Host cell lysis and recovery of the mycobacteria

After the required incubation time, cultures treated or non-treated were harvested, centrifuged (850 \( \times \) g, 5 min) and lysed by means of freeze thawing several times as previously described. Following lysis, 100 µl of 20% BSA (Boehringer Mannheim, Germany) in PBS was added and the mycobacterial suspension in the cell lysate was vortexed and sonicated (Sandelin Electronic UW 2070, Berlin, Germany) at 50% power for 10 seconds. Recovered mycobacteria were washed twice with Middlebrook 7H9 media, centrifuged (18 894 \( \times \) g, 10 min), the final pellet resuspended in 1 ml Middlebrook media and either allowed to grow for 7 days in 96-well plates or plated on agar. To assess viability
of recovered *M. tb* after 7 days, we once again utilized the MABA. Fluorescence was measured 1 h after the addition of Alamar Blue. Supernatant from HIV-infected cell lysates as well as 100% reduced Alamar Blue and media only were included as necessary controls.

**Assessing colony forming units**

Two dilutions of the recovered H37Rv *M. tb* were done in Middlebrook media and 100 µl of each dilution was plated, in duplicate, on 7H10 agar plates consisting of Middlebrook 7H10 agar medium (Difco laboratories, Detroit, MI) supplemented with 0.5% glycerol (Sigma chemical company, St. Louis, MO) and 10% Middlebrook enrichment (Merck, Darmstadt, Germany). The plates were incubated in the inverted position for 4 weeks in a humidified atmosphere (37°C, 5% CO2) after which the numbers of individual colonies visible were counted. Mycobacterial concentration (CFU/ml) was calculated according to the formula:

\[
[M. tb] \text{(CFU/ml)} = \frac{\text{number of colonies counted} \times \text{dilution factor}}{\text{Volume plated (ml)}}
\]

6. **Statistical analysis**

Statistical analysis and graphs were done using Microsoft® Excel 98 (Microsoft Corporation, Redmond, WA). Data were analyzed for statistical significance by Student’s *t* test using simple interactive statistical analysis (SISA) at http://home.clara.net/sisa/. Results with *P* < 0.05 were considered significant. Significance of differences was determined as follows: co-infected compared to HIV-infected cells, and all treated cells were compared to co-infected cells.
Results

Section A
Experimental settings and preliminary studies

1. Study population

Blood samples were collected on a randomized basis where patients, chosen by chance and on their own choice agreed to be part of the study. All patients completed a consent form (see addendum) after agreeing to participate in the study. Only then, 8 ml of blood sample was collected from the infected individuals. Out of 63 HIV-infected people who participated in this study, 71.43% (45/63) was female and 28.6% male while the population composition was as follows: 2 white (3.2%), 5 colored (7.9%) and 56 black (88.9%; Fig. 3.1-A).

The mean and median ages were 36 and 34 respectively with the dispersion in age showing that a range of age groups in the population is affected, young (min 24) as well as older people (max 63). However, those most affected are between 20-to-40 age group (considered to be the economically active population, Fig. 3.1-B and -C). The age group [20-30] consisted almost entirely of females (20.6%; 13/63) while most infected males were in the age group [30-40] (20%; 12/63) implicating that younger women are infected by older men (UNAIDS/WHO, 2004). This reflects the scenario common to Africa presently, where older men marry younger women.

It also important to distinguish between mammalian and bacterial cells as pathogen hosts. Although bacteria serve as hosts for diverse pathogens, the word host in this study refers to human PBMCs or monocytes as host cells for bacterial or viral infection.
Figure 3.1. Graphic representations of the study population stratified by race (A), age group and gender (B), and age distribution (C). Analysis was done on 63 individuals, mostly blacks. The graphs show all age groups being affected in particular the economically active section of the population ([20-50]). Frequency represents the number of individuals.
2. Reagents and methods

Most of the reagents used are commonly available.

For acute infection of cells, non-syncytium inducing Du151 a reported HIV-1 subtype C isolate, was used because subtype C isolate accounts for more than 95% of infections in the southern African region. Secondary objectives of this study were (1) to mimic the real picture experienced in sub-Saharan Africa, where the deadly trilogy of HIV, *M. tb* infection and Fe overload predominate and (2) to provide solutions/alternatives to reduce the therapeutic challenges facing the continent. The strain of *M. tb* used in the study was H37Rv. This virulent strain is susceptible to antitubercular drugs and amenable to genetic manipulation.

The method used for isolation of cells from infected and non-infected blood, was based on the density gradient of ficoll-histopaque. An average of $5.35 \times 10^6$ cell/ml were obtained per 8 ml infected blood ($\geq 92\%$ viable) and $8.8 \times 10^6$ cell/ml for uninfected blood (97% viable) as determined by the trypan blue exclusion dye method.

Infection of cells with Du151 was achieved due to (1) the presence of both specific and required receptors on both PBMCs and monocytes and (2) the infection protocol used where concentrated stock virus was directly in contact with pelleted cells. The addition of PHA in cultures was crucial on the day of PBMC isolation and followed by addition of IL-2 prior to experiment extended the life span of cells. Any changes to this protocol negatively affected the outcome of experiments; we standardized our protocol for experimentation with IL-2 where no stimulation was done before experiment.

3. Preliminary studies

Described here are the necessary parameter refinements for the study.

3.1 CCR5 receptor expression on PBMC

Since the viral isolate utilized (Du 151) requires the co-receptor CCR5 on the host cell surface, variation (if any) of the levels of expression of this receptor on PBMCs in different individuals is of importance, we assessed the expression of CCR5 using CCR5-FITC
antibody. Data indicates that CCR5 is constitutively expressed in PBMCs (20% - 55%, Fig. 3.2 and Fig. 3.3-B) with a lower fluorescence in control (cell with no antibody) than test samples (cells with antibody). Having shown the presence of CCR5 on PBMCs, we determined whether two different individuals could have the same level of CCR5 expression. In Fig. 3.3-A, one individual displayed higher expression than the other. This was expected because in a similar study done by Wu et al. (1997), it was found that CCR5 was better expressed in cells with longer exposure to IL-2 and also this group studied many individuals where no two people had the same expression levels. Moreover, we showed that CCR5 expression could be induced by external factors such as mitogen (Fig. 3.3-B). A dose response was then performed where the expression with 5µg PHA was about 68.4%, while 10 µg of PHA increased expression of CCR5 to 74.3%, higher concentration of PHA (≥15µg) tapered expression off to between 75 – 79% only suggesting a threshold of expression when using concentrations of mitogen greater than 10 µg/ml.

Figure 3.2. Dot plots showing the presence of CCR5 on PBMCs.
The presence of the CCR5 chemokine receptor on the surface of the 3-day PHA-stimulated PBMCs was confirmed by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody against the CCR5 receptor. A hundred percent of the cell populations were gated. Autofluorescence of cells was measured in control samples while CCR5 receptors were detected on test samples.
Figure 3.3. Variation in the levels of expression of CCR5 receptors on PBMCs between two individuals.
Flow-cytometric analysis of surface CCR5 expression with FACS Ab-CCR5 on non-stimulated (no PHA treatment) PBMCs of two individuals (A) and on PBMCs stimulated with different concentrations of PHA (B). The level of CCR5 receptor expression varied between individuals and reached a maximum expression level at [PHA] of 20μg/ml and higher.
3.2 Screening reagents for toxicity: Concentration study

The results obtained with Alamar Blue assay were more reliable and consistent than those in the MTT assay. The dose response curves of reagents screened in both assays are shown in Fig. 3.4. At concentrations of iron greater than 500\( \mu \)M, the MTT assay clearly showed that there is toxicity (as one might expect). There was no toxicity with DFO at any concentration. And importantly, the chelation of iron with DFO was able to prevent any toxicity associated with high doses of iron. Data obtained with the Alamar blue assay were not as clear as with the MTT assay, but did demonstrate the increased toxicity (seen as decreased fluorescence) with high doses of iron. Once again, DFO had no toxic effect at any concentration tested.

3.3 Assays performed extracellularly on pathogens

3.3.1 Effect of iron overload and its chelation on mycobacterial viability using a micro plate based Alamar Blue assay (MABA).

In order to determine whether iron influences mycobacterial growth by directly exerting its effect on the bacilli, we analyzed the extracellular growth of \( \text{M.t.b} \) with 500 \( \mu \)M of Fe and/or DFO (Fig. 3.5, similar studies were done with iron but not DFO by Serafin-Lopez et al., 2004; Collins and Franzblau, 1997). \( \text{M.t.b} \), at a concentration of \( 2.5 \times 10^6 \) CFU/ml (of media), were exposed to Fe and/or DFO for 4 days in RPMI or 7H9 Middlebrook media. Fluorescence was measured 24 h after the addition of the dye (Fig. 3.5). In both media, in the presence of excess Fe, bacterial viability was increased by 13.6\% in RPMI and a significant 34% \( (P=0.009) \) in Middlebrook 7H9. In RPMI, DFO and [Fe+DFO] decreased viability by 33\% and 39.5\% respectively. Whereas in Middlebrook 7H9 media, DFO caused a significant inhibition of bacterial viability by 64.3\% \( (P=0.00134) \). These results showed that iron enhanced bacterial replication and chelation of excess iron by DFO can counteract this effect and correlated to those shown in Fig 3.6 and Fig 3.8. The difference in the significance of the data is obviously the fact that Middlebrook 7H9 is the preferred culture medium for bacteria, while RPMI is the preferred one for mammalian cell culture. RPMI was used to mimic the milieu in which bacteria-infected or non-infected mammalian cells are grown in vitro while cultivation of bacteria (in the absence of mammalian cells) is done in Middlebrook 7H9 media.
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Figure 3.4. Dose response curves of Fe and/or DFO on cells, assayed with MTT (A) and Alamar Blue (B). Cells were exposed to various concentrations of Fe and/or DFO for 4 days. Absorbance (MTT) / fluorescence (Alamar Blue) was read following a 4-h exposure to the dyes. Generally, a decrease in absorbance/fluorescence occurs as concentration of reagents increases.
Figure 3.5. Effect of reagents on extracellular growth of *M. tb*. Bacteria were grown in RPMI or Middlebrook 7H9 media with the addition of 500μM Fe and/or DFO for 4 days. Fluorescence of bacterial cultures was measured 24 h after the addition of Alamar Blue and Tween 80. Controls included media, autoclaved bacteria and non-treated bacteria. The values are shown as the mean ± SD of three separate experiments done in triplicate. Highly statistically significant effects with *p*≤0.01 are indicated with two asterisks, as compared to bacteria. Iron increased while DFO decreased bacterial viability.
3.3.2 Effect of iron overload and its chelation on reverse transcriptase directly

