Development of novel molecular and microfluidics tools for identification and characterization of latent HIV-1 reservoir

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Populärvetenskaplig sammanfattning

Trots att antiretroviral behandling (ART) har visat sig väsentligt minska HIV och progressionen av HIV-sjukdom, finns än idag ingen bot for HIV. Förekomsten av en latent HIV-1-reservoar (LR) hos alla HIV-1-infekterade patienter tjänar som ett stort hinder för fullständigt botande av en HIV-1-infektion. Hittills har det dock inte funnits någon analysmetod som ger en noggrann mätning av reservoarstorleken. Syftet med den här avhandlingen är att ta itu med denna utmaning från olika aspekter med flera nya teknologier varav både molekylära och mikrofluidikbaserade verktyg används. För att hitta ett lämpligt verktyg att identifiera latent HIV-1-reservoarer med optimerades och utvärderades (i artikel I och II) LIPS-, RNAflow- och RNAscope-analys för indirekt och direkt LR-detektering. Resultaten indikerade att LIPS-metoden kan vara otillräcklig för LR-detektering, däremot föreslås den för indirekt kvantifiering av reservoaren. Vidare presterade den optimerade RNAscopetekniken bättre än RNAflow för transkriptions- och translationskompetent LR-identifiering. RNAscope visade sig även vara oberoende av HIV-1-subtypen och kan tillämpas på patientprover vid encellsnivå. Eftersom det för närvarande inte finns några tillgängliga ytbiomarkörer för LR fastställdes i artikel III en transkriptomik- och proteomikbaserad analysmetod för hög-kapacitetselektion av potentiella biomarkörer, som sedan tillämpades i olika patientgrupper. Tolv membranproteinkodande gener identifierades som nedreglerade i den patientgrupp som var hypotiserad att ha en mindre latent reservoar. Dessa proteiner kan ha potential att användas som ytbiomarkörer för LR. CD4+ T-celler, monocyter/makrofager och NK-celler antas vara den huvudsakliga källan för HIV-1-reservoarer i perifert blod. I artikel IV utvecklades ett mikrofluidik-chip för att simultant isolera dessa tre mononukleära leukocyter direkt från helblod. Mikrofluidik-metoden minskar behovet av provvolym och är ett lovande verktyg för LR-studier. Sammanlagt, även om ytterligare förbättringar och klinisk verifikation är nödvändig, har arbetet i denna avhandling bidragit till framsteg i LR-karakterisering och kan komma att underlätta den framtida utvecklingen av LR-targeting- och clearingsmetoder med det ultimata målet att bota HIV-1-infektion.

Nyckelord HIV-1, latent HIV-1-reservoar, mikrofludik, molekylär detektion

Abstract

The existence of latent HIV-1 reservoir (LR) in all HIV-1 infected patients serves as a major obstacle to completely cure HIV-1 infection. However, up to now there is still no available assay that provides an accurate measurement of the reservoir size. This thesis aims to address this challenge from different aspects with several novel technologies, using both molecular and microfluidics-based tools. To find a proper tool to identify the latent HIV-1 reservoir, in Paper I and II, LIPS assay, RNAflow, and RNAscope assay were optimized and evaluated for indirect and direct detection of latent HIV-1 reservoir. The results indicated the LIPS method might not be sufficient for latent HIV-1 reservoir detection, although it has been proposed to quantify the latent HIV-1 reservoir indirectly. Furthermore, the optimized RNAscope technique performed better than RNAflow for transcription and translation competent latent HIV-1 reservoir identification. The RNAscope was also found to be independent of the HIV-1 subtype and can be applied to patient samples at single cell level. As there are currently no available surface biomarkers for latent HIV-1 reservoir, in Paper III, transcriptomics and proteomics-based analysis method for high-throughput selection of potential biomarker were established and applied to different patient groups. Twelve membrane protein-coding genes were identified as downregulated in the patient group who were hypothesized to have lower latent reservoir. These proteins might have the potential to be used as surface biomarkers for latent HIV-1 reservoir. CD4+ T cells, monocyte/macrophages, and natural killer cells are believed to be the primary source for HIV-1 reservoirs in peripheral blood. In paper IV, a microfluidic chip was developed to simultaneously isolate these three mononuclear leukocyte cell types directly from whole blood. The microfluidic method reduces the sample volume requirement and is a promising tool for latent HIV-1 reservoir study. Together, though further improvement and clinical verification are necessary, the work in this thesis has contributed to the advancement of latent HIV-1 reservoir characterization and may facilitate future development of the latent HIV-1 reservoir targeting and clearance methods with the ultimate goal – to cure HIV-1 infection.

Keywords HIV-1, Latent HIV-1 reservoir, HIV-1 characterization, Microfludics, Molecular detection

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List of Publications

I **Wang Zhang**, Sara Svensson Akusjärvi, Anders Sönnerborg and Ujjwal Neogi. "Characterization of inducible transcription and translation-competent HIV-1 using the RNAscope ISH technology at a single-cell resolution." Frontiers in Microbiology 9 (2018): 2358.

II **Wang Zhang**, Mohammed M. Morshed, Kajsa Noyan, Aman Russom, Anders Sönnerborg, Ujjwal Neogi. "Quantitative humoral profiling of the HIV-1 proteome in elite controllers and patients with very long-term efficient antiretroviral therapy." Scientific reports 7.1 (2017): 666.

III **Wang Zhang***, Anoop T.Ambikan*, Maike Sperk*, Robert van Domselaard Piotr Nowak, Kajsa Noyan , Aman Russom. Anders Sönnerborg, Ujjwal Neogi."Transcriptomics and targeted proteomics analysis to gain insights into the immune-control mechanisms of HIV-1 infected elite controllers." EBioMedicine 27 (2018): 40-50.

IV **Wang Zhang**, Zenib Aljadi, Neogi Ujjwal, Russom Aman. "Microfluidic-based immune cell subset isolation".Manuscript

The contributions of Wang Zhang to the different papers:

Paper I: major part of method development, experiments performance, data analyzing and part of writing

Paper II: major part of method development, experiments performance, data analyzing and part of writing

Paper III: major part of method development, experiments performance, part of data analyzing and writing

Paper IV: major part of method development, experiments performance, data analyzing and writing

* Shared first authorship

Other contributions not included in this Thesis

V Maike Sperk, **Wang Zhang**, Piotr Nowak and Ujjwal Neogi."Plasma soluble factor following two decades prolonged suppressive antiretroviral therapy in HIV-1-positive males: A cross-sectional study." Medicine 97.5 (2018).

VI Banerjee Indradumna, Aralaguppe Shambhu G, Lapins Noa, **Wang Zhang**, Kazemzadeh Amin, Sönnerborg Anders, Neogi Ujjwal, Russom Aman."Microfluidic Centrifugation Assisted Precipitation for DNA Quantification on a Lab-on-DVD platform."Submitted

List of Abbreviations

HIV	Human Immunodeficiency Virus
AIDS	Acquired immune deficiency syndrome
SIV	Simian Immunodeficiency Virus
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic acid
cART	Combination Antiretroviral therapy
LRs	Latent HIV-1 reservoirs
ELISA	Enzyme-linked immuno sorbent assay
RODT	Rapid Orientation and Diagnostic Tests
LTNP	Long Term Non Progressor
PDCs	Plasmacytoid dendritic cells
GALT	Digestive lymphoid tissue
PBMCs	peripheral blood mononuclear cell
CCR5	C-C chemokine receptor type 5
CXCR6	C-X-C chemokine receptor type 6
CXCR4	C-X-C chemokine receptor type 4
QVOA	Quantitative Viral Outgrowth Assay
PHA	Phytohemagglutinin
IUPM	Infectious Unit Per Million
LRAs	Latency-Reversing Agents
TCMs	Central Memory T cells
CNS	Central Nervous System
LTR	Long Terminal Repeat
SCA	Single-Copy Assay
LIPS	Luciferase Immuno-Precipitation Systems
ECs	Elite Controllers
PHI	Primary HIV-1 infection
msRNA	Multiply Spliced HIV-1 mRNA
usRNA	unspliced HIV-1 mRNA
TILDA	tat/rev-induced limiting dilution assay
FISH	Fluorescence *insitu* Hybridization
bDNA	branched DNA amplification
NK cells	Natural killer cells
FDR	False Discover Rate

PCA	Principal Component Analysis
RNAflow	PrimeFlow™ RNA Assay
RNAscope	RNAscope® ISH
HC	Healthy controls
VP	Viremia patient
FACS	Fluorescence Activated Cell Sorter
MACS	Magnetic Activated Cell Sorting

Chapter 1

Introduction

Viruses are obligatory parasitic particles, which replicate only in the host cells by inserting its nucleic acid into the genome of the host cells. Many viruses, which plague human are derived from animal population, periodically leap across the human population. In the case of Human Immunodeficiency Virus (HIV), this jump seems to have occurred during mid 20^{th} century and emerged as a devastating infectious disease.

HIV infection weakens the immune system by targeting and destroying immune cells, mainly the CD4+ T cells. As a result, the individual will become immuno deficient and vulnerable to a wide range of immunological abnormalities, contributing to secondary infection and oncological diseases [1]. As the infection progresses over several months to years, it will lead to Acquired immune deficiency syndrome (AIDS).

The first report of AIDS was made in 1980, as a unique illness in homosexual men [2]. In 1983, HIV-1 virus, a retrovirus was identified as the causative agent for AIDS [3] [4], an illness that is still haunting several million people. According to WHO 2017 report on HIV, around 36.9 million individuals live with HIV, as shown in Figure1.1. Additionally, the new infection and HIV related death has been significantly reduced by -14% and -34%, respectively, relative to 2010 [5].

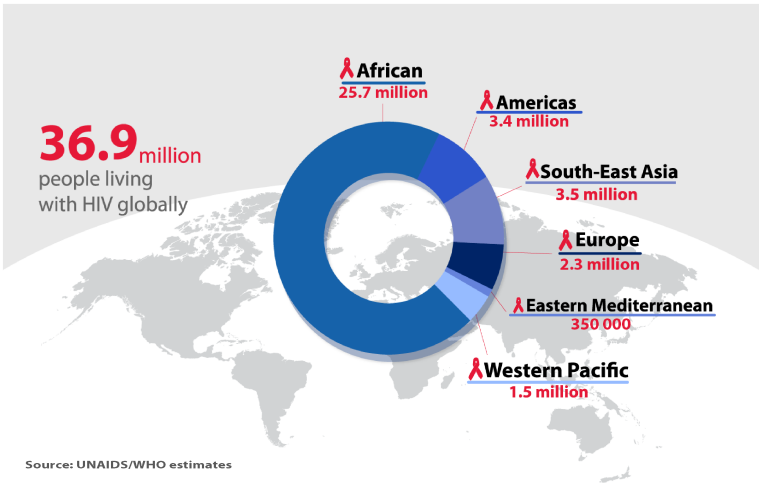

Figure 1.1: *People living with HIV by WHO region. Source: UNAIDS/WHO estimates*

HIV is a retrovirus composed of Ribonucleic acid (RNA) genome; the virus has an extraordinary capacity to copy RNA into Deoxyribonucleic acid (DNA) and insert DNA into host cells, which will integrate into the host genome. Particularly, viruses in the new host with no previous major adaptation are potentially dangerous. Consequently, integration of viral nucleic acid into the host genome remains permanently. There are two types of HIV: HIV-1 and HIV-2 based on the difference in genetic characteristics and viral antigens [6]. HIV-1 was first isolated in 1983 by French scientists Francoise Barrre-Sinnoussi and others [3], whereas HIV-2 was isolated in 1986 from a western African patients with AIDS-like symptom [7]. HIV-1 accounts for most severe infection and more death than HIV-2, in this thesis only HIV-1 was studied.

The discovery of Combination Antiretroviral therapy (cART) transformed HIV-1 infection from a lethal disease into a long-term chronic disease. cART is substantially effective at reducing HIV-1 replication, transmission and the progression of HIV-1 disease by suppressing the viral replication [8]. cART can effectively reduce HIV-1 virus load to undetectable levels through disturbing the viral machinery during infection. However, HIV-1 infection cannot be completely cleared by cART suppression of viral replication and the virus reappears rapidly after treatment interruption [9]. The rebounding of HIV-1 virus is mainly due to the latent infection in some long-lived cells, especially in resting memory CD4+ T cells that harbor the latent HIV-1 reservoirs, which is composed of inactive HIV-1 DNA integrated into the genomes of these resting cells [7].

Latent HIV-1 reservoirs (LRs) is a group of HIV-1 infected cells in resting state, in which no viral particles are actively produced. It is one of the several mechanisms used by HIV-1 for its persistence within the infected host, from which the infection could be reactivated under certain conditions [11]. Though cART can eliminate cell-free viruses, it is ineffective against proviruses integrated into the host chromosome. Hence, the main obstacle for a HIV-cure is this persistence of the latent reservoir as transcriptionally silent and integrated proviruses of which 90% are non-functional [12]. Therefore, identifying the size and dynamics of the latent HIV-1 reservoir during cART and how best to target it, is crucial for understanding the barriers to curing HIV-1 infection and HIV-cure research. However, after a decade's research there are still no available surface biomarkers and proper established method to identify, detect and isolate the latent HIV-1 reservoirs. Hence, there is an urgent need for identification of new surface biomarkers and assays to characterize the latent HIV-1 reservoirs in HIV-1 infected patients.

1.1 Objectives

In this thesis work, different molecular tools and microfluidic technology were employed to explore the potential latent HIV-1 reservoir biomarker, and developing new methods to identify the latent HIV-1 reservoir.

1. **Development of a detection method for latent HIV-1 reservoir**
   There is an urgent need for sensitive and specific assays to identify and detect the latent HIV-1 reservoir containing inducible proviruses.

2. **Development of a high-throughput approach to explore and identify potential surface biomarkers for the latent HIV-1 reservoir.**
   Surface biomarkers are extremely important for in vitro latent HIV-1 reservoir research; a high throughput approach is necessary to identify the potential surface biomarkers to characterizes the latently infected cells. Furthermore, surface biomarkers can also be used to selectively target and killing of latent HIV-1 reservoir in vivo without the need for reactivation aiming towards HIV cure.

3. **Development of sample preparation devices for latent HIV-1 reservoir study.**
   CD4+ T cells, monocyte/macrophages and natural killer cells are believed to be the main source for latent HIV-1 reservoir in peripheral blood. Microfluidics has the potential to efficiently isolate cell subsets from small volumes of blood enabling latent HIV-1 reservoir research.

Above-mentioned objectives were achieved and contributed through the following studies:

1. **Optimization and evaluation of three novel molecular assays for indirect and direct characterization of latent HIV-1 reservoir**
   An antibody-profiling assay was optimized and tested for profiling the antibody against HIV-1 proteome in a well-characterized group of Swedish patients, aiming to indirectly identify the size of latent HIV-1 reservoir by the antibodies profiling signature (paper I). Furthermore, two novel signal-amplification molecular assays were optimized and investigated for the applicability of direct identification of transcription and translation competent latent HIV-1 reservoirs (Paper II).

