HIV reservoir size in relation to the expression and usage of coreceptors

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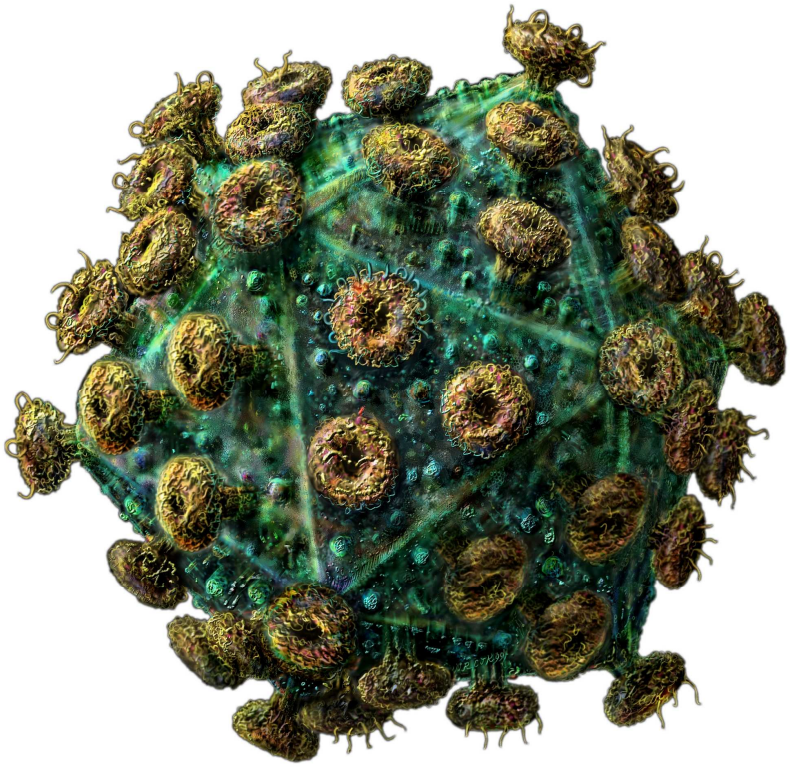

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Abstract

Previous findings indicate that the majority of HIV-1 (subtype B and C) infections occur via CCR5 coreceptor usage. Among other factors, this may be related to the selective expression of CCR5 coreceptor on the surface of $CD4^+$ target cells in the genital mucosa (HIV-1 is often sexually transmitted). Using this CCR5 coreceptor HIV-1 is able to establish viral reservoirs during the early onset of primary infection in both myeloid as well as lymphoid lineages and are widely disseminated throughout the whole body. During the chronic phase, the largest reservoir resides in resting $CD4^+$ memory T cells, which have a long life-span, creating a solid barrier for virus eradication. There are indications that R5 viruses have the ability to switch to usage of an alternative co-receptor CXCR4, which is primarily determined by the V3 loop of glycoprotein 160. Since CXCR4 is expressed on different cells, this may play a role in the size and composition of the viral reservoir. With this study, we aim to gain more insight in the dynamics of the HIV reservoirs in relation to the use of different coreceptors in separate compartments

HIV latency is established in cellular- and anatomical reservoirs. Several T cell subsets, mainly resting $CD4^+$ T memory cells are considered to be the largest (cellular) reservoir. Anatomical reservoirs include the GALT, genital tract, central nervous system, lungs, liver and kidneys. HIV uses reservoirs to avoid viral cytopathic effects and host immune clearance, since no viral protein is produced in latently infected cells.

Viremia is suppressed by combined antiretroviral treatment, which is an intensive targeting combination of three different antivirals, to below a detection limit of 20-50 copies/ml plasma. However antiretroviral therapy is not sufficient to eliminate HIV reservoirs. Therefore reactivation (followed by elimination) of latent HIV provide promising perspectives. However total eradication of HIV is an extremely challenging process due to the stability of reservoirs, variation in reservoir sizes and the possible effects of the potential coreceptor switch to CXCR4.

Gaining profound knowledge on reservoir dynamics may provide clues towards the development of novel therapies to not only suppress viral replication, but to eventually target and perhaps successfully eliminate HIV worldwide in the future.

List of abbreviations

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
C1-5	Conserved region 1-5
CA	Capsid protein
CCR5	C-C motif chemokine receptor 5
CD4	Cluster of differentiation 4
CD4i	CD4-induced epitope
CNS	Central nervous system
CTL	$CD8^+$ cytotoxic T-lymphocyte
CXCR4	C-X-C motif chemokine receptor 4
DC-SIGN	Dendritic cell specific ICAM-3-grabbing non-integrin
Env	Envelope protein
ESCRT	Endosomal sorting complexes required for transport
FPR	False positive rate
GalCer	Galactoside ceramide
GALT	Gut-associated lymphoid tissue
Gp 41	Glycoprotein 41
Gp120	Glycoprotein 120
Gp 160	Glycoprotein (polyprotein)
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HPC	Hematopoietic progenitor cells
HR-C	Carboxy-terminal heptad repeat
HR-N	Amino-terminal heptad repeat
HSPGs	Heparin sulphate proteoglycans
HTLV	Human T cell leukemia virus
IgG	Immunoglobulin G
IN	Integrase
IL-2	Interleukin 2
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
LTR	long terminal repeats
MA	Matrix protein
MBL	Mannose-binding lectin
MIP1	Macrophage inflammatory protein 1
nABs	Neutralizing antibodies
NC	Nucleocapsid
NF-Kβ	Nuclear factor Kβ
NKs	Natural killer cells
PIC	Pre-integration complex
PNGS	N-linked glycosylation sites
PR	Protease

RANTES	regulated upon activation, normal T cell expressed and presumably secreted, CCL5
RT	Reverse transcriptase
RTC	Reverse transcription complex
SIV	Simian immunodeficiency virus
SDF-1	Stromal-derived factor 1
ssRNA	Single-stranded RNA
ST	Strand transfer
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{TM}	Transitional memory T cells
tRNA	Transfer RNA
vDNA	Viral DNA
VLP	Virus-like particles
vRNA	Viral RNA
V1-3	Variable loop 1-3
Vpr	Viral protein R
Vpu	Viral protein Unique

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1. Introduction

Since the start of the Human immunodeficiency virus (HIV) pandemic over 25 million people have died due to AIDS-related pathologies worldwide and up to 34 million people are currently infected with HIV (*see **figure1** below*). The majority of these patients are living in sub-Saharan Africa and Asia. Rapid evolution and high genetic variability has caused a wide variety in viral species in the pandemic spread of HIV[1]. HIV is believed to be derived from simian immunodeficiency virus (SIV) and spread via zoonotic transmission from primates into humans in West- and Central Africa. Transmission likely occurred after hunting and /or consuming primates. Phylogenetic research has led to classification of independent zoonotic transmissions which have rendered multiple HIV lineages, namely HIV-1 and HIV-2. HIV-1 is divided in the following subgroups: M-P and HIV-2 in A-H. In this review we will elaborate on subtype C from the Major (M) Group in HIV-1, as it accounts for 50% of HIV infection worldwide[1,2].

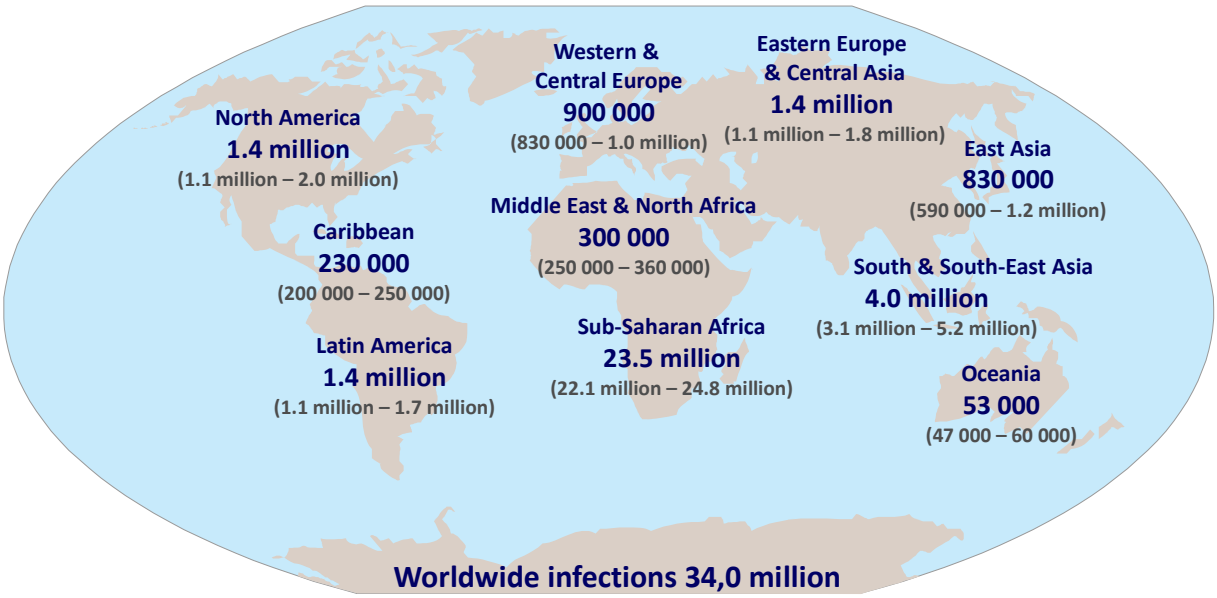

Figure 1: Worldwide prevalence of HIV in 2011.
There are 34.0 million people infected worldwide with HIV-1. Prevalence seems to stabilize over the past few years due to the use of antiretrovirals. South Africa covers 16.5% of all infections worldwide with approximately 5.6 million infections[3].

