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HIV persistence in subsets of CD4+ T cells: 50 shades of reservoirs

Rémi Fromentin[1], **Nicolas Chomont**[1,2,*]

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Montreal, Quebec, Canada

[2]Department of Microbiology, Infectiology and Immunology, Université de Montréal, Montreal, Quebec, Canada

Abstract

Antiretroviral therapy controls HIV replication but does not eliminate the virus from the infected host. The persistence of a small pool of cells harboring integrated and replication-competent HIV genomes impedes viral eradication efforts. The HIV reservoir was originally described as a relatively homogeneous pool of resting memory CD4+ T cells. Over the past 20 years, the identification of multiple cellular subsets of CD4+ T cells endowed with distinct biological properties shed new lights on the heterogeneity of HIV reservoirs. It is now clear that HIV persists in large variety of CD4+ T cells, which contribute to HIV persistence through different mechanisms. In this review, we summarize recent findings indicating that specific biological features of well-characterized subsets of CD4+ T cells individually contribute to the persistence of HIV. These include an increased sensitivity to HIV infection, specific tissue locations, enhanced survival and heightened capacity to proliferate. We also discuss the relative abilities of these cellular reservoirs to contribute to viral rebound upon ART interruption. Together, these findings reveal that the HIV reservoir is not homogeneous and should be viewed as a mosaic of multiple cell types that all contribute to HIV persistence through different mechanisms.

Graphical abstract

*Corresponding author: Centre de Recherche du CHUM, 900 rue St-Denis, Montreal, H2X 0A9, QC, Canada, Phone: +1 514-890-8000 #31266, nicolas.chomont@umontreal.ca.

Author contributions

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Competing interests

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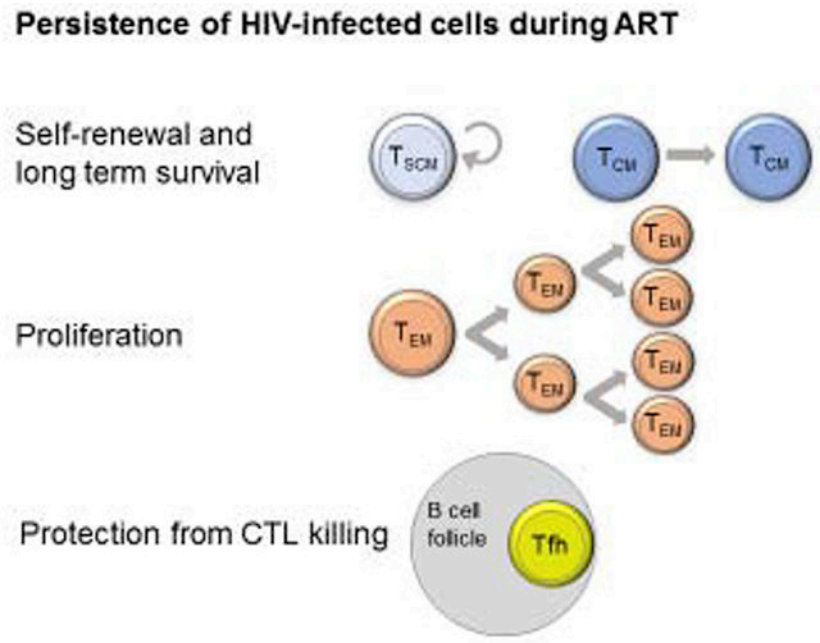

Keywords

HIV reservoir; CD4+ T cells; tissues; Tfh; central memory cells; latency

1. Introduction

More than 20 years ago, the discovery that HIV had the ability to persist in resting CD4+ T cells provided a likely explanation for the inability of ART alone at eradicating the virus [1–3]. It became rapidly clear that a viral reservoir from which HIV replication can reignite when the therapeutic pressure is withdrawn would represent a formidable challenge to HIV eradication efforts. The HIV reservoir was originally described as a small pool of resting CD4+ T cells harboring transcriptionally silent proviruses (“the latent HIV reservoir”) [4–6]. Since then, years of research revealed the complexity of cellular subsets and it is now clear that HIV persists in multiple types of cells that are endowed with distinct biological features and in which proviruses are expressed at different levels [7], further complicating the development of a safe and scalable cure for all people living with HIV (PLWH). Cellular reservoirs are numerous and different in nature. Myeloid reservoirs such as tissue macrophages remain understudied, primarily because they are difficult to access and because obtaining a sufficient number of pure myeloid cells for *in vitro* culture to demonstrate their potential as clinically relevant reservoirs remain technically challenging [8, 9]. Similarly, while circulating CD4+ T cells can be easily isolated from the blood, CD4+ T cells residing in tissues are difficult to study [10–13]. Recently, the use of nonhuman primate models of SIV infection [14–16] and the development of less invasive procedures to collect cells from tissues in PLWH [17] revealed the major contribution of cells residing in various tissues/lymphoid organs to viral persistence. The identification of these new viral reservoirs was largely made possible through the characterization of novel subsets of CD4+ T cells

revealed by the field of human T cell immunology. These discoveries resulted in multiple classifications of CD4+ T cells, which are based on their functions, localization and memory differentiation status.

