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Studies on the role of IL-37 in the pathogenesis of HIV infection

by Ayoub Abulkhir

Department of Microbiology, Infectiology and Immunology
Faculty of Medicine

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Résumé

L'IL-37 fait partie de la sous-famille de l'IL-18 et, de façon plus large, se classe avec les cytokines de la famille IL-1. L'IL-18, tout comme l'IL-1β, est une cytokine pro-inflammatoire puissante, tandis que l'IL-37 a des effets anti-inflammatoires. Les deux cytokines, IL-18 et l'IL37, utilisent la chaîne IL-18Rα pour la liaison initiale à la surface des cellules cibles. Cependant, ces deux cytokines se distinguent par rapport au recrutement et l'utilisation des chaînes secondaires de leurs récepteurs. L'IL-18 utilise la chaîne IL-18Rβ, tandis que l'IL-37 utilise SIGIRR ("Single Immunoglobulin and IL-1 Receptor-Related") pour la signalisation intracellulaire. Jusqu'à récemment, SIGIRR était considéré comme récepteur orphelin. L'IL-37 inhibe l'activation des inflammasomes ainsi que les effets biologiques d'un grand éventail de médiateurs inflammatoires tels que l'IL-1β, l'IL-6 et le TNF-α, parmi d'autres. De plus, ces deux cytokines lient la protéine de liaison à l'IL-18 (IL-18BP) avec des effets différents. Il est connu que la concentration d'IL-18 est plus élevée dans la circulation des personnes VIH-positives. Cette cytokine joue un rôle important dans l'activation aberrante globale du système immunitaire et l'inflammation remarquée chez ses individus. Cependant, très peu est connu sur le rôle de l'IL-37 lors de l'infection au VIH. Ce projet de maîtrise s'est concentré sur cette question.

Les résultats présentés dans ce mémoire démontrent que les niveaux circulants de l'IL-37 sont plus élevés dans le sérum d'individus VIH-positifs sous traitement antirétroviral ainsi que d'individus non-progresseurs à long-terme comparativement aux personnes VIH-positives naïves au traitement antirétroviral. Notamment, les niveaux d'IL-37 sériques sont significativement plus élevés chez les non-progresseurs à long-terme comparé aux témoins VIH-négatifs. De façon surprenante, nos résultats démontrent aussi une baisse significative de l'expression du SIGIRR à la surface de divers types cellulaires contenus dans la population des cellules mononuclées du sang périphérique chez les personnes VIH-positives. De plus, nous avons observé une augmentation significative de la concentration de SIGIRR soluble dans les individus infectés par le VIH. Fait intéressant, nous avons remarqué que le SIGIRR soluble neutralise les effets anti-inflammatoires de l'IL-37 sur les cellules humaines. En outre, nous démontrons que l'IL-37 humain recombinant cause une baisse de la réplication du VIH dans les lymphocytes humains stimulés à l'aide de la phytohémagglutinine, ainsi que de

l'expression du corécepteur VIH (CXCR-4) et du PD-1, marqueur classique d'épuisement, dans les cellules CD4+ et CD8+ de personnes VIH-positives. Pris ensemble, ces résultats suggèrent que les fonctions anti-inflammatoires de l'IL-37, malgré ses concentrations accrues dans la circulation, sont atténuées chez les personnes qui sont dépisté Séropositives. La restauration de ces fonctions peut pallier l'inflammation et l'activation immunitaire chez ces patients.

Mots-clés : SIGIRR, IL-37, VIH, IL-18Rα, IL-1, IL-18.

Abstract

IL-37 belongs to the IL-18 subfamily and to the larger IL-1 family of cytokines. While IL-18, like IL-1β, is a potent pro-inflammatory cytokine, IL-37 exerts anti-inflammatory effects. Both IL-18 and IL-37 use the IL-18Rα chain for initial binding to the surface of target cells. However, they differ in the recruitment and use of the secondary chains of their receptors. IL-18 uses the IL-18Rβ chain, whereas IL-37 uses SIGIRR (Single Immunoglobulin and IL-1 Receptor-Related) for transducing intracellular signals. Until recently, SIGIRR was known as an orphan receptor. IL-37 inhibits activation of inflammasomes, as well as the biological effects of a wide range of inflammatory mediators such as IL-1β, IL-6 and TNF-α, etc. Furthermore, the two cytokines bind IL-18 Binding Protein (IL-18BP) with divergent effects. It is now well established that the concentration of IL-18 is increased in the circulation of HIV-infected individuals. It plays an important role in overall aberrant immune activation and inflammation in these individuals. However, little is known about the role of IL-37 in this infection; and thus, is the focus of this master's thesis.

In this thesis, we demonstrate that circulating levels of IL-37 are higher in the serum of HIV-infected individuals on anti-retroviral treatment (ART) and in HIV-infected long term non-progressors (LNTP), as compared to HIV-infected individuals naïve to ART. More importantly, serum levels of IL-37 are significantly higher in LTNP as compared to healthy controls. Surprisingly, the results also show that a significant decrease in the surface expression of SIGIRR occurs in different types of peripheral blood mononuclear cells of HIV-infected individuals. Furthermore, we found a significant increase in the circulating concentrations of soluble SIGIRR in HIV-infected individuals. Interestingly, we also noted that soluble SIGIRR neutralizes the anti-inflammatory effects of IL-37 on human cells. We also show that recombinant human IL-37 reduces HIV replication in human phytohaemaglutinin blasts. It also reduced expression of the HIV co-receptor CXCR4 and of PD-1, a classical marker of exhaustion, on the surface of CD4+ and CD8+ T cells from HIV-infected individuals. Taken together, these results suggest that anti-inflammatory functions of IL-37, despite its increased concentration in the circulation, are dampened in HIV-infected individuals. Restoring these functions may attenuate inflammation and immune activation in these patients.

Keywords: HIV, SIGIRR, IL-37, IL-18Rα

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List of Abbreviations

AIDS: Acquired immune deficiency syndrome

ART: Antiretroviral therapy

cART: Combination anti-retroviral therapy

CCR5: C-C chemokine receptor type 5

cDNA: Complementary DNA

CTLA: Cytotoxic T-lymphocyte

CTLA4: Cytotoxic T-lymphocyte associated protein 4

CVD: Coronary vascular disease

CXCR4: CXC chemokine receptor type 4

DCs: Dendritic cells

DNA: Deoxyribonucleic acid

dsRNA: Double-stranded RNA

EC: Elite controllers

ELISA : Enzyme-linked immunosorbent assay

ENV: HIV envelope glycoprotein

Gag: Group-specific antigen, coding for structural proteins

GALT: Gut-associated lymphoid tissue

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GIT: Gastro-intestinal tract

Gp: Glycoprotein

HAART: Highly aggressive anti-retroviral therapy

HC: Healthy controls

HIV-1: Human immunodeficiency virus type 1

HIV-2: Human immunodeficiency virus type 2

HIV: Human immunodeficiency virus

HTLF: Helicase-like transcription factor

HVL: High viral load

IBD: Inflammatory bowel disease

ICI: Immune checkpoint inhibitors

IEC: Intestinal epithelial cells

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL-1R: IL-1 receptor

IL: Interleukin

IL1R: Interleukin 1 family receptor

LAG-3: Lymphocyte-activation gene 3

LPS: Lipopolysaccharide

LTNP: Long-term non-progressors

MHC: Major histocompatibility complex

mRNA: Messenger RNA

ND: Non-detectable

Nef: Negative factor

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

NFAT: Nuclear factor of activated T cells

NKs: Natural killer cells

NLR: Nod-like receptors

PBMC: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

PD-1: Programmed cell death-1

PHA: Phytohaemaglutinin

PHI: Primary HIV infection

PI: Protease inhibitor

Pol: DNA polymerase

Rev: Regulator of expression of viral proteins

RNA: Ribonucleic acid

RT: Reverse-transcriptase

RT-PCR: Real-time polymerase chain reaction

SIGIRR: Single immunoglobulin and toll-interleukin 1 related receptor

SIV: Simian immunodeficiency virus

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

STIs: Sexually transmitted diseases

TAT: Trans-activator protein

TGF-β: Transforming growth factor beta

TIR-8: Toll interleukin-1 receptor 8

TLR: Toll-like receptors

TNF-α: Tumor necrosis factor alpha

Vif: Viral infectivity factor

VIH: Virus de l'immunodéficience humaine

Vpr: Viral protein R

Vpu: Viral protein unique

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Introduction & Review of the Literature

The aim of my master's thesis project was to investigate the role of Interleukin-37 (IL-37) in HIV-1 infection.

A brief introduction of HIV infection and IL-37 is provided in the following section.

1. REVIEW OF LITERATURE

1.1 Human Immunodeficiency Virus (HIV)

1.1.1 The virus and related diseases

HIV-1 is the underlying cause of AIDS and AIDS-related disease; however, its origin remains obscure. Firm serological evidence of the infection can be found on the east and west coasts of the USA as of the mid-1970s, but HIV infection in central Africa may have predated the AIDS epidemic in North America (Clavel, Mansinho et al. 1987). Phylogenetic analysis of the HIV-1 genome suggests it originated in chimpanzees; whereas HIV-2, which shows similarity to the simian immunodeficiency virus (SIV) genome, suggests a source in sooty manage monkeys (Marx, Li et al. 1991, Jaffar, Grant et al. 2004). Confined to West Africa, HIV-2 infection is less virulent than HIV-1 infection (Clavel, Mansinho et al. 1987, Sharp and Hahn 2011). Like some other RNA viruses, HIV appears to have evolved rapidly, mutating and shifting its host range and virulence as a result of its error-prone reverse transcription enzyme (St Clair, Martin et al. 1991). This high rate of mutation explains how a new pathogenic retrovirus could arise in human. The United Nations Programme on HIV/AIDS (UNAIDS) reports that there are currently thirty-six million people living with HIV worldwide (UNAIDS 2017).

Retroviruses are named as such because their genomes encode an unusual enzyme, Reverse Transcriptase, which allows DNA to be transcribed from an RNA template (Preston, Poiesz et al. 1988, Roberts, Bebenek et al. 1988). Thus, HIV can make copies of its genome in host cells, such as the human CD4+ "helper" T lymphocyte, which is the main target of HIV-1 infection (Dragic, Litwin et al. 1996). Other antigen presenting cells (APCs) such as macrophages, dendritic cells (DC), and monocytes, are also targets of HIV infection (Janeway 2001, Stevenson 2003). A hallmark characteristic of HIV infection is the integration of the newly synthesized viral DNA into the genome of the infected lymphocyte. Integration of the viral DNA into the host genome allows the viral DNA to evade immune detection, thereby forming the basis of chronic HIV infection and creating the greatest obstacle for viral eradication (Schröder, Shinn et al. 2002). Many anti-retroviral treatments suppress replication of the virus but do not eliminate the integrated viral DNA (provirus) from infected cells.

Nonetheless, advanced therapies, which include combinations of nucleoside analogs and protease inhibitors, have transformed the prognosis for carriers of HIV. Treatment with these combination anti-retroviral (cART) therapies often leads to a sustained decrease of virus in the blood, helping to restore levels of the central target cell (CD4+ lymphocytes) to near normal (Sabin and Lundgren 2013, Sabin, Reiss et al. 2016). Despite these efforts, the inherent variability of the HIV genome and failure of the human host to produce sufficient amounts of neutralizing antibodies against the virus, as well as concerns surrounding safety, have continued to frustrate attempts to develop a reliable vaccine (Titti, Cafaro et al. 2007).

1.1.2 Transmission of the virus

HIV has been isolated from several bodily fluids including semen, cervical secretions, lymphocytes, cell-free plasma, cerebrospinal fluid, tears, saliva, urine, and breast milk (Sabin and Lundgren 2013). However, not all these fluids transmit infection due to the considerable variation in their viral concentration. The predominantly infectious fluids are semen, blood, and possibly cervical secretions. Transmission of HIV-1 and HIV-2, known as the major and minor human AIDS viruses, respectively, is similar to that of other retroviruses and can be classified in two ways: horizontal and vertical. Epidemiological observations have shown that, globally, the most common mode of HIV transmission is through unprotected anal or vaginal intercourse (Pantaleo and Fauci 1995). Transmission through sexual intercourse or through the exchange of infected blood or fluids (e.g. via transfusion, organ donation, sharing or reusing of contaminated needles between infected drug users or during therapeutic procedures) is classified as horizontal transmission, given the transfer between individuals of the same generation (Lipsitch, Siller et al. 1996). Vertical transmission, on the other hand, occurs across two generations, as it refers to the transmission of the virus from mother to offspring. Vertical transmission occurs *in utero* and has also been shown to transfer through breast milk (Kreiss 1997).

Several factors affect the transmission of HIV, notably the concentration of HIV in bodily fluids, secondary infection of the genital tract, the efficiency of epithelial barriers, and the efficiency of immune function against HIV infection. The stage of the disease and

associated viral load are essential in determining infectivity (Hollingsworth, Anderson et al. 2008).

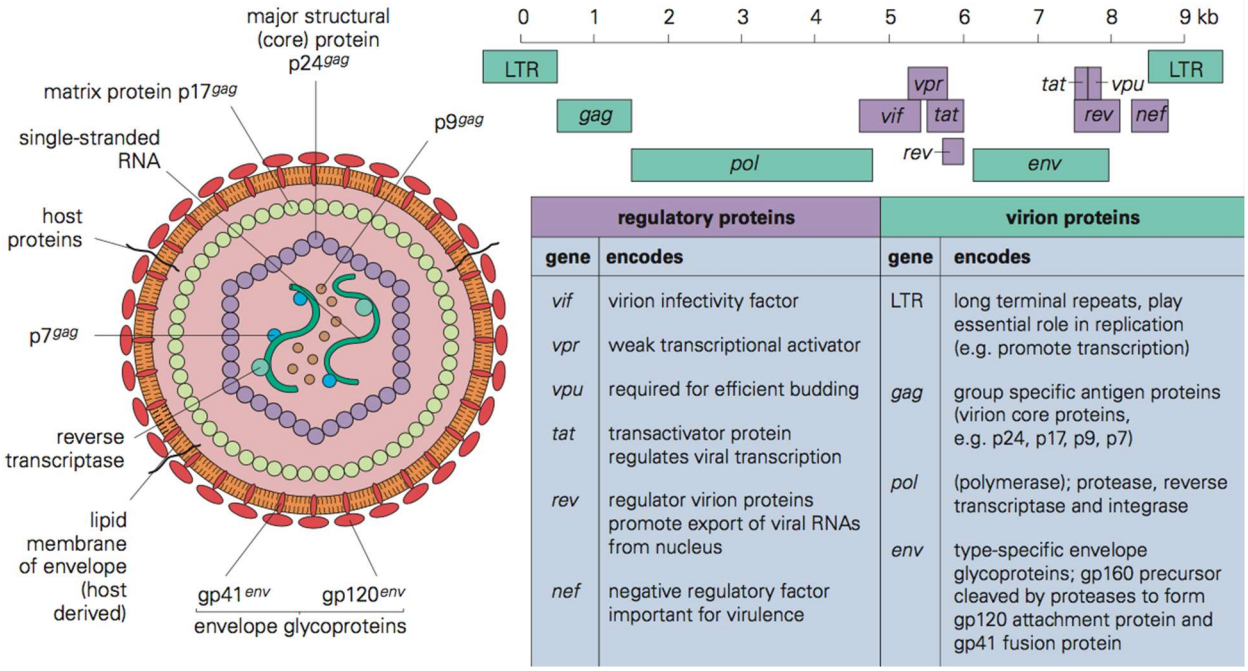
1.1.3 HIV-1 structure

HIV is a spherical enveloped virus belonging to the lentivirus subgroup of retrovirus. The lentivirus subgroup includes other retroviruses that cause slow progressing diseases in humans and animals (Cullen 1991, Klimas, Koneru et al. 2008). As illustrated in Figure 1, HIV particles are approximately 110 nm in diameter and consist of a lipid membrane bilayer known as the envelope. The viral envelope is formed from the plasma membrane of the host cell and two viral proteins, the external glycoprotein (gp) 120 and transmembrane gp41, which are non-covalently attached (Chan, Fass et al. 1997, Kwong, Wyatt et al. 1998). The virus uses these glycoproteins to infect target cells. The viral envelope encloses a viral genome comprised of two identical single-stranded positive-sense RNA strands and numerous copies of viral enzymes, which are pivotal for the infection of host cells. In order to complete the life cycle of the virus, the viral genome must be transcribed from RNA to DNA within the infected cells. This process is known as reverse transcription and is catalysed by the viral reverse transcriptase enzyme (Sarafianos, Marchand et al. 2009). It is because of this enzyme that HIV is classified as a retrovirus. In addition to the basic retroviral genes (envelope (*env*), group antigen genes (*gag*), and polymerase (*pol*)), the virus has additional genes that are important for infection. These genes include regulator of expression of virion proteins (*rev*), viral infectivity factor (*vif*), viral protein U (*vpu*), viral protein R (*vpr*), negative factor (*nef*), and trans-activator of transcription (*tat*). *Tat* and *rev* are essential regulatory genes coding for accumulating proteins and increase production of the viral genome (Malim and Bieniasz 2012).

Each protein plays an essential role in viral structure and/or infectivity. The Tat protein plays a positive feedback role in the transcription of viral messenger RNA (mRNA), while the Rev protein splices transcripts from the nucleus and ensures their transport from the nucleus to the cytoplasm (Langer and Sauter 2016).

Another important protein in the life cycle of the virus is Nef. The Nef protein sustains T cell activation during the early phases of the viral cycle by decreasing the threshold of T cell activation. It achieves this through the down-regulation of cytotoxic T lymphocyte associated protein 4 (CTLA-4), which is a negative regulator of T cell activation (El-Far, Isabelle et al. 2013). Nef also reduces the expression of both major histocompatibility complex (MHC) class 1 and 2 on the surface of host cells; therefore, cytotoxic T cells and antiviral immune response are less likely to be induced by the actively infected cells. A third function of Nef is to enhance the degradation of surface CD4+ molecules, thereby blocking reinfection of the cell (Piguet and Trono 1999, Pawlak and Dikeakos 2015).

The Vpu protein has a unique role in HIV-1. Its function is to increase the intracellular degradation of CD4 and it is also responsible for releasing newly produced virus particles into the cytoplasm (Pham, Lukhele et al. 2014, Apps, Del Prete et al. 2016). The Gag protein is essential for the formation of the viral core and matrix proteins (PAL, REITZ JR et al. 1990). Vif inactivates the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC-3G), a cytidine deaminase that catalyzes the conversion of deoxycytidine into deoxyuridine in reverse transcribed viral complementary DNA (cDNA) (Donahue, Vetter et al. 2008). This inactivation triggers ubiquitination and degradation of the deaminase and prevents modifications of the viral cDNA (Nakashima, Ode et al. 2016). Vpr is important for HIV-1, as it allows for the infection of non-dividing cells, causing cell cycle arrest in G2M (Elder, Benko et al. 2002, Richard, Sindhu et al. 2010). Independently, it also downregulates helicase-like transcription factor (HTLF), which is a DNA translocase that functions to repair damaged replication forks (Zhou, DeLucia et al. 2017). The Pol protein, which is arguably the most important structural HIV-1 protein, results in the expression of three enzymes critical for viral replication: reverse transcriptase, integrase, and protease. Reverse transcriptase allows the viral RNA to be transcribed into viral DNA, which then integrates into the host DNA with the help of the integrase enzyme. Viral protease is essential for maturation of the virus particle, as it cleaves polyproteins into active functional proteins (Baldwin and Linial 1998). Finally, the Env protein precursor, gp160, is cleaved by the cellular enzyme Furin into gp120 and gp41. Gp120/41 use CD4+ as a receptor and both chemokine receptors CXCR4 and CCR5 as co-receptors to infect human cells (Malim and Bieniasz 2012).



regulatory proteins		virion proteins	
gene	**encodes**	**gene**	**encodes**
vif	virion infectivity factor	LTR	long terminal repeats, play essential role in replication (e.g. promote transcription)
vpr	weak transcriptional activator		
vpu	required for efficient budding	*gag*	group specific antigen proteins (virion core proteins, e.g. p24, p17, p9, p7)
tat	transactivator protein regulates viral transcription		
rev	regulator virion proteins promote export of viral RNAs from nucleus	*pol*	(polymerase); protease, reverse transcriptase and integrase
nef	negative regulatory factor important for virulence	*env*	type-specific envelope glycoproteins; gp160 precursor cleaved by proteases to form gp120 attachment protein and gp41 fusion protein

Figure 1. **HIV structure**

This figure illustrates the structure of HIV and describes the enzymes essential for replication. Originally published in (Murphy and Weaver 2016) and reproduced with permission from the publisher.

