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Minimal Requirements for Primary HIV Latency Models Based on a Systematic Review

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Abstract

Due to the scarcity of HIV-1 latently infected cells in patients,* in vitro *primary latency models are now commonly used to study the HIV-1 reservoir. To this end, a number of experimental systems have been developed. Most of these models differ based on the nature of the primary CD4+ T-cell type, the used HIV strains, activation methods, and latency assessment strategies. Despite these differences, most models share some common characteristics. Here, we provide a systematic review covering the primary HIV latency models that have been used to date with the aim to compare these models and identify minimal requirements for such experiments.

***A systematic search on PubMed and Web of Science databases generated a short list of 17 unique publications that propose new* in vitro *latency models. Based on the described methods, we propose and discuss a generalized workflow, visualizing all the necessary steps to perform such an* in vitro *study, with the key choices and validation steps that need to be made; from cell type selection until the model readout.* (AIDS Rev. 2016;18:171-83)**

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Key words

HIV-1. Viral latency. Primary cell. In vitro. Model. Systematic review.

Introduction

Combination antiretroviral therapy (cART), introduced in 1996 to combat HIV-1 infection, was a game changer. It turned a lethal disease into a manageable chronic disease, with life expectancy currently matching the one of healthy individuals[1, 2]. However, small populations of extremely long-lived, latently infected central memory T-cells characterized by a half-life of 40 weeks persist under cART. These cells are the major reason why latent infection has still not been eradicated. Current notion suggests that due to the longevity of latently infected cells, eradication of the entire HIV-1 reservoir would take over 70 years[3]. However, a recent report indicates that the actual size of the reservoir may be even 60-fold bigger[4].

Efforts to study the latent reservoir are hampered by the scarcity of latently infected cells *in vivo,* combined with the inability to selectively isolate latently infected cells from the blood of HIV-1-infected patients. No phenotypic markers to target latently infected cells have been described so far, and the identification of such cells can only be performed by an extensive T-cell

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activation or by PCR-based assays. These, on the other hand, involve cell lysis or lead to cell death. Consequently, HIV latency models appear to be the best way to study the reservoir.

The *in vitro* latency models described so far can be divided into two major subgroups, depending on their origin and features. Latently infected cell lines are the earliest and most used models to study latency. However, latent cell lines mainly consist of clonally proliferating cells, most often isolated from leukemic patients. Moreover, the latent provirus is generally clonally amplified in these cells as well, increasing the risk of biasing research results due to the nature of the cells or the limited number of proviral integration sites. Models based on primary cells form a very appealing alternative for *in vitro* research as these cover the wide heterogeneity of the T-cell populations and of different integrations[5].

In this study, we aimed at identifying the minimum requirements for HIV latency models using primary cells based on a systematic review. A systematic literature screen was used to identify original HIV latency models with primary T-cells described to date.

Methods

Searching strategy and exclusion criteria

PubMed and Web of Science databases were screened with the terms "HIV latency model" and "HIV latently infected primary cell". Search results were then narrowed down to articles published in English from 1 January 2000. To identify original research articles, the exclusion of reviews was applied with filters available for both databases. The search results were then exported to EndNote and the removal of duplicates yielded 489 unique articles.

Further, papers focusing on primate and mouse models as well as SIV (simian immunodeficiency virus), MLV (murine leukemia virus), EBV (Epstein-Barr virus), FIV (feline immunodeficiency virus), TB (tuberculosis) and other pathogens and diseases were excluded. Abstracts or full texts of the remaining 379 articles were screened for their relevance. Papers proposing mathematical models, investigating drugs and latency reversing agents (LRA) and remaining reviews, posters, and conference reports were eliminated from the list. Next, we excluded clinical studies and studies employing patient cells or cell lines only. The remaining articles were assessed in the context of their focus on HIV latency and subsequently on their relevance for the study design: the use of primary HIV latency models. Finally, the list was narrowed down to original articles proposing new models rather than referring to models described by other authors. Applying these search terms and exclusion criteria, we identified 17 relevant articles. A further literature search to find potentially omitted publications did not identify additional hits. The study selection workflow is presented in figure 1.

Results

Several primary *in vitro* latency models have been described. They implement a variety of cell subsets, viral and vector strains, infection methods, cell preconditioning, and latency reactivation strategies. It is clear that none of these models entirely represents the viral reservoir, but the different models led to independent discoveries, facilitating our understanding of the extremely complex matter of HIV latency.