Because of the nature of the pathogen, bacterial viability could be directly assessed in the absence of host influence. The only way to do a related study on HIV is by assessing experimental conditions on viral enzymes directly. Reagents were tested for their ability to stimulate or inhibit HIV Reverse Transcriptase (RT) in a colorimetric ELISA (Fig. 3.6). None of the results obtained here were significant, with iron marginally increasing (6%) and both DFO and [Fe+DFO] inhibiting RT activity by respectively 14% ($P=0.059$) and 12.89% ($P=0.09$). In this assay, the enzyme is a recombinant enzyme in the absence of all virus and neither iron nor DFO had a clear effects on it.

![Graph](image)

**Figure 3.6. Inhibitory effect of DFO on recombinant HIV RT.**

Reagents were tested at a final concentration of 500 μM on viral enzyme as described in materials and methods. The percent activity was obtained by comparison to the positive control (which represents the activity of a recombinant HIV-I Reverse Transcriptase) and then subtracted from 100 to give the percent inhibition. Values represent mean ± SD of two experiments done in triplicate. Iron slightly stimulated while DFO reduced directly viral enzyme activity.
3.4 Evaluating the ability of Alamar Blue and MTT dyes to assess viability of *M.tb*-infected cells

3.4.1 Increased Alamar Blue reduction during co-infection

Section 3.3.1 clearly shows the ability of *M.tb* to reduce Alamar Blue and that excess Fe increases this reduction ability of the bacteria. It is obvious then to anticipate a further increase in Alamar Blue reduction in the presence of bacteria and host cells. When bacteria were added to HIV-infected cells, the fluorescence intensity increased (Fig. 3.7). Alamar Blue reduction was increased and had demonstrated a linear increase as the incubation period increased. The data obtained here regarding bacteria only are comparable to those seen with bacteria in RPMI media (Fig. 3.5).

![Figure 3.7. Time kinetic on the intensity of Alamar Blue reduction by bacteria in the presence or absence of host cells.](image_url)

Alamar Blue (AB, 10% v/v) was added to cells (HIV-infected only and Co-infected cells) and fluorescence measured after 4 h following 0, 4 and 6 days incubation. Media only, media containing AB, reduced AB (autoclaved) and bacteria alone were included as controls. An increased in the reduction of Alamar Blue when HIV-infected cells were co-infected *in vitro* with bacteria was observed.
3.4.2 Reduction of dyes after lysis of cells

Viability dye assays work on the principle of active mitochondria in host cells able to reduce dyes to a different colour. We wanted to see if the dye could be reduced in the absence of host cells. Upon lysing the cells, it is logical to expect that the cell lysates would contain fragmented membranes, free proteins and obviously the pathogens under evaluation.

There are no reports on the ability of HIV to reduce Alamar Blue or any other viability dyes as does *M. tb* on its own. To pinpoint possible external factors contributing to dyes reduction, after 4-day incubation, host cells were lysed, the lysates resuspended in RPMI media and re-incubated for 7 days. Reduction was observed after addition of 10% (v/v) dye to cultures of cell lysates and readings taken following 4 hours incubation. The controls include dye in media and totally reduced dyes (achieved by autoclaving dyes in media). Fig. 3.8 is representative of three experiments showing significant reductions of dyes by lysates. However, the reduction of the dyes by lysates was variable with MTT showing the most variation (high standard deviation) and highest values (0.8 absorbance units). Alamar Blue reduction was only around 0.4 (absorbance units). The assumption was made that the reduction of dyes was caused by recovered bacteria however it is also possible that the reductions (especially the variations noticed with MTT) are due to enzymes (involved in the mechanism of dyes' reduction), released in the media after destruction of PBM cellular walls.
Figure 3.8. Histograms showing reduction of MTT (A) and Alamar Blue (B) by lysates of non-treated (HIV-infected and co-infected) and treated co-infected cells.

Cells were exposed to 500 μM of reagents for 4 days, washed and lysed by freeze-thawing method. Lysates were resuspended in media and cultures re-incubated. At day 7, 10% of dyes were added and absorbance read at corresponding wavelengths. Data represents the mean ± SD of 3 independent experiments done in triplicate.
4. Summary thus far

According to the data collected in this study, several age groups of the population are being affected by HIV-1 (Fig. 3.1-C) the majority 20-40 and being females aged 20-40 (Fig. 3.1-B). CCR5 is the receptor of choice for HIV subtype C (Morris et al., 2001) but this subtype also tends to use CXCR4 as co-receptor (Cecilia et al., 2000). In this study, we found that CCR5 receptor on the surface of PBMCs was constitutively expressed and this expression could be further influenced by stimulation with PHA-P (Fig. 3.2 and 3.3). Inter-individual variation in the level of CCR5 receptor expression was seen (Fig. 3.3-A) This motivates our choice to work with PBMCs and monocytes, which can easily be infected with both HIV (Du151) and M. tb (H37Rv) because of the presence of necessary co-receptors on their surfaces (for HIV) and their phagocytic function (for entry of M. tb) respectively.

Fe and DFO were screened for their toxicity on host cell by conducting a concentration range study (Fig. 3.4). As demonstrated before, iron concentrations greater than 500 μM resulted in toxicity (Traoré and Meyer, 2002). Thus, 500 μM of iron and DFO were consequently used for the study.

It was seen that iron increased M. tb replication (Fig. 3.5) extracellularly as expected. DFO could counteract the effects of iron and to a lesser extend HIV. Iron and DFO had no significant effects on HIV RT activity (Fig. 3.6). The media of choice for bacterial culture was found to be Middlebrook media instead of RPMI (Fig. 3.5).

Finally, when assessing viability of cells during co-infection (Fig. 3.7), we found increased Alamar Blue reduction implicating interference by mycobacteria during host cell reduction of the dye. To include more controls, co-infected cells were lysed by means of freeze thawing and the resulting lysates were exposed to dyes and re-incubated for 4 h. There was reduction of dyes (Fig. 3.8), which could mostly be ascribed to: (1) enzymes (involved in cellular metabolism) released in the media after host cell membrane disruption and (2) mycobacterial-mediated reduction of dyes.
Section B

Host cell defence responses: Biochemical and immunological analyses.

Host defence mechanisms against infectious pathogens include the secretion of specific cytokines, induction of apoptosis and the production of nitric oxide. In this section, the following aspects will be dealt with: viability of host cells, flow-cytometric analyses of cell death, cytokines secretion and nitric oxide production of cells.

1. Assessing cell viability

The viability data are represented with box and whisker plots. These plots are helpful in interpreting the distribution of data and allow one to see patient variation. The significance of data was calculated using summarized viability data (not shown). Since, Alamar blue assay can measure both host cells and bacteria viability, thus confounding interpretation of viability data, we will only be discussing the MTT assay data. Following recommendation of one of the thesis examiner, viability results obtained with the Alamar blue assay are shown in the addendum section (Fig. A1 - A2). In part 2, we discussed the flow-cytometric data. PBMCs and monocytes isolated from blood collected from HIV-positive individuals were co-infected in vitro with *M.tb* H37Rv. Following the necessary incubation period, cell viability was assessed by MTT. In Fig 3.9, it can be seen that co-infection did not significantly reduce the viability of host cells. However in the presence of excess iron, there was a very clear and profound reduction in host cell viability with 36% (*P*<0.01) and 41% (*P*=0.055) decreases for PBMCs and monocytes respectively. DFO decreased viability significantly by 28% (*P*=0.01) for PBMCs and surprisingly increased viability of monocytes by 23% (*P*=0.165). The unexpected increase in co-infected monocyte viability in the presence of DFO was seen 10 times (this experiment was repeated as many times). The reason for this phenomenon is not clear to us but we speculate that the effect of DFO varies between cell types (van Asbeck *et al.*, 2001) with lymphocytes being more sensitive to iron chelation than other cells. Importantly, the presence of DFO with excess iron could reverse the effects of excess iron and restore host cell viability to levels comparable to that in the absence of excess iron.
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Figure 3.9. Box and whisker plots to compare cell viability of chronically HIV-infected cells co-infected in vitro with M.tb. Host cell (PBMCs and monocytes) viability was assessed using MTT. Fe and/or DFO represent treated co-infected cells. The box boundaries indicate the 25th percentile (closest to zero) and the 75th percentile (farthest from zero) while the line within boxes represents the median. Whiskers to the left and right of the boxes are the 5th and 95th percentiles. The results indicate the inhibitory effect of co-infection on host viability.
Regarding acutely infected cells, PBMCs and monocytes were sequentially infected in vitro with both HIV-1 Du151 (subtype C isolate) and *M.tb* H37Rv (virulent strain), and their viability determined once again with MTT (Fig. 3.10). Co-infection (the presence of the 2 microorganisms in concert) decreased viability by 45.9% and 44.9% for PBMCs ($P<0.0001$) and monocytes ($P=0.0873$) respectively. Addition of excess Fe to co-infected cells, showed a significant decrease in viability of PBMCs (40.6%, $P=0.0012$) but not in monocytes (3.75%, $P=0.367$). Treating co-infected cells with DFO did not affect viability of both monocytes and PBMCs ($P=0.49$). Fe chelation by DFO prior to treating co-infected cells led to stimulation of PBMCs (44.7%, $P=0.026$) and monocytes (19%, $P=0.2$).

