AMERICAN SOCIETY FOR MICROBIOLOGY | Clinical Microbiology Reviews

Virology | Review

Experimental models for HIV latency and molecular tools for reservoir quantification—an update

Divyadarshini Angamuthu,[1] Sandhya Vivekanandan,[1] Luke Elizabeth Hanna[1]

AUTHOR AFFILIATION See affiliation list on p. 14.

SUMMARY 1
INTRODUCTION 2
***IN VITRO* MODELS OF HIV LATENCY** 2
T_{CM} cell model of HIV latency 2
Monocyte-derived macrophage (MDM) model of HIV latency 3
Stable J-mC cell model 4
Jurkat/NL model/WIPE model 5
R2N3-LTR model 6
pMorpheus-V5, quadrapule HIV reporter vector model 6
***IN VIVO* MODELS OF HIV LATENCY** 7
HI.fate. SFFV model- an effective tool to monitor HIV-1 fate *in vitro* and *in vivo* 7
SHIV.C.CH848 model 7
TKO-hu-PBMC mouse model 8
NOVEL METHODS FOR QUANTIFYING HIV LATENT RESERVOIRS 9
Triplex digital PCR 10
Multiplex droplet digital PCR—a combination of two triplex digital PCRs 10
ALU-5′ LTR PCR, a standard curve-free PCR assay 11
Duplex digital PCR assay 11
VIP spot assay 11
Integrated site-specific PCR 12
IMMUNE-BASED THERAPIES TARGETING LATENT HIV RESERVOIRS 12
CONCLUSION 13
AUTHOR AFFILIATION 14
FUNDING 14
AUTHOR CONTRIBUTIONS 14
REFERENCES 14
AUTHOR BIOS 17

SUMMARY A major impediment for HIV cure is the ability of the virus to integrate its genome in the form of replication-competent proviral DNA into the cellular genome of the host and remain transcriptionally silent and hidden from the host's immune defense mechanisms in latent reservoir cells. These latent reservoirs are highly heterogeneous, long-lived cells that are capable of reactivating to restore the viremic stage in virally suppressed individuals upon treatment interruption, thus necessitating life-long antiretroviral treatment. Latency reversal has become one of the most explored therapeutic approaches for eliminating HIV reservoirs and effecting HIV cure. Various aspects governing the establishment, maintenance, and reversal of HIV latency continue to be an enigma and warrant further research. Quantifying the size of the latent reservoir pool is also a challenge as these cells are very few in number and cannot be easily differentiated from uninfected cells. This article provides a comprehensive review of the *in vitro* and *in vivo* models currently available for studying HIV latency as well as the recently developed molecular tools for detection and quantification of latent viral reservoirs.

Editor Jennifer Dien Bard, Children's Hospital Los Angeles, Los Angeles, California, USA

Address correspondence to Luke Elizabeth Hanna, hannatrc@yahoo.com.

Divyadarshini Angamuthu and Luke Elizabeth Hanna contributed equally to this article. Sandhya Vivekanandan contributed to image processing. Author order was determined on the basis of their contribution to this manuscript.

The authors declare no conflict of interest.

See the funding table on p. 14.

Published 15 November 2023

KEYWORDS Human immunodeficiency virus, latency, *in vitro* and *in vivo* models, reservoir quantification

INTRODUCTION

Antiretroviral therapy (ART) in HIV-positive individuals has constrained the replication of HIV and increased the life expectancy of people living with HIV. Regardless of the effect of ART in quashing HIV and its replication, eradication of HIV continues to be a daunting challenge due to the persistence of transcriptionally silent, yet replication-competent, provirus, especially in resting $CD4^+$ memory cells (1). By integrating its genome into the host's cellular DNA and remaining silent within resting cells, the virus remains hidden from the host's immune surveillance mechanisms and inaccessible to conventional ART which chiefly targets the enzymes of HIV. The latently infected cells act as viral sanctuaries and can reconstitute the viraemic stage in ART-suppressed individuals at any given time (2, 3). Latently infected $CD4^+$ cells are long-lived cells with a life span of a few years (4) and can easily access any part of the body through the blood stream and lymphatic system (5).

Owing to the stability of viral reservoirs and their potential to reactivate, HIV-infected individuals need to be on uninterrupted lifelong antiretroviral therapy. Currently, there is no way of differentiating latently infected cells from uninfected cells as there is very minimal viral gene expression and no specific cellular marker that can be used to identify them. The current strategy to eradicate the dormant $CD4^+$ cells is to activate them to produce virus/viral protein and kill them either via viral cytopathic effect or immune-mediated viral clearance. In this context, HIV-1 latent cell models serve as invaluable tools for understanding the mechanisms underlying latency establishment and maintenance, which could unveil useful clues for designing effective strategies to overcome latency. HIV latency poses several daring challenges. The event of proviral integration is a rare phenomenon occurring in one out of a million resting $CD4^+$ cells (3) and is a major hindrance to dissecting the molecular mechanisms governing HIV-1 latency. The first *in vitro* model for studying HIV-1 latency was established using primary $CD4^+$ lymphocytes by Sahu et al. (6, 7). Since then, many *in vitro* HIV-1 primary latency models have been developed and reported.

Several methods have also been developed for the quantification of viral reservoirs, including the Quantitative Viral Outgrowth Assay (QVOA) and Tat/Rev-Induced Limiting Dilution Assay (TILDA). Notwithstanding the method, viral reservoir size estimation remains laborious, and its practical utility is limited to identification of functional replication-competent intact proviruses from the circulating reservoir pool, thus misleading accurate measurements. Quantification of tissue-resident viral reservoirs is another major impediment due to the difficulty in accessing samples. Novel detection tools are still needed for effective detection and quantification of the difficult-to-access reservoir pools in HIV-infected persons.

We provide here an updated comprehensive review on the available cell models for studying HIV-1 latency, the methodologies for establishment of *in vitro* and *in vivo* HIV-1 latency models, reporter vectors and latent cell lines, and the methods for quantification of latent viral reservoirs.

IN VITRO MODELS OF HIV LATENCY

T_{CM} cell model of HIV latency

HIV is known to majorly infect $CD4^+$ T cells, the critical immune cells responsible for eliciting an effective immune response. These cells functionally differentiate into various subsets, some of which can also serve as HIV reservoirs. Despite the fact that HIV establishes latency in various $CD4^+$ T cell subsets, resting memory cells predominate the latent pool. Sarabia et al. (8) developed a primary T_{CM} model of HIV latency using NL4-3, a subtype B/X4 tropic virus (Fig. 1). Subsequently, this primary cell model was extrapolated to include a R5 tropic subtype C virus. In this model, the latently infected cells

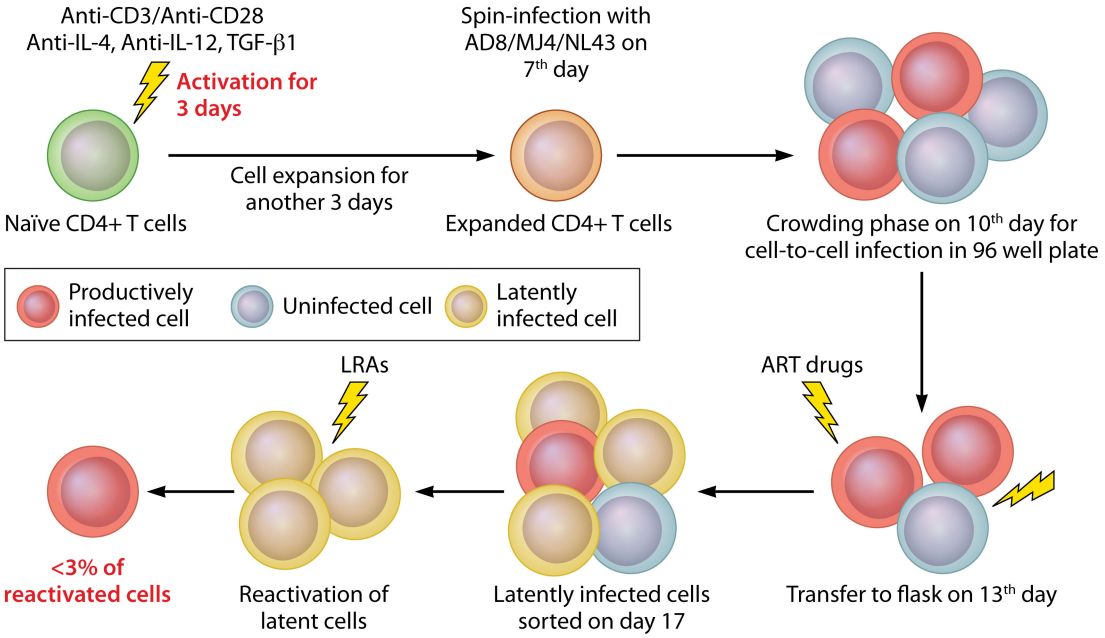

FIG 1 T_{CM} cell model of HIV latency. AD8, an R5 subtype B virus with envelope derived from HIV-1 ADA derivative AD8.1 with NL4-3 backbone; MJ4, an R5 chimeric infectious molecular clone of HIV-1 subtype C from Botswana; NL4-3, a subtype B CXCR4 (X4) —tropic virus; ART, antiretroviral therapy; LRA, latency reversal agents.

were enriched via negative selection by eliminating productively infected cells through magnetic sorting of p24 positive CD4 negative cells from infected cell cultures. Using the R5/non-B subtype virus-based latency model, the investigators demonstrated that percent reactivation was lower for AD8, an R5/subtype B virus, carrying fewer number of total *gag* copies than MJ-4, an R5/subtype C virus as well as NL4-3, an X4/Subtype B Virus. This latency model, however, is heterogeneous as it contains intact as well as defective proviruses, with the least number of intact proviral DNA copies in AD8 followed by MJ4 and NL4-3. Induction of productive infection through IL-2/CD3/CD28 stimulation was found to be very difficult in this latency model, regardless of the viral subtype it contained, and conventional latency reversal agents were found to be ineffective in stimulating the intact provirus. This model, however, serves as a suitable proxy for dissecting the latency-inducing behavior of HIV-1 as the *in vitro* results are consistent with that seen *in vivo*.