2. **Establishment of a transcriptomics and proteomics-based analysis method for high-throughput selection of potential latent HIV-1 reservoir biomarker**
   A high-throughput transcriptomics and proteomics-based analysis method was established and applied in a well-characterized group of Swedish HIV-1 patients (paper III). Twelve different membrane protein coding gene were identified and might be used as biomarkers of latent HIV-1 reservoir identification.

3. **Development of microfluidic-based cell subsets isolation device for latent HIV-1 reservoir study**
   A microfluidics chip was developed for rapid isolation of three mononuclear leukocytes separately as single cell-subset from a small volume of whole blood. Furthermore, integration of microfluidics with the technologies developed in Paper 1 and paper II might enable accurate identification and characterization of HIV-1 reservoir at the single cell level.

1.2 Thesis Structure

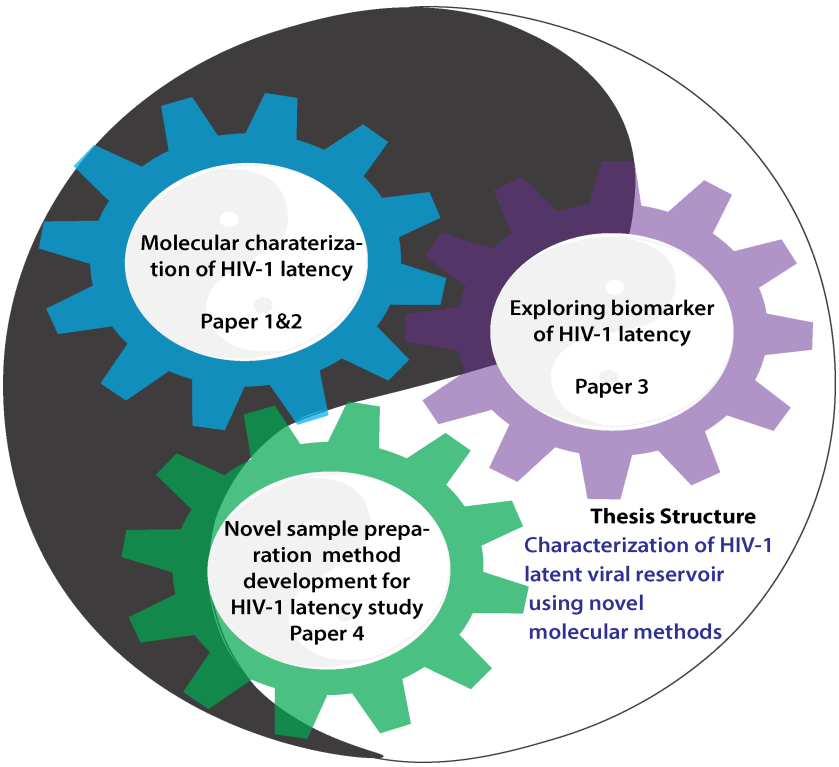

Figure 1.2: *Overview of thesis structure*

Chapter 2

Human Immunodeficiency virus-1 (HIV-1)

The HIV virus belongs to the family of Retroviridae [13], which contains non-segmented RNA genome, essentially characterized by its mode of replication. The virus replication depends on the hallmark enzyme: the reverse transcriptase that has the ability to transcribe a single-stranded RNA into a double-stranded DNA [14]. HIV can be vertically transmitted prenatally, perinatally and postnatally and horizontal transmission and can occur as a cell free virus or as infected cells [15].

HIV has a remarkable genetic diversity. It is linked to numerous errors of incorporation of nucleotides by the reverse transcriptase, as the dynamic of viral replication is intense (1 to 10 billions of viruses a day) [16] [17] [18]. There are two types of HIV: HIV-1 and HIV-2 based on the difference in genetic characteristics and viral antigens [6]. HIV-1 was first isolated in 1983 by the French scientists Francoise Barrre-Sinnoussi and others [3], whereas HIV-2 was isolated in 1986 from a western African patients with AIDS-like symptom [7]. HIV-1 accounts for a more severe infection and more HIV-related death cases than HIV-2. HIV-1 is composed of four different groups: major (M), non-major (N), outlier (O) and group P based on four independent cross-species transmissions from a simian immunodeficiency virus (SIV) [13]. As the name suggests, the HIV-1 major group is responsible for the major epidemic. This group is further phylogenetically classified into the subtypes A, B, C, D, F, G, H, J, and K as shown in Figure 2.1.

2.1 HIV-1 Structure

Electron microscopy image of mature HIV-1 particle show that the virion is spherical shape with a diameter of 80 to 120nm [19]. Infectious HIV-1 has an envelope derived from cytoplasmic membrane from the host cells as the outermost layer that carries glycoproteins (gp) spicules and structural Gag proteins, matric P17, P24

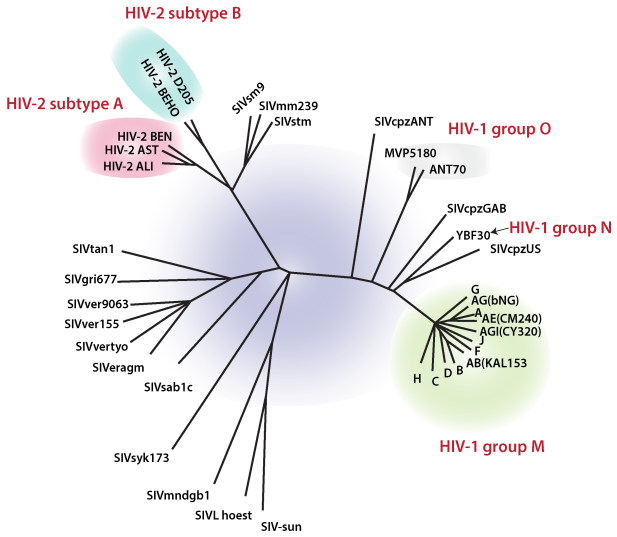

Figure 2.1: *Phylogenetic tree showing HIV-1 subgroups and distance between HIV-2 subtypes. Reproduced from [13]*

and P7 [20] as shows in Figure 2.2. Each spicule in the membrane is composed of a transmembrane glycoprotein called gp41 and surface glycoprotein gp 120. The envelope surrounds the cone shaped viral capsid, which consists of the capsid protein p24. The capsid core contains two identical single strands of RNA, which are bound to two nucleocapsid proteins (p6/p7), as well as the viral enzymes, reverse transcriptase, integrase, and viral protease. The reverse transcriptase is an RNA-dependent DNA polymerase that is characteristic for retroviruses. The integrase is necessary for the integration of proviral DNA into the host genome and the viral proteases are responsible for cleaving certain precursors polypeptides during post-translational modifications [20].

The viral genome encodes for nine open reading frames and has three structural genes: *gag, pol* and *env* [19] as depicted in Figure 2.3. *Gag* encodes the proteins for the nucleocapsid (p7), the capsid (p24) and the matrix (p17), while pol encodes reverse transcriptase, viral proteases and integrase, The *env* encodes for the envelope proteins (gp41 and gp120). The cleavage of *gag* and gag-pol polypeptide precursors is carried out by the viral proteases, whereas cleavage of the env polypeptide precursor by a cellular proteases. Additionally, the viral genome also includes accessory proteins Vif, Vpr, Nef and two regulatory genes *Tat and Rev* that plays an essential role in the pathogenicity of the virus. Further the viral protein Vpu required for virion assembly [20].

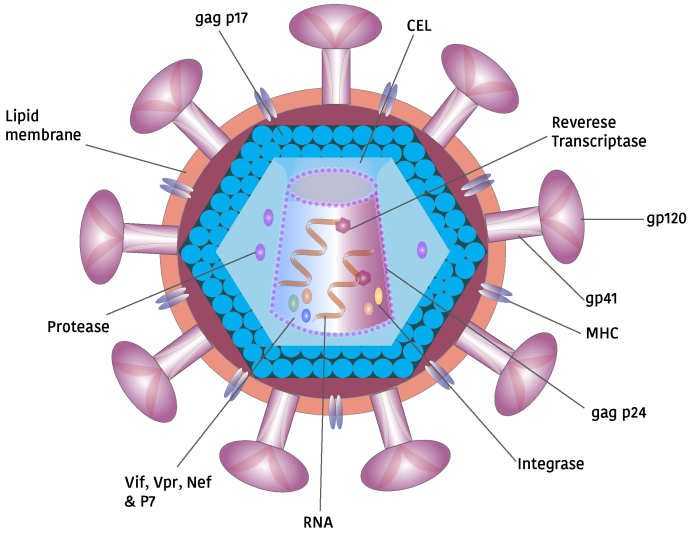

Figure 2.2: *Structure of a HIV-1 virion particle*

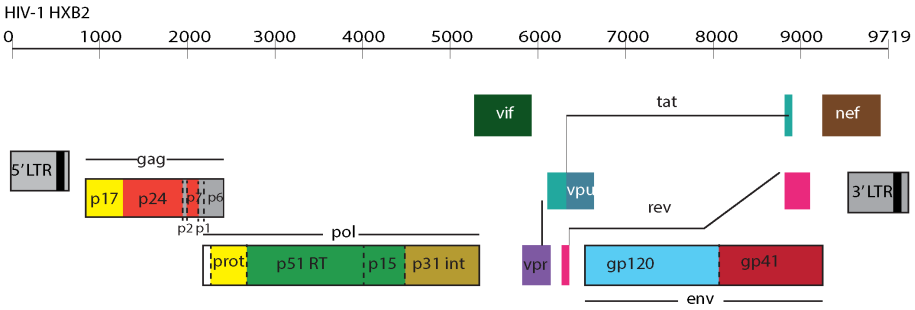

Figure 2.3: *Genomic organization of HIV-1(Ref Foley B T, Korber B T M, Leitner T K, et al. "HIV Sequence Compendium 2018", Los Alamos National Lab.(LANL), Los Alamos, NM (United States),2018.)*

2.2 HIV-1 transmission and life cycle

Sexual transmission is considered to be one of the main routes for HIV-1 transmission, either as cell-free or cell-associated in semen or mucosal surfaces. There are several other routes of transmission such as exposure to infectious blood or blood components through blood transfusion, horizontally from infected mothers to infants or by shared use of needles for drug injections [22]. Short viral generation time and error prone HIV-1 reverse transcriptase increase the probability of genetic changes resulting in extremely diverse viral populations within and between HIV-1

infected individuals [22].

When the route of infection is through sexual transmission, it is unknown which HIV-1 phenotype will be selected as a transmitter virus. However, the diversity of HIV-1 is decreased when the infection occurs via blood. The recipient's mucosal membrane might act as a bottleneck for the entry of the virions or the infected cells that enter the recipient's genital organ [23]. It is, however, unknown how the early events after the HIV transmission occur in humans. Studies from SIV about mode of early transmission provides a role of partially activated CD4+ T cells of the recipient's genital mucosa [23] as shown in Figure 2.4.

HIV-1 might gain access to the recipient's immune system with the assistance and protection of the dendritic cells, langerhans cells and macrophages. The virus crosses the epithelial layer [22] as shown in Figure 2.4 and binds to and enters memory CD4+ T cells, its main target cell, where it initiates HIV replication.

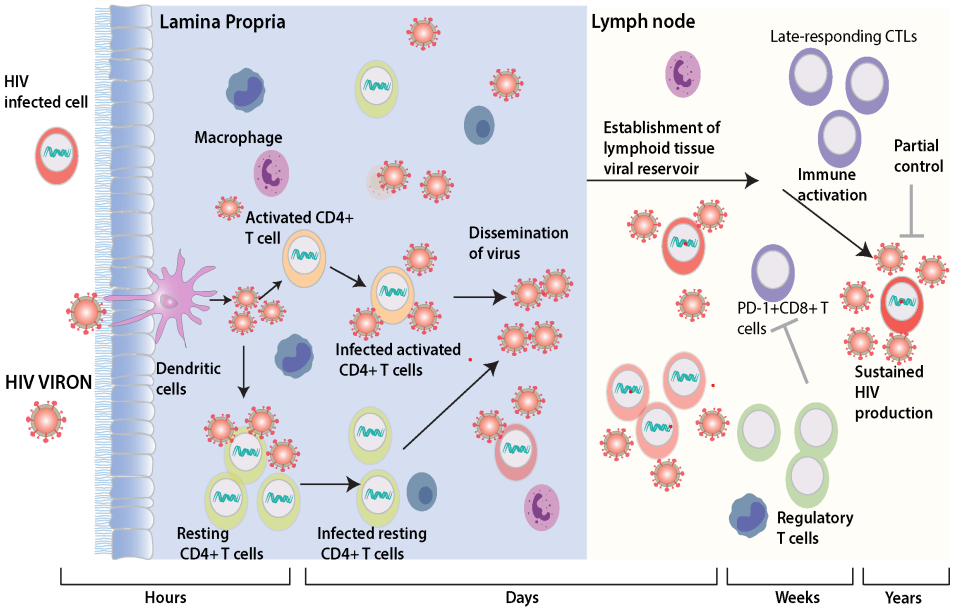

Figure 2.4: *Phases of infection following exposure to HIV-1 virus. Reproduced from [23]*

2.3 HIV-1 replication

The replication cycle includeds different steps as shown in Figure 2.5.

i) Fixation and penetration of the virus: Entry of HIV-1 into CD4+ T cells starts at the early phase of replication and requires a high affinity interaction between one of the viral Env glycoprotein (gp120) to the CD4+ T cell surface proteins CD4 and chemokine receptors CCR5, CXCR6 or CXCR4 [24] [25]. Successful binding will trigger the cascade of reactions shown in Figure2.5 that results in conformation changes of several proteins. The virus to cell interactions leads to the the fusion of virus and cell membranes, which will result in releasing of the viral content into the cell [24].

ii) Decapsidation and eclipse: After decapsidation, based on the state and maturity of the infected T cells, the reverse transcriptase transcribes the viral RNA into double stranded DNA. Thereafter, newly synthesized viral DNA, called proviral DNA, is transported into the nucleus as a pre-integration complex composed of viral proteins and cellular protein. It is then integrated into the host genome by viral integrase. In the late phase of replication, the neogenome is then transcribed into pre-messenger RNA by the transcription machinery of the host cells. The pre-messenger RNA matures and is spliced to become the messenger RNA, which then migrates into the cytoplasm to be translated into polypeptide precursors [20] [26].

iii) Morphogenesis and release of new viral particles: The first viral polypeptide precursors (gag, env) are cleaved by viral protease and become functional structural and enzymatic viral proteins, also env polypeptide precursors, the origin of envelope viral proteins, are subsequently cleaved by cellular proteases. All these elements are assembled before the new virus particles are released into the extracellular medium by budding [27]. Different stages of replication cycles are under the control of cellular and viral factors and in particular of HIV regulatory proteins rev and tat.