Looking at data obtained with MTT, the patterns between acute and chronically infected cells are not similar with difference in responses. Possible reasons could be that (1) PBMC’s are composed of a mixture of cells whereas monocytes are one cell type with differentiated and undifferentiated cells; (2) combining 10 people’s data versus looking at individual pattern; (3) the extent of HIV infection, etc.

The effects of iron and DFO were done primarily in chronically HIV-infected cells resulting in interesting and significant results. The results obtained were then experimentally reproduced and validated by acutely infecting cells from normal subjects (secondary infection). The reason for the inclusion of acutely infected HIV-infected cells was to determine whether marked difference occurred if the viral infection was not already established before *M.tb* infection and subsequent excess Fe or DFO treatment.
Figure 3.10. Box and whisker plots to compare cell viability of acutely HIV-infected cells co-infected in vitro with M.tb. Host cell (PBMCs and monocytes) viability was assessed using MTT. Fe and/or DFO represent treated co-infected cells. The box boundaries indicate the 25th percentile (closest to zero) and the 75th percentile (farthest from zero) while the line within boxes represents the median, Whiskers to the left and right of the boxes are the 5th and 95th percentiles.
2. Monitoring cell death by flow-cytometry

In an attempt to clarify some of the ambiguous data observed with viability dyes, the more accurate flow cytometric analysis method was employed. HIV-infected cells were co-infected in vitro with \textit{M. tb} and exposed to Fe and/or DFO for 4 days. Untreated and treated cells were sequentially stained with Annexin V-FITC and PI and then analyzed by flow cytometry. Data plots (expressed as percentages) were generated from analysis of ungated data (3.11). Early apoptotic cells staining with Annexin V-FITC, but not PI appeared in the lower right quadrant of data plots. Necrotic cells appear in the upper right quadrant, staining with both PI and Annexin V-FITC (Fig. 3.11) when 10,000 events are collected which is typical of flow cytometry (Bradbury \textit{et al.}, 2000).

The percentages of viable and non-viable (apoptotic or necrotic) cells are presented in Figure 3.12 (A, B and C). In Fig. 3.12-A, co-infection of chronically-infected PBMCs with \textit{M. tb} resulted in a significant decrease in viability (25%; \textit{P}=0.038) with an increase in percentages of necrosis (28%; \textit{P}=0.051) and apoptosis (7.9%; \textit{P}=0.063). After treatment of cells with 500 \textmu M Fe, a decrease in both viability (28%; \textit{P}=0.069) and apoptosis (54.8%; \textit{P}<0.01), whereas a further increase in necrosis (51.2%; \textit{P}<0.01) of cells were obtained. With DFO, there was a decrease in viability (16%; \textit{P}=0.261) and an increase in apoptosis (38%; \textit{P}=0.11) compared to co-infected cells. Regarding [Fe+DFO], chelation decreased apoptosis but not necrosis meaning cells that appear viable with viability dyes may already be necrotic (Traoré and Meyer, 2002).

It was observed that co-infection of HIV-infected monocytes \textit{in vitro} was associated with a marked increase in necrosis (Fig. 3.12-B and C). In Fig. 3.12-B, excess Fe led to a decrease in viability of 27%, an increase in necrosis and a slight induction of apoptosis. DFO induced a low level of apoptosis and inhibited necrosis (44.5%, \textit{P}=0.074). Here, [Fe+DFO] displayed the same pattern as Fe on co-infected cells. In Fig. 3.12-C, there was no statistically significant results observed. Both Fe and DFO further decreased viability of co-infected cells (6% and 3% respectively) and increased the percentage of necrotic cells (18% and 14% respectively).
Figure 3.11. Contour diagram of Annexin V/PI flow cytometry of HIV-infected cells co-infected in vitro with M.tb. Cells were treated with 500 μM of Fe and/or DFO for 4 days. Following incubation, cells were incubated with both Annexin V and PI and analyses done by fluorescence-activated cell sorter. Lower left quadrants represent viable cells (PI/Annexin V). Lower right quadrants, early apoptotic cells (PI/Annexin V'). The upper right quadrants (PI'/Annexin V') contain nonviable, late apoptotic/ necrotic cells.

Chelation of Fe with DFO prior to exposure to cells, resulted in the basal levels (viable and necrotic cells) obtained with co-infected cells. This can be seen as the inability of both chemicals to induce their individual effects.

Cell death caused by co-infection, excess iron or DFO tends to be more due to necrosis rather than apoptosis. The general picture obtained is that data in Fig.3.12 are consistent with the MTT data, showing fewer viable cells when excess iron was added to co-infected cells. However, the number of events collected made the protective effect of DFO not readily apparent. If more events were collected here, the pictures would have been clearer.
Figure 3.12. Detection of apoptosis and necrosis (percentage) of co-infected cells.

Cells were treated as described in 3.11. Data (means ± SD from three individuals) are shown for: chronically infected PBMCs (A), monocytes (B) and acutely infected monocytes (C). Fe and/or DFO are treated co-infected cells. Statistically significant effects with $p \leq 0.05$ are indicated in the figures by one asterisk, highly statistically significant effects with $p \leq 0.01$ are indicated with two asterisks, as compared to HIV-infected or co-infected cells. Statistical analysis was done using Student's $t$-test.
3. *In vitro* cytokine production

The importance of cytokines in regulating different cellular functions, as well as their important role during infections is well known. We therefore analyzed the secretion of TNF-α and IL-6 in culture supernatants of co-infected cells. TNF-α is an extremely potent peptide cytokine that serves as an endogenous mediator of inflammatory, immune and host-defence functions and is produced by activated cells in response to various stimuli including viral and *M. tb* infections. A TNF-α ELISA kit was used to quantify secreted TNF-α in supernatant of cells treated or not with Fe and/or DFO. There were statistically significant differences in secreted TNF-α concentrations in co-infected cells (Fig. 3.13). Also a trend was seen for TNF-α concentrations to be more elevated in chronically infected cells (A) than acutely infected ones (B). Since HIV-infected individuals (seen as chronic infection) secrete TNF-α (Boue *et al.*, 1992) and *in vitro* studies have shown that *M. tb* induces TNF-α. Therefore, these mechanisms could contribute to high levels of TNF-α observed in chronically infected cells. Co-infection induced secretion of TNF-α in both PBMCs and monocytes. However, treatment of cells with excess iron and/or DFO showed a decrease in secretion, lower than basal levels obtained with HIV-infected cells (control). Since the secretion of IFN-γ and TNF-α often go hand-in-hand, the results obtained are consistent with prior reports (Shapshak *et al.*, 2004; Vincendeau and Daulouede, 1991) showing inhibition of IFN-γ secretion in the presence of excess iron.

Because IL-6 is induced by exposure of cells to HIV, we measured the level of secreted IL-6 in chronically and acutely infected cells co-infected *in vitro* with *M. tb* using an IL-6 ELISA kit. There was no significant difference in secreted IL-6 concentrations between conditions (treated or non-treated cells) in both chronically (Fig. 3.14-A) and acutely infected cells (Fig. 3.14-B). Higher levels of IL-6 were secreted by monocytes in both types of infection. This was expected because IL-6 is a monocytic cytokine (Le Meur *et al.*, 1999). Co-infection of cells induced secretion of the cytokine; excess Fe stimulated its secretion more while DFO inhibited it to a level lower than control. In [Fe+DFO]-treated cells, IL-6 secretion was maintained at the same level of expression as HIV-infected cells. The same pattern of secretion was observed in both types of infections (acute and chronic).
Figure 3.13. Secretion of TNF-α in culture supernatant of co-infected cells. Chronically (A) and acutely HIV-infected cells were co-infected with M.tb H37Rv and were cultured in RPMI media with 500 µM Fe and/or DFO. TNF-α production was determined in supernatants using ELISA. Data obtained were all highly statistically significant with $p \leq 0.01$, as compared to HIV-infected or co-infected cells. The values are given as mean ± SD of three experiments (representing three individuals) done in duplicate.
Figure 3.14. Secretion of IL-6 in culture supernatant of co-infected cells. Chronically (A) and acutely HIV-infected cells were co-infected with *M. tuberculosis* H37Rv and were cultured in RPMI media with 500 μM Fe and/or DFO. IL-6 production was determined in supernatants using ELISA. Data obtained were all highly statistically significant with p<0.01, as compared to HIV-infected or co-infected cells. The values are given as mean ± SD of three experiments (representing three individuals) done in duplicate.
These results demonstrate the association between HIV infection and elevated levels of pro-inflammatory cytokines.

