REVIEW

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The active human immunodeficiency virus reservoir during antiretroviral therapy: emerging players in viral persistence

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Purpose of review
To discuss the role of CD4$^+$ T cells with active Human immunodeficiency virus (HIV), meaning infected cells with transcriptional and/or translational viral activity during antiretroviral therapy (ART), focusing on new technologies for its detection, potential cell markers for its characterization, and evidences on the contribution of the active HIV reservoir to long-term viral persistence.

Recent findings
HIV-infected cells expressing viral ribonucleic acid are systematically detected in subjects on long-term ART. In recent years, powerful new tools have provided significant insights into the nature, quantification, and identification of cells with active HIV, including the identification of new cell markers, and the presence of viral activity in specific cell populations located in different cellular and anatomical compartments. Moreover, studies on viral sequence integrity have identified cell clones with intact viral genomes and active viral transcription that could potentially persist for years. Together, new investigations support the notion that the active reservoir could represent a relevant fraction of long-term infected cells, and therefore, the study of its cell sources and mechanisms of maintenance could represent a significant advance in our understanding of viral persistence and the development of new curative strategies.

Summary
The presence of HIV-infected cells with viral expression during ART has been traditionally overlooked for years. Based on recent investigations, this active viral reservoir could play an important role in HIV persistence.

Keywords
active reservoir, cell markers, human immunodeficiency virus, latency, viral persistence

INTRODUCTION

Human immunodeficiency virus (HIV) establishes a persistent infection for which nowadays there is not an available cure. Combined antiretroviral therapy (ART) strongly inhibits viral replication and stops disease progression but is unable to fully eliminate the virus, which accumulates and persists long-term in cell reservoirs [1]. The classical view of the HIV reservoir consists of resting memory CD4$^+$ T cells harboring replication-competent virus, located in different cellular and anatomical compartments, that could reignite systemic infection when therapy is interrupted [2,3]. The frequency of such cells is very low; first approximations estimated that only ~1 out of a million infected CD4$^+$ T cells contain replicative viral forms [4,5]. Initially, this low proportion of cells was considered one of the main obstacles to cure the infection, but soon after it was recognized that this number, most likely, underestimated the real frequency of cells with potential to produce infectious virus [6,7]. Additionally, a bigger fraction of cells, roughly 2–18% of all infected cells have been detected to transcribe HIV and produce viral proteins during ART [8▪▪,9,10]. Transcriptionally and translationally competent HIV-infected cells are considered the active viral reservoir. Importantly, the extent to which some

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KEY POINTS

- The HIV reservoir during antiretroviral therapy is comprised of infected cells containing virus in a silent or transcriptionally active state.
- New methods have allowed us to identify markers of the active HIV reservoir and its localization in anatomical compartments.
- Cells expressing viral RNA could contain intact HIV sequences, persist long-term, and contribute to residual viremia.
- Targeting the active viral reservoir might significantly impact HIV persistence.

of these cells contribute to long-term HIV persistence has been poorly defined. In the past, cells with HIV proviral activity were systematically excluded from reservoir studies. This fact was due to several assumptions; (i) activated cells, usually linked to HIV expression, are thought to be short-lived and therefore, will not represent a long-term reservoir for HIV, and (ii) transcriptionally active cells producing viral particles will be eliminated by viral cytotoxicity and/or immune recognition [11]. However, investigations indicate that cells with resting phenotypes can also transcribe HIV [12,13], that transcriptionally active HIV-infected cell clones can be maintained for years [8▪▪,9,14,15▪▪,16], and that active reservoirs might be the source of residual viremia [14]. These studies uncover the active reservoir as an important component of the long-term reservoir, and therefore, convene the study of this particular cell reservoir for comprehensive approaches directed to target viral persistence. Here, we will review new technological advances that have been instrumental to study the active HIV reservoir, the cell sources and markers of active HIV-infected cells, and the potential role of the active reservoir in long-term viral persistence.

IDENTIFICATION OF ACTIVE HUMAN IMMUNODEFICIENCY VIRUS-INFECTED CELLS

A detailed review of methods used to quantify the different forms of the HIV reservoir can be found elsewhere [17]. In this review, we will focus on approaches used for the identification and characterization of the active HIV reservoir, meaning cells with transcriptionally and/or translationally active HIV.