A number of strategies have been investigated in depth to mimic the likely mechanism of latency establishment *in vivo* by infection of activated T-cells during their transition to cellular quiescence. To that end, several pre-activation methods, HIV strains (wild type or engineered to carry mutations or reporter genes), infection methods as well as activation compounds have been tested on a variety of cell types. There are many differences between the proposed strategies, all sharing the final goal to mimic *in vivo* latency as closely as possible, and to achieve sufficient levels of latent infection in quiescent cells. For the purpose of the study, we define "latency levels" as the difference in the amount of positive signal before cellular activation, further referred to as "background", and after cellular activation aimed at disrupting latency, consisting of background active infection and reversed latency, measured by flow cytometry, ELISA, or PCR-based methods. The outline of the *in vitro* HIV latency models described in this study is presented in figure 2.

Activated T-cells

Since resting cells are difficult to infect, strategies involving the initial activation of target cells are expected to overcome the natural blocks of infection posed by resting cells. Additionally, such preconditioning often leads to increased cell proliferation, which in turn generates large amounts of cells.

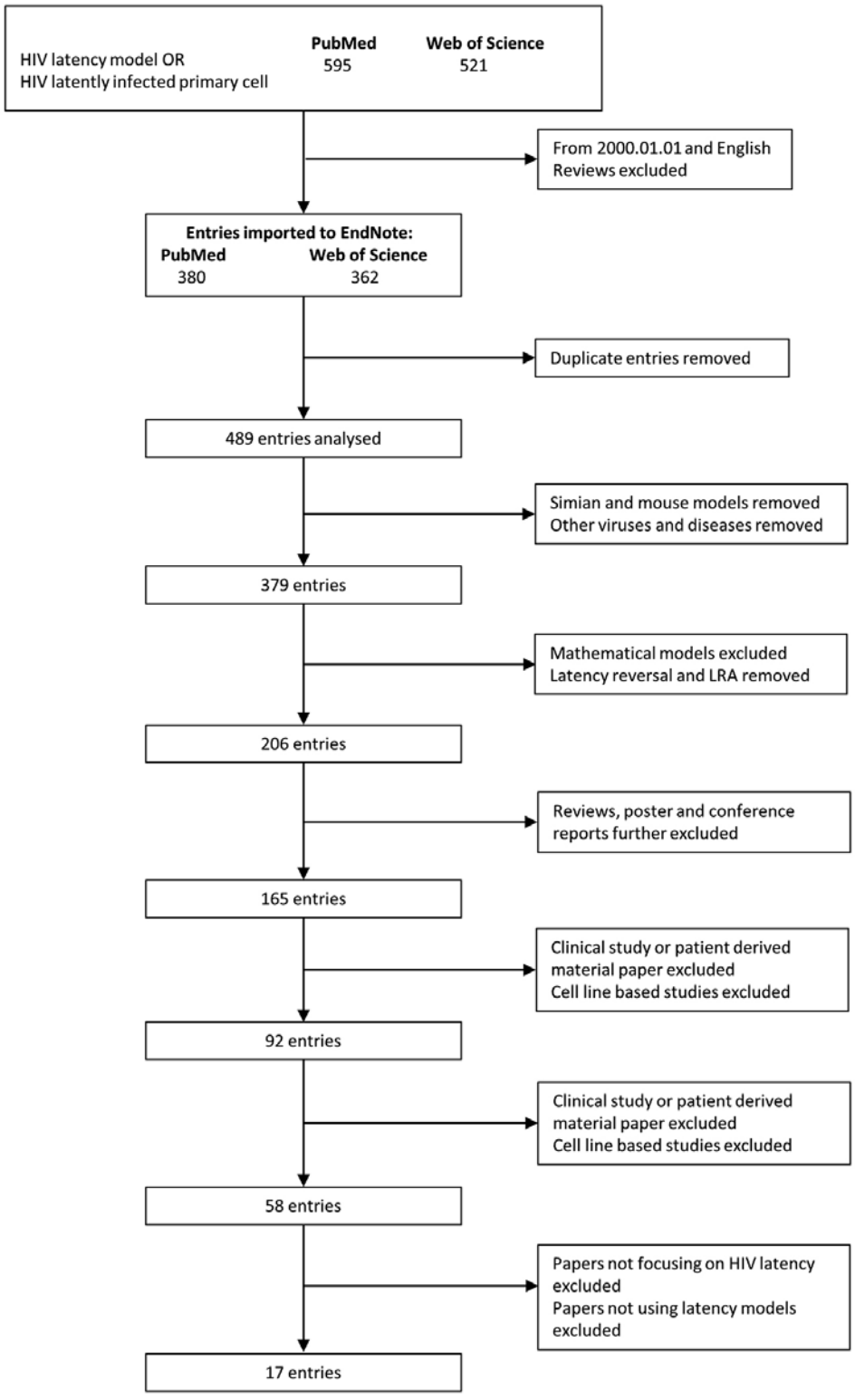

Figure 1. *The study selection workflow.*

Model	Author	Year published	Cells					Virus used		Block of viral replication		Time of ARV treatment	Reactivation strategy	Evaluation of viral production			TAT	Minimal requirements
			Primary Cell type	Cell pretreatment	*in vitro* differentiated cell type	Activation markers checked	Cell phenotype	Replication competent HIV	Single-round HIV	Pseudotyping	ARVs			Flow cytometry	PCR	other		
TNP model	Bosque et al.	2009	Naïve T-cells	CD3/CD28	Non polarized T-cells	Ki67, CD25	CD25 expressing cells	/	Δenv HIV + HIV env	Yes	/	/	CD3/CD28	p24/GFP	/	/	1 month	8/10
	Martins et al.	2014	Naïve T-cells	CD3/CD28	Non polarized T-cells	None	Resting, depletion of activated cells	NL4.3	/	/	INI	4d pre-activation, 6 days	CD3/CD28	p24/GFP	/	/	1 month	8/9
	Lusic et al.	2013	Naïve T-cells	CD3/CD28	Non polarized T-cells	None	Resting, depletion of activated cells	/	Δenv NL4.3 + VSVG	Yes	/	/	CD3/CD28	/	CA-RNA	/	1 month	7/10
PHA-activated cells	Gondois-Rey et al.	2001	CD4+ T cells	PHA-activation	N/A	CD25, CD69, HLA-DR	Resting cells, activated cells depleted	NL4.3	/	/	/	/	/	p24/gag	gag-DNA, unspliced RNA	/	1 month	8/9
Activated naïve CD4+ T cells	Burnett et al	2010	Naïve T-cells	CD3/CD28 + IL-2	N/A	None	Resting cells by applying resting conditions	/	EGFP-encoding vector + VSV-G	Yes	/	/	CD3/CD28	EGFP	/	/	1 month	7/10
