MAKERERE **UNIVERSITY**

VARIATIONS IN *TRIM5α* AND *CYCLOPHILIN A* GENES AMONG HIV-1 ELITE CONTROLLERS AND NON CONTROLLERS IN UGANDA

BY

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August 2019

DECLARATION

I Amanya Sharon Bright declare that this study is original and has not been submitted for any other degree award to any other University before.

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LIST OF ABREVIATIONS

APOBEC3: Apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like 3

ART: Antiretroviral Therapy

Cyclophilin A: CypA

DNA: Deoxyribonucleic Acid

HIV: Human Immunodeficiency Virus

MJAP: Makerere University Joint Aids Program

MJAP: Makerere University Joint AIDS program

Nef: Negative Regulatory Factor

PBMC: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

RNA: Ribonucleic Acid

SAMDH1: Sterile α motif domain-, HD domain-containing protein 1

SNP: Single Nucleotide Polymorphism

TLR: Toll-like receptor ligand

TRIM5α: Tripartite Motif Containing 5 alpha

Vif: Virion infectivity factor

Vpr: viral protein R

Vpu: Viral protein U

WHO: World Health Organization

OPERATIONAL DEFINITIONS

Elite Controllers: Individuals who maintain undetectable HIV-1 viral load (<50 copies/ml) for more than 5 years without Anti-Retroviral Treatment (ART).

Non Controllers: HIV-1 positive adults who are well controlled on ART as defined by $CD4^+$ T cell count of >500 cells/ml and absence of opportunistic infections.

Restriction factors: These are intrinsic anti-viral proteins that inhibit HIV at distinct stages of the viral life cycle.

ABSTRACT

Background:

Human immunodeficiency virus type-1 (HIV-1) elite controllers represent a unique population that controls viral replication in the absence of antiretroviral therapy (ART). Various genetic, immunologic and virologic factors have been implicated as mechanisms involved in their viremic control. Host restriction factors such as TRIM5α and Cyclophillin A have been reported to influence HIV susceptibility and disease progression and that polymorphism in their genes may contribute to the viral suppression phenotype exhibited by HIV-1 elite controllers. However, little is known about the occurrence of these polymorphisms among Ugandan HIV-1 elite controllers and non-controllers.

Objective: To characterize the variations in TRIM5α and CypA genes among Ugandan HIV-1 elite controllers and non-controllers.

Methods: Upon obtaining ethical clearance from SBS-HDREC, samples were retrieved from the ELITE study sample repository. These were thawed and $CD4^+$ T cell isolated using $CD4^+$ T Cell enrichment magnetic kit. After 2-hour incubation, $CD4^+$ T cells were cultured under 4 conditions; a) unstimulated, b)Anti-CD3 & CD28, Anti-CD3/28/Imiquimod, and c) Imiquimod alone. Cells were stained for surface markers (CD3, CD4, CD38, and PD-1) and analyzed by flow cytometry, while the supernatant was stored for use in measuring cytokines(Th1, Th2, and Th17 cytokines) by Luminex. RNA was extracted using Quick-RNA™ Whole Blood kit, quality assessed using QIAxcel gel analyzer and RT qPCR done using QuantiTect Probe RT-PCR Kit in a Rotor gene Q real-time PCR machine. mRNA was quantified using delta CT relative quantification method. DNA was extracted using Qiagen Blood Genomic DNA Kit, PCR amplified and cleaned with exosap and stored at -20^0C awaiting DNA sequencing.

Results: Upon stimulation, non-controller cells expressed significantly more PD-1 compared to the elite controllers, with the differential expression being significantly more in CD3/CD28 stimulated cells(p=0.0315), unstimulated (p=0.0401) and Anti-CD3/28/imiquimod (p=0.0565). There was no statistical difference in the expression of CD38. The genes of the intrinsic cellular defense including TRIM5α and CypA were more expressed among elite controllers, but this differential expression was not significant both in the Anti-CD3/28 stimulated (TRIM, p=0.6695 & CypA p=0.6340) and Anti-CD3/28/Imiquimod stimulated (TRIM5α, p=0.4879 & CypA p=0.7632).

Conclusion and recommendation: This study highlights differential PD-1 expression among elite controllers and non-controllers. This may have implications for potential immune checkpoint therapies as adjuncts in HIV-1 therapy. Additionally, the nondifferential expression of TRIM5α and CypA genes may imply that elite controllers have unique TRIM5α and CypA that have more capacity of innate immune signaling, not necessarily expressing more of the protein. Further investigations need to be done to establish the uniqueness of TRIM5α and CypA proteins expressed by the elite controllers.

CHAPTER I

INTRODUCTION

1.10 **Background**

Since its discovery in 1983, Human Immunodeficiency Virus (HIV) has claimed lives of more than 35 million people globally. Currently, there are 36.7 million people living with HIV of which 70% are from the WHO African region (1). To date, there is no documented cure. Rather, HIV infected individuals are enrolled in lifelong Anti-retroviral Treatment (ART) which enables them to live long healthy lives (1). In addition to limited access, there are concerns such as; viral latency, drug side effects and, resistance associated with long-term ART (2). This creates a need to study host immune factors, restriction factors that enable host cells to resist HIV replication among elite controllers as an approach to host-directed therapy.

Restriction factors, dominantly acting proteins that function in a cell-autonomous manner to suppress HIV viral replication at distinct stages have been reported to influence HIV susceptibility and disease progression (3). These include; Tripartite Motif-containing 5α (TRIM5α), Apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like 3 (APOBEC3), Tetherin/bone marrow stromal cell antigen (BST2) (3), Myxovirus resistance protein 2 (MxB), and Sterile α motif domain-HD domain-containing protein 1(SAMDH1) (4). TRIM5α, a member of the tripartite motif-containing family of proteins is reported to restrict HIV by interfering with viral capsid uncoating hence terminating downstream processes that facilitate HIV genome integration (5). It is also implicated in the modulation of innate immune signaling via nuclear factor kappaB (NF-kB) and activator protein 1 (AP-1) leading to the production of inflammatory cytokines such as interleukin-2 (IL-2) & interferon-gamma (IFN-γ) various cell surface markers(6).

Whereas the role of innate immune proteins in restriction of HIV is widely recognized, HIV has evolved proteins to antagonize the restriction effect. Accordingly, viral proteins including Vpu, Vpx, and Vif. Todate can antagonize the effect of Tetherin, SAMDH, and APOBEC respectively (4). To date, no viral protein has been reported to antagonize the restriction activity of TRIM5α and therefore representing a good candidate for host-directed therapy. Recent studies have reported polymorphism in the human TRIM5α gene to affect susceptibility to and progression of HIV infection. For example, R136Q single nucleotide polymorphism has been associated with

resistance to HIV (7) while the defective H43Y mutation is reported to increase progress in HIV infection within the population (8).

The antiviral activity of TRIM5α is potentiated by Cyclophillin A (CypA), which acts by inhibiting nuclear import of the HIV pre-integration complex (9). Consequently, polymorphisms in CypA gene have also been documented to influence susceptibility to HIV-1 infection (10). The presence of HIV elite controllers, individuals who maintain undetectable viral load for more than 5 years without anti-retroviral therapy is proof that there are unique genetic, immunologic and virologic mechanisms which are protective to these people and would, therefore, be critical in developing effective host-directed therapies. Exploring variations in TRIM5α and CypA genes among HIV-1 elite controllers is therefore essential to identify protective mutations that can be used as target molecular markers for host-directed therapy and screening tools for targeted anti-HIV-1 therapy. In this study, we report on the differential expression of cell surface markers among elite and non-controllers as well as nondifferential expression of TRIM5α and CypA genes among elite and non-controllers in Uganda.

1.20 **Problem statement**

In the Ugandan population, there is a small proportion of HIV infected people (0.26%) who maintain undetectable HIV viral load for more than 5 years without treatment (elite controllers) (11). Additionally, elite controllers also maintain high $CD4^+$ counts with less possibility of developing acquired immune deficiency syndrome (AIDS) and eventually death. Accordingly, the viral suppression mechanisms used by these elite controllers are being aggressively studied to identify potential candidates for use in HIV therapeutic vaccine development (12). Whereas their viral suppression mechanisms have been attributed to so many virologic, immunologic and genetic factors, the specific mechanism(s) through which elite controllers achieve viremic control remain largely undefined (13).

TRIM5α, one of the restriction factors is known to inhibit HIV replication at the stage of capsid uncoating while Cyclophilin A inhibits HIV nuclear import in a cell type-dependent manner. Furthermore, TRIM5α as a cellular sensor activates innate immune signaling leading to the production of various cytokines and cell surface markers important in cellular defense. Despite the overwhelming evidence on the influence of TRIM5α and CypA on HIV susceptibility and disease progression, data on variations of the respective genes between elite controllers and non-controllers in Uganda is scarce, especially in the context of Uganda’s subtypes A and D dominated HIV-1 epidemic. Our study documents the expression patterns of TRIM5α and CypA

genes among HIV elite controllers and non-controllers in Uganda as well as the influence of these variations on $CD4^+$ T cell function.

1.30. Objectives

1.31. General Objective

To characterize the variations in TRIM5α and CypA genes among HIV-1 elite controllers and non-controllers in Uganda

1.32. Specific Objectives

1. To quantify *TRIM5α* and *CypA* mRNA among HIV-1 elite controllers and non-controllers in Uganda
2. To identify sequence variations in *TRIM5α* and *CypA* genes among HIV-1 elite controllers and non-controllers in Uganda
3. To determine the relationship between *TRIM5α* variations and $CD4^+$ T cell function among HIV-1 elite controllers and non-controllers in Uganda

1.4. Significance

Understanding the role of TRIM5α and CypA in HIV-1 suppression among elite controllers is critical to combating the virus. The finding that non-controllers express more inhibitory receptor PD-1 compared to elite controllers shows that the PD-1 pathway in elite controllers plays a significant role in viral suppression. This implies that PD-1 suppressing agents could be potent adjuvants in HIV therapy. Our study also shows that elite controllers do not differentially express TRIM5α and CypA genes, which could imply that another mechanism, perhaps the uniqueness of the respective protein could be responsible for the viral suppressive phenotype exhibited by elite controllers and needs to be investigated further.

1.5. Justification

HIV remains a pandemic of great public health concern yet efforts to develop curative and preventive therapies remain largely ineffective. Elite controllers are able to suppress HIV viral load for years without ART (14). Different mechanisms have been postulated to be responsible for this viral suppression including lack of HIV susceptibility by $CD4^+$ T cells, infection by a replication-defective HIV Virus, and immune control of viral replication (13). Whether these factors contribute to the viral suppression phenotype exhibited by elite controllers in Uganda

remains largely unknown. Our study provides valuable data on some of the immunologic and genetic factors that could play a role in viral suppression by Ugandan elite controllers.

