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SUPPLEMENT ARTICLE

Measuring the Size of the Latent Human Immunodeficiency Virus Reservoir: The Present and Future of Evaluating Eradication Strategies

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One of the major barriers to the successful design and implementation of human immunodeficiency virus (HIV) curative strategies is the limited ability to sensitively, specifically, and precisely quantify and characterize the whole-body burden of replication-competent HIV in individuals on effective antiretroviral therapy. Here, we review the development and validation of assays that directly and indirectly measure the size and distribution of the reservoir in blood and tissues. We also discuss the role that treatment interruptions will have in validating these assays and ultimately as a "proof of cure."

Keywords. Human Immunodeficiency Virus (HIV); HIV reservoirs; HIV persistence; quantitative assays; antiretroviral treatment interruption.

Despite the overwhelming success of antiretroviral therapy (ART) to routinely achieve complete or near-complete human immunodeficiency virus (HIV) suppression, residual virus that integrates into host cell genomes prior to ART initiation persists indefinitely [1–3]. These infected cells constitute the HIV reservoir and may remain quiescent and evade detection and clearance from host immune mechanisms. Following cessation of ART, plasma viremia rebounds rapidly within several weeks in most individuals [4, 5]. Blood-derived resting CD4$^+$ T cells comprise one of the most characterized reservoirs of latent HIV, and integrated viral DNA can exist at frequencies below one copy per million resting CD4$^+$ T cells [1, 6–8]. However, cellular reservoirs also persist for unknown duration in various tissues, such as lymph nodes, gut-associated lymphoid tissue, spleen, liver and potentially the central nervous system [9–12]. These tissue-based cells constitute a significant portion of the viral reservoir [13–18], but are far less characterized than peripheral CD4$^+$ T cells given the difficulties associated with accessing tissues from a wide-variety of anatomical sites and immunological microenvironments. Myeloid cells including monocyte-derived macrophages may also harbor HIV and contribute to HIV persistence on ART [19]. Current HIV curative strategies are focused on targeting infected cells in these various tissues and aim to enhance immunological recognition and control of quiescent or HIV transcriptionally reactivated cells [20].

One of the major barriers to the successful design and implementation of HIV curative strategies is the limited ability to sensitively, specifically, and precisely quantify and characterize the whole-body burden of residual replication-competent HIV in the setting of ART. In states of very low levels of residual HIV (eg, after stem cell transplantation or early ART initiation), an analytical treatment interruption (ATI) is the only current method to definitively determine if replication-competent HIV persists [21–24]; such interruptions are logistically challenging and pose significant risks to the study participant and his or her sexual partners.

There is an expanding number of interventions that could potentially reduce the reservoir or control HIV postinterruption. Each will need to be well-characterized in the clinic. Progress in developing these interventions will be dramatically accelerated by the development and validation of assays that quantify the size and distribution of the reservoir or that can predict the time to viral recrudescence or viral set points following ART cessation. In this review and summarized in Table 1, we discuss the pros and cons of a growing number of research assays that have been developed to quantify HIV reservoir size.

CURRENT APPROACHES AND LIMITATIONS

Nucleic Acid Measures of HIV

Sensitive and specific quantitative measurements of HIV DNA and RNA from peripheral blood mononuclear cells, tissue, plasma, and other fluids, such as cerebrospinal fluid, are now commonplace, relatively inexpensive to perform on a large

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Table 1. Advantages and Limitations of Strategies to Quantify and Characterize the Human Immunodeficiency Virus Reservoir