Thymocyte model	Burke et al.	2007	CD4+CD8+ thymocytes	CD3/CD28	N/A	CD25, CD38, CD69, HLA-DR	Resting cells	/	Δenv EGFP-Luc vector + VSV-G	Yes	/	/	CD3/CD28	EGFP	Alu-PCR	Luciferase assay	1 month	8/10
Activated T cells co-cultured with feeder cells	Sahu et al	2006	CD4 T cells + H80 feeder cell line	CD3 + IL-2	N/A	CD25, CD69, HLA-DR	CD69 expressing cells	JRCSF	/	/	PI/RT inhibitor	Not stated	Prostratin	/	/	Elisa/p24	2 months	9/9
	Tyagi et al	2010	CD4 T cells + H80 feeder cell line	CD3/CD28	N/A	Ki67, CD25, CD38	CD38 expressing cells	/	Δenv Δnef egfp+ VSV-G	Yes	/	/	CD3/CD28 or TNF-α	EGFP	/	/	2 months	8/10
	Mohammadi et al	2014	CD4 T cells + H80 feeder cell line	CD3/CD28	N/A	CD25, CD69, HLA-DR	Resting, low-level transcription of HIV	/	Δgag Δvif Δvpr Δvpu Δenv Δnef egfp+	Yes	/	/	CD3/CD28 + IL-2	EGFP	/	NGS, HIV RNA	2 months	8/10
Bcl-2 transduced cells	Yang et al	2009	Bcl-2 transduced CD4+ T cells	Bcl-2 transduction, CD3/C28	N/A	CD25, CD69, HLA-DR	Mostly resting	/	Δgag Δvif Δvpr Δvpu Δenv Δnef egfp+	Yes	/	/	CD3/CD28	EGFP	single-spliced HIV RNA	/	1 month	8/10
DC-stimulated T cells	Marini et al.	2008	Naïve CD4+ T cells activated by MDDCs	DC-induced preactivation of T cells	N/A	Ki67, CD25, CD69, HLA-DR	Not fully resting (CD25)	IIIB	/	/	NRTI	Not stated	CD3/CD28	p24	/	Elisa/p24	2 months	9/9
DC-infected T cells	Evans et al.	2013	Naïve CD4+ T cells co-cultured with DCs	DC-induced preactivation of T cells	N/A	CD25, CD69, HLA-DR	Resting, activated cells depletion	EGFP+ NL4.3	/	/	INI	At the moment of activation	PHA	EGFP	Alu-PCR, RT	/	1 month	9/9
Primary resting T cells	Swiggard et al.	2005	Resting CD4+ T cells	None	N/A	CD25, CD69, HLA-DR	Resting cells	IIIB	/	/	INI	At the moment of activation	IL-7 or CD3/CD28	p24	Alu-PCR	/	1 week	9/9
CCR7-stimulated resting cells	Saleh et al.	2007	Resting CD4+ T cells	CCL19 and CCL21	N/A	Ki67, CD25, CD69, HLA-DR	Resting cells	Δnef EGFP+ NL4.3 or AD8	/	/	INI	Before activation	IL-2 + PHA	/	Alu-PCR, RT	/	2 weeks	9/9
Directly infected resting cells	Lassen et al.	2012	Resting CD4 T cells	None	N/A	CD25, CD69, HLA-DR	Resting cells	EGFP+ or Luc NL4.3	/	/	PI + INI	3d before activation	CD3/CD28 or PMA	EGFP	/	Luciferase assay	1 week	9/9
Co-culture of infected cells with resting cells	Spina et al.	2013	Activated CD4+ T cells co-cultured with resting T cells	CD3/CD28	N/A	None	Nondividing cells	NL4.3	/	/	INI	2d before activation	CD3/CD28	p24	Alu-PCR, CA-RNA	/	1 month	8/9
Resting cells infected with a dual-reporter virus	Chavez et al.	2015	CD4+ T cells	IL-7 and CCL19	N/A	CD25, CD69	Resting cells	/	Dual-reporter vector + VSV-G	Yes	INI	Before activation	CD3/CD28	EGFP/mCherry	Alu-PCR	/	1 month	10/10