1.1 The role of HIV in the development of AIDS

HIV-I was first documented in current Kinshasa, Democratic Republic of Congo in 1959. Due to the fact that a wide range of virus sequences have been retrieved from this area, it is highly likely that a great part in the early evolution of the virus took place here. The majority of HIV-1 infections are currently observed in sub-Saharan Africa[4].

In 1981 a large group of young homosexual men in the United States were dying from opportunistic infections due to a severe suppressed, dysfunctional immune system. During this period, AIDS appeared as a chronic disease with a remarkable long lag time after infection. It seemed that a specific subgroup of $CD4^+$ T cells were decreased in affected patients. Shortly after documentation of patients in the US a retrovirus, human T cell leukemia virus (HTLV) was identified[5]. The hypothesis arose that AIDS is also caused by a retrovirus due to similar routes of transmission:

through blood (transfusion and intravenous drug use) and sexual contact (in semen and vaginal secretions), from mother to infant (blood contact during birth and breast feeding). Furthermore, transmission through filtered clotting factors in donor blood to patients with hemophilia strongly supported this hypothesis[1-2,4].

Linking HIV to the development of AIDS started in 1983 when a retrovirus was isolated from the lymph node (LN) of a patient with lymphadenopathy in Paris. Although it was also found in patients with Kaposi's sarcoma in the US, it differed in morphology and antigenicity from HTLV. This fortified the hypothesis of a retrovirus that causes AIDS. HIV-1 was isolated from patients years after infection and despite the fact that numerous other infections were acquired by these patients over time, repeated isolation of HIV-1 from AIDS patients established a clear link between HIV-1 and AIDS. Later on in 1984 reproducible blood tests were developed for blood transfusion clinics, which actually contributed to the molecular characterization process of the HIV-1 genome[5-7].

Two decades later HIV-2 was described, a second strain that shares a large percentage of homology with HIV-1 (40-60%). HIV-2 is thought to have originated from West Africa, as it was identified in 1985 and was even earlier described. HIV type 2 is closely related to type 1, however serological testing and genomics have revealed that HIV-2 is even closer related to the simian immunodeficiency virus, the primate lentivirus. In comparison, HIV-2 is less pathogenic than HIV-1. It is less easy to be transmitted, has a slower disease progression and appears to be confined to West Africa. Further on we will solely discuss HIV-1[7-8].

Objectives and scope

Nowadays researchers are getting closer to curing human immunodeficiency virus infections. However persistence and latency are a major problem in eradication of HIV as the virus resides in inactive cells with a long lifespan, such as resting $CD4^+$ memory T cells. In addition, we believe that the coreceptor switch (usage from CCR5 to CXCR4) during disease progression may potentially influence the stability of HIV reservoirs. Therefore coreceptor usage in separate compartments and ultimately the switch of coreceptor usage in relation to the stability of HIV reservoirs urges to be further investigated. This may provide us with great insight in terms of targeting HIV-1 by antiretrovirals. Thus gaining profound knowledge on reservoirs and coreceptor usage will help us to get one step further in total eradication of HIV infections.

In this review we have underlined several aspects of HIV-viral entry, including potential target cells and their (co)receptors, which contribute greatly to the manifestation of different HIV reservoirs.
Reactivation of these reservoirs appears to be a challenging due to the several possible compartments within the human host. Moreover the possible coreceptor switch from CCR5 to CRCR4 may have an impact on the size and composition of the reservoir.

2. HIV-1 virus structure and replication

2.1 Virus structure

HIV-1 is an enveloped retrovirus from the lentivirus genus and its complex genome consists of two copies of single-stranded (ss) RNA. The viral membrane contains approximately 10 trimeric envelope proteins and is constructed of a lipid bilayer, which is derived from the host cell membrane and a mature HIV-1 particle has a distinct conical capsid core (*see **figure 2** below)*.

The genome contains three structural proteins Gag, Pol and Envelope (Env). Gag is a polyprotein which carries matrix proteins (MA), capsid proteins (CA), Nucleocapsid proteins (NC), P6 and two small proteins P1 and P2. Pol polyprotein contains reverse transcriptase (RT), integrase (IN) and protease (PR). Env codes the gp160 glycoprotein, which derives the gp120-gp41 complex that serves as the binding- and fusing mechanism to a target cell.

Additionally the genome also contains regulatory proteins such as Tat (transactivation) and Rev (regulation of mRNA expression), which are essential for viral replication. HIV-1 also carries the following accessory proteins: Nef (negative factor; increase or decrease replication), Vif (viral infectivity factor; increases infectivity), Vpr (viral protein R; assists replication), and Vpu (viral protein Unique; assists in release) which are not essential for replication[6,9].

The following figure represents the schematic virion structure of HIV-1.

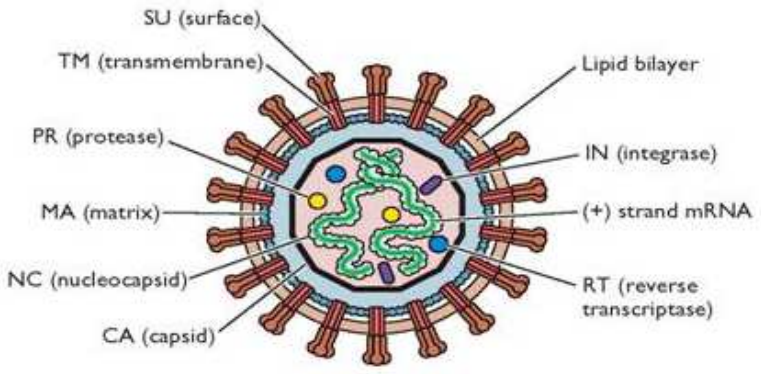

Figure 2: Schematic representation of the HIV-1 virion structure.

HIV-1 is a spiked enveloped retrovirus with two copies of ssRNA. The following structural proteins are depicted in the figure: MA, CA, NC (Gag proteins), PR, IN, RT (Pol proteins) and Env proteins[6].

2.2 HIV-1 replication Cycle

Replication starts with viral attachment and entry, which occurs through a two-staged strategy of receptor interaction. First attachment of envelope (Env/gp120) proteins to CD4 and the CCR5 and/or CXCR4 coreceptor of the host target cell will occur. When this is established, a unique fusion strategy by HIV-1 is utilized which provides a highly effective mechanism to conceal conserved neutralization epitopes to evade the host immune system. As mentioned previously in ***2.1 virus structure***, spikes are natively expressed trimeric envelope (Env) proteins on the surface of the virion. This Env protein is heavily glycosylated and is comprised of a trimeric gp120 and heterodimeric gp41 complex. The gp160 polyprotein is a precursor, which derives the protein gp41-gp120 complex through proteolytic cleavage by the cellular convertase furin into separate gp41 and gp120 subunits. The gp120 surface subunit is non-covalently associated with the gp41 transmembrane subunit and forms a tripod-like complex. Gp120 acts as the external subunit that mediates attachment between the virion and its

target, which is anchored by the transmembrane gp41 subunit that mediates fusion with the cellular target. Once the virus and host membranes have fused, the capsid is released into the host cell, this viral capsid is then able to uncoat and release its RNA and proteins into the cytoplasm of the host cell. When the RNA and proteins are released, reverse transcription will take place in the cytoplasm of the host cell to yield DNA. Simultaneous to this step the pre-integration complex (PIC) is formed and the DNA is translocated into the nucleus[10-12].

Upon translocation the DNA can integrate into the host genome and subsequently be transcribed and translated to initiate formation of new viral RNA and proteins respectively. The newly created products are assembled into immature, non-infectious virions which will be released through budding. The last step of the replication cycle is maturation. During this essential step, rearrangement of the genome will occur and mature Gag proteins will be formed through cleavage of structural polyproteins by HIV protease to finally form mature virions[10-12]. The individual steps of the replication cycle will be discussed in detail further on.

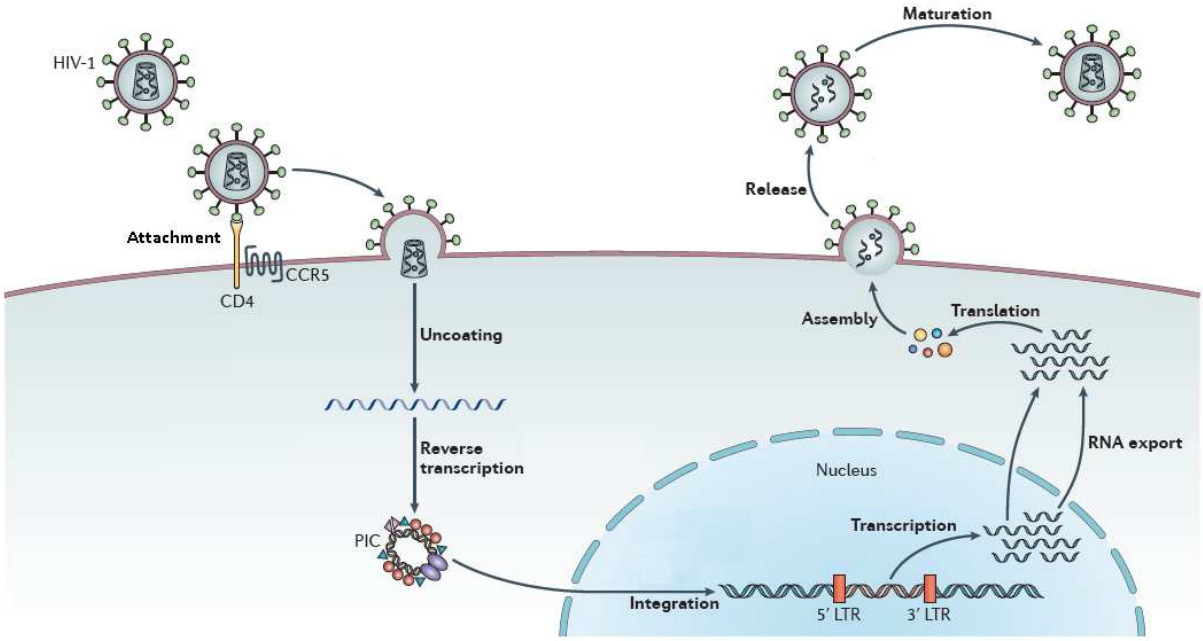

Figure 3: Overview of the replication cycle of HIV-1.