The understanding of the composition and location of active viral reservoirs during ART has been possible by the development of more specific and sensitive novel assays [9,18–21]. The traditional polymerase chain reaction (PCR)-based assay, and more recently the droplet digital PCR approach, allow quantifying different cell-associated HIV viral transcripts, including spliced and unspliced ribonucleic acid (RNA) molecules, in bulk cell populations. This methodology can be combined with the quantification of total viral HIV deoxyribonucleic acid (DNA) to obtain a relative measure of the transcriptional activity of HIV in infected cells [22–24]. This method *per se* does not allow the identification of cells producing HIV-RNA neither the quantification of the transcriptional activity in individual cells; however, it could be used to assess the transcriptional activity of specific cell subpopulations after cell isolation. Notably, in recent years, novel *in situ* hybridization (ISH)-based techniques have allowed the detection of intracellular HIV RNA molecules at the single-cell level with high sensitivity and specificity. The Prime Flow RNA assay (fluorescence in situ hybridization-flow) and the HIV-RNA scope ISH technologies have permitted the identification of different subpopulations carrying transcriptionally active provirus in blood and tissue specimens in patients on ART [19,25▪▪,26]. Additionally, these techniques have been used to measure the frequency of cells with active HIV upon exogenous cell stimulation or addition of latency reversal agents [20,25▪▪,27,28]. Of relevance, these approaches have also provided valuable insights into the variable response of different cell subpopulations to drugs intended to stimulate HIV expression [20,27,28]. Other induction-based viral RNA reactivation assays, including the tat/rev induced limiting dilution assay [29,30], have been used to quantify cells that can reactivate HIV transcription upon cell stimulation. However, recent efforts have been directed to develop new methodologies to provide information on the proviral sequences in cells with transcriptional viral activity. In this sense, the cell-associated HIV RNA and DNA single-genome sequencing method was developed to investigate the fraction of proviral expression [9]. This method revealed that identical HIV RNA from infected cell clones could arise from multiple single cells and across multiple times. Currently, new approaches comprising technological advances and aimed at assessing the intactness of proviral DNA, integration sites, viral expression, and the biology of cells harboring HIV at the single-cell level, are being developed [16,31,32▪] and will certainly provide relevant information about the nature and significance of the active HIV reservoir.

CELL RESERVOIRS FOR ACTIVE HUMAN IMMUNODEFICIENCY VIRUS

Curing HIV most likely will require the elimination of all infected cells with potential to initiate new

rounds of infection, thus the identification and characterization of reservoir cells in patients on ART have represented an important goal during the last decade. The main cell type supporting long-term HIV persistence are $CD4^+$ T cells; although macrophages containing integrated HIV DNA, RNA, viral proteins, and intact virions in virus-containing compartment-like structures have also been described in tissues [33]. Resting memory phenotypes have been the most studied subpopulations due to their higher intrinsic capacity to harbor silent HIV and therefore, their likelihood to constitute a long-term niche for the virus through the evasion of the immune system and the action of antiretroviral drugs [5,34]. However, $CD4^+$ T cells are intrinsically a heterogeneous population and are defined by differential expression of cell surface receptors associated with different stages of cell maturation, activation, differentiation, function, and cell turnover. In blood, subpopulations of $CD4^+$ T cells showing central memory (T_{CM}), transitional memory (T_{TM}), and effector memory (T_{EM}) phenotypes comprise the largest proportion of HIV proviruses [35,36]. A detailed review of the major cell subpopulations that compose the total HIV reservoir can be found here [37]. From the total pool of reservoir cells, only a small fraction of cells contain intact viral forms [6,7,38,39]; higher proportions of intact viral regions seem to be located, in some studies, in the T_{EM} subset [7,15▪▪,38,40], but naïve $CD4^+$ T cells could also contain intact HIV [41,42]. Importantly, only a small fraction of infected cells contain active HIV without previous cell activation [8▪▪,9]. In blood, T_{EM} and T_{TM}, cells with relatively short half-lives, are the main contributors to HIV transcription [19,43,44▪]. Grau-Expósito *et al.* found T_{EM} $CD4^+$ T cells as a major niche for HIV transcription in ART-treated patients [19], whereas Yukl *et al.* found enrichment in HIV transcriptional activity in T_{EM} but also in T_{TM} cells [43]. Similarly, T_{EM} cells which is the subpopulation with the fastest replacement rates were found highly enriched for HIV-RNA and contained the most clonal proviral expansion [44▪]. However, viral burden in several tissues, including lymph nodes (LN), cervix, and the gut-associated lymphoid tissues is systematically higher than in blood [26,43,45–47]. Indeed, $CD4^+$ T-follicular helper (T_{FH}) cells found in LN are highly enriched in viral RNA and replication-competent HIV [47,48]. Similarly, in the female genital tract, tissue-resident memory $CD4^+$ T cells (T_{RM}) contain transcriptionally active HIV and are the highest contributors to the total pool of HIV-infected cells [46]. Some of these cellular subsets and anatomical compartments might constitute sanctuaries for HIV persistence under ART [46,48,49] and might promote viral rebound upon treatment interruption [40]. Collectively, active cellular reservoirs for HIV are widely distributed in different anatomical and cellular compartments, which might vary inter-patient, and represent potential sources of replication-competent virus that, eventually, might fuel systemic viral replication after ART discontinuation.