1.1.4 The HIV replication cycle

The HIV life cycle is comprised of various steps including entry, reverse transcription, integration, transcription, and translation (KARAGEORGOS, LI et al. 1993). The virus targets the CD4 molecule, which is mainly expressed on CD4+ T cells and, to a lesser extent, on macrophages and DCs. As depicted in Figure 2, the entry of HIV into the host cell begins with the high affinity binding of gp120 to the CD4 molecule. Before the virus can enter the host cell, gp120 must also bind to either the CCR5 or CXCR4 chemoreceptor on the host cell surface. CCR5 or CXCR4 serve as co-receptors (Gomez and Hope 2005, Panos and Watson 2015). CCR5 is predominantly expressed on effector memory CD4 T cells, macrophages, and DCs (Zaitseva, Blauvelt et al. 1997). In contrast, CXCR4 is expressed by naïve and central

memory T cells (Nicholson, Browning et al. 2001). After gp120 binds the co-receptors, it undergoes a conformational change which exposes the fugenic peptide of gp41 and then fuses with the plasma membrane of the host cell. This process enables the viral nucleocapsid, viral genome, and associated proteins to enter the target cell's cytoplasm (Gomez and Hope 2005, Panos and Watson 2015). Once the virus has entered the host cell, the nucleocapsid disintegrates, releasing two strands of viral RNA and essential viral enzymes into the cytoplasm and begins to replicate. As a first step, the reverse transcriptase transcribes the viral RNA into cDNA, which encodes nine genes. The newly synthesized cDNA is then transported into the cell nucleus, where it is integrated into the host genome by the viral integrase enzyme. This integrated viral cDNA is called the provirus (Panos and Watson 2015).

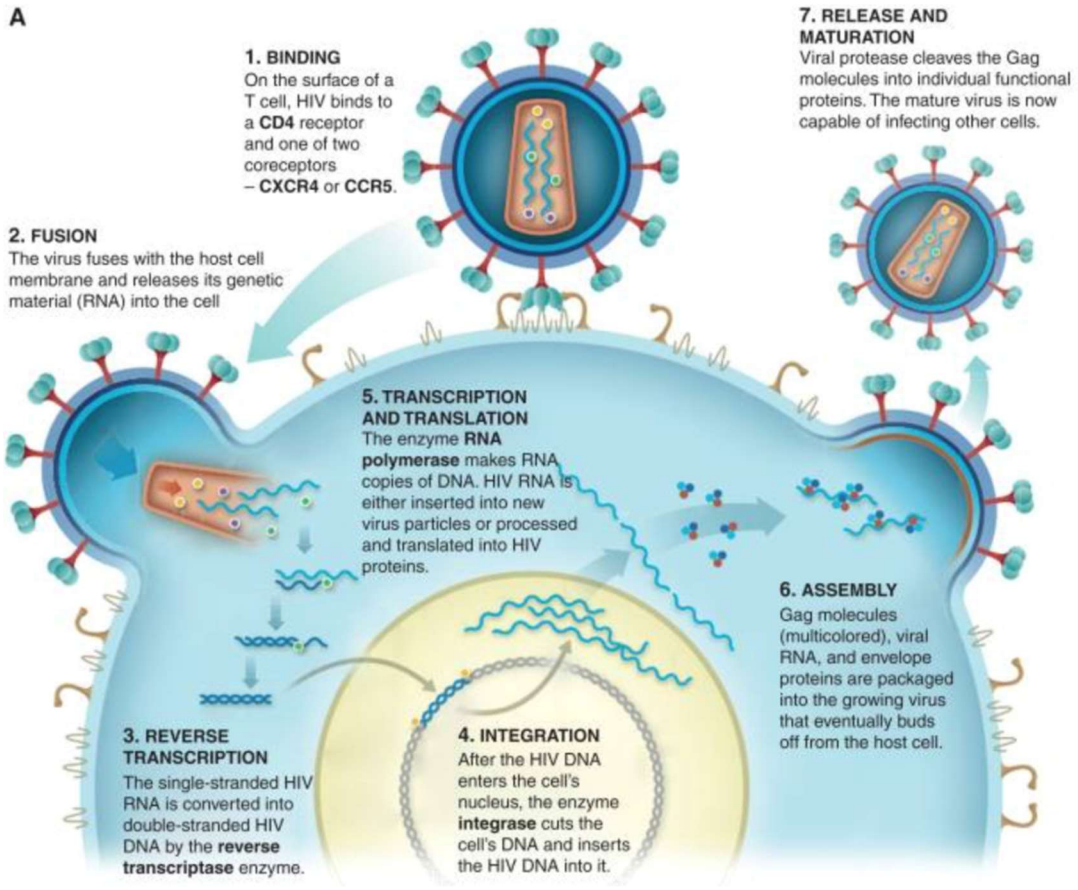


Figure 2. **Stages of the viral replication cycle**

This figure shows how free virus can fuse with the host cell, where it releases its genetic material. The viral RNA genome is reverse transcribed into DNA by the reverse transcriptase enzyme, after which the newly synthesized viral cDNA inserts into the host cell's DNA. Integration allows for the production of viral proteins by the host cell's own transcription/ translation machinery, after which the proteins come together to form an immature virus particle. Finally, this form of the virus is pushed out through the host cell membrane, taking the lipid bilayer with it, enveloping the viral components and creating a mature virus particle. Originally published in (Vanpouille, Arakelyan et al. 2012) and reproduced with permission from the publisher.

In order to begin the replication process and to form infectious virions, HIV requires activation of the target cell. The virus hijacks the target cell's transcriptional machinery and activates the host cells' own transcription factors. Two important transcription factors required for virus production are nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and nuclear factor of activated T-cells (NFAT) (Murphy and Weaver 2016). Although NF-kB is expressed in all immune cells infected by HIV, NFAT is primarily activated in CD4 T cells (Murphy and Weaver 2016).

1.1.5 Infection with HIV

Many of the clinical features of HIV infection can be linked to the profound immune deficit that develops in infected individuals. HIV is immunosuppressive, as it infects the CD4+ T cells of the immune system and ultimately destroys them (McCune 2001, Brenchley, Schacker et al. 2004). An understanding of this process is helpful in interpreting tests that monitor the disease and may also explain the failure of immunotherapy, as well as the difficulties in developing vaccines for HIV (Gomez and Hope 2005).

HIV's primary target is a subset of thymus-derived (T) lymphocytes carrying the CD4 surface molecule receptor. These cells have been shown to bind with gp120 on the HIV envelope, thus beginning the fusion process (Mohan, Bhatnagar et al. 2014). CD4 is also expressed on a large number of monocytes, macrophages, Langerhans cells of the skin, and DCs in all tissues (Bernstein, Plasterer et al. 2006, Zhen, Krutzik et al. 2014). More recently,

it has become clear that entry of the virus also requires the function of co-receptors, most of which are members of the seven transmembrane-spanning G protein-coupled receptor (GPCR) family. In the immune system, GPCRs principally function as chemokine receptors capable of regulating migration, differentiation, and function of leucocytes during immune responses and inflammation (Lodowski and Palczewski 2009). The two co-receptors, CCR5 and CXCR4, are of great importance (Mohan, Bhatnagar et al. 2014). CCR5 (R5) is widely expressed on lymphocytes, macrophages, DCs, and on cells of the rectal, vaginal, and cervical mucosae. Viral strains capable of infecting primary macrophages (M or R5 tropic viruses) use CCR5 as their co-receptor. Only R5 strains are detected in the early stages immediately after infection, while both R5 viruses and strains that infect T cells and use CXCR4 (T or X4 tropic viruses) are found in later-stages of infection (Trkola, Kuhmann et al. 2002, Cicala, Arthos et al. 2011). These data suggest that R5 strains are necessary for the transmission of HIV, while X4 variants evolve in the infected host and may be responsible for T cell loss and disease progression (Bednar, Hauser et al. 2015, Sironi, Malnati et al. 2015). Even stronger evidence that CCR5-using M-tropic viruses transmit infection comes from the observation that individuals homozygous for a 32-base pair deletion of CCR5 showed substantially increased resistance to HIV infection (Balotta, Bagnarelli et al. 1997, Suppiah, Armstrong et al. 2013).

1.1.6 Immunopathology

CD4 lymphocytes are known as “leaders of the immunological orchestra” owing to their central role in the induction of an immune response. Stimulation of these cells through antigen presentation results in cell division and in the production of lymphokines such as interferons (IFN), interleukins (ILs), tumor necrosis factor (TNF), and chemoattractant chemokines (Mohan, Bhatnagar et al. 2014). Lymphokines function as local hormones controlling migration, growth, maturation, and behavior of other lymphocytes; especially of the cytotoxic/suppressor (CD8) T cells and of antibody-producing B lymphocytes (B cells). Lymphokines also affect the maturation and function of monocytes, tissue macrophages, and DCs (Corinti, Albanesi et al. 2001).

Macrophages and DCs are antigen-presenting cells that play a critical role in the initiation of the immune response (Banchereau and Steinman 1998, Geissmann, Manz et al. 2010). Monocytes, especially inflammatory ones, are the precursors to some glial cells. After HIV infection, their abnormal lymphokine production may have harmful effects on neural tissue, which may result in encephalopathy (Imran, Manzoor et al. 2016).

Early after HIV infection, antibody responses are not impaired. In fact, the presence of antibodies in the blood, targeting the viral envelope and core proteins, is the crucial evidence required to diagnose HIV infection; the development of these antibodies persists until death (Lange, Paul et al. 1986, Walker and Burton 2010). In adults, enormous activation of B lymphocytes is characterized by an increase in serum immunoglobulin (Ig) levels, perhaps because of the direct and indirect activation of the B cells by HIV (Imran, Manzoor et al. 2016). This massive polyclonal activation explains, in part, the reason for false positive serological tests seen in HIV infection. Interestingly, in young children, the reverse may be seen. Low Ig levels seen in children can sometimes necessitates intravenous replacement therapy (Cheynier, Langlade-Demoyen et al. 1992). Within days or weeks following infection, there may be a decrease in the number of CD4+ T lymphocytes and an increase in the number of CD8+ cytotoxic/suppressor T cells (Migueles, Osborne et al. 2008). Depletion of CD4+ T cells can occur via two mechanisms: direct cytopathology of HIV-1 or immune responses. The host immune responses activate the elimination of infected cells through the action of Natural Killer (NK) and cytotoxic CD8+ T lymphocytes (CTL); thus explaining the rise in CTLs (Cloyd, Chen et al. 2001, McCune 2001, Brenchley, Schacker et al. 2004). Following acute infection, healthy seropositive individuals may still have a reasonable level of CD4+ T lymphocytes; however, the number of CTLs frequently remains high (Betts, Nason et al. 2006). This happens, in part, due to reduced production of the lymphokine IL-2. Infected individuals may remain healthy for extended periods of time (several years). A hallmark of disease progression, often before the development of clinical symptoms, is a decrease in the number of CD4+ T lymphocytes. As CD4+ T cells are the main mediators of immune responses, their depletion due to HIV infection increases the vulnerability of infected individuals to opportunistic infection and to death (Okoye and Picker 2013). In AIDS, the number of CTLs is also reduced (Mohan, Bhatnagar et al. 2014, Imran, Manzoor et al. 2016).

1.1.7 Specific immune responses to HIV

Even though HIV-infected individuals show a deficit in their immune function, as described above, their immune system can directly target HIV itself. While serum reactivity to viral proteins is detectable, virus neutralizing titers are low and directed against the immunizing viral strain. Antibodies against HIV might even facilitate infection of new cells via binding to Ig (Fc) receptors on target cells (Mohan, Bhatnagar et al. 2014).

CTLs capable of killing HIV-infected target cells are detected in most HIV-infected individuals and are host-protective. This protective nature of CTLs is evidenced by the observation that viremia declines at the time when CTLs are first detected in the peripheral blood following infection, as well as in patients with stable disease (Borrow, Lewicki et al. 1994, Kiepiela, Ngumbela et al. 2007). Moreover, it is interesting to note that individuals who have been regularly exposed to HIV, but who remain seronegative and without a detectable viral load, do have detectable levels of HIV-specific CTLs (Pereyra, Palmer et al. 2009). In addition to killing the infected cells directly, CTLs may contribute to protection by producing several chemokines such as CD8 T cell antiviral factor (CAF), which actively suppresses viral replication in CD4+ T cells (Levy 2003). All these findings suggest that CTLs are an efficient protective mechanism. Nevertheless, because reverse transcription is highly error-prone, mutations of the viral genome occur frequently and allow the virus to escape the CTL response. Moreover, these mutants themselves not only avoid immune detection, but they may also inhibit the recognition of unmutated virus (Imran, Manzoor et al. 2016) .

A hallmark of HIV infection is the substantial depletion of CD4+T cells. HIV can trigger host cell immune activation in two ways: directly through antigens, and indirectly through the activation of other viruses, such as the cytomegalovirus (Hussey, Richardson et al. 1988, Cloyd, Chen et al. 2001). After an individual is infected with HIV, the primary targets of the virus are the CCR5 CD4+ activated T cells (Siliciano and Siliciano 2000). The majority of these T cells are located in lymphoid tissues, such as the lymph nodes; and in mucosal lymphoid tissues, like the gastrointestinal tract (Reynes, Portales et al. 2001). During the acute infection phase, HIV induces a significant T cell response, especially of CTLs, and the

response can last well into the chronic phase of the disease due to persistent viral replication (Douek, Brenchley et al. 2002). HIV pathogenesis can be divided into three groups based on the duration of the infection, viral load, CD4+ T cell counts, and presenting clinical symptoms. These three groups are illustrated in Figure 3 and are discussed below:

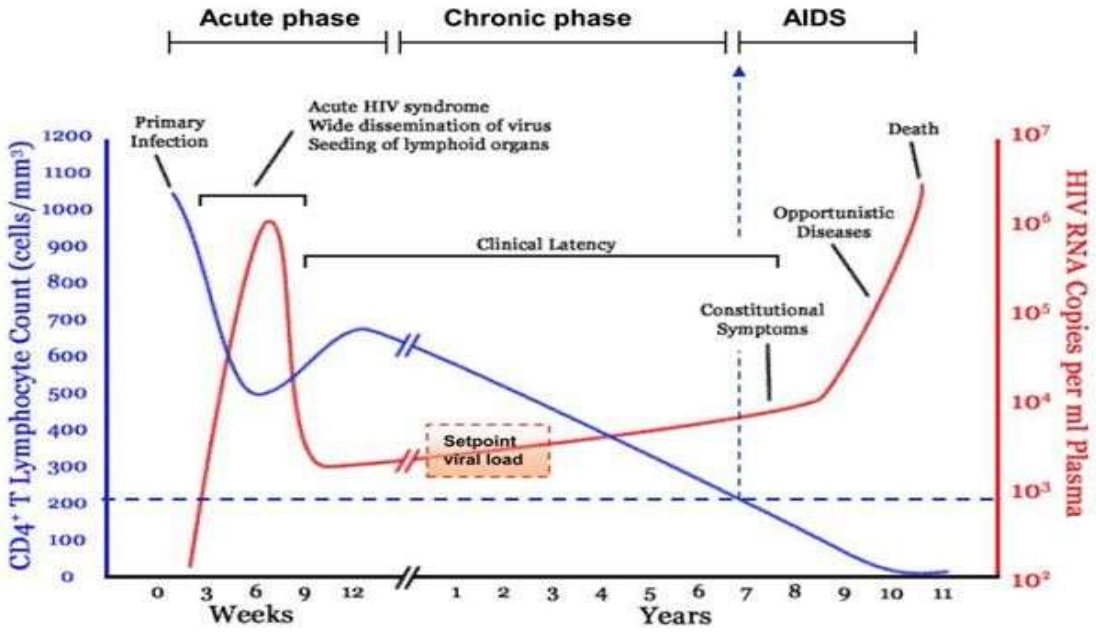


Figure 3. **Clinical stages of HIV infection**

HIV infection is divided into three different stages: (i) primary/acute stage, (ii) chronic stage, and (iii) AIDS. This division is based on the duration of the infection, the number of circulating CD4+ T cell, the viral load in the blood, and the clinical symptoms that infected individual's experience. Chronic infection, also known as the period of clinical latency, is characterized by a period where the infection is asymptomatic. Originally published in (An and Winkler 2010) and reproduced with permission from the publisher .

Group I: Primary HIV infection

Primary HIV infection (PHI) is also known as seroconversion illness or as acute HIV infection (Schacker, Collier et al. 1996, Schacker, Hughes et al. 1998). It represents the stage of infection after initial acquisition of the virus and is characterized by the development of

viral antibodies. It often lasts two to four weeks following initial infection, and between 25% and 65% of infected individuals present with symptoms at the time of seroconversion. The symptoms can be flu-like, with a mild to moderate glandular fever-like illness similar to an encephalopathy. Nevertheless, severe symptoms are rarely seen (Kahn and Walker 1998).

During the acute phase of HIV infection, sometimes there is a high rate of viral replication which leads to a transient rise in viral load due to a temporary fall in the CD4+T cell count. This interplay between increased viral production and decreased immune response might affect the outcome of HIV-related diseases (Meulendyke, Croteau et al. 2014).

The best diagnostic tests used during this stage are those that detect HIV antibodies and antigens. If the results of these tests are negative, but PHI is suspected, a more definitive test is an HIV RNA PCR, as it is the most sensitive test for the detection and quantification of the virus.

Group II: Asymptomatic chronic infection

Following the acute phase, HIV antibodies continue to be detectable in the blood. The amount of virus in the blood and lymphoid tissues decreases to very small amounts and the rate of HIV replication is reduced, but not eliminated (Goodsell 2015). This is also referred to as the chronic, asymptomatic phase. During this stage, CD4+ T cell counts are within normal range, generally above 350 cells/mm^3. However, due to immune exhaustion from the major activity of the acute phase, the immune system becomes persistently activated, leading to high levels of circulating pro-inflammatory cytokines (Brenchley, Price et al. 2006). This chronic immune activation is thought to cause cell death (Douek, Picker et al. 2003, Ford, Puronen et al. 2009). This step can persist for up to ten years or more (An and Winkler 2010).

Group III: Symptomatic HIV infection before the development of AIDS

The progression of HIV infection is a result of the depletion of immune competence, which happens after increased replication of latent HIV virus sites. Occasionally, the latent virus undergoes reactivation; however, the exact mechanism of this reactivation remains

unknown. Because of the variability of disease progression, HIV-infected individuals may exhibit different symptoms that may range from skin disorders and mouth ulcers, to hematological disorders; many of which are not difficult to treat or alleviate. A drop in viral load due to the introduction of antiretroviral therapy often leads to a complete or partial resolution of such symptoms (Goodsell 2015). Nevertheless, during this period, infected individuals become much more vulnerable to various opportunistic conditions.

1.1.8 The effect of antiviral therapy

Viral replication leads to the depletion of CD4 T cells and to immune dysfunction. If not treated, in most infected individuals, this process gradually and slowly results in fatal immunodeficiency. Thus, many drugs have been developed to inhibit HIV replication and they have led to significant improvements in the outcome of this deleterious infection. As illustrated in Figure 4, there are different mechanisms through which these drugs inhibit viral replication. Antiretroviral therapies can target the viral life cycle at five different steps. The first wave of successful drugs was developed to interfere with the reverse transcriptase enzyme, which functions to convert viral RNA to cDNA (De Clercq 1998, Butanda-Ochoa, Hernández-Espinosa et al. 2017). These nucleoside reverse transcriptase inhibitors served as chain terminators of DNA synthesis, prematurely ending transcription of the viral genome. Soon after, non-nucleoside reverse transcriptase inhibitors were introduced (De Clercq 1998). These molecules induced a conformational change in the HIV reverse transcriptase enzyme, preventing its retro-transcription activity. The second generation of medication was developed to inhibit activity of the viral protease required to cleave the precursor Gag-Pol protein (De Clercq 2002, Murphy and Weaver 2016); thus preventing assembly of functional virus particles. The mechanism of third-generation agents was to block fusion of the virus with the cell membrane, via gp41, thereby preventing viral entry into the host cell (Murphy and Weaver 2016). The fourth group of treatments targeted the CCR5 chemokine receptor (Lederman and Margolis 2008, Cummins and Badley 2010). By binding to the receptor, these molecules induce conformational changes preventing attachment of gp120, which is required for entry into the host cell. Finally, the latest antiviral group of drugs was developed to

interfere with the viral integrase, which is required for the insertion of the viral genome into the infected host cells' own DNA (De Clercq 2004, Murphy and Weaver 2016). Integrase inhibitors prevent close interaction with the host DNA, which is required to initiate integration. Initially, when used separately, these medications were very efficient at inhibiting viral replication; however, with time and due to its mutagenic nature, the virus developed resistance to these drugs (Murphy and Weaver 2016). Resistance significantly hinders the treatment process, rendering it little or completely ineffective. Thus, a new approach known as highly active antiretroviral therapy (HAART) was developed. HAART refers to therapy that combines two or more drug classes previously described. In most cases, cART therapy appears to overcome the ability of the virus to mutate, which is the primary cause of drug resistance (Cao, Mehraj et al. 2015). HAART has been shown to lower the viral load in the plasma to levels that cannot be detected by current tool and methods. The success of using HAART against HIV infection has increased the length of time over which patients progress to the AIDS stage; thus, converting HIV infection into more of a chronic and manageable disease (Estes, Haase et al. 2008). However, in order to maintain this chronic state, infected individuals must take HAART for life.

While antiviral therapies have revolutionized the war against this harmful virus and have suppressed viremia in many infected individuals, they have also markedly reduced the death rate from AIDS and improved the quality of life of HIV-infected individuals. However, abnormalities of the immune system are still observed in virus-infected individuals. These individuals show low-grade chronic inflammation and immune activation, which leads to many side effects including enhanced aging, weakness, cardiovascular disease, bone abnormalities, and cancers to name a few (Schneider, Birger et al. 2016).

