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HIV reservoir quantification and eradication

Current status and advances

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1. INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) is a lentivirus, a specie of retrovirus, which has caused one of the worst global pandemics in human history (Peeters et al., 2013). It is characterized as a lentivirus due to its long incubation period. Usually called just HIV, this virus is responsible for the infection of over 74.9 million people and 32.0 million deaths (ONUAIDS statistics, 2019).

HIV deteriorates the host immune system attacking CD4+ T cells, until the patient reaches the condition of AIDS (acquired immunodeficiency syndrome). AIDS is the final stage of infection with HIV and it opens the door to a wide range of opportunistic infections and neoplasms, which lastly causes death (Nishijima *et al*., 2020).

The isolation and description of the retrovirus was made in 1983 (Barré-Sinoussi *et al*., 1983) and it took a year for the first diagnostic test to be released (Schupbach *et al*., 1984). When the first reports of AIDS appeared, clinicians could only treat the opportunistic infections associated with the disease with quite low success. That changed with the development of the first antiretrovirals, which highly reduced mortality (Connor *et al*., 1994). However, HIV soon created resistance to the treatment, and positive results started to drop. In order to limit the development of resistance, a major breakthrough was made with the introduction of a therapy that combined several drugs. That therapy was named as combination antiretroviral therapy (cART) or highly active antiretroviral treatment (HAART), currently known just as antiretroviral therapy (ART).

ARTs have not stopped improving and nowadays they are able to reduce HIV presence in blood below detectable levels. Besides, it has been confirmed that undetectable levels equal untransmittable, thus, an infected person could have unprotected sexual intercourse without transmitting the virus (Rodger *et al*., 2016). There are different groups of antiretrovirals and each of them focuses on different steps of HIV life cycle in order to stop its replication (Pau & George., 2014). For example, protease inhibitors avoid the excision of precursor proteins by viral protease and stop the production of viral particles and the continuation of the life cycle. HIV life cycle is composed of seven steps detailed in **Figure 1**.

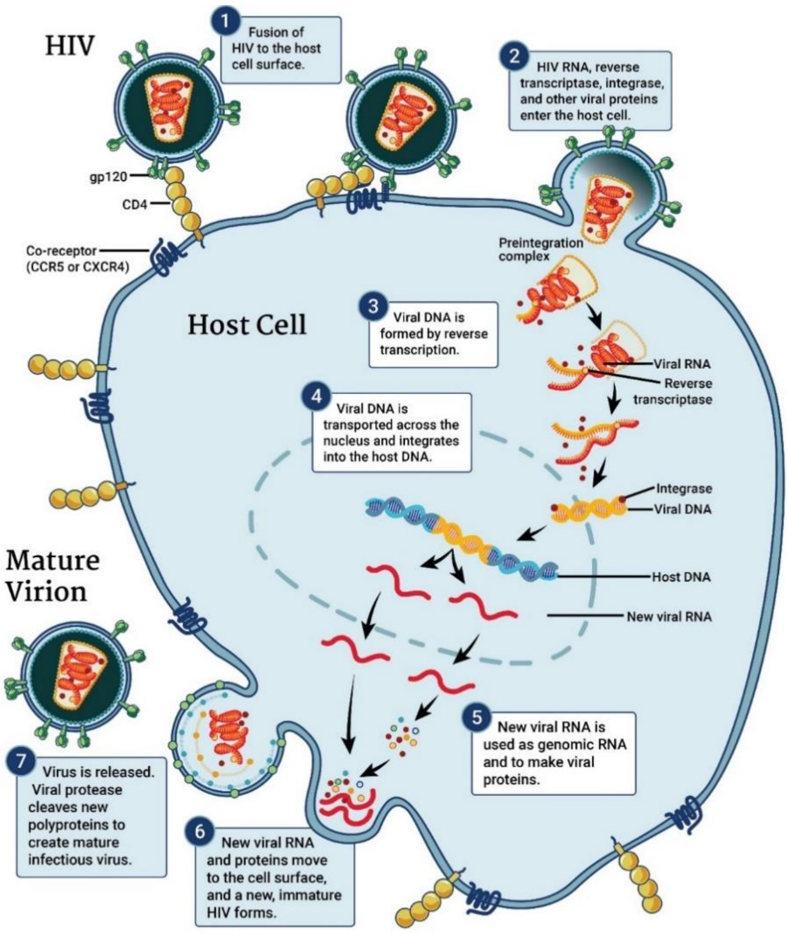

Figure 1: Illustration of HIV life cycle. HIV life cycle begins with the attachment of the virus to the surface of the host cell. As membranes are fused, the viral capsid is introduced inside the cell, containing the virus's genome and proteins. The shell of the capsid is disintegrated in contact with the cytoplasm and HIV protein reverse transcriptase transcribes the viral RNA into DNA. The viral DNA is transported through the nucleus, and using the viral protein integrase it is integrated into the host's DNA. Once integrated, the host's normal transcription machinery transcribes HIV DNA into multiple copies of new HIV RNA. Some of this RNA will eventually become the genome of a new virus, while other copies of the RNA will be used to make new HIV proteins. Manufactured products move to the surface of the cell, where they form a new immature HIV form. Finally, the virus is released from the cell, and the HIV protease cleaves newly synthesized polyproteins to create a mature infectious virus. Figure from NIAID, HIV Replication Cycle, 2018.