Approach	Advantages	Limitations
Total and integrated HIV DNA by quantitative PCR (eg, real-time, digital PCR)	Highly sensitive, able to be performed on tissues and cells; inexpensive; high throughput	The majority of HIV DNA is defective and does not constitute replication-competent virus; variation in measurements across assays and laboratories; PCR inhibitors exist in various biofluids
Cell-associated HIV RNA by quantitative PCR	Highly sensitive, can be used to detect the entire spectrum of HIV transcript species within cells; inexpensive; high throughput	Usually measured from bulk cell extracts; sensitive to time of sampling and time from sampling to processing; variation in measurements across assays and laboratories; PCR inhibitors exist in various biofluids
Ultrasensitive measurement of residual plasma HIV RNA	Highly sensitive, may reflect the "active" HIV reservoir	Remains to be determined if replication-competent virus is exclusively characterized
qVOA	Provides measurement of replication-competent HIV reservoir	Expensive; time consuming; requires large numbers of cells; variation in results across assays and laboratories; primarily used on cells obtained from peripheral blood; challenging to obtain sufficient viable cells from tissues; some genetically intact viruses may not grow in culture
Inducible measures of HIV reactivation (eg, TILDA)	Provides measure of the percentage or number of cells in which HIV reactivates upon maximal stimulation; relatively high throughput; requires fewer cells than traditional qVOA	Does not provide measures of replication competence as RNA can be generated from defective viral genomes; challenges with downstream isolation and characterization of genomic DNA or mRNA from individual cells
Viral protein quantification (eg, HIV p24)	Measures virus with sufficient genetic integrity to drive transcription, translation, and downstream processing	May overestimate viral reservoir size as replication-incompetent viruses may still generate protein
PET-based imaging/nuclear medicine	Has the potential to survey the whole-body HIV reservoir in various tissues and anatomical locations	In development; requires expression of viral protein and may lack sensitivity required to detect latently infected cells or low levels of viral transcriptional and translational activity in antiretroviral-treated individuals; potential for low signal to noise ratios; expensive; involves in vivo radiation exposure
Single-cell HIV reservoir characterization	Potential to lead to a greater understanding of the genomic and transcriptional differences between actively infected, latently infected and uninfected cells	Commercially available platforms are expensive and lack the throughput to characterize millions of cells that may be required given low frequency of latently infected CD4 T cells in individuals on ART; higher throughput assays still in development
Measurement of anti-HIV immune responses (indirect marker)	Titer and avidity of HIV antibodies may represent whole-body, tissue-based HIV persistence and be useful in predicting HIV recrudescence following ATI	Heterogeneous responses; larger longitudinal and cross-sectional studies required to rigorously associate immune responses with reservoir size and HIV rebound dynamics

Abbreviations: ART, antiretroviral therapy; ATI, analytical treatment interruptions; HIV, human immunodeficiency virus; mRNA, messenger RNA; PCR, polymerase chain reaction; PET, positron emission tomography; qVOA, quantitative viral outgrowth assay; TILDA, Tat/Rev-induced limiting dilution assay.

number of samples, and provide an estimate of the burden of HIV (as extensively reviewed in [25]). A simplified diagram of the HIV life cycle that highlights major "points" at which HIV has been measured is illustrated in Figure 1. Viral nucleic acids may be quantified using real-time quantitative polymerase chain reaction (qPCR) methods or by newer digital PCR methods [26–29]. DNA measurements may be performed on total DNA, integrated DNA or episomal DNA (eg, 2-LTR circles) [30]. RNA measurements include those that quantify total RNA, spliced versus unspliced RNA, or the entire spectrum of transcript species.

DNA quantification from cells is highly sensitive but overestimates the size of the replication-competent HIV reservoir, as the majority of integrated DNA in circulating $CD4^+$ T cells is defective [31]. More specifically, HIV DNA may contain various nucleotide insertions (potentially causing frameshifts), deletions, mutations, or APOBEC3-induced hypermutation that prohibit assembly of infectious virions, although defective proviruses are able to undergo various degrees of transcription and translation [32] and may contribute to continued inflammation and pathology during ART [33].

RNA assays provide either measures of HIV transcriptional activity within a cell [34, 35], or the detection of virion-associated RNA or free RNA in plasma using highly sensitive assays that are able to detect <1 HIV RNA copy [36, 37]. HIV produces numerous RNA transcripts, in various unspliced and spliced forms. Spliced RNA encodes the regulatory proteins of HIV required for the assembly of mature virions. Increases in multiply spliced RNA (msRNA), such as a *tat/rev* spliced form, are seen earlier in the HIV life cycle and precede increases in unspliced RNA (usRNA) transcripts, which emerge very late in the life cycle (during the virus budding phase) [35, 38–40]. Interestingly, higher usRNA/msRNA ratios correlate with increased lymphocyte activation and death, decreased $CD4^+$ T-cell immune recovery following ART initiation, and more rapid HIV disease progression [39, 41, 42]. Conversely, usRNA/msRNA ratios are also lower in HIV controllers compared with ART-suppressed patients [43], indicating that usRNA/msRNA ratios may provide useful insights into HIV pathogenesis and persistence.

