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# Exploring the role of IL-32 in premature age-related cardiovascular diseases in HIV-infected individuals

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# Exploring the role of IL-32 in premature age-related cardiovascular diseases in HIV-infected individuals

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# Résumé

Malgré l'introduction de la thérapie antirétrovirale combinée (cART), l'infection par le VIH continue à accélérer les comorbidités non infectieuses telles que les maladies cardiovasculaires (MCV). Cependant, seule une sous-population de patients infectés par le VIH et traités est sujette à ces complications. Par conséquent, il est important de comprendre la pathogenèse de ces maladies cardiovasculaires précoces afin d'identifier les marqueurs prédictifs pouvant être utilisés pour le diagnostic précoce et les stratégies de prévention. Dans cette étude, nous investiguerons l'utilisation potentielle de la cytokine inflammatoire IL-32 comme biomarqueur des maladies cardiovasculaires. L'IL-32 humaine est une cytokine polyfonctionnelle clé. Des études antérieures ont attribué à cette nouvelle cytokine des fonctions intracellulaires antivirales contre le VIH-1 mais aussi des propriétés immunosuppressives dues à l'induction d'interférons de type I et de type III et à la création d'un environnement favorable à la réplication du VIH-1, respectivement. L'IL-32 est impliquée dans plusieurs pathologies infectieuses et non infectieuses avec une expression accrue dans diverses maladies auto-immunes inflammatoires, certains cancers, maladies pulmonaires, ainsi que dans le cas d'infections virales. Elle peut induire d'autres cytokines inflammatoires telles que l'IL-6, l'IL-8 et le TNF-α. Elle est exprimée à la fois dans les cellules immunitaires et non immunitaires. Actuellement, aucun récepteur membranaire cellulaire d'IL-32 n'a été identifié. L'expression de l'IL-32 peut être induite par les mitogènes, les bactéries et les virus. Un seul gène code pour l'IL-32 et un épissage alternatif conduit à plusieurs isoformes d'ARN messager : pro-inflammatoire (α & γ), anti-inflammatoire (β & θ) et régulatoire (δ). Il est intéressant de noter qu'IL-32δ est connue pour sa capacité à se lier à IL-32β et inhiber ses fonctions anti-inflammatoires. La contribution de ces isoformes aux maladies inflammatoires et infectieuses est mal comprise. Nous avons préalablement démontré qu'IL-32 est augmentée dans l'infection au VIH-1 et corrèle avec l'inflammation persistante et la progression des maladies. L'inflammation chronique associée aux MCV représente un des phénotypes de vieillissement prématuré associés au VIH, justifiant ainsi notre intérêt pour l'étude de l'IL-32 dans la progression des MCV.

Nos résultats montrent une augmentation significative des niveaux d'IL-32 dans le plasma des patients VIH+ (traités avec cART) par rapport aux contrôles non infectés. Nous avons également montré pour la première fois une modulation dans l'expression différentielle d'IL-32 basée sur l'âge et le sexe chez les personnes infectées par le VIH. Les femmes infectées présentent des niveaux d'IL-32 plasmatique supérieurs aux hommes. Cependant, les femmes dont l'âge est inférieur à 50 ans ont des niveaux nettement plus élevés par rapport à celles de plus de 50 ans alors que l'inverse a été observé chez les hommes. Au niveau transcriptionnel, toutes les isoformes d'IL-32 sont exprimées à des niveaux plus élevés chez les individus VIH+ par rapport aux contrôles non infectés. En mesurant les isoformes d'IL-32 des patients traités pour lesquels nous disposons d'imagerie cardiaque, nous avons montré que le ratio entre certains isoformes d'IL-32, particulièrement les isoformes D et beta (ratio D/β), corrèle positivement et de manière significative avec le volume total de plaque d'athérome. Étant donné le rôle de l'IL-32β dans l'induction de la cytokine anti-inflammatoire IL-10, nos données suggèrent un rôle protecteur d'IL-32β contre les maladies cardiovasculaires mais un rôle délétère de l'IL-32D. Nos données montrent que l'infection par le VIH augmente sélectivement l'IL-32D, tôt après l'infection.

Enfin, le résultat de cette étude montre pour la première fois que le ratio des isoformes IL-32D/β pourrait être utilisé comme un biomarqueur de la formation des plaques coronariennes et des maladies cardiovasculaires chez les sujets VIH+ traités par cART.

**Mots-clés**: VIH-1, Interleukine-32, MCV

# Abstract

Despite the introduction of combined antiretroviral therapy (cART), HIV infection continues to accelerate non-infectious comorbidities such as premature-age associated cardiovascular disease (CVD). However, only a subpopulation of HIV-infected and treated patients is prone to these complications. Therefore, it is important to understand the pathogenesis of these accelerated disorders and to identify predictive markers of premature CVD that can be used for early diagnosis and prevention strategies. In the current study, we investigated the potential use of the pro-inflammatory cytokine IL-32 as a biomarker for CVD. Human IL-32 is a key polyfunctional cytokine. Previous studies have attributed both intracellular anti-viral functions against HIV-1 but also immunosuppressive properties to this novel cytokine due to the induction of type I and type III interferons and through the creation of an environment conducive to HIV-1 replication, respectively. IL-32 is involved in several infectious and non-infectious pathologies with increased expression in various inflammatory autoimmune diseases, certain cancers, pulmonary diseases, and viral infections. It can induce other inflammatory cytokines such as IL-6, IL-8 and TNF-$\alpha$. It is expressed in both immune and non-immune cells. However, IL-32 cell surface receptors have yet to be identified. IL-32 expression can be induced by mitogens, bacteria and viruses. IL-32 is encoded by a single gene that produce several isoforms by multiple splicing. These isoforms were recently described to exhibit distinct immune-regulatory potential: pro-inflammatory ($\alpha$ & $\gamma$), anti-inflammatory ($\beta$ & $\theta$), and regulatory ($\delta$). Of note, IL-32$\delta$ is known to bind IL-32$\beta$ and inhibits its anti-inflammatory functions. The contribution of these isoforms to infectious and inflammatory disease is poorly understood. Our group has previously shown that IL-32 is upregulated during HIV-1 infection and that it correlates with persistent inflammation and disease progression. Given that chronic inflammation is associated with CVD, a condition with increased prevalence in the HIV-infected population, we sought to characterize the role IL-32 in CVD progression.

Our results showed significantly high levels of total IL-32 (pool of all isoforms) in the plasma of HIV+ (cART-treated) individuals compared to non-infected controls. We were the first to show that there is a differential expression of IL-32 levels depending on sex and age in HIV infected individuals. Infected women were found to have higher levels of IL-32 in their

plasma, when compared to men. Furthermore, women under the age of 50 had significantly higher levels of IL-32 compared to women over 50, whereas the opposite was observed in men (although age-associated difference in this group did not reach statistical significance). At the transcriptional level, all IL-32 isoforms were highly expressed in HIV+ individuals compared to non-infected controls. By comparing IL-32 isoforms from treated subjects with cardiovascular imaging, we show that the ratio between certain IL-32 isoforms, particularly the D and beta isoforms (D/β ratio), positively and significantly correlates with the total atherosclerotic plaque volume of the coronary artery. Given the role of IL-32β in the induction of the anti-inflammatory cytokine IL-10, our data suggests a protective role for IL-32β against CVD but a deleterious role for IL-32D. Furthermore, our data showed that HIV infection selectively upregulates IL-32D early post-infection.

Overall, the results of this study are the first to show that the ratio IL-32D/β can be used as a predictive biomarker for coronary artery plaque formation and CVD in HIV+ ART-treated subjects.

**Keywords**: HIV-1, Interleukin-32, CVD

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# List of abbreviations

ABC: Abacavir

ADCC: Antibody-dependent cellular cytotoxicity

ART: Anti-retroviral therapy

AIDS: Acquired immunodeficiency syndrome

Akt: Protein Kinase B

ALG-2: Apoptosis-linked gene 2

APOBEC-3G: Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-like 3G

AZT: Azidothymidine (Zidovudine)

bDNA: branched DNA)

cART: Combination anti-retroviral therapy

CCR5: C-C chemokine receptor type 5

cDNA: Complementary DNA

CI: Chronically infected

CTL: CD8+ T lymphocytes

CTLA-4: Cytotoxic T-Lymphocyte Associated Protein 4

CVD: Cardiovascular Disease

CXCL: Chemokine (C-X-C motif) ligand

CXCR: Chemokine C-X-C Motif Receptor

d4T: Stavudine

DC: Dendritic cells

ddI: Didanosine, marketed under trade name Videx, used to treat HIV/AIDS

d4T: Dstavudine, used to treat HIV/AIDS

ddN: 2',3'dideoxynucleoside

DNA: Deoxyribonucleic Acid

ENV: HIV envelope glycoprotein

ESCRT: Endosomal sorting complexes required for transport

FDA: Food and Drug Administration

Gag: Group-specific antigen, coding for structural proteins

GI: Gastro-intestinal

Gp: Glycoprotein

HAART: Highly aggressive anti-retroviral therapy

HC: Healthy controls

HDL-C: High density lipoprotein- cholesterol

HIV-1: Human immunodeficiency virus type 1

HIV-2: Human immunodeficiency virus type 2

HTLV-I: Human T-Lymphotropic Viruses

IBD: Inflammatory bowel disease

IDO: Indoleamine 2,3-Dioxygenase

IgG1: Immunoglobulin G1

IFN: interferon

ICAM-1: Intercellular Adhesion Molecule 1

IL: Interleukin

INI: Integrate inhibitors

JAK-2: Janus kinase-2

JNK: c-Jun N-terminal kinase

LAV: Lymphadenopathy-Associated Virus

LDL-C: Low density lipoprotein- cholesterol

LPS: Lipopolysaccharide

LTNP: Long-term non progressors

LTR: L-terminal repeat

MAPKs: Mitogen-activated protein kinases

mCD14: Membrane CD14

mRNA: messenger RNA

NEF: Negative Regulatory Factor

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NLR: Nod-like receptors

NNTRI: Non-nucleotide reverse transcriptase inhibitors

NOD: nucleotide-binding oligomerization domain-like receptors

NRTI: Nucleotide reverse transcriptase inhibitors

PBMC: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

PD-1: Programmed cell death-1

pDC: Plasmacytoid dendritic cells

PHA: Phytohaemagglutinin

PHI: Primary HIV infection

PI: Protease inhibitor

PI3K: Phosphatidylinositol 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

Pol: DNA polymerase

PR: Protease

PRR: Pattern recognition receptors

RA: Rheumatoid arthritis

RER: Rough endoplasmic reticulum

REV: Regulator of expression of viral proteins

RLH: RIG-like helicases

RNA: Ribonucleic acid

RRE: Rev Responsive Element

RT: Reverse-transcriptase

RTI: Reverse-transcriptase inhibitor

sCD: soluble cluster of differentiation

SiRNA: Small interfering RNA

SIV: Simian immunodeficiency virus

SIVcpz: Simian immunodeficiency virus infection of Chimpanzees

SIVcpzPt: SIV of chimpanzees Pan troglodyte troglodytes

SIVgor: SIV of gorilla

SNP: single-nucleotide polymorphism

STAT-3: Signal transducer and activator of transcription-3

SU: Surface unit

TAT: Trans-activator protein

TH: T helper

TLR: Toll-like receptors

TCR: T cell receptor

TM: Transmembrane

TNF-α: Tumor necrosis factor alpha

Vif: Viral infectivity factor

Vpr: Viral protein R

Vpu: Viral protein U

VSV: Vesicular stomatitis virus

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# Chapter 1:

# LITERATURE REVIEW

## 1.1. Epidemiology of HIV-AIDS

HIV-1 and HIV-2 are both members of the *Retroviridae* family and have the capacity to infect and replicate in the human body. However, HIV-1 is the etiological agent responsible for the majority of acquired immunodeficiency syndrome (AIDS). According to the most recent United Nations Programme on HIV/AIDS (UNAIDS), there are currently 36 million people living with HIV worldwide, among whom 34.5 million are adults (UNAIDS, 2017). Each year, there are more than 1.8 million new infections worldwide, which stresses the fact that HIV/AIDS remains a global concern (WHO, 2018). The highest population of individuals living with the viral infection are native to sub-Saharan African communities. Epidemiological observations have shown that the most common and causative factor for the transmission of novel HIV infection is through unprotected sexual intercourse (HIV.gov, 2017). However, HIV can also be transmitted by other horizontal means such as through transfusions with infected blood or through the sharing of infected needles (Day et al., 2008). Additionally, vertical transmission of HIV, from mother to child during pregnancy, labour, delivery or breastfeeding, has been on the rise in the absence of anti- retroviral therapy (King et al., 2013). Over the years, the rate of people with access to anti-retroviral therapy has increased to reach 19.5 million in 2016, compared to 17.1 million in 2015 and just 7.7 million in 2010. This encouraging data reveals that access to effective medications is improving and is, in part, responsible for the 11% decrease in new HIV infections reported among adults in 2016. More encouragingly, the number of deaths due to AIDS-related illnesses has fallen by 48%, reaching one million in 2016 compared to 1.9 million in 2005 (UNAIDS, 2017). However, global efforts are still needed to help make anti-retroviral treatments more accessible to infected people in countries with limited resources.

## 1.2. The origin and discovery of HIV

### 1.2.1. Origin of HIV

HIV is thought to have originated in monkeys, resulting from adaptation of the simian immunodeficiency virus (SIV) following transmission to humans (Sharp et al., 2010).

Interestingly, there are four distinct groups of HIV-1, which have been classified based on four independent events resulting in transmission to humans. These groups are the M (Major), O (Outlier), N (New) and P (Putative) (Charneau et al., 1994; Wainberg, 2004). The most common group worldwide, the M group, was discovered in 1981 at the origin of the pandemic. It has 9 subtypes (A, B, C, D, F, G, H, J, K), of which subtype B is the most prevalent in North America (Plantier et al., 2009). With only 10-1000 reported cases, Group O was discovered in 1990 in Central Africa (De Leys et al., 1990). In 1998, the N group was identified in Cameroon, reporting only a dozen known cases (Simon et al., 1998). Similarly, the P group was recently identified after being isolated from two individuals in Cameroon (Plantier et al., 2004).

However, phylogenetic studies of these groups suggest two possible origins of HIV. Evolutionary relationships trace the origin of the HIV-1 genome to the Pan troglodyte troglodytes SIV (SIVcpzPtt) in chimpanzees (Gao et al., 1999), whereas HIV-2 appears to be derived from the SIV of sooty mangabey ($SIV_{SM}$) lineages (Sharp et al., 2010, 2011). HIV-2 is confined to West Africa and is less virulent compared to HIV-1 infection (Sharp et al., 2011). These phylogenetic analyses have also allowed researchers to approximate how long the virus made its transmission to humans. The transmission of the first three groups (M, N & O) took place at the beginning of the 20th century. In contrast, the date the P group was introduced in humans remains unknown (Wertheim et al., 2009). Regardless of the origin, HIV evolves rapidly due to a high rate of mutation resulting from the error-prone nature of its reverse transcriptase enzyme and to recombination events in its human host.

### 1.2.2. Discovery of HIV

During the Summer of 1981, clinicians in New York and California were reporting high incidence of unusual cancers such as Kaposi's sarcoma and of other opportunistic infections such as *Pneumocystis jiroveci*, among the homosexual community (Gottlieb et al., 1981). Some of these patients were also affected by persistent lymphadenopathy and had significantly low CD4+ lymphocyte counts. These observations led clinicians to agree that these patients suffered from a common immunodeficiency and prompted the quest to identify the etiologic agent responsible for this disease. Because of its high prevalence among gay men, it was first hypothesized to be a result of sexual transmission (Brennan et al., 1981; Friedman-Kien et al.,

1981). However, this theory came into question with a study in the United States where the syndrome was observed in various social groups, suggesting transmission through blood (Masur et al., 1981)(Masur et al., 1981). In 1983, the virus in question was isolated from an infected lymph node by a group of researchers led by Françoise Barré-Sinoussi and her mentor Luc Montagnier at Institut Pasteur in France (Barré-Sinoussi et al., 1983). This infectious pathogen was named lymphadenopathy-associated virus (LAV). Interestingly, this virus exhibited a striking similarity to the Type I and Type III Human T-Lymphotropic Viruses (HTLV-I and III) previously identified by Robert Gallo in the United States (Poiesz et al., 1980; Gallo et al., 1983). It turned out that both LAV and HTLV-III were actually the same virus and it was renamed human immunodeficiency virus (HIV) in 1986 (Coffin et al., 1986).

## 1.3. Morphological structure of HIV-1

HIV-1 viral particles measure around 80-120 nanometers (nm) in diameter and are enclosed by a lipoprotein membrane made up of 72 glycoprotein (gp) heterodimers that are integrated into the lipoprotein membrane (Murray et al., 2009). The membrane heterodimers are comprised of gp120 and transmembrane gp41. During the budding process, HIV incorporates various host cell membrane proteins into its lipoprotein envelope. Among these host cells proteins are the intercellular adhesion molecule 1 (ICAM-1), which accelerates adhesion to the target cells, and the human lymphocyte antigen class I and II proteins (Paquette et al., 1998). The viral matrix consists of p17 protein and links the lipoprotein membrane of the retrovirus to its viral core (Dolcetti et al., 2015), which is comprised of the primary antigen (p24). The p24 core houses two copies of single-stranded HIV-1 RNA and the p7 nucleoprotein. The core also contains the enzymatic makeup of the virus; notably protease p11, integrase p32, and the p66 reverse transcriptase (RT). These enzymatic elements are essential for early viral replication. Figure 1 illustrates the structure of the HIV-1 virus and its key replication factors (Hill et al., 2005).

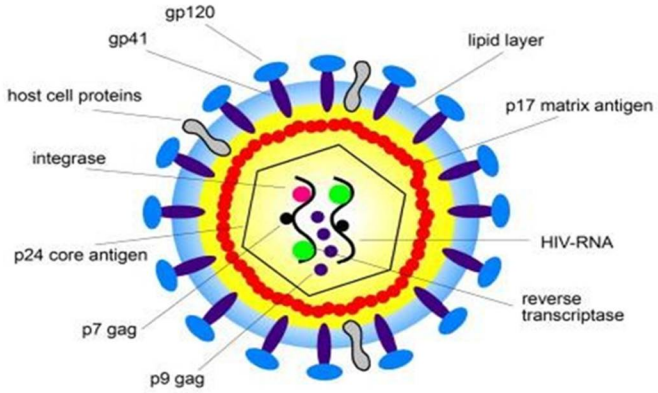



**Figure 1. Structure and proteins of HIV-1.** Reproduced from (Behrens, 2011), no permission required.

## 1.4. HIV-1 genome

The HIV-1 genome, which is about 9 kb of RNA, encodes for 15 proteins (Frankel et al., 1998). HIV replication relies on two types of genes: structural and auxiliary genes. The three distinct structural genes necessary for building a new virus particle include the envelope gene *(env)*, the polymerase gene *(pol)*, and the group antigen genes *(gag)*. The auxiliary genes include two essential regulatory genes: the trans-activator of transcription *(tat)* and the regulator of expression of virion proteins *(rev)*, as well as four important accessory genes: viral infectivity factor *(vif)*, viral protein U *(vpu)*, viral protein R *(vpr),* and negative factor *(nef)* (Behrens, 2011; Karn et al., 2012). Figure 2 illustrates the HIV-1 genome and the critical genes it codes for.

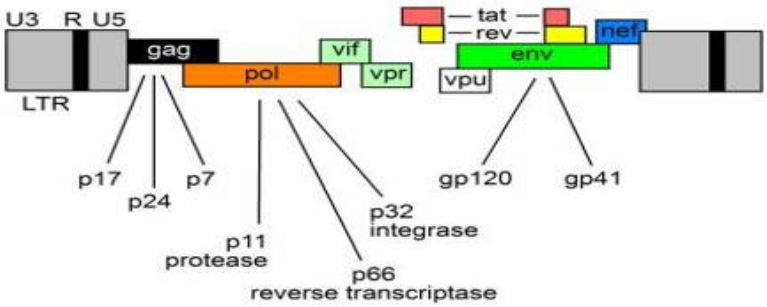



**Figure 2. Genomic organization of HIV-1 proviral DNA.** Reproduced from (Behrens, 2011), no permission required.

### 1.4.1. Structural genes

The classical framework of the viral genome is represented by 5'LTR-gag-pol-env-3'LTR structure. The long-terminal repeats (LTRs), which do not code for any retroviral proteins, flank genome and contain the HIV promoters required for viral expression (Frankel et al., 1998). Both *gag* and *env* code for the glycoprotein and nucleocapsid components of the retroviral membrane. During maturation, activated viral proteases cleave the Gag polyprotein into the matrix protein (p17), core proteins such as the capsid protein (p24), the spacer peptides 1 and 1 (sp1 and sp2), the nucleocapsid protein (p7), and the late-assembly p6 protein which is involved in virion budding (Votteler et al., 2011). The *env* gene encodes the gp160 homotrimer, which is then cleaved into gp120 and gp41. These active fusion proteins are critical for internalization of the virus. Gp120 binds to C C chemokine receptor 5 (CCR5) and to cluster of differentiation 4 (CD4) on the host cell, while gp41 plays a key role in the fusion and internalization process (Morikawa et al., 1993; Alkhatib, 2009). Arguably the most important structural HIV-1 gene is the pol gene, which codes for key enzymes that are critical for viral replication, reverse transcriptase (RT), and protease enzymes. These viral enzymes are produced following cleavage of the Gag-Pol precursor polyprotein and are translated following a shift in the reading frame of the ribosome, near the 3' end of the *gag* gene. RT is critical for the retro-transcription of viral RNA into DNA, which then integrates into the host genome through the action of the viral integrase. HIV protease is equally important, as it cleaves other polyproteins such a Gag into active and functional proteins essential for the virion's maturation and infection (Jacks et al., 1988; Greene et al., 2002; Jeang et al., 2007).

### 1.4.2. Auxiliary genes

The HIV-1 genome codes for six other genes*: nef, vif*, *vpr, rev, tat,* and *vpu*. Although *vpu, vif, nef,* and *vpr* are considered to be accessory genes because they are not needed for viral replication *in vitro*, their functions and regulations are significantly important for HIV pathogenesis *in vivo (*Malim et al., 2008*)*. The two regulatory genes, *tat* and *rev*, code for accumulating proteins that regulate the expression of other viral proteins by binding to the retroviral RNA genome. The Tat protein plays a substantial role in the transcription of viral

messenger RNA (mRNA) by binding to the transactivation-response element (TAR) located in LTR. In the complex containing the transcriptional positive elongation factor b (P-TEFb), through the help of cyclin T1, binding of Tat onto TAR allows for the recruitment of the CDK9 cell cyclin on the LTRs. Through the kinase activity of CDK9, the C-terminal phosphorylation of the large subunit of RNA polymerase II, allows for initiation of genomic transcription. When Tat is not present, RNA polymerase II is unable to synthesize full-length viral transcripts (Fisher et al., 1986; Berkhout et al., 1989; Bannwarth et al., 2005). On the other hand, Rev expression activates expression of structural and enzymatic genes, including *gag, pol and env* of proviral DNA. It binds the RRE (Rev responsive element) and ensures the transport of partially spliced and un-spliced HIV-1 mRNA from the nucleus to the cytoplasm during viral replication, where it allows for the translation and stabilization of the mRNA. Rev is also responsible for the transport of the gag transcript to the cytoplasm (Malim et al., 1988; Malim et al., 1989; Zapp et al., 1989).

Accessory gene, *vif,* encodes a 23 kilo Dalton (kDa) Vif protein, which is transcribed during the late stage of the virus life cycle. Vif binds the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC-3G) and induces degradation of the enzyme via the proteasomal pathway. This degradation prevents the hypermutation effects of APOBEC-3G and leads to the subsequent enhancement of viral replication (Miyagi et al., 2014). Vpu is a specific protein involved in the degradation of the CD4 molecule in the endoplasmic reticulum (Willey et al., 1992). Vpu is also involved in releasing the newly produced virions attached to Tetherin protein (BST-2) into the cytoplasm by inducing the formation of pores in the cell membrane (Strebel, 2014). This BST-2-induced host-protective mechanism can be overcome by HIV-1 via ubiquitination (Laplana et al., 2012). Nef represents a critical protein in the HIV-1 life cycle. In addition to its key role in the down-regulation of CD4 and MHC-I molecules to protect infected cells from cytotoxic killing by CD8 T-cells (Schaefer et al., 2008), the Nef protein also sustains T-cell activation during the initial stages of the HIV life cycle (Das et al., 2005). Nef does this by decreasing the threshold of T-cell activation through, among other mechanisms, down-regulation of the negative regulators of T-cell activation such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (El-Far et al., 2015). Nef also downregulates CD4 molecules on the surface of infected host cells, thereby blocking re-infection of the cell and intracellular binding of newly synthesized viral Env proteins, allowing for proper viral assembly

(Li et al., 2005a). Finally, Vpr plays a pivotal role in transcription activation of the LTR (Kogan et al., 2011). Vpr also promotes viral replication by allowing the viral transactivation of cellular genes and cell differentiation (Romani et al., 2009).

## 1.5. HIV-1 replication cycle

### 1.5.1. Early phase

The initial stage of the HIV replication cycle involves binding of the virus to the surface of the host CD4+ T-lymphocyte via the CD4 receptor and fusing with the cellular membrane (Goodsell, 2015). As illustrated in Figure 3, the HIV gp120 attaches to the CD4 molecules, as well as to the CCR5 or CXCR4 co-receptors on the host cell before initiating the fusion process. Following penetration of the fusion peptide into the cell membrane, the two helices of gp41 fold back onto themselves, allowing fusion of cellular and viral membranes (Weissenhorn et al., 1997), and releasing the nucleocapsid into the host-cell's cytoplasm. Once inside the cell, the nucleocapsid disintegrates, liberating two strands of viral RNA and essential viral enzymes. Using its own reverse transcriptase and by hijacking some of the host cell's replication machinery, the viral RNA is reverse-transcribed into single-stranded and then into double-stranded DNA (Farnet et al., 1996). More specifically, transcription is initiated using a host cell-derived transfer RNA (tRNA) as a primer. The viral RNA is then reverse transcribed into the first strand of cDNA. During cDNA synthesis, the template RNA is completely degraded by RNAse H, except for two short purine-rich sequences (PPT for poly-purine tracts). These PPTs serve as a primer for synthesis of the second DNA strand, which is also carried out by the reverse transcriptase (Basu et al., 2008). The newly synthesized viral DNA is then transported into the cell nucleus, where it integrates into the host genome through the action of the viral integrase enzyme. This integrated DNA is referred to as the provirus, which can remain inactive for several years, forming a few to no replicates of the virus (Miller et al., 1997).

### 1.5.2. Late phase

The transcription of mRNAs by the RNA polymerase enzyme takes place during the late stage of the viral replication cycle. In the host cell nucleus, viral mRNA is spliced and exported to the cytoplasm for translation by the ribosomes. This process results in the synthesis of the

first viral proteins Nef, Tat, and Rev. Gag, Gag-Pol and Env (gp160) precursor proteins are later transcribed from an unspliced or mono-spliced mRNA, as their export is dependent upon the expression of Rev (Suhasini et al., 2009; Checkley et al., 2011). Integrase, protease, and reverse transcriptase enzymes are translated from the same Gag-Pol transcript, using a frame-shifting process.

Virion assembly is mediated by conformational changes within Gag. The Gag domain binds to the plasma membrane, through interaction of its amino-terminal domain with the lipid bilayer of the cell membrane (Sundquist et al., 2012), where it recruits the viral Env protein. The Env proteins traffic through the secretory pathway of the rough endoplasmic reticulum (RER), to the Golgi body and vesicles, until they reach the plasma membrane. There, the Env protein induces endosomal sorting, which is needed for viral budding and transport, while Gag engages both the endosomal sorting complex required for transport (ESCRT), and the apoptosis-linked gene 2 (ALG2)-interacting protein (Usami et al., 2009; Sundquist et al., 2012). These glycoproteins play a key role in enabling budding of the virus, so the new particles can bind co-receptors and infect CD4+ T-cells (Figure 3). Also, at the plasma membrane, small protein chains associate with copies of the HIV RNA and enzymes to form new virus particles ready for release during the budding stage (Sundquist et al., 2012). During the budding process, the new virus emerges from the cell, taking with it part of the host cell membrane and incorporating expression of certain co-stimulatory molecules such as CD40L from the viral envelope membrane (Imbeault et al., 2011).

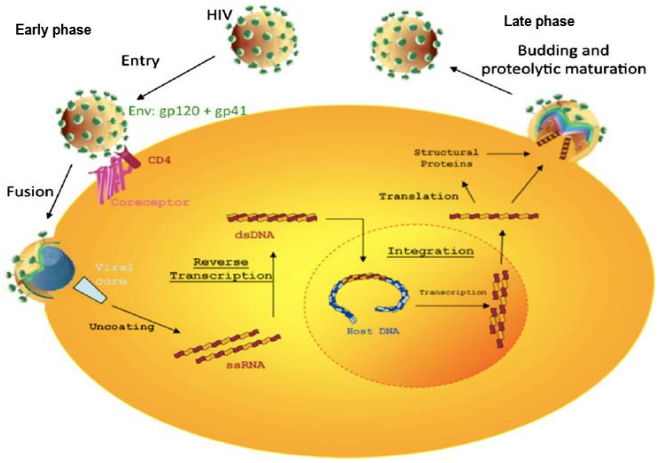



**Figure 3. The Lifecycle of HIV.** Reproduced from (Greene et al., 2008) with permission from Elsevier: Anti-viral Research, copyright 2008.

## 1.6. Anti-retroviral therapy to HIV-1 infection

HIV treatment experienced a breakthrough in the mid-1990s and has since become easier to manage with the emergence of combined therapies. There are currently six classes of anti-viral drugs classified based on how they affect the viral life cycle.

### i) Nucleoside reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first drugs available against HIV infection. In 1986, the United States Food and Drug Administration (FDA) approved the first ever anti-retroviral medicine, zidovudine (AZT), which targets replication of HIV (Broder, 2010) by suppressing the activities of the reverse transcriptase.

Various nucleoside analogs such as zalcitabine (ddC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), abacavir (ABC), and tenofovir (a nucleotide RTI) were added to the anti-retroviral arsenal. They are 2', 3'dideoxynucleoside (ddN) analogues that compete with normal cellular deoxynucleoside triphosphates during transcription. They act as chain terminators of DNA synthesis by incorporating into the ongoing synthesis of the DNA strand, leading to pre-mature termination of the reaction and subsequent blocking of viral replication (Broder, 2010; Arts et al., 2012).

### ii) Non-nucleoside reverse transcriptase inhibitors

The class of non-nucleoside reverse transcriptase inhibitors (NNRTIs) was introduced in 1996. First generation NNRTIs include nevirapine, delavirdine, and efavirenz. The second generation has an improved resistance profile and includes etravirine and rilpivirine (Usach et al., 2013). These inhibitors function by inducing a conformational change in the HIV reverse transcriptase enzyme by binding to the hydrophobic pocket of its P66 subunit, away from the active site of the enzyme (Sluis-Cremer et al., 2004).

### iii) Protease inhibitors

Protease inhibitors (PIs) are different from reverse transcriptase inhibitors. Instead of interfering with transcription, PIs are used to inhibit the activity of HIV protease in host cells already infected with the virus, thereby preventing further viral replication (Lv et al., 2015). They prevent the proteolytic cleaving function of the protease enzyme, preventing the generaton of functional protein units (Lv et al., 2015) from their large non-functional polyproteins precursors (Gag and Gag-Pol). This category of drugs includes ritonavir, indinavir, lopinavir, nelfinavir, saquinavir, amprenavir, atazanavir, and darunavir.

**iv) Integrase strand transfer inhibitors**

Integrase strand transfer inhibitors (INSTIs) include Raltegravir and Elvitegravir introduced in 2007, as well as Dolutegravir introduced in 2013 (Cooper et al., 2008; Cahn et al., 2013; Gallant et al., 2013). Additionally, there are two new INSTIs (bictegravir and cabotegravir) that are currently in clinical trials. Bictegravir can be given once a day, while cabotegravir is a long-acting agent that can be dosed once a month because of its exceptionally long half-life (Han et al., 2017). INSTIs prevent integration of the viral genome into the host DNA of infected cells (Dow et al., 2014). INSTIs achieve this by binding metallic ions in the active site of the integrase, preventing the formation of a covalent bond with host DNA, and further inhibiting the strand transfer reaction (Hazuda et al., 2000).

