



**Evolution of Anti-Tat Antibodies and Its Role in
Developing Prophylactic and Therapeutic HIV-1 Vaccine.**

By

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PREFACE

The research detailed in this thesis was conducted at the HIV Pathogenesis Programme, College of Health Sciences, Nelson R. Mandela School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Durban, South Africa. The timeframe for the experimental work was extended from February 2022 to January 2024 and supervised by Dr NP Mkhwanazi. The author asserts that this work is original and has not been previously presented for any degree or diploma at any educational institution. Proper acknowledgment is given in the text for utilising others' work.

DECLARATION

IThandeka Innocentia Kubheka

Declare that:

- (i) The research reported in this dissertation/thesis is my original research, except where otherwise indicated.
- (ii) This dissertation/thesis has not been submitted for any degree or examination at any other university.
- (iii) This dissertation/thesis does not contain other persons' data, pictures, graphs, and other information unless expressly acknowledged as being sourced from other persons.
- (iv) This dissertation/thesis does not contain other persons' writing unless expressly acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
 - a) Their words have been rewritten, but the general information attributed to them has been referenced.
 - b) Where their exact words have been used, their writing has been placed inside quotation marks and referenced.
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Dr N.P Mkhwanazi (Supervisor).

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Date

DEDICATION

I want to express my dedication for this dissertation to my family and friends. A deep appreciation goes to my supportive parents, Mr. and Mrs. Kubheka, who encouraged and inspired me to pursue this degree. Special acknowledgment is extended to my sisters, Nompumelelo and Andile Kubheka, who have consistently stood by me and hold a significant place in my heart.

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LIST OF ABBREVIATIONS

AIDS- Acquired immunodeficiency syndrome

Abs- Antibodies

AGID- Agar gel immunodiffusion

ARM- arginine-rich motif

ART- Antiretroviral therapy

AZT- Azidothymidine

bNAbs- Broadly neutralising antibody

BSA- Bovine serum albumin

CA- Capsid

cART- Combined antiretroviral therapy

Cas9- CRISPR-associated protein 9.

CCR5- C-C chemokine receptor type 5

CDK9- Cyclin-dependent kinase 9

CFT- Complement fixation test

CO₂- Carbon dioxide

CRISPR- Clustered regularly interspaced short palindromic repeats

CXCR4- C-X-C chemokine receptor type 4

DC- Dendritic cells

DDPCR- Digital droplet polymerase chain reaction

DMEM- Dulbecco's Modified Eagle Medium

DNA- Deoxyribonucleic acid

DNMTi- DNA methyltransferase inhibitors

EC- Endothelial cell

ELISA- Enzyme-linked immunosorbent assay

Elispot- Enzyme-linked immune absorbent spot

Env- Envelope

ESCRT- endosomal sorting complex required for transport

FBS- Fetal bovine serum

FDA- Food and Drug Administration

FRESH- Female Rising through Education, Support and Health

Gag- group-specific antigen

Gp- Glycoprotein

gRNA- genomic RNA

H- Hydrogen

H₂O₂- Hydrogen peroxide

H₂SO₄- Sulphuric Acid

HAART- Highly active antiretroviral therapy

HAI- Haemagglutination inhibition

HAND- HIV-associated Neurocognitive Disorder

HDACi- Histone deacetylase inhibitors

HERV- Human endogenous retroviruses

HIV- Human Immunodeficiency Virus

HLA- Human leukocyte antigen

HMTi- Histone methyltransferase inhibitors

HRP- Horseradish peroxidase

IFAT- Indirect fluorescent antibody

IgA- Immunoglobulin A

IgG- Immunoglobulin G

IgM- Immunoglobulin M

IL- Interleukin

INSTI- Integrase single transfer inhibitor

IFN- γ - Interferon-gamma

IPDA- Intact proviral DNA assay

KS- Kaposi's sarcoma

LAT- Latex agglutination test

LPA- Latency promoting agent

LRA- latency-reversing agent

LTR- Long terminal repeat

MA- Matrix

MAT- Microscopic agglutination test

Nef- Negative factor

NC- Nucleocapsid

NK- Natural killer

NNRTI- Non-nucleoside reverse transcriptase inhibitor

NRTI- Nucleoside-analogue reverse transcriptase inhibitor

Nt- Nucleotide

OPD- Ortho-phenylenediamine

O- Oxygen

PBMC- Peripheral blood mononuclear cell

PBS- Phosphate-buffered saline

PCR- Polymerase chain reaction

PLWH- People living with HIV

PM- Plasma membrane

PI- Protease inhibitor

Pol- Polymerase

P-TEFb- Positive transcription elongation factor

Q4PCR- Quadruplex polymerase chain reaction

qPCR- quantitative PCR

qVOA- Quantitative viral outgrowth assay

Rgp160- Recombinant glycoprotein 160

RLU- Relative luminescence units

RNA- Ribonucleic acid

RNAP II- RNA polymerase II

SA- South Africa

SAT- Serum agglutination test

TALEN- Transcription-activator-like effector nucleases

TAR- Trans-activation response element

Tat- Trans-activator of transcription

Th1- T- helper type 1

TLR7- Toll-like receptor 7

TNF- α - Tumor necrosis factor α

Vif- Virion infectivity factor

VL- Viral load

VNT- Virus neutralisation test

Vpr- Viral protein R

Vpu- Viral protein U

UNAIDS- United Nations Programme on HIV/AIDS

WIHS- Women's Interagency HIV Study

ABSTRACT

The introduction of the antiretroviral drugs has reduced the burden of HIV-1 by reducing the mortality rate of people living with HIV-1. In addition, HAART and cART were introduced and capable of virological suppression (<400 copies ml^{-1}), and widespread uptake quickly led to dramatic reductions in morbidity and mortality in the developed world. However, antiretroviral therapy cannot eradicate/ cure the virus. This is due to the viral reservoirs, drug resistance, and toxicity development. Therefore, up to date there is no cure for HIV and effective preventative vaccine. Hence, there is a need to continue to search for the effective HIV-1 vaccine to prevent the new infections. HIV-1 regulatory protein known as Tat have been found to play an important role in disease progression. Due to this knowledge Tat has been identified as a potential target for the development of prophylactic and therapeutic vaccination for cART intensification. In this study, we investigated the influence of HIV-1 anti-Tat antibodies on HIV-1 disease progression, and the evolution of HIV-1 anti-Tat antibodies from early-treated individuals throughout 12 months of HIV-1 infection. We also measured the total anti-Tat specific antibodies titers in early treated individuals from the FRESH cohort and associate them with the size of the reservoirs at 12 months. Secondly, we evaluated the role of anti-Tat immunity in immune activation in early-treated individuals. Lastly, we investigated how the HIV-1 Tat protein impacts the anti-Env antibody neutralization ability, thus preventing the infection. Anti-clade C Tat IgG, IgM and IgA titers were assessed in plasma samples from 34 HIV-1 early treated individuals from FRESH cohort using ELISA and associated them with duration of treatment and CD4⁺ T cell count. Digital droplet PCR was used to measure size of the reservoirs and CD4⁺ and CD8⁺ T cell activation was measured using HLA-DR and CD38 markers using flow cytometry. TZM-bl cells were used to measure the antibody neutralising titers. The present study found that, Anti-clade C tat IgA, IgG and IgM were associated with the duration of treatment as the presence of anti-tat antibodies tends to disappear over time. Anti-Tat antibodies correlates positively with the size of the reservoirs irrespective of CD4⁺ T cell counts. Anti-Tat antibodies were associated with low CD4⁺ T cell activation and correlated positively with CD8⁺ T cell activation. Anti-Tat antibodies correlates negatively with neutralisation antibody titers. This data suggests that Tat-specific IgM and IgG antibodies in combination with cART could be a better strategy to improve HIV-1 vaccine.

CHAPTER 1: INTRODUCTION

1.1 Background

Human Immunodeficiency Virus (HIV) remains one of the most severe global health challenges since its discovery in 1981 by French scientists (Barré-Sinoussi et al., 1983). The Human Immunodeficiency Virus infects the white blood cells, called CD4⁺ T cells and in the absence of antiretroviral treatment, it results in acquired immunodeficiency syndrome (AIDS). Human Immunodeficiency Virus is transmitted between individuals via blood transfusion, tissue/organ transplantation, breastfeeding, and unprotected sexual intercourse (Kalinichenko et al., 2022). Within the host, the virus can spread by utilising either cell-free or cell-to-cell modes of transmission (Kalinichenko et al., 2022; Sattentau, 2008).

Approximately 39 million people are living with HIV-1 (including 1.5 million children), with a global prevalence of 77% among adults (UNAIDS, 2023). According to UNAIDS 2023, 4000 people become infected with HIV-1 every day, including 1100 young people (aged 15 to 24 years) (UNAIDS, 2023). In South Africa, approximately 8.2 million people are living with HIV-1, an estimated 13.9% of the population. In 2021, about 67% (5.5 million) of people living with HIV-1 were accessing ART in South Africa (McInziba et al., 2023; Stats SA, 2021). Antiretroviral drugs lowered the burden of HIV-1 by decreasing the mortality rate of people living with HIV-1. In addition, they were capable of inhibiting the virus to undetectable viral load (<400 copies ml⁻¹) (Tseng et al., 2015; Palella et al., 1998). However, combination antiretroviral therapy cannot eradicate/ cure the virus. This is due to the presence of viral reservoirs (Sankaranantham, 2019), drug resistance and toxicity developments (Maldarelli, 2011; Desai et al., 2012). Therefore, up to date, there is no HIV-1 cure or HIV-1 vaccine; hence, there is a need to continue to search for an effective HIV-1 vaccine to prevent new infections.

Several HIV-1 vaccine trials have failed to give positive results (Ng'uni et al., 2020). The difficulties associated with developing an effective/successful vaccine have increased since the initial clinical trials of the Microgenex vaccine up to the widely publicised Vaxgen trial (Robinson, 2007; Letvin, 2006; Billich, 2004). The HIV-1 env-based vaccines were designed to induce neutralising antibody responses, given previous findings demonstrating that the passive transfer of neutralising antibodies could protect primates from infection. Unfortunately, these initial trials were unsuccessful, because inducing neutralising antibodies is a daunting task at which more than one group has failed (Burton et al., 2004). Other reasons for these

failures involve the genetic diversity of viral envelope proteins, enabling the virus to escape neutralising antibodies. Furthermore, the challenge lies in identifying immunogens and immunisation platforms that consistently induce antibodies with the ability to neutralise various HIV clades (Phogat et al., 2007). HIV-1 prophylactic vaccines based on the HIV-1 Env antigen have provided minimal protection (Monini et al., 2012), showing that Env alone is insufficient to protect against HIV-1 infection and that requires new approaches. AIDSVAX was the first Env-based vaccine tested for efficacy (Ensoli et al., 2021; Rerks-Ngarm et al., 2009). The vaccination with this AIDSVAX did not improve the viral loads and CD4+ T cell counts. These were prophylactic vaccines based on two recombinant Gp120 surface proteins from different HIV-1 strains (Pitisuttithum et al., 2006). However, it failed to induce protective, neutralising humoral responses, most likely due to the high variability of Env in the circulating virus variants (Ensoli et al., 2021). DNA-based vaccines are less effective in protecting against HIV-1 infection because of their poor ability to enter cells (Chhatbar et al., 2011). Loret et al (2016) showed that Tat based vaccine (Tat oyi) in association with cART may provide an efficient means of controlling the HIV-infected cell reservoir (Loret et al., 2016). In the current study, we investigated the role of HIV-1 Anti-Tat antibodies in developing prophylaxis and therapeutic vaccines.

HIV-1 Trans-Activator of Transcription, known as Tat, is a 14-kDa regulatory protein produced very early during the infection. It comprises of 86 to 101 amino acids depending on the subtype (Bagashev and Sawaya, 2013; Campbell and Loret, 2009). It is released into the extracellular space from cells with HIV-1 infected cells into neighbouring uninfected cells even without active HIV-1 replication and viral production due to effective antiretroviral therapy (ART) (Nicoli et al., 2013). The major function of Tat was found to stimulate transcriptional elongation rather than initiation (Rice, 2017; Kao et al., 1987). Tat protein is also essential in controlling latency and viral rebound following the interruption of combined antiretroviral treatment (Jin et al., 2020). HIV-1 Tat has attracted more attention in viral latency by ensuring high levels of viral transcription during the virus life cycle (Das et al., 2011). It could be therapeutically beneficial to prevent or at least reduce the size of the established latent reservoir to a large extent. Evidence indicates that, sufficient Tat quantities, may counteract the establishment of HIV-1 latency by promoting transcriptional initiation or elongation (Donahue et al., 2012; Pearson et al., 2008).

HIV-1 Tat activity also induces the release of pro-inflammatory cytokines and up-regulation of transcription factors involved in T cell activation, contributing to hyperactivation and

dysfunction of T cells (Nicoli et al., 2016). Moreover, HIV-1 Tat interacts with various co-infecting opportunistic pathogens and is directly implicated in the pathogenesis of AIDS-related Kaposi's sarcoma and AIDS-associated neurocognitive (HAND) (Chang et al., 2011; Ensoli et al., 1990). Furthermore, HIV-1 Tat protein is actively released from infected cells and then interacts with the cell surface receptors of other uninfected cells (immune cells) in the brain leading to cellular dysfunction (Chang et al., 2011). By binding to heparan sulphate proteoglycans with its basic region, extracellular Tat accumulates in tissues (Chang et al., 1997) where it exerts effects on both the virus and the immune system (Bellino et al., 2014; Fanales-Belasio et al., 2009; Ensoli et al., 1993), making it an optimal target for an immune intervention based on antibody (Ab) responses (Ensoli et al., 2010; Bachler et al., 2013). Furthermore, extracellular Tat binds HIV-1 Env spikes, forming the virus entry complex that favours infection of dendritic cells and efficient transmission to the dendritic cells, thus evading neutralisation by anti-Env antibodies. However, it can be restored and further increased by anti-Tat antibodies either present in natural infection or induced by vaccination (Monini et al., 2012).

Anti-Tat antibodies are produced by only a small fraction of individuals (Bellino et al., 2014; Demirhan et al., 2000; Krone et al., 1988). When Tat is present, anti-Tat antibodies correlates with the asymptomatic state and lower disease progression (Bellino et al., 2014). In particular, a higher prevalence of anti-Tat antibodies has been shown in asymptomatic non-progressors HIV-1-infected individuals as compared to patients with advanced disease or fast progressors (Rezza et al., 2005; Ensoli et al., 2021). In addition, anti-Tat immunity might counteract Tat-mediated immune dysregulation and hence play a role in controlling HIV-1 infection and comorbidity (Nicoli et al., 2016). Bellino et al. (2014) have shown that IgG and IgM against Tat are associated with slow disease progression in clade B HIV-1 infected individuals. However, anti-Tat IgG alone is not protective in non-clade B infected subjects unless concomitant with IgM, suggesting a protective role of persistent anti-Tat IgM irrespective of the infecting clade (Nicoli et al., 2016). Although Tat activities strictly depend on the viral clade, knowledge about the importance of anti-Tat antibodies in non-clade B HIV-1 infection has not been studied (Nicoli et al., 2016). This study investigated the association of anti-Tat antibodies with disease progression in clade C HIV-1 infected individuals.

The prophylactic vaccine is a preventive HIV-1 vaccine given to people who do not have HIV-1 to prevent HIV-1 infection in the future. The vaccine teaches the person's immune system to recognise and effectively fight HIV-1 in case the virus ever enters the person's body, whilst a

therapeutic HIV-1 vaccine is a vaccine that is designed to improve the body's immune response to HIV-1 in a person who already has HIV-1 (Hargrave et al., 2021). Developing prophylactic and therapeutic vaccines that block or target functionally essential parts of HIV-1 Tat protein may be a good strategy since it plays a critical role in HIV-1 transcription and replication (Jin et al., 2020). Therefore, we hypothesized that Tat-based vaccines might help in blocking the virus replication by infected cells by inhibiting viral transcription. This study investigated the effect of anti-Tat antibodies during early HIV-1 treatment up to the late stage of treatment and its association with the size of the reservoirs, immune activation and how HIV-1 Tat interferes with Env neutralisation ability.

1.2 Rationale/problem statement

HIV-1 vaccine development efforts have not yet proven successful. The extraordinary diversity of HIV-1, the capacity of the virus to evade adaptive immune responses, the inability to induce broadly reactive antibody responses, the early establishment of latent viral reservoirs, and the lack of clear immune correlates of protection represent unprecedented challenges for vaccine development. Anti-Tat antibodies have been found to play a crucial role in controlling HIV-1 infection and co-morbidity in clade B HIV-1 infection. However, knowledge about the importance of anti-Tat antibodies in non-clade B HIV-1 infection has not been studied. A study by Tripiciano, reported that Anti-Tat immunity is significantly associated with higher nadir CD4⁺ T cell numbers, control of low-level viremia and long-lasting CD4⁺ T-cell recovery, but not with decreased immune activation in long-term cART treated individuals. Hence, this study investigated the role of anti-tat antibodies in association with the size of the reservoirs, immune activation and how anti-tat antibodies restore the neutralisation by anti-env antibodies in clade C HIV-1 early treated individuals.

1.2 Hypothesis

The persistence of anti-Tat antibodies in the early treated HIV-1 infected individuals is associated with the reduced size of the viral reservoirs and low immune activation.

1.3 The main of the study

The study aims to investigate the evolution of anti-Tat antibodies and their role in HIV-1 pathogenesis and vaccine design from early treated individuals throughout 12 months of HIV-1 infection.

1.4 Specific objectives

1. To investigate the evolution of HIV-1 anti-Tat antibodies from HIV-1 early treated individuals using ELISA and associate them with the size of the reservoirs at 12 months.
2. To evaluate the role of anti-Tat immunity in residual viremia and immune activation in early-treated individuals.
3. To investigate how the HIV-1 Tat protein impacts the anti-Env antibody neutralisation ability.

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CHAPTER 2- LITERATURE REVIEW

2.1 HIV-1 History and Epidemiology

Human Immunodeficiency Virus has remained a global health issue since it was discovered by French scientists in 1981 (Barré-Sinoussi et al., 1983; Gao et al., 1999). It is thought to have occurred after people ate chimpanzees that were carrying the Simian Immunodeficiency Virus (SIV), meaning it was transmitted by primates to human zoonotic transmission (Gao et al., 1999).

The high prevalence of HIV in South Africa has been attributed to various biological, socio-behavioural, contextual, and structural drivers (Madiba and Ngwenya, 2017; Conroy et al., 2017; Pitpitan et al., 2016; Zungu et al., 2016). The majority of HIV transmission in South Africa is through heterosexual transmission, including through commercial sex. However, HIV prevalence is also increasing among men who have sex with other men (UNAIDS, 2017). Socio-behavioural factors have been associated with HIV vulnerability, age of sexual debut among youth and age-disparate relationships. Age-disparate relationships between young women and older men have been driving the high prevalence of HIV in the country, especially among those aged 15–24 years (Zuma et al., 2022) and a group of black Africans aged 25–49 (Burger et al., 2022). Burger et al., 2022, conducted a study and revealed that the rollout of free ART has reduced annual mortality by 27% and decreased the likelihood of reporting poor health by 36% for black Africans aged 25–49.

The human immunodeficiency virus infects the host's CD4⁺ T cells using CCR5 and CXCR4 receptors. HIV-1 untreated infection causes progressive and critical damage to the immune system, rendering the host susceptible to potentially fatal opportunistic infections and cancers (Fisher et al., 2006). During disease progression to AIDS, CD4⁺ T cell counts fall below 200 cells/mm³ and present a high viral load. The infection may lead to acquired immunodeficiency syndrome (AIDS), a chronic stage of HIV, which is symbolised by opportunistic infections such as cancer, TB, etc (Deeks et al., 2015). The human immunodeficiency virus (HIV) is grouped into the genus *Lentivirus* within the family of *Retroviridae*, subfamily *Orthoretrovirinae* (Transfus Med Hemother, 2016).