1.6 Conceptual Framework

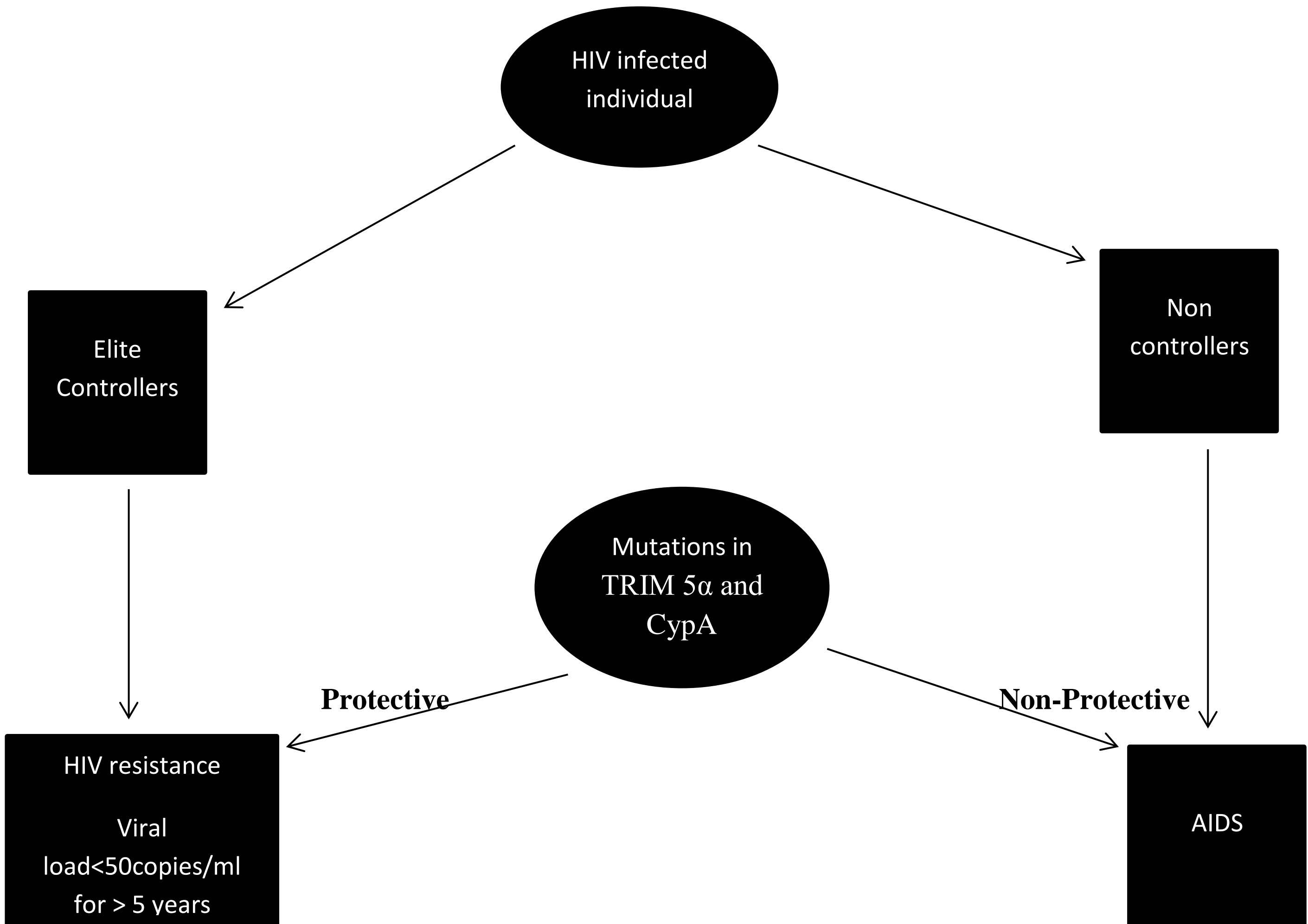

Figure 1: *The conceptual framework: Elite controllers exhibit HIV viral suppression phenotype which could be explained by host genetic or immunologic factors*

1.7 Hypothesis

We hypothesize that polymorphisms in TRIM5α and CypA genes, as well as their influence on $CD4^+$ cell function, contribute to HIV-1 viral suppression among Ugandan elite controllers.

1.8 Scope

This was a nested case-control study conducted at Makerere University, Molecular and Immunology Laboratories. A sample size of 21 PBMCs samples from 13 elite controllers and 8 non-controllers was utilized to characterize the variations in TRIM5α and CypA genes.

CHAPTER II

LITERATURE REVIEW

2.1. HIV Burden

HIV has claimed lives of more than 35 million people globally (15). Currently, there are 36.7 million people living with HIV of which 70% are from the WHO African region (15). At the moment, there is no documented cure for HIV. Infected individuals are therefore enrolled on a life long Antiretroviral Treatment(ART) which enables them to live long healthy lives. Of the infected people, only 54% of the adults and 43% of children living with HIV have been enrolled on ART treatment (1). Early initiation of ART is able to suppress HIV virus to very low levels thus preventing transmission (16), however, this can be reverted within weeks in case treatment is stopped (2). More so, challenges related to drug side effects and cost remain a major concern especially in low-income countries such as Uganda that shoulder a heavy burden of HIV. This creates a need to study Host factors that can eliminate HIV infection from Host cells as an approach to host-directed therapy against HIV-1.

2.2. Viral Restriction Factors

Viral restriction factors are intrinsic anti-viral proteins that inhibit HIV at distinct stages of the viral life cycle (17). These include; TRIM5α (capsid uncoating), APOBEC3 and SAMHD1 (reverse transcription), MxB (nuclear import and integration), Schlafen 11 (translation), and Tetherin/BST2 (budding) (3, 4, 17). These proteins such as TRIM5α also induce innate immune signaling which also contributes to their restriction activity (17, 18). Unfortunately, the HIV virus has evolved a wide array of proteins including Vif, Vpu, Vpx/Vpr, Nef, among others to evade the restriction activity of these restriction factors (3, 17). For TRIM5α however, there has been no documented counter protein to its restriction activity which makes it a good candidate for host-directed therapy against latent HIV.

2.3 TRIM5α

TRIM5α is encoded for by the TRIM gene located at 11p 15.4 position on chromosome 11 with 12 exons (19). It belongs to the family of TRIM proteins which take part in essential cellular processes such as cell proliferation, differentiation, development, apoptosis, oncogenesis and innate immunity (20, 21). The proteins consist of the Ring domain comprised of conserved cysteine and histidine residues that bind two zinc atoms. This has E3 ubiquitin ligase activity and is important for the flexibility of TRIM proteins (21). This is followed by B-Box domain which contains cysteine and histidine residues. TRIM proteins usually contain B1 and B2 domains although some members contain only B2 domain. The B-Box is important in protein-protein interaction as well as TRIM oligomeration. TRIM also has the coiled-coil domain which drives the formation of dimers and the C-terminal domain which determines the species specificity of the virus restricted (4, 20, 21).

Over 100 TRIM proteins exist, of these, TRIM5α, TRIM11, TRIM15, TRIM31, TRIM1, TRIM28, TRIM 22 (21) and TRIM 37 (22) have been documented to have antiretroviral activity. Of these, TRIM5α anti-retroviral restriction activity has been widely studied. Although not well understood, anti-retroviral restriction activity of TRIM5α is thought to be executed by the following methods; a) premature disassembly of the viral capsid lattice hence blocking reverse transcription (17) and b) targetting the viral capsid for degradation by proteosomes via the proteasome component PSMC2 or adaptor protein p62/sequestosome 1 (SQTM-1) (21). Another intracellular protein, CypA is reported to potentiate the action of TRIM5α (21) through blocking the nuclear import of the pre-integration complex (9).

Expression of TRIM5α is interferon-inducible (23), and is expressed ubiquitously in various tissues including the urinary bladder, endometrium, thyroid, spleen, appendix, gall bladder among others (19). Additionally, it’s expressed in various cells of the immune system including $CD4^+$ T cells and $CD14^+$ monocytes. It’s expression in $CD4^+$ T cells and $CD14^+$ monocytes has been documented to be similar, and mRNA levels correlate with protein levels (24).

TRIM5α gene polymorphism has been reported to influence susceptibility to HIV infection and progression of HIV disease (21, 25). In a study to determine single nucleotide polymorphisms (SNPs) responsible for HIV resistance among Pumwani sex workers, 13 SNPs were identified in Exon 2 that encodes for the Ring domain. Of these, R136Q (10.8%), H43Y (4.84%) and V112F (1.21%) were the most common. R136Q was associated with resistance to HIV among sex workers (7), while the H43Y was found to accelerate HIV disease progression among

homosexual men enrolled in the Amsterdam Cohort studies (ACS) between October 1984 and March 1986 (25). Exon 2 of TRIMα encodes for most of the important protein components that contribute to its antiviral activity (Fig 2).

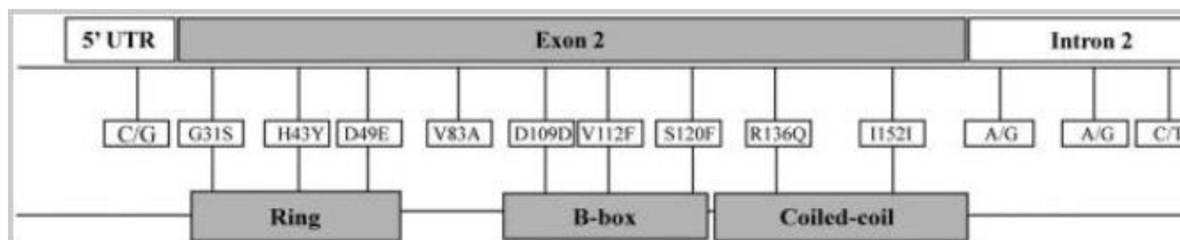

Figure 2: *Figure 2: TRIM5α exon 2 gene map showing SNPs that have been identified to influence HIV susceptibility and disease progression (17)*

Additionally, SNP2 rs16934386 T/C and SNP3 rs7127617 T/C located in Exon 1 and Intron 1 respectively were found to be associated with susceptibility to HIV infection. SNP5 (H43Y) and SNP7 (R136Q) located in the exon 2 were more in high-risk exposed HIV-1-uninfected individuals (HREU) and HIV-1 seronegatives (SN), and therefore thought to be protective against HIV infection (26). In another study, polymorphism in the promoter rather than the coding region including G-184A and C-737T reduced viral infectivity and were associated with increased TRIM5α expression levels (27). Tapping into the potential of TRIM5α HIV-1 protective polymorphisms poses a great opportunity for host-directed therapy development. However, research on TRIM5α polymorphism among Ugandan HIV infected individuals as well as its impact on viral suppression is scarce.

2.4. TRIM 5α and T cell function

Host cells express a wide range of pattern-recognition receptors (PRRs) that recognize various pathogen components called pathogen-associated molecular patterns (PAMPs) which may include lipids, lipoproteins, proteins and nucleic acids. PRRs such as Toll-like receptors (TLR) and RIG-I-like receptors (RLRs) activate intracellular signaling pathways that result in the induction of inflammatory cytokines (IL-2, IFNγ), chemokines and type I interferons (IFNs), as well as the upregulation of co-stimulatory molecules. Although TLR family is mostly expressed by antigen-presenting cells such as dendritic cells (DCs) and macrophages, recent studies show that TLR1, 2, 3, 4, 5, 7 and 9 are also expressed by T cells at varying levels (28). TLRs take up various locations on the cell depending on the PAMPS specificity. For example, TLR1, TLR2, TLR4–TLR6 and TLR11 located on the cell surface where they recognize extracellular

organisms. TLR3 and TLR7–TLR9 are expressed exclusively in intracellular vesicles such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, where they encounter microbial nucleic acids and trigger anti-viral innate immune responses by producing type I IFN and inflammatory cytokines.