4. Nitric oxide production as a host defence mechanism

NO is produced in large amounts during both host defence and immunological reactions. Certain evidences have demonstrated a relation between increased NO release and high viral load in HIV-infected individuals (Hermann et al., 1997). We evaluated NO production during co-infection with HIV and M.tb. Cells (HIV-infected and co-infected) were plated in RPMI-10% FBS with or without excess Fe and/or DFO for 4 days. Accumulated NO was measured after the 4-day experimental time using a NO colorimetric assay. Background NO levels in RPMI media were determined by sampling fresh media with no chemicals or cells added, and these values were subtracted from the data obtained for each condition. In chronically infected cells, NO levels in co-infected cells (Fig. 3.15-A) was significantly inhibited in PBMC ($P=0.0007$) and elevated in monocytes ($P=0.002$) compared to baseline NO levels in non-treated HIV-infected cells. Upon treatment with excess Fe, accumulated NO was increased in both PBMCs (4 times, $P=0$) and monocytes (1.5 times, $P=2 \times 10^{-4}$) relative to co-infected cells. While DFO-treated cells showed an increase of NO level in monocytes (1.15 times, $P=0.0023$), in PBMCs a complete inhibition was achieved ($P<0.001$). Fe chelation by DFO prior to addition of complex to cells also induced a significant amount of accumulated NO in monocytes (1.26 times, $P=8 \times 10^{-4}$) and PBMCs (4.4 times, $P=0$).

NO accumulation was also measured in acutely infected cells where the same protocol was carried out. Acutely infected cells exposed to M.tb in vitro had significant decreases of NO levels (Fig. 3.15-B) in monocytes (38%, $P=1 \times 10^{-6}$) and PBMCs (3%, $P=0.0087$). The cells exposed to excess Fe or DFO had their levels of accumulated NO significantly lower than that of co-infected cells, with a complete inhibition obtained with DFO, observed in both PBMC ($P=0$) and monocytes ($2 \times 10^{-5}$). The combination of Fe and DFO resulted in the only induction of NO accumulation in PBMCs (32%, $P=1 \times 10^{-5}$) and monocytes (69%, $P=0$).
Figure 3.15. Nitric oxide (NO) production in chronically (A) and acutely (B) HIV-infected cells co-infected in vitro with *M. tb*. Cells were co-infected with *M. tb* H37Rv and were cultured in RPMI media with 500 μM Fe and/or DFO. NO production was determined in supernatants using a colorimetric assay. Data obtained were all highly statistically significant with *p<0.01*, as compared to HIV-infected or co-infected cells. Each bar represents the mean ± SD of three independent experiments (individuals) done in duplicate (n=2). Increased levels of NO in chronically infected cells whereas inhibition of NO observed in acutely infected monocytes.
5. Summary thus far

*In vitro* co-infection of HIV-infected cells with *M.tb*, led to an inhibition of cellular viability as seen with MTT (Fig. 3.9 - 3.10) and flow-cytometry (Fig. 3.12). This inhibition of host cell viability in turn, resulted in an increase of (1) cell death (necrosis, Fig. 3.12); (2) cytokine secretion (Fig. 3.13 and 3.14) and (3) cell defence mechanism (Nitric Oxide production; Fig. 3.15).

Decreased host cell viability of co-infected cultures was especially pronounced in the presence of excess iron. On the other hand, DFO inhibited all host cell defence/immune responses as well as viability.

Complexing Fe with DFO reversed the effects of the individual reagents. This was seen as a restoration of (1) cell viability with both MTT and Alamar Blue, (2) host immune responses (cytokines) and (3) host defence mechanism (Nitric Oxide). We were not able to see any restored viability with flow-cytometry. Instead, chelation of Fe showed a decrease in host cell viability with an increase in necrosis. However, it is of importance to note that inhibition of host cell viability by chelation of iron is simply due to cell death.

The results obtained showed the deleterious effect of Fe-overload on host cells in favour of pathogens.
Section C
Pathogen behaviour: Microbiological analyses

This section describes the results obtained as a measure of responses from the two pathogens to iron and its chelation during co-infection of host cells.

1. HIV quantification

In order to investigate the effects of excess Fe and DFO on viral particle production, during *in vitro* co-infection with *M. tb*, p24 release and reverse transcriptase (RT) activity were measured in acutely and chronically infected PBMCs and monocytes.

1.1 p24 production

The extent of virus replication during co-infection was determined by measurement of core protein (p24) antigen in culture supernatant on Day 4 (Fig. 3.16). It can be seen that in chronically infected cells (Fig. 3.16-A), co-infection had opposite effects on p24 production with a 2.3 times increase (*P*=0.03116) in PBMCs and a 57% decrease (*P*=0) in monocytes was observed. Excess Fe increased p24 of both co-infected PBMCs (26%, *P*=0.116) and monocytes (133%, *P*=0). DFO and [Fe+DFO] significantly inhibited p24 production of co-infected PBMCs by 94% (*P*=0.0079) and 89% (*P*=0.0159) respectively, while stimulating viral release by 25% (*P*<0.001) and 146% (*P*=0) in co-infected monocytes. Inhibition of p24 suggests survival of host cells but it should be kept in mind that induction of necrosis could be a reason as to why a decline in p24 is observed.

P24 values for acute infection were higher than those of chronic infection (Fig. 3.16-B), co-infection led to insignificant increases in p24 production in PBMCs (19%, *P*=0.26) and monocytes (9%, *P*=0.25). As expected, DFO inhibited viral p24 production by 27% (*P*=0.151) and 67% (*P*=0.0019) in PBMCs and monocytes respectively, whereas treatment of these cells with excess Fe resulted in induction in p24 release of 9% (*P*=0.42) and 21% (*P*=0.247) respectively. DFO’s inhibitory effect on viral release could not be prevented by addition of Fe.
Figure 3.16. Core protein (p24) content as a measure of viral replication in chronically (A) and acutely (B) HIV-infected cells co-infected in vitro with M.tb.

Viral replication was measured as p24 release in supernatants of co-infected cells 4 days after in vitro co-infection. Statistically significant effects with \( p < 0.05 \) are indicated in the figures by one asterisk, highly statistically significant effects with \( p < 0.01 \) are indicated with two asterisks, as compared to HIV-infected and co-infected cells. Data show level of p24 antigen, expressed as mean ± SD.
Low p24 values in chronic infection do not suggest that the patient’s immune system was controlling viral load. Sometimes, low viral load means a depleted immune system where only a small amount of p24 can be detectable (Ribas et al., 2003; Prado et al., 2004). Results obtained in Fig 3.16 during co-infection with HIV and M.tb were opposite in PBMCs Vs. monocytes. The data suggest that TB co-infection particularly enhances viral replication in a non-monocyte (T helper cell) population.

1.2 RT activity
The effects of Fe and DFO on RT activity (Fig. 3.17) of co-infected cells were determined as a measure of viral production and as an attempt at confirming what was observed with p24 analysis. A stimulation of RT activity during co-infection in both chronically infected PBMCs and monocytes was observed (46%, \( P=0.0062 \) and 53%, \( P<0.001 \) respectively; Fig. 3.17-A). Treatment of cells with excess Fe further induced RT activity in co-infected PBMCs (55%, \( P=0.0014 \)) while slightly inhibiting it in monocytes (5%, \( P=0.19 \)). DFO and [Fe+DFO] resulted in significant inhibition of viral enzyme activity in PBMCs (69%, \( P=0.00137 \) and 10%, \( P=0.24 \) respectively) and monocytes (78%, \( P=9 \times 10^{-5} \) and 74%, \( P=1 \times 10^{-4} \) respectively).

In Fig. 3.17-B (acute infection of cells), viral activity was also stimulated by co-infection as seen in chronic infection but not statistically significant (46%, \( P=0.129 \) in PBMCs and 21.6%, \( P=0.2 \) in monocytes). Adding excess Fe to co-infected cells led to 8% inhibition \( (P=0.37) \) of viral activity in PBMCs, whereas a 34% stimulation \( (P=0.042) \) was achieved in monocytes. DFO completely inhibited viral enzymatic activity in PBMCs \( (P=0.019) \) and monocytes \( (P=0.0027) \). The inhibitory effect of DFO on RT activity was not abrogated when Fe was chelated to DFO before addition to co-infected cells.

Overall during co-infection, Fe-loading induced both release of more viral particles and viral enzymatic activity while DFO caused an inhibition of viral replication machinery.
Chapter 3: Results

Figure 3.17. Reverse transcriptase activity in co-infected cells.
Chronically (A) and acutely HIV-infected cells were co-infected with *M. tuberculosis* H37Rv and were cultured in RPMI media with 500 μM Fe and/or DFO. RT activity was assayed using an RT ELISA. Statistically significant effects with *p*≤0.05 are indicated in the figures by one asterisk, highly statistically significant effects with *p*≤0.01 are indicated with two asterisks, as compared to HIV-infected or co-infected cells. Data are shown as mean ± SD of three different experiments (three individuals) done in duplicate.
2. Evaluation of bacterial survival

This part of the study was done in conjunction with the TB research group at the Biochemistry Division of the Rand Afrikaans University led by Prof. Liza Bornman.

Chronically HIV-infected monocytes were co-infected with Mtb at an infection ratio of 1:10 (cells to mycobacteria) and exposed to excess Fe and/or DFO for 4 days. Following lysis of cells by means of freeze-thawing, the viability of the recovered mycobacteria was measured by use of a micro plate Alamar Blue assay (MABA) and bacterial growth was determined on agar plates by counting colony forming units (CFUs) after 4 weeks.

