CHARACTERIZATION OF HIV-1 RNA AND DNA DURING LONG-TERM SUPPRESSIVE THERAPY

Susanne von Stockenström

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Characterization of HIV-1 RNA and DNA during Long-Term Suppressive Therapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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By

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ABSTRACT

Human immunodeficiency virus (HIV) is the virus causing acquired immune deficiency syndrome (AIDS). Since the discovery of HIV/AIDS over three decades ago, this disease has claimed millions of lives. One of the major accomplishments of modern history came in 1996, when combination antiretroviral therapy (cART) was introduced. Treatment with cART caused the death rates from AIDS to decrease dramatically. Although current cART is effective in suppressing HIV type 1 (HIV-1) it is not curative and therefore meticulous lifelong therapy is necessary. To effectively target HIV-1 persistence with the goal of achieving a cure, it will be important to determine the source and dynamics of persistent viremia. In the work presented in this thesis we compare the different approaches for measuring the persistent HIV-1 reservoir. We also use highly sensitive assays to genetically characterize intracellular HIV-1 within a broad spectrum of cells sorted from unique tissue samples from patients on long-term suppressive cART.

In paper I we compare eleven different approaches for quantifying persistent HIV-1. Results from this study showed major differences among the assays. The viral outgrowth assay, which is a culture-based assay that quantifies replication competent virus, resulted in measurements of replication competent virus that were at least 300-fold lower compared to PCR-based methods which measured total and/or integrated HIV-1 DNA. The differences between these methods may reflect the number of defective viral genomes in cells. Overall, the study reveals many difficulties in measuring the latent reservoir and shows that there is currently no assay that will accurately measure the latent reservoir during clinical trials of curative strategies.

In papers II-IV we genetically characterized intracellular HIV-1 DNA within a broad spectrum of cells sorted from different anatomical compartments of eight patients on long-term cART. In paper II we investigated whether CD34+ hematopoietic progenitor cells (HPCs) from the bone marrow serve as an HIV-1 reservoir. In this study we did not detect HIV-1 DNA in CD34+ HPCs indicating that this cell type is not a source of persistent HIV-1.

In papers III and IV we genetically characterized intracellular HIV-1 in different cell types from peripheral blood, gut-associated lymphoid tissue (GALT) and lymph node tissue. We found that the majority of HIV-1 DNA was detected in memory CD4+ T cells and that participants who initiate therapy during early infection have a lower intracellular HIV-1 infection frequency. These results imply that despite several years of therapy, memory CD4+ T cells serve as an important reservoir and that early initiation of therapy results in a smaller latent reservoir. In paper III we used phylogenetic analyses to study the genetic evolution of HIV-1 between samples isolated before initiation of therapy and several years after suppressive therapy. Our studies revealed a lack of substantial HIV-1 genetic evolution during cART which strongly suggests that ongoing replication is not a major cause of viral persistence in memory T cells.

In paper IV we evaluated the longitudinal stability of the HIV-1 reservoir and the role of cellular proliferation in maintaining persistent HIV-1 during cART. Our results show that memory T cells retained a relatively constant HIV-1 DNA integrant pool that was genetically stable during long-term cART. These DNA integrants appear to be maintained by cellular proliferation and longevity of infected cells, rather than by ongoing viral replication.

In conclusion, the work presented in this thesis has helped us to gain a fuller appreciation for the range of cells and tissues containing HIV-1 DNA in patients on long-term cART and a better understanding as to how intracellular HIV-1 DNA is maintained in different tissues.
LIST OF SCIENTIFIC PAPERS


3.4.3 Measurements of Diversity and Evolution

3.5 Statistical Analyses

4 RESULTS AND DISCUSSION

4.1 Comparative Analyses of Measures of the HIV-1 Reservoir

4.2 Cellular and Anatomical Sites of Persistent HIV-1

4.3 Stability and Maintenance of HIV-1 Reservoir

5 CONCLUSIONS AND FUTURE PERSPECTIVES

6 ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>APOBEC</td>
<td>Apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
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<tr>
<td>cART</td>
<td>Combination antiretroviral therapy</td>
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<tr>
<td>Bp</td>
<td>Base pairs</td>
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<tr>
<td>CCR5</td>
<td>CC chemokine receptor type 5</td>
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<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>CXCR4</td>
<td>CXC chemokine receptor type 4</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ddPCR</td>
<td>Digital droplet PCR</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
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<tr>
<td>Env</td>
<td>Envelope</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>Gag</td>
<td>Group specific antigen</td>
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<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
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<tr>
<td>HIV-2</td>
<td>Human immunodeficiency virus type 2</td>
</tr>
<tr>
<td>HPC</td>
<td>Hematopoietic progenitor cell</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<tr>
<td>IN</td>
<td>Integrate</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
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<tr>
<td>ML</td>
<td>Maximum likelihood</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative factor</td>
</tr>
<tr>
<td>NJ</td>
<td>Neighbor joining</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
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<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of virion expression</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
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<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
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<tr>
<td>SCA</td>
<td>Single-copy assay</td>
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<tr>
<td>SCS</td>
<td>Single-cell sequencing</td>
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<tr>
<td>SGS</td>
<td>Single-genome sequencing</td>
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<td>SPS</td>
<td>Single-proviral sequencing</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>Vif</td>
<td>Virion infectivity factor</td>
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<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>Central memory CD4&lt;sup&gt;+&lt;/sup&gt; T cell</td>
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<tr>
<td>T&lt;sub&gt;EFF&lt;/sub&gt;</td>
<td>Effector CD4&lt;sup&gt;+&lt;/sup&gt; T cell</td>
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<tr>
<td>T&lt;sub&gt;SCM&lt;/sub&gt;</td>
<td>Stem cell memory CD4&lt;sup&gt;+&lt;/sup&gt; T cell</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>T&lt;sub&gt;TM&lt;/sub&gt;</td>
<td>Transitional memory CD4&lt;sup&gt;+&lt;/sup&gt; T cell</td>
</tr>
<tr>
<td>QVOA</td>
<td>Viral outgrowth assay</td>
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<tr>
<td>Vpr</td>
<td>Viral protein R</td>
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<tr>
<td>Vpu</td>
<td>Viral protein U</td>
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1 AIMS

The general aim of this thesis was to characterize HIV-1 populations in patients on long-term suppressive therapy. More specifically, the objectives of this thesis were:

PAPER I: To compare different methods used to measure the viral reservoir during eradication studies.

PAPER II: To investigate whether hematopoietic progenitor cells from bone marrow tissue are an important viral reservoir in patients on effective therapy.

PAPER III: To genetically characterize HIV-1 DNA within T cell subsets sorted from peripheral blood and gut-associated lymphoid tissue in patients on effective therapy and to explore whether this reservoir is replenished by residual HIV-1 replication.

PAPER IV: To define the stability of the intracellular HIV-1 DNA pool during effective antiretroviral therapy and to explore the role of T cell proliferation as a cause of HIV-1 persistence.
2 THE HUMAN IMMUNODEFICIENCY VIRUS

2.1 THE HIV PANDEMIC

2.1.1 The Discovery of HIV

Over three decades have passed since acquired immune deficiency syndrome (AIDS) was recognized as a new disease. The first documentation of AIDS was in 1981 when previously healthy young homosexual men from Los Angeles and New York were treated for opportunistic diseases such as Kaposi’s sarcoma and Pneumocystis carinii pneumonia [1-3]. Subsequently, several similar cases were reported throughout the United States and the first AIDS cases were documented in other countries [4-7]. At this time, the disease did not have a name and the cause was unknown. Organizations referred to the disease in different ways e.g. lymphadenopathy (swollen glands), Kaposi’s sarcoma and Opportunistic Infections (KSOI), gay-related immune deficiency (GRID) or “gay-cancer” [8-11]. In December 1982, a child who received a blood transfusion died of an AIDS-related infection. This was the first clear evidence that the disease was caused by an infectious agent [12]. The causative agent, now known as human immunodeficiency virus (HIV), was subsequently identified in 1983 by doctors at the Pasteur Institute in France [13]. Drs. Francoise Barré-Sinoussi and Luc Montagnier were awarded the Noble Prize for their finding in 2008.

2.1.2 The Origin and Classification

The origin of HIV has been traced to the simian immunodeficiency viruses (SIV) naturally infecting African primates. The SIV variants were introduced to humans through several independent cross-species transmissions, with each successful zoonotic transmission resulting in different lineages of HIV [14, 15]. HIV is divided into HIV type 1 (HIV-1) groups M, N, O and P and HIV type 2 (HIV-2) groups A-H. HIV-1 originates from SIVcpz found in the West-Central African chimpanzees and probably also from SIVgor found in western gorillas [16], whereas HIV-2 has been linked to the transmission of SIVsmm found in sooty mangabeys [17, 18]. HIV-1 is more infectious and causes a faster progression to AIDS compared to HIV-2 [19, 20]. HIV-1 group M is responsible for over 95% of infections worldwide and can be further divided into nine subtypes (A, B, C, D, F, G, H, J and K) and many circulating recombinant forms (CRFs) [21]. Subtype C is the most prevalent subtype worldwide whereas subtype B is the most dominant subtype in Europe, the United States, and Australia [22]. As a result, most research has been done on HIV-1 subtype B, including the studies conducted for this thesis.

2.1.3 The Global Situation of HIV/AIDS

In the last 15 years alone, around 38 million people have become infected by HIV and the disease has claimed more than 25 million lives. In 2014 approximately 37 million people globally were living with HIV (Figure 1). The prevalence of the disease varies greatly between regions with Sub-Saharan Africa being the most severely affected region accounting for about 70% of all HIV-infected individuals. Fortunately, the number of newly infected individuals is declining in most parts of the world, but infection rates are still high. During 2014, 2 million individuals became newly infected worldwide. This is a great reduction from 2000, when 3.1 million people became newly infected [23].
2.2 HIV-1 VIROLOGY

2.2.1 The HIV Virion

2.2.1.1 Structure

HIV-1 and HIV-2 are members of the Lentivirus genus of the Retroviridae family. The HIV-1 particle is approximately 120 nm in diameter and roughly spherical. Similar to other lentiviruses, HIV is enveloped by an outer lipid bilayer derived from the host cell membrane. Trimeres of the viral envelope surface glycoprotein (SU, gp120) are anchored to the virus via interactions with the envelope transmembrane protein (TM, gp41). Lining the inner surface of the viral membrane is the matrix (MA, p17). A cone-shaped capsid formed by the capsid protein (CA, p24) surrounds the viral genome, which forms a stable complex with the nucleocapsid protein (NC, p7). The nucleic acid consists of two copies of non-covalently linked, positive-sense single-stranded RNA (+ssRNA) molecules. The capsid also contains the three viral enzymes that are essential for replication: reverse transcriptase (RT, p66/p51), protease (PR, p10) and integrase (IN, p32). HIV has evolved a variety of regulatory and accessory proteins that modulate the replication cycle and the host immune responses. Packaged in the HIV-1 particles are the accessory proteins Nef (p27), Vif (p23) and Vpr (p15). Additional viral proteins that function in the host cell are Rev (p19), Tat (p14) and Vpu (p16), however, these are not present in the virion [24] (Figure 2).
2.2.1.2 Genomic Organization

The genomic size of HIV is approximately 10 kilobases (kb) containing open reading frames encoding for several viral proteins. The three major structural genes, common for all retroviruses, are group specific antigens (gag), polymerase (pol) and envelope (env). The primary transcript of HIV-1 is a full-length mRNA, which is subsequently translated into the Pol and Gag proteins. The pol-gene is highly conserved and encodes the viral enzymes RT, PR and IN. These enzymes are essential for transcription, integration and proteolytic processing of viral proteins. Initially the viral enzymes are produced as a Pol precursor polyprotein and auto-cleaved by the PR region into enzyme products. The gag-gene encodes four proteins needed for the structural elements. The precursor protein is post-translationally cleaved and modified by the viral protease into capsid protein, matrix protein, nucleocapsid protein and a protein having a role in budding (p6). The env-gene encodes the envelope glycoproteins that are essential for recognition, binding and entry into target cells as it contains the binding sites for the CD4 receptor as well as co-receptors. The precursor gp160 is proteolytically cleaved by cellular proteases into gp120 and gp41. The surface of gp120 has five variable loops (V1-V5), which are carbohydrate rich. In addition to the major structural genes, HIV-1 has two regulatory genes (tat and rev) and four accessory genes (vif, vpr, vpu and nef), which are important for regulation of the viral life cycle and immune evasion [24] (Figure 2).

2.2.2 Replication

Similar to other retroviruses the HIV-1 replication cycle involves reverse transcription of the RNA viral genome to form double-stranded DNA. The viral DNA can subsequently be integrated stably into the chromosomal DNA resulting in the provirus.

2.2.2.1 Binding and Entry

The initial step in the HIV-1 replication cycle is a high-affinity binding of the surface glycoprotein gp120 to the cellular receptor CD4. The binding results in conformational changes in gp120 leading to exposure of new epitopes thereby allowing binding to the co-receptor CC chemokine receptor 5 (CCR5) and/or CXC chemokine receptor 4 (CXCR4). Thereafter a second conformational change occurs in gp120, which triggers the fusion peptide in gp41 to penetrate the cell membrane. Consequently, the virion and the host cell
membrane are brought close together, allowing fusion of the membranes and the capsid can be released into the cytoplasm [25, 26].