Binding to TLR leads to dimerization that results in recruitment of TIR-domain-containing adapter proteins, including myeloid differentiation primary response gene 88 (MyD88). MyD88 is recruited on all TLRs except TLR 3 and activates NF-κB and mitogen-activated protein kinase (MAPK) to control inflammatory responses. TLR3 signals through TIR domain-containing adaptor inducing IFN-Beta (TRIF) to activate NF-kB as well as interferon regulatory factor 3 (IRF3)(29). TRIM 5 activates TAK1 via generation of K63-linked polyubiquitin chains and its overexpression activates signaling pathways that lead to the activation of both NF-κB and AP-1(6).

Of the intracellular TLRs, TLR3 recognizes viral double stranded RNA as well as its synthetic analog, dsRNA polyinosinic–polycytidylic acid (poly IC), TLR7 recognizes guanosine-rich and uridine-rich ssRNA derived from human immunodeficiency virus (6). Engagement or binding of TLR7 by its agonist/ligand, imiquimod is reported to enhance T cell function in acute viral infection while it causes anergy of $CD4^+$ T cells during chronic infection with RNA viruses such as HIV (30, 31), suggesting a possible viral suppression effect. Since Elite controllers are known to suppress HIV, we investigated $CD4^+$ T cell response upon engagement by TLR7 agonists, and correlated it with TRIM5a expression among Elite controllers and non-controllers. We assessed the expression of cytokines such as IL-2, IFNγ, IL-10, anergy markers such as PD1 and activation markers such as CD38.

Programmed Death 1(P-1) also known as CD CD279 is a It is a 55.0-kDa type I transmembrane glycoprotein of the CD28 superfamily. It adopts an immunoglobulin structure with an Ig variable (V) distal region and an Ig constant (C) proximal region in its extracellular domain(32) (Fig 3). It is expressed as an inhibitory receptor on T cells and its ligands PD-L1 and PD-L2 are that are mainly expressed on antigen presenting cells. D-1 plays as an immune checkpoint plays a major role in induction and maintenance of peripheral tolerance, dampening infectious immunity and tumor immunity, and protecting tissues from immune attack(33). In chronic HIV infection, PD-1 expression is reported to be associated with high viremia.

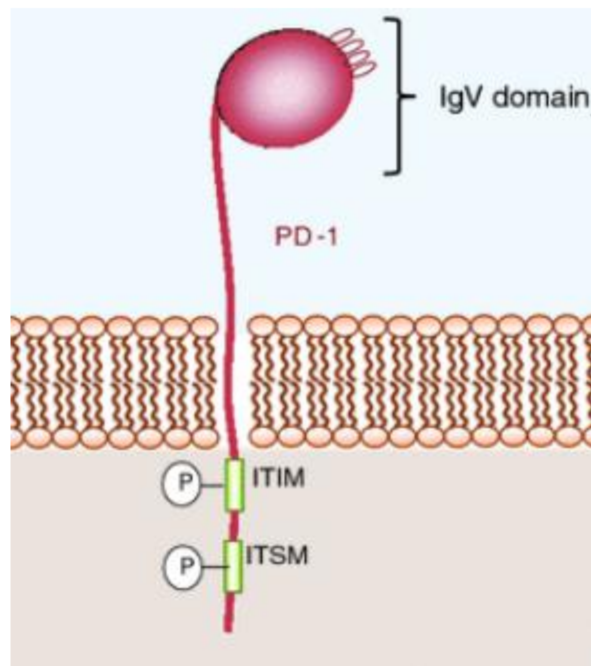

Figure 3: Programmed death-1 (PD-1) structure

2.5. Cylophillin A

CypA protein belongs to the cyclophilins family. In humans, the family has 16 members, the most ubiquitously expressed being CypA (34). The protein is encoded by the peptidyl prolyl isomerase A (PPIA) gene found on chromosome 7 (35). The protein has peptidyl prolyl isomerase activity that facilitates the isomerization of peptide bonds from trans-form to cis-form at proline residues (34, 35). CypA has multiple cellular functions including protein folding, trafficking, and T-cell activation among others (34). It has also been implicated in influencing susceptibility to HIV infection (36).

CypA interacts with the HIV viral Capsid (CA), a phenomenon that has raised controversial research findings among researchers. Some have documented that CypA-CA interaction increases susceptibility to HIV infection and disease progress (34). Others have documented that CypA inhibits HIV infection by potentiating the activity of TRIM5α. A study conducted to ascertain the mechanism of CypA dependent TRIM5α anti-HIV-1 activity found out that CypA inhibits nuclear import of the HIV pre-integration complex (9). Additionally, other researchers have found out that CypA could sense HIV-1 CA and subsequently initiate an interferon type I (IFN-I) response through activation of interferon regulatory transcription factor 3 (IRF3) (34).

Polymorphism in the non-coding region of peptidyl-prolyl isomerase A gene has also been reported to influence susceptibility to infection as well as the slow disease progress. Rits and colleagues in their study on polymorphisms in the regulatory region of the CypA gene and its

influence on the susceptibility to HIV-1 infection identified SNPs C1604G and A1650G in the promoter region. C1604G was associated with increased HIV progression (10) with CD 4+ cell count <200cells/ml (36), while SNP A1650G was associated with reduced HIV-1 infectivity (10). In another study, however, SNP A1650G was associated with increased HIV susceptibility as it associated more with seropositive participants compared to high-risk exposures to HIV-1 who remain uninfected (HREU) (36). Fig 3 shows SNPs in CypA gene that are associated with HIV disease progression.

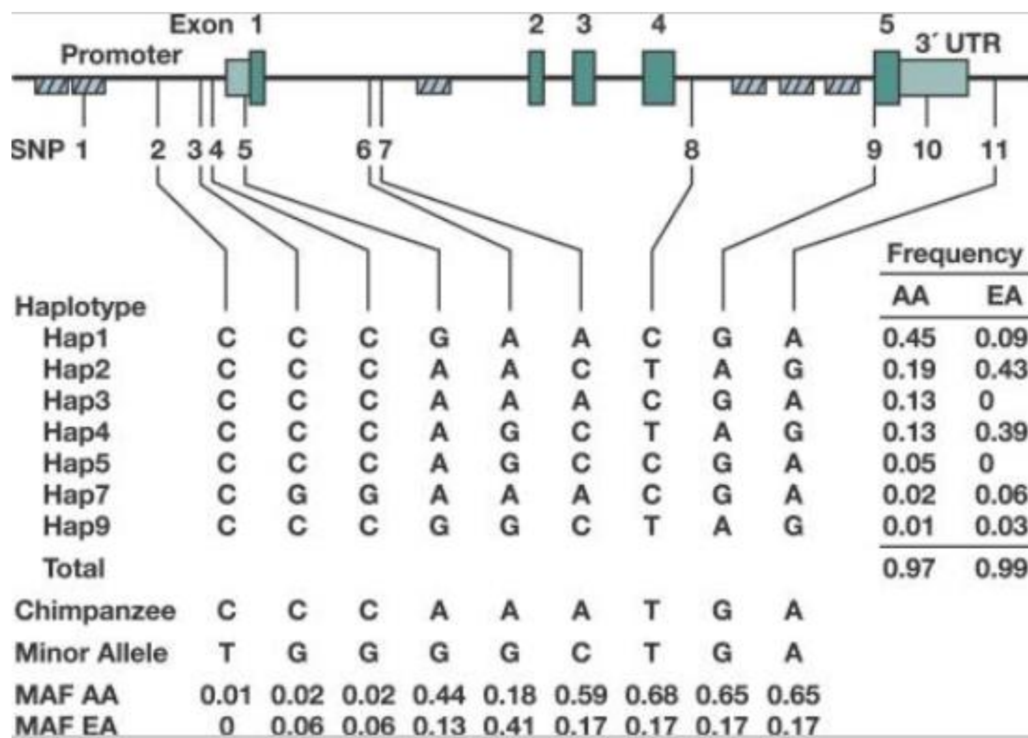

Figure 4: *Gene map of PPIA gene showing SNPs reported to influence HIV susceptibility and disease progression(35)*

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CHAPTER III

METHODOLOGY

3.1 Research Design

This was a nested case-control study utilizing PBMC samples from the Elite study cohort. The cases were elite controllers (undetectable viral load with >5 years in care) and the controls were non-controllers (HIV infected individuals well controlled on ART).

3.2 Study Area

The laboratory experiments were conducted at Makerere University College of Health sciences, Molecular and Immunology Laboratories located in Kampala district, upper Mulago Hill. The immunology Lab is a large research laboratory with the capacity to conduct various immunological assays including cell cultures, ELISAs, lateral flow assays among other immunological assays. It has a repository for sample storage, where the Elite study samples were stored. The molecular lab is also a large research facility with a capacity to do several molecular assays ranging from nucleic acid extraction to whole-genome sequencing. It houses the biorepository for the Human Hereditary and Health in Africa (H3Africa). The other molecular assays were conducted at CFAR labs at Joint clinical research center (JCRC) which is accredited by the college of American Pathologists (CAP).

3.3. Study Population

Samples from HIV-1 Elite controllers and Non-controllers obtained from the ELITE study cohort were utilized.

3.3.1 The Elite Study

The Elite study objective was to examine the role of host genes in T cell resistance to HIV among Elite and Viremic controllers in Uganda which was conducted at Makerere University Joint Aids Program (MJAP) ISS clinic. The clinic is located in Kampla, upper Mulago hill near Mulago Hospital complex and currently serves 14,492 HIV postive adults who routinely access HIV care. Under this study, clients were recruited basing on the following criteria;

3.3.1.1 Selection Criteria for the Parent Study

Inclusion

From the parent study, participants were selected basing on the following inclusion criteria;

Cases

- HIV-1 postive adults (≥18years) who have beeen comfirmed by HIV RNA PCR using Abbort real time HIV-1 Assay(Abbott Molecular, USA)
- ART naïve for ≥ 5 years with $CD4^+$ T cell count of ≥500cells/ml
- Have viral load of <50copies/ml
- Hemoglobin consentration >10g/dl
- Able to give written informed consent

Exclusion

- Active opportunistic infections e.g Pneumocystis jiroveci pneumonia (PJP), Tuberculosis(TB)
- Platelets <50
- Bleeding disorder

Control

- HIV-1 postive adults who are well controlled on ART. In this study, well controlled on ART means HIV positive individuals who have $CD4^+$ T cell count of >500 cells/ml and no opportunistic infections.

3.4. Selection Criteria for the Current study

3.4.1 Inclusion criteria:

- All samples with a storage viability ≥80%

3.4.2 Exclusion criteria

- All samples with a storage viability ≤80%

3.5 Sampling procedure

All the 21 samples that met the eligibility criteria for the study were included. This included 13 elite controllers and 8 non-controllers.