2.1 Micro plate Alamar Blue assay

To examine the effect of test reagents on the viability of mycobacteria post-infection the MABA was also applied here. Viability of the recovered mycobacteria was measured on day 7 post-recovery (when bacteria would have grown enough; Fig. 3.18). Middlebrook 7H9 media, unreduced Alamar blue, 100% reduced Alamar Blue and lysates from non-treated HIV-infected cells were included as necessary controls. As expected the fluorescence of the dye reduced by the mycobacteria recovered from the untreated co-infected cells were higher than the fluorescence of unreduced Alamar Blue (6.8 times, \( P=0.00026 \)). The reduction of dye by lysates of cells infected by HIV only is possibly due to the presence of enzymes (in the cell lysates), active enough to reduce Alamar Blue. There is no significant difference between the fluorescence of recovered mycobacteria and the fluorescence of HIV-infected cells only (36%, \( P=0.0724 \)). When looking at the effect of test reagents on bacterial viability, there is an increase in fluorescence with excess Fe (26%, \( P=0.064 \)) and a decrease in fluorescence with DFO (19%, \( P=0.149 \)). There is only a marginal decrease (3%, \( P=0.485 \)) in fluorescence when Fe was complexed to DFO. In summary, excess iron increased bacterial viability while DFO decreased it, \([\text{Fe+DFO}]\) had very little effect when compared to co-infected cells for reference purposes. These results are related to those seen in Fig. 3.5.
2.2 **Quantitative cultures: Plate counting**

In order to look at the effect of Fe and DFO on mycobacterial growth, a quantitative culturing method was used and the concentration of *M. tb* in each sample determined. After lysis of cells, samples were diluted ($10^2$ and $10^5$) in order to get the optimal dilution where countable colonies could be obtained. The diluted samples were plated on Middlebrook 7H10 agar, colonies were allowed to grow for a period of 4 weeks and the concentration of mycobacteria in each condition calculated (Table 3.1). The plates from the $10^2 \times$ dilutions were overgrown with colonies, whereas the $10^5 \times$ dilutions of samples resulted in the growth of individual colonies. As expected, lysates from HIV-infected cells alone showed...
no colonies and DFO treatment resulted in reduction of the number of colonies (34.7%, \(P=0.018\)). Fe increased the number of colonies by 47% \((P=0.0136)\), while a slight decrease in growth/concentration of the bacteria was observed with lysates from [Fe+DFO]-treated cells (6%, \(P=0.23\)). This moderate difference between co-infected cells alone and cells treated with [Fe+DFO] suggests that when used collectively, one chemical abrogates the effect of the other.

Overall, DFO inhibited bacterial viability and colony growth whereas excess Fe assisted bacterial survival and caused multiplication of colonies.

**Table 3.1. Quantitative analysis of mycobacterial concentration.** After a 4-day incubation period, treated and non-treated monocytes were lysed by freeze-thawing, two dilutions of samples were made and each dilution was plated on Middlebrook 7H10 agar. Colonies were counted after 4 weeks incubation and the mycobacterial concentration (CFU/ml) was calculated \((n = 2)\) for the 10^5 dilution which resulted in the growth of individual colonies.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Dilution factor</th>
<th>Average number of colonies ((n = 2))</th>
<th>Mycobacterial concentration (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infected monocytes (^1)</td>
<td>10^5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Co-infected monocytes</td>
<td>10^5</td>
<td>61</td>
<td>6.1 \times 10^7</td>
</tr>
<tr>
<td>Fe</td>
<td>10^5</td>
<td>89</td>
<td>8.9 \times 10^7</td>
</tr>
<tr>
<td>DFO</td>
<td>10^5</td>
<td>40</td>
<td>4.0 \times 10^7</td>
</tr>
<tr>
<td>Fe+DFO</td>
<td>10^5</td>
<td>57</td>
<td>5.7 \times 10^7</td>
</tr>
</tbody>
</table>

1. Dilution of 10^5 produced uncountable, overgrown colonies
2. Indicates situations where no bacteria was added
3. Summary thus far

Viral replication was higher in acutely infected cells (Fig. 3.16-B) than in chronically infected ones (Fig. 3.16-A). This could be because of 2 reasons, (1) low viral load in the chronically infected patients selected for the study and (2) high amounts of virus was used to infect cells in vitro (500 x TCID₅₀ this number agrees with routine values from the literature). Both co-infection and Fe stimulated p24 expression (Fig. 3.16) and RT (Fig. 3.17) while DFO inhibited viral replication via inhibition of viral enzyme activity and induced cell death. Fe chelation by DFO showed a restoration of p24 expression to the level of co-infected cells but unexpectedly, it inhibited RT activity.

Excess Fe induced an increase in bacterial viability (as measured with MABA, Fig. 3.18) and in bacterial colonies (Table 3.1). On the contrary, DFO inhibited both bacterial viability and colony size.

These results show that Fe overload during co-infection appears to be beneficial to both HIV and M.tb.
Chapter 4: Discussion

Discussion

1. Introductory comments

Opportunistic infections (OIs) are still plaguing individuals with HIV infection even while highly active antiretroviral therapy (HAART) lowers viral load. NIAID in a 2001 document commented that HAART has been extremely successful, not only in treating primary HIV infection, but in reducing the incidence of many OIs as well. Nonetheless, many HIV-infected adults and children remain vulnerable to OIs, especially those who are not receiving HAART and those for whom HAART has failed. These opportunistic infections include *Mycobacterium avium* causing mycobacterium avium complex (MAC) disease (a form of tuberculosis; Denis and Ghadirian, 1994), Pneumocystis carinii (Israel-Biet et al., 1993) causing pneumonia, herpes simplex virus-1, commonly associated with skin lesions (Heng et al., 1994) and *Mycobacterium tuberculosis* (*M.tb*), which is the most common HIV-related opportunistic infection and one of the initial manifestations in people dying from AIDS (Shattock et al., 1993; Scharer and Mc Adam, 1995; Imperiali et al., 2001). Concurrent infections of HIV and *M.tb* have been reported to enhance HIV replication (Juffermans et al., 2000).

Sub-Saharan Africa is facing parallel epidemics of Acquired immunodeficiency syndrome (AIDS) and Tuberculosis (TB). A reciprocal influence exists between *M.tb* and HIV. While HIV increases the speed, at which mycobacterial infection becomes TB, *M.tb* infection results in acceleration of HIV disease progression (Kirschner, 1999; Biswas et al., 2003). Furthermore, excess iron in the system (also common to sub-Saharan Africa), assists microorganism replication and may be detrimental to the defence mechanisms of the host (Weiss et al., 1992). Therefore, to control the AIDS-TB dual epidemic in Africa one needs to account for the additional challenge, of the existence of excess iron in the system (Fe overload) due to dietary habits or hereditary causes. This then becomes a triad epidemic, which will require a triad strategy - treating both TB and iron overload and preventing new infections with HIV.
Chapter 4: Discussion

Even though clinic records indicated all the persons involved in the study to be HIV positive, we confirmed a positive antibody response by utilizing rapid screening tests. Rapid tests produce results very quickly, usually between 5 to 30 minutes.

The escalating and uncontrollable spread of HIV/AIDS in Africa has tremendous political and economical consequences. One of the issues associated with the epidemic is the impact on families and social structure (Sleasman and Goodenow, 2003). In general, AIDS results in loss of young adults who are supposed to contribute to social support and economic stability of the continent (Sleasman and Goodenow, 2003). That is the economically active section of the population is affected by HIV/AIDS was noticed though be it to a small extent, during this research. In this study, females contributed 71.43% of the participants (Fig. 3.1-B) and the most affected people were those between 20-to-40 years of age (considered the economically active population, Fig. 3.1-B and -C).

Although  \textit{M. tb}, HIV and Fe-overload are common in sub-Saharan Africa, to our knowledge, no \textit{in vitro} studies have ever been conducted investigating the three conditions in concert. This observation is supported by reviews authored by Lounis et al. (2001) and Moyo et al. (1997). Evidence provided here include that (1) Fe' overload contributes to stimulation of HIV replication during concomitant infection with  \textit{M. tb} and (2) iron deprivation plays a role in inhibiting pathogen growth. Because previous studies in the division of Biochemistry evaluated the effect of iron on HIV- or  \textit{M. tb}-infected cells respectively and demonstrated an increase in both HIV replication and \textit{M. tb} growth by excess iron (Traoré and Meyer, 2004; Cronjé and Bornman, 2004) no further mention of the individual studies is made here. The bulk of the HIV-\textit{M. tb} literature demonstrates an unquestionable effect of one organism on the other. The work presented here now shows that excess iron augments/worsens this effect.

To initiate the study (hypothesis, objectives and outlines previously stated), various parameters were evaluated to optimize conditions. The data from the preliminary studies are described in section A (2). Host cell responses/defences during co-infection with the two pathogens (HIV and \textit{M. tb}) in the presence of excess iron were studied and these
results are presented in section B (3). Finally, in section C (4), we discuss the effect of iron overload on the replication of both pathogens. This chapter is ended by providing a conclusion (5) and future direction (6) for the research.

2. Section A: Experimental settings and preliminary studies

The studies undertaken here include defining iron overload, motivating the use of 4 days incubation time and the choice of cell types used.

Previous studies in the lab (Traoré and Meyer, 2002) confirmed the uptake and increase in cellular iron levels when adding 500 μM FeSO$_4$ in vitro. This was done using Inductively Coupled Plasma (ICP)-Atomic Emission Spectrometry (AES). Growing (culturing) mammalian cells in vitro, requires the addition of ~ 10% foetal calf serum (FCS) because of the valuable growth factors, nutrients and minerals including iron it contains. We wanted to determine how much iron was naturally found inside 10% FCS and once again using ICP-AES it was shown to be 237 μM (considered as normal and necessary for growth). Therefore, adding 500 μM FeSO$_4$ in addition to 10% FCS will amount to iron overload in vitro. All the necessary controls were taken care of to make sure that we did not have excessive background readings caused by the residual iron in the media.

