REVIEW

OPEN

The multifaceted nature of HIV tissue reservoirs

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Purpose of review
To underline the complexity and the heterogeneity of the HIV reservoir.

Recent findings
While lymphoid tissues (spleen, lymph nodes, gut-associated lymphoid tissue) harbor specific subsets of specialized $CD4^+$ T cells enriched in HIV-infected cells, non-$CD4^+$ T cell reservoirs such as tissue-resident macrophages and dendritic cells have also been implicated to contribute to viral persistence. Moreover, studies have applied highly sensitive tools to detect transcriptional activity within HIV-infected cells during prolonged ART and revealed a broader spectrum of transcriptional activity for proviruses than previously thought. Finally, while a combination of factors might be involved in the regulation of HIV persistence within different tissues and remains to be fully elucidated, recent results from autopsy samples of HIV-infected ART suppressed individuals indicate extensive clonality of HIV reservoirs in multiple tissues and suggest that the recirculation of HIV-infected cells and their local expansions in tissues may also contribute to the complexity of the HIV reservoirs in humans.

Summary
HIV persistence in blood and multiple tissues despite long-standing and potent therapy is one of the major barriers to a cure. Given that the HIV reservoir is established early and is highly complex based on its composition, viral diversity, tissue distribution, transcriptional activity, replication competence, migration dynamics and proliferative potential across the human body and possible compartmentalization in specific tissues, combinatorial therapeutic approaches are needed that may synergize to target multiple viral reservoirs to achieve a cure for HIV infection.

Keywords
compartmentalization, dendritic cells, HIV persistence, HIV reservoir, HIV transcription, lymph node, replication competent HIV, T-follicular helper cells, tissue HIV reservoir

INTRODUCTION

Although antiretroviral therapy (ART) is undeniably effective at blocking HIV replication to levels below the detection limit of conventionally available assays [1,2], neither early [3] nor prolonged treatment is sufficient to cure HIV infection [4–6]. Indeed, upon treatment interruption, plasma viral rebound occurs in majority of HIV-infected individuals within a relatively short time frame, around 14–21 days [7,8], demonstrating that HIV persists despite ART. Historically, the quantification of HIV-infected cells relied mainly on either PCR-based methods measuring viral nucleic acids [9–11] and/or on viral outgrowth assays assessing viral competency [12,13]. These assessments supported the paradigm that viral persistence was primarily associated with quiescent yet inducible HIV infection of long-lived resting memory $CD4^+$ T cells in the blood, referred to as the 'latent reservoir' [4,5,14] which was largely unchanging and stable over-time [6,15]. In contrast to peripheral blood, secondary lymphoid organs (such as the spleen, lymph nodes, and gut-associated lymphoid tissues), where viral replication predominantly occurs, remained less extensively investigated [16,17]. This was mainly attributed to two major reasons: limited accessibility to tissue samples from individuals on ART due to ethical considerations, and tissues were believed to harbor HIV-infected cells actively expressing HIV genes and viral transcripts, constituting an 'active reservoir' susceptible to elimination – either directly through viral cytopathic effects or through cell-mediated immunity [18]. However, recent studies

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KEY POINTS

- Despite recent technological breakthroughs allowing the single-cell proteo-genomic profiling of HIV-infected cells, no evidence till date supports the presence of a single phenotypic marker capable of effectively distinguishing virally infected cells from uninfected cells.
- Increasing amount of studies support to presence of replication-competent inducible HIV reservoir within macrophages and dendritic cells isolated from distinct tissues in the body, highlighting, the need of considering these cellular reservoirs in cure-oriented clinical trials.
- Viral rebound upon ART cessation can originate from multiple tissues in the body. In this context, multiple factors can contribute to viral replication following ART cessation, including anatomical and microanatomical locations, the infected cell type, cellular phenotype, half-life, the nature of the provirus, the potential for transcriptional activity given the specific integration site, and/or distribution of antiretroviral drugs within tissues.

have played a pivotal role in unravelling the complexity of the HIV reservoir in terms of its cellular composition, tissue distribution, and transcriptional activity [19].