3.6 Summary of Methods

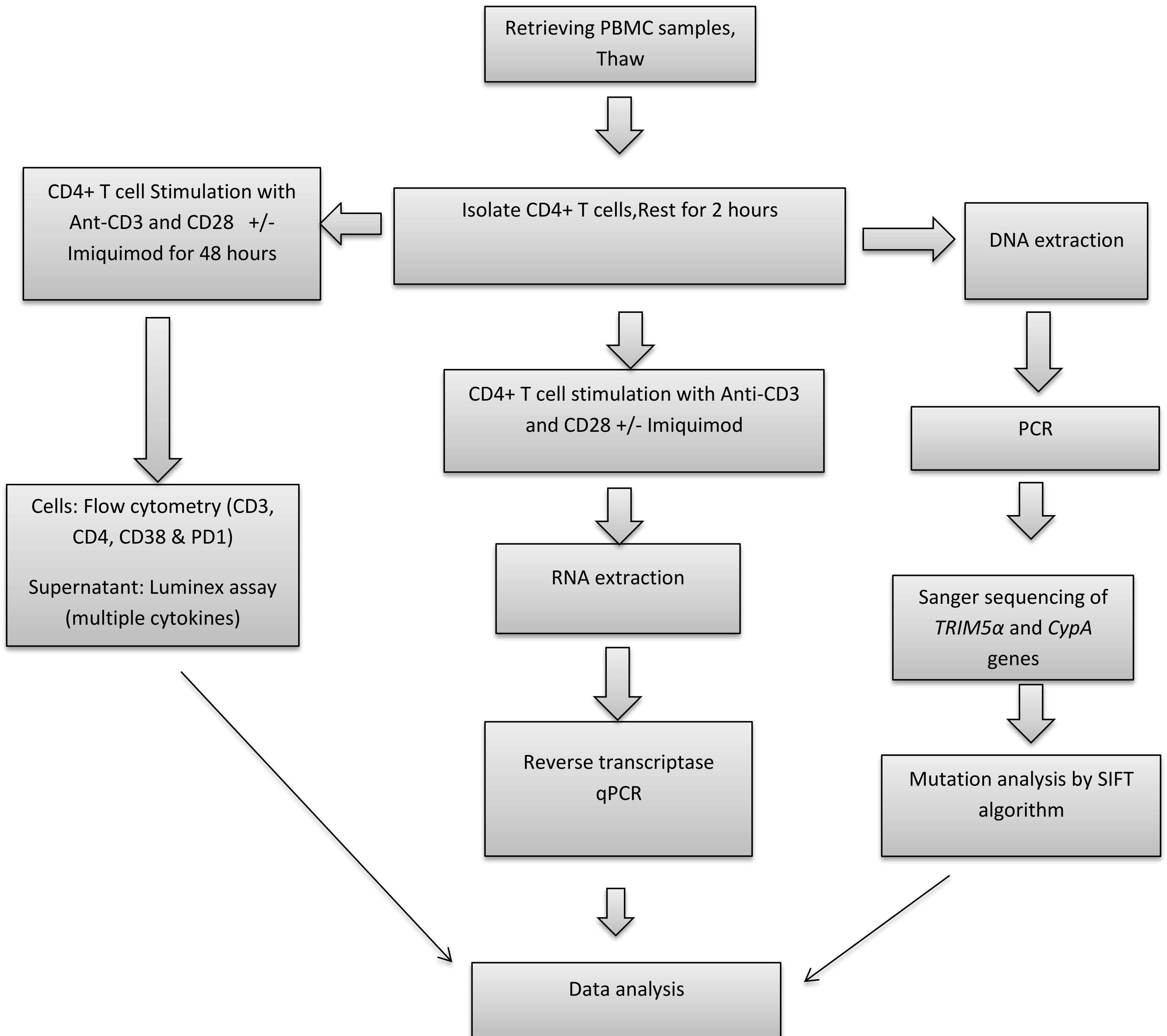


Figure 5: ***Summary and flow of the laboratory methods***

3.7 Data collection procedure

3.7.1. Sample Processing and Thawing

PBMCs were retrieved from liquid nitrogen and immediately thawed in a water bath set at 37^0C. The samples were transferred into 10ml of R-10 media and then centrifuged at 1500rpm for 10 minutes. The supernatant was decanted, and the pellet resuspended in 5ml R-10 media for counting. The cells were stained with trypan blue and counted using an automatic cell counter (Invitrogen, Carlsbad, CA, USA). 1ml of the sample was removed for DNA downstream procedures.

3.7.2. CD4+ T cell Isolation

The thawed PBMCS were subjected to $CD4^+$ T cell isolation using human $CD4^+$ T cell enrichment magnetic kit following the manufacturers intsuctions (StemCell Technologies, Vancouver, Canada). The cells were centrifuged at 1500rpm for 10 minutes, decanted and the pellet resuspended in 1ml of 2% FBS containing 0.5% EDTA. The samples were transferred into FACs tubes from where 50µl of the enrichment cocktail were added and then incubated at room temperature for 10 minutes. Thereafter, 100 µl of the magnetic beads were added and the sample incubated at room temperature for 5 minutes. The sample tube (lid removed) was then placed in the EasySep magnet and incubated at room temperature for 5 minutes. In one continous motion, the sample (isolated $CD4^+$ T cells) was poured into a second tube after the 5 minutes incubation. The isolated $CD4^+$ T cells were washed in 1ml PBS, centrifuged at 1500rpm for 10 minutes. These were resuspended in 2ml R-10 media, stained for counting with trypan blue and then incubated at 37^0C on a 24 well plate for 2 hours in a CO_2 incubator. The cells were also stained for purity using anti-CD3, and anti-CD4 and ran on a BD FACS Canto II (BD Biosciences, Franklin lakes, New Jersey, USA)

3.7.3. CD4+ T cell Stimulation

A 96-well plate coated with 100µl of 5µg/ml of Anti-CD3 (eBioscience Clone CD28.2) was incubated at 37^0C for 2 hours in CO_2 incubator. For negative control wells, 100µl of PBS was added. After the 2 hour incubation, the plated was bloated.

In each well, 100,000 cells from the sample was added and topped up with R-10 media containing 5 µg/ml of anti-CD28 (eBioscience clone OKT3), with or without 5 µg/ml of

Imiquimod (Invivogen, Cat tlrl-imqs) to make 200 µl per well. For negative control wells, 110µl of PBS was added. The plate was incubated at 37^0C for 48 hours in a CO_2 incubator.

3.7.4 Objective I Methods:

3.8.1 RNA extraction

RNA was extracted using Quick-RNA™ Whole Blood kit (Zymo Research, CA, U.S.A) following manufacturer's instructions. The $CD4^+$ T cell samples previously suspended in RNAlater were centrifuged at 10.000g for 1 minute and then decanted. The pellet was re-suspended in 300µl of DNA/RNA Shield™ then 30µl PK digestion buffer and 15µl Proteinase K added to the sample and mixed well. The mixture was incubated at 55°C for 30 minutes. After incubation, the sample was vortexed and then centrifuged at 16,000g for 2 minutes. The supernatant was transferred into RNase-free eppendoff tubes. To the supernatant, 350µl of RNA recovery buffer was added and mixed well, transferred into a Zymo-Spin™ IIICG Column in a Collection Tube and centrifuged at 16,000g for 30 seconds. To the filtrate, 700µl of 100% ethanol was added and mixed well. The mixture was transferred into a Zymo-Spin™ IC Column in a Collection Tube, centrifuged at 16,000g for 30 seconds and then the filtrate discarded. This was followed by DNase treatment to remove extra traces of DNA in the column. To achieve this, the column was washed with 400 µl RNA wash buffer and centrifuged at 16,000g for 30 sec and thereafter the filtrate discarded. A Mixture of 5µl DNase and 35µl DNA digestion buffer was made and added directly to the column matrix. The column was incubated at room temperature for 15 minutes. After DNase treatment, 400 µl RNA prep buffer was added to the column and centrifuged at 16,000g for 30 seconds. The filtrate was discarded, and 700 µl RNA wash buffer added to the column and centrifuge at 16,000g for 30 seconds. The filtrate was discarded, 400 µl RNA wash buffer added and then centrifuged for 2 minutes at 16,000g. The column was then transferred into an RNase free eppendoff tube, thereafter, 15µl DNase/RNase-free water added directly onto the column matrix to elute RNA. The eluted RNA was quantified by Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA, USA). The RNA was then immediately stored at -80^0C prior to downstream processes.

3.8.2 cDNA synthesis and Reverse transcription PCR

Extracted RNA was subjected to cDNA synthesis and real time PCR using QuantiTect Probe RT-PCR Kit (Qiagen Inc., Valencia, CA, USA) as described in the manufacturers instructions. A 50µL reaction volume was used for the PCR. Primers and probes used were obtained from a previous study (37) and are summarized in table 1. For each gene to be measured, separate mastermix containing; a) 25µL 2x QuantiTect Probe RT-PCR Master Mix(HotStarTaq® DNA

Polymerase, QuantiTect Probe RT-PCR Buffer, dNTP mix, including dUTP, ROX™ passive reference dye, and MgCl2), b) 2µL of each of the forward and reverse primers, c)1µL of the probe, d) 0.5µL of the QuantiTect RT Mix, and e) 12 µL of the RNase free water. In every PCR tube, 42µL of the master mix was added, and then 4µL of RNA template added in 3 tubes containing master mix of the 3 respective genes namely; GAPDH (reference gene), Cyclophillin A (target gene), and TRIM5α (target gene). For each of the genes, a negative control was added in each of the experiments containing mastermix and PCR water but no RNA template added. The PCR tubes were loaded into the Rotor gene Q real-time PCR machine (Quiagen Inc, Valencia, CA, USA) and PCR set using the following conditions; reverse transcription (cDNA synthesis) at 55^0C for 30 minutes, PCR initial activation at 95^0C for 15 minutes, followed by 45 cycles of denaturation at 94^0C for 15 seconds, and combined annealing and extension 60^0C for 60 seconds. Ct values for each gene were obtained ad analysed using delta CT relative quantification method to determine the fold change in gene expression.