2.2.2.2 Reverse Transcription

Following the release of the capsid, the viral RT enzyme directs the synthesis of a linear double-stranded DNA (dsDNA) copy from the +ssRNA genome. This complex process, called reverse transcription, is performed inside a partially opened capsid. The process is primed by a human transfer RNA (tRNA) that is bound to the 5’ end of the RNA genome. During reverse transcription the RT enzyme jumps between the two +ssRNA templates [27]. If the two templates are genetically distinct this leads to a new recombinant virus. Recombination significantly contributes to HIV genetic variation and evolution (see section 2.3.1). The RT enzyme contains a DNA polymerase domain and a ribonuclease H (RNAse H) domain. The RNAse H activity degrades the RNA template immediately after its transcription to the first complementary DNA strand, which then serves as template for the synthesis of the second DNA strand. During the formation of dsDNA, long terminal repeats (LTRs) are generated in the 5’ and the 3’ ends of the genome, which are necessary for subsequent integration and transcription.

2.2.2.3 Integration

The dsDNA forms a pre-integration complex (PIC) together with the viral enzyme integrase (IN), p17 and Vpr. The PIC is actively transported from the cytoplasm to the nucleus. Here the IN catalyzes integration of the full-length dsDNA into the host chromosome [28]. Integration primarily occurs into transcriptionally active regions of the cellular genome [29, 30], but can also be take place in resting cells [31, 32]. After entry into the cellular nucleus through the nuclear pore, HIV-1 DNA is then integrated into an active chromatin site close to the nuclear pore complex. [33]. The integrated viral DNA is referred to as a provirus or proviral DNA and persists until the cell dies [34, 35]. Because the provirus is integrated into the host genome, it is also passed on to daughter cells during normal cell division.

2.2.2.4 Transcription and Translation

The proviral DNA can enter a latent state where it is not transcribed or, more commonly, be transcribed into viral mRNA by the host RNA polymerase II. This transcription is initiated at the promoter in the 5’LTR region, where several binding sites for cellular transcription factors are found. The cellular RNA polymerase II transcribes a full-length copy of the viral RNA that can be spliced into several mRNA species or directly packaged into new virion particles as HIV-1 genomes. Initially the primary transcript is spliced into short mRNAs and translated into the regulatory proteins Tat and Rev. Tat binds to the transactivation response region (TAR) and promotes the elongation phase of HIV-1 transcription, thereby increasing the production of viral mRNA. Rev binds to the Rev response element (RRE), which facilitates the export of unspliced and incompletely spliced viral RNAs out of the nucleus for translation. Thereby Rev facilitates the switch from production of early regulatory proteins to production of late structural proteins. Nef increases the rate of CD4 endocytosis and lysosomal degradation as well as down-regulates the expression of major histocompatibility complex (MHC) class I molecules. These events facilitate both viral production and evasion of the host immune response. During the late transcription stages, the predominant mRNA species are unspliced or incompletely spliced. This involves the expression of the longer Gag, Gag-Pol and Env as well as Vif, Vpr and Vpu mRNAs. The mRNAs are translated in the cytoplasm near the endoplasmatic reticulum (ER). The Env protein (gp160) is synthesized in ER, and migrates through the Golgi complex where it undergoes glycosylation, which is essential for infectivity. Cellular proteases cleave gp160 into the surface proteins gp41 and gp120. Finally, the glycoproteins are transported to the plasma membrane [36, 37].
Assembly of the components is initiated near the plasma membrane of the host cell. When the HIV-1 particle buds from the cell it is immature and has poor ability to fuse with target cells. After release the PR cleaves the Gag and the Gag-Pol polyproteins into functional proteins forming the matrix, capsid and nucleocapsid as well as the viral enzymes. The maturation of the virion is final step of the HIV-1 replication cycle and the mature virions can infect other cells [38].

2.3 HIV-1 INFECTION

2.3.1 Transmission

HIV-1 is transmitted through sexual contacts, maternal-infant exposure, blood transfusions and contaminated injection equipment. Most transmissions occur through sexual contact, and heterosexual contact accounts for the majority of sexual transmissions [39]. The sexual transmission rates for HIV-1 from untreated infected persons ranges from 10% per exposure down to less than 0.1% [40, 41]. This wide range in transmission risk is influenced by many biological and behavioral factors. A major role determining the risk of transmission is the level of infectious virions that are present. During the early and late clinical stage of HIV-1 infection the viral load is very high and therefore patients are more infectious during these stages. To establish a systemic infection only one or a few infectious virions need to succeed and in most cases HIV-1 infection is initiated with a single virion infecting a single target cell [40, 42]. A factor that can increase the risk of transmission is the presence of co-infections such as sexually transmitted diseases and genital ulcers [41, 43]. Behavioral factors are of importance for the actual transmission risk. For instance, when condoms are used correctly and when injecting drug users use sterile injection equipment the transmission risk is close to zero. Successful antiretroviral therapy (ART) has been shown to lower the level of viral load to undetectable levels and thereby the risk of HIV-1 transmission from individual patients as well as the spread of the infection at a population level is zero or extremely low (see section 2.6).

2.3.2 Pathogenesis

In the absence of antiretroviral therapy, the course of HIV-1 infection goes through different stages, eventually ending in AIDS and death (Figure 3). After transmission there is an initial stage, referred to as the “eclipse phase”, during which the infection is established. During this phase, which lasts up to 10 days, the virus replicates and spreads from the site of entry to other tissues and organs. Next is the primary (or acute) infection phase, where HIV infection spreads to the lymphoid tissues and the systemic circulation of HIV virions takes place. At this stage viral replication increases rapidly and the viral load reaches very high levels (up to $10^7$ copies of RNA per milliliter of blood). As a result of immune responses directed against this HIV-1 infection, flu-like symptoms may appear during this stage. Primary infection is also characterized by a transient decline in the number of primary target cells, i.e. the CD4$^+$ T cells, which is caused by the viral cytopathic effect and the host immune response. Due to control by the immune response and possibly also the exhaustion of activated target cells, the primary phase ends with a decline in viral load to the so called viral set-point. At this point the levels of CD4$^+$ T cells have been partially restored [44, 45]. The next phase is referred to as chronic infection or clinical latency. During this phase the viral load is stable or slowly increasing whereas the CD4$^+$ T cell levels decline slowly in a roughly linear manner. At this point, patients typically are asymptomatic and unaware of their infection. Although this stage is clinically quiet, the infection is highly dynamic, with large numbers of CD4$^+$ T cells being infected and killed each day. AIDS is the final stage of HIV-1 infection and takes place, on average, 8 years after infection. At this stage the virus has defeated the immune system,
which is unable to control viral replication. This occurs gradually when the CD4⁺ T cells drops below 200 cells/µL. However, when the CD4 counts are 200-500 cells/µL early symptoms of immunodeficiency may appear. During the AIDS stage the CD4⁺ T cells continue to decline and the viral load rises. At the same time the infected individual may experience several different opportunistic infections, including pneumocystis pneumonia and fungal infections, as well as other diseases, including dementia and virus-induced tumors [45].

Figure 3. Typical course of HIV-1 infection.

2.4 CELLS AND TISSUES INFECTED BY HIV-1

2.4.1 Cell Types

As previously described (2.2.2 Replication), HIV-1 initially attaches to the CD4 receptor, which is primarily expressed on CD4⁺ T cells, mainly on naïve, activated and memory T cells. To infect the target cell HIV-1 also requires the engagement of the co-receptor CCR5 and/or CXCR4. Other co-receptors have been identified in vitro, but only CCR5 and CXCR4 have been documented as co-receptors in vivo [46]. A viral classification based on co-receptor usage has been established: R5 viruses (CCR5), X4 viruses (CXCR4) and R5X4 viruses (CCR5 and CXCR4). The majority of infections are established by R5 viruses and during the course of infection X4 viruses can emerge, which happens in 50% of patients [47, 48]. Activated and memory CD4 T cells primarily express CCR5 and are therefore thought to be the initial cells that are infected through mucosal transmission. Monocytes, macrophages and dendritic cells (DCs) also express CCR5, but to a lower degree [49-52]. Monocytes circulate in the peripheral blood and migrate to various tissues where they differentiate into macrophages. Conflicting results have been reported whether monocytes are infected. Several studies indicate that monocytes can be infected in vivo [53-55] whereas other studies show that this cell type is rarely found to be infected [56]. Several studies have shown evidence that different tissue-specific macrophages can become infected in vivo [57-60]. Discussions whether DCs are susceptible to HIV-1 infection or if they merely capture and transport the virions are ongoing [61, 62]. Hematopoietic stem cells (HPCs) express CD34 and are capable of long-term self-renewal and differentiation into either myeloid or lymphoid lineages. HPCs have also been proposed to be infected by HIV-1 in vivo [63]. Whether this cell type is infected or not is discussed in paper I.
2.4.1.1 CD4+ T Cells

The memory CD4+ T cell populations have a significant role in the HIV-1 infection. During an individual’s lifetime the frequency of memory T cells undergo dynamic changes (Figure 4). The different cell types express a distinct set of surface molecules, can be located in different tissues, and have various functions. At birth, all T cells in the peripheral blood are naïve and subsequently memory T cells are generated following antigen exposure [64]. During the primary immune response, antigen-specific naïve T cells (TNA) migrate to the T cell area of secondary lymphoid tissues to search for antigens presented by dendritic cells. When the TNA cells are exposed to an antigen they will undergo proliferative expansion and differentiation into cells with effector capacities. The activated effector T cells (TEFF) will migrate to infection sites to assist with the clearance of infection by orchestrating adaptive immune responses [65, 66]. While the majority of the TEFF cell population dies during clearance of infection the remaining cells will transition into various antigen-specific memory T cell subsets. These memory T cells have been divided into subsets characterized by their phenotypic and functional profiles, including central memory (TCM), transitional memory (TM) and effector memory (TEM). The main subsets, TCM and TEM, have distinct homing capacities and effector functions [67, 68]. The TCM subset, which expresses the lymph node homing receptor CCR7, have the ability to home to secondary lymphoid organs and have an increased capacity to survive and proliferate after activation [68]. The TCM transitions into TEM after T cell receptor (TCR) triggering or, to a lesser extent, in response to homeostatic cytokines such as interleukin 7 (IL-7) and interleukin 15 (IL-15) [69]. The TEM subsets, which do not express CCR7, have direct effector functions after antigen stimulation. The TEM subset displays functional and transcriptional characteristics that are intermediate to the central and effector subset characteristics. TEM subsets express CCR7 as well as a marker important for the long-term maintenance of memory called CD27 [70]. During adulthood the levels of memory T cells are maintained through homeostasis. Thereafter, the memory T cells undergo the last phase referred to as immunosenescence. This phase starts around age 65 and is characterized by an altered proportion and functionality of memory T cells [64]. Recently, a new subset of memory T cells was observed in viral and tumor-reactive T cell populations, the T memory stem cell (TSCM). They have the ability to rapidly release cytokines on activation and a high proliferative capacity in response to IL-15. TSCM cells are the least differentiated memory T cell population and have the capacity to self-renew and generate all memory and effector T cell subsets [71]. In addition, studies on mouse models have established the existence of another new subset referred to as tissue-resident memory T (TRM) cell subset. This memory T cell subset is a non-circulating subset that resides in peripheral tissue sites. TRM cells have been shown to be important to antiviral host defense in epithelial tissues and these cells have also been found in lung where they are designated to respond to pathogens previously encountered through lung mucosa [64, 72].

![Figure 4. A schematic model for T cell differentiation.](image-url)
2.4.2 Tissue Compartments

If HIV-1 is transmitted through sexual contact the virus will first replicate locally at the site of infection and thereafter migrate to lymph nodes where the replication intensifies due to a higher abundance of target cells. After the virus has established itself in lymph nodes it is broadly disseminated to the rest of the body where it establishes infection in cells located within various tissues. Most studies investigating HIV-1 infection have relied on analyses of peripheral blood, which is the most accessible compartment. However, several studies have shown that other tissue compartments harbor the majority of the virus as the majority of lymphocytes are distributed in tissues such as lymph nodes, spleen and gut-associated lymphoid tissues (GALT) [73, 74]. The peripheral blood compartment harbors a low percentage of the total lymphocytes found in the body [75]. In contrast, the GALT compartment, which is the largest lymphoid organ in the body, harbors 60% of the lymphocytes, 40% of them being CD4+ T cells [74, 76, 77]. Due to the large amount of lymphocytes that are located in the GALT compartment it is not surprising that the GALT compartment plays an important role in HIV-1 infection. Studies have shown that this compartment is the primary site of viral replication and that during acute/early infection up to 60% of the mucosal memory T cells are infected. Moreover, this compartment is thought to encounter the most substantial CD4+ T cell depletion during all stages of the disease [77]. As mentioned above, dendritic cells and other antigen presenting cells migrate to the lymph nodes where infection of CD4+ T cells occurs. Some studies suggest that the lymph node has a higher amount of HIV-1 RNA and DNA compared to peripheral blood [78, 79]. In addition to lymphoid tissue, virus can be detected in the central nervous system (CNS) throughout HIV infection [80-83].

2.5 HIV-1 GENETIC VARIABILITY

2.5.1 Sources of Genetic Variation

HIV-1 has the capacity to rapidly develop a genetically diverse population from initially one or a limited number of infectious particles. This feature provides the virus with the ability to evade the host immune system, develop drug resistance during suboptimal antiretroviral therapy, and hinder the development of successful vaccines. Several unique mechanisms contribute to this effect: high viral turnover, a high mutation rate during viral replication, viral recombination and immune evasion.