**v) Viral entry inhibitors**

The entry of HIV-1 into the target cell can be blocked at several stages during HIV entry into cells. These inhibitors include CD4-binding inhibitors, co-receptor inhibitors, and fusion inhibitors (Wilen et al., 2012).

**A) Attachment inhibitors**

Also known as CD4-gp120 inhibitors, attachment inhibitors interfere with the viral gp120. Four types of drugs with different mechanism of action belong to this category of inhibitors: PRO-542 (CD4-IgG2) which acts by mimicking the CD4

receptor, TNX-355 which acts by competing with HIVgp120 for CD4 binding, CADA which downregulates CD4 receptor expression and, lastly, BMS-806 which prevents the conformational changes of gp120 that take place after binding of gp120 to the CD4 receptor (Briz et al., 2006). Fostemsavir is a gp120 attachment drug that has recently shown promise in clinical trials involving heavily treated individuals.

**B) Co-receptor binding inhibitors**

Another name for this class of drugs is chemokine receptor antagonists (CCR5 antagonists). This class is comprised of small molecules that, by binding to the receptor, induce conformational changes of the extracellular loops (allosteric mechanism) in the protein, preventing attachment of gp120. It includes Maraviroc, which interferes with viral entry into susceptible cells by targeting the CCR5 chemokine co-receptors. Maraviroc works well; however, resistance mechanisms are known. The most common type of resistance is the emergence of X4 virus (Westby et al., 2006). The second mechanism of resistance developed by HIV is the ability to use the co-receptor in its compound-bound form (Westby et al., 2007). It is only effective against R5 viruses.

**C) Fusion inhibitors**

Fusion inhibitors (FIs) were approved in 2003. They are directed against viral gp41, specifically the formation of the 6HB structure responsible for fusion. FI drugs such as Enfuvirtide (the only fusion inhibitor currently marketed) function by blocking viral fusion and subsequent entry into the cell (Lalezari et al., 2003; Lazzarin et al., 2003). Although Enfuvirtide is the only FDA approved fusion inhibitor, its use is limited because of the side effects such as severe local injection site reaction (Ball et al., 2003).

## 1.6.1. Importance of using combined anti-retroviral therapy

Since the mid-90s, Highly Active Anti-Retroviral Therapy (HAART) has been the standard of care to block the active replication of HIV (Arts et al., 2012). HAART refers to

combination therapy, which includes the use of protease inhibitors, integrase inhibitors, non-nucleoside, and nucleoside analogue drugs, given in a wide range of combinations (Arts et al., 2012). It had previously been shown that treatment with a single protease inhibitor and two other anti-retroviral therapies had a ripple effect in reducing viral replication and copies of HIV in the blood. Thus, combination therapy has become the standard of care for patients living with HIV/AIDS as it targets multiple stages of the HIV-1 replication cycle and reduces the chances of the virus developing resistance to the various treatments (Staszewski et al., 1996; Arts et al., 2012).

Viral replication occurs at a high rate. Several billion copies of the retrovirus are created and destroyed on a daily basis. This high turnover is one of the mechanism by which HIV develops drug resistance. Due to the high rate of replication and a highly error-prone HIV-reverse transcriptase, random mutations are introduced into the HIV RNA. These genetic mutations lead to changes in viral proteins targeted by anti-retrovirals, decreasing their efficacy. Resistance significantly complicates the treatment process, rendering the medication provided either less or completely ineffective. Once resistance develops to a particular drug, the virus is likely to become resistant to drugs in the same class. Thus, it has become clear that a single-agent against HIV would have limited ability to control such a rapidly replicating virus. Expansion of the anti-retroviral drug classes has made it possible to combine treatments. Combination therapy can more effectively suppress the mutated forms of the retrovirus.

## 1.7. Natural history of HIV-1 infection

HIV infection is predominantly characterized by the depletion of CD4+ T lymphocytes (Février et al., 2011). Since these cells are one of the main mediators of immune responses, HIV-mediated depletion of CD4+ T-cells increases the vulnerability of infected individuals to opportunistic infections and death (Okoye et al., 2013). As with other lentiviruses, HIV infection can have slow periods of replication, leading to chronic disease (Okoye et al., 2013). Some individuals may show significant progression throughout the course of the disease; However, less than 5% of infected individuals do not show disease progression over several decades. These individuals are called long term non-progressors (LTNP) (Kumar, 2013; Gurdasani et al.,

2014). The course of HIV-1 infection can be divided into three phases: acute, chronic, and AIDS phase (Figure 4). Each phase is determined by its duration and specific clinical characteristics.

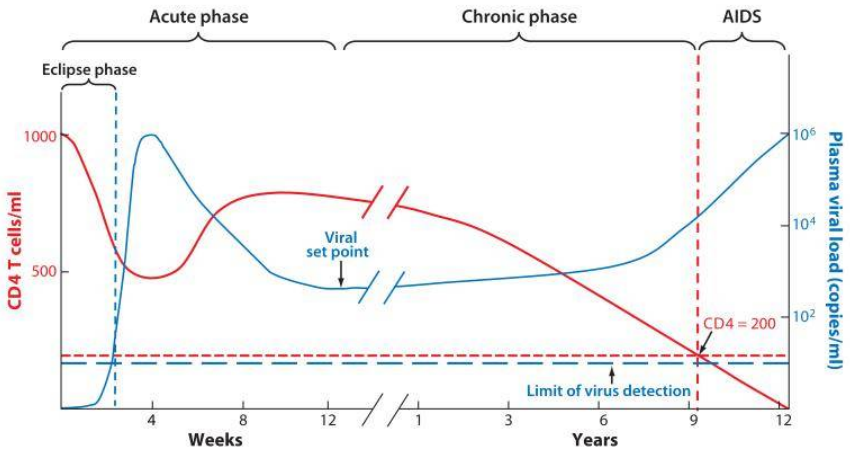



**Figure 4. Clinical Stages of HIV Infection.** The clinical features of the different phases of the pathogenesis of HIV-1. Reproduced from (Bashirova et al., 2011) with permission from Elsevier: Cytokine, copyright 2012.

## 1.7.1. Acute phase

The acute phase occurs within the first two to four weeks of HIV-1 infection. This stage is characterized by a high plasma viral load due to the significant amount of virus being produced. The body naturally responds to this vigorous HIV replication, leading to flu-like symptoms which include but are not limited to swollen glands, asthenia, joint aches, weight loss, and sore throat (Kahn et al., 1998). Most of the virus particles identified in this phase show R5 tropism (i.e. binding to CCR5 co-receptor), whereas switching to CXCR4 viruses may occur during the later-stage of infection (Scarlatti et al., 1997).

During the acute phase of HIV infection, activated target host cells (CD4+ CCR5+ T lymphocytes) begin to overexpress early tissue-resident marker CD69 and activation markers such as CD25 and HLA-DR receptors (Kunkel et al., 2002; Douek et al., 2003). At the same time, viral accessory proteins Nef, Vif, Vpr and Vpu, bypass typical T cell receptor (TCR)-mediated activation and contribute to the activation of these cells (Unutmaz et al., 1999;

Simmons et al., 2001). As HIV infects and depletes CD4+ T cells, CD4 counts are significantly reduced, particularly in the lymphoid organs associated with the genitals and gastrointestinal tract (Kahn et al., 1998). Two mechanisms are responsible for the depletion of CD4+ CCR5+ T cells: direct cytopathology of HIV-1 or immune responses. The host immune responses lead to destruction of infected cells through the action of natural killer (NK) cells and cytotoxic CD8+ T lymphocytes (Douek et al., 2003; Vieillard et al., 2005), and work to reduce the viral load to a stable level (Meulendyke et al., 2014). At the viral set point (i.e. the end of the acute phase, which is characterized by a marked reduction in viral load), the CD4+ T cell count starts to increase again. This viral set point varies from one individual to another, which will remain the same throughout the chronic phase (Koup et al., 1994; Safrit et al., 1994; Huang et al., 2012).

### 1.7.2. Chronic phase

The chronic phase of HIV infection is the longest and can take place over several years or decades. It starts after HIV-specific antibodies are developed. Due to the exhaustion of the immune system and bacterial translocation at the gastrointestinal mucosal barriers, the immune system becomes persistently activated; leading to high levels of circulating pro-inflammatory cytokines (Brenchley et al., 2006). HIV infection *per se* is not solely responsible for the massive depletion of CD4+ T cells. Chronic immune activation is largely believed to lead to compromised cellular metabolism and, consequently, to cell death (Douek et al., 2003; Ford et al., 2009). Several mechanisms are involved in the events leading to depletion of CD4+ T cells. These mechanisms include (i) attrition of the memory cell pool due to persistent immune activation, (ii) extensive activation of naïve T cells entering the pool of memory cells, (iii) reduced number of reserve cells in steady state due to recurrent stimulation, and (iv) lower supply of naïve T cells due to HIV-mediated decreased thymic output (Douek et al., 2003). Infected patients with a high level of HIV RNA proceed to the symptomatic stage at a faster rate than patients with low RNA levels (Watanabe et al., 2015). At this stage, CD4 cells continue to decline at a progressive rate (Streeck et al., 2009). Some patients experience organ dysfunction as a direct result of the virus rather than as a consequence of defective cell-mediated immunity (Naif, 2013); while other individuals may develop generalized lymphadenopathy.

### 1.7.3. AIDS phase

The AIDS stage of the HIV-1 infection occurs once the human immune system is completely damaged and the infected individual becomes vulnerable to various opportunistic infections, most notably Tuberculosis, *Pneumocystis jiroveci*, Cryptococcal meningitis, and Cytomegalovirus infections (Jung et al., 1998; Corbett et al., 2003). It is also possible to observe neoplasias such as Kaposi's sarcoma (Chu et al., 2011). During this stage, CD4+ T cell counts fall below 200 cells/µl and there is an increase in viremia (Leng et al., 2001). There can also be a change in viral tropism from R5 to X4 (Douek et al., 2003), which promotes rapid spreading of the virus. Without anti-retroviral therapy (ART) medication, HIV-1 infected individuals can only survive for a few years.

## 1.8. Immune response to HIV infection

### 1.8.1. Innate immune response

Innate immune responses represent the first line of defense of the human immune system. The primary components of the innate immune system include the skin, mucosal lining, and epithelial layers of the gastrointestinal tract (GI). These elements act as either chemical or physical barriers to illnesses (Riera Romo et al., 2016). The innate immune response is largely based on the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR)s. Thus, during viral evasion, the inflammatory response causes the recruitment of innate immune system cells, including neutrophils, NK cells, monocytes, and plasmacytoid dendritic cells (pDCs). These cells express several PRRs, which act as sensors capable of detecting viral nucleic acids (PAMPs) and initiating a response to type 1 interferon (α and β); thereby preventing spreading of the virus. Recognition of HIV by host cells, primarily CD4+ T cells, can occur at many stages of the replicative cycle. Recognition can occur through detection of the products of reverse transcription, and also through interaction of the virion, capsid, or viral RNA with the different PRRs. Among other things, two types of intracellular PRRs allow for the detection of retroviral DNA in the cytoplasm: IFI16 (interferon inducible protein 16) (Jakobsen et al., 2013) and cGAS (cyclic GMP-AMP synthetase) (Gao et al., 2013). TLRs (toll-like receptors), receptors on the cell surface and in endosomes, are also involved in

the recognition of HIV. As a result, gp120 is recognized by TLR2 and 4 on the surface of mucosal epithelial cells (Nazli et al., 2013) and viral genomic RNA is recognized by endosomal TLRs 7 and 8 (Schlaepfer et al., 2006). Neutrophils express TLR 1-9 and respond to pathogens by phagocytosis, producing oxidative compounds and releasing antimicrobial peptides such as Trappin, α-1, -2 and -3 defensins (Chang et al., 2004; Klotman et al., 2006). These defensins are also known as human neutrophil peptides (HNP 1, -2 and -3). They interact directly with the virus and inhibit viral replication (Wang et al., 2004; Klotman et al., 2006). As a result of this recognition, activation of the transcription factor and nuclear factor kappa B (NFκB) signaling cascade occurs, leading to the expression of costimulatory molecules and to the downstream production of several pro-inflammatory cytokines (Kawai et al., 2007; Henrick et al., 2015). During the innate immune response, NK cells have the ability to recognize infected cells with weak MHC class I (MHC-I) expression. Through their cytotoxic function, NK cells lyse infected cells detected via killer immunoglobulin receptor (KIR) inhibitors that interact with MHC-I (Alter et al., 2009). NK cells can also kill virus-infected cells in the absence of antibodies through an antibody-independent mechanism. This mechanism causes NK cells to express the Fas Ligand molecule (CD 178) on their surface and can induce the death of Fas-expressing target cells (CD95) as soon as they adhere to it (Yoon et al., 2015).

## 1.8.2. Adaptive immune response

Adaptive immune responses are mediated by both B and T lymphocytes.

*1.8.2.1. Humoral immunity (B lymphocytes)*

The humoral immune response is mediated by antibody-producing B cells. During HIV-1 infection, these cells control the infection by producing and secreting a variety of HIV-specific antibodies (IgA, IgD, IgG, and IgM) (Moir et al., 2009). The first sign of B cell response detected in the plasma are complexes of antibody-virions (Tomaras et al., 2008). A few days later, specific antibodies against gp41 are detected, followed by specific antibodies for gp120 (mainly against the V3 loop) and antibodies specific to other viral proteins such as p24 (Derdeyn et al., 2004; Gray et al., 2007). During primary HIV-1 infection, recent studies have shown that gp41-specific IgM and IgD initial antibodies do not significantly affect the dynamics of viral

load (Tomaras et al., 2008). Moreover, during the acute phase, IgA antibodies specific to gp41 can be detected in mucosal secretions. However, it appears that the initial IgA response is non-neutralizing (McMichael et al., 2009).

Owing to the frequent mutations in the variable region of the envelope, HIV-1 escapes detection by the humoral antibodies, thereby preventing neutralization of the pathogen. While the conserved regions of the envelope, such as the CD4 attachment site, do not undergo mutation (Burton et al., 2015), neutralizing antibodies specifically directed against the constant regions are only produced 20-30 months after infection (Burton et al., 2015). Thus, antibodies produced at a given moment do not neutralize the contemporary virus particles, but rather are directed against earlier variants. Consequently, newer virus particles are capable of escaping the humoral anti-viral response (Albert et al., 1990; Moog et al., 1997). Therefore, fast-progressing individuals rarely have these antibodies during the course of infection (Tomaras et al., 2008; Kwong et al., 2013).

There are five different epitopic regions of the HIV envelope spike identified by broadly neutralizing antibodies 1) the CD4-binding site (CD4bs), 2) the V2 proteoglycan moiety loop, 3) the V3 proteoglycan moiety loop, 4) the membrane proximal external region (MPER) of the Env transmembrane domain, and 5) the gp120-gp41 interface epitopes (Sahay et al., 2017). These neutralizing antibodies are generated from multiple hypermutations (Kwong et al., 2013), and their proper production remains the major obstacle for inducing protective immunity by vaccination.

During the chronic phase, persistent immune activation, antigenic pressure, and the progressive decline of CD4+ T cells (less help from CD4) can lead to B cell dysfunction (e.g hyper-gammaglobulinemia). This immune dysfunction eventually leads to B cell depletion, compromiseing the humoral response with loss of immunological memory of the B lymphocytes (De Milito, 2004).

Studies are currently underway to develop a vaccine against HIV-1 by generating specific neutralizing antibodies against both the conserved and variable regions of the HIV-1 genome (Virgin et al., 2010; Haynes et al., 2012; Burton et al., 2015).

*1.8.2.2. Cell mediated immunity*

*HIV-1-specific CD4+ T cells*

CD4 T cells are the primary target of HIV-1 infection. They play a critical role in the establishment and maximization of the immune system's ability to control a pathogen by serving as helper cells. CD4+ T lymphocytes are divided into different populations based on their location, function, and the type of cytokines they secrete. The activation and differentiation of a naive CD4+ T lymphocyte in either of these populations will depend on the environment of cytokines and costimulatory molecules produced by DCs. The first two types of CD4+ T cells discovered are T helper (Th) 1 and Th2 (Mosmann et al., 1986). CD4+ Th1 T cells produce INF-γ and are involved in cytotoxic cellular responses, as well as in the effector functions of NK, CD8+ T cells and macrophages (Younes et al., 2003; Crotty, 2015). Therefore, they promote a response to intracellular pathogens. CD4+ Th2 T cells secrete IL-4 and IL-13. This provides a second signal for the activation of B cells and promotes their production of IgG4 and IgE. Upon viral antigen stimulation from either the envelope or by the Nef, Gag and Pol proteins, the viral antigen peptide is presented to T cells via the MHC-II complex. The Th cells recognize and bind to the pathogen presented by the MHC-II complex through their T cell receptors (TCRs) (Mohan et al., 2014) and express the surface protein CD40L and several cytokines such as IL-4 and IL-21. Expression of these factors provides the necessary co-stimulation to activate B cells by binding to their CD40 cell surface receptor (Crotty, 2015). It is now known that there are two other major types of CD4+ T cells. The Th17-type expresses the IL-23 receptor (IL-23R) and CCR6. These CD4+ T cells produce IL-17, IL-1, IL-6, IL-21, IL-22, and TNF-α, and are essential in maintaining intestinal homeostasis. As a result, they are involved in the control of the integrity of the intestinal epithelium and in the production of pro-inflammatory cytokines that counteract the invasion of extracellular bacteria and fungi (Bettelli et al., 2008). Finally, Tregs have anti-inflammatory properties and participate in the control of immune tolerance (Costantino et al., 2008). These cells secrete IL-10 and TGF-β (Sempere et al., 2007; Février et al., 2011), which inhibit the proliferative response of HIV-1-specific CD4+ T cells. Although all types of CD4+ T cells can be infected with HIV, HIV preferentially infects activated CD4+ T cells or memory CD4+ T cells expressing the CCR5 receptor (Grossman et al., 2006).

Several viral protein epitopes recognized by specific CD4+ T cell clones have been well

documented. These viral epitopes from the Gag-Pol polyprotein, Nef, and Env protein (Zaunders et al., 2006; Yue et al., 2008) lead to clonal expansion and heterogeneity based on the stage of infection. During the acute phase, HIV-1 specific CD4 T cells are compromised and express low levels of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein. Decreased expression of Bcl-2 makes them more vulnerable to apoptosis, thus contributing to the overall decline of the total CD4 T cell population (Zaunders et al., 2005). As previously mentioned, HIV infection is accompanied by the significant depletion of mucosal CD4+ T cells, particularly of Th17 lymphocytes, which express high levels of CCR5 (Brenchley et al., 2008b). This depletion occurs not only at the level of peripheral CD4 + T cells but mainly in lymphoid tissue associated with the gastrointestinal tract (GALT, gut-associated lymphoid tissue) (Brenchley et al., 2004), where nearly 30 to 60% of CD4 + T cells become productively infected and are eliminated in just four days in *in-vivo* studies in a model of SIV infection in macaques (Li et al., 2005b; Mattapallil et al., 2005). Th17 infection cannot fully explain this depletion. However, the pro-inflammatory environment and the presence of viral proteins is likely to contribute to apoptosis of these cells (Douek et al., 2003). Moreover, because of their important role in maintaining the integrity of the mucosal epithelial barrier, loss of Th17 cells leads to an increase in microbial translocation (Eggena et al., 2005).

In addition to viral- and apoptosis-mediated cell death, pyroptosis, another form of programmed cell death, contributes to the depletion of CD4+ T cells during HIV infection. Pyroptosis accounts for approximately 95% of CD4+ T cell death localized in the lymphoid tissues (Doitsh et al., 2014). It occurs due to release of several inflammatory cytokines such as IL-1β and IL-18 (Fink et al., 2005). Release of these cytokines leads to cellular swelling, rupture, and to the subsequent release of intracellular contents, thereby creating an environment conducive to the death of surrounding cells (Doitsh et al., 2014). During abortive HIV-1 infection, which represents the majority of infected cells in vivo, incomplete reverse transcription products are released in the cytosol. As a result, the cytosolic DNA sensor and interferon-γ-inducible protein 16 (IFI16) detect these products and lead to activation of caspase-1 and to the assembly of an inflammasome (Monroe et al., 2014). Activation of caspase-1 causes cell death by pyroptosis (Bergsbaken et al., 2009). Factors that bias CD4 responses include the upregulation of negative immune regulators such as Cytotoxic T-Lymphocyte Antigen 4

(CTLA) and Programmed cell death-1 (PD1) on HIV-1 specific CD4+ T cells (Kaufmann et al., 2007; El-Far et al., 2008).

*HIV-1 specific CD8 + T lymphocytes*

Cytotoxic CD8+ T lymphocytes play an important role in the control of HIV-1 infection (Migueles et al., 2008). CD8+ T cells recognize specific antigens (small viral peptides 8-13 amino acids in length) with the help of MHC-I present on the surface of all nucleated cells, with the exception of germ cells (Lieberman, 2003). Once activated, antigen-specific cytotoxic CD8+ T lymphocytes lyse infected cells through the action of various enzymes (perforin, granzyme A and granzyme B). This enzyme-mediated process leads to apoptosis of target cells through activation of the cell death pathway known as the caspase cascade (Mahajan et al., 2003). It is believed that failure of CD8+ T cell maturation has a direct effect on the production of perforin (Migueles et al., 2002; Halwani et al., 2006).

HIV-1 specific CD8+ T cells have non-cytolytic anti-viral properties. Like NK cells, CD8+ T cells can produce chemokines that sequester the CCR5 ligand, such as β-chemokines macrophage-inflammatory proteins (MIP-1α, MIP-1β) and RANTES. Since these suppressive factors compete to bind to CCR5 and CCR3, they block the entry of R5 HIV into the target cell, resulting in the subsequent reduction of viral replication (Le Borgne et al., 2000). Another non-cytolytic anti-viral activity of CD8+ T cells involves secretion of the thermostable soluble cell anti-viral factor (CAF) (Walker et al., 1986). CAF can block HIV transcription, thus inhibiting viral replication (Shridhar et al., 2014). Upregulation of immunosuppressive PD-1 on the surface of HIV-1 specific CD8+ T cells is an indicator of chronic activation of the immune system. Subsequent cell exhaustion and lack of specific CD8+ T cell function differentiation, correlates with accelerated disease progression (Petrovas et al., 2006; El-Far et al., 2008; Kaufmann et al., 2009).

In the acute phase of HIV infection, the CD8 + T response is restricted to very few epitopes, mostly present in Env and Nef. Nevertheless, this narrow response gives rise to an effective response, causinga significant decrease in viremia (Goonetilleke et al., 2009). During the chronic phase, it is still possible to detect a CD8 + T response that is extended to a wider variety of epitopes (Addo et al., 2003). However, this response results in selective

immunological pressure on the virus. HIV, having the ability to mutate easily due to its DNA polymerase, eventually escapes the CD8 response (Addo et al., 2003). CD4+ T cells indirectly activate CD8 + T cells by stimulating DCs to produce IL-12, a cytokine essential for initiating the CD8 response. However, it is known that CD4+ T cells are affected by HIV which could prevent the development of a new CD8 response against mutated virions that have escaped the cytotoxic response (Hall et al., 2001; Walker et al., 2012). Following early infection (within the first month) and continued administration of ART for one year, a reduction in CD8+ T-cell activation was reported; similar to levels found in uninfected controls (Chevalier et al., 2013; Markowitz et al., 2014). However, other studies have reported higher levels of activated CD8 + T cells compared to the healthy donors (Jain et al., 2013; Vinikoor et al., 2013). This might be explained by the differences in CD4+ T cell levels and time of ART initiation (days or months) of these studies.

## 1.9. Immunopathogenesis of HIV-1

### Chronic immune activation

Chronic activation of the immune system in HIV-1 infected individuals is an integral part of pathogenesis and is indicative of clinical progression (Hazenberg et al., 2003; Deeks et al., 2004). Key characteristics of chronic immune activation include polyclonal activation of B lymphocytes (Lane et al., 1983) and high turnover of CD4+ T and CD8+ T cells accompanied by increased expression of their activation molecules (Hellerstein et al., 1999) such as CD38 and HLA-DR (Giorgi et al., 1993; Ho et al., 1993), and high concentrations of pro-inflammatory cytokines and chemokines (Valdez et al., 1997). Several studies have shown that the presence of these activation markers and the plasma levels of IL-6, IFNγ, TNF receptor and coagulation markers are important indicators of accelerated aging and subsequent progression to AIDS (Liu et al., 1997; Sokoya et al., 2017). Several potential factors have been studied to explain how chronic activation of the immune system is established. As previously mentioned, the rapid destruction of memory CD4+ T cells at the GALT level, causing a breach in the mucosal barrier, and the subsequent microbial translocation could greatly contribute to this chronic activation (Brenchley et al., 2006). To explain the conditions responsible for the alteration of the epithelial

barrier, Estes et al. showed that the strong microbial translocation in rhesus macaques infected with SIV was the result of damage to the epithelial barrier of the gastrointestinal tract; under these conditions, the host's immune system could not eliminate the large number of transposed bacteria, leading to persistent immune activation (Estes et al., 2010). This gap allows for the translocation of microbial products, such as lipopolysaccharide (LPS), from the intestinal flora into the systemic circulation. This causes immune and proinflammatory cell (pDC, neutrophils, monocytes) activation to the detriment of regulatory cells essential for maintaining the integrity of the epithelial barrier, thereby accentuating the translocation of microbial products and the activation of the immune system, particularly in the binding of receptors such as TLRs (Klatt et al., 2013; Paiardini et al., 2013). These TLRs recognize specific molecular patterns associated with pathogens. Activation of these receptors promotes local inflammation, as well as systemic activation of the immune response (Brenchley et al., 2006; Brenchley et al., 2008a; Baeten et al., 2013). However, HAART, which reduces viral load (Evans et al., 1998), appears to lower the presence of activation markers. This suggests that active replication of the virus is also involved in establishing chronic activation of the immune system (Tilling et al., 2002).

## Immune exhaustion and immunosenescence

Persistent exposure to HIV antigens and sustained activation of the immune system lead to gradual fatigue of the immune response directed against the virus. In particular, there is over-expression of different negative immune regulators such as PD-1, CTLA-4, T cell immunoglobulin mucin 3 (TIM-3), and lymphocyte activation gene 3 (GLAC-3) (Khaitan et al., 2011). These negative immune regulators inhibit T cell activation through inhibition of TCR signalling pathways, rendering them unable to proliferate or to secrete cytokines following antigenic stimulation of the T cell (Xu et al., 2017).

Despite early introduction of HAART, HIV-infected individuals experience early age-related changes to their immune system, known as immunosenescence. During immunosenescence, T cells (mostly effector memory T cells) lose the expression of co-stimulatory receptors CD27 and CD28, which are required for optimal T cell activation. T cells also experience reduced expression of homing receptors such as CCR7 and CD62L (Sokoya et al., 2017), and an increase in expression of senescence markers CD57 and Killer cell lectin-like receptor sub family G (KLRG-1) (Xu et al., 2017). Immunosenescent T cells are unable to

proliferate but can induce a pro-inflammatory state by secreting pro-inflammatory cytokines (Xu et al., 2017) and, thus, are believed to contribute to cardiovascular diseases (Yu et al., 2016).

## 1.10. Restoration of immune response with ART

### 1.10.1. Timing of HAART initiation

The current recommendation of the World Health Organization (WHO) for the treatment of HIV infection is to start ART as early as seroconversion is confirmed, regardless of CD4 counts (WHO, 2015). Early initiation of treatment protects against significant CD4 T cell decline and reduces the risk of developing related opportunistic infections. Additionally, it reduces the potential of establishing a large HIV reservoir (Thorner et al., 2003). Early initiation is also recommended as a part of a comprehensive prevention program. This recommendation was based on several trials conducted to examine the efficacy of early ART initiation, notably Strategies for Management of Anti-Retroviral Therapy (SMART), Strategic Timing of Anti-Retroviral Therapy (START), Early Anti-retrovirals and Isoniazid Preventive Therapy in Africa (TEMPRANO), and HIV prevention network trial 052 (HPNT052). The results of the SMART study, which examined continuous *versus* CD4+ count guided interrupted use of ART, favoured continued ART regardless of CD4 counts to lower the risk of opportunistic infection and death (Smart-Study-Group et al., 2006). Both the START and TEMPRANO studies for early initiation and continued use of ART reported a decreased risk of HIV transmission, death and AIDS related morbidity and mortality by 40% (Siedner, 2016). The HPTN 052 trial was conducted in nine countries and examined the efficacy of using anti-retroviral therapy in people living with HIV-1 in serodiscordant couples. This trial showed that early initiation and use of anti-retroviral therapy in this group, until achieving sustained viral suppression status, was greatly effective at reducing sexual transmission of HIV infection to their partners, as they were no longer contagious (Cohen et al., 2016).

### 1.10.2. Anti-retroviral therapy as prevention against HIV

Administration of anti-retrovirals to uninfected individuals who are at high risk of developing HIV, such as serodiscordant heterosexual partners and men who have sex with men (MSM), can protect them from developing HIV infection. This refers to the concept of pre-exposure prophylaxis (PrEP) (Baeten et al., 2013). The combination of two NRTIs, emtricitabine and tenofovir disproxil fumarate, was recently approved for use as PrEP. According to clinical trials such as the Ipergay trial (an intermittent PrEp use study) and the Proud trial (a continuous PrEp use study), administration of PrEP prior to sexual activity reduces the relative risk of HIV transmission by 86% (Spinner et al., 2016). Moreover, the use of PrEP has been proven to be cost-effective (Ouellet et al., 2015).

## 1.11. HIV Reservoirs and Other Obstacles to the Treatment of HIV-1

HIV infects activated cells (Oswald-Richter et al., 2004). Although the majority of activated CD4+ T cells die during the contraction phase of immune response, a subset of them become quiescent; these are the memory lymphocytes ("resting memory"). During the resting state, memory cells stop active production of viral RNA and HIV-1 establishes a viral reservoir, where its integrated proviral DNA remains transcriptionally inactive (latent) in the host cell. This latency permits HIV to avoid recognition by the immune system and can persist for many years due to the intrinsic capacity of memory CD4 T cells to live for a prolonged period of time (Coiras et al., 2009). Although it has been shown that both central memory CD4+ T cells (CD45RA-CCR7 + CD27 +) and transitional memory CD4+ T cells (CD45RA-CCR7-CD27+) are the major cell subsets harbouring HIV reservoirs in subjects receiving anti-retroviral therapy and having undetectable plasma viral load (Chomont et al., 2009), they can also be found in other cell types and tissue sanctuaries.

Even though the use of anti-retroviral therapy has its indisputable advantages, it does not eradicate the virus that persists in latent cell reservoirs. This latent reservoir presents a major

obstacle to the treatment and development of a potential cure of infection, as it has the capacity to reinitiate infection once treatment is stopped.

While HIV persistence in immune cells under treatment represents a major hurdle for the development of a potential cure for HIV, other viral sanctuaries where HAART cannot exert its activities, such as the brain, also present an obstacle. Various studies have reported that HIV-1 can cross the blood brain barrier and that it targets astrocytes and microglia (Price et al., 1988; Gorry et al., 1999; Archin et al., 2014b). However, this physiological barrier can prevent anti-viral drugs from reaching brain cells that harbor the HIV genome (Ene et al., 2011), thereby preventing complete eradication of the virus.

The field has made significant attempts to establish more practical means to eradicate the latent HIV reservoir without harming infected individuals (Dahabieh et al., 2015). Multiple anti-latency compounds have been developed and clinical trials are in progress, with the goal of re-activating dormant HIV-infected cells and rendering them susceptible to both anti-viral drugs and the immune system. This strategy is known as "*shock and kill*" (Archin et al., 2014a). Among the potential anti-latency agents being studied are histone deacetylase inhibitors (HDACi), bromodomain inhibitors, extra terminal proteins inhibitors, and Protein Kinase C (PKC) activators (Archin et al., 2014a). HDACi, such as vorinostat, cause disruption of viral latency and promote HIV transcription (Archin et al., 2012). Bromodomains act by interacting with acetylated lysine residues on both histone and non-histone proteins, leading to histone acetylation (Bartholomeeusen et al., 2012).