Table 1: Primers and probes used in reverse transcriptase PCR to quantify expression of TRIM5α, CypA and GAPDH

Protein	**Primers and probes(Tamra)**
TRIM5α F	5'- TGCCTCTGACACTGACTAAGAAGATG
TRIM5α R	5'- GGGCTAAGGACTCATTCATTGG
TRIM5α Probe	5'- (6-Fam)AAGCTTTTCAACAGCCTTTCTATATCATCGTGTGATA
CypA F	5'- GGCCGCGTCTCCTTTGA
CypA R	5'- AATCCTTTCTCTCCAGTGCTCAGA
Probe	(6-Fam)TGCAGACAAGGTCCCAAAGACAGCAG
GAPDH F	5'- ACCCCTGGCCAAGGTCATC
GAPDH R	5'- AGGGGCCATCCACAGTCTTC
Probe	5'- (6-Fam)AGGACTCATGACCACAGTCCATGCCA

3.9 Objective II Methods:

3.9.1 DNA extraction

DNA was extracted using the Qiagen Blood Genomic DNA Kit (QIAamp DNA kit; Qiagen, Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions as used in the previous studies (38). 20 µl of Qiagen Protease was pippetted into the bottom of a 1.5 ml microcentrifuge tube, then 200 µl sample added. 200 µl Buffer AL was then added to the sample and mixed by pulse-vortexing for 15s. The mixture was incubated at 56°C for 10 min and centrifuged to remove drops from the inside of the lid. 200 µl ethanol (96–100%) were added to the sample, and

mixed again by pulse-vortexing for 15 s. After mixing, the tube was again centrifuged to remove drops from the inside of the lid. The reaction mixture was applied to the QIAamp Mini column, centrifuged for 6000g for 1 minute and the filtrate discarded. The column was placed in a clean 2ml collection tube. 500 µl of Buffer AW1 were then added to the QIAamp Mini column and centrifuged at 6000g for 1 minute. The tube containing the filtrate was discarded and the column placed in a new clean collection tube. 500µl Buffer AW2 was also added, centrifuged at 20,000g for 3 minutes and the tube containing filtrate discarded. The column was placed in a new collection tube, centrifuged at 20,000g for 1 minute and the tube containing filtrate discarded. The QIAamp Mini column was then placed in a clean 1.5 ml microcentrifuge tube and 200 µl Buffer AE added. The mixture was incubated at room temperature for 1 minute and then centrifuged at 6000xg for 1 min to elute DNA. The extracted DNA was stored at -80°C prior to PCR amplification.

3.9.2 PCR Amplification

3.9.2.1 Exon 2 of TRIM5α gene

PCR amplification of exon 2 of TRIM5α gene was carried out with 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 68°C for 45 s using SuperScript III platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) in the presence of 2X reaction buffer, 5mM MgCl with primers summarized in Table 2 as described in a similar study (26).

Table 2: Table 2: Primers for amplification of exon 2 of TRIM5α gene

Location	Primer
Exon 2, F	TGCAGGGATCTGTGAACAAG
Exon 2, R	CCATCTGGTCCCATTTTCTG

3.8.2.2 Cyclophillin A gene promoter

PCR amplification of the Cyclophilin A gene was carried out with 40 cycles of denaturing at 95 °C for 30 s, annealing at 65°C for 45 s, and extension at 68 °C for 45 s using SuperScript III platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) in the presence of 2X reaction buffer, 5mM MgCl with primers summarized in Table 3 as described in a similar study (39).

Table 3: Table 3: Primers used for amplification of Cyclophillin A promoter

Location	Primer

C1604G-F	GCACTGTCACTCTGGCGAAGTCGCAGAC
P4H-R	GCCGAGCACGTGCGTCGGACAGGAC

3.9.3 PCR Clean up

From all samples positive on gel electrophoresis that have a single band, 10ul was aliquoted into a new PCR tube and 2ul of ExosapIT reagent added. The tubes were then transferred into a thermocycler (Applied Biosystems, California, United States) and ran under the following conditions: 37^oC for 45 minutes, 80^oC for 45 minutes and held at 4 ^oC. Thereafter, PCR products were stored at -20^0C prior to Sanger sequencing.

3.11 Objective 3: $CD4^+$ T cell function assays

After 48-hour incubation in a CO_2 incubator, $CD4^+$ T cells were carefully harvested and transferred into FACs tubes. The samples were centrifuged at 1500rpm for 10 minutes, the supernatant kept for cytokine assay while the cells used for downstream processes of flow cytometry. The pellet was re-suspended in 400µl of FACs buffer (1% FBS) then centrifuged at 1800 rpm for 5 minutes, decanted and the pellet stained with 1µl Zombie Aqua for live dead (Biolegend, San Diego, CA, USA). The samples were incubated in the dark at room temperature for 10 minutes. Thereafter, the samples were stained with 5 µl of Anti-CD3, Anti-CD4, Anti-CD38, and anti-PD-1 on Percyp Cy 5.5, APC, PE Cy7, and BV421 (all from BD Biosciences) respectively. The stained samples were incubated in the dark at room temperature for 30 minutes and then washed with 400µl of FACS buffer. The pellet was re-suspended in 400µl of FACs buffer and acquired on a FACSCanto II (BD Biosciences, Franklin Lake, New Jersey, USA) for analysis. The supernatant is currently stored at -80^0C awaiting cytokine assay.

3.12 Data Management

3.12.1 Data entry

Data was entered in excel and exported to GraphPad prism v8 for analysis.

3.12.2 Data analysis

$CD4^+$ T cells were analyzed on an 8-laser FACS Canto II (BD Bioscience). Approximately 50,000 events were recorded per specimen. In addition, antibody capture beads (BD Bioscience) were used for compensation and prepared individually by separate staining of all the antibodies used in the experiment. FlowJo X 10.6 (Treestar) was used for gating analysis, and statistical analysis was performed with GraphPad Prism 6.0. For mRNA quantification, relative quantification using the obtained CT value was done using the delta CT method. Statistical

differences between the different groups were determined using the unpaired t-test in Graph pad prism v8.

3.12.3 Data security

Hardcopy data was stored under key and lock, while soft copy data was stored in a password-protected laptop.

3.12.4 Data Dissemination

Findings from the study will be submitted to the department of medical microbiology for the award of MSc. Immunology and clinical microbiology. It will also be published in peer-reviewed journals and presented at various scientific meetings. Sequences of the respective genes will also be submitted to databases.

3.13 Ethical Consideration

Ethical clearance was obtained from the School of biomedical sciences Higher Degree Research and Ethics Committee (SBS-HDREC) prior to the study. Administrative clearance was sought from the Elite Study before samples were used.

CHAPTER IV

RESULTS

4.1 Demographic characteristics of participants

A case-control study of 21 HIV-1 chronically infected individuals was conducted. These included 13 elite controllers HIV plasma viral load <50 viral RNA (vRNA) copies ml^{-1}) and 8 non controllers (ART controlled) whose demographic characteristics including years in care, CD4 counts, blood pressure and Body Mass Index (BMI) are summarized in table 4.

Table 4: Table 4: Demographic and clinical characteristics of study participants

PT ID	Age	Sex	CD4 count[1]	Duration in Care (Years)	VL	Months between VLs	Systolic BP	Diastolic BP	BMI[2]
Elite controllers									
1	53	F	1245	10	Undetectable	8	129	93	33.9
3	32	F	1008	5	Undetectable	9	124	77	31.8
4	38	F	919	9	Undetectable	12	104	68	18.9
7	36	F	1188	7	Undetectable	8	103	80	38.5
13	56	M	833	7	Undetectable	9	101	64	17.2
15	42	F	909	5	Undetectable	9	missing	missing	31.8
16	30	F	1050	5	Undetectable	10	102	73	29.3
21	37	F	728	6	Undetectable	9	127	74	23.9
22	39	F	650	9	Undetectable	9	missing	missing	Missing
35	40	F	994	10	Undetectable	9	141	90	32.3
36	41	M	778	9	Undetectable	12	128	81	25.2
37	37	F	1063	6	Undetectable	8	121	90	26.1
42	28	F	653	8	Undetectable	10	missing	missing	Missing
Non-controllers									
2	40	M	920	6	10500	6	101	74	27.2
8	41	F	1192	6	2840	10	109	78	37.5
11	40	F	940	5	10800	15	119	83	26.3
18	29	F	747	5	14800	8	103	72	
25	43	F	781	8	2310	8	130	90	32.7
28	38	F	589	5	75100	10	117	81	21.4
30	42	F	1021	8	5250	6	118	59	21.3
31	41	F	852	10	2850	7	103	67	30.2

[1]Baseline CD4 at time of recruitment [2]BMI denotes body mass index

4.2 Gene Expression

4.2.1 qPCR

Reverse transcriptase PCR were successful, with clear sigmoid curves formed on qPCR.

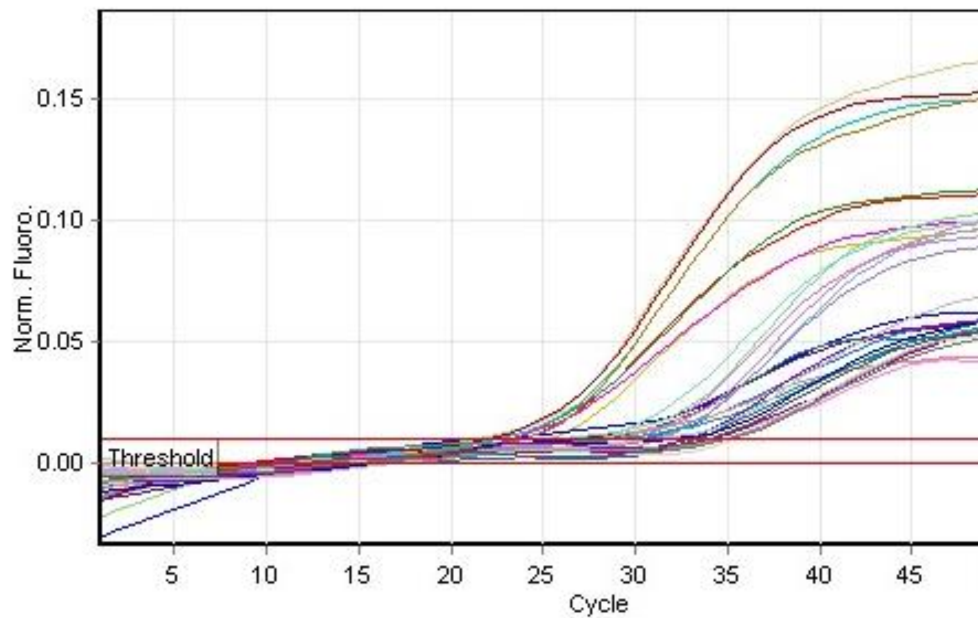


Figure 6: *Figure 5: Clear sigmoid curves shown on qPCR*

4.2.2 TRIM5α gene expression

There was overall high expression of TRIM5α among HIV-1 elite controllers, with the effect most pronounced among cells stimulated with Anti-CD3/CD28 (Fig 6). However, the difference was not statistically significant (p=0.669).