Despite the potency of current antiretroviral regimens, HIV doesn´t disappear in infected patients, it just gets reduced to very low levels as mentioned above. As it is illustrated in **Figure 1**, HIV has the ability to integrate its genome inside the host DNA, forming a provirus. Proviruses have the ability to stay in a resting latent state, without producing viral RNA and, with that ability, they become inaccessible for ART and form what is called a viral reservoir. Reservoirs stay immune to ART limiting their viral replication, waiting for the treatment to end. As soon as treatment is interrupted, the virus starts to replicate again restabilising pre-treatment viral levels (Colby *et al*., 2018). So that the viral suppression with current treatment is mantained, chronic therapy must be indefinitely used.

However, as it happened with the first antiretrovirals, ART could become unviable over the long term due to the development of resistant HIV variants (Van and Kalayjia, 2017). Therefore, a complete understanding of HIV reservoirs is urgent in order to find a real cure that could stop the pandemic.

2. OBJECTIVES

Along this project, it will be provided an overview of current research in HIV reservoirs. First, reservoirs will be defined and the different types explained. The next step will be analyzing the strategies for its detection and quantification. After that, developing strategies for the eradication or control of the latent provirus will be covered and finally, the current situation of HIV research will be discussed.

3. HIV RESERVOIRS

First of all, it is necessary to understand the importance of reservoir analysis in order to eliminate the virus. As mentioned before, HIV is able to integrate its genetic material into target cells' DNA and stay in a latent state under the pressure of antiretrovirals forming a reservoir. Neither the immune system nor ART can detect and suppress HIV reservoirs; therefore, HIV can survive. (Ganor *et al*., 2019).

HIV-1 expresses the *env* gene that is used to infect cells. *Env* codes the gp160 precursor and then is spliced into gp120 and gp41 glycoproteins. Gp120 is the only protein present in the surface of the lipid membrane and is used to link with the cellular CD4 membrane glycoprotein receptor. Then, it helps to infiltrate the target cell membrane by fusion, and consequently all cell types expressing the CD4 marker could be infected. However, the spectrum of target cells is modified by interaction of gp41 with CD4 in combination with the co-receptors CXCR4, CCR5 and in a less amount CCR3, enabling the infection of other type of cells (Myszka *et al*., 2000). Those cells could later become a reservoir and an obstacle for HIV elimination. All affected cells are shown in **Annexe 1**.

Among all the cells that can be infected by HIV, memory T cells are considered the main reservoir (Melkova *et al*., 2017), mostly because they can stay in a resting state during their extremely long lifespan. Their ability to stay in a latent state during decades limits the effectivity of current antiretrovirals.

Hematopoietic stem and progenitor cells (HSPCs) can also be infected by HIV *in vitro* and *in vivo.* These cells have the capacity for lifelong survival, self-renewal, and the generation of daughter cells and, therefore, could limit the effectivity of therapies focusing only on differentiated cells (Zaikos *et al.*, 2018).

Additionally, cell types that lack CD4 receptors, including epithelial cells and astrocytes, can also become infected, in this case by syncytial fusion with infected CD4+ cells. However, the significance of these infections for long term stability of the viral reservoir in patients on ART has not been determined (Barat *et al.*, 2018).

Besides, monocytes are responsible for the expansion of the infection throughout the body. They migrate to a variety of tissue compartments where they differentiate into macrophages and dendritic cells and also cross the blood–brain barrier as differentiated microglial cells. They are not considered a main reservoir because the life span of these cells is much shorter than the one of T memory cells.

There are some tissues in the body where ART cannot reach and HIV could freely replicate. However, recent research shows that the contribution of viral replication in those sanctuary sites, where ART is not effective, is negligible. After 1 year of treatment, researchers estimate that more than 99% of infected cells are members of clonal populations coming from infected cell proliferation (Reeves *et al*., 2018). Therefore, even with low or none viral replication, researchers estimate that considering the long life of memory cells, 88 years of treatment would be required for this population to decay to a level that would be equivalent to a cure using current ART regimens (Hill, 2017). Those results push inevitably to the development of new strategies to eliminate infected cells.

In the history of HIV, there have only been two reports of people cured from the virus, the cases known as the Berlin patient and the London patient. After being diagnosed and treated for a blood cancer, a bone marrow transplant became the only way to replenish the blood stem cells destroyed during chemotherapy. In both cases, doctors used bone marrow cells from a donor who was immune to HIV. Less than 1% of population is immune to HIV due to a CCR5 deletion and those are called HIV-controllers. Thanks to the stem cell transplant, both patients were no longer infected with HIV nor having cancer (Gupta *et al.*, 2019).

Unfortunately, this strategy cannot be applied to the vast majority of patients due to its high mortality, and other approaches should be researched.

To sum up, ART has evolved enough to control the infection and allow patients to have a normal lifespan whenever they take proper daily treatment. The problem resides in the number of infected cells staying in a latent state, which can survive for long periods due to their long life span plus the proliferation of the host cells. Consequently, the development of a cure will inevitably require strategies to quantify the exact reservoir size, target it and eradicate it, all before HIV strains acquire resistance to current ART.

4. QUANTIFICATION OF HIV RESERVOIRS

First, it is important to distinguish between clinical tests used to detect HIV and reservoir quantification assays. The former is used to detect HIV infection in patients without antiretroviral treatment that suspect being infected. The most common methodology used is searching for HIV-specific antibodies performing a screening ELISA complemented with a confirmatory western blot to avoid false positives. When those assays are not enough to detect the presence of HIV, as in the cases of patients under ART, intracellular HIV DNA quantification assays can also be performed. Nevertheless, this review will focus in the latter, in the quantification assays of HIV reservoirs found in patients under ART, known as suppressed patients.