2.5.1.1 High Viral Turnover and Mutations

During untreated HIV-1 infection the virus has high replication capacity and turnover rate, which contributes to the rapid evolution of HIV-1. The time from the release of a virion until it infects another cell and causes the release of a new viral particle is called the generation time. For HIV-1 the generation time is estimated to be approximately 2 days and the replication rate is thought to be as high as 10^{10} new viral particles per day in untreated infected persons [29, 84, 85].

During the HIV-1 replication cycle, point mutations are spontaneously generated throughout the viral genome by several mechanisms. The vast majority of genetic diversity is introduced by the HIV reverse transcriptase (RT) when it transcribes the viral RNA into dsDNA. Since the viral RT enzyme lacks proofreading mechanisms, these mutations will remain uncorrected. The overall mutation rate has been estimated to generate an average of 3.4x10^{-5} mutations per base pair per replication cycle. Considering that the HIV-1 genome is approximately 10 kb this equals 0.3 mutations per genome and replication cycle [86, 87]. A second source of viral diversity takes place during the transcription of the viral genome which is mediated by the host cellular RNA polymerase II (RNAPII) complex. However, this
mechanism contributes to less than 3% of all mutations during replication and is therefore not a major contributor to the genetic diversity [88]. Another mechanism contributing to the genetic diversity of HIV-1 are the APOBEC3 host proteins: APOBEC3G and APOBEC3F. These host restriction factors function as innate inhibitors of retroviral replication by introducing G-A mutations. These APOBEC3G-induced G-A mutations are believed to occur during a single replication cycle and can result in stop codons within the HIV-1 genome and replication incompetent viruses – a process called hypermutation [89, 90]. However, the viral protein Vif counteracts the antiviral effects of APOBEC3G and F by targeting these proteins for degradation.

2.5.1.2 Retroviral Recombination

During reverse transcription the RT enzyme switches between the two ssRNA genome templates. By using information from both templates the process results in a hybrid viral DNA. This event is estimated to occur between 2 to 30 times per replication cycle. Although recombination occurs in all replication events, making all HIV-1 DNA molecules recombinants, a single cell must be infected with two or more genetically distinct viruses to generate a recombinant that is genetically different from either of the two parental templates [91-93]. However, the majority of CD4+ T cells in peripheral blood contain only one copy of the HIV-1 DNA molecule implying a limited potential for virus recombination in these cells [56]. Despite being a rare event, recombination contributes significantly to HIV-1 diversity as evidenced by the existence of circulating recombinant forms.

2.5.1.3 Immune Evasion

After primary infection, the host immune system exerts considerable selection pressure on the infecting HIV-1 population. In fact, this immune pressure continues throughout the course of HIV-1 infection. Through Darwinian selective pressures the best-adapted, most “fit” genetic variants, are favored. Therefore, due to immune pressure, escape mutations arise which allow HIV-1 to evade the host immune system. These escape mutants have been shown to be transmitted from one host to another, thereby driving evolutionary changes also at a population level [94-96].

2.5.2 Methods to Study HIV-1 Genetic Diversity

Phylogenetic analysis is the study of evolutionary relationships through sequence comparison. By studying the relatedness between viral variants one can attempt to reconstruct the evolutionary history and explain the observed diversity. The sequencing data, retrieved through extraction of genetic material from viral RNA or DNA, can be used to construct phylogenetic trees and measure the genetic distance between viral sequences.

2.5.2.1 Phylogenetic Analyses

Phylogenetic analysis is a process whereby the use of mathematical algorithms, statistical methods and software programs are used to construct a phylogenetic tree that attempts to represent the evolutionary and/or genetic relationship between sequences. A phylogenetic tree, also called an evolutionary tree, is a branching diagram which shows the inferred genetic relationship between different sequences (see Figure 5 and Textbox 1 “Tree Terminology”). There are several approaches to construct a phylogenetic tree. The four major methods that can be used are distance, parsimony, maximum likelihood (ML) and Bayesian. The constructed phylogenetic tree is unlikely to fully reproduce the “true” tree that represents the actual evolutionary relationship and each method has its advantages and weaknesses. Both the parsimony and the distance-based methods, such as neighbor-joining (NJ), are relatively fast and work well on closely related sequences. In contrast, ML and Bayesian methods are
usually more accurate, but slower because they are computationally more complicated. For NJ, the optimal tree is generated by first creating a pair-wise distance matrix to estimate the evolutionary distances between the sequences. These matrices are then used to build a tree. While NJ does not explore several tree options, the parsimony method considers the optimal tree to be the tree requiring the fewest nucleotide or amino acid substitutions required over time to fit the sequences in the dataset. The maximum likelihood method explores how likely the sequences are to have evolved in a particular tree. An evolutionary model is used to assess the probability of a particular mutation. Thereby, the optimal tree has the highest likelihood of producing the observed data (see section below). Finally the Bayesian method uses a statistical model to search the phylogenetic tree by calculating the likelihood of a tree itself.

![Figure 5. Description of a phylogenetic tree.](image)

Evolutionary models are statistical descriptions of the process of nucleotide changes in the tree. To estimate the genetic distances between sequences in an alignment the models measure the nucleotide substitutions per site that have occurred and their most recent common ancestor. There are several models of nucleotide evolution. The simplest models, such as Jukes-Cantor assumes the following: 1) there are equal proportions of all nucleotides; 2) that the ratio of transitions and transversions equals 1; and 3) that the probability of a base changing into any of the other three is equal. Since these assumptions do not reflect the true evolutionary path several more complex models have been developed. The general time-reversible (GTR) model assumes that all substitutions have their own rate, a symmetric substitution matrix \((A \rightarrow T = T \rightarrow A)\) and variable base frequencies. In addition, there are models to describe the rates of variation among sites in a sequence such as the gamma distribution model which assumes that substitution rates vary between sites in a gamma-distributed manner. Sometimes the models also allow a proportion of sites in the sequence are invariable. Reliability tests are used to evaluate the constructed trees robustness or accuracy. There are a number of measures for this with the most common being a resampling method called bootstrapping. By creating pseudo-replicate datasets through resampling with replacement, bootstrapping allows you to assess the trees reliability. Each node is given a number which represents the reliability of the placement of individual branches in the optimal tree given the sequence data and the assumptions used to construct the tree.
Textbox 1. Tree terminology

- **Tree topology** is the branching pattern.
- The **branches** define the relationship between the sequences.
- The **branch length** represents the number of genetic changes that have occurred between each sequence referred to as the genetic distance. The longer the branch, the greater the genetic distance is between two sequences.
- The **taxa** are the tips of the tree and in our case represent the actual viral sequence. Can be specified with a specific symbol to represent a different cell type and tissue.
- A **clade** is a grouping of an ancestor and all of its descendants.
- The **node** is the point where the branches are connected and represent the assumed ancestral sequence from which the tips or taxa descend.
- A **distance scale** is used to show the amount of genetic change in the number of nucleotide substitutions per site, i.e. the number of changes or substitutions divided by the length of the sequence.
- A **rooted tree** suggests that the path from the root to a node represents an evolutionary path, with the root being the common ancestor of all taxa.
- An **unrooted tree** shows the genetic relationship between taxa or sequences.
- An **out-group** can be included in unrooted tree, which is a one or more sequences that falls outside the group of sequences being studied. All sequences of interest are more closely related to each other than they are to the out-group.

2.5.2.2 Average Pairwise Distance

Average pairwise distance (APD) (used in paper I) is a method used to estimate the genetic diversity between sequences by calculating the proportion of nucleotide differences between each pair of sequences. The method can be used to compare the genetic diversity of HIV within different cell types, compartments or at different time points. For example, the viral populations in acute/early HIV-infected patients typically have low genetic diversity and APD, consistent with the infection by a single or small number of viral variants [97]. In contrast, the viral populations within an HIV-infected patient who has been untreated for several years will have a high genetic diversity and APD. However, one should keep in mind that pairwise methods simply evaluate the number of differences and, therefore, this can be an underestimate of the true evolutionary distance. Also, if a patient is infected with two or more diverse HIV-1 variants, the genetic diversity and APD will be high during acute infection.

2.5.2.3 Compartmentalization

A viral subpopulation can become compartmentalized if trafficking and gene flow is significantly restricted between the different subpopulations. Due to the high mutation rate of HIV-1, the genetic distance between subpopulations can increase rapidly. Factors such as different selective pressures by the immune system and suboptimal drug distribution can increase the compartmentalization of viral populations. The analyses used to detect compartmentalization of viral populations between different compartments can also be used to detect compartmentalization between different time points. There are two different types of compartmentalization analyses (used in papers III and IV) 1) tree-based, which use a phylogenetic tree to detect compartmentalization and 2) distance-based, which are based on pair-wise distance. These compartmentalization analyses are only as reliable as the topology of the phylogenetic tree (tree-based) or as reliable as the pairwise distance values (distance-based).
2.5.2.4 Evolutionary Rate Estimations

Another way to study genetic diversity and genetic evolution is to measure the rate of mutation accumulation. Due to features such as high error rate and high turnover, HIV-1 viral populations accumulate many mutations over time. By comparing molecular sequence data from two time points the evolutionary rate can be estimated. This estimation can be done using different statistical methods such as linear regression, maximum likelihood and Bayesian inference. A challenge with these estimations is that single nucleotide sites may experience sequential mutations. These processes are difficult to discern, especially over a longer evolutionary time.

2.6 ANTIRETROVIRAL THERAPY

2.6.1 History and Current HIV-1 Disease Management

Almost all untreated HIV-1 infected patients will develop AIDS. With a mortality rate well over 95%, this makes HIV-1 one of the most lethal diseases known to mankind. Although no cure or effective HIV vaccine exists, the remarkable development of HIV-1 treatment has dramatically increased the life expectancy of millions of HIV-1 infected individuals. By 1987, a total of 71,751 AIDS cases had been reported to the World Health Organization (WHO) and 5 to 10 million people were estimated to be infected with HIV worldwide [98]. During 1987 the first antiretroviral drug, azidothymidine (AZT), became available. However, it took several years until the death rates from AIDS dramatically fell for the first time. The explanation for this decrease was the development of new drugs and the introduction of a more powerful combination therapy regimen using a “cocktail” of drugs. This new treatment strategy, often referred to as highly active antiretroviral therapy (HAART) or combination antiretroviral therapy (cART), was introduced in 1996. Today there are six distinct classes of antiretroviral drugs. By giving a combination of drugs simultaneously from two or more different classes, long-term viral suppression can be achieved, as treatment with cART stops or significantly reduces viral replication and prevents the evolution of drug resistance [99-102]. If treatment is successful viral replication is suppressed and the plasma viral load is reduced to less than 50 RNA copies/mL. However, despite several years of successful therapy one can still detect low-levels of viremia [103]. Virological treatment failure and development of drug resistance occurs when the viral load rebounds or if the level is not decreased sufficiently despite cART. Factors causing virological failure include lack of adherence, poor drug tolerability and drug-drug interactions [84].

As of March 2015, 15 million people living with HIV were accessing antiretroviral therapy, which is an increase from 13.6 million in June 2014 [23]. In 2013 a worldwide treatment target, called 90-90-90 was developed to help end the AIDS epidemic. This strategy, proposed by UNAIDS, has the ambitious target that by 2020 90% of all individuals living with HIV will be aware of their HIV status, 90% of all individuals diagnosed with HIV will receive antiretroviral treatment (ART) and 90% of all individuals receiving ART will have viral suppression [104]. The recent 2014 progress report from European centre for disease prevention and control (ECDC) showed that Sweden is the only country in Europe and central Asia which is currently reaching all three targets [105].

2.6.2 Available and Potential Antiretroviral Drugs

Although there are many potential targets in the HIV-1 replication cycle that could be possible for therapeutic interventions, only a few targets have led to successful drugs. Today there are about 25 antiretroviral drugs that have been approved for treatment of HIV-1 infection. Out of the six different classes of antiviral drugs the majority of antiretroviral compounds belong to three classes: nucleoside reverse transcriptase inhibitors (NRTIs); non-
nucleoside reverse transcriptase inhibitors (NNRTIs); and protease inhibitors (PIs). The reverse transcriptase inhibitors, NRTIs and NNRTIs, target the HIV-specific RT enzyme and affect the DNA polymerization activity of the enzyme and block the generation of full-length viral DNA. NRTIs, such as abacavir (ABC), lamivudine (3TC), and tenofovir (TDF), are dNTPs lacking the 3-hydroxyl group that is necessary for DNA elongation. The NRTIs function as substrates for the RT during reverse transcription and thereby terminate the DNA strand of HIV-1 during transcription [102, 106], hence they are sometimes called chain terminators. The other subclass of RT inhibitors is the NNRTI subclass, which by non-competitive binding to the RT enzyme inhibit the enzyme activity. Efavirenz (EFV) is an example of a NNRTI which is often chosen as part of first-line cART today [102, 107]. The PIs interfere with the essential viral enzyme protease and inhibit the maturation of viral particles. PIs block the gag-pol polyprotein cleavage, thereby leaving them non-infectious. Atazanavir (ATV) and darunavir (DRV) are examples of two PIs currently being recommended as first line therapy options [102, 108]. Another drug class is aimed at blocking viral entry. This step can be targeted by attachment inhibitors, chemokine receptor antagonists and fusion inhibitors. To date there are two approved drugs: maraviroc (MVC) and enfuvirtide (T-20). MVC is a small molecule CCR5 chemokine receptor antagonist, which prevents the binding of gp120 to the co-receptor and hence prevents viral entry. T-20 is a peptide, which binds to the HIV-1 gp41 protein and thereby prevents the fusion of the virus to the host cell [109, 110]. Integration is another step in the viral replication cycle which can be targeted by drugs. Raltegravir (RAL) inhibits strand transfer and blocks integration of the viral genome into the cellular DNA, by binding to a specific complex between the viral integrase and the viral genome [102, 111].