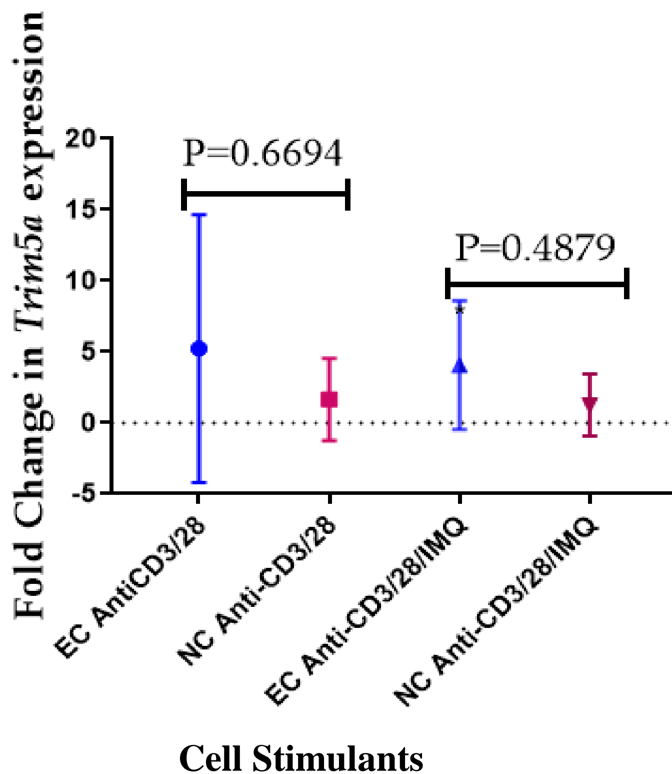


Figure 7: Fold change in expression of TRIM5α gene among HIV-1 elite and non-controllers in Uganda.

We conducted reverse transcription real time PCR to quantify TRIM5α gene expression in CD4+ T cells of elite controllers compared to those of non-controllers. P-values for fold change in gene expression induced by various stimulants were obtained by t-test.

4.2.3 Cyclophillin A gene expression

Elite controllers expressed more cyclophillin A particularly in Anti-CD3/CD28/Imiquimod stimulated cells compared to the non-controllers (Fig 7). Still, this difference in expression was not statistically significant (p=0.8632). There was considerably more expression of cyclophillin A when cells were stimulated with CD3/28 in addition to Imiquimod compared to CD3/28 alone.

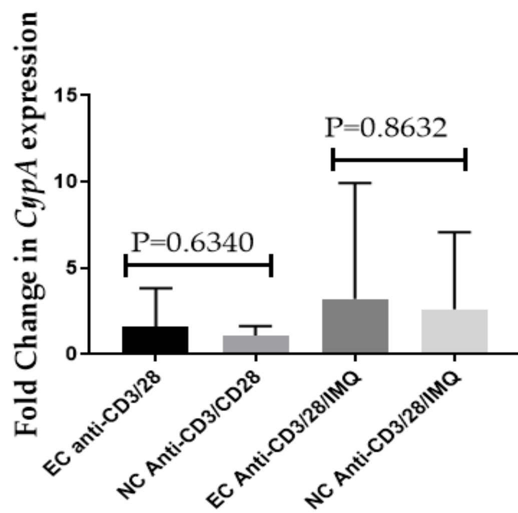


Figure 8: Fold change in expression of cyclophillin A gene among HIV-1 elite and non-controllers i Cell Stimulants

We conducted reverse transcription real time PCR to quantify Cyclophillin A gene expression in CD4+ T cells of elite controllers compared to those of non-controllers. P-values for fold change in gene expression induced by various stimulants were obtained by t-test.

4.3.1: DNA analysis

DNA extraction was successful, and the target for both genes was effectively amplified (Fig 8a and b)

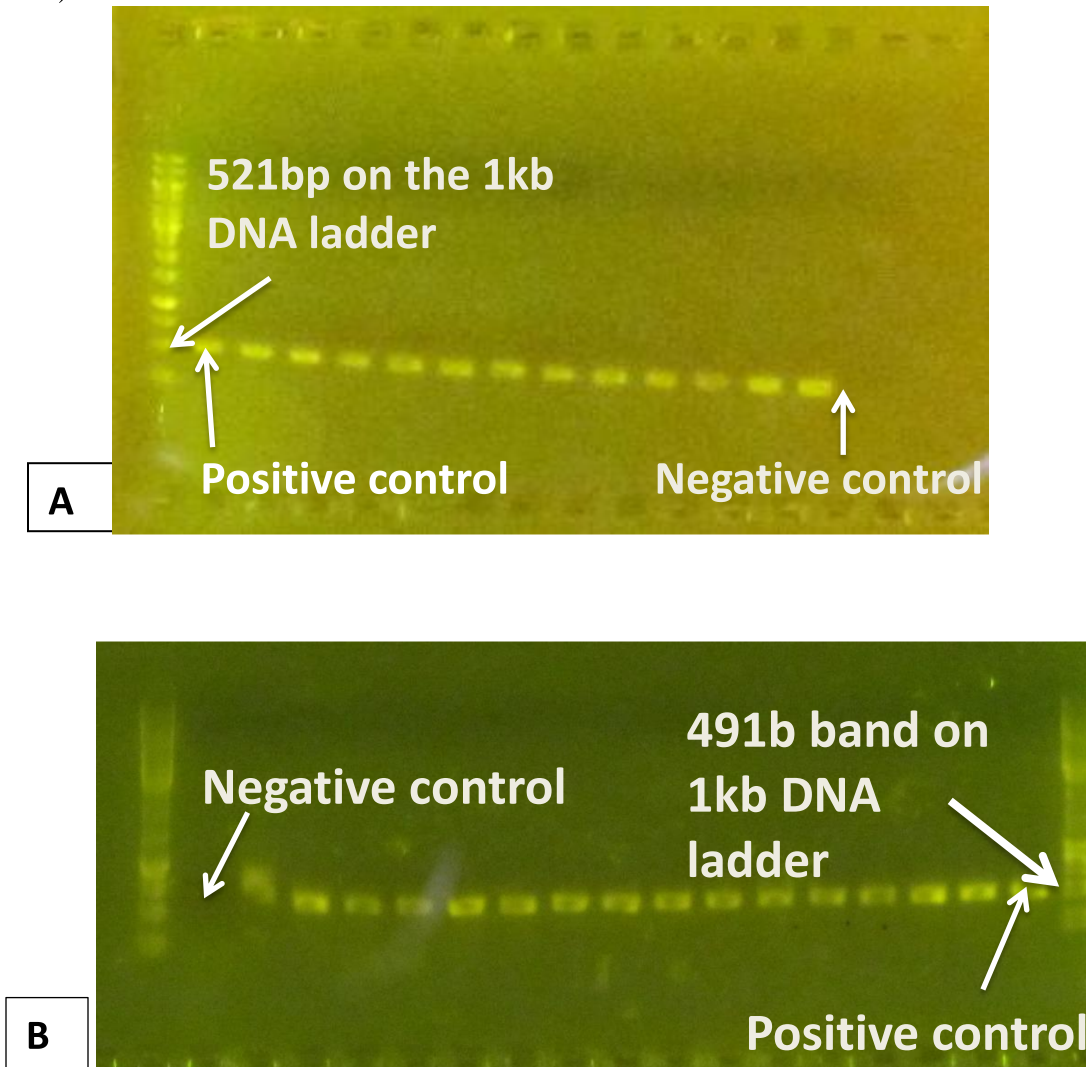


Figure 9: A) Gel image of TRIM5α gene with target band of 521bp Gel and B) Image of Cyclophillin A gene with a target DNA band of 491 base pairs.

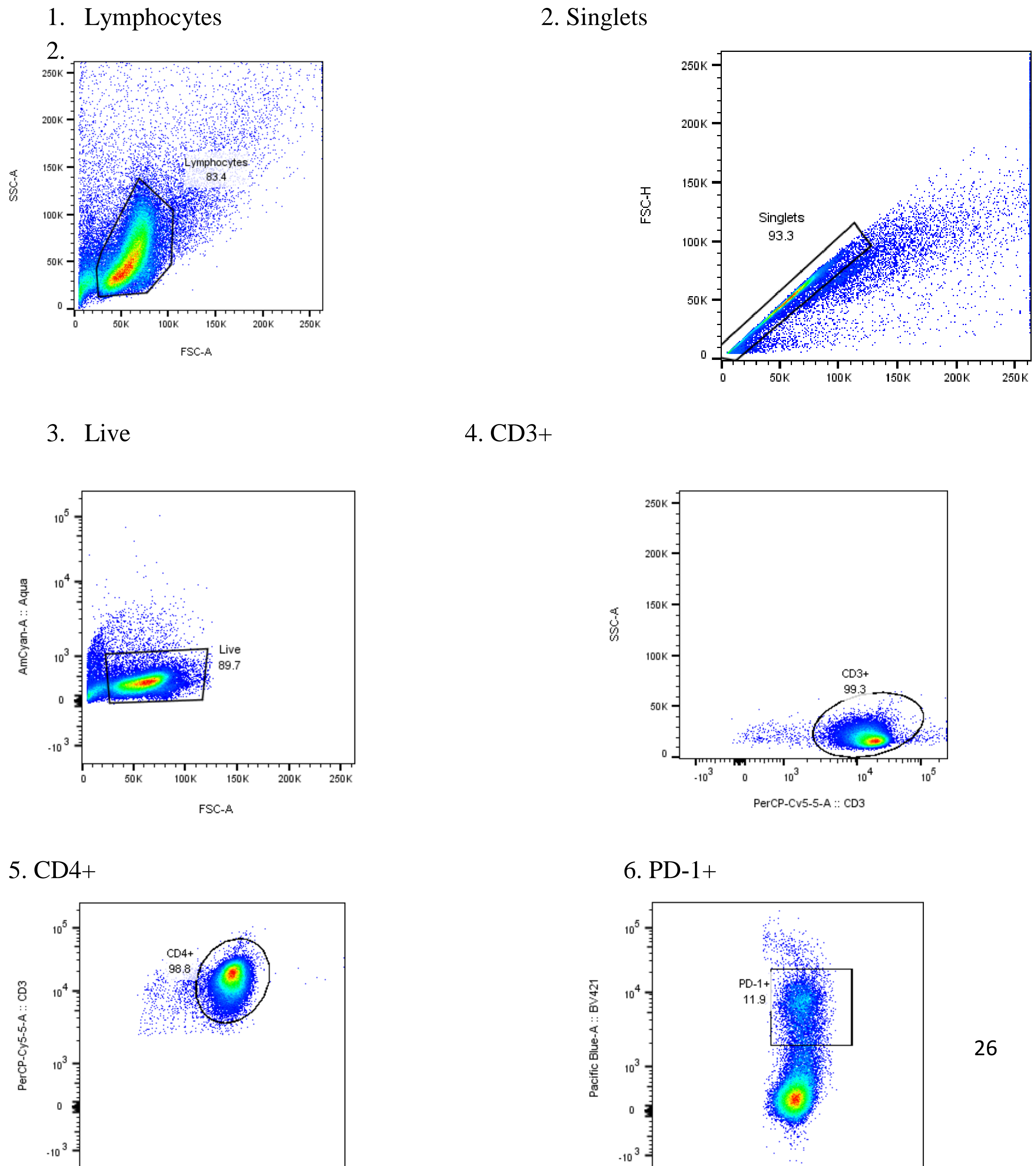
The images were obtained by gel electrophoresis of PCR amplification products of the TRIM5α exon 2 and CYCLOPHILLIN A promoter regions.

4.4. CD4$^+$ T cell function and HIV viremia

To establish the CD4$^+$ T cell functions that distinguish progressive from controlled HIV infection, we cultured CD4$^+$ T cells from the respective groups under four conditions namely; a) unstimulated, b) anti-CD3 and anti-CD28, c) anti-CD3, anti-CD28 and Imiquimod (IMQ), and d) Imiquimod alone. Imiquimod is a TLR-7 ligand, that mimics HIV stimulation of the cells. After 48-hour incubation, cells were stained for activation and exhaustion markers (CD3, CD4, CD38, and PD-1).