Being imperative to achieve the eradication of the reservoirs, it is previously necessary to quantify them precisely. In order to accomplish that goal, it is essential to identify HIV reservoirs and differentiate them from non-infected cells. Although HIV reservoirs are antigenically indistinguishable from non-infected cells, some evidence suggests that most latently infected cells produce sporadic occasional viral transcripts. Those transcripts may maintain low levels of gene products that could produce a unique cellular identity, either directly or indirectly by affecting the expression of host cell proteins (Lusic & Giacca, 2015). Therefore, adequate techniques to detect and quantify reservoirs are crucial in order to attack the resting HIV. It also should be taken into account that the vast majority (93-98%) of HIV proviruses are highly defective and incapable of replication (Churchill *et al.*, 2016) and, therefore, quantification assays could easily overestimate the competent reservoir.

Moreover, HIV quantification assays are not only essential for reservoir targeting and elimination, but also as a post-treatment test in order to analyze the effect of the strategy used. A reliable and reproducible measure is needed to compare how much effect new treatments and therapies have on the size of the latent reservoir. However, up to date, selecting the optimal method of measuring the HIV reservoir size remains controversial. As CD4+ cells form the vast majority of HIV reservoirs identified up to date, most quantification assays focus on the detection and measuring of those cells and will be the target of the strategies that will be covered. The information presented in the next section about quantification assays is based on a comprehensive review (Sharaf & Jonathan, 2017) and complemented with reports that are more recent. Overall, there are several categories of HIV reservoir quantification assays and each of them focuses in the analysis of a different target: 1) intracellular HIV DNA, 2) cell-associated HIV RNA, 3) ultrasensitive plasma viremia, 4) viral outgrowth, 5) inducible HIV RNA, and 7) murine viral outgrowth. Each of those assays has its advantages and drawbacks and they will be briefly covered:

4.1 INTRACELLULLAR HIV DNA QUANTIFICATION ASSAYS

Using this kind of assays, all the HIV DNA inside the cells can be detected and quantified. It is also an interesting strategy to control the pathogenesis of the virus, as it can give an overall view of its spread through the body (Avettand-Fènoël *et al.*, 2016).

There are three types of HIV DNA: circular unintegrated, linear unintegrated and linear integrated viral DNA. When calculating total HIV DNA, the sum of all three forms is being calculated. While the replication capacity of unintegrated forms is very limited and therefore are almost inexistent in HIV reservoirs, they conform the majority of the total HIV DNA in non-supressed patients (Koelsch *et al.*, 2008). When monitoring the reservoir size in non-suppressed patients, the excess of unintegrated HIV DNA could confuse the interpretation of total DNA results. There is a special assay to selectively quantity integrated DNA used in these cases called Alu-*gag* PCR. In patients under ART, total HIV DNA is reflective of the total viral reservoirs, but as it also quantifies the defective provirus, it over-estimates the actual replication competent reservoir.

In order to detect total HIV DNA in the cells, the conserved Long Terminal Repeat (LTR) of the viral DNA inside the cells is targeted and amplified using different PCR strategies.

LTRs are identical sequences of DNA repeated hundreds or thousands of times that are found at the end of proviral DNA, and they are used by viruses to insert their genetic material into the host genomes. In order to calculate the HIV DNA copy number per cell and the cell number assayed, a parallel measurement of a control gene is performed (*CCR5*, *albumin*…) (Malnati *et al*., 2008).

Besides, it has recently been reported that some inaccuracy in the quantification could arise from the high genetic heterogeneity of HIV-1 that is reflected by the various HIV-1 strains and subtypes among patients and the high variation of sequences within patients (Rutsaert *et al*., 2018).

A recent variant for HIV DNA quantification is using next generation sequencing (NGS) based assays, and the most advanced in the topic will be revised.

4.1.1 Full-Length Individual Proviral Sequencing (FLIPS) assay

FLIPS assay is designed to amplify and sequence single, near full-length (intact and defective), HIV-1 proviruses. FLIPS is efficient and high-throughput and allows the determination of the genetic composition of integrated HIV-1 within a cell population and it is the evolution of the older single-proviral sequencing (SPS) assay (Hiener *et al*., 2018).

FLIPS is a next-generation sequencing (NGS)-based assay that limits the number of primers utilized; therefore, it decreases the chance of primer mismatches and limits the unintentionally introduced defects into a viral sequence. FLIPS is also less technically challenging than most of the assays explained below and involves 6 main steps: 1) lysis of HIV-1 infected cells isolated from peripheral blood, 2) amplification of single HIV-1 proviruses via nested PCR (a modified PCR to reduce non-specific bindings) performed at limiting dilution using primers specific for the highly conserved HIV-1 5' and 3' U5 LTR region (**Figure 2A**), 3) purification and quantification of amplified products, 4) library preparation of amplified proviruses for NGS, ligating specialized adapters to both fragment ends of DNA amplicons, 5) NGS (Illumina) and 6) *de novo* assembly of sequenced proviruses using a software that links amplified small sequences to obtain contigs of each individual provirus.

Sequences generated by FLIPS can undergo a stringent process of elimination to identify those that are genetically intact and potentially replication-competent (**Figure 2B**).

Genetically intact proviruses lack all known defects, which result in generation of a replication-competent provirus.