Based on genetic characteristics and differences in viral antigens, HIV is classified into types 1 and 2 (HIV-1, HIV-2). HIV-1 is the most common and responsible cause of AIDS throughout most of the world. HIV-2 is found primarily in Western Africa and is much less common

(Waymack and Sundareshan, 2023). HIV-1 is subdivided into the groups M, N (Simon et al., 1998), O, and P (Plantier et al., 2009), with group M being the most widely spread. HIV-1 group M viruses are subdivided into nine subtypes: A to D, F to H, J and K. In evolutionary terms, groups A and D, are the oldest viruses (Ward et al., 2013).

Combination antiretroviral therapy (cART) has controlled the HIV-1 epidemic to become a manageable chronic illness, allowing people living with HIV to live long and healthy lives (Margolis et al., 2020). However, ART can prevent the virus by inhibiting the replication of the virus in the plasma but not in the HIV-1 reservoirs (Margolis et al., 2020). The development of viral latency, drug resistance, and toxicity hinders the success story of antiretroviral therapy. In addition, there is no approved effective preventative HIV-1 vaccine despite the numerous HIV-1 vaccine research conducted. Therefore, the continuous search for an effective therapeutic vaccine for HIV-1 is in demand.

2.2 HIV-1 genome

HIV-1 genome contains two identical single-stranded ribonucleic acids (RNA), which hold HIV's genetic material (Moore and Hu, 2009). The HIV-1's RNA comprises nine genes that encode fifteen viral proteins (Frankel and Young, 1998). Figure 1 shows the genome of the human immunodeficiency virus type 1 (HIV-1). There are three significant genes, *gag*, *pol* and *env*, which code for structural proteins. HIV-1 *gag* plays crucial functions in HIV-1 replication, notably during the assembly, budding (Sundquist and Krausslich, 2012), and release of infectious particles and codes for structural proteins: matrix (MA), nucleocapsid (NC), p6 and capsid (CA). Each of these domains performs specific functions in the assembly process either alone or in collaboration with the other domains. The MA domain is implicated in the targeting and binding of Gag to the plasma membrane (PM). It also binds RNAs and mediates the incorporation of Env in virions (Klingler et al., 2020). The capsid (CA) domain mediates Gag multimerisation. The NC domain is essential for stabilisation of selection and packaging of genomic RNA (gRNA) inside virions and the stabilising Gag oligomers (Klingler et al., 2020). Finally, p6 is vital for recruiting the endosomal sorting complex required for transport (ESCRT) machinery, a mandatory step for effective budding from the PM, while SP1 seems to play a role in immature particle assembly (Klingler et al., 2020).

Polymerase (*pol*) gene codes for reverse transcriptase, protease and integrase- which catalyse the steps of retroviral replication (Li et al., 2015). These enzymes are critical for synthesising and integrating viral DNA into the host genome and generating capsid proteins. HIV-1 uses

reverse transcriptase to convert its RNA into viral DNA, a process discovered by Howard Temin and David Baltimore known as reverse transcription (Baltimore, 1970; Mizutani et al., 1970). Protease cuts up large precursor proteins into smaller proteins (Li et al., 2015). These smaller proteins combine with HIV's genetic material to form new HIV-1. HIV-1 uses integrase to insert (integrate) its viral DNA into the DNA of the host CD4 cell (Li et al., 2015).

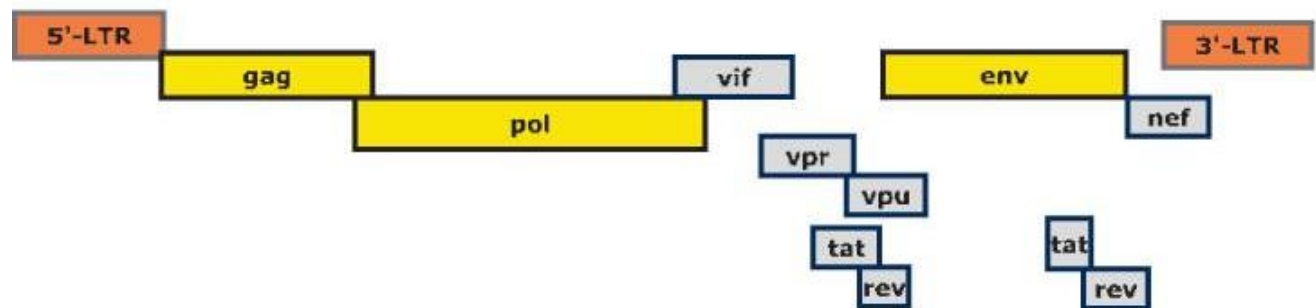


Figure 1: HIV Genome showing nine HIV-1 genes (Transfus Med Hemother, 2016). There are two long terminal repeats (LTRs) of about 600nt long at the 5' and 3' ends. Fully spliced mRNAs encode the accessory proteins Rev, Tat, and Nef. Incompletely spliced mRNAs encode env, which is cleaved into the envelope proteins and accessory proteins vif, vpu, and vpr. Unspliced full-length mRNA serves as genomic RNA packaged into virions and as a template for translating the gag and gag-(pro)pol polyproteins (ribosomal frameshift).

Env gene codes for gp120 and gp41, which interact with the CD4+ T cell receptors CCR5 during infection (Ahmad et al., 1993). The HIV-1 regulatory proteins are Tat and Rev, and essential accessory proteins are Nef, Vpr, Vif, and Vpu. Accessory proteins are dispensable for virus replication *in vitro* but play vital roles in efficient viral spread, maintenance, and pathogenicity *in vivo* (Malim and Emerman, 2008; Kirchhoff, 2010). HIV-1 Tat is a protein that plays a pivotal role in HIV-1 replication because it enhances transcription by binding to the TAR hairpin at the five' end of newly formed RNA transcripts (Das et al., 2011). Rev is responsible for the splicing length of the newly formed HIV RNAs. This enables the production of regulatory proteins early during replication, followed by translation of structural proteins in the late stage of the replication cycle (Transfus Med Hemother, 2016). Nef enhances viral replication and promotes immune escape of HIV-1 infected cells but lacks intrinsic enzymatic activity (Staudt et al., 2020). It also downregulates CD4 receptor on the cell surface of infected T cells, downregulates HLA (Geyer et al., 2001) and downregulates SERINC 5, thus increasing the ability of the virus to replicate and infect other cells (Shi et al., 2018). Vpr facilitates HIV-1 infection of nondividing cells by contributing to the nuclear transport of the preintegration complex (Hrimech et al., 1999). Vpu promotes virion release by counteracting

host restriction factors, downregulating CD4⁺ T cells during the late stages of HIV-1 infection, and inhibiting NF- κ B activation (RüCker et al., 2004).

2.3 HIV-1 replication cycle

The first phase of the HIV-1 viral replication cycle begins with the adherence of the virus to the host CD4⁺ T cell. The HIV-1 envelope fused with the host's CD4⁺ T cell membranes with subsequent delivery of the viral core into the cytoplasm (Wilén et al., 2012). Figure 2 shows the seven steps of the HIV-1 replication cycle, which are: binding, fusion, reverse transcription, integration, replication, assembly, and budding.

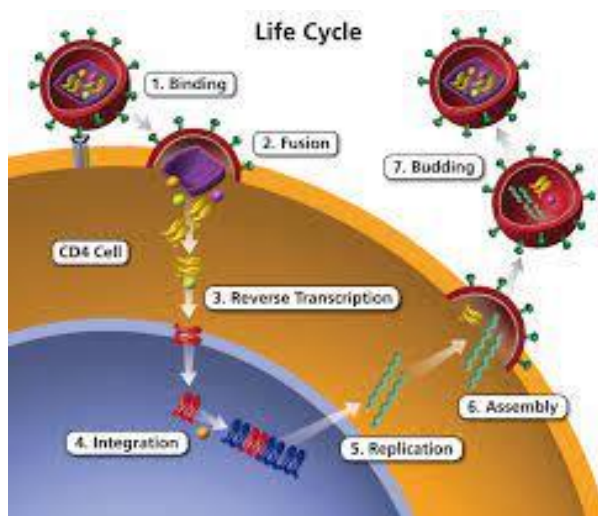


Figure 2: HIV-1 replication cycle (Clinic info HIV- HIV.gov): 1.HIV binds to the receptors on the surface of a CD4⁺ T cell. 2, HIV envelope and CD4⁺ T cell membrane fuse, allowing HIV to enter the CD4⁺ T cell. 3, In the CD4⁺ T cell, HIV releases and uses reverse transcriptase to convert HIV RNA into HIV DNA, thus allowing HIV to enter the CD4⁺ T cell nucleus and combine with cell DNA. 4, HIV releases integrase and uses integrase to insert its viral DNA into the DNA of the CD4⁺ T cell. 5, HIV begins to use the machinery of the CD4⁺ T cell to make long chains of HIV proteins (building blocks for more HIV). 6, New HIV proteins and HIV RNA move to the cell's surface and assemble into immature HIV. 7, Newly formed immature HIV pushes itself out of the host CD4⁺ T cell. The new HIV releases protease to break up the long protein chains, creating the mature virus.

Initially, the HIV protein envelope (Env) binds to the primary cellular receptor CD4⁺ T cell and then to a cellular coreceptor (CCR5 or CXCR4). After HIV-1 attaches itself to a host CD4⁺ T cell, the HIV-1 viral envelope fuses with the CD4⁺ T cell membrane (Wilén et al., 2012). Once inside the CD4⁺ T cell, the virus releases HIV-1 RNA and HIV-1 enzymes, such as reverse transcriptase and integrase (Hamid et al., 2017). Reverse transcription occurs in the cytoplasm, where HIV-1 RNA is converted to DNA. This process is facilitated by reverse transcriptase (Hu and Hughes, 2012). The conversion of HIV-1 RNA to HIV-1 DNA allows HIV-1 to enter the CD4⁺ T cell nucleus and combine with the cell's genetic material. The HIV-

1 DNA is transported into the cell nucleus where it integrates into the host's genome. Integrase facilitates the integration of viral DNA into the host genome (Craigie and Bushman, 2012). Once HIV-1 is integrated into the host CD4+ T cell DNA, the virus begins to use the machinery of the CD4+ T cell to replicate long chains of HIV-1 proteins- a process called replication (Louten, 2016). The protein chains are the building blocks for more HIV-1. Then new HIV-1 RNA and HIV-1 proteins made by the host CD4+ T cell move to the cell's surface and assemble into immature (non-infectious) HIV-1. During budding, immature HIV-1 pushes itself out of the host CD4+ T cell. Once outside the CD4+ T cell, the new HIV-1 releases protease, an HIV-1 enzyme. Protease breaks up the long protein chains in the immature virus, creating the mature (infectious) virus (Louten, 2016).

2.4 HIV-1 antiretroviral therapy classes

Antiviral drug therapy has reduced the HIV-1 pandemic into a clinically managed chronic disease. Antiviral drugs can prevent the virus from replicating, reduce viral loads, help to restore CD4+ T counts and immune function, reduce complications from HIV-1 and improve survival, and reduce the transmission of HIV-1 to the next person (Moore and Chaisson, 1999). The discovery of HIV-1 as the causative agent of AIDS, together with an ever-increasing understanding of the virus replication cycle have been instrumental in the drug discovery efforts focused on targeted inhibition with specific pharmacological agents. To date, an arsenal of 24 Food and Drug Administration (FDA)-approved drugs are available for the treatment of HIV-1 infections (Arts and Hazuda, 2012). These drugs are distributed into six distinct classes based on their molecular mechanism, namely: nucleoside-analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors (PIs), fusion inhibitors, and coreceptor antagonists (Arts and Hazuda, 2012). Different classes of HIV-1 drugs improved by 24 Food and Drug Administration are shown in Table 1 below.

Table 1: Different classes of HIV drugs improved by 24 Food and Drug Administration (Kemnic and Gulick, 2023)

Class of ARVs	Drugs and Year of Approval	Function
Nucleoside reverse transcriptase inhibitors (NRTIs)	Azidothymidine (1987) Zalcitabine (1992) Stavudine (1994) Didanosine (1991)	Block reverse transcriptase and reverse transcription to prevent HIV from replicating

	<p>Abacavir (1998)</p> <p>Tenofovir disoproxil fumarate (2001)</p> <p>Tenofovir alafenamide (2016)</p> <p>Lamivudine (1995)</p> <p>Emtricitabine (2003)</p>	
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	<p>Nevirapine (1996)</p> <p>Etravirine (2008)</p> <p>Delavirdine (1997)</p> <p>Rilpivirine (2011)</p> <p>Efavirenz (1998)</p>	<p>NNRTIs non-competitively bind to the reverse transcriptase enzyme, altering its conformation to prevent DNA binding.</p>
Integrase single transfer Inhibitors (INSTIs)	<p>Dolutegravir (2013)</p> <p>Bictegravir (2018)</p> <p>Elvitegravir (2014)</p> <p>Raltegravir (2007)</p> <p>Cabotegravir (2021)</p>	<p>Prevent HIV from being able to make integrase.</p>
Protease Inhibitors (PIs)	<p>Amprenavir (1999)</p> <p>Atazanavir (2003)</p> <p>Tipranavir (2005)</p> <p>Darunavir (2006)</p> <p>Fosamprenavir (2003)</p> <p>Indinavir (1996)</p> <p>Lopinavir (2000)</p> <p>Nelfinavir (1997)</p> <p>Ritonavir (1996)</p> <p>Saquinavir Tipranavir (1995)</p>	<p>Block the enzyme “protease,” which HIV cells need to develop and mature. Blocking protease prevents the virus from making copies of itself.</p>
Fusion Inhibitors	<p>Enfuvirtide (2003)</p>	<p>Blocks the HIV envelope from merging with the host CD4 cell membrane (fusion). This prevents HIV from entering the CD4 cell.</p>
Chemokine receptor antagonist (CCR5)	<p>Maraviroc (2007)</p>	<p>Bind to the CCR5 chemokine coreceptor on host cells, inducing a conformational change that impedes CCR5 interaction with</p>

		HIV gp120, thereby preventing HIV entry into host cells.
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In 1987, the nucleoside reverse transcriptase inhibitor Azidothymidine was the first FDA-approved drug to inhibit HIV-1 (Holec et al., 2018; Maga et al., 2010; Fischl et al., 1987). However, there are limitations to single-drug treatment regimens. These include the inability to adjust dosages of components of the regimen for drug-drug or drug-food interactions. In particular, patients with preexisting renal impairment (estimated CrCl < 50 mL/min) have limited options and safety data for single-drug treatment regimens. Also, single-drug treatment regimens do not exist currently for all NRTI-anchor drug pairings (Cutrell and Bedimo, 2016). HIV-1 mutates rapidly and prone to drug escape (Johnson et al., 2022). Drug resistance mutations that confer resistance to an antiretroviral drug can evolve quickly. In some people taking AZT alone, drug resistance develops in a matter of days (Pennings, 2013). By 1996, research showed the advantages of combining medicines to treat HIV (Kemnic and Gulick, 2023; Gulick et al., 1997). Combination antiretroviral therapy (cART) inhibits HIV-1 replication in plasma. However, it cannot eradicate the virus from viral reservoirs (Arts and Hazuda, 2012). In addition, previous studies showed that HIV-1 treatment reduces the amount of replicating HIV-1 (viral loads) in the blood and increases CD4+ T cell count, making viral loads undetectable (Ostrowski et al., 2005; Ramratnam et al., 2000; Barry et al., 1998). Moreover, Cohen (2016) observed zero linked sexual transmission among HIV-1 discordant couples with viral suppression (Cohen et al., 2016). The complications associated with HIV-1 drugs are a potential cause of toxicities and the development of HIV-1 drug resistance. Some strategies have been developed to eradicate the virus from the viral reservoirs, which are “shock and kill”, “block and lock” and gene therapy (CRISP/Cas technology). However, none of these strategies has been successful. Therefore, there is an urgent need to develop an HIV cure that will eliminate HIV and effective therapeutic/preventative vaccines to prevent further infection.

2.5 Strategies to eradicate the HIV-1 reservoirs

Among various discussed remedies to counter the disease, several approaches are being considered to be supplemented with existing cART.

2.5.1 “Shock and kill”

“Shock and kill” strategy aims to reactivate the latent reservoir by latency-reversing agents (LRAs) and allow the elimination of these cells by immune system-mediated clearance or HIV-1 related cytopathic effects (Deeks, 2012). Figure 3 shows the shock and kill pathway, in this strategy integrated HIV-1 DNA remain latently in the CD4⁺ T cell. Latency reversing agent (LRA) activates these cells and become exposed in the peripheral blood where ART can inhibit it. This strategy is performed in individuals on ART to prevent new rounds of infection.

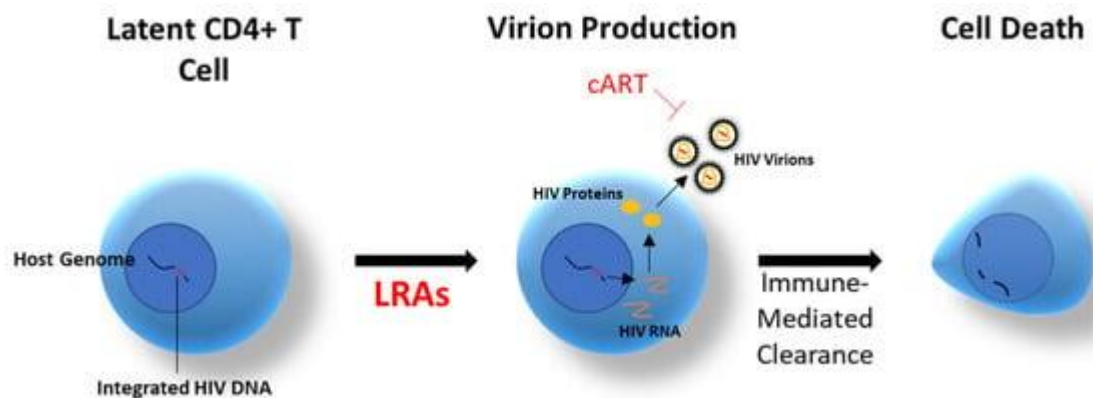


Figure 3: The “Shock and Kill” Strategy: Latency Reversing Agents (LRAs) increase HIV transcription, protein expression and virion production. The cell may die through virus-mediated cytopathic events or immune-mediated clearance (Weichseldorfer et al., 2021).

One class of LRAs are epigenetic modifiers, such as histone deacetylase inhibitors (HDACi), histone methyltransferase inhibitors (HMTi), and DNA methyltransferase inhibitors (DNMTi), which all act on reversing the repressive epigenetic marks present in the HIV-1 promoter during latency (Spina et al., 2013). These LRAs result in global activation, relaxing epigenetic marks in not just the HIV-1 promoter but any promoter that is epigenetically silenced by these mechanisms. Another challenge of LRAs is the variability of effect depending on the specific cell model (Spina et al., 2013). Successful reactivation by most LRAs *in vitro* has failed to induce sufficient reactivation to make a detectable impact on the HIV reservoir *in vivo* or *ex vivo* in patient latently infected cells (Spina et al., 2013).

Additional improvements in the ability to kill reactivated cells are also likely to be needed, such as a broadly neutralising antibody (bNAb) PGT121 and a Toll-like receptor 7 (TLR7) agonist (Borducchi et al., 2018). Moreover, the “shock-and-kill” approach is unsuitable for all cell types harbouring latent viruses, such as microglial cells in the brain, reviewed by Wallet

et al. (2019). This is due to the reactivation of microglial cell reservoirs resulting in neuroinflammation, a key component of HIV-1-associated neurocognitive disorders (HAND) (Wallet et al., 2019). As demonstrated by several studies, it is unlikely that targeting a single mechanism of HIV-1 latency will be sufficient to reactivate the majority of the virus reservoir (Ait-Ammar et al., 2019; Das et al., 2018; Rochat et al., 2017; Jiang et al., 2015). Instead, a combination of LRAs targeting multiple mechanisms of HIV-1 latency is likely to be required for an effective sterilising cure without ART. Currently, latency-reversing agents are still under investigation and have not been approved by the Food and Drug Administration (FDA).