Monocyte-derived macrophage (MDM) model of HIV latency

Myeloid cells represent an important pool of viral reservoirs, particularly the tissue macrophages in the brain, lungs, gut, liver, and anogenital tract. Though these cells are said to be analogous to T cells in terms of longevity, their unique ability to resist the cytopathic effect of the virus due to their innate immune function, and ability to survive the cell-mediated response generated by NK cells and cytolytic CD8+T cells, makes them ideal targets for HIV-1 latency (9, 10). However, despite these features, the contribution of tissue macrophages in rebuilding active viraemia remains inadequate, chiefly due to their poor accessibility. Monocyte-derived macrophages (MDM) differ from tissue macrophages in their life span as they are quite short-lived, yet MDM reservoirs have been identified even in individuals on long-term ART, which might possibly have arisen as a result of residual *de novo* infection (11).

Wong et al. (12) developed an *in vitro* model of latently infected MDMs (Fig. 2) by infecting MDMs from healthy volunteers with a GFP-tagged HIV reporter virus derived from NL4-3, with the GFP sequence inserted in the *nef* gene under the control of the HIV-1 LTR promoter to represent an early transcription event, and the *nef* gene

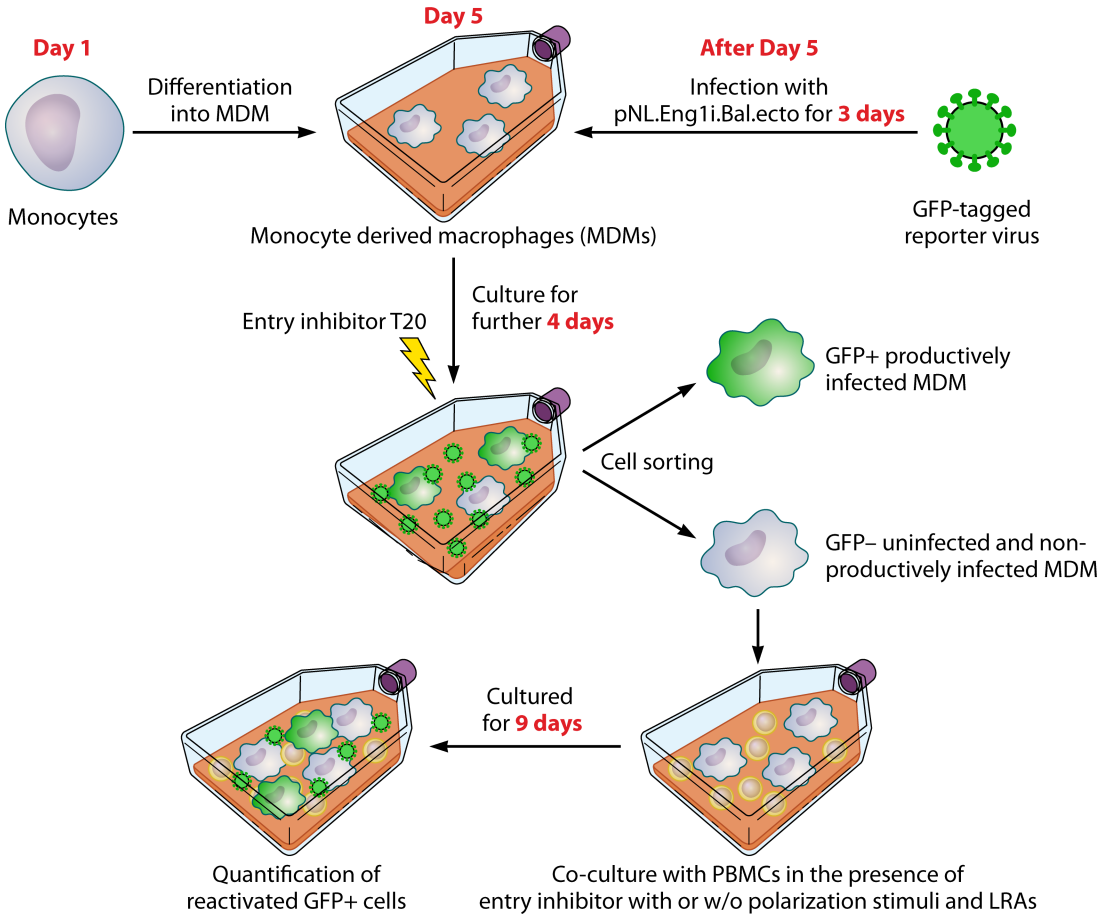

FIG 2 MDM latency model. MDM, monocyte-derived macrophages; pNL.Eng1i.Bal.ecto, a macrophage tropic GFP reporter HIV with NL4-3 backbone and envelope sequence derived from BaL HIV; GFP, green florescent protein.

re-inserted downstream of the IRES promoter to represent *tat*-mediated transcription. Using this *in vitro* latency model, the researchers demonstrated that latency could be achieved in MDMs under *in vitro* conditions, by showing that both HIV LTR DNA and HIV p24 levels were significantly higher in GFP +ve (productively infected) cells than in GFP –ve (latently infected) cells. They further demonstrated spontaneous reactivation of viral transcription from latently infected MDMs, indicating that MDMs harboring the latent provirus can instinctively switch to the productive phase of infection in persons on long-term ART. They further showed that the spontaneously reactivated viruses were replication-competent and capable of infecting T cells. The investigators also demonstrated that the polarization state of the MDMs impacted the level of HIV replication within them. Since MDMs are short-lived cells, considering them as true and stable reservoirs of HIV continues to be a point of contention. However, the authors who developed the MDM latency model declare that the lack of appropriate *in vitro* models to investigate the effect of agents that modulate latency in macrophages, prompted them to develop this model as a surrogate for studying latent HIV-1 infection in primary human macrophages.

Stable J-mC cell model

The identification of green fluorescent proteins (GFP) has allowed its use as a tool for easy identification and localization of various viral proteins within infected cells, thus enabling us to study virus-host cell interactions. The first infectious molecular clone of HIV successfully tagged with GFP was HIV Gag-iGFP, a replication-competent clone derived from pNL4-3, where the GFP sequence was inserted between the matrix (MA)

and capsid (CA) domains of Gag (13). Dual-color fluorescence-tagged HIV reporter virus systems were developed later to help distinguish between latently and productively infected cells. The dual-fluorescent HIV reporter system has two different fluorescent proteins, one under the control of the HIV promoter to detect productively infected cells and the other under the control of an HIV-independent constitutive promoter to identify latently infected cells. Stable latently infected Jurkat cells (J-mC cells) were generated through natural infection of Jurkat cells with the dual reporter virus. The J-mC model is based on the principle that when productively infected (GFP and mCherry double positive) cells revert to a resting/latent state viral gene expression shuts down resulting in lack of GFP expression. Thus, the single positive (mCherry+) cells comprise the population of latently infected cells, while the double positive (GFP+ mCherry+) subset represents productively infected cells. Though a number of dual fluorescent HIV reporter systems are now available (14–17), many of these systems are associated with seeming flaws in terms of efficacy pertaining to their identification, latency establishment, and reactivation potential.

Cai et al. (18) designed a dual fluorescent virus, called DFV-B (18), having GFP and mCherry under the control of two distinctive promoters, namely, the HIV LTR and EF-1α, to aid in the clear distinction between productively (double positive-DP) and latently infected (mCherry positive-mC) cells (Fig. 3). The investigators demonstrated that whereas the mC+/single positive as well as mC+ GFP+/double positive cells had comparable amounts of proviral DNA indicating no difference in virus integration, the single (mC) positive cells did not express viral mRNA or proteins, providing evidence to suggest that this subset of cells constitute the quiescent/latently infected population. The investigators went on to show that DFV-B could induce a latent reservoir state in effector-to-memory transitioning (EMT) $CD4^+$ T cells, though with poor infectivity. Stable J-mC cells are considered to be a suitable model for studying HIV-1 latency-associated mechanisms as it has a number of integration sites (n = 5,154) that are concentrated in the intronic sites, reflecting the heterogeneity *in vivo* (19, 20). The integration sites are frequently found to be enriched in genes involved in cancer-related pathways (21).

Jurkat/NL model/WIPE model

Matsuda et al. (22) also generated a chronic HIV-1 infected T cell line model called the Jurkat/NL model, by infecting Jurkat cells with NL4-3. This model is far more heterogeneous than the other conservative latent cell models as there are 2,933 different integration

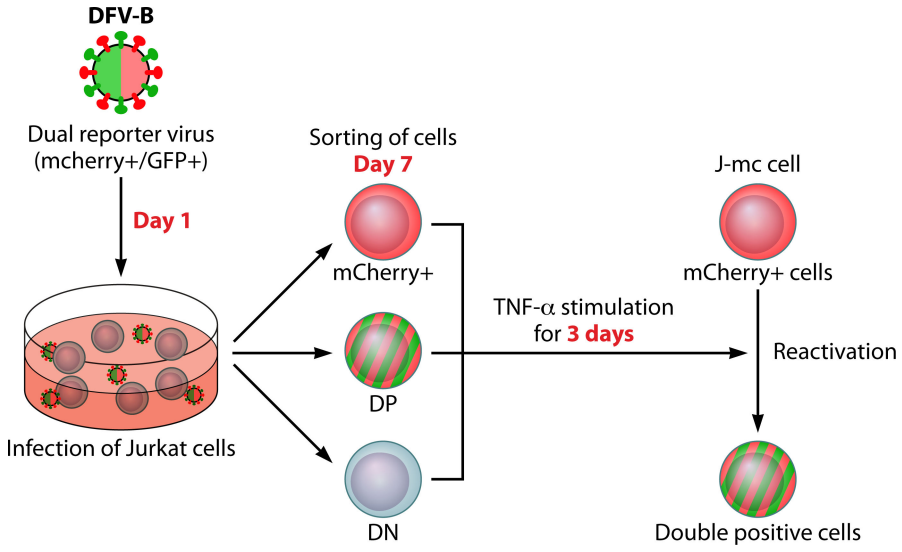

FIG 3 Stable J-mC model of latency. DFV-B, a dual fluorescent reporter virus; DP, double positive; DN, double negative; J-mC, a stable latently infected cells obtained by infection of Jurkat cells with DFV-B; TNF-α, tumor necrosis factor-alpha.

sites (IS) in these cells, as is seen in HIV-1-infected individuals. Interestingly, the IS exhibits common features as observed *in vivo* and lies in the same orientation as the host transcription unit. This long-term latent cell model is called the "widely distributed intact provirus elimination" (WIPE) model and is recommended as a suitable experimental prototype for evaluating the combined effect of antiretroviral drugs and latency reversal agents. Using this model, the investigators demonstrated that the antiretroviral agent EFdA-Islatravir used along with a latency reversal agent, PEP005, was highly effective in reversing the latent phase of infection. Mathematical modeling and experimental data generated with the WIPE assay support the reliability of this *in vitro* model and underline the possibility of HIV latency reversal by the addition of an LRA to anti-retroviral therapy.