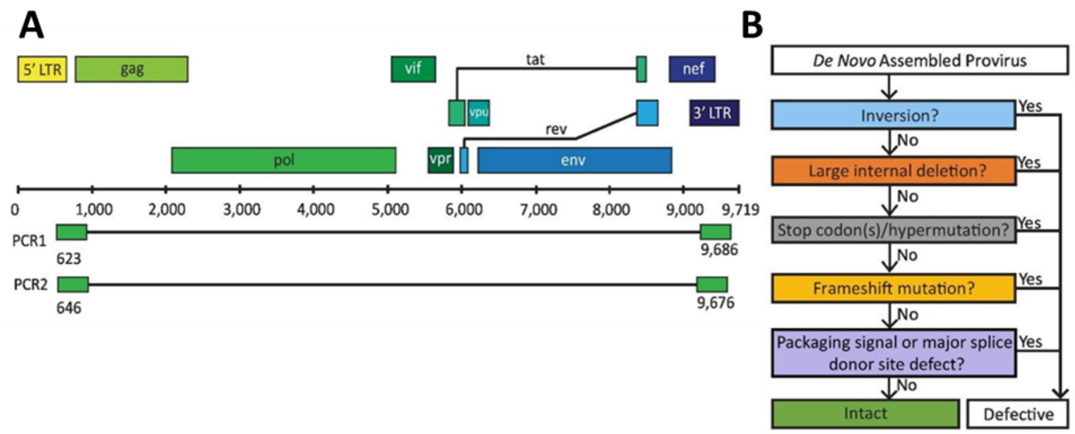

Figure 2: Critical steps in the full-length individual proviral sequencing (FLIPS) assay. (**A**) HIV-1 DNA genome with primer binding sites in 5' and 3' U5 LTR regions used by FLIPS to amplify near full-length (defective and intact) HIV-1 proviruses via nested PCR. (**B**) Process of elimination used to identify genetically intact, and potentially replication-competent, HIV-1 proviruses. This figure has been adapted from Hiener *et al*., 2018.

4.2 CELL-ASSOCIATED HIV RNA QUANTIFICATION ASSAYS

In this case, viral RNA from HIV-infected cells is going to be analysed. To express all its genes, HIV produces a large number of differentially spliced transcripts collectively termed "cell-associated (CA) HIV RNA"; among them we find: multiply spliced (ms), incompletely spliced (is) and unspliced (us) RNA (Purcell & Martin, 1993). After viral integration into the host cell msRNAs are formed to encode viral regulatory proteins, such as tat and rev. Later on, in the infection process, there is a shift towards isRNA and usRNA production, in order to form the rest of the viral proteins. Therefore, in intracellular HIV RNA quantification assay, the degree of ongoing HIV replication is calculated and the results reflect the activity of the HIV provirus.

To date, the most used methods to measure the intracellular transcripts have been based in PCR amplification using primers and probes targeting the functional long terminal repeat (LTR) region (Pasternak *et al*., 2008).

A recently developed variant is called Prime Flow RNA Assay, which uses fluorescence in-situ RNA hybridization. It allows the detection of as low as one infected cell out of 10^5, either spontaneously releasing virions or inducible by latency reversing agents (LRA) (Romerio *et al*., 2015). Latency reversing agents are compounds able to awake the latent virus from its dormant state with the purpose of making infected cells visible to the immune system. LRAs are both used in quantification and also in reservoir eradication strategies that will later be revised in section 5 of this review. Nevertheless, the ability of LRAs to aim different types of HIV-containing cells apart from CD4+ cells is currently unknown (Grau-Expósito *et al*., 2019).

A potential limitation of RNA assays in supressed patients is that if LRAs are not used, the amount of cells producing RNA transcripts is not representative of the real competent-reservoir. Therefore, in patients under ART, this assay is mostly used with the application of LRAs, and that strategy will later be revised in section 4.5. In non-supressed patients this assay is more used as a complementary HIV detection test or after ART interruption to detect viral rebound. In addition, although less than DNA-based assays, these assays could overestimate the number of cells that can express replication-competent HIV, in this case because they can also detect RNA production from defective virus. Finally, CA-RNA assays require large volumes of blood or difficult-to-sample tissue sections.

4.3 ULTRASENSITIVE PLASMA RESIDUAL VIREMIA ASSAYS

Ultrasensitive plasma residual viremia assays measure the amount of HIV RNA in patient's plasma. Some reports indicate both a correlation between the level of residual plasma viremia and the frequency of CD4+ T cells reservoirs carrying HIV proviral DNA (Chun *et al*., 2011), and a strong correlation between plasma viremia and pre-treatment HIV levels (Riddler *et al*., 2016). Plasma residual HIV RNA levels suffer a slow decay in patients with long-term ART and that decline seems to keep on until a permanent basal viremia level is reached. Those results confirm that cells capable of expressing HIV RNA do not persist indefinitely and are reduced with ART. However, it is still not certain the source and duration of this basal viremia. To characterize residual plasma viremia several assays have been developed and the most remarkable one will be covered:

4.3.1 Single copy assay (SCA)

SCA is the most used assay to measure plasma viremia due to its broad dynamic range (1–10^6 copies/ml) and a limit of detection (LOD) down to one copy of HIV RNA per ml of plasma. To monitor the step of viral RNA recovery from the plasma, samples are marked with an internal control standard, a specific amount of replication competent avian sarcoma (RCAS) virions.

Using a proteinase K digestion of proteins, nucleases are inhibited and nucleic acids are precipitated. HIV-1 RNA is quantified via an RT-qPCR reaction using primers and a probe targeting the conserved integrase region of *pol* (Cillo *et al*., 2014a).