2.5.2 “Block and lock”

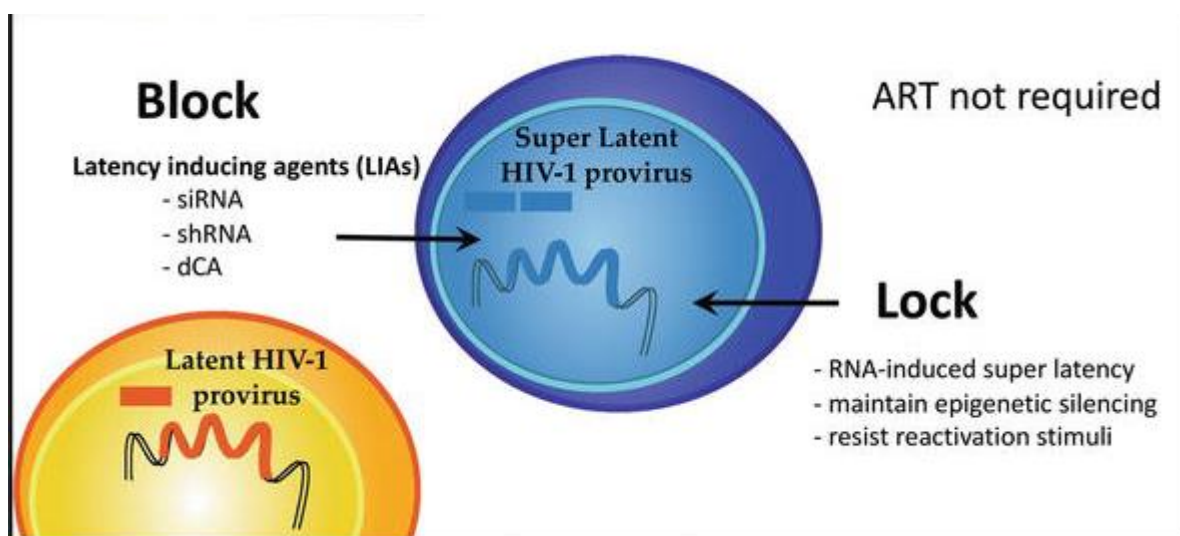


Figure 4: The “Block and lock” strategy: Latency-inducing agents (LIAs) mediate the process by “blocking” virus replication and “locking” the virus genome in an induced “super-latency” that is refractory to reactivation (Ahlenstiel et al., 2020).

The “Block and lock” functional cure strategy permanently silence the latent reservoir. Figure 4 shows the “block and lock” strategy, which uses the latency promoting agents (LPAs) to “block” virus transcription and “lock” the virus promoter in a latent state via repressive epigenetic modifications (Ahlenstiel et al., 2020). Permanent control of the HIV-1 promoter means ART is no longer required. The “block and lock” approach mimics natural virus latency by inducing a state of latency, described recently by the terms “super latency” or “deep latency” (Ahlenstiel et al., 2020). A precedent for forcing HIV-1 into a permanently silenced state via the block and lock epigenetic silencing approach has been set by the many ancient, epigenetically silenced human endogenous retroviruses (HERVs) that comprise ~8% of the human genome (Lander et al., 2001). This supports the feasibility and potential longevity of the block and lock approach. When mediated by RNA therapeutics, a significant benefit of this

curing approach is that precise sequence targeting is required. However, it is challenging to silence all provirus permanently with a block-and-lock approach (Vansant et al., 2020).

2.5.3 Gene therapy

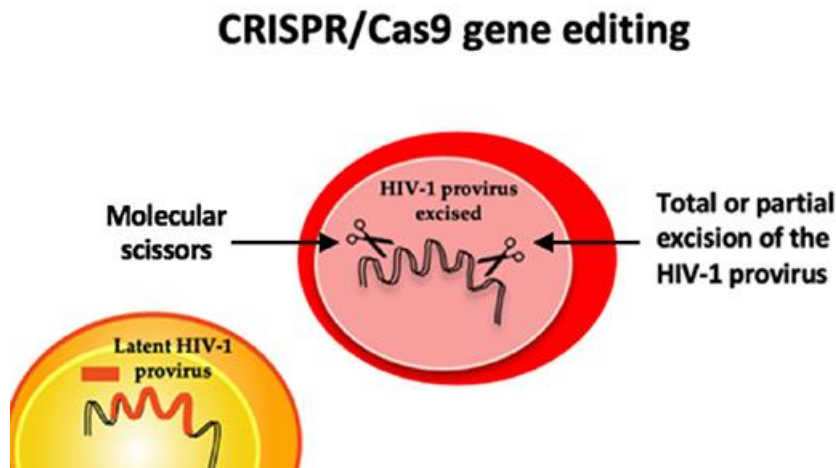


Figure 5: Gene therapy (Oggenovska et.al., 2018): CRISPR gene therapy uses modified cell therapies to target the latent reservoir.

Gene therapy cure approaches also aim to eradicate the integrated latent reservoir and use several nuclease-mediated gene editing tools, i.e., molecular scissors, that cut genomic DNA in a particular manner. Some examples include clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9) technologies and the transcription-activator-like effector nucleases (TALEN) (Ahlenstiel et al., 2020; Shi et al., 2017), gene editing to excise the HIV-1 genome from the host genome. Figure 5 shows the gene therapy strategy that is used to eradicate HIV-1 latent reservoirs. A benefit of these gene editing sequence approaches is the high specificity required to match the target sequence. However, this also means that due to the extreme sequence diversity in the HIV-1 genome, a combination of multiple sequences will be required to ensure sequence diversity and the potential for future virus mutations in the target site is addressed. Additionally, off-target effects and virus escape have been reported (Wang et al., 2016). Delivery of gene editing therapies to the target site is also a significant challenge. Multiplexing of several RNA therapeutics targeting different sites in the virus genome will be necessary to address the global sequence diversity of HIV-1 (Pang et al., 2018; Ahlenstiel et al., 2015).

2.5.5 Stem cell therapies

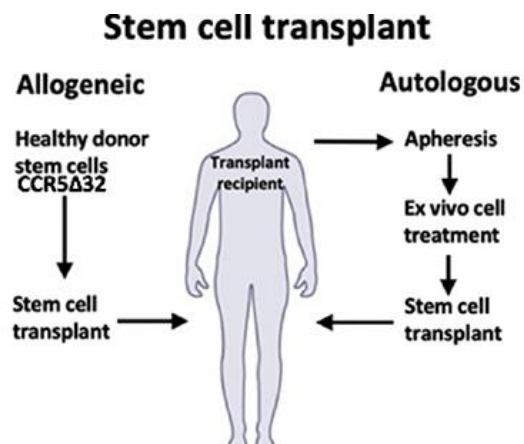


Figure 6: Stem cell therapy (Ahlenstiel et al., 2020): Transplant recipients receive CCR5 Δ 32 from the health donor.

A stem cell transplant is a medical procedure by which healthy stem cells are transplanted into your bone marrow or blood. There are so far two examples of people living with HIV-1 being successfully cured of HIV, with both cases utilising cell therapy with stem cell transplantation, i.e., the Berlin and London patients (Gupta et al., 2019; Hütter et al., 2009). Figure 6 illustrates a stem cell therapy strategy to cure HIV-1 patient. This approach employed naturally HIV-resistant donor CCR5 Δ 32 stem cells for transplantation in patients treated for associated malignancies, i.e., undergoing myeloablative/chemotherapy treatments (Gupta et al., 2019; Hütter et al., 2009). This approach is not currently scalable, feasible, or desirable for the general population of PLWH who do not require such heroic treatment for malignant disease. This is due to the high risk of undertaking an allogeneic stem cell transplantation.

The main challenge in all strategies lies in reaching and affecting each cell containing replication-competent provirus. If not, each infected cell is targeted, HIV replication will eventually rebound (Vansant et al., 2020). Moreover, efforts to eradicate the reservoir via recent strategies have proven difficult and unsuccessful. Therefore, more research needs to be done recently on an alternative functional cure strategy, including the therapeutic HIV-1 vaccine.

2.6 HIV-1 vaccines

An effective vaccine could prevent people from HIV-1 infection, further reducing the number of people who can pass the virus on to others (Verma et al., 2016). Vaccines are biological agents that elicit an immune response to a specific antigen derived from an infectious disease-

causing pathogen (Czochor and Turchick, 2014). They contain weakened or inactive parts of a particular organism (antigen) that trigger an immune response within the body (Czochor and Turchick, 2014). Vaccines historically have been the most effective means to prevent and even eradicate infectious diseases by helping your immune system fight infections faster and more effectively (Pollard and Bijker, 2021). They are safe and cost-effective in preventing illness, disability, and death. A therapeutic vaccine varies from a prophylactic vaccination in that preventive vaccines are given to individuals as a preventative strategy to avoid infection or disease. In contrast, therapeutic vaccines are given to persons after the disease or infection. A prophylactic vaccine is an option for combating HIV-1 since most HIV-1 positive individuals are on treatment due to the “Test and treat” policy (Sela and Hilleman, 2004). The prophylactic vaccine is a preventive HIV-1 vaccine given to people who do not have HIV-1 to prevent HIV-1 infection in the future. The vaccine teaches the person's immune system to recognise, and effectively fight HIV-1 in case the virus ever enters the person's body, whilst a therapeutic HIV-1 vaccine is designed to improve the body's immune response to HIV-1 in a person who already has HIV-1 infection. It also creates memories for the pathogen; if the person gets infected, the immune system fights the pathogen. (Hargrave et al., 2021).

Antibodies are proteins immune cells (B lymphocytes) make to block viruses and other infectious agents. Antibodies interrupt the transmission of viruses by binding to surface proteins needed for cell entry (Nicholson, 2016). The different strategies that viruses use to enter cells all depend on specific molecular recognition. Interfering with this specific binding process will stop the virus (Nicholson, 2016). In the case of HIV-1, people who are infected typically produce antibodies to the virus. However, because the virus mutates and replicates rapidly, antibodies are largely ineffective at controlling the virus (Klein and Bjorkman, 2010). After years of infection, though, some people produce highly potent antibodies called broadly neutralising antibodies (bnAbs) that, in laboratory tests, can neutralise a wide variety of HIV-1 strains (Landais and Moore, 2018). Identifying of such antibodies has transformed the field of HIV-1 prevention research for two reasons. Firstly, it provides information to guide the design of vaccines that could elicit bnAbs for protection. Secondly, it has opened the door to a new prevention modality: the administration of HIV bnAbs to prevent infection (Morris and Mkhize, 2017). Furthermore, bNAbs have been shown to have modest antiviral effects in HIV-1 infected humans, both in reducing viremia and delaying viral rebound after interruption of antiretroviral treatment (Morris and Mkhize, 2017).

The administration of antibodies to prevent infection is known as passive immunisation, in contrast to active immunisation, which occurs due to vaccination (Marcotte and Hammarström, 2015). The development of a vaccine or the use of passive immunisation to prevent HIV-1 infection remains our best hope for an AIDS-free future. In active immunisation, vaccination stimulates antibodies that correlate with a reduced risk of HIV-1 infection. This is being tested in HVTN 702. In passive immunisation, pre-formed neutralising antibody VRCO1 is infused to protect against HIV-1 infection (Morris and Mkhize, 2017). This is being tested in HVTN 703.

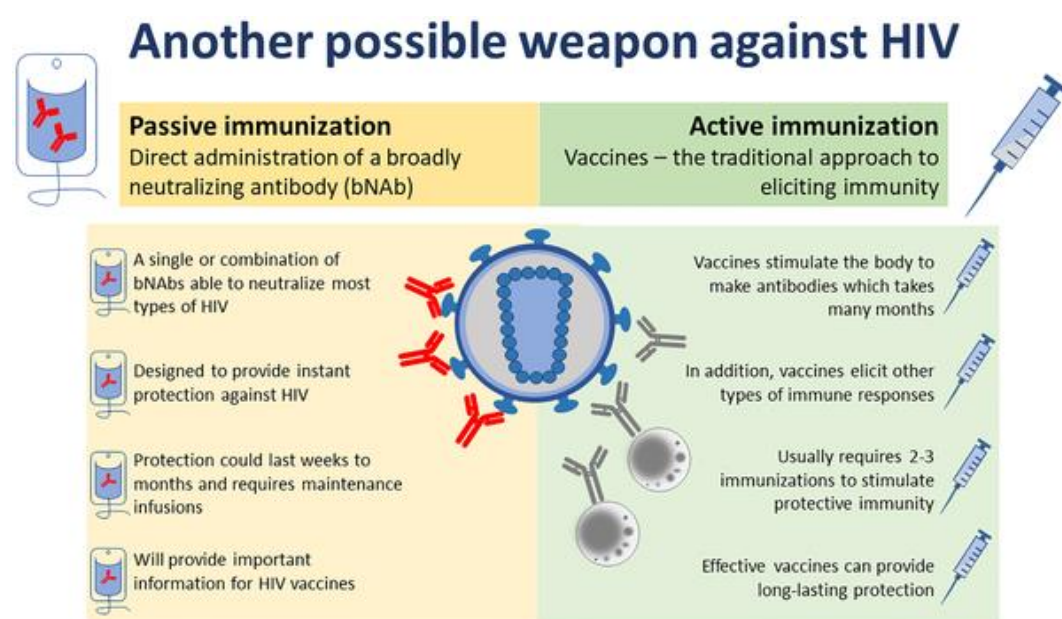


Figure 7: The principles of using a monoclonal antibody as passive immunisation to prevent HIV infection, as compared with the more traditional vaccine approach of active immunisation (Morris and Mkhize, 2017).

Scientists have been trying to develop vaccines for HIV-1 but the way forward toward an effective vaccine has been prolonged. The greatest challenge in developing an effective HIV vaccine has been the high rate of mutation and recombination during viral replication (Ng'uni et al., 2020). Table 2 summarises unsuccessful HIV-1 vaccines since early year 2000 (Ng'uni et al., 2020). The rapid replication rate of HIV-1 replicates approximately 10^{10} copies per day resulted in the unsuccessful HIV-1 vaccine development. At such a high replication rate, the virus changes its composition, leading to mutation, and the changes are irreversible (Williams and Loeb, 1992). Changes in the genetic sequence of the viral protein can block the effectiveness of antiviral drugs to fight HIV-1. Vaccines function by training the body's

immune system to find and fight pathogens if they enter the body and prevent sickness by introducing antigens into the body, which imitate an infection and prime the immune system to respond. Another concern is that high mutation rate leads to the development of multiple strains and threatens the development of drug resistance (Desai et al., 2012). Different vaccines must be developed to target, destroy, and work effectively against all subtypes. A therapeutic vaccine would help to prevent and control HIV-1 by priming the immune system to recognise and kill HIV-1 infected cells. Another concern is that HIV-1 has many subtypes found in many parts of the globe, circulating recombinant forms, and continuous viral evolution within populations and individual hosts. It has been shown that amino acid variations within subtypes can be as high as 30%, with those between subtypes reaching as high as 42%. These amino acid variations are based on the subtypes and region of the genome being examined (Korber et al., 2001; Hemelaar et al., 2011).

Table 2: HIV vaccine trials (Ng'uni et al., 2020).

Vaccine trial	Year	Site	Target group	Vaccine	Immune response	Result
VaxSyn	1987	Canada (Clade B)	72 adults	Recombinant envelope glycoprotein subunit (rgp160) of HIV	Neutralising antibodies were detected	No vaccine efficacy
HIVAC-1e	1988	USA (Clade B)	35 male adults	Recombinant vaccinia virus designed to express HIV gp160	The vaccine was unable to confer protection against HIV	No vaccine efficacy
Vax004	1998-2002	North America (Clade B)	5,417 MSM and 300 women	AIDSVAX B/B gp120 with alum	The vaccine was unable to confer protection against HIV	No vaccine efficacy
Vax003	1999-2003	Thailand (Clade B/E)	2,545 men and women IDUs	AIDSVAX B/E gp120 with alum	The vaccine was unable to confer protection against HIV	No vaccine efficacy
HVTNN 505	2009-2013	United States (Clade B)	2,504 men or transgender women who have sex with men	Three vaccinations with DNA encoding HIV	Vaccine was unable to prevent infection or	No vaccine efficacy

				clade B <i>gag</i> , <i>pol</i> and <i>nef</i> as well as <i>env</i> from HIV clades A, B and C, followed by Ad5 vector-based vaccine encoding clade B <i>gag</i> and <i>pol</i> as well as <i>env</i> from clades A, B and C	decrease viral load in vaccinated volunteers	
STEP/HVTN 502 trial	2004-2007	North America, the Caribbean, South America, and Australia (Clade B) South Africa (Clade C)	3000 MSM and heterosexual men and women	MRKAd5 HIV-1 <i>gag/pol/nef</i> trivalent vaccine	The vaccine was unable to confer protection against HIV	No vaccine efficacy
Phambili/HVTN 503 trial	2003-2007	South Africa (Clade C)	801 adults	rAd5 (<i>gag/pol/nef</i>)	The vaccine was unable to confer protection against HIV	No vaccine efficacy
RV144	2003-2009	Thailand (Clade B)	16,402 community-risk men and women	ALVAC-HIV (Vcp1521) and AIDSVAX B/E vaccines	IgG antibody avidity for <i>env</i> in vaccine recipients with low IgA	31.2% vaccine efficacy at 42 months
HVTN 305	2012-2017	Thailand (Clade B/E)	162 women and men	ALVAC-HIV and AIDSVAX B/E		No vaccine efficacy
HVTN 306	2013-2020	Thailand (Clade B/E)	360 men and women aged 20-40 years	ALVAC-HIV and AIDSVAX B/E	The vaccine was unable to confer protection against HIV	No vaccine efficacy
HVTN 097	2012-2013	South Africa (Clade B/E)	100 black Africans (men and women) aged 18-40 years	ALVAC-HIV (Vcp1521) and AIDSVAX B/E	Induction of CD4+ T cells directed to HIV-1 Env	No vaccine efficacy
HVTN 100	2015-2018	South Africa (Clade C)	252 men and women	ALVAC-HIV (Vcp2438) and	CD4+ T cell responses and gp120 binding	No vaccine efficacy

				bivalent subtype C gp120/MF59	antibody responses	
HVTN 702	2016-2020	South Africa (702Clade C)	5400 men and women	ALVAC-HIV (Vcp2438) and bivalent subtype C gp120/MF59	Vaccine was unable to confer protection against HIV	No vaccine efficacy