Figure 2. *Overview of primary HIV latency models with their major features. The table lists the* in vitro *HIV latency models, their authors and year of publication, cell type used together with pre-treatment strategies, the activation markers assessed and the cell phenotype, the virus strain used, the strategy to block viral replication in the case of infection with replication-competent strains, timing of antiretroviral treatment, compounds used to disrupt latency, the method of latency levels readout, the turnaround time of an experiment and the number of minimal requirements met in each study (cfr. Fig. 3). N/A: not available (to indicate no* in vitro *differentiation of target cells upon their isolation); /: not relevant, either no particular treatment or assay was performed or no type of virus was used in the study; INI: integrase inhibitor; PI: protease inhibitor; RT: reverse transcriptase: NRTI: nucleoside reverse transcriptase inhibitor; CA-RNA: cell-associated RNA; TAT: turnaround time.*

Phytohemagglutinin-activated cells (Gondois-Rey, et al. 2001)

Gondois-Rey, et al. created the first *in vitro* HIV latency model based on primary cells in 2001[6]. Their approach involves pre-treatment of peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) followed by full-length NL4.3 HIV infection. These cells are then cultured for three weeks before the depletion of activated cells based on the expression of activation markers, i.e. CD25, CD69, and HLA-DR. Despite the purification of resting cells, ongoing viral production was observed in the model, indicating that most cells did not establish true latency.

Activated naive CD4+ T-cells (Burnett, et al. 2010)

The approach described by Burnett, et al. involves the use of CD4+ naive T-cells isolated from PBMCs, which are stimulated with CD3/CD28 and interleukin-2 (IL-2) and expanded for seven days[7]. Such treatment makes these cells susceptible to infection with VSV-G transduced lentiviral vector carrying a green fluorescent protein (GFP) reporter gene, referred to as LGIT. After the infection, the cells were cultured for seven days in the presence of IL-2. Then, the levels of IL-2 were reduced and minimal doses of IL-7 were added to the culture medium to promote cell viability and their return to the resting state for an additional two weeks until a panel of LRAs was tested in the system.

This approach was of use to test a panel of compounds and assess their ability to disrupt latency. However, the actual levels of latency were not presented[7]. The low multiplicity of infection (MOI) of the lentiviral vectors used to transduce the activated cells suggests that these levels were not very high. Additionally, the use of mutated HIV sequence pseudotyped with vesicular stomatitis virus glycoprotein G (VSV-G) that overcomes the need for CD4 receptor for viral entry makes this model less physiologically relevant.

Thymocyte model (Burke, et al. 2007)

The model developed by Burke, et al. is derived from a severe combined immunodeficient humanized (SCID-hu) mouse model previously proposed by the lab[8]. In this model, quiescent latently infected lymphocytes were generated *in vivo* during thymopoiesis, recapitulating the *in vivo* events. The human fetal thymic tissues serve as a source of CD4+CD8+ thymocytes that are infected with a reporter HIV carrying several mutations, including a deletion in nuclear factor kappa-B (NF-κB) binding site to promote latency establishment. The cells undergo a maturation process and differentiation to resting latently infected CD4+ T-cells.

This approach has been invaluable for identifying the role of NF-κB signaling for the reactivation of latent HIV[8]. However, the workflow is relatively expensive, labor-intensive, and it generates mostly naive T-cells, whereas *in vivo* it is believed that the major HIV reservoir resides in central and transitional memory T-cells[9].

Activated T-cells co-cultured with feeder cells (Sahu, et al. 2006; Tyagi, et al. 2010; Mohammadi, et al. 2014)

Sahu, et al. proposed a model of HIV latency employing primary CD4+ T-cells infected with a replication-competent strain of the virus and supported with H80 cells[10]. This brain tumor-derived cell line is of use for extending the lifespan of T-cells without the need for externally supplemented cytokines.