2. Flavors of CD4+ T cells

2.1 Functions of CD4+ T cells

Upon activation through TCR, naïve CD4+ T cells differentiate into lineage specific T helper (Th) subsets. Each subset produce distinct sets of cytokines that activates downstream signal transducer and activator of transcription (STAT) signaling proteins and dictates lineage commitment by expressing unique master transcription factors [18]. Th cells are typically classified by the cytokines they produce upon TCR engagement and were originally divided into two subsets named Th1 and Th2 [19]. Th1 cells, which are prominent during infections by viruses and intracellular bacteria, produce IFN-γ and IL-2, induce the differentiation and proliferation of cytotoxic T lymphocytes (CTL) and contribute to the activation of macrophages. In contrast, Th2 cells coordinate the immune response to large extracellular pathogens such as parasites and helminths and are characterized by IL-4 production, which contributes to the differentiation of B cells and the development of humoral immune responses. In 2005, Harrington [20] and Park [21] identified a third and distinct subset of Th effector cells named Th17 due to their capacity to produce IL-17. Under physiological conditions, Th17 cells reside mainly in the lamina propria of the small intestine and contribute to the integrity of the mucosal barrier [22]. During infection they are induced at other mucosal sites and contribute to the immune control of a variety of pathogens including Staphylococcus aureus, Citrobacter rodentium, and Salmonella [23], primarily through the recruitment of neutrophils. More recently, Th9 cells have been described a new lineage involved in the development of immune responses to helminthic infections through the production of IL-9 [24]. They also contribute to the development of allergic inflammatory diseases and play a role in anti-tumor immune responses [25]. The most recent members of the effector CD4+ T cells family are Th22 cells, which were first identified in skin tissues of patients with inflammatory skin diseases, in which they produce IL-22 [26]. Th22 cells resemble Th17 cells, but unlike Th17 cells, which produce IL-17 either alone or concomitantly with IL-22, the Th22 subset completely lacks expression of IL-17 [27]. Regulatory T cells (Tregs) represent another subset of CD4+ T cells that are induced during virtually all infections and contribute to tumor progression by suppressing anticancer immunity [28]. They control the magnitude of adaptive immune responses by producing the immunoregulatory cytokines IL-10 and TGF-β and contribute to the maintenance of self-tolerance to prevent auto-immune disease [29]. Finally, T follicular helper cells (Tfh), are located in the B cell follicles of secondary lymphoid organs and contribute to the maturation of B cells through the production of IL-21 and IL-4 [30]. Therefore, they are likely involved in humoral adaptive immune responses in all infectious diseases [31].

2.2. Anatomic locations of CD4+ T cells

The case of Tfh is the best illustration that the classification based on the function of CD4+ T cells somewhat overlaps with another classification that uses their specific anatomical

locations. Although Tfh cells circulating in the periphery can be detected [32], they primarily exert their B cells helper function in the B cell follicles located in lymph nodes, the spleen and Peyer patches. Other examples are given by Th17 cells which primarily reside in the lamina propria of the gut during homeostasis, and Th22 cells which are essentially recruited to the skin. From the recent discovery that a subset of CD4+ T cells have the ability to persist in tissues without recirculating emerged the concept of tissue resident memory T cells (Trm) [33]. Recent studies suggest that a significant fraction of CD4+ Trm cells derive from effector Th17 cells [34], indicating that once again, these classifications may largely overlap.

2.3 Memory status of CD4+ T cells

The classifications described above are mainly based on the functions and locations of CD4+ T cells when they exert their effector functions. After the antigen is cleared, a fraction of these cells persist as memory cells which are maintained for decades in response to homeostatic signals such as IL-7. The memory compartment is heterogeneous, and two main subsets of memory cells named central (T_{CM}) and effector (T_{EM}) memory cells can be distinguished by multiple criteria: (i) the absence (T_{CM}) or presence (T_{EM}) of immediate effector functions [35]; (ii) the expression of the homing receptor CCR7 that allows cells to migrate to secondary lymphoid organs (T_{CM}) *versus* nonlymphoid tissues (T_{EM}) [36]; (iii) the capacity to produce IL-2 (T_{CM}) or IFN-γ (T_{EM}) upon antigen stimulation [37]; (iv) the prevalence of a pro-survival (T_{CM}) or pro-apoptotic program (T_{EM}) [38]. Upon antigenic stimulation, T_{CM} differentiate into T_{EM} cells, whereas T_{EM} cannot revert back to a T_{CM} phenotype [39]. Although the memory subsets are largely defined by their capacity to migrate to secondary lymphoid organs and to have immediate effector functions, it is important to keep in mind that when activated (i.e. upon secondary stimulation), they will exert specific effector functions and could be re-classified as Th1, Th2, Th9, Th17, Th22, Tfh or Treg cells according to the cytokines they produce.

Therefore the definition of CD4+ T cells based on their function, location and memory status are largely overlapping, which complicates the identification of a particular subset as a preferential cellular target or a preferred cellular reservoir for HIV. Theoretically, a cell that could serve as a long-lived viral reservoir should present at least two characteristics: 1) being susceptible to HIV infection up to the integration step and 2) having the ability to persist during ART. Both parameters greatly vary between subsets and CD4+ T cells and should be investigated independently.