R2N3-LTR model

The major governing factor for HIV transcription is the long-terminal repeat (LTR) region that directs viral gene expression. The transcription factor-binding sites (TFBS) within the LTR play an important role in regulating HIV transcription by recruiting transcription activators or repressors, which ultimately decide whether the fate of the infection would be productive or latent. Subtype-specific variations in the configuration of the TFBS and other controlling elements within the viral promoter can also impact viral latency (23–26).

R2N3-LTR is an HIV-1C promoter variant possessing two RBEIII motifs (RBEIII motif duplication) and three NF-κB sites and having the ability to promptly establish latency (27). Ali et al. (28) evaluated the potential of R2N3-LTR as a reporter vector and found that it exhibited less transcriptional noise as compared to the canonical RN3 and R2N4-LTR variants and that the latency establishment potential of R2N3-LTR was superior to that of the other two LTR-variants. It was observed that the R2N3-LTR variant was highly resistant to latency reversal when testing with a cocktail of potent cell activators like TNF-α and PMA, suggesting that multiple molecular mechanisms contribute to the establishment of HIV latency and that a combination of compounds targeting different mechanisms of action may have synergistic effects in activating latent HIV expression. Though Protein Kinase C (PKC) agonists are believed to be highly promising candidates for HIV eradication in view of their potency to reactivate latent HIV, the specific configuration of transcription factor-binding sites (TFBS) in the R2N3-LTR variant, like the presence of an additional copy of RBEIII motif in the absence of co-duplication of NF-kB, possibly contributes to the resistance to reactivation due to the involvement of multiple factors and mechanisms. Besides, different PKC activating compounds have been shown to vary in their potency to reactivate HIV provirus in cell culture models and HIV-infected humanized mice model. Given these features, the R2N3-LTR variant is thought to be an excellent model for studying HIV-1 latency and for identifying highly potent LRAs as it is capable of maintaining stringent latency.

pMorpheus-V5, quadrapule HIV reporter vector model

Kim et al. (29) hypothesized that the existing dual reporter HIV vectors developed for studying HIV latency might suffer from promoter interference as the reporter genes are placed in close proximity to the HIV LTR and LTR-dependent regions making it difficult to distinguish between productive and latent infection. They developed a novel quadrapule HIV reporter vector which they called pMorpheus-V5, to enable easy identification of latently infected cells with minimal promoter interference, by positioning the reporter expression cassette at distal sites. Unlike the previously established dual reporter HIV vectors, the quadrapule reporter HIV vector contains all HIV accessory genes including *nef*, which is the conventional site for tagging fluorescent proteins. pMorpheus-V5 is enabled with four reporters, of which V5-NGFR is LTR-independent and is expressed by the PGK promoter. The V5-NGFR is inserted into the *env,* and its function is compensated by pseudotyping with a different HIV envelope. In addition, it contains two LTR-dependent reporters—HSA and mCherry, inserted upstream of IRES-Nef. HSA and V5-NGFR are membrane-bound fluorescent tags. The V5-NGFR localizes on the cell surface upon

proviral integration enabling easy identification of latently infected cell populations through flow cytometry. This reporter virus will generate productively infected cells expressing all four reporters (V5-NGFR driven by PGK, HSA, and mCherry driven by HIV-1 LTR) and latent cells expressing V5-NGFR alone.

The pMorpheus-V5 vector displays minimal interference between the LTR-dependent and LTR-independent promoters as evidenced by the absence of HSA+mCherry+ double-positive cells. This could possibly be due to the ability of the PGK promoter to master the epigenetic factors involved in LTR silencing. This reporter system allows accurate identification of latently infected and productively infected cells using flow cytometry or cytometry by time of flight analysis (cyTOF) based on the expression of viral reporters and/or viral proteins while levels of viral integration are similar between latently and productively infected cells. The absence of multiply spliced *tat* and *rev* RNA confirm establishment of appropriate latency. The expression of cell activation markers such as CD69/HLA-DR further assures the successful generation of latently infected cells. Using this lentivirus reporter, the investigators went on to demonstrate that latency establishment occurred at a higher frequency in $CD4^+$ T_{EM} cells and T_{REG} cells.

Though the pMorpheus-V5 reporter system is proposed as an effective tool for interrogating HIV-1 latency at the level of single-cell resolution, there are certain limitations that need to be addressed in future. The region deleted from the *env* ORF happens to be a cryptic exon coding for a fusion protein called Tnv of unknown property, and the role of this protein in the absence of PGK-driven nef expression remains to be elucidated since an IRES preceeds *nef*. Besides, results generated from this experiment are based out of two healthy donors and need further confirmation in additional samples.

IN VIVO MODELS OF HIV LATENCY

HI.fate. SFFV model- an effective tool to monitor HIV-1 fate *in vitro* and *in vivo*

Ratnapriya et al. (30) developed an improved reporter vector designated as HI.fate for studying HIV replication and latency *in vitro* and *in vivo*. HI-fate is derived from NL4-3 backbone and contains two different fluorescent tags, E2-crimson and ZS-green, under the control of the HIV-1 LTR and an internal promoter EF1α-HTLV for studying HIV transcription and integration. This reporter is highly genetically stable with reduced loss of internal promoter-mediated transcription but shows considerable variation between diverse latent and productively infected cell types. These variations are thought to be associated with certain exclusive cell environments. Further optimization with stronger internal promoters (PGK and UB) and an alternate viral promoter (SFFV) increased the sensitivity of identifying latent cells with minimal interference from the LTR promoter. The HI.fate. SFFV reporter system is thought to be suitable for performing trancriptomics analysis in productively and latently infected cells. This system can also be used for screening of compounds that can inhibit HIV-replication and for monitoring the fate of HIV-1 infection in humanized BLT mouse model.

SHIV.C.CH848 model

Chimeric Simian/Human Immunodeficiency Virus (SHIV) has been extensively used for studying HIV pathogenesis. SHIV was originally derived from subtype B HIV and includes SHIVsf262P and SHIVAD8. Despite their various applications, they differ from subtype C SHIV in terms of co-receptor usage and have their own limitations like low persistence and immune-associated viral clearance which restricts their applicability in studying viral persistence (31, 32). To overcome these issues, a subtype C-specific CCR5-tropic SHIV (SHIV.C.CH848) was developed in 2008 with a single amino acid substitution at position 375 in a subtype C founder virus Env to increase the susceptibility of macaques to infection with this virus. This clone precisely mimics HIV replication in humans and is, thus, highly useful for HIV research.

Ziani et al. (33) used SHIV.C.CH848 to investigate the immunological and virological events associated with SHIV.C.CH848 infection in rhesus macaques (Fig. 4). They

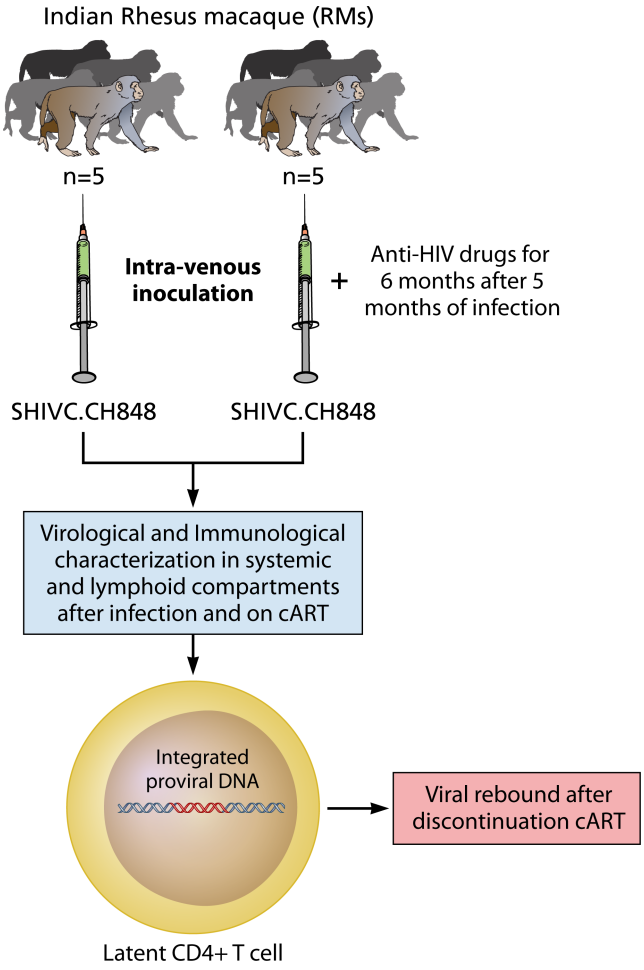

FIG 4 *In vivo* latency model in Rhesus Macaques. SHIVC.CH848, a CCR5 utilizing clade C Simian/Human immunodeficiency virus clone carrying a clade C Env with an amino acid substitution at residue 375 to infect Rhesus Macaque CD4.

observed a rapid increase in plasma viral load and decrease in CD4 count following 14 days of intravenous inoculation with the virus. The viral load dropped below the limit of detection within 2 weeks of initiating antiretroviral therapy, but upon cessation of therapy, the plasma viral load was restored. In addition to recapitulating the key virological and immunological characteristics of infection, viral reservoirs were also established in CD4+ cells, as evidenced by the detection of SHIV RNA and DNA in CD4+ cells in PBMCs and lymph node-derived mononuclear cells. ART for 6 months reduced the levels of unspliced SHIVC RNA in CD4+ cells among PBMCs but not among lymph node-derived mononuclear cells. These results reveal that SHIV.C.CH848 established stable, latent viral reservoirs in CD4+ cells that can rebuild the viraemic condition after discontinuation of ART. Increased levels of autologous neutralizing antibodies were observed against SHIV.C.CH848 in infected animals after 5 months of infection. These observations demonstrate the promising role of SHIV.C.CH848 as a valuable tool for exploring the boundaries of viral sanctuaries and persistence *in vivo*.