Other experimental approaches to more effectively treat HIV include: permanent HIV suppression strategies by targeting the elongation process of HIV-1 transcription by p-TEFb (CDK9/Cyclin T1), or reactivation of HIV-1 latency by PIM-1 kinase, and activation of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) by Heat-Shock Protein 90 and, consequently, HIV-1 transcription following activation of T cells (Dahabieh et al., 2015). Moreover, it has been shown that, within the memory CD4 T cell compartment, cells expressing high levels of the negative immune regulator PD-1 harbour more integrated proviral DNA (Chomont et al., 2009). This molecule, together with other negative immune regulators such as CTLA-4, is believed to contribute to the latency mechanisms employed by HIV to persist and escape immune surveillance. Thus, functional treatment through immunotherapy was suggested by blocking these negative receptors in order to promote T cell activation and HIV reactivation

(Kaufmann et al., 2009). Leading candidates such as the anti-CTLA-4 ipilimumad and anti-PD-1 nivolumab antibodies are currently under investigation (Wykes et al., 2017).

Therapeutic HIV vaccines, active immunization strategies using HIV-derived immunogens, are also under investigation. These vaccines aim to slow down HIV progression and to maintain undetectable levels of HIV viremia, thereby eliminating the need for HIV-infected individuals to strictly adhere to HAART (Mylvaganam et al., 2015). While there is currently no FAD-approved therapeutic vaccine, clinical trials on a Tat vaccine have shown a reduction in both viral load and immune activation, and with restoration of immune cells (Gray et al., 2016). These promising results, among others, open the door for further exploration of vaccines as a therapeutic option.

## 1.12. Consequences of chronic HIV infection

The majority of HIV-infected individuals taking HAART achieve complete viral suppression (Havlir et al., 2016). Consequently, AIDS-related conditions have significantly diminished, as evidenced by the drastic drop in mortality rates; especially in developed countries with access to treatment. However, despite the success of HAART in controlling HIV infection, treatment does not fully restore the health of HIV-infected individuals, thus they must adhere to treatment as a lifelong therapy.

Many patients who have received HAART have a higher risk of non-AIDS-related morbidities. Some of the most common HIV-associated non-infectious co-morbidities include but are not limited to cardiovascular illness, lung and liver disease, kidney impairment, cancer, and neurocognitive decline (Deeks et al., 2013).

### Gut and liver diseases in HIV

Chronic HIV infection is commonly associated with diarrhea in about 50-70% of infected individuals (Crum-Cianflone, 2010). HIV enteropathy (i.e., direct HIV infection of gut cells) follows alteration of the GI-tract function and structure as either a direct or indirect effect of HIV infection. This destabilization of the gut happens as a result of ionic balances in the epithelium and apoptosis of enterocytes due to the induction of local cytokines such as IL-6, IL-

10 and TNF (Schmitz et al., 2002; Kotler, 2005). This leads to chronic diarrhea, bacterial overgrowth and altered bowel motility. HIV patients are also at increased risk of opportunistic infections and malignancies of the GI tract, such as Candidiasis, Kaposi's sarcoma and lymphoma (Anwar et al., 2012; Yanik et al., 2016). Hepatic cells are also direct targets of HIV infection, as infection lowers the number of liver Kupffer cells which further affects the elimination of microbial translocation products by the liver (Housset et al., 1990; Schmitt et al., 1990). Due to increased rates of chronic hepatitis B co-infection in HIV patients, rates of liver cirrhosis are increased, and decompensated liver failure is accelerated (Sun et al., 2014).

## HIV and lung diseases

Compared to the general population, HIV-infected individuals have a higher risk of developing chronic obstructive pulmonary disease (COPD) and lung cancer. While this is partly due to increased prevalence of traditional non-HIV-related risk factors such as smoking, drug abuse and alcohol, infected individuals are prone to *Pneumocystis*, a yeast-like fungal infection, and to persistent inflammation by HIV and as a consequence of antiretroviral therapy (Morris et al., 2011). Although the rate of HIV-associated opportunistic lung diseases has decreased, bacterial pneumonia is still prevalent. Thus, vaccinations against influenza and pneumococcal diseases are recommended to prevent possible complication (Aberg et al., 2013).

## Renal complications

HIV-1 infected individuals are highly likely to experience a renal disorder throughout the course of their treatment (Winston et al., 2008). Several factors are associated with an increased risk of chronic kidney disease, for instance being of black race, of an older age, having a hepatitis C (HCV) co-infection, having a CD4 count less than 200 cells per $mm^3$, and having HIV viremia (Lucas et al., 2014). Renal disease impacts the metabolism of vitamin D, resulting in bone disorders (Atta, 2010).

## HIV and Thrombosis

HIV-infected individuals are at higher risk of developing venous thrombosis, with up to a 10-fold increase compared to the general population (Bibas et al., 2011). Ongoing HIV

infection can also affect the coagulation profile of the host, resulting in an imbalance between the activities of the anti- and pro-coagulation factors. This imbalance can lead to an increased risk of developing thrombosis (Baker, 2013). Additionally, in a study conducted by (Maggi et al., 2012), it was found that expression of pro-coagulating agents is upregulated by monocytes during HIV infection. Many factors, such as age, intravenous drug use, low CD4 count, protein C and protein S deficiency contribute to the development of venous thromboembolism (Bibas et al., 2011).

## HIV-positive individuals and cardiovascular disease

Even in the era of HAART, cardiovascular disease (CVD) remains the major cause of death among HIV-infected individuals. Although HIV-infected individuals under treatment experience a longer life expectancy, as a consequence, their risk of developing CVD has also increased (de Gaetano Donati et al., 2010). CVD is now a major concern for HIV patients and their caregivers. In a study carried out in the USA listing the causes of death among 6,945 HIV patients from 1996 to 2004, there was an increased proportion of non-AIDS deaths from 13% to 42% during the study period. Cardiovascular diseases accounted for up to 25% of total deaths, which was the most common cause of non-AIDS related deaths (Palella Jr et al., 2006). In chronic SIV infected macaques, studies by Shannon et al., showed that coronary arteriopathy was seen in nine out of fifteen infected macaques and myocardial related death occurred in around two-thirds of them (Shannon RP et al., 2000). According to the international Data Collection on Adverse Events of anti-HIV Drugs (D: A: D) study, aimed at determining the risk factors for developing CVD in HIV-infected subjects under anti-retroviral therapy, researchers reported that exposure to abacavir, lopinavir-ritonavir, indinavir and didanosine either alone or in combination was associated with a greater risk of developing acute myocardial infarction (AMI). This study included 33,347 HIV positive participants from 11 cohorts from across the USA, Europe and Australia (Friis-Møller et al., 2003; Worm et al., 2010). While low levels of nadir CD4 cells (< 200) increases risk of AMI in HIV-infected people (Silverberg et al., 2014), in a study carried out on 82,459 veterans from a veterans aging cohort study virtual cohort (VACS-VC), researchers found that low levels of HIV-RNA (< 500 copies/ml) in HAART treated patient was associated with an increased risk of AMI in HIV+ individuals compared to

the general population (Freiberg et al., 2013). In 2011, Durand et al., conducted a study using a dataset from the Régie de l'assurance maladie du Québec, which showed an increased incidence of AMI in HIV-infected individuals by 3.88/1000 patient-years compared to 2.11/1000 patient years in the non-infected group. In this study, exposure to anti-retroviral drugs such as abacavir, efavirenz, lopinavir and ritonavir were associated with a higher risk of AMI (Durand et al., 2011).

People living with HIV experience up to a two-fold increase in relative risk of developing AMIs, coronary heart disease, ischemic stroke, and heart failure (Triant et al., 2007; Freiberg et al., 2013; Marcus et al., 2014) compared to more than two- to three-folds increased risk of AMI in smoker HIV infected patients (Calvo-Sanchez et al., 2013). Some HIV-infected adults show signs of accelerated aging and encounter comorbid diseases such as renal impairment, CVD, and metabolic syndrome 5 - 10 years earlier than age- and sex-matched non-infected persons (Erlandson et al., 2014; Greene et al., 2015). In a study investigating the prevalence of AMI in HIV-infected patients, it was reported that HIV-infected individuals had increased rates of AMI with age compared to their age-matched non-infected peers, as shown in Figure 5 (Schambelan et al., 2008).

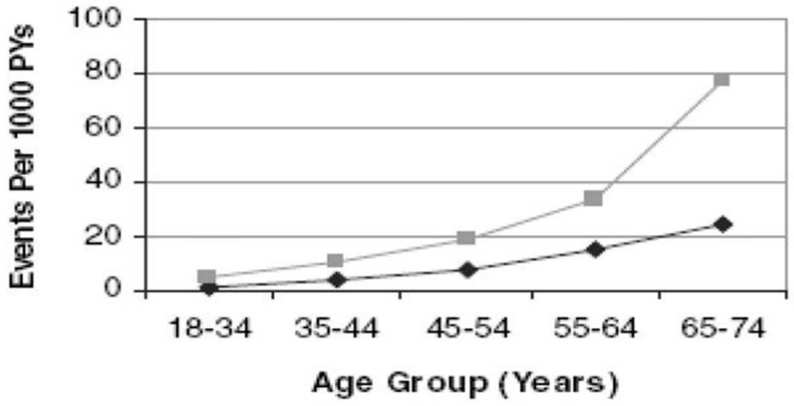



**Figure 5. Rates of AMI among the HIV-infected and non-infected population.** This figure represents the rate of AMI *per* 1000 individuals *per* year (PY) in HIV positive individuals (dark line), compared to age-matched uninfected patients (grey line). Reproduced from (Schambelan et al., 2008) with permission from Oxford University Press: The Journal of Clinical Endocrinology & Metabolism, copyright 2007.

## 1.13. Inflammation and cardiovascular disease

Several factors contribute to the persistence of the inflammatory state in HIV-infected patients. For example, HIV reservoirs and residual viremia are believed to play a role in the increased risk of cardiovascular events and complications (Martinez-Picado et al., 2016) as it maintains a state of persistent immune activation and chronic inflammation. Biased gut mucosal barrier immunity and bacterial translocation across a compromised epithelial barrier and inflammation (El-Far & Tremblay, 2017; Hadigan et al., 2017) are also involved in persistent immune activation. For instance, activation of monocytes as a result of microbial translocation increases the release of CD14, which is associated with cell death, and of CD163, which is associated with higher risk of non-calcified coronary artery plaque progression and atherosclerosis (Burdo et al., 2011; Fitch et al., 2013).

It is widely accepted that inflammation is a major mediator of CVD (Hadigan et al., 2017). Over the past years, several studies have shown that people living with HIV have CVD associated with several non-infectious immune-mediated chronic inflammatory diseases such as COPD, inflammatory bowel disease (IBD), and rheumatoid arthritis (RA) (Damen et al., 2017a). In fact, people living with HIV have higher levels of biomarkers and cytokines associated with endothelial dysfunction and accelerated atherogenesis (Kearns et al., 2017).

Several traditional risk factors such as sex (male), smoking, sedentary lifestyle, high blood pressure and dyslipidaemia, contribute to the increased risk of developing CVD in patients living with HIV, as well as in the general population. However, their prevalence, especially smoking and dyslipidaemia, seems to be higher in HIV individuals than in non-infected individuals, which contributes to raising their cardiovascular risk (Friis-Møller et al., 2003; Durand et al., 2011).

### 1.13.1. Endothelial cell dysfunction and association with carotid intima media thickness and arteriosclerosis

HIV infection results in the activation of multiple inflammatory pathways. This activation is attributed to the release of cell adhesion molecules (CAD) by HIV proteins (Tat and gp120), which further promotes adhesion and the transmigration of leukocytes to the

endothelium. Also, Tat helps to attract monocytes to the site of infection by inducing monocyte chemo-attractive protein-1 (MCP-1) (Maniar et al., 2013). There is a close connection between immune activation and endothelial dysfunction. MCP-1 induced cytokines such as TNFα and IL-6 to trigger the activation of the endothelial lining (Maniar et al., 2013), thus changing its functionality. Moreover, HIV-1 directly damages endothelial cells, thereby promoting apoptosis by increasing permeability of the cell. Nef can induce the release of inflammatory factors, such as IL-6 and TNFα, IL-1β and macrophage inflammatory protein 1, by activated macrophages (Olivetta et al., 2003), while Tat can induce IL-6 production by monocytes (Zauli et al., 1993). These events lead to an increase in adhesion molecules, such as intercellular adhesion molecule (ICAM)-1 and endothelial adhesion molecule (E-selectin), and of inflammatory cytokines, such as IL-6 and TNFα, which further promote the development of plaque formation, arteriosclerosis and subsequent CVD (Figure 6) (Mu et al., 2007).

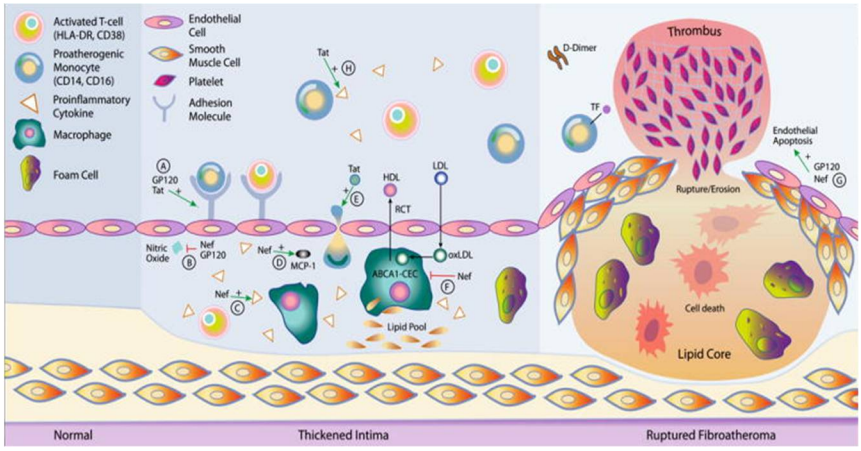



**Figure 6. HIV-mediated changed in arterial structure and function leading to atherosclerotic plaque formation.** Reproduced from (Nou et al., 2016) with permission from Wolters Kluwer Health, Inc, AIDS, copyright 2018.

Furthermore, in endothelial cell lines, HIV gp120 protein and TNFα decrease nitric oxide (NO) levels, which further impacts vascular tone, platelet adhesion, and aggregation (d'Ettorre et al., 2016). Increased viral load is associated with dysfunction of the endothelial layer and with subsequent increase in risk of CVD (Tawakol et al., 2017). Endothelial dysfunction is associated with increased frequency of occurrence of carotid intima media thickness (cIMT), a validated measure of sub-clinical atherosclerosis. CIMT is highly correlated with the standard risk factors

for atherosclerosis, such as hyperlipidemia, hypertension, diabetes, smoking, obesity and, in particular, cardiovascular illnesses (Albuquerque et al., 2013). A negative correlation was observed between cIMT and $CD4^+$ T cell counts in HIV+ subjects with $CD4^+$ counts <200 cells/$mm^3$ compared to an age matched non-infected population. Moreover, compared to the general public, the occurrence of cIMT is more frequent in HAART treated HIV+ patients (Maniar et al., 2013).

### 1.13.2. Cardiac imaging for coronary heart disease

Various non-invasive imaging modalities are now available to assist physicians in establishing early diagnosis of cardiovascular diseases and improving outcomes.

Two imaging types, functional and anatomical, are currently available to diagnose coronary artery disease (CAD) (Schuijf et al., 2005). Functional imaging helps evaluate the haemodynamic of CAD, such as nuclear cardiology and stress echocardiography; whereas anatomical imaging, such as magnetic resonance imaging (MRI) and multi-slices computed tomography (CT) scan, help to visualize the coronary artery tree (Schuijf et al., 2005). Non-contrast cardiac CT (CAC scan) helps identify calcifications in the coronary artery and provides calcium score measurement for early detection of sub-clinical atherosclerosis (Weber et al., 2015) and epicardial fat. Sub-clinical atherosclerosis can also be assessed non-invasively, through direct visualization of the carotid artery for cIMT, using carotid ultrasound (Weber et al., 2015; Kim et al., 2017). Moreover, coronary computed tomographic angiography (CCTA) scanning can be used to evaluate the atherosclerotic plaque and correlate it with immunological data. Use of CCTA allows for screening and early detection of coronary plaque volume and of lipid-rich low attenuation plaque volume, a plaque vulnerability marker, which are considered to be indicators of CAD (Chen et al., 2016). In this project, coronary computed tomographic angiography (CCTA) scanning will be used to evaluate the atherosclerotic plaque and correlate it with the collected immunological data.

## 1.14. Highly expressed inflammatory factors in HIV infection

Several biomarkers have been found to be associated with a risk of CVD in patients infected with HIV. For instance, C-reactive protein (CRP) and IL-6 have been associated with a 6-fold increase in rate of mortality in patients with unstable angina, compared to the 3.5-fold increase observed with TNFα (Koukkunen et al., 2001). In contrast, there was no relationship between the inflammatory markers (CRP and IL-6) and cIMT, calcium score or coronary stenosis (Vos et al., 2016). Therefore, these markers may not predict all cardiac events in HIV+ patients, especially in those with cIMT (Vos et al., 2017). However, it may be beneficial for these markers to join the list of other predictors of CVD, such as those described below:

IL-1β is a member of IL-1 family. This pro-inflammatory cytokine is produced by many immune cells, such as eosinophils, macrophages, T cells, and DCs (Turner et al., 2014). Although the exact mechanism is not fully clear, the expression of IL-1β is upregulated during HIV infection. In a study conducted on human monocytes, it was reported that maturation of IL-1β via the NLRP3 inflammasome-dependent mechanism and the expression of pro-IL-1β, which is required for the production of IL-1β, are induced by HIV-1 infection (Guo et al., 2014). In turn, IL-1β enhances the expression of HIV-1 (Granowitz et al., 1995). Moreover, IL-1β enhances systemic inflammation. It was observed that Caco2 colorectal adenocarcinoma cells treated with IL-1β produce other inflammatory cytokines, such as TNFα and IL-8 (Leonard et al., 2010). IL-1β mainly targets the endothelium, which then increases endothelial production of IL-6, which increases endothelial permeability, of prostaglandin E2 (PGE2), an enhancer of platelet aggregation and vascular permeability, of fibrinolysis inhibitors, and of adhesion molecules that enable leukocyte adherence to endothelial cells (Mu et al., 2007). All together, these inflammatory cytokines play an important role in atherogenesis (Mu et al., 2007).

IL-6 is a pro-inflammatory cytokine that plays a key role in both acute phase response and in the transition from acute to chronic inflammation (Heinrich et al., 1990). Upon HIV infection, IL-6 can be secreted by monocytes (Nakajima et al., 1989) and macrophages (Breen et al., 1990), and its levels are not normalized; not even under HAART treatment (Neuhaus et al., 2010). At physiological levels, IL-6 expression plays a role in acute phase responses. However, dysregulated expression of IL-6, resulting in persistently high levels, is associated with adverse effects and pathogenic roles, especially in CVD (Hartman et al., 2014; Reiss et al.,

2017)(Hartman et al., 2014, Reiss et al., 2017). For example, continuous activation of gp130, a signal-transducing receptor component for IL-6-related cytokines, causes myocardial hypertrophy in humans and in mice (Eiken et al., 2001; Ancey et al., 2003). IL-6 has a significant influence on the cells of the immune system, as well as on other non-immune cells by exerting hormonal features capable of impacting the homeostatic process (Hunter et al., 2015). High levels of IL-6 are related to several conditions including older age, low nadir CD4+ cell count (i.e., predictor of HIV neurocognitive impairment), use of protease inhibitors (i.e. anti-retroviral and anti-cancer treatments), and HIV replication (Borges et al., 2015). Additionally, independent of the traditional cardiovascular risk factors in HIV, IL-6 is strongly associated with and predictive of the development of CVD (Duprez et al., 2012).

IL-8 belongs to the intracrine α group. It is a chemotactic element produced from multiple cell types in response to a particular inflammatory stimulus and functions by attracting T cells, basophils, and neutrophils. However, IL-8 does not attract monocytes (Pananghat et al., 2016). High levels of IL-8 are found in the lymphoid tissue of AIDS patients (Lane et al., 2001). Moreover, HIV-1 replication can be stimulated by IL-8 in macrophages and T lymphocytes (Lane et al., 2001). IL-8 is produced by neutrophil granulocyte cells, including macrophages, keratinocytes, endothelial cells, and mast cells (Turner et al., 2014). Many studies have reported that high levels of IL-8 are present and produced by macrophages in atherosclerotic plaques. However, vascular smooth muscle cells (VSMCs) and epithelial cells are also potential sources of IL-8 (Apostolakis et al., 2009). Angiotensin II stimulates the induction of IL-8 in VSMCs, which might lead to exacerbation of atherosclerosis (Ito et al., 2002).

TNFα binds to TNFR1, receptors found in all cell types, and to TNFR2, present only in immune and endothelial cells, to initiate its cellular signalling pathway. It triggers different signalling pathways, such as apoptotic pathways, NF-κB, p38 MAPK and JNK. Compared to the general population, high levels of TNFα have been reported in HIV-infected patients by several studies (Kumar et al., 2016). These levels are not normalized even after HAART treatment (Kaplan et al., 2012). TNFα also plays a pivotal role in the pathogenesis of HIV-1. During chronic HIV-1 infection, TNFα enhances transcription and replication of HIV-1 and helps HIV-1 to expand its reservoir through use of the TNFα signalling pathway (Kumar et al., 2016; Pasquereau et al., 2017). However, another study reported that in freshly infected Peripheral Blood Mononuclear Cells (PBMCs) and alveolar macrophages, HIV-1 replication

can be inhibited by TNFα (Lane et al., 1999). Moreover, TNFα plays a role as a pro-atherogenic cytokine and its high levels could predict CVD risks in both HIV-infected and non-infected subjects (Kaplan et al., 2012; Bae et al., 2017).

CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein systematically expressed on the surface of multiple cells like macrophages, keratinocytes, endothelial cells, mast cells, DCs, neutrophils, and B cells. CD14 exists in two forms: membrane-bound mCD14, and soluble sCD14. Several inflammatory diseases of different origin, such as infectious and autoimmune diseases, are associated with increased levels of sCD14 in human blood (Cauwels et al., 1999). Persistently high levels of sCD14 were reported in HIV-infected subjects even after treatment. However, it has been formally linked to a higher rate of HIV morbidity and mortality (Leeansyah et al., 2013). HIV-infected individuals with CVD have also shown high plasma levels of sCD14 (Shive et al., 2015); thus, there is a strong link between increased levels of plasma sCD14 and incidence of cIMT (Leeansyah et al., 2013).

# 1.15. IL-32 as a potential player in CVD in HIV infection

## 1.15.1. Discovery and general properties of IL-32

IL-32 is a protein of approximately 27 kDa first discovered in 1992. Formerly known as NK transcript 4 (NK4), it is significantly expressed in activated T and NK cells (Kim, 2014). The IL-32 gene is located on chromosome 16, it is comprised of eight exons, a tyrosine sulfation, various putative phosphorylation sites, and three potential N-myristylation sites. IL-32 is expressed in nine different isoforms generated by the alternative splicing of the longest IL-32γ isoform (Hong et al., 2017). Figure 7B shows the various IL-32 isoforms, which include IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε, IL-32ζ, IL-32η, IL-32σ, and IL-32θ (Ribeiro-Dias et al., 2017).

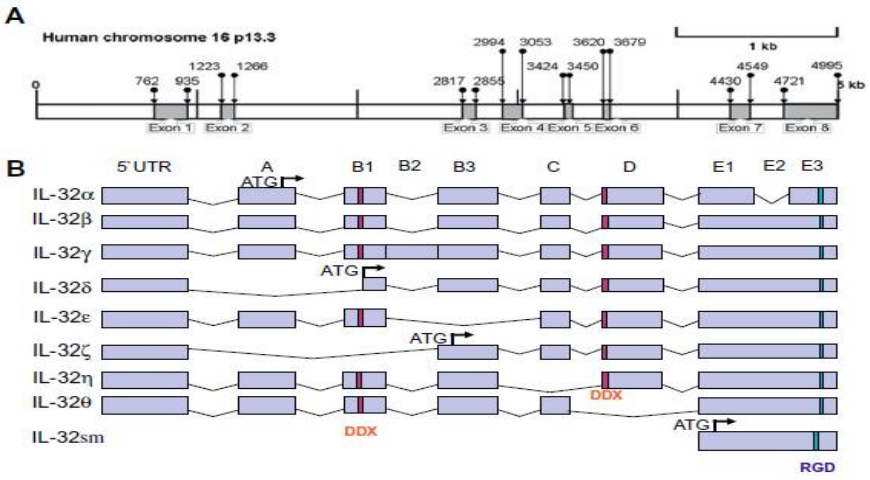



**Figure 7. The IL-32 Genome and Isoforms.** A) Organization of the IL-32 genome; B) Major isoforms of IL-32 (i.e., IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε, IL-32ζ, IL-32η, IL-32θ, and IL-32sm). Reproduced from (Hong et al., 2017) with permission from Elsevier: Pharmacology & Therapeuutics, copyright 2017.

When looking at the gene alignment, IL-32β is most homologous with equine IL-32. Both IL-32ε and IL-32ζ were first observed in activated T cells, whereas IL-32sm was isolated from activated Jurkat T cells (Hong et al., 2017). While it is expressed in various animals, like horses and pigs, IL-32 has not been identified in rodents such as mice and rats (Joosten et al., 2013). Although the IL-32 gene has been cloned from activated T cells, its expression has also been identified in other non-immune tissues such as muscle, liver, placenta, heart, colon, prostate, small intestine, lung, spleen, thymus, brain, and pancreas (Hong et al., 2017; Ribeiro-Dias et al., 2017). IL-32 is mainly cell associated cytokine as it doesn't possess a transmembrane domain except for the gamma isoform. IL-32 γ possesses a hydrophobic signal peptide in its N-terminus hence it is the only secreted isoform (Netea et al., 2006; Hong et al., 2017)

The process through which IL-32 performs its signalling functions remains poorly characterized. However, the protein could stimulate the secretion of various pro-inflammatory cytokines, including IL-6 and TNF-α, which are both critical inflammatory cytokines. Although receptors for IL-32 have yet to be established, recombinant human (rhu) IL-32 has demonstrated the ability to activate human PBMCs and murine macrophages cell lines. While the IL-32 gene is not present in rodents, studies investigating its biological activities have been carried out using

transgenic mice. For instance, expression of IL-32 has been shown to be upregulated in PBMCs after stimulation by phytohemagglutinin (PHA) (Ribeiro-Dias et al., 2017).

IL-32γ acts on monocytes and plays a key role in cell activation and death. Additionally, IL-32γ can splice into two less active pro-inflammatory isoforms, IL-32α and IL-32β, as shown in Figure 8 (Heinhuis et al., 2011b). These two isoforms serve a protective function by reducing inflammation (Damen et al., 2017a).

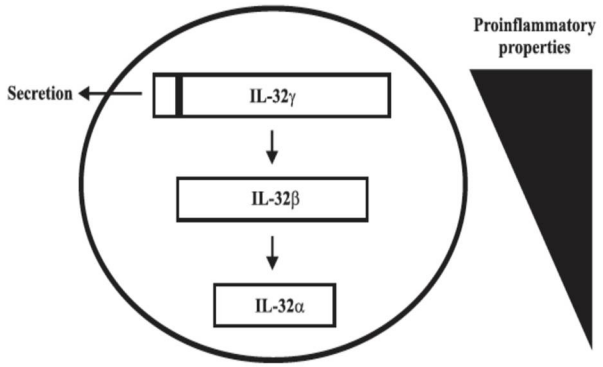



**Figure 8. IL-32 isoform splicing. Splicing of IL-32γ generates two isoforms (IL-32α, IL-32β) with diminished pro-inflammatory properties.** Reproduced from (Heinhuis et al., 2012b) with permission from Elsevier: Cytokine, copyright 2012.

When evaluating the different IL-32 isoforms, it is evident that rhuIL-32γ, compared to IL-32δ, IL-32α, and IL-32β, is the most active isoform. It is responsible for activation of the murine macrophage and PBMC cell lines, and for activation of the chemokines (Choi et al., 2009). The effects of rhuIL-32 are mediated by the activation of NF-κB, as well as by the phosphorylation of p38 mitogen-activated protein kinase (MAPK) (Kim et al., 2005).

## 1.15.2. IL-32 receptors

While several studies have focused on the IL-32 cytokine *per se*, the precise receptors of IL-32 remain unknown. Studies have reported that this cytokine is capable of binding to integrins αVβ3 and αVβ6 on the cell surface, thus identifying these integrins as plausible receptors for exogenous IL-32 (Kim, 2014). In addition, IL-32 can bind to proteinase 3 (PR3),

a neutrophil serine protease, which results in the cleavage of IL-32 and further enhancement of its biological activities (Novick et al., 2006; Kim et al., 2008).

### 1.15.3. IL-32 induction mechanisms

IL-32 transcripts are expressed in various immune cells such as monocytes, NK cells, DCs, T cells and B cells (Shoda et al., 2006; Kobayashi et al., 2009). Cytokines like IL-1β, IL-12, IL-18, and TNFα can induce the production of IL-32. These cytokines are responsible for the activation of the Protein Kinase C delta (PKCδ) pathway and of Protein Kinase B (Akt) transcription factors like NF-κB and AP-1, which stimulate IL-32 in various immune and non-immune cells. For instance, in macrophages, DCs, and monocytes, the activation of NF-κB is capable of inducing IL-32 mRNA through the mediation of both nucleotide-binding oligomerization domain-containing protein 2 (NOD2) agonists and TLR2 and TLR4 (Schenk et al., 2012). Moreover, the expression of intra-cellular IL-32 mRNA (particularly IL-32β) in human T cells can be induced by several stimuli such as anti-CD3 antibody, HIV and mitogens such as concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA) plus ionomycin. However, IL-18, TNFα and IL-23 stimulate IL-32 mRNA expression in peripheral $CD4^+$ T cells. In B cells, IL-32 mRNA can be induced by ConA and activated T cells (Hong et al., 2017). In non-immune cells such as endothelial cells and myofibroblasts, IL-1β induces IL-32 mRNA via activation of ERK, AKT, AP-1 and NF-κB pathways as illustrated in Figure 9.

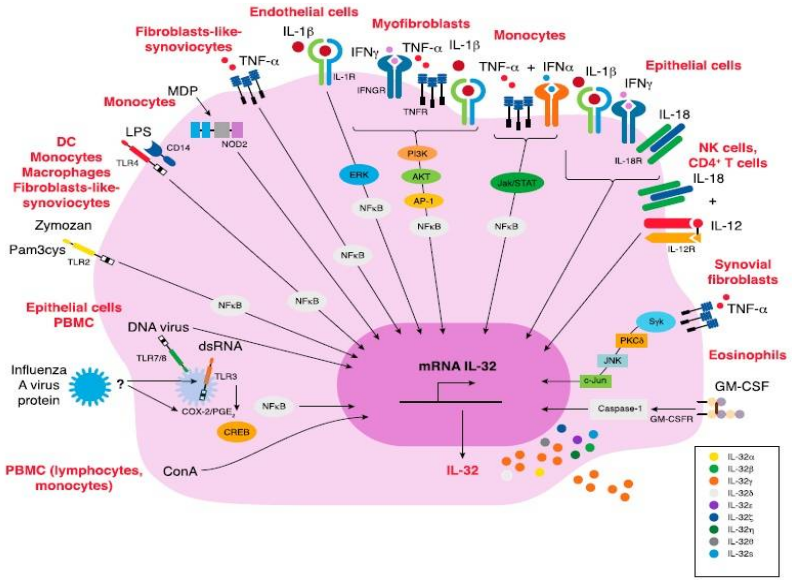


**Figure 9. Inducers of IL-32.** Reproduced from (Ribeiro-Dias et al., 2017) with permission of John Wiley and Sons, Journal of Leukocyte Biology, copyright 2016.

Several signalling pathways leading to the expression of IL-32 have been proposed. For example, *Mycobacterium tuberculosis* induces the expression of the IL-32 gene in PBMCs through the IL-18, caspase-1 and IFNγ signalling pathways (Netea et al., 2006). According to (Xu et al., 2012), hepatitis B virus (HBV) stimulates IL-32 expression via activation of NF-κB. This further enhances the rate of apoptosis, fibrosis and inflammation in hepatocytes infected with HBV or HCV (Xu et al., 2012).