Replication occurs in sequential steps namely attachment (binding and fusion) of the virus with CD4 (primary receptor) and a coreceptor CCR5/CXCR4, fusion with the host cell membrane, viral capsid uncoating and release of viral RNA and proteins into the cytoplasm, reverse transcription and integration into the host genome, transcription, translation, assembly, release of new particle and finally maturation[13]*.

2.2.1 Attachment to host cell

Attachment is driven by specific interactions between the trimeric glycoprotein surface unit of Env, gp120 and the primary cellular receptor CD4. Interaction will in turn mediate other gp120 interactions with chemokine receptors such as CCR5 and CXCR4, which are particularly significant in leading to fusion of viral and host cell membrane. As mentioned above, gp120 binds to the primary receptor, cellular CD4. Before the actual binding of CD4, several aspecific interaction events occur together with alternative "attachment receptors". These receptors are virtually present on all cells and include mannose-binding lectin (MBL), dendritic cell specific ICAM-3-grabbing non-integrin (DC-SIGN), heparin sulphate proteoglycans (HSPGs) and the epithelial surface receptor galactoside ceramide (GalCer)[14]. Gp120 contains five conserved domains, C1-5, which are interspersed with five variable loops, V1-5. These variable loops containing minor variations in amino acid sequences and

length. These properties appear to be correlated to pathogenesis, disease progression and sensitivity to neutralizing antibodies (nABs)[15-16].

Binding of CD4 results in rearrangement of variable loops 1-3 and V3 plays an important role in primary receptor tropism[11]. V1 and V2 are believed to take part in shielding binding sites of nABs to protect the virion from the host immune system. The length of V1 and V2 regions, as well as potential N-linked glycosylation sites (PNGS) appears to significantly increase during chronic infection. V1-2 and PNGS decrease again in a later stage of infection[15]. Fluctuation of V1-2 and PNGS lengths contributes to nABs resistance of the virus.

Initial binding of CD4 triggers a change in conformation of Env which results in partial exposure of the coreceptor binding site on gp120. The binding of Env triggers rearrangements of the conserved regions C1-5 into a core structure that is organized into an inner domain and outer domain (*see **figure 4** below*). The inner- and outer domain are connected by the five surface exposed V1-5 and the bridging sheet, which is also formed after binding of CD4 and enables formation of two pairs of β-strands to form a four-stranded β-sheet and also repositioning of V3, which plays a crucial role in attachment to a target cell, resulting in binding of the coreceptor[10,12,14].

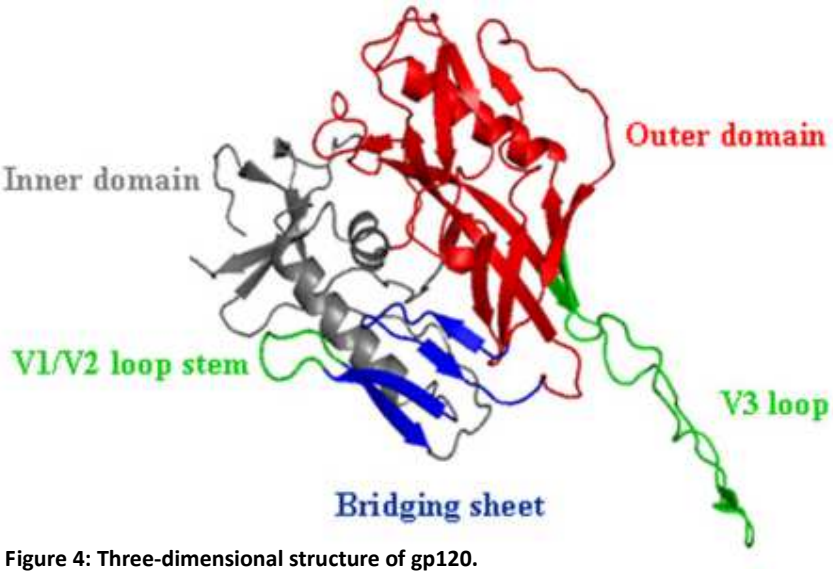

Figure 4: Three-dimensional structure of gp120.
In this CD4-bound conformation, gp120 is constructed of an inner- and outer domain (C1-5 folded core structure), V1/V2 stem loop, the V3 loop and the bridging sheet. The bridging sheet is induced by CD4 binding and consists of four β-sheets. The V-loops and bridging sheet all contribute to the coreceptor interaction and selectivity[14].

2.2.2 Fusion with the host cell

The gp120-gp41 fusogenic complex catalyzes a pH-independent fusion between the Env-containing viral membrane and the target host cell. The actual fusion process is triggered by binding to CD4 and a coreceptor (CCR5/CXCR4) (*depicted in **figure 5** below*). HIV-1 maintains its highly conserved coreceptor-binding surface (V3 and the CD4 induced epitope (CD4i), a four-stranded β-sheet) shielded and is able to present this surface solely after binding of gp120 to the CD4 on the target cell[14].
The process of gp120 binding to the primary CD4 receptor elicits further conformational modification of gp120 of Env. This process induces the activation of the gp41 fusion peptide. Gp41 is now able to bridge the amino-terminal heptad repeat (HR-N) and the carboxy-terminal heptad repeat (HR-C) by folding at its hinge region to form a six-helix bundle (6HB). Gp41 will insert and anchor into the cell membrane to activate fusion through zippering, a strategy to pull the viral- and host membrane towards each other for internalization of the virus[14,12,15].

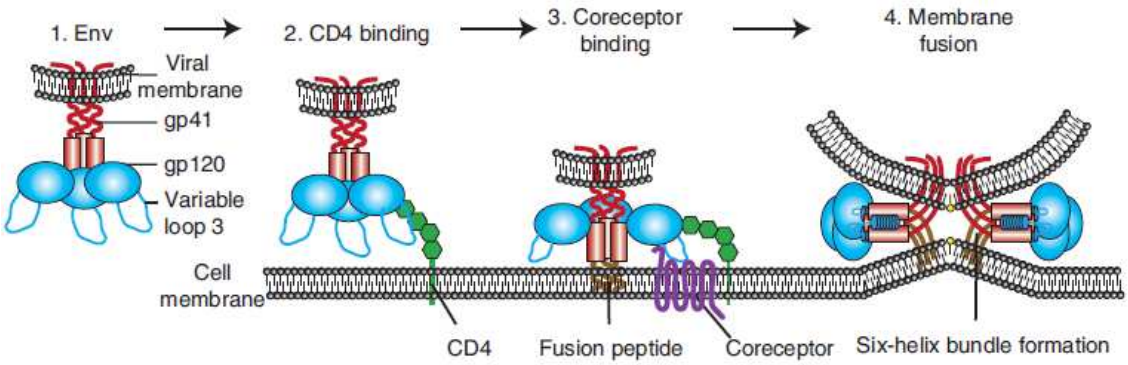

Figure 5: HIV entry into a host cell.

1 and 2. HIV attaches to the host cell via Env interaction (gp120) with CD4.

3. After attachment a conformational change in Env unmasks the V3 regional loop, which in turn can bind the coreceptor

4. Coreceptor binding facilitates membrane fusion by triggering gp41 to form a six-helix bundle. This process results in fusion with the cell membrane through zippering of the gp41 subunit[12].

2.2.3 Viral capsid uncoating and release of viral RNA into cytoplasm

Uncoating of the viral capsid is the next step in the viral life cycle of HIV-1. There are different theories regarding the timing of RNA release (directly after entry or during migration to the nucleus). Although timing is not completely understood, uncoating certainly occurs after fusion into the cytoplasm of the host cell. This step leads to the formation of the reverse transcription complex (RTC) and is important for transportation into the nucleus[17].

2.2.4 Reverse transcription and integration into host genome

Uncoating of the viral capsid occurs simultaneous with the formation of the RTC and pre-integration complex (PIC) during early replication. The RTC is an HIV-1 complex that undergoes reverse transcription to yield double-stranded (ds) DNA. This intermediate complex may contain ssRNA, RNA-DNA or dsDNA. In contrast to RTC, PIC solely contains dsDNA and is completely competent of integrating efficiently into the host genome. Upon DNA synthesis, PICs are formed and translocated into the nucleus to become an integrated provirus. Therefore the formation of PICs is a very crucial step in the development of latent reservoirs[17].

Integration is established in two catalytic steps, 3'-end processing of the long terminal repeat (LTR) through hydrolysis of an OH-group followed by strand transfer (ST), the *trans*-esterification step that initiates covalent insertion of vDNA into the host genome (*see **figure 6** below*).

The first step occurs before the actual integration; the viral DNA (vDNA) is primed in the cytoplasm by integrase (IN) via endonuclease activity (cleavage) of the vDNA (PIC) and processing of two nucleosides of the long terminal repeats (LTR) at the 3'-ends. The PIC is supported by several proteins, such as RT, NC, matrix proteins (MA), and viral protein R (Vpr). These proteins coordinate transportation of the PIC into the nucleus.