CELL TYPES

Research efforts over the past 30 years have sought to understand the complexity of the HIV reservoir in order to facilitate the development of interventions that could eradicate the HIV-infected cells (a cure) or enable HIV seropositive individuals to maintain suppressed viremia in the absence of ART (a functional cure) [20,21]. In this context, the qualitative and quantitative assessment of the HIV reservoir in ART treated individuals has been conducted by various research groups employing diverse assays to measure the presence of viral nucleic acids (such as total and integrated DNA, or various forms of RNA) through PCR-based assays either alone or with proviral sequencing, viral protein (p24) detection and/or with functional assessments measuring viral competence through culture-based viral outgrowth assays. In this regard, pioneering studies demonstrated the presence of HIV-infected cells (HIV DNA^+ and/or RNA^+), albeit at different frequencies, within various blood $CD4^+$ T cell subsets [9–12,22–27]. However, it is important to note that while PCR-based assays are highly standardized, robust, and require fewer cells as compared with culture-based methods, they fail to distinguish between defective and replication competent viruses [28].

Alternative approaches, such as the use of culture-based quantitative viral outgrowth assays (Q-VOAs) to assess the frequencies of HIV-infected cells harboring inducible replication-competent proviruses in treated individuals demonstrated a notable enrichment within blood $CD4^+$ T cells expressing CXCR3 [24,29] and CD32a [30]. Similarly, enrichment was observed within lymph node (LN) $CD4^+$ T cells expressing PD-1 [23], that is, LN Tfh cells (Fig. 1) and CD32a [31] in the same individuals during ART, although at significantly higher frequencies within lymph nodes. Notably, Q-VOAs are highly robust and could be instrumental for measuring reservoir size in cure-oriented clinical trials. However, Q-VOAs rely on large cell inputs of highly purified cell populations in the limiting-dilution format, the necessity for specific stimulations to induce viral production, and potentially underestimate the viral reservoir size due to the assay's sensitivity (reviewed in Eriksson *et al.* [28]). Furthermore, elegant studies have demonstrated that while around 10% of HIV-infected cells may harbor intact proviruses in treated individuals, only a small fraction (<5%) is induced to produce replication-competent viruses *in vitro* under routine VOA laboratory settings [32].

Given these technical limitations, recent developments focused on alternative experimental strategies targeted at the combined evaluation of both the phenotype of HIV-infected cells and cellular transcriptome at single cell level, as seen in the PheP-seq assay [33]. Notably, these new advancements provided an unprecedented high-resolution analysis of proviral landscape of individual HIV-infected cells during ART [33]. This analyses revealed the presence of intact viruses within both tissue-resident memory $CD4^+$ T cells - expressing CD127 and $CD69^+$ T cell population and within circulating resting $CD4^+$ T cells (central memory and effector memory T cells) in lymph node tissues of treated individuals [33], suggesting the capacity of these cells to disseminate infection to other lymphoid organs. Of note, these analyses did not provide evidence supporting the presence of a single phenotypic marker capable of effectively distinguishing virally infected cells from uninfected cells. Therefore, future studies probing the efficacy of these approaches to better identify and define the viral reservoir characteristics would be crucial.