4.4 QUANTITATIVE VIRAL OUTGROWTH ASSAYS (QVOA)

QVOA has historically been the gold standard to detect replication-competent HIV latent provirus. This assay comes to fix the problem that previous strategies assays have, the overestimation of replication-competent reservoir. In QVOA a limiting dilution culture is used to measure the number of wells, containing detectable HIV-derived p24 antigen released in the supernatant. Before the analysis, resting cells are subjected to one round of stimulation with LRAs (Finzi *et al*., 1997).

The major advantage of this assay is the capacity to detect only the replication-competent virus reservoir. Nevertheless, QVOA has the opposite drawback of previous assays, it underestimates the reservoir size by approximately 25-fold. This happens because after one round of stimulation, not all of the replication-competent reservoir is activated (Ho *et al*., 2013). Therefore, QVOA should be thought of as the lower-bound estimate of the replication-competent reservoir. Other limitations of QVOA include the requirement of a large sample volume and that it is both time- and resource-intensive.

Currently researchers are trying to increase QVOAs sensitivity to detect a bigger portion of replication competent provirus. However, their main concern is that a bigger sensitivity for virion components in the culture supernatant could also detect low levels of induced extracellular virions that are not replication competent (Richman *et al*., 2019).

4.5 INDUCIBLE HIV RNA QUANTIFICATION ASSAYS

The inducible HIV RNA quantification assays introduced before, are an improved mixture between the nucleic acid-based measurements of the HIV reservoir and QVOA. They provide a more accurate reading of the inducible HIV reservoir than the cell-associated HIV RNA (CA-RNA) assay and are faster and more scalable than QVOA.

In these assays, resting or total CD4+ T cells from virally suppressed individuals are activated using phytohemagglutinin (PHA), CD3/CD28 beads or LRAs in order to make them detectable. Then, HIV RNA is extracted from cells (CA-RNA) or cell supernatants (cell-free RNA, cf-RNA) (Plantin *et al*., 2018). A newer variant of these assays is the *tat/rev* Induced Limiting Dilution Assay (TILDA) which doesn´t involve an RNA extraction step.

4.5.1 Inducible cell-associated HIV RNA (CA-RNA) quantification assay

Levels of usRNA and msRNA can be measured using RT-qPCR (Cillo *et al*., 2014b) and HIV-1 transcription after activation is based on levels of msRNA, like *rev* and *tat*. As this assay requires RNA extraction, potential loss of viral RNA may occur. Moreover, although this assay reflects transcriptionally competent provirus, it also detects defective genomes that can be partially or entirely transcribed.

4.5.2 Cell free RNA (cfRNA) quantification assay

cfRNA quantification assay measures the production of viral products in culture supernatants of stimulated cells (Cillo *et al*., 2014). At different time points post-stimulation, cfRNA is isolated using a commercial RNA isolation kit and levels of HIV RNA are assayed using RT-qPCR. As a limitation, this assay also requires an RNA extraction step.

4.5.3 Tat/rev Induced Limiting Dilution Assay (TILDA)

TILDA is currently the best inducible HIV RNA quantification assay. It is performed quantifying tat/rev transcripts that are required (but not sufficient) for the production of viral particles using a limited dilution and the ultrasensitive detection of msRNA (Procopio *et al*., 2015). As tat/rev transcripts are formed after splicing of full-length viral transcripts, TILDA reduces the probability of quantifying proviruses with large internal deletions.

Although closer to real counts than other HIV RNA assays, TILDA could still measure defective proviruses, since infected cells can sometimes produce tat/rev transcripts, but might still be unable to produce infectious viral particles due to other defects outside the this region.

TILDA measures are between those of QVOA and HIV DNA analyses. TILDA is not sample-intensive (10mL of blood), is extremely reproducible, has a wide dynamic range and can be completed in two days (Procopio *et al*., 2015). However, compared to nucleic acid-quantification assays, this technique is more resource-intensive.

4.6 MURINE VIRAL OUTGROWTH ASSAY (MVOA)

The murine viral outgrowth assay (MVOA) is a binary end-point assay that uses a mouse model to determine whether the cells of our patient harbour infectious virus or not (Metcalf Pate *et al*., 2015). In this assay, whole Peripheral Blood Mononuclear cells (PBMCs) or CD4+ T cells are injected into immunodeficient knockout mice. After several weeks, HIV RNA from the plasma of mice is isolated and quantified by RT-qPCR.

The assay has been successful in several try-outs recovering virus from patient cells, including a HIV elite controller, who had negative QVOA results (Metcalf Pate *et al*., 2015). However, a more recent study doubted the sensibility limit of MVOA after having a negative result analysing the cells of four patients who received a blood stem cell transplant (Schmitt & Akkina, 2018). As it is unprovable that all four patients were cured of HIV, it was supposed that the assay did not have enough sensitivity to detect the latent reservoir in those patients.

MVOA can be used to survey a large number of patient cells, requiring one mouse per 10-50 million CD4+ T cells. The major disadvantages of this method are the lack of quantification capacity, the amount of time required (weeks) and the need of living animals and special facilities for them.

4.7 COMBINATION OF QUANTIFICATION ASSAYS

Currently, all HIV reservoir quantification assays have drawbacks such that combinations of assays are needed to obtain the most precise view of the HIV reservoir.

On the one hand, techniques that quantify levels of HIV cell-associated DNA by PCR are high-throughput, but considerably over-estimate the size of the true viral reservoir, as they also detect defective provirus. Therefore, those assays should be used as a maximum estimate of latent provirus and also as an advanced clinical test to detect HIV presence due to the low time they need.

On the other hand, as previously mentioned, QVOA assay has historically been considered the gold standard for measuring the size of the replication-competent reservoir, as it is the only assay that is able to quantify just and only the replication competent provirus. However, as it underestimates the real amount of the reservoir, it could be used for determining its lower bound.