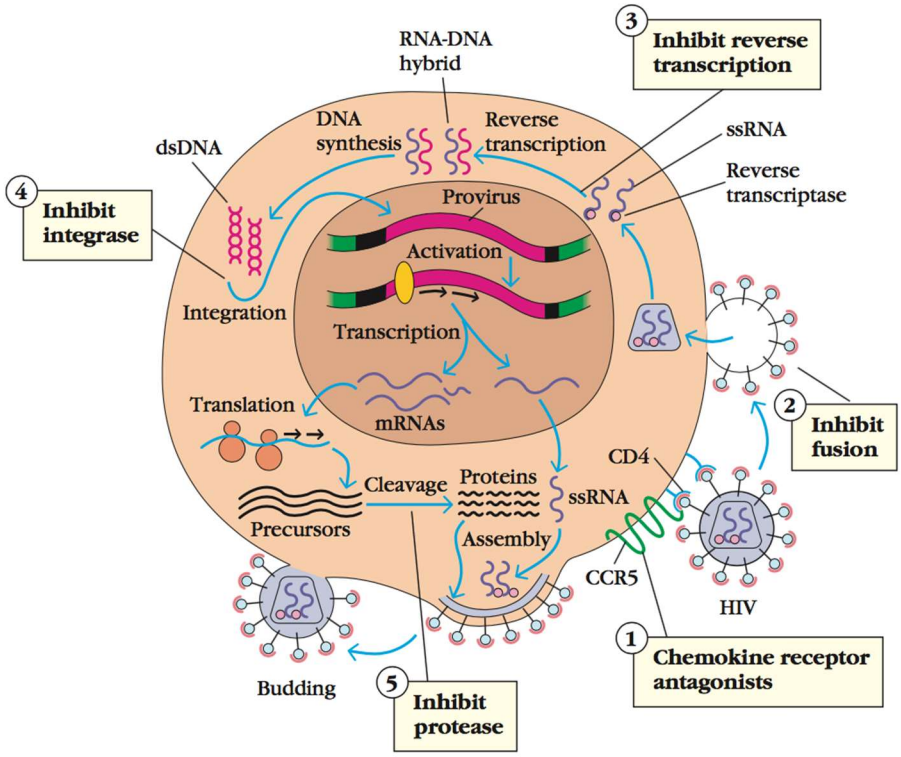


Figure 4. **Stages of the viral life cycle which may provide targets for HIV therapy**

As demonstrated in the figure above, there are five different points to target HIV activity: (1) the first, to interfere with co-receptor attachment; (2) the second, to inhibit fusion with the cell membrane; (3) the third, through medication that can interfere with reverse transcription of viral RNA to cDNA; (4) the fourth, through drugs that interfere with viral integrase, necessary for generation of the pro-virus; (5) finally, through medicines that block the viral protease that cleaves precursor proteins into the peptides needed to assemble new virions. Originally published in (Owen, Punt et al. 2013) and reproduced with permission from the publisher.

1.1.9 Consequences of chronic infection

Decreased numbers of CD4+ T cells

A hallmark of HIV infection is the depletion of CD4+ T cells. The mechanism by which CD4+ T cell numbers are decreased has been investigated extensively, revealing two reduction mechanisms: (i) directly, by the virus itself, and (ii) indirectly by the non-specific

response to infection and immune activation (Douek, Picker et al. 2003, Ford, Puronen et al. 2009).

Lymphoid tissue pathology

Persistent antigen stimulation and inflammation lead to enlargement of the lymphoid tissues, ultimately changing the architecture of the lymph nodes (Orenstein, Feinberg et al. 1999, Biancotto, Grivel et al. 2007, van Grevenynghe, Halwani et al. 2008). Chronic activation also promotes the deposition of collagen in lymphoid tissues, disrupting their fibro-reticular network (Ulrickson, Press et al. 2012). These effects reduce proliferation of naïve T cells by lowering access to IL-7 and by leading to both apoptosis and to the depletion of T cells (Gannon, Khan et al. 2011).

Lymphoma associated with HIV

Importantly, HIV *per se* is not known to be a cancer-causing virus, but it has been shown to facilitate the development of lymphoma through the indirect mechanisms of chronic infection, aberrant immune activation, inflammation, and immunodeficiency (Adle-Biassette, Chretien et al. 1999). Furthermore, HIV can reactivate other directly oncogenic viruses, such as Epstein-Barr virus and Human Herpes-virus 8. Fortunately, cART leads to a significant decrease in the incidence of lymphoma linked to HIV infection (Bonnet and Cheˆne 2008).

HIV and neurological problems

HIV is associated with minor to severe neurocognitive disorders (Heaton, Franklin et al. 2011, Zanni and Grinspoon 2012). Local chronic infection of the neural tissues results in ongoing activation of the macrophages and microglia, which can lead to severe neurological disorders (Cota-Gomez, Flores et al. 2002, Heaton, Franklin et al. 2011).

HIV and cardiovascular disease (CVD)

The incidence of CVD is significantly increased in HIV-infected individuals and is one of the main causes of death among these patients. Moreover, such individuals have also demonstrated an increase in the traditional factors that predispose to CVD, such as dyslipidemia, diabetes, hypertension, and central obesity (Freiberg, Chang et al. 2013). Immune activation stimulates the endothelial cells of the coronary artery to produce more chemokines and adhesion molecules, which facilitate the development of atherosclerosis (Woollard, Haqshenas et al. 2008). The Tat proteins can also upregulate the expression of adhesion molecules (Funderburg, Mayne et al. 2010). Furthermore, the incidence of acute myocardial infarction is 50% higher in HIV-infected patients (Giralt, Domingo et al. 2011).

HIV and thrombosis

Ongoing infection leads to an imbalance between pro- and anti-coagulation factors, which results in the increased incidence of thrombosis. Moreover, another study has shown that monocytes of HIV infected individuals upregulate the expression of pro-coagulating agents (Atta 2010).

Diabetes mellitus

Chronic infection with HIV is associated with lipodystrophy, which involves the redistribution of adipose tissue, mitochondrial dysfunction, and increased lipolysis due to apoptosis of adipocytes (Barbaro and Iacobellis 2009, Caron-Debarle, Lagathu et al. 2010). Consequently, adipokine secretion is altered, resulting in the release of pro-inflammatory cytokines and free fatty acids, which exacerbate chronic inflammation, dyslipidemia, and insulin resistance (Vescini, Cozzi-Lepri et al. 2011). Additionally, HIV patients receiving antiretroviral treatment, particularly protease inhibitors, are likely to become insulin resistant (Aboud, Elgalib et al. 2007).

Renal and hepatic complications

HIV infection, itself, can cause renal disorders (Glassock, Cohen et al. 1990); and hepatic cells are a direct target of HIV infection, leading to chronic liver inflammation. Furthermore, kidney and liver problems are associated with the use of antiviral therapies (Palella Jr, Baker et al. 2006, Deeks and Phillips 2009). Renal disease might affect vitamin D metabolism, resulting in reduced activation of vitamin D, which may then lead to bone disorders (Borderi, Gibellini et al. 2009).

HIV and gastrointestinal tract

Gut-associated lymphoid tissue (GALT) is a site of massive HIV replication in infected patients (Nilsson, Kinloch-de-Loes et al. 2007, Chun, Nickle et al. 2008). Replication of the virus is accompanied by a drastic depletion of CD4+ T cells in the gut, leading to increased intestinal permeability (Brenchley and Douek 2008). Consequently, bacterial products such as lipopolysaccharide (LPS), bacterial fragments, and even intact bacteria can gain access to the intestinal tissue and systemic circulation (Canny and McCormick 2008), causing intense activation of the immune system. This immune activation further enhances viral replication. HIV-associated intestinal pathology is a common finding in HIV-infected individuals, especially during later stages of the infection. Increased intestinal permeability, microbial translocation, and enteropathy are also seen in the simian model of SIV infection (Brenchley, Price et al. 2006, Marchetti, Tincati et al. 2013). It is evident that persistent immune activation and inflammation are two fundamental driving forces in the loss of CD4 T cells, and in the progression to AIDS and other HIV-related complications (Brenchley and Douek 2008).

Therefore, new immunotherapeutic strategies are needed to seek a cure or, at least, to normalize the immune status (Lafeuillade 2011, Lewin and Rouzioux 2011, Deeks, Autran et al. 2012). Two suggested approaches to achieve this normalization of the immune system are described here. The first approach suggests a sterilizing cure by eradicating the virus. This proposed strategy is based upon observations following the transplantation of CD34+ hematopoietic stem cells into a so-called ‘Berlin’ patient from a donor homozygous for the

CCR5 Δ32 gene (Allers and Schneider 2015). In this situation, the HIV-infected patient was also suffering from leukemia. Transplantation resulted in the apparent eradication of HIV, as the virus was no longer detectable in the blood of the patient, without use of any anti-retroviral therapy (Palella, Delaney et al. 1998). A second approach is known as a functional cure. The purpose of this approach is not to eliminate the virus, but rather to develop active host immunity to keep viral replication under control so that it does not cause any pathology. The concept of this approach came from studies of elite controllers and long-term non-progressors (LTNP) who were infected with HIV and who continued to show an effective anti-viral response, remaining healthy for more than seven years and maintaining undetectable viral loads and normal CD4+ T cell counts, without taking any anti-retroviral therapy (Chomont, El-Far et al. 2009).

1.1.10 Immune activation increases susceptibility to HIV infection

It is well known that sexually transmitted infections (STIs), which lead to micro-lesions caused by the pathogen and to recruitment of activated immune cells to the site of the infection, increase susceptibility to HIV infection (Stoiber, Banki et al. 2008). On the other hand, natural protection against HIV infection in HIV-exposed seronegative sex workers has been linked to a lower level of immune activation and decreased expression of pro-inflammatory cytokines (Kaul, Pettengell et al. 2008, McLaren, Blake Ball et al. 2010). Based on these findings, inducing immune quiescence, thereby decreasing the presence of HIV target cells in the genitourinary tract, has been suggested as a novel concept for the prevention of HIV infection (Lajoie, Mwangi et al. 2017).

1.1.11 Viral reservoirs

In ART-treated individuals, latent infection of a small population of quiescent cells persists and these cells are resistant to anti-viral therapy (Archin, Liberty et al. 2012). These latently infected quiescent cell populations are called ‘viral reservoirs’ (Siliciano and Siliciano 2004, Swiggard, Baytop et al. 2005) and are found in immune privileged sites within the body

called ‘viral sanctuaries’. These reservoirs can reinitiate new rounds of infection if anti-retroviral therapy is interrupted or stopped. These viral reservoirs, pools of potentially active virus, are established within days following primary infection and are unaffected by the antiviral immune response or current treatment regimens (Chun, Garrett et al. 2002). Thus, the complete eradication of HIV through the use of antiretroviral drugs seems like an impossible task because the virus persists, lying dormant in these viral reservoirs (Strain, Little et al. 2005).

The central dynamics of seeding the viral reservoir have not been identified but are thought to occur at peak viremia during acute HIV-1 infection (Cockerham and Deeks 2014). Resting memory CD4+ T cells, especially central and transitional memory CD4+ T cells, are considered to be the primary source of the latent viral genomes. Hence, they represent the main viral reservoirs (von Stockenstrom, Odevall et al. 2015). It is believed that CD4+ T cell depletion is significantly associated with the size of the viral reservoir (Chomont, El-Far et al. 2009). After infection, when HIV RNA becomes detectable, the number of virus particles increases dramatically. As do the viral reservoirs, demonstrating about a 100-fold rise occurring in the subsequent two weeks, and peaking approximately four weeks after initial infection (Fromentin et al., 2016). However, early initiation of HAART is associated with a decrease in HIV reservoirs (Jain, Hartogensis et al. 2013). Several studies have shown that the size of the viral reservoir is strongly associated with the low-grade immune activation that persists during ART, suggesting that HIV persistence and residual inflammation are unlinked (An and Winkler 2010). Interestingly, one study reinforced the idea of memory CD4 T cell proliferation as the mechanism of viral persistence in the blood and lymphoid tissues (Chun, Carruth et al. 1997, Finzi, Blankson et al. 1999). This study suggested a connection between HIV persistence and immune checkpoints such as programmed cell death-1 (PD-1), lymphocyte-activation gene 3 (LAG-3), and CTLA-4 (Surh and Sprent 2008). More studies are needed to identify and to validate this connection.

1.1.12 Cytokines and HIV infection

Cytokines are small molecular weight gps that are secreted by cells to communicate with other cells in the body. They also exert biological effects in an autocrine fashion. Dysregulated production of several cytokines has been reported in HIV infection (Catalfamo, Le Saout et al. 2012, Reuter, Pombo et al. 2012). In general, HIV infection causes an increase in the expression of pro-inflammatory cytokines such as TNF-α, IL-6, IL-32, and IL-18, etc. (Kedzierska and Crowe 2001, Torre and Pugliese 2006, Lester, Yao et al. 2008, Nold, Nold-Petry et al. 2008, Desai and Landay 2010). Additionally, there is a decrease in the production of other immune cytokines such as IL-2, IL-21, IL-13, IL-27 and IFN-1 (Kedzierska and Crowe 2001, Iannello, Tremblay et al. 2008). Interestingly, while the production of some cytokines (IL-7, IFN-γ and TGF-β) may be increased, this increased production is often accompanied by resistance to the cytokine. For example, although IL-7 concentrations are usually increased in the tissues and circulation of HIV-infected individuals, the cells do not respond to it. Interestingly, the level of this cytokine was found to have a strong correlation with the depletion of CD4+ T cells (Torheim, Ndhlovu et al. 2009). The cytokine dysregulation observed in HIV infection is very complex and does not involve a simple T helper (TH) 1- to TH2-type cytokine switch. More importantly, dysregulation plays an important role in the pathogenesis of HIV-induced AIDS through several mechanisms:

1. It increases HIV replication by activating cellular transcription factors such as NF-κB, Activator Protein-1 (AP-1), and Activating Transcription Factor-2 (ATF-2), etc. (Copeland 2005);
2. It causes intense immune activation and inflammation in the body, resulting in immunosuppression and metabolic changes, and predisposes infected individuals to opportunistic infections and rare cancers. The γ-chain using cytokines such as IL-7, IL-15, and IL-21, etc., induce expression of PD-1 and programmed cell death ligand-1 (PDL-1) on CD4+ and CD8+ T cells, causing their exhaustion (Boasso, Hardy et al. 2008, Vandergeeten, Fromentin et al. 2012). Exhausted T cells lose their antiviral effector functions. Once PD-1 binds to PDL-1, it induces and upregulates the expression of IL-10, resulting in reduced T cell proliferation (Boasso, Hardy et al. 2008). Furthermore, cytokine

dysregulation also has an effect on differentiation of different TH subsets. For example, an imbalance in TH17/Treg cell ratios in the gut has been reported in HIV-infected individuals (Bettelli, Oukka et al. 2007);

3. Cytokine dysregulation contributes towards the establishment and persistence of viral reservoirs, i.e. the presence of latently infected cells in immune-privileged sites in the body (Vandergeeten, Fromentin et al. 2012). These reservoirs present a major obstacle to developing an effective 'cure' for the infection using powerful anti-inflammatory drugs;

4. Anti-retroviral drugs suppress viral replication to undetectable levels, restoring cytokines to their physiological levels. However, this restoration is not complete. The levels of several pro-inflammatory cytokines remain above or within upper limits of their physiological ranges. Consequently, the virus-infected individuals suffer from chronic low-grade inflammation, which predisposes them to immune-senescence, accelerated aging, metabolic syndrome, and cancers (Pawelec, Goldeck et al. 2014).

Several members of the IL-1 family such as IL-1α, IL-1β, IL-18, and IL-33 have been studied in HIV infection (Catalfamo, Le Saout et al. 2012). Among these cytokines, IL-18 has consistently been shown to be increased in HIV-infection. Increased levels of IL-18 in the circulation of HIV-infected individuals, even after ART, suggests treatment failure (Iannello, Tremblay et al. 2008). Formerly known as IFN-γ inducing factor, IL-18 is produced by an array of immune and non-immune cells in the body in response to microbial infections and stress. IL-18 stimulates IFN-γ production via NK cells and can stimulate the development and differentiation of both TH1- and TH2-type cells in a context dependent fashion (Iannello, Samarani et al. 2009).

There has only been one study investigating the regulation of IL-37 in HIV-infected individuals (Hojen, Rasmussen et al. 2015). It is worth noting that IL-37 is closely related to IL-18 structurally, and that the two cytokines are members of the IL-18 subfamily. Unlike IL-18, IL-37 exerts anti-inflammatory effects on human cells and tissues and attenuates immune

activation. Given that inflammation and immune activation are the main processes that drive disease progression in HIV infection, and that IL-37 could potentially oppose these processes, it would be very important to understand how this anti-inflammatory cytokine is regulated in HIV-infected individuals. In the following section, a brief overview of IL-37 biology is provided.

1.2 IL-37

1.2.1 Discovery of IL-37

IL-37, formally known as interleukin-1 family member 7 (IL-1F7), was discovered *in silico* by three groups in 2000 (Kumar, McDonnell et al. 2000, Nold, Nold-Petry et al. 2010). It is a member of the IL-1 family, which is comprised of pro-inflammatory cytokines IL-1α, IL-1β, IL-18, IL-33, IL-36 α, β, and γ, as well as anti-inflammatory members such as the IL-1R antagonist, IL-36Ra, IL-37, and IL-38 (Garlanda, Dinarello et al. 2013). All members of the IL-1 family share the same β-trefoil structure consisting of 12 β-strands. IL-37 is a member of the IL-1 family due to its function as a broad and general inhibitor of innate immunity. It can reduce activation of and cytokine production in different innate immune cells. IL-37 also exerts immune deviatory, immunosuppressive, and metabolic effects (Chen and Fujita 2015, Dinarello, Nold-Petry et al. 2016). Members of the IL-1 family are known to be produced as precursor proteins, which contain pro-piece/pro-domains of different lengths. The pro-piece is cleaved in the cytoplasm by a protease, which converts the precursor into its biologically active mature form. IL-1F members are divided into three sub-families based on the length of the pro-piece: IL-1, IL-18, and IL-33. The IL-18 subfamily contains IL-18 and IL-37. These two cytokines are closely related to one another in their amino acid sequences and three-dimensional protein structures (Dinarello, Nold-Petry et al. 2016). It is also worth noting that IL-37 is the only gene of the IL-1 family that is represented by a pseudogene in mice. No functional homolog of human IL-37 has yet to be discovered in this species.

1.2.2 Expression of IL-37

IL-37 is expressed in small quantities in different tissues and cells in the body. Tissues in which IL-37 is expressed include the lymph nodes, thymus, bone marrow, intestines, placenta, airways, uterus, testis, prostate, breast, and kidney, etc. (Nold, Nold-Petry et al. 2010, Dinarello, Nold-Petry et al. 2016). In these tissues, IL-37 is expressed in a wide range of cells such as monocytes, activated B cells, plasma cells, DCs, macrophages, epithelial cells, CD4+ regulatory T cells (Tregs), and in keratinocytes (Fonseca-Camarillo, Furuzawa-Carballeda et al. 2015, Shuai, Wei-Min et al. 2015, Dinarello, Nold-Petry et al. 2016). Among those cells, the myeloid (m)DC constitutively secrete IL-37 upon activation, while monocytes mainly store it and release it rapidly upon encountering inflammation (Rudloff, Cho et al. 2017). Despite widespread expression of the IL-37 gene, healthy individuals have less than 100 pg/ml of IL-37 in their circulation. Due to the presence of an instability motif in exon five, the IL-37 mRNA has a short half-life and is rapidly degraded (Bufler, Gamboni-Robertson et al. 2004). Stabilization of this mRNA occurs after deletion of exon 5. Toll-like receptor (TLR) stimulation by specific ligands such as LPS, flagellin, CpG, PAM3CSK4, etc., activates myeloid cells. This activation results in stabilization of the IL-37 mRNA and in increased production of the cytokine. Thus, treatment of human cells with these ligands leads to enhanced production of IL-37 (Bufler, Gamboni-Robertson et al. 2004). Interestingly, TGF-β is a significant inducer of IL-37 in human cells and tissues (Chen and Fujita 2015).

1.2.3 Alternate splice variants and isoforms of IL-37

In humans, like other members of the IL-1 family, the IL-37 gene is located on chromosome 2q12.21 and has six exons as described in Figure 5 (Busfield, Comrack et al. 2000, Taylor, Renshaw et al. 2002). Due to alternate splicing of its pre-mRNA, IL-37 exists in five different isoforms, named IL37a-e. Among these variants, IL-37b is the most studied and most abundant isoform. As illustrated in Figure 5, IL-7b contains five of the six exons that comprise the gene, while exon 3 is spliced out. It is interesting to note that exons 4, 5, and 6 are predicted to form a β-trefoil structure, which is characteristic of the IL-1 family. The isoforms that lack any of these exons, for example isoforms c and e, are predicted to be non-

functional proteins. While each isoform does not have a signal peptide, which is needed for secretion, they do contain a pre-domain encoded in exon 1 and 2. This pre-domain is cleaved by proteases intra- and extra-cellularly (Boraschi, Lucchesi et al. 2011). The only isoform that lacks exon 1 and 2 is IL-37a, but it is also the only isoform that has exon 3. The protein sequences encoded by exon 3 contain the cleavage site for elastase (Boraschi, Lucchesi et al. 2011).