Beyond characterization of usRNA and msRNA variants, recent efforts have cataloged the HIV transcriptome ex vivo in unprecedented resolution, focusing on a broad spectrum of transcript species including short, aborted transcripts [44]. This highly detailed map of HIV transcriptional patterns in the

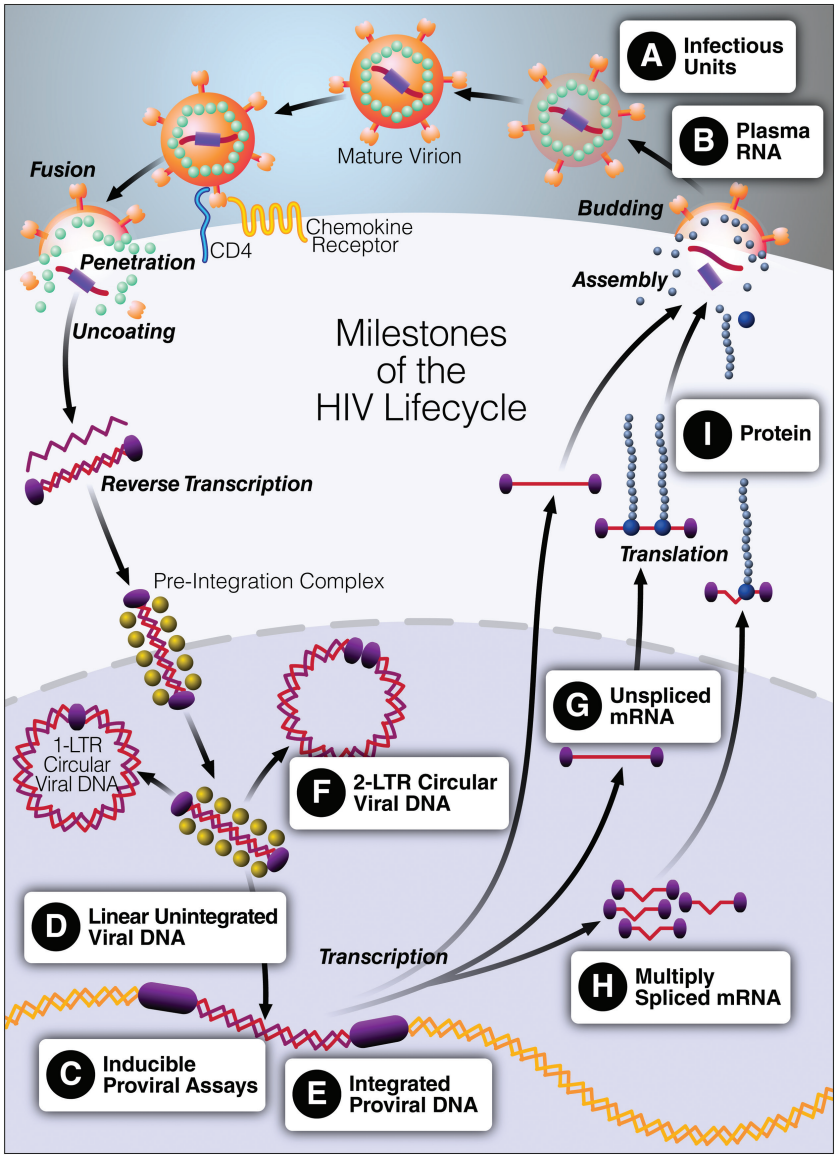

Figure 1. Milestones of the human immunodeficiency virus (HIV) life cycle targeted by HIV reservoir assays. Viral biomarkers of the viral reservoir include infectious units in plasma (A); plasma viral RNA or antigens (B); inducible proviral assays (C); linear unintegrated viral DNA (D); integrated proviral DNA (E); 2-LTR (long terminal repeat) circular viral DNA (F); unspliced viral messenger RNA (mRNA) (G); multiply spliced viral mRNA (H); viral protein (I).

setting of ART and pharmacologically induced viral reactivation may yield critical new insights into the molecular determinants and signatures of HIV latency.