Freshly isolated CD4+ T-cells are activated with CD3 antibodies and IL-2 before the infection and subsequently co-cultured with H80 cells. This procedure is expected to help the cells transition to the resting state. However, several weeks later the T-cells still expressed the early activation marker CD69 and produced progeny HIV particles. This indicates that not all the cells in culture were truly quiescent and truly latently infected.

The laboratory of Jonathan Karn followed the idea of supporting the T-cells with the H80 feeder cell line[11]. Primary CD4+ T-cells were activated by T-cell receptor (TCR) stimulation and infected with a VSV-G pseudotyped viral strain encoding enhanced green fluorescent protein (EGFP) reporter. Cells positive for EGFP are then sorted out, expanded by CD3/CD28 stimulation, and co-cultured with H80 cells for several weeks. Similarly to the results of Sahu, et al., the resulting cells still express activation markers (CD25).

CD3/CD28 stimulation led to NF-κB recruitment and conversion of heterochromatin, in the proximity of the integrated provirus, to transcriptionally accessible euchromatin. The lack of response to tumor necrosis factor-α (TNF-α) was explained by restriction in the levels of positive transcriptional elongation factor b (P-TEFb). Together, this model proved that a combination of the state of the chromatin environment and P-TEFb levels can be a mechanism inducing latency in this primary CD4+ T-cell system[11].

The biggest advantage of this approach is the generation of large amounts of cells with central memory phenotype. On the other hand, the mechanism of latency achieved with a VSV-G pseudotyped HIV[11] may not fully recapitulate the *in vivo* scenario, as the method of cellular infection differs between a VSV-G and a natural HIV envelope[12]. The constant expression of activation markers also indicates that these latently infected cells differ from fully resting cells *in vivo.* Nevertheless, this approach has been of use for determining the role of several epigenetic modifications in HIV latency[11].

An analogous approach to generate latently infected T-cells was recently described by the group of Ciuffi[13]. Similar to the workflows of Tyagi, et al. and Sahu, et al., $CD4^+$ T-cells isolated from the blood of HIV-naive donors were activated with CD3/CD28 stimulation prior to infection. Here, the authors used a crippled NL4.3-based vector, carrying a *gfp* reporter gene and mutations in *gag, vif, vpr, vpu, env,* and *nef* to prevent high levels of ongoing infection. Forty-eight hours after infection, cells positive for EGFP were sorted out and expanded in the presence of H80 cells for 10 weeks in order to facilitate reversion back to the quiescent state.

In the model by Mohammadi, et al. latency was targeted with several reversing agents including suberoylanilide hydroxamic acid (SAHA), (5'-azacytidine (AZA), disulfiram (DSF), CD3/CD28, and IL-7. However, only TCR stimulation was efficient enough to induce an increase in EGFP levels compared to background[13]. The CD3/CD28 stimulation, SAHA, and DSF caused elevated levels of transcription of HIV genes, while the other reagents had minor or no effect. Constant low-level transcription of HIV genes without LRA treatment indicates that at least some of the cultured cells are not truly latent. This effect, although not desired in the context of *in vitro* models, may resemble the *in vivo* scenario with possible ongoing transcription in patients on cART. Of note, a recent report describes ongoing HIV replication as a potential mechanism of reservoir maintenance in patients on cART[14]. The major shortcoming of the experimental method introduced by Mohammadi, et al. is the type of vector used to infect the cells. A vector only expressing EGFP, Tat, and Rev may simplify the complexity of latency establishment and maintenance. Basal levels of transcription are unlikely to resemble those achieved in experiments taking advantage of full viruses, and this could be reflected in latency levels as well[13].

B-cell lymphoma 2-transduced cells (Yang, et al. 2009)

The approach of Mohammadi, et al. resembles the model developed by the group of Siliciano. Here, a two-step transduction protocol was implemented to generate latently infected cells[15]. First, the primary $CD4^+$ T-cells are transduced with a lentiviral vector coding for B-cell lymphoma 2 (Bcl-2), an anti-apoptotic gene to promote their survival. Next, after their return to the quiescent state, these cells are activated by CD3/CD28 stimulation and transduced with a replication-deficient NL4.3-derived HIV strain lacking several genes *(env, gag, nef, vpr, vpu* and *vif)* but carrying an *egfp* gene (the same as used by Mohammadi, et al.). This is followed by up to four weeks of culture during which the cells return to quiescence and latency is established.

