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MINIREVIEW

HIV-1: towards understanding the nature and quantifying the latent reservoir

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Summary. – The human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) pandemic constitutes one of the greatest public health issues, since 36.9 million people worldwide were living with HIV in 2017 and 940,000 died from AIDS- related illnesses in the same year. One of the main obstacles in the effort to achieve viral eradication or long-term virologic remission is the existence of the HIV-reservoir. Except for resting memory CD4+ T cells there is a plethora of innate immunity cells including macrophages, dendritic cells, follicular T helper cells and NK cells which are now considered to play a role in viral latency and persistence. Hematopoietic precursor cells and progenitor mast cells, astrocytes, fibrocytes, renal and liver epithelial cells could also contribute to the reservoir, but their role remains controversial. Tissue reservoirs, such as the central nervous system (CNS), lymphoid tissue, adipose tissue and the gut-associated-lymphoid-tissue (GALT) are usually referred to as anatomic sanctuaries, where it is difficult to achieve high concentration and efficacy of antiretroviral agents. Accurate quantification of this reservoir is of the utmost importance and multiple assays have been developed for this purpose. The role of several cell populations in viral latency needs to be clarified by further studies. Furthermore, there is an urgent need for new assays, which will accurately measure the size of the reservoir, which plays a key role in predicting the timing of viral rebound upon cessation of antiretroviral treatment, since the currently available ones either overestimate or underestimate the size and have significant limitations.

Keywords: HIV-1; cellular reservoirs; tissue reservoirs; quantification

1. Introduction

The introduction of highly active antiretroviral therapy (HAART) in 1996 was a milestone in the effort to cure HIV-1 infection. It has increased survival thus transforming HIV-1 infection into a chronic condition, improved the quality of life and succeeded in suppressing viremia to clinically undetectable levels (<50 copies ml^{-1}). However, HIV-1 infection remains one of the leading causes of morbidity and mortality worldwide. The main reason is the existence of a pool of latently infected cells (commonly referred to as the HIV-1 reservoir) which are insensitive to antiretroviral therapy (ART) and undetectable by the

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Abbreviations: ART = antiretroviral therapy; CNS = central nervous system; DCs = dendritic cells; FDCs = follicular DCs; GALT = gut-associated lymphoid tissue; HAART = highly active ART; HIV = human immunodeficiency virus; LAG-3 = lymphocyte activation gene 3; mDCs = myeloid DCs; NGS = next generation sequencing; PD-1 = programmed cell death protein 1; pDCs = plasmacytoid DCs; RT-qPCR = quantitative real-time PCR; QVOA = quantitative viral outgrowth assay; T_{FH} = follicular helper T cells; γδ T cells = gamma-delta T cells

immune system, thus making the eradication of the virus a rather challenging affair (Chun *et al*., 2015; Kulpa and Chomont, 2015; Melkova *et al*., 2017) with resting memory CD4+ T cells being the most significant cell population that contributes to viral persistence (Chun *et al*., 1998). A long nonproductive phase of infection caused by the administration of ART is followed by a reactivation of the replication competent viral forms resulting in viral rebound upon discontinuation of ART. As a result, lifelong treatment is considered mandatory. The initiation of ART is followed by a decline of plasma viremia and HIV-1 infected cells in peripheral blood according to a well-described pattern with four phases. The existence of the reservoir is proven by the very existence of phase IV, which is characterized by a stable low-level viremia, which does not decrease anymore (Hilldorfer *et al*., 2012; Melkova *et al*., 2017).

2. Cellular reservoirs

Apart from CD4+ T cells many other cell populations have been proven to contribute to viral latency and persistence. Of note, it has recently been discovered that the low-affinity receptor for the immunoglobulin G Fc fragment, CD32a, is highly induced only in quiescent HIV-infected cells and neither in cells in a productive phase of infection nor in bystander cells thus marking the latently infected ones (Descours *et al*., 2017). Furthermore, Fromentin *et al.* (2016) showed that the majority of cells with inducible HIV genomes express at least one of the markers: programmed cell death protein 1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and the protein encoded by lymphocyte activation gene 3 (LAG-3). These findings are very promising, since blockers directed against these molecules may prove to be useful tools in the effort to target the latently infected cells.