IL-32 expression is also induced in several non-infectious inflammatory diseases. For example, in RA, the stimulation of synovial fibroblasts with TNFα induces the expression of IL-32 (α, β, γ and δ) through the activation of the Syk/PKC-δ/JNK/c-Jun pathways (Mun et al., 2009). In IBD patients, various pro-inflammatory cytokines such as IL-1β, IFN-γ and TNFα induce the expression of IL-32α mRNA in colonic epithelial cells (Shioya et al., 2007). In COPD patients, TNFα induces the expression of IL-32 in human lung fibroblasts via the NK/c-Jun pathways and its expression is further enhanced by the combination of IFN-γ and TNFα (Li et al., 2015).

## 1.15.4. Downstream effects of IL-32

Various effects of IL-32 have been reported on different cell types. For instance, stimulation of monocytes, THP-1 derived macrophages and monocyte-derived macrophages with rhuIL-32γ involves secretion of inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and chemokines, CCL2 and CXCL2, along with the anti-inflammatory cytokine IL10-through the activation of ERK1/2, Akt and Fyn protein tyrosine kinase phosphorylation signalling pathways (Choi et al., 2014). In vesicular stomatitis virus (VSV) infection, IL-32 was found to protect epithelial cells by preventing the cytopathic effect of VSV through the stimulation of MxA and IFN-α production (Zepp et al., 2011). Conversely, high levels of IL-32 in synovial fluid correlates with the level of inflammation and expression of pro-inflammatory cytokines, TNFα and IL-1β (Heinhuis et al., 2012). In general, the pro-inflammatory activity of most cells is exacerbated when exposed to overexpression of IL-32 (Joosten et al., 2013).

## 1.15.5. Role of IL-32 in Pathogen Infection

IL-32 is involved in many inflammatory illnesses, as illustrated in Figure 10. Levels of IL-32 have been shown to increase at both the protein and mRNA level in individuals with RA, graft versus host disease and atherosclerosis (Kobayashi et al., 2009; Heinhuis et al., 2011b). IL-32 expression is greatly increased in patients infected with *Mycobacterium avium* compared to non-infected subjects (Bai et al., 2011). Additionally, the intracellular multiplication of *Mycobacterium avium* is reduced in monocytes derived from human macrophages exposed to rhuIL-32γ. Increased apoptosis in these infected cells would partly explain the anti-mycobacterial effects of IL-32γ (Bai et al., 2011). Interestingly, the same effect was observed in the THP-1 human macrophage cell line stimulated with recombinant IL-32 and infected with *Mycobacterium tuberculosis*, resulting in apoptosis of the THP-1 cells due to increased levels of caspase-3 (Bai et al., 2010).

Influenza A virus activates the C-AMP Response Element-Binding protein (CREB) and stimulates the NF-κB pathway, as well as the cyclooxygenase (COX) 2 pathway, leading to overexpression of IL-32. By means of a negative feedback loop, IL-32 negatively regulates prostaglandin $E_2$-($PGE_2$) induced COX2 production (Li et al., 2008). IL-32 also mediates NO and inducible NO (iNO) expression resulting from influenza infection. Additionally, the influenza virus can stimulate the sIL-6Ra pathway, which up-regulates IL-32 by trans-signalling.

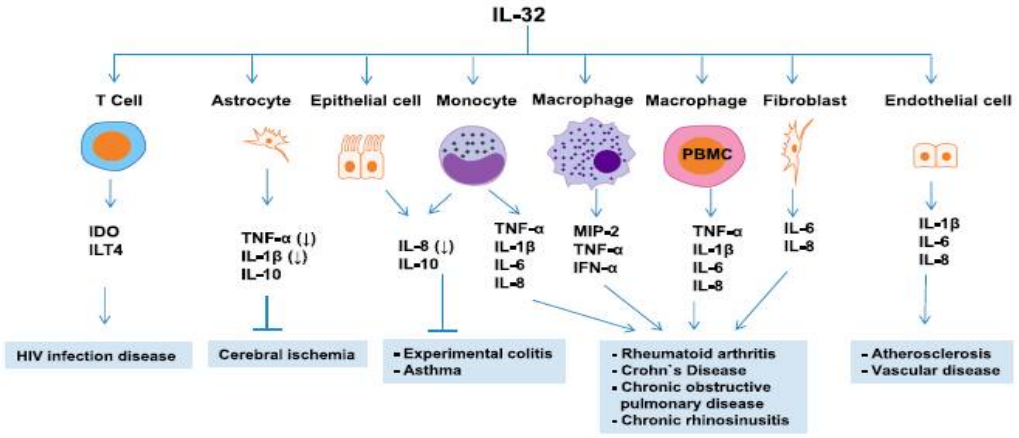



**Figure 10. Roles of IL-32 in Different Inflammatory Diseases**. This figure depicts the different mechanisms by which IL-32 causes several inflammatory diseases. Reproduced from (Hong et al., 2017) with permission from Elsevier: Pharmacology & Therapeuutics, copyright 2017.

## 1.15.6. Effect of HIV infection on the induction of IL-32

According to Rasool et al., (2008), IL-32 levels remain consistently higher in HIV-infected individuals compared to non-infected individuals. Interestingly, it has been observed that IL-32 plays an anti-viral role against HIV-1. Experiments using small interfering RNA (siRNA) to inhibit the expression of endogenous IL-32 in freshly infected PBMCs resulted in increased HIV-1 replication. However, overexpression of the recombinant IL-32γ isoform decreased, but did not inhibit HIV-1 proliferation (Nold et al., 2008). It has also been shown that inhibiting the biological activity of both IFNα and IFNβ in PBMCs previously exposed to rhuIL-32γ treatment accelerates HIV-1 replication. Therefore, it seems that type I IFNs mediate the anti-viral function of IL-32 by interferon-stimulated genes (ISG) (Nold et al., 2008).

## 1.15.7. IL-32 in HIV-1 infection

In 2017, Ribeiro-Dias et al., provided a hypothetical model for the role of IL-32 in HIV infection. This framework is described in Figure11.

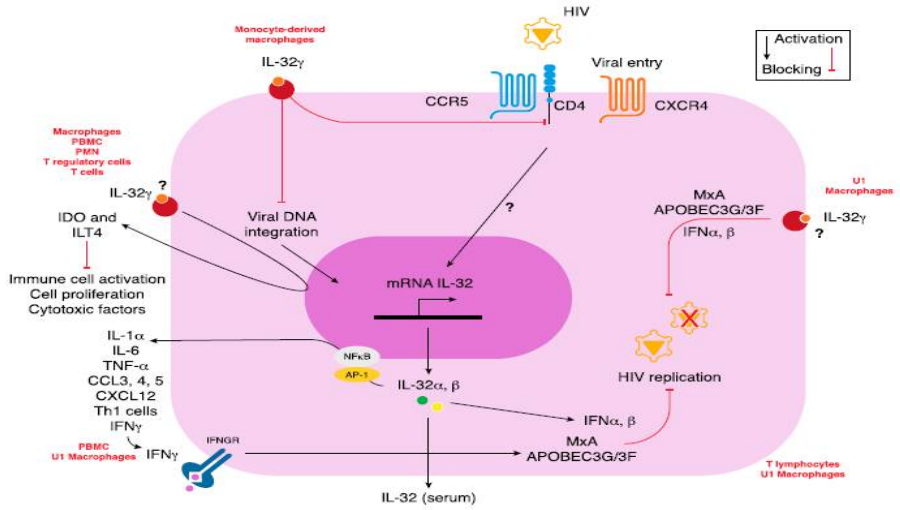



**Figure 11. The role of IL-32 in HIV Infections. HIV induce the expression of endogenous IL-32 in T-lymphocytes**. Additionally, the cytokine inhibits replication of the virus through a process that relies on IFN-α, Th1, and other inflammatory cytokines. Reproduced from (Ribeiro-Dias et al., 2017) with permission of John Wiley and Sons, Journal of Leukocyte Biology, copyright 2016.

In HIV infection, IL-32 has both anti-viral and immune-suppressive roles. IL-32 exerts its anti-viral function during the innate immune response, as soon as NOD-2 becomes involved and serves as a cytoplasmic viral PRR, recognizing viral peptides. This process leads to activation of IFN's regulation factors (IRFs) and to the subsequent increase in levels of IFNα and IFNβ. IFNα/β stimulates the production of IFN stimulated genes (ISGs) required for the inhibition of HIV-1 replication, and in the increased production of type I IFNs (Heinhuis et al., 2012).

Monteleone et al., (2014), have shown a negative correlation between the IL-32 gene expression and plasma viral RNA in chronically-infected individuals. PBMCs obtained from HIV-negative patients previously treated with the IL-32γ isoform demonstrated increased levels of restriction factors that control HIV-1 replication, such as proteins APOBEC3G/3F and MxA, compared to untreated PBMCs. The mRNA levels of IL-32α and IL-32 non-α in PBMCs from HIV-1 positive subjects correlate positively with expression of the genes coding for MxA and APOBEC3F proteins (Monteleone et al., 2014).

IL-32 induces immunosuppressive molecules Ig-like Transcript 4 (ILT4) and indoleamine 2,3-dioxygenase 1 (IDO-1) (Smith et al., 2011). This was observed in mucosal sites of HIV-infected subjects during the three major stages of infection (Smith et al., 2011). IL-32 can also induce the anti-inflammatory cytokine IL-10 (Kang et al., 2009). Together, these functions may compromise the immune response against HIV and, rather than serving as an anti-viral molecule, may favour HIV replication. The later hypothesis is supported by observations by El-Far et al., (2016), who reported a significant positive correlation between cytoplasmic levels of IL-32 and the HIV viral load, and negative correlations with CD4 and CD4/CD8 ratio (El-Far et al., 2016).

As mentioned earlier, various non-infectious inflammatory diseases such as COPD, IBD and RA induce the expression of IL-32. These chronic inflammatory diseases also cause activation of a wide range of several immune cells leading to enhanced expression of a variety of cytokines, such as IL-6, TNF-α, IL-1β, IL-17, and IL-32, which contribute to the persistent pro-inflammatory state of the immune system. This chronic non-resolved inflammatory response leads to altered lipid levels and composition, which further increases the risk of CVD (Nathan et al., 2010; Maclay et al., 2013; Damen et al., 2017a), as illustrated in Figure 12.

Furthermore, in addition to the antiviral role of type I interferons, they are also considered as important mediators in atherosclerosis by promoting endothelial dysfunction. Plasmacytoid dendritic cells producing IFN I was specifically found in rupture-prone plaque areas (Goossens et al., 2010).

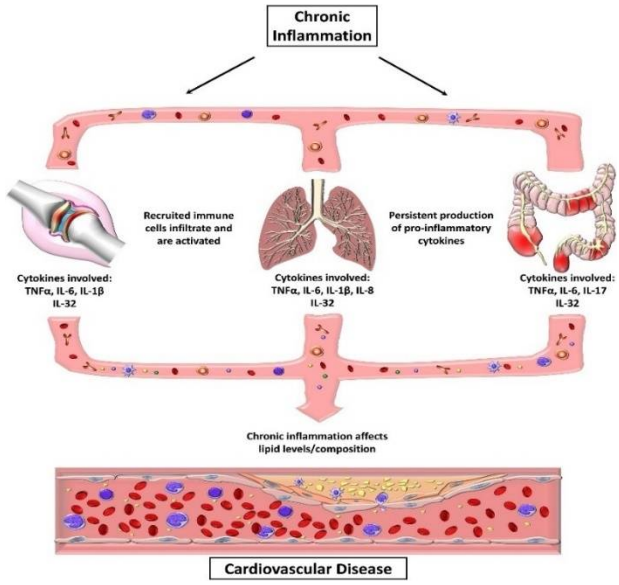



**Figure 12. Role of several inflammatory diseases where IL-32 may play a central role in the development of CVD**. Reproduced from (Damen et al., 2017a), no permission required.

For instance, in RA, several inflammatory mediators are known to contribute to its pathophysiology, among which IL-32 has been observed to play a key role (Damen et al., 2017b). However, the exact mechanism linking IL-32 to CVD has not been elucidated (Damen et al., 2017a). Studies on the blood of RA patients have investigated the impact of the genetic variants of IL-32 on CVD and lipid profiles. It was reported that in matching the IL-32 genotypes to the lipid profiles, the allelic distribution was found to be similar in all tested groups. Moreover, there is a single-nucleotide polymorphism (SNP) which impacts the function and effect of IL-32 on lipid profiles in RA patients (Damen et al., 2017b).

## Multiple roles of the different IL-32 isoforms

The main characteristic property of human IL-32 is the pro-inflammatory function mediated through IL-32α and IL-32γ. These isoforms can enhance the production of pro-inflammatory mediators such as IL-6, IL-8, IL-1β and CXCL8 (Heinhuis et al., 2011a; Kang et al., 2012). Nevertheless, there is emerging evidence linking certain IL-32 isoforms with cellular mechanisms involved in counteracting inflammation. For instance, IL-32β exerts an anti-inflammatory role by enhancing the secretion and expression of IL-10, which is known to play an important anti-inflammatory role (Kang et al., 2009). Although the exact function of IL-32ε is not known, a number of inter-isoform interactions between the different IL-32 isoforms add more complexity to its biological roles, as shown in Figure 13. Auto-regulatory functions of IL-32 were suggested through the binding of IL-32δ to IL-32β, to inhibit IL-32β-mediated induction of IL-10 (Kang et al., 2013). Similarly, counteracting functions on the induction of TNFα by two different IL-32 isoforms (γ and θ) was also observed. On one hand, induction of TNFα is enhanced by IL-32γ through the activation of NF-κB and p38 MAPK (Park et al., 2014; Kim et al., 2015), while on the other hand, IL-32θ blocks PKCδ, p38 MAPK and p65 NF-κB, and further inhibits secretin of TNFα (Kim et al., 2015). Given the important role of TNFα in inflammation and in CVD, and since IL-32 isoforms have different roles in the induction or inhibition of TNFα and other inflammatory cytokines, it is important to study their expression and roles in the context of HIV infection in association with CVD.

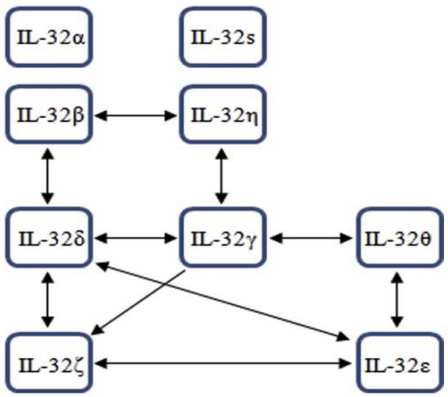



**Figure 13. Model for IL-32 isoform-interaction mapping.** Reproduced with permission from (Kang et al., 2014). This figure illustrates ten different interaction pairs among the various IL-32 isoform

# Chapter 2:

# RATIONALE, HYPOTHESIS AND OBJECTIVES

## 2.1. RATIONALE

HIV infection is associated with persistent inflammation and immune dysfunction that leads to premature aging phenotypes such as CVD. These complications are particularly observed in individuals who do not restore their CD4 T cell count despite the control of viral replication with anti-retroviral therapy (ART), as evidenced by increased levels of pro-inflammatory cytokines such as IL-6 and TNF-α. Novel inflammatory players that are likely upstream of IL-6 and TNFα are also upregulated in HIV infection and the potential role they play in CVD remains unknown. Interestingly, expression of IL-32 is detectable in the human atherosclerotic arterial vessel wall with enhanced expression of IL-32β and IL-32γ mRNA (Heinhuis et al., 2013). Enhanced expression of IL-32β and IL-32γ are markers of control failure and correlate with inflammation (Kobayashi et al., 2009; Nold-Petry et al., 2013).

This thesis builds on observations previously published by our laboratory, showing that IL-32 is linked to persistent inflammation, and to loss of virological and immunological control in slow progressing HIV subjects (El-Far et al., 2016). Moreover, IL-32 could predict the decline of important clinical parameters such as CD4 counts, CD4/CD8 ratio and increased inflammation. However, IL-32 is expressed in multiple isoforms, some of which are pro-inflammatory, and others which are anti-inflammatory or regulatory.

## 2.2. HYPOTHESIS:

We hypothesize that the balance between IL-32 pro- and anti-inflammatory isoforms plays a pivotal role in the fine-tuning of chronic inflammation and impacts the increase in age-associated CVD during HIV infection. Understanding the mechanism(s) of IL-32 modulation in the elderly may lead to novel therapeutic targets aiming to predict and/or prevent CVD in HIV patients.

## 2.3. OBJECTIVES:

**Objective # 1:** Study the expression of IL-32 in HIV+ aging individuals

**Objective # 2:** Develop isoform-specific quantitative tools for the different IL-32 isoforms (IL-32 is expressed in at least 9 isoforms) and identify the abundant IL-32 isoforms in subjects infected with HIV

**Objective # 3:** Characterize the role of IL-32 in accelerated CVD in HIV-infected and aging subjects

## Note:

Mohamed EL-Far contributed to the design of IL-32 primer sets to quantify the different isoforms, optimizing the PCR conditions and generation of the standard protocols. Etienne Larouch-Anctil helped in sample preparations for the whole cohort and measurements of total IL-32 protein by ELISA.

# Chapter 3:

# MATERIAL & METHODS

## 3.1. Study population and biological specimens

PBMCs and plasma samples were used from HIV-infected subjects enrolled in the Canadian HIV and Aging Cohort Study (CHACS). This cohort follows longitudinally 800 individuals living with HIV and 200 non-infected controls in 10 sites across Canada. A complete medical history is recorded prospectively. Complete haematological, biochemical, metabolic and immunological profiles along with anthropometric measurements were collected in a centralized, electronic data platform. The samples were taken with the informed written consent signed by all participants. The study was approved by the Institutional Review Boards (IRB) of the Centre Hospitalier de l'Universite de Montreal Research Center (Ethical approval #: CE.11.063). Samples were collected at study entry (baseline), year 1, 2, and 5 study visits. A subgroup of participants without overt CVD (n = 200) underwent cardiac computed tomography scan (CT Scan) with injection of contrast media and measurement of total atherosclerotic coronary plaque volume. Plasma samples were obtained after centrifugation of whole blood. Peripheral Blood Mononuclear Cells (PBMCs) were isolated and frozen in liquid nitrogen. A total of (n = 715; women n = 92 and men n = 623) participants (HIV+ and $HIV^{neg}$) with available blood samples at baseline visit were used in this study. The study participant demographics and clinical data are shown in Table I:

| SEX | Women | Men |
|---|---|---|
| **Number of participants** | 92 | 623 |
| **Age (Average ±SD)** | 52.6 ± 8.7 | 55.8 ± 9.2 |
| **Viremics (average age±SD)** | 49.96 ± 5.5 | 56.8 ± 11.9 |
| **Aviremics (average age±SD)** | 52.75 ± 9.4 | 54.97± 8.4 |
| **Duration of infection (Average ±SD)** | 15.6 ± 6.7 | 18.6 ± 8.2 |
| **Number of subjects on treatment** | 50(71.4%) | 347 (64.4%) |
| **Number of subjects with CT Scan** | 12 | 121 |
| **Average viral load of viremic patients under treatment** | 1044.4 | 45622.18 |
| **Average viral load of aviremic patients under treatment** | 39.07 | 39.45 |

**Table I. Demographics and clinical data of the study participants**

## 3.2. Sample preparation for ELISA

The available plasma samples from the baseline visits of both HIV+ and $HIV^{neg}$ subjects were inactivated using disruption buffer (DB) (PBS, 0.05% Tween-20, 2.5% Triton X-100, 0.02% thimerosal). The composition of the disruption buffer is described in Table II. Eight hundred (800) µl of plasma from each patient and 200 µl of disruption buffer were added to a deep 96-well plate (stackable volume of 1ml per well) and incubated for one hour at room temperature. Inactivated samples were then aliquoted in 10 aliquots for subsequent single use of each aliquot (to avoid multiple freeze/thaw conditions) and stored at -80°C.

| Description | Final concentration (5X) | Volumes |
|---|---|---|
| **PBS 10X** | 1X | 10ml |
| **Tween-20** | 0.05%(v/v) | 50µl |
| **Triton X-100** | 2.5% (v/v) | 2.5ml |
| **Trypan blue** | 1% (v/v) | 1ml |
| **Thimerosal 10%** | 0.02%(v/v) | 200µl |
| **ddH2O** | | Complete to 100ml |

**Table II. Composition of Disruption buffer (5X) and preparation of (1X) DB**

## 3.3. ELISA technique

IL-32 levels in plasma were measured by enzyme-linked immunosorbent assay (ELISA) using the human IL-32 ELISA kit (Cat # DY3040-05, Lot # P118541, R&D System Inc.) that uses the sandwich ELISA technique. Since this set allows the measurement of the main isoforms of IL-32 (α, β, γ and δ isoforms), it will be referred to it as total IL-32. Briefly, the capture antibody specific for the α, β, γ and δ isoforms of IL-32 was added to the 96-well ELISA plate (0.8 µg/ml in PBS; 100 µl/well), and the whole plate was incubated overnight. The next day, plates were washed three times with 300 µl/well of wash solution composed of PBS (pH between 7.2 and 7.4) and 0.05% Tween20. The plate was then incubated for one hour with a solution consisting of PBS (pH between 7.2 and 7.4) and 1% BSA in order to block any uncoated

areas of the ELISA plate from binding non-specifically to samples and secondary antibodies. The reference positive protein used to generate the standard curve was made from a known recombinant human IL-32 standard concentration solution; serial half dilutions ranging from 5000 pg/ml to 78.1 pg/ml were carried out using the 1% BSA in PBS solution. Plasma samples, diluted 1:3 in the PBS 1% BSA solution, were centrifuged at 1500 rpm/30 seconds. Next, the samples as well as the diluted standard reference protein were added to the plate and incubated for two hours at room temperature. After washing, the Biotinylated detection antibody (200 ng/ml; 100 µl/well) was added, followed by a two-hour incubation. Streptavidin conjugated with the HRP enzyme (horseradish-peroxidase) (100 µl /well; dilution 1:40) was added after washing five times and the plate was incubated in the dark for 20 minutes. The specific substrate of the enzyme (a mixture of $H_2O_2$ and Tetramethylbenzidine; 100 µl/well) was added followed by a 20-minute incubation in the dark. Finally, the reaction was stopped with a 2N sulfuric acid solution (50 µl/well). The plate was read using a spectrophotometer at dual wavelengths (450 nm and 570 nm) to correct for optical imperfections in the plate by subtracting the readings at 570 nm from the readings at 450 nm. The IL-32 concentration of the samples was deduced from the optical density and the standard curve. All incubations were performed at room temperature (20-25 °C).

IL-32α and IL-32 αβδ from Biolegend were also used to measure the plasmatic levels of IL-32 sandwich ELISA technique. Briefly, the 96-well plate was pre-coated with either IL-32α (Lot # B176144; 2 µg/ml) or IL-32 αβδ (Lot # B215986; 5 µg/ml) antibodies diluted in a coating buffer (Lot # B226015; 5x) and incubated at 4 °C overnight. The next morning, the plate was washed four times with a solution composed of PBS (pH between 7.2 and 7.4) and 0.05% Tween20 then blocked with 200 µl of Assay Diluent B solution (Lot # B213481;1 x) for one hour to block any uncoated areas of the plate. The standard range was calculated from a known recombinant human IL-32 standard concentration solution from which several binary dilutions ranging from 500 pg /ml to 7.8 pg /ml were carried out using the Assay Diluent B solution. The samples were diluted to half in the Assay Diluent B solution and centrifugated quickly at 1500 rpm. Next, the samples as well as the diluted standard range were added to the plate and incubated for two hours at room temperature (RT). After washing, the detection antibody (Biotin anti-human IL-32 αβγδ; Lot # B205099; 0.5 µg/ml) was added followed by a two-hour

incubation at RT. Streptavidin (HRP Avidin; Lot # B224078; 1: 2500 concentration) was added after washing, and the plate was incubated in the dark for 30 minutes followed by five washes of the plate. The specific substrate of the enzyme was added (TMP Peroxidase Substrate; Lot # D16031401; Fitzgerald co.) followed by 15 minutes incubation in the dark. Finally, 100 µl of 2 N sulfuric acid was added to stop the reaction. A spectrophotometer at 450 and 570 nm was used to detect the optical density values of the plates

Competitive ELISA was used for the detection of the alpha isoform (IL-32α) using the My BioSource Human Interleukin 32 alpha (IL-32α) kit (Cat # MB S283809). Briefly, samples were diluted as (1 : 2) in PBS (pH between 7.0 and 7.2) and centrifuged at 1500 rpm for 30 seconds. Next, the samples as well as the standard range consisting of different dilutions of recombinant human IL-32α or IL-32 γ standard solutions with concentrations ranging from 0 pg / ml to 1000 pg / ml were added to the plate pre-coated with anti-IL-32α specific antibody or anti-IL-32 γ for gamma detection. The blank is represented by PBS (pH 7.0 and 7.2). The 50 µl / well of conjugate provided with the kit was then added to all wells except the blank control well. After mixing, the plate was coated and incubated for one hour at 37 °C. The plate was then washed five times by adding 300 µl / well of the wash solution provided with the kit. After adding the substrate (100 µl / well), the plate was coated and incubated in the dark for 10 to 15 minutes at 37 °C. The reaction was stopped, and the optical spectra densities were measured by the spectrophotometer at 450 nm. The IL-32α concentration of the samples was deduced from the measured optical densities and the standard curve using linear correlation. The results were calculated using four-parameter logistic curve fit (4 - PL). The standard curve and the equation were generated online using the https://www.mycurvefit.com/ website.

## 3.4. Non-quantitative RT-PCR

IL-32α, β, δ, and ε were amplified using primer sets described in (Huang et al. 2015). IL-32 γ and θ were amplified with an in-house designed primer sets based on γ and θ transcript reference sequences with the GenBank accession numbers NM_001308078 and FJ985780, respectively. The sequences of the oligonucleotide primers (forward and reverse) are included in Table III:

| Oligo name | Oligo sequence in 5'-3' orientation |
|---|---|
| **28S sense**<br>**28S anti-sense** | **CGA GAT TCC CAC TGT CCC TA**<br>**GGG GCC TCC CAC TTA TTC TA** |
| **β-glucuronidase sense**<br>**β-glucuronidase anti-sense** | **TCTGTCAAGGG CAGTAA CCTG**<br>**GCCCACGACTTTGTTTTCTG** |
| **hIL-32A (Alpha) sense**<br>**hIL-32A (Alpha) anti-sense** | **CACCCAGAGCTCACTCCTCT**<br>**GGCTCCGTAGGACTTGTCAC** |
| **hIL-32B (Beta) sense**<br>**hIL-32B (Beta) anti-sense** | **GAAGACTGCGTGCAGAAGGT**<br>**CTTTCTATGGCCTGGTGCAT** |
| **hIL-32C (Epsilon) sense**<br>**hIL-32C (Epsilon) anti-sense** | **AGGCCCGAATGGTGATGT**<br>**GGCACCGTAATCCATCTCTT** |
| **hIL-32D (D) sense**<br>**hIL-32D (D) anti-sense** | **AGGACGTGGACAGGACGACT**<br>**AGGAGTGAGCTCTGGGTGCT** |
| **hIL-32G (Gamma) sense**<br>**hIL-32G (Gamma) anti-sense** | **TACTTCTGCTCAGGGGTTGG**<br>**TGGGTGCTGCTCCTCATAAT** |
| **hIL-32 (Theta) sense**<br>**hIL-32 (Theta) anti-sense** | **GAGCTGGAGGAGCTCACTCC**<br>**GAGCTCTGACAGAGAGCAGC** |

**Table III. Different Oligonucleotide primers (forward / reverse) for IL-32 isoforms**

(α, β, γ, D, ε and θ). The expression of each gene was normalized relative to internal controls, either the housekeeping gene 28S rRNA or the β-glucuronidase.

Non-quantitative RT-PCR was used for optimization and testing of the primer sets. Total RNA was extracted from total PBMCs using RNAeasy plus mini kit from Qiagen (Catalog No. 74134). Briefly, primary cells were lysed with the buffer RLT (composed of Guanidine thiocyanate + t-Octylphenoxypolyethoxyethanol and β-mercaptoethanol) provided in the kit. Total RNA was isolated using affinity columns. The concentration of the eluted RNA from the column was measured by a Nanodrop spectrophotometer (ND-1000). Complementary DNA (cDNA) was generated by reverse transcribing 25ng RNA obtained from the total PBMCs with the RT-PCR kit from Qiagen (RNase-Free DNase Set (50); Cat. No. 79254) using GeneAmp®

PCR System-9700 machine. The reaction was carried out in a total volume of 20µl. The cycling conditions were according to the protocol summarized in Table IV.

| Number on cycles | Temperature | Time |
|---|---|---|
| **1 cycle** | 50ºC | 40 minutes |
| **1 cycle** | 95ºC | 15 minutes |
| **30 cycles** | 94ºC | 15 seconds |
| | 56ºC | 30 seconds |
| | 72ºC | 30 seconds |
| **Store at** | 4ºC | |

**Table IV. Run protocol of non-quantitative PCR.**

To show the specific amplification of IL-32 mRNA, about 8 - 10 µl of each PCR reaction was analysed by DNA electrophoresis on 1.5 % agarose gel. The DNA bands were revealed by incorporation of ethidium bromide and exposure to UV light using the Bio-Rad Gel Doc XR System.

## 3.5. Quantitative RT-PCR by SYBR Green

Total RNA was extracted from PBMCs of HIV-infected aviremic subjects with cardiac imaging as well as HIV non-infected controls as explained in Section 3.4. Isoform specific RT-PCR amplification of IL-32 isoforms (α, β, γ, δ, ε and θ) was carried out on all samples with cardiac measures using one-step SYBR Green real-time RT-PCR in a LightCycler 480 II (Roche) using Qiagen reagents (RNase-Free DNase Set (50); Cat. No. 79254) as per manufacturer's recommendations. The PCR mixture for quantitative and non-quantitative PCR and the protocol (cycling conditions) for Quantitative RT-PCR are summarized in Tables V and VI respectively.

| QuantiTect primers | N = 1 | Final concentration |
|---|---|---|
| **2X Master mix** | 10 µl | 1X |
| **Primers FW 10uM (20X)** | 1 µl | 0.5 µM |
| **Primers R 10uM (20X)** | 1 µl | 0.5 µM |
| **QuantiTect RT mix** | 0.2 µl | |
| **Template RNA** | 5 µl | 25ng total |
| **H2O** | 2.8 µl | |
| **Total:** | 20µl | |

**Table V. Reaction mixture for quantitative PCR.**

| Number on cycles | Temperature | Time |
|---|---|---|
| **1 cycle** | 50ºC | 40 minutes |
| **1 cycle** | 95ºC | 15 minutes |
| **40 cycles** | 94ºC | 15 seconds |
| | 56ºC | 30 seconds |
| | 72ºC | 30 seconds |
| | 75ºC | 30 seconds |
| **Cool down at** | 40ºC | |

**Table VI. The cycling conditions of quantitative RT-PCR**

## 3.6. *In vitro* infection of cells with HIV-1

The non-stimulated PBMCs were infected *in vitro* with P86.9 Dual Tropic virus by spinoculation (O'Doherty et al. 2000) using 50 ng HIV p24 per one million cells. The plasmids containing the infectious clone of p89.6 HIV were obtained through the National Institute of Health (NIH) repository reagent program. The prepared virus was kindly provided by Dr. Nicolas Chomont's laboratory (CRCHUM). Briefly, HIV was added to PBMCS in a total volume of 200 µl (100 µl virus + 100 µl of cells (cell concentration of 10 x 10E6 / ml)) in flat-bottom 96-well plates. Cells were centrifuged at 1200 xg / 2 hours at 22 ºC. Afterwards, cells were cultured in the presence of virus for different time intervals; 3 hours, 6 hours and 24 hours at 37 °C. After each time point, supernatant was collected, and cells were pelleted for the ELISA of cell-associated IL-32 and/or IL-32 transcription by RT-PCR assay, respectively.