There are several potential steps in the viral replication cycle which are under intense investigation for future drug discovery. For example, during transcription, the HIV-1 regulatory protein Tat binds to the viral RNA element TAR. Since this mechanism is unique to HIV-1 it is a highly desirable step to target in the HIV-1 replication cycle. Although several small-molecule inhibitors targeting HIV-1 transcription have been identified, none have passed phase I clinical trials. Another potential target is virus assembly and viral release. Progress have been made in this area but unfortunately insufficient antiviral activity have terminated these compounds in early phase trials [102].

2.7 THE HIV-1 RESERVOIRS

Although HIV-1 viremia can be suppressed and maintained at low levels for prolonged periods of time, cART alone cannot eradicate HIV-1 in infected individuals. Latency is a common feature of retroviruses and in the case of HIV-1 enables the virus to survive and persist despite host immune response and antiviral therapy. During the latent state, the integrated HIV-1 virus is transcriptionally silent and persists solely as information. However, if the latently infected cell is activated, e.g. during suboptimal treatment or treatment interruption, the latent state is reversed and leads to rapid viral rebound. The latent HIV-1 reservoir is defined as a cell type or anatomical site where a replication-competent form of the virus can accumulate and persist stably during optimal cART. When HIV-1-infected individuals stop treatment they exhibit plasma viral rebound which arises from replication-competent virus that persists within these latent reservoirs. Therefore, defective proviral sequences do not represent the true latent reservoir as cells containing defective proviral sequences cannot produce HIV-1 upon reactivation [112]. These long-lasting latent reservoirs are the main impediment to a cure for HIV-1, and therefore, there is great interest in identifying which cell types and anatomical sites act as reservoirs during cART (see section 2.7.2).
2.7.1 Dynamics during Antiretroviral Therapy

In 1995, studies showed that suppressive therapy caused plasma viral loads to decrease exponentially [85, 113]. Hopes for HIV-1 eradication arose when cART was introduced and showed a reduction of plasma viral load to below limit of detection [114-116]. But unfortunately, it was soon evident that the decay rate of the pool of latently infected cells was extremely slow in patients treated with antiretroviral therapy. Statistical analysis suggest that these cells, with a half-life of approximately 44 months, would require over 60 years of suppressive cART to be eradicated [117-119]. The viral decay after initiation of cART can be divided into four phases. The different phases represent the death or elimination of different infected cell types. The first phase after initiation of cART is characterized by its rapid drop in plasma viral load, reflecting the short half-life (1-2 days) of the activated virus-producing CD4+ T cells [85, 113]. The second phase of decay is slower and represents the release of virus from other cell populations with a half-life of about two weeks. The cells thought to be responsible for this decay are partially activated CD4+ T cells and macrophages [116, 120-122]. However, this is uncertain and other cells have also been proposed to be linked to the second phase of decay. During phase three the viral decay is much slower with a half-life of 39 weeks and subsequently during phase four the HIV-1 RNA levels are stable at very low levels with no perceptible viral decay. Despite years of successful cART, sensitive assays with limits of detection down to less than 1 copy of HIV-1 RNA/ml revealed traces of viremia in many patients with a median viral RNA level of 3 copies/ml [103, 123, 124]. This residual viremia has a very short half-life and therefore it is continuously replenished by some mechanism [29]. However, the origin of this residual viremia during cART is a matter of controversy and several hypotheses have been suggested (Figure 6). The first hypothesis suggests that ongoing HIV-1 replication occurs in sanctuary sites where drug penetration is low or absent. A second explanation is that cART inhibits all or almost all viral replication and that the residual viremia is released from long-lived T cells that are reactivated. Another hypothesis suggests that low-level of HIV-1 RNA is released from other undiscovered reservoirs (discussed in 2.7.2). However, recent studies suggest that the reservoir is not only driven by T cell survival but is also maintained by homeostatic proliferation of latently infected CD4+ T cells. These cells may be the source of the low-level of HIV-1 RNA found in patients on cART [125] (paper IV).

Figure 6. Mechanisms of HIV-1 persistence.
2.7.1.1 Viral Replication during Suppressive Therapy

There are several approaches that can be used to investigate whether ongoing replication occurs during suppressive therapy. One approach is to study genetic change during suppressive therapy. The detection of viral evolution during therapy would be a clear indication that cART does not completely stop viral replication. However, analyses of this type can be complicated if patients are not fully adherent to cART. Therefore, the detection of HIV-1 evolution in a small subset of patients could rather be evidence of poor adherence than evidence of ongoing replication. Several studies fail to detect strong evidence for evolution in the majority of patients on cART [46, 126, 127] (papers III and IV), but there are also studies that support the hypothesis that replication occurs during suppressive therapy [128]. One report, with results implying ongoing viral replication in the GALT, showed a decrease in the amount of un-spliced HIV-1 RNA in CD4\(^+\) T cells isolated from the terminal ileum during raltegravir intensification [129]. Another study by Fletcher and colleagues, revealed that replication continues in the lymph nodes of some individuals receiving successful treatment. According to this study, the ongoing replication is explained by the lower concentrations of antiviral drugs in these tissues compared to peripheral blood [130].

Another approach to study whether replication occurs during cART is to use certain virologic measures to detect unintegrated forms of the viral genome. Detection of linear unintegrated HIV-1 DNA would indicate recent infection since this form of DNA is targeted by exonucleases and is labile until integrated into cellular DNA [131, 132]. When integration is blocked, circular forms of the HIV-1 genome, especially 2-LTR circles, can arise [133, 134]. The circular forms are dead ends with respect to replication but could also indicate recent infection. However, the stability of these HIV-1 DNA forms is still under discussion and therefore the significance of finding these forms of HIV-1 DNA during treatment remains unclear [135, 136].

Another way to test the hypothesis that viral replication continues during suppressive therapy would be to demonstrate that intensification of standard cART would further reduce the level of viremia. Several studies, using different drugs, have shown that intensification of cART does not affect the level of persistent viremia [129, 137-139]. A study by Buzón and colleagues did however show increased levels of episomal viral cDNA during raltegravir intensification. This study indicates that ongoing replication may occur during cART and that replication is completely or partially blocked during intensification [140]. The CNS has been proposed to be a possible sanctuary site where HIV-1 can continue to replicate despite cART. Since the CNS is separated from the circulating blood by the blood-brain barrier, some antiviral drugs penetrate the CNS poorly. Antiviral therapy is known to reduce the HIV-1 RNA levels in the cerebral spinal fluid (CSF) in most patients [82, 141, 142]. However, recent intensification studies could not detect a reduction in CSF viral loads indicating that there is little or no ongoing replication in this compartment [143].

2.7.2 Source of Persistent HIV-1 during Antiretroviral Therapy

The establishment of latently infected cells is a rare event. However, although the pool of latently infected cells is very small it is extremely important since without life-long cART viral rebound occurs within weeks of treatment interruption [144]. One well-defined reservoir is a small pool of latently infected resting memory CD4\(^+\) T cells [145, 146]. When resting memory CD4\(^+\) T cells are activated in response to an antigen they undergo a burst of cellular proliferation and differentiation, giving rise to effector cells. Although most effector cells die quickly a subset reverts to a memory state. A plausible hypothesis is therefore that HIV-1 latency is established when activated CD4\(^+\) T cells, which are highly susceptible to HIV-1 infection, become infected by HIV-1 and survive long enough to revert back to a resting state [85, 113, 147]. Out of the memory CD4\(^+\) T cell subsets, \(T\text{CM}\) and \(T\text{Tm}\) have been shown to
contribute most to the HIV-1 reservoir during therapy [125] (discussed in paper III). In a recent study it has been shown that the stem cell-like memory T cell subset, T_{SCM}, despite their low frequencies, may be of importance for HIV-1 persistence due to their ability to self-renew, resist apoptosis and survive for long periods of time [148]. Another T cell subset that has been demonstrated to contain HIV-1 DNA in patients on long-term suppressive therapy is T_{NA} cells. Compared to memory T cell subsets this population contains a lower frequency of HIV-1 DNA [149] (papers III and IV).

Some phylogenetic analyses indicate that the majority of the low-level HIV-1 RNA isolated and sequenced from the plasma during therapy is genetically identical. Interestingly, these plasma-derived HIV-1 RNA sequences are not often found in intracellular HIV-1 DNA isolated and sequenced from circulating resting CD4^+ T cells [150]. Hypotheses for the origin of these clonal plasma-derived sequences are: 1) they represent virus being continuously released from a long-lived cell which was infected before initiation of cART; 2) they represent virus released when a large subset of cells, which were infected by the same variant before initiation of cART, are reactivated and release virions; or 3) they represent progeny virus being released from an infected progenitor cell as it divides. Progenitor cells that have been suggested to be infected are the HPCs [63]. However, whether this cell population is infected or not is unclear [151] (paper II). Other cell types that have been proposed to play an important role in maintaining the HIV-1 reservoir in patients on cART are monocytes and macrophages. In some studies these cells have been shown to contain HIV-1 DNA [152-154]. However, to date, it is unclear what role these cells play in maintaining the HIV-1 reservoir in patients on long-term treatment (discussed in papers III and IV).

Beside peripheral blood and bone marrow, a number of anatomical sites have been proposed to act as reservoirs including the gastrointestinal (GI) tract, lymph nodes, central nervous system (CNS), genital tract, semen and the lung. If these anatomical compartments have suboptimal drug penetration or are non-permissive to immune surveillance viral replication may take place at these sites. Studies have shown that the majority of the lymphocytes are sequestered in the GI tract and thereby it is not surprisingly GALT has been proposed as a major HIV-1 reservoir in patients on long-term cART [155-159]. Lymph node tissue, which also contains numerous memory CD4^+ T cells, may be another important anatomical compartment that may serve as an important reservoir. In untreated patients, HIV-1 infection is detected in microglia and perivascular cells [160]. If these cells or other cells in the CNS act as a latent reservoir during cART, they also need to be targeted in order to cure HIV-1.

2.7.3 Methods to Study HIV-1 Reservoirs

HIV-1 persistence is the major obstacle to eradication and finding strategies to reduce or totally eradicate HIV-1 reservoirs is a major challenge. Therefore, it is crucial to find methods that accurately measure the HIV-1 reservoirs. There are several factors that must be taken into account when measuring persistent HIV-1 infection. Most of the work investigating the reservoir has relied on studies of peripheral blood. However, findings suggest that the reservoir is largely established and maintained in tissues, and that the infected cells circulating in blood may not necessarily be representative of the much larger population of infected cells in tissue. Therefore, it is important to decide in which body compartment and cell type one should measure the HIV-1 reservoir. Another critical issue is the status of the virus. If the virus is replication-competent it has the ability to replenish the reservoir if cART is interrupted. However, if the virus is replication-incompetent it is dead-end virus and may not need to be targeted during eradication attempts. A final aspect to take into account is whether the infected cell is quiescent or activated.
Currently, there are several methods used to study the reservoir. The different methods often address different questions such as the size of the reservoir, the composition of these reservoirs, or the capacity of latent reservoirs to produce virus. All these assays provide new insights and are valuable for studying the reservoir. However, each method has its advantages and disadvantages (Table 1). Therefore, it is crucial to recognize what the assay is measuring and what the goal is for a particular clinical study.

### Table 1. Advantages and disadvantages.

<table>
<thead>
<tr>
<th>Measurement (Assay)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma-Derived HIV-1 RNA (SCA) * [123]</td>
<td>-Measures ongoing viral production -Relatively inexpensive</td>
<td>-Does not directly detect the frequency of latently infected cells -Patients on long-term cART close to limit of assay detection</td>
</tr>
<tr>
<td>Replication competent virus (QVOA) * [117, 147, 161]</td>
<td>-Direct measurement of replication competent virus, or number of proviruses capable of productive infection</td>
<td>-Requires large quantities of cells -Expensive -Time consuming (2-3 weeks) -Limited dynamic range -Does not detect all functional proviruses</td>
</tr>
<tr>
<td>Total HIV-1 DNA (qPCR or ddPCR) * [162, 163]</td>
<td>-Inexpensive -Easy and quick -For ddPCR absolute quantification</td>
<td>-Also detects defective proviruses which may not pose a barrier to a cure</td>
</tr>
<tr>
<td>Integrated HIV-1 DNA (Alu-PCR) * [164]</td>
<td>-Excludes unintegrated HIV-1 DNA -More accurate than QVOA</td>
<td>-Detects defective proviruses -Does not detect proviruses too far from Alu sequence (a correction factor can be used to account for this issue)</td>
</tr>
<tr>
<td>2-LTR circles (qPCR or ddPCR) * [165, 166]</td>
<td>-Used to measure ongoing replication based on the assumption that these circles are labile -For ddPCR absolute quantification</td>
<td>-Does not detect integrated proviruses</td>
</tr>
<tr>
<td>Cell-associated HIV-1 RNA [167-169]</td>
<td>- Direct measures of cell associated HIV-1 RNA, unspliced RNA or multispliced RNA - Inexpensive, easy, and quick - Measures intracellular HIV transcription</td>
<td>-Detects abortive and defective transcripts which may not result in viral proteins</td>
</tr>
</tbody>
</table>

*Method described in method section.
2.7.3.1 Novel Assays to Study the HIV-1 Latent Reservoir