3. Susceptibility of CD4+ T cells to HIV infection

Early studies on the susceptibility of different cell subsets to HIV infection revealed that infection of activated CD4+ T cells is much more efficient than resting cells [40–42]. Unlike that of activated CD4+ T cells, the viral genome is not completely reverse transcribed in quiescent cells [43], suggesting that a minimal state of activation is required to establish infection. Such cells with minimal levels of activation are found in tissues even in homeostatic conditions, which is in line with the high susceptibility of CD4+ T cells from the gut to HIV infection [44]. Among those, Th17 cells have been repeatedly shown to be

preferentially infected by HIV [45–47]. Similarly, CD4+ T cells expressing the gut homing marker α4β7 and tissue resident memory CD4+ T cells all show enhanced susceptibility to HIV infection [48]. A high expression level of CCR5 is also a hallmark of enhanced susceptibility to HIV [49], particularly in tissues [50], which is in line with the observation that T_{EM} cells, which express high levels of this HIV coreceptor, are preferentially infected during acute HIV infection [51]. This also partially explains the relative resistance of naïve cells to HIV infection by CCR5-using viral strains [52, 53], since this chemokine receptor is expressed at very low levels on these cells [54]. In addition to the activation status, Th17 lineage and CCR5 expression, the anatomic location of some CD4+ T cell subsets may contribute to their preferential infection. This is best exemplified by the case of Tfh cells, which are known to be major producers of HIV particles during untreated HIV infection [55], possibly because they are relatively protected from HIV-specific CTLs [56].

The antigen specificity of CD4+ T cells also influences their susceptibility to HIV infection. While CMV-specific CD4+ T cells may be relatively protected through the autocrine production of CCR5 ligands [57], CD4+ T cells specific to HIV [58], mycobacterium tuberculosis [59] as well as tetanus toxoid and *Candida albicans* [60] have been shown to be preferentially targeted by HIV. Whether the increased susceptibility of these cells to HIV infection depends on their function or anatomical location remains unclear.

4. Generation of latently infected cells

Unlike productively infected cells which are primarily found during untreated HIV infection, proviruses integrated in persistently infected CD4+ T cells identified in PLWH on ART display low to no transcriptional activity [61]. Whether these cells are derived from previously productively infected cells that reverted back to a resting state or whether they were directly infected as resting cells and immediately established latency is still a matter of debate. These two models of establishment of HIV latency are known as the post- and the pre-activation latency models, respectively.

4.1 Post-activation latency

Post-activation latency is based on the idea that the transition from an activated and productively infected CD4+ T cells to a resting memory state is accompanied by HIV transcriptional silencing. The transition from an activated state to quiescence may offer a narrow window of opportunity that permits HIV silencing and persistence of the infected cells [62]. During the contraction phase of the immune response, when the antigen load decreases and activated cells transition from an effector to a memory phenotype, a rare subset of cells expressing CCR5 are still permissive to HIV infection but also are transcriptionally programmed to become quiescent, a state that is favorable to HIV latency [62]. In addition, the strength of TCR stimulation is key to influence the generation of memory CD4+ T cells [63]. Analogously, intermediate and low TCR signals predispose cells towards latent infections that are refractory to reversal [64].

Post-activation latency is likely to be an active rather than a passive phenomenon: During the resolution of immune responses, several pathways are known to dampen T cell activation and consequently could trigger HIV latency [65, 66]. T cell activation and proliferation can

be modulated by anti-inflammatory cytokines such as TGF-β and IL-10. TGF-β acts on TCR-induced activation [67] but also on the proliferation induced by γ-c cytokines [68]. Although the role these immunomodulatory cytokines may exert on HIV latency has not been formally demonstrated *in vivo*, *in vitro* evidence are emerging: Combination of TGF-β, IL-10 and IL-8 induces T cell quiescence and HIV latency in differentiated effector CD4+ T cells [69], suggesting that HIV latency can be established in Th1, Th2, Th17 and Treg cells post-activation. Additionally, T cell activation can be dampened by the engagement of immune checkpoint molecules such as PD-1, CTLA-4, TIGIT, LAG-3 and TIM-3 [70]. For instance, PD-1 is actively promoting HIV transcriptional silencing in productively infected cells [71, 72]. Consequently, PD-1 expressing memory CD4+ T cells are more likely to become latently infected and persist during ART [72–74].

4.2 Pre-activation latency

An alternative way to generate latently infected cells is to increase susceptibility of resting CD4+ T cells to HIV infection. Resting CD4+ T cells are largely refractory to productive HIV infection due to blocks at the levels of entry, reverse transcription, nuclear import, and viral gene expression [43, 75, 76]. However, CCL19 and CCL20, two chemokines involved in the trafficking of cells to lymph node and the gut-associated lymphoid tissues (GALT) via CCR7 and CCR6 respectively, enhance HIV infection of resting CD4+ T cells by modifying the actin cytoskeleton, thereby increasing nuclear entry and integration of the viral DNA [77]. These findings from an *in vitro* model are in line with the important contribution of CCR7 expressing cells, such as T_{CM} cells, to HIV reservoirs during ART [73]. An *in vitro* model of HIV latency that recapitulates the complex dynamics of the establishment and maintenance of the latent reservoir in different memory T cell subsets was recently developed [78]. Interestingly, the generation of latent cells in this LAtency and Reversion Assay (LARA) does not require polyclonal T cell activation before infection but only exposure of resting CD4+ T cells to TGF-β, IL-7 and conditioned medium containing TGF-β, IL-9 and IL-21 to promote the survival of infected cells in long-term culture. In this model, latently infected cells display various memory status and functions including T_{CM}, T_{EM}, Th1, Th2 and Th17 cells [78]. In addition, IL-7, a cytokine involved in T cell homeostasis, modulates the activity of the restriction factor SAMHD1 and increases the permissiveness of resting CD4+ T cells to HIV infection [79, 80]. It is important to note that CD127, the α chain of the IL-7 receptor, was recently identified as a marker of susceptibility to latent HIV infection of memory CD4+ T cell isolated from tissues [81]. This particular tonsillar memory subset (CD57-CD127+) is endowed with the transcriptional signature of quiescent T cells which prompts infected cells to HIV transcriptional silencing.