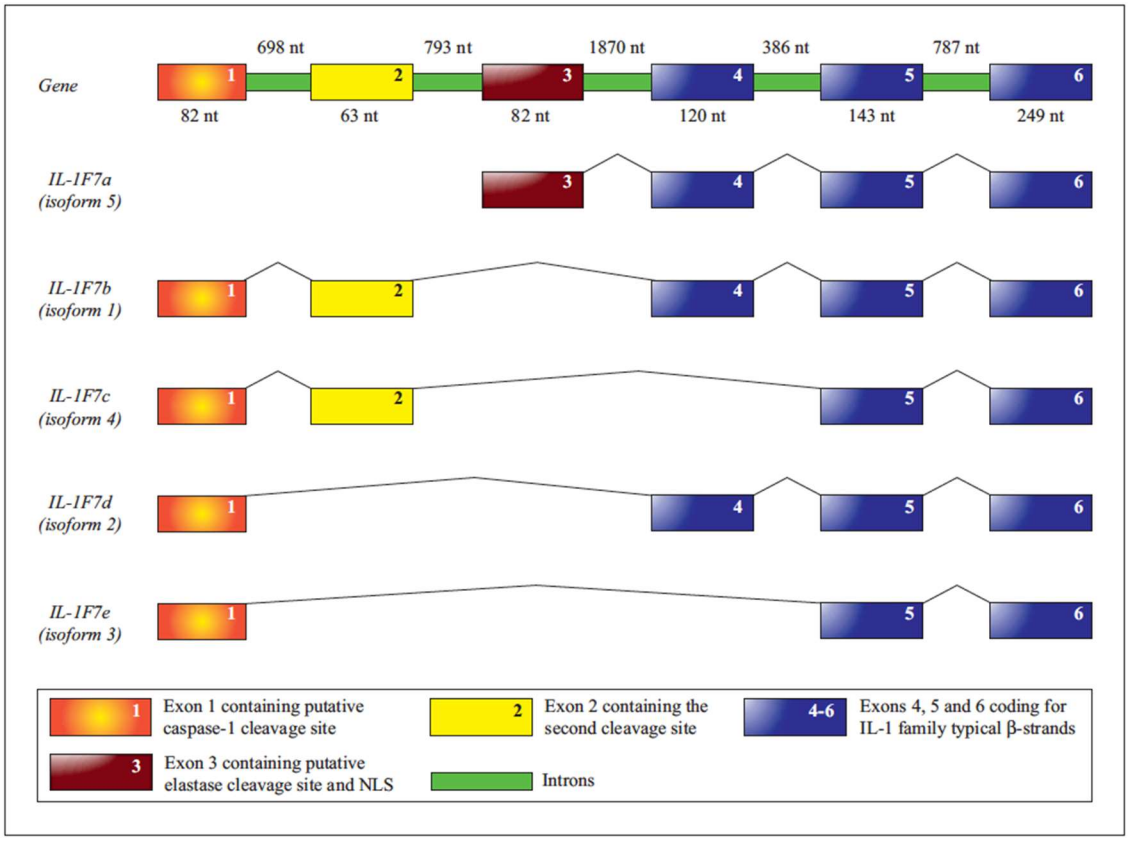


Figure 5. **Gene structure and splice variants (isoforms) of IL-37**

This figure shows the exon-intron structure of the IL-1F7 gene and its different isoforms (a to e). NLS stands for nuclear localization signal. Originally published in (Boraschi, Lucchesi et al. 2011) and reproduced with permission from the publisher.

1.2.4 Processing of IL-37

As described in section 1.2.3 Alternate splice variants and isoforms of IL-37, IL-37 is produced as a precursor protein with no signal peptide. Due to the absence of this signal peptide, the precursor protein cannot enter the endoplasmic reticulum and thus cannot be secreted through the classical secretory pathway.

Caspase-1 and caspase-4 can process the precursor IL-37 into its mature form (Kumar, Hanning et al. 2002). However, caspase-3 cleaves and inactivates it. Although, the IL-37 precursor is biologically active, the mature IL-37 is biologically more active than the precursor form of the cytokine.

1.2.5 Secretion of IL-37

Both the mature and immature (precursor) forms of the cytokine are secreted from the cells through non-classical mechanisms. However, these mechanisms are still not fully understood. The release of the mature form, but not of the precursor variant, requires activation of Caspase-1, which in turn requires assembly of an inflammasome (Bulau, Nold et al. 2014, Dinarello, Nold-Petry et al. 2016). Interestingly, the immature form can be released either upon cell death or loss of membrane integrity. It is worth noting that about a quarter of the cleaved mature form of IL-37 binds with SMAD-3 in the cytoplasm, translocate to the nucleus, and reduce the transcription pro-inflammatory cytokine and chemokine genes (Figure 6) (Sharma, Kulk et al. 2008, Bulau, Nold et al. 2014).

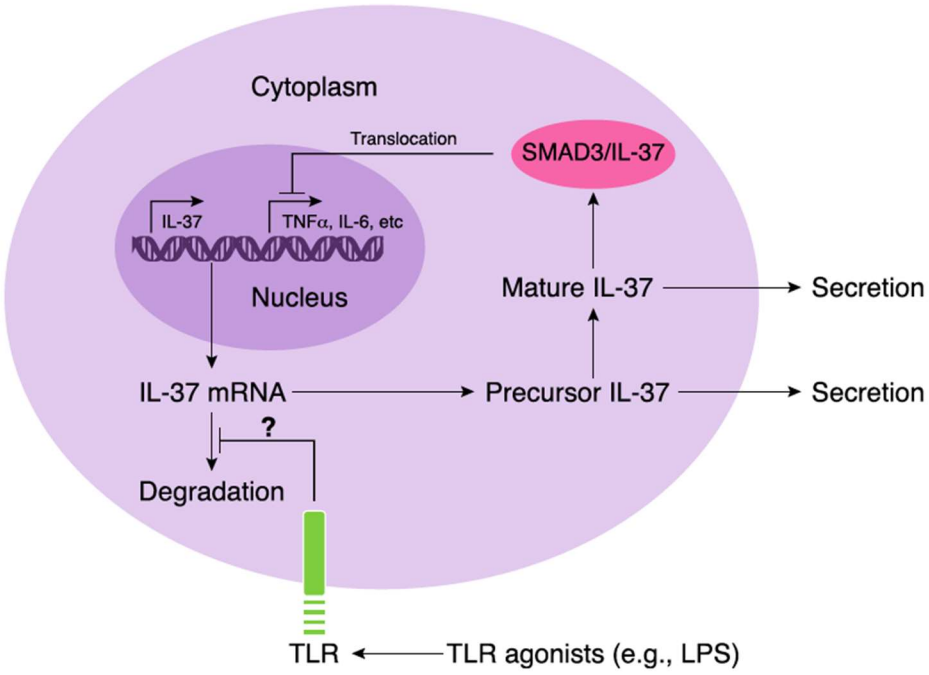


Figure 6. **Production and secretion of IL-37**

Pro-inflammatory stimuli, like those induced by TLR agonists, inhibit the degradation of cytokine mRNA and promote production of the precursor form. The immature cytokine is processed by Caspase-1 and by other proteases inside the cytoplasm. Both the precursor and mature form of IL-37 are secreted from the cell via non-classic secretory pathways. A fraction of the cleaved form binds phosphorylated SMAD-3, which translocates it to the nucleus, and inhibits transcription of the genes of the pro-inflammatory mediators. Originally published in (Abulkhir, Samarani et al. 2017) and reproduced with permission from the publisher.

1.2.6 IL-37 receptor

It is well known that cytokines of the IL-1 family bind to different members of the IL-1 receptor family (IL-1RF). Figure 7 describes the IL-1F members and their specific receptors.

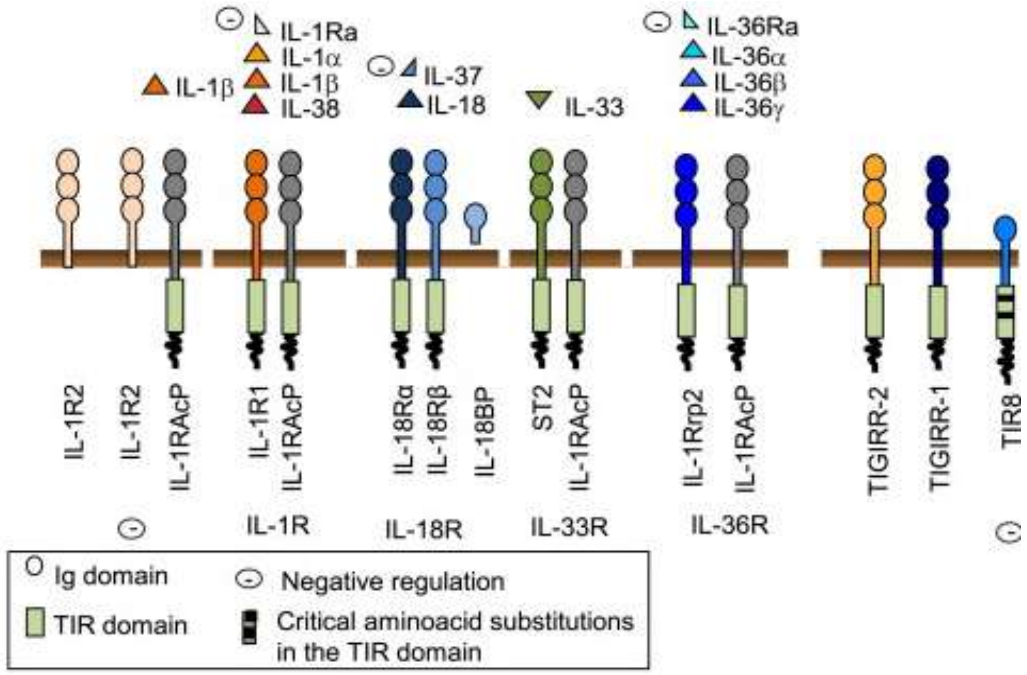


Figure 7. **IL-1 family receptors**

This figure shows the different members of the IL-1FR and their respective ligands. As illustrated above, TIR-8 has a single Ig-like domain in its extra-cellular region and an atypical intracellular TIR domain with a long cytoplasmic tail, giving this receptor a unique structure. As it can be appreciated in this figure, all but two members of the IL-1RF have three Ig domains in the extracellular region. After binding with IL-18Rα, IL-37 recruits TIR-8. Originally published in (Garlanda and Mantovani 2013) and reproduced with permission from the publisher.

A typical IL-1RF usually has three Ig-like domains in the extracellular region, which bind its cytokine. It also has a Toll-IL-1R (TIR) domain in the cytoplasmic region to mediate intracellular signals. IL-37 was shown to bind the IL-18Rα chain, also known as IL-1RF5 (Kumar, Hanning et al. 2002, Boraschi and Tagliabue 2013). Interestingly, IL-18 also binds to IL-18Rα. However, the affinity of IL-18 for IL-18Rα is much higher than that of IL-37 for this chain (Novick, Kim et al. 2013, Nold-Petry, Lo et al. 2015). After binding IL-18Rα, IL-18 recruits the IL-18Rβ chain (IL-1RF7) and exerts its pro-inflammatory functions. On the other hand, when IL-37 binds to IL-18Rα, it does not recruit IL-18Rβ, but instead recruits

another member of the IL-1RF known as TIR-8 (Lunding, Webering et al. 2015, Nold-Petry, Lo et al. 2015). TIR-8 is also known as IL-1R8 and Single Ig and IL-1R Related (SIGIRR).

First identified in 1999, in humans, the TIR-8 gene is located on chromosome 11p15.5 (Thomassen, Renshaw et al. 1999). Surprisingly, in humans, the genes for other members of the IL-1R family are located on chromosome 2. TIR-8 is a unique member of the IL-1RF that has only one Ig domain in the extracellular region and a long cytoplasmic tail that contains an atypical TIR domain (Thomassen, Renshaw et al. 1999).

TIR-8 is expressed in a broad range of tissues and cells in the human body, which include DC, T cells, B cells, NK cells, neutrophils, macrophages, neurons, astrocytes, epithelial cells of the airways, gastro-intestinal tract, and renal tube, etc. (Molgora, Barajon et al. 2016). It is worth noting that the inflammatory stimuli that stabilize mRNA and enhance the production of IL-37 also reduce the expression of TIR-8 on human and murine cells by preventing the binding of specificity protein-1 to the TIR-8 promoter (Ueno-Shuto, Kato et al. 2014).

The expression of TIR-8 in human tissues and cells plays an important physiological role. For instance, its downregulation via TLR-2 activation in microglia has been implicated in the development of Alzheimer disease (Costello, Carney et al. 2015). On the other hand, its increased expression in DC causes tolerance to gut-dwelling bacteria (Davies, MacSharry et al. 2010).

1.2.7 IL-37-mediated signaling pathways

IL-37 is a major anti-inflammatory cytokine of the IL-1 family. There are two mechanisms of its unique functions and they are shown in Figure 8, below. The first mechanism involves binding of the secreted IL-37 to its receptor IL-18Rα/SIGIRR (Lunding, Webering et al. 2015, Nold-Petry, Lo et al. 2015, Dinarello, Nold-Petry et al. 2016). Although both mature and immature forms of the cytokine are biologically active, the mature form binds to its receptor more efficiently than the full-length precursor form (Nold-Petry, Lo et al. 2015). The second mechanism involves binding of the cleaved IL-37 to SMAD-3 in the cytosol and

its subsequent translocation to the nucleus. The complex then inhibits transcription of many pro-inflammatory genes (Shi and Massague 2003, Nold-Petry, Lo et al. 2015). Interestingly, the mutant form of IL-37 cannot be cleaved by Caspase-1, and thus does not bind SMAD-3 or translocate to the nucleus (Sharma, Kulk et al. 2008). Both signaling pathways work in parallel and cause the anti-inflammatory effects of IL-37. IL-37 activates the MER-PTEN-FOXO-STAT-3 and Dok-1 pathway; however, it down regulates Fyn and TAK-1 kinases upstream of NF-kB and MAPK. The DOK family members serve as negative regulators of immunoreceptor-mediated activating signaling (Mashima, Hishida et al. 2009). The cytokine's metabolic effects include inhibition of mTOR and activation of AMPK. Overall, the cytokine induces pseudo-starvation (Zeng, Dang et al. 2016). It inhibits TLR and IL-1RF-mediated signaling, as well as the assembly of inflammasomes (O'neill and Hardie 2013, Zeng, Dang et al. 2016). *In vitro*, IL-37 inhibits LPS-induced production of pro-inflammatory mediators from macrophages and DC (Nold, Nold-Petry et al. 2010, Boraschi, Lucchesi et al. 2011). IL-37 neutralizing antibodies enhance LPS-induced cytokine production from macrophages (Bulau, Nold et al. 2014). Although, mice lack a functional IL-37 gene, human IL-37 is functional in murine cells. Consequently, IL-37 transgenic mice are hypo-responsive to LPS and are relatively resistant to Con-A-induced hepatitis and collagen-induced arthritis (Quirk and Agrawal 2014).

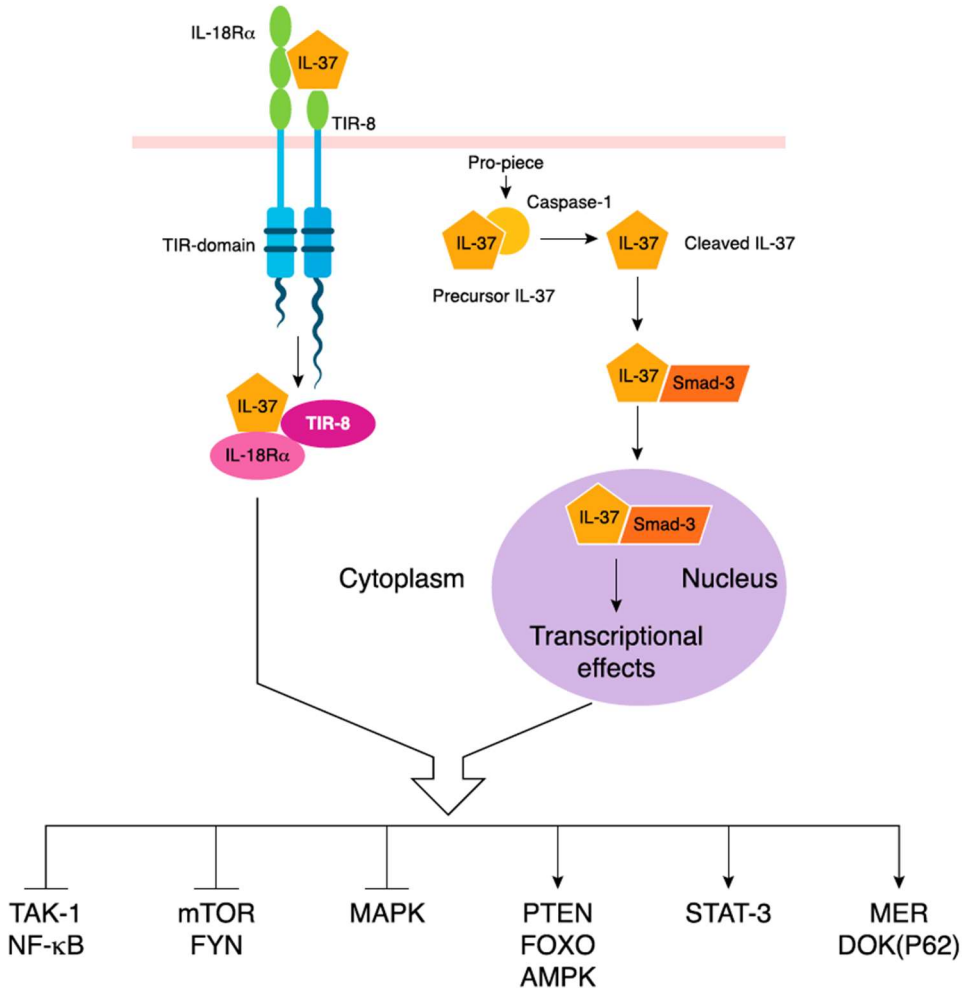


Figure 8. **IL-37-induced signaling pathways**

This figure illustrates the cytokine-induced signaling pathways mediated by (i) the exogenous cytokine via IL-18Ra/SIGIRR complex, and (ii) the mature endogenous cytokine via SMAD-3. The two pathways lead to the anti-inflammatory, immune-deviatory, and metabolism-regulating effects of IL-37. The arrows at the bottom point to enzymes/transcription factors activated by IL-37-mediated signaling. Originally published in (Abulkhir, Samarani et al. 2017) and reproduced with permission from the publisher .

1.2.8 Biological function of IL-37

IL-37 is a negative regulator of innate and adaptive immune responses. The cytokine decreases production of IL-1, IL-6, and TNF-α, etc., from LPS-activated human peripheral blood mononuclear cells (PBMCs) and macrophages (Aggarwal and Pittenger 2005, Quirk and Agrawal 2014). These inhibitory effects were seen upon activation of human and murine cells with other TLR ligands and pro-inflammatory cytokines of the IL-1 family. The cytokine also inhibits assembly and activation of inflammasomes and leads to decreased maturation and release of pro-inflammatory cytokines such as IL-1β and IL-18 (Zeng, Dang et al. 2016). On the other hand, downregulating IL-37 synthesis causes increased production of several pro-inflammatory cytokines such as IL-1R antagonist, IL-1α, IL-1β, IL-6, IL-12, IL-17, IL-18, IL-23, G-CSF, GM-CSF, and TNF-α, etc., and chemokines such as MIP-2/CXCL-2, BLC/CXCL-13 and IL-8/CXCL-8, etc. (Sharma, Kulk et al. 2008, Zeng, Dang et al. 2016). Also, worth noting is that, depending on cellular context, IL-37 has either minimal or no effect on the production of IL-10 and TGF-β (Godsell, Rudloff et al. 2016, Zeng, Dang et al. 2016). Moreover, Il-37 inhibits immune responses and attenuates development and differentiation of TH1, TH2, and TH17 cells (Garlanda, Anders et al. 2009). Due to the fact that IL-37 is able to enhance antigen-specific T-regs, IL-37 and its receptor play a significant role in peripheral tolerance and in the production of autoimmune diseases (Xu, Zhao et al. 2015). The IL-37/TIR-8 complex plays two critical roles in bacterial and fungal infections; on the one hand, it attenuates resistance of the mice to these pathogens, and on the other, it can reduce inflammation and pathology of microbial infections (Blok, Van Lieshout et al. 2014). The absence of the TIR-8 cytokine receptor has been shown to promote inflammatory response in local (e.g., lungs) and systemic infections of mice with *Pseudomonas aeruginosa* (Veliz Rodriguez, Moalli et al. 2012). Interestingly, TIR-8 knock-out mice experience exacerbated responses in the asthma, neuroinflammation, and spinal injury mouse models, suggesting that IL-37 plays a protective role in these diseases (Watson, Costello et al. 2010, Coll-Miro, Francos-Quijorna et al. 2016). In the case of rheumatoid arthritis, IL-37 levels were highly increased compared to healthy controls. Patients with increased disease activity demonstrated significantly higher levels of the cytokine, whereas patients with less severe disease had lower levels (Xu, Zhao et al. 2015). Notably, the cytokine has been shown to inhibit mTOR and

GSK3α/β, while it activates AMPK, which results in pseudo-starvation (Gulen, Kang et al. 2010). In this context, the effects of the cytokine are similar to those of metformin, which means that IL-37 might be useful in metabolic syndrome.