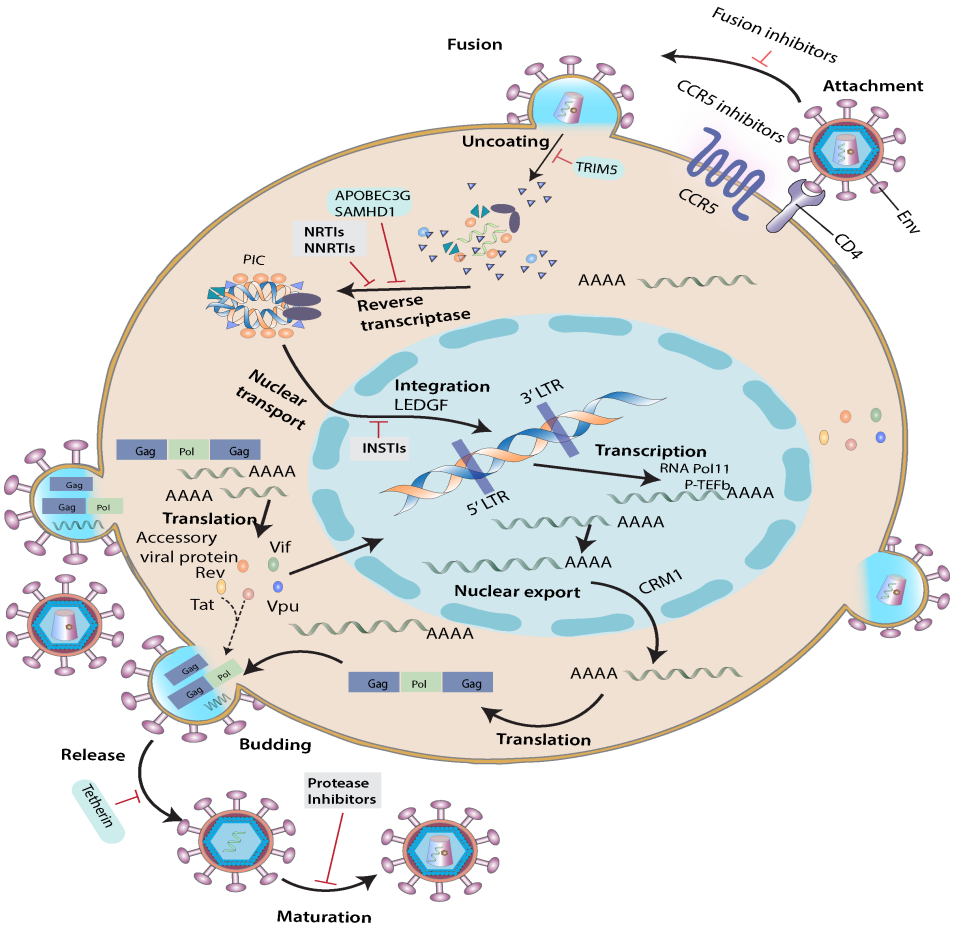

Figure 2.5: *Life cycle of HIV-1 infection to CD4 T cell. Reproduced from [27]*

2.4 Clinical and biological evolution of HIV-1 infection

In the absence of treatment, the HIV-1 infection progressively evolves. Three phases can be distinguished during the process of progression: primary infection, clinical latency or asymptomatic disease, and AIDS.

i) The primary infection phase: Primary infection is the period of invasion of the virus into the recipient body. The viral replication is intense, the latent HIV-1 viral reservoir is constituted and an antiviral immune response appears gradually. Clinically, the symptoms occur on average two to four weeks after exposure. All of these symptoms define the primary infection syndrome.

A persistent acute viral syndrome is, most often described as when a patient presents with fever, myalgia and arthralgia and poly-lymphadenopathy, mainly cervical, axillary and inguinal. Cutaneous manifestations (angina, pharyngitis, oral or genital ulcers) and digestive symptoms (diarrhea) are frequently associated with primary HIV-1 infection. More rarely neurological symptoms (meningo encephalitis, lymphocytic meningitis, mononeuropathy) occur [28].

Most of these symptoms gradually disappear; the lymphadenopathies can still persist for weeks or even months. Biologically, primary infection syndrome is accompanied by hematological abnormalities: thrombocytopenia, leukopenia, and neutropenia. Hyperlymphocytosis, a mononucleosis syndrome or early lymphopenia can be demonstrated. Hepatocytes anomalies,that mark asymptomatic and anicteric cytolysis of the liver and can be revealed by a moderate elevation of transaminases may also occur [28].

ii) Latency phase: The latency phase follows after phase of infection and is paucisymptomatic or asymptomatic. This clinical latency masks the virological evolution with persistence of infected lymphocytes, attenuated viral replication but constant presence of viruses in the blood. Clinically, cutaneo-mucous signs (shingles, warts, condyloma) can be observed [29].

iii) AIDS phase: The ultimate phase of HIV-1 infection is the acquisition of immunodeficiency syndrome. It is defined by all the opportunistic pathologies (infections and tumors) related to HIV-1-induced immunosuppression. Recurrent oral or genital candidias), digestive (chronic diarrhea) or general symptoms (alteration of the general state, abundant night sweats, feverishness etc) can sometimes appear or reappear.

2.5 HIV-1 Diagnosis

Clinical signs present in approximately 50% of patients with primary HIV-1 infection as the consequences of activation of inflammation. They appear most often within 10 to 15 days, and are similar to a pseudo syndrome that may persist for up to 10 weeks after infection [30]. Nevertheless, clinical presentations depends on individual variation and the intensity of symptoms is correlated with the HIV-1 RNA burden [31]. The level of HIV-1 DNA in peripheral blood mononuclear cells (PBMCs) is inversely correlated with the number of CD4+ T cells [32]. The severity of the symptoms is correlated with the rapid evolution of the pathology. Rapid HIV-1 infection is predicted by high level of HIV-1-RNA, HIV-1-DNA and low primary CD4+ T cell counts [33]. Progressively, the intensity of the immune reaction and the viral multiplication attenuate gradually and an immune-virological equilibrium state is established to lead to a latent biological and clinical phase [34].

The Enzyme-Linked Immuno Sorbent Assay (ELISA) is remain the reference method for HIV-1 detection and are based on solid phase (beads or microplate wells) on which antigens are adsorbed for HIV 1 and 2. The so-called 4th generation ELISA tests allow the detection of antibodies and also P24 antigen enabling earlier detection of the infection (after 4 days on average). Rapid Orientation and Diagnostic Tests (RODT) are recommended for screening for HIV-1 infection. There are different tests (such as immunochromatography, agglutination) that can be performed on different biological fluids (plasma, whole blood, saliva). However, they are less sensitive than the combined ELISA tests in the case of sampling during the seroconversion phase and their performance is lower on a salivary specimen [35].

In case of a positive ELISA test, a confirmation test will be performed. A second blood test (ELISA) must then be performed. The combined tests may be positive after 15 days infection by detection of p24 antigen and will require serological testing by ELISA 15 days later. The confirmatory test is either an immunoblot or a western blot (prior fractionation of viral proteins, denaturing electrophoresis on polyacrylamide gel and transfer on a support type nylon, nitrocellulose and revelations with labeled specific antibodies), which are more specific than the ELISA. This makes it possible to detect antibodies directed against different constitutive proteins of HIV-1 or HIV-2. A result is positive, if at least two bands are found among the envelope glycoproteins (glycoproteins, gp41, gp120, gp160). The presence of these anti-envelope protein antibodies may or may not be associated with antibodies directed against the gag and / or pol gene proteins. If the Western blot is negative or indeterminate additional analyzes can be performed in case of suspicion of primary infection [36].

Besides, p24 antigen is a viral replication marker that can be detected early by a specific test, two to three weeks after the infection and can detect HIV-1 primary infection in the case of western blot negative or indeterminate western blot result. The quantification of viral RNA by polymerase chain reaction (PCR) in real time can detect the presence of virus as early as seven to ten days after infection. The kinetics of appearance of the antibodies, coupled with the quantification of HIV-1-RNA and / or HIV-1-DNA (especially in the newborn) [36] as well as quantification of the p24 antigen made it possible to define criteria of biological diagnosis of primary infection with classification.

Furthermore, acute infection can be defined by the presence of a negative or weakly positive HIV-1 ELISA (3rd or 4th generation), with western-blot HIV-1 negative or incomplete ($\leq$1 antibody) and a positive RNA-HIV and / or p24 positive antigen (I, II, III stages). A recent infection can be defined by HIV1-positive ELISA test with incomplete HIV-1 Western blot ($\geq$2 and $<$5 antibodies with presence of anti-p24 antibody, associated with the anti-gp160 or anti-gp120 antibody or anti-gp41) and a positive RNA-HIV load (IV, V stages) [36]

Chapter 3

Latent HIV-1 Reservoir

At present, across the world, more than 35 million people are infected with HIV-1. Thanks to the development of antiretroviral therapy (ART), the plasma viral loads in infected individuals could be reduced to undetectable levels, and thus allowing treated patients to have an average life expectancy [37]. Nevertheless, complete cure of HIV-1 is still not achieve. If the ART is stopped, viremia will rebind within a few weeks, even in long-term ART-treated patients.

The primary mechanisms that hinder HIV-1 eradication is the presence of and residual replication of virus from a latent HIV-1 reservoir [38]. HIV-1 latency can be defined as individual on treatment with ART for HIV-1 carrying subpopulation of infected cells containing replication-competent HIV-1. Presently, latent HIV-1 reservoir has been detected in blood [39], lymph nodes [40], brain [41], and other immune-privileged sites within different cell subsets [42], while the most well-studied latent HIV-1 reservoir exists in resting memory CD4+ T cells [43] [44].

3.1 Molecular basis of latent HIV-1 reservoir

As mentioned earlier the HIV-1 provirus mainly depends on the host cellular machinery for production of virions. Therefore, the viral genome preferentially integrates into the transcriptionally active region in the host genome [45]. However, there are mechanisms down-regulating the expression of specific viral genes that might intervene in the establishment and maintenance of viral latency. These types of mechanisms can be divided in three groups [46] ,latency (trans acting effect) regulating factors, viral gene deficiencies (cis-acting effector) factors or a combination of these factors as shown in Figure 3.1.

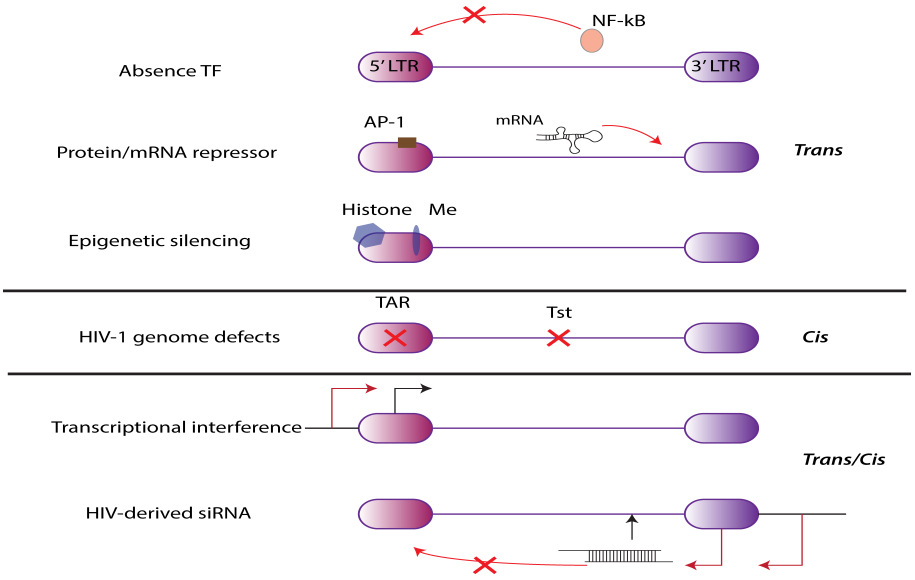

Figure 3.1: *Potential mechanisms of the latent HIV-1 reservoir at the molecular level. Reproduced from [48]*

The Trans acting effect is an important mechanism for maintenance and establishment of the latent HIV-1 reservoir, It is mainly regulated by the activation state of the cell, availability and accessibility of transcription factors together with signaling from environmental clues. Compaction of chromatin in nucleosomes opposite to the viral promoter prevents the transcription factor access to the viral promoter site. These changes are related to the state of methylation of polycomb protein group [47], sumoylation, ubiquitinylation, phosphorylation and acetylation. Chromatin in the quiescent cells blocks the transcription in the non-acetylated form. During activation of a cell, the chromatin opens up allowing easier accessibility for transcription factors [48].

According to the state of cell activation there will be variation in the level of transcription factors NF-kappa Beta and SP1, and these levels may be insufficient to initiate the transcription at the promoter level [46]. In unactivated T-cells, these are predominantly intra cytoplasmic.

The Cis acting effects mainly depend on the integration site of the provirus, chromatin structure and the genome environment. Latency can be induced by mutation in the viral genes including the tran-activating response element (tar) gene. Moreover, tat protein is necessary for the elongation phase, hence the transcription of HIV-1 integrated genome might be prevented by the mutations in the tar gene [49].

The Cis/Trans acting effect plays an important role in integration and maintenance of latency by intervening transcription depending on the polarity of virus integration. When the virus integrated in the same orientation as the cellular genes, the weaker promoter will exhibit a decrease in activation. Another mechanism is the overexpression of miRNA capable of preventing the translation of tat or rev protein in quiescent host CD4 T cells [50]. Maintenance of viral latency results from complex intracellular mechanisms that are regulating the expression and transcription of the viral genome. Maintenance of the long-lived reservoir is one of the key factors responsible for persistent residual replication and progressive destruction of CD4 T cell. This is one of the major obstacles for eradicating the body of HIV-1 from the body [51].

3.2 Dynamics of circulating lymphocyte subpopulations

After the early HIV-1 infection, latent HIV-1 reservoir might be establised in CD4+ T cells. Majority of theses cells die shortly after infection due to virus-mediated cytotoxicity or immune-mediated killing. Nevertheless, a small part of infected CD4+ T cells are capable of reversing to a resting memory state, and become silent in producing viral particles. Since they have limited virus transcription and viral proteins expressing, these cells are missed by the immune system or ART treatment, and they can maintain or expand through cell proliferation of the infected cells that still retains the proliferation ability. Therefore, these resting memory CD4+ T cells are widely recognized as the main cellular latent HIV-1 reservoir nuturing latent provirus [44].

The presence of subpopulations of memory lymphocytes represents a high level of infection and makes important supplement to the latent HIV-1 reservoir. However infected naive or effector T cells have low frequency of infection and contribute less [52]. In addition, lower antigenic stimulation, long half-life and proliferation capacity plays a key role in maintaining the reservoir for long term [53]. Low infection of effector T cells by R5 tropism virus might be due to weak expression of these receptors on the T effector cell surface [54].