Altogether, these studies suggest that within the memory compartment, T_{CM} CD4+ T cells displaying a CCR7+/CD127+/CCR5+ may represent a subset particularly favorable to the establishment of latent infection. Given the plasticity of CD4+ T cells, it is difficult to determine if the cells in which HIV latency is established retain their phenotype after prolonged ART. As discussed below, the number of cell types in which HIV persist may be even larger than the number of subsets in which latency can be efficiently established.

5. Persistence of HIV in CD4+ T cell subsets during ART

5.1 Dynamics of the HIV reservoir during ART

Following ART initiation, only a minute fraction of productively HIV-infected CD4+ T cells survive and are maintained as persistent and long-lived latently infected cells [51, 61]. Whereas some studies suggest that the bulk of the persistent reservoir is established at this time [82], others have reported the presence of archived sequences corresponding to transmitted founders in CD4+ T cells persisting on ART [83]. Therefore, the reservoir is likely made of a mix of cells infected at different times before ART initiation. Whether the reservoir is replenished through de novo infection of CD4+ T cells during ART remains controversial [84–87]. Independently of the possible generation of newly infected cells, several lines of evidence indicate that the reservoir is highly dynamic in virally suppressed individuals [88]. This dynamic is attributed to sustained as well as sequential clonal expansions of infected cells which are attributed to i) proviral integration in genes controlling cell growth [89, 90], ii) homeostatic proliferation [73, 91] and iii) clonal expansions of infected CD4+ T cell clones following antigenic stimulation [92]. Here, we describe these mechanisms contributing to HIV persistence during ART and elaborate on the cell subsets in which they are more likely to occur (Figure 1).

5.2 HIV persistence in memory CD4+ T cells

HIV-infected cells need to survive for long periods of time to persist during ART. In the memory compartment, T_{CM} CD4+ T cells, which are phenotypically defined as CD45RA−/CD27+/CCR7+, show exquisite survival and self-renew abilities [38, 93] and have a long half-life [94, 95]. Accordingly, T_{CM} are a key player in HIV persistence, as they highly contribute to the pool of HIV-infected cells [73, 96]. In addition, T_{CM} cells are the source of the more differentiated T_{EM} cells (CD45RA−/CD27−/CCR7−) which are rapidly generated upon antigen stimulation [97]. Although T_{EM} CD4+ T cells contribute less than T_{CM} cells to the pool of cells harboring HIV DNA [73], they account for the majority of clonal expansions in the reservoir as a result of their elevated proliferative capacity [98, 99]. In addition, T_{EM} cells may play a critical role in viral rebound since they harbor higher frequencies of intact and inducible genomes [78, 98, 100–102] (see section 6). Although memory CD4+ T cell subsets are the main reservoirs for HIV during ART, naïve cells may also contribute to HIV persistence [103, 104]. A limitation to these findings stems from the difficulty in defining the phenotype of truly naïve cells (i.e. non antigen-experienced cells). In the two studies mentioned above, the combination of three cell surface markers CD45RA+, CD27+, CCR7+ does not allow to distinguish stem-cell like memory CD4+ T cells (T_{SCM}), which are known to contribute to HIV persistence [105, 106]. Zerbato et al. isolated rare naïve cells, from which T_{SCM} were excluded by depleting CD95-expressing cells, and from which replication-competent HIV was detected [107]. These studies raise the question of the nature of the mechanisms by which naïve CD4+ T cells, which are resting CD4+ T cells expressing extremely low levels of CCR5, get initially infected. They also emphasize the importance of combining several cell-surface markers to precisely define antigen-naïve cells and of using flow cytometry cell sorting to obtain highly pure cellular populations.

T_{SCM} cells own unique sternness properties which contribute to their ability to serve as a stable reservoir for HIV [108–110]. Since T_{SCM} cells (and to a lower extent, T_{CM} cells) have the ability to self-renew and to generate a progeny of more differentiated cells, they could represent an infinite source of infected cells. Interfering with the Wnt/β-catenin signaling pathways to induce the differentiation of T_{SCM} and T_{CM} cells has recently been proposed as a possible eradication strategy [111].

5.3 HIV persistence in CD4+ T cells expressing immune checkpoint molecules

As mentioned above (section 4.1), immune checkpoint molecules, and particularly PD-1, actively promote HIV latency [71, 72]. These receptors may also favor the persistence of HIV-infected cells over time by preventing reactivation of the latent provirus. Indeed, PD-1, LAG-3, TIGIT and CTLA-4 have been identified as markers enriching in HIV/SIV infected cells during ART, both in peripheral blood and tissues [74, 101, 112]. Of note, PD-1 is also a marker of T-cell activation and infected cells expressing PD-1 cells may also represent a labile pool of activated infected cells, particularly during the first months of ART [113]. After prolonged ART, HIV genomes found in the less differentiated memory subsets (T_{CM} and T_{TM}) expressing PD-1 may have a selective advantage to persist over time compared to cells that do not express this molecule [74]. Importantly, CD4+ T cells co-expressing multiple immune checkpoint molecules (PD-1, LAG-3 and TIGIT) are further enriched for integrated viral genomes, suggesting an enhanced capacity to persist during ART. Since the co-expression of these molecules is a hallmark of profound immune exhaustion, it is possible that infected cells expressing multiple immune checkpoint molecules harbor deeply latent proviruses.