## 3.7. Bradford Technique

This technique was performed to measure total protein in cell lysates using the Bradford Protein Assay Quick Start Kit (BioRad® Hercules, CA, USA). Briefly, PBMCs from *ex-vivo* samples of study participants were lysed with a lysis buffer composed of a solution of RIPA 1X (Cell Signaling®, Cat # 9806), Protease inhibitor (Roche® Cat # 04 693 159 001), and phosphatase inhibitor (PhoSTOP 10X Roche® Cat # 04 906 837 001) was added to prevent protein degradation during the extraction. Serial half dilutions of the BSA protein (Bovine Serum Albumin) were performed to form the standard eight-point curve with the highest concentration of 2 mg / mL. The lysis buffer was used as the blank. Dilutions of BSA as well as protein lysates and Coomassie blue dye (Biorad® Cat # 1610406) were added to a 96-well plate. Coomassie brilliant blue is an acidic solution, which due to its physicochemical properties has the capacity to bind to the proteins present in the lysate and turns into a blue colour that can be measured at $A^{max}$ = 595 nm by spectrophotometers. The density of the colour reflects the concentration of protein. The protein concentration of the samples was deduced from the measured optical densities and the standard curve.

## 3.8. FACS cell sorting:

PBMCs were isolated from fresh blood or leukapheresis by Ficoll-Paque centrifugation. The expression of IL-32 isoforms in different subsets of PBMCS such as T cells, B cells, NK cells, NKT cells, and monocytes was determined after sorting these subsets by flow cytometry (BD FACSAria; BD Biosciences). For labelling purposes, cells were incubated with fluorochrome-conjugated antibodies as described below for 45 minutes at room tempreture. The samples were then washed and centrifuged at 1500 rpm / 5 minute. The antibodies used are Pacific Blue™ Mouse Anti-Human CD3 (Clone UCHT1) (0.3 µl / million cells) (BD biosciences®, Cat # 558117), Alexa Fluor® 700 Mouse Anti-Human CD4 (Clone SK3)(0.3 µl/million cells) (BD biosciences®, Cat # 566318), APC-H7 Mouse anti-Human CD8 (Clone SK1) (0.5 µl / million cells) (BD biosciences®, Cat # 560179), APC Mouse Anti-Human CD14 (Clone M5E2) (1 µl / million cells) (BD biosciences®, Cat # 555399), PerCP-Cy™5.5 Mouse Anti-Human CD19 (Clone HIB19)(1 µl / million cells) (BD biosciences®, Cat # 561295), PE Mouse Anti-Human CD56 (Clone B159) (2

µl/million cells) (BD biosciences®, Cat # 561903) and FITC Mouse Anti-Human CD16 (Clone 3G8) (1.5 µl / million cells) (BD biosciences®, Cat # 555406). Monocytes ($CD3^{neg}$, $CD4^{lo}$, $CD14^{+,}$ $CD16^{+/-}$), T cells ($CD3^{+}$, $CD56^{neg}$), B cells ($CD19^{+}$, $CD3^{neg}$, $CD56^{neg}$), NK cells ($CD3^{neg}$, $CD56^{+}$, $CD19^{neg}$), and NKT cells ($CD3^{+}$, $CD19^{neg}$, $CD56^{+}$). The antibodies were identified using a strategy of selection (gating strategy). An example is explained in Figure 14. Quality control analysis post-sort indicated an average purity of > 95%.

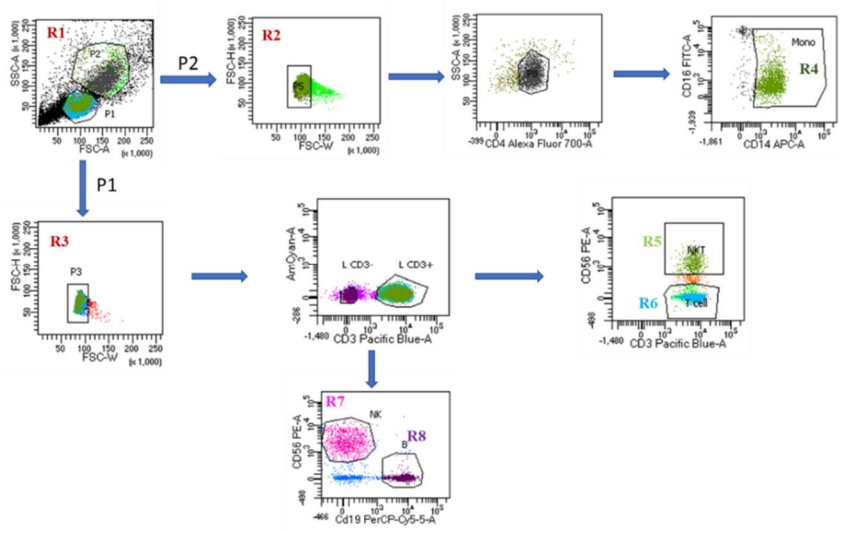



**Figure 14. Gating strategy for cell sorting of the major immune cell types in PBMCs.** As an example of cell type selection, the lymphocyte population (P1) and the monocyte population (P2) were selected based on SSC (side-scattered) and FCS (Forward scattered) (R1). As a result of eliminating the doublets (R2 and R3), the following populations were identified: ($CD3^{neg}CD4^{lo}CD14^{+}CD16^{+/-}$) monocytes (R4), ($CD3^{+}CD19^{neg}CD56^{+}$) NKT cells (R5), ($CD3^{+}CD56^{neg}$) T lymphocytes (R6), $CD3^{neg}CD56^{+}$ NK positive cells (R7), and ($CD3^{neg}CD56^{neg}CD19^{+}$) B-cells (R8).

## 3.9. Statistical analysis

The statistical analyses were carried out using the Prism 7 software. Differences between groups were deemed significant when the p value was $< 0.05$. Differences in the mean of data sets were assessed using the Student *t*-test for paired (from the same donor sample) or unpaired (from different samples). Mann-Whitney nonparametric tests were used for unpaired observations from different groups. Linear regression analysis and Spearman nonparametric tests were used for correlation studies.

# Chapter 4:

# RESULTS

## 4.1. Schematic representation of baseline samples

In this study, the available samples (n = 740) collected from baseline visits from Canadian HIV and Aging cohort were analysed. A total of (n = 107) samples were from $HIV^{neg}$ donors and (n = 608) HIV-infected subjects. Out of these available samples, 25 samples were excluded due to missing data on HIV status and sex. The HIV positive group was stratified into HIV+ females (n = 70) and HIV+ males (n = 538). Samples were divided into viremics and aviremics in both HIV positive male and female groups. Total numbers of subjects were as follows: (n = 19) HIV+ viremic women and women with unknown viremic load (VL), (n = 51) HIV+ aviremic women, (n = 210) HIV+ viremic men and men with unknown VL, and finally, (n = 328) HIV+ aviremic men. A subgroup of individuals in the HIV+ aviremic male p-group (n = 86) had cardiac measures (Figure 15). Due to lack of certain clinical parameters for some individuals, the number of analysed samples differs from one variable to another.

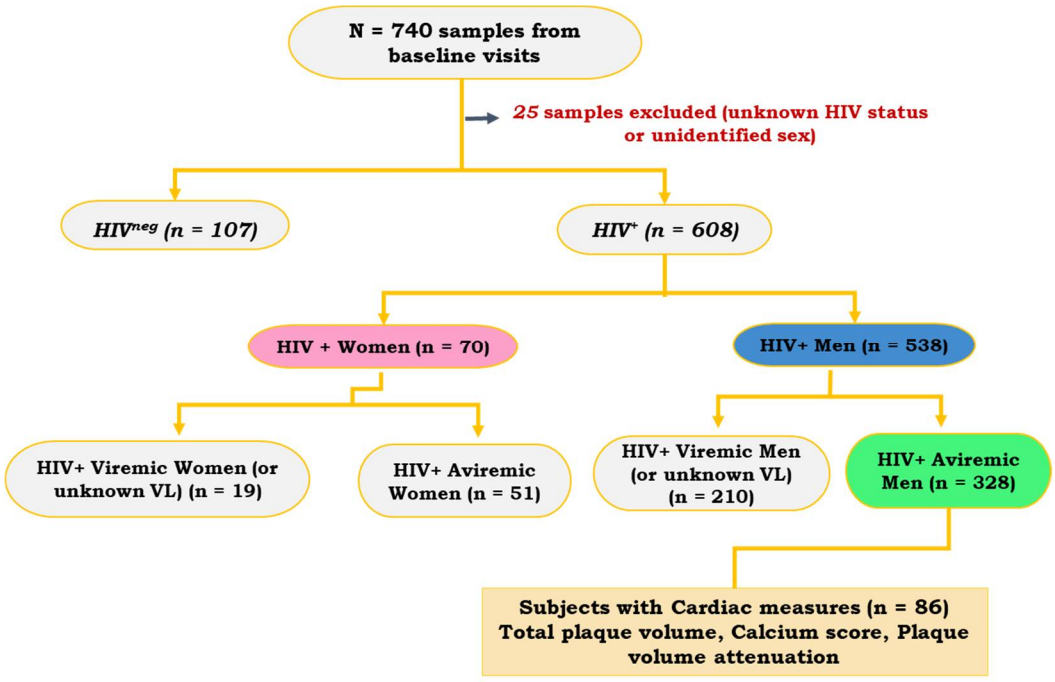



**Figure 15. Schematic representation for the number of available samples at baseline visits used in the current study.** Subjects are stratified by HIV status, sex and viral load.

## 4.2. IL-32α and IL-32αβδ isoforms

The first objective was to develop quantitative tools for IL-32. In order to measure IL-32 protein, the commercially available IL-32 antibodies were examined for quantitative assays by ELISA. The IL-32α isoform was tested at the protein level using ELISA sets from Biolegend and MyBiosource suppliers. In the first case (i.e. antibodies from BioLegend), lower concentrations were observed (close to the background levels). However, in the second case (i.e. antibodies from MyBioSource), there was a significant level of variability between experiments as shown in Figure 16. This was not due to the sample preparation nor to the standard protocol used nor the ELISA set since one-time thawed aliquots from the same original plasma sample were used to repeat the IL-32α measures and the same ELISA set lot. The variability in measures might be due to instability of the recombinant human IL-32α protein provided by the supplier to be used as a reference protein for the standard curve. A significant variability was observed using this recombinant protein that impacted the generation of the standard curve and subsequently the absolute quantification of IL-32α in the biological samples (Figure 17, left panel) compared to standard curves for IL-32 total protein (Figure 17, right panel). As shown in Figure 17 (generated by raw data of standard curves from all IL-32 ELISA tests ran in the lab), there was a significant variability in optical density measures for the recombinant proteins used to generate the standard curves for IL-32α. As the IL-32α ELISA is a competitive assay the intensity of the color is inversely proportional to the IL-32α concentration.

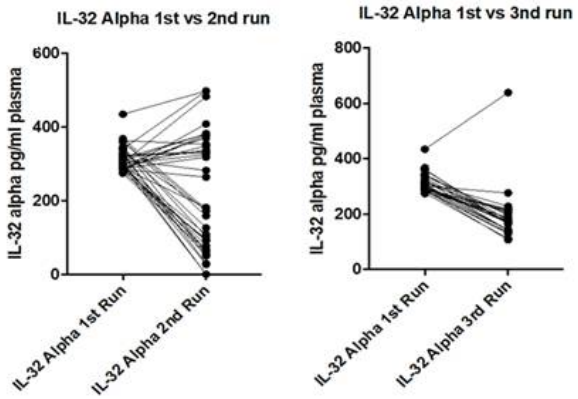



**Figure 16. Significant levels in experiment-to-experiment variations in measures of IL-32α but not total IL-32.** Testing the variability in readings between experiments using IL-32α kit on HIV+ subjects. Left panel shows the variability in readings between 1st and 2nd run (n = 55), while the right panel depicts the variability in readings between 1st and 3rd run (n = 31).

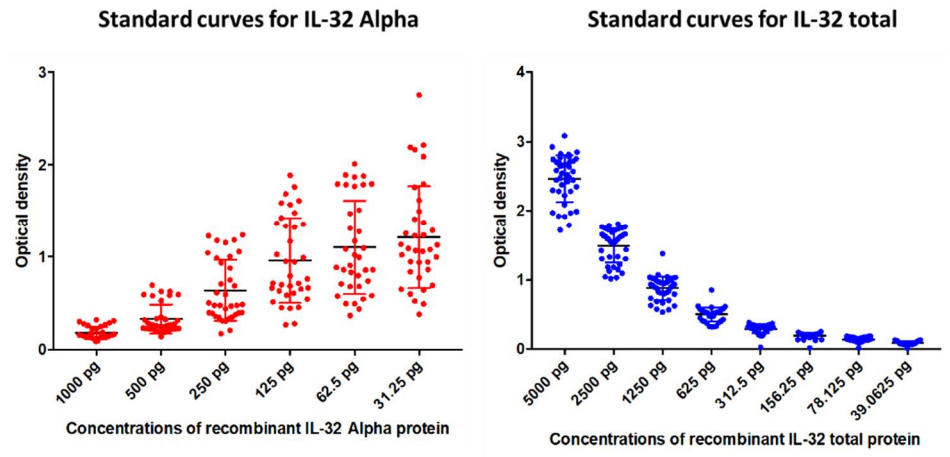



**Figure 17. Comparison of standard curve stability between IL-32α and IL-32 total proteins.** Each dot represents an independent experiment. This figure depicts a wide range of variability in readings for the same concentration of the different IL-32α standard protein concentrations (left panel) compared to standard curves for IL-32 total protein (right panel).

Antibodies against IL-32α, β, and δ, from Biolegend, were also tested. However, the results showed significant variability in measurements with very low detection values; therefore, these ELISA sets were not considered for further analysis.

## 4.3. Total levels of IL-32 in HIV-infected and non-infected subjects.

Due to the variability in IL-32α and IL-32 αβδ soluble measures, the assays mainly focused on using the standard total pool of IL-32 ELISA sets to test levels of IL-32 in plasma from HIV+ and aging subjects. The experiment-to-experiment variation was first tested in measures on n = 32. As shown in Figure 18, there was a limited variability (fold change of 0.65 ± 0.39) compared to fold change in the IL-32α measures (n = 55, fold change of 3 ± 2.49) ($p < 0.0001$). The total IL-32 ELISA set detects and quantifies IL-32α, β, γ and δ isoforms; therefore, it will be referred to as the total IL-32 in the rest of results.

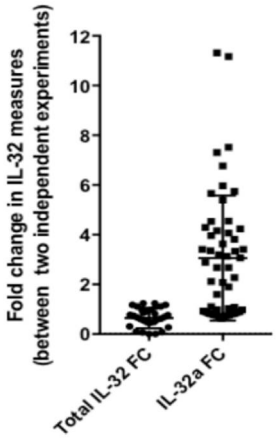



**Figure 18. Comparison of fold changes in experimentto-experiment variations between measurements of IL-32α and total IL-32.** The figure depicts the fold change in readings between independent experiments using IL-32α ELISA kit on n = 55 HIV+ subjects and total IL-32 ELISA kit on (n = 32) HIV+ subjects.

In order to study the expression of IL-32 in HIV+ aging individuals and compare it to the general population, the total IL-32 levels were measured in plasma from all available samples at the baseline visits. Similar to earlier results (Rasool et al., 2008; El-Far et al., 2016), the total IL-32 level remained higher in HIV-positive subjects compared to HIV-negative controls (Figure 19).

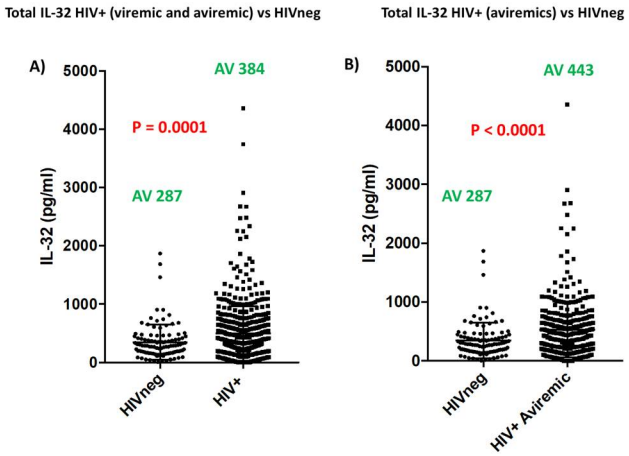



**Figure 19. Plasmatic levels of total IL-32 protein (pool of all isoforms) in HIV+ subjects.** A) IL-32 plasmatic levels in the total population of HIV+ subjects, n = 608 (viremic and aviremic) or B) only aviremic subjects (viral load < 50 copies/ml) with (n = 379) compared to non-infected controls (n = 107). P values are calculated by non-parametric Mann-Whitney test. AV = average.

## 4.4. Correlations between IL-32, common CVD markers, and viral load.

The total plasma IL-32 levels were determined in viremics (10% of the cohort) and aviremic subjects at the baseline visits. They were also correlated with CD4/CD8 ratio, an independent CVD biomarker (Huang et al., 2015), D-dimer, and LDL/HDL ratio (unpublished data provided by the CHACS cohort). The results showed a negative correlation between IL-32 levels and the CD4/CD8 ratio in the total population (viremics and aviremic) (Figure 20A). However, when limiting the analysis to the aviremic subjects (treated subjects with suppressed viremia), there was a tendency for a negative correlation without reaching statistical significance (Figure 20B). This is likely due to recovery of the CD4 counts and the decreased IL-32 levels. In support of this assumption, a significant negative correlation between IL-32 and the CD4/CD8 ratio (Figure 20C) as well as a highly significant positive correlation with HIV viral load (Figure 20D) was observed when limiting the analysis to the viremic subjects.

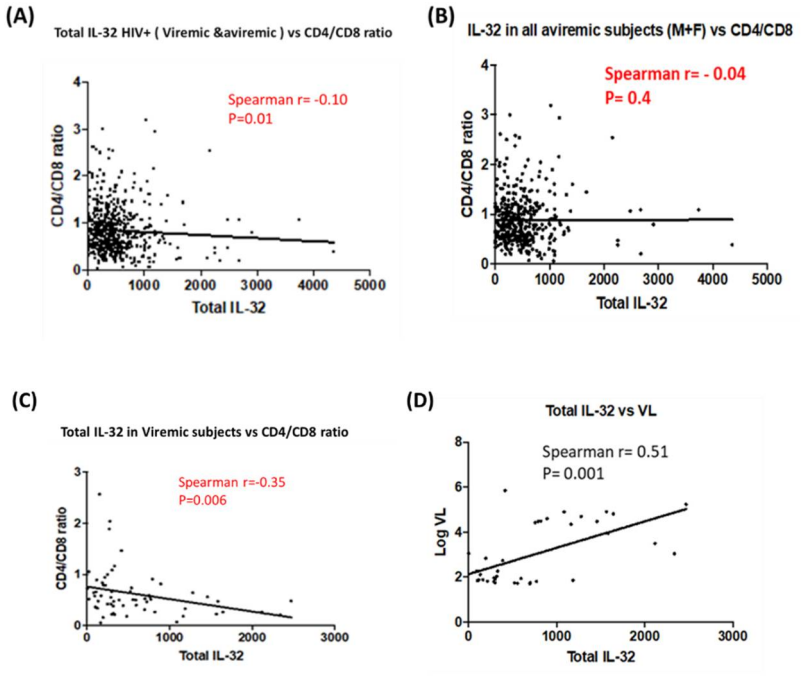



**Figure 20. Correlations among IL-32 levels, CD4/CD8 ratio, and viral load (VL).** (A) Correlation of total plasmatic levels of IL-32 in the total population (male and female; n = 608) to CD4/CD8 ratio. (B) Correlation of total IL-32 in HIV-infected and aviremic subjects (male and female; n = 355) to CD4/CD8 ratio. (M = Male), (F = Female). (C) Correlation of total IL-32 in HIV-infected and viremic male subjects (n = 61) to CD4/CD8 ratio. (D) Correlations between IL-

32 levels and HIV viral load (VL) n = 37 male subjects. The increase in viral load is associated with an increase in plasma total IL-32. The Spearman correlation test was used to assess the significance correlations of total IL-32 with CD4/CD8 ratio and viral load.

## 4.5. Age- and sex-associated differential expression of IL-32 in HIV+ aviremic subjects

The total plasma IL-32 levels were analysed based on sex and age between male and female groups of the aging cohort (Figure 21). The data showed that the average levels of total IL-32 were significantly higher in the female group (n = 51, AV = 571.9) compared to the male group (n = 328, AV = 426) with P value = 0.0036 (Figure 21A). Subsequently, the total IL-32 levels were compared in each group between subjects based on age (< 50 years of age compared to those > 50 years). The results showed that in the male group, (Figure 21B) there was a tendency for higher IL-32 levels with age (men < 50 years, n = 86, AV = 366.6 pg/ml *versus* men > 50 years n = 211, AV = 439.4 pg/ml), however without reaching statistical significance. In contrast, in the female group, IL-32 was significantly higher (p = 0.0092) in younger women (n = 18, AV = 734 pg/ml) compared to older women (n = 30, AV = 477.1 pg/ml) (Figure 21C). Data on age were missing for n = 31 aviremic men and n = 3 aviremic women.

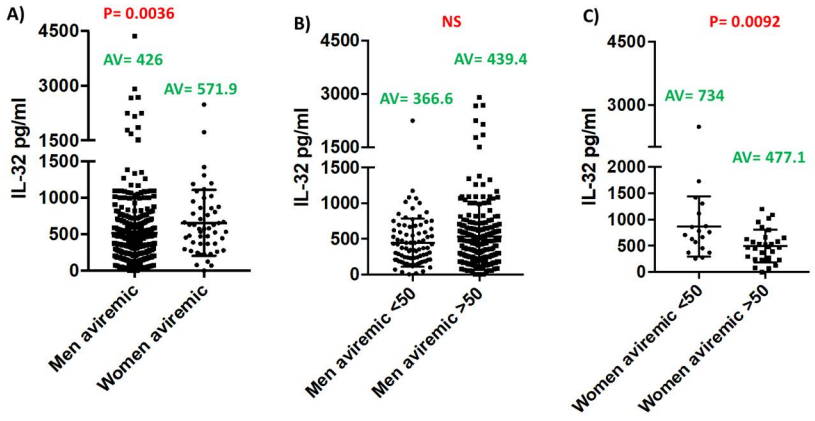



**Figure 21. Plasmatic levels of total IL-32 protein (pool of all isoforms) in ART-treated HIV+ men and women (aviremic) in association with age.** (A) Comparison between HIV+ men (n = 328) and women (n = 51). (B) IL-32 plasmatic levels in men < 50 years of age (n = 86) *versus* men > 50 (n = 211). (C) IL-32 plasmatic levels in women < 50 years of age (n = 18) *versus* women > 50 years (n = 30). AV = Average, NS = non-significant. P values are calculated by non-parametric Mann Whitney test.

Although IL-32 levels significantly decreased following HAART initiation, these levels were not normalized compared to the general population (El-Far et al., 2016). To test whether the significant differences between men and women were due to the duration of treatment, the correlation between IL-32 and duration of HAART were studied in both groups. A tendency for negative correlation was observed within the male group (n = 250, r = -0.11) but not within the female group (n = 41, r = +0.19, p = 0.26). There was no significant difference when comparing the average ART duration between men and women (Figure 22, average duration in the male group was 14.1 years and women, 13.2 years). These results suggest that the differential expression between HIV+ men and women was not associated with the duration of suppressed viral replication. However, as the number of women participants was limited compared to men, further analysis on a larger number of female subjects is still needed to confirm these results. In addition, as IL-32 was expressed in different isoforms and a set of ELISA antibodies was used to quantify the total pool of IL-32 isoforms, it remains important to determine which of these isoforms are dominantly and differentially expressed in men *versus* women.

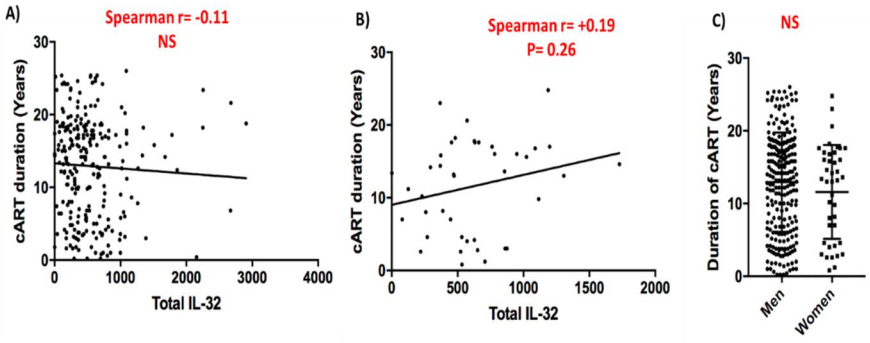



**Figure 22. Correlations between IL-32 and the average of HAART duration.** (A) Correlations between IL-32 levels and duration of HAART in HIV+ men (n = 250). (B) Correlations between IL-32 levels and duration of HAART in HIV+ women (n = 41). (C) Comparison of the HAART duration between HIV+ men and women, (n = 250) (n = 41) respectively.

## 4.6. Correlation of total IL-32 with the CVD markers D-dimer and LDL/HDL ratio

Since a differential expression of IL-32 was observed between men and women and the cohort is mainly composed of men participants (86% male, 13% women), the focus of subsequent analysis was

primarily on the male group. The analysis was carried out to examine the correlation between the plasma levels of total IL-32 from HIV-infected aviremic men with typical risk markers of CVD such as D-dimers and the LDL/HDL ratio (increased levels of both markers are typically associated with increased CVDs (Triant 2013, Kunutsor et al., 2017). The data showed that there was a significant negative correlation with D-dimers (n = 141, r = -0.16 and p = 0.04). Similarly, there was a negative correlation between plasmatic IL-32 levels and the ratio LDL/HDL (n = 322, r = -0.12 and p = 0.038). Although the main role of IL-32 was described to be pro-inflammatory, these data suggest that IL-32 or either of its dominant isoforms may also play a protective role since it is negatively correlated with the above-mentioned risk factors (Figure 23).

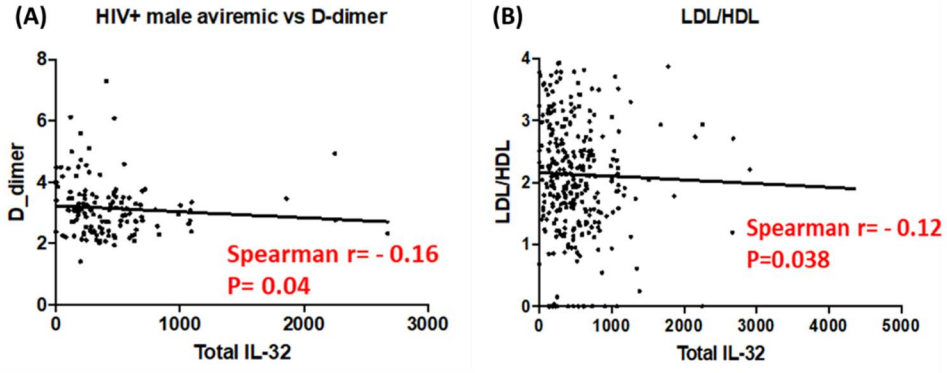



**Figure 23. Correlation between total IL-32 levels and CVD biomarkers (LDL/HDL ratio and D-dimer).** Total IL-32 levels were measured using commercially available kits from R&D on HIV-infected and aviremic male samples available at the baseline visits. (A) A significant negative correlation between IL-32 levels and D-dimer (n = 141) as well as between IL-32 levels and LDL/HDL ratio, which is a biomarker for CVD (n = 322) (B). The Spearman correlation test was used to assess the significance correlations between IL-32 and LDL/HDL ratio.

## 4.7. PCR Amplification of IL-32 isoforms

To characterize the role of IL-32 in accelerated CVD in HIV-infected and aging subjects, an isoform-specific PCR was developed for the different IL-32 isoforms. The isoform-specific primer sets generated DNA sizes corresponding to spliced mRNA and not genomic DNA as shown in Figure 24. The quantity of total cellular mRNA template isolated from total PBMCs was optimized and a minimum of 25 ng mRNA per PCR reaction was determined to successfully amplify all of the IL-32 isoforms. The primer-sets and mRNA template concentrations were further employed to establish a quantitative RT-PCR. The data on the use of two independent

housekeeping genes, 28S and β-glucuronidase, as internal control references for the assays showed that the 28S reached the amplification plateau (saturation) at early cycles under the current conditions of 25 ng RNA template/reaction. However, the amplification plateau for β-glucuronidase was in the same range as the rest of IL-32 isoforms as shown in Figure 25. Therefore, the β-glucuronidase was considered for all the subsequent quantitative RT-PCR experiments. Each RT-PCR reaction was performed in duplicates. Samples without a template or without reverse transcriptase were included in parallel for each gene master mix as negative controls.

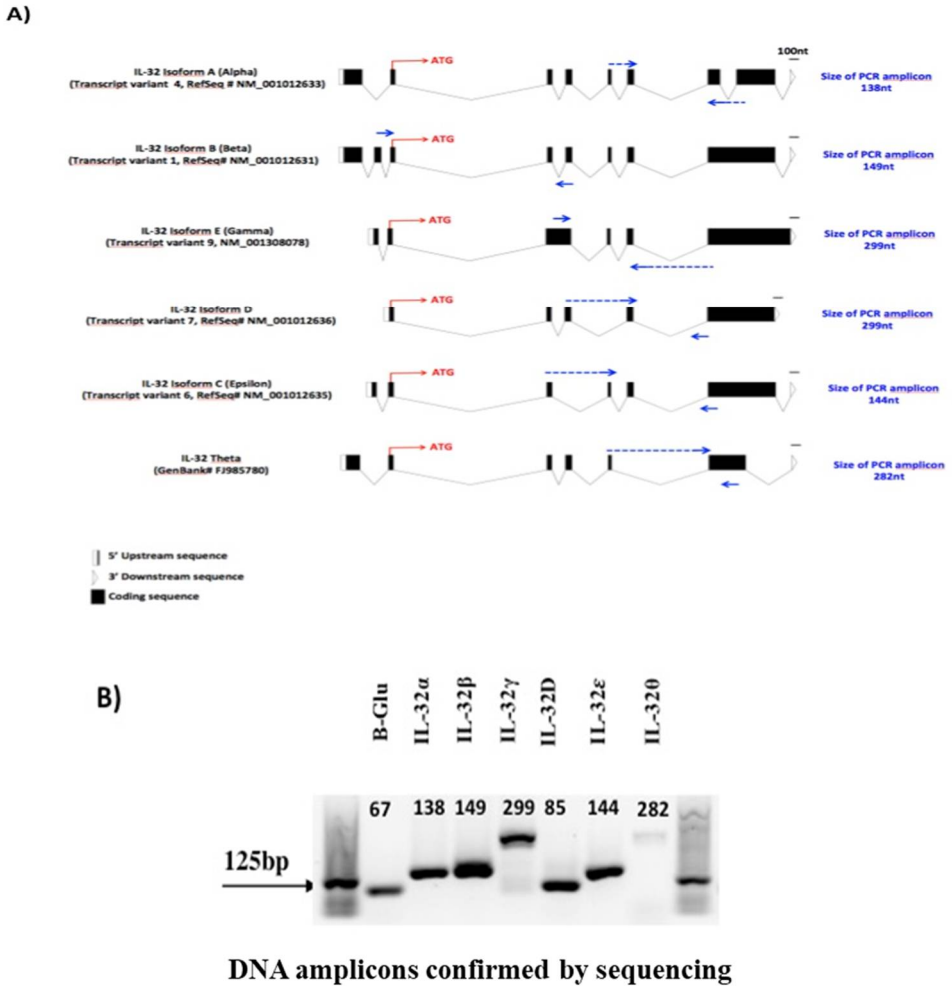



**Figure 24. Isoform-specific PCR amplification of IL-32 isoforms (α, β, γ, D, ε, and θ) in addition to the housekeeping gene β-glucuronidase.** (A) Schematic representation for the IL-32 exons within each of the individual IL-32 isoforms detected and quantified in the current study. Sequence blast was done using individual IL-32 transcript variant with the NCBI RefSeq numbers noted to the left of each isoform and IL-32 gene on chromosome 16 (RefSeq# NG_029254.1).

Forward and Reverse Primer positions are indicated with blow arrows (dashed arrows indicate the primer sequence was designed to overlap the extremities of two adjacent exons (B) PCR products (amplicons) for each individual isoform analyzed on agarose gel (1.5%). Numbers above the DNA bands represent the size of each isoform. Nt = nucleotide, β-glu = House Keeping gene β-glucuronidase.