HIV RNA assays have been particularly useful in quantifying changes in viral transcription following latency reactivation strategies applied ex vivo [45–47]. However, caveats emerge with the application of these assays to in vivo clinical studies. For example, in a recent study of disulfiram, an antialcoholism drug experimentally administered to reactivate latent HIV, increases in usRNA were observed following administration of drug to ART-suppressed participants [48]. Interestingly, usRNA from the third of 3 baseline samples taken prior to disulfiram administration was significantly higher than the prior 2 measurements. Although factors such as time of sampling, time from sampling to processing, and clinical stress response markers were not significantly associated with usRNA levels, factors such as these may have contributed to the variation in HIV usRNA observed [48], confounding assessment of a major clinical endpoint.

Further caution should be used when comparing nucleic acid measures of the HIV reservoir using different assays, and even when similar assays are performed in different laboratories on different days using different sets of DNA or RNA standards. Assays using different primer or probe sets, or different sets of standards (known copy numbers of a particular target nucleic acid sequence) that are subject to dilutional error can lead to differences in

quantification readouts. In a systematic comparison study of various PCR-based and other methods to quantify HIV reservoirs, an integrated HIV DNA assay demonstrated overall higher levels of DNA than various measures of total HIV DNA from the same patient samples [30]. As it is impossible to have more integrated than total DNA, this finding suggests that variation in assay measurements may be driven by method-intrinsic variability and bias in addition to biological differences.

Cell-associated RNA or DNA can be normalized between samples using simultaneous quantification of a human housekeeping gene or by enumeration of input cell numbers using a DNA housekeeping gene from genomic DNA simultaneously extracted from the same cell lysate. Each method has its own advantages and disadvantages. For example, there can be variation in transcriptional activity of housekeeping genes that may be influenced by various in vivo or in vitro activation strategies [49–51], whereas extraction efficiencies may differ between DNA and RNA, even when simultaneously extracted. Last, PCR inhibition is likely to be more of a factor in certain anatomical sites and biofluids, further limiting application of PCR-based methods to whole-body reservoir characterization.

Quantitative Viral Outgrowth/Co-culture Assays

As nucleic acid measures of cell-associated HIV overestimate the size of the replication-competent reservoir, the quantitative viral outgrowth assay (qVOA) has been developed to measure the amount of intact replication-competent virus that can be produced by a particular pool of cells. These assays typically involve ex vivo co-culture of resting $CD4^+$ T cells with $CD4^+$ T-cell blasts or other laboratory-derived feeder cells under highly activating conditions [6, 30, 52]. Serial dilutions of input cells allow for quantitative measurements to be calculated using maximum likelihood statistics (typically reported as infectious units per million cells [IUPM]) [53]. These qVOAs have proved to be extremely important in characterizing the nature of HIV persistence in the setting of suppressive ART. However, despite recent advances in streamlining these assays [54], these assays remain time-consuming and expensive and require large numbers of input cells in order to make cross-sample comparisons with adequate statistical power. Given these issues, many studies have been unable to incorporate IUPM as a primary endpoint. Moreover, in contrast to nucleic acid measures, these assays may underestimate the size of the replication-competent reservoir, as genetically intact proviruses (evaluated through full-genome sequencing) may not produce virus in response to stimulation in vitro [55]. In addition, there may be a stochastic component to viral reactivation in vitro; even after maximal in vitro stimulation over a period of weeks, previously nonproductive cells can begin to produce infectious virus after repeated cycles of stimulation/activation [55]. The assays have also been used predominately in cells obtained from peripheral blood, as obtaining sufficient numbers of viable cells from tissue sampling is challenging. This is an important limitation, as it is becoming increasingly evident that the latent HIV reservoir largely resides in lymphoid tissues, rather than peripheral blood.

Inducible Measures of HIV Reactivation and Transcriptional Activity

Techniques to quantify the frequency or percentage of cells that become transcriptionally active following ex vivo stimulation have recently been developed, including the Tat/Rev-induced limiting dilution assay (TILDA) [56]. TILDA provides a measure of the percentage of $CD4^+$ T cells that produce HIV type 1 msRNA after ex vivo stimulation with potent mitogens (phytohaemagglutinin and ionomycin) using serial dilutions of input $CD4^+$ T cells [56]. TILDA does not provide a measure of replication competence, but is high throughput and can be completed on the order of days rather than weeks with fewer input sample cells. Although the assay is able to provide an estimate of the number or frequency of cells that become transcriptionally active through similar dilutional methods incorporated into qVOAs [53], the current strategy does not allow for isolation and downstream characterization of individual infected cells and may overestimate the size of the replication-competent reservoir [32]. In its current form, it is restricted to msRNA, as using primers specific for other usRNA sequences on bulk cell lysates would also amplify HIV DNA.