HIV DNA has also been readily detected within local macrophages resident in several mucosal tissues of ART treated individuals such as the gastro intestinal tract [34–36], penile urethra [37], testes [38] and vaginal tissues [39,40] or in tissue-resident macrophages such as Kupffer cells in liver [41], alveolar macrophages in lungs [42] and within

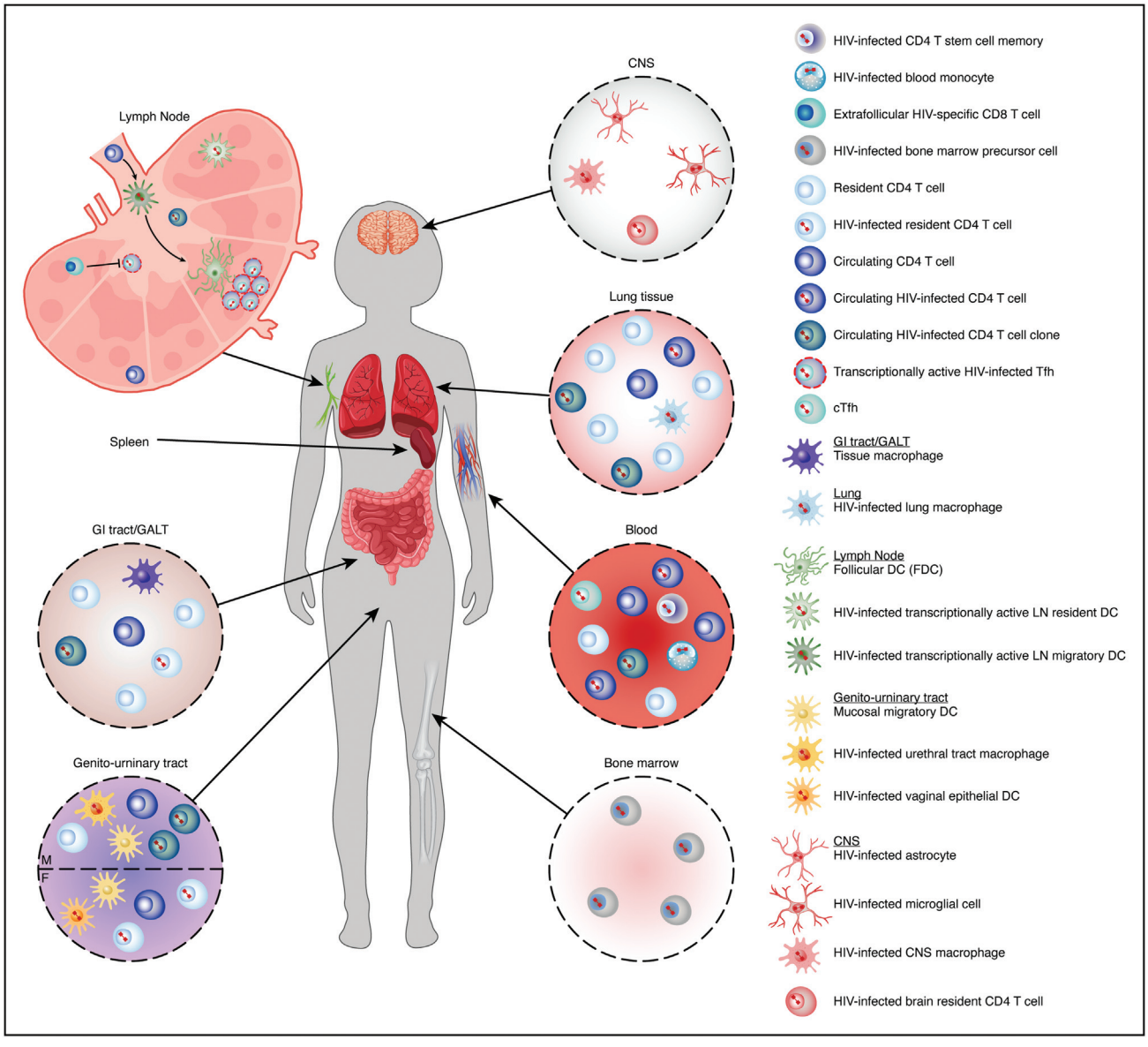

FIGURE 1. Schematic representation of potential HIV tissue reservoirs in the body: The figure represents the complexity of the HIV reservoir in terms of its cellular composition, broad anatomical distribution and transcriptional status during ART. Major HIV reservoirs depicted include: blood, lymphoid tissues, i.e. spleen, lymph nodes, gut-associated lymphoid tissues (GALT), bone marrow, lungs, genitourinary systems and central nervous system (CNS). Proposed cellular reservoirs within blood and tissues are depicted. Cell types are color-coded; HIV-infected cells are depicted harboring integrated HIV DNA; transcriptionally active HIV-infected cells are shown with dashed red lines. 'cTfh' refers to circulating T follicular helper cells; 'GI' refers to gastrointestinal tract. 'M' refers to male genitourinary system and 'F' refers to female genital tract.