The second step, integration, takes place in the nucleus and is activated by cleaved vDNA and creates a flanking 5 nucleotide base pair overhang at both LTRs. Integration is completed by host DNA repair enzymes which facilitate the ligation process[18].

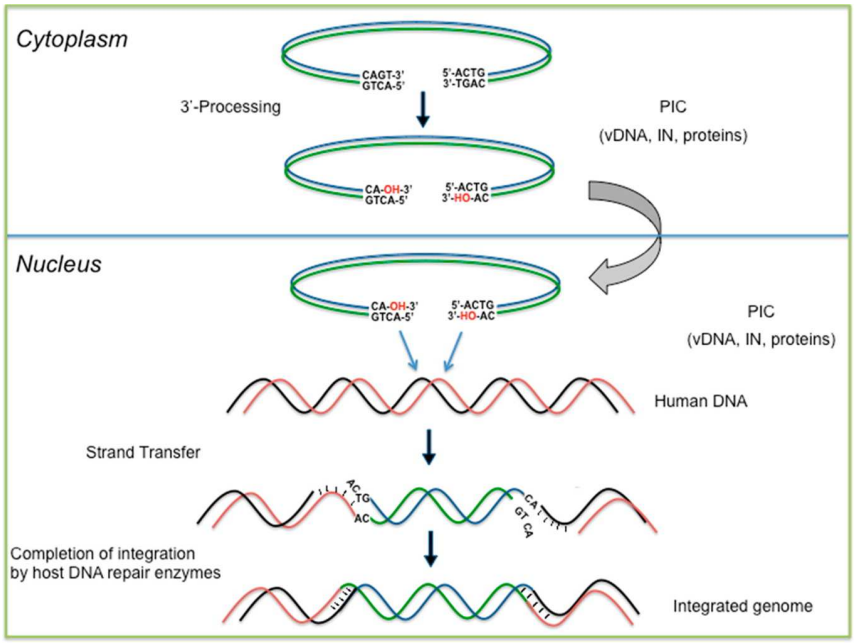

Figure 6: ***In vivo integration of HIV-1*** Integration of viral DNA is established in two steps. The first step is 3'-end processing of the LTR through hydrolysis of an OH-group in the cytoplasm (upper panel). This step is followed by strand transfer, which initiates covalent insertion of vDNA into the infected host (lower panel)[18].

2.2.5 Assembly

Virion assembly occurs at the plasma membrane of the host cell. During assembly of new virion particles the following components that are required for infectivity are packaged: cellular transfer RNA (tRNA), two copies of positive sense viral RNA (vRNA), Env protein, Gag polyprotein and viral enzyme sequence domains Gag-Pro-Pol (protease, reverse transcriptase and integrase). As mentioned before, Gag polyprotein has multiple domains. The three structural proteins are MA, CA and NC. Gag also contains shorter peptides SP1, SP2 and p6. All steps in assembly are mediated by Gag, which has the ability to bind to and fuse with membranes to form spherical virus-like particles (VLPs), mediate in packaging of genomic vRNA (via interaction with RNA packaging sequence Ψ) and concentration of Env (which recruits itself towards the site of assembly). Although Gag initiates spherical formation, the endosomal sorting complexes required for transport (ESCRT) pathway is required for budding[9,19].

2.2.6 Release

The immature virion, mostly consisting of Gag polyproteins, releases through budding. The ESCRT pathway of host cell is utilized in this process for termination of Gag polymerization resulting in release. ESCRT proteins are also involved in endosomal sorting, exosome biogenesis, cytokinesis and shedding[20].

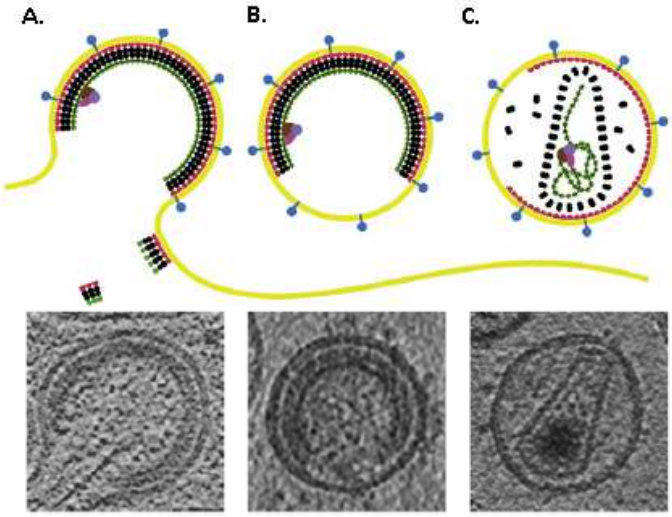

Figure 7: Budding and maturation of HIV-1.
The upper panel represents a schematic overview of HIV-1 budding and maturation. The lower panel depicts sections of cryo-electron tomograms of HIV-1 budding and maturation[9]*.
A. End stage of assembly and initial budding
B. Immature, non-infectious virus-like particle
C. Mature, infectious HIV-1 particle

2.2.7 Maturation

Initially the virus undergoes formation into a non-infectious immature virus particle. After major rearrangement and processing of the structural proteins through cleavage by the viral protease at several specific sites, the virus particle becomes infectious (*overview of budding and maturation are depicted in **figure 7** above*). The viral protease is essential for the maturation process as it, among which, yields structural proteins and viral enzymes. Additional factors also seem to be important for infectivity, such as the packaging of cyclophilin A, a cellular protein[9].

Additionally, the MA and CA domains of Gag mediate formation of the hexameric polyprotein. However during cleavage the N-terminal domain of CA is also able to form pentamers, which result in the distinct conical capsid shape of the mature virion[20].

2.3 Antiretroviral treatment

There are several potential targets in the HIV replication cycle for antiretroviral drugs[21]. Currently three compounds are used in a combined regimen to inhibit the activity of viral enzymes and can decrease viremia below a detection limit of 20-50 copies/ml plasma. Combined antiretroviral therapy (cART)/ highly active antiretroviral therapy (HAART) is composed of FDA approved compounds belonging to the following drug classes[22]:

Table 1: classes of antiretroviral therapy.

Treatment	Mechanism	Drug
1. Nucleoside reverse transcriptase inhibitors (NRTIs)	Competitive inhibition of HIV reverse transcriptase and DNA chain termination[21]	Abacavir, Didanosine, Emtricitabine, Lamivudine, Stavudine, Tenofovir and Zidovudine[22]
2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	non-competitive binding of reverse transcriptase. Binding causes a conformational change, which interferes with the enzymes activity[23]	Delavirdine, Efavirenz, Nevirapine, Etravirine and Rilpivirine[22]
3. Protease inhibitors (PIs)	Interference through binding of protease, which inhibits cleavage of polypeptides[24]	Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Nelfinavir, Saquinavir and Tipranavir [22]
4. Integrase inhibitors (IIs)	Interference of strand transfer through binding of metallic ions in the active site[25]	Raltegravir, Elvitegravir and Dolutegravir[22]
5. Fusion inhibitors (FIs)	Extracellular prevention of HIV fusion to the target cell[26]	Enfuvirtide[22]
6. Chemokine receptor antagonists (CRAs)	Reversible binding of the CCR5 coreceptor, which blocks V3 interaction with, and fusion of target cell. This antagonist only prevents CCR5-tropic HIV strains[27]	Maraviroc[22]

3. Tropism

The viral preference for a particular host cell is determined by the interaction of viral structures with the cellular receptor. HIV-1 uses the CD4 glycoprotein (a member of the immunoglobulin superfamily) as its primary receptor and a 7-transmembrane-domain chemokine coreceptor. Although all viral strains use CD4, coreceptor preference may differ. Macrophage-tropic strains of HIV-1 are able to use C-C motif chemokine receptor 5 (CCR5), which is present on macrophages and most T cell subsets, as a coreceptor. Post-infection, CCR5 tropic strains may evolve towards using C-X-C motif chemokine receptor 4 (CXCR4)[28-29]. CCR5 is a member of the CC-receptor family, which contains a double C-C bond, and has three natural ligands, namely RANTES (regulated upon activation, normal T cell expressed and presumably secreted or called CCL5), Macrophage inflammatory protein 1α (MIP1α) and Macrophage inflammatory protein 1β (MIP1β). These ligands may serve as natural inhibitors of HIV-1. CXCR4 is an α-chemokine receptor, which has one known natural ligand, stromal-derived factor 1 (SDF-1), also called CXCL12[13]. There are alternative receptors, among which CCR1-3 and CXCR6 that may act as coreceptors for HIV-1, HIV-2 and SIV strains. However this is mostly seen in *in vitro* studies of virus entry in transfected cell lines[30].

CD4 and the coreceptors are clinically significant as they play a crucial role in viral entry and pathogenicity. Therefore viral strains are classified based on coreceptor tropism: R5 variants are able to use CCR5, X4 variants are able to use CXCR4 and R5X4 dual-tropic variants have the ability to use both coreceptors. The dual-tropic variants that have a higher efficiency in binding CCR5 are further classified as Dual-R and dual-tropic variants that bind CXCR4 with higher efficiency are classified as Dual-X. In some cases various phenotypes are present in one mixture. These mixtures are classified as dual mixed (D/M)[29].