Stimulation with different LRAs, including CD3/CD28, phorbol myristate acetate (PMA), and PHA, is able to disrupt latency, proving that the Bcl-2 transduction does not interfere with the pathways responsible for establishment and reversal of latency. The levels of latent infection reach 6%, and are sufficient for low-throughput screening of compounds to reactivate the virus from latency. In fact, this model was used to identify disulfiram and 5-hydroxyapthoquinone as LRAs[16].

Of note, this approach generates primarily effector memory T-cells as characterized by expression of CD45RO in the absence of CCR7 and activation markers.

Dendritic cell-stimulated T-cells (Marini, et al. 2008)

The approach by Marini, et al. was the first aimed at reconstruction of the *in vivo* interplay between $CD4^+$ T-cells and dendritic cells (DC)[17]. Naive T-cells were activated by the autologous DCs and afterwards infected with wild-type HIV. This was followed by culturing in the presence of low doses of IL-7 to enable the cells to return to the quiescent state. Most cells showed the central memory phenotype and expressed no activation markers (CD25, HLA-DR).

The major drawback of this model is the requirement for IL-7, which next to inducing the transition of effector T-cells to memory T-cells, is also known to reactivate latently infected cells. Massive cell death is the reason why only approximately 20% of the initial number of cells survives the workflow. On the other hand, the

model implements wild-type HIV and dendritic cells to generate latently infected T-cells with central memory phenotype.

Dendritic cell-infected T-cells (Evans, et al. 2013)

Upon initial HIV infection, DCs are the first cellular subset to encounter HIV in the mucosa, and their ability to transfer the virus to CD4+ T-cells makes them an interesting topic in the setting of *in vitro* latency models[18,19]. It can be speculated that mimicking the DC-mediated HIV transmission to T-cells recapitulates the establishment of the early latent reservoir better than other methods of infection, and this is why HIV latency models implementing DCs emerge and are gaining increased appreciation.

Regardless of the role of DCs in latency reactivation[20], the laboratory of Sharon Lewin recently introduced a model employing dendritic cells to infect T-cells by direct contact, exploring the largely elusive mechanism of trans-infection[21]. In this workflow, CD4+ T-cells are treated with a proliferation dye, co-cultured with DCs for 24 hours, infected with a reporter HIV strain carrying EGFP, and cultured for an additional five days. This was followed by sorting out of productively infected and proliferating cells, while the remaining population was activated to reveal the levels of latent infection.

These efforts proved that close contact between myeloid dendritic cells and CD4+ T-cells promotes the establishment of latency in resting T-cells. It has also hinted that the importance and potential of DCs in HIV latency models has been overlooked and urges for an in-depth investigation. Unfortunately, the levels of latency established with this approach are very low, making downstream applications of this model severely limited.

Activated non-polarized T-cells (Bosque, et al. 2009)

The model described by Bosque and Planelles is based on naive CD4+ T-cells that are differentiated into non-polarized T-cells (T_{NP}), the *in vitro* equivalent of central memory T-cells[22]. T-cell receptor stimulation in the presence of IL-4 antibodies, IL-12 antibodies, and transforming growth factor beta (TGF-ß) leads to the generation of large pools of T_{NP} with a memory phenotype. After activation, these cells are expanded for four days in the presence of IL-2 and infected by spinoculation. Seven days later, the cells are stimulated with CD3/CD28 again to reveal the latent infection.

The original model involves the use of a single-round HIV-1-derived construct. A 600 bp deletion in *env* sequence was introduced into the full-length HIV sequence, and this plasmid was complemented with an intact env supplied on a separate plasmid during co-transfection. Cellular activation leads to approximately 50% of positive signal, supposedly originating from reactivated latently infected cells with only 1% of background active infection[22,23].

However, we previously showed that the replication-deficient vector becomes replication-competent due to recombination between the wild-type envelope and the *env*-deficient sequences[24]. Spreading infection in the model urged for the re-analysis of the data obtained in previous studies. The percentage of latently infected cells observed in the model is lower than initially stated, as P24 antigen staining visualizes the latently infected cells, but also the overlooked cells carrying productive infection[23]. The workflow of this model was modified to still yield latently infected cells with a memory phenotype by introducing replication-competent laboratory strains of HIV without reporter genes to better mimic the *in vivo* scenario, or with EGFP to facilitate tracking the infection levels. The spreading infection is stopped 24 hours pre-activation by integrase and/or protease inhibitors, and reactivation reveals post-integration events rather than new integrations induced by the response to the strong stimulation agents. Optional steps include cell crowding; changing cell density by culturing them in U-bottom rather than flat-bottom plates or sorting based on CD4 or EGFP expression to maximize the levels of latency and exclude productively infected cells, respectively[25]. The new approach routinely leads to generation of latency levels of approximately 6-8%; however, the strength of the model lies in the phenotype of the cells that very closely resemble the most relevant *in vivo* reservoir: central memory T-cells.