DNA-based vaccines are less effective in protecting against HIV-1 infection because of their poor ability to enter cells (Robinson, 2002). HIV-1 prophylactic vaccines based on the HIV-1 Env antigen have provided minimal protection, showing that Env alone is insufficient to protect against HIV-1 infection and that requires new approaches (Monini et al., 2012; Haynes et al., 2012). The first vaccine candidates were focused on the glycoproteins of the virus envelope, which mediate the attachment and entry of HIV-1 into the cell (Esparza, 2013). AIDSVAX, a bivalent subunit vaccine composed of recombinantly expressed envelope proteins (gp120) was the first Env-based vaccine to be tested in efficacy (Ensoli et al., 2021; Pitisuttithum et al., 2006; Flynn et al., 2005). However, it failed to induce protective, neutralising humoral responses, most likely due to the high variability of Env in the circulating virus variants (Yates et al., 2018; Araújo and Almeida, 2013). The phase IIb HVTN 502 (STEP trial) was stopped due to evidence of enhanced risk of acquisition of infection, especially in those uncircumcised and with pre-existing antibodies to the vector. Because of this serious safety concern, the companion Phambili trial (HVTN 503) conducted in South Africa was also stopped (Buchbinder et al., 2008; Johnson et al., 2009). Bellino et al. (2014) reported that anti-Tat antibodies in asymptomatic and treatment-naïve HIV-infected subjects is associated with containment of CD4+ T-cell loss and viral load and with a delay of disease progression. In fact, no subjects with high anti-Tat Ab titers initiated antiretroviral therapy during the three years of follow-up. In contrast, no significant effects were seen for anti-Env and anti-Gag Abs. The increase of anti-Env Ab titers was associated with a reduced risk of starting therapy only in the presence of anti-Tat Abs, suggesting an effect of combined anti-Tat and anti-Env Abs on the Tat/Env virus entry complex and on virus neutralization (Bellino et al., 2014). In addition, Loret et al (2016), the Tat Oyi vaccine preparation was administered with informed consent to 48 long-term HIV-1 infected volunteers whose viral loads had been suppressed by antiretroviral therapy (cART). These volunteers were randomised in a double-blind method into four groups (n = 12) injected intradermally with 0, 11, 33, or 99 µg of synthetic Tat Oyi proteins in buffer without adjuvant at times designated by month 0 (M0), M1 and M2, respectively. The volunteers then underwent a structured treatment interruption between M5

and M7 (Loret et al., 2016). The outcomes of this phase I/IIa clinical trial were safety and lowering the extent of HIV-1 RNA rebound after cART interruption. The 33µg dose was most effective at reducing the importance of HIV-1 RNA and DNA rebound (Mann and Whitney test, $p = 0.07$ and $p = 0.001$). Immune responses against Tat were increased at Month 5, which correlated with a low HIV RNA rebound at M6 ($p = 0.01$). This study suggests *in vivo* that extracellular Tat activates and protects HIV-1 infected cells. The Tat Oyi vaccine in association with cART, may provide an efficient means of controlling the HIV-infected cell reservoir (Loret et al., 2016). Consistent with other earlier research, a study conducted by Cafaro et al. (2019) provided experimental and epidemiological evidence that antibodies against HIV-1 Tat, which are not commonly found in spontaneous infections, protect against the disease's progression and that cART can be intensified by a vaccine that targets Tat. Their findings showed that, Tat vaccination of subjects on suppressive cART in Italy and South Africa promoted immune restoration, including CD4⁺ T-cell increase in low immunological responders. Tat vaccine promoted a reduction of proviral DNA even after six years of cART, when both CD4⁺ T-cell gain and DNA decay have reached a plateau (Cafaro et al., 2019). Of note, DNA decay was predicted by the neutralization of Tat-mediated entry of Env into dendritic cells by anti-Tat antibodies, which were cross-clade binding and neutralizing. Anti-Tat cellular immunity also contributed to the DNA decay. The Tat therapeutic vaccination has been reviewed by Ensoli et al. (2021) and their findings suggest that it may enhance immune system homeostasis and functionality. It further reduces the size of the reservoirs in virologically suppressed vaccinees and assists in the establishment of key determinants for combination ART intensification and functional cure. Based on these data, they proposed the Tat therapeutic vaccine as a pathogenesis-driven intervention that effectively intensifies cART and it may lead to a functional cure, providing new perspectives and opportunities also for prevention and virus eradication (Cafaro et al., 2019).

Prophylactic vaccines remain the best approach for controlling or preventing HIV-1 transmission. Developing preventive and therapeutic vaccines that would block or target functionally important parts of HIV-1 Tat protein may be a good strategy since Tat protein plays a critical role in HIV-1 transcription and replication. Tat-based vaccines might help block virus production by infected cells by inhibiting HIV-1 replication and viral transcription. In addition, promising efficacy data were obtained in non-human primates, where prevention or containment of infection at the portal of entry or reduction of peak viral load and CD4⁺ T cell decay were observed in monkeys immunized parenterally with Tat combined with Env and

challenged with SHIV89.6P or SHIVSF162 (Ferrantelli et al., 2011; Demberg et al., 2007; Ensoli et al., 2005).

2.6.1 Tat-based vaccine and clinical trial.

Despite the fact that vaccines were typically safe and immunogenic, most trials yielded little to no evidence of their effectiveness. Novel strategies are needed to intensify combination antiretroviral therapy (cART), as it only partially restores immune functions and does not reduce the latent HIV reservoir. A notable exception is the therapeutic Tat vaccine approach showing promising results of cART intensification, promoting immune restoration, CD4 T-cell increase and reduce the virus reservoirs well beyond those afforded by cART alone (Cafaro and Ensoli, 2022). The authors suggest the Tat vaccine as a promising vaccine candidate for cART intensification toward HIV reservoirs depletion, functional cure, and eradication strategies. Suggesting that targeting a key protein in the virus life cycle is pivotal to success (Cafaro and Ensoli, 2022; Moretti et al., 2020). Previous studies in natural HIV-1 infection, indicated that the presence of a Tat-specific immune response correlates with a lower incidence and reduced risk of progression to AIDS as compared to anti-Tat negative individuals suggesting that an immune response to Tat may exert a protective role and control the progression to AIDS *in vivo* (Cafaro et al., 2019; Bellino et al., 2014). Based on Ensoli et al., 2021 epidemiological evidence and its key role in the HIV-1 life cycle and disease pathogenesis, the Tat protein was chosen as a vaccine candidate for preclinical and clinical development for the prevention and treatment of HIV-1 infection (Ensoli et al., 2021). Table 3 shows clinical trials on Tat-based vaccines.

Table 3: Clinical trials on Tat based vaccines.

Code (Clinicaltrials.gov identifier)	Study	Type	Country	No of volunteers	Reference
ISS P-001 (NCT00529698)	Phase I preventative trial (Tat)	Randomised, double blind, placebo-controlled	Italy	20	Ensoli et al., 2021
ISS T-001 (NCT00505401)	Phase I therapeutic trial (Tat)	Randomised, double blind,	Italy	27	Ensoli et al., 2021

		placebo-controlled			
ISS T-002 (NCT00751595)	Phase II therapeutic trial (Tat)	Randomised, open label	Italy	168	Ensoli et al 2010
ISS T-003 (NCT01513135)	Phase II therapeutic trial (Tat)	Randomised, double blind, placebo-controlled	South Africa	200	Ensoli et.al., 2016
ISS P-002 (NCT01441193)	Phase I preventative trial (Tat + Env)	Randomised, open label	Italy	11	Gavioli, et al., 2008.

Preventative and therapeutic, double-blind, placebo-controlled phase I trials with the biologically active Tat (ISS P-001, ClinicalTrials.gov Identifier: NCT00529698; ISS T-001, ClinicalTrials.gov Identifier: NCT00505401) were conducted in Italy, meeting both primary (safety) and secondary (immunogenicity) endpoints (Bellino et al., 2009; Ensoli et al., 2008). The Tat vaccine was safe and, in the HIV-1 infected volunteers, did not induce virus replication, as indicated by preservation of CD4+ T cell counts and by the absence of significant plasma viremia rebounds. The long-term follow-up (ISS OBS P-001, ClinicalTrials.gov Identifier: NCT01024764) showed the persistence of anti-Tat Abs up to five years after the first immunisation (Bellino et al., 2009; Ensoli et al., 2008).

A phase I preventive trial was also conducted with the combination of Tat and V2-loop-deleted Env (Δ V2-Env) in Italy (ISS P-002, ClinicalTrials.gov Identifier: NCT01441193) (Table 3). This was a multicentric, open-label, phase I trial conducted in healthy volunteers to evaluate the safety and the immunogenicity of the vaccine based on the association of HIV-1 Tat and Δ V2-Env proteins, as compared to vaccination with single proteins (Gavioli et al., 2008). Tat and Δ V2-Env proteins, either in association or as single components, were administered by a prime-boost regimen consisting of three intradermal priming doses and two intramuscular boosting injections. The Tat/ Δ V2-Env vaccination was safe and immunogenic, as indicated by the development of Ab responses to the vaccine antigen(s) in all participants (Ensoli et al., 2021). Importantly, while the highest anti-Tat antibodies response was detected in the subjects vaccinated with Tat alone, the highest anti-Env Ab responses were also detected in the volunteers co-immunized with Tat/ Δ V2-Env (Ensoli et al., 2021). These results were consistent

with previous studies showing better priming provided by Tat-mediated entry of Env in dendritic cells (Monini et al., 2012; Gavioli et al., 2008).

The therapeutic vaccination was prioritised over the preventive one as a shorter, more cost-effective route to proof of efficacy (Ensoli et al., 2014). Therapeutic phase II trials for cART intensification were conducted in Italy and South Africa in patients on successful cART. The Italian phase II study (ClinicalTrials.gov Identifier: NCT00751595) was an exploratory phase II open-label therapeutic trial, randomised on the different regimens utilised (Ensoli et al., 2015; Ensoli et al., 2010). It enrolled 168 HIV-1 infected (B clade) anti-Tat Ab-negative adults on long-term (mean >6 years) cART who were virologically suppressed, with CD4⁺ T cell counts ≥ 200 cells/mm³, and were vaccinated with 7.5 or 30 μ g of the Tat protein (clade B) without adjuvant, administered intradermally 3 or 5 times, one month apart. Both primary (immunogenicity) and secondary (safety) endpoints were met. No increase in virological biomarkers was observed. The results also showed a reduction in immune activation and durable increases in CD4⁺ T cells, B cells, NK cells, and CD4⁺ and CD8⁺ T central memory cell subsets, with a reduction in effector memory cells, indicating a shift of the immune response towards homeostasis (Ensoli et al., 2010). None of these changes were observed in anti-Tat-Ab-negative subjects on effective cART enrolled in a parallel observational study at the same clinical centres (ISS OBS T-002) (ClinicalTrials.gov Identifier: NCT01024556) (Ensoli et al., 2015; Ensoli et al., 2010). Of note, Tat immunisation induced a reduction of HIV-1 DNA load in blood, especially in volunteers receiving 30 μ g of Tat, given three times, that continued throughout the 8-year follow-up (ClinicalTrials.gov Identifier: NCT02118168) (Sgadari et al., 2019; Ensoli et al., 2015). Tat-specific cellular responses also contributed to HIV-1 proviral DNA reduction. Furthermore, the induction, upon vaccination, of CD38⁺/HLA-DR⁺ CD8⁺ T cells and natural killer (NK) cells endowed with killer activity against virus-infected cells may have also contributed to the reduction of the virus reservoir (Ensoli et al., 2021). cART intensification by the Tat vaccine was durable, as the increase in CD4⁺ T cells persisted during the 8-year follow-up and was accompanied by a progressive, slow decrease in HIV-1 proviral DNA in the peripheral blood, which became undetectable in 34% of all vaccinees and 48% of volunteers in the group receiving 30 μ g of Tat three times (Sgadari et al., 2019). These results indicate that the induction of anti-Tat immune responses intensifies cART efficacy and attacks the cART-resistant virus reservoir.

A confirmatory randomised, double-blind, placebo-controlled, safety and immunogenicity phase II therapeutic trial (ISS T-003, ClinicalTrials.gov Identifier: NCT01513135) was then

conducted in South Africa in 200 HIV-1 infected (C clade) anti-Tat Ab-negative adults who were virologically suppressed, with CD4⁺ T cell counts ≥ 200 cells/mm (Ensoli et al., 2016). The clade-B Tat vaccine (30 μ g) was administered intradermally thrice monthly. The vaccine was safe and induced durable and high titres of anti-Tat Abs that were capable of cross-recognising the Tat protein from different HIV-1 clades and cross-neutralising both clade B and C HIV-1 viruses. Cross-recognition and cross-neutralisation correlated with increased CD4⁺ T cell counts, a key target for cART intensification (Ensoli et al., 2016). Of note, vaccination contained the VL rebound. It maintained CD4⁺ T cell counts above the baseline levels in subjects who were noncompliant with therapy compared to (noncompliant) placebo, suggesting that Tat vaccine intensification of cART may counterbalance incomplete adherence to treatment (Ensoli et al., 2016). An extended follow-up study of this trial (ISS T-003 EF-UP) is underway. Overall, the Tat vaccine study shows for the first time that cART can be intensified by therapeutic immunisation and that the proviral DNA load can be progressively lowered.

2.7 Role of HIV-1 Tat in HIV pathogenesis

HIV-1 Tat (Trans Activator of Transcription) is a regulatory protein that enhances the efficiency of viral transcription. It is the “trans-activator of transcription”, consisting of 86 to 101 amino acids depending on the subtypes (Jeang et al., 1999). The basic region of HIV-Tat protein is suggested to form an alpha helix. The basic region is involved in RNA (TAR, trans-activation response element) binding, and Tat proteins thus belong to the family of arginine-rich motif (ARM) RNA binding proteins (Tahirov et al., 2010). HIV-1 Tat is released extracellularly space from the cells with HIV-1 infected cells, meaning that the protein is released by infected cells in culture and is found in the blood plasma of HIV-1 infected patients; also plays a critical role in the HIV-1 pathogenesis (Nicoli et al., 2013). It can be absorbed by cells that are not infected with HIV and can act directly as a toxin-producing cell death via apoptosis in uninfected "bystander" T cells (Pre-existing memory CD8⁺ T cells that are not specific for the infection pathogen), assisting in the progression toward AIDS (Nicoli et al., 2013; Campbell et al., 2004; Xiao et al., 2000).

Before Tat is present, a small number of RNA transcripts will be made, which allow the Tat protein to be produced. The function of Tat during viral transcription is to stimulate transcription from the Viral long terminal repeat (LTR) promoter by binding to the trans-activating response (TAR) hairpin at the 5' end of newly formed RNA transcripts (Das et al.,

2011). In attaching to TAR, Tat alters the properties of the transcription complex, recruits positive transcription complex (P-TEFb) of cellular CDK9 and cyclin T1, and hence increases the production of full-length viral RNA (Molle et al., 2007). Without Tat, HIV transcription by RNA polymerase II (RNAP II) is very inefficient and viral RNA transcripts corresponding to TAR RNA are less than 60 nucleotides long (Jin et al., 2020; Laspia et al., 1989; Feinberg et al., 1991). Important features in the TAR hairpin are the highly conserved 3-nucleotide (nt) pyrimidine bulge that binds the Tat protein and the apical 6-nt loop to which the transcriptional elongation factor pTEFb binds in a Tat-dependent manner (Das et al., 2011). Upon TAR binding, the kinase component of pTEFb, cyclin-dependent kinase 9 (CDK9), can phosphorylate the C-terminal domain of RNA polymerase II, which enhances the processivity of the elongating polymerase (Bieniasz et al., 1999). pTEFb also directs the recruitment of TATA box binding protein to the LTR promoter and thus stimulates the assembly of new transcription complexes (Raha et al., 2005).

Tat protein is also essential in controlling latency and viral rebound following combined antiretroviral therapy treatment (Jin et al., 2020; Hill et al., 2016). Viral latency is a reversible state whereby a pathogenic virus becomes dormant (latent) during the viral life cycle in individual cells. HIV-1 may either actively replicate to produce progeny virions or can enter a viral latency, from which it may later be subsequently reactivated (Kamori and Ueno, 2017). The mechanisms for the establishment and maintenance of HIV-1 latency mainly operate at the transcriptional level by both viral and host machinery and occur at the levels of transcription, chromatin modification, and epigenetic regulations (Donahue and Wainberg, 2013; Coiras et al., 2009). HIV-1 latency is primarily found within resting memory CD4⁺ T cells, microglia cells, and others, which intrinsically have a long half-life *in vivo* (Kamori and Ueno, 2017; Chavez et al., 2015). Because the expression level of the viral proteins is absent or poorly expressed and also the existence of immune escape mutations, the latently infected cells are much less susceptible to be recognised and cleared by the host immune system, viral cytopathic effects or currently available antiretroviral drugs (Deng et al., 2015). Thus, to date, the latently infected viral reservoir is one of the fundamental limitations toward HIV-1 cure (Kamori and Ueno, 2017). It could be therapeutically beneficial to prevent or at least reduce the size of the established latent reservoir to a large extent. Previous studies indicate that, sufficient Tat quantities may counteract the establishment of HIV-1 latency by promoting transcriptional initiation or elongation (Donahue et al., 2012; Pearson et al., 2008). Donahue et al. (2012) demonstrated that fewer latently infected cells were established in Jurkat cells that stably

expressed Tat compared to cells that did not express Tat (Donahue et al., 2012). These findings showed the contribution of Tat and its abundance in the prevention of the establishment of viral latency. HIV-1 Tat mutations severely impair viral replication, which agrees with the transcriptional requirement for Tat. This dominant negative effect makes it difficult to study other Tat-mediated processes in the viral replication cycle (Das et al., 2011). Nullbasic is a mutant HIV-1 Tat protein, that has anti-HIV-1 activity through mechanisms that include inhibition of Rev function and redistribution of the HIV-1 Rev protein from the nucleolus to the nucleoplasm and cytoplasm (Lin et al., 2012). Rustanti et al. (2017) showed that null basic inhibits HIV-1 replication by testing null basic against representative HIV-1 strains from subtype C, D, and A/D recombinant (Rustanti et al., 2017). It was observed that Nullbasic inhibits Tat-mediated transactivation and virus replication of all the HIV-1 strains tested in TZM-bl cells (Rustanti et al., 2017). Moreover, several natural polymorphisms, including P10S, W11R, K19R, A42V, and Y47H, that were observed in 5 HIV-1 infected subjects at the acute infection stage, demonstrated impaired transactivation activity and were statistically enriched in latently infected CD4 T cells (Yukl et al., 2009).

By targeting cells expressing RGD-binding integrin receptors, such as dendritic cells, macrophages and activated endothelial cells via its RGD-binding site, extracellular Tat enters them very efficiently (Fanales-Belasio et al., 2009). Tat activates the proteasome, increasing antigen processing and presentation, thus contributing to Th-1 cell activation (Gavioli et al., 2008). At the same time, via induction of TNF α , Tat induces the maturation of dendritic cells toward a Th-1 phenotype, again increasing T cell responses. Tat also activates the expression of cytokines with critical immunomodulatory effects and can trigger HIV-1 gene expression (Ensoli et al., 2021). Extracellular Tat also induces HIV-1 co-receptor expression, activating virus replication, rescuing defective provirus, and facilitating virus transmission to neighbour cells. Of note, the Tat protein is detected in highly purified virions, further supporting its crucial role in virus transmission and establishment of infection (Ensoli et al., 2010).

Tat plays key roles any time the virus needs to establish or to reactivate infection, i.e. at the acquisition of infection or under HAART-mediated viral suppression, both of which are accompanied by the presence of unintegrated proviral DNA expressing regulatory gene products and RGD-containing Tat protein isoforms (Ensoli et al., 2010).

Extracellular Tat may lead to the reactivation of opportunistic diseases. Tat uptake has been shown to activate several transcription factors through phosphorylation or other means

(Montano et al., 1997; Karn and Stoltzfus, 2012). Tat-induced activated transcription factors like Sp1 and others have been shown to modulate the expression of both HIV-1 and host genes. In the absence of the virus, soluble Tat has been shown to cause induction of apoptosis, release of neurotransmitters, oxidative stress, and inflammation (Clifford and Ances, 2013; Anand et al., 2018). Tat modulation of several genes involved in the processes contributes to chronic inflammation in people with HIV-1. It has been linked to several comorbidities observed in the HIV-infected population, including HIV-1 associated neurocognitive (HAND) and cardiovascular disease impairment. Kaposi's sarcoma (KS) is also associated with HIV-1 infection. A study by Ensoli et al (1990) was conducted on the Tat protein of HIV-1 stimulates the growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Briefly, supernatants from HIV-1-infected T cells carrying the CD4 antigen promote the growth of cells derived from KS lesions of AIDS patients (AIDS-KS cells), and the HIV-1 *tat* gene, introduced into the germ line of mice, induces skin lesions closely resembling KS (Ensoli et al., 1990). It was reported that the *tat* gene product (Tat) was released from both HIV-1-acutely infected H9 cells and *tat*-transfected COS-1 cells (Ensoli et al., 1990). These Tat-containing supernatants promoted the growth of AIDS-KS cells inhibited by anti-Tat antibodies. Therefore, a viral regulatory gene product can be released as a biologically active protein and directly as a growth stimulator (Ensoli et al., 1990). These and previous data indicated that extracellular Tat could be involved in the development or progression of KS in HIV-1-infected individuals.