Besides their role in T cell exhaustion, some immune checkpoint molecules are constitutively expressed by subsets of cells in which HIV persists, independently of the functional role played by these receptors. For instance, PD-1 and TIGIT are markers of Tfh cells [32, 114], which are major cellular reservoirs for HIV during ART [115]. In addition to their high susceptibility to HIV infection [55], productively infected Tfh may escape CD8+ T cell killing by being localized in the germinal centers within the lymph node B-cell follicles, from which CTL are largely excluded [56, 116]. Additional factors may contribute to HIV persistence in Tfh cells, since their circulating counterparts (CXCR5+/PD-1+/CXCR3−) are also enriched in HIV [117]. Another example is CTLA-4, which identifies CD4+ T cell with regulatory properties [118, 119]. Using a model of virally suppressed SIV-infected rhesus macaques, McGary et al. recently characterized the contribution of CTLA-4 expressing T cells to viral persistence [112]. CTLA-4+/PD-1− memory CD4+ T cells residing outside of the lymph node follicle were enriched in replication-competent virus. Their ability to support viral persistence was not related to spatial escape from CD8+ T cell killing but more likely to increased potential of survival (high Bcl-2 expression) and homeostatic proliferation (high levels of phosphorylated STAT5). Whether these cells expressing CTLA-4 exert regulatory functions remains to be determined. Indeed, the contribution of Tregs in HIV persistence remains unclear: Initial studies of the latent HIV reservoir were performed using “resting CD4+ T cells” from which CD25+ cells were depleted, which obviously excluded Tregs from these analyses. More recently, several studies highlighted that Treg cells (typically identified as $CD25^{hi}$/CD127lo) are enriched

in HIV DNA and have the ability to produce infectious virus [120–122]. Since Tregs are hyporesponsive to stimulation and relatively resistant to killing, they may represent a particularly challenging reservoir to eliminate [123]

5.4 HIV persistence in functional CD4+ T cell subsets

As discussed above (section 2.1), CD4+ T cells can be defined by their functional properties. Although the spectrum of cytokines they produced remains the gold standard way to characterize these subsets, expression of chemokine receptors are commonly used as surrogate markers to identify functionally polarized CD4+ T cell subsets such as Th1 (CXCR3+/CCR4−/CCR6−), Th2 (CXCR3−/CCR4+/CCR6−), Th17 (CXCR3−/CCR4+/CCR6+) and Th1/Th17 cells (CXCR3+/CCR4−/CCR6+) [124]. Extensive work using CD4+ T cells isolated from the blood of ART-treated PLWH allowed the identification of CCR6 as a marker of HIV susceptibility and persistence during ART [125, 126]. In addition, CCR6+ cells are imprinted with gut homing properties, which is reflected by preferential persistence of HIV in this subset in the gut [11, 47]. Th17, and by extension Th1/Th17, are relatively heterogenous and plastic in their fate. Thus, a fraction of Treg and Th1 cells could be the progeny of subsets of Th17 cells [127, 128]. In addition, Th17 cells are endowed with sternness properties supporting their long-lived capacity [128, 129]. Such properties support the ability of Th17 cells to serve as long-lived viral reservoir for HIV. Of note, CD161, a marker of Th17 and Th17 precursor cells [130], identifies HIV-infected cells which have the ability to persist through proliferation during ART [131]. Remarkably, a recent study characterized HIV persistence in polarized CD4+ T cell subsets defined by their cytokines expression [132]. Th9 cells, specialized in antitumor immune responses [25], were enriched for HIV genomes, but these were mostly defective, while Th1 cells harbored clonally expanded intact HIV genomes. Interestingly, despite their relatively short half-life, Th1 cells may significantly contribute to HIV persistence through antigen-induced proliferation. This is well supported by a recent study in which antigen induced clonal expansion of HIV proviruses was observed in HIV- and CMV-specific CD4+ T cells [92]. A broader assessment of the contribution of different antigens to HIV persistence will be key to the development of targeted HIV cure strategies.

5.5 Additional cellular markers associated with HIV persistence

In addition to the subsets described above and that are usually defined by combinations of cellular markers, individual markers highly expressed at the surface of HIV-infected CD4+ T cells persisting during ART have been identified. Some of these receptors can be targeted by antibodies to specifically deplete the infected cells, which make them particularly attractive for HIV eradication strategies: This is the case of CD20-expressing cells which can be depleted by rituximab [133] and CD30-expressing cells which are targeted by brentuximab vedotin [134]. Interestingly, the latter is a marker of transcriptionally active HIV-infected cells persisting during ART, highlighting the potential and controversial contribution of leaky latency to HIV persistence [135, 136]. Finally, CD32a, also known as Fc gamma receptor IIa (FcγIIa), is expressed on rare CD4+ T cells which are enriched for HIV DNA at a unprecedently reported high level (up to a 1,000-fold enrichment when compared to their negative counterparts) [137]. Although the role of CD32-expressing CD4+ T cell in the persistence of the latent and replication-competent HIV reservoir remains controversial

[138–142], several reports indicate that CD32 may be preferentially expressed by HIV-infected and transcriptionally active CD4+ T cells, particularly in tissues [143, 144].