TKO-hu-PBMC mouse model

Animal models represent an important tool in medical research for studying *in vivo* pathogenesis and for evaluating the safety and potency of therapeutic drugs and vaccines. The first humanized mouse model was developed in 1988 by McCun (34), and it has since been a recognized animal model for HIV research as it supports HIV

replication and pathogenesis (35–37). Though this model has the fastest engraftment rate with PBMCs, the associated graft-versus-host-disease (GVHD) remains one of the major restriction factors complicating experimental results.

The Triple Knock Out (TKO) mouse developed from the mouse strain B6.129S-Rag2^{tm1Fwa} CD47^{tm1Fpl} Il2rg^{tm1Wjl}/J by Lavender et al. (38) has the ability to overcome GVHD for up to 45 weeks. Holguin et al. (39) established TKO mice for HIV research by transplanting human PBMCs into 4- to 6-week-old TKO mice by intraperitoneal injection (Fig. 5). The TKO-hu-PBMC animal model has several advantages over the NSG (NOD SCID gamma mouse model) as it can easily engraft hu-PBMCs and strongly support replication of CD4+ T cells within 6 weeks of intraperitoneal engraftment, as compared to 8 weeks in the case of NSG mice. In addition, lymphoid tissues obtained from various anatomical sites of the TKO mice were enriched with CD4+ and CD8+ T cells, and there was delayed onset of GVHD as compared to NSG mice. TKO-hu-PBMC rapidly support HIV-1 replication within NSG-hu-PBMC mice, as seen by augmentation of plasma viral load following CD4 depletion irrespective of their route of infection. ART with nucleoside RT inhibitors and integrase inhibitors resulted in a rapid decline of plasma viral load and an associated increase in CD4 cell count. However, rapid viral rebound and CD4 depletion occurred following cessation of therapy.

NOVEL METHODS FOR QUANTIFYING HIV LATENT RESERVOIRS

HIV research focusing on latency reversal demands the availability of robust reservoir quantification methods to elucidate the potency of LRAs. Clinically, the quantification of latent reservoirs helps determine the long-term effect of ART and support treatment interruption and cessation trials. Older methods of latent reservoir estimation relied on quantification of HIV DNA. However, these techniques are not very successful in estimating the size of latent reservoirs as they tend to overestimate the latent population. One of the major reasons for this is the production of a large number of defective viral genomes that are unable to replicate due to the activity of HIV's error-prone reverse transcriptase (40). Further, it may be assumed that not all integrated proviral genomes without mutations would necessarily rebound to the productive phase of infection. The occurrence of tissue-resident anatomical latent reservoirs and their poor accessibility also contributes to miscalculation of the size of the viral reservoir. All of these stratified challenges in estimating the actual size of the latent reservoir obscure the suitability of the earlier developed molecular techniques and necessitate the development of highly reliable robust methods for reservoir estimation in HIV-positive persons on ART.

The Quantitative Viral Outgrowth Assay (QVOA) quantifies viral reservoirs based on cells that are reactivated upon stimulation. Ever since its introduction, the QVOA has

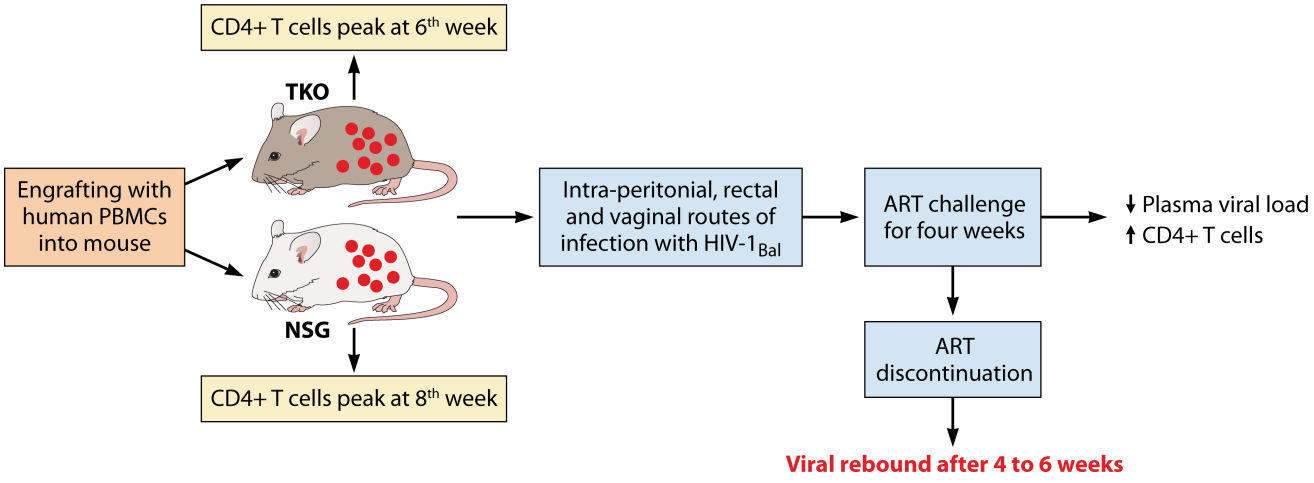

FIG 5 *In vivo* latency model. TKO, triple knockout mice, mice resistant to graft-versus-host disease (GVHD) due to the knockout of Rag1, IL2rg, and CD47; NSG, NOD, SCID gamma mice, immunodeficient laboratory mice; HIV-1_{Bal}, Human immunodeficiency virus type 1 Bal strain; ART, antiretroviral therapy.

remained the method of choice for reservoir quantification due to its high specificity in identifying cells carrying inducible proviral DNA. Regardless of this, QVOA still has limited applications in conventional clinical practice due to the need for an ample amount of sample, highly intensified labor and cost, and contradictory results obtained with HIV DNA PCR (41). This assay as well as its modified version, Q2VOA (42), cannot easily distinguish between intact and defective viral genomes as in the case of the former molecular methods; however, the advantage and distinctiveness of Q2VOA lies in its ability to retrieve the sequence information along with the size of the reservoir. This reservoir estimation method assesses the number of cells that are reactivated upon stimulation (43, 44).

The limitations of QVOA drove the emergence of the Intact Proviral DNA Assay (IPDA) in the year 2019. This is a novel digital droplet PCR-based assay that targets the conserved unique regions of the packaging signal (Ψ) that lies in proximity to the 5′ end and the Rev Responsive Element (RRE) that lies in the *env* gene, allowing to a certain extent the distinction between intact inducible proviral HIV genomes and integrated defective ones (45). Though IPDA is reported to have 97% specificity and requires lesser sample volume, resources, and time than QVOA, polymorphisms due to the extensive genetic diversity of HIV within the same host can lead to substantially higher rates of assay failure resulting in erroneous interpretation of results (46). IPDA can also exaggerate the actual size of the latent population since the probes can bind to both intact and defective provirus as it does not involve any sequence verification technique (47).

Quadruplex PCR is another molecular technique that employs a combination of quantitative PCR and next-generation sequencing, with four different qPCR probes covering the packaging signal (PS), group-specific antigen (*gag*), polymerase (*pol*), and envelope (*env*) genes of HIV to detect proviral genomes. This technique has the additional advantage of sequence verification to distinguish between intact and defective viral genomes based on full-length genome sequencing. This method is also suitable for analyzing the dynamics of latent reservoirs over time in HIV patients on ART (48). Though Q4PCR is highly specific in detecting intact proviruses, this assay suffers from low throughput due to the limitations of NGS. Comparison of Q4PCR and IPDA showed a fair correlation between the methods.

Triplex digital PCR

Van Snippenberg et al. (49) developed a triplex digital PCR for reservoir estimation by merging IPDA with Q4PCR on a three-color digital PCR system. The HIV-1 triplex assay was designed by incorporating primers and probes targeting *gag* and/or *pol* regions along with IPDA probes targeting the *PSI* and *env* regions with a slight modification of omitting the probe for hypermutating APOBEC3G in the e*nv* region. It was found that most of the sequences identified by the triplex assay were intact with very minimal variation between the *gag* and *pol* triplex assay though the triplex *pol* assay had a slightly more robust performance than the triplex *gag* assay in terms of precision and repeatability. The results obtained with the triplex *pol* assay were found to commensurate with that of IPDA. Studies have shown that taking care of DNA shearing during quantification using the IPDA/triplex digital PCR assay increases the scalability of HIV DNA estimation in reservoir cells. Validation of the triplex assay in patient samples detected HIV DNA in all the samples. No dropout was observed in the triplex *gag* or *pol* assays, as compared to the *env* and *PSI* assays. The latter scenario underlines the possible presence of corresponding deletions/mutations in the samples that were categorized as falsely negative. Hence, it would be advantageous to include an additional assay, like for instance, the IPDA or any other molecular technique, along with the triplet digital PCR assay while estimating the size of the reservoir cell population in HIV patients on ART.