2.8 The Role of Anti-Tat Antibodies in HIV-1 disease progression and immune activation

Extracellular Tat can also bind to HIV-1 Env spikes, forming the virus entry complex that favours infection of dendritic cells and transmission to the dendritic cells (Bellino et al., 2014), decreasing recognition of C-type lectin receptors and promoting engagement of RGD binding integrins, which are expressed by inflammatory DCs, macrophages and endothelial cells (ECs) present at the site of infection. As a result, virions escape neutralisation by anti-Env Abs directed against high mannose determinants (are much less effective) and enter target cells upon binding to RGD-binding integrins (Monini et al., 2012). In the presence of Tat, antibodies against both Tat and Env restore and further increase the neutralisation of HIV-1 entry into DCs and cell infection. They are preventing virus entry through RGD-binding integrins (Monini et al., 2012).

Anti-Tat antibodies are uncommonly found in the body after HIV-1 infection and present in about 20% of asymptomatic individuals. Anti-Tat antibodies were reported to play a very

protective role against HIV-1 progression to AIDS in humans and were shown to inhibit HIV-1 infection *in vitro* (Re et al., 2001). Therefore, novel vaccine strategies combining Tat and Env antigens are being developed to induce broad cellular and humoral immune responses able to kill early the infected cells, as well as to neutralise infectious virions, acting together to dampen or block initial HIV-1 infection and dissemination (Monini et al., 2012).

Evidence of the occurrence of anti-Tat Abs in the course of HIV-1 infection was reported, and it soon became apparent that, unlike Abs against HIV-1 structural proteins (Rezza et al., 2005; Krone et al., 1988). Antibodies against Tat were only present in a minority of infected individuals, and there was an inverse relationship between progression to disease (associated with p24 antigenemia, plasma viral load (VL) and CD4⁺ T-cell loss) and anti-Tat seropositivity (Re et al., 1995). Rezza et al., (2005) showed the relationship between the anti-Tat serostatus and the risk of progression to disease over the 14-year relationship between the anti-Tat serostatus and the incidence and risk of progression to disease over an observation time of up to 14 years (median follow-up time: 7.2 years). The risk of progression was found to be lower in anti-Tat Ab-positive subjects as compared to anti-Tat Ab-negative individuals (Rezza et al., 2005). No progression was observed in the persistently anti-Tat Ab-positive subjects. However, progression occurred in those who lost anti-Tat Ab-reactivity and was even faster in the persistently anti-Tat Ab-negative subjects. These observations suggested a close association between the anti-Tat serostatus and disease progression (Rezza et al., 2005). Anti-Tat antibody mouse monoclonal raised against amino acids 6-12 of Tat of HIV-1 origin and it has been recommended to detect a Tat protein of HIV -1 origin. Anti-Tat antibodies are very protective against HIV-1 progression (Gray et al., 2016). Antibodies were shown to be associated with slow disease progression in HIV-1 chronic individuals (Nicoli et al., 2013). According to Nicoli et al (2016), chronically HIV-1 individuals positive with anti-Tat IgM⁺ and are developing IgG responses are protected from the rapid loss of CD4⁺ T cells. However, contrary to findings with clade B HIV-positive persons, anti-Tat IgG prevalence did not vary between patients stratified according to CD4⁺ T cell counts, in contrast to observations made with clade B HIV infected individuals (Ensoli et al., 2006; Rodman et al., 1992). In addition, Anti-Tat IgG alone are not protective in non-clade B infected subjects, unless concomitant with IgM, suggesting a protective role of persistent anti-Tat IgM irrespective of the infecting clade (Nicoli et al., 2016). This implies that an association of anti-Tat IgG with progression to AIDS could depend on the HIV clade and/or the presence of multiple anti-Tat isotypes. Tripiciano et al (2021) conducted a study to examine the effects of anti-Tat immunity on low-level viremia, persistent immune

activation and CD4⁺ T-cell recovery in people living with HIV who are on long-term cART. The results showed that anti-Tat immunity is significantly associated with higher nadir CD4⁺ T-cell numbers, control of low-level viremia and long-lasting CD4⁺ T-cell recovery, but not with decreased immune activation (Tripiciano et al., 2021). These results argue against anti-Tat antibodies as a mechanism for eliminating HIV Tat-driven low-level inflammation. Therapeutic approaches aiming at reinforcing anti-Tat immunity should be investigated to improve immune reconstitution in people living with HIV on long-term cART.

However, there may also be other potential mechanisms that could partially account for the observed findings. For instance, another cause of the positive association between levels of anti-Tat antibodies and nadir (the lowest recorded value) CD4⁺ T cell count could be that anti-Tat antibodies are a marker of intact and well-preserved B cell functions. It is well-documented that PLWH who start ART early after the time of infection have better-preserved immune functions than those who start ART late after the time of infection (Søgaard, 2021; Lundgren et al., 2015; Grulich et al., 2007; El-Sadr et al., 2006). Nadir CD4⁺ T cell count, which is used as a marker of immune dysfunction prior to starting ART, was in fact higher among the anti-Tat antibody positive compared to the anti-Tat antibody negative group. This seems to indicate that the levels of anti-Tat antibody might depend on the level of immune dysfunction prior to starting ART (Søgaard, 2021). The authors propose, future clinical trials could test therapeutic strategies such as vaccination using an HIV Tat-protein based vaccine or administration of anti-Tat antibodies to determine whether anti-Tat humoral or cellular immunity can lower residual viremia and restore immune functions in PLWH and ultimately reduce non-AIDS-related comorbidity (Søgaard, 2021).

A study by Ensoli et al 2010, showed that cellular and anti-Tat Abs responses exert protective roles to control virus replication and to delay disease progression, both in humans and monkeys (Ensoli et al., 2010). Recently, retrospective analysis on 112 monkeys with 67 vaccinees and 45 controls indicated that vaccination with Tat has statistically significant protective effects against infection acquisition. In viraemic monkeys, it reduces significantly set-point viral load and CD4⁺ T cell decline (Cafaro et al., 2010). Not surprisingly, anti-Tat Abs are produced by a small fraction (20%) of HIV-1 infected individuals in the asymptomatic phase and are lost during progression (Ensoli et al., 2010). In contrast, high Ab titres are produced against all viral products at all infection stages (Reiss et al., 1991). Studying Anti-Tat antibodies and Tat-based vaccines is very important because it may lead to a functional cure of HIV-1, providing opportunities and strategies for preventing and eradicating HIV-1.

2.9 Principles of methods

2.9.1 Detection of antibodies

The antibody is a protein naturally produced by plasma cells within the human body to mediate an adaptive immune response against invading pathogens (Aziz et al., 2023). Therefore, antibodies are useful research tools in diagnosis and therapy, as they can recognize and bind specifically and strongly with respective antigens (Litman et al., 1993). Antibody detection assays are very crucial for disease diagnosis, because of their practicality, ease of sample collection and preparation, generally good diagnostic performance characteristics, suitability for automation (high-throughput), low cost and fast turn-around time (WOAH (OIE), 2008). They are particularly useful for processing large numbers of samples in epidemiological and population studies, or for mass diagnosis and surveillance programmes.

The most common antibody detection methods are classical virus neutralisation test (VNT), enzyme-linked immunosorbent assay (ELISA) (Tripiciano et al., 2021), haemagglutination inhibition (HAI) (Spackman and Sitaras, 2020) and the complement fixation test (CFT) (Thacker and Talkington, 2000). Other, less common, antibody detection tests are the agar gel immunodiffusion (AGID) (Jenson, 2014), the indirect fluorescent antibody test (IFAT) (Yoon et al., 1992), the latex agglutination test (LAT) (Büscher et al., 1999), and the microscopic agglutination test (MAT) (Chirathaworn et al., 2014). More recent novel methods include biosensors, bioluminometry, fluorescence polarisation, chemoluminescence and lateral flow devices also known as point of care or pen-side tests (WOAH (OIE), 2008).

Enzyme-linked immunosorbent assay is the mostly used assay for the detection of antibodies, and we also used it for the present study. The principle of an ELISA relies on the specific binding between an antibody and antigen (Hayrapetyan et al., 2023; Sakamoto et al., 2018). There are four main general steps to completing an ELISA immunoassay. These steps are: coating (with either antigen or antibody), blocking (typically with the addition of bovine serum albumin [BSA]), detection and final read (Alhajj et al., 2023). There are four major types of ELISA: Direct ELISA (antigen-coated plate; screening antibody), Indirect ELISA (antigen-coated plate; screening antigen/antibody), Sandwich ELISA (antibody-coated plate; screening antigen), Competitive ELISA (screening antibody) (Sakamoto et al., 2018). The main difference between direct and indirect ELISA is that direct ELISA relies on the binding of the enzyme-conjugated primary antibodies to the antigen-coated plates. Indirect ELISA introduces

enzyme-linked secondary antibodies specific to the primary antibodies bound to the antigen-coated plates (Hayrapetyan et al., 2023).

ELISAs are performed in polystyrene plates, typically 96-well plates coated to bind protein strongly (Engvall, 2010). Depending on the ELISA type, testing requires a primary and/or secondary detection antibody, analyte/antigen, coating antibody/antigen, buffer, wash, and substrate/chromogen (Shah and Maghsoudlou, 2016). The primary detection antibody is a specific antibody that only binds to the protein of interest. In contrast, a secondary detection antibody is a second enzyme-conjugated antibody that binds to a primary antibody that is not enzyme-conjugated (Konstantinou, 2017). ELISA exhibits the following advantages: Simple procedure, high specificity, and sensitivity, because of an antigen–antibody reaction, high efficiency, as simultaneous analyses can be performed without complicated sample pre-treatment, generally safe and eco-friendly. No hazardous or radioactive substances and large amounts of organic solvents are not required, Cost-effective assay, as low-cost reagents are used (Sakamoto et al., 2018). However, the disadvantages of ELISA are as follows: labour-intensive and expensive to prepare antibody because it is a sophisticated technique, High possibility of false positive or negative results because of insufficient blocking of the surface of microtiter plate immobilized with antigen. Antibody instability because an antibody is a protein that requires refrigerated transport and storage (Sakamoto et al., 2018).

2.9.2. Techniques of Size of reservoirs measurements

HIV-1 reservoirs are group of immune system cells or tissues in the body that are infected with HIV-1 but are not actively producing new HIV-1 (Eisele and Siliciano, 2012). There are different assays that can be used to measure the size of the reservoir in infected individuals. The quantitative viral outgrowth assay (qVOA) measures the frequency of resting CD4⁺ T-cells that produce infectious virus after a single round of maximum *in vitro* global T-cell activation but underestimates the true size of the reservoir (Ho et al., 2013; Siliciano et al., 2003; Finzi et al., 1997). Novel approaches such as intact proviral DNA assay (IPDA), Quadruplex PCR (Q4PCR) and Tat/Rev induced limiting dilution assay (TILDA) represent major advances in quantifying and characterizing intact/replication-competent HIV-1 reservoir (Belmonti et al., 2021).

Emerging PCR-based techniques used to quantify HIV-1 DNA such as the intact proviral DNA assay (IPDA) and quadruplex quantitative PCR (Q4PCR), distinguish intact proviruses from defective ones, therefore providing a better resolution for studying the dynamics of defective

and intact HIV-1 proviral DNA and requiring a relatively small number of cells (Belmonti et al., 2021). The simplest and most cost-effective way to measure the frequency of HIV-1 infected cells is to use PCR-based assays that quantify HIV-1 genomes in a given number of cells, usually PBMCs or isolated CD4⁺ T cells (Roux et al., 2023). Several quantitative PCR-based approaches have been developed to quantify various HIV DNA forms including integrated HIV-1 DNA, 1- and 2-long terminal repeat (LTR) circles and unintegrated linear HIV DNA (Roux and Chomont, 2023). Quantification of “total HIV DNA” (which includes all forms) is typically performed by amplifying a short and highly conserved region of the HIV genome (usually LTR or gag) (Roux and Chomont, 2023). It has been extensively used to quickly estimate the magnitude of the HIV-1 reservoir easily. Because they are fast, simple, and target the most abundant form of HIV-1 persistence, quantitative PCR (qPCR) can be used on a small number of cells and have been the standard in extensive clinical studies for years (Leyre et al., 2020; Luzuriaga et al., 2014).

Most HIV-1 genomes in people on long-term ART are integrated, and measurements of integrated proviruses may represent a better surrogate of the HIV-1 reservoir (Leyre et al., 2020). Notably, people taking integrase inhibitors or on relatively short-term ART in whom unintegrated genomes are still detectable at substantially high levels (Leyre et al., 2020). Quantification of integrated HIV-1 DNA can also be achieved by Alu-gag PCR assay, by combining an HIV-specific primer with primers specific for *Alu* regions that are distributed throughout the entire human genome (Roux and Chomont, 2023; Vandergeeten et al., 2014). However, a significant limitation of both total and integrated HIV-1 DNA qPCR-based approaches is that they greatly overestimate the frequency of cells harbouring genetically intact HIV-1 proviruses. This is due to the amplification of only tiny region of the viral genome that was primarily selected for its limited genetic diversity among different variants (Vandergeeten et al., 2014). Intact proviral DNA assay (IPDA) and digital droplet PCR (ddPCR) are using strategically positioned primer sets in the *env* and the Ψ regions to discriminate between intact and defective proviruses (Roux and Chomont, 2023). The IPDA provides a much more accurate estimate of the frequency of cells harbouring potentially intact proviruses than regular PCR assays. As was verified by sequencing, 70% of the proviruses considered intact by the IPDA were shown to lack defects, whereas this value is less than 10% for regular PCR-based assays (Bruner et al., 2019).

In the present study, we used ddPCR, the third generation of PCR that enables absolute quantification through partitioning the reaction (Day et al., 2013). We used this assay to

measure HIV-1 DNA overestimate the true size of the latent reservoir of replication-competent proviruses, as they will also amplify defective or deleted proviruses which are not capable of producing virus (Anderson and Maldarelli, 2018; Eriksson et al., 2013). Droplet digital PCR exhibits the following advantages: it provides an absolute quantification of target molecules and does not require use of a standard curve; in contrast, traditional qPCR methods employ a relative quantification, using a standard curve to determine DNA copies. Secondly, the sensitivity of ddPCR makes it an ideal platform for studying rare event molecules in the presence of an extensive background of unaffected molecules (Oellerich et al., 2017). Thirdly, ddPCR has a broad linear range of quantification, with sensitive two-fold detection of differences, permitting the resolution of small changes in abundance (Hindson et al., 2011). Finally, ddPCR detection can be multiplexed for simultaneous detection of two targets. This advantage is beneficial for estimates of the proportion of HIV-1 proviruses that contain deletions; for instance, the finding of HIV-1 LTR without detection of an internal portion of the genome (e.g., gag, tat, and rev) immediately determines that the HIV-1 provirus is deleted (Anderson and Maldarelli, 2018). However, ddPCR exhibits the following disadvantages: narrow dynamic range and high cost (Mao et al., 2019).

2.9.3 Immune activation.

Immune activation is a pathologically appropriate response to invading pathogens, including viral infection (Spudich, 2016). Moreover, T cell activation is a process in which developed T cells can express antigen-specific T cell receptors on their surface to recognise their cognate antigens and respond by entering the cell cycle, secreting cytokines or lytic enzymes, and initiating the cell-based functions of the immune system (Majedi et al., 2019). Immune activation can be measured using various assay such as enzyme-linked immune absorbent spot (Elispot) and flow cytometry. Enzyme-linked immune absorbent spot is a quantitative method for measuring relevant parameters of T cell activation using PBMCs (Ranieri et al., 2014). The sensitivity of Elispot allows the detection of low-frequency antigen-specific T cells that secrete cytokines and effector molecules, such as granzyme B and perforin (Ranieri et al., 2021). The most utilised ELISPOT assay is the interferon-gamma (IFN- γ) test, a marker for CD8(+) CTL activation. Still, Elispot can also be used to distinguish different subsets of activated T cells by using other cytokines such as T-helper (Th) 1-type cells (characterised by the production of IFN- γ , IL-2, IL-6, IL-12, IL-21, and TNF- α), Th2 (producing cytokines like IL-4, IL-5, IL-10, and IL-13), and Th17 (IL-17) cells (Ranieri et al., 2014). Flow cytometry is a laser-based

technique used to detect and analyse cells or particle's chemical and physical characteristics. It is a technology that rapidly analyses single cells or particles as they flow past single or multiple lasers while suspended in a buffered salt-based solution. Each particle is analysed for visible light scatter and one or multiple fluorescence parameters (McKinnon, 2018).

In the present study, we used PBMCS to determine the proportion of T cells expressing activation (HLA-DR and CD38) markers. Using flow cytometry to look at uniform cell populations has the benefit of always highlighting any non-uniformity. It also removes debris or dead cells when providing the final data. This level of accuracy beats that of the competition (Cole, 2018). Unfortunately, there are some disadvantages. Flow cytometry sorters are very accurate and purify small or complex subpopulations. But even a high-speed sorter is, at times too slow to achieve the desired results. For instance, a pair of cells is often discarded because the sorter cannot distinguish between them in time (Cole, 2018).

2.9.4 Antibody Neutralisation assay.

The virus neutralisation test detects antibodies capable of neutralising the infectivity of the virus (Manenti et al., 2021). Serial dilutions of serum are mixed with a reference strain of viable virus and incubated to allow any antibody present to bind and neutralise the virus infectivity, and then dilutions are inoculated onto cells. The presence or absence of viral growth is observed. The titer is the highest dilution (i.e., the least amount of antibody) that neutralises infectivity. In the present study, we used TZM-bl neutralisation assay to detect the capability of anti-Tat antibodies to neutralise the virus by preventing envelope binding. This assay measures neutralisation in TZM-bl cells as a function of a reduction in Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection (Sarzotti-Kelsoe et al., 2014; Montefiori, 2009).

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CHAPTER 3

Anti-Tat antibodies and their role in developing prophylactic and therapeutic HIV-1 vaccine.

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3.1 Abstract

Background: Highly Active Antiretroviral therapy has reduced morbidity and mortality in people living with AIDS globally. There is no cure for HIV and no effective preventative vaccine. Hence, there is a need to continue to search for it to prevent new infections. HIV-1 Tat has been identified as a potential target for developing prophylactic and therapeutic vaccination. Therefore, we investigated the evolution and development of anti-Tat antibodies (IgA, IgG, IgM) from early-treated individuals throughout 12 months of HIV-1 infection, associating them with CD4+ T cell count, size of reservoirs and T cell activation and how the HIV-1 anti-tat antibodies neutralise envelope binding.

Methods: Anti-Tat C IgG, IgM, and IgA titers were measured longitudinally by enzyme-linked immunosorbent assay in plasma samples of 34 HIV-1 early-treated individuals from the FRESH cohort. We determined the association between anti-Tat antibodies (IgA, IgG, and IgM) and CD4+ T cell counts throughout 12 months of infection. Digital droplet PCR (ddPCR) was used to measure the size of reservoirs and associated with them with anti Tat antibodies. T-cell activation was measured using HLA-DR and CD38 markers using flow cytometry and analysed using FlowJo version 10.8.1. The immune activation levels were associated with the presence of anti-Tat antibodies. TZM-bl cells were used to measure the % env neutralisation by anti-Tat antibodies, thus preventing virus infection.