To date, a single study has investigated the expression of IL-37 in HIV-infected individuals (Hojen, Rasmussen et al. 2015). In a cross-sectional study, the authors showed that steady state levels of IL-37 mRNA are significantly increased in PBMCs from virus-infected individuals when compared to healthy controls. Among the infected individuals, cART responders had the highest levels of the cytokine mRNA. Interestingly, the mRNA levels correlated with sCD14 (a marker of inflammatory monocytes) but not with sCD163 (a marker of T cell activation). These two markers have previously been linked to increased morbidity and mortality in HIV infections (Kelesidis, Kendall et al. 2012). Furthermore, the authors showed a strong positive correlation between IL-37 mRNA and HIV DNA in PBMCs. Thus, the mRNA level of IL-37 may be indicative of the size of the entire viral reservoir in the infected host (Hojen, Rasmussen et al. 2015).

2 HYPOTHESIS & OBJECTIVES

2.1 Hypotheses

We hypothesize that production and/or functional activities of IL-37 become dysregulated in HIV infection. Restoring the cytokine's functional activities may attenuate inflammation and immune activation, thereby slowing the progression of HIV infection into AIDS.

2.2 Rationale

As discussed above, IL-37 is an anti-inflammatory cytokine belonging to the IL-1 family. It attenuates pro-inflammatory signaling mediated by TLRs and IL-1R family members. Furthermore, it also inhibits inflammasome assembly, maturation, and release of pro-inflammatory cytokines such as IL-1β and IL-18, etc. Thus, it may play an important role in terminating and resolving the inflammatory response. It is biologically plausible that the cytokine's functional activities are neutralized early in the induction of the inflammatory response and become active in the later phase of the response, when resolution and return to homeostasis is required. The regulation of the cytokine's functional activities in inflammatory diseases such as HIV infection remains under-investigated, since signaling unit of the cytokine's receptor was only very recently identified. Knowledge-based use of this cytokine may help to resolve the inflammation and to attenuate the cellular activation that accompanies viral infection. However, to achieve that end, basic knowledge of the regulation of this cytokine and its biological activities, in the context of this infection, are required. This gap in the research and literature is addressed in this study.

2.3 Main objective

The main objective of this study is to investigate how IL-37 and its receptor, which is required to mediate the cytokine's effector functions, are regulated in HIV infection.

Furthermore, we will also investigate how the cytokine affects viral replication, the virus-induced inflammatory response, and cell activation.

2.4 Specific aims

The specific aims of our study are to:

1. investigate the how the levels of IL-37 differ in HIV-infected individuals compared to healthy controls and to explore how IL-37 affects the expression of HIV-related chemokines and ligands;
2. examine the levels of soluble SIGIRR in the cells of HIV-infected individuals compared to healthy controls and to understand the effects of SIGIRR on IL-37;
3. investigate the effect of exogenous IL-37 on HIV replication and on the expression of CXCR4 in CD4+ T cells;
4. examine the effect of exogenous IL-37 on the expression of PD-1 on CD4+ and CD8+ T cells;
5. identify the IL-37 isoforms in HIV-infected individuals versus healthy control.

3 MATERIALS & METHODS

3.1 Antibodies and reagents

The reagents used in this study included: Recombinant Human IL37B catalog # 7585-IL-025, From R&D Systems, NE, Minneapolis, USA, rabbit anti-human IL-37 polyclonal antibody (catalog# MBS668098 from My BioSource, San Diego, CA, USA), rabbit anti-human IL-1F7 polyclonal IgG antibody (catalog# GTX108419, from GeneTex, Irvin CA, USA). Rat IgG2bκ anti-human CD4 Alexa Fluor 700, clone A161A1, catalog# 357418 from BioLegend. PerCP-eFluor 710-conjugated mouse anti-human CD3, clone OKT3 (IgG2a, kappa) from eBioscince (San Diego, CA, USA) (catalog# 46-0037-42). Mouse IgG1 anti-human CD14 PerCP- eFluor 710, clone 61D3 (IgG1, kappa) and the reference# 46-0149-42 from eBioscince Dx (San Diego, CA, USA). Mouse IgG1 kappa, anti-human CD56 PE-Cy7, clones CMSSB, reference# 25-0567-42 from eBioscince. Mouse IgG2aκ anti-human CD3 APC/CY7, clone HIT3a, catalog# 300317 from BioLegend. Mouse IgG1 κ, 301024 anti-human CD8a, Alex Fluor 488, clone RPA-T8, catalog# 301024 from BioLegend. Mouse IgG1κ 302217anti-human CD19 APC/CY7, clone HIB 19, catalog# 302217 from BioLegend. Mouse IgG2bκ anti-human CD20 PE-Cy7, clone 2H7, catalog# 25-0209-42 from eBioscince. Mouse IgG1 anti-human CD19 eFluor 450, clone HIB19, reference# 48-0199-42 from eBioscince. Mouse IgG2aI, anti-human SIGIRR APC, clone 162201 catalog# FAB990A, from R&D systems. Mouse IgG2a κ anti-human CD184 (CXCR4) APC, clone 12G5, catalog# 306509 from BioLegend. Mouse IgG1, κ, anti-human CD279 (PD-1) Brilliant Violet 421, clone EH12 2H7, catalog# 329919 from BioLegend Live/Dead fixable Aqua dead cell stain kit, reference# L34965 from LifeTechnologies, Eugen, OR, USA; LPS from Sigma-Aldrich lot #011M4008VMissouri, USA; Recombinant Human SIGIRR Lot# 0917R360 from Leinco Technology Fenton, USA.

3.2 Isolation of peripheral blood mononuclear cells (PBMCs)

For *in vitro* experiments, PBMCs were isolated from peripheral venous blood samples collected from healthy volunteers in Heparin-containing collection tubes (Vacutainer, B&D).

Briefly, the blood samples were diluted with an equal volume of Phosphate Buffered Saline (PBS; pH 7.2), and the cells were isolated by centrifugation on Ficoll-Hypaque density gradients (Sigma-Aldrich, Montreal, QC, CA). The buffy coats were obtained and processed as described earlier (Ahmad, Sindhu et al. 2002). PBMCs were washed twice with PBS and re-suspended in the culture medium described below.

3.3 Cell culture

PBMCs and other cells used in this study were cultured using Roswell Park Memorial Institute (RPMI)-1640 culture medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) containing 100 U/mL of penicillin and 100 µg/mL of streptomycin (Thermo Fisher Scientific). The cell cultures were incubated at 37°C in 5% CO_2 and 85% humid atmosphere. The culture medium was changed after every 3-4 days. To obtain phytohemagglutinin (PHA) blasts, PBMCs were cultured in the culture medium for 72 hours in the presence of 10 U/mL of recombinant human IL-2 (Sigma Aldrich) and 10 µg/ml PHA (Sigma-Aldrich).

3.4 Virus preparation

The T-tropic viral strain NL4.3 was prepared as described previously (Iannello et al., 2009). Briefly, the provirus was amplified from the pNL4.3 plasmid using the Midiprep kit (Qiagen, Germantown, MD, USA). One µg of the plasmid DNA was transfected into HEK293T cells, an Adenovirus-transformed human embryonic kidney cell line expressing the SV-40 large T antigen (ATCC, CRL-11268) using polyethylenimine (catalog# 408712 from Sigma-Aldrich) using a standard protocol (Iannello, Samarani et al. 2009). Transfected cells were incubated for 24 hours in the culture medium at 37°C in humidified 5% CO_2 atmosphere. Thereafter, culture supernatants were collected and centrifuged at 14,000*g* for 30 minutes to remove cellular debris. Clarified supernatants were titrated for p24 contents using an ELISA kit from ABL (Rockville, MD, USA). The supernatants contained 98.0 ng/ml of p24. They

were aliquoted and stored at -80°C until use. Similarly obtained culture supernatants from mock-transfected HEK293T cells were used for mock infections.

3.5 *In vitro* infection of cells with HIV-1

PHA blasts were infected *in vitro* with a T-tropic HIV-1 strain (NL4.3), as described in (Iannello, Boulassel et al. 2010). Briefly, $5x10^6$ cells were cultured for two hours at 37°C with 200 µl of the viral preparation (containing 98 ng of p24 per ml) or with the same volume of mock viral preparation in 48-well micro-culture plates. Infected cells were washed twice with culture medium to remove residual virus. To investigate its effects on HIV replication, recombinant human IL-37 (5 ng/ml) was added to the cell cultures at the time of addition of the viral preparations. The cells were cultured at 37°C for 24 hours, after which culture supernatants were collected and their p24 contents were determined using an ELISA kit (ABL, Rockville, MD, USA).

3.6 Measuring IL-37 and SIGIRR concentrations

The concentration of IL-37 in serum samples was determined using a commercial ELISA kit (Ebioscience catalog# 88-52103) this kit to detect polyclonal IL-37. The lower detection limit of the kit was 31.3 pg/ml. For determining soluble SIGIRR concentrations in the serum samples, a commercial kit (My BioSource catalog# MBS9318786) was used as per manufacturer's recommendations. The detection range for the kit was 31.2 pg/ml-1000.0 pg/ml.

3.7 Measuring TNF-α concentrations

TNF-α levels were determined from culture supernatants of THP-1 cells after stimulation with LPS (10 µg per ml) in the presence or absence of soluble SIGIRR. For these determinations, a commercial ELISA kit from BioLegend (catalog# 430204) was used. The lower detection limit of the kit was 2.0 pg/ml.

3.8 Flow cytometry

Expressions of SIGIRR, CCR4, PD-1, and PDL-1 in different subsets of PBMCs were determined using multicolor flow cytometry. First, cells were treated with FcR blocker (eBioscince, catalog# 14-9161-73). After washing with PBS containing 0.5% FBS, cells were incubated on ice for 30 minutes with marker-specific primary antibodies. After washing, the cells were stained with Fluorochrome-conjugated secondary antibodies. Stained cells were then washed with PBS containing 0.5% bovine serum albumin (BSA) and stained with different fluorochrome-conjugated anti-human CD3, CD4, CD8, CD14, CD19, and CD56 for gating CD3+CD4+ T cells, CD3+CD8+ T cells, CD3-CD14+ monocytes, CD3-CD19+ B cells, and CD3-CD56+ NK cells. The stained cells were treated with Aqua-Blue (reference# L34965) purchased from Life Technologies (Eugen, OR, USA) to distinguish between live and dead cells. Finally, cells were washed with PBS and re-suspended in 2% paraformaldehyde (PFA). Cells were then analyzed using the BD-LSR Fortessa (BD Bioscience, San Jose, CA). Data were acquired using FACS Diva (BD Bioscience, San Jose, CA) and analyzed using FlowJo (Treestar, OR, USA) software.

3.9 Qualitative PCR

The different isoforms of IL-37 and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control were determined using Real-Time Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using Trizol reagent (Life Technology Catalog# 15596026) following the manufacturer's recommendations. Then cDNA was synthesized by reverse transcription using a QIAGEN One Step RT-PCR kit (Qiagen, Valencia, CA, USA, Catalog# 210210). RT-PCR was performed using QIAGEN PCR Master. The cDNA from the transcripts was amplified by PCR using published gene-specific primers (Table 1) . The cDNA sequences were retrieved from the NCBI database and designed using Oligo Primer Analysis 4.0 software. We also performed RT-PCR for the transcripts of GAPDH genes as positive and negative controls on cDNA from PBMCs, since the transcripts for this gene are present in human PBMCs. A volume of 5-10 µl of each PCR reaction was analyzed by electrophoresis on 1% agarose gel, looking for the bands of expected DNA fragment size. The

DNA bands were revealed by ethidium bromide and photographed using the Gene Genius Bio-Imaging System (Syngene, CA, USA).

Table 1. The sequences of the primers used in RT-PCR to identify IL-37 isoforms

Genes and splice variants		Sequences of primers
IL37A	sense	5′-ATGTCAGGCTGTGATAGGAG-3′
	antisense	5′-TTAGTGAGCAGGTTTGGTGT-3′
IL37B	sense	5′-CTCTGCGGAGAAAGGAAGT-3′
	antisense	5′-GCTGAAGGGATGGATGAC-3′
IL37C	sense	5′-GCCCAAGCCTCCCCACCAT-3′
	antisense	5′-GAGCCGCCGACTCCAGCAT-3′
IL37D	sense	5′-AATGGGCTCTGAGGACTG-3′
	antisense	5′-GCTGAAGGGATGGATGAC-3′
IL37E	sense	5′-CTTCATTCCATTTCTGTTG-3′
	antisense	5′-CTCTGGGCGTATGTAGTT-3′
GAPDH	sense	5′-CGGGAAACTGTGGCGTGAT-3′
	antisense	5′-A AAGGTGGAGGAGTGGGTG-3′

3.10 Study participants

The study was conducted using biological samples from a cross section of 79 HIV-infected individuals, 33 Long Term Non-Progressors (LTNP) and 42 HIV-seronegative healthy volunteers. LTNP were defined as HIV-infected individuals who had been infected with the virus for 7 or more years, were not receiving any anti-retroviral treatment (ART) and were free from any AIDS-defining condition. Of the HIV-infected individuals, 37 had not received and 42 had been receiving ART, which consisted of different regimens that included ≥1 nucleoside and/or nonnucleoside reverse-transcriptase inhibitor in combination with 1 or 2 protease inhibitors or the CCR-5 antagonist.

For IL-37 measurements, we used serum from 26 individuals with primary HIV infection (PHI; defined as being within 6 months from the date of infection), 42 patients with chronic HIV infection (CI; having the infection for more than 6 months and not treated with ART), 22 patients with chronic HIV infection receiving ART, 22 patients with chronical infection who were naïve to ART; while for SIGIRR measurements, we used 36 patients with primary HIV infection, 21 patients on ART, 15 PHI non received ART, 33 patients as LTNP (infected for more than 7 years without receiving ART and not having any AIDS-defining condition), and 42 HIV seronegative healthy controls. Concentrations of soluble SIGIRR were determined in the sera of 11 CI patients treated with ART, 17 CI patients not receiving ART, 21 PHI receiving ART, 15 PHI patients not receiving ART, 6 LTNPs, and in 7 healthy control donors.

Table 2. Demographic and clinical parameters of the study participants

Category (numbers)	Age Range	CD+4 Counts Range	CD+8 Counts Range	VL (log10) Range
PHI ART- (15)	22-44	380-1037 (402)	520-2510 (980)	3.50-4.275 (4.222)
PHI ART+ (21)	28-57	240-900 (367.5)	360-1080 (519)	1.60-3.41 (2.10)
CI ART- (22)	22-56	201-1037 (430)	480-2180 (760)	4.376-5.684 (5.189)
CI ART+ (20)	22-56	400-780 (710)	360-1080 (1065)	1.602-2.822 (1.65)
LTNP (33)	-	-	-	ND

PHI: Primary HIV infection, CI: Chronic HIV infection, CD4+, and CD8+ counts represent counts of the respective T cells per mm^3, VL represents copies of HIV RNA per ml in Log10, ND: Non-detectable. A dash (-) means unknown.

3.11 Statistical analysis

Non-parametric tests were used to compare differences between groups. Two groups were compared using Mann-Whitney U test and multiple groups were compared using a one-way non-parametric ANOVA (Kruskal-Wallis test) followed by post-hoc comparisons. Analyses were performed using SPSS (IBM, Chicago, IL, USA). Differences were deemed significant at $p \leq 0.05$.

3.12 Ethical statement

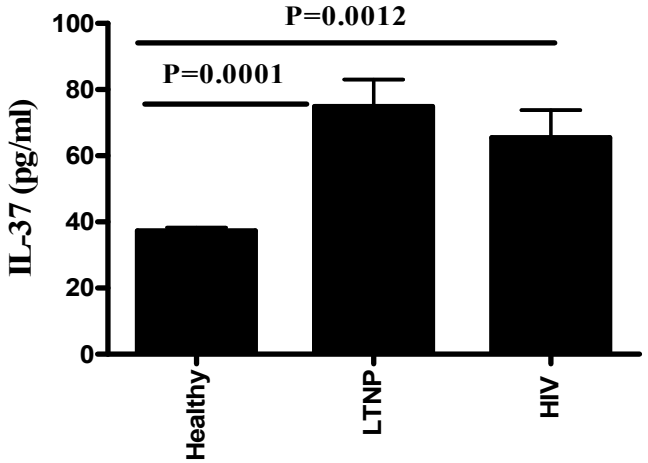
The research involving human participants was approved by the Institutional Ethics Committee (CER; Committee for Ethics in Research of the CHU Sainte-Justine, Montreal, Québec, Canada). The clinical investigations were conducted following the principles of the Declaration of Helsinki. Written informed consent was obtained from all study participants. The samples were collected after obtaining written informed consent from the study participants.

4 RESULTS

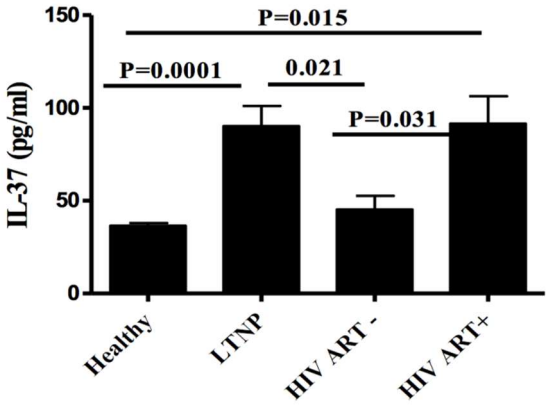
4.1 Levels of IL-37 in HIV-infected individuals

We determined protein concentrations of the IL-37 by using a commercial ELISA kit, as described in the Materials & Methods. Our results show that the concentrations of IL-37 show a significant increase (p=0.0001; Figure 9A) in LTNPs then followed by HIV- infected individuals where is the p=0.0012 as compared with those in healthy donors. Furthermore, a significant difference was observed in cART-treated vs treatment-naïve patients (Figure 9B). Treated patients showed significantly higher concentrations compared with those from the treatment-naïve patients (p=0.031), as well as from healthy controls (p=0.015; Figure 9B). However, when primary infection and chronic infection patients were further stratified with respect to ART (Figure 9C), those receiving the treatment had significantly higher (PHI ART+: p=0.046, CI ART+: p=0.05) concentrations of the cytokine in their serum samples. Serum concentrations in treatment-naïve patients were not significantly different (p>0.05) from those of healthy donors.

A



B



C

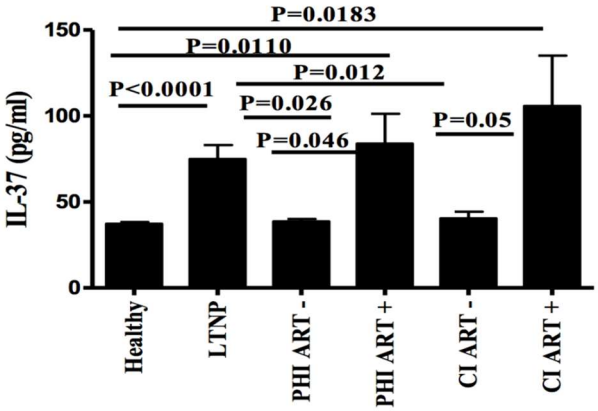


Figure 9. **Concentration of IL-37 in the circulation of HIV-infected individuals**

IL-37 concentrations were measured using a commercial IL-37 ELISA kit. The number of donors in each category included: healthy controls (HC; 42), long-term non-progressors (LTNP; 33), chronically infected anti-retroviral therapy+ (ART+; 22), chronically infected anti-retroviral therapy- (ART-; 22), primary infection without ART (PHI ART-; 15) and primary HIV infection with ART (PHI ART+; 21). This figure depicts mean ± SE. Data were analyzed using one-way ANOVA and means were compared with Tukey's post-hoc tests. The figure panels show the concentrations of IL-37 with and without stratification of the patients with respect to cART and type of the infection (chronic vs acute).

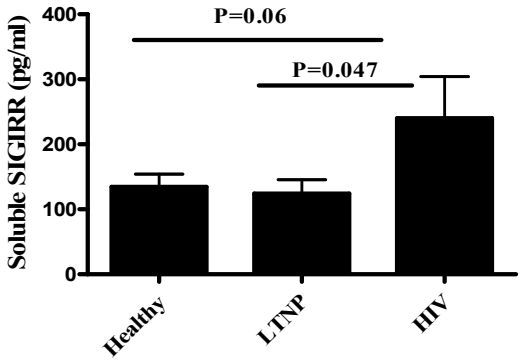
A. Shows the concentrations of IL-37 in healthy, HIV-infected patients, and in LTNP.
B. Illustrates the difference between healthy, LTNP, HIV ART+ (treated) and HIV ART- (non-treated) individuals.

C. Describes the difference between healthy, LTNP, Primary Infection non-treated and treated, as well as Chronic Infection non-treated and treated patients.