The contribution of macrophages in HIV persistence has generated a great deal of heated debate, since their role as phagocytes may account for the presence of HIV nucleic acids and proteins in these cell (Calantone *et al*., 2014). However Baxter *et al*. showed that macrophages can capture HIV-1 infected CD4+ T cells resulting in macrophage infection (Baxter *et al*., 2014). Studies using humanized myeloid-only mice have proven HIV persistence in tissue macrophages during ART (Honeycutt *et al*., 2016, 2017), thus changing our perspective and drawing researchers' attention to this underestimated reservoir. Another interesting aspect is the contribution of macrophages to viral spread through trans infection. Myeloid-lineage cells, such as macrophages and dendritic cells can capture HIV-1 without necessarily being infected and then transfer it to CD4+ T cells (Sattentau and Stevenson, 2016).

Dendritic cells (DCs) are a very diverse group of innate immunity cells, which includes plasmacytoid (pDCs), myeloid (mDCs), Langerhans and follicular dendritic cells (FDCs). The first three categories serve as antigen-presenting cells, whereas FDCs lack this ability. Both CD123+ pDCs and CD11c+ mDCs express CD4, CCR5 and CXCR4, receptors involved in HIV-1 infection. These infected DCs transfer the virus to autologous CD4+ T cells during antigen presentation via an infectious synapse. It is particularly interesting that myeloid and plasmacytoid DCs transfer the virus preferentially to antigen-specific CD4+ T cells (Loré *et al*., 2005).

Follicular dendritic cells, located in B cell follicles of secondary lymphoid organs, have the unique ability to maintain large quantities of HIV on their surface without being infected themselves (Smith-Franklin *et al*., 2002).

These cells are in close proximity to follicular T helper (T_{FH}) cells thus allowing effective viral transmission (Heesters *et al*., 2015). Furthermore T_{FH} express CXCR5 as well as PD-1 and are highly susceptible to HIV-1 infection. The phenotype of the cell changes as the virus replicates resulting in downregulation of PD-1 and to a lesser extent of CXCR5 (Kohler *et al*., 2016).

Recent studies have demonstrated that hematopoietic precursor cells (HPCs) as well as progenitor mast cells could also contribute to the reservoir (Alexaki and Wigdahl, 2008; Bannert *et al*., 2001; Carter *et al*., 2010; McNamara, Collins, 2011). Active and latent infection of CD34+ cells could account for the hematopoietic abnormalities observed in HIV-1 infected patients. However, Josefsson *et al*. (2016) concluded that HPCs in the bone marrow are not a source of persistent HIV-1 during long-term suppressive therapy, while Zhang *et al*. (2007) showed that hematopoietic stem cells resist infection via the cyclin-dependent kinase inhibitor p21.

Astrocytes are the most abundant cell type in the brain and their role in HIV-1 latency is highly controversial. A very promising study is the one conducted by Luo and He (2015), which showed that, apart from the gp120-human mannose receptor mediated endocytosis, a cell-to-cell viral transfer from infected CD4+ T lymphocytes is also possible and that HIV-1 successfully establishes latency in astrocytes. Chauhan *et al*. concluded that pH-dependent endocytosis is the only natural way of HIV infection in astrocytes and causes minimal but productive infection (Chauhan and Khandkar, 2015) and that the endosomal internal machinery is crucial for the successful establishment of infection (Chauhan *et al*., 2014). On the other hand Russell *et al*. found that astrocytes cannot be infected by HIV-1 by cell-free or cell-to-cell routes but are able to engulf infected macrophage material (Russell *et al*., 2017). Further studies need to be conducted in order to determine the exact role of astrocytes in HIV infection.

Other cells that could be part of the HIV-1 reservoir include liver and renal epithelial cells, fibrocytes and NK cells (Kandathil *et al*., 2016).