In untreated chronic patients, there will be a reduction in effector T cells and inferior to uninfected patients, which may be explained by progressive fibrosis of lymphoid tissues [55]. It has been reported that effector T cell lymphocytes contribute poorly to low-frequency reservoir of infection [56], and play a vital role in maintaining homeostasis in congenital SIV-infected monkeys [57]. Higher levels of infection was observed in central memory CD4+ T lymphocytes than in the memory effectors predominantly represent the reservoirs [58]. It has been shown in chronic patients under treatment that the viral load was undetectable, but the frequency of infection of memory, central, transitional and effector subpopulation cells were equivalent with a dominant contribution of effector cells in homeostatic

proliferation [59].

Moreover, in case of long term non-progressor patients central memory cells are protected from infection and transitional subpopulation of cells contributes for most to the reservoir. Also, the preservation of the number of central memory T cells (TCMs), seems to be an ineffective element in the control of the viral infection.

3.3 Other cells involved in the latent HIV-1 reservoir

Existence of a latent HIV-1 reservoir in other cells than the quiescent CD4+ T cells has been reported in treated patients with residual viral load. This is partly related in virus production from other cells than CD4+ T cells [60].

Monocytes

Monocyte precursors of macrophages express the CD4 and CCR5 / CXCR4 receptors of HIV-1 and can be infected with the HIV-1 Virus. They are highly resistant to the cytopathic effects of viruses and antiretrovirals do not easily enter these cells [61]. It has been reported that the integrated HIV-DNA has been detected in the monocyte cells of HIV-1 infected patients [62]. Their ability to produce virus has also been demonstrated in patients with AIDS and 10% of macrophages can be infected. In the chronic patient, they contribute less than 1% to peripheral blood HIV-1 DNA [42] and the viral clones are phylogenetically distinct from those present in CD4+ T cells [63]. Their short half-life of a few days before their macrophage differentiation and their significant turnover of macrophages suggest that these cells represent a temporary reservoir of virus but are important because of their tissue dissemination [64]. Macrophages can persist for years in compartments such as the central nervous system and concentrate particularly in the mucosa of the gastrointestinal tract [64]. Macrophages are very migratory species, which gives them an important role in maintaining the reservoir.

Dendritic cells

Dendritic cells that express low levels of elective receptor for HIV-1 CXCR4, CD4 and CCR5 can capture the virus on their cell surface through surface lectin presented on the cells. There are different types of dendritic cells, i.e. myeloid cells that are present in ganglia, thymus, lympnodes and blood. Plasmacytoid dendritic cells (PDCs) located in the mucousal membrane can be infected and produce virus. Follicular dendritic cells, despite their short half-life have a high migratory property and can spread the infection rapidly thus, playing a dynamic role for the latent HIV-1 reservoir [65].

Natural Killer cells

Natural Killer (NK) cells have been poorly studied during HIV-1 infection [48]. A small fraction of NK cells express the CD4 receptor on their cell surface [67]. Longitudinal analysis of HIV-1 DNA in NK cells have indicated that NK cells can become infected. However, another study found no evidence of NK cells infection after 7 years of ART. The involvement of NK cells in latent HIV-1 reservoir persistence remains to be elucidated [68].

B lymphocytes Some studies have shown evidence that B cells can become infected in vitro if they express low levels of CD4 and CXCR4. Using a similar mechanism as dendritic cells, B cells can capture the HIV-1 virus on their cell surface and transfect CD4 T cell. The role of B lymphocytes and their role in establishment of the HIV-1 reservoir remains unresolved [22].

In tissues

One of the major anatomical HIV-1 reservoirs is the lymphoid tissue, comprising up to 98% of concentrated lymphocytes. CD4+ T cells are mainly found in the secondary lymphoid organs. Basal ganglia play a vital role in the amplification of virus considered as major reservoirs of HIV-1. Gut associated lymphoid tissue (GALT) is another main reservoir composed of secondary lymphoid structures such as Peyer's patch and it contains macrophages, dendritic cells, plasma cells and 40 to 60% of the lymphocytes. Here, HIV-1 has access to a dense population of CD4+ T cells that are more susceptible to infection than circulating lymphocytes by the expression of CCR5 or CXCR4 co-receptors of HIV-1. Intestinal CD4 cells are therefore particular targets of HIV-1 being activated almost permanently because of their exposure to a multitude of antigens. This allows for a potential spread of the infection at the systemic level [69].

In humans, a 10-fold higher HIV-1 RNA burden has been reported in lymphoid organs compared to peripheral blood. In treated chronic patients biopsies showed 3 times higher infection in duodenum and up to 10 times higher in the rectum than in PMBCs [70] [49]. Moreover, the viral clone compartmentalization is different from rectal colnes linked to viral replication in GALT [71] and plasma viral clones. Therefore, GALT contributes to long-term viral production and remains an important reservoir. The genital compartment is another source for persistence of the virus. HIV-1 is found as free particle in the seminal fluid and embedded in the infected leukocytes and can easily be detected in untreated patient. Even in the female genital tract, HIV-1 is presented as free or integrated virus. It has been shown that in 5 to 10% of patients the viral particle can persist in sperm after 6 month even with effective ART, suggesting that the genital compartments probably are a distinct reservoir site [72].

A pathological finding from autopsies of the AIDS patient shows the presence of virus in the central nervous system (CNS). In CNS several cell types can express the CCR5 and CD4 receptors e.g. astrocytes, perivascular macrophages and activated glial cells [73]. How macrophages and glial cells contributes to the latent HIV-1 reservoir is not well-studied [74]. Phylogenetic analyses revealed compartmentalization in macrophages and brain astrocytes of clones different from circulating ones [75]. These clones were found in the meninges suggesting the production of virons capable of migrating out of the brain brain [76]. Also, the poor penetration of anti-retrovirals into cerebral parenchyma allows the viral protection and acts as a reservoir.

3.4 Latent HIV-1 reservoir detection methods

Accurate quantification of the latent HIV-1 reservoir in HIV-1-infected individuals is required for evaluating HIV-1 eradication strategies and for deciding on whether and when ART interruption is legitimate. However, quantifying the size of the latent HIV-1 reservoir is extremely challenging due to its rarity (only 10–100 in 10^6 of CD4+ T cells) and lacking detectable surface biomarkers [12], [77]. To date, many molecules related to different stages of the HIV-1 replication cycle and the response of the host's immune system have been used as the biomarker for the latent HIV-1 reservoir detection, and the corresponding detection methods have been developed to quantitate the latent HIV-1 reservoir in HIV-infected individuals after long-term ART therapy (Figure 3.2).

3.5 Assays for Measuring the HIV-1 Latent reservoir

3.5.1 Viral load rebounding time

Typically, after cessation of ART, the HIV-1 virus will start to replicate again from the latent HIV-1 reservoir and release to the circulatory system within just a few weeks. Therefore, the time from ART interruption to viral load reach to detection limit again was believed reflecting the size of latent HIV-1 reservoir [78]. The two most famous examples were the 'Boston patients' [79] and the 'Mississippi baby' [80]. The 'Boston patients' were two HIV-1 infected patients on ART treatment received stem cell transplants from a CCR5 wild-type donors. They had an undetectable level of HIV-1 DNA after successful transplants, hence they were believed to have a smaller latent HIV-1 reservoir than normal treated patients. Consequently, these two patients kept the viral load under detection limit for up to 8 months after stopping ART, while it usually was just few weeks for normal patients [79]. In the same way, the 'Mississippi baby,' who was given 18 months of ART treatment as early as 30 hours after birth from her HIV-1 positive mother, enjoyed an even longer non-viremia time (27 months) without ART due to much smaller HIV-1 latent reservoir size [80].

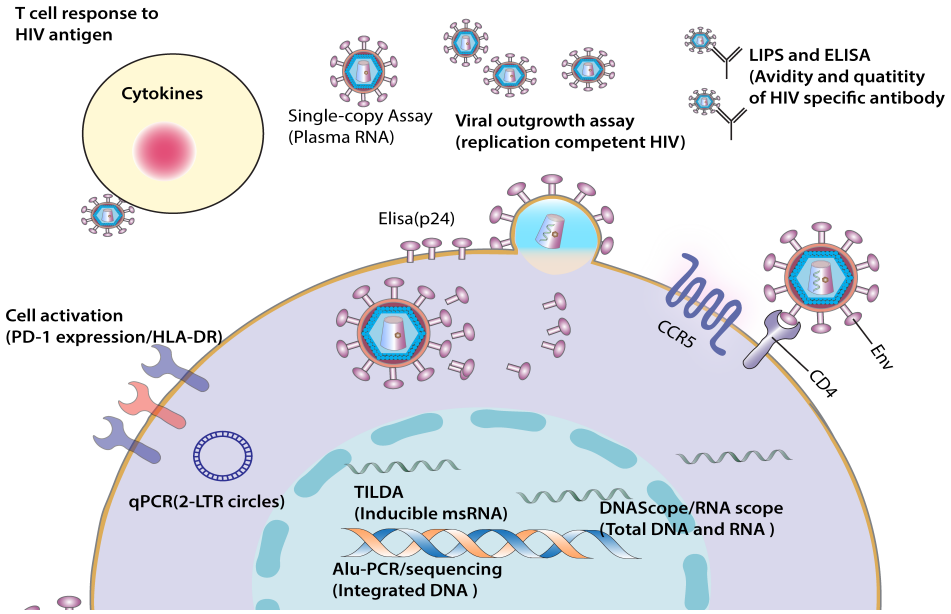

Figure 3.2: *Many molecular related to different stages of the HIV-1 replication cycle and the response of the host's immune system were used as the biomarkers for latent HIV-1 reservoir detection and the corresponding detection method were developed to quantitate the latent HIV-1 reservoir in HIV-infected individuals after long-term ART therapy. Reproduced from [11]*

However, there are not only method performance related problems but also ethical issues need to be carefully considered before using this method, although treatment interruption seems a simple way to estimate the size of latent HIV-1 reservoir. The first limitation is the enormous variability in time to rebound. For example, stochastic viremia rebound might happen if the latent HIV-1 reservoir is decreased dramaticlly in size but not completely cleared, resulting in unpredictable rebounding time after ART interruption [81]. Drug resistance is another drawback since it can occur if the drug exists in the body at suboptimal drug concentrations for a significant period [82]. As a consequence, compared to those on continuous ART, the patients who interrupte ART treatment might have higher levels of morbidity, opportunistic diseases, and malignancies [83]. Therefore, treatment interruption is problematic in measuring latent HIV-1 reservoir size.

3.5.2 Viral outgrowth assay

The quantitative viral outgrowth assay (QVOA) (Figure 3.3) was the first assay developed to identify and detect the size of the latent HIV-1 reservoir by measuring

the frequency of cells that containing the replication-competent virus. To date, it is still considered the gold standard for latent HIV-1 reservoir measurement [84]. QVOA typically starts with purifying the resting CD4+ T cells by density gradient centrifugation and negative selection from HIV-1 infected individuals on ART. After isolation, the resting CD4+ T cells are diluted in fivefold serial and stimulated with phytohemagglutinin (PHA), followed by adding irradiated lymphoblasts from the healthy donor to amplify virus replication. After 14 days incubation, the positive wells are determined by detecting HIV-1 p24 antigen in the supernatant of each well by ELISA. Finally, the frequency of cells containing the replication-competent virus in initial sample is estimated based on maximum likelihood methods and expressed as infectious unit per million (IUPM) [84].

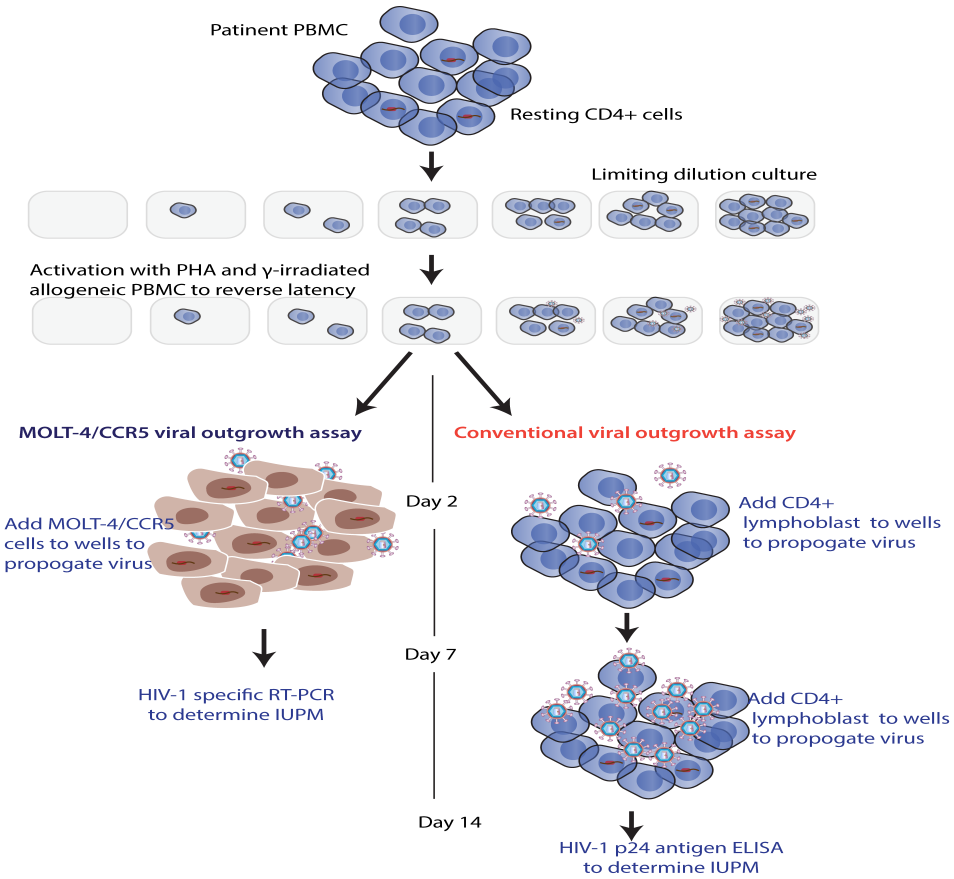

Figure 3.3: *The workflow of standard QVOA and the MOLT-4/CCR5 VOA. Reproduced from [86]*

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The QVOA has several advantages; the top one is the capacity to measure individual latently infected cells that harbor the replication-competent virus. Another advantage is the released viruses in positive wells can be isolated and characterized by sequencing and functional studies [38]. By utilizing QVOA, several studies successfully revealed the frequency of latent HIV-1 reservoir in the blood of HIV-1 infected patients on ART were about one latently infected cell per million CD4+ T cells.

However, from the view of feasibility, QVOA is still far from being widely applied. The most notable, QVOA tends to underestimate the size of by 60-300 fold, resulting from the inefficiency of a single round of PHA stimulation to reactivate all intact provirus. The studies by Ho et al. [12]] showed that 98.5% of the viral reservoir was not stimulated by one round activation, and 10–12% of non-induced proviruses contain intact genomes and are potentially replication competent. Thus, QVOA can only give a minimal estimation of the size of latent HIV-1 reservoir and relies on the ability of latency-reversing agents (LRAs) to efficiently induce viral expression from the latent HIV-1 reservoir [12]] [85]. Other reasons that hinder QVOA translating from lab to clinic include intensive labor work and require a large volumes of blood (120ml) and a longtime culture in a BSL3 lab (2 weeks), which are not easy to get access.