4.2 Levels of soluble SIGIRR in HIV-infected individuals

Many cell surface-expressed receptors (e.g., CD16) are known to be cleaved by matrix metalloproteases (MMP) and shed in the circulation (Romee, Foley et al. 2013). Since the expression of SIGIRR was reduced on the surface of different immune cells in HIV-infected individuals, we investigated whether the co-receptor was shed in the circulation of HIV-infected individuals. Therefore, we determined the concentration of SIGIRR in the circulation of HIV-infected individuals using a commercial ELISA kit. As shown in Figure 10A the concentrations of soluble SIGIRR increased significantly in the circulation of HIV-infected individuals compared with those in LTNPs (p=0.047) but not in healthy controls (p=0.06). When stratified with respect to cART (Figure 10B and C), concentrations were significantly increased in the cART-treated individuals compared with those of treatment naïve individuals.

A



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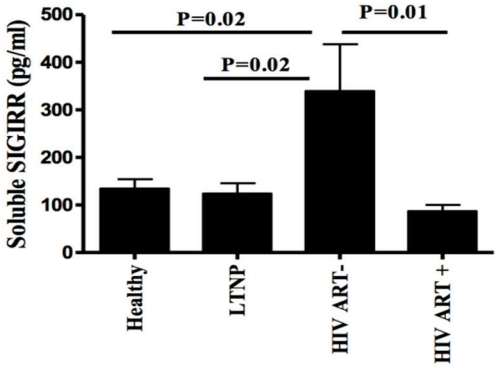

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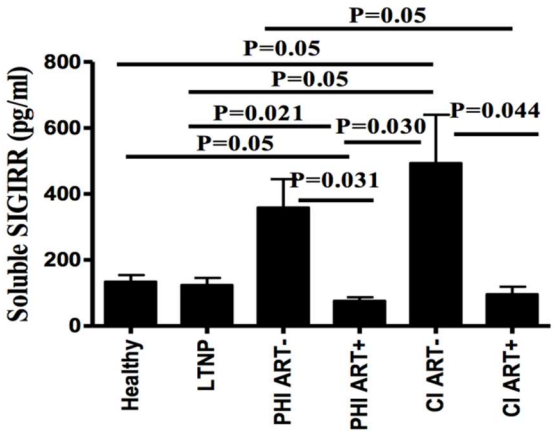


Figure 10. **Comparison of SIGIRR concentrations between ART+ and ART- HIV infected individuals**

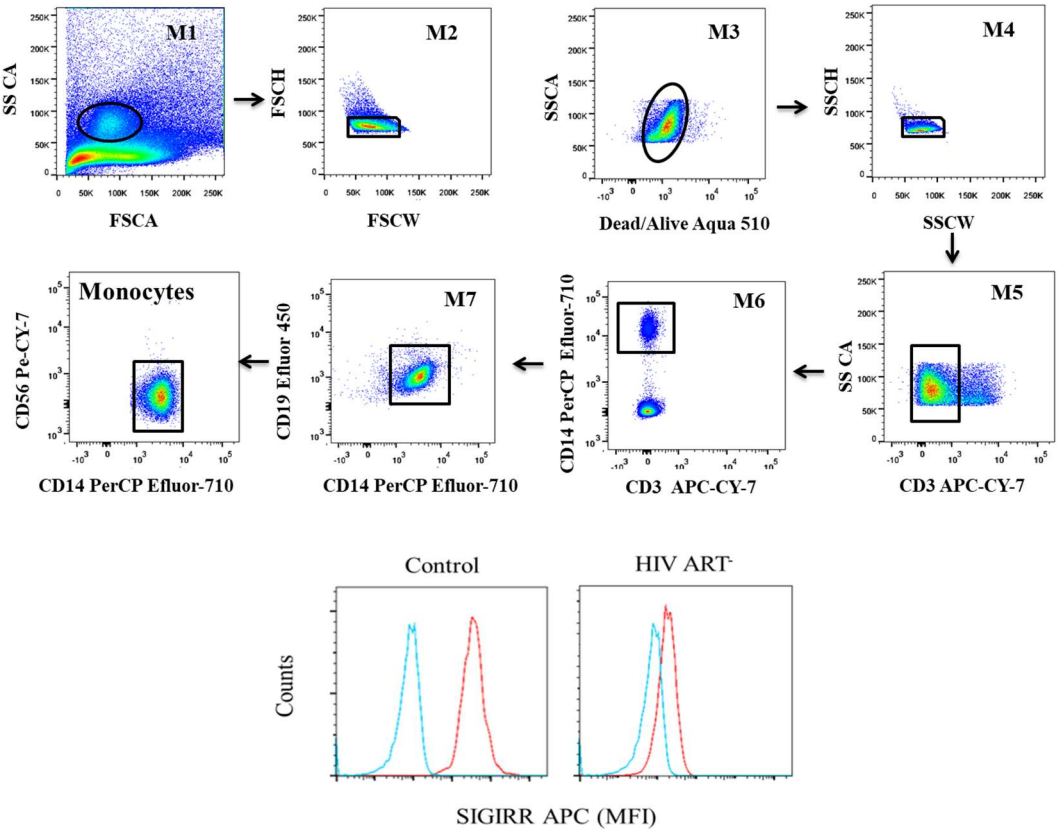
SIGIRR concentrations were measured using a commercial ELISA kit. Concentrations of soluble SIGIRR (mean ± SE) in the sera of healthy controls (HC; n= 7), long-term non-progressors (LTNP; n= 6), chronically infected anti-retroviral therapy+ (CI ART+; n= 11), chronically infected anti-retroviral therapy- (CHI ART-; n= 17), primary infection without ART (PHI ART-; n= 15) and primary HIV infection with ART (PHI ART+; n=21). One-way ANOVA. The results show that concentrations of soluble SIGIRR increase in the circulation of HIV-infected individuals.

A. Shows that soluble SIGIRR is high in HIV-infected individuals compared to LTNP and healthy individuals.
B. Shows that patients who are ART- have the highest level of soluble SIGIRR.
C. Shows that CI individuals who are ART- have the highest level of soluble SIGIRR; higher than PHI ART-, while treated individuals have the lowest level of soluble SIGIRR.

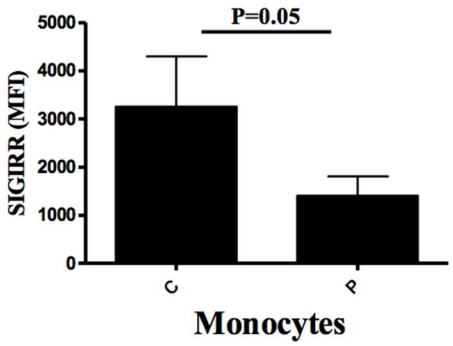
4.3 Expression of SIGIRR in HIV-infected individuals

As SIGIRR acts as the signaling subunit for IL-37 receptor, we investigated its expression on the surface of PBMCs using flow cytometry. The expression was determined on different cell types using specific antibodies. The results shown below demonstrate that the expression of SIGIRR is significantly downregulated on the surface of monocytes (Figure 11B; p=0.05), NK cells (Figure 11F; p=0.012), and B cells (Figure 11F; p=0.037) in treatment-naïve HIV-infected individuals. The expression also tended to be downregulated on CD4+ and CD8+ T cells, but the differences were not significant (p>0.05) compared with that on the PBMC of healthy controls. Interestingly, ART tended to increase the expression in HIV-infected individuals.

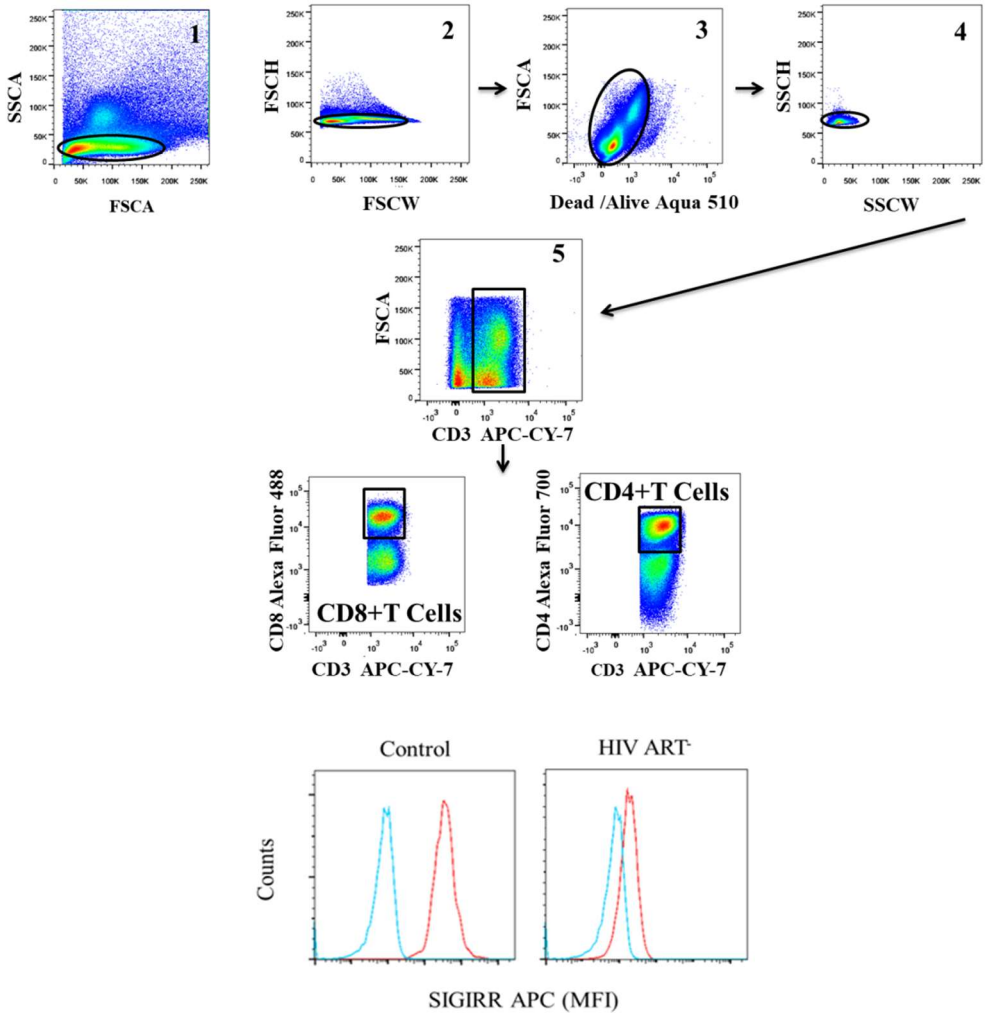
A



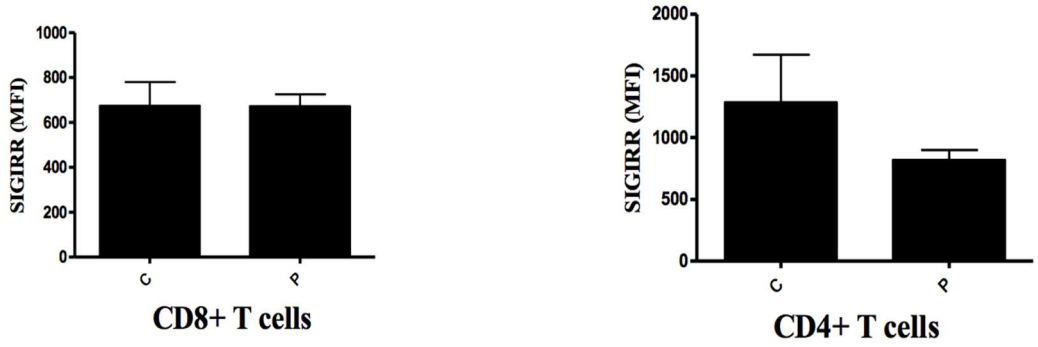
B


P=0.05
SIGIRR (MFI)
5000
4000
3000
2000
1000
0
C
P
Monocytes

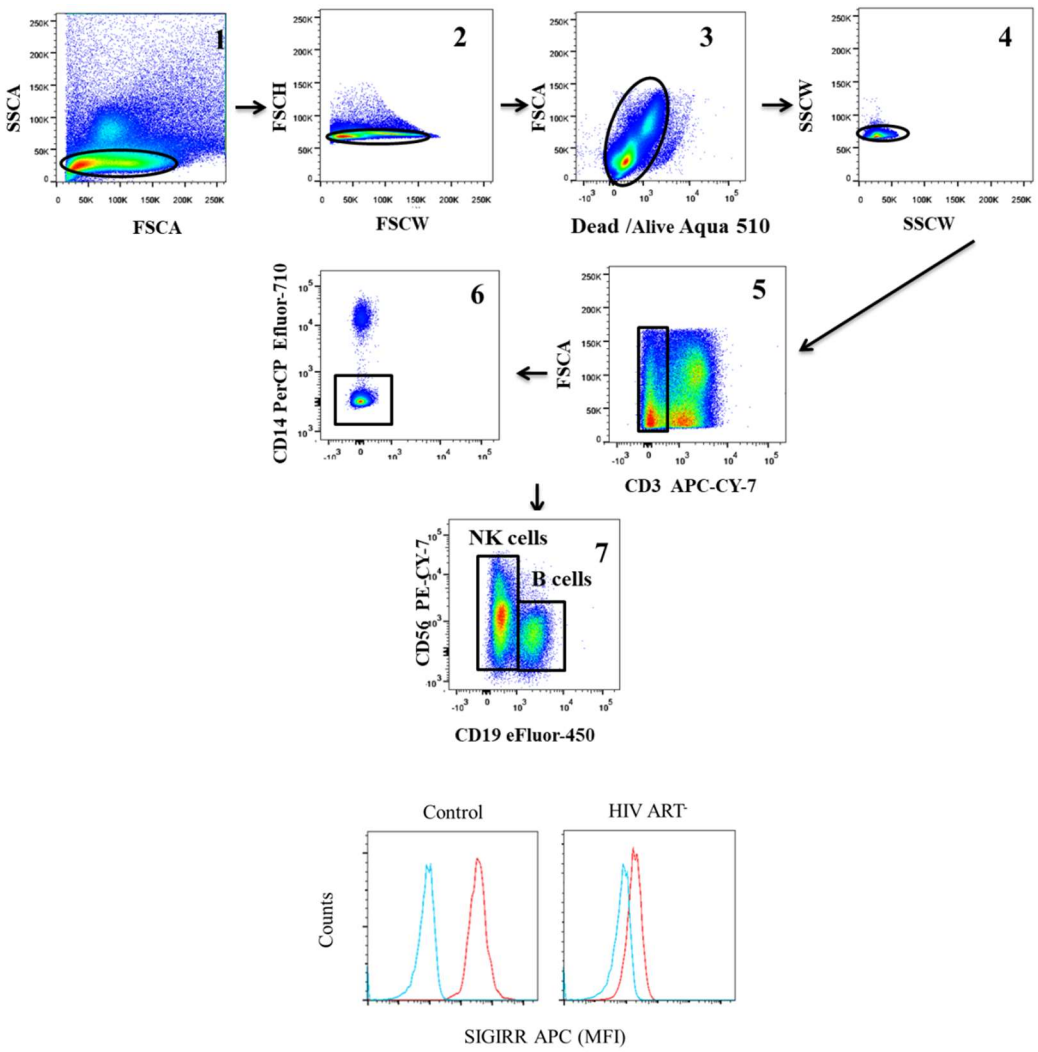
C


1
SSCA
FSCA
2
FSCH
FSCW
3
FSCA
Dead /Alive Aqua 510
4
SSCH
SSCW
5
FSCA
CD3 APC-CY-7
CD8 Alexa Fluor 488
CD8+T Cells
CD3 APC-CY-7
CD4 Alexa Fluor 700
CD4+T Cells
CD3 APC-CY-7
Control
HIV ART-
Counts
SIGIRR APC (MFI)

D


SIGIRR (MFI)
1000
800
600
400
200
0
C
P
CD8+ T cells
SIGIRR (MFI)
2000
1500
1000
500
0
C
P
CD4+ T cells

E


1
SSCA
FSCA
2
FSCH
FSCW
3
FSCA
Dead /Alive Aqua 510
4
SSCW
SSCW
5
FSCA
CD3 APC-CY-7
6
CD14 PerCP Efluor-710
7
NK cells
B cells
CD56 PE-CY-7
CD19 eFluor-450
Control
HIV ART-
Counts
SIGIRR APC (MFI)

F

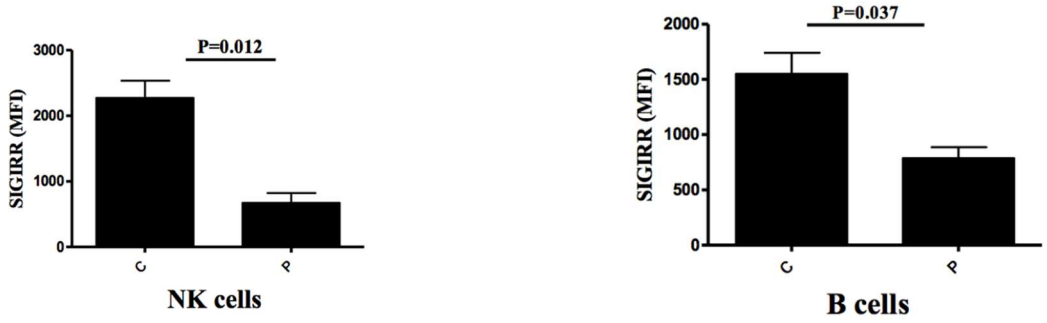


Figure 11. **Expression of SIGIRR on different immune cells**

A. The panels show the strategy used to gate different types of cells from healthy controls and HIV-infected individuals. Lymphocytes were gated by FSCA and SSCA (panel 1), doublets were eliminated using FSCW and SSCA (panel 2), alive cells were selected by using Blue Aqua 510 (panel 3) and then doublets were again eliminated (panel 4).

B. Shows the gating of CD3-CD14+ monocytes and their expression of SIGIRR. It also shows the mean fluorescence intensity (MFI), measured in arbitrary units, of SIGIRR on monocytes from 4 healthy individuals and from 4 HIV-infected individuals. The mean values were compared using an unpaired test with Welch's correction, which does not assume equal variance.

C. Shows the gating of CD3+CD4+, CD3+CD8+ T cells and their expression of SIGIRR. The figure also shows gating of CD3-CD19+ B cells and their expression of SIGIRR, as well as the gating of CD3-CD56+ NK cells and their expression of SIGIR.

D. Shows that there is no difference in the expression of SIGIRR on the CD8+ T cells of control and HIV-infected individuals. However, on CD4+ T cells there is a slight decrease in the expression of SIGIRR in HIV-infected individuals compared to healthy controls.

E. Shows the gating of CD3+CD4+, CD3+CD8+ T cells and their expression of SIGIRR. The figure also shows gating of CD3-CD19+ B cells and their expression of SIGIRR, as well as the gating of CD3-CD56+ NK cells and their expression of SIGIR.

F. Shows how on NK cells there was a significant decrease ($p=0.012$) in the expression of SIGIRR in HIV-infected individuals, while on B cells was a statistically significant dramatic reduction ($p=0.037$) in the expression of SIGIRR in HIV-infected individuals.

4.4 Soluble SIGIRR neutralizes the function of IL-37

To determine whether soluble SIGIRR modulates the anti-inflammatory effects of IL-37, we investigated its effect on the cytokine's ability to inhibit LPS-induced TNF-α production in THP-1 cells, a human cell line of monocyte origin. For this purpose, the cells were stimulated with LPS in the presence or absence of sera from HIV-infected individuals with known concentrations of soluble SIGIRR. One hour later, IL-37 was added to the micro-cultures. Supernatants were collected 24 hours later, and their TNF-α levels were determined. The results of this experiment are shown in Figure 12.

As expected, addition of IL-37 reduced the production of TNF-α in THP-1 cells, whether they were treated with LPS or not. The addition of sera from HIV-infected individuals with high concentration of soluble SIGIRR (HS) suppressed the inhibitory effect of IL-37. The effect of the sera with low concentrations of soluble SIGIRR (LS) was not as suppressive as that of HS. These data suggest that soluble SIGIRR present in the sera suppresses the anti-inflammatory effects of IL-37.

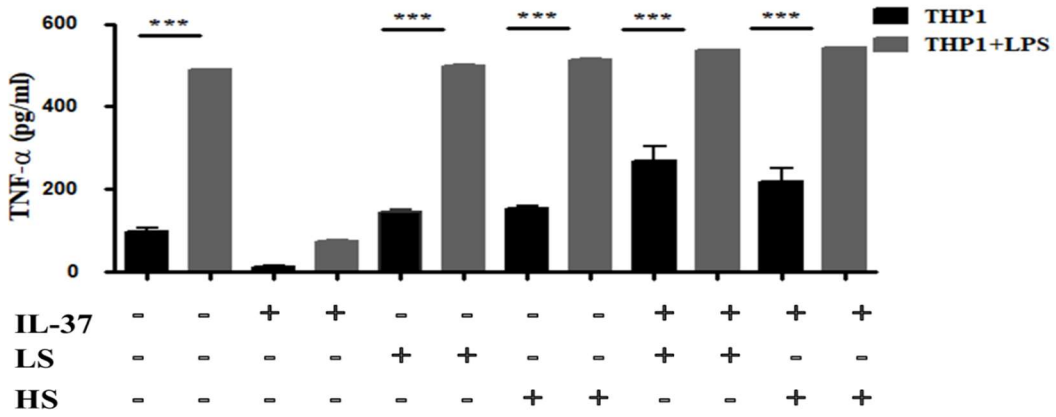


Figure 12. **The effect of soluble SIGIRR on the anti-inflammatory function of IL-37 in the sera of HIV-infected individuals**

THP-1 cells ($5x10^4$) were cultured in 200 µl culture medium, with and without LPS stimulation (10 µg per ml). To the micro-cultures, 100 µl of sera from HIV-infected individuals containing low concentration of soluble SIGIRR (LS; 60 pg per ml) or high concentrations of soluble SIGIRR (HS; 213 pg per ml) was added, followed one hour later by the addition of recombinant human IL-37 (5 ng per ml). After 24 hours, the culture supernatants were collected and their TNF-α content was determined using an ELISA test. Data were analyzed using ANOVA and Tukey's post-hoc tests.