3. Tissue reservoirs

Significant tissue reservoirs, usually referred to as anatomic sanctuaries for HIV-1 in ART- treated patients, include CNS, lymphoid tissue, adipose tissue and GALT. The CNS is considered to be an immune privileged site due to the presence of the blood-brain barrier and the reduced antiretroviral drug efficacy and concentration in the brain (Asahchop *et al*., 2017). There are three types of CD4 + cells in the CNS: CD4+ T cells, macrophages and microglia. CD4+ T cells are present in very low concentrations, while macrophages and microglia express low densities of CD4 (Wang *et al*., 2002). It has been observed that viruses replicating in the CNS make an evolutionary transition and get accustomed to infecting cells with low surface levels of CD4. The presence of these M-tropic HIV-1 lineages that are found late in disease makes macrophages and microglia the most significant targets of HIV-1 in the CNS (Joseph *et al*., 2015). The use of quantitative viral outgrowth assay (QVOA) confirmed the presence of latently infected brain macrophages, which harbor replication competent virus (Avalos *et al*., 2017).

Preadipocytes and adipocytes cannot be infected with HIV-1 *in vivo* due to the extremely low surface levels of CD4, CXCR4 and CCR5, which make the entry of the virus impossible (Munier *et al*., 2003). However the adipose tissue also contains a plethora of innate immunity cells, including CD4+ T cells, CD8+ T cells, T regulatory cells, NK cells and B cells (Grant, Dixit, 2015). These CD4+ T cells have an activated (CD69+) memory phenotype (Sathaliyawala *et al*., 2013). Couturier *et al*. showed that adipocytes can stimulate CD4+ T cell activation and HIV replication in the presence of IL-2, IL-7 and IL-15 (Couturier *et al*., 2016), while Damouche *et al*. confirmed the presence of replication competent virus in the adipose tissue. The authors found elevated adipose density, enhanced activation of adipose tissue-resident immune cells and/or inflammation profile in SIVmac251 infected macaques (Damouche *et al*., 2015). These findings suggest that HIV/SIV infection has similar effects with obesity on the adipose tissue, since both cause influx of immune cells and increased levels of pro-inflammatory cytokines (Iyer *et al*., 2010). Further studies need to be conducted, in order to determine whether the same pharmacological approaches could prove beneficial for obese as well as for HIV-1 infected patients, the exact effects of HIV/SIV infection on adipogenesis and the role of adipose tissue-resident macrophages in HIV persistence.

The lymphoid tissue is a significant cofactor in HIV-1 latency and persistence partly due to the low penetration for many antiretroviral agents (Fletcher *et al*., 2014). The lymph nodes constitute a unique environment due to the presence of FDCs and T_{FH}, which are, as mentioned above, a well described reservoir. Fukazawa *et al*. were the first to show that B cell follicle areas function as a sanctuary for SIV despite cytotoxic T lymphocytes responses in the lymph nodes (Fukazawa *et al*., 2015). Virus-specific CD8+ T cells are normally excluded from this site, since they lack the expression of the CXCR5 (Ansel *et al*., 2000). Furthermore, HIV-specific CD8+ T cells in the lymphoid tissue have reduced cytolytic activity due to reduced expression of perforin and granzymes (Reuter *et al*., 2017).

Finally, target cells in the gut include a variety of innate immunity cells: macrophages, regulatory T cells (Treg), interleukin-17 producing helper T cells (T_H17), interferon gamma and interleukin 17 producing helper T cells (T_H1/T_H17), interleukin 22 producing helper T cells (T_H22), follicular helper T cells (T_{FH}), transitional memory T cells (T_{TM}), tissue resident memory T cells (T_{RM}), gamma-delta T cells ($\gamma\delta$ T cells), stem cell memory T cells (T_{SCM}) and central memory T cells (T_{CM}) (Khan *et al*., 2017). Interestingly it has been found that rectal macrophages express higher levels of CCR5, which suggests that the most distal parts of the gut are particularly vulnerable to HIV-1 infection (McElrath *et al*., 2013) and that PD-1 can serve as a marker of HIV persistence in rectal tissue (Khoury *et al*., 2017). Furthermore, it has recently been discovered that T regulatory cells suppress viral replication in T cells via a cAMP-dependent pathway thus contributing to viral persistence (Li *et al*., 2017) and that HIV-1 selectively targets gut-homing CCR6+CD4+ T cells via mTOR-dependent mechanisms (Planas *et al*., 2017). However, more evidence is required in order to determine the extent to which each of the aforementioned cell populations contributes to the reservoir, as their role is still controversial.