Recently, several modifications of this assay have been reported. One of them is using MOLT-4/CCR5 cells [86] (Figure3.3) or TZM-bl cell line [87] to replace the donor cells for viral expansion. By this method, the volume of blood sample required is decreased as well as culture time, while no significant difference or higher sensitivity was observed on detected latent HIV-1 reservoir when compared with QVOA. Furthermore, ELISA-based P24 protein detection after 14 days can be replaced by quantitative PCR (qPCR) based HIV-1 RNA detection after seven days, which can be used to reduce the total time [87].

Recently, another variant of QVOA using humanized mice (MVOA) was reported, and it was also the first in vivo model, in which it was able to detect the latent HIV-1 reservoir, enabling prediction of viral rebound time without harming the patient status [88]. In this assay, up to 60 million isolated CD4+ T cells/PBMCs from HIV-1 positive patients can be injected into one or several humanized mice, followed by HIV-1 RNA monitoring with RT-PCR [88]. Pate et al. studies shows that HIV-1 could be detected in mice after injecting cells isolated from participants on ART, who have a viral loads under detection limit (<50 copies/ml). Hence, MVOA is a relatively simple and sensitive tool for determining whether the latent HIV-1 reservoir harbor the replication competent virus is present or not. However, the MVOA assay cannot give the quantitative information of latent HIV-1 reservoir, and it has a high requirement for experiment condition (such as BSL3 animal facility, animal handling and trained presonal), making it not sutiable for clinics.

3.5.3 Monitoring of the HIV-1 DNA

In the HIV-1 replication cycle, there are three forms of cell-associated HIV-1 DNA created that can be targeted individually or totally to estimate the size of latent HIV-1 reservoir in HIV-1 infected patients, including integrated HIV-1 DNA, unintegrated linear HIV-1 DNA and 2-Long terminal repeat (2-LTR) DNA circles [88]. PCR-based assays are commonly used to identify or quantify each HIV-1 DNA form [89]. Most of the tests depend on the quantitative real-time PCR, while some new methods have been introduced recently based on the targeted forms, such as droplet digital PCR (ddPCR) for absolute quantification of HIV-1 total DNA [90] and Alu-PCR for accurate measuring the integrated HIV-1 DNA [91].

So far, these approaches have been widely applied in various immune cell types and tissue biopsies [92], which provide a quicker and simpler way to study viral persistence, and have become a complementary method to the QVOA.

The most common target for PCR-based latent HIV-1 reservoir detection is the total HIV-1 DNA in isolated immune cells or tissue [92]. The estimated frequency of cells harboring HIV-1 DNA can be calculated by combining the total HIV-1 DNA copy number with the total number of cells present. In blood, one study showed that the central memory CD4+T cells contributed most of the total pool of HIV-1-infected cells, by using real-time PCR for the assessment of total HIV-1 DNA [39]. Within tissues, quantification of total HIV-1 DNA further showed residual HIV-1 infection in the gut and rectum of patients under ART [92]. Recently, droplet digital PCR was introduced and compared with traditional quantitative RT-PCR in monitoring of total HIV-1 DNA in patients on ART. The result of this study showed that the signal to noise ratio was increased by droplet digital PCR and the limit of detection was about three copies per million cells. Furthermore, ddPCR proved to be more robust in targeting HIV-1 sequence variations [90].

Though the total HIV-1 DNA-based method is simple and robust, it cannot discriminate between integrative and non-integrative forms of HIV-1 DNA. Stevenson et al. showed that in resting CD4+ T cells from viremic patients, the majority of the HIV-1 DNA is linearly unintegrated, and are the end products of reverse transcription before integration [93]. Furthermore, it was shown by several studies that the linear unintegrated HIV-1 DNA is labile and replication-defective [94] [95]. Thus, cells with un-integrated HIV-1 DNA should not be included as latent reservoir.

Except the linear unintegrated HIV-1 DNA, 2-LTR and 1-LTR should also not be considered as part of the LR. 2-LTR and 1-LTR normally generated when linear unintegrated HIV-1 DNA fail to integrate within the cell, followed by nonhomologous or homologous end joining between the LTRs [97]. These two HIV-1 DNA forms cannot integrate into host genome and produce infectious virus, hence they

are normally not being considered as biomarkers of the LR, although 2-LTR circles have been used as a biomarker for recent infection or ongoing replication because of instability [98].

In order to solve the interference problem between linear unintegrated HIV-1 DNA and 2-LTR HIV-1 DNA, Alu-PCR (Figure3.4) has been developed to distinguish the integrated HIV-1 DNA from unintegrated genomes. In the first PCR step, integrated HIV-1 genomes was selectively amplified by using one human genome specific Alu elements primer along with another HIV-1-specific primer that bind to the gag region. After this primary amplification, a second nested real-time PCR is then applied by targeting the HIV-1 long terminal repeat (LTR). Finally, with the help of the control which excludes the Alu primer, the detected signal can then be demonstrated whether coming from integrated HIV-1 DNA or not [99].

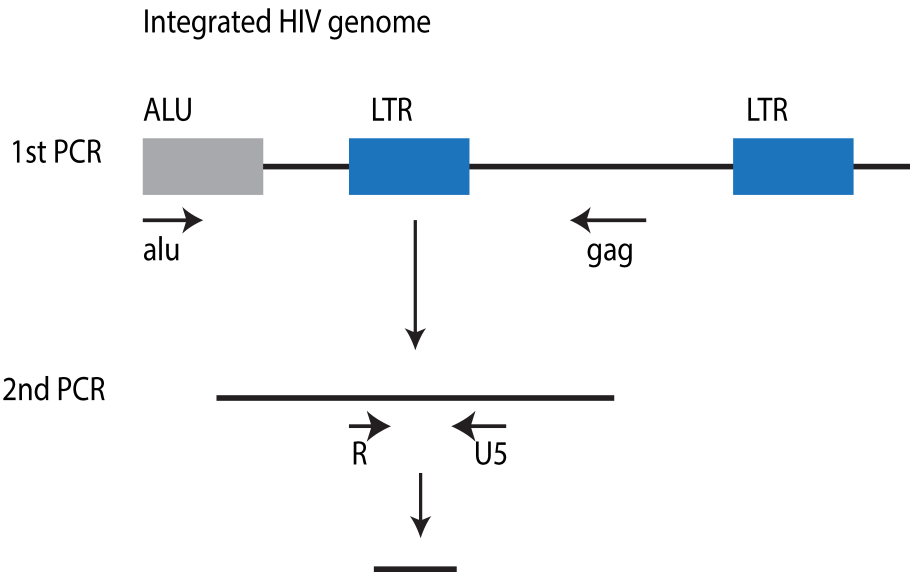

Figure 3.4: *Alu-PCR principle. Reproduce from bitesizebio.com*

Recently, Eriksson et al. compared many LR detection methods and found there was a strong correlation between the result of integrated HIV-1 DNA by using Alu PCR and total HIV-1 DNA by adopting ddPCR in patients on suppressive ART, showing that most of the HIV-1 DNA in the integrated form in the ART patients [100]. This finding is further supported by others studies, showing that in patients on suppressive ART, low levels of unintegrated HIV-1 DNAs are typically found [101]. However, a recent full-length characterization of the integrated HIV-1 DNA revealed that more than 88% of such genomes were defective and unable to produce replication competent virus [12]. Therefore, though Alu-PCR can provide

more accurate latent HIV-1 reservoir size measurement by targeting integrated HIV-1 DNA, but real size of the latent HIV-1 reservoir is overestimated much higher compared to QVOA [100], resulting from quantifying both cells containing replication competent and defective virus.

3.5.4 Testing of residual viremia

HIV-1 residual viremia can still be measured in the plasma of ART treated patients using the single-copy assay (SCA). Although ART can effectively suppress the viral load to below the detection limit of clinical methods [102]. SCA is a highly sensitive real-time RT-PCR based assay that uses primers targeted at a conserved region of HIV-1 integrase and gag. It can detect as few as 1 copy of HIV-1 RNA in 1ml of plasma [103].

Several previous studies analyzed the residual viremia by sequencing and confirmed that the residual viremia mainly resulted from the virus production from an established reservoirs [104]. In this sense, it might be able to be used as a measure of the latent HIV-1 reservoir. However, the relationship between residual viremia and the latent HIV-1 reservoir is complex and still unclear [38]. Furthermore, SCA cannot distinguish the latent HIV-1 reservoir and replication-competent virus. Therefore, the SCA method may not accurately reflect the dynamic of the latent HIV-1 reservoir in patients on ART therapy, though it is a powerful tool to measure the residual viremia in plasma.

3.5.5 Measurement of the immune response

The host immune response to HIV-1 infection, especially the antibodies targeting different HIV-1 antigens, can also be used as potential biomarkers to reflect the latent HIV-1 reservoir size. A recent study reported that the anti-HIV-1 antibody assay "luciferase immuno-precipitation systems (LIPS)" could exticate the HIV-1 infected individuals harboring different sizes of the latent HIV-1 reservoir. They performed the quantitative humoral profiling of the presumably "cured" Berlin patient, who has no or very limited latent HIV-1 reservoir, and the result revealed that there were no antibodies against HIV-1 p24, matrix, nucleocapsid, integrase, protease, and gp120, while low levels of antibodies against reverse transcriptase, tat, and gp41 were found [105]. In contrast, the antibody levels persisted in most well treated patients, while a small number untreated elite controllers (EC) had a similar antibody pattern as the Berlin patient [105]. Furthermore, this finding were supported by another study, in which it is shown that the anti-HIV-1 antibody level decline to an undetectable level by immunoassay in primary HIV-1 infection (PHI) treated patients [106]. Therefore, the levels of HIV-1 antigen-specific antibodies may be a surrogate biomarker of the latent HIV-1 reservoir size. However, further validation is required.

3.5.6 Detection of the cell-associated HIV-1 mRNA

In the HIV-1 life cycle, integrated HIV-1 DNA can transcripts to cell-associated multiply spliced HIV-1 mRNA (msRNA) and the unspliced HIV-1 mRNA (usRNA) in nucleus. The former one encoding the viral accessory proteins during early infection, such as *Tat, Rev* and *Nef*, while the latter one encoding the viral structural proteins at later stages of the infection, including the Gag–Pol precursor protein. With the help of *Rev* all cell-associated HIV-1 mRNA are then exported into the cytoplasm, where translation, assembly and release of the new viral particle from the cells [107] (Figure3.5). Recently, in a comapartive study it has been shown that virion release with intracellular mRNA measurements after stimulation, the result revealed that only 1.5% of proviruses could be reactivated to produce HIV-1 virions (replication-competent latent HIV-1 reservoir), while 6.8% and 8.2% of proviruses from two different patients could be reactivated to produce cell-associated mRNA (translation-competent LR) [85]. Hence, cell-associated viral mRNA can be considered as a more sensitive and accurate biomarker for latent HIV-1 reservoir detection than others biomarkers.

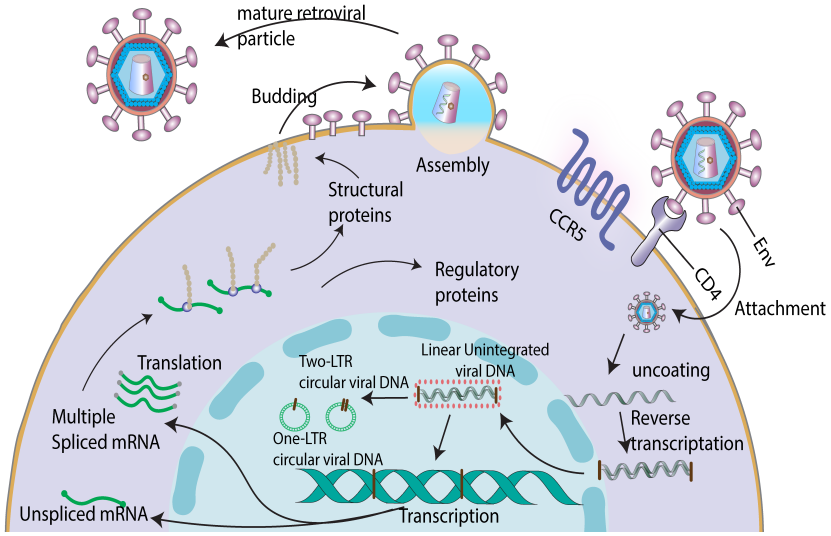

Figure 3.5: *The cell-associated mRNA in the life cycle of HIV-1, Reproduced from [106]*

Recently, two novel cell-associated viral mRNA based detection methods were developed to identify the size of translation-competent latent HIV-1 reservoir: tat/rev-induced limiting dilution assay (TILDA) [108] and the RNAflow [109]/RNAscope [110]] single cell assay.

TILDA measures the CD4+ T cells that produce cell-associated tat/rev HIV-1 msRNA after maximal cell activation (Figure 3.6A). First, total CD4+ T cells are isolated, followed by 12 h of stimulation with phorbol myristate acetate/Ionomycin, follow by serially diluted in 22–24 replicates. After that, copy number of tat/rev HIV-1 msRNA in each well will be detected by RT-PCR, and the latent HIV-1 reservoir will be estimated by maximum likelihood method. As shown in this study [108], the size of latent HIV-1 reservoir measured by TILDA in ART-treated patients was estimated 24/million CD4+ T cells, 48-times larger than QVOA, and lower than PCR based total viral DNA detection, supporting its ability to close the gap between these two standard latent HIV-1 reservoir detection assays [108].