6. Contributions of CD4+ T cell subsets to HIV rebound

Although persistently infected macrophages [8] and viral particles retained in follicular dendritic cells [145] can contribute to rebound, HIV-infected CD4+ T cells persisting represent a likely source of viral recrudescence upon ART cessation. The identification of the CD4+ T cells subsets from which infection reignites will be key to develop eradication strategies based on the prevention of burst of viral replication. Obviously, only cells harboring intact HIV genomes can contribute to viral rebound. Recent studies indicate that these cells have a shorter half-life than those carrying defective proviruses [146, 147]. Whether this difference is due to intrinsic properties of the cells harboring intact genomes (shorter half-life) or to a greater immune pressure that negatively select for defective viruses over time remains to be determined. In any case, viral rebound may originate both from latently infected cells or from cells harboring transcriptionally active proviruses, as long as they carry intact genomes.

6.1 Transcriptionally active cells as a source of HIV rebound

Transcriptionally active HIV-infected cells persist during ART and may represent the first cells to fuel viral rebound. Indeed, the size of the active reservoir, as measured by cell-associated viral RNA, predicts time to viral rebound [148, 149]. Phylogenetic studies identified these transcriptionally active cells present before analytical treatment interruption as the source of plasma viral rebound [150, 151]. Interestingly these cells tend to harbor clonally expanded proviruses suggesting that proliferating cells are more likely to be the source of rebounding viruses. In addition, a single-genome sequencing approach combined with quantification of cell-associated HIV RNA revealed the transcriptional activity of expanded proviruses [152]. Although, the phenotype of HIV-infected cells was not determined in this study, this active reservoir maybe less stable [153]. T_{EM} cells own these characteristics (active viral transcriptional and proliferation), suggesting their potential prominent role in viral rebound, although this remains to be formally demonstrated (Figure 2). Interestingly, CD32a and CD30 identify actively transcribing cells persisting in blood and tissues during ART [134, 143], but whether the viral genomes persisting in these cells are intact and can produce replication-competent HIV is unknown. Circulating CD4+ T cells expressing CD32a display a T_{EM} phenotype and co-express multiple markers of T cell-activation such as CD69, CD25, HLA-DR, CD38 and Ki67 [143]. Remarkably, the frequency of CD30+ CD4+ T cells increases before viral rebound, suggesting that CD30 may represent a surrogate marker of early replication or transcriptional activity during analytical treatment interruption (ATI) [154]. To characterize the source of viral rebound phenotypically and virologically, an SIV barcoded virus, which allows infection of rhesus macaques with more than a thousand different viral variants, has recently been developed [155]. This novel tool will certainly be used in the near future to molecularly track viral rebound after ART cessation and to characterize the phenotype of the cells responsible for the initial burst of replication A way to assess the potential ability of a viral genome to generate replication-competent HIV particles is to evaluate the intactness of the provirus

using near full length genome sequencing [156]. During the past few years, several groups characterized the phenotype of CD4+ T cell subsets harboring intact proviruses [98, 99, 132]. Collectively, the results from these studies indicate that T_{EM} cells (CD45RA−/CD27−/CCR7−), Th1 cells (IFN-γ+) and activated CD4+ T cells (HLA-DR+) are enriched in intact genomes. Of note, the markers used in these studies are not mutually exclusive and their combination may identify a subset of proliferating cells enriched in intact genomes [157] and from which infection may reignite. The possibility that viral recrudescence may originate from multiple tissues [158] and that recombinant viruses may contribute to viral rebound [159] complicate the efforts to identify the cellular sources of HIV rebound.

6.2 Latently infected cells as a source of HIV rebound

A prerequisite to viral rebound from latently infected cells is an efficient viral reactivation of the latent provirus to generate infectious viral particles. Several studies identified T_{EM} cells as the memory subset harboring the highest frequency of inducible proviruses [78, 100, 101]. These findings have recently been challenged by a study suggesting that all subsets have an equal ability to generate infectious HIV particles upon activation [160]. However, the exclusion of CD69+/CD25+/HLA-DR+ CD4+ T cells, which are enriched in intact genomes [99] and from which latent HIV may preferentially reactivate [102], provides a possible explanation for the discrepancy with the aforementioned studies.

An additional layer of complexity emerged from a recent study that combined integration sites and near full length proviral sequencing. This approach revealed that intact HIV genomes are characterized by a particular integration landscape and are more frequently found in non-genic chromosomal positions, in opposite orientation relative to host genes and distant from accessible chromatin regions [161]. These observations suggest that intact proviruses integrated in more silent regions of the host genome may be selected over time, resulting in a viral reservoir characterized by a deeper degree of viral latency after prolonged ART.

Altogether, these studies suggest that in addition to the intactness of the HIV genomes, their inducibility (i.e their capacity to produce viral particles upon stimulation) should be assessed to better identify potential sources of rebound upon ATI.

7. Perspective: Single cell approaches to study HIV cellular reservoirs

Most studies describing the phenotypic heterogeneity of HIV-infected cells during the course of HIV infection mainly used well-characterized cell subsets to identify distinct cellular reservoirs. This approach may not suffice to grasp the complex heterogeneity of HIV reservoirs during ART. During the past five years, single-cell approaches opened new avenues to analyze the HIV reservoir dynamics with an unprecedented depth.

Single-cell transcriptomic studies identified a new type of cell (CD25+CD298+CD63+BST-2+) highly permissive to HIV infection *in vitro* [162]. This cell subset expressing activation markers is imprinted with a downregulated interferon-mediated response and low expression levels of several known restriction factors. Single-cell RNA sequencing analysis using *in vitro* models of HIV latency highlighted the heterogeneity of

the cell types in which HIV latency is established and from which HIV can be reactivated by latency-reversing agents (LRAs) [163–165]. *Ex vivo* studies identifying HIV-infected cells through the detection of viral proteins or transcripts confirmed the downregulation of cellular antiviral immunity pathways and the presence of pro-survival factors as common features of persistently HIV-infected cells [166, 167]. Of note, a limitation to these studies is the need for a stimulation step to reveal latently infected cells, which likely induces transcriptomic and phenotypic changes.