Given the highly diverse sources of persistent cell reservoirs, finding a unique, or a set of markers, able to identify cells carrying replication-competent virus could significantly facilitate the development of new strategies directed to cure HIV [50]. Several surface molecules, such as CD2high, CXCR3, CD32a, and CD161 [51–54] or immune exhaustion markers like PD-1, TIGIT, CTLA-4, and TIM-3 [55,56], have been reported to identify latent cells enriched in inducible proviruses. Recently, cells expressing the activation marker human leukocyte antigen – DR were shown to contain high levels of intact HIV [57,58]. Importantly, similar viral transcriptional levels are found in these cells and their negative counterparts, suggesting that phenotypic markers of cell activation are not necessarily surrogates for cells with a transcriptionally active viral status [58]. Besides, other molecules, such as CD30 and CD20 have been able to identify transcriptionally active cells, and the targeting of these active reservoirs with specific antibodies *ex vivo* and *in vivo* have showed reduction in the HIV cellular burden [25▪▪,59]. Among cell markers, CD32a probably remains one of the most promising candidates, since it was shown to contain latent HIV [52,60] and to be enriched in transcriptionally active HIV in several compartments; CD32a was detected in infected cells in blood and main tissue reservoirs like the LN and the gastrointestinal tract in people on ART [61–63]. Although the value of CD32a as a marker of the HIV reservoir was challenged by early investigations denoting technical artifacts [64,65], the isolation of *bona fide* $CD32^+$ $CD4^+$ T cells has pointed out the usefulness of CD32a as a relevant marker of HIV reservoir cells [46,60,61]. Moreover, CD32a is also expressed on cells enriched in molecules related to HIV susceptibility and long-term maintenance [46,61], and importantly, the possible interaction of CD32a with immune complexes might confer to the infected cell an escaping mechanism from immune surveillance [66,67]. Together, up to date, several markers have been reported to identify active HIV in $CD4^+$ T cells. To what extend the combination of these markers or their expression kinetics during the HIV infection cycle might represent a more accurate measure to identify active reservoir cells warrants further investigation. A summary of the main cell markers proposed for the study of the active HIV reservoir, as well as the main cell