microglial cells and perivascular macrophages in the brain [43] by RNA and DNAscope *in situ* approaches. However, viral RNA has been demonstrated predominantly within vaginal [39] and urethral macrophages [44] of ART suppressed individuals. Moreover, cells harboring inducible replication competent HIV has been shown to be specifically enriched within urethral macrophages isolated from ART suppressed individuals undergoing elective gender reassignment surgery [44] as compared to $CD3^+$ penile urethral cells of the same individuals, suggesting the major contribution of these cells to the total reservoir in these tissues (Fig. 1). Interestingly, recent evidences indicated that tissue macrophages may persist long-term through self-renewing capacity [45], were relatively resistant to viral cytopathic effects [46] and to cytotoxic T lymphocytes (CTL) killing [47], and could contain inducible replication competent viruses, highlighting their contribution as relevant tissue reservoirs [37].

The data regarding dendritic cells (DCs) as potential cellular reservoirs are rare, mainly due to the low frequencies and scarce availability of tissues, and therefore the high reliance on experiments performed either on blood or on *in-vitro*-derived cells. Notably, within lymph nodes, two distinct populations of myeloid DCs can be identified on the basis of their original tissue location, that is, 'resident DCs' and 'migratory DCs'. In particular, LN resident DCs differentiate in, and spend their entire lives within LN tissues. On the other hand, migratory DCs can migrate from peripheral tissues (e.g., from the genital mucosa to the inguinal draining LNs) bearing antigens. In this context, a recent study evaluated the presence of HIV-infected LN-derived myeloid DCs during HIV infection and under suppressive ART. The study demonstrated that despite detectable levels of antiviral restriction factors within LN DC subpopulations, a large number of individual proviral DNA sequences isolated from LN DCs were genome intact. Moreover, HIV-infected LN DCs harboring inducible and infectious replication-competent HIV could be detected despite years of suppressive therapy, highlighting their potential role as a yet underestimated cellular source of HIV within LN tissues (Fig. 1) [48▪▪]. Moreover, $CD1a^+$ vaginal epithelial DCs harboring integrated HIV DNA could be detected in female genital tract of virologically suppressed women, suggesting that tissue DCs could also serve as potential cellular reservoirs *in vivo* [49]. However, the potential mechanisms associated with the detection of HIV-infected DCs despite ART remain to be elucidated with possibilities including replenishment through the proliferation of precursor DCs.

HIV infection of hematopoietic precursor or progenitor cells capable of differentiating into distinct cell lineages have also been described [50] (Fig. 1) (discussed in Herd *et al.* [51]). However, the data regarding the potential susceptibility of hematopoietic precursor or progenitor cells isolated directly *ex vivo* from bone marrow tissue to HIV infection has long been debated mainly due to conflicting results obtained in part because of insufficient samples, insufficient cell purities, different experimental approaches, the use of differentiation and growth factors, HIV vectors used, infection strategy, and HIV readouts used in different studies [51]. Notably, a recent study detected identical genome intact sequences in hematopoietic progenitor cells isolated directly *ex vivo* from ART treated individuals using near-full genome proviral sequencing, and suggested their survival to be linked with cellular proliferation [52]. Moreover, clonal proviral sequences obtained from progenitor cells and their daughter cells showed a large homology to virion-sequences obtained from residual plasma viremia, suggesting the contribution of this compartment to residual plasma viremia during ART [52]. Although few, these studies support the possibility that bone marrow compartment may serve as a relevant and a less explored tissue reservoir in ART treated HIV-infected individuals.