CHALLENGES AND FUTURE DIRECTIONS

Sensitivity and Detection of Low-Level Reservoirs

One of the limitations of current assays is ultrasensitive detection of HIV-infected cells, given that HIV in the setting of suppressive ART is a very rare target. While PCR-based methods have become quite sensitive, often able to detect <3 copies of a target sequence in a given reaction well, assay linearity and accuracy tend to be reduced at these extremely low levels. As few of the established strategies allow for accurate, reliable, and scalable quantitation of replication-competent HIV within a sample, new molecular methods are currently in development to address this need.

Quantifying Protein Rather Than Nucleic Acid Levels

Given the limitation inherent in measuring nucleic acids, there are increasing efforts aimed at directly quantifying viral proteins such as the p24 viral capsid protein within the infected cell. The logic here is straightforward; in contrast to HIV messenger RNA that can still be readily generated by defective viral genomes, a stable, correctly folded protein is unlikely to be encoded by a heavily mutated, replication-incompetent genome. Exploratory studies demonstrate that ultrasensitive enzyme-linked immunosorbent assay (ELISA) can be used to measure p24 in infected cells, and measurements track with the results of established assays [57]. "Immuno-PCR" and "digital ELISA" approaches also show promise in measuring intracellular p24. Unless the entire viral proteome is characterized, however, this approach will likely overestimate reservoir size to some degree.

Quantifying and Characterizing the Whole-Body Reservoir

Regardless of the ability of any single assay to detect very low levels of HIV nucleic acids, proteins or replication-competent virus, current assays only allow for detection of HIV from the cells directly surveyed. HIV largely resides in organized lymphoid tissues outside of the peripheral circulation, and numerous anatomical compartments that have the capacity to harbor HIV are inaccessible to routine sampling. Other tissue sites and cell types may also contribute to persistence of HIV in the setting of ART, such as the brain, or tissue-resident myeloid cells [12]. Recent studies also suggest that HIV replication or transcriptional activity persist to some degree in lymphoid tissues, which may be due, in part, to particular immune and structural tissue microenvironments. For example, B-cell follicles appear to be an important sanctuary for simian immunodeficiency virus (SIV)–infected $CD4^+$ T lymphocytes. Follicular helper (T_{FH}) cells are particularly enriched in cell-associated HIV DNA and RNA, and support high levels of replication in vitro [18, 58, 59]. The virus that resides in these tissues may not freely circulate, suggesting that blood may ultimately prove to be a poor surrogate for what is happening systemically [60].

For obvious reasons, only a small amount of tissue from a limited number of sites can be realistically obtained from study participants. As a result, there is a very limited understanding of the total-body burden of HIV infection. Alternative approaches to quantifying and characterizing HIV infection and persistence include noninvasive strategies such as positron emission tomography (PET)–based imaging. A recent PET–computed tomography imaging study of SIV envelope gp120 protein expression in infected macaques with varying degrees of immune and viremic control and in the setting of ART provided initial proof-of-concept data that areas of active SIV reservoirs can be visualized [61]. As expected, lymphoid-rich areas were predominate sites of persistent SIV protein expression. It is unknown, however, if detectable signal would be identifiable after longer-term ART in either SIV or HIV infection. As a result, using PET-labeled antibodies specific for HIV proteins expressed on the cell surface has inherent challenges, and techniques to increase sensitivity and decrease signal-to-noise ratios are needed.

The identification of nonviral markers of HIV infection is on the forefront of HIV curative research, and potential markers could be readily co-opted for use in PET-based imaging techniques. Concomitant imaging of nonspecific markers of immune exhaustion or activation that have been associated with HIV burden (eg, PD-1 [59, 62]) may enable more specific identification of areas supporting residual active HIV transcription and translation on ART, or help to define radiographic regions with a high burden of persistent infection. Regardless of the sensitivity limitations of whole-body PET imaging, these methods could be particularly useful when applied to a clinical intervention such as the "shock and kill" approach.