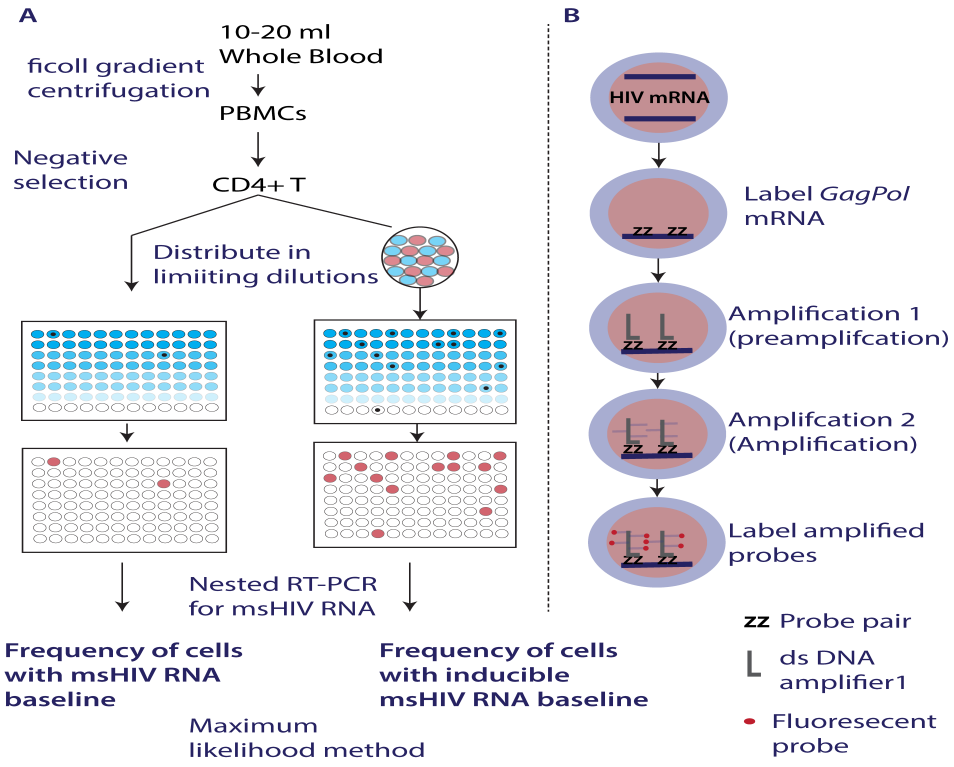

Figure 3.6: *The workflow of TILDA (A) and RNAflow (B) for HIV-1 latent HIV-1 reservoir detection. Reproduced from [109] [12]*

RNAflow/RNAscope is another novel CA HIV-1 mRNA detection method by combining fluorescence in situ hybridization (FISH) technique and branched double-stranded DNA signal amplification technology (Figure3.6b), and it normally also coupled with concurrent antibody staining for phenotypic markers [109]. Briefly, the target sequences of HIV-1 CA viral mRNAs are identified and bound by multiple specific probes pairs, which make sure high specificity. This initial reorganization signal is amplified and identified with fluorescent detection probes to drastically increase the sensitivity, follow by result reading with FACS or microscope.

Consequently, CA HIV-1 mRNA and protein expression information of the latent HIV-1 reservoir is acquired at a single-cell level with a high throughput, high specificity and sensitivity. Therefore, rare events and different gene expression pattren can be identifed. This technology has been successfully used to detect the latent HIV-1 reservoir in tissue samples [111] and isolated PBMCs [109] from infected individuals.

These two novel technologies are highly attractive, not only because of the high sensitivity, specificity and reproducibility, but also since they requires low input numbers of CD4+ T cell and the results can be obtained in 2 days without any cell culture or RNA extraction. Unlike others method, these two assays can be used to study the latent HIV-1 reservoir at the single cell level and retain localization information and provides more information about the heterogeneity of the latent HIV-1 reservoir.

However, CA HIV-1 mRNA have similar drawback as QVOA. Since some defective integrated HIV-1 DNA may still capable to transcribe mRNA and even able to produce virions despite it is not infectious. Additionally, mRNA based assays mentioned above, only apply single round stimulation, thus they also unable to detect intact noninduced proviruses within the LR. Besides, TILDA uses tat/rev region as target, which is highly varible, thus introduces bias in viral detection. Nevertheless, resulting overestimation of the LR by CA HIV mRNA-based assay is lower than DNA based qPCR assay.Therefore, CA HIV-1 mRNA-based assay appears as an appealing alternative to QVOA and PCR-based assays.

3.6 Latent HIV-1 reservoir and HIV-1 cure

There are two major concepts for HIV Cure, a sterilizing cure and a functional cure. A sterilizing cure would be achieved by a complete elimination of the HIV virus from the body. A functional cure on the other hand seeks to control the HIV infection without anti-retroviral therapy. This would be achieved by strengthening the immune system to naturally control remission.

Several strategies have been developed in hope of reducing and being able to con-

trol the HIV reservoir. Strategies involve early initiation of ART, latency reversing agent, immune therapy and gene therapy. These strategies can be divided into the categories of expose, clear and protect. Expose means inducing the latent cells to express HIV, thereby exposing to the immune system.. Some of the latency inhibitor agents are HDAC inhibitors such as Vorinostat, Panobinostat [112]. A single dose of these agents is enough to activate the latency so that the immune system can recognize HIV-1. The clearing strategy is improving clearance of infected cells. Broadly neutralizing antibodies targeting HIV-1 and neutralizing the viruses, it has been tested for prevention and cure. Not a single type of antibody have shown enough efficiency in neutralizing HIV-1. However, it will require a combination of different type of antibodies due to high variability of HIV-1. Interferon-alpha is another agent that boosts the immune system [113]. Another way is to develop therapeutic vaccine so that boost antibodies upon infection. However, combination of expose and curing strategies are required to control HIV-1 [113]. Modifying, CD4 cells in another strategy making these cells impervious to HIV-1, one way could be infusion of CD4 cells lacking CCR5 receptor or using CRISP/CAS gene therapy to protect the cells. The recent finding that viral transcription does not halt at the begin rather at downstream stage [114]. This result might provide better insight for development of therapies to completely cure HIV infection. Therefore, increasing the knowledge of how to measure and reduce the HIV-1 reservoir is needed to cure HIV-1.

Several studies aim towards finding phenotypic biomarker for infected latent cells have failed. Recent finding showed that CD32 generally expressed in myeloid rather than lymphoid cells might be used as marker of infected cells enriched in HIV patient by the ART treatment [113]. However, this result has not been reproduced in other studies. Therefore, a biomarker that could predict the clinical endpoint would revolutionize HIV cure strategies..

3.7 Challenges

After the finding of the latent HIV-1 reservoir in resting CD4+ T cells and its long-term lifecycle, many novel approaches to target, activate and eliminate the latent HIV-1 reservoir have been developed, for instance “shock-and-kill” strategy and different kinds of detection methods as discussed above. However, due to the rarity and the lacking of available surface biomarkers of latent HIV-1 reservoir, the identification of the real size of the latent HIV-1 reservoir is still very challenging.

As shown in the 3.7, the current gold standard method QVOA tend to underestimation the reservoir size, by which a treatment interruption might be misled and result in virus rebound. On the other hand, overestimation of the reservoir size by HIV-1 DNA based method might results in overdose of treatment and leading to unnecessary side effects. Therefore, in order to identify the true size of latent HIV-1

reservoir, more research is required to identify surface biomarkers of latent HIV-1 reservoir and develop corresponding high-throughput, low cost and well-validated detection assay.

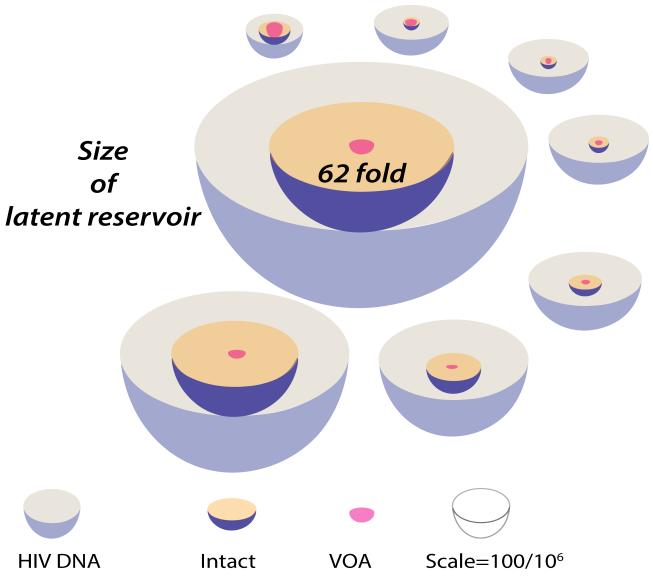

Figure 3.7: *Size of the latent HIV-1 reservoir from different methods. Recent studies indicate that most of the HIV proviruses detected by PCR based assays (yellow) are defective. Replication-competent proviruses are detected by the viral outgrowth assay (VOA, pink). However, single genome analysis of proviruses indicates that the actual size of the reservoir of intact proviruses (blue) is larger that indicated by the VOA by an average of 62 fold. These intact proviruses represent that true barrier to curing HIV infection. Reproduced from [12]*

Chapter 4

Microfluidics and HIV-1

Microfluidics is a term used to describe the studies of transport of fluids in channels of transverse dimensions ranging from a few micrometers to a few hundred micrometers. At this scale, the characteristic of fluid flow is defined by dimensionless Reynolds number as given in the (Equation 4.1).

$$Re = \frac{\rho U D_h}{\eta} \tag{4.1}$$

where U is the fluid drag velocity, D_h = characteristic length depends on channel geometry, η= dynamic viscosity, ρ=density.

Typically, the Reynolds number (which describes the ratio of inertial forces/ viscous force) is often very low in microfluidics, often less than 1. In a pressure driven flow in microchannels the flow is typical laminar in microfluidics, such that the layers of fluid flow in parallel and has the highest velocity at the middle of the straight channel forming a parabolic velocity profile [115], as shown in figure 4.1.

Due to the laminar flow behavior, precise control of individual cells in blood can be effectively accomplished at the microscale level. Hence, microfluidic lab-on-a-chip devices have the potential to enable the miniaturization of complex reaction processes into small, self-contained packages. The most attractive features of microscale approaches for blood analysis include the requirement for only microliter blood volumes obtainable without venipuncture, and simple operation by minimally trained personnel.

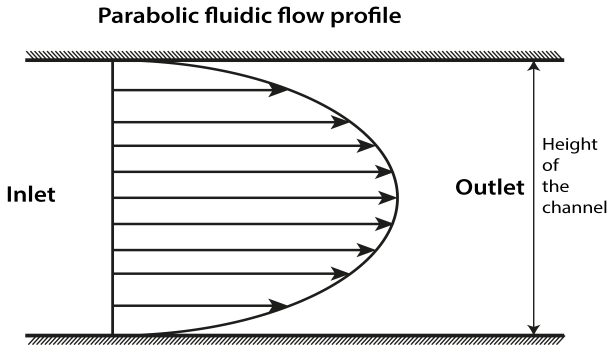

Figure 4.1: *Laminar flow within a microfluidic channel has a parabolic flow profile.*

There are a number of HIV screening tests for adults, such as enzyme-linked immunosorbent assays (ELISAs) and simple/rapid assays often in the form of lateral flow strips or cassettes. However, for staging and ongoing monitoring of HIV, the "gold standard" tests today are direct viral load quantification and CD4+ T cells counting.

In the interest of improving the accessibility and affordability of high-quality ART, there is a growing demand for simple, affordable, reliable and quality-assured point-of-care (POC) diagnostics for use in resource-limited settings. For resource-limited settings, it is often suggested that diagnostic tests for use at the point of patient care should meet the ASSURED criteria for the ideal rapid test. The ASSURED criteria [116], coined by WHO, are as follows: Affordable by those at risk of infection, Sensitive (few false-negatives), Specific (few false-positives), User-friendly (simple to perform and requiring minimal training), Rapid (to enable treatment at first visit) and Robust (does not require refrigerated storage), Equipment-free (minimum equipment) and Delivered to those who need it.

With the capacity of precisely control and manipulation of small volume of fluids, microfluidics technology offers new opportunities to develop ASUURED-fulfilled point-of-care devices for HIV-1 diagnosis, such as CD4+ T cells counting [117–122] viral particle isolation [122,123,130,135,138], viral load quantification [90,141–143] and HIV-1 latent reservoir detection [144].

Since CD4+ T cells are the primary targets of HIV-1, the CD4+ T cells number becomes an important diagnostic indicator for HIV-1 diseases progress and treatment management. The CD4+ T cells count for a health person is normally between 500 and 1500 in per milliliter whole blood, while normally below 200 cells/ml blood for AIDS patients without treatment and over 500 cells/ml for well-controlled HIV-1 infected patients [119]. Recently, several microfluidic-based devices for immune-

capturing and enumeration of CD4+ from HIV-1 patient blood sample were reported [117–122].For instance, a inexpensive, rapid and portable lab-on-DVD microfluidic platform together with a modified commercial DVD reader was developed to perform fully automatic CD4 cell count [118]. Besides, differential shear stress [120] and counting algorithms [117] were also developed for capturing and counting of CD4+ T cells on the microfluidics device, make it more accurate and efficient than manual counting. Furthermore, using a size-coded mixture of multiple affinity-capture microbeads and an inertial microfluidic particle sorter device, low abundance antibodies specific to different HIV antigens and rare HIV-specific cells from blood were isolated from HIV-1 infected patients [130].

In addition to CD4+ T cells counting, viral load quantification is another important measure for HIV-1 disease progress. Today there are many devices available for molecular based HIV-1 diagnosis, such as Alere q system, Cobas Liat, Eoscape HIV-1 rapid RNA assay system and GeneXpert HIV-1 viral load test and ExaVir load viral RNA assay. However, these diagnostic methods require expensive instrument and highly trained personnel. Often each of these methods follows the same elaborate three-step process. First, HIV particles in plasma are separated from other components of blood and lysed to release viral RNA, which is then purified. Second, the purified RNA is amplified by several orders of magnitude to allow for fluorescence-based detection. Third, the amplified nucleic acid is detected, either in "real-time" simultaneous with amplification, or following amplification, using fluorescence detection methods. Recently, several microfluidics device were reported to be able to isolate viral particles with high efficiency [138] or amplify HIV-1 nucleic acid with isothermal amplification methods such as LAMP [142]. Furthermore, recently, the entire process including cell lysis, extraction of DNA, polymerase chain reaction (PCR), and optical detection have been successfully integrated on one microfluidic system, in which equipped with a sample pretreatment device and a nucleic acid amplification device for the rapid and automated diagnosis of HIV-1 viral load [143].

Droplet microfluidic is a new technique, which uses two-phase system, generating aqueous droplets in an immiscible oil medium [145]. Each droplets act as a single reaction container, thus enabling high throughput analysis of single cells or molecules [146] Recent studies showed that droplet microfluidics technology could be used to sort millions of HIV-1 particles with >99% efficiency [138], and to perform digital PCR for absolute quantification of HIV nucleic [90]. Moreover, a microfludic single-cell-in-droplet (scd) PCR assay were developed for direct measurement of latent HIV reservoir after external activation of infected CD4 cells from HIV patients, and suggested that HIV latency reversal is high heterogeneous [144].

Chapter 5

Present Investigation

This thesis is based on the structure as shown in the Figure1.2 and this chapter describes the results presented in paper 1-4.

Firstly, I discuss the applicability of three novel molecular assays for latent HIV-1 reservoir characterization. In Paper I, an antibody profiling method was optimized and used to verify the hypothesis that the anti-HIV-1 proteome antibodies signature in HIV-1 infected patients could indirectly reflect the size of HIV-1 reservoir. In paper II, we optimized and investigated the capacity of two branched DNA amplification (bDNA)-based methods, that target to cellular HIV-1 mRNA in the latent HIV-1 reservoir after activation, to directly detect inducible transcription and translation-competent latent HIV-1 reservoir.

Secondly, in paper III, I present a transcriptomics and proteomics-based high-throughput approaches, which were established and utilized to study the immune-control mechanisms of HIV-1 infected elite controllers, as well as identifying potential biomarker for latent HIV-1 reservoir.

Finally, in paper IV, I presents the development of an integrated microfluidics chip to isolate three primary mononuclear leukocyte cell subsets that can harbor the latent HIV-1 reservoir, namely monocyte cells, CD4+ T-lymphocytes, and natural killer (NK) cells, from a small volume of whole blood.