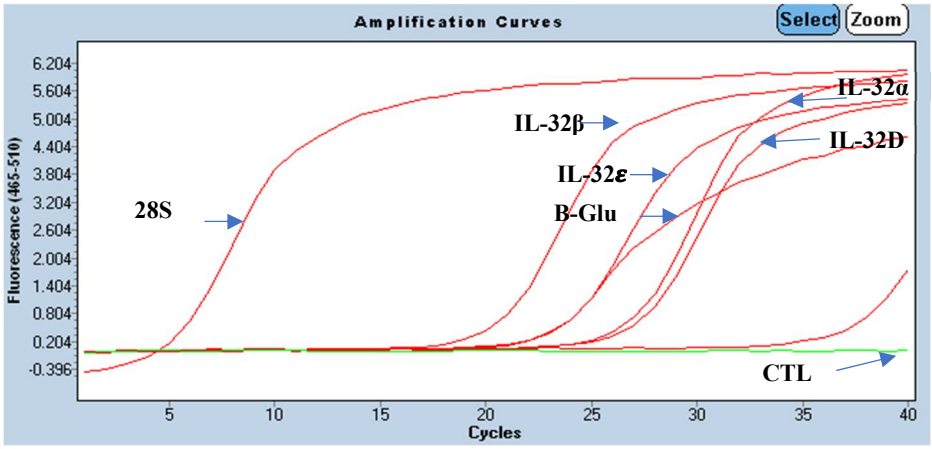



**Figure 25. Amplification curves and cycle threshold for IL-32 isoforms and housekeeping genes produced by LightCycler 480.** CTL = Control. B-Glu = β-glucuronidase

Using these conditions, it was determined that the total pool of IL-32 mRNA (sum of all relative isoforms measured at RNA level from PBMCs) positively and significantly correlated with the cell-associated IL-32 protein (measured by ELISA from the total cell lysate of PBMCS and normalized to total amount of cellular protein (Figure 26).

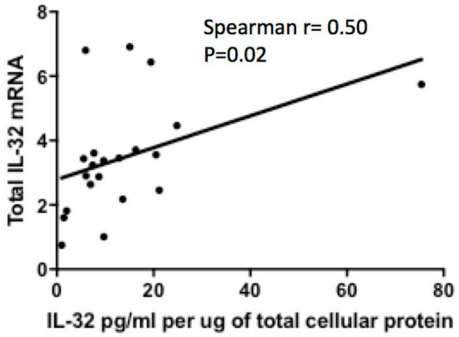



**Figure 26. Correlation between cell-associated total IL-32 protein (measured by ELISA) and total IL-32 mRNA isolated from the same PBMCs** (n = 21, 15 HIV+ and 6 $HIV^{neg}$).

## 4.8. Differential expression of IL-32 isoforms

At the transcriptional level, the results (Figure 27) confirmed the observations at the protein level in that the total pool of IL-32 mRNA was still upregulated in HIV-infected aviremic subjects (n = 81, p = 0.005) compared to non-infected controls (n = 29) (A). However, it is important to identify the contribution of each of the IL-32 isoforms to the total pool of this cytokine and to determine the dominant expressed isoform under HIV infection. IL-32 has isoforms that are highly inflammatory, such as the γ isoform (Heinhuis et al., 2011a), whereas others are anti-inflammatory, like IL-32β, through the induction of the IL-10 (Kang et al., 2009) and the IL-32θ isoforms which block the induction of TNFα (Khawar et al., 2017). Therefore, IL-32 can act as a pro- and anti- inflammatory cytokine at the same time by inducing TNF through the γ isoform and blocking it by the action of θ isoform (Heinhuis et al., 2011a; Heinhuis et al., 2011b; Khawar et al., 2017). Moreover, IL-32δ binds to the β isoform and blocks its functions in the induction of the anti-inflammatory cytokine IL-10 (Kang et al., 2013). In this study, it was observed that IL-32β isoform was the most expressed isoform in HIV-infected and non-infected subjects (B). This is followed by the expression of IL-32ε and IL-32γ and finally IL-32α, IL-32D, and IL-32θ.

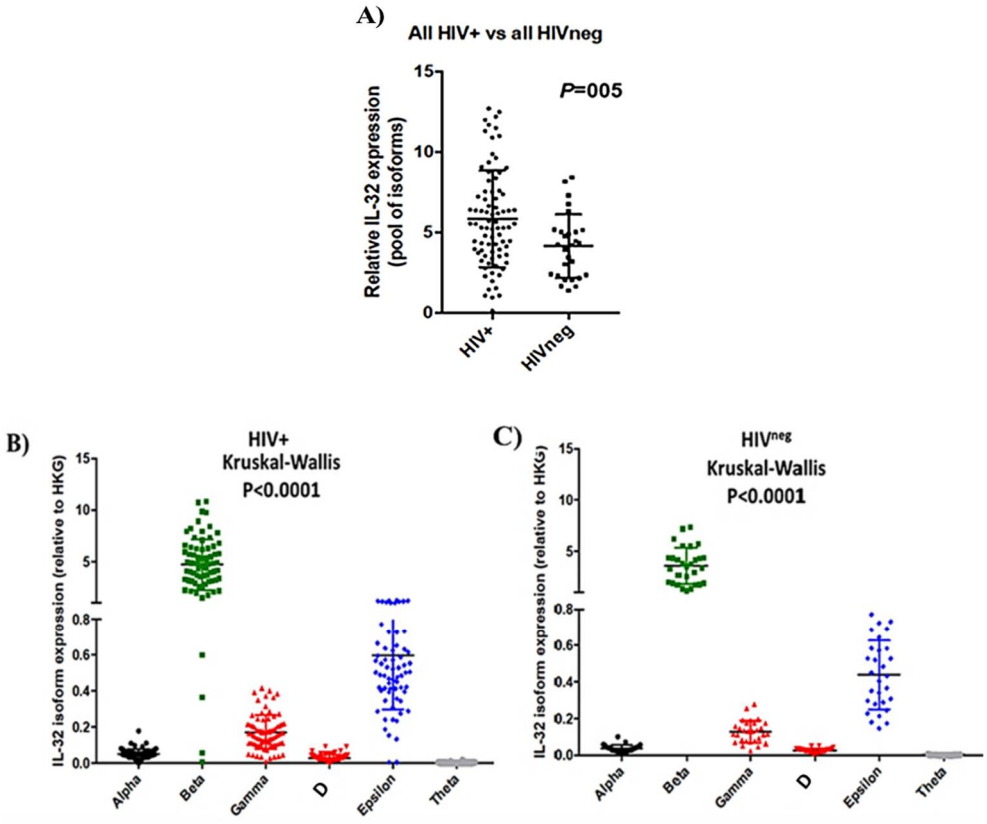



**Figure 27. IL-32 mRNA expression in HIV+ and HIV$^{neg}$ subjects.** The figure depicts the expression of the different IL-32 isoforms in RNA isolated from total PBMCs from HIV+ (n = 81) or HIV$^{neg}$ (n = 29) subjects. Isoform expression levels were normalized to the internal control housekeeping gene β-glucuronidase. A) Comparison between IL-32 mRNA levels (Total pool of all isoforms) in the HIV+ infected and non-infected subjects. B) Abundant expression of IL-32β in both HIV+ subjects (left panel) and HIV$^{neg}$ donors (right panel). P values are calculated by non-parametric Mann-Whitney test in (A) and by Kruskal-Wallis test in (B) and (C).

The individual IL-32 isoforms were further compared in HIV positive subjects with their counterparts from the HIV$^{neg}$ controls. All individual IL-32 isoforms remained significantly higher in HIV infection with the exception of IL-32D which showed a tendency for a higher expression, but with no statistical significance (Figure 28).

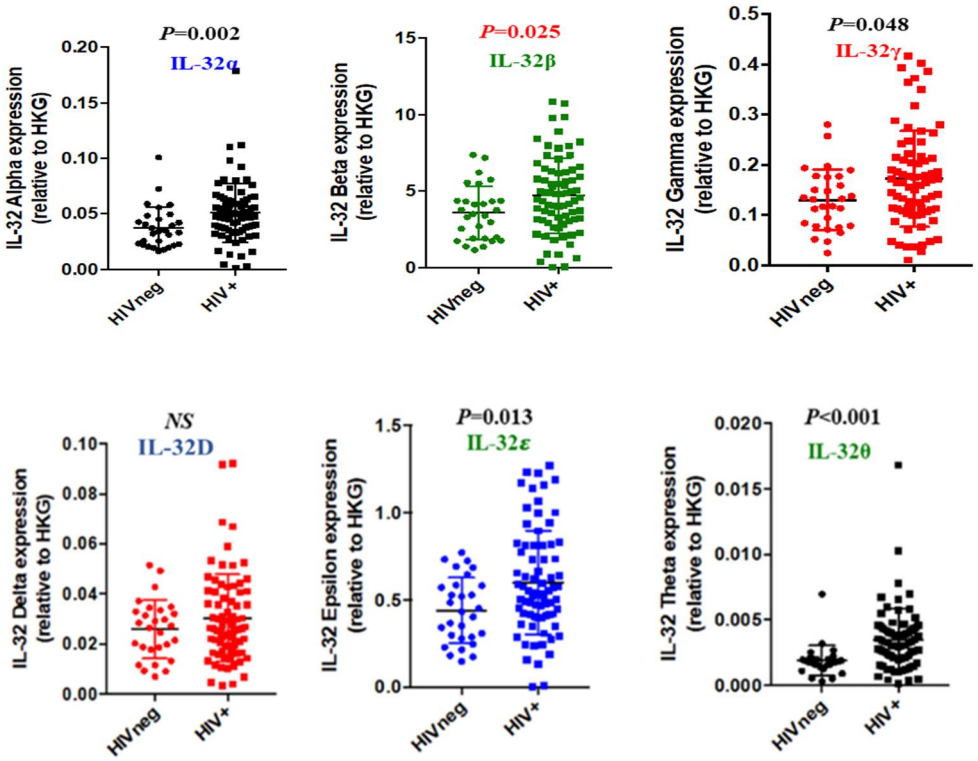



**Figure 28. Comparison between the level of each individual isoform of IL-32 mRNA in HIV+ subjects and HIV$^{neg}$ controls.** Comparative graphs showing individual expression of IL-32 isoforms in HIV-infected (n = 81) *versus* HIV non-infected controls (n = 29). RNA was isolated from total PBMCs of HIV+ and HIV$^{neg}$ controls and used for the one-tube single assay qRT-PCR. Isoform expression levels were normalized to the internal control housekeeping gene β-glucuronidase. P values are calculated by non-parametric Mann Whitney test.

## 4.9. Individual expression of IL-32 isoforms in PBMCs from HIV+ with coronary artery atherosclerosis

This work further focused on a subgroup of HIV+ aviremic men subjects, who underwent total atherosclerotic plaque volume (TAPV) measures; they were either positive (TAPV > 0) or negative (TAPV = 0). The results showed that there was no significant difference in the expression of the total pool of IL-32 mRNA or the individual IL-32 isoforms among subjects with TAPV$^{+}$ compared with those who were TAPV$^{neg}$ with the exception of the IL-32D and IL-32ε isoforms. IL-32D was highly expressed in TAPV$^{+}$ subjects compared to TAPV$^{neg}$ counterparts while the opposite was observed with IL-32ε (Figure 29).

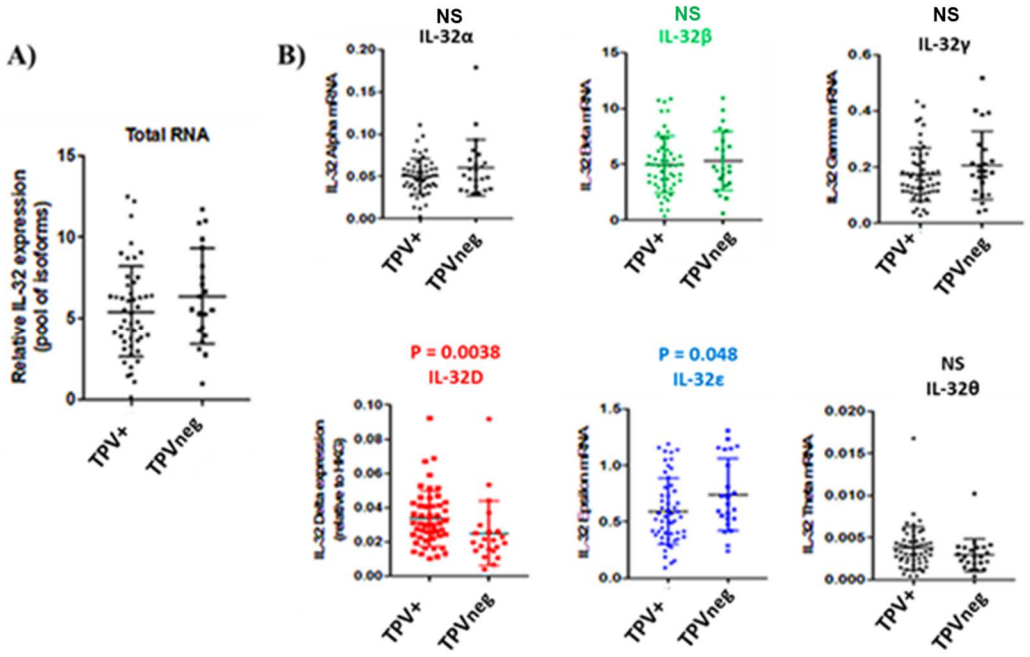



**Figure 29. Relative expression of IL-32α, β, γ, δ, ε and θ mRNA in total PBMCs isolated from HIV+ male aviremic subjects with or without TAPV.** A) Comparison between relative expression of total pool of IL-32 isoforms between $TAPV^{+}$ (n = 54) and $TAPV^{neg}$ HIV (n = 23) positive subjects (P = NS). B) Comparative graphs showing individual expression of IL-32 isoforms in HIV-infected aviremic male subjects with either $TAPV^{+}$ or $TAPV^{-}$. P values were calculated by non-parametric Mann-Whitney test.

## 4.10. Higher IL-32D levels positively correlate with the coronary artery plaque volume

As mentioned earlier, IL-32**δ** is a regulatory isoform as it binds to IL-32β and inhibits its anti-inflammatory role (induction of IL-10) (Kang et al., 2009). Interestingly, a positive correlation was observed between IL-32D and total coronary plaque volume (Figure 30). Moreover, by considering the ratio between IL-32D and IL-32β isoforms, the results showed that the most significant correlations were observed between the D and β isoforms as well as the D isoform to the total plaque volume. The data showed that higher plaque volume is associated with a higher IL-32D to IL-32β ratio or higher IL-32D level in the total pool of isoforms (which is mainly composed of IL-32β).

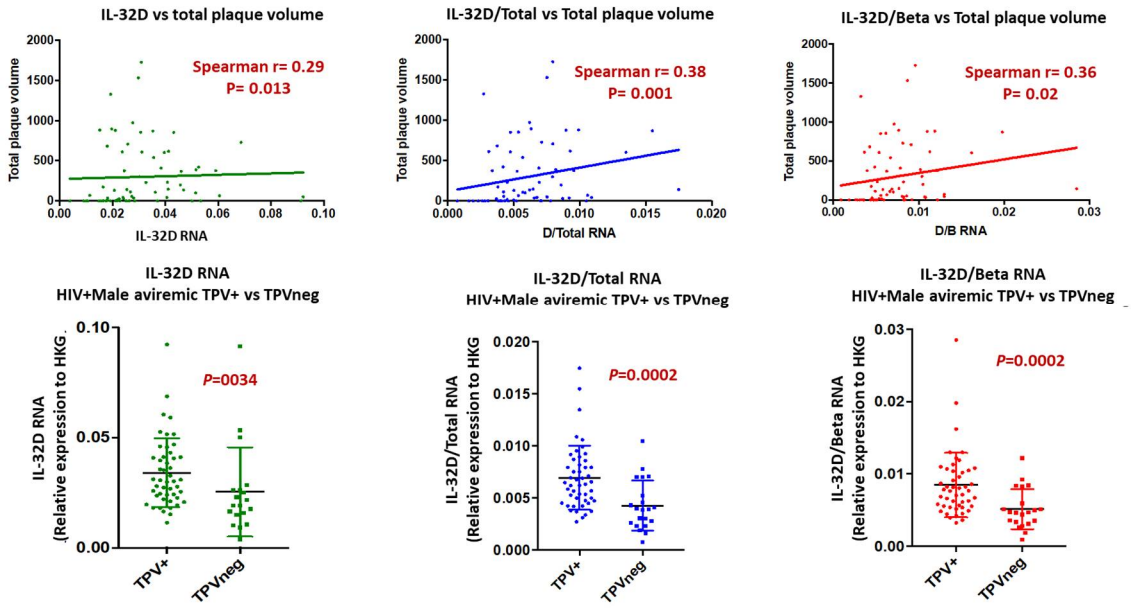
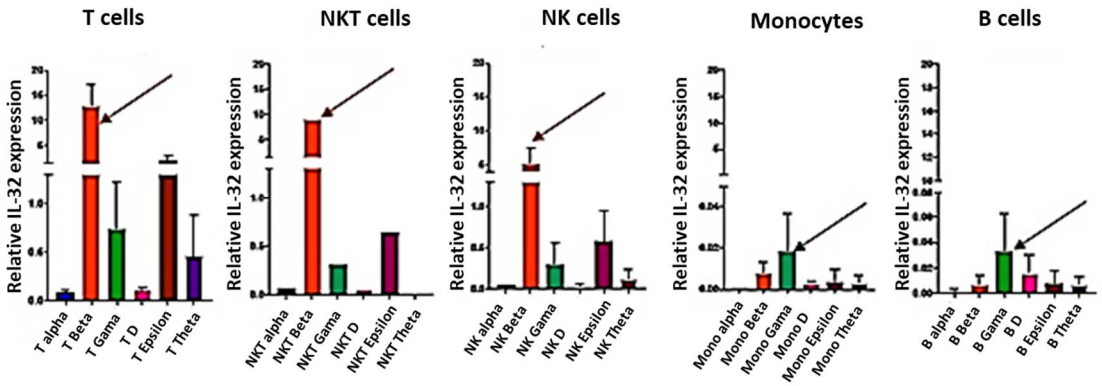



**Figure 30. The ratio between IL-32D and IL-32β predicts the total coronary artery plaque volume (TAPV) in HIV+ ART-treated subjects.** A) Correlations among IL-32D (left panel), IL-32D/total IL-32 RNA (middle panel) or IL-32D/IL-32β (right panel) and TAPV. B) Comparison of IL-32D (left panel), IL-32D/IL-32β (right panel) or IL-32D/total IL-32 RNA (middle panel) levels between $TPV^{+}$ and $TPV^{neg}$ subjects from the same subjects shown in A. P values are calculated by non-parametric Mann-Whitney test in A and Spearman correlation test in B.

## 4.11. Differential expression of IL-32 isoforms in immune cells

To further determine the major cellular source of IL-32 and to determine the cells expressing IL-32D, T cells, NKT cells, NK cells, B cells and monocytes were sorted from n = 3 HIV+ and n = 3 $HIV^{neg}$ controls. The results suggested that T cells express the highest levels of IL-32 as shown in (Figure 31); this is followed by NKT cells, NK- cell, monocytes and lastly B-cells. In both HIV groups, the β isoform dominated the IL-32 expression in all subjects, whereas the γ isoform is the dominant one in monocytes and B cells in both $HIV^{neg}$ (A) and HIV+ subjects (B) thus confirming the observation on the quantification of IL-32 from the total PBMCs in terms of IL-32β expression.

**(A)**




**(B)**

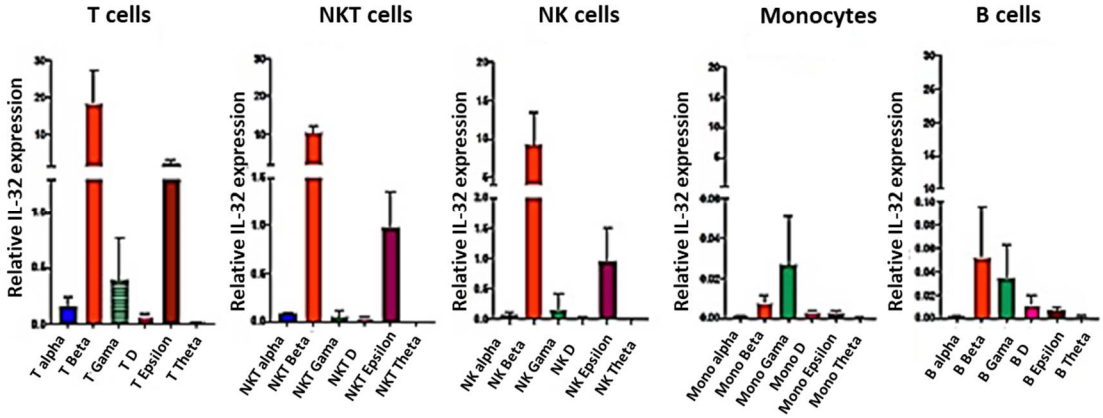



**Figure 31. IL-32 isoform expression by different immune cells.** Comparative graphs showing the expression of IL-32 isoforms in different immune cells ($CD3^{positive}$ $CD56^{neg}$ T lymphocytes, ($CD3^{positive}$ $CD56^{positive}$) NKT cells, $CD3^{neg}CD19^{neg}CD56^{+(hi/lo)}$ NK cells, $CD3^{neg}CD4^{lo}CD14^{positive}CD16^{+/-}$ $CD14^{positive}$ Monocytes and $CD3^{neg}CD56^{neg}CD19^{positive}$ B cells) sorted from (n = 3) HIV non-infected donors(A) and (n = 3) HIV infected subjects(B).

Next, the primary cell subsets (T cells, NKT cells, NK cells, monocytes, and B cells) were compared in terms of IL-32D expression to pinpoint where most of the IL-32D comes from. Although NK and NKT cells produce high levels of IL-32D, the contribution of T cells to the production of IL-32D was the most significant among other cell types in both HIV+ and $HIV^{neg}$ groups (Figure 32).

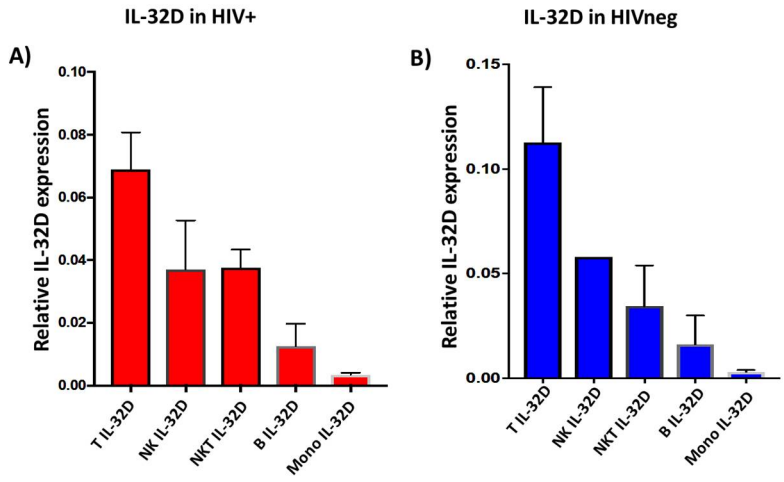



**Figure 32. IL-32D expression by immune cells in HIV positive and HIV negative donors.** Comparative graphs showing the expression of IL-32D by different immune cells (T cells, NKT cells, NK cells, monocytes, and B cells) from HIV-infected (n = 3) and HIV non-infected subjects (n = 3).

## 4.12. *In vitro* infection of PBMC

In order to investigate whether IL-32D is directly induced by HIV-1, an *in vitro* assay was established to infect non-stimulated (resting) PBMCs from $HIV^{neg}$ subjects (n = 10) to examine the impact of HIV on the differential expression of IL-32 isoforms. Although HIV replicates in activated cells, the resting cells were infected as cell activation modulates IL-32 expression. A dual tropic R5X4 isolate of HIV (p89.6) was used to infect both CCR5+ and CXCR4+ cells. The FACS blots and histograms in (Figure 33) show the infectivity of dual-tropic HIV. The RNA expression of different IL-32 isoforms was measured at early time points, 6 hours and 24 hours after infection in infected cell lysates. The results show that the D isoform was upregulated as early as 6 hours post infection; however, the modulation of β isoform was very transient (Figure 34).

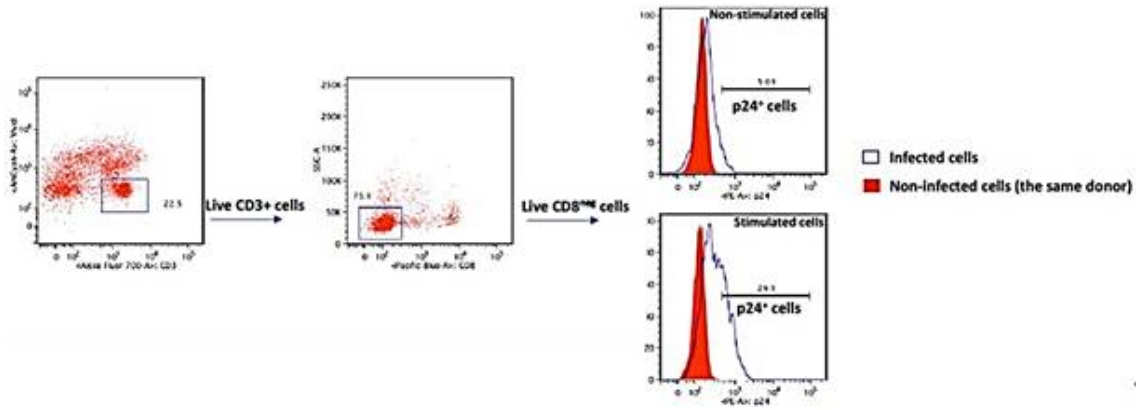



**Figure 33. Infection rate in total PBMCs using the dual tropic virus p89.6.** Left and middle panels: FACS blots showing the gating on CD3+, followed by CD8neg cells. Right panels: Overlapping histograms showing the frequency of p24+ (infected) cells (empty histograms) in either resting (upper panel) or PHA and IL-2 activated cells (1ug/ml and 10ng/ml, respectively) (lower panel) compared to non-infected control cells (Red).

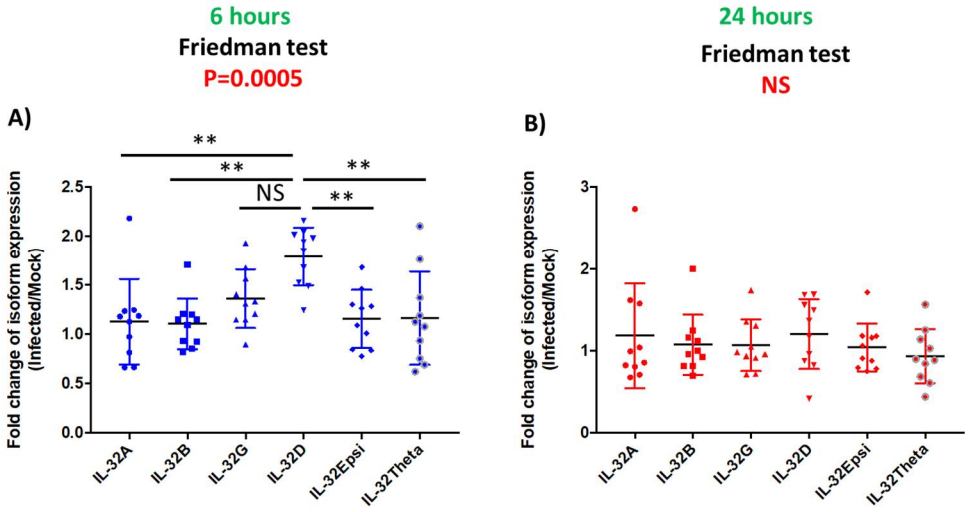



**Figure 34. Differential expression of IL-32 isoforms in response to HIV infection.** This figure illustrates the significant expression of IL-32 isoforms from α down to θ relative to the housekeeping gene β-glucuronidase 6 hours post infection in panel A compared to non-significant results at 24 hours post infection in panel (B) at the mRNA level. The Friedman test was used to analyze matched measures (Alpha, Beta and gamma, D, epsilon and theta) quantified from infected and non-infected mock cells from the same donor)

# Chapter 5:

# DISCUSSION AND CONCLUSIONS

## 5.1. Discussion

Despite the successful introduction of HAART with its role in suppressing viral replication and enhancement of the life expectancy of people living with HIV, inflammation persists. Therefore, several non-AIDS related chronic diseases, such as CVD, have emerged in the aging HIV population as a result of persistent immune activation and low-grade chronic inflammation (Durand et al., 2017). Among HIV-infected individuals, CVD is the leading cause of mortality in developed countries (Shahbaz et al., 2015); therefore, it is important to understand the underlying pathogenesis and to identify predictive markers to help in the early diagnosis and prevention of premature CVD. In this study, the potential use of the novel pro-inflammatory cytokine IL-32 was investigated (a marker that was previously shown to correlate with disease progression in HIV infection) as a biomarker for age- and sex-dependent systemic inflammation and their impact on the development of CVD in HIV-infected individuals.

IL-32 was suggested as being a contributing inflammatory factor (Damen et al., 2017a), as it induces various pro-inflammatory cytokines implicated in the pathogenesis of several chronic inflammatory disease like RA, COPD, and IBD (Damen et al., 2017a). These inflammatory diseases are also associated with increased risk of developing atherosclerosis and CVDs. For instance, a positive inflammatory loop may take place between IL-32 and TNFα as they can induce each other resulting in a state of non-resolving inflammation which further accelerates the immune senescence especially under HIV infection (Nixon et al., 2010; Damen et al., 2017a). In addition, IL-32 is present in endothelial cells (Nold-Petry et al., 2009) and contributes to atherosclerosis through induction of pro-inflammatory cytokines and synthesis of matrix metalloproteinases (Heinhuis et al., 2013). Therefore, it becomes more interesting to investigate how HIV impacts IL-32, how IL-32 impacts inflammation and how inflammation contributes to CVDs.

Previous studies highlighted the role of IL-32 in HIV-1 infection. It was established that IL-32 levels are increased in HIV-1 seropositive individuals compared to HIV-negative donors (Rasool et al., 2008). Upregulation of IL-32 was then linked with modest intracellular anti-viral functions that are mediated by interferon-stimulated genes (Nold et al., 2008). Intriguingly,

several studies have subsequently shown that higher and persistent levels of IL-32 are associated with immune suppression, persistent inflammation and disease progression (Smith et al., 2011; El-Far et al., 2016) and even enhancement of HIV replication (Palstra et al., 2018). The immunosuppressive function of IL-32 is mediated through the induction of negative regulatory proteins such as Ig-like transcript 4 (ILT4) and the IDO-1 in the lymphoid tissues that blunts the T cell responses with further enhancement of viral replication and CD4+ T cell depletion (Smith et al., 2011).

Similarly, several studies have reported either positive or negative functions for IL-32 in cancer development. For instance, IL-32 enhances the growth and invasion of osteosarcoma cells as well as breast cancer cells via AKT pathway-mediated MMP-13 expression (Wang et al., 2015; Zhou et al., 2015). However, in melanoma, exogenous IL-32α administration inhibits the melanoma human cell line proliferation invitro (Nicholl et al., 2016). Together, these opposite observations in both HIV infection and cancer suggest that IL-32 expression may play multiple functions based on the type of expressed isoform.

Currently there are at least nine different isoforms expressed by the IL-32 gene that are associated with different functions. Some of these isoforms are pro-inflammatory such as IL-32γ isoform, and others are anti-inflammatory like IL-32β, which induces the anti-inflammatory cytokine IL-10 (Kang et al., 2009), and IL-32θ, which inhibits the expression of TNFα through the inhibition of P38 MAPK pathway (Khawar et al., 2017). Some other isoforms of IL-32 may play an autoregulatory role as they modulate the functions of IL-32 *per se.* In this regard, earlier studies showed that IL-32δ binds to IL-32β and disrupts its association with PKCδ around the nuclear membrane, therefore inhibiting its anti-inflammatory functions by suppressing the induction of IL-10 (Kang et al., 2013). However, this study was done using a modified IL-32δ protein in which IL-32D (the isoform quantified in our current studies) was amplified by a specific set of PCR primers intended to remove exon 2 and to generate a protein sequence similar but not identical to the IL-32δ (personal communication by Kang et al.). The multitude of IL-32 functions are clearly linked with its large number of expressed isoforms that, together with the lack of clear knowledge on the interplay between these isoforms, represents a major hurdle towards a better understanding of IL-32 roles in different pathologies.