Characterization of the Spatial Distribution of the HIV Reservoir

Although unable to directly measure replication competence, application of in situ hybridization techniques to localize and quantify HIV RNA and DNA in various tissues with single-cell resolution is experiencing a scientific renaissance [63, 64]. These methods involve hybridizing and amplifying chromogenic or fluorescent probes to targeted HIV RNA and DNA sequences to be visualized by microscopy. Amplification of the probe allows for very sensitive detection of single nucleic acid sequences, and the technique has already been applied to characterize SIV-infected cells within the architectural context of organized lymphoid tissues [58]. Increasing throughput, reducing cost, and expanding the capabilities of in situ hybridization techniques is quickly becoming a research priority.

Characterization of Replication-Competent HIV Reservoirs

Unlike nucleic acid measures of HIV, quantitative measures of replication-competent portions of the reservoir (eg, qVOA) have been performed predominately on cells obtained from peripheral blood. Given the importance of tissue-based reservoirs, adaptation of existing assays and development of higher-throughput assays with very high reproducibility for application to tissues is of high importance. Several approaches to improving measures of replication-competent HIV burden are being developed. Some of these approaches aim to increase sensitivity of the traditional qVOAs, such as transplanting human donor cells from HIV-infected individuals into humanized mice to allow for viral expansion and subsequent detection in a more physiologic environment. While early research suggests that these humanized mouse outgrowth models may be more sensitive than in vitro assays [65], they have obvious limitations as to efficiency and cost. Furthermore, given variable engraftment of transplanted cells into humanized mice, quantification of IUPM is challenging. Nonetheless, these models may play an important role in investigating samples lacking detectable HIV by other traditional methods (eg, in the setting of stem cell transplantation or very early ART initiation).

Another approach to improve measures of replication-competent HIV in cells and tissues with higher throughput and reduced input cell requirements is to implement microfluidic chip-based or other in vitro culture conditions that allow for close control of microenvironments in self-contained systems. These microfluidic-based platforms already allow for the growth of human cells, tissues, and complex tissue systems with organ functions (aka "organ on chip" technologies) [66–68], but have yet to be widely adapted for use in HIV quantification and characterization. Overall, on-chip experiments that mimic partial tissue conditions may lead to greater assay sensitivity, require fewer input cells, increase automation, and assay reproducibility.

Other approaches aim to go beyond co-culture/outgrowth assays and directly characterize the integrity of the viral genome.

Sequencing and analysis of the HIV genome may enable differentiation between and specific quantification of replication-competent and incompetent proviruses. Inactivating mutations appear to be nonrandomly distributed across the HIV genome and many defective proviruses share similar genetic deficiencies [32], suggesting that allele-specific qPCR-based approaches may be useful in assessing frequency of intact virus. It may additionally be feasible to measure replication-competent virus by performing high-throughput, single-molecule sequencing of HIV genomes, followed by application of bioinformatic methods to predict replication competence (examining sequences for frameshifts, gross deletions, premature stop codons, and hypermutation). This may be achieved by using microfluidics to efficiently dilute viral genomes or transcripts to the single molecule level, coupled with ultra-deep sequencing to comprehensively characterize viral genetic variation [69]. These modalities may be of significant value to the cure field after necessary optimization and validation.

Single Cell Approaches to Quantifying and Characterizing the HIV Reservoir

The capacity to quantify and characterize viral reservoirs on a single-cell level is of high importance. For example, the popular "shock and kill" approach requires that infected cells that reactivate following latency reversal are targeted and cleared by the immune system or other secondary targeting agent [48, 70–75]. Increases in cell-associated and plasma HIV RNA have been identified following drug administration, but it is unclear if reactivation of HIV reservoirs leads to an increase in the number of transcriptionally active cells, or substantial increase in per-cell transcription in a small number of highly activated cells. Furthermore, the ability to identify and isolate latently or actively infected cells that harbor HIV DNA or RNA has the potential to lead to a greater understanding of the genomic and transcriptional differences between actively infected, latently infected, and uninfected cells. Single-cell approaches may involve a range of techniques such as encapsulating individual cells in isolated microenvironments [76] followed by viral target identification [76], or by flow cytometric analysis and sorting of individual cells identified by intracellular protein production or expression of HIV RNA or DNA by similar hybridization experiments as detailed above. For example, a recent study implemented a flow cytometry–based assay with fluorescent in situ RNA hybridization and detection of HIV protein to demonstrate that various T-cell immune subsets respond differently to latency reversing agents [77]. Fields outside of HIV, such as oncology, have rapidly been integrating single-cell assays.