Multiplex droplet digital PCR—a combination of two triplex digital PCRs

Levy et al. (50) described a multiplex droplet digital PCR consisting of two triplex digital PCRs that together can detect five targets across the HIV-1 genome in reservoir cells.

This technique employs primers and probes targeting the HIV-1 *env* in each of the triplex PCR assays along with the 3′ end of *pol* and *tat* in assay 1, and the long-terminal repeat (LTR)/*gag* and 5′ end of *pol* in assay 2. These two triplex droplet digital PCR assays use the same dye for detecting two of the three targets in each assay but at varying concentrations, enabling specific target separation and quantification of targets even if they are in random combinations. This method has a high sensitivity of detecting even scanty proviral DNA copy numbers (≤10 per reaction) and can, therefore, be an appropriate method for detecting proviral DNA in those on ART where the copy number is below the limit of detection. Thus, the enormous volume of sample required in the previously discussed methods is not required here. The high degree of specificity and sensitivity of the assay ensures the reliability of reservoir quantification. Furthermore, the method employs selective DNA digestion resulting in minimal DNA shearing leading to precise quantification of intact proviral DNA. The reliability of the assay was demonstrated by comparing the replication-competent reservoirs quantified by multiplex droplet digital PCR with the results obtained from QVOA.

ALU-5′ LTR PCR, a standard curve-free PCR assay

The binding efficiency of the primers and probes to the target region, successful amplification, and appropriate usage of the PCR reagents is ensured by means of a standard curve during quantification in a PCR reaction. Malatinkova et al. (51) suggested a few refinements to increase the sensitivity of reservoir pool quantification directly from PBMCs, by executing a standard curve-free PCR called *Alu*-5′ LTR PCR. The *Alu*-5′ LTR PCR is a modified version of the previously described standard curve-free, *Alu-gag* PCR assay that relies on amplifying the junction between the human *Alu* sequence and HIV-1 genome at the integration site. The sensitivity of the *Alu-gag* PCR was further improved by replacing the *gag* primer with an already validated primer from other reported studies that could recognize a 647-bp region in close proximity to the 5′ LTR end and the splice 1 donor site in the HIV-1 genome. This approach enhanced the sensitivity of the assay by two- to fivefold with an LOD of 0.25 proviruses in 10,000 genomes, which is much higher than the previously reported *Alu-gag* PCR assay, further minimizing sample requirement. Since this method can be applied directly on PBMCs without the need for a CD4 purification step and can perform quantification based on Poisson principle, there is no need to include standards in each and every run. Though the results of this assay were validated using integrated standards from different J-Lat clones, the results obtained with this technique were not compared with the QVOAs which remains as one of the limitations of the method.

Duplex digital PCR assay

Chung et al. (52) published a duplex droplet digital PCR method for the sensitive detection of total HIV-1 DNA and integrated proviral DNA from the brain tissues of HIV-1-infected individuals. They developed this assay by combining ddPCR with nested PCR using *Alu* and HIV-1 *gag* primers which increased the sensitivity of detection to a very low copy number in the brain tissue of ART-suppressed individuals (~4 copies) and HIV-1-infected individuals with encephalitis despite the plasma viral load being below the limit of detection. The assay was able to quantify HIV-1 DNA in brain tissues where qPCR failed to detect the virus.

VIP spot assay

Puertas et al. (53) developed and validated another novel and sensitive assay called the viral protein spot (VIP-SPOT) assay for measuring the size of the functional reservoir at the single cell level. This assay is analogous to the classic ELIspot assay and uses a combination of anti-CD3 and anti-CD28 antibodies for activating the cells, and anti-p24 antibodies for capturing the cells producing HIV-1 p24 antigen. This concept of using specific monoclonal antibodies increased the sensitivity of identifying reactivated

cells carrying replication-competent provirus and precluded the requirement of pooled human serum from HIV-1-infected individuals with an absolute serological pattern for immunoblotting as observed in the previously reported assays (54, 55). The VIP-SPOT assay requires a very low volume of sample, and hence, the assay can be scaled up to analyze tissue resident reservoirs.

Although defective proviruses persisting in ART-suppressed individuals can express viral proteins and be responsible for triggering an immune response in the absence of active viral replication (56), the results of the VIP-SPOT assay correlated with that of IPDA implying that cells producing HIV-1 p24 antigen after activation carry an intact provirus. This assay can be used to screen the activity of LRAs with different kinetics of response and can also quantify the frequency of T cells producing HIV-1 antigen upon stimulation. However, further phenotypic analysis cannot be attempted on reactivated cells, which is one of the major limitations of this assay.

Integrated site-specific PCR

Brandt et al. (57) developed the integrated site-specific PCR (IS-qPCR) assay that can measure the frequency of individual clones in genomic DNA extracted from PBMC or CD4 cells of ART-suppressed individuals. This assay was designed using primers and probes targeting the NT5C3 gene in ACH2 cells and is highly sensitive and accurate in quantifying the number of proviral copies integrated in ACH2 cells, which is equivalent to the number of input cells. This assay is free of off-target amplification and is able to detect integrated proviruses in HIV-positive donors. The clone frequencies obtained with IS-qPCR when used along with deep integration site sequencing can serve as a complementary tool for characterizing the HIV-1 reservoir. Thus, the assay can be used to differentiate between clones carrying defective and intact proviruses in T cells and in tissues. The assay can also furnish details on the dynamics of replication-competent clones in infected cells and their response to therapeutic interventions. However, the assay can analyze only a few proviruses at a given time point provided the integration site is known and validated in patient-derived proviruses, which is the major limitation of this method. However, the technique can be performed with low input of samples and does not exhibit much difference in recognizing integration sites in PBMC and CD4 cells, which highlights the suitability of the assay in several areas of HIV research.

IMMUNE-BASED THERAPIES TARGETING LATENT HIV RESERVOIRS

Immune-based therapies appear to be the most effective means of eliminating HIV from the body. Broadly neutralizing antibodies (bNAbs) targeting the HIV envelope are being explored as promising prophylactic as well as therapeutic tools against HIV infection. Several bNAbs are currently being tested in clinical trials either alone or in combination with cART. Studies are also underway to investigate the effect of bNAbs in clearing the latent cellular reservoirs.

Sneller et al. (58) investigated the combinatorial effect of two bNAbs, 3BNC117 and 10-1074, in delaying plasma viraemia in HIV patients. Passive administration of the bNAbs in eight infusions over a period of 6 months helped achieve virological suppression for upto 43 weeks (58). The potential therapeutic effect of these two bNAbs in delaying the viral rebound was found to be successful when there were no antibody-resistant viruses at baseline, and ART is initiated soon after the last infusion of bNAbs.

Romidepsin, a latency reversal agent used in combination with 3BNC117, did not significantly reduce the size of the HIV reservoir. This treatment strategy also did not enhance the CD8 T cell response that plays a vital role in HIV clearance (59). This seems to suggest that bNAbs cannot be a potential surrogate for ART for eradication of HIV (60). Effective killing of latently infected cells through latency reversal requires CD8+ cytolytic lymphocyte-mediated or NK cell-mediated killing. However, latently infected cells can evade antibody-dependent cell cytotoxicity through the expression of the Fcγ receptor, CD32, on its surface that could potentially confer an added advantage for cell survival,

allowing these cells to proliferate in the presence of immune complexes and decrease their potency to bind to HIV-specific antibodies (61).

CD8+ cell-mediated killing of infected cells via the T cell receptor (TCR) involves the recognition of short peptide fragments of HIV proteins presented with MHC class I molecules. HIV infection results in compromised CD8+ T cell function which ART fails to restore completely. This leads to compromised efficacy of LRAs in eliminating viral reservoir cells. Besides, latent reservoirs may have proviruses containing mutations in CTL epitopes that prevent HIV clearance. Newer methods to revert hypofunctionality of cytolytic T lymphocytes using pMHC-single chain dia-body backbone (scDb) have recently been published. Sengupta et al. (62) illustrated that TCR mimicking (TCRm) bispecific antibodies like HA29 and HI12, produced through phage display technique, target HIV-1 peptide MHC complexes (pMHC) and promote targeted cell lysis by linking CTLs following latency reversal. Of these two antibodies, one binds to epitopes in Gag and reverse transcriptase, while the other binds to CD3ε, an essential signal transduction subunit of TCR. The high specificity and high-affinity binding of pMHC can prevent immune escape of HIV and elicit CTL activation at very low antigen density. However, it must be kept in mind that repeated infusion of bispecific antibodies may lead to the development of anti-drug antibodies which can limit their efficacy as seen in cancer immunotherapy clinical trials (62).

In addition to bNAbs and CTLs, anti-HIV polyclonal antibodies that can induce antibody dependent cell cytotoxicity (ADCC) via NK cell-mediated immune clearance have also been proposed as an effective strategy for eliminating latent reservoirs. Recent studies have shown that the HIV Env can activate proviral DNA expression from latent reservoirs and also initiate a CTL response to achieve killing of reactivated reservoirs (63). Suryawanshi et al. (64) reported that anti-HIV-ADCC antibodies and HIV Env work hand-in-hand to reduce latent reservoirs and proposed HIV Env as a promising latency reversal agent (64). Purified IgG from plasma of ADCC responders has been shown to have the ability to lyse-infected ACH2 cells as well as primary CD4 and resting memory cells from HIV-infected individuals that were reactivated by HIV-1 subtype C Env protein, along with a significant increase in the number of activated NK cells. Though the study did not address the neutralization potential of ADCC antibodies, it highlights the dual benefit of ADCC antibodies in neutralizing the virus as well as in immune boostering by inducing humoral and cellular antiviral responses. The investigators hythothesized that stimulation of latently infected cells with HIV-1 subtype C Env for a short period of around 3 h resulted in the presentation of MHC II-bound peptide to HIV-specific CD4 cells by dentritic cells via the TCR. This activation could induce multiple signal transduction pathways by sequestering HIV transcription factors such as NF-kβ and P-TEFB, leading to the reactivation of provirus through an ERK-dependent pathway and ADCC IgG-mediated lysis of reactivated cells. Nevertheless, the suitability of HIV Env protein in latency reversal needs to be evaluated *in vitro* in ART-suppressed individuals, for its potential in latent reservoir eradication.