4.4.1. Gating strategy

After compensation in Flow jo software, data was analyzed using the following gating strategy (Fig 9)



7. CD38

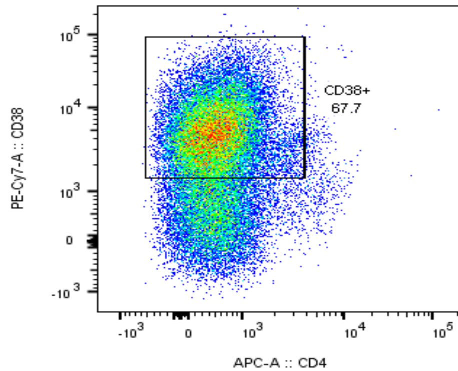


Figure 10: Gating strategy used for data analysis in Flow Jo software

From the Lymphocytes, we gated out single cells followed by the live cells. From the live cells, we gated out CD3+ cells, then CD4+ cells followed by those expressing PD-1. CD4+ cells were also gated for CD38.

4.4.2 PD-1 expression

Overall, high PD-1 expression was noted among HIV-1 non-controllers compared to the elite controllers. Non-controllers significantly expressed more PD-1 when stimulated with Anti-CD3/CD28 (p=0.0315), and even when at baseline (unstimulated) (p=0.0401) compared to the elite controllers. Expression was still more when stimulated with AntiCD-3/CD28 along with Imiquimod (p=0.0565) and when imiquimod was used alone (p=0.0907), although this difference was not statistically significant. Findings are summarized in Fig 10 below.

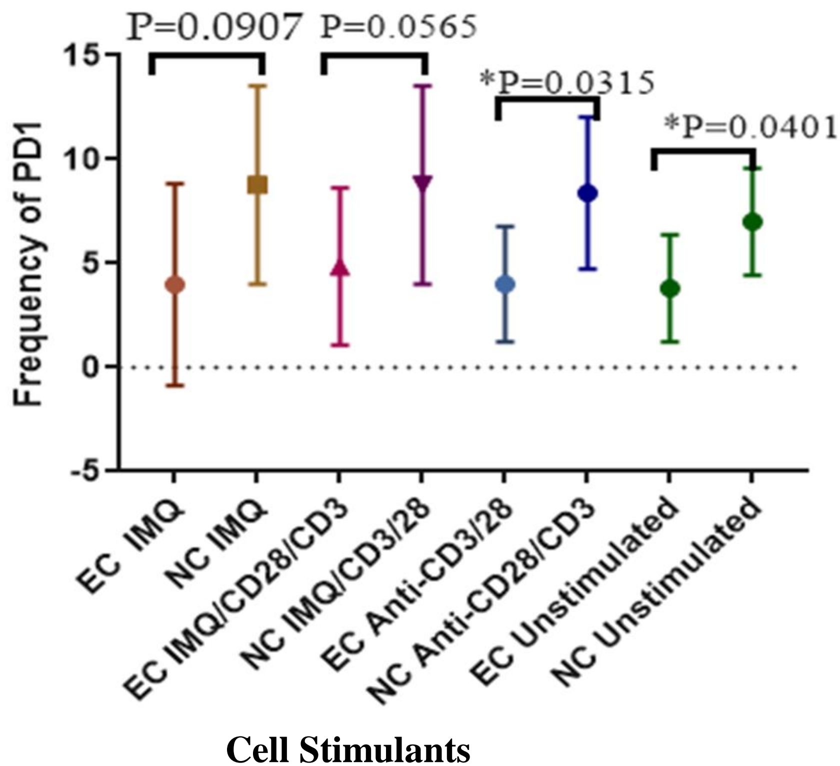
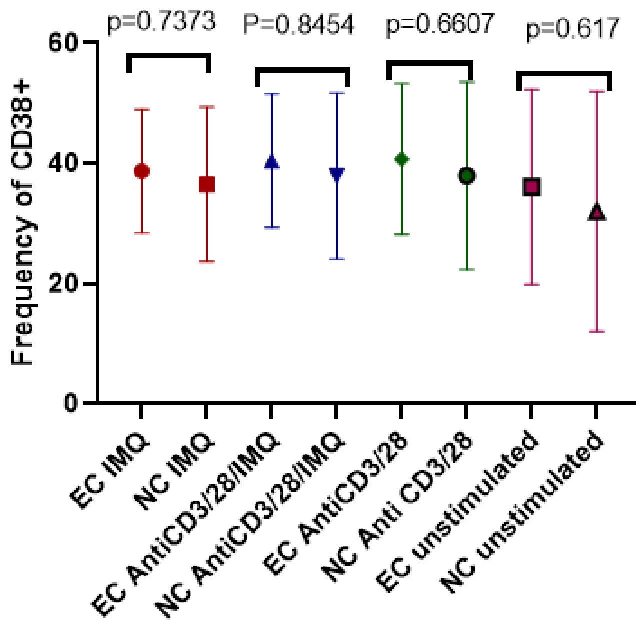


Figure 11: PD-1 expression among HIV elite controllers (13) and non-controllers (8)

Cells were stimulated with Imiquimod (IMQ), Anti-CD3/28/Imiquimod, Anti-CD3/CD28 and unstimulated (PBS), cultured for 48 hours and then stained for surface markers analyzed for flow cytometry. P-values were obtained by t-test

4.4.3 CD38 expression

CD38 was expressed more among elite controllers with the highest expression in the anti-CD3/28 group. However, these differences in expression among elite controllers and non-controllers were not statistically significant (Fig 11).



Cell Stimulants

Figure 12: CD38 expression among HIV elite controllers (13) and non-controllers (8).

Cells were stimulated with Imiquimod (IMQ), Anti-CD3/28/Imiquimod, Anti-CD3/CD28 and unstimulated (PBS), cultured for 48 hours and then stained for surface markers analyzed for flow cytometry. P-values were obtained by t-test

CHAPTER V

DISCUSSION

5.1 Gene expression

5.1.1 Cyclophillin A gene expression

Cyclophillin A has long been reported as a co-factor required for HIV infection(10, 40). However, the discovery of TRIM5-CypA fusion proteins as restriction factors together with Rhesus TRIM5(41) has motivated the research agenda in this area. In this present study, we investigated the potential role of TRIM5α and Cyclophillin A in the viral suppression phenotype exhibited by HIV-1 elite controllers in Uganda. Our study findings show that TRIM5α and Cyclophillin A are highly expressed among elite controllers compared to the non-controllers. However, this difference was not statistically significant(TRIM5α p=0.6694 and CypA p=0.6340). These findings agree with those from previous studies(40). Vigneault et al in their transcriptional profiling study of $CD4^+$ T Cells among HIV-1 patients noted that gene transcripts known to be involved in intrinsic cellular defense against retroviruses, such as the TRIM, tetherin/BST2 , cyclophilin A, and other genes were not differentially expressed among elite controllers compared to ART controlled HIV positive individuals (42). These findings could mean that viral suppression effect exhibited by elite controllers could be due to other mechanisms, not necessarily increased expression of the respective genes. Nonetheless, other studies have found a correlation between elevated expression of Cyp A and HIV disease progression(43).

Regarding Cyclophillin A influence in HIV infection, various models have been postulated (9, 42). One model suggests increased cyclophillin A (CypA) binding to the capsid increases sensitivity to innate sensing (44), and also inhibits nuclear import and promotes TRIM5α binding (9). However, another proposes that CypA binding coordinates virus capsid uncoating, reverse transcription, and nuclear import of the pre-integration complex (45), all to minimize the exposure of viral nucleic acids to cytosolic sensors, thus promoting HIV infectivity. Whereas it is clear that HIV-1 utilizes a variety of mechanisms to evade T cell activation, divisive questions still exist. These controversial reports could be due to the use of different cell types, and the differential use of clinical HIV-1 isolates, replication-competent lab strains, or pseudotyped virus. It is essential to confirm experimental findings with primary cells that closely demonstrate *in vivo* HIV infection. However, we note that our study was limited by number of study participants, therefore, additional studies will be necessary to analyze expression signatures of HIV-1 resistance or dependence genes in large study cohorts.

5.1.2. TRIM5α Expression

There was no significant difference in TRIM5α gene expression among elite controllers and non-controllers seen in our study. These findings are in agreement with findings of Vigneault et al (2011) who reported no differential expression of genes involved HIV restriction among elite controllers and ART treated HIV individuals (42). In another study to investigate the effect of levels of TRIM5α expression on prevention or control of HIV-1 infection in South Africa reported that TRIM5α mRNA levels were lower among HIV+ individuals compared to uninfected subjects and that seroconverters had lower pre-infection TRIM5α mRNA levels compared to non-seroconverters. The study further showed that TRIM5α mRNA levels did not change significantly after infection, and that there was no correlation between expression levels and HIV viral loads or CD4 counts (46). Findings in this study are comparable to our findings and could mean that high expression of TRIM5α is associated with reduced susceptibility to HIV-1 infection, but TRIM5α expression levels do not contribute to the control of primary HIV-1 viremia.

However, other studies have demonstrated that genes of the intrinsic cellular defense against retroviruses (TRIM5α, TRIM22, TRIM19/PML, APOBEC3G, APOBEC3F, APOBEC3H, PPIA/Cyclophilin A, BST2/Tetherin) are all upregulated with increasing viral load, a finding that is consistent with their general dependence on the interferon pathways (47). The controversy in these findings could be due to the difference in methods used. For example, the South African study measured levels of TRIM5α expression in using quantitative real time PCR while Rotger et al's study utilized Human-6 expression bead chips (Illumina) to measure expression levels. Whether TRIM5α is differentially expressed among elite and non-controllers of HIV remains a controversial subject that requires further investigations.

Furthermore, in individuals that have the HLA B27 or B57 allele, HIV-1 control is associated with mutations in viral proteins that are a product of immune pressure from cyctotoxic T cells. Accordingly, HIV viral proteins in these subjects are more sensitive to detection by TRIM5α. Consequently, cells have decreased permissiveness to subsequent HIV-1 infections, an occurrence that could contribute to the decreased disease progression observed in these HIV elite controllers (48, 49). Taken together, our findings could mean that TRIM5α ability to detect HIV-1 virus contributes viral suppression as opposed to high expression of the protein.

5.2 PD-1 Expression

Increased expression of inhibitory receptors such as PD-1 on T cells is linked to poor control of chronic viral infections and cancers. Accordingly, inhibitory receptor blocking agents are being aggressively studied for potential therapeutic benefits in chronic viral infections and cancers (50, 51). However, most of these studies have been done in $CD8^+$ T cells, as opposed to $CD4^+$ T cells which are normally infected by the virus, and their response influence the anti-viral activity of CD8+ T cells during HIV infection. In this study, we demonstrate that HIV-1 non-controllers have significant elevation in PD-1 expression (p=0.003) on $CD4^+$ T cells which is much more when cells are stimulated with Anti-CD3/CD28 and Anti-CD3/CD28 plus Imiquimod, a TLR-7 ligand. These findings of increased levels of $PD-1^+$ $CD4^+$ T cells among non-controllers correlate with HIV disease progression, and are consistent with previous studies of HIV+ adult subjects (52, 53). More so, PD-1 expressing $CD4^+$ T cells have been reported to be enriched for persistent HIV during ART therapy (53). This could explain the high viral loads of non-controllers in our study despite the ART therapy (table 4).