Analytical Treatment Interruption and Clinical Markers of Treatment Responses

Currently, interrupting ART for a certain duration is the only definitive approach to determining the efficacy of a particular HIV cure strategy. Analytical treatment interruptions may involve restarting ART as soon as HIV has recrudesced (also now referred to as a monitored antiretroviral pause [MAP]) or after rebounded virus has achieved a certain viremic threshold. The cases of the Mississippi child and the "Boston patients" [21, 78], in which virus rebounded months to years after cessation of ART despite lack of detectable HIV reservoirs in blood or tissue, highlight the need for ATIs. As a result, there has been recent interest in identifying markers that predict ATI outcomes and potentially act as surrogate endpoints to clinical trials. This approach may avoid unnecessary harm to study participants while improving feasibility of clinical studies. Traditional nucleic acid and immunological markers have already been correlated with time to HIV rebound following ATI. For example, higher cell-associated usRNA levels correlated with faster time to rebound in one post hoc study of several interruption trials [79], and higher HIV DNA was associated with shorter time to rebound in a cohort of patients who started ART early in infection [80]. Interestingly, pre-ART cell-surface immune checkpoint/exhaustion markers (PD-1, Tim-3, Lag-3) predicted time to viral rebound in the same early-treatment cohort [81].

Studies are now being developed in which well-characterized individuals interrupt ART and are carefully followed until the virus rebounds. Such studies have the potential to allow investigators to discover, characterize, and validate measurements at baseline that predict the time to rebound. If such a biomarker proves to be able to consistently predict the period that a person can safely remain off therapy, and if treatment-mediated changes in this biomarker predict longer periods of aviremia off ART, then the biomarker might be considered a true surrogate marker, thus allowing curative studies to be performed efficiently. From a regulatory perspective, the development of a true surrogate marker for cure research would almost certainly stimulate massive interest in the industrial sector, just as the development of HIV and hepatitis C virus RNA measurements stimulated antiviral drug development.

There are a number of obstacles that might prevent the development of viable biomarkers in a treatment interruption protocol. A large majority of ART-suppressed individuals who have detectable HIV by a traditional assay experience viral rebound within a few weeks after stopping therapy [4, 5], and markers of viral rebound may only predict differences of short intervals of time following a clinical intervention. Each of the current methods to quantify HIV reservoirs characterize different but informative aspects of viral persistence, and may be less useful alone than in the context of other assays. Characterizing combination approaches will be challenging given the number of variables that might be measured and the number of subjects that can be realistically enrolled.

Nonviral Markers of HIV Reservoirs and Future Directions

As the HIV cure field shifts increasingly toward a "functional cure," or longer-term ART-free remission, and durable ART-free virologic

suppression in the absence of complete viral eradication, it is expected that host immune markers will be integrated into patient monitoring following curative interventions. Chosen markers may include host immune determinants that directly suppress viral replication and prevent recrudescence, as well as indirect markers that serve as proxies for effective viral suppression. In regard to the former, it remains to be elucidated which host immune mechanisms underlie durable virologic suppression following cessation of ART. Detailed analyses of clinical cohorts enriched for "posttreatment controllers" including the VISCONTI (Virological and Immunological Studies in CONtrollers after Treatment Interruption) cohort may reveal nonviral biomarkers that enable prediction of viral rebound. Studies of HIV controllers suggest that diverse immunologic mechanisms may contribute to viral suppression in vivo, including $CD8^+$ T-cell responses, neutralizing antibodies, antiviral cytokines, and cell-intrinsic immune factors. It is therefore likely that more than one branch of host immunity will need to be surveyed to accurately determine if extended ART-free remission is likely following an intervention. This is of special relevance to the "shock-and-kill" strategy; most efforts to date have monitored the "shock" component by directly measuring induced viral expression in cells and biofluids. The field will now have to focus intensely on characterizing and quantifying the "kill" side of the equation, especially given accumulating data suggesting that viral reactivation on its own may not result in death of the infected cell. Multiplex droplet digital PCR may enable efficient, simultaneous characterization of both viral and host immune activation in a single reaction following latency reactivating agent administration [82]. The shock and kill approach, along with the diagnostic modalities used to gauge intervention efficacy, will have to shift focus to immunomodulatory effects, clearance of infected cells, and host-mediated suppression of viral replication. The multitude of novel molecular advances, planned curative interventions, ATI protocols, and associated biomarker development efforts have the potential to revolutionize the search for an HIV cure in the near future.

Notes

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