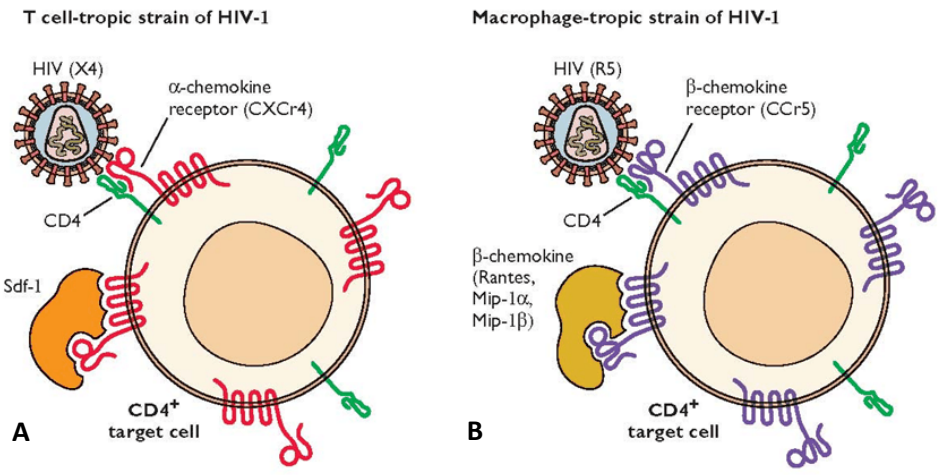

Figure 8: Coreceptor tropism in HIV-1.

A. T cell-tropic strain. This strain generally uses the CXCR4 coreceptor of $CD4^+$ target cells, which is used by its natural ligand Sdf-1[6].

B. Macrophage-tropic strain. This strain generally uses the CCR5 coreceptor of $CD4^+$ target cells, which is naturally used by ligands Rantes, Mip-1α and Mip-1β[6].

3.1 Pathogenesis of HIV-1

There are three different stages of HIV-1 pathogenesis, namely acute, chronic and the AIDS phase. The virus initially targets activated $CD4^{hi}$ T cells with a short half-life and may evolve later on during disease progression to be able to enter host cells with lower CD4-levels than during the initial stage of infection. This leads to the infection of a wider range of target cells[13].

The acute phase is characterized by a decrease in $CD4^+$ T cells due to rapid viral replication (at the mucosal tissue proximal to the site of infection) followed by partial recovery of these cells. Viral entry of $CD4^+$ T cells mainly occurs through CCR5 coreceptor usage (*this mechanism is illustrated in more detail in **Tropism** above*). Initial rapid replication causes mutations in the genome which results in evolution of the virus and immune escape. After the acute phase, the host immune system partially recovers and becomes homeostatic with the viral replication process (which varies per patient). This phase is characterized by an undetectable virus level leading to the chronic phase where more $CD4^+$ T cells are affected due to continuous virus replication and immune activation[13].

During the chronic phase, the virus disseminates further throughout the body. Most of the viral replication takes place in the lymphoid tissue, where a major part of the $CD4^+$ T cell pool resides. During this phase the virus switches coreceptor tropism towards usage of the alternative co-receptor CXCR4 in 50% of the patients, which correlates with an accelerated disease progression. In some cases a very low viral load is reported, which may imply that viral reservoirs can also be formed during this stage in addition to initial formation in the acute phase[13].

The AIDS phase is portrayed by a severe decreased level of $CD4^+$ T cells (<400 cells/mm^3). Generally the viral population becomes relatively homogeneous and CXCR4 tropic. Lymphoid tissue is severely damaged by replication and may be also indirectly affected by chronic inflammation. During this final stage of AIDS the patient will succumb to the consequences of opportunistic infections if the infection is not treated or if the virus is resistant to treatment[13,20,31].

3.2 Disease progression and coreceptor tropism

During the course of infection, disease progresses in three sequential stages. The first, acute stage is characterized by a high viral load of R5 progenitor virus. R5 progenitor viruses target T cells with a high concentration of CD4. During the chronic/latent phase both R5 and X4 viruses are evolved from the R5 progenitor. However most infections remain R5-tropic during this stage and $CD4^+$ T cells partially recover (*earlier mentioned in section **3.1 Pathogenesis of HIV-1** above*). Eventually the slow process of $CD4^+$ T cells decrease in the chronic phase changes into a rapid, severe decrease when the AIDS phase is reached and the virus changes receptor tropism towards CXCR4. The switch to CXCR4 is paired with the ability to enter cells with a low CD4 surface expression (*events are depicted* **figure 9 below**)[28]**.**

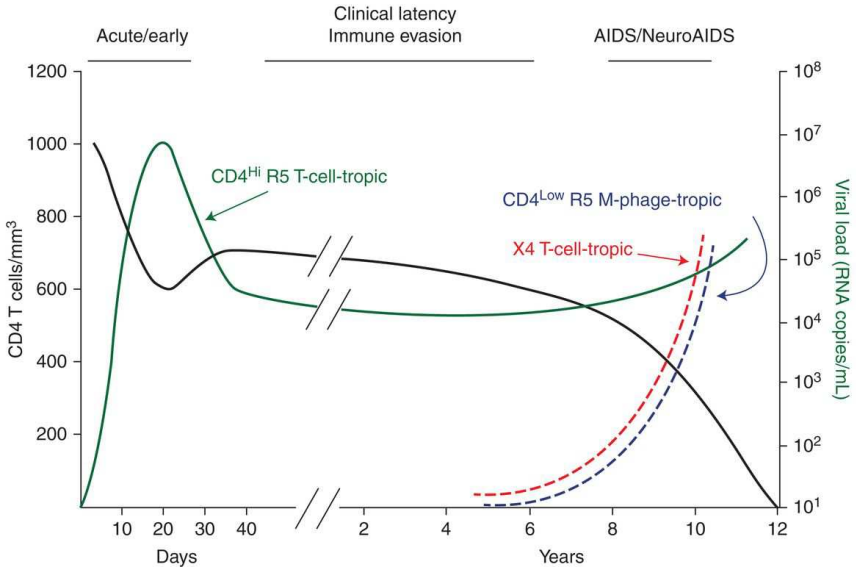

Figure 9: Disease progression of a typical HIV-1 infection.
The figure depicts disease progression over time. The initial infection starts with high $CD4^+$ R5 tropic viremia during initial phase which stabilizes during clinical latency (**green line**). **The black line** indicates an initial, rapid decrease of $CD4^+$ T cells, followed by a partial restabilization and a slow decay of target cells. During the final (AIDS) phase, HIV-1 may evolve to binding of cells with a lower level of CD4 (**blue line**) or changes tropism towards binding of[28].

Determinants of coreceptor tropism

Previous studies show that R5 viruses are able to evolve into X4 variants and as such, alter coreceptor tropism. Tropism is determined by several factors, which are embedded in the viral envelope protein (gp160). As mentioned before (*in* ***2.2 Replication cycle***) Env is a complex of mature surface glycoprotein gp120 and the transmembrane gp41(heterodimer). It contains 5 variable loops that are interspersed with five conserved regions (C1-5). V1 and V2 contain more conserved binding sites (20-35 N- and O-linked glycan residues), which serve as a shielding mechanism from the host immune system.

The primary determinant of co-receptor usage is the gp160-V3 loop, which is in direct contact with the extracellular loop 2 of the viral co-receptor. In general one can state that the net charge of the V3-loop of an X4-using virus is more positively charged than the V3-loop of an R5-tropic virus, enabling binding to the negatively charged extracellular loop 2 of the CXCR4 coreceptor[14,29]. Nowadays the CCR5 coreceptor has become an important target of antiretroviral compounds, such as Maraviroc. To predict the efficacy of this CCR5 antagonist in inhibition of HIV-1 entry, coreceptor usage can be determined through several predicting algorithms. A commonly used bioinformatics tool is the Geno2pheno-false positive rate (FPR) predictor, which indicates the probability of false prediction as CXCR4-using[32].

3.3 HIV-1 target cells

HIV infections are characterized by impairment of the cellular immunity. The severe consequences of a dysfunctional immune system are responsible for opportunistic infections ultimately resulting in death. The following cell types are directly affected (target cells) and indirectly affected (due to an increased immune activation) by HIV-1):

Directly affected (target) cells

$CD4^+$ T-lymphocytes

The majority of infected cells in the circulation consist of $CD4^+$ T cells. These cells are killed directly by viral replication and CD4 is downregulated by Nef. Nef down regulates CD4 expression on the cell surface in order to inhibit T cell activation. Another viral protein Vpu, mediates in release of new virions by disrupting the Env-CD4 complex resulting in degradation of CD4. Infected $CD4^+$ T cells are also recognized by the host immune system, which also plays an important role by $CD8^+$ cytotoxic T-lymphocyte (CTL) mediated killing of infected cells[6].

Monocytes and macrophages

A low percentage of circulatory monocytes and macrophages are infected. However HIV-1 infection has a great effect on functionality of these cells, among which the impairment of chemotaxis, the inability stimulate T cell proliferation and a dysfunctional complement-mediated clearance. Chemotactic impairment results in abnormal secretion of chemokines and may lead to a disturbed proliferation and also apoptosis of $CD4^+$ T cells[6].

In the periphery tissue macrophages and microglial cells (central nervous system) are potential targets. The central nervous system is composed of chemokine receptor-expressing neurons and microglial cells. Astrocytes make up for the majority of the microglial cell population and contribute greatly to the maintenance of the blood-brain barrier. Previous studies have suggested a correlation between chemokine receptor and HIV infection of astroglial cells. However direct evidence is not shown yet[33].

B cells

B cells proliferate at an abnormal rate upon exposure to virus particles and also as an effect of increased proliferation in the lymph nodes. This leads to hyper production of immunoglobulin G (IgG) IgA and IgD followed by poor responses to regular antigen signalling and a severe loss of immunological helper function. Eventually during the final stage of the disease, B cell levels will decrease and result in more immune dysfunction[6].