In the current study, in order to quantify IL-32 isoforms in the aging cohort samples, several commercially available antibodies for the different IL-32 isoforms were considered. We tested different sets of antibodies against either IL-32α alone, mixed IL-32αβδ antibody sets from Biolegend, and total IL-32 ELISA kit from R&D (a set of antibodies that recognizes all isoforms of IL-32, total pool of IL-32). By measuring IL-32α levels using commercial ELISA sets from MyBiosource and Biolegend, a significant experiment-to-experiment variation in measurements was encountered along with very low or near background detection values. Similarly, low detectability was also observed in case of IL-32αβδ measurements. However, this variability was not observed using the ELISA sets detecting the total plasmatic IL-32 (pool of all isoforms). Although the low detection levels of IL-32α alone or mixed IL-32αβδ might be explained by the fact that these proteins are known to be more cell-associated rather than secreted compared to other isoforms such as IL-32γ (Hasegawa et al., 2011), the experiment-to-experiment variations were unexpected and might be due to the instability of the recombinant proteins used as a standard reference for the assay. The instability of the recombinant IL-32α protein used for the quantification assays may also apply for IL-32α protein in the biological samples (plasma and serum); therefore, it is important for future studies to address this question before reaching conclusions on the contribution of IL-32α in diseases, mainly CVD. For these reasons, the IL-32α and IL-32αβδ protein measures were not considered for further analysis while only the total IL-32 measurements were maintained.

To test our proposed hypothesis on the role of IL-32 in accelerating inflammation with age, our studies were carried out on the (CHACS). This cohort includes 1000 participants, of whom 200 are $HIV^{neg}$. Comprehensive clinical data including cardiovascular risk factors, complete haematological, biochemical, metabolic and immunological profiles were collected in a centralized electronical data platform. Other important subclinical atherosclerosis indicators, such as calcium scores, cIMT and CT scanning, epicardial fat volume, and coronary artery TAPV, were also measured for 200 patients.,

Total IL-32 levels were previously shown to correlate with and predict typical inflammatory markers such as IL-6 and sCD14 in slow progressor HIV+ individuals (El-Far et al., 2016). Both markers are also associated with increased risk of CVD (Nou et al., 2016; Vos et al., 2017). Compared to healthy individuals, biopsies from lymph nodes and gut of HIV

infected patients showed higher levels of IL-32 expression at all stages of HIV infection (Smith et al., 2011). Similar to these observations, the current results on HIV-infected and aging individuals (viremic and aviremic) confirmed that the total IL-32 levels are significantly higher in HIV+ individuals compared to non-infected controls at the protein level. In addition, total plasmatic IL-32 levels in viremic subjects correlated negatively with the CD4/CD8 ratio which is an independent biomarker of CVD (Huang et al., 2015).

An interesting observation of this study was that total IL-32 plasmatic levels were differentially expressed in HIV+ women *versus* men. IL-32 was significantly higher in women compared to men. Moreover, age-associated differences were also observed, where women under 50 years had higher IL-32 levels compared to women over 50. However, this was not observed in the men. One of the possible mechanisms underlying the decline in IL-32 levels in aged *versus* young participants could be related to the duration of treatment. However, no significant differences were observed when comparing the average cART duration between men and women; this reflects that age-related decline in IL-32 in women, is sex-associated. IL-32 is considered as pro-inflammatory cytokine, and younger women are known to show a better level of protection against CVD compared to male counterparts (Garcia et al., 2016). However, due to the lack of specific tools to distinguish among the different isoforms at the protein level within the total pool of plasmatic IL-32 and due to small sample size of women in this analysis, it may not be possible to draw conclusions concerning the differences between women and men. Therefore, the exact mechanism underlying these significant changes in IL-32 levels based on sex and age remains an open question until a significant number of female samples from the Women's Interagency HIV Study (WIHS) cohort are analyzed (the lab has now received n = 500 plasma samples and n = 72 PBMCs samples from the WIHS cohort to validate the age and sex-associated differential expression of IL-32).

To support a potential role for IL-32 in CVD during HIV infection, we investigated the correlation between IL-32 and common CVD biomarkers. This study showed a significant negative correlation between total IL-32 levels and D-dimer and LDL/HDL cholesterol ratio. D-dimer is a strong predictive biomarker for CVDs (Triant, 2013), and its plasmatic levels are elevated with advanced aging in untreated individuals living with HIV. However, it was found that early initiation of HAART normalizes the plasma levels of D-dimer (Sereti et al., 2016).

HIV-infected subjects under treatment are more prone to develop dyslipidemia as a complication of HAART. For instance, use of protease inhibitors is associated with increased levels of small, dense LDL (atherogenic lipoprotein phenotype) and endothelial dysfunction. These atherogenic lipoproteins are elevated in serum from atherosclerotic patients and they are more prone to chemical modifications. These chemical changes predispose patients to atherogenesis and CVD (Steinberg et al., 1989; Rizzo et al., 2007; Shahbaz et al., 2015). Additionally, a study carried out by Kunutsor et al., (2017) showed that high levels of serum LDL/HDL ratio in middle-aged men are strongly associated with sudden cardiac risk. These significant negative correlations between IL-32 and these last CVD biomarkers further suggest that the dominant IL-32 isoform under HAART treatment is likely anti-inflammatory and either IL-32 or one of it's dominant isoforms might play a protective role in CVD. This observation led us to think about what actually was measured in plasma. Since what measured in plasma was the total pool of IL-32 isoforms (Pro- and anti-inflammatory isoforms) and due to lack of suitable tools to detect these isoforms at the protein level, we decided to explore the RNA expression of this cytokine and detect these isoforms by PCR to confirm this hypothesis.

To better dissect and understand the roles of different IL-32 isoforms in the development of CVDs in HIV-infected and aging individuals and to identify the dominant isoforms, an isoform-specific quantitative RT-PCR was developed as described in Chapter 3. Consistent with the soluble measures of IL-32 protein by ELISA, it was observed that total IL-32 mRNA measured in PBMCs (the sum of all relative isoforms) was higher in HIV+ *vs* $HIV^{neg}$ subjects. Furthermore, cell associated IL-32 protein was measured to confirm what we observed at IL-32 mRNA level by qRT-PCR. Our validation assays demonstrated positive correlation between IL-32 mRNA levels and cell-associated total IL-32 protein, thus confirming IL-32 RNA is translated into IL-32 protein.

At the single IL-32 isoform levels, all isoforms were highly expressed in HIV+ compared to $HIV^{neg}$ subjects. In addition, the data at the transcriptional level showed for the first time that IL-32β is the dominant isoform in PBMCs from both HIV-infected and non-infected individuals. Interestingly, IL-32β was found to play a dual immune role. IL-32β can induce the pro-inflammatory cytokine TNFα, (Shoda et al., 2006); however, it can also induce the anti-inflammatory cytokine (IL-10) (Kang et al., 2009). In addition, data generated in the lab and

included in the "Manuscript in Preparation" annexed to this thesis, showed that both IL-32α and IL-32β but not IL-32γ can induce IL-10 expression in activated primary $CD4^+$ T-cells. Although it is not yet clear in the literature how IL-32 functions *in-vivo*, the fact that IL-32β induces anti-inflammatory cytokine (IL-10) may reflect the complexity of its functions particularly in the context of co-expression with other isoforms such as IIL-32D and L-32δ which may render IL-32β more pro-inflammatory than anti-inflammatory (Kang et al., 2013).

We further investigated the correlation between the expression of individual IL-32 isoforms and coronary artery atherosclerotic plaque volume as a main sub-clinical marker for CVD. Coronary TAPV, measured by coronary computed tomography angiography (CCTA) scanning, is used to help in early assessment and detection of subclinical atherosclerosis (Chen et al., 2016). People living with HIV have an increased prevalence of subclinical atherosclerosis (Lo et al., 2010). This is as a result of a chronic inflammatory process together with lipid deposition in the coronary artery walls which lead to formation of atherosclerotic plaques and subsequent development of CAD and atherosclerosis (Lo et al., 2010). In a sub-study carried by Durand et al., (2017) on the CHAC, results based on studying five different cardiac imaging modalities to assess atherosclerosis showed that the presence and composition of atherosclerotic plaque is different between HIV+ and $HIV^{neg}$ individuals, this study did not give further details concerning the differences in plaque sites and composition. In our study, the mRNA expression of each individual IL-32 isoform, which may be different than the protein expression, was investigated in HIV positive subjects with TAPV compared to infected subjects with no sign of atherosclerosis (TAPV = 0). Our data showed that subjects with coronary artery plaques had significantly higher levels of the IL-32 isoform D compared to their counterparts without atherosclerotic plaques. These levels of IL-32D positively and significantly correlated with the plaque volume. Of particular interest, Kang et al., (2013) reported a significant interplay and regulation between IL-32δ (a modified form of IL-32D isoform where the first exon was excluded) and IL-32β. IL-32δ exerts its modulatory effect by interacting with IL-32β and preventing its binding to PKC$\delta$ and subsequently suppressing the expression of the anti-inflammatory cytokine IL-10 production (Kang et al., 2013). Therefore, the balance between IL-32δ (modified IL-32D isoform) and IL-32β may dictate the IL-32-mediated inflammatory outcome. This last hypothesis was supported by the observation that the IL-32D / IL-32β ratio

positively correlated with TAVP. These data suggest a protective role for IL-32β and, in contrast, a negative role for IL-32D. The results may bear important implications for IL-32D in clinical practice as they also show for the first time that the IL-32D / IL-32β ratio might be used as a predictor marker for coronary plaque formation and CVDs in HIV+ subjects.

The function of IL-32β on the induction of the anti-inflammatory cytokine IL-10 (Kang et al., 2009), together with the dominance of IL-32β at the transcriptional level, may in part explain our observation at the protein level where IL-32 protein negatively correlated with typical CVD markers such as D-dimer and the LDL / HDL ratio. It may also partly explain the decline in total IL-32 levels with age in HIV+ women. In this regard, earlier studies suggested that women are less likely to develop CVDs prior to menopause compared to their age-matched men. However, during the postmenopausal period the sex-gap diminishes and both genders tend to possess almost equal risk of developing CVD (Garcia et al., 2016). This is believed to be due to hormonal effects (estrogen) on the vascular function (Vaccarino et al., 2011). This may suggest that the dominance of IL-32β in younger women might contribute to their protective immunity against CVD at a younger age. Further studies are still needed to investigate the dynamics of expression of IL-32 isoforms with age in the female group. This hypothesis was not tested in our CHAC cohort as it is mainly composed of men. However, studies are planned in the lab to carry out similar analyses on women participating in the WIHS cohort (the largest HIV-infected women cohort in USA with cIMT measurements) (Shendre et al., 2017).

We further extended our studies to determine which cells are expressing the IL-32D isoform. For these experiments, different immune cells; T cells, B cells, monocytes, NK cells and NKT cells were sorted from HIV+ and $HIV^{neg}$ donors. The focus was on these cells as they showed important relevance to the pathogenesis of atherosclerosis. For instance, NK cells and NKT cells are involved in atherogenesis through their cytotoxic effect by inducing apoptosis of target cell and subsequent accumulation of necrotic and apoptotic cells in atherosclerotic lesions (Lotze et al., 2009; Selathurai et al., 2014). This cytotoxic activity is mediated by granzyme B and perforin secretory granules (Selathurai et al., 2014). B cells were found to have two different effects on the development of atherosclerosis based on its subsets in both human and mice. On one hand, B1 B-cell subset (present in spleen) plays a protective role against atherosclerosis and on the other hand, the B2 subset, which is originated from bone marrow, augments atherogenesis

(Tsiantoulas et al., 2014; Ammirati et al., 2015). T cells in humans, specifically CD8+ cytotoxic T cells, are abundantly present in atherosclerotic plaques specifically in unstable plaques (Ammirati et al., 2015). Their atherogenic effect is mediated by inducting cell death (Ammirati et al., 2015). Moreover, $CD4^+$ T helper (Th) cells are also present in atherosclerotic plaques and their role in atherogenesis depends on the type of CD4+ T cell subsets. Some are atheroprotective cells such as $T^{regs}$, whereas others are atherogenic like Th1 (Ammirati et al., 2015). Th1 cells secretes IFNγ, which mediates its atherogenic functions by promoting inflammation, development and destabilization of atherosclerotic lesions (Ammirati et al., 2015). High levels of inflammatory (CD14++CD16+) and patrolling (CD14+CD16++) monocytes have been reported in untreated people infected with HIV as well as non-HIV patient with acute coronary syndrome (Nou et al., 2016). In response to pro-atherogenic stimuli, monocytes transmigrate to the intima and further propagate inflammatory process by secreting pro-inflammatory molecules such as IL-6 and TNFα mediated through stimulation of TLR4, as well as enhancing plaque neovascularization (Jaipersad et al., 2014). Our data on the expression of IL-32 isoforms in these above-mentioned cell types showed that IL-32β is the dominant expressed isoform in T cells, NK-T and NK cells, whereas the IL-32γ is dominant in monocytes in both groups within cell types. In addition, T cells have the most contribution to express higher levels of IL-32D in both HIV+ and HIV negative subjects among other tested immune cells. Therefore, it was highly interesting to investigate how IL-32D production is modulated in these T cells. To answer this question, an assay of *in vitro* infection of resting cells was established using a dual-tropic virus that infects both CCR5+ and CXCR4+ cells. Our focus was on resting cells to avoid interfering with the total levels of IL-32 by activated cells due to differences in IL-32 expression levels in stimulated *versus* resting cells. Therefore, the goal was to investigate if the exposure of quiescent cells to HIV would induce the expression of IL-32, particularly IL-32D. The results showed that the only isoform that was significantly upregulated early post infection (starting 6 hours post infection) was the IL-32D isoform. However, it remains unknown how HIV specifically induces the overexpression of the IL-32D and whether it is viral proteins or RNA sensing by innate receptors that is involved. Future studies using truncated HIV (viruses with deleted proteins) to infected primary cells together with cell-stimulation with viral RNA may answer these questions.

The observations on the aging cohort that the D isoform is upregulated in only a subgroup of HIV+ and treated subjects (with coronary plaque volume) suggest that this subgroup might be experiencing residual viral replication. Further studies on the detection of residual viral replication are thus needed to validate this hypothesis. Since our *in vitro* studies on these infections were done on resting non-activated cells that are typically resistant to productive HIV infection (Pan et al., 2013), and as abortive infection *in vivo* is known to induce cell death by pyroptosis leading to inflammasome assembly (Doitsh et al., 2014), it is highly interesting to study the role of IL-32 isoform D in this context. Interestingly cell death either by apoptosis or pyroptosis is associated with overexpression of IL- 32 (Netea et al., 2006; Bai et al., 2015).

The upregulation of IL-32D might also be due to gut dysbiosis (imbalance of gut microbiota between commensal and pathogenic bacteria) (Brenchley et al., 2006; El-Far & Tremblay, 2017) or to low level viremia in HIV infected individuals receiving effective antiretroviral therapy (Palmer et al., 2008). In a study carried out by Boyd et al., on the association of residual HIV viremia and cIMT, high levels of cIMT were observed in HIV infected and treated individuals with residual viremia (Boyd et al., 2014). Given the potential role of IL-32D and IL-32δ in binding to and diminishing the anti-inflammatory functions of other IL-32 isoforms such as IL-32β towards prominent pro-inflammatory functions, the upregulation of IL-32D may then contribute to the persistent inflammatory status observed in a subgroup but not all HIV+ cART-treated individuals. However, *in vitro* studies are still needed to validate the regulatory functions of IL-32D. Furthermore, the link between the expression IL-32D and gut dysbiosis is still missing. However, studies on gut dysbiosis in correlation with IL-32 expression already started in the lab with (n = 100) stool collections already done on 2017 - 2018.

In summary, in this study we investigated the potential use of IL-32 as a biomarker for early prediction of CVDs within aging HIV-infected population, in which inflammation is a major health concern. While the overall expression of IL-32 is associated with inflammatory conditions, emerging evidence suggests IL-32 expression is tightly regulated through splicing of the IL-32γ (most active isoform) into a less active isoform IL-32β, an isoform that also induces the anti-inflammatory cytokine IL-10. However, in HIV infection and under conditions

that are yet to be determined, other isoforms of IL-32 such as IL-32D and/or δ may be upregulated and bind to and inhibit the IL-32β-mediated expression of IL-10 while maintaining the production of the proinflammatory mediator TNFα, which would promote inflammation through activation of multiple cell types known to respond to IL-32 and to contribute to inflammation-induced CVD (summarized in the model Figure 35). Therefore, it is likely that the balance between IL-32 isoforms may dictate the overall outcome of inflammation. Here we aimed to determine the expression of the different isoforms of IL-32 in the aging HIV+ population. An isoform-specific qRT-PCR was developed to quantify most IL-32 isoforms, at the RNA level, together with using the commercially available ELISA sets to quantify the soluble protein. Although IL-32 level remains high in HIV+ subjects and IL-32β is the most expressed isoform, the data suggested a protective role for IL-32β and a negative role for IL-32D. Our data also show for the first time that the ratio IL-32D/IL-32β might be used as a predictor marker for coronary plaque formation and CVD in HIV+ subjects. Overall, careful interpretation for role of IL-32 in inflammation remains difficult without having proper tools to precisely quantify the most dominantly expressed isoforms at the protein level. Furthermore, knowledge on the IL-32 receptors is still lacking. It is not yet known whether or not these receptors are modulated by HIV infection and inflammation. Finally, mechanistic studies to dissect the role of IL-32 in atherogenic mechanisms and plaque formation are still needed.

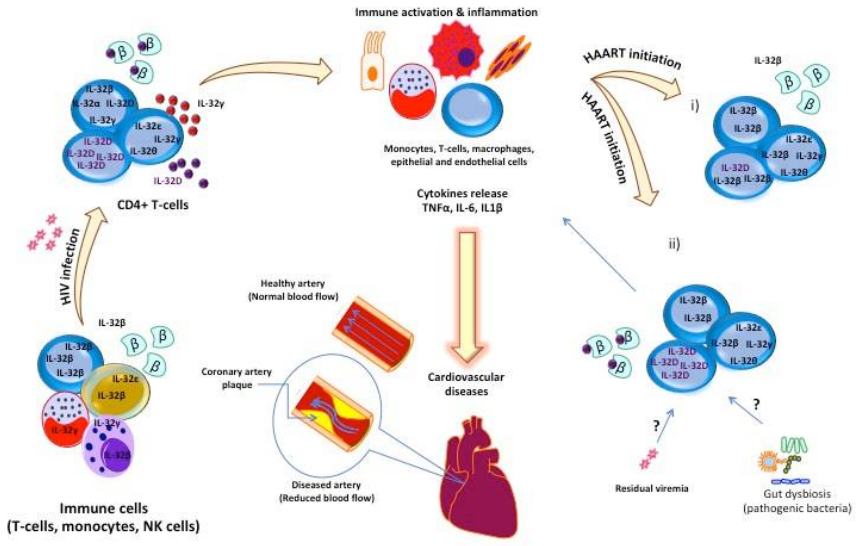



**Figure 35. A hypothetical model for the role of L-32 in CVD in aging HAART-treated HIV+ individuals.** All IL-32 isoforms are expressed in immune cells (T-cells, monocytes, NK

cells) at physiological levels prior to HIV infection, particularly the less inflammatory isoform IL-32β (that also holds an anti-inflammatory potential through the induction of IL-10). Following HIV infection, expression of IL-32 isoforms is enhanced, particularly IL-32D. HAART initiation decreases IL-32 levels, however without normalization compared to the $HIV^{neg}$ general population (i). In certain individuals (ii) higher levels of IL-32D persists, likely due to residual HIV replication and/or age-related dysbiosis of gut microbiota (remains to be validated). Binding of IL-32D to IL-32β diminishes IL-10 production and favours the inflammatory functions, such as the production of the master inflammatory cytokine TNFα (Kang et al., 2013). IL-32 inflammatory isoforms activate different cell types known to play key roles in inflammation such as monocytes, T-cells, epithelial and endothelial cells. Activation of these last cells contributes to persistent inflammation and atherogenesis leading to CVD.

## 5.2. Strengths and Limitations

The major strength of this project was the exploration of the balance between pro- and anti-inflammatory and regulatory isoforms of IL-32 might dictate the inflammatory outcomes, especially CVD, in HIV infection. To establish this concept, we had access to biological samples from a large number of participants from the CHAC with detailed epidemiological data and a biobank with plasma and stored cells. A significant subgroup of subjects has complete medical records of cardiovascular risk factors and cardiac scans. However, our Canadian cohort is mainly comprised of men with a less than 12% of women participants. The low number of women participants in this study represents a limitation and therefore results on the difference between men and women for the levels of IL-32 should be interpreted with caution. Our laboratory has now received plasma (n = 500) from the Women Interagency HIV study (WIHS Cohort) to measure IL-32 and other inflammatory markers associated with CVD. Data from this number of women participants will help to draw statistically significant conclusions as to the difference between men and women for the IL-32 expression. Further limitations were due the absence of specific antibodies to validate the expression of the deleterious IL-32 isoform D at the protein level. Future studies are also planned in the lab to generate these antibodies and also to over-express this isoform (that is not yet characterized) and to study its impact on cell activation and functions. Moreover, over-expression of IL-32D.

## 5.3. Conclusion

Extensive studies have been conducted concerning IL-32 since the discovery of its biological properties, mainly pro-inflammatory. Several studies have shown the involvement of IL-32 in various inflammatory, neoplastic, and infectious diseases (Kobayashi et al., 2009; Bai et al., 2011; Heinhuis et al., 2011b; Yang et al., 2015). The research shows that:

**At the protein level:**

1) The total pool of IL-32 remains higher in HIV+ ART-treated and aging subjects compared to the general $HIV^{neg}$ population even after several years of treatment.
2) Total IL-32 protein (plasmatic IL-32) is differentially expressed in HIV+ women versus men (women have significantly higher levels compared to men).
3) Age-associated differences were also observed; women < 50 have higher IL-32 compared to women >50.

**At the transcriptional level:**

4) IL-32β is the most abundant IL-32 isoform in different primary cell types.
5) All isoforms of IL-32 were significantly increased in PBMCs from $HIV^{+}$ and treated subjects.
6) The β isoform dominates IL-32 expression in T, NK-T, and NK cells, whereas the γ isoform is the dominant one in monocytes and B cells.
7) T cells and NK/NKT cells contribute the most for the D isoform of IL-32.
8) Early modulation of IL-32D and IL-32α in *in-vitro* HIV infection.

- Overall, the data strongly suggest that correlative studies with IL-32 are difficult to interpret without dissecting the role of each single isoform and study the balances among these isoforms to each other. It would be highly desirable to do mechanistic studies to dissect the role of IL-32D on T cell activation and inflammatory cytokine secretion.

# Chapter 6: Appendix

## Article: Differential expression and functions of IL-32 isoforms in HIV+ individuals under cART

**Differential expression and functions of IL-32 isoforms in HIV+ individuals under cART**

**Running title # 2 Upregulation of IL-32 in HIV-infected and treated individuals: Potential role in persistent inflammation and reactivation of HIV reservoir**

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**Abstract**

Human IL-32 is a key polyfunctional cytokine that was initially reported to play anti-viral functions in HIV-infected cells through the induction of type I interferons. However, recent data suggest that certain IL-32 isoforms may enhance HIV replication *in vitro*. Furthermore, we have previously shown that upregulation IL-32 expression is associated with suppression of immune responses and correlates with disease progression in HIV-infected individuals. As IL-32 is expressed in multiple isoforms (α, β, γ, δ, ε and θ), some of which are proinflammatory such as IL-32γ while others are anti-inflammatory (θ), we aimed in the current study to quantify the expression of these individual isoforms in circulating blood from HIV-infected individuals receiving combined therapy. Our data showed that all IL-32 isoforms were significantly upregulated in HIV+ individuals receiving cART with IL-32β representing the dominantly expressed isoform. At the functional level, our data showed that both IL-32β&γ significantly enhance the expression of proinflammatory cytokines, such as IL-6 and IFNγ in TCR-stimulated CD4 T-cells. However, both IL-32α&β but IL-32γ induce the expression of the anti-inflammtory cytokine IL-10. Interestingly, IL-32γ, but IL-32α enhanced the reactivation of latent HIV reservoir as measured by HIV release from in CD4+ T-cells isolated from cART-treated individuals. Together, our data highlight the potential role of IL-32 in persistent inflammation and reactivation of viral reservoir in HIV infection. Further studies are needed to investigate whether IL-32 might contribute to the replenishment of viral reservoir under cART treatment.

## Introduction

Combined Anti-Retroviral Therapy (cART) has greatly diminished the rapid disease progression in HIV-infected patients by suppressing active viral replication to undetectable levels. This remarkable control of viral infection underlies the significant decline in AIDS-related comorbidities and the increased life expectancy [1] [2]. However, non-AIDS malignancies remain common within the cART-treated population, which clearly compromises the quality of life of individuals living with HIV [3]. Among key mediators of these comorbidities are chronic immune activation and inflammation, both are mainly driven by persistent viral infection and disturbed immunity that is not fully restored even under treatment [4, 5]. Chronic perturbations in the cytokine network, particularly in the proinflammatory cytokines such as TNFα, IFNα/β and IL-6, largely contribute to the compromised immunity in the cART era [6] [7] [8]. While the contribution of these cytokines to HIV pathogenesis and disease progression is relatively well documented, the role of novel cytokines known to be dysregulated during infection such as the human interleukine 32 (IL-32) remains elusive. IL-32 is a proinflammatory mediator that induces other inflammatory cytokines such as TNFα, IL-8, IL-1β, IFNγ and IL-6 [9] [10] [11]. Early studies on IL-32 showed that this cytokine is upregulated in HIV infection and is associated with intracellular antiviral responses through the induction of type I interferon responses (IFNα/β) [12] [13]. This was supported by the enhanced HIV replication upon inhibition of endogenous IL-32 expression. Meanwhile, recent data showed that treatment of activated cells with human IL-32γ may induce HIV replication by enhancing CD4 activation [14].

Furthermore, other studies including ours, also showed that upregulation of IL-32 in un-treated HIV-infected individuals is associated with inflammation and immune suppression and disease progression [10] [15]. Interestingly, the study by Smith et al., ([15]) showed that IL-32 is highly expressed in the lymphatic tissues and that the gamma isoform (the most biologically active isoform of IL-32 [16] can significantly induce the immunosuppressive enzyme Indoleamine 2,3-dioxygenase (IDO1). Increased IDO1 expression

is associated with dysregulatd intestinal mucosal immunity [17], immune exhaustion [18] and the overall disease progression. Together, these studies suggest that IL-32 may exert multiple functions in HIV infection. These diverse functions might be explained by the expression of multiple IL-32 isoforms (α, β, γ, δ, ε, ζ, η, ς and θ), which are produced by alternative splicing [11, 19]. Interestingly, while the IL-32γ induces a panel of proinflammatory cytokines including TNFα and IFNγ [9], IL-32β specifically induces the anti-inflammatory cytokine IL-10 [20, 21]. Moreover, the functions of IL-32γ on the induction of TNFα through the activation of NF-kB and p38 MAPK, is counteracted by the newly identified IL-32θ isoform that diminishes these effects through the interaction and blockade of PKCδ, an upstream activator of both NF-kB and MAPK [22]. IL-32θ also inhibits the PMA-induced expression of IL-1β through the interaction with PKCδ [23]. Similarly, while IL-32β significantly increases the expression of IL-10, the δ isoform of IL-32 can bind to and inhibits IL-32β functions on IL-10 [20].

Due to these multiple functions and counter functions of IL-32 isoforms, it was then important to identify the dominantly expressed IL-32 isoforms and their functional consequences in HIV-infected individuals receiving therapy. In the current study, IL-32 isoforms were quantified from circulating blood of HIV-infected individuals participating in the Canadian HIV and Aging Cohort Study.

## Material and Methods

### Study cohort

Biological samples (plasma, sera and peripheral blood mononuclear cell, PBMCs) collected from HIV-infected and non-infected individuals enrolled in the Canadian HIV and Aging Cohort Study (CHACS) were used in this study. In this cohort, 800 HIV-infected participants and 200 non-infected controls were followed longitudinally for 5 years in ten sites across Canada. Tested samples belong to baseline visits. Demographic as well as biological data are collected in a centralized, electronical data platform. Written

informed consent was obtained from all participants (all are adults participants) included in this study. The study was approved by Institutional Review Boards (IRB) of the Centre Hospitalier de l'Universite de Montreal Research Center (Ethical approval #: CE.11.063).

**Measurements of soluble IL-32, IL-10 and TNFα proteins**

Plasmatic levels of human IL-32 (total pool of isoforms) were quantified using the human IL-32 ELISA kit (R&D System Inc., Cat # DY3040-05) as per manufacturer's recommendations on the available samples from baseline visits of HIV+ and $HIV^{neg}$ individuals. Samples of both HIV+ and $HIV^{neg}$ were inactivated using disruption buffer (DB) [PBS; 0.05%, Tween-20, 2.5% Triton X-100, 0.02% thimerosal and 1% Trypan blue] with a ratio of 1:4 (DB: Sample). IL-10 and TNFα were measured using human ELISA sets from R&D and PeproTech (Cat # DY-217B-05 and 900-M25, respectively) according to the supplier's protocol. Cell-associated IL-32 protein was measured as previously described [10].

**Quantification of IL-32 by Real-time quantitative SYRR Green PCR**

The total RNA from PBMCs was isolated using RNAeasy plus mini kit from Qiagen (Catalog No. 74134) as per manufacturer's protocol. Quantification of IL-32 isoforms (α, β, γ, δ, ε and θ) was carried out using one-step SYBR Green quantitative real-time PCR, which was performed on the LightCycler 480 II (Roche) apparatus using QIAGEN reagents (RNase-Free DNase Set (50); Cat. No. 79254). A total 25ng RNA (25ng) were used per reaction and each real-time PCR reaction was performed in duplicates. Template controls were included in parallel for each gene master mix. All fold changes in gene expression were normalized to the housekeeping gene β-glucuronidase. Primer sets for IL-32α (transcript variant 4 RefSeq # NM_001012633), β (transcript variant 1 RefSeq # NM_001012631), D (transcript variant 7 RefSeq # NM_001012636), ε (C) (transcript variant 6 RefSeq # NM_001012635) and β-glucuronidase have been described earlier (Huang, et al., 2015), while primer sets for IL-32 γ (transcript variant 9 RefSeq #

NM_001308078) and θ (GenBank # FJ985780) were designed based on γ and θ transcript reference sequences as follows: hIL-32GF 5'-TACTTCTGCTCAGGGGTTGG-3' and hIL-32GR 5'-TGGGTGCTGCTCCTCATAAT-3'and hIL-32 θF, 5'-GAGCTGGAGGAGCTCACTCC-3' and hIL-32 θR 5'-GAGCTCTGACAGAGAGCAGC-3'.

**Cell sorting and FACS**

To study the expression of IL-32 isoforms in different cell type, we sorted T-cells, B-cells, NK cells and monocytes from total PBMCs of HIV positive and HIV negative subjects using flow cytometry (BD FACSAria; BD Biosciences). Briefly, cell Surface staining was performed with fluorochrome-conjugated antibodies; Pacific Blue™ Mouse Anti-Human CD3 (Clone UCHT1, Cat # 558117), Alexa Fluor® 700 Mouse Anti-Human CD4 (Clone SK3, Cat # 566318), APC-H7 Mouse anti-Human CD8 (Clone SK1, Cat # 560179), APC Mouse Anti-Human CD14 (Clone M5E2, Cat # 555399), PerCP-Cy™5.5 Mouse Anti-Human CD19 (Clone HIB19, Cat # 561295), PE Mouse Anti-Human CD56 (Clone B159, Cat # 561903) and FITC Mouse Anti-Human CD16 (Clone 3G8, Cat # 555406). Average purity of sorted cells was of >95% as determined by post sorting FACS quality control analysis.