There is an intense interest in developing new highly sensitive methods that can be useful in curative interventions and/or to identify HIV-1 infected individuals with such a low reservoir size that they may be able to interrupt antiviral therapy. One such novel approach utilizes the host immune response against HIV-1 antigens to quantify the latent reservoir. This method, called luciferase immunoprecipitation systems (LIPS), detects HIV-1 specific antibodies against antigens such as gp120, gp41, RT and p24. In a recently published paper, Burbele and colleagues showed that a loss of antibodies against p24 and lower responses to gp41 was associated with an HIV-1 curative state [170]. The so called murine viral outgrowth assay (MVOA) is a new method used to evaluate curative strategies. In this assay PBMCs or purified CD4+ T cells from HIV-1 infected participants with undetectable plasma viral loads are transferred into immunocompromised mice to see whether this results in viral amplification. This method could also be used to detect viral reservoirs within the peripheral blood of SIV infected macaques used in eradication studies. In the published study, replication-competent HIV-1 was successfully detected in all studied HIV-1 infected participants as well as from all elite controllers (HIV-1-infected individuals who naturally maintain undetectable viral loads and high CD4 counts without receiving cART). Interestingly, replication-competent virus was amplified from one elite controller using the MVOA but not when using the standard QVOA, indicating that the MVOA may be more sensitive than the QVOA [171]. However, a disadvantage with the MVOA assay is that it is expensive compared to PCR-based assays. Another promising novel approach to quantify latently infected cells is the so called tat/rev induced limiting dilution assay (TILDA). This assay measures the frequency of cells with inducible tat/rev multiply spliced HIV-1 RNA, which are usually absent in latently infected cells but induced when viral reactivation occurs. The assay combines sensitive detection of tat/rev multiply spliced HIV-1 RNA and maximal activation of CD4+ T cells in a limiting dilution format. Results from a recent study of this assay showed that the frequency of latently infected cells estimated using TILDA was almost 50 times higher than the frequency measured using QVOA, and 6-27 times lower than the frequency measured using PCR-based assays. Although this assay may be an overestimate of the true latent reservoir it is a promising assay due to its attractive characteristics such as it is sensitive, quick, covers a wide dynamic range and only requires 10 ml of blood [172].

2.7.4 Eradication Strategies

Due to the success of cART, the life expectancy of an individual living with HIV-1 in a high income country is close to life expectancy of uninfected people. However, life-long therapy and stigmatization remain. Furthermore, the access to care for all HIV-1 individuals is a tough challenge. Another issue with the need for life-long cART is that little is known about the long-term toxicity (>20 years) of antivirals. Finally, the lifetime treatment costs associated with cART represents another argument for developing a cure for HIV-1 infection. Eradication of HIV-1 is one of the major challenges of our time. In developing new curative strategies there are two forms of a cure which may be achieved: a sterilizing cure or a functional cure. A sterilizing cure is the ideal model in which all traces of the virus would be eradicated from the body. With functional cure, also referred to as viral remission, the aim is to give individuals living with HIV-1 the possibility to stop treatment, at least for a defined period, and still be without risk for transmission and/or significant plasma viral rebound. In this case HIV-1 DNA can still be detected, indicating that replication-competent virus may be present as a latent reservoir.

The interest for an HIV-1 cure intensified after an infected man named Timothy R. Brown, also known as the ‘Berlin patient’, exhibited evidence of an HIV-1 cure after receiving an allogeneic hematopoietic stem cell transplant. Brown was diagnosed with HIV-1 in 1995.
After many years of successful therapy he was diagnosed with acute myeloid leukemia (AML) and in 2007 he underwent an allogeneic hematopoietic stem cell transplantation from a donor homozygous for a deletion in the gene that encodes for CCR5 [173]. Brown stopped cART approximately seven years ago and has had no detectable virus in his plasma by standard assays [174], i.e. appears to be cured. Although, Timothy Brown is a proof of concept for a curative strategy other studies have given some hopeful results. Recently Henrich and colleagues conducted a study where two HIV-1 positive lymphoma patients in Boston received allogeneic hematopoietic stem cell transplants using cells from wild-type CCR5 donor [175]. Unfortunately, both individuals demonstrated viral rebound 3 and 8 months after cession of cART [176], indicating that these transplants delayed viral rebound but did not result in a cure. Other studies have used approaches to genetically modify CD4+ T cells [177, 178] or stem cells [179] in order to make the cells resistant to HIV-1. However, stem cell transplants and gene modifying strategies are expensive and possibly not scalable to resource limited areas.

Since HIV-1 typically has the ability to rebound soon after cART interruption sustained viral remission is a challenging goal. Studies have shown that very early initiation of cART reduces the frequency of cells containing HIV-1 DNA integrants [180, 181]. Unfortunately, recently it became evident that virus can remain hidden for years before eventually rebounding. This was shown in a study of a baby from Mississippi, who initiated therapy within 30 hours after birth [182]. The infant stopped cART after 18 months because the baby was lost to follow-up care and seemed to be cured but eventually after 27 months off cART viral rebound occurred [183]. Recently, a study by Saez-Cirion and colleagues presented promising results which showed that early treatment (<10 weeks after primary infection), for a median of 3 years resulted in virological control following cART cessation for an average time of nine years. Replication-competent virus has been isolated from these patients but viral production appears to be controlled by the immune system [184]. However, relying on early cART to achieve a possible functional cure means that diagnosis and treatment must occur very soon after infection, which rarely is the case.

A current focus of HIV-1 cure-research is called “shock and kill”. This strategy is based on the hypothesis that cART prevents infection of new cells and aims to eliminate all latently infected cells capable of producing infectious virions by treating patients on cART with latency reversing agents. In short, by reactivating all latently infected cells in patients on cART it is presumed that these cells will die from viral cytopathic effects or be killed by the immune response directed against them. In theory, this strategy will eliminate the latent reservoir. Histone deacetylase is involved in maintaining HIV-1 latency and therefore histone deacetylase inhibitors (HDACi) are candidates as latency reversing agents. The findings that the HDACi, vorinostat, can reactivate HIV-1 transcription in patients on suppressive therapy showed that latency is not completely irreversible [185]. Other studies using the same molecule or a molecule from the same family have also shown that latency reversing agents induce HIV-1 transcription in latently infected cells. But unfortunately, these studies do not seem to significantly reduce the size of the reservoir [186, 187]. A recent in vitro study revealed that virus reactivation did not cause the elimination of latently infected cells by viral cytopathic effect or cytolytic T lymphocyte response. This finding indicates that for latency reversing agents to be effective, sufficient immune response is needed. Therefore, one approach to eliminate HIV-1 infection would be to induce transcription from quiescent HIV-1 proviruses followed by targeted immunotherapy to clear infected cells [188].

Ongoing potential strategies to eliminate latently infected cells utilize cytomegalovirus as a novel persistent vector to deliver HIV-1 antigens [189] and highly broadly neutralizing antibodies [190]. Moreover, immune modulatory therapies used in cancer treatment may be able to activate antigen specific T cells. Ongoing research show that so-called immune
checkpoints, such as programmed death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), expressing cells are enriched for HIV-1 DNA and may be used as biomarkers to identify the viral reservoir. Furthermore, these ligands inhibit viral reactivation and thereby have a role in maintaining the HIV-1 reservoir. By blocking these molecules with immune checkpoint blockers, HIV-1-specific immunity is presumed to be restored and viral replication reactivated [191-193]. This was shown in a study where PD-1 blockade enhanced SIV-specific immunity in Rhesus macaques [194]. To date, no single drug or intervention has resulted in anything near an effective sterilizing or functional cure. Therefore, effective eradication of HIV-1 from a chronically infected person will most likely require several non-overlapping interventions, including: (1) long-term suppressive therapy, (2) agents aimed at directly reversing transcriptional latency (e.g., with drugs such as the HDAC inhibitors and other chromatin modifiers), and (3) immunotherapy including agents that reverse the inflammatory and immunoregulatory signals that promote latency and viral persistence. An example of a potential alternative to eliminating infected cells or latent viral reservoirs is to use RNA-based anti-HIV-1 gene therapeutics to achieve long-term silencing of the HIV-1 genome.
3 MATERIALS AND METHODS

3.1 STUDY DESIGN AND PATIENT MATERIAL

All participants in the studies presented in this thesis are part of two well-established cohorts at University of California, San Francisco. The OPTIONS Cohort, is an ongoing longitudinal observational cohort of individuals enrolled within six months of HIV-1 antibody seroconversion and followed approximately every third month throughout the course of HIV disease. The evaluation of the participants with a possible acute/early infection includes detailed HIV-1 testing, exposure history and several laboratory analyses such as CD4+ T cell count. The second cohort is the SCOPE cohort, also an ongoing longitudinal study of about 1,500 HIV-1 infected and uninfected individuals. All participants are seen and interviewed at four-month intervals to perform plasma HIV-1 RNA level measurements and routine T cell immunophenotyping as well as to ascertain four criteria: (1) current medication, (2) adherence to medication, (3) recent intercurrent illnesses, and (4) recent diagnoses or hospitalizations. For each participant cohort, the date of HIV infection was estimated as the midpoint between the last HIV-1 negative test and the first HIV-1 positive test.

In paper I peripheral blood samples from 30 participants were analyzed. For 19 participants GALT samples were analyzed. For this study we included 10 participants who initiated therapy during acute/early infection (initiated therapy within 3 months after infection) and 20 participants who initiated therapy during chronic infection. All participants maintained HIV-1 RNA levels below 40 copies/mL in the year preceding the blood draw. At all sample collections, the CD4+ T cell counts ranged from 400 to 1200 cells/μL. For detailed patient characteristics and therapeutic regimens please see paper I.

The samples analyzed in papers II-IV were from the same 8 HIV-1 subtype-B-infected individuals. For these studies we included five participants who initiated therapy 1–3 months after estimated time of infection (acute/early infection group; participants 1–5) and three who initiated therapy >1 year after estimated time of infection (chronic infection group; participants 6–8). All participants had undetectable viremia (i.e., <40–75 HIV-1 RNA copies/mL) for at least three years after the initiation of therapy. At the on-therapy sample collection time points, their CD4+ T cell counts ranged from 500 to 1500 cells/μL. For detailed patient characteristics and therapeutic regimen please see papers II-IV.

- In paper II paired bone marrow and peripheral blood samples were analyzed.
- In paper III paired GALT and peripheral blood samples were analyzed. These samples were isolated at the same time point as the samples analyzed in paper II. In addition, plasma samples before initiation of therapy were analyzed. The plasma samples were collected 0-180 days before initiation of therapy except for one participant whose plasma sample was collected 14 days after initiation of therapy.
- In paper IV additional paired GALT and peripheral blood samples were analyzed. These samples were collected 7-9 months after the samples described in papers II and III. In addition we analyzed lymph node tissue specimens from two of the participants.

3.2 ETHICAL CONSIDERATIONS

All participants in the studies presented in this thesis provided written informed consent prior to participation in the study. The studies were approved by the institutional review boards at the Karolinska Institutet, Stockholm (papers I-IV), the University of California, San Francisco (papers I-IV), the John Hopkins University School of Medicine, Baltimore (paper I) and the Western Sydney Health Department for the Westmead Millennium Institute for Medical Research, Sydney (paper IV).
3.3 METHODS

To quantify and genetically characterize HIV-1 DNA from cells and HIV-1 RNA from plasma we used different methods described briefly below. For more details please see the material and methods section for each paper.

3.3.1 Fluorescence Activated Cell Sorting

To characterize HIV-1 genetic populations in papers II-IV, a broad spectrum of cells were isolated using fluorescent activating cell sorting (FACS). FACS is a method which enables a mixture of different cells to be divided into specific cell types according to the specific phenotype of the cells. Different cell types have different antigenic and other markers on their surface that can be tagged using fluorescent probes, which are typically monoclonal antibodies that have been conjugated to fluorochromes. The principle of FACS is that a stream of single cells flow in a liquid medium channel past an excitation light source where the specific light scattering and fluorescent characteristics and wavelength for a specific cell type are measured. This light emission data is recorded by an optical and an electronic system which translates the light signals into digital information enabling the sorting of specific cell types of interest.