ANATOMIC SITES

The majority of studies addressing the HIV reservoir have been performed in peripheral blood $CD4^+$ T cells from treated individuals (Fig. 1) [6,9,22,28, 32,53,54]. Although these studies have yielded key insights into the HIV reservoir, circulating $CD4^+$ T cells represent <2% of total body $CD4^+$ T cells at a given instance [55], with the majority of them located within secondary lymphoid organs. In this context, there is a growing recognition of the significance of tissue microenvironments in studying the viral reservoir and assessing cure interventions. Indeed, HIV-infected cell fate (survival, proliferation, elimination, migration and renewal-capacity) within different tissues might be influenced by multiple parameters which may be specific to tissues and distinct from blood [7,17,19,56,57]. In particular, different tissues may harbor specific spatial organization, cellular and cytokine milieu [58]; variation in viral expression levels [23,48▪▪,59,60]; anatomical compartments harboring specific immune effector mechanisms to clear infected cells expressing viral RNA and/or protein [61–63] and finally differential ART drug penetration [59,64].

The characterization of tissue reservoirs has been performed using multiple approaches including either through virological and/or phenotypic evaluation of HIV-infected cells using flow/mass cytometry [23,24,31,48▪▪,65], imaging platforms [7,60,66] and/or through alternative approaches after direct isolation *ex vivo* under various scenarios, from SIV-infected macaques [60] or HIV-infected individuals on ART [23,24,48▪▪,65], SIV-infected macaques [66] or HIV-infected individuals after analytical treatment interruption [7], and more recently, in autopsy samples from HIV-infected individuals [67▪,68]. Notably, these studies have demonstrated that HIV DNA and/or RNA are detected within multiple tissues, consistent with the infection of multiple tissue-resident and circulating cell types. However, lymphoid tissues (lymph nodes, GALT and spleen) are the predominant sites of HIV infection and persistence of cells with replication competent virus.

Lymph nodes (LNs) represent distinct compartments containing phenotypically and functionally specialized cell subsets as compared to blood. LNs

are dynamic and highly structured tissues, consisting of strategically prepositioned LN resident cells within micro-anatomical niches and recirculating cells. The differential location of LN cell subsets within the micro-anatomical niches is associated with distinct cell phenotypes and molecular and functional signatures [19] and different ART drug penetration [59]. In this context, classical *in situ* hybridization-based approaches in ART SIV NHP models [60], and more recently, the profiling of phenotype of HIV-infected cells and cellular transcriptome at single cell level have indicated that while HIV DNA harboring cells can be detected within both LN extra-follicular and follicular areas [33], transcriptionally active SIV/HIV RNA$^+$ cells are mainly detected within B cell follicles in LNs. Interestingly, SIV/HIV RNA$^+$ was associated with two distinct cell types including [23,69–71]: follicular dendritic cells (FDCs) that are not infected but can bind and retain intact HIV virions within a nondegradative cycling compartment for prolonged periods of time and transmit infectious particles to CD4$^+$T cells [70,71] or HIV/SIV infected T follicular helper cells (Tfh) (Fig. 1). Notably, SIV/HIV infected Tfh cells were enriched in multiple virological setting including in HIV viremic controllers [72], SIV-infected elite controller macaques [69], HIV viremic individuals and ART-treated aviremic HIV-infected individuals [23,31]. The enrichment of HIV-infected Tfh cells potentially involves nonmutually exclusive phenomena that are not yet fully identified, including: heterogeneous ART drug penetration (particularly protease inhibitors) into distinct areas of LN tissues with limited combined exposure to all infected cells, potentially allowing for environments where low-level, intermittent viral replication can occur [59]; likelihood of maintenance of HIV-infected cells containing replication-competent viruses through both long-term survival and clonal expansion [53,73–76], without necessarily inducing viral expression; relatively low accessibility of GCs to HIV/SIV-specific cytotoxic CD8$^+$T cells, thereby allowing viral reservoirs that reside in these microenvironments to escape CTL elimination [73,74,76–78], and enrichment of regulatory T cells and DCs expressing immunomodulatory molecules cytokines such as TGF-β and interleukin (IL)-10 and plasmacytoid DCs secreting type I interferon that contribute to promote viral latency in the LN paracortex and relative exclusion from germinal center areas (Fig. 1) [19].