Indirectly affected cells

$CD8^+$ Cytotoxic T-lymphocytes

During the acute phase of infection, the number of $CD8^+$ CTLs rapidly increases in order to destroy infected $CD4^+$ T cells. After the acute phase CTLs are downregulated due to disruption of immune homeostasis. This is caused by several factors, including a depletion of $CD4^+$ T cells (which are required for immune activation) and direct infection and depletion of CTL progenitor ($CD4^+/8^+$) cells[6].

Natural killer cells

The immune system is further degraded by the loss of function of Natural killer cells (NKs). Depletion of the $CD4^+$ T cells pool has a direct effect on the cytotoxicity of NK cells. T cells produce interleukin 2 (IL-2), which is required to activate NK cells. Therefore another segment of cellular immunity is affected[6].

4. HIV Reservoirs

Antiretroviral treatment (ART)/ highly active antiretroviral therapy (HAART), which is an intensive targeting combination of three different antivirals, can decrease viremia below a detection limit of 20-50 copies/ml plasma. Because virus levels are efficiently decreased, treatment also restrains immune deterioration. Nevertheless in the ideal situation ART alone is solely proficient in suppressing viral replication, yet incapable of stopping viral production and eliminating the viral reservoir[34-35]. Interruption of antiretroviral treatment results in a swift rebound in viremia[36].

HIV latency in the viral reservoirs is one of the major challenges of eradicating HIV-1. Viral reservoirs are established during the early onset of primary infection in both myeloid as well as lymphoid lineages and are widely disseminated throughout the whole body. Several T cell subsets can be latently infected and serve as a viral reservoir as they carry integrated viral genomes, which are replication competent. The resting $CD4^+$ memory T cell pool is considered to be the largest and moreover most consistent latent reservoir[34,37].

Viral reservoirs can be established when activated $CD4^+$ T cells are infected prior to their natural transition into a quiescent state. Naïve T cells become cellular reservoirs when infection occurs during differentiation in thymopoiesis. The actual process of latency is mainly regulated by Tat, a viral activator. An additional mechanism regarding to the establishment of reservoirs is the responsiveness of the HIV LTR promoter to several signals, such as counter-regulatory repression of cellular transcription as well as activation through cell signalling[38].

It has been proposed that residual viremia is present during latency. Persistent virus replication might be caused by several mechanisms, among which very low levels of replication despite antiretroviral therapy or reactivation of a minor pool of latently infected T cells[39].

Reactivation of latent reservoirs is considered to be a suitable approach for purging viruses to exit the host cell and eventually eradication of the complete reservoir. Latency can be reversed upon by T cell activation, which requires the addition of transcription factors by administering drugs or by activation of the T cell receptor (TCR). There is a high risk potential for new infections in reactivation of the viral reservoir (and moreover proliferation of latently infected cells), and a combination of immune suppression (to prevent replenishment of the reservoir) and selective killing of virus infected cells must also be taken into account[39].

4.1 Forms of latency

Latency may occur pre-integration as well as post-integration. Post-integration latency is the most common and stable form of latency.

Pre-integration latency is facilitated by a low metabolic state of the infected cell, as free virus is unable to integrate into the host genome. Nevertheless the virus is capable of forming a PIC in the cytoplasm of infected cells where it resides. This pre-integration complex mediates integration upon activation of the host cell, but will be degraded within a few days if there is no host cell activation[27,32].

Post-integration latency is characterized by successful integration of the provirus into the host genome. However it is not able to activate transcription of its own genome, thus causing the virus to remain blocked on a transcriptional level[32]. There is evidence that proviruses can express some gene products during transcriptional silence. This may be caused when activated cells transform into a resting memory state. Before the cell can reach this state, it has to survive homeostatic mechanisms in which the majority of activated cells are depleted by cytopathic effects and virus-specific cytolytic host effector mechanisms[31-40].

Other factors include transcriptional interference and chromatin restrictions. Furthermore HIV RNA export appears to be inhibited when the host T cell is in a resting state. Quiescent cells lack co-activating factors, such as nuclear factor Kβ (NF-Kβ) [41-42]. Transcriptional interference is established when HIV-1 is integrated within an intron of an actively transcribed gene, resulting in prevention of viral transcription at the HIV LTR promoter. However inhibition of upstream expression allows HIV transcription when the provirus is integrated in the opposite orientation from the host gene. Given the fact that the transcriptional mechanism of the host is a very complex mechanism, and proviruses are able to integrate into different integration sites and potentially may do so in different orientations, viral eradication may be very difficult.

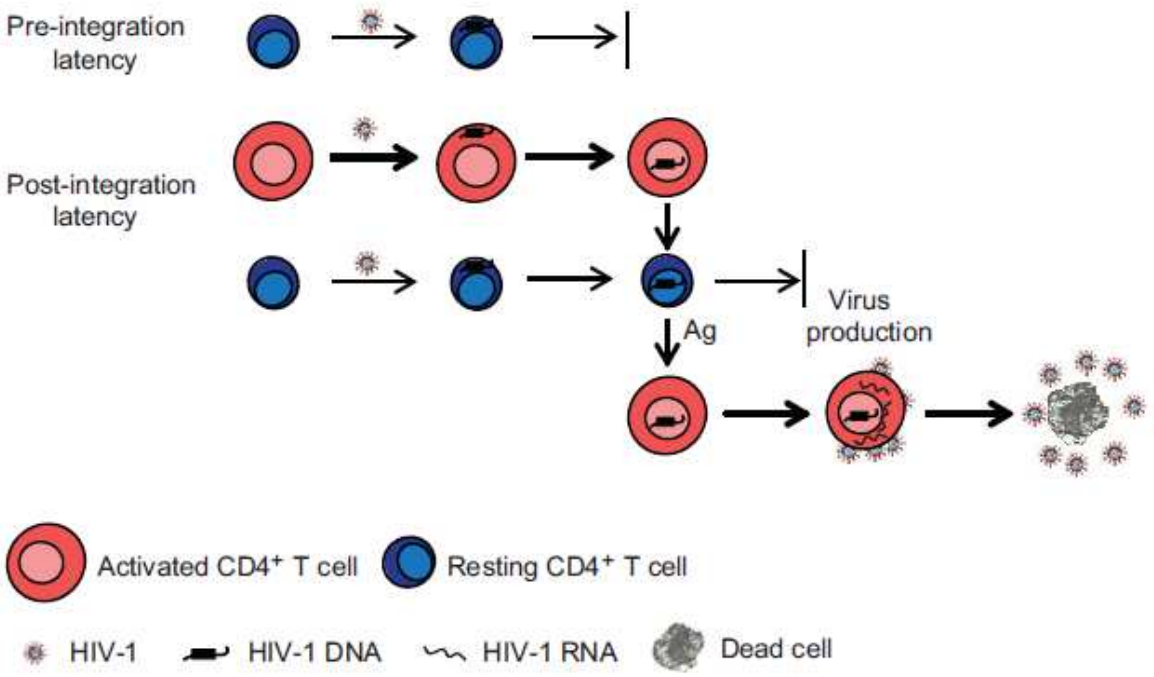

Figure 10: Latency in HIV-1.

Pre-integration latency may occur when HIV-1 is successfully reverse transcribed into vDNA. However it fails to integrate into the host genome[36].

Post-integration latency is characterized by successful integration into the host genome. When a resting T cell (blue) is activated, it will also transcribe viral products[36].

4.2 Cell and anatomical reservoirs and coreceptor tropism

The largest barrier in eradication of HIV-1 is the viral reservoir. Target cells contribute to the survival of reservoirs due to the long life-span of $CD4^+$ memory- and naïve T cells. Furthermore, clonal proliferation of infected cells also contributes to the viral reservoir.

Table 2: an overview of receptor expression in HIV target cells

	CD4	CCR5	CXCR4
Astrocytes	+	+	+
$CD4^+$ Thymocytes	+	-	-/+
$CD8^+$ Thymocytes	-	-	-/+
$CD4^+CD8^+$ thymocytes	+	-/+	+
Dentritic cells	+	+	-/+
HPCs	-/+	+	+
Macrophages	-/+	-	+
Microglia	+	+	+
Monocytes	+	-	+
Natural killer cells	+	+	+
Perivascular macrophages	+	+	+
T_0	+	-	+
T_{CM}	+	+	-/+
T_{EM}	+	+	-/+
T_{TM}	+	+	-/+
T_{SCM} (memory T stem cell)	+	+	-

Cellular reservoirs

T cell subsets

Naïve T cells (T_0) are a significant reservoir for X4 viruses, as they express the CXCR4 coreceptor (some clusters are $CD4^-$ $CCR5^+$), and are infected during thymopoietic differentiation. Moreover the replenishment of $CD4^+$ T cells after initiation of HAART is dependent on the slow increase of naïve T cells. This restoration of the T cell pool probably contributes to the size of the viral reservoir[43].

$CD4^+$ memory cells all express CCR5 and can be subdivided into the following subsets: central memory cells (T_{CM}) which express CCR7 and migrate through the lymphoid tissue through CCR7-CCL21 interaction, effector memory cells (T_{EM}) which do not express CCR7 and migrate to non-lymphoid tissues and secrete effector cytokines and transitional memory cells (T_{TM}) which is an intermediate subtype[44-45].

Resting $CD4^+$ T memory cells are responsible for accommodating the majority of the HIV-1 reservoir. This pool consists of T_{CM} that migrate to lymphoid tissue and T_{TM} in the periphery where they both serve as hosts for persisting integrated vDNA[34,44,46]. The majority of reservoirs predominantly consist of integrated R5-tropic viruses when highly active antiretroviral therapy (HAART) is initiated shortly after infection. HAART is able to reduce plasma vRNA levels below detection limit of sensitive clinical assays (20-50 copies/ml). However treatment interruption results in viremia rebound[34].