For our studies, using monoclonal antibodies, we sorted different cell populations from bone marrow tissue, GALT, lymph node tissue and peripheral blood. Table 2 shows the different panels used in papers II-IV.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Compartment</th>
<th>Cellular Population</th>
<th>Sorting Strategy</th>
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<tr>
<td>II</td>
<td>Bone marrow</td>
<td>HPCs</td>
<td>Lin / CD34</td>
</tr>
<tr>
<td></td>
<td>Bone marrow</td>
<td>HPCs</td>
<td>Lin / CD34</td>
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<tr>
<td></td>
<td>Bone marrow</td>
<td>Bone marrow cells</td>
<td>Lin / CD4</td>
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<tr>
<td></td>
<td>Bone marrow</td>
<td>Bone marrow cells</td>
<td>Lin / CD4</td>
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<tr>
<td></td>
<td>Blood</td>
<td>*Memory CD4+ T cells</td>
<td>CD45RO / CD27+</td>
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<tr>
<td></td>
<td>Blood/GALT</td>
<td>*TNA</td>
<td>CD45RO / CD27+</td>
</tr>
<tr>
<td></td>
<td>Blood/GALT</td>
<td>*TCTM</td>
<td>CD45RO / CD27+</td>
</tr>
<tr>
<td></td>
<td>Blood/GALT</td>
<td>*TEM</td>
<td>CD45RO / CD27+</td>
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<td></td>
<td>Blood/GALT</td>
<td>Myeloid cells</td>
<td>CD45/ CD3/ CD4</td>
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<td></td>
<td>Blood/GALT</td>
<td>Myeloid cells</td>
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<td></td>
<td>Blood/GALT</td>
<td>Myeloid cells</td>
<td>CD45/ CD3/ CD4</td>
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<tr>
<td></td>
<td>Blood/Lymph node</td>
<td>*TNA</td>
<td>CD45RO / CD27 / CCR7 / CD57</td>
</tr>
<tr>
<td></td>
<td>Blood/Lymph node</td>
<td>*TCM</td>
<td>CD45RO / CD27 / CCR7</td>
</tr>
<tr>
<td></td>
<td>Blood/Lymph node</td>
<td>*TEM</td>
<td>CD45RO / CD27 / CCR7</td>
</tr>
<tr>
<td></td>
<td>Blood/Lymph node</td>
<td>*TEM</td>
<td>CD45RO / CD27 / CCR7</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>Myeloid cells</td>
<td>CD45 / CD3 / CD14</td>
</tr>
<tr>
<td></td>
<td>GALT</td>
<td>*TCTM</td>
<td>CD45RO / CD27+</td>
</tr>
<tr>
<td></td>
<td>GALT</td>
<td>*TEM</td>
<td>CD45RO / CD27+</td>
</tr>
<tr>
<td></td>
<td>GALT</td>
<td>Myeloid cells</td>
<td>CD45 / CD3 / CD13</td>
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HPC, hematopoietic progenitor cells; GALT, gut-associated lymphoid tissue
*T cells also include CD45/ CD3/ CD4/ CD8

3.3.2 Single-Genome Sequencing (SGS)

To analyze viral HIV-1 RNA from plasma collected before initiation of antiretroviral therapy and during therapy we used single-genome sequencing. This method developed by Dr. Sarah Palmer and co-workers at the NIH, has the advantage of detecting minor variants while
avoiding PCR errors such as resampling and PCR induced recombination [195]. Depending on the plasma volume or HIV-1 RNA level, plasma samples are either micro-centrifuged or ultracentrifuged to spin down and concentrate HIV-1 RNA. The viral RNA is then converted to cDNA using random primers, or in the case of low viral loads gene specific primers. Thereafter, the sample is serially diluted (1:3 to 1:729 in 5mM Tris-HCL) over a 96-well polymerase chain reaction (PCR) plate to ensure that each well contains one HIV-1 cDNA molecule. According to Poisson distribution, the serial dilution yielding amplified product in less than 30% of the wells contains one HIV-1 cDNA copy at least 80% of the time. Amplification was done using a nested PCR procedure with primers flanking the gag-pol region (p6 through nucleotides 1-900 of reverse transcriptase, 1.3 kb fragment, papers I-IV) or the env region (V1-V3, 0.8 kb fragment, paper IV). Generated PCR products were detected by gel electrophoresis and subsequently the viral DNA amplicons were sent for sequencing.

3.3.3 Single-Proviral Sequencing (SPS)

We used a method called single-proviral sequencing to quantify and genetically analyze the HIV-1 DNA integrants in cells isolated from peripheral blood, bone marrow tissue, gut-associated lymphoid tissue, and lymphoid tissue [56]. Using FACS (see section Fluorescent activating cell sorting) a broad spectrum of cells were sorted into vials, lysed and amplified using nested PCR. According to the same procedure described for single-genome sequencing the cellular lysate was diluted to obtain PCR products derived from single HIV-1 DNA molecules.

3.3.4 Quantitative Viral Outgrowth Assay (QVOA)

The QVOA is a limiting dilution virus culture assay that is used to quantify and analyze replication-competent HIV-1. In paper I, the QVOA was used as a standard for comparison. Replication-competent HIV-1 was isolated from purified resting CD4+ T cells from peripheral blood. The purification of resting CD4+ T cells was carried out using a two-step magnetic bead depletion protocol. Briefly, five-fold serial dilutions of highly purified resting CD4+ T cells were stimulated with the mitogen phytohaemagglutinin (PHA) and a ten-fold excess of irradiated allogenic healthy donor PBMCs. The stimulation of PHA and PBMCs induces global T cell activation which reverses latency at least in a fraction of cells carrying integrated HIV-1 DNA. CD4+ healthy donor lymphoblasts were added to detect the released replication-competent virus. The HIV-1 virus replicates in the CD4+ lymphoblasts and after 2-3 weeks of culture the virus is detected using an enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24 antigen (please see paper I for more details).

3.3.5 Droplet Digital PCR (ddPCR)

A ddPCR procedure was used to measure HIV-1 DNA and 2-LTR circles in PBMCs and in resting CD4+ T cells (paper I). Cellular DNA was extracted, ethanol precipitated and thereafter eluted to increase the concentration. The DNA concentration was measured using a spectrophotometer to ensure the desired concentration. The extracted DNA was heated and then digested by a restriction enzyme. Plasmids encompassing the entire viral genome (pNL4-3) or 2-LTR junction were used as standards. Two sets of primers were used: primers flanking the pol region (HXB2 positions 2536-2662) or the LTR (HXB2 positions 9585-51). The PCR reaction mixture was loaded in an emulsification device and droplets were formed. Thereafter, the content was transferred to 96-well plates and the DNA was amplified using PCR. After cycling, the droplets were analyzed. The number of template copies per unit volume μ was estimated from the number of positive events detected in the corresponding channel and the number of total accepted droplets by maximum likelihood.
3.3.6 Alu PCR for integrated DNA

Alu PCR was used to measure integrated HIV-1 DNA in PBMCs and in purified resting CD4+ T cells (paper I). The DNA was isolated from PBMCs and resting CD4+ T cells, diluted, and assayed in replicates for integrated HIV-1 proviruses. In the first round of amplification the Alu primer is paired with an HIV-1 gag primer and in the second round of amplification a sequence in the HIV-1 LTR is targeted. To estimate integrated HIV-1 genomes the detection signal is compared to a standard curve that correlates cycle thresholds with integration standard copy number.

3.3.7 Quantitative PCR (qPCR)

qPCR was used to analyze HIV-1 RNA and DNA in rectal biopsy samples from 19 participants (paper I). Using FACS (see section Fluorescent activating cell sorting), CD4+ T cells were extracted and enumerated. Results were expressed as copies of HIV-1 RNA measured by RT-qPCR or DNA measured by qPCR per CD4+ T cell. Primers and a probe from the LTR region were used for detection. Three replicate samples of 500 ng of RNA and DNA from each donor were assayed. External standards for HIV-1 RNA or DNA were included.

3.3.8 Single-Copy Assay (SCA)

The current lower-limit of detection for clinical assays varies between 20-50 copies of HIV-1 RNA/mL. The SCA, developed by Dr. Sarah Palmer and co-workers, can quantify HIV-1 RNA concentrations in plasma down to 0.3 copies HIV-1 RNA/mL (paper I) [123]. To control for recovery of HIV-1 each plasma sample, containing a starting volume of 7 mL, is spiked with a known amount of an internal control virus derived from an unrelated retrovirus, the replication competent avian sarcoma-leucosis retrovirus vector (RCAS). Using ultracentrifugation the virions are pelleted and the total RNA is extracted. HIV-1 RNA and RCAS RNA are then converted to cDNA and quantified by real-time PCR. By using a specific primer and probe set, HIV-1 RNA can be amplified from nearly 90% of those infected with HIV-1 subtype B. For each plasma sample, three independent reactions are run with HIV-1 gag-specific primers encompassing a highly conserved coding region in gag (79 bp) and two with RCAS gag-specific primers. PCR products are detected using fluorescent probes. The quantity of HIV-1 RNA in the original plasma sample is then calculated from a standard curve generated for each assay using serial dilutions of in vitro transcripts of the same region of gag.

3.4 PHYLOGENETIC ANALYSES

3.4.1 Alignment

Using single-proviral sequencing and single-genome sequencing approximately 30 single genome sequences were obtained from each cell type and plasma sample. The 1.3kb large fragments, constructed using four overlapping primers, were assembled using an in house Perl script. The 1.3 kb contigs were thereafter aligned into multiple alignments files using MAFFT version 6.0 (http://mafft.cbrc.jp/alignment/software/). To ensure high quality data sequences all low quality sequences were discarded both automatically and manually. Each sequence was required to have the following: 1) electropherograms with a mean quality score of Q20 (99% accuracy), 2) bidirectional sequence reads of at least 1.1kb, and 3) no more than one single-nucleotide polymorphism (SNP) (>1 SNPs indicates amplification of more than one cDNA template). In addition all contigs were carefully scanned by eye to detect suspicious base calls and gaps which were trimmed, edited and/or discarded when considered
necessary. Finally, to discover potential contamination or mislabeling, phylogenetic trees were constructed including all patient-derived sequences as well as several laboratory strains.

### 3.4.2 Phylogenetic Inference

Since G-A mutations (discussed in 2.3.1.1) can be misinterpreted as sequence evolution it is crucial to identify hypermutant sequences. Therefore, before constructing each phylogenetic tree the sequence alignments were scanned for hypermutants using the on-line tool “Hypermut” at the Los Alamos HIV database (www.hiv.lanl.gov) [196]. When using the “Hypermut” program all sequences with a p-value less than 0.05 are considered to be a hypermutant and discarded. In paper III, alignments were inspected for recombination using Split Tree or the genetic algorithm for recombination detection (GARD). The identified recombinants were divided into non-recombinant fragments and analyses were conducted on both full-length sequences and non-recombinant fragments. Phylogenetic trees were constructed using Maximum Likelihood in MEGA 5.0 and 5.1 [197]. The evolutionary model best fitting the dataset was selected using the on-line tool “FindModel” at Los Alamos HIV database [198]. Statistical branch support of the tree structure was obtained by 100 (paper II) and 1000 (papers III and IV) bootstrap replicates. In paper II, pNL-43 was chosen as the outgroup whereas in papers III and IV, where sequences sampled from more than one time point were included, no outgroup was included as it might be wrongly interpreted as the root of the tree.

### 3.4.3 Measurements of Diversity and Evolution

In paper II, average pairwise distance was used to assess the genetic HIV-1 diversity between the sequences for each patient. This was calculated using the p-distance model in MEGA 5.0.

Compartmentalization analyses, both tree-based and distance-based, were conducted in papers III and IV to assess the degree of compartmentalization between sequences derived from different cell subsets and/or from different time points. [199]. The Simmonds association index (AI; tree-based) evaluates the deviation from randomness in the position of sequences derived from different cell subsets and/or different time points on the phylogenetic tree. In order to evaluate the degree of compartmentalization the method considers the contribution of each internal node based on its depth and it evaluates the robustness of both the population structure and the shape of the phylogenetic tree by considering the bootstrap analysis as implemented. Nodes with sequences from different samples are given a grouping value of 1, while nodes with descendants from the same sample are given a grouping value of 0. A phylogenetic tree showing a high degree of compartmentalization of sequences will mostly contain nodes with sequences from the same sample and will be given a lower grouping value compared to one where each descendant node contained mixed variants. To evaluate the significance of the observed values, compartmentalization has to be demonstrated by comparing the grouping values with the obtained values calculated for a control dataset that has the same phylogeny. For this method this is achieved by randomly reassigning the sequence grouping labels in the dataset, while still retaining the relative numbers of sequences from each compartment and the tree structure [199, 200]. For papers III and IV, AI statistical support was obtained using 1000 bootstrap-resample replicates of the sequences, each with 10 sample label reassignments. Bootstrap values above 0.95 were considered significant.

Wright’s measure of population subdivision ($F_{ST}$; distance-based) uses nucleotide distances to evaluate the degree of compartmentalization. The method compares the mean pairwise genetic distances between two sequences derived from two different time points to the mean pairwise genetic distances between two sequences derived from the same time point. The
distances were measured using a maximum likelihood approach (GTR nucleotide substitution model) and statistical significance was obtained by a permutation test (10,000 permutations) which randomly allocates the sequences into time points. P-values <0.05 were considered significant evidence for a genetic subdivision of sequences isolated at different time points.

To further evaluate the degree of evolution during suppressive therapy, we also investigated the correlation of genetic divergence and time (paper III) between two time points (between pre-therapy samples and on-therapy samples as well as between two pre-therapy samples were analyzed). This analysis, which is based on the estimated phylogenetic tree, was evaluated using linear regression analysis (root-to-tip analysis as implemented in Path-O-Gen) [201]. A strong correlation between genetic divergence and time implies that viral evolution has occurred between the viral sequences isolated at the different time points, thereby suggesting a model in which there are productive cycles of viral replication during treatment.

As a final analysis of the dynamics of HIV-1 during suppressive therapy, we estimated the evolutionary rate (papers III and IV) using a “root to tip regression analysis”. The evolutionary rates (nucleotide substitutions per site per year) were estimated between pre-therapy samples and on-therapy samples (papers III and IV) and between on-therapy samples collected 7-9 months apart (paper IV).

For more in-depth explanation of the methods used to measure diversity and evolution, please see the methods section for each scientific paper.