The initial model, biased by active replication, was used to identify nuclear factor of activated T-cells (NFAT) as the major transcription factor involved in latent HIV reactivation[22]. Productive infection in the model necessitates reassessment of the data due to the involvement of NFAT in the kinetics of HIV replication[26]. Similarly, the role of the JAK-STAT pathway[27] and PIM-1[28] in replication of the virus needs to be clarified. The effect can result from the inhibition of latency reactivation or inhibition of replication kinetics. The study investigating the integration sites in latently

infected cells from several different models can also be affected by the spreading infection[5]. Despite the sorting strategy aimed at removing the productively infected cell from the culture, a pool of cells at an early stage of infection that did not express EGFP at that point could be present and influence the data collected.

Directly infected resting T-cells

Resting memory T-cells constitute the major part of the latent reservoir *in vivo* and this is why several groups proposed *in vitro* latency models based on resting cells. Direct infection of such cells is possible, but highly inefficient. Although it may be more relevant physiologically, due to the multiple blocks following reverse transcription, the levels of latency proposed by the following approaches are less appealing than those that can be achieved in pre-activated T-cells.

Primary resting T-cells (Swiggard, et al. 2005)

The earliest latency model based on resting T cells was proposed by Swiggard, et al.[29]. It involves removal of activated CD4+ T-cells as determined by CD69 and HLA-DR expression followed by wild-type HIV infection mediated by spinoculation. Infection by spinoculation triggers changes in the actin environment of target cells, rendering them susceptible to HIV infection[30].

This workflow leads to the establishment of low levels of latent infection in a mixed population of naive, effector, and memory T-cells. However, direct infection results in a relatively small population of cells for further experiments. Since the cells are cultured without any cytokines that could promote cell survival after infection, their lifespan is a limiting step as well.

The application of this model led to several important findings. This model for the first time indicated that *in vitro* latently infected cells can still produce HIV protein Gag without producing viral particles, providing a new and unconventional definition of latency[31,32]. This phenomenon has potential therapeutic applications for HIV cure as strategies targeting Gag-producing cells may also target Env-expressing latently infected cells[31]. The primary resting CD4+ T-cell model was also used to investigate integration sites in a larger study comparing several latency models[5]. Additionally, a similar approach, although not focusing strictly on latently infected cells, emphasized the stability of 2-long terminal repeat (2LTR) circles in HIV-infected cells[33] in culture. Finally, the widespread assay for detecting integrated copies of HIV makes use of cells infected with this method as an internal standard to validate the PCR reactions[34].

CCR7-stimulated resting cells (Saleh, et al. 2007)

The group of Sharon Lewin modified the approach of Swiggard, et al. by implementing pre-treatment of resting CD4+ T-cells with CCR7 ligands CCL19 and CCL21[35,36]. This procedure does not lead to cellular activation but, similar to spinoculation, induces cytoskeleton rearrangements which facilitate the establishment of latency[30,37]. Stimulation with different LRAs leads to the reversal of latency as measured by reverse transcriptase activity in the culture medium.

This model was a strong confirmation that latency can be induced directly through infection of resting memory T-cells. It is important to note that unlike naive and central memory, effector memory T-cells do not express CCR7, suggesting that these cells may have not been affected by CCL19 or CCL21 treatment.

Directly infected resting cells (Lassen, et al. 2012)

The laboratory of Warner Greene further modified the approach of Swiggard, et al. by introducing reporter HIV strains[38]. The EGFP HIV strain is suited for assessing absolute levels of latently infected cells, while the *Δnef/luc* HIV can be used to measure the induction of transcription due to the latency reversing agent stimulation. The workflow involves infection by spinoculation, activation in the presence of antiretrovirals three days later, and the readout after an additional three days. Next to the short duration of the experiment, another major advantage of the model is the latency level, which routinely reaches 5-10%.

Co-culture of infected cells with resting cells (Spina, et al. 2013)

The model proposed by Spina, et al. combines the approach utilizing direct infection of resting cells with dividing, productively infected cells that mediate the direct transfer of the virus instead of spinoculation. Autologous CD4+ T cells are pre-activated with CD3/CD28 stimulation and infected with HIV before a co-culture with resting, uninfected cells is initiated. After four days of co-culture, the pre-activated cells are

removed and the remaining cells are maintained for an additional three days. Activation in the presence of ART reveals from 0.5 to 5% of inducible provirus, while a total of 1-12% of cells contain integrated HIV. This approach leads to the establishment of latency in naive, effector, and central memory T-cells[39].

Despite the variability between donors, the latency percentage is relatively high and the method of latency establishment can be considered physiologically relevant.