A better approach for the real estimate of the HIV reservoir could be given by the inducible HIV RNA assays, even if some defective proviruses are also measured. Nevertheless, it has an important limitation: it could be affected by target sequence variations between different individuals when targeting msRNA (*tat/rev*), a highly variable region of the HIV genome (Plantin *et al*., 2018). This problem shouldn´t affect that much to other assays measuring more conserved regions such as the *gag* or *pol* genes, which is the case of plasma residual viremia quantification assays. Even though plasma viremia is still far from being completely understood, it seems a promising field of research.

When the objective is just to determine whether a patient is infected or not, murine viral outgrowth assay should be the most sensitive and reliable assay. Nevertheless, it cannot be applied as a widely-used clinical test due to the weeks needed for the result. It should be used in patients where other quantification assays cannot detect HIV and especially as a future use as a test to check if after a novel treatment a patient is fully cured.

Quantifying the number of intact proviruses by sequencing appears to provide the best current estimate of the HIV reservoir's potential true size, but this assay is still relatively new, and is both labour-intensive and expensive, calling into question its scalability in large clinical studies.

To sum up, when the combination of assays is recommended, it is referred to the use of different techniques in order to obtain contrasted results that do not just rely in a single technique. In order to choose the correct strategy many parameters should be considered: time, cost, precision needed, HIV subtype, stage of the disease…

Once finished with this section and after a precise determination of the reservoir is achieved, it is the turn to control and eradicate the latent provirus. It is equally or more important than the determination and quantification of the reservoir the ability to reduce it from infected patients, in order to progressively be closer to the complete eradication of the virus.

5. ERADICATION OF THE VIRUS

ART has shown to be highly effective in the control of viral replication; however, due to the existence of reservoirs, a diminishing of the provirus below certain levels is impossible with the only use of antiretrovirals. Besides, as mentioned before, HIV could develop resistance to ART in the long-term. Furthermore, ART has further drawbacks such as long-term possible toxicity and social stigma. All those reasons push researchers to find the best strategy to reduce the latent HIV reservoir.

Nowadays, the most efficient method to reduce HIV reservoirs in patients is the early initiation of ART, within the first weeks of infection. That way, reservoir amount is significantly decreased and, in some cases, patients maintain viral levels under control after ART removal. Results seem especially effective in patients who maintain therapy during longer time prior to treatment interruption. Those patients are called post-treatment controllers (PTCs) in reference to HIV-controllers. However, only 1% of infected individuals are natural HIV-controllers, and less than 15% of patients treated in the early stage of the virus become PTCs (Martin and Frater, 2018). There is still a lot of research needed to understand what makes PTCs and HIV-controllers unique, so that new therapies can be developed to replicate their especial characteristics and make patients be immune to the virus.

However, there are a lot of different approaches and developing strategies in order to control and eradicate HIV reservoirs, and the most promising ones will be revised below.

5.1 SHOCK AND KILL

This strategy is based on the induction of HIV expression (Shock) using latency reversing agents introduced in the previous section combined with ART and immune therapies to eliminate (Kill) HIV reservoirs. When this strategy was developed, it was assumed that after viral reactivation, the provirus would be eliminated through cytopathic effects caused by HIV or killed by immune cells after their detection. As the assumption was proved wrong and inefficient, other techniques were developed to eliminate the previously activated reservoir.

Currently there are 4 main categories of Latency Reversing Agents (LRAs) with the potential for viral induction applied in the “Shock” step (Sadowski & Hashemi, 2019):

1. Cytokines receptor agonists: in order to induce the activation of latent cells, either cytokines or receptor agonists for other cytokines can be used, in order to upregulate several genes and their transcription factors.

2. Epigenetic modifiers: Composed mainly by histone deacetylases (HDAC), and histone methyltransferases (HMT). They alter the structure of chromatin and its transcriptional activity such as reducing DNA methylation activating gene expression.

3. Intracellular signalling modulators: includes compounds that modulate protein kinases in signalling pathways upstream of transcription factors that bind the LTR, and are normally regulated by cytokine signalling or T cell receptor engagement.

4. Transcriptional elongation regulators: they target molecules that inhibit transcriptional elongation; thus, they have an anti-inhibition effect, therefore producing HIV transcription. They produce a strong synergistic response with a variety of other latency-reversing agents.

In addition, new more potent “Shock”-strategies are being developed like combining recombinant macromolecules with the LRAs mentioned. For example, a recombinant HIV TAT can be utilized in exosomes to cause a strong activation of provirus expression in latently infected cells (Tang *et al*., 2018). Similarly, gene-editing tools like CRISPR/Cas9 and designer zinc finger proteins have been developed to force induction of HIV provirus, where the methodology is to direct a transcriptional activation domain fusion to highly conserved elements on the 5’ LTR to cause constitutive expression (Bialek *et al.*, 2016).

As regards the “Killing” part of the strategy, it is not enough with the cytopathic effects caused by HIV or the recognition and killing by immune cells; together with the newer “shocking” strategies more potent clearance functions will need to be used, likely provided by diverse immune therapies (Sadowski & Hashemi, 2019) such as:

- Antibody drug conjugates (ADC) using viral gene-specific antibodies coupled to toxic effectors such as doxorubicin or 5-fluorouracil.

- Chimeric antigen receptor (CAR)-expressing CD8+ T cells that target HIV gene products. For example, transduction of CD8+ T cells with engineered T cell receptor genes with specificity redirected towards HIV antigens.