During activation of effector T cells, the majority of the total T cell population dies in the process of immunological response. A very small percentage of the total population develops into a memory T cell pool and slips into an arresting state. The survival of memory T cells contributes highly to the stability of the viral reservoir due to the homeostatic proliferative capacity and long life span of this particular cell type[39,47]. The variety in different stages of disease progression and timing of therapy initiation is responsible for the variety in affected T cell subsets.

Macrophages

Macrophages can be infected by macrophage tropic R5 variants (requiring low levels of CD4 for entry) and have showed to be more resistant to cytopathic effects than activated $CD4^+$ T cells *in vivo*[48]. Infection of these cells occurs particularly during the late stage of infection when most $CD4^+$ T are depleted as a result of viral replication. Although macrophages arise from the hematopoietic stem cells (HSCs), they could be infected during three differentiation stages, namely during differentiation in the bone marrow, as monocytes in the circulation (even though HIV DNA is extremely low in monocytes) and as tissue macrophages. HIV mainly prevails in macrophages when viremia is suppressed by HAART[48].

Hematopoietic progenitor cells

A potential reservoir could be $CD34^+$ hematopoietic progenitor cells (HPCs), as they express low amounts of CD4 and also expresses the CXCR4 coreceptor. Most studies have concluded that HPCs do not carry HIV-1 *in vivo* in a latent manner. However $CD34^+/CD133^+$ cord blood cells also appear to be susceptible to infection *in vitro* with X4 viruses only. Contradictory results and perhaps insufficient knowledge on HPCs create uncertainties regarding to HPCs as a potential solid reservoir for HIV-1[48].

Anatomical reservoirs

GALT and the gastrointestinal tract

Gut-associated lymphoid tissue (GALT) makes up for the largest lymphoid tissue in the whole body and $CD4^+$ cells present in this tissue expresses the CCR5 coreceptor[43]. Lymphocytes are present in the epithelium and activated T cells are present in the Peyer patches, lamina propria and intraepithelial cells of the intestinal mucosa throughout the gut[48-49]. Earlier studies show a severe loss of cells in the lamina propria during a very early stage of infection, which express CCR5 on the surface[48]. The gastrointestinal tract is considered to be a potential multi-compartment reservoir for quasispecies[49].

Genital tract

The reservoir in the genital tract seems to be different in males and females. It is suggested that the compartments are selective in terms of tropism and place of origin of the virus. Presumably X4 viruses are originated from peripheral blood and differs from virus strains found in genital mucosa. Compartmentalization is probably caused by selective expression of CCR5 on the surface of target cells in the genital tract. Male seminal secretions may contain either R5- or X4-tropic viruses in infected individuals. Overall R5-tropic viruses are more likely to persist in the genital tract, as genital mucosa selectively expresses CCR5 on the cell surface[43].

Central nervous system

The central nervous system (CNS) serves as a sanctuary, an anatomical site where drug penetration may be suboptimal. Viral replication can have a (rare) pathological effect in the brain, namely HIV-associated neurological diseases (HAND). Perivascular macrophages and microglial cells (which include astrocytes) both express CD4 and CCR5 on their surface. The long life span and slow turn-over rate of microglial cells are mainly responsible for harboring of latent HIV-1[40]. R5-tropic viruses are predominant in the central nervous system as monocytes and macrophages are prevalent in brain tissue. This theory is supported by characterization of viral isolates from patients with HIV-associated dementia and encephalitis, which turned out to be mostly R5-tropic[43]. The virus is likely to enter through the blood-brain barrier via infection of capillary endothelial cells or infected macrophages. Upregulation of β-chemokines show an increased transendothelial migration. The precise harboring of HIV-1 is yet to be determined[40,48].

Lungs

A high percentage of alveolar macrophages reside in the lungs. Therefore this macrophage-rich tissue is considered to be an important cellular reservoir. The lungs are also a highly potent target site for opportunistic infections, such as tuberculosis. This particular compartment is limited by means of viral exchange with the circulation. The majority of the viruses in this compartment are R5-tropic since monocytes and macrophages express high levels of CCR5 on the cell surface[28,43].

Kidneys

Viral dissemination into the kidneys may significantly influence end-stage renal disease (HIV-associated nephropathies). Nef (viral) protein sequences have been detected in renal epithelial cells, indicating cellular impairment through interaction with cell signalling[28].

Liver

Kupffer cells in the liver may be a potential target for HIV-1 as these cells are derived from the macrophage lineage. Coinfection of HIV-1 and hepatitis C virus (HCV) is correlated with accelerated liver disease. An additional proposed mechanism is apoptotic induction in hepatocytes resulting from exposure to HCV E2 and HIV gp120[28,50].

4.3 Dynamics of the reservoir

The size of the viral reservoir differs greatly between patients which is in part due to timing of HAART initiation of HAART. When infection is left untreated, high replication levels result in lymphocyte death and epithelial damage in the GI tract. This epithelial damage, in turns facilitates bacterial infection and may render systemic immune activation[47].

The HIV reservoir develops during a very early stage of primary infection. During this acute stage the viral replication level is very high, enabling proviral integration. This usually occurs prior to the initiation of HAART[47]. As mentioned earlier, viral reservoirs are able to form when naïve $CD4^+$ T cells are differentiating during thymopoiesis. Activated $CD4^+$ T cells are infected prior to their natural transition into a resting state[38]. The long life-span of quiescent $CD4^+$ T cells and their proliferative capacity are considered to account for the stability of the viral reservoir in patients despite long term treatment[47].

The reservoir remains a large barrier in terms of eradication, as each patient differs greatly in HIV latency. Nevertheless patients with a large reservoir can provide more insight in the measurement of treatment impact, yet total eradication of HIV in these patients is troublesome. In contrast, patients with a smaller reservoir and a healthier immune status due to early treatment, might be less challenging to sterilize, however it is far more difficult to assess reservoir size in these patients[47].

Measurement of the reservoir size

Sensitive and reproducible quantification assays are a necessity for aiming at eradication of HIV as latency resides throughout the body. The following assays are used in current studies to measure the size of the reservoir[47]. PCR based methods are not able to distinguish between replication competent and non-replication competent viral genomes[34]:

1. Viral outgrowth assay

The size of the reservoir is commonly measured through the standardized viral outgrowth assay (VoA), where the presence of latently infected cells and persistence in patients undergoing antiretroviral therapy is measured. This is carried out through activation of quiescent T cells. These cells are cultured in limited dilutions in the presence of HIV negative donor cells and subjected to activation by a mitogen (phytohemagglutinin). The cells will produce virus after stimulation. After two weeks, free virus is measured in the supernatant of the culture through ELISA assays for the antigen, p24. The mean frequency of infected cells with replication-competent virus in patients on ART is determined at approximately $1x10^6$ cells[47,51-52].

2. Total HIV-DNA

A less time consuming method to measure reservoir size is by analyzing total cell-associated HIV-DNA. This method includes measurement of both integrated and non-integrated vDNA through real-time PCR and is highly convenient as it can be used as a marker in different cell types and tissues. For an even higher sensitivity and accuracy digital PCR dPCR and droplet digital PCR (ddPCR) could be used for quantification of markers. The cell- and tissue types that can be used to quantify the reservoir are whole blood, purified T cells, tissue biopsies and also cell pellets[47].

3. *Alu* PCR

Post-integrated viruses have the potential to reactivate and the number of integrated HIV variants can be measured in this PCR based assay. However this method is labour intensive and not all integrated viruses turn out to be replication competent[47].

4. 2-LTR circle quantification

Analysis of unintegrated, linear DNA formed as a by-product from integration (non-homologous end joining yields 2-LTR circles (resp. 1% of total product)) indicate persisting viral replication. Results vary and therefore could hamper an accurate read out of this assay. In addition, this method appears difficult to interpret, due to controversies around the actual half-life of 2-LTRs (which is unknown) [53].

5. Cell associated HIV-RNA single copy assay

The HIV-RNA single copy assay (SCA) is a highly sensitive method for quantification of low-level residual vRNA in plasma. It provides detection levels that are below the levels of conventional methods. Residual viremia indicates ongoing viral reproduction. Intensification of current treatment does not reduce residual viremia. However this assay could provide more insight in treatment and eradication strategies[47].

The effect of antiretroviral therapy on the stability of reservoirs

Initiation of antiretroviral therapy can inhibit several steps of the viral replication cycle if it is administered in a timely and consistent manner. Multiple studies indicate a larger effect on virus control in patients when it is induced during acute phase of infection[29]. The following ***figure 11*** depicts the course of viremia during- and post treatment with ART: an immediate suppression of viremia occurs after administration and a rapid viral rebound occurs as a result from treatment interruption. Induction of ART during early acute phase can lead to control of infection after treatment interruption in a minority of patients. A higher efficacy is reached when therapy is initiated during the early acute phase[35].

Although antiretrovirals inhibit new infections and free virus decays rapidly, it is not capable of eliminating latently infected cells. Latent viruses persist to produce low level viremia, but levels off to below detection limit and are estimated to persist at an insignificant rate of 1 copy/ml[40,51].