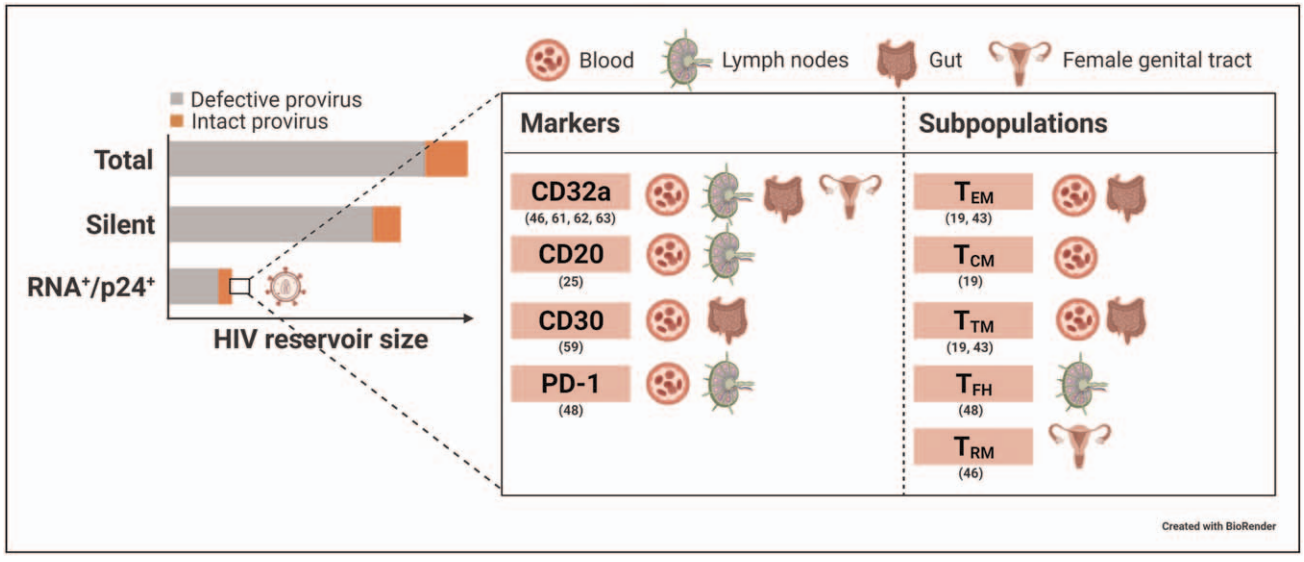

FIGURE 1. Active HIV reservoirs identified in individuals on ART. Illustration representing the relative contributions to the total HIV reservoir of both, the silent and the active viral fractions, highlighting the main different cell markers, cell subsets and anatomical compartments that might support HIV transcription during ART.

subpopulations and anatomical compartments showed to contribute to HIV expression in subjects on ART, is illustrated in Fig. 1.

THE ACTIVE HUMAN IMMUNODEFICIENCY VIRUS CELL RESERVOIR AND LONG-TERM VIRAL PERSISTENCE

The mechanisms driving HIV latency in vivo are currently not well defined; the existence of a pool of cells with different blocks in several steps of the HIV replication process precedes the high heterogeneity in viral silencing observed in cell reservoirs [24,68,69]. Although HIV transcription and production of viral particles do not necessarily mean infectious competence, here we summarize some data supporting the importance of characterizing the active cell reservoirs during ART.

In viremic patients, the clinical significance of measuring the intracellular levels of viral RNA soon became apparent by the direct association between intracellular HIV-RNA quantification and $CD4^+$ T cell counts [70–72]. Moreover, the level of viral transcription has been associated with virological failure in patients on ART, suggesting the frequency of HIV RNA^+ cells as a potential biomarker of therapy success [22]. Importantly, in several investigations, viral expression predicted time to viral rebound after treatment interruption [73▪▪,74]. During ART, however, only a small fraction of the total pool of reservoir cells are able to transcribe HIV [8▪▪,9,75]. The fraction of these cells containing replication-competent HIV and therefore, representing a potential target for cure approaches, has not been very well established. The disconnection between the assays used to detect different forms of the viral reservoir has not allowed to estimate the fraction of transcriptionally active cells with potential to produce infectious viral particles on a per cell level basis. Recently, however, several investigations found the existence in some ART-treated individuals, of persisting cell clones expressing HIV that represented the source of residual viremia, being closer to a replication-competent state [14]. Furthermore, new methodologies based on the simultaneous genome DNA and RNA viral sequencing in single cells have demonstrated that intact genomes could also express viral RNA [15▪▪,16], and importantly, that intact HIV located in cell clones could transcribe HIV during long periods of time [8▪▪,9,14,15▪▪]. Whether or not these specific cell clones have a constitutive or intermittent expression of viral RNA, and/or are properly targeted by the immune system, are still open questions. Likewise, further research on T cell clones and residual viremia in patients on ART might be very informative [76], since viral blips and low-level viremia might affect long-term dynamics of the HIV reservoir, slowing down the reduction of its size [77].

Besides, recent studies have also demonstrated the presence of defective provirus encoding translationally competent HIV-RNA transcripts [78–80]. These transcriptionally and translationally competent cell reservoirs could be responsible for the persistent immune activation and cell exhaustion

evidenced in ART-treated and some Elite controllers patients [81–83]. The direct relationship between the expression of viral products and viral-driven pathogenesis during ART evidences the need to pay more attention to the existence and the impact of the active reservoir. It is tempting to speculate that the elimination of RNA-expressing cells or the acceleration of the active viral reservoir decay, could have a positive effect not only on systemic immune activation, but also on shifting the reservoir toward a profound dormant state as recently shown in elite controllers [32▪,84]. However, to accomplish this goal will likely require the restoration of relevant antiviral immune responses and revert possible immune resistant mechanisms raised in reservoir cells, as recently suggested [67,85].

CONCLUSION

The role of the active viral reservoir on HIV persistence has not been completely considered until recently. During ART, HIV can be found in a transcriptionally and translationally active state, comprising a small proportion of the total reservoir cells. Recently, new technological advances have allowed characterizing the reservoir landscape in depth, and have identified the presence of active reservoir cells with intact genomes with potential to survive long-term. Based on these recent findings, we believe that a more profound study of the active reservoir might lead to the development of new targeted strategies for the continuous elimination of relevant reservoir cells over time. Elimination of cells with viral transcriptional activity could significantly impact viral persistence.

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Conflicts of interest

There are no conflicts of interest.

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