- Recombinant Dual Affinity Retargeting Antibodies (DART), bispecific antibodies that simultaneously recognize two different viral epitopes like the CD3 receptor in combination with HIV-specific gag or env antigens expressed on reactivated CD4+T cells.

Although being promising strategies, they are complex to develop, and the efficacy of immunotherapy could be reduced by the immunomodulatory activities of some epigenetic LRAs (Chomont *et al.*, 2018).

Apart from the choice of LRAs, it has been addressed above that the timing of drug administration is critical for the clearance of the reservoir. A new possible strategy could be the administration of a LRA soon after the infection, at the time of ART initiation, in the "window of opportunity", where the immune responses are more present and the latent reservoir may be easier to reactivate (Chomont *et al.*, 2018).

However, eliminating the latent reservoir is not the only possibility to control HIV and improve the current chronic treatment situation. When the aim is to disable the replication ability of the latent provirus forever, two approaches can be made: eliminating the provirus, or assuring that even with no ART used the virus will no replicate again. As the first approach has just been revised, the strategy focused on the control will be now covered.

5.2 LOCK AND BLOCK

An opposing strategy to "Shock and Kill" includes the control of the capacity of the provirus to re-emerge from latency, known as "Lock & Block". The use of this strategy could avoid the use of ART in patients with HIV, locking down the viral reservoir and preventing it to wake up and spread the infection.

In order to "Lock and Block", the HIV provirus should be repressed and reactivation should be blocked specifically. To this aim, small molecule compounds can be developed; for example, some compounds inhibit the basal or signal-induced activity of NFκB (Nuclear transcription factor kappa B), a protein complex that controls DNA transcription and protects cells from apoptosis (Wang *et al*., 2014). However, as LTR enhancer has binding sites for numerous transcription factors, it is hard to lock down expression just inhibiting one of its activator like NFκB. Therefore, a general strategy to "lock down" HIV provirus expression might be more effective by encouraging transcriptional repression, through recruitment of molecules such as histone deacetylases, histone methyltransferases, DNA methyltransferases, or polycomb repressive complexes.

In a recent research, it has been found that the molecule Didehydro-Cortistatin A (dCA) can inhibit HIV-1 *tat* in humanized Bone Marrow Liver Thymic (BLT) mice (Kessing *et al.*, 2017). Tat is a viral protein that activates viral transcription by penetrating in it and transactivating its LTR promoter. Combining dCA with ART accelerates the suppression of HIV-1 and prevents viral reactivation after treatment is interrupted. dCA increases nucleosomal occupancy at Nucleosome-1, disabling RNA Polimerase II atachment to the HIV-1 promoter and limiting transcription.

Lastly, there is a promising new strategy to eliminate the latent HIV reservoir, which is gaining strength with the development of new technologies: the use of gene therapy.

5.3 GENE THERAPY

Although conceptualized decades ago, it has not been until these last years that gene therapy has started to be applied clinically. Gene therapy is based on the introduction of desired genetic material into a target cell, with the aim of provoking a specific effect. New gene editing tools such as CRISPR-Cas9 have enabled gene therapy to be applied clinically in a consistent way.

Gene therapy has recently been applied in the oncology field, with remarkable results. Therefore, the knowledge acquired from gene therapy clinical trials already performed could be used not only for the elimination of cancerous cells, but also for HIV.

Gene therapy could be used with different approaches. In fact, HIV target cells can be gene edited to A) become resistant to HIV infection by HIV co-receptor gene knockout, B) purge the cell from infection by permanent gene disruption of the HIV genome or C) have an adoptive cellular immune therapy. Now all those three strategies will be analyzed.

5.3.1 Knockout of HIV co-receptors

As mentioned before, allogeneic stem cell transplantations hold great risk with very high-observed mortality rates, limiting the applicability of this strategy only to cancer patients for which it is considered a last option therapy. An alternative could be the modification of autologous cells to eliminate the *CCR5* gene and make CD4+ T cells genetically resistant towards CCR5-tropic HIV variants. In the beginnings of the development of this strategy zinc finger nucleases were used but nowadays thanks to new gene-editing tools like transcription activator-like effector nuclease (TALEN) and CRISPR-CAS9 this strategy is being further developed. Using CRISPR it has been achieved a simultaneous knockout of *CCR5* and *CXCR4* in CD4+ T cells (Liu *et al*., 2017), contributing to a wider protection against all HIV strains. In addition, recently, this strategy was used to obtain CCR5-ablated hematopoietic stem and progenitor cells (HSPCs). However, the amount of CCR5 disruption in lymphocytes was only of a 5%, which shows that further research is needed.

As for its weak points, this method is highly dependent on the efficiency to generate a biallelic knockout of the target genes to fully eliminate their expression, but the strategy will further improve as well as new gene editing and current techniques are developed.

5.3.2 Viral Genome Disruption

Similarly, targeting the HIV provirus within infected cells presents an interesting alternative strategy that could make the virus permanently replication incompetent. CRISPR-Cas9 technology was successfully used to suppress viral replication in an *ex vivo* primary CD4+ T cell model (Kaminski *et al*., 2016). Interestingly, no off-target or adverse effects were observed during the analysis. In a more recent report, a small size Cas9 from Staphylococcus aureus (SaCas9) showed better precision and was successful to excise the latent HIV-1 provirus and suppress provirus reactivation using CRISPR (Wang *et al*., 2018)

However, due to the size of viral reservoir and its distribution in different anatomical compartments, a major challenge will be to target hidden HIV reservoirs in tissues. Further research is still needed to improve target precision and efficiency.