5.1 Paper I and II

5.1.1 Optimization and evaluation of three novel molecular assays for indirect and direct characterization of latent HIV-1 reservoir

After more than 30 years of study, identifying the size and dynamics of the latent HIV-1 reservoir during cART and how to target it, is still an unsolved problem and have become an obstacle for the design of strategies to eliminate the latent HIV-1 reservoir and cure HIV-1 infection. Therefore, there is an urgent need for an assay that can identify the size of the latent HIV-1 reservoir and distinguish the inducible latent HIV-1 reservoir from defective proviruses with high precision.

Several previous studies have reported that the anti-HIV-1 antibodies profiling can reflect the size of the latent HIV-1 reservoir in HIV-1 infected individuals [139] [105]. In paper I, we adapted and optimized the antibody-profiling assay "luciferase immunoprecipitation systems (LIPS)". Based on this method, we screened the antibody levels against the HIV-1 proteome in a well-characterized group of Swedish patients and verified the hypothesis that the anti-HIV-1 antibody level in HIV-1 infected patients can indirectly reflect the size of the HIV-1 reservoir.

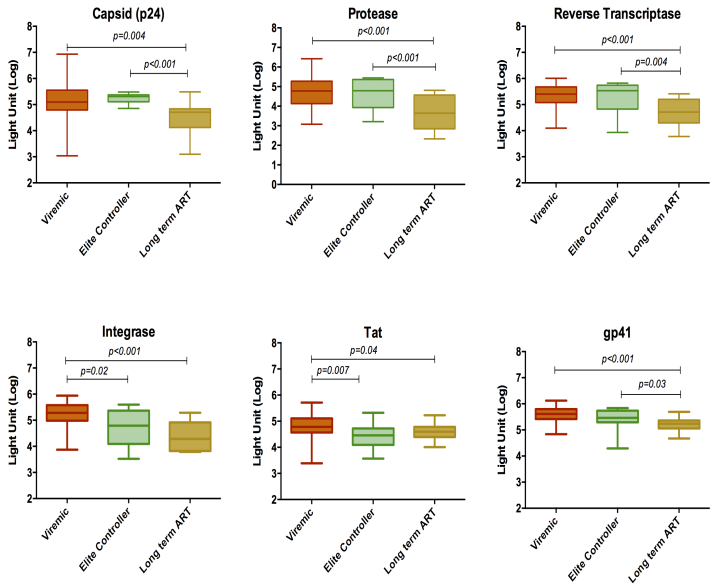

Figure 5.1: *The result of antibodies profiling against sex HIV-1 proteome in a well-characterized group of Swedish patients. Lines, boxes and whiskers represent median, interquartile ranges, and range respectively.*

The antibodies against HIV-1 RT, Tat, p24, gp41, protease and integrase were profiled using optimized LIPS assay in in a well-characterized group of Swedish patients, included 19 elite controllers (ECs), 38 viremic progressors and 19 patients with fully suppressive long-term antiretroviral therapy (ART)(mean 17 years).

As a result, the long-term suppressive ART patients contain lower antibody levels to gp41, reverse transcriptase, p24 and protease, compared to the viremic patients and the ECs. Nevertheless, between viremic patients and the ECs, we did not observe a statistically significant difference. For the antibodies against HIV-1 integrase and tat, compared to viremic patients, they were found to have a significantly lower level in the ECs and the patients with long-term ART. (Mann Whitney U test; $p<0.05$ for all analysis) (Figure 5.1)

The PCA and hierarchical clustering analysis results (Figure5.2) further indicated that the antibodies against HIV-1 replication enzymes (reverse transcriptase, protease and integrase)might perform better than the HIV-1 structural proteins (e.g Gag or Env proteins) for disease state classification as well as identification of size of the latent HIV-1 reservoir.

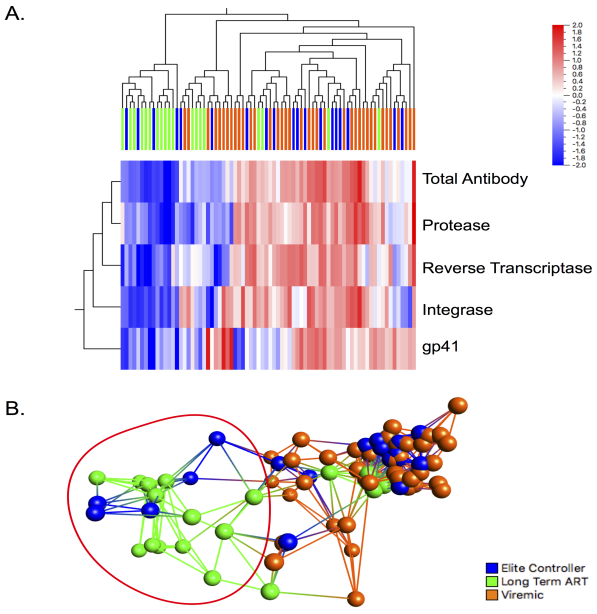

Figure 5.2: *The PCA and hierarchical clustering analysis results based on the four HIV-1 antigens and the total antibody response.*

In summary, our data indicated that the anti-HIV-1 proteome antibodies level was low in the HIV-1 patient who received long-term cART treatment, which was expected and can be explained by the limited latent HIV-1 reservoir and low antigen expression. However, beyond our expectation, this method failed to distinguish HIV-1 elite controllers (EC) expected to have a low level of HIV latent reservoir from viremic patients expected to have a high level of HIV latent reservoir. Though these ECs in our study were selected with strict criteria and followed for more than 15 years and were expected have smallest latent HIV-1 reservoir as well as lowest anti-HIV-1 antibodies level. Hence, we concluded that there is certainly room for this method to improve, especially in sensitivity and specificity.

The primary aim of Paper II was to optimize and evaluate two novel branched DNA amplification (bDNA)-based molecular assays, namely PrimeFlow™ RNA Assay (RNAflow) and RNAscope® ISH (RNAscope), for identification of the transcription and translation competent latent HIV-1 reservoir through detection of cellular HIV-1 specific mRNA after latency reversal agent activation.

The workflow is shown in Figure5.3. Based on three latent HIV-1 reservoir cell models (J-Lat cell model, clones 6.3, 9.3 and 10.6), four latency reversal agents were optimized with respect to the combination and dose to achieve maxium latent HIV-1 reservoir activation. Subsequently, under the optimized condition, the RNAflow and RNAscope assays were evaluated for sensitivity, specificity, and lower detection limit for the identification of the translation-competent HIV-1 reservoir. The HIV-1 subtype specificity of the RNAscope assay was also verified using patient-derived subtype A1, B, C and CRF01 AE recombinant plasmids following transfection in 293T cells. Finally, as a proof-of-concept, optimized RNAscope was applied in patient derived peripheral blood mononuclear cells (PBMCs) to indentify the translation-competent HIV-1 reservoir after activation.

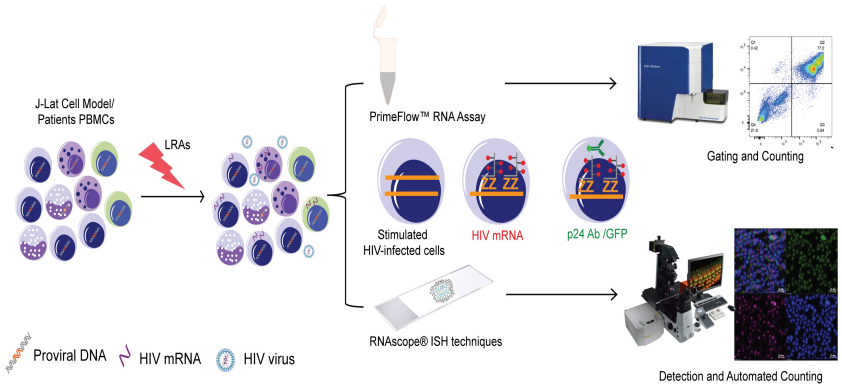

Figure 5.3: *The schematic workflow for RNAflow and RNAscope.*

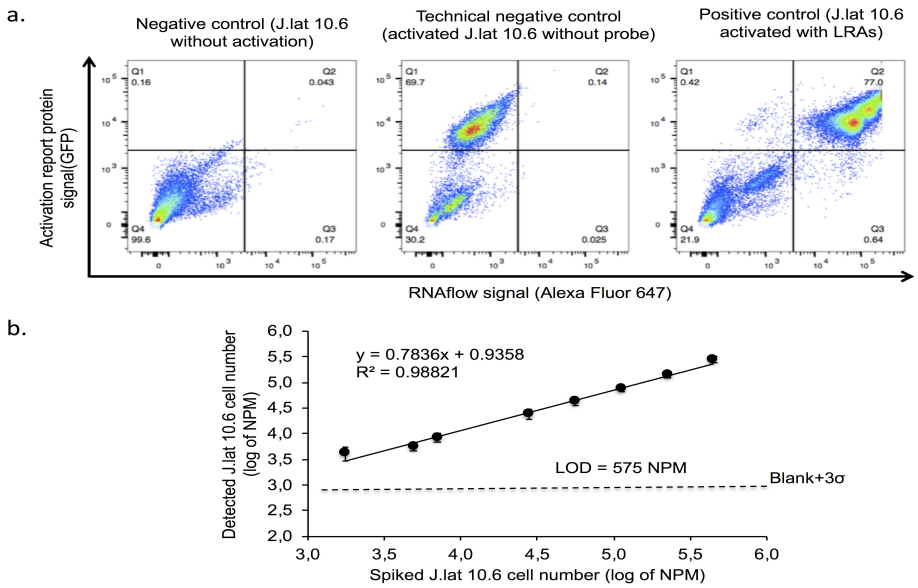

Figure 5.4: *Gating strategy (a) and determination of the lower detection limit of RNAflow linear equation (y = meanblank + 3SDblank).*

As shown in Figure 5.4 and Figure 5.5, RNAflow has 575 HIV-1 infected cells/million as the lower detection limit. In contrast, the lower detection limit for optimized RNAscope was 45 cells/million, a 10-fold lower than the former and with higher specificity and less cell loss. Furthermore, we also showed that the RNAscope method can also detect several other patient-derived HIV-1 subtypes (HIV-1A1, HIV-1B, HIV-1C, 01 AE), though the probes for RNAscope were developed based on HIV-1B. Finally, we also showed that the translation-competent HIV-1 reservoir could be detected by this optimized RNAscope method in patient derived PBMCs after activation (Figure5.6), and allow single cell characterization of HIV-1 RNA and protein simultaneously.

In summary, this study compared the performance of two novel and sensitive molecular assays in translation-competent HIV-1 reservoir detection. The result indicated that the optimized RNAscope techniques has a higher sensitivity and less cell loss than the RNAflow, and is independent on the HIV-1 subtype. We also showed that RNAscope could directly identify and quantify the individual translation-competent latent HIV-1 reservoir, which is expressing HIV-1 mRNA after activation. Hence, this optimized RNAscope method can be a valuable means for characterization of the translation-competent latent HIV-1 reservoir at single-cell resolution, not only for quantification but also for single-cell transcriptomics and proteomics study, when combined with a proper single-cell isolation method.

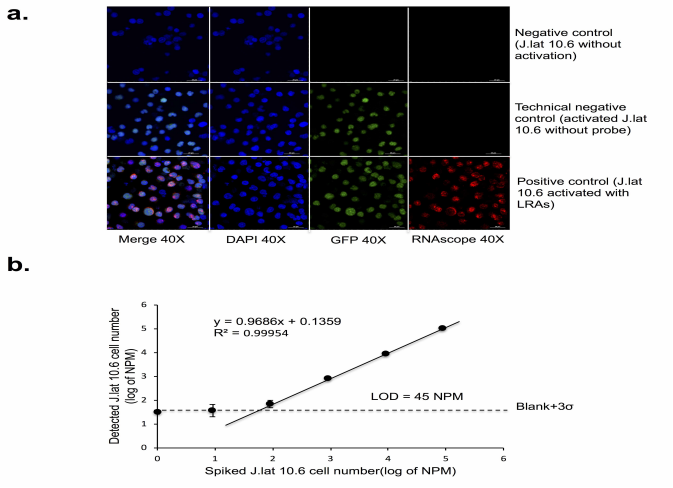

Figure 5.5: *Determination of the specificity (a) and the lower detection limit (b) of RNAscope.*

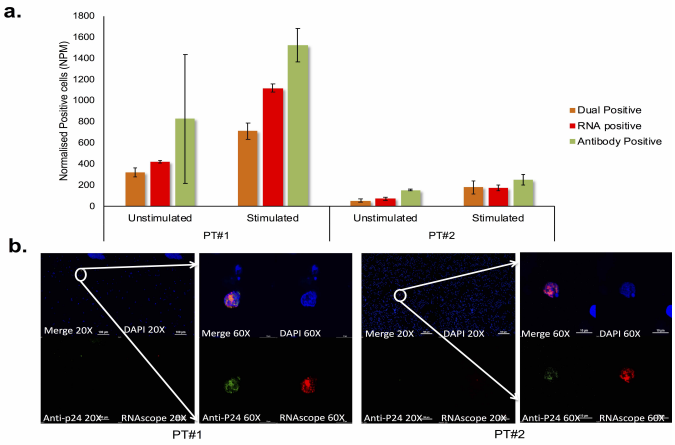

Figure 5.6: *Counting of activated HIV-1 latent cells in patients derived PBMC by RNAscope, error bars represent the standard deviation among three independent replicates.*

5.2 Paper III

5.2.1 Establishment of a transcriptomics and proteomics-based analysis method for high-throughput selection of potential latent HIV-1 reservoir surface biomarkers

To date, there is still no available surface biomarkers for the latent HIV-1 reservoir. One main reason is because of the lack of sufficient identification and isolation methods for latent HIV-1 reservoir, which we tried to adress in paper II. Another reason is the low-throughput of traditional FACS-based surface biomarker selection methods. Thanks to the development of the next generation sequencing, searching and exploring the potential surface biomarkers at gene and protein level with high-throughput may offer more hints than tranditional methods. Hence, in paper III, we tried to establish a transcriptomics and proteomics-based analysis method for high-throughput selection of potential latent HIV-1 reservoir surface biomarker at gene and protein level.

As a model, we established a research pipeline that combining transcriptomics with targeted proteomics analysis based on the isolated PBMCs from a well-defined Swedish HIV-1 infected patients cohort with up to 20 years of clinical follow-up data (Fig 5.7), with the aim to apply this method after we achieve isolation the latent HIV-1 reservoir from patient blood sample. In this study, 19 untreated elite controllers (ECs, the group of individual who control viral replication spontaneously and hypotheised to be have low latent reservoir), 32 treatment naïve patients with viremia (VP, who were hypotheised to be have high latent reservoir) and 23 HIV-1-negative healthy controls (HC) were assayed with this approach.