Single-cell flow cytometry based analysis of the phenotype of HIV-infected cells is currently going through a revolution supported by the usage of multiparametric flow cytometry and mass cytometry (CyTOF) associated with high-dimensional analysis. They confirmed CD127 as a marker of cells permissive to latent HIV infection [81, 168] and TIGIT as a cellular marker of persistently infected cells [169]. The heterogeneity of different cellular reservoirs in response to a variety of LRAs was formally demonstrated by single-cell flow cytometry based studies [170–172]. These studies revealed that CD4+ T cells displaying a T_{EM} phenotype are generally more responsive to current LRAs when compared to T_{CM} cells. Single-cell epigenetic studies using ATAC-seq should help to further understand the molecular mechanisms responsible for these differential responses to LRAs.

In situ hybridization methods to visualize single infected cells have also been developed and present the advantage of visualizing HIV-infected cells in the context of a preserved tissue architecture [14, 173]. Remarkably, the development of new whole body imaging positron emission tomography coupled with magnetic resonance imaging allows the visualization of foci of HIV-infected cells which co-localize with activated T cells in lymph nodes of PLWH [174, 175]. Although this novel technology will require an increased resolution to detect HIV at the single-cell level, it will certainly contribute to a better understanding of the biology of persistent HIV reservoirs in the near future.

More than 20 years of research on HIV reservoirs have revealed the heterogeneity of the cells in which HIV persists during ART. Although these discoveries may be seen as an increased level of complexity and an additional obstacle to the development of a cure for HIV infection, they also reveal common features of HIV-infected cells shared by cells displaying distinct phenotypes. Therefore, rather than adding more cellular reservoirs to the list, it is now time to identify shared cellular markers, metabolic pathways and functions that are hallmarks of persistently infected cells. The development of single cell approaches that can identify reservoir cells with an unprecedented level of specificity and which allow the combination of multiple parameters will certainly help in this endeavor.

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Highlights

- HIV persists in a large variety of CD4+ T cells
- Cellular reservoirs contribute to HIV persistence through different mechanisms
- Tissue locations, survival and capacity to proliferate promote HIV persistence
- Cellular reservoirs differ in their ability to cause viral rebound
- Novel single-cell approaches revolutionized this area of research