5.3.3 Adoptive Immune Therapy

Adoptive immune therapy, originally developed in the oncology field, involves genetic engineering of host cells with genes encoding new immune functionalities. As an example, in adoptive T cell therapy (ACT) T cells can be redirected towards specific targets by the integration of genes encoding either artificial T cell receptors (TCRs) or Chimeric Antigen Receptors (CARs).

Shortly, adaptive immunity and especially ACT has several beneficial properties (Perica *et al*., 2015) that make it an interesting strategy:

- T cell responses are specific, and can thus potentially distinguish between healthy and viral cells.

- T cells responses are robust, undergoing up to 1,000-fold clonal expansion after activation.

- T cell response can traffic to the site of antigen, suggesting a mechanism for eradication of distant reservoirs.

- T cell responses have memory, maintaining the therapeutic effect for many years after initial treatment. In the case reservoirs survived and were able to replicate again, adoptive T cells would continue attacking and controlling the infection.

Recent reports remark the importance of applying this strategy without a previous activation of the latent reservoir; in doing so, the proliferation and engraftment potential of the modified T cells is higher, increasesing the efficacy of this therapy at a reduced cost (Ahlenstiel et al., 2019).

To finish the eradication strategies section, it is important to remember that if agents are unable to activate and then eliminate all HIV-1 in the reservoir, much of the provirus that remains may be capable of reinitiating and sustaining infection. Therefore, reservoir eradication is a process that must be mastered, and until a completely efficient technique is developed, it will be difficult to get close to a cure.

In the short term, it seems more promising to improve Lock and Block strategies in order to eliminate chronic treatment, even though it means maintaining small amount of virus inside the body and periodical monitoring of patients.

Finally, gene editing seems the most precise strategy, with the development of new editing tools like CRISPR/Cas9 the modification of target genes is easier and more precise nowadays. Nevertheless, genetic modification techniques are still of high-cost, only affordable in advanced countries, making it difficult this way to create a cure for AIDS accessible for all.

6. FINAL CONCLUSIONS AND FUTURE PROSPECTS

Current situation of HIV is unsustainable over time, being over 37.9 million people infected worldwide (ONUAIDS statistics, 2019). The number keeps increasing in millions every year, making it almost certain that we will reach 40 million infected this 2020.

Although being ART essential nowadays to prevent death only 2/3 of those infected can access to ART, provoking hundreds of thousands of deaths annually (ONUAIDS statistics, 2019). Furthermore, ART continues to be too expensive, and its chronic feature and daily usage need makes it difficult to be a treatment consistently applied worldwide. Long-acting medications effective for weeks or months could make it easier for patients to gain adherence to the chronic treatment and could improve cost-effectiveness (Ross *et al.*, 2015).

Both HIV detection and control methods are being constantly updated and upgraded. It is already possible to detect and quantify quite efficiently HIV reservoirs combining detection strategies and setting top and bottom estimates. However, as regards the next step, if all latent HIV is not precisely controlled or eradicated, a viral rebound is prone to happen without ART. Therefore, eradication strategies need still further development, and gene therapies will probably deliver promising results next few years.

Besides, as mentioned before, there are other approaches that could be researched in order to reduce HIV viral load in patients. Those approaches include the deep study of post-treatment controllers (PTCs). PTCs have not been yet intensively studied, with only a few reports performed in the topic (Etemad *et al.*, 2019). Lowering provirus levels to the smallest amount with the combination of better ART and initial treatment protocols could be effective to consistently create PTCs and maintain the epidemic under control.

To conclude, in the next years, new techniques will be developed, while current ones get either improved or discarded. Furthermore, strategies that nowadays are considered cost-demanding (FLIPS, gene therapy…), may get affordable and widely used. Therefore, although all the advances made, the time left for a final cure for the HIV remains uncertain.

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Annexe 1. HIV target cell types, markers, tissue distribution and life span.

Cell lineage	*Markers*	*Tissue reservoirs*	*Life span*
CD4+ T lymphocytes	CD4, CD45, CXCR4, CCR5/CCR3	Peripheral blood, lymphatic tissue, gastrointestinal tract	1-3 years
Cytotoxic $CD8^{+}$ T lymphocytes	CD8	Peripheral blood, lymphatic tissue, gastrointestinal tract	1-3 years
Monocytes	CD4, CD14, CD16, CD52, CXCR4	Peripheral blood, lymphatic tissue	4-7 days
Macrophages	CD4,CD13,CD11b, FcγR	Peripheral blood, lymphatic tissue	2-24 months
Dendrocytes	CD4, CD16, CD14, CD1c, CD141	Peripheral blood	2-14 days
Folicular dendrocytes[a]	CD4, CD14, CD1c, CD141	Lymphoid tissue	2-14 days
Microglia	CD4, CD45, CD11b, P2RY12	Central nervous system	3-10 years
Astrocytes[b]	CD44, GLAST, ACSA	Central nervous system	Months
Perivascular Macrophages	CD4, CD45, CD206	Central nervous system	Months
Adipose macrophages	CD4, CD206, CD14	Adipose tissue	2-24 months
Kupfer cells	CD4, CD68, CD11b	Liver	3-4 days
Epidermal Langerhans	CD4, CD1a, CD207	Skin epidermis, genital tract	Months
HSPCs	CD4, CD34, CD133	Bone marrow	Years
Epithelial cells[b]	CD146, CD326	Genital tract, mammary tissue	Years

[a]Can maintain virus on surface without becoming infected [b] May become infected by syncytia formation.

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