3.5 STATISTICAL ANALYSES

We estimated the HIV-1 infection frequency in each cell type using a maximum likelihood statistical analysis. For further information concerning the statistical analyses used in papers I-IV please see the relevant sections for each scientific paper.
4 RESULTS AND DISCUSSION

A major barrier to HIV-1 eradication is the establishment of latent HIV-1 reservoirs. To achieve an HIV-1 cure several critical steps are needed: 1) determining which cell types contain persistent HIV-1 during suppressive therapy; 2) determining which compartments contain these cells; and 3) determining how the persistent HIV-1 reservoir is maintained. Since many different methods are used to measure HIV-1 reservoirs we compared the different approaches for quantifying the reservoir in paper I. In papers II-IV we used unique and sensitive assays to analyze defined cell subsets from different tissues collected from eight participants on long-term suppressive therapy: five participants who initiated therapy during early/acute infection when the virus typically is homogeneous; and three participants who initiated therapy during chronic infection when the virus is more genetically diverse. These analyses allowed us to determine how HIV-1 is subdivided among different cells and tissues, how the timing of treatment initiation (during early/acute versus chronic infection) influences the size and diversity of the viral reservoir in these tissue sites and allowed us to estimate the relative contributions of viral replication and viral production. Furthermore, the longitudinal study of persistent HIV-1 reservoirs allowed us to investigate the effects of cellular proliferation, differentiation and expansion on the maintenance and genetic composition of HIV-1 populations within specific T cells (paper IV).

4.1 COMPARATIVE ANALYSES OF MEASURES OF THE HIV-1 RESERVOIR

Today there are several different assays used to study and measure the HIV-1 reservoir. In paper I we compared eleven different approaches for quantifying persistent HIV-1 in patients on cART. The study involved seven analytical approaches and four different kinds of tissue samples. In this study 30 well-characterized participants were included, 10 who initiated cART during acute/early infection and 20 who initiated cART during chronic infection. The goal of this study was to provide insights into how reservoirs should be evaluated in future clinical trials aimed at eradicating HIV-1 infection.

Results from the different assays were compared to the QVOA, which is currently considered the most sensitive assay for quantifying replication competent virus [145, 147, 161]. When measuring HIV-1 DNA in unfractionated PBMCs and purified resting CD4+ T cells, using ddPCR, we detected HIV-1 DNA in 28/30 and 14/16 participants, in PBMCs and resting CD4+ T cells respectively. These ddPCR assays showed more than 100-fold higher mean infected cell frequency compared to the QVOA. One likely reason for these differences is that the PCR assays detect defective viral genomes such as cells containing HIV DNA which was hypermutated by APOBEC3G [89, 90]. If the proportion of defective viral genomes was known then the PCR measurements might provide a reliable measure of the latent reservoir size. However, no correlation between the PCR assay and the QVOA was shown. Therefore, it may be possible that the defective HIV-1 genomes accumulate at different rates in different patients and thereby no correlation between the methods was found. We further evaluated a well-established Alu PCR assay which is specific for stably integrated viral genomes and ddPCR that does not distinguish integrated from unintegrated viral genomes [164]. The comparison of infection frequencies of cells between the Alu PCR and ddPCR showed similar results and significant correlation (r=0.85, P=0.0079). These assays correlated well due to the fact that unintegrated LTR circle forms of HIV-1 DNA only represent a small fraction of the total HIV-1 DNA and unintegrated HIV-1 DNA have a short half-life and are more prevalent in untreated patients [131, 132]. Despite this, the Alu PCR assay used to measure HIV-1 DNA in PBMC showed the best correlation with the QVOA, among the approaches evaluated (r=0.70, P=0.0008). The correlation was not as strong when Alu PCR and QVOA were performed on the same sample of resting CD4+ T cells, which may be due
to the small number of samples and genomes available for analysis which affected the power to detect correlation.

In addition, we measured HIV-1 DNA and RNA levels in rectal biopsy samples. The frequency of infected CD4\(^+\) T cells in the GALT were significantly higher compared to the frequencies of these infected cells in the blood. This is consistent with some earlier studies [157, 159], but does not agree with other studies (see paper IV) [125]. HIV-1 DNA levels in the GALT and blood showed a modest correlation. However, these HIV-1 DNA levels did not correlate with the QVOA. These results suggest that GALT may serve as an important HIV-1 reservoir and that it is necessary to examine multiple anatomical sites to properly measure the reservoir.

Furthermore we used the SCA as a measure of persistent viremia [123]. HIV-1 RNA was detected in 20/30 study participants but the results from SCA did not correlate with the QVOA. If the activation of latently infected CD4\(^+\) T cells was the major source of residual viremia a correlation may be expected. However, recent studies show that HIV-1 RNA clones in plasma are rarely found in memory CD4\(^+\) T cells from blood, suggesting that residual viremia may come from infected cells not analyzed in this study such as cells located in lymph node follicles or cells from the central nervous system [202].

Overall, this study revealed several difficulties in measuring the reservoir. First of all, none of the currently available assays provide an accurate measurement of the reservoir size. Another important result is that the number of resting CD4\(^+\) T cells with defective proviruses may be, on average, 300-fold higher compared to the number of cells harboring replication-competent virus. Thereby, no PCR-based assay provides a precise and consistent indication of the amount of replication-competent HIV-1 DNA in resting CD4\(^+\) T cells. The great difference between the QVOA and the PCR-based methods may, in most part, be due to cells containing defective genomes, such as those genomes which are hypermutated or genomes with large deletions which would not result in replication-competent virus. A key finding from this study is that while PCR-based assays may overestimate the reservoir size, the QVOA provides only a minimal estimate of the frequency of cells harboring replication-competent virus.

**4.2 CELLULAR AND ANATOMICAL SITES OF PERSISTENT HIV-1**

In 2010 Carter and colleagues published a study showing that HIV-1 can infect and establish a latent cellular reservoir in multipotent progenitor cells. They detected HIV-1 in four out of nine patients analyzed with a frequency of one HIV-1 DNA molecule per 10,000 CD34\(^+\) HPCs [63]. If HIV-1 has the ability to infect HPCs this would be yet another roadblock for an HIV-1 cure as these proliferative cells would generate an expanded set of progeny cells that contain and release genetically identical virus. In paper I we therefore aimed to answer the question whether HPCs function as a viral reservoir after several years of suppressive therapy. We sorted and analyzed different cell subsets isolated from bone marrow of eight participants enrolled in the Options Cohort who had been on successful cART for 3-12 years. From each subject 100,000-870,000 Lin /CD34\(^+\) were analyzed and none of these cells contained HIV-1 DNA. This result provides strong evidence that if Lin /CD34\(^+\) HPCs are infected in patients on long-term cART, their infection frequency is very low. To further evaluate the viral content in more developed cells from the bone marrow we analyzed Lin /CD34\(^-\), Lin /CD4\(^+\) and Lin\(^+/\)CD4\(^+\). The number of Lin /CD34\(^-\) sorted was 580,000-5,900,000 per subject, and we did not find a single HIV-1 DNA molecule. The absence of HIV-1 DNA in these cells indicate that their infection frequency is zero or very low. When analyzing Lin /CD4\(^+\) cells from bone marrow we found no HIV-1 DNA in any of the cells from participants who initiated therapy during acute/early infection (370,000-1,400,000 cells
per subject). However, we did detect HIV-1 DNA in the Lin−/CD4+ cells isolated from participants who initiated cART during chronic infection and we also found HIV-1 DNA in the Lin+/CD4+ cells from both patient groups. The purity of Lin−/CD4+ cell sample was 72-85%, therefore we cannot rule out the possibility of the sample containing a low number of Lin+/CD4+. Using phylogenetic analyses we compared the HIV-1 genetic populations in Lin−/CD4+ and Lin+/CD4+ from bone marrow to HIV-1 populations isolated from contemporaneous memory CD4+ T cells sorted from peripheral blood. The results showed genetic similarity between the HIV-1 populations in cells from bone marrow and peripheral blood implying an active cellular exchange between the compartments or that cells from peripheral blood were present in the bone marrow samples. The discrepancy between the Carter study and our study could be explained by different sorting strategies and differences in treatment periods. Overall the findings from paper II strongly suggest that HPCs are not a viral reservoir in patients on long-term suppressive therapy.

To further characterize the HIV-1 reservoir we analyzed cells isolated from peripheral blood, GALT and lymph node tissue collected from the same eight participants studied in paper II. In papers III and IV we found that persistent HIV-1 infection takes place primarily in memory CD4+ T cells sorted from these compartments. By conducting single-HIV-1 DNA sequencing on specific numbers of each cellular subset, we could estimate the HIV-1 integrant frequency of each cell type in each compartment. We found the HIV-1 integrant frequency of memory CD4+ T cells sorted from blood and GALT were similar. This was shown in both papers III and IV and is consistent with a study by Chomont et al. [125], but in disagreement with another study [159]. The differences between studies can be explained by the limited availability of cells from GALT and isolation from different anatomical sites of the GI tract. In further agreement with the study performed by Chomont and colleagues we found that HIV-1 integrant frequencies in naïve and memory T cells were similar in lymph node tissue specimens compared to peripheral blood (paper IV) [125]. We also calculated the contribution of each cell type to the overall reservoir. In paper III we found that the central and transitional memory CD4+ T cell (T_{CTM}) population was the major T cell contributor to the HIV-1 reservoir in peripheral blood whereas effector memory T cells were the major T cell contributor to the HIV-1 reservoir in GALT during long-term cART. However, in paper IV the T_{TM} population was found to be the major contributor and T_{CM} the second largest contributor to the T cell HIV-1 reservoir in peripheral blood. The difference in the results between these studies can be explained by the different sorting strategies. For paper III the central and transitional CD4+ T cells were sorted as a combined cell type whereas in paper IV the two cell types were sorted separately. In addition to the memory CD4+ T cells, we also isolated and analyzed T_{NA} and found that these cells also contained HIV-1 DNA, although at much lower frequencies.

Myeloid cells have been proposed as a possible reservoir in patients on cART in several studies [152-154]. Therefore, we sorted and analyzed myeloid cells from peripheral blood and GALT in papers III and IV. In paper III we detected HIV-1 DNA in blood myeloid cell preparations from three of eight participants and in GALT myeloid cell preparations from four of the eight participants analyzed. In paper IV we analyzed 6-7 million myeloid cells from blood specimens and found an HIV-1 DNA integrant frequency of 0.00002% in two of the eight participants. From GALT specimens we detected HIV-1 DNA in myeloid cells in one of the eight participants. To investigate the possibility of contaminating T cells in the myeloid cell preparations we performed T cell receptor (TCR) sequencing. We identified TCRs in all myeloid samples where HIV-1 DNA had been detected, indicating the presence of low proportions of T cells. Therefore, we cannot rule out the possibility that the HIV-1 DNA came from T cells. However, if myeloid cells contain HIV-1 DNA the frequency is extremely low and their importance as a latent reservoir may be limited.
Consistent with earlier studies, results from **papers II, III and IV**, show that the frequency of HIV-1 DNA within cells from peripheral blood, GALT and lymph node is lower in participants who initiate cART during acute/early infection compared to participants who initiate cART during chronic infection [182, 184, 203]. These results provide evidence that the initiation of therapy during acute/early infection will influence the magnitude and composition of the viral reservoir.

### 4.3 STABILITY AND MAINTENANCE OF HIV-1 RESERVOIR

A critical issue is how the HIV-1 reservoir is maintained. Is viral replication completely stopped by cART? Is ongoing HIV-1 replication maintaining the reservoir or is it maintained in a different way? In **papers III and IV** we used different phylogenetic analyses to evaluate whether ongoing HIV-1 replication takes place during suppressive therapy. We analyzed the genetic composition of HIV-1 DNA sequences in cells isolated from peripheral blood, GALT and lymph node. We then compared these tissue-derived sequences to plasma-derived pretherapy RNA sequences and to contemporaneous plasma-derived RNA sequences collected after 4-12 years of cART. In **paper IV** we also conducted phylogenetic analyses of viral evolution on HIV-1 DNA sequences isolated from cells collected 7-9 months apart. If ongoing replication is maintaining the reservoir during cART one would expect to detect viral evolution between pre-therapy HIV-1 RNA sequences and the intracellular HIV-1 DNA sequences after long-term cART. In both studies we found that the phylogenetic distribution of on-therapy intracellular HIV-1 DNA sequences intermingled with HIV-1 RNA sequences isolated from plasma specimens obtained before therapy. The compartmentalization analysis, Wright’s measure of population subdivision (\(F_{ST}\)), in **paper III** showed evidence for genetic compartmentalization between sequences isolated before initiation of therapy and after several years of therapy in four of the eight patients. However, the corresponding AI values revealed that the degree of compartmentalization was low. This finding indicates that the HIV-1 reservoir during long-term cART is stable and not maintained by ongoing replication. Lack of substantial ongoing replication was also shown by an analysis investigating the correlation of genetic divergence and time between pre- and on-therapy sequences. This analysis showed a limited correlation in all patient samples. A lack of temporal correlation was also shown in **paper IV** when we conducted regression analysis on on-therapy sequences from cells collected 7-9 months apart. This was true for the gag-pol and the env region. Furthermore, when estimating the evolutionary rate of the HIV-1 sequences between the pre-therapy and on-therapy time points, we found a very low evolutionary rate, indicating an extremely low but not zero, directional nucleotide change during the years on cART. A reason why the results do not show a total lack of viral evolution could be that we cannot exclude the occurrence of small bursts of viral replication, for instance during short periods of lower adherence, which would result in a few genetic changes. Another limitation in this study is that the pre-therapy samples for some patients were collected 12-180 days before the initiation of cART. The period when participants were not on therapy could allow for the accumulation of nucleotide substitutions. However, the phylogenetic analyses conducted in **papers III and IV** suggest that viral replication is not a major contributor to persistent HIV-1 in patients receiving effective therapy.