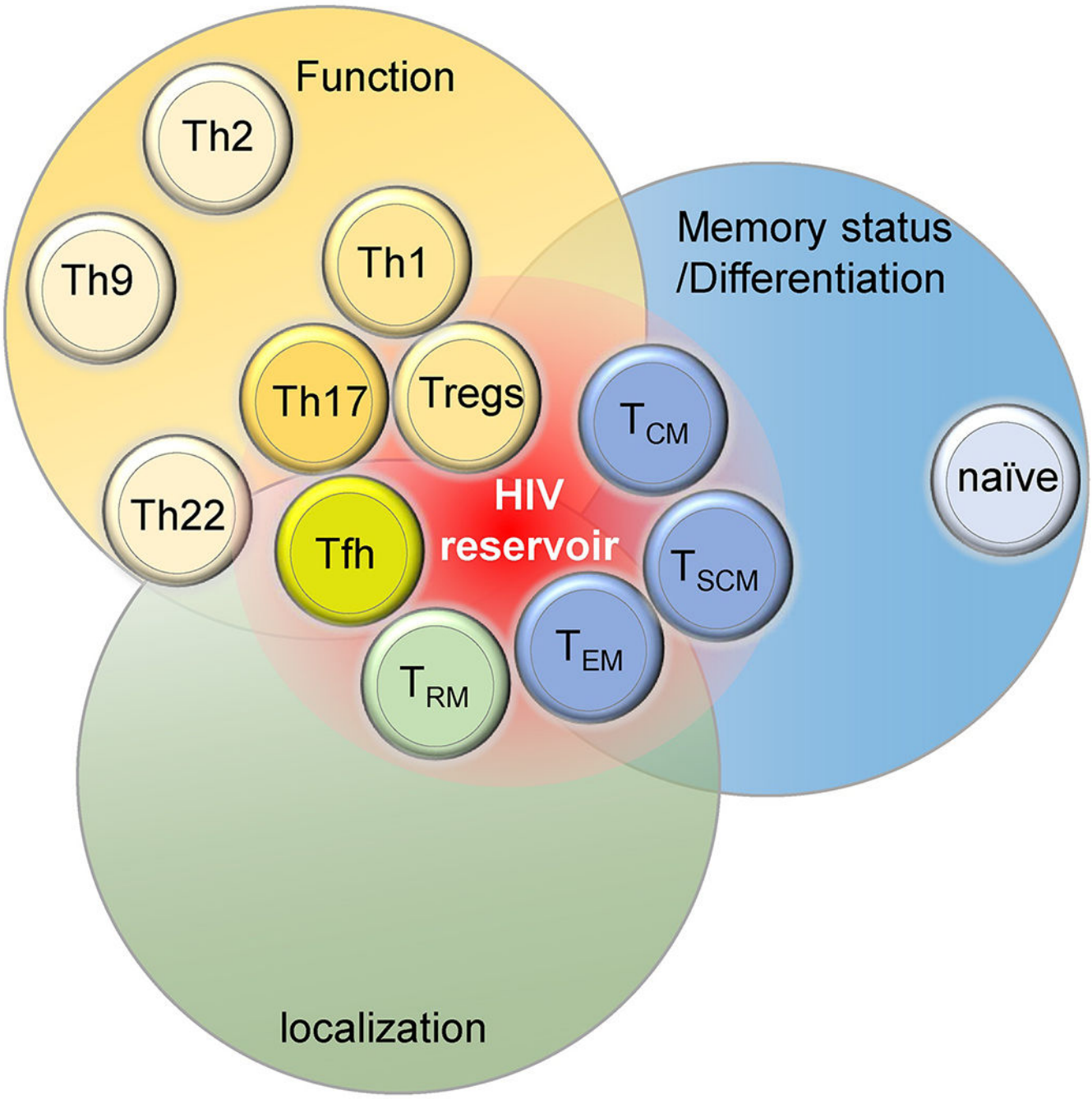

Figure 1. Contributions of CD4+ T cells subsets to HIV persistence.

CD4+ T cell subsets can be classified according to their functions (yellow), memory status (blue) and localization (green). Some of these classifications largely overlap. The relative contributions to viral persistence in depicted by their proximity to the red zone representing the HIV reservoir.

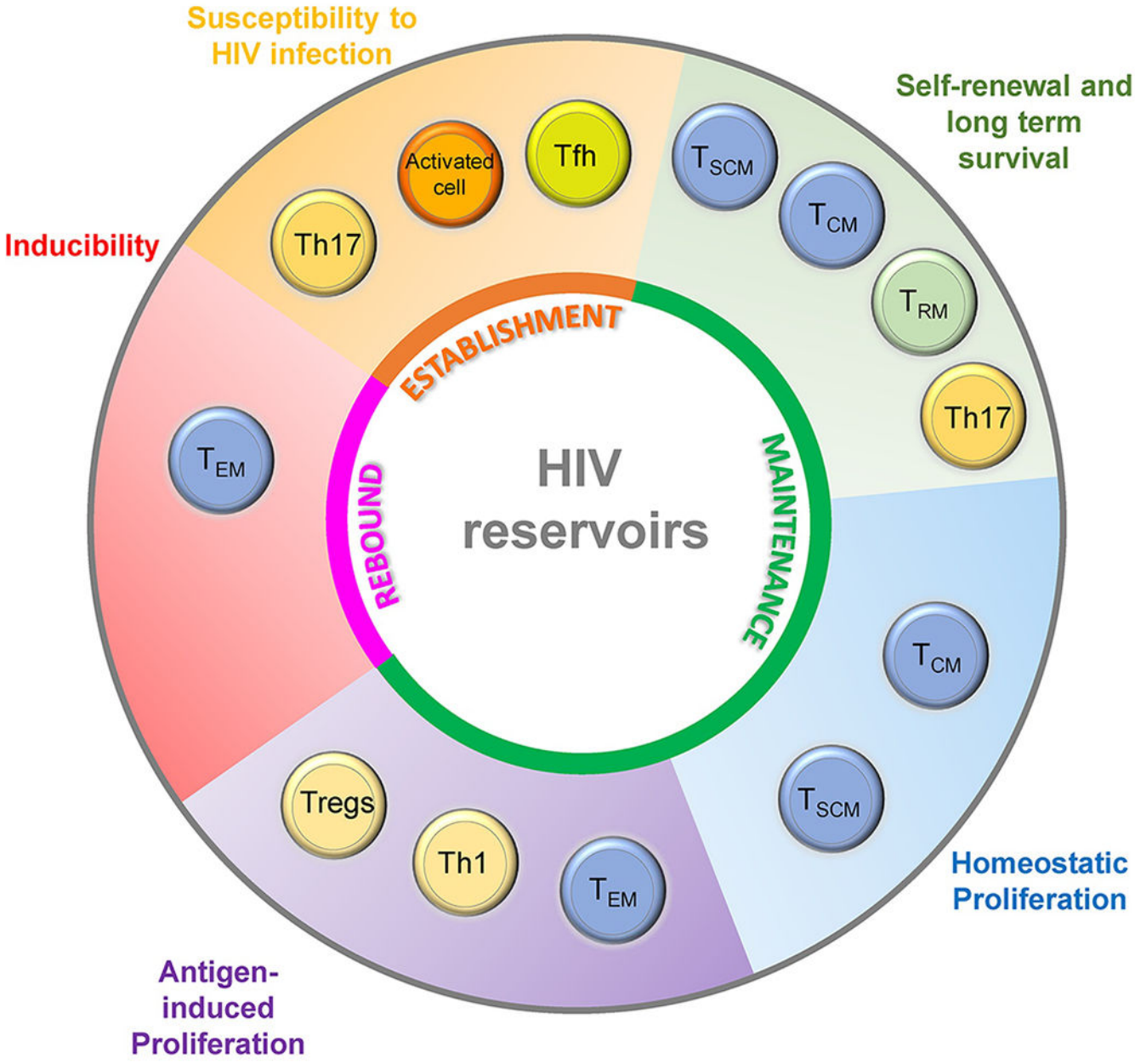

Figure 2. Cellular features involved in the establishment and maintenance of HIV reservoirs and in viral rebound.

Common features of HIV-infected cells (outer cyle) are required for the establishment, maintenance and rebound of the HIV reservoirs (inner cycle). Multiple CD4+ T cell subsets likely contribute to each phenomenon.