CONCLUSION

HIV's persistence in reservoir cells in ART-suppressed individuals poses a great challenge to achieving complete cure, as the latent state can switch back to the viraemic condition following interruption of antiretroviral treatment. Latently infected cells cannot be easily distinguished from uninfected cells as the integrated virus remains transcriptionally silent and protected from the host's immune surveillance system. Hence, there is an urgent need to design novel strategies for identifying and targeting the latent viral reservoirs for immune-mediated clearance. This effort will depend on the availability of a robust HIV latency model, as latent cells are very sparse in HIV-infected persons. Several *in vitro* and *in vivo* latency models have been established over the decades to help in the screening of novel latency reversal agents and for the identification of host factors involved in the establishment of HIV latency. Inspite of these efforts, latency reversal has

continued to remain unachievable. Thus, novel research perspectives focusing on HIV latency and its mechanisms are needed to address this research gap.

Quantification of reservoirs is key to assessing the effectiveness of novel therapeutics aimed at latency reversal. Over the last few decades, a number of reservoir quantification methods have been developed and improvised. However, many of these methods still lack the ability to differentiate between defective and intact proviruses, necessitating further improvements in procedure and development of more robust *in vitro* techniques for efficient and accurate estimation of reservoir cells harboring replication-competent intact provirus. In the recent years, a number of newer molecular techniques such as ASAPseq (antigen profiling by sequencing), PRIP-seq (parallel analysis of HIV-1 RNA), and Chip-Seq have been developed for more efficient and precise quantification of reservoir cells harboring replication-competent intact proviruses. ASAPseq leverages a single-cell sequencing assay for Transposase Accessible Chromatin (scATACseq) and surface marker profiling to provide an unbiased, single-cell epigenetic profile and can, therefore, assign cellular identity to cells with and without detectable provirus (65). PRIP-seq employs profiling of transcriptional activity and chromosomal location of individual proviruses to capture transcriptionally active viral reservoirs (66). ChIP-seq (chromatin immunoprecipitation-high-throughput parallel DNA sequencing) is based on the analysis of histone modifications and their effect on latency establishment and maintenance. These newer methodologies can be exploited to obtain valuable insights into the intricate mechanisms of latency establishment, maintenance, and reversal.

AUTHOR AFFILIATION

[1]Department of Virology & Biotechnology, ICMR-National Institute for Research in Tuberculosis, Chennai, Tamil Nadu, India

AUTHOR ORCIDs

Divyadarshini Angamuthu http://orcid.org/0000-0003-2468-5617
Sandhya Vivekanandan http://orcid.org/0000-0002-5854-249X
Luke Elizabeth Hanna http://orcid.org/0000-0001-7338-1631

FUNDING

Funder	Grant(s)	Author(s)
Indian Council of Medical Research (ICMR)		Divyadarshini Angamuthu

AUTHOR CONTRIBUTIONS

Divyadarshini Angamuthu, Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review and editing | Sandhya Vivekanandan, Validation, Visualization, Writing – review and editing | Luke Elizabeth Hanna, Conceptualization, Supervision, Validation, Visualization, Writing – review and editing

REFERENCES

1. Wong JK, Hezareh M, Günthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278:1291–1295. https://doi.org/10.1126/science.278.5341.1291
2. Carroll RG, Folks TM, Japour AJ. Identification of a reservoir for HIV-1 in patients 6.
3. Chun T-W, Stuyver L, Mizell SB, Ehler LA, Mican JAM, Baseler M, Lloyd AL, Nowak MA, Fauci AS. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci U S A 94:13193–13197. https://doi.org/10.1073/pnas.94.24.13193
4. Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, Kovacs C, Gange SJ, Siliciano RF. 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nat Med 9:727–728. https://doi.org/10.1038/nm880
5. Wong JK, Yukl SA. 2016. Tissue reservoirs of HIV. Curr Opin HIV AIDS 11:362–370. https://doi.org/10.1097/COH.0000000000000293
6. Sahu GK, Lee K, Ji J, Braciale V, Baron S, Cloyd MW. 2006. A novel *in vitro* system to generate and study latently HIV-infected long-lived normal CD4+ T-lymphocytes. Virology 355:127–137. https://doi.org/10.1016/j.virol.2006.07.020
7. Van Lint C, Bouchat S, Marcello A. 2013. HIV-1 transcription and latency: an update. Retrovirology 10:67. https://doi.org/10.1186/1742-4690-10-67