Since it is quite possible that some factor(s) other than soluble SIGIRR present in the sera of HIV-infected individuals may be responsible for suppressing the effects of IL-37 in LPS-stimulated THP-1 cells, we investigated the effects of recombinant soluble human SIGIRR on the anti-inflammatory properties of IL-37. For this purpose, we repeated the experiments described above, this time adding different concentrations of recombinant soluble human SIGIRR in three concentrations: low 200 pg per ml, medium 2 ng per ml, and high 10 ng per ml, instead of using sera from HIV-infected individuals. The results of this experiment are shown in Figure 13. The recombinant soluble human SIGIRR suppressed the anti-inflammatory effects of IL-37 in THP-1 cells in a dose dependent manner.

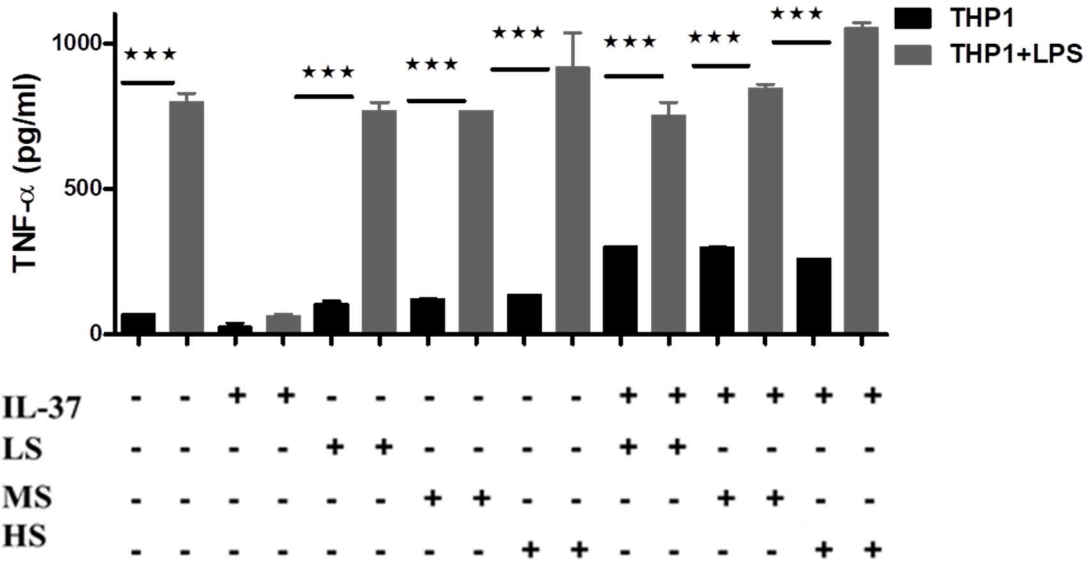


Figure 13. **Effect of recombinant SIGIRR on the anti-inflammatory effects of IL-37**
The experiment was designed as described in the legend of Figure 12, except that different concentrations of recombinant soluble human SIGIRR were added instead of sera from HIV-infected individuals. LS, MS and HS refer to low (200 pg/ml), medium (2 ng/ml) and high (10 ng/ml) concentrations of soluble SIGIRR. The results depict that soluble SIGIRR acts as a decoy receptor and that, at least, *in vitro* conditions attenuate the ant-inflammatory effects of IL-37 as determined by the production of TNF-α.

4.5 The effect of different concentrations of IL-37 on TNF-α

In order to determine the optimal concentration(s) of IL-37 required for its anti-inflammatory effects, we activated PBMCs using LPS. After an hour we added different IL-37 concentrations as follows: 0 pg, 10 pg, 100 pg, 1 ng, 5 ng, 7 ng, 10 ng, and 15 ng. After 24 hours of incubation, the culture supernatants were collected and TNF-α concentrations were determined using an ELISA test. Data were analyzed using ANOVA and Tukey's post-hoc tests. As illustrated in Figure 14, the optimal dose of IL-37 resulting in the greatest reduction of TNF-α production was 5 ng, followed by 1 ng and then by 7 ng.

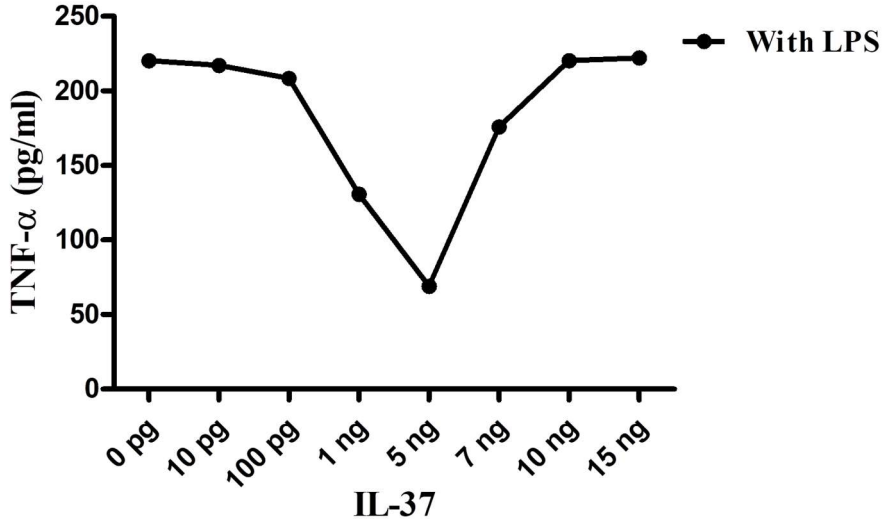


Figure 14. **Effect of different concentrations of IL-37 on the expression of TNF-α**

This experiment was performed to determine the optimal dose of IL-37 to inhibit inflammation. PBMCs were collected from healthy individuals using ficole, and the cells were stimulated with LPS. After an hour, different concentrations of IL-37 were added and cultured over night for 24 hours. The following day, the concentration of TNF-α was determined by ELISA. As illustreated above, 5 ng of IL-37 has the most suppressive effect on the production of TNF-α, followed by 1 ng and 7 ng respectivally; while concentrations of 10 pg, 100 pg, 10 ng and 15 ng of IL-37 had almost no effect on the secretion of TNF-α.

4.6 The effect of different concentrations of IL-37 on HIV replication

After determining the optimal concentration of IL-37 required to reduce inflammation, we used the two most effective concentration of IL-37, 5 ng and 1 ng, to determine their effects on HIV replication in human PHA blasts. The cells were infected with NL4.3, a T-tropic HIV strain. Culture supernatants were obtained 2 and 3 days post-infection and quantified for p24 content. As shown in Figure 15, after 48 hours of treatment with 5 ng of IL-37, the p24 contents in the culture supernatants was significantly decreased ($p<0.005$). On the contrary, 1 ng of IL-37 had little effect on HIV replication over same time period ($p<0.036$). After 72 hours, both concentrations of 5 ng and 1 ng IL-37 showed minimal suppression of HIV replication ($p<0.015$ and $p<0.017$, respectively), as measured by p24.

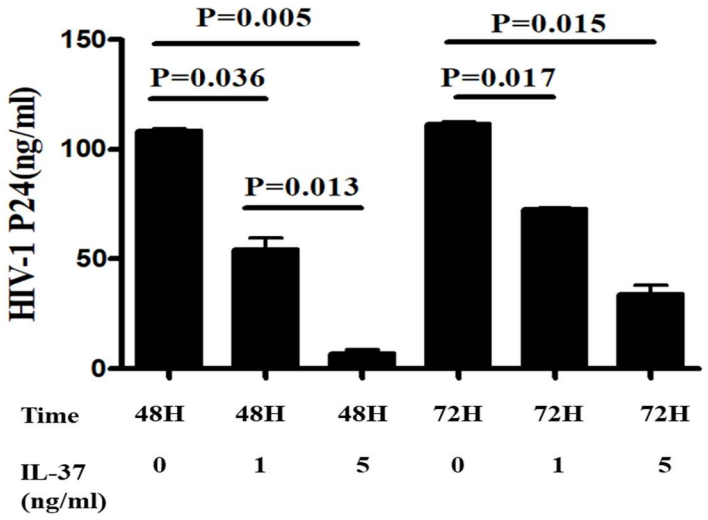
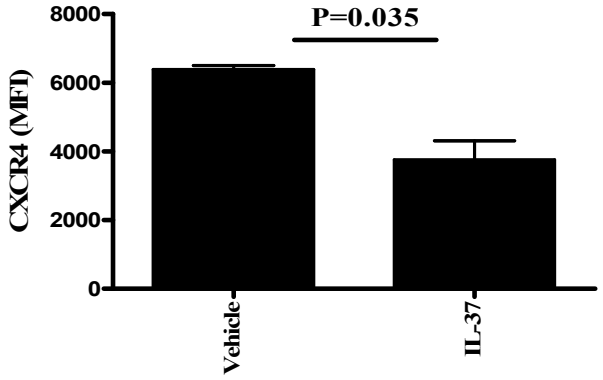


Figure 15. **Effect of different concentrations of IL-37 on HIV replication**
IL-37 was added to PHA blasts infected with NL4.3. Two- and three-days post-infection, culture supernatants were collected, centrifuged and quantified for their p24 contents. This figure shows mean ± SE of p24 contents from three independent experiments. Data were analyzed using one-way ANOVA tests and Tukey's post comparisons. The result show that recombinant human IL-37 (b isoform) inhibits HIV replication in PHA blasts. Given that the cytokine inhibits cell activation by reprogramming its metabolism and by inhibiting production of pro-inflammatory cytokines and chemokines, the results were as expected.

4.7 The effect of IL-37 on expression of the HIV chemokine receptor CXCR4

Given that the main co-receptor used by HIV-1 for entry into CD4+ T cells is CXCR4, we investigated whether IL-37 had any effect on the expression of this co-receptor. For this purpose, we used flow cytometry and determined the expression of CXCR4 on CD3+ CD4+ T cells in PBMCs. PBMCs from healthy donors were cultured for 24 hours in the presence of low doses of IL-2 (10 units per ml) with and without IL-37 (5 ng per ml). Thereafter, cells were washed, stained for CD3, CD4 and CXCR4, and analyzed by flow cytometry. As shown in Figure 16A, cytokine treatment significantly decreased expression of the chemokine receptor on CD3+CD4+ T cells. The decreased expression from a typical donor is shown in Figure 16B.

A



B

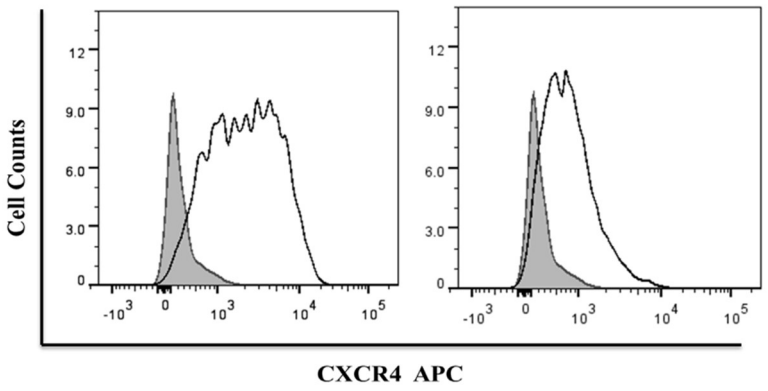


Figure 16. **Effect of IL-37 on expression of the HIV chemokine receptor CXCR4**

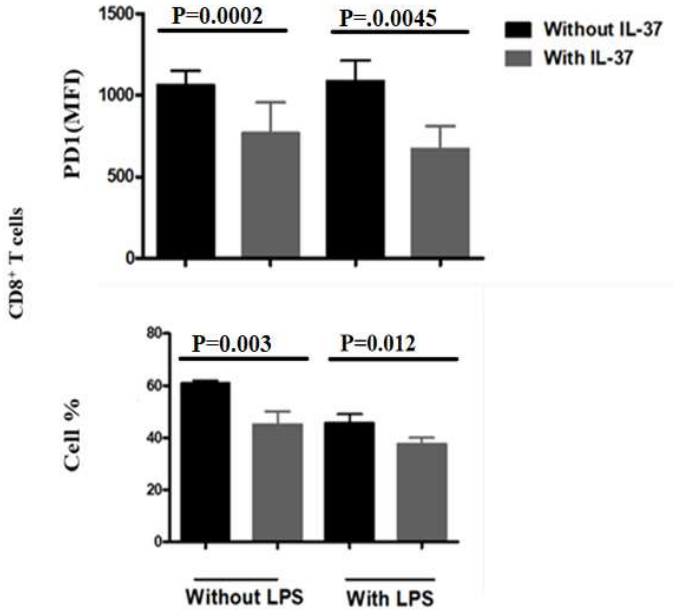
A. Depicts mean ± SD of MFI (in arbitrary units) of expression of CXCR4 on CD3+CD4+ T cells. Means were compared using a T test.
B. Is a typical histogram of the expression of CXCR4 on CD3+CD4+ T cells in the absence of IL-37 (left panel) and in the presence of IL-37 (right panel).

4.8 The effect of IL-37 on markers of exhaustion

It has been shown that T cells in HIV-infected individuals become relatively non-functional due to exhaustion (El-Far, Halwani et al. 2008). Exhausted cells express different inhibitory molecules, so-called markers of exhaustion. One such molecule is PD-1 (Khaitan and Unutmaz 2011, Catakovic, Klieser et al. 2017). Since immune hyper-activation and inflammation play an essential role in the induction of these markers, we sought to investigate whether IL-37, which attenuates immune activation as well as inflammation, can inhibit the expression of these markers on T cells. For this purpose, we cultured PBMCs from healthy control subjects in culture medium containing low doses of IL-2 (10 units per ml) in the presence and absence of LPS (10 μg per ml) and recombinant human IL-37 (5 ng per ml). After 24 hours of culture, the expression of PD-1 was determined on CD3+CD4+ and CD3+CD8+ T cells using multicolor flow cytometry. The results of these experiments are shown in Figure 17 below. The treatment of PBMCs from HIV-infected individuals significantly reduced the expression of PD-1 in both subsets of T cells.

In CD8+ T cells, IL-37 was shown to significantly reduce the expression of PD-1 ($p=0.0002$), especially in the absence of LPS. In fact, with exposure to IL-37, the percentage of cells expressing PD-1 decreased significantly ($p=0.003$) in the absence of LPS, and ($p=0.012$) in the presence of LPS (Figure 17A). Moreover, IL-37 was shown to significantly reduce the expression of PD-1 in CD4+ T cells from HIV-infected individuals ($p<0.0001$). Similar to the CD8+ T cells, the percentage of CD4+ T cells expressing PD-1 decreased significantly ($p<0.0001$), and ($p=0.027$) in the presence of LPS (Figure 17B).

A



B

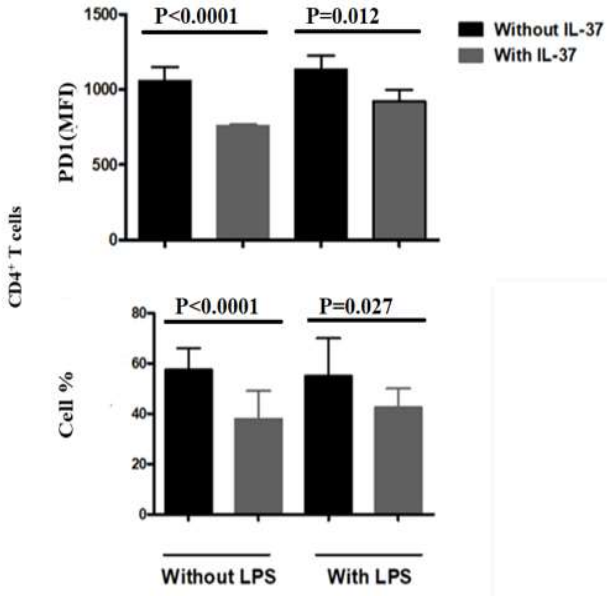


Figure 17. **Effect of IL-37 on expression of PD-1 in T cells**

PBMCs from 4 HIV-infected and individuals were cultured in medium containing 10 U per ml of IL-2 with and without the presence of LPS and IL-37. After 24 hours, CD4+ and CD8+ T cells were examined for the expression of PD-1 by flow cytometry. The cells were gated as shown in Figure 11c

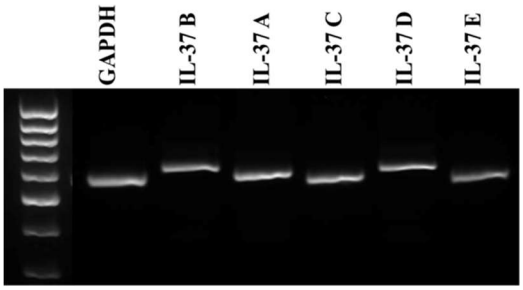
A. Illustrates the mean ± SD of MFI (in arbitrary units) of the effect of IL-37 on the expression of PD-1 on CD8+ T cells (on the top) and the percentage of cells that expressed PD-1 (on the bottom).
B. Illustrates the mean ± SD of MFI (in arbitrary units) of the effect of IL-37 on expression of PD-1 on CD4+ T cells (on the top) and the percentage of cells that expressed PD-1 (on the bottom).

4.9 IL-37 isoforms in HIV-individuals

We investigated the expression of all five isoforms of IL-37 in the PBMCs of HIV-infected and healthy individuals. As no isoform-specific antibodies and ELISA kits are available for the different isoforms, we used RT-PCR using isoform-specific primers as described in Materials & Methods. The results are shown in Figure 18. Whereas transcripts for all five isoforms could be detected in the PBMCs of healthy and LTNPs, we noticed the absence of isoforms b and d in treatment-naïve HIV-infected individuals. Interestingly, only b isoform transcripts were missing in cART-treated individuals. The data suggests that the transcripts for the most functional isoform (b isoform) is missing in the PBMCs of HIV-infected individuals irrespective of their treatment status.

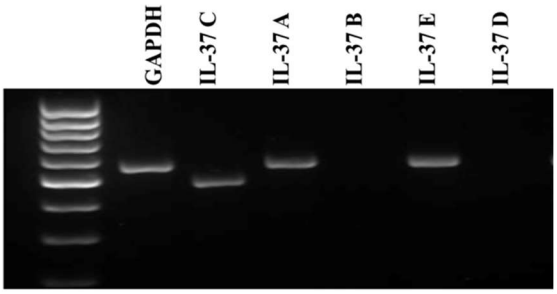
A

Control



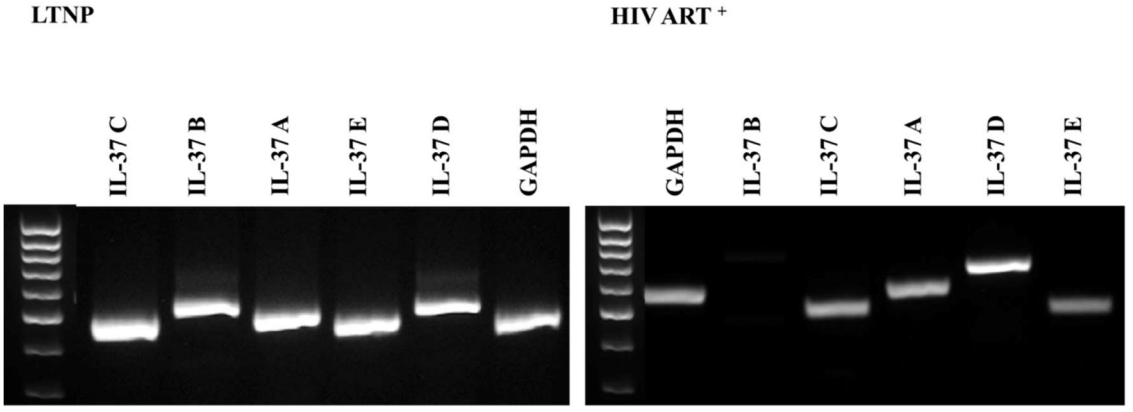
Controls	IL-37B	IL-37A	IL-37C	IL-37D	IL-37E
1	+	+	+	+	+
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+

HIV ART -



HIV ART -	IL-37C	IL-37A	IL-37B	IL-37E	IL-37D
1	+	+	-	+	-
2	+	+	-	+	-
3	+	+	-	+	-
4	+	+	-	+	-
5	+	+	-	+	-

B



LTNP	IL-37C	IL-37B	IL-37A	IL-37E	IL-37D
1	+	+	+	+	+
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+

HIV ART+	IL-37B	IL-37C	IL-37A	IL-37D	IL-37E
1	-	+	+	+	+
2	-	+	+	+	+
3	-	+	+	+	+
4	-	+	+	+	+
5	-	+	+	+	+

Figure 18. **Isoforms of IL-37 expressed in HIV individuals**

Total RNA was collected from PBMCs of different HIV-infected categories (5 patients from each group: HIV ART-, HIV ART+, LTNP and 5 healthy people as controls), and cDNA was made by reverse transcribing 0.5 µg RNA. The cDNA from the transcripts was then amplified by PCR using published gene-specific primers. We also performed RT-PCR for the transcripts of GAPDH genes as both positive and negative controls in the cDNA from PBMCs. The PCR reaction was analyzed by electrophoresis on 1% agarose gel for the expected DNA band size. The DNA bands were revealed using ethidium bromide and photographed using the Gene Genius Bio-Imaging System (Syngene). Interestingly, the different isoform bands showed that healthy individuals and LTNP (also referred to as elite controllers) express all the isoforms, while HIV-infected individuals treated with ART do not express isoform b. HIV-infected individuals negative to ART did not express both isoform b and d. These data strongly suggest that isoform b is the functional isoform among all the other IL-37 isoforms.