PBMCs (composed of lymphocytes and monocytes) and monocytes (efficient at phagocytosing $M. tb$, Goletti et al., 1996; Mancino et al., 1997) have a record of successful use in HIV studies (Gandhi et al., 1998; Sonza et al., 1995). Monocytes/macrophages have a dual role, being involved in the pathogenesis of tuberculosis and HIV-1 infection and have been reported to change their functional activities after infection with HIV and $M. tb$ (Bonecini-Almeida et al., 1998; Herbein et al., 2002). These cells also serve as reservoirs of both viral and bacterial infections (Shattock et al., 1993) and their dysfunction contributes to CD$^+$ T-cell dysfunction as well as to impaired host defence responses against intracellular pathogens such as $M. tb$ (Weiss, 2002). According to Urmc (2004), primary cells such as PBMCs, are the most suitable to use when studying the effects of virus infection on cellular metabolism,
differentiation, function and survival because continuous cell lines are so different from the cells that the virus might normally infect in vivo.

The literature revealed that in in vitro studies of co-infection with both HIV and M.tb, incubation times varied from 1 day (Meylan et al., 1992; Shattock et al., 1993) to 16 days (Goletti et al., 1996) with 4 days being the norm (Zhang et al., 1995; Mancino et al., 1997; Toossi et al., 1997; Walder et al., 1997). Based on previous studies (Traoré, 2001), we used a period of 4 days incubation for most of our analysis because it agrees with the normative time reported in the literature but also because after this time period the cells would be viable enough to show some defence responses.

For the viral source an HIV-1 Subtype C isolate was used because it accounts for more than 95% of infections in the southern African region where CCR5 is the co-receptor preferred over CXCR4 (Morris et al., 2001). The expression of CCR5 on PBMCs can vary and be influenced by many factors including culture conditions. To ensure adequate expression of CCR5 for successful infection with macrophage tropic HIV-1 subtype C viral isolate DU-151a2-P2, we assessed the CCR5 levels on PBMCs (Fig 3.2 and 3.3). In PBMCs, CCR5 was found to be constitutively expressed (Fig. 2) and could be over expressed in stimulated cells. This expression correlated with increases in PHA (a lymphocyte growth stimulator) concentrations with a threshold expression reached when PHA was greater than 10 µg/ml (Fig. 3.3-B). There was also individual-to-individual variation in the expression of CCR5 on PBMCs (Fig. 3.3-A). Indeed, PBMCs treated the same but collected from different individuals, clearly demonstrated different levels of CCR5 expression. Therefore, due to the importance of CCR5 as a co-receptor for HIV-1, variability in CCR5 expression may have major consequences for susceptibility to HIV-1 or AIDS pathogenesis (Wu et al., 1997; Zamarchi et al., 2002). In a similar study, Wu et al. (1997) reported a correlation between low levels of CCR5 receptor and a decrease in infectability of cells with HIV in vitro where they showed CCR5 being abundant on long-term activated, IL-2-stimulated T cells, on a subset of effector/memory T cells in blood, and on tissue macrophages. We saw a similar abundance of CCR5 on stimulated PBMCs.
The concentration study (Fig. 3.4) showed fluctuations in viability but generally, 500 μM of reagents was not overly cytotoxic so that the effect of co-infection would still be noticeable. This was previously seen in the laboratory (Traoré and Meyer, 2002). Iron and DFO were then screened for their anti- or pro-viral and bacterial activities in a host cell free environment. Iron exhibited pro-activities for both M.typhus (Fig. 3.5) and HIV (Fig. 3.6) seen as increases in either viral enzyme activity or bacterial viability. DFO, an iron chelator exhibited anti-viral and anti-bacterial activities, presumably due to its iron depletion function (Voest et al., 1994). The results obtained with DFO show that the survival of any pathogen depends on its ability to secure iron from its environment (Weinberg, 2000a). When screening the effect of a complex of Fe/DFO on M.typhus, RPMI was added as an additional control and used to mimic the in vitro milieu in which bacteria-infected or clean eukaryotic cells are grown. For cultivation of bacteria (in the absence of eukaryotic host), Middlebrook 7H9 media is recommended due to its specificity for bacterial cultures.

The use of MTT and Alamar Blue as a means of assessing host cell viability in the presence of pathogens (such as M.typhus) can be conflicting since both dyes can also measure bacterial viability (Mshana et al., 1998; Franzblau et al., 1998). In this study, viability data were depicted as absorbance or fluorescence as a measure of an increase or decrease in cell growth or proliferation. An increase in fluorescence of Alamar Blue was observed when assessing viability of co-infected cells (Fig. 3.7). In general, reduction of Alamar Blue is due to an enzyme called diaphorase found in both mammalian cells and bacteria (O'Brien et al., 2000). Therefore, an increase in intensity of Alamar Blue reduction can be expected when cells are infected with M.typhus (even if cell viability is low). In order to exclude the influence of host cells and/or to determine the extent of bacteria-induced reduction of dye, the mammalian host cells were lysed and lysates exposed to dyes for 4 hours. Lysates showed reduction of dyes (Fig. 3.8), which we assumed to be due to mycobacteria released into the lysate after destruction of their hosts. It can also be argued that remaining activity of mammalian cell mitochondrial enzymes could have played a role in dye reduction, but the process of lysis most probably caused enzyme degradation.
3. Section B: Host cell defence responses

According to Goletti et al. (1996), the stimulatory effect of *M.tb* on HIV replication occurs by a variety of mechanisms. In our study, we found that co-infection of HIV-infected cells with *M.tb*, *in vitro* (1) increased HIV replication and (2) led to necrosis of cells. These results were firstly seen as a decrease in host cell viability as assessed with MTT (Fig. 3.9 & 3.10) and similar to the decrease in host cell viability during dual infection reported by Newman et al., (1993). The decrease in viability was also seen with flow-cytometry (Fig. 3.12). Iron overload and DFO further decreased host cell viability demonstrated by both viability dyes and flow-cytometry. In normal quantities, iron is essential for a variety of cellular processes and a competent immune response (Walker and Walker, 2000) since it is involved in host macrophage-mediated cytotoxicity (Gordeuk *et al.*, 2001) as a means for host cell defences against microorganisms. On the other hand, iron overloading could become harmful to the host (Doherty *et al.*, 2001; Boelaert *et al.*, 2003) and leads to large quantities of the metal being available to pathogens during infection, for replication (Traoré and Meyer, 2004). The mechanism by which excess iron is deleterious to cells may involve oxidative stress through the iron-catalyzed Fenton reaction (Crichton *et al.*, 2002; De Freitas and Meneghini, 2001). In this study, the effects of DFO varied between the two cell types used but the general finding was a decrease in host cell viability. DFO is known to inhibit normal cell growth (Lederman *et al.*, 1984) by reducing iron-content of cells and consequently decreasing host cell viability. However, treating co-infected monocytes with DFO led to an increase of viability. It is possible that the chelator's effect on the pathogens overshadowed its effect on the host cells. Another reason could be that cell types might respond to DFO differently with lymphocytes being more sensitive to iron chelation than other cells (van Asbeck *et al.*, 2001). In addition, besides being an iron chelating agent (thus protecting from free radical-mediated damage), DFO can have an additional role, that is its reaction with *trans*-peroxynitrous acid (Denicola *et al.*, 1995) which allows for protection in animal models of human diseases (Halliwell, 1989). Moreover, DFO has also been reported to have a low toxicity in vivo (Soriani *et al.*, 1993). Importantly, chelation of iron with DFO before addition to cells reversed the effects of excess iron on host cell viability and restored viability to the level of co-infected cells in the absence of iron. This
implies that chelation alone is not enough to restore host cell viability to basal level of HIV-infected cells.

Analysing the data collected by using the PBMCs/monocytes from several individuals by means of box and whisker plots makes it possible to see interindividual variability of cellular immune responses. Indeed, the reproducibility of experiments was influenced by the fact that cells were obtained from different individuals (Fig. 3.9 & 3.10). However, a similar pattern was observed for all individuals but with different levels of responses. Generation of effective responses to infections may be influenced by several intrinsic technical factors including diet, general health and living conditions, age, and immune system. In addition, it is possible that individual variation in the genetic make-up of humans will affect the interaction between host and disease (Jin et al., 2004; den Uyl et al., 2004; Yang et al., 1997). Given the setting in which we work; (collecting blood from individuals attending an HIV clinic which is open once a week) it should be obvious that negotiating for blood from the same individual is difficult. Ethics rules does not allow us to pay individuals for being part of the study, which would certainly serve as an incentive for returning to the clinic. Another disadvantage that indirectly influence our study is that no ART was provided by the clinic (during the time of our study) meaning people living with AIDS could get treated for some opportunistic infections and receive vitamins but very little was being done for their real problem. Understandably, CD4 counts were low and viral loads high. Therefore, differences between individuals should be taken into consideration when any extrapolation is made from \textit{in vitro} study to \textit{in vivo} condition.