8. Sarabia I, Huang S-H, Ward AR, Jones RB, Bosque A. 2021. The intact non-inducible latent HIV-1 reservoir is established in an *in vitro* primary T_{CM} Cell model of latency. J Virol 95:e01297–20. https://doi.org/10.1128/JVI.01297-20
9. Clayton KL, Mylvaganam G, Villasmil-Ocando A, Stuart H, Maus MV, Rashidian M, Ploegh HL, Walker BD. 2021. HIV-infected macrophages resist efficient NK cell-mediated killing while preserving inflammatory cytokine responses. Cell Host & Microbe 29:435–447. https://doi.org/10.1016/j.chom.2021.01.006
10. Clayton KL, Collins DR, Lengieza J, Ghebremichael M, Dotiwala F, Lieberman J, Walker BD. 2018. Resistance of HIV-infected macrophages to CD8+ T lymphocyte–mediated killing drives activation of the immune system. Nat Immunol 19:475–486. https://doi.org/10.1038/s41590-018-0085-3
11. Graziano F, Aimola G, Forlani G, Turrini F, Accolla RS, Vicenzi E, Poli G. 2018. Reversible human immunodeficiency virus type-1 latency in primary human monocyte-derived macrophages induced by sustained M1 polarization. Sci Rep 8:14249. https://doi.org/10.1038/s41598-018-32451-w
12. Wong ME, Johnson CJ, Hearps AC, Jaworowski A. 2021. Development of a novel *in vitro* primary human monocyte-derived macrophage model to study reactivation of HIV-1 transcription. J Virol 95:e0022721. https://doi.org/10.1128/JVI.00227-21
13. Hübner W, Chen P, Del Portillo A, Liu Y, Gordon RE, Chen BK. 2007. Sequence of human immunodeficiency virus type 1 (HIV-1) gag localization and oligomerization monitored with live confocal imaging of a replication-competent, fluorescently tagged HIV-1. J Virol 81:12596–12607. https://doi.org/10.1128/JVI.01088-07
14. Calvanese V, Chavez L, Laurent T, Ding S, Verdin E. 2013. Dual-color HIV reporters trace a population of latently infected cells and enable their purification. Virology 446:283–292. https://doi.org/10.1016/j.virol.2013.07.037
15. Dahabieh MS, Ooms M, Simon V, Sadowski I. 2013. A doubly fluorescent HIV-1 reporter shows that the majority of integrated HIV-1 is latent shortly after infection. J Virol 87:4716–4727. https://doi.org/10.1128/JVI.03478-12
16. Battivelli E, Dahabieh MS, Abdel-Mohsen M, Svensson JP, Tojal Da Silva I, Cohn LB, Gramatica A, Deeks S, Greene WC, Pillai SK, Verdin E. 2018. Distinct chromatin functional states correlate with HIV latency reactivation in infected primary CD4+ T cells. Elife 7:e34655. https://doi.org/10.7554/eLife.34655
17. Kim Y, Cameron PU, Lewin SR, Anderson JL. 2019. Limitations of dual - fluorescent HIV reporter viruses in a model of pre - activation latency. J Intern AIDS Soc 22. https://doi.org/10.1002/jia2.25425
18. Cai J, Gao H, Zhao J, Hu S, Liang X, Yang Y, Dai Z, Hong Z, Deng K. 2021. Infection with a newly designed dual fluorescent reporter HIV-1 effectively identifies latently infected CD4+ T cells. Elife 10:e63810. https://doi.org/10.7554/eLife.63810
19. Han Y, Lassen K, Monie D, Sedaghat AR, Shimoji S, Liu X, Pierson TC, Margolick JB, Siliciano RF, Siliciano JD. 2004. Resting CD4Ȩ T cells from human Immunodeficiency virus type 1 (HIV-1)-Infected individuals carry integrated HIV-1 genomes within actively transcribed host genes . J VIROL 78:6122–6133. https://doi.org/10.1128/JVI.78.12.6122-6133.2004
20. Cohn LB, Silva IT, Oliveira TY, Rosales RA, Parrish EH, Learn GH, Hahn BH, Czartoski JL, McElrath MJ, Lehmann C, Klein F, Caskey M, Walker BD, Siliciano JD, Siliciano RF, Jankovic M, Nussenzweig MC. 2015. HIV-1 integration landscape during latent and active infection. Cell 160:420–432. https://doi.org/10.1016/j.cell.2015.01.020
21. Wagner TA, McLaughlin S, Garg K, Cheung CYK, Larsen BB, Styrchak S, Huang HC, Edlefsen PT, Mullins JI, Frenkel LM. 2014. Proliferation of cells with HIV integrated into cancer genes contributes to persistent infection. Science 345:570–573. https://doi.org/10.1126/science.1256304
22. Matsuda K, Islam S, Takada T, Tsuchiya K, Yang Tan BJ, Hattori S-I, Katsuya H, Kitagawa K, Kim KS, Matsuo M, Sugata K, Delino NS, Gatanaga H, Yoshimura K, Matsushita S, Mitsuya H, Iwami S, Satou Y, Maeda K. 2021. A widely distributed HIV-1 provirus elimination assay to evaluate latency-reversing agents *in vitro* Cell Rep Methods 1:100122. https://doi.org/10.1016/j.crmeth.2021.100122
23. Naghavi MH, Salminen MO, Sönnerborg A, Vahlne A. 1999. DNA sequence of the long terminal repeat of human immunodeficiency virus type 1 subtype A through G. AIDS Res Hum Retroviruses 15:485–488. https://doi.org/10.1089/088922299311240
24. Jeeninga RE, Hoogenkamp M, Armand-Ugon M, de Baar M, Verhoef K, Berkhout B. 2000. Functional differences between the long terminal repeat transcriptional promoters of human immunodeficiency virus type 1 subtypes A through G. J Virol 74:3740–3751. https://doi.org/10.1128/jvi.74.8.3740-3751.2000
25. Bachu M, Yalla S, Asokan M, Verma A, Neogi U, Sharma S, Murali RV, Mukthey AB, Bhatt R, Chatterjee S, Rajan RE, Cheedarla N, Yadavalli VS, Mahadevan A, Shankar SK, Rajagopalan N, Shet A, Saravanan S, Balakrishnan P, Solomon S, Vajpayee M, Satish KS, Kundu TK, Jeang K-T, Ranga U. 2012. Multiple NF-κB sites in HIV-1 subtype C long terminal repeat confer superior magnitude of transcription and thereby the enhanced viral predominance. J Biol Chem 287:44714–44735. https://doi.org/10.1074/jbc.M112.397158
26. Chakraborty S, Kabi M, Ranga U, Silvestri G. 2020. A stronger transcription regulatory circuit of HIV-1C drives the rapid establishment of latency with implications for the direct involvement of tat. J Virol 94:e00503–20. https://doi.org/10.1128/JVI.00503-20
27. Bhange D, Prasad N, Singh S, Prajapati HK, Maurya SP, Gopalan BP, Nadig S, Chaturbhuj D, Jayaseelan B, Dinesha TR, et al. 2021. The evolution of regulatory elements in the emerging promoter-variant strains of HIV-1 subtype C. Front Microbiol 12:779472. https://doi.org/10.3389/fmicb.2021.779472
28. Ali H, Bhange D, Mehta K, Gohil Y, Prajapati HK, Byrareddy SN, Buch S, Ranga U. 2021. An emerging and variant viral promoter of HIV-1 subtype C exhibits low-level gene expression noise. Retrovirology 18:27. https://doi.org/10.1186/s12977-021-00572-2
29. Kim EH, Manganaro L, Schotsaert M, Brown BD, Mulder LCF, Simon V. 2022. Development of an HIV reporter virus that identifies latently infected $CD4^+$ T cells. Cell Rep Methods 2:100238. https://doi.org/10.1016/j.crmeth.2022.100238
30. Ratnapriya S, Harris M, Chov A, Herbert ZT, Vrbanac V, Deruaz M, Achuthan V, Engelman AN, Sodroski J, Herschhorn A. 2021. Intra- and extra-cellular environments contribute to the fate of HIV-1 infection. Cell Rep 36:109622. https://doi.org/10.1016/j.celrep.2021.109622
31. Miller CJ, McChesney MB, Lü X, Dailey PJ, Chutkowski C, Lu D, Brosio P, Roberts B, Lu Y. 1997. Rhesus macaques previously infected with simian/human immunodeficiency virus are protected from vaginal challenge with pathogenic SIVmac239. J Virol 71:1911–1921. https://doi.org/10.1128/jvi.71.3.1911-1921.1997
32. Jayaraman P, Mohan D, Polacino P, Kuller L, Sheikh N, Bielefeldt-Ohmann H, Richardson B, Anderson D, Hu S-L, Haigwood NL. 2004. Perinatal transmission of SHIV-Sf162P3 in macaca nemestrina. J Med Primatol 33:243–250. https://doi.org/10.1111/j.1600-0684.2004.00079.x
33. Ziani W, Bauer A, Lu H, Wang X, Wu X, Bar KJ, Li H, Liu D, Shaw GM, Veazey RS, Xu H, Kirchhoff F. 2021. Immune responses and viral persistence in simian/human immunodeficiency virus SHIV.C.CH848-infected rhesus macaques. J Virol 95:e02198–20. https://doi.org/10.1128/JVI.02198-20
34. Abeynaike S, Paust S. 2021. Humanized mice for the evaluation of novel HIV-1 therapies. Front Immunol 12:636775. https://doi.org/10.3389/fimmu.2021.636775
35. Hessell AJ, Haigwood NL. 2015. Animal models in HIV-1 protection and therapy. Curr Opin HIV AIDS 10:170–176. https://doi.org/10.1097/COH.0000000000000152
36. Victor Garcia J. 2016. Humanized mice for HIV and AIDS research. Curr Opin Virol 19:56–64. https://doi.org/10.1016/j.coviro.2016.06.010
37. Deruaz M, Tager AM. 2017. Humanized mouse models of latent HIV infection. Curr Opin Virol 25:97–104. https://doi.org/10.1016/j.coviro.2017.07.027
38. Lavender KJ, Pang WW, Messer RJ, Duley AK, Race B, Phillips K, Scott D, Peterson KE, Chan CK, Dittmer U, Dudek T, Allen TM, Weissman IL, Hasenkrug KJ. 2013. BLT-humanized C57BL/6 Rag2−/−γc−/−CD47−/− mice are resistant to GVHD and develop B- and T-cell immunity to HIV infection. Blood 122:4013–4020. https://doi.org/10.1182/blood-2013-06-506949
39. Holguin L, Echavarria L, Burnett JC. 2022. Novel humanized peripheral blood mononuclear cell mouse model with delayed onset of graft-versus-host disease for Preclinical HIV research. J Virol 96:e0139421. https://doi.org/10.1128/JVI.01394-21