Additional cell types, such as tissue resident macrophages in other sites (such as microglial cells in brain), astrocytes, hematopoietic progenitor cells, among others, in which HIV DNA or RNA has been detected however inducible replication-competent HIV has only been recovered in limited scenarios and with extreme difficulty (Fig. 1) [52,79,80]. In this context, it is important to note that because of limited access to human tissue, there is sparse sampling of many tissue types (i.e., spleen) that may also harbor CD4$^+$T cells with replication-competent virus [67▪] and addition technical complexities associated with sampling of tissues and the assessment of replication competence by Q-VOAs, have limited the sensitivity of assessments performed to date in various tissues. Therefore, understanding the composition and frequency of the replication-competent reservoirs across different anatomic sites remains a critical issue for future cure-oriented interventional studies.

SPECTRUM OF TRANSCRIPTIONAL ACTIVITY AND REPLICATION COMPETENCE

HIV-infected cells in blood were frequently considered as 'transcriptionally silent', primarily due to the absence of detectable HIV RNA in most instances, as assessed by PCR methodologies. However, recently, breakthrough assays were developed that either allowed the profiling of individual proviral chromosomal integration site and transcriptional activity with increased sensitivity (PRIPseq) [81▪▪], evaluation of transcriptional activity in individuals under temporary ART initiated during primary HIV infection by distinct cell-associated HIV RNA-based measurements [82], or evaluation of transcriptional activity directly *ex vivo* in CD4$^+$T cells from fully suppressed individuals at single-cell level by RNA-flow-FISH [83▪▪]. These studies have independently supported the detection of a broad spectrum of transcriptional activity directly *ex vivo* under these conditions. Furthermore, they have also highlighted the power of assessing quantitative and qualitative nature of transcriptional activity directly *ex vivo* as a tool to predict features of HIV-specific immune responses *in vivo* and viral rebound post-ART cessation [82,83▪▪].

Transcriptional activity within infected CD4$^+$T cells at a specific point is suggested to be associated with several factors including the presence or not of transcriptional blocks arising at various stages during transcription – such as in initiation, elongation and in multiple splicing in resting CD4$^+$T cells, due to both host-related and virus-related factors [84]. Based on these observations, it is conceivable that diverse mechanisms influencing the levels of transcriptional activity may exist within distinct cell types, tissues, and anatomical compartments as compared to blood (Fig. 1).

Multiple mechanisms have been proposed for the persistence of transcriptionally active HIV-infected $CD4^+$ T cells under long-term during ART. Amongst these, proviral integration within nongenic locations has been strongly associated with significantly weaker viral transcriptional activity, likely because of nonpermissive features of the chromatin at or near the chromosomal integration site as opposed to those integrations within chromosomal locations surrounded by activating epigenetic chromatin signals in their immediate chromatin proximity (observed for both intact and defective proviruses) [85]. The authors highlighted an intriguing aspect, emphasizing the probability of detecting an expanded clone that contained both transcriptionally silent and active proviruses [81▪▪] in suppressed individuals. This suggests the possibility that the transcriptionally active cells may survive over-time by outcompeting host-related immune selection forces despite ART or through intrinsic features of survival and reduced susceptibility to host-mediated elimination [81▪▪,85].