**Isolation and stimulation of CD4 + T cells**

Total $CD4^+$ T cells were sorted from PBMCs by negative selection using EasySep Human $CD4^+$ T Enrichment Kit (StemCell® Cat # 19052) according to the supplier's protocol. Purity of isolated $CD4^+$ T cells were on average 99%. Stimulation of purified cells took place in a pre-coated plate with anti-CD3 antibodies (1 μg / ml) (BD biosciences®, Cat # 555329) and soluble anti-CD28 antibodies (0.5 μg / ml) (BD biosciences®, Cat # 5555726) with or without 500ng IL-32 isoforms from R&D (IL-32α # 3040-IL-050, IL-32β cat# 6769-IL-025 and IL-32γ cat# 4690-IL-025/CF). Cells were cultured at 37 °C for 2 days and secreted cytokines in the supernatant were measured by ELISA as mentioned above.

**Statistical analysis**

Differences between groups were considered statistically significant at values of $p < 0.05$. Mann-Kruskal-Wallis and Dunn's subtest were used to analyse more than one group, non-parametric Mann-Whitney was used for unpaired samples and nonparametric Spearman test was used for correlation studies. All analyses were performed using Prism 7 software (GraphPad, San Diego, CA, USA).

**Results and Discussions**

***Persistent upregulation of IL-32 under cART treatment***

Infection with the human immunodeficiency virus-1 (HIV-1) is associated with the upregulation of pro- and anti-inflammatory cytokines, most of which are not normalized even under intensified therapy [24]. Dysregulation of these cytokine networks contributes to the persistent immune activation and diseases progression. Our earlier longitudinal studies on a limited number of $HIV^+$ subjects showed that IL-32 is highly expressed during the primary phase of HIV infection and is not normalized following a complete year of treatment with cART [15] [10]. In the current study, we first investigated the expression of IL-32 in HIV infected individuals receiving cART for extended periods of time (average: 12,7+/- 6,7 Yrs) by direct measure of plasmatic IL-32 (total pool of all IL-32 isoforms). IL-32, levels remained significantly higher ($p < 0.0001$) in HIV-infected individuals (n = 379) (IQR: 437.4 pg/ml, Q1-Q3: 229,9-667.3 pg/ml) compared to the general non-infected population (n = 107) (IQR: 254.14 pg/ml, Q1-Q3: 162,5-416.7 pg/ml). However, these levels were significantly lower compared the $HIV^+$ viremic individuals (n = 46) (IQR: 783, Q1-Q3: 301.6-1085.2 pg/ml), **Figure 1A**. As we have previously shown [10], IL-32 levels positively correlated with the HIV viral load ($r = 0.488$, $p = 0.0006$) and negatively with the ratio CD4/CD8 ($r = -0.316$, $p = 0.032$) in the HIV+ viremic individuals (**Figure 1B**, left and middle panels, respectively). However, in virally-suppressed individuals, the correlation between IL-32 and CD4/CD8 ratio was non-

significant (**Figure 1B**, right panel), likely due to the recovery of the CD4 counts following cART treatment, even in the presence of low-grade inflammation.

***Dominant expression of IL-32β isoform***

Our data showed that circulating levels of total IL-32 remain upregulated in $HIV^+$ cART-treated individuals. However, as IL-32 is expressed in different isoforms for which specific antibodies are currently unavailable and since these isforms have distinctive functions, it is important to identify the expression of these individual isoforms. The balance between these single isoforms may dictate the overall impact of IL-32 on persistent inflammation in HIV infection. For instance, dominant expression of IL-32γ, the most active and pro-inflammatory isoform [16] could be deleterious for the immune response. Therefore, regulation of IL-32 isoform expression was suggested as a rescue mechanism to control inflammation. In this regard, IL-32γ is known to undergo multiple splicing into shorter and less inflammatorty isoforms such as IL-32α & β to prevent deleterious inflammatory effects [19]. Furthermore, smaller isoforms of IL-32 such as IL-32θ were also shown to counteract the inflammatory nature of IL-32γ by specifically inhibiting TNFα production [22]. Therefore, to investigate which of the IL-32 isoforms is expressed under cART treatment, we developed an isoform-specific RT-PCR to quantify the individual IL-32 isoforms (α, β, γ, D, ε, and θ) from total PBMCs of both $HIV^+$ and $HIV^{neg}$ individuals. The specificity of our RT-PCR is shown in **Figure 2**, where the amplified complementary DNA corresponded to the expected spliced transcripts of the different IL-32 isoforms that was further confirmed by DNA sequencing (data not shown). Similar to the total pool of IL-32 protein, the total pool of IL-32 mRNA (a sum of relative expression of all isoforms amplified from PBMCs) was significantly higher in $HIV^+$ cART treated ($n = 10$) compared to the non-infected controls ($n = 10$) (**Figure 3A**, left panel, $p < 0.0001$). As expected, the total pool of IL-32 mRNA positively and significantly correlated with the total pool of cell-associated IL-32 protein measured, by ELISA from total cell lysates (**Figure 3A** right panel, $n = 21$, $r = 0.5$, $p = 0.02$).

At the single IL-32 isoform level, IL-32β dominated the expression of the other isoforms, particularly the IL-32γ that represents the most inflammatory isoform in both $HIV^{neg}$ (in blue) and $HIV^{+}$ virally suppressed individuals (in red), **Figure 3B**. Interestingly, with the exception of the IL-32D, all of the IL-32 isoforms were highly and significantly expressed in the $HIV^{+}$ cART treated individuals compared to the $HIV^{neg}$ controls ($p = 0.023$, $p < 0.0001$, 0.0068, 0.089, $p < 0.0001$, and $< 0.0001$ for α, β, γ, D, ε, and θ, respectively).

***Dominant expression of the proinflammatory isoform IL-32γ in monocytes***

As the last data were generated on total peripheral mononuclear cells (PBMCs), we next aimed to determine the expression of individual IL-32 isoforms in different cell subsets and to determine the major cellular source of IL-32. We used classical phenotypic lineage markers for T-cells ($CD3+CD56^{neg}$), B-cells ($CD3^{neg}CD56^{neg}CD19^{+}$), NK cells ($CD3^{neg}CD19^{neg}CD56^{+(hi/lo)}$) and monocytes ($CD3^{neg}CD4^{lo}CD14^{+}CD16^{+/-}$) to isolate these cell subsets by flow cytometry cell sorting (**Figure 4A**). Our data on quantification of the different IL-32 isoforms from these cell subsets showed that both T-cells and NK cells express high levels of IL-32 compared to B cells or monocytes. Furthermore, the IL-32β was the dominant isoforms in the former two cell types followed by IL-32ε and IL-32γ then IL-32α, IL-32D and IL-32θ (**Figure 4B**). Of note, IL-32γ, the most active isoform, was the dominant isoform in monocytes and B cells. Dominant expression of IL-32γ in the former cells may have important implications in HIV infection in the context of persistent inflammation due to the highly plastic nature of monocytes and their potential to differentiate into inflammatory macrophages. Interestingly, upregulation of IL-32, particularly the γ isoform, in macrophages from lymph nodes and gut from HIV-infected individuals was previously shown to be associated with enhanced expression of the immunosuppressive enzyme IDO1 and correlated with disease progression [15]. However, whether IL-32 can directly impact the differentiation of monocytes to macrophage and whether the different isoforms of IL-32 can mediate such differentiation is not yet known.

Further studies are then warranted to address these questions and to understand the uncommon nature of IL-32 expression in a multitude of isoforms.

### *Differential functions of IL-32 isoforms*

The results mentioned above clearly showed that isoform β is the most abundant isoform of IL-32 followed by IL-32ε and IL-32γ. While functions of the IL-32ε is not yet known and that IL-32γ plays a potent proinflammatory role [10, 16], the dominant IL-32β has the potential to dampen inflammation as it induces the expression of the anti-inflammatory cytokine IL-10 [25]. However, this later function is counteracted by co-expression of other isoforms such as the IL-32δ that may regulate the production of the IL-32β-induced IL-10. This regulation is mediated by direct binding of IL-32δ to the IL-32β together with inhibition of IL-32β to PKCδ (necessary for IL-10 production) [25].

As our data showed that T-cells are the major source for IL-32 production, we then aimed in the current work to investigate the impact of the commercially available recombinant IL-32 isoform proteins (IL-32α, β and γ) on these cells. We first investigated the impact of IL-32α, β and γ on the induction of IL-10 *in vitro*. Primary CD4+ T-cells, isolated by negative selection from PBCS of healthy donors, using IL-32α, β, γ simultaneously with TCR-activation as described earlier [10]. Our data showed that, both IL-32α, and IL-32β, but not IL-32γ induced IL-10 production in TCR-activated CD4+ T-cells (**Figure 5A**, n = 22, p = 0.0096, data are normalized to the levels of IL-10 induced by TCR stimulation from each single donor). Paired analysis for IL-10 production in the same donor cells by IL-32 isoforms showed that IL-32α was a stronger inducer compared to IL-32β and IL-32γ (Figure 5B, p = 0.013 and p = 0.0029, respectively) and that IL-32β had better capacity to induce IL-10 compared to IL-32γ (p = 0.0043). In contrast to IL-10 induction, only IL-32β and IL-32γ but not IL-32α induced IL-6 (Figure 5B, n = 14, P = 0.0008) and IFNγ (Figure 5C, n = 11, p = 0.01). These results, in addition of confirming earlier reports describing IL-32γ as

a potent inducer of proinflammatory cytokines compared to IL-32α [16], show for the first time that IL-32α, and to lower extent IL-32β, may favor an anti-inflammatory response by inducing IL-10 from activating CD4+ T-cells. However, as we used total CD4 cells and since IL-10 production by T cells is mainly secreted by Th2 and $T_{reg}$ [26] it remains unknown which of these two cell subsets is specifically activated by IL-32α and/or IL-32β. Such a differential cellular response to IL-32 isoforms may explain the multitude of IL-32 biological roles and suggest that the overall functions of IL-32 should be interpreted with caution.

***Differential impact of IL-32 isoforms on reactivation of HIV reservoir in CD4$^+$ T cells from cART-treated individuals.***

Given the role of IL-32 isoforms on activating $CD4^+$ T cells, cellular compartments harboring the major HIV reservoir under cART therapy [27], and given the recent reports showing a significant capacity of IL-32 isoforms enhance HIV replication *in vitro* [14], it was then tempting to investigate the capacity of IL-32 isoforms to reactivate latent HIV reservoirs. CD4+ T cells were isolated from peripheral blood of cART successfully treated HIV+ individuals with undetectable viremia (n=8). Following stimulation with IL-32 isoforms α and γ and incubation for 6 days to allow reactivation and production of viral particles, quantification of HIV RNA was done on supernatant of activated cells. As shown in Figure 6, IL-32γ but not α (with the exception of one out of 8 individuals) could readily activate HIV reservoir compared to the non-stimulated conditions on the CD4 T cells from the same donor (Matched pairs analysis, p=0.015 and p=0.6, respectively).

Together, our data suggest that at least at the peripheral blood level, IL-32 isoforms are differentially expressed and that the IL-32β is the dominant isoforms in T cells and NK cells under cART treatment. However, IL-32γ, the most biologically active isoform, is dominant in circulating monocytes. While IL-32γ showed a typical proinflammatory nature by inducing IL-6 and IFNγ in activated T cells, IL-32α and

IL-32β induced the anti-inflammatory cytokine IL-10. The proinflammatory nature of IL-32γ was consistent with its capacity to reactivate latent HIV reservoir in CD4+ T-cells from individuals receiving cART treatment. These observations may have clinical consequence *in vivo* under cART treatment as persistent high levels of IL-32, may contribute to continuous HIV reactivation and fueling the HIV reservoir especially in lymphoid tissue sanctuaries where HIV drugs may have limited access [28]. Therefore, a better understanding of IL-32 expression and the balance between its isoforms together with tissue distribution of this expression are needed before developing specific inhibitors to those IL-32 isoforms involved in disease progression.

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**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors' Contributions**

S. Z. and L. L., planned, performed the experiments and wrote the manuscript, E.L.A., R.B. M.S. and A.C. contributed to the soluble and PCR measures, C.C.A., P.A., N.C., R.K., A.L., and M.D. contributed to the patient selection and study design, J.P.R., B.T., J.G.B., P.M., S.T. provided patient samples. C.T. and M.E. supervised the study, planned the experiments, analyzed data and wrote the manuscript.

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**Figure legends:**

**Figure 1: Persistent high levels of total IL-32 in plasma from cART-treated HIV+ individuals.** A) Comparison between total plasmatic levels from HIVneg (n = 107), HIV+ untreated (viremic) individuals (n = 46) and HIV+ virally suppressed individuals (n = 379). B) Correlation between plasmatic IL-32 and Log10 viral load (VL) (left panel), or CD4/CD8 ratio (middle panel) in untreated viremic individuals or CD4/CD8 ratio in cART-treated individuals (right panel). Data were analyzed by the non-parametric Kruskal-Wallis and Dunn's sub-test for multiple comparisons.

**Figure 2: Isoform-specific PCR amplification of IL-32 isoforms (α, β, γ, D, ε, and θ) in addition to the housekeeping gene β-glucuronidase.** Isoform-specific PCR amplification of IL-32 isoforms (α, β, γ, D, ε, and θ) in addition to the housekeeping gene β-glucuronidase. A) Schematic representation for the IL-32 exons within each of the individual IL-32 isoforms detected and quantified in the current study. Sequence blast was done using individual IL-32 transcript variant with the NCBI RefSeq numbers noted to the left of each isoform and IL-32 gene on chromosome 16 (RefSeq # NG_029254.1). Forward and Reverse Primer positions are indicated with blow arrows (dashed arrows indicate the primer sequence was designed to overlap the extremities of two adjacent exons (B) PCR products (amplicons) for each individual isoform analyzed on agarose gel (1.5%). Numbers above the DNA bands represent the size of each isoform. Nt = nucleotide, β-glu = House Keeping gene β-glucuronidase.

**Figure 3: Dominant expression of less inflammatory isoforms of IL-32 in HIV+ individuals under cART treatment.** A) Comparison between the relative expression of total IL-32 mRNA (pool IL-32; sum of relative expression of all isoforms) from $HIV^{neg}$ (n = 10, in blue) and $HIV^{+}$ cART-treated (n = 10, in red) (left panel) and correlation between cell-associated IL-32 total mRNA and cell-associated protein

(measured by ELISA on total cell lysate), n = 21 (right panel). B) Relative expression of individual IL-32 isoforms from in total PBMCs from $HIV^{neg}$ (n = 10, in blue) and $HIV^{+}$ cART-treated individuals (n = 10, in red). Data were analyzed by non-parametric Mann-Whitney test in A (left panel) and B and Spearman correlation (A, right panel).

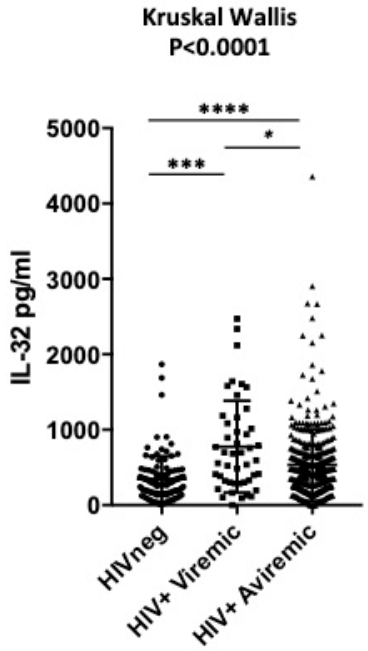
**Figure 4: Higher expression of IL-32 in T and NK cells compared to monocytes and B cells.** A) Gating strategy to isolate the different cell types by FACS cell sorting. B) Relative expression of the individual IL-32 isoforms in sorted T, NK, B cells and monocytes sorted from n=4 individual's PBMCs (n = 2 HIV+ and n = 2 $HIV^{neg}$).

**Figure 5: Induction of human IL-10 by IL-32.** A) IL-10 production by TCR-stimulated cells in the presence of IL-32α, IL-32β or IL-32γ (Left panel, n = 22). Middle and right panels: matched pair analysis for IL-32 isoforms. B&C) IL-6 (n = 14) and IFNγ (n = 11) production as in A. Purified $CD4^{+}$ T cells were stimulated with plate-coated anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence or absence of IL-32α, IL-32β or IL-32γ (500 ng/ml). Data were analyzed by the non-parametric Kruskal-Wallis for multiple comparisons (A, B and C left panels) and Wilcoxon for matched pair analysis (A, B and C, middle and right panels).

**Figure 6: Reactivation of HIV reservoir CD4 T cells from HIV+ cART-treated aviremic individuals**. A) HIV RNA quantified by PCR from supernatant of activated $CD4^{+}$ T cells (expressed as HIV copies per PCR reaction). NS represents non-stimulated control cells from the same individuals. Cells treated with anti-CD3 and anti-CD28 were used as positive control for HIV reactivation. B) Matched pair analysis for IL-32α- and IL-32γ-treated cells relative to non-stimulated (NS) cells. Matched pair analysis was done by Wilcoxon test.

**Figure 1** **Zaidan, Leyre et al.,**

**A)**

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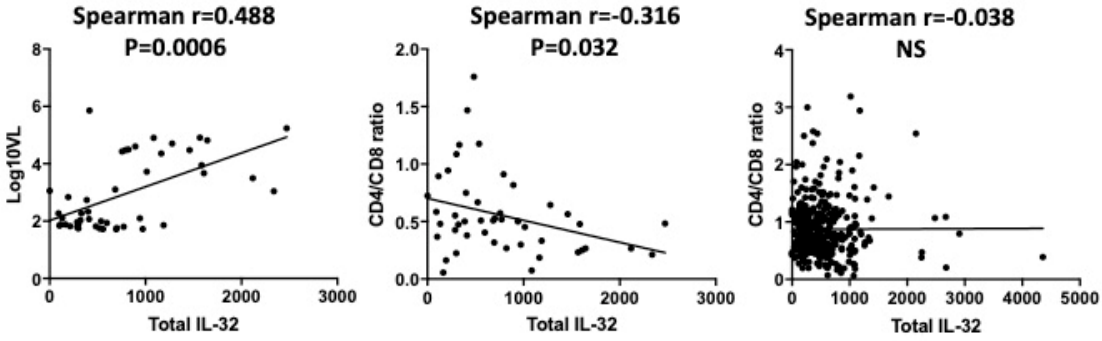

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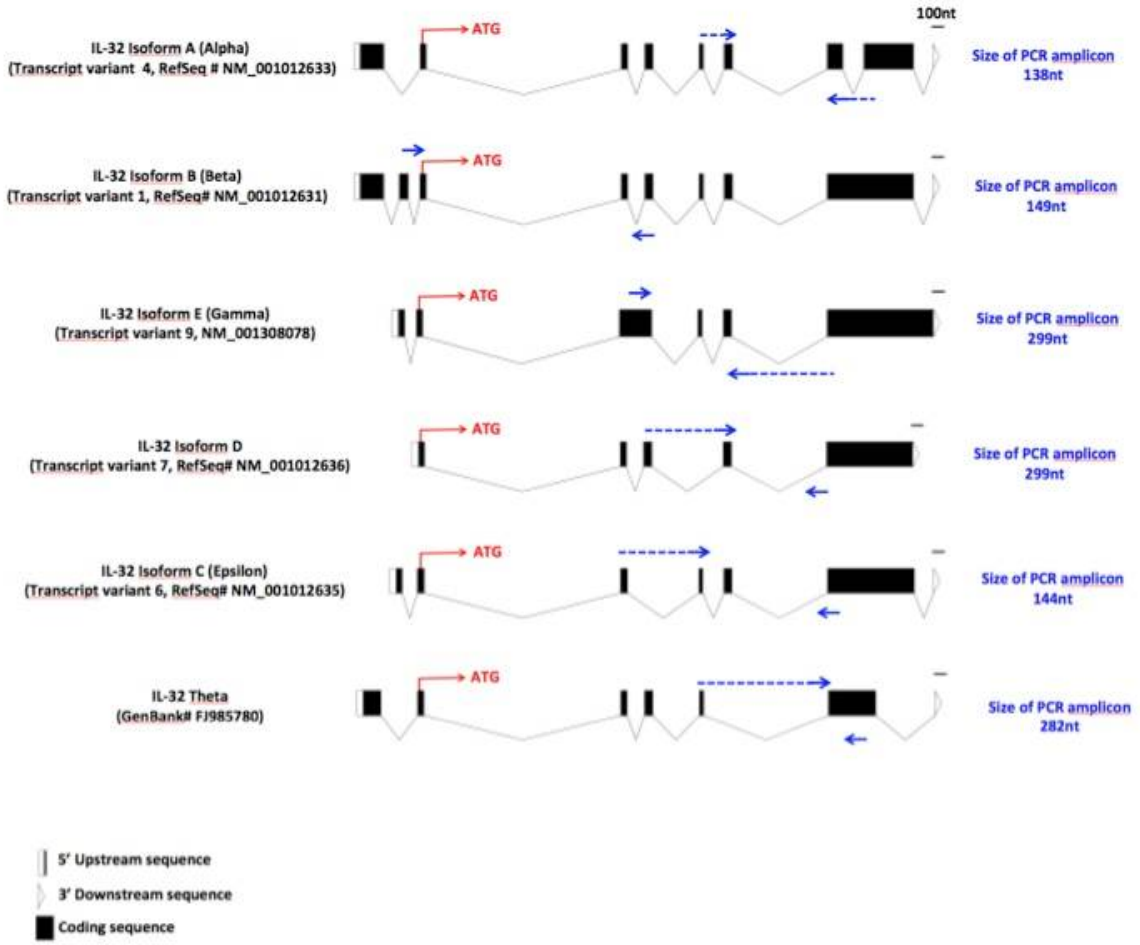


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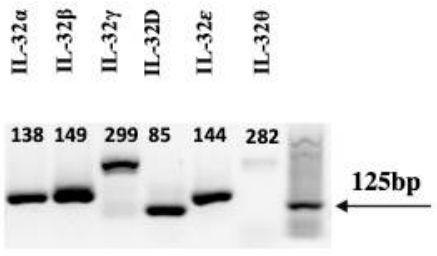

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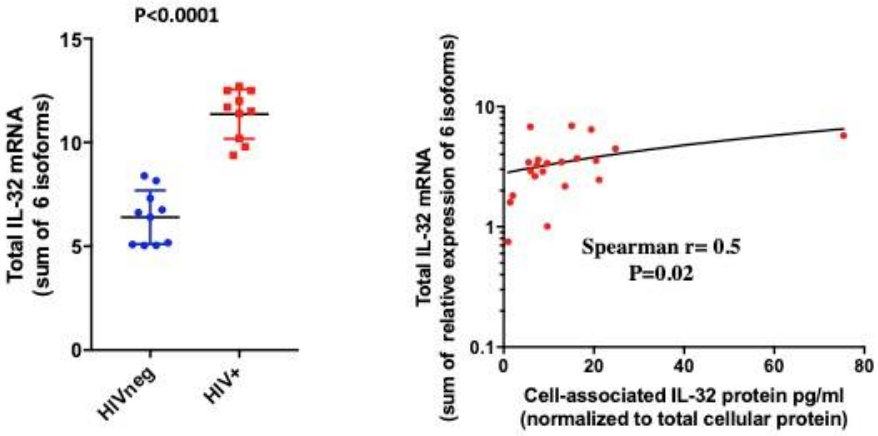


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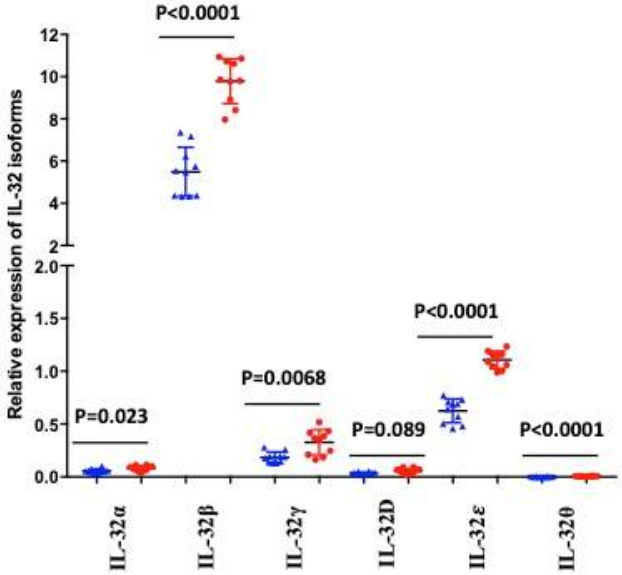

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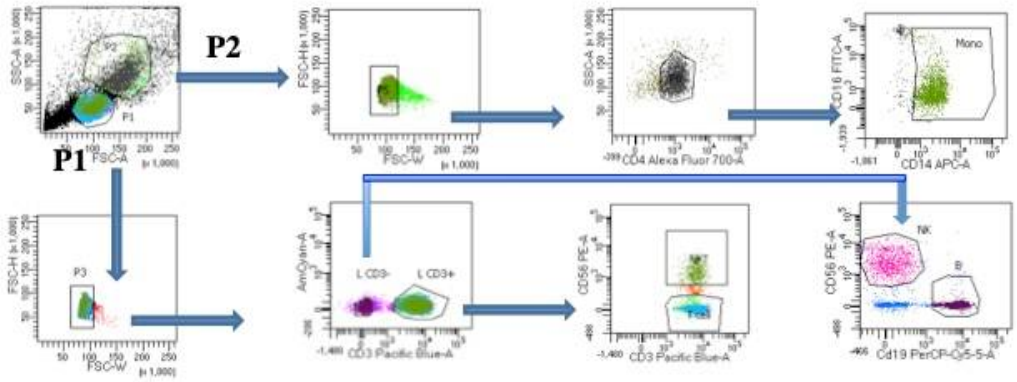
**A)**

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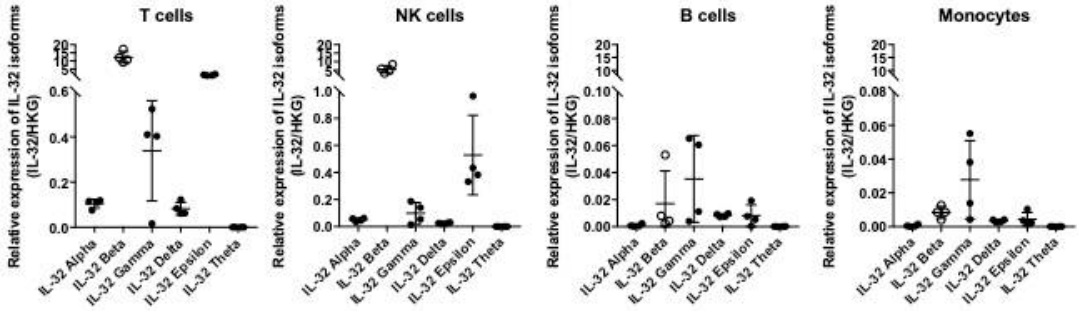

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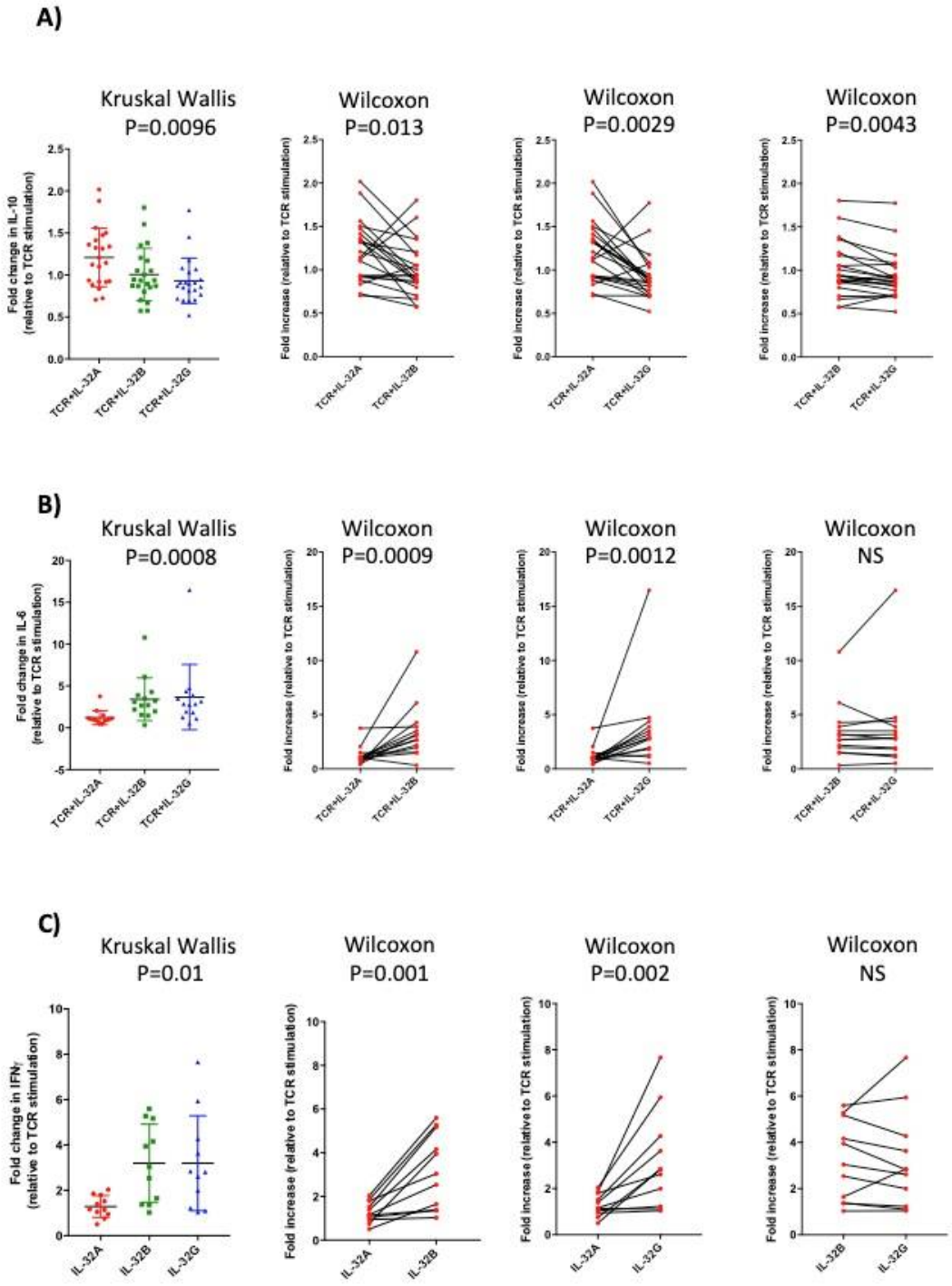

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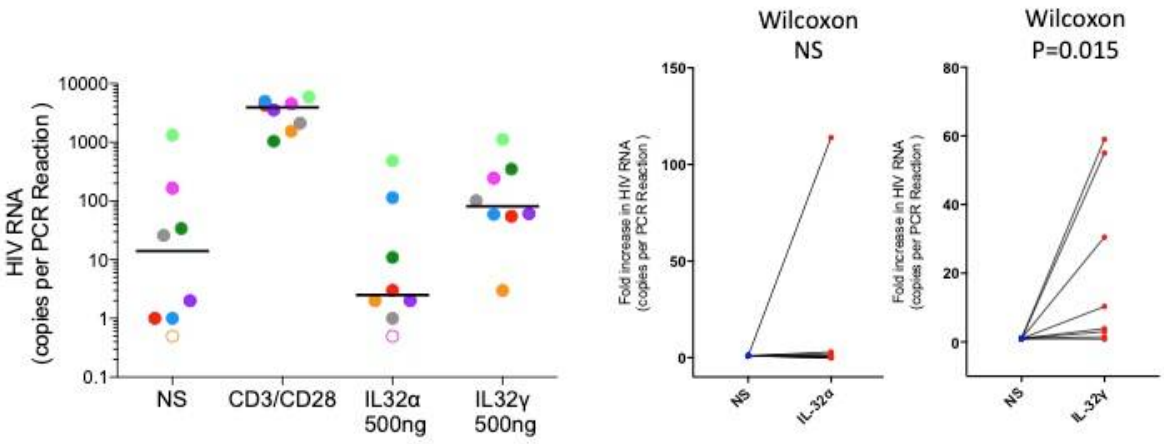
**A)**



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