40. Bruner KM, Murray AJ, Pollack RA, Soliman MG, Laskey SB, Capoferri AA, Lai J, Strain MC, Lada SM, Hoh R, Ho Y-C, Richman DD, Deeks SG, Siliciano JD, Siliciano RF. 2016. Defective proviruses rapidly accumulate during acute HIV-1 infection. Nat Med 22:1043–1049. https://doi.org/10.1038/nm.4156
41. Eriksson S, Graf EH, Dahl V, Strain MC, Yukl SA, Lysenko ES, Bosch RJ, Lai J, Chioma S, Emad F, Abdel-Mohsen M, Hoh R, Hecht F, Hunt P, Somsouk M, Wong J, Johnston R, Siliciano RF, Richman DD, O'Doherty U, Palmer S, Deeks SG, Siliciano JD. 2013. Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. PLoS Pathog 9:e1003174. https://doi.org/10.1371/journal.ppat.1003174
42. Lorenzi JCC, Cohen YZ, Cohn LB, Kreider EF, Barton JP, Learn GH, Oliveira T, Lavine CL, Horwitz JA, Settler A, Jankovic M, Seaman MS, Chakraborty AK, Hahn BH, Caskey M, Nussenzweig MC. 2016. Paired quantitative and qualitative assessment of the replication-competent HIV-1 reservoir and comparison with integrated proviral DNA. Proc Natl Acad Sci U S A 113:E7908–E7916. https://doi.org/10.1073/pnas.1617789113
43. Ho Y-C, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DIS, Lai J, Blankson JN, Siliciano JD, Siliciano RF. 2013. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. Cell 155:540–551. https://doi.org/10.1016/j.cell.2013.09.020
44. Hosmane NN, Kwon KJ, Bruner KM, Capoferri AA, Beg S, Rosenbloom DIS, Keele BF, Ho Y-C, Siliciano JD, Siliciano RF. 2017. Proliferation of latently infected CD4+ T cells carrying replication-competent HIV-1: potential role in latent reservoir dynamics. J Exp Med 214:959–972. https://doi.org/10.1084/jem.20170193
45. Bruner KM, Wang Z, Simonetti FR, Bender AM, Kwon KJ, Sengupta S, Fray EJ, Beg SA, Antar AAR, Jenike KM, et al. 2019. A quantitative approach for measuring the reservoir of latent HIV-1 proviruses. Nature 566:120–125. https://doi.org/10.1038/s41586-019-0898-8
46. Kinloch NN, Ren Y, Conce Alberto WD, Dong W, Khadka P, Huang SH, Mota TM, Wilson A, Shahid A, Kirkby D, Harris M, Kovacs C, Benko E, Ostrowski MA, Del Rio Estrada PM, Wimpelberg A, Cannon C, Hardy WD, MacLaren L, Goldstein H, Brumme CJ, Lee GQ, Lynch RM, Brumme ZL, Jones RB. 2021. HIV-1 diversity considerations in the application of the intact proviral DNA assay (IPDA). Nat Commun 12. https://doi.org/10.1038/s41467-020-20442-3
47. Gaebler C, Falcinelli SD, Stoffel E, Read J, Murtagh R, Oliveira TY, Ramos V, Lorenzi JCC, Kirchherr J, James KS, Allard B, Baker C, Kuruc JD, Caskey M, Archin NM, Siliciano RF, Margolis DM, Nussenzweig MC, Silvestri G. 2021. Sequence evaluation and comparative analysis of novel assays for intact Proviral HIV-1 DNA. J Virol 95. https://doi.org/10.1128/JVI.01986-20
48. Cho A, Gaebler C, Olveira T, Ramos R, Saad M, Lorenzi JCC, Gazumyan A, Moir S, Caskey M, Chun TW, Nussenzweig MC. 2022. Longitudinal clonal dynamics of hiv-1 latent reservoirsmeasured by combination quadruplex polymerase chain reaction and sequencing. Proc Natl Acad Sci U S A 119:e2117630119.
49. van Snippenberg W, Gleerup D, Rutsaert S, Vandekerckhove L, De Spiegelaere W, Trypsteen W. 2022. Triplex digital PCR assays for the quantification of intact proviral HIV-1 DNA. Methods 201:41–48. https://doi.org/10.1016/j.ymeth.2021.05.006
50. Levy CN, Hughes SM, Roychoudhury P, Reeves DB, Amstuz C, Zhu H, Huang M-L, Wei Y, Bull ME, Cassidy NAJ, McClure J, Frenkel LM, Stone M, Bakkour S, Wonderlich ER, Busch MP, Deeks SG, Schiffer JT, Coombs RW, Lehman DA, Jerome KR, Hladik F. 2021. A highly multiplexed droplet digital PCR assay to measure the intact HIV-1 proviral reservoir. Cell Rep Med 2:100243. https://doi.org/10.1016/j.xcrm.2021.100243
51. Malatinkova E, Thomas J, De Spiegelaere W, Rutsaert S, Geretti AM, Pollakis G, Paxton WA, Vandekerckhove L, Ruggiero A. 2021. Measuring proviral HIV-1 DNA: hurdles and improvements to an assay monitoring integration events utilising human *Alu* repeat sequences. Life (Basel) 11:1410. https://doi.org/10.3390/life11121410
52. Chung HK, Hattler JB, Narola J, Babbar H, Cai Y, Abdel-Mohsen M, Kim W-K. 2022. Development of droplet digital PCR-based assays to quantify HIV proviral and integrated DNA in brain tissues from viremic individuals with encephalitis and virally suppressed aviremic individuals. Microbiol Spectr 10:e0085321. https://doi.org/10.1128/spectrum.00853-21
53. Puertas MC, Bayón-Gil Á, Garcia-Guerrero MC, Salgado M, Urrea V, Morón-López S, Peña R, Jiménez-Moyano E, Clotet B, Prado JG, Martinez-Picado J. 2021. VIP-SPOT: an innovative assay to quantify the productive HIV-1 reservoir in the monitoring of cure strategies. mBio 12:e0056021. https://doi.org/10.1128/mBio.00560-21
54. Corne P, HuguetMF, Briant L, Segondy M, Reynes J, Vendrel JP. 1999. Detection Andenumeration of HIV-1-producing cells by ELISPOT (Enzyme- linked Immunospot) assay. J Acquir Immune Defic Syndr Hum Retrovirology 20:442–447.
55. Fondere J-M, Planas J-F, Huguet M-F, Baillat V, Bolos F, Reynes J, Vendrell J-P. 2004. Enumeration of latently infected CD4+ T cells from HIV-1-infected patients using an HIV-1 antigen ELISPOT assay. J Clin Virol 29:33–38. https://doi.org/10.1016/s1386-6532(03)00083-0
56. Imamichi H, Smith M, Adelsberger JW, Izumi T, Scrimieri F, Sherman BT, Rehm CA, Imamichi T, Pau A, Catalfamo M, Fauci AS, Lane HC. 2020. Defective HIV-1 proviruses produce viral proteins. Proc Natl Acad Sci U S A 117:3704–3710. https://doi.org/10.1073/pnas.1917876117
57. Brandt LD, Guo S, Joseph KW, Jacobs JL, Naqvi A, Coffin JM, Kearney MF, Halvas EK, Wu X, Hughes SH, Mellors JW. 2021. Tracking HIV-1-infected cell clones using integration site-specific qPCR. Viruses 13:1235. https://doi.org/10.3390/v13071235
58. Sneller MC, Blazkova J, Justement JS, Shi V, Kennedy BD, Gittens K, Tolstenko J, McCormack G, Whitehead EJ, Schneck RF, Proschan MA, Benko E, Kovacs C, Oguz C, Seaman MS, Caskey M, Nussenzweig MC, Fauci AS, Moir S, Chun T-W. 2022. Combination anti-HIV antibodies provide sustained virological suppression. Nature 606:375–381. https://doi.org/10.1038/s41586-022-04797-9
59. Gruell H, Gunst JD, Cohen YZ, Pahus MH, Malin JJ, Platten M, Millard KG, Tolstrup M, Jones RB, Conce Alberto WD, Lorenzi JCC, Oliveira TY, Kümmerle T, Suárez I, Unson-O'Brien C, Nogueira L, Olesen R, Østergaard L, Nielsen H, Lehmann C, Nussenzweig MC, Fätkenheuer G, Klein F, Caskey M, Søgaard OS. 2022. Effect of 3BNC117 and romidepsin on the HIV-1 reservoir in people taking suppressive antiretroviral therapy (ROADMAP): a randomised, open-label, phase 2A trial. Lancet Microbe 3:e203–e214. https://doi.org/10.1016/S2666-5247(21)00239-1
60. Scheid JF, Horwitz JA, Bar-On Y, Kreider EF, Lu C-L, Lorenzi JCC, Feldmann A, Braunschweig M, Nogueira L, Caskey M, et al. 2016. HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption. Nature 535:556–560. https://doi.org/10.1038/nature18929
61. Astorga-Gamaza A, Grau-Expósito J, Burgos J, Navarro J, Curran A, Planas B, Suanzes P, Falcó V, Genescà M, Buzon MJ. 2022. Identification of HIV-reservoir cells with reduced susceptibility to antibody-dependent immune response. Elife 11:eLife https://doi.org/10.7554/eLife.78294
62. Sengupta S, Board NL, Wu F, Moskovljevic M, Douglass J, Zhang J, Reinhold BR, Duke-Cohan J, Yu J, Reed MC, Tabdili Y, Azurmendi A, Fray EJ, Zhang H, Hsiue EH-C, Jenike K, Ho Y-C, Gabelli SB, Kinzler KW, Vogelstein B, Zhou S, Siliciano JD, Sadegh-Nasseri S, Reinherz EL, Siliciano RF. 2022. TCR-mimic bispecific antibodies to target the HIV-1 reservoir. Proc Natl Acad Sci U S A 119:e2123406119. https://doi.org/10.1073/pnas.2123406119
63. Shete A, Thakar M, Singh DP, Gangakhedkar R, Gaikwad A, Pawar J, Paranjape R. 2012. Short communication: HIV antigen-specific reactivation of HIV infection from cellular reservoirs: implications in the settings of therapeutic Vaccinations. AIDS Res Hum Retroviruses 28:835–843. https://doi.org/10.1089/aid.2010.0363
64. Suryawanshi P, Bagul R, Shete A, Thakar M. 2021. Anti-HIV-1 ADCC and HIV-1 Env can be partners in reducing latent HIV reservoir. Front Immunol 12:663919. https://doi.org/10.3389/fimmu.2021.663919
65. Wu VH, Nordin JML, Nguyen S, Joy J, Mampe F, Del Rio Estrada PM, Torres-Ruiz F, González-Navarro M, Luna-Villalobos YA, Ávila-Ríos S, Reyes-Terán G, Tebas P, Montaner LJ, Bar KJ, Vella LA, Betts MR. 2023. Profound phenotypic and epigenetic heterogeneity of the HIV-1-infected CD4+ T cell reservoir. Nat Immunol 24:359–370. https://doi.org/10.1038/s41590-022-01371-3
66. Einkauf KB, Osborn MR, Gao C, Sun W, Sun X, Lian X, Parsons EM, Gladkov GT, Seiger KW, Blackmer JE, Jiang C, Yukl SA, Rosenberg ES, Yu XG, Lichterfeld M. 2022. Parallel analysis of transcription, integration, and sequence of single HIV-1 proviruses. Cell 185:266–282. https://doi.org/10.1016/j.cell.2021.12.011

AUTHOR BIOS

Divyadarshini Angamuthu completed her Ph.D. in Microbiology on studying the antiviral effects of medicinal plants and their compounds on Human Herpes Virus-3 at Presidency college, Chennai in 2018. She did her Master's in Applied Microbiology and was one among the top university rank holders in her Bachelor's degree. With over 8 years of professional experience, Dr. Angamuthu has contributed significantly to the field of microbiology holding various positions in prestigious institutions and organizations. Currently, she is working as an ICMR-Research Associate Fellow in the Department of Virology & Biotechnology at the National Institute for Research in Tuberculosis (ICMR-NIRT) in Chennai, from June 2022. Her current research focus is on HIV latency. During her research career, she has contributed several publications in renowned scientific journals. Some of her notable publications include studies on *in vitro* antiviral activity against dengue virus, HHV-3, and *in silico* screening of potential phytochemicals against HHV-3.

Sandhya Vivekanandan is a full-time research scholar and DBT-SRF at ICMR- NIRT. She completed her B. Tech Biotechnology from Sree Sastha Institute for Engineering and Technology, Chennai and M. Tech Biotechnology from Sri Venkateswara College of Engineering, Chennai. She was awarded gold medal for her academic excellence in graduation and post-graduation. She is currently working on isolation of broadly neutralizing antibodies from elite neutralizers. She has also acquired research training in Tuberculosis and animal studies. She has published two review and research articles, as of now. She is a passionate researcher who actively participates in paper and poster presentations. Some of her research work were awarded with best poster awawds. She recently won the "Paul Ehrlich award" for her research presentation in the National Immunology Conference.

Luke Elizabeth Hanna obtained her Ph.D. in immunology at the ICMR-National Institute for Research in Tuberculosis, Chennai, India, in 2000, and has been working at this Institute since then. She was awarded a fellowship under the Fogarty AIDS International Training Program in 2004 to do her post-doctoral research in the Department of Microbiology & Immunology at the Albert Einstein College of Medicine, where she was trained in molecular virology. She has more than 25 years of research experience and has published over 100 research articles on the immunopathogenesis of HIV infection, HIV-TB co-infection and HIV virology. Her current research focuses on HIV latency, HIV drug resistance and isolation of broadly neutralizing antibodies against HIV-1 subtype C virus. She heads the Department of Virology and Biotechnology and is in-charge of the Regional Reference Labs for molecular diagnosis of HIV infection and HIV-1 viral load testing for the National HIV Program as well as the Viral Research and Diagnostic Lab at NIRT.