Results: Anti-Tat C immunity (IgA, IgG and IgM) is significantly associated with the duration of treatment ($p=0.0001$) and correlates positively with the size of the reservoirs irrespective of CD4+ T cell counts. Anti-Tat antibodies were associated with low CD4+ T cell activation and correlated positively with CD8+ T cell activation. Anti-Tat antibodies correlates negatively with neutralisation antibody titers.

Conclusion: Anti-Tat immunity is essential in the therapeutic approach to improve immune reconstitution in early-treated individuals: Tat-specific IgM and IgG antibodies in combination with cART lower the disease progression. Additionally, earlier initiation of cART can reduce the size of the reservoirs, which may have a functional cure.

3.2 Introduction

HIV-1 infection is a global epidemic, and currently, over 39 million people are living with the virus (UNAIDS, 2023). The introduction of the combined antiretroviral therapy (cART) has reduced the burden to manageable chronic infection. Antiretroviral therapy effectively decreases viral loads to undetectable levels, delay disease progression, restore T cell immune function, prevent transmission to uninfected individuals and reduce the burden of HIV-1 by decreasing the mortality rate of people living with HIV-1 (Castro-Gonzalez et al., 2018; Tseng et al., 2015). However, the inability of antiretroviral therapy in curing the virus, and the development of HIV-1 drug resistance and drug toxicity poses the challenge in ARV success story. The major barrier to the complete eradication of HIV-1 from the host cell is the presence of viral reservoirs of latently infected cells (Chen et al., 2022; Ho et al., 2013; Finzi et al., 1997). These cells are not recognised by the immune system, and ART does not inhibit their replication (Stevenson et al., 2021). Therefore, up to date, there is no cure for HIV-1 cure and an effective preventative vaccine; hence, there is a need to continue to search for an effective HIV-1 vaccine to prevent the new infections.

Several HIV-1 vaccine trials have been tested and failed to protect against HIV-1 (Ng'uni et al., 2020). The difficulties associated with developing an effective/successful vaccine have increased since the initial clinical trials of the Microgenex vaccine up to the widely publicised Vaxgen trial (Robinson, 2007; Letvin, 2006; Billich, 2004). A study by Loret et al. (2016), revealed that the combined use of Tat-based vaccine (Tat Oyi) and cART could offer an effective approach to manage the reservoir of HIV-1 infected cells (Loret et al., 2016).

HIV-1 regulatory protein known as Tat has been found to increase the viral transcription of HIV-1 and play an important role in disease progression. The major function of Tat was found to stimulate transcriptional elongation rather than initiation (Rice, 2017; Kao et al., 1987). Tat protein is also essential in controlling latency and viral rebound following the interruption of combined antiretroviral treatment (Jin et al., 2020). Evidence indicates that, when present in sufficient quantities, Tat may counteract the establishment of HIV-1 latency by promoting transcriptional initiation or elongation (Donahue et al., 2012; Pearson et al., 2008). Tat protein

is released into the extracellular space from cells with HIV-1 infected cells into neighbouring uninfected cells even without active HIV-1 replication and viral production due to effective antiretroviral therapy (ART) (Nicoli et al., 2013). Extracellular Tat released by infected neighbour cells binds to trimeric Env on HIV-1, promoting engagement of RGD binding integrins, expressed by inflammatory DCs, macrophages and endothelial cells (ECs) present at the site of infection. As a result, virions escape neutralisation by anti-Env Abs and enter target cells upon binding to RGD-binding integrins. Anti-Tat Abs neutralise this binding, preventing virus entry through RGD-binding integrins (Monini et al., 2012).

Anti-Tat immunity might counteract the Tat-mediated immune dysregulation and play an important role in controlling HIV-1 disease progression and co-morbidity (Cafaro et al., 2019; Nicoli et al., 2016). A study by Nicole et al. (2016) has shown that anti-Tat IgM and IgG are more frequently found in the early stage of the disease than during the symptomatic stages and in non-progressors and are associated with slow disease progression in cART-naïve HIV-1 infected individual (Nicoli et al., 2016). Although Tat activities strictly depend on the viral clade, knowledge about anti-Tat antibodies in non-clade B HIV infection has not been studied.

Due to this knowledge, Tat has been identified as a potential target for developing prophylactic and therapeutic vaccination for cART intensification (Loret et al., 2016). Here, our goal was to understand the influence and the evolution of HIV-1 anti-Tat antibodies on HIV-1 early-treated individuals. The present study investigated the evolution of HIV-1 anti-Tat antibodies from early treated HIV-1 individuals throughout 12 months of HIV-1 infection and measures the total anti-Tat specific antibodies titers in early-treated individuals in the FRESH cohort and associate them with the size of the reservoirs at 12 months. Secondly, it evaluated the association of anti-Tat immunity in T cell immune activation in early-treated HIV-1 individuals. Lastly, investigated how the HIV-1 anti-Tat antibodies impact the env binding, thus preventing virus infection.

3.3 Materials and methods

3.3.1 Study design and participants

Female Rising through Education, Support and Health (FRESH) is an ongoing cohort investigation conducted in KwaZulu-Natal, South Africa (Dong et al., 2018). Its purpose is to diagnose acute HIV infection in young women at risk, collecting blood and mucosal samples before and after infection, and enable study of HIV pathogenesis and biological and

behavioural risk factors for HIV acquisition, as well as research on vaccine and cure strategies. Eligible women were HIV uninfected, aged 18–23 years, sexually active, not pregnant, non-anaemic (haemoglobin ≥ 10 g/L), without other barriers to participation (serious chronic illness, enrolment in another study, or family responsibilities), and gave written consent to enrolment. Participants were informed about the importance of the study, helping researchers find better ways to prevent, detect, or treat HIV and AIDS. We targeted disadvantaged, at-risk women, favouring the enrolment of those who were unemployed and not attending school. Participants were recruited at local sites frequented by young people, including cafes, nightclubs, and shopping malls. To promote peer support, women were enrolled in groups of 30–35 participants, forming cohorts whose visits were scheduled together throughout the surveillance period. Surveillance duration was initially 96 weeks, shortened to 48 weeks on May 1, 2015, 29 months after study launch. HIV-1 RNA testing during surveillance was done by finger prick twice a week and was modelled on a previously described study. Unique to the FRESH study design was the incorporation of a socioeconomic intervention developed to address challenges affecting young women that might contribute to HIV acquisition risk. The intervention's twice-weekly classes, done at the study site, coincided with the HIV surveillance schedule. The study was approved by the biomedical research ethics committee of the University of KwaZulu-Natal.

In the current study, human plasma samples from 34 HIV-1 early treated individuals from the FRESH cohort were included in the analyses. FRESH cohort is described in detail above. Participants were given confidential identity, which was not disclosed in the current study. We stratified according to CD4⁺ T cell count and viral load under effective cART (treated the next day after tested positive). Participants were followed for up to 12 months (CD4 T cell counts U/L (median= 768 (IQR=1423.5-954); Viral loads cps/ml (median= 29000 (IQR=147500-6525)).

3.3.2 Enzyme-linked immunosorbent assay (ELISA)

Human anti-Tat IgG, IgM and IgA were measured in plasma by ELISA using plasma samples collected from the FRESH cohort. Ninety-six well Nunc™ MaxiSorp™ ELISA plate (BioLegend, US) were coated with 100 μ l/well of HIV-1 clade C Tat (Biorbyt, UK) and resuspended in PBS (Gibco, US), overnight at 4°C. Plates were then washed three times with PBS containing 0.05 % Tween-20 (Sigma Aldrich, US) and then blocked with PBS containing 5% BSA (Thermo Fisher, SA) for 2 hours at room temperature. Plates were washed three times,

and 100 µl/well of appropriate dilutions of each plasma diluted in PBS were dispensed in duplicate wells and then incubated for 2 hours at room temperature. Plates were washed three times before the addition of 100 µl/well of HRP-conjugated anti-human IgG (Sigma Aldrich, US), HRP-conjugated anti-human IgA (Sigma Aldrich, US), or HRP-conjugated anti-human IgM (Sigma Aldrich, US) diluted 1:1, 1:10 and 1:100, in PBS and incubated for 1 hour at room temperature. After incubation, plates were washed three times and 50µl of the substrate (1 o-phenylenediamine dihydrochloride (OPD) tablet (Thermo Fisher, SA) + 11ml of phosphate citrate buffer (Sigma Aldrich, US) +4.4 µl H₂O₂ (ReAgent, UK)) was added for detection. 50 µl of H₂SO₄ was added to stop the reaction. Absorbance in optical density was measured at 490 nm using a Victor Nivo multimode plate reader (PerkinElmer, US). HIV-1 negative patients were used as a negative control and cART-naïve patients with chronic HIV-1 infection were used as a positive control. The cut-off value was estimated as the mean absorbance of 3 negative control plasma. Cut-off values were subtracted from the absorbance value of each sample to obtain net absorbance values. A plasma sample with a higher value than the cut-off value was considered positive for anti-Tat antibodies. Using GraphPad prism 5, titers were calculated using different dilutions to get area under the curve.

3.3.3 Measurement of the size of the reservoirs by digital droplet PCR

Total peripheral blood mononuclear cells (PBMCs) collected from each patient at each time point were independently subjected to DNA extraction using DNeasy Blood & Tissue Kits (QIAGEN, USA). Total HIV-1 DNA and host cell concentrations in the DNA extracts were estimated using BIO-RAD ddPCR, using primers and probes covering HIV-1 5' LTR-gag HXB2 coordinates 684–81037 (forward primer 5'-TCTCGACGCAGGACTCG-3', reverse primer 5'-TACTGA CGCTCTCGCACC-3' probe/56-FAM/CTCTCTCCT/ZEN/TCTAGCCTC/31ABkFQ/, and human RPP30 gene forward primer 5'-GATTTGGACCTGCGAGCG-3', reverse primer 5'-GCGGCTGTCTCCACAAGT-3', probe/56 FAM/CTGACCTGA/ZEN/AGGCTCT/31ABkFQ/). Digital droplet PCR was performed using the following thermocycler program: 95 °C for 10 min, 45 cycles of 94 °C for 30 s, and 60 °C for 1 min, and 72 °C for 1 min. Figure 8 shows the digital droplet PCR workflow. The BIO-RAD QX100 droplet reader (BIO-RAD, USA) subsequently read the droplets and data were analysed using QuantaSoft software (BIO-RAD) edition 1.2.

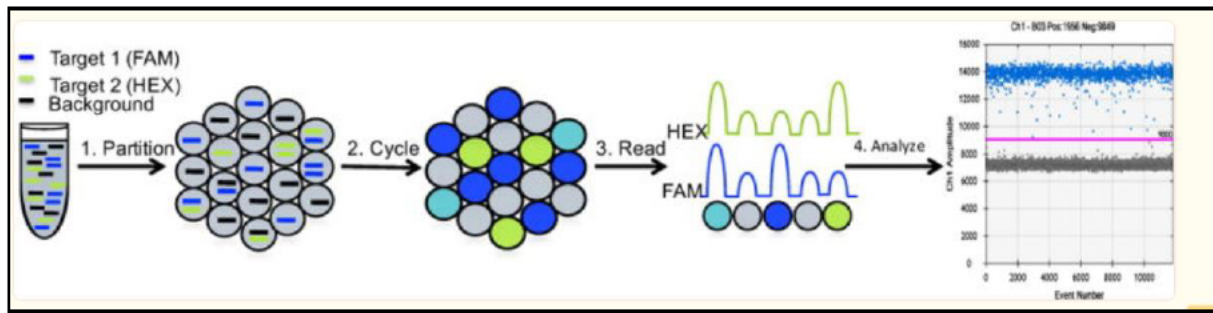


Figure 8: Digital droplet PCR workflow: First, a PCR mix containing target DNA along with primers and fluorescently labelled probes is partitioned into around 20,000 nanolitre-sized droplets with the QX100 droplet generator. Then these droplets are cycled to an endpoint on a thermal cycler. Next the droplets are read with the BIORAD QX100 Droplet Reader which sips each well, singulates the droplets and passes them by a two-colour detection system. The data generated can finally be analysed in the Quantasoft software edition 1.2 where thresholds should be set between the negative and positive droplets.

3.3.4 Measurement of immune activation by flow cytometry

Peripheral blood mononuclear cells (PBMCs) from FRESH cohort were isolated by Ficoll density gradient centrifugation and cryopreserved until use. Cryopreserved PBMC samples were thawed and resuspended in R10 medium (RPMI (Sigma- Aldrich, US), 5.5 ml L-glutamine (Gibco, US), 5.5 ml Penicillin- Streptomycin (Thermo Fisher, SA), 5.5 ml Hepes (Thermo Fisher, SA), 10% FBS (Gibco, US). Cells were rested for 2 hours at 37⁰ C before staining. For immunophenotyping one million cells were stained and incubated for 20 minutes using the following fluorochrome-labelled monoclonal antibodies (MAbs): CD3- BV650/BV786, CD4- APC, CD8 FITC, CD56- BV510, CD38- BV711, HLA-DR- PECF594 (BD Bioscience) and viability dye (Thermo Fisher, SA), before the 20 minutes fixation with fix and perm medium A. Acquisition was performed on a LSR Fortessa (BD Biosciences). Compensation was conducted with antibody capture beads stained separately with the individual antibodies used in the test samples. Flow cytometry data was analysed using FlowJo version 10.8.1. Figure 9 shows the gating strategy for assessing T cell activation markers.

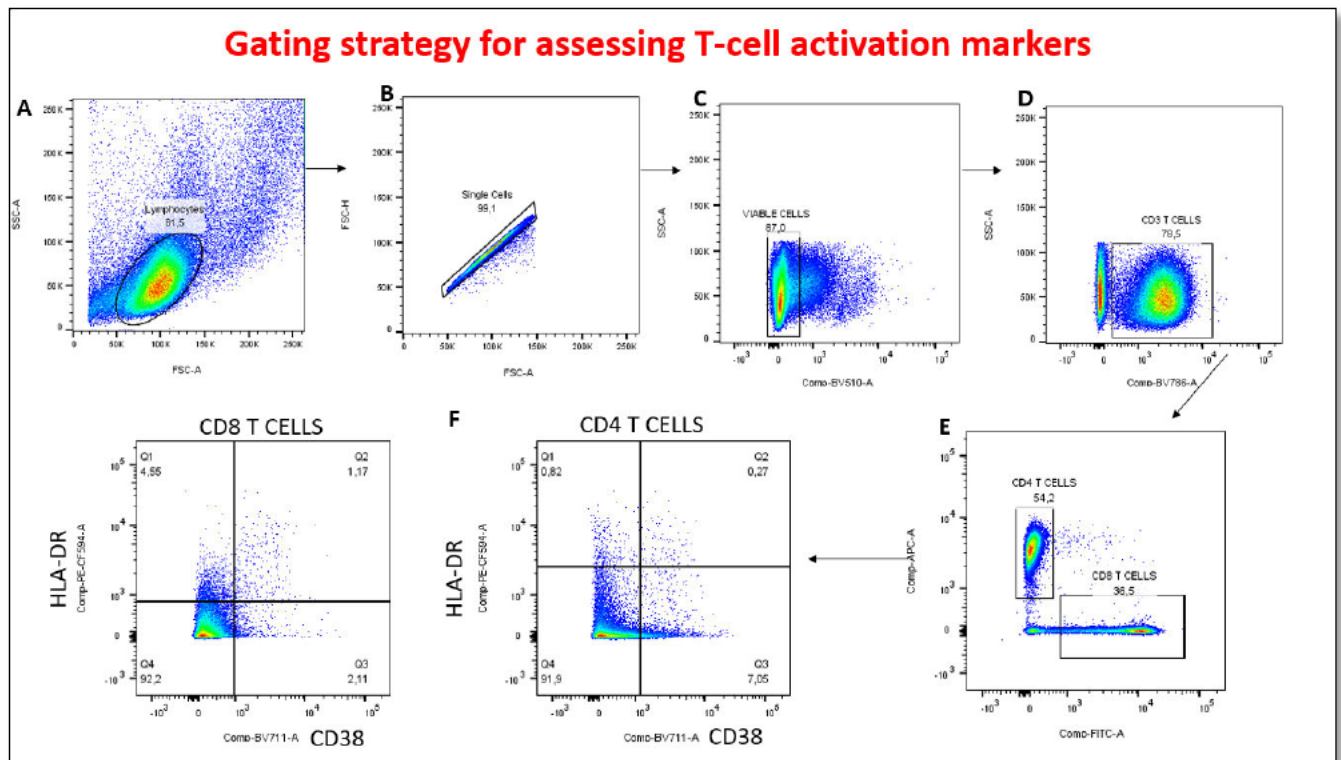


Figure 9: Gating strategy for assessing T cell activation markers: Frequencies of CD4⁺ and CD8⁺ T cells expressing activation markers in the PBMCs; Shown are representative plots demonstrating the gating strategy for the expression of activation (CD38 and HLA-DR). Samples were initially gated on lymphocytes based on SSC-A/FSC-A gating (A). Single cells were gated using FSC-H/FSC-A gating (B). Dead cells and other unwanted cells were excluded (C). T cells were gated as CD3⁺ cells (D). Subsets of T cells were further gated based on CD4 and CD8 expression (E). T cell activation was determined using activation markers; HLA-DR⁺ CD38⁺ on CD4⁺ and CD8⁺ T cells (F).

3.3.5 Cell culture maintenance

293 T cells and TZM-bl cells were cultured in different culture flasks using 10ml of Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 2% Penicillin-Streptomycin (Thermo Fisher, SA), 25mM Hepes buffer (Thermo Fisher, SA) and incubated in a humidified incubator set at 37⁰ C and 5% CO₂. The old medium was carefully removed from the culture flask, and the cells were rinsed with 4ml of PBS (Gibco, USA). PBS was then discarded, and 1.5 ml of trypsin was added for 3-5 minutes to allow the cells to detach from the surface. Following the trypsinisation, trypsin was carefully removed, and 10ml of DMEM was added and mixed until cells were consistently spread through the solution. The cell suspension was repeatedly sucked into the pipet and then expelled into the flask, thus breaking up cell chains and groups. Cells were counted using TC20, and a calculated aliquot of cell suspension and fresh culture medium was pipetted into the new, unused cell culture flask and incubated in a humidified incubator set to 37⁰ C and 5% CO₂. This method was repeated until the cells were ready to be used.

3.3.6 Virus Production

The virus was generated by transfecting 293 T cells with NL4.3 plasmid DNA using transfection reagent, Eugene (Promega, US). Transfected 293 T cells were set for 48 hours at 37°C, and then the supernatant was harvested and filtered. The virus harvested was titrated in TZM-bl cells using Nunc 96 well plates (Thermo Fisher, SA) and set for 48 hours at 37°C. The infection was monitored using Brightglo luciferase reagent (Promega, US) in a Victor Nivo multimode plate reader (PerkinElmer, US). The level of viral replication was expressed in Relative Luminescence units (RLU). The presence of light will mean the virus generated is infectious. For infectious viruses, an RLU greater than 50,000 is expected.