The extent of cell death induced by co-infection with \textit{M.tb in vitro} was then investigated (Fig. 3.11 & 3-12). Increases of both necrotic and apoptotic cells were observed when comparing co-infected cells to HIV-infected cells only. This implies that \textit{M. tb} induces cell death of HIV-infected cells. Treating cells with iron overload induced a decrease in apoptosis but further increased necrosis of both acutely and chronically infected cells. Here increased necrosis of cells can be the result of Fe overload being detrimental to the host (via production of hydroxyl radicals) rather than as a host cell defence response to infection. DFO led to apoptosis rather than necrosis of cells. In general, apoptosis is an
essential process for cellular homeostasis (Samuilov et al., 2000) and its induction is seen as part of the host defence strategy to reduce survival of any pathogens. It has been reported that HIV-1-related proteins such as gp120 could induce apoptosis of bystander cells and recently, monocytes infected with recombinant HIV-1 tat have been shown to kill uninfected CD4+ cells by a Tumour Necrosis Factor (TNF)-related apoptosis-inducing mechanism (Bouzar et al., 2004). The mechanism of cell death during HIV infection is not completely understood and is linked to considerable controversy. Many scientists believe that HIV induces necrosis (Bolton et al., 2002) rather than apoptosis (Bouzar et al., 2004). In the case of mycobacterial infection, cell death mainly occurs via necrosis (Placido et al., 1997) because of bacterial burden. In our study, the flow-based assay did not show the protective effect of DFO as seen with viability data. The discrepancy between MTT data and flow cytometric data might be the results of (1) one of the two assays being less sensitive than the other (2) and too fewer events were evaluated by flow cytometry to acquire statistically meaningful data. However, the former is the most likely explanation as flow cytometry can also be accurate to low quantity of cells (Bradbury et al., 2000). It is necessary to note that apoptosis and necrosis are two different and mutually exclusive modes of cellular death. The two types of cell death are characterized by distinct morphologic and biochemical changes. Induction of either necrosis or apoptosis may depend on various factors including infection by a virus or any other pathogen (Samuilov et al., 2000) or level of ATP depletion (Paxian et al., 2003). Furthermore, necrosis of cells is mainly mediated by the release of TNF-α (Biswa et al., 2003), which is exactly what we saw when measuring cytokine production (discussed in more detail later). We noticed low levels of apoptosis; here cell death occurred mainly by necrosis, especially in acutely infected monocytes (where viral load was high). There may be a shift in the balance between apoptotic and necrotic cell death with transition through early and late stages (Sarafian et al., 2001). Consequently, cells detected as viable by MTT may be in a late apoptotic phase (Traoré and Meyer 2002). Furthermore, the difference observed between PBMCs and monocytes with regard to cell death was of concern since both cells were primary isolated cells. A possible explanation could be that since these cells have different and distinct functions they may also have distinct pathways of death (Richardson and Milnes, 1997). In order to
better distinguish between apoptosis and necrosis, the detection of DNA fragmentation (Kiss et al., 2004) may have provided clearer data than flow cytometry employed here. A critic expected would be that fixation of cells using paraformaldehyde should show all cells as PI positive. Fortunately, we used Annexin V in conjunction with PI to assess if the mode of cell death was a defence response from the host cell or because of dual infection.

In addition to inducing necrosis of HIV-infected cells and loss of viability, *M. tb* can also affect viral replication by immune activation (Goletti et al., 1996) and the induction of some cytokines (Toossi et al., 1999). Certain evidence implies that specific immune responses to HIV may be harmful to the host (Cohen and Fauci, 2001). During HIV infection, dysregulation of cytokines production is a main characteristic of this phenomenon (Cohen and Fauci, 2001) and seems to contribute to the stimulation of viral replication by suppressing any efficient antiviral response. Moreover, it has been suggested that dual infection might also modify the profile of cytokine expression (Bonecini-Almeida et al., 1998). Indeed *in vitro* studies have shown that phagocytosis of *M. tb* induces macrophage activation and production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (secreted by PBMCs and monocytic cells). In order to understand the mechanism involved in response to dual infection, ELISAs were performed to analyze the secretion of soluble factors such as IL-6 and TNF-α and NO. In this regard, we have shown an increase in both TNF-α and IL-6 secretions (Fig. 3.13 and 3.14) in co-infected cells. These proinflammatory cytokines, particularly TNF-α, are the most potent HIV-inducing cytokines (Cohen and Fauci, 2001) and are also produced to kill mycobacteria by exhibiting both anti-microbial activity and formation of granulomas (Kaushal Sharma et al., 2003; Imperiali et al., 2001) as inhibitors of mycobacterial growth. The increased levels of pro-inflammatory cytokines seen here concur with results obtained by Choi et al. (2004) in a study where they demonstrated that bacteria in chelating host iron (via secretion of siderophores) for their growth, contribute to the evocation of host inflammatory responses. Fe-overload resulted in a decrease of TNF-α but increased IL-6 secretion. Possible explanations for the former observation include the fact that (1) TNF-α secretion by cells is down regulated by iron (Gordeuk et al., 1992;
Serafin-López et al., 2004), (2) and negative stimulators of TNF-α production including IL-10 and IFNγ may counteract the pro-inflammatory effects of TNF-α (Kaushal Sharma et al., 2003). Indeed, the viral-suppressive activity of some cytokines such as IL-10 is mainly due to their ability to inhibit the secretion and activity of HIV-inducing proinflammatory cytokines (Cohen and Fauci, 2001). DFO-treated cells had inhibited secretion of both cytokines directly as a consequence of DFO-related decreased host viability. Iron in combination with DFO did not abrogate the effect of Fe alone in chronically infected cells. Whereas in acutely infected cells, both chemicals balanced each other’s effect. An increase in NO production during in vitro co-infection of HIV-infected PBMCs and monocytes with M. tb (Fig. 3.15) was noticed. Fe-overload showed a further increase in NO production. This increased NO was likely due to a combination of iron and co-infection. Similar studies have shown that a stimulation of NO was observed during co-infection with both HIV and M. tb (Torre et al., 1996a and b) or with HIV-1 infection (Pietraforte et al., 1994). The ability of iron to reduce NO production is well-documented (Serafin-Lopez et al., 2004; Harhaji et al., 2004). However, excess iron during co-infection with HIV and M. tb had an opposite effect, inducing a large amount of NO production. The same results were recently obtained by Serafin-Lopez et al. (2004) during mycobacterial infection in the presence of iron. A possible reason is that pathogens consuming iron would contribute to increased formation of NO and therefore to enhanced cytotoxicity towards themselves (Weiss et al., 1992). An interesting result obtained here was the inhibition of NO production after treatment of co-infected cells with DFO (Fig. 3.15). This inhibition may relate to the fact that limitation of iron by DFO consequently inhibits NF-κB, which in turn decreases iNOS activity. NO, abundantly produced during host defence responses (Torre and Ferrario, 1996) is released in vitro by human monocytes in response to various stimuli (Vullo et al., 1998). However, NO can have a dual action in host defence mechanism: its production can restrict pathogen growth (Nathan and Xie, 1994a) and lead to various intracellular pathogens’ killing (Hibbs et al., 1987). In contrast, excessive production of NO (as a means of defence against infections) can lead to tissue damage (Nathan and Xie, 1994b). Finally, Fe complexed to DFO prior to treating the cells decreased the inhibitory effect seen with DFO alone and this attached DFO limited Fe’s stimulating effects, seen as a restoration of...
host defence response to baseline/co-infected cells alone. The increased NO production correlated with elevated concentrations of secreted TNF-α and IL-6 (Fig. 3.13 & 3.14) and induction of cell death (Fig. 3.12; Brüne et al., 1998). A potential role exists for these cytokines in stimulating NO production (Torre et al., 2002) that in turn resulted in host cells undergoing necrosis (Hibbs et al., 1987; Hermann et al., 1997).

4. Section C: Pathogen behaviour

Various authors have suggested an enhancement in viral replication during concurrent infection with *M. tb* (Weiden et al., 2000; Nakata et al., 1997; Toossi et al., 1997; Ho, 1996; Zhang et al., 1995). In this work, we looked at whether excess iron was a co-factor to *M. tb*-induced HIV replication and conversely, to HIV-induced *M. tb* growth. Co-infection of cells resulted in a stimulation of viral infection seen as an increase in both p24 production (Fig. 3.16) and viral reverse transcriptase (RT) activity (Fig. 3.17). This enhanced replication of HIV by *M. tb* may result from various mechanisms including activation of HIV long terminal repeats (LTR; Toossi et al., 1999; Zhang et al., 1995) and host immune activation (Goletti et al., 1996; Ho, 1996). Indeed, activation of host transcription factors, specifically nuclear factor-κB (NF-κB), which increases HIV transcription by interaction with HIV LTR, is critical (Alcami et al., 1995). *M. tb* was reported to induce HIV replication in chronically and acutely infected cells (Lederman et al., 1994; Zhang et al., 1995) through activation of NF-κB. Another possible mechanism through which *M. tb* induces HIV replication is by specific recognition of mycobacterial antigens (Ag) through the release of soluble factors (Biswas et al., 2003; Zhang et al., 1995). Consistent with our observation, are the increases in both pro-inflammatory cytokines and NO leading to decreases in host cell viability and ultimately to necrosis of cells. TNF-α is well established as the main HIV-inducing cytokine in HIV-infected cells and its capacity in enhancing HIV is attributed to its activation of HIV LTR through NF-κB (Toossi et al., 1999). Conversely, the effect of this cytokine on HIV production may be bi-directional. In addition to the transcriptional up-regulation of HIV by TNF-α, an inhibitory effect of this cytokine on mechanisms preceding proviral integration was reported (Toossi et al., 1999). Further, another pro-inflammatory cytokine, namely IL-6 appears to be involved in transcriptional or translational activation of HIV and is
abundantly present in mycobacterial cultures. Moreover, the large amount of NO produced in the study correlated to p24 production and in our case, its role in HIV infection is easily predictable. NO is reported to activate NF-κB, which in turn increases the replication of HIV-1 but can also inhibit the replication of other viruses (Torre and Ferrario, 1996). Interestingly, strengthening our observation is that *M. tb*-induced HIV replication could be decreased via inhibition of pro-inflammatory cytokines by the addition of soluble receptors or following exposure to exogenous IL-10 (Goletti *et al*., 1998). Fe-treated cells (that were also co-infected) displayed a large amount of p24 particles and had a higher viral enzyme activity. However, there were differences between p24 productions in PBMCs compared to monocytes. The decrease in p24 noticed in monocytes correlates to that reported by Sterling (2000) who concluded that (1) *M. tb* may decrease HIV-1 replication *in vitro* by inducing Interferon (IFN) and (2) INF-β may be clinically useful in preventing HIV-1 replication during opportunistic infections. In addition, Bernstein *et al.* (1991) demonstrated that bacterial lipopolysaccharide (LPS) down-regulates HIV in monocytes through activation of a nuclear factor that negatively affects HIV LTR. These data imply that co-infection with *M. tb* particularly enhances viral replication in a non-monocyte population. The increase in p24 observed in excess iron treated co-infected cells can be explained by the increase in ROS, accumulated from Fe-related Fenton reaction and produced by *M. tb*-infected monocytes as a defence mechanism by the host against micro organism (Mancino *et al.*, 1997). DFO inhibited HIV replication and this was seen as a decrease in viral p24 production and inhibition of RT activity. A link has been established showing iron-modulated HIV replication (Boelaert *et al*., 1996; Traoré and Meyer, 2004), given that DFO targets a variety of Fe-containing molecules including ferritin and ribonucleotide reductase. Consequently, the removal/unavailability of iron (by for example a chelator) will result in a lack of deoxyribonucleotides for DNA synthesis (Richardson and Milnes, 1997) of host cells which in turn will lead to inhibition of host cell proliferation. Therefore, synthesis of incorporated viral genetic material will not occur. The combination of iron and DFO prior to their addition to cells allowed for the abrogation of the individual effects of chemicals on p24 production but not RT activity. These results suggest that, the mechanism for DFO’s inhibitory effect on HIV replication is not via inhibition of RT but rather through
a different route. It is possible that DFO does not directly attack the virus but rather kills infected cells by withholding iron from normal host cell functions. Furthermore, there were discrepancies between the levels of p24 in acutely and chronically infected cells. However, the p24 values obtained for acutely infected cells were predictable because of the TCID50 used to infect cells in vitro. Whereas, in the chronically infected cells, levels of viral core protein release would be determined by external factors such as whether the patient was receiving HAART, the viral load and the stage of disease. The low values obtained were seen by other researchers (Ranjbar et al., 2003). Indeed, high viral loads characterize the acute phase of HIV infection whereas the latent phase displays a steady viral load (Rosenberg et al., 1997) where only a small amount of p24 can be detectable (Ribas et al., 2003; Prado et al., 2004). Furthermore, secondary factors including viral strain, genetic factors, and coexistent infections could have affected the results (Feinberg et al., 1998).