In **paper IV** we further evaluated the stability of the HIV-1 DNA integrant pool during effective long-term cART by comparing the HIV-1 integrant frequencies estimated in **paper III** to integrant frequencies in cells isolated from the same patients 7-9 months later. The comparison showed that the amount of HIV-1 DNA had changed by ≤4 fold between the two time points for all cells and compartments, indicating that the pool of naïve T cells and memory CD4+ T cells containing integrated DNA did not change dramatically over a period of 7-9 months. The detection of some fluctuation may reflect contractions and expansions of different cell types.
In paper IV we examined the distribution of identical HIV-1 intracellular env and gag-pol sequences isolated from participants who initiated therapy during chronic infection (paper IV). The results from this analysis showed that the virus populations in these participants contained up to 73% identical sequences and that only 3 of the variants involved in these expansions were found in pre-therapy plasma sequences. These findings indicate that the sequence expansions were most likely present due to cellular proliferation that occurred during cART rather than because they were deposited in multiple cells prior to therapy. An interesting finding is that T_{EM} cells are more likely to contain identical HIV-1 sequences (2≥ genetically identical sequences) compared to T_{CM} and T_{TM} cells. The higher proportion of identical HIV-1 sequences found in T_{EM} cells can be explained by the different rates of cellular proliferation. T_{EM} cells have been shown to have the highest proliferation rate [125, 204, 205] and therefore this cell type would have a greater likelihood of harboring clonal viral genomes. In paper II we identified the expansion of identical sequences from cells isolated from bone marrow and peripheral blood from one subject who initiated therapy during chronic infection. This clonal sequence contains a large deletion in the gag-pol viral gene region essentially eliminating the protease from this HIV-1 population, indicating that this viral population is not replication competent. This clone was also detected in paper III and IV. Results from the longitudinal study (paper IV) show that the sequence containing this deletion had increased from 82% to 92% among the T_{EM} cells over a period of 8 months. This indicates that this deletion mutant expanded through cellular proliferation and/or clonal cell expansion with integrated virus and not through viral replication as the variant is unable to replicate without a functional protease.
5 CONCLUSIONS AND FUTURE PERSPECTIVES

With the discovery of the latent reservoir and the recognition of its long-term stability, there has been a constant search for curative strategies to eliminate it. In order to measure the effectiveness of these new curative strategies, high throughput assays will be needed. In paper I we compare eleven different approaches for quantifying persistent HIV-1, and compare the effectiveness of each approach to the QVOA, which has been considered the gold standard. The QVOA assay has been very valuable for characterizing the latent reservoir in resting memory CD4⁺ T cells, but it is very laborious and costly. PCR-based assays, which are easier to perform and require fewer cells, unfortunately quantify both defective and replication-competent viral populations. Overall, the results from this study showed that PCR-based assays did not correlate well with the QVOA assay, indicating that no PCR-based assay provides a precise and consistent measurement of replication-competent HIV-1 in memory CD4⁺ T cells.

The study also shows that there are major differences among the assays. The major difference was that infected cell frequencies determined by PCR-based methods are on average 300-fold higher than frequencies of replication-competent viral populations detected by the QVOA. However, it was recently shown that the QVOA underestimates the true replication-competent reservoir [206]. When examining the HIV-1 sequences which did not give rise to viral outgrowth following cellular activation Ho and colleagues found that while many genomes were massively deleted or mutated approximately 12% appeared intact. Thus, the true size of the latent reservoir may be approximately 60-fold greater than estimated by the QVOA. Although each method alone may not be sufficient to measure the latent reservoir, together these models can be used to study many important characteristics of HIV-1 reservoirs including location, size and persistence.

In conclusion, the data presented in paper I indicate that no assay accurately measures the latent reservoir during trials of new curative strategies for HIV-1 infection. Therefore, the most reliable test of whether a patient is cured or not is to stop therapy. However, this must be done with careful planning and monitoring due to the fact that viral rebound can occur months and possibly years after remission and that drug resistance may develop if virus replication is initiated before drugs levels have dropped to zero. Nevertheless, there are many promising new approaches in development which will soon result in an assay for quantifying the latent reservoir. It is important that this assay is less-costly, sensitive, efficient, and provides an accurate measurement of the replication-competent latent reservoir. The TILDA assay is the most promising assay since it could be easily adapted for use in a clinical setting. Although it may overestimate the reservoir, this is more desirable than an assay giving an underestimation of the latent reservoir since that could lead to viral rebound.

In papers II-IV we analyze different cell types containing intracellular HIV-1 DNA from unique tissue samples from a well-characterized cohort. We studied the genetic composition of HIV-1 DNA integrants and investigated how the HIV-1 DNA pool is maintained during effective cART. HIV-1 infection of HPCs in patients on long-term suppressive therapy, if it occurs, will greatly impede the possibility of a cure for HIV-1 infection. Using the newly developed single-proviral sequencing method we investigated whether HPCs contain HIV-1 DNA during long-term cART. In this study we did not detect a single CD34⁺ HPC containing HIV-1 DNA. The lack of infected HPCs provides strong evidence that if these cells are infected in patients on long-term cART, their frequency is very low. The more differentiated HIV-1 populations analyzed from bone marrow were phylogenetically similar to sequences derived from contemporaneous memory CD4⁺ T cells from blood. This result shows that HIV-1 populations from bone marrow are not unique, indicating an exchange of cells containing HIV-1 DNA between bone marrow and the peripheral blood. As bone marrow is
highly vascularized, we cannot rule out that the CD4$^+$ cells containing HIV-1 DNA in bone marrow actually originated from blood. To formally prove that HPCs do not serve as a latent reservoir a much larger study is needed. However, it is difficult to find a large group of study participants that meet the required criteria (such as treatment period, well monitored, and fully suppressed patients etc.) and are willing to go through the painful procedure of bone marrow aspiration. The findings from paper II together with earlier studies strongly suggest that HPCs are not a viral reservoir in patients on long-term cART [151, 207]. This finding, weighs against conducting a larger study including more patients to more powerfully prove a negative. Questions will always remain: how many participants are needed to prove that HPCs are not infected? Can we be sure that the samples will not be contaminated with CD4$^+$ T cells? Also, a large bone marrow study would be very expensive, resources which would be more usefully devoted toward developing curative treatment strategies within the HIV research field.

In papers III and IV we continued characterizing the integrated HIV-1 DNA pool by investigating the genetic composition of intracellular HIV-1 DNA in cells sorted from peripheral blood, GALT and lymph node tissue collected from the same 8 participants studied in paper II. Consistent with other studies, we found that the majority of HIV-1 DNA in all tissues (blood, GALT and lymph node) and both patient groups (acute/early and chronic) was located in the memory CD4$^+$ T cells. In addition, we found that $T_{NA}$ cells also contained HIV-1 DNA, although at a much lower frequency, indicating that this cell type may be a viral reservoir in patients on long-term suppressive therapy. Another cell type that may contribute to the latent reservoir is myeloid cells. In papers III and IV we analyzed myeloid cells from blood and GALT and found a few cells containing HIV-1 DNA. Since we found the presence of TCRs in all these myeloid cell lysates, we cannot rule out that the HIV-1 DNA found in these myeloid lysates was due to low-level T cell contamination. Our results indicate that if these cells are infected in blood and GALT the frequency is extremely low. However, since we have analyzed up to 7 million cells per patient, at two different time points, and as we detected TCRs in the few HIV-1 positive myeloid lysates, it is highly unlikely that myeloid cells are infected in patients on long-term suppressive therapy.

In both studies we found that the frequency of cells containing HIV-1 in blood was similar to GALT. However, with relatively limited GALT cells available to us, our analysis was restricted to very low number of cells. When we analyzed lymph node tissue we found that the frequency of HIV-1 DNA was similar to the frequencies found in peripheral blood. Both these results indicate that memory cells circulate between these tissue compartments. Our longitudinal analysis of HIV-1 in specific T cell subsets collected 7-9 months apart showed that the levels of memory T cells containing intracellular HIV-1 DNA are relatively stable. Further studies are required to fully understand how the GALT and lymph node tissue serves as a reservoir. However, owing to the painful procedures required to obtain GALT samples, participants are understandably reluctant to provide such samples. Furthermore, due to the complex anatomical nature of GALT and lymph node tissues different areas in these tissues may have different viral burdens.

A consistent result found in papers II-IV is that the participants who initiate therapy during early infection have a lower frequency of intracellular HIV-1 DNA implying that early initiation of therapy results in a smaller latent reservoir. Therefore, early initiation of therapy will most likely be beneficial for future research and efforts aimed at HIV-1 remission or cure. Therefore as shown in the study by Saez-Cirion and colleagues, very early initiation of therapy may hold the greatest promise as a curative strategy. For this strategy to work early and accessible testing is needed for diagnosis as well as early initiation of therapy, which is very difficult. However, this strategy will not benefit all newly infected individuals as many
are unaware of their infection. Furthermore, this curative strategy of treatment during acute infection will not benefit chronically infected individuals.

When cART was introduced it was possible to reduce plasma HIV-1 to below the detection limit and more recently researchers started to hope that it will be possible to achieve HIV-1 remission and/or eradication. Despite successful treatment low-level viremia can be detected in most patients, indicating ongoing viral production. What is unclear is whether this viremia is from latently infected cells or from ongoing replication. Using phylogenetic analyses we studied viral evolution and genetic change in cells from participants on long-term suppressive cART. Results from our studies (papers III and IV) show very little evidence for viral evolution and genetic change. The minor evolutionary change shown by the evolutionary rate analyses can be explained by the accumulation of HIV-1 genomic nucleotide changes when participants were not fully suppressed. However, if low-level ongoing replication occurs, it is unknown whether this contributes to the latent viral reservoir. Overall these findings, which are in agreement with many earlier studies, strongly suggest that ongoing replication is not the major cause of viral persistence in these cells [46, 126, 127]. When cART is taken meticulously viral replication does not contribute to HIV-1 persistence. Rather, as examined in paper IV, the reservoir is primarily maintained by long-lived cells and the proliferation and expansion of CD4+ T cells. Here we evaluated the role that cellular proliferation plays in maintaining persistent HIV-1 during cART. In typical patients treated during chronic infection the viral population is genetically diverse and few sequences are identical. However, when studying the genetic composition of HIV-1 in the participants who initiated therapy during chronic infection we found that up to 73% were identical sequences. Interestingly, we found that these clonal sequences were more enriched in TEm which is a more differentiated cell type. These cells have the highest proliferation rate and therefore are more likely to contain clonal viral genomes [125, 204, 205]. This indicates that a small number of proliferating cells harboring HIV-1 DNA are contributing to the persistence of HIV-1 in TEM cells. The hypothesis that TEM cells are maintained through cellular proliferation was further proven in a subject who had many identical HIV-1 sequences in TEM. We found that this clonal species, which contained a 380bp deletion, essentially eliminating the protease gene, increased over time. This further indicates that HIV-1 persistence during effective cART is driven in large part by the proliferation, differentiation and expansion of cell populations with HIV-1 infection and sustained and durable regenerative potential, rather than ongoing viral replication. These findings reveal that it will be crucial to not only block all infection of new cells, but also find strategies to block homeostasis of HIV-1 infected cells non-selectively and/or find strategies for purging the latent HIV-1 reservoirs at a greater efficiency than cellular proliferation. However, if the proliferating cells contain defective viral genomes that cannot be transcribed and produce proteins these should be invisible to the immune system and will therefore not be a concern for new rounds of infection.

The development of cART for the treatment of HIV-1 remains one of the great triumphs of modern medicine. However, despite its success, this therapy has limitations. Effective therapy requires meticulous life-long adherence, which many HIV-infected patients find challenging. In addition, antiretroviral therapy is expensive and cannot be delivered sustainably to everyone in need. Importantly, since HIV-1 DNA persists as an integrated genome in long-lived or slowly-dividing cellular reservoirs, current therapeutic approaches are not proven curative. Although several different cellular reservoirs have been suggested, it is likely that the relevant cells that need to be targeted for future aims at HIV-1 eradication are different T cell subsets, such as naïve T cells, memory T cells and potentially other T cells such as follicular helper T cells. A sterilizing cure may be impossible given that all cells containing HIV-1 must be eliminated. Nonetheless, there is great hope for an HIV/AIDS free generation within the near future. It is unlikely one specific therapeutic regimen will confront and defeat
the HIV epidemic. To reach the goal of an HIV/AIDS free generation will require not only curative therapies but universal access to testing services to make all HIV-1 infected individuals aware of their infection and ensure access to appropriate medical care and treatment.

In light of many challenges aimed at HIV-1 remission there are several strategies that stand out as highly promising. These include shock and kill strategies which employ HDACi and immune checkpoint blocker strategies. Even if some cells containing HIV-1 are not eradicated, these strategies may eliminate a sufficient amount of infected cells to achieve HIV-1 remission.

In conclusion, in the work presented in this thesis we show that there is still a need for high-throughput assays that accurately measure the latent reservoir (paper 1). In papers II-IV we analyze HIV-1 RNA and DNA from unique patient samples using well-validated and sensitive techniques, developed by our lab. The work has helped us to gain a fuller appreciation for the range of cells and tissues containing HIV-1 DNA in patients on long-term cART and a better understanding as to how the pool of these HIV-1 DNA integrants is maintained in cell subsets from different tissues. Although our SPS assay is limited in its ability to distinguish replication-competent from replication-incompetent virus, these studies of the integrant HIV-1 DNA pool in patients on cART bring us several steps closer to understanding the HIV-1 reservoir.

While a difficult challenge, finding the key to HIV-1 remission and/or eradication is well worth the effort!
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