3.3.7 TZM-bl Neutralisation assay

Using the format of a Nunc 96-well flat bottom culture plate (Thermo Fisher, SA), 150µl of DMEM medium was placed in all wells of column 1 (cell control). Then, 100µl of DMEM was placed in all wells of columns 2-12 (virus control). An additional 40µl DMEM was added in all wells of columns 3-12, row H (to receive test samples). 11 µl of plasma with/without anti-tat antibodies (test sample) was added in duplicate. The samples were mixed at least 5 times, and 50µl was transferred to the next row. The mixing and transferring of 50µl was repeated through row A (these were serial 5-fold dilutions). After the final transfer and mixing was complete, 50µl was discarded from the wells. The thawed virus was mixed in a conical tube and diluted with DMEM prior to plating to achieve a TCID range of approximately 50,000-150,000 RLU. 50 µl of the virus/DMEM suspension was dispensed to all wells, excluding the cell control wells. The plate was covered with the lid and incubated at 37°C for 1 hour. TZM-bl cell suspension was prepared at a concentration of 100,000 cells/ml in DMEM during the incubation. DMEM and DEAE-Dextran (Thermo Fisher, SA) were added to the cell suspension prior to plating. 100 µl of the prepared cell suspension (10,000 cells per well) was dispensed to each well in columns 1-12. The plate was covered and incubated for 48 hours. After incubation, the plate was removed from the incubator. For the luciferase reaction, 150µl of culture medium was removed from each well and discarded, leaving approximately 100 µl in the well. 100 µl Bright-Glo luciferase reagent (Promega, US) was dispensed to each well. The plate was then incubated away from light at room temperature for 2 minutes to allow complete cell lysis. The mixture was mixed by pipettor action (at least two strokes), and 150 µl was transferred to a corresponding 96-well black plate. Read the plate in a Victor Nivo multimode

plate reader (PerkinElmer, US). The level of viral replication was expressed as a percentage of the HIV-1 inhibition using the following equation:

$$\% \text{ HIV inhibition} = \frac{(\text{Average sample} - \text{Average control})}{(1 - (\text{Average viral control} - \text{Average control}))} \times 100$$

Neutralising antibody titers (ID₅₀ values) were expressed as the reciprocal of the plasma dilution required to reduce RLU by 50%.

Statistical Analysis

Data analyses were performed using Prism version 5 (GraphPad Inc.), Microsoft Excel (Microsoft). Groups were compared using the Mann-Whitney U-test. For association analyses, the Spearman rank correlation was determined. P-values ≤ 0.05 were regarded as statistically significant.

CHAPTER 4- RESULTS

4.1. To investigate the evolution of HIV-1 anti-Tat antibodies from HIV-1 early treated individuals using ELISA and associate them with the size of the reservoirs at 12 months.

4.1.1 Longitudinal development of anti-Tat antibodies in HIV-1 early-treated individuals

Plasma samples from early treated HIV-1 individuals were first tested for anti-Tat IgA, IgG and IgM recognising clade C Tat (Figure 10). Figure 10 shows the association between different isotypes of anti-Tat antibody titers and the duration of treatment. The cut-off value was estimated as the mean absorbance of 3 negative control plasma. Negative control plasma samples were randomly selected from HIV-1 negative subjects in the FRESH cohort. Cut-off values were subtracted from the absorbance value of each sample to obtain net absorbance values. Optical density from screening ELISA tests for each patient after cut-off subtraction. Cut-off values were included in the following values: Absorbance of 0.03 for IgA, 0.046 for IgG and 0.062 for IgM (Supplementary figure 1). A plasma sample with a higher Δ value than the cut-off value was considered positive for anti-Tat antibodies. Then, we calculated anti-Tat antibody titers for all patients. The mean for anti-Tat IgA detected on 1st day of treatment was 183.3 Ab titer, at 2-3 months of treatment, it was 84.62 Ab titer, and at 6-12 months of treatment, it was 132.1 Ab titer ($p=0.0001$) (Figure 10a). The mean for anti-Tat IgG detected on 1st day of treatment was 1026 Ab titer; at 2-3 months of treatment, it was 207.9 Ab titer and at 6-12 months of treatment, it was 120.0 Ab titer ($p=0.0001$) (Figure 10b). The mean for anti-Tat IgM detected on 1st day of treatment was 1564 Ab titer, at 2-3 months of treatment, it was 363.2 Ab titer and at 6-12 months of treatment, it was 143.5 Ab titer ($p=0.0001$) (Figure 10c). The antibody titer cut-off value included were as follows: 78.4, 103.8, and 143.2 for IgA, IgG, and IgM, respectively. Plasma sample that was higher than the cut-off value was considered positive for anti-Tat antibodies. These results indicate that anti-Tat antibodies are associated with the duration of treatment. The presence of anti-Tat antibodies tends to disappear over time ($p=0.0001$) (Figure 10). These analyses demonstrate that a large proportion of the 34 HIV early HIV- treated individuals had plasma anti-tat antibody responses dominated by IgG or IgM.

Association between anti-Tat antibodies and duration of treatment.

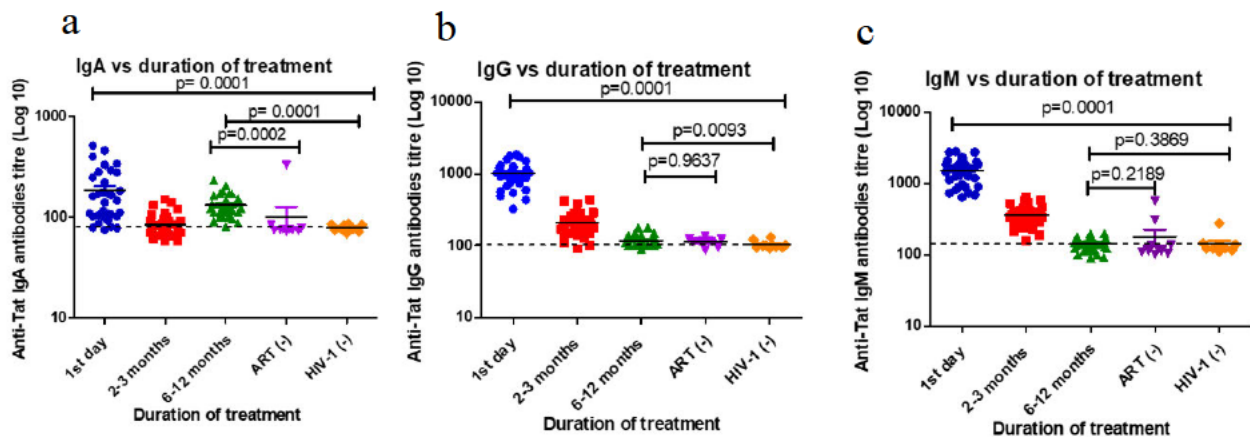
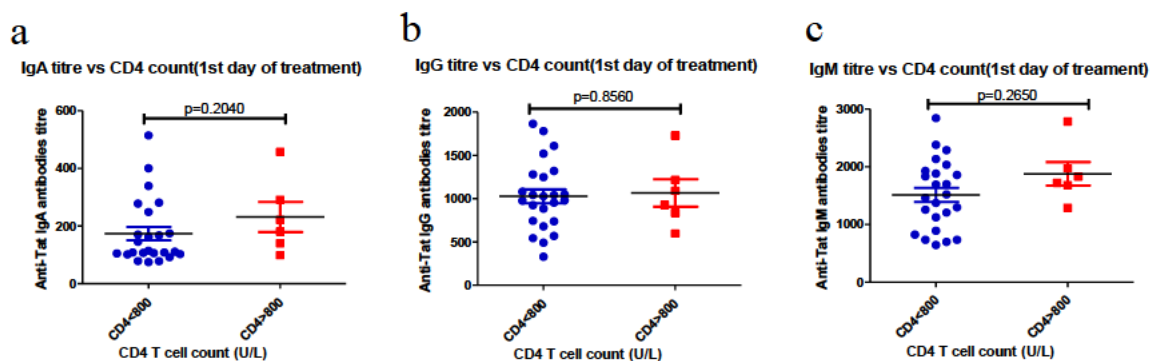


Figure 10: Association between different isotypes of anti-Tat antibody titres and the duration of treatment. Antibody isotypes compared were (a) IgA, (b) IgG and (c) IgM. Y-axes represent anti-tat antibody titres and x-axes represent different time points of treatment. Circles highlighted in blue, red, green, purple, and orange represent 1st day, 2-3 months and 6-12 months of treatment, HIV-1 untreated, and HIV-1 negative respectively. Lines represent the mean value. Statistical comparisons were made using the Kruskal-Wallis statistic.

We further analysed the association between anti-Tat antibody isotypes and CD4+ T cell count. We selected plasma samples from different time points, according to their duration of treatment. We compared each Anti-Tat isotype titre against CD4+ T Cell counts less than 800 U/L and CD4+ T cell counts greater than 800 U/L. No effect was observed (Figures 11a-i). We compared anti-Tat isotypes against CD4+ T cell less/greater than 800 U/L because all participants had high CD4+ T cell count since they were given treatment earlier. Additionally, they all maintained undetectable viral loads.

Association between anti-Tat antibodies and CD4+ T cell count



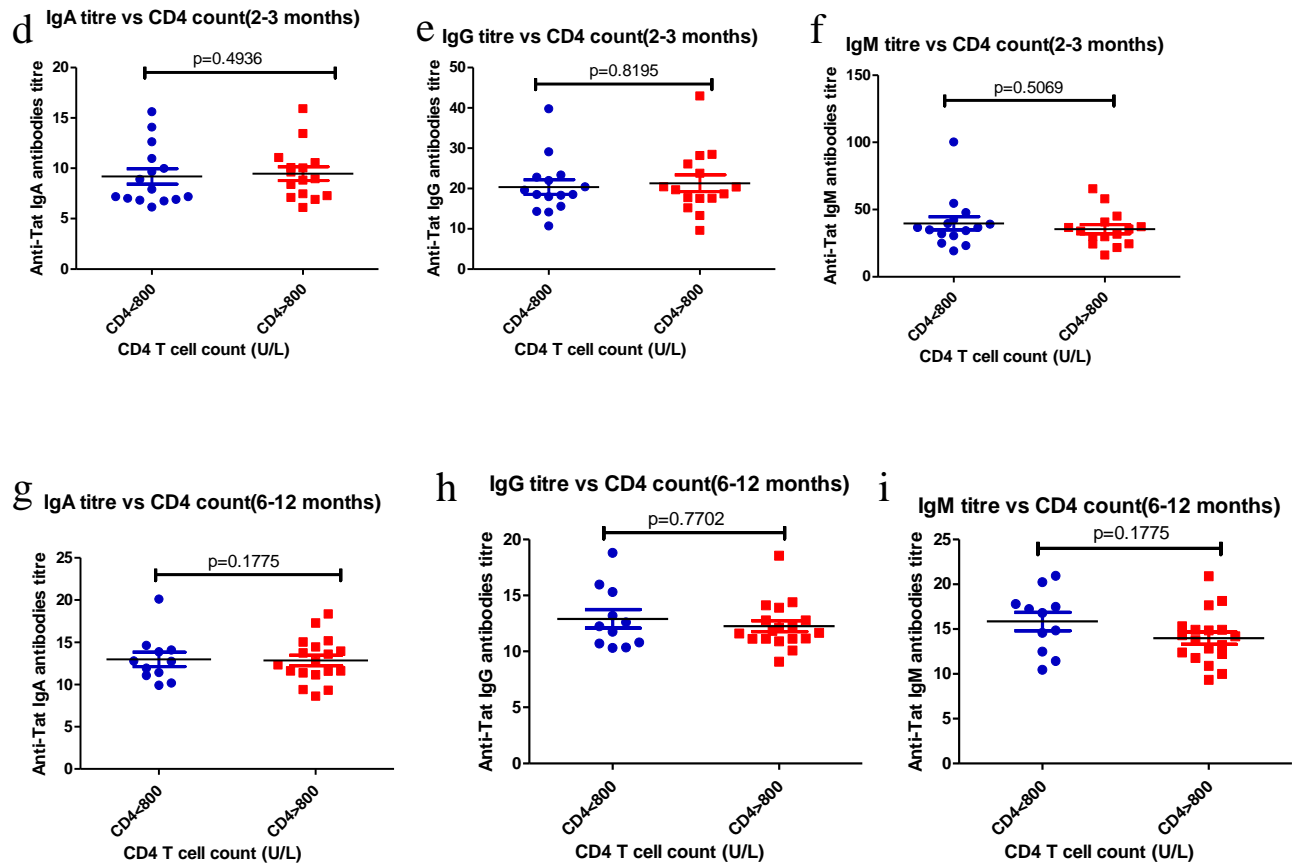


Figure 11: Association between different isotypes of anti-tat antibodies titres with CD4 T cell count. Subgroups were compared for (a-c) 1st day of treatment, (d-f) 2-3 months of treatment and (g-i) 6-12 months of treatment. Y-axes represent anti-tat antibody titres and x-axes represent CD4 T cell count (U/L). Lines represent the mean value. Circles highlighted in blue and red represent CD4 T cell count less than 800, and CD4 T cell count greater than 800, respectively. Statistical comparisons were made using the Mann-Whitney test.

We further analysed the association between anti-Tat antibodies and the size of the reservoirs. Total HIV DNA (Log copies/ 10^6 cells) was measured from selected plasma samples that had all anti-Tat antibodies at 1st day and 12 months of treatment using ddPCR. Figure 12 shows that the size of the reservoirs correlates positively with anti-Tat antibodies. The detection of anti-Tat antibodies decreases as the total HIV DNA decreases.

Association between anti-Tat antibodies and size of the reservoirs

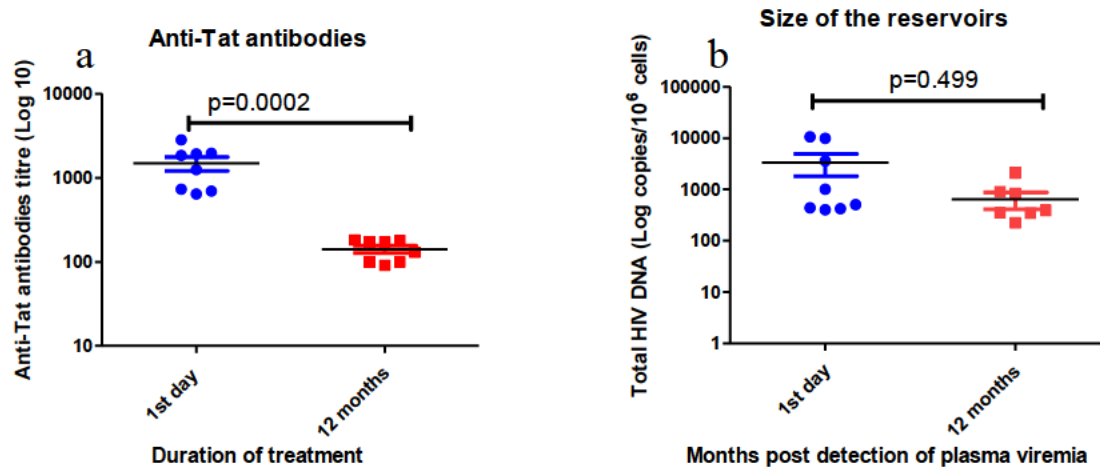


Figure 12: Association between anti-Tat antibodies (a) with the size of the reservoirs (b). Subgroups were compared for 1ST day of treatment and 12 months of treatment. Y-axes represent Total HIV DNA and x-axes represent months post detection of plasma viremia. Lines represent the mean values. Circles highlighted in blue and red represent 1st day post-infection and 12 months post-infection, respectively. Statistical comparisons were made using the Mann-Whitney test.

4.2 To evaluate the role of anti-Tat immunity in immune activation in early-treated individuals.

4.2.1 Association between anti-Tat antibodies and T cell activation

HIV -1 disease progression is characterised by T cell abnormalities such as T cell activation. The proportion of T cells expressing activation (HLA-DR and CD38) markers was determined in PBMCs. In Figure 13 a and b, we evaluated the activation profiles of CD4⁺ and CD8⁺ T cell activation by assessing CD38 and HLA-DR co-expression as shown in Figure 13. In Figure 13 a and b, patients with positive IgG, IgM and IgA responses in 1-3 months displayed almost similar frequencies of activated CD4 T cells compared to HIV-negative individuals. Higher CD4 T cell activation was observed in 6-12 months compared to HIV-negative individuals. Figure 13b, Patients displayed higher CD8 T cell activation on 1st day of treatment compared to HIV-negative individuals. Following the initiation of therapy, CD8⁺ T cell activation was reduced such that the frequency of activated cells in 6-12months was significantly lower than in HIV-negative patients and were statically different to those on 1st day of treatment (IgG and IgM, $p=0.0129$ and IgA, $p=0.0578$). Overall, these results indicate that the high response of different isotype anti-Tat antibodies is associated with low CD4⁺ T cell activation irrespective of the duration of treatment ($p\text{-value}>0.05$). Anti-Tat antibodies correlated positively with CD8T cell activation.

Association between anti-Tat antibodies and T cell activation

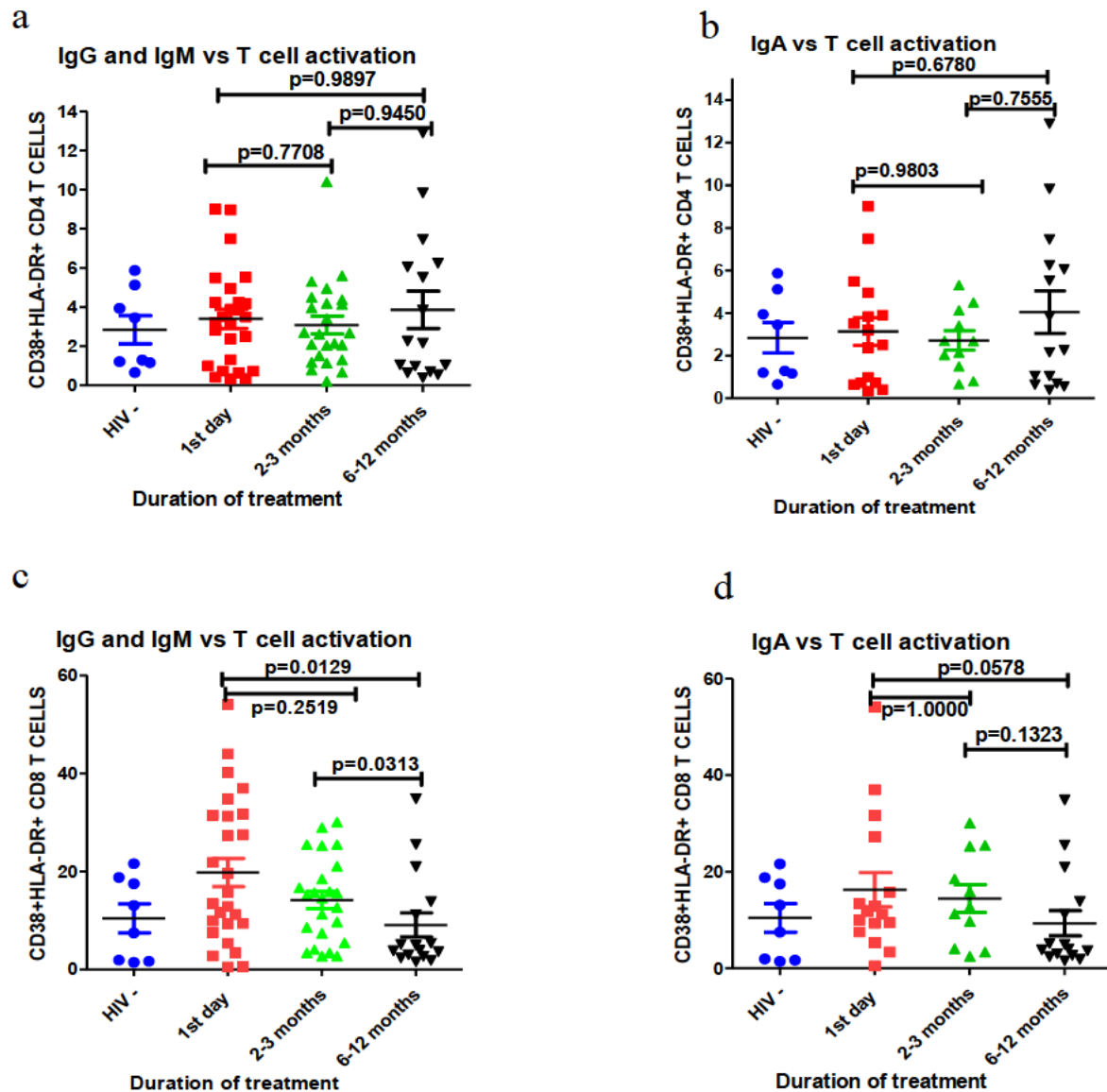


Figure 13: Association between different isotypes of anti-tat antibodies titres with T cell activation. Subgroups were compared for percentages of (a-b) CD8⁺HLA-DR⁺ on CD4⁺ T cells and (c-d) CD8⁺HLA-DR⁺ on CD8⁺ T cells. Y-axes represent frequencies of T cell activation and x-axes represent the duration of treatment. Lines represent the mean value. Circles highlighted in blue, red, green and black represent HIV- patients, 1st day, 2-3 months and 6-12 months of treatment. Statistics comparisons were made using the Mann-Whitney test.