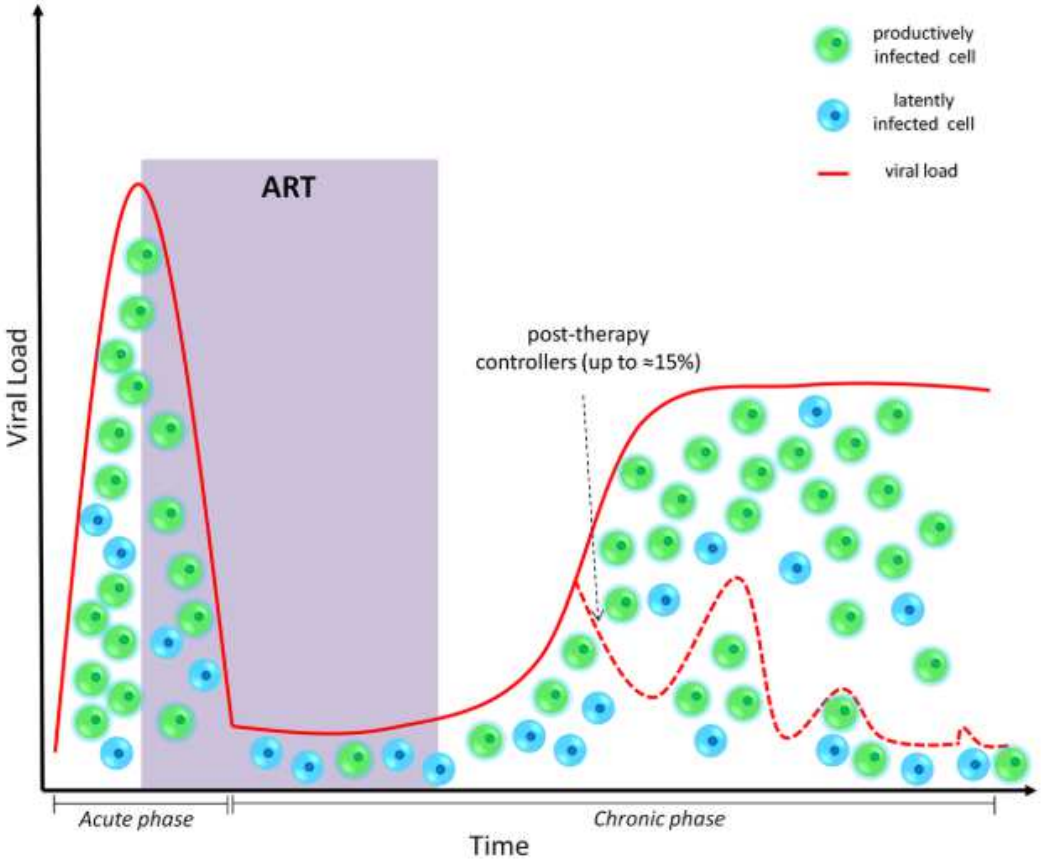

Figure 11: The effect of ART during acute infection.

Initiation of ART during primary phase of HIV-1 infection results in rapid decrease of viral load below threshold of clinical assays. After treatment interruption most of the cases experience rapid viral rebound (**solid red line**), in contrast to a minority that seem to control the infection after treatment interruption (**red dotted line**)[35].

4.4 Reactivation of the latent reservoir

Reactivation of latent virus is a promising tool for the ultimate goal: complete viral eradication. Latency is a major barrier in curing AIDS since no viral protein is produced in latently infected cells. HIV avoids both viral cytopathic effects and host immune clearance, therefore reactivation is necessary

HIV-1 latency can be purged through activation of infected cells. Although activation of T cells results in cell death, not all latently infected cells are eliminated due to survival of a small fraction. Moreover global T cell activation may cause significant adverse effects, such as systemic induction of proinflammatory cytokine release and a high rate of cell death of uninfected cells. Moreover attempts to purge latently infected cells through global T cell activation (through TCR stimulation with IL-2 and α-CD3) have not yet been successful[36].

Other attempts to reactivate latent reservoirs also failed. It was proposed that perhaps inducing viral gene expression without activating T cells could be a possibility. Several patients on ART show no complete clearance after administering HDAC (histone deacetylase) inhibitors (allowing histone acetylation, therefore transcription) due to quantitative defects in CTL response that is HIV-1 specific. *In vitro* stimulation of $CD8^+$ T cells with Gag peptides could reverse these defects, proposing a combination-therapy of reservoir purging with therapeutic vaccination[51].

Another possible candidate to trigger reactivation through activation of integrated proviral genomes by induction of NF-Kβ, which is a transcription factor for HIV-1. This has been attempted with αCD3 monoclonal AB OKT3. This method also failed due to probable causes such as dose-dependent toxicity. Pilot studies have showed progress in reactivation by using combination therapy. The multi-drug approach consists of a histone deacetylase, a methyltransferase inhibitor and protein kinase C, which resulted in increasing levels of plasma vRNA[37].

Nevertheless infected cells are rare in *in vivo*, approximately 1 per $1x10^6$ resting $CD4^+$ T cells, they are not detectable and more importantly not targetable for elimination. Distinguishing these cells from healthy, uninfected cells is still impossible. Furthermore coreceptor tropism may evolve to X4-tropic, which may require a different approach for reactivation. Screening of cell surface markers could be a potential manner for hunting down reservoirs in the near future[51].

4.5 Viral eradication

Although reactivation is not completely succeeded yet in clinical trials, anticipation is rekindled by the remarkable case of one patient whom is considered to be the first to be cured from (R5-tropic) HIV. This patient had developed acute myeloid leukemia (AML) and was treated with myeloablative therapy. Additionally, this patient received a hematopoietic stem cell transplant from a donor with homozygous base pair deletion on the CCR5 coreceptor (CCR5Δ32). After the stem cell transplant the patient withdrew from HAART completely and his HIV-1 infection remained undetectable in peripheral blood, bone marrow and rectal mucosa for years[54].

It remains unclear whether the myeloablative therapy itself was sufficient to eliminate HIV or if the CCR5Δ32-containing stem cell transplantation was responsible for eradication[36]. Furthermore several therapeutic targeting strategies are investigated in currently ongoing trials. For instance development of drugs targeting CCR5 and integrase and intensification of HAART implicate a decreasing reservoir size in patients mainly in the acute, primary phase of infection[39].

Initial approaches to eliminate viral reservoirs consisted of non-specific approaches to activate T cells. Activation occurred through stimulation of CD3 with antibodies or IL-2 stimulation. This severe immune activation is paired with high levels of toxicity and cytokine storm[51]. Non-specific activation may be not the only factor that decrease efficacy of purging. Another important factor is the effect of a potential tropism switch to CXCR4, which may cause an alternation of target cells.

Another previous study aims at eradication through immune intervention. The investigators genetically modified HSCs to differentiate into mature cytotoxic T lymphocytes (CTLs) that are targeted against HIV-infected cells. This was done by cloning a HIV-specific TCR that is derived from $CD8^+$ T cells recognizing Gag and transducing this into HSCs. The modified cells resulted in suppressive polyclonal CTL responses against infected cells, which are very promising[53].

Several other studies aim at a multi-tiered approach which combine reactivation and elimination strategies[37]. However the majority of the patients do not reach a target not detected (TND) level, which implies that not only latency needs to be reversed and eliminated, but also ongoing replication needs to be completely inhibited in order to prevent replenishment of the latent reservoir.

Discussion and future perspectives

We have witnessed the conversion of HIV infection from a relatively rapid death sentence into a chronic disease over the past three decades. There are currently over 34 million people living with HIV-1 worldwide and more than 25 million people have already passed away due to the consequences of AIDS. In addition HIV-related pathologies are still persistent. This includes HIV-induced cancer, an overall higher risk of cardiovascular diseases and other co-morbidities. It is financially not achievable to distribute antiretrovirals worldwide, let alone the additional costs that are required to treat AIDS related diseases. Therefore a different approach is required, for instance a multi-hit treatment which focuses on complete and simultaneous eradication of different reservoirs, perhaps through a unique cellular marker.

Even though many efforts are undergoing to eventually cure AIDS, there is still a lot more to be investigated in terms of latent reservoirs. There is evidence that HAART fortifies the stability of cellular reservoirs by suppressing the few infected cells that are present, allowing them to minimally replicate below detection limit and replenish the reservoir. Long-term combined treatment may also assist in progressive immune dysfunction due to the potential toxicity of the compounds. This may cause persistent systemic inflammation.

Although antiretroviral treatment strongly prevents new $CD4^+$ T cells from becoming infected, coreceptor tropism remains a major factor that might skew the viral reservoir towards a different composition. HIV-1 also persists to evolve during disease progression. Switching coreceptor usage from CCR5 to CXCR4 during disease progression is seen in 50% of subtype B infected patients. This evolving process may be caused by several aspects, among which inhibition of the CCR5 receptor (Maraviroc). In addition, CCR5 inhibition allows R5 tropic HIV-1 strains to switch to CXCR4 usage and interruption of CCR5 inhibition shows re-using of the originally used coreceptor. Nevertheless spontaneous tropism switching might indicate that not only viral factors are involved, but also cellular factors may play a role. It remains unclear whether patients that carry viruses that has undergone a switch to X4-tropic differ in viral reservoirs and size compared to non-switched patients . It seems very likely that X4 viruses develop slightly different reservoirs in comparison with R5 viruses, as both variants target different cells. The size of the reservoir remains very difficult to measure, as suppressed viral replication levels stay below detection limits. This creates yet another barrier in terms of detection, reactivation and eradication. Besides it is also very doubtful that success rates will be similar in patients carrying different subtypes of HIV-1.

Proper characterization and targeting of viral reservoirs, perhaps through a specific cellular marker that is present on all infected cells, is a suggested focus of the near future. Thus gaining profound knowledge on reservoirs and coreceptor usage may provide clues towards the development of novel therapies to not only suppress viral replication, but hopefully also take us one step further in total eradication of HIV infection worldwide.

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