5 DISCUSSION

The results of this study show that concentrations of the anti-inflammatory cytokine IL-37 increase in the serum of HIV-infected individuals. This increase was significant only in virus-infected individuals undergoing ART and not in individuals naïve to therapy. Interestingly, IL-37 concentrations in the serum were significantly higher in LTNP than in healthy controls. They were even higher compared to levels in treatment-naïve virus-infected individuals. To the best of our knowledge, this is the first report describing IL-37 concentrations in the sera of HIV-infected individuals. So far, there is only one study on the regulation of IL-37 in HIV-infected individuals (Hojen, Rasmussen et al. 2015), in which the authors of the study investigated IL-37 at the mRNA level and not at the protein level. The study showed that steady state levels of the cytokine mRNA increase in HIV-infected individuals compared to healthy control individuals. It is noteworthy that the mRNA of the cytokine contains an instability motif in sequences transcribed from exon 5 of the gene. The mRNA is stabilized and translated into protein under inflammatory conditions (Bufler, Gamboni-Robertson et al. 2004). Since HIV infection is accompanied by an inflammatory response, it is conceivable that the mRNA of the cytokine is stabilized during this infection and is translated, thereby increasing concentrations of the cytokine at the protein level. In this regard, our results are in accordance with those of Hojen et al. (2015). However, we only observed increased concentrations of the cytokine in HIV-infected individuals who were receiving anti-retroviral therapy (ART), whereas Hojen et al. (2015) observed that the levels of IL-37 mRNA decreased in infected individuals that were receiving ART. In fact, the increases observed by Hojen et al. (2015) were in individuals with primary HIV infection, as well as in individuals with chronic infection. Moreover, they observed increases in the sera of patients who were not receiving ART, but these increases were not statistically significant.

SIGIRR is an atypical member of the IL-1R family (Thomassen, Renshaw et al. 1999, Molgora, Barajon et al. 2016). It has only one Ig-like domain in its extracellular region, whereas other members of the IL-1RF, as well as TLRs, carry three such domains in this region. Furthermore, SIGIRR has an atypical TIR domain in its cytoplasmic tail. This atypical TIR domain cannot recruit the same signaling partners as the typical TIR domains of other IL-1R family members and TLRs do. Thus, it is not surprising that SIGIRR induces

anti-inflammatory signaling. SIGIRR has been recognized as an orphan receptor with no known ligand. It was only recently that three independent studies identified SIGIRR as the signaling subunit of the IL-37 receptor (Lunding, Webering et al. 2015, Nold-Petry, Lo et al. 2015). It was demonstrated that IL-37 binds to the IL-18Rα chain, which is also shared by IL-18. When IL-37 binds this chain, it recruits SIGIRR, thereby inducing intracellular signaling that culminates in anti-inflammatory effects. On the other hand, when IL-18 binds the IL-18Rα chain, it recruits the IL-18Rβ chain and exerts pro-inflammatory effects. This happens in humans who carry a functional IL-37 gene. Unlike humans, mice do not carry a functional IL-37 gene and lack IL-37. In this species, SIGIRR functions to attenuate the pro-inflammatory effects mediated by TLRs and IL-1R family members. SIGIRR knock-out mice show exaggerated pro-inflammatory responses (e.g., production of TNF-α) in response to TLR ligands such as LPS. Interestingly, human IL-37 is functional in mice, as it can bind the murine IL-18Rα chain, as well as SIGIRR. Thus, IL-37 transgenic mice are hypo-responsive to LPS (McNamee, Masterson et al. 2011). Interestingly, our results show that the expression of SIGIRR is significantly reduced in several types of PBMCs isolated from HIV-infected patients such as NK cells, B cells, monocytes, and T cells. These results suggest that these immune cells may be less responsive to the anti-inflammatory effects of IL-37. Thus, despite an increase in the concentration of IL-37 in the circulation of HIV-infected individuals, the cytokine may not be exerting its anti-inflammatory effects due to decreased expression of the signaling partner of its receptor. We had previously reported no decrease in the expression of the IL-18Rα chain in the PBMCs of HIV-infected individuals (Iannello, Samarani et al. 2009). Given that increased concentrations of IL-18 have been found in the circulation of HIV-infected individuals (Ahmad, Sindhu et al. 2002, Torre and Pugliese 2006, Allam, Samarani et al. 2017), these data suggest that immune cells may be fully responsive to the pro-inflammatory effects of IL-18 and of various TLR agonists such as LPS; and that they may be less responsive to the anti-inflammatory effects of IL-37. Thus, the homeostatic role of IL-37 may be lost in this infection. Since increased intestinal para-cellular permeability, increased microbial translocation, and consequently systemic inflammatory response immune activation, have been well documented in HIV-infected individuals (Marchetti, Tincati et al. 2013), decreased expression of SIGIRR may contribute to the inflammatory response and immune activation observed in HIV-infected individuals.

The exact mechanism(s) responsible for decreased expression of SIGIRR in different cell types in the PBMCs of HIV-infected individuals remain unknown. However, expression of various immune receptors is known to be decreased due to their cleavage and shedding in soluble forms into circulation. A good example of this is CD16, the low-affinity receptor for the Fc region of IgG expressed in NK cells and monocytes, among other cells (Romee, Foley et al. 2013). CD16 is involved in antibody-dependent cell-mediated cytotoxicity (ADCC), which has recently gained importance in HIV infection, as it was demonstrated that HIV-specific ADCC antibodies play a role in the protection afforded by anti-HIV vaccine trials (Wren and Kent 2011). Upon cell activation, this receptor is cleaved and shed into the circulation by the metalloprotease ADAM-17 (Romee, Foley et al. 2013). We investigated concentrations of soluble SIGIRR in the sera of HIV-infected individuals. Our results show that soluble SIGIRR concentrations increase significantly in the sera of HIV-infected individuals. These results strongly suggest that SIGIRR is shed from the surface of the cell in HIV-infected individuals. Our *in vitro* studies in THP-1 human monocyte cell line (Chanput, Mes et al. 2014) showed that when these cells are stimulated with LPS, their surface expression of SIGIRR decreases. Further studies are required to understand the mechanism behind the shedding of SIGIRR from the PBMCs in HIV-infected individuals. Furthermore, it would also be interesting to investigate whether any protease other than ADAM-17 is implicated in the cleavage and shedding of this signaling subunit of the IL-37 receptor. Such a discovery would have important implications for HIV-infected individuals. For example, cell permeable, protease-specific inhibitors may be used to prevent shedding of SIGIRR, thereby restoring the anti-inflammatory effects of IL-37.

In this study, we investigated the potential effects of soluble SIGIRR on the anti-inflammatory effects of IL-37. The results presented here show that soluble SIGIRR inhibits the anti-inflammatory effects of IL-37. We also investigated the potential effects of soluble SIGIRR on IL-37's ability to inhibit production of TNF-α in LPS-stimulated THP-1 cells. As expected, addition of recombinant human IL-37 inhibited production of TNF-α in both LPS-stimulated and non-stimulated THP-1 cells. Moreover, addition of sera from HIV-infected individuals containing low, medium, and high concentrations of soluble SIGIRR inhibited the suppressive effects of IL-37 on the production of TNF-α in THP-1 cells. These data suggest

that soluble SIGIRR inhibits the anti-inflammatory effects of IL-37 on human cells. Since it is possible that factors other than soluble SIGIRR present in the sera of HIV-infected individuals may have caused increased production of TNF-α in the THP-1 cells, we sought to determine the direct effects of recombinant soluble human SIGIRR on the anti-inflammatory effects of IL-37. We repeated the experiments, adding different concentrations of soluble recombinant SIGIRR to the THP-1 cell cultures and found that, just like the soluble SIGIRR in the sera, recombinant SIGIRR inhibited the anti-inflammatory effects of IL-37 on the production of TNF-α in THP-1 cells. Overall these data suggest that soluble SIGIRR inhibits the anti-inflammatory effects of IL-37. Thus, the shedding of SIGIRR neutralizes the anti-inflammatory effects of IL-37 in two ways: (i) firstly, the cytokine cannot exert its biological effects due to a loss/decrease in expression of the signaling subunit of the cytokine's receptor, and (ii) secondly, soluble SIGIRR antagonizes the anti-inflammatory effects of the cytokine. Although further studies are required to understand the exact mechanism by which soluble SIGIRR neutralizes the anti-inflammatory effects of IL-37, we hypothesize that it likely competes with the membrane-expressed SIGIRR for recruitment to the cytokine/receptor complexes and thus attenuates the cytokine's intracellular anti-inflammatory signaling.

The results presented herein show that recombinant human IL-37 inhibits HIV replication in human PHA blasts. For these experiments, we used a T-tropic viral strain NL4.3. Different doses of recombinant cytokine were added to cell cultures after *in vitro* infection with the virus. Although further studies are required to understand the mechanism by which the cytokine inhibits replication of the virus, IL-37 most likely inhibits cell activation and availability of the transcription factors required for this process. It is known that transcription factors such as AP-1, Sp-1 and NF-κB are required for transcription and replication of the virus (Schiralli Lester and Henderson 2012). Furthermore, IL-37 may also affect other steps of the replication process such as nuclear transport of the viral pre-integration complex, maturation of different viral proteins, viral assembly and/or release of virions from the cell surface (budding), etc. Further studies are required to learn more on the exact role and involvement of the cytokine.

IL-37 inhibits replication of T-tropic HIV strain (NL4.3) once it has entered the cells, thus it is also very likely to inhibit replication of M-tropic and dual tropic HIV strains. While

these strains differ mainly in their mode of entry, (T tropic viral strains mainly use CXCR4 as co-receptors for entry into human cells, M-tropic strains use CCR5 and dual tropic strains can use both) (Woodham, Skeate et al. 2016), once in the host cell their replication process is essentially similar. The viral replication assay used in this study was inherently incapable of detecting the effects of the cytokine on viral entry into human cells. Therefore, we investigated the potential effects of the cytokine on the expression of CXCR4 on human cells. The results suggest that the cytokine reduces the expression of CXCR4 on human cells. These data support the hypothesis that IL-37 is likely to reduce entry of HIV-1 into human cells by reducing the expression of CXCR4 on human cells. It is worth noting that these co-receptors are absolutely essential for entry of the virus into human cells (Woodham, Skeate et al. 2016). These results have other implications for HIV infection other than viral entry into target cells. CXCR4-tropic viruses evolve during the course of the infection (Borggren and Jansson 2015). Secondly, CXCR4 and CCR5 are used for the recruitment of new target cells to the site of infection and inflammation (Olsson, Poles et al. 2000, Borggren and Jansson 2015). This process fuels the infection process and augments viral replication in an infected host. The cytokine may attenuate this process by reducing the expression of CXCR4 on human cells, thereby reducing their chemo-attraction and recruitment to the site of infection within an infected individual. In the future, it would be interesting to investigate the effects of IL-37 on the expression of CCR5 on human T cells.

HIV infection is known to induce a strong anti-viral cellular immune response (Genesca 2011). This response is known to play a protective role in the host and to delay progression of the infection into full-blown AIDS. Nevertheless, the virus has evolved several strategies to evade and subvert the host's anti-viral immune responses (Klenerman, Wu et al. 2002). Thus, the host is unable to control and eradicate the infection. Persistent viral replication and inflammation cause immune activation. One of the deleterious effects of persistent immune activation is the exhaustion of anti-viral T cells. The cells express a variety of inhibitory molecules such as PD-1, CTLA-4, LAG-3, 2B4 and TIM-3, etc. (Catakovic, Klieser et al. 2017). These molecules are commonly referred to as immune checkpoints (IC). The purpose of these inhibitory molecules in anti-viral T cells is to protect the host from tissue destruction. However, exhausted T cells are unable to exert their effector functions and to kill

virus-infected cells. The exhaustion does not limit itself to T cells, B cells are also exhausted during HIV-infection (Moir, Ho et al. 2008, Moir and Fauci 2014). The phenomenon of exhaustion is not restricted to HIV infection; in fact, it is manifested in all chronic viral infections, as well as in cancer (Kahan, Wherry et al. 2015). It has been demonstrated that γ chain-using cytokines (IL-2, IL-4, IL-15 and IL-21, etc.) play an important role in the expression of these IC in immune cells. Furthermore, activation of GSK-3β has been shown to play an important role in the induction of PD-1 in T cells. It has been demonstrated that blocking these checkpoints using small molecule inhibitors or molecule-specific monoclonal antibodies (so-called immune checkpoint inhibitors or ICI) can restore anti-cancer immunity and cause the regression of several types of cancers (Mahoney, Freeman et al. 2015). The only caveat is that long-term usage of these ICI can cause autoimmune phenomenon such as colitis. It would be better to decrease the expression of these IC using anti-inflammatory drugs or biological substances that reduce cell activation. As IL-37 exerts anti-inflammatory effects and inhibits cell activation (Abulkhir, Samarani et al. 2017), we sought to determine the effects of the cytokine on these IC. For this purpose, we cultured PBMCs from HIV-infected individuals in culture medium and in the presence and absence of human recombinant IL-3. After 24 hours, we determined the expression of PD-1 on CD4+ and CD8+ T cells. The treatment of the cells with the cytokine significantly reduced their expression of PD-1. Further studies are necessary to investigate whether IL-37 can reduce the expression of other ICs in the T cells of HIV-infected individuals. An important question in this regard would be whether reduced expression of ICs would not affect the effector functions of T and immune cells. It is very unlikely, as inhibitors of GSK-3β have been shown to reduce expression of PD-1 and to increase cytotoxic potential of NK cells (Fionda, Malgarini et al. 2013). However, IL-37 is likely to exert broader effects on immune cells than GSK-3β inhibitors do.

A previous study has shown that steady state levels of IL-37 mRNA correlate with those of viral DNA in the PBMCs of HIV-infected individuals (Hojen, Rasmussen et al. 2015). The authors concluded that the mRNA levels of the cytokine are indicative of the magnitude of HIV reservoirs in the infected individuals. Since the cytokine gene is induced and its mRNA is stabilized by inflammatory stimuli, which also activate immune cells making them more susceptible to infection, this may explain the correlation between the mRNA levels

of the cytokine and the magnitude of pro-viral DNA in the PBMCs. However, the magnitude of viral DNA in the PBMCs does not reflect the magnitude of viral reservoirs. These reservoirs represent latently infected cells resting in immune privileged sites such as the gut, brain, eye, and testis, and other tissues where anti-retroviral drugs do not diffuse efficiently (Alexaki, Liu et al. 2008, Massanella, Fromentin et al. 2016). The reservoir is established early in the course of infection and is not affected by the use of ART in the later stages; thus presenting a major hurdle in the eradication of the virus from ART-treated individuals. Occasional fluctuations in the replication of residual virus maintain viral reservoirs. Upon interruption of ART, the residual virus in the reservoirs begin to replicate rapidly (Chun, Moir et al. 2015). Because of this, HIV-infected individuals are required to take ART for the rest of their life. Various strategies are being employed to eradicate or to reduce these reservoirs in HIV-infected individuals taking ART. The most investigated strategy has been the so-called 'shock and kill' approach (Kimata, Rice et al. 2016) whereby the latent virus is first activated by a variety of stimuli and then the virus-infected cells are killed using ART. However, this strategy has not been very successful in reducing HIV reservoirs (Kimata, Rice et al. 2016). For this reason, scientists are now proposing a different strategy aimed at reducing inflammation and cell activation (Kimata, Rice et al. 2016, Massanella, Fromentin et al. 2016). One can hope that through this strategy, viral replication will be reduced, and viral reservoirs will be not replenished. In such a scenario, IL-37-based therapy may prove useful. The therapy may be used in the form of recombinant cytokine, restoring the cytokine's effector function by reducing the shedding of SIGIRR and/or neutralization of soluble SIGIRR. Further studies are required to decipher the exact mechanism and to validate this hypothesis. Treated individuals would require close monitoring for opportunistic infections, due to the fact that IL-37 expression may also lead to immune quiescence. Moreover, the cytokine-based therapy would have additional benefits in HIV-infected individuals. For example, IL-37 has been shown to improve insulin sensitivity and to reduce inflammation-induced fatigue (Ballak, Degens et al. 2014, Onuora 2017).

IL-37 is expressed by intestinal epithelial cells (IEC) (McNamee, Masterson et al. 2011). The IEC-produced cytokine maintains tolerance at the interface between gut and gut-dwelling microbiota (Lopetuso, Chowdhry et al. 2013). A lack of this tolerance may lead to

local inflammation and production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-18, etc. The cytokines disrupt the intestinal barrier function and lead to enhanced para-cellular permeability of the intestinal epithelial monolayer, causing microbial translocation i.e., translocation of bacterial products, fragments, and even whole bacteria into intestinal tissues and systemic circulation (Marchetti et al., 2013). The increased microbial translocation occurs early in the course of HIV infection and has been thought to be an important factor in HIV-induced enteropathy and disease progression (Poles, Alvarez et al. 2014). It would be highly desirable to investigate how the production of this cytokine is regulated in the intestine and in other mucosal tissues during HIV infection. Localized delivery of the cytokine in the form of multi-layered biodegradable nanoparticles may improve gut inflammation and intestinal barrier function in HIV-infected individuals. More studies are required to investigate this further.

Our results show that PBMCs from healthy individuals, as well as from LTNPs, express transcripts for all five isoforms of the cytokine; whereas the transcripts for two functional isoforms (b and d) were not expressed in the cells obtained from treatment-naïve HIV-infected patients. Interestingly, the transcripts for only one functional isoform b were not expressed in the PBMCs obtained from patients receiving ART. These results suggest the viral infection and the treatment may also regulate production of the cytokine at the isoform level. Inhibition of the production of the functional isoforms of the cytokine suggest its protective role in the context of the infection. It would be highly desirable to measure concentrations of the isoforms directly. For this, we may have to wait for the development of isoform-specific monoclonal antibodies and ELISA protocols.

Overall the results presented in this thesis suggest that although IL-37 is induced in HIV-infected individuals, it may not be fully functional due to the shedding of its signaling subunit, SIGIRR, from the cell surface. As a result, immune cells become less responsive to the cytokine's anti-inflammatory effects. Furthermore, soluble SIGIRR also neutralizes the cytokine's biological effects by attenuating cytokine-induced signaling. Because of the cytokine's ability to inhibit HIV replication, reduce expression of viral co-receptors, and

reverse expression of immune checkpoints in anti-viral T cells, Il-37 could potentially be useful in reducing inflammation and immune activation in HIV-infected individuals.

6 CONCLUSIONS

Many studies have investigated the regulation of different cytokines over the course of HIV infection. These cytokines include IL-2, IL-7, IL-10, IL-12, IL-15, TGF-β, IL-21, and IL-18, etc. (Catalfamo, Le Saout et al. 2012). However, very little remains known about the regulation and functional activity of IL-37 in HIV-infected individuals. IL-37 is an important anti-inflammatory cytokine belonging to the IL-1 family. So far, only one study has investigated the regulation of this cytokine at the mRNA level in HIV-infected individuals, and has shown increased mRNA levels of IL-37 in PBMCs from HIV-infected individuals compared to healthy control subjects. We have investigated the expression of IL-37, as well as the expression of the signaling subunit of its receptor, at the protein level. Our results show that, although cytokine levels increase in the circulation of HIV-infected individuals, the biological activities of the cytokine are likely to be dampened. This dampening happens for two reasons: (i) firstly, the expression of the signaling subunit of the cytokine receptor, SIGIRR, decreases in immune cells such as monocytes, NK, B and T cells, and (ii) secondly, soluble SIGIRR, whose concentration increases in the circulation of HIV-infected individuals, attenuates the anti-inflammatory signaling induced by the cytokine. The decreased functional activities of the cytokine contribute to inflammation and immune activation; hallmarks of this viral infection. Moreover, decreased functional activities of IL-37 contribute towards increased viral replication, establishment, and replenishment of viral reservoirs, and to immune exhaustion. It has been well established that chronic low-grade inflammation induced by clinically undetectable replication of residual virus, as well as the toxic side effects of ART, cause several non-AIDS related clinical co-morbidities such as accelerated aging, CVD, insulin resistance, increased susceptibility to cancer and frailty, etc. In this context, strategies aimed at restoring the anti-inflammatory biological functions of IL-37 would be desirable.

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