PD-1 has been reported as one of the inhibitory co-receptors expressed on exhausted $CD4^+$ T cells that show progressive loss of effector functions (52), which eventually lead to poor quality CD8+ T cell response. Velu and others in their study about the role of PD-1 co-inhibitory pathway in HIV infection noted that PD-1 high $CD8^+$ T cells lack the expression of CD28 co-receptor, effector functions including perforin and granzyme-B secretion and express low levels of CCR7 and IL-7 receptor which are important in maintenance of memory T cells (54). Our findings and that of previous scholars taken together could mean that high viremia among non-controllers may be attributed to high PD-1 expression. Additionally, engagement of TLR7 ligand (Imiquimod) that mimics HIV engagement in our study showed increased expression of PD-1 (p=0.0536). This could be due to the fact that engagement of TLR 7 induces calcium-NFATc2-driven CD4+ T cell anergy, which leads to expression of inhibitory receptors and decreased production of pro-inflammatory cytokines among non-controllers as demonstrated by a previous study(31). This could imply that HIV recognition by TLR 7 causes a cascade of events leading to calcium-NFATc2-driven $CD4^+$T cell anergy. However, we are further going to investigate the cytokines produced by these cells to see if cytokine production is affected by TLR 7 engagement.

Furthermore, gene expression profiles from HIV-specific T cells show that PD-1 upregulates a transcription factor basic leucine transcription factor ATF-like (BATF) which then impair T cell proliferative and cytokine production abilities. Silencing BATF in T cells from chronic viremic patients reverses the HIV-specific T cell function (55). Gay and colleagues in their study to

evaluate the immune exhaustion reversal with an anti-PD1 antibody demonstrated that the immunologic checkpoint inhibitor (BMS-936559) single dose infusions enhanced HIV-1–specific immunity in study participants (56). This further supports the role of PD-1 in HIV disease progression, justifying its high expression among the non-controllers compared to the elite controllers.

Interestingly, elite controllers in our study expressed significantly low PD-1 levels compared to the non-controllers. These findings are in agreement with those of Noyan et al where they demonstrated that elite controllers are able to maintain low level of $CD4^+$ T cell exhaustion as demonstrated by low PD-1 expression despite the years of HIV replication without treatment (57). Moreover, the elite controllers are reported to express PD-1 on both $CD4^+$ and $CD8^+$ T cells in levels that are similar to those found in HIV-uninfected individuals (58). These findings could mean that inhibitory receptor levels are not associated with excessive immune activation in elite controllers, rather a special mechanism elite controllers use to maintain 'normal state' during chronic HIV infection. These further support the use of anti-PD-1 agents as potential therapeutics in HIV treatment.

5.3 Conclusion

In summary, expression of PD-1 was significant among non-controllers compared to the elite controllers and it correlates with HIV disease progression. This reveals that elite controllers are able to maintain low expression of inhibitory receptors on $CD4^+$ T cells, despite ongoing viral infection for a prolonged period of time. Additionally, these results indicate that low $CD4^+$ T cell exhaustion is associated with effective control of HIV-1, which could be useful as a potential therapeutic target in HIV treatment. Furthermore, there was slightly higher TRIM5α and cyclophillin A gene expression among elite controllers as opposed to non-controllers although this difference was not statistically significant. This could imply that the elevated levels of genes involved in cellular intrinsic protective mechanisms against HIV may play a role in viral suppression exhibited by elite controllers and this effect needs to be investigated further with a large cohort of participants.

5.4 Recommendations

This study highlights a need for further investigation into the precise role of the PD-1 pathway in elite controllers to shade more light on the use of PD-1 blockers as potential adjunct therapies in HIV treatment.

There should be a more extensive study in the expression patterns and genetic variations in TRIM5α and cyclophillin A genes among elite controllers to elucidate their role in viremic control phenotype exhibited by the elite controllers.

5.5 Limitation of the study

The study utilized a small sample size whose findings may not be generalized to the entire population.

The study also used relative quantification method of gene expression which could not give copy number of the genes expressed. More studies done in this area should explore use of absolute quantification methods that are able to tell the exact copies of the gene.

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Appendix

Appendix 1: Ethical approval for the current study

P.O. Box 7072 Kampala, Uganda
E-mail: biomedicalresearch62@gmail.com
Phone: +256752575050
Fax: 256 414 532204

COLLEGE OF HEALTH SCIENCES
SCHOOL OF BIOMEDICAL SCIENCES
HIGHER DEGREES RESEARCH AND ETHICS COMMITTEE

19th Nov 2018

File: SBS-605

To: Ms. Amanya Sharon Bright
Principal Investigator
Department of Microbiology
Makerere University

Category of review
[X] Initial review
[ ] Continuing review
[ ] Amendment
[ ] Termination of study
[ ] SAEs

Decision of the School of Biomedical Sciences Higher Degrees Research and Ethics Committee (SBS-HDREC) following its 87th REC meeting held on 18th Oct 2018.

In the matter concerning the review of a research proposal entitled **"Variations in Trim5α and Cyclophilin A genes among HIV-1 elite controllers and non-controllers in Uganda" SBS-605**

The investigator has met all the requirements as stated by the **SBS-HDREC** and therefore, the protocol is **APPROVED.**

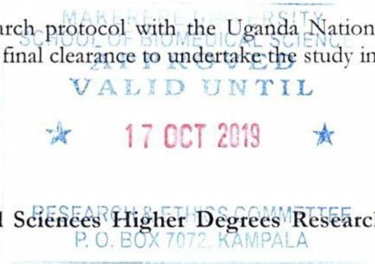
The approval granted includes all materials submitted by the investigator for the **SBS-HDREC** review unless otherwise stated; and is valid until **17th Oct 2019.**

Any problems of a serious nature related to the execution of the research protocol should be promptly reported to the **SBS-HDREC**, and any changes to the research protocol should not be implemented without approval from the **SBS-HDREC**, except when necessary to eliminate apparent immediate hazards to the research participant(s)

Please note that the annual report and the request for renewal where applicable should be submitted to the **SBS-HDREC** office at least six (**6**) weeks before expiry date of approval.

You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

Signed.



Dr. Erisa Mwaka
Chairperson, School of Biomedical Sciences Higher Degrees Research and Ethics Committee.

Appendix 2: Ethical approval for the parent study

MAKERERE UNIVERSITY

P.O. Box 7072 Kampala, Uganda
E-mail: irbbiomedicalsciences@gmail.com
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COLLEGE OF HEALTH SCIENCES
SCHOOL OF BIOMEDICAL SCIENCES
HIGHER DEGREES RESEARCH AND ETHICS COMMITTEE

24th Nov 2016

SBS-372

To Mr. Kayongo Alex
Principal Investigator
Department of Medicine

Category of review
[X] Initial review
[ ] Continuing review
[ ] Amendment
[ ] Termination of study
[ ] SAEs

Decision of the School of Biomedical Sciences Higher Degrees Research and Ethics Committee (SBS-HDREC) at its 60th REC meeting held on 25th Aug 2016.

In the matter concerning the review of a research proposal entitled, **"Host genes responsible for T-cell resistance to HIV in Ugandan elite and viremic controllers." SBS-HDREC - 372**

The investigators have met all the requirements as stated by **SBS-HDREC** and therefore, the protocol is **APPROVED.**

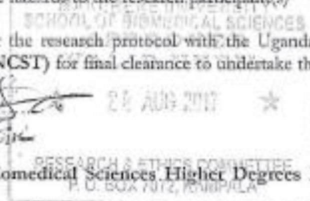
The approval granted includes all materials submitted by the investigators for **SBS-HDREC** review unless otherwise stated; and is valid until **24th Aug 2017.**

Please note that the annual report and the request for renewal where applicable, should be submitted six weeks before expiry date of approval.

Any problems of a serious nature related to the execution of the research protocol should be promptly reported to the **SBS-HDREC**, and any changes to the research protocol should not be implemented without approval from **SBS-HDREC**, except when necessary to eliminate apparent immediate hazards to the research participant(s).

You are required to register the research protocol with the Uganda National Council for Science and Technology **(UNCST)** for final clearance to undertake the study in Uganda.

Signed



Dr. Erisa Mwaka
Chairperson, School of Biomedical Sciences Higher Degrees Research and Ethics Committee.

1

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS 2169

3rd April 2017

Prof. Moses Joloba
Principal Investigator
Makerere University
Kampala

Dear Prof. Joloba,

Re: Research Approval: **Host Genes Responsible for T Cell Resistance to HIV in Uganda Elite and Viremic Controllers**

I am pleased to inform you that on **20/02/2017**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period **20/02/2017** to **20/02/2018.**

Your research registration number with the UNCST is **HS 2169**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority.
4. Unexpected events involving risks to research subjects/participants must be reported promptly to the UNCST. New information that becomes available which alters the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. A progress report must be submitted electronically to UNCST within four weeks after every 12 months. Failure to do so may result in termination of the research project.

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1.	Research Proposal	English	3.0	January 2017
2.	Storage Consent Form	English and Luganda	3.0	January 2017
3.	Informed Consent Document for Participation	English and Luganda	3.0	January 2017
4.	Genetics Consent Form	English and Luganda	3.0	January 2017

Yours sincerely,

Hellen N. Opolot
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Copied to: Chair, Makerere University School of Biomedical Sciences, Research Ethics Committee

LOCATION/CORRESPONDENCE

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P. O. Box 6884
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COMMUNICATION

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EMAIL: info@uncst.go.ug
WEBSITE: http://www.uncst.go.ug

Appendix 3: Waiver of consent

COLLEGE OF HEALTH SCIENCES

SCHOOL OF BIOMEDICAL SCIENCES

HIGHER DEGREES RESEARCH AND ETHICS COMMITTEE

30th Nov 2018

Ms. Amanya Sharon Bright
Department of Microbiology

Dear Sharon,

RE: APPROVAL OF CONSENT WAIVER

In your letter dated 4th Dec 2018, you requested the committee to grant you a waiver of consent for the study entitled **"Variations in Trim5α and Cyclophilin A genes among HIV-1 elite controllers and non-controllers in Uganda"**

The committee has waived the requirement for consent because it has noted that the study will be using samples obtained under the study entitled "**Host genes responsible for T-cell resistance to HIV in Ugandan elite and viremic controllers"** whose participants consented to future use of their samples and the PI has also granted permission to access and use the samples. Please ensure that the study is conducted according to the established ethical guidelines.

On behalf of the committee, I am glad to inform you that the committee has granted a waiver of the informed consent process.

Yours sincerely,

Dr. Erisa Mwaka
Chairperson, School of Biomedical Sciences Higher Degrees Research and Ethics Committee