4. Quantification of the latent reservoir

The size of the reservoir is considered to be a predictor of the time of viral rebound after the cessation of HAART (Li *et al*., 2016). It is therefore crucial, in order to estimate the time of viral rebound and in order to evaluate the efficacy of therapeutic strategies, to be able to quantify the latent reservoir and achieve a better understanding of its characteristics. Multiple assays have been developed for this purpose.

Quantitative viral outgrowth assay (QVOA), which aims to identify the cells that carry replication-competent virus, is currently the gold standard. However, there are multiple drawbacks. QVOA requires 14–21 days to be

completed and large numbers of cells and a biosafety level 3 laboratory, is expensive, and has been proven to underestimate the size of the reservoir (Henrich *et al*., 2017; Hodel *et al*., 2016; Wang *et al*., 2018). One of the basic problems of the QVOA protocol is that only a portion of the cells with replication-competent proviruses is activated with a single round of stimulation. Ho *et al*. (2013) analyzed 213 non-induced proviral clones and demonstrated that 11.7% had intact genomes and were in fact replication-competent. These findings suggest that QVOA only provides a minimal estimate of the size of the latent reservoir (Wang *et al*., 2018). Several modifications of the gold standard have been proposed. Alternative activation methods have been tested with CD3/CD28 costimulation showing the most promising results (Beliakova-Bethell *et al*., 2017). Furthermore, MOLT4/CCR5 viral outgrowth assay constitutes an improvement of the classic QVOA. MOLT4/CCR5 T-cell lines, which are highly susceptible to HIV-1 infection, are used instead of large number of cells from uninfected donors, thus reducing the needs in cells, time and labor (Hodel *et al*., 2016). Since the HIV-1 p24 antigen ELISA used in QVOA is particularly time-consuming, quantitative real-time PCR (RT-qPCR) detection of HIV-1 RNA at assay day seven and deep sequencing have also been used to detect viral outgrowth (Wang *et al*., 2018). TZM-bl cell based assay (termed TZA) is a novel assay, which measures replication-competent HIV-1 in the TZM-bl cells after induction of HIV-1 production by a potent latency reversing agent, such as anti-CD3/CD28 activating antibodies. Compared to QVOA a 70-fold larger reservoir was detected (Gupta *et al*., 2017).

PCR- based assays are widely used with the main limitation being that they are unable to distinguish between intact and defective sequences, thus overestimating the size of the reservoir (Hodel *et al*., 2016). Total HIV DNA, including integrated and unintegrated forms, is considered to be a marker of the latent reservoir, which can be quantified easier, faster and with satisfactory sensitivity with RT-qPCR based assay being the most frequently used method (Rouzioux and Avettand-Fenoel, 2018). Digital PCR and especially droplet digital PCR (ddPCR), despite the issue of the unexplained false-positive partitions, are constantly gaining ground, as mismatches between the target sequence and the primer/probe are better tolerated than in qPCR and the need for a standard curve for DNA quantification is eliminated (Rutsaert *et al*., 2018).

Lee *et al*. (2017) and Hiener *et al*. (2017) were the first to use next generation sequencing (NGS), in order to genetically characterize proviruses and predict replication competency. Full-length sequencing constitutes a very appealing approach given the heterogeneity of proviruses and the significance of differentiating between intact and defective sequences.

Finally, the tat/rev-induced limiting dilution assay (TILDA) which measures CD4+ T cells that produce viral tat/rev HIV-1 msRNA upon activation, the inducible cell-associated RNA expression in dilution (iCARED) assay which measures viral RNA and Simoa digital ELISA which detects proteins are the so-called next generation assays, which are easy, fast and highly sensitive and require minimal amounts of blood. These novel assays aim to bridge the gap between the underestimation of the HIV reservoir caused by culture-based assays and the overestimation caused by PCR-based assays (Hodel *et al*., 2016).

5. Conclusions

It is, therefore, evident that the currently available assays fail to determine the true size of the reservoir with culture-based assays underestimating and PCR-based assays overestimating the size. New assays are constantly being developed in order to achieve high throughput, sensitivity and specificity. NGS represents an extremely promising approach, since the differentiation between replication-competent and defective proviruses is of the utmost importance, when trying to estimate the time to viral rebound upon cessation of ART and determine the efficacy of strategies aiming at viral eradication or long-term virologic remission. Finally, understanding the characteristics and measuring tissue reservoirs constitutes another major challenge.

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