4.3 To investigate how the HIV-1 Tat protein impacts the anti-Env antibody neutralisation ability.

4.3.1 TZM-bl neutralising antibodies

We next measured plasma-neutralising antibodies using the TZM-bl against anti-Tat antibodies. There was no significant difference in neutralisation titers between individuals on 1st day, 2-3 months and 6-12 months of treatment detected all anti-Tat antibodies (IgA, IgG and IgM) (Figure 14a). Neutralisation titers in individuals that detected IgM and IgG only were significantly lower on 1st day of treatment compared at 2-3 and 6-12 months ($p=0.0062$ and $p=0.0180$), respectively (Figure 14b). Neutralisation titers at 2-3 months of treatment were lower than those at 6-12 months. However, there was no significant difference between the two groups ($p=0.8252$) (Figure 14b). At 6-12 months of treatment, all anti-Tat antibodies were detected in some of the participants and were not detected in some of the participants. Therefore, we further compared neutralisation titers between the two groups (anti-Tat antibodies positive and anti-Tat antibodies negative) at 6-12 months. Figure 14c shows that individuals with anti-Tat antibodies had low neutralisation titers compared to individuals without anti-Tat antibodies. However, there was no significant difference between the two groups ($p=0.3829$).

Association between anti-Tat antibodies and neutralisation antibody titers.

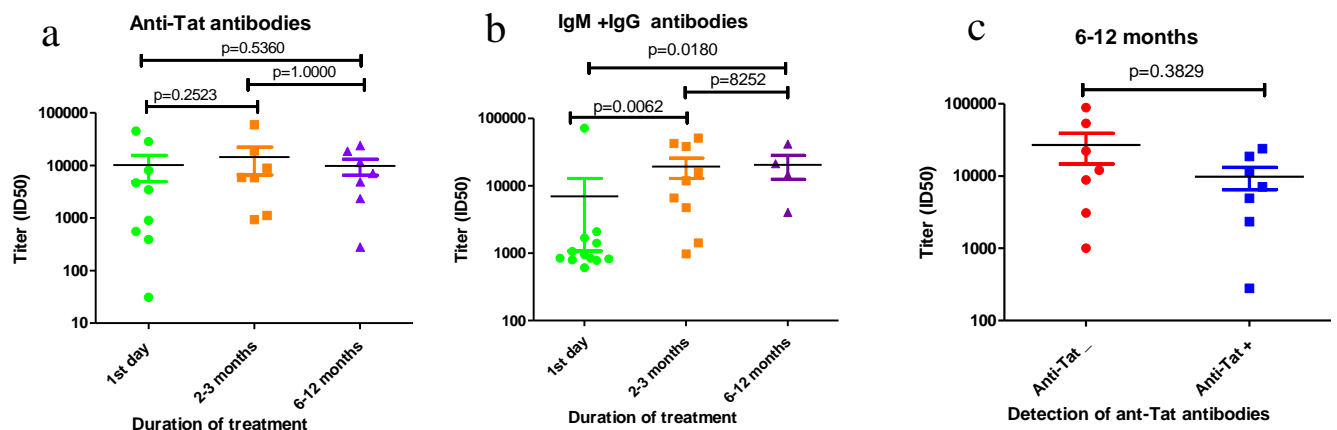


Figure 14: TZM-bl neutralising antibodies: Subgroups were compared between patients detected a) all anti-Tat antibodies, b) IgM and IgG, and c) Patients at 6-12 (Anti-Tat abs negative and positive). Y-axes represent the titer (ID50), and x-axes represent the duration of treatment. Lines represent the mean value. Circles highlighted in green, orange, and purple represent patients on 1st day, 2-3 months, and 6-12 months of treatment. Comparisons of statistics were made using the Mann-Whitney test.

4.4 Discussion

Combination antiretroviral therapy (cART) suppresses HIV-1 replication to levels that are undetectable in the peripheral blood, a low level of plasma viremia (<50 copies per mL) however, it cannot eradicate the virus entirely even after several years of treatment. Tat-specific antibodies have been found to be associated with lower disease progression in untreated patients (Nicoli et al., 2016). In this study, the longitudinal evolution of Tat-specific antibodies from early treated people living with HIV-1 was determined from the first day to 12 months of infection. Consistent with Keating et al., (2020) and Nicoli et al., (2016), the present study reports that HIV-1 early treated individuals with Anti-Tat clade C antibodies (IgM, IgG and IgA) are significantly associated with the duration of treatment and correlate positively with the size of the reservoirs irrespective of CD4⁺ T cell counts. Anti-Tat antibodies were associated with low CD4⁺ T cell activation and correlated positively with CD8⁺ T cell activation.

Enzyme-linked immunoassay test performed to measure the presence of anti-Tat clade C antibodies in early-treated individuals displayed that anti-Tat antibodies are directly related to the duration of treatment (Figure 10). In early treated individuals, anti-Tat IgM and IgG showed almost similar prevalence (mean ~1000) in the earliest stages of HIV-1 ART, while anti-tat IgA detection had lower responses (mean ~200). This suggests that ART have effects on the detection of anti-Tat antibodies. Keating et al., (2020) measured HIV-1 antibody concentrations longitudinally at years 1, 4 and 6–15 in chronic HIV-1 infected individuals after ART initiation. There was a strong correlation between antibody level and avidity at each on-treatment time point (Keating et al., 2019). In agreement with our results, the study revealed that HIV-1 antibody levels declined significantly with increasing time on ART. Together with our study we can conclude that a decline in anti-Tat antibodies throughout 12 months after infection is associated with earlier antiretroviral treatment (ART) initiation (Eshleman et al., 2019).

The current study shows that IgM and IgG responses are dominated in the early stages of treatment (Figure 10 b and c). IgM (unswitched antibody) is the first antibody isotype produced during an immune response, followed by class-switching antibodies: IgG and IgA. Moreover, IgM functions as a primary barrier against HIV-1 and regulates immune responses. IgM is highly efficient in activating the complement system and inhibiting virus entry by directly interacting with HIV-1 co-receptors (Lobo et al., 2008). The persistence of IgM during chronic

infection is interesting and has been recently described for other diseases (Figure 10) (Skountzou et al., 2014). In addition, anti-Tat IgM has been observed to also persist in Tat-vaccinated subjects, suggesting that Tat-specific IgM⁺ memory B cells are long-lived (Ensoli et al., 2010). Moreover, individuals with IgM and IgG antibodies detected high responses at early stages of treatment, which declined over time.

Interestingly, IgA responses reduced from the initiation of treatment to 2-3 months of infection and after six months responses rebounded (Figure 10a). The role of serum HIV-specific IgA has been debated before. Mazzoli et al. (1999) reported that serum IgA might display neutralising activity in the serum of exposed seronegative partners of HIV-seropositive persons (Mazzoli et al., 1999). HIV-infected individuals with anti-Tat IgA showed significantly higher pVL and activation of CD8⁺ T cells and lower CD4⁺ T cell counts (Nicoli et al., 2016). There was no evidence of accelerated progression in these subjects, although the follow-up period was limited (1 year). The fact that anti-Tat IgA detected were low in early treated patients infected for less than six months, this observation indicates that this isotype may not necessarily favour disease progression but rather represents a marker of late progression.

Previous studies have shown that anti-Tat immunity is significantly associated with higher nadir CD4⁺ T cell counts and control of low viral load in PLWH on long-term cART (Tripiciano et al., 2021). Which is in argue with Nicoli et al (2016), where they reported that anti-Tat IgM correlated with higher CD4⁺ T cell counts and lower viral loads irrespective of the duration of infection in cART- naive chronically (Nicoli et al., 2016). In this study, we reported no statistical significance between anti-tat antibodies and CD4⁺ T cell count, suggesting that no effect was observed (Figure 11 a-i). This may be because participants-initiated treatment early, regardless of their CD4⁺ T cell count (Test and Treat-policy). The viral loads of the present study participants have remained less than 20 copies/ml. Thus, we cannot conclude that anti-tat immunity had an effect on the drastic decrease of the viral load in HIV-1 early-treated individuals.

The establishment and maintenance of latent infection have been found to be influenced by HIV-1 Tat protein defects (Chen et al., 2022). Hence, we determined the association between anti-Tat antibodies and the size of the reservoirs (Figure 12). The latent reservoir is established during acute infection (Chun et al., 1998). There is evidence suggesting that Tat, when present in sufficient quantities, could potentially counteract the development of HIV-1 latency by

facilitating transcriptional initiation or elongation (Donahue et al., 2012; Pearson et al., 2008). In the present study, we reported that the size of the reservoirs at the acute stage of the infection was higher compared to 12 months post-infection and correlated positively with the presence of anti-Tat antibodies (Figure 12). Because latent proviruses do not produce viral gene products, they are protected from both antiretroviral drugs and the host immune response. The long-lived, latently infected host cell remains unaffected by viral cytopathic effects (Joos et al., 2008). Hence, we can conclude that the decrease of the size of the reservoirs is associated with the earlier ART initiation. Additionally, the establishment of the size of the reservoirs may be counteracted by Tat activity, as anti-Tat antibodies declines overtime due earlier cART initiation (Eshleman et al., 2019). Our finding is in line with promising data from Saez-Cirion et al. (2013), indicates that early treatment with cART can reduce the reservoir size and may facilitate a “functional cure”, where viremia is controlled by yet unknown immune processes (Sáez-Cirión et al., 2013)

This study shows that anti-Tat antibodies are associated with low CD4⁺ T cell activation and high CD8⁺ T activation in the early treated HIV-1 cohort (Figure 13). Likewise, a study by Nicoli et al. (2016) showed anti-Tat antibodies associated with low T cell activation in chronically infected individuals (Nicoli et al., 2016). Consistently, we observed that patients with positive IgG, IgM and IgA responses in 1-3 months displayed almost similar frequencies of HLA-DR⁺CD38⁺ CD4⁺ T cells compared to HIV-1 negative individuals. Higher HLA-DR⁺CD38⁺ CD4⁺ T cells were observed at 6-12 months (Figure 13 a and b). This may be due to low responses of anti-Tat antibodies as shown in figure 10. In agreement with Naidoo et al. (2021), hyperacute infections have low CD4⁺ and CD8⁺ T cell activation compared to HIV-1 negative individuals. In addition, anti-Tat antibody titres correlated positively with HLA-DR⁺CD38⁺ CD8⁺ T cells (Figure c-d). High CD8⁺ T cell activation is observed compared to CD4⁺ T cell activation (Figure 13). CD8⁺ T cells are critical in the natural and cART-induced control of viral replication; however, CD8⁺ T cells are highly affected by the persistent immune activation and exhaustion state driven by the increased antigenic and inflammatory burden during HIV-1 infection, inducing phenotypic and functional alterations and hampering their antiviral response (Perdomo-Celis et al., 2019). Together, these observations suggest that responses that of anti-Tat antibodies that are higher than the cut-off values (78.4, 103.8, and 143.2 for IgA, IgG, and IgM, respectively) response reduces T cell activation and counteract disease progression, according to reports from HIV-1 early treated individuals from the FRESH

cohort. This implies that high anti-Tat antibody titers with cART provide potential clinically significant HIV-1 vaccine.

A study by Monini et al. 2012, revealed that, the existence of Tat prevents the neutralisation of Env entry and the infection of dendritic cells by HIV-1 sera that do not have anti-Tat antibodies. Nonetheless, when anti-Tat antibodies are present, neutralisation is not only restored but further increased. The same is evident in sera obtained from monkeys vaccinated against both Tat and Env (Monini et al., 2012). Hence, we evaluated the neutralisation activities of anti-Tat antibodies and early-treated plasma patient against NL4.3 on TZM-bl cells. The present study, reported that, there was no significant differences in neutralisation titers throughout 12 months of treatment (Figure 14a). Lower neutralisation titers were observed in patients with anti-Tat antibodies compared to those without anti-Tat antibodies at 6-12 months of treatment (Figure 14c). However, there was no significant difference between the two groups. Together, this data suggest that anti-Tat antibodies correlate negatively with neutralisation titers, as anti-Tat antibodies decrease over time, due to earlier antiretroviral therapy initiation (Eshleman et al., 2019) (Figure 10), while there is an increasing trend in neutralisation titers throughout 12 months of treatment (Figure 14). Additionally, the increase of neutralisation titers is not associated with the presence of anti-Tat antibodies but associated with the earlier ART initiation.

SUPPLEMENTARY FIGURES

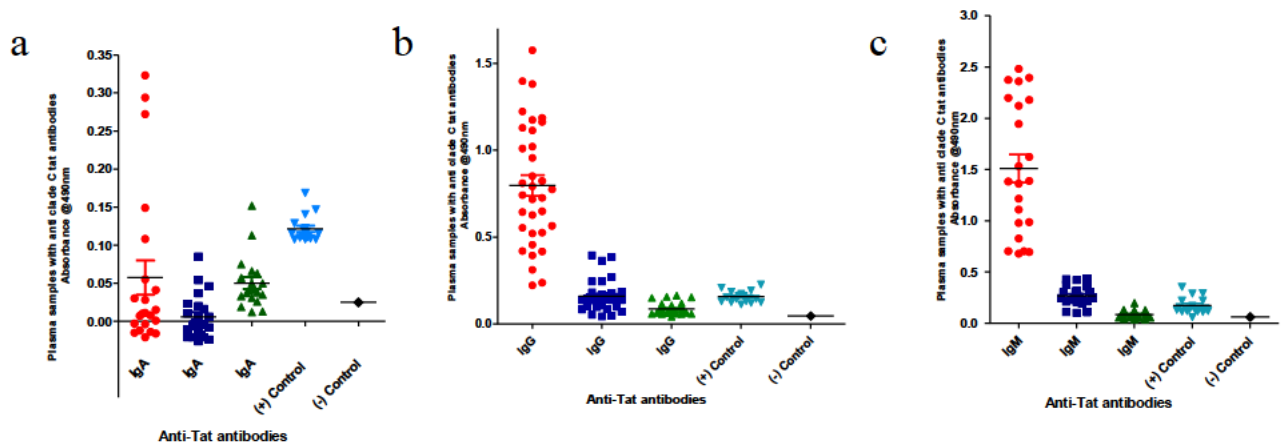


Figure 1: Plasma with anti-clade C Tat IgA, IgG and IgM or anti-clade C Tat negative of Different time point. and the duration of treatment. Optical density of anti-Tat antibody isotypes compared were (a) IgA, (b) IgG and (c) IgM. Y-axes represent anti-tat antibody OD @490nm and x-axes represent anti-Tat antibodies. Circles highlighted in Red, blue, green, turquoise blue, and black represent 1st day, 2-3 months and 6-12 months of treatment, positive control, and negative control, respectively. Lines represent the mean value.

Thirty-four patients from different time points were first tested for antibodies recognising clade C Tat. The cut-off value was estimated as the mean absorbance of 3 negative control plasma. Negative control plasma was randomly selected from HIV-negative subjects enrolled in the FRESH cohort. cART-naïve patients with chronic HIV infection were used as positive control. Cut-off values were subtracted from the absorbance value of each sample to obtain net absorbance values. This figure shows the optical density from screening ELISA tests for each patient after cut-off subtraction. Cut-off values were included in the following values. 0.03 for IgA, 0.046 for IgG and 0.062 for IgM. A Plasma sample with higher Δ value than the cut-off value was considered positive for anti-Tat antibodies.

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CHAPTER 5

5.1 General discussion

The present study, showed that, high responses of anti-Tat antibodies is associated with low CD4⁺ T cell activation and correlates positively with CD8⁺ T cell activation in early-treated HIV-1 infected individuals (Figure 13). This observation suggests that anti-Tat immunity have the potential to intensify cART and slower the disease progression. Our study results are in line with a report published by Nicoli et al. (2016), where they reported that Anti-Tat IgM were preferentially detected in cART-naïve chronically HIV-infected subjects with low T cell activation and correlated with higher CD4⁺ T cell counts and lower viral loads (Nicoli et al., 2016). It further showed that IgM and IgG are dominated in the early stages of treatment compared to IgA, which rebounded at 6-12 months of infection. This implies that IgM and IgG play an essential role in protecting against HIV-1 and IgA may not necessarily protect against HIV-1 rather represent marker of late progression. IgM is the first antibody to be produce during early HIV-1 infection because IgM can be expressed without isotype switching (Janeway et al., 2001). IgM has high avidity, allowing effective binding to the antigen, even with binding sites of relatively low affinity. Additionally, it can mobilise other components of the immune system, such as complement and various Fc receptors (Schroeder and Cavacini, 2010).

5.2 Conclusion

In this study, the contribution of anti-Tat immunity in disease progression in early-treated HIV-1 clade C infected individuals for intensifying cART as a strategy in the development of HIV-1 vaccine was investigated for the first time. Our study confirms that high responses of anti-Tat antibodies particularly, Tat-specific IgM and IgG antibodies in combination with cART lower immune activation and associated with the reduced size of reservoirs. In conclusion, anti-Tat immunity is essential in the therapeutic approach to improve immune reconstitution in early-treated individuals.

5.3 Limitations and recommendations

Limitations of this study include a small sample size, a limited number of samples in some of the objective analysis, and a short duration of longitudinal follow-up of early treated HIV-1

individuals. These limitations call for caution of interpreting our data and make it difficult to generalise our findings. For example, the short duration of longitudinal follow-up resulted in us being unable to observe IgA rebound after 6-12 months post-infection. A limited number of samples available restricted us from comparing T cell activation of patients that did not detect all anti-Tat antibody isotypes with those that detected anti-Tat antibody isotypes. In addition, age differences in HIV-1 infection and markers of HIV-1 disease progression have been reported (Pirrone et al., 2013), and future studies will therefore need to consider age. Additionally, future studies should long duration of longitudinal follow up and compare T cell activation of patients with and without anti-Tat antibodies.

Nevertheless, longitudinal samples from early-treated HIV-1 individuals (FRESH cohort) allowed us to observe the evolution of anti-Tat antibodies and the association between anti-Tat antibodies and size of the reservoirs, and immune activation. Moreover, we explored the effect/impact of anti-Tat antibodies in env binding.

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APPENDICES

Appendix 1: Ethics approval



24 August 2023

Miss Thandeka Innocentia Kubheka (217016774)
School of Laboratory Medicine & Medical Science
Medical School

Dear Miss Kubheka,

Protocol reference number: BREC/00004500/2022

Project title: The evolution of anti-Tat antibodies and its role in the development of prophylactic and therapeutic HIV-1 vaccine.

Degree: MMedSci

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 18 August 2023

Expiration of Ethical Approval: 18 August 2024

I wish to advise you that your application for recertification for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC). The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 12 September 2023.

Yours sincerely

Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
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Website: <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

Appendix 2: FRESH cohort ethics approval



12 June 2023

Prof. T Ndung'u
HIV Pathogenesis Programme
Medical School
University of KwaZulu-Natal

Dear Prof Ndung'u

PROTOCOL: Short Title: (FRESH) Females Rising with Education, Support, and Health study: Establishment and Long-term follow-up of a cohort of HIV negative women in Umlazi, South Africa.
BREC REF: BF131/11

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 25 July 2023
Expiration of Ethical Approval: 24 July 2024

I wish to advise you that your application for Recertification for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.






If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 July 2023.

Yours sincerely



Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

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