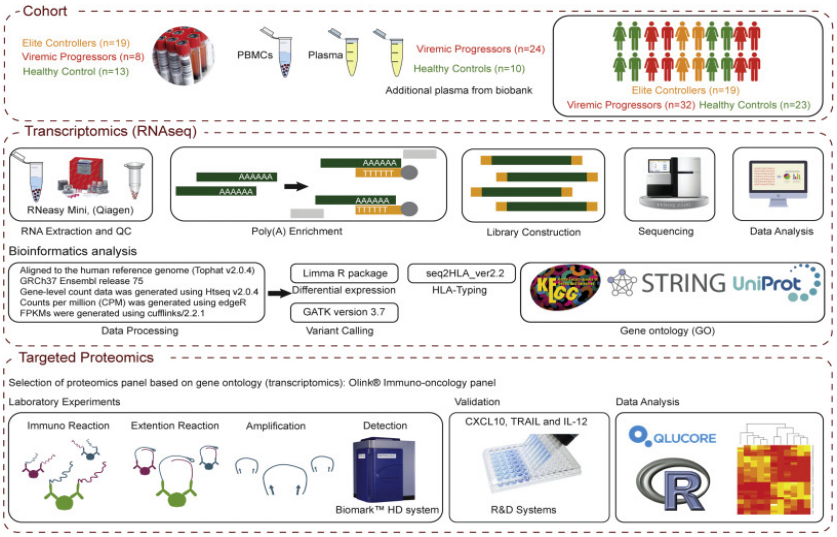

Figure 5.7: *The patient cohort, study designing and analysis plan of this transcriptomics and proteomics study.*

As shown in Figure 5.8, out of 159 up-regulated protein coding genes, 12 membrane protein-code genes *(CCR5, MELK, CAV1, PTPN3, TNFRSF17, CXCR6, SSPN, GPRC5D, TSHR, SLC16A14, SIGLEC1 and ABCG2)* were found up-regulated in treatment naïve patients with viremia who were hypothesized to have high latent reservoir compare to elite controllers, who were hypothesized to have low latent reservoir, and might have the potential to serve as biomarkers for LR. Further study is till on going to validate this finding.

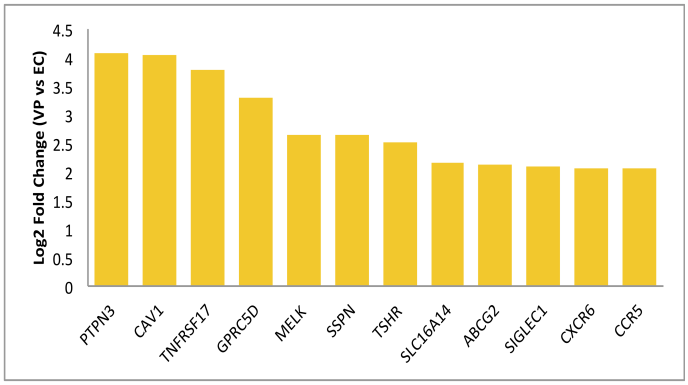

Figure 5.8: *The fold change value of twelve membrane-protein coding genes,VP against EC*

5.3 Paper IV

5.3.1 Development of microfluidics-based cell subsets isolation device for latent HIV-1 reservoir study

CD4+ T cells, monocyte/macrophages and natural killer cells are believed to be the main source for HIV-1 reservoirs in peripheral blood. However, current HIV latency studies are mainly based on PBMCs or only CD4+ T cells, due to the lack of appropriate cell subset isolation method. These cells were normally isolated by conventional cell isolation techniques, such as fluorescence-activated cell sorter (FACS), magnetic activated cell sorting (MACS) and centrifugation systems. However, these techniques offer several limitations, such as the requirement for large sample volumes, high reagent consumption, cross contamination of samples and expensive equipment cost. Hence, in paper IV, we developed an integrated microfluidics chip to isolate three primary mononuclear leukocyte cell subsets that can harbor HIV latency, namely monocyte cells, CD4+ T-lymphocytes, and natural killer (NK) cells, from a small volume of whole blood in short time.

In order to achieve high capture efficiency as well as purity, the shear stress force and immunoaffinity were combined and optimized before the final design of the mononuclear leukocyte-capturing chip. The three cell lines mixture (in which has the same proportion as in blood) was pumped into antibody-immobilized Hele–Shaw chips, which allows the analysing of the cell adhesion over a range of shear stresses in a single experiment (Fig 5.9). As shown in Fig 5.10, 1.7 dyn/cm^2, 1.1 dyn/cm^2 and 0.9 dyn/cm^2 were determined as the optimal shear stresses for CD4+ T cells, NK cells and monocyte cells capture in microfluidic chip. After optimization of the shear stress for cell capture using Hele-shaw chip, the capturing device was designed and fabricated with standard lithography technology. The surface of the three chambers in this integrated microfluidic device is coated with anti-CD4, anti-CD56 and anti-CD14 antibodies respectively, to specifically capture CD4+ lymphocytes, natural killer cells and monocytes. Eventually, this chip was tested with 200 µl of cell mixture suspension and whole blood sample.

When tested the capturing in cell line mixture suspension sample, the results show that the average number of CD4+T cells captured in the CD4 capture chambers of the chip was (131490) with 85% of purity. While, the average number of monocyte captured cells in monocyte chambers was (44082) and the capture purity was 64%, the mean capture of NK cells in NK chambers was (49749) with 67% purity as shown in (Figure.5.11 A and B). For whole blood sample, the highest cell subset capture was in lymphocytes (106382) with 76% purity. The average number of monocyte capture was (4696) and the capture purity was 41%, while, the capture of other leukocytes was higher in monocyte chambers than other chip chambers. NK cells capture average from whole blood was (13115) and the capture purity was 47% as seen in (Figure.5.11 C and D).

In summary, we demonstrated a simple, quick and integrated microfluidic device based on immunoaffinity capture of three subpopulations of mononuclear leukocytes (lymphocyte, monocyte and NK cells), from small volumes of whole blood, within 20min. The device operated under controlled shear stress. To our knowledge, this is the first design that can isolate the three mononuclear cell populations, which are involved in the HIV latency mechanism in a single step. In future, this microfluidic chip can be combined with the technology we developed in the paper I and III, to characterize the HIV-1 latent reservoir in an isolated single cell subset.

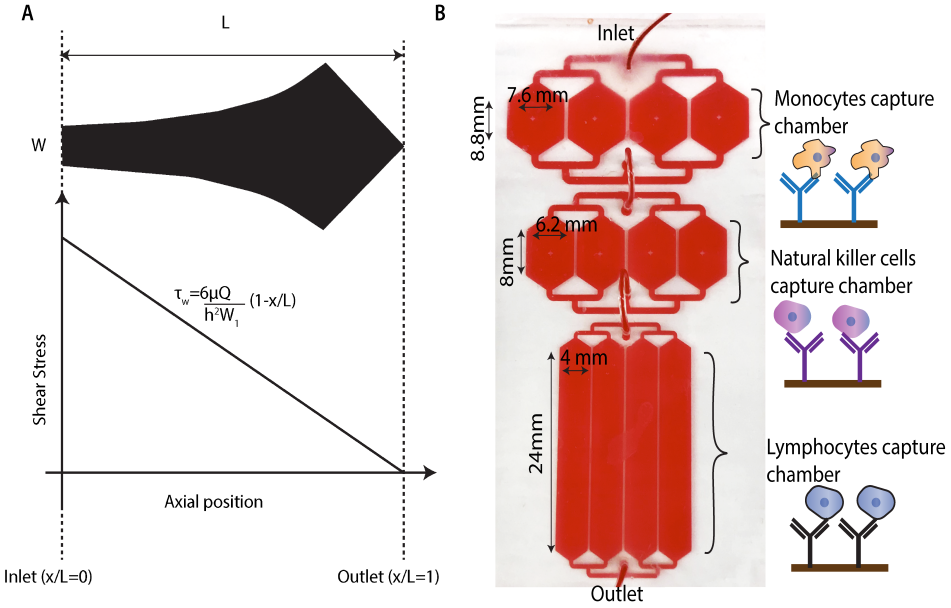

Figure 5.9: *The Microfluidic chips design. (A) Geometry of the Hele–Shaw device. The Hele–Shaw device offers a linear variation of shear stress along its central line. It was used in this study to determine the optimal shear stress for different cell capture. (B) Cell capturing device, the chamber bottom surface was modified and immobilized with neutravidin and specific biotinylated antibody to capture target cells from whole blood*

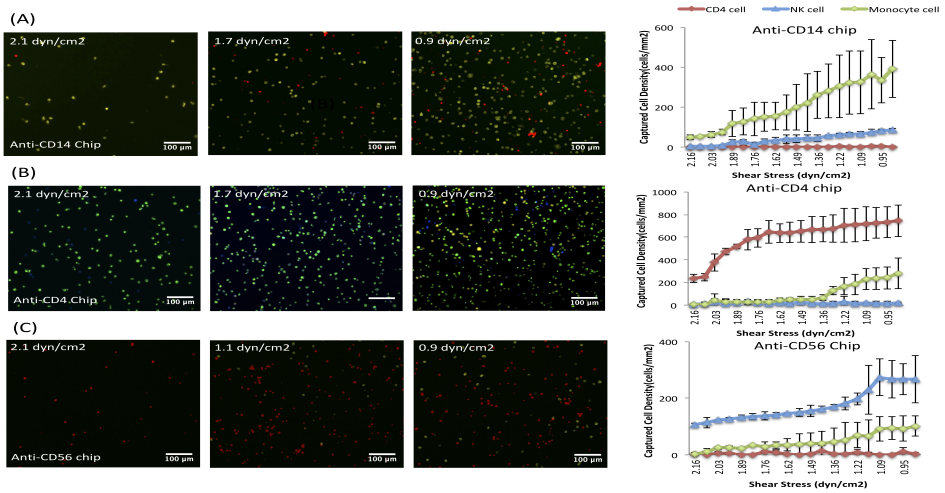

Figure 5.10: *The shear stress optimization result based on Hele–Shaw chip experiment. The representative images of captured and stained cells (CD4: green, Monocyte: yellow, NK cell: Red) in the Hele–Shaw chamber at different locations corresponding to different shear stresses. (A, B, C) The relationship between shear stress and captured cell density of CD14+cells (monocytes), CD4+ T cells and CD56+ cells (NK cells) in the Hele–Shaw chip. Error bars represent the standard deviation among three independent replicates*

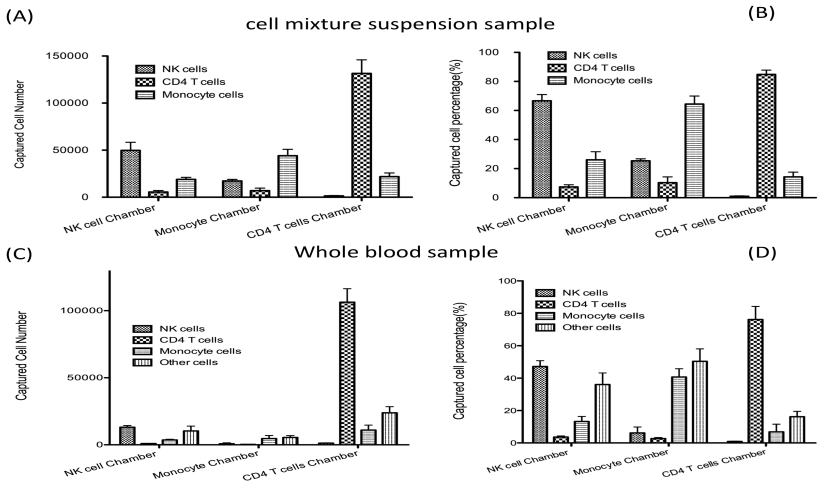

Figure 5.11: *Capture of CD4+T cells, monocytes and NK cell lines in the capturing device from cell line suspension and whole blood. Error bars represent the standard deviation among three independent replicates*

Chapter 6

Conclusion and outlook

Due to the rarity and lack of detectable surface biomarkers, to develop robust assays that can characterize the latent HIV-1 reservoir is still a major challenge in HIV-cure research. The papers presented in this thesis aims to address this challenge from different aspects using different novel technologies.

In order to find a proper tool to identify the latent HIV-1 reservoir, three molecular assays were optimized and evaluated for indirect and direct characterization of latent HIV-1 reservoir. First, in Paper I, LIPS assay was optimized and tested to profiling the antibody against HIV-1 proteome in a well-characterized group of Swedish patients, aiming to indirectly identify the size of latent HIV-1 reservoir by the antibodies profiling signature. However, though LIPS has been proposed for the indirect quantification of latent HIV-1 reservoir, our study showed it might not be a sufficient method for latent HIV-1 reservoir characterization, as it failed to distinguish the HIV-1 positive elite controller (EC) who possesses low level of latent reservoir from the viremic patients who has high level of latent HIV-1 reservoir. Besides, from Paper I, we also can conclude that EC may not be an ideal model for HIV-1 cure due to its heterogeneity. Rather long term successfully treated ART group can be a good model of HIV-1 cure.

Secondly, in Paper II, RNAflow and RNAscope assay were further investigated for identification of transcription and translation competent latent HIV-1 reservoir. Our result indicated that our optimized RNAscope technique performed better than RNAflow for transcription and translation competent latent HIV-1 reservoir identification, especially in a small volume of samples due to the less cell loss. The RNAscope was also found to be independent of the patient-derived HIV-1 subtype. Besides, the optimized RNAscope also have the potential to combine with laser cutting to isolate single latent HIV-1 reservoir for downstream analysis. However, since the lower detection limit of RNAscope is still higher than the number of mathematically predicted replication-competent HIV-1 reservoir (10-100 copies/10^6 PBMC),

the RNAscope method still need further improvement.

As there were no available surface markers for latent HIV-1 reservoir cells, in paper III, a transcriptomics and proteomics-based analysis method for high-throughput selection of potential biomarkers were established and applied in different patient groups. 12 membrane protein-coding genes were identified as upregulated in the patient group who were hypothesized to be have low latent reservoir, and might have the potential to serve as surface biomarkers for the latent HIV-1 reservoir. Nevertheless, given the small number of samples, it was difficult to validate. A larger sample size will be needed to prove it, a work is presently ongoing. Besides, it could also be beneficial to apply this method on individual cell populations, by combine with our cell subset isolation microfluidic chip in paper IV, as the expression of those markers differs between the cell populations.

CD4+ T cells, monocytes/macrophages and natural killer cells are believed to be the main source for HIV-1 reservoirs in peripheral blood. In paper IV, a microfluidics chip was developed to isolate these three mononuclear leukocytes as single cell-subset from whole blood samples in short time. More effort is needed to improve the capture purity and yield from blood samples. In future, this microfluidic chip can be combined with the technology we developed in the paper II and III, to characterize the HIV-1 latent reservoir in isolated single cell subset with less sample consumption.

Together, though further improvement and clinical verification is needed, I believe the described achievements can contribute to the improvement of latent HIV-1 reservoir characterization, and may facilitate future development of the latent HIV-1 reservoir targeting and clearance method with the ultimate goal – to complete cure HIV-1 infection.

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