Indeed, the transcriptional status of a cell may change over-time, impacting the fate of an HIV-infected cell and therefore the frequency of total cells harboring transcription at a given time. Cellular factors such as activation (spontaneous or antigen-driven) and/or migration through tissues may induce a threshold of transcription adequate for virus-mediated cytolysis or immune recognition, and therefore promote the elimination of infected cells. On the contrary, homeostatic or antigen-driven clonal expansion of cells harboring or not transcriptional activity may in-turn increase the frequency of HIV-infected cells that may or may not at a later stage become transcriptionally active. Taken together, it appears that the dynamic and evolving nature of transcriptionally active proviruses under the influence of host-mediated selection pressure necessitates longitudinal evaluation.

COMPARTMENTALIZATION

The various immune environments, immunological selection forces and ART drug penetration capacities within specific tissues (such as within LNs, GALT, and CNS) may foster viral compartmentalization as well as evolution of viral sequences, allowing for new cell types to be infected. In this regard, to better understand the relationship between circulating proviruses and those present in tissues, studies have focused on the assessment of phylogenetic relationship between individual proviral sequences isolated from the tissues and from blood $CD4^+$ T cells [8,24,65,67▪]. Notably, these studies have indicated the possibility of three states of virus in tissue when compared to the blood: equilibrated (where virus in the blood and tissue are very similar), compartmentalized (where blood and tissue viral populations are distinct, indicating separately evolving populations in these compartments), and clonal amplification (where a single variant is greatly expanded within a compartment). In this context, while proviral compartmentalization has been observed in some studies (liver [86], testes [87], female genital tract [88], central nervous system (CNS) [89]), many others did not observe viral compartmentalization (gut [90], LNs [24,65], CNS [91]). The contrasting results obtained might be explained at least in part by the compartments compared the type of samples compared, that is, cells isolated from ART treated individuals or biopsies [91] and/or the techniques used, that is, bulk versus single genome proviral sequencing of a part of viral genome (HIV *env* [68,92] versus other regions [93,94]) or near full-length sequencing [67▪,91]. Importantly, an elegant study performed an extensive characterization of genetic compartmentalization of proviral sequences present in multiple tissues obtained from autopsies of HIV-infected individuals using near full-length proviral genome sequencing [67▪]. Notably, compartmentalization analyses restricted to distinct proviruses per tissue, revealed no evidence for compartmentalization [67▪]; on the other hand, several analyzed tissue reservoirs (secondary lymphoid organs, lungs, liver and genital tract) harbored large clones (both defective and intact proviruses) that were often shared between distinct tissues (such as lymph nodes, GALT, spleen, liver, lungs; Fig. 1), suggesting a re-circulation of HIV-infected cells between lymphoid and effector tissues even after prolonged ART [67▪].

CONCLUSION

More than 30 years of research demonstrated that the HIV reservoir is heavily complex in nature and involves multiple cell types located in multiple tissues. In addition, the HIV reservoir is probably heavily dynamic in terms of renewal potential, cell migratory potential but also in terms of transcriptional activity. In this context, the recent development of single-cell and single-viral genome approaches, will probably bring valuable insights into tissue reservoirs.

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Conflicts of interest

There are no conflicts of interests.

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- ■ of special interest
- ■■ of outstanding interest

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This study developed a novel multidimensional assay for HIV reservoir cell profiling that simultaneously captures the transcriptional activity, the sequence, and the chromosomal integration site of single HIV proviruses. The study demonstrated that large, transcriptionally active proviral clones persisted during long-term ART through clonal expansion, evading negative host selection forces.

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This study employed a multiplexed single-cell RNAflow-fluorescence *in situ* hybridization (FISH) approach to profile HIV reservoir cells in individuals on suppressive ART. The study revealed the presence of phenotypically diverse HIV-infected cells expressing viral RNA. While predominantly defective, the magnitude of these reservoirs correlated with HIV-specific T cell responses, indicating the potential role of transcriptionally active reservoir cells in sustaining immune responses *in vivo*.

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