HIV is known to induce M.tb growth by compromising host’s resistance to mycobacteria (Meylan et al., 1992; Lawn et al., 2002). For instance, a reduced CD4+ cell count may be seen as a deficient antimycobacterial immune response. The effect of excess iron on M.tb in co-infected cells resulted in growth of the bacteria (Fig. 3.18) as seen in Fig. 3.5. We also evaluated bacterial growth using an agar-based technique (Table 3.1), which enabled us to determine the concentration of bacteria in each sample. Fe-treated samples were found to have the highest concentration of bacteria, which was in agreement with results obtained in Figures 3.5 and 3.7. In addition to measuring bacterial viability, counting colonies further strengthens the observation of the stimulating effect of iron on M.tb growth. These findings show that iron not only act directly on the growth of M.tb but also affect indirectly some mechanisms of the macrophage that regulate intracellular growth of M.tb (Serafin-Lopez et al., 2004). Lounis et al. (2001) reported the enhancing-growth ability of iron on mycobacterial species while reviewing its effect on M.tb to be varied. Indeed, some authors reported an enhanced growth of M.tb in the presence of iron (Rook et al., 1986; Gobin and Horwitz, 1996; Rodrigue and Smith, 2003); others showed an inhibition of growth (De Voss et al., 2000; Byrd, 1997). These differences in observations were due to differences in host cell type, iron concentration, and pre-
treatment of cells for example with cytokines (Zwilling et al., 1999; Lounis et al., 2001). In order to understand the host defence response to *M. tb* during dual infection with HIV, secretions of pro-inflammatory cytokines and NO were assessed. *M. tb* infection induced large amounts of NO production in the absence and presence of iron and induced both IL-6 and TNF-α. This ability of *M. tb* to induce cytokines has been reported by others (Nakata et al., 1997; Zhang et al., 1995). Normally, TNF-α stimulates macrophage-dependent bacteriostatic and bactericidal activity against *M. tb* (Champsi et al., 1995). However, in dual infection, TNF-α seems to loose this activity indicating that HIV infection affects normal cell function (Imperiali et al., 2001). Consequently, a positive feedback mechanism ensued, in which the growth of *M. tb* is maintained by enhanced TNF-α production and vice versa. Moreover, HIV-1-induced cell alterations could explain the different effect of IL-6 and NO (Imperiali et al., 2001). *M. tb* infection is characterized by an increased NO production (Anstey et al., 1996; Valone et al., 1988). Therefore, during concurrent infections with HIV and *M. tb*, host specific responses to bacterial infection induce immune activation. HIV takes advantage of this activated state to replicate resulting in depletion of cells and overgrowing of bacteria. The lowest number of bacterial colonies was obtained in DFO-treated samples, suggesting that withholding iron leads to inhibition of bacterial growth. As seen in table 1, the highest sample dilution gave single countable colonies while a lower dilution would result in an overgrowth of colonies. With respect to limitations, the agar based technique was found to be very long (4 weeks) and dependant upon meticulous culture technique.

5. Conclusion

The findings in this study further indicate that HIV replication is extremely influenced by iron overload during concomitant infection with *M. tb*. Acutely and chronically (latent) infected cells (PBMCs and monocytes) were induced by *M. tb* in vitro to increase production of HIV p24. This effect correlated with (1) decreasing host viability, (2) increasing host defence responses and (3) causing necrosis of cells. Activation of host defence responses against mycobacteria infection induced HIV replication. TNF-α, IL-6 and NO appear to have a major role in the enhanced growth of *M. tb*. Here, for the first time we demonstrated the deleterious effect of iron on the host in vitro while it remains
beneficial to the pathogens during co-infection with *M. tb* and HIV. In addition, DFO as a possible therapeutic agent in Fe overload conditions counteracted the effects of excess iron. It would therefore be appropriate to propose for a combination therapy in co-infected individuals with excess levels of iron comprised of DFO and antiretroviral agent(s).

6. Future prospects

To get a better idea of the synergy between HIV, *M. tb* and excess iron, the blood of HIV-infected individuals naturally co-infected with *M. tb*, should be evaluated by measuring the level of serum ferritin saturation as a marker of total body iron. In this kind of analysis, the transferrin levels would provide valuable information about *in vivo* iron status. In general, iron overload is characterized by increased serum transferrin saturation (Beutler *et al.*, 2003). For a better understanding of the role of the host immune system when exposed to dual infection under conditions of iron overload, a detailed characterization of cytokine production should be done. Indeed, The T<sub>H1</sub>/T<sub>H2</sub> balance during host immune responses, even though controversial in HIV-1 infection alone, is modified during co-infection with HIV and *M. tb* (Manetti *et al.*, 1996; Benecini-Almeida *et al.*, 1998). T<sub>H1</sub> CD4+ T cells produce IL-2 and IFN-γ, cytokines supporting CTL, NK-cells and macrophages (effector functions of the immune system) while T<sub>H2</sub> cells predominantly produce IL-4, IL-10 and IL-6, which are cytokines that favour the development of a humoral immune response (Rubbert and Ostrowski, 2003). A decrease in T<sub>H1</sub> cytokines profile in favour of increase in T<sub>H2</sub> is expected during co-infection with HIV and *M. tb*. 
References


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The following websites were accessed in October 2004:

http://biology.fullerton.edu
www.Avert.org
www.NIAID.org
www.people.virginia.edu
www.thebody.com/cdc/rapidfaq.html
www.research.bidmc.harvard.edu
www.UNAIDS.com
www.urmc.rochester.edu
INFORMED CONSENT FORM

TITLE: Obtaining biological HIV isolates for *in vitro* studies

I hereby confirm that I have been informed by the investigator, Dr. of the nature and purpose of being involved in this study. I understand that the blood specimens taken from me will be used in HIV research.

I am also aware that the results of the study, including personal details regarding my age, sex, race, diagnostic and risk factors may be anonymously processed into a publishable report.

I may at any stage, without prejudice, withdraw my consent and participation in this study.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient:
Signature: Date:

Witness:
Signature: Date:

Dr who obtained informed consent:
I hereby confirm that the above patient has been fully informed about the nature, purpose and risk of the above study.

Signature: Date:

Name (please print):
Table A1: List of HIV positive patients

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Figure A1. Modification of Fig. 3.4. including Alamar Blue absorbance readings. Cells were exposed to various concentrations of Fe and/or DFO for 4 days. Absorbance/fluorescence was read following a 4-h exposure to the dyes. Generally, a decrease in absorbance/fluorescence occurs as concentration of reagents increases.
Figure A2: Box and whisker plots to compare cell viability of chronically HIV-infected PBMCs co-infected \textit{in vitro} with \textit{M.tb}. Host cell viability was assessed with the Alamar blue assay. Data are shown as absorbance (A) and fluorescence (B). Fe and/or DFO represent treated co-infected cells.
Figure A3: Box and whisker plots to compare cell viability of chronically HIV-infected monocytes co-infected in vitro with *M. tb*. Host cell viability was assessed with the Alamar blue assay. Data are shown as absorbance (A) and fluorescence (B). Fe and/or DFO represent treated co-infected cells.
Figure A4: Box and whisker plots to compare cell viability of acutely HIV-infected PBMCs co-infected in vitro with *M. tb*. Host cell viability was assessed with the Alamar blue assay. Data are shown as absorbance (A) and fluorescence (B). Fe and/or DFO represent treated co-infected cells.
Figure A5: Box and whisker plots to compare cell viability of acutely HIV-infected monocytes co-infected in vitro with M.tb. Host cell viability was assessed with the Alamar blue assay. Data are shown as absorbance (A) and fluorescence (B). Fe and/or DFO represent treated co-infected cells.