Resting cells infected with a dual-reporter virus (Chavez, et al. 2015)

The group of Eric Verdin recently published a novel approach to generate resting latently infected cells[40]. Upon isolation of $CD4^+$ T-cells from donor blood, the cells are either treated with CCL19 and IL-7 or non-treated and infected by spinoculation. The virus used is a dual-reporter strain with *egfp* under the control of LTR promoter and mCherry driven by elongation factor 1-α (EF1α) promoter. Productively infected cells express both fluorescent proteins, while latently infected cells are $mCherry^+$ only. These primary cells can also be activated with CD3/CD28 stimulation and challenged with HIV at different time points following the activation.

Consistently with previous reports, it has been observed that IL-7 and CCL19 (C-C motif ligand 19) treatment increases the permissivity of $CD4^+$ T-cells for HIV-1 infection, boosting the levels of both active and latent infection several fold. Importantly, latent infection was established both in directly infected resting cells and in pre-activated cells. The ratios between latent and active infection in cells with different activation status suggest that quiescent cells are more prone to harbor latent infection, while productive infection is more likely in activated cells. Additionally, the ratios of latent to active infection established in activated cells during the transition to the resting phenotype support this notion. These *in vitro* findings support both hypotheses of the mechanism of latency establishment: direct infection of resting cells and infection of activated cells before and during their transition to the quiescent phenotype, the former being more supportive in terms of latency levels.

The biggest drawback of the model is the low percentage of latently infected cells, which in none of the scenarios exceeds 1%. Sorting of $mCherry^+$ cells can be a way to overcome it. The dual-reporter virus pseudotyped with VSV-G envelope does not fully mimic the natural route of infection, but the results obtained by the group support the theories of latency establishment very well.

Comparison of models

Most laboratories focusing on HIV latency research need an *in vitro* model to study the topic, while the multitude of described models and the variability between them can be puzzling. The above-mentioned systems differ drastically and the decision of which model to use appears to be a difficult one. Two recent studies aimed at comparing the most commonly used approaches in terms of integration sites[5] and response to stimuli[39].

The study conducted by Spina, et al. was a multi-institutional effort aimed at investigating the response of the primary cell models by Lassen, et al., Saleh, et al., Bosque and Planelles, Yang, et al. and Spina et al., J-Lat cell line (Verdin, et al.) and *ex vivo* material from infected patients. A panel of 13 compounds including CD3/CD28 stimulation, PHA, PMA, hexamethylene bisacetamide (HMBA), TNF-α, SAHA, and bryostatin was tested across the models to assess the ability of these LRAs to reactivate latent infection.

Despite the fact that the models tested vary, it was possible to cluster them into three significant subsets. The first cluster included the models of Saleh, et al., J-Lat clone 5A8 and the viral outgrowth assay. Another cluster consisted of Bosque and Planelles, Yang, et al. and Lassen, et al. models, while the final one, differing from the closely related previous two clusters, was composed of three other J-Lat clones (6.3, 8.4, and 11.1). This analysis confirms the natural hypothesis that primary cell models (with the notable exception of J-Lat clone 5A8) would cluster together and be separated from a cluster of cell lines in terms of responsiveness to activating stimuli. Such clustering may also indicate the advantage of primary cell models based on mostly resting, *ex vivo* T-cells over proliferating, leukemia cell lines in this setting[39].

Another analysis in the same study focused on clustering the activating compounds. CD3/CD28 stimulation and PMA cluster together, which is consistent with the fact that they induce the strongest activation in most models, while PHA was the only compound reactivating latency in all the analyzed systems. It is important to note that not a single latency model fully responded to the tested panel of agents. This is due to the differences in experimental design between

proposed models, which translate into different mechanisms of latency establishment and patterns of reversal.

While little is known about the preference for proviral integration, it has been postulated that insertion of the viral genome into specific sites of the host cell may be a mechanism governing latency[5]. Sherrill-Mix, et al. extensively analyzed HIV integration sites in VSV-G pseudotyped HIV-infected Jurkat cells, J-Lat cells, Bcl-2 transduced CD4+ T-cells, the directly infected resting CD4+ T-cells, and infected primary central memory T-cells. The infected cells were separated into two subgroups based on the possibility to induce the provirus and over 6,000 integration sites from every group were analyzed. This study showed that slight differences in integration site distribution could be noticed between latently and productively infected cells. However, these differences were not conserved from model to model, and they appeared to be rather model-specific. Hotspots for integration leading to latency were not identified, although the previous notion that integration into alphoid repeats frequently lead to latency[41] was confirmed.

These two studies emphasize the notion that while a single latency model can be used for initial screening of compounds to target the reservoir or to study the mechanisms controlling latency, no single model is truly representative of the *in vivo* scenario. The extreme complexity of multiple pathways putatively governing latency cannot be fully recapitulated by any of these models. Only the concerted efforts of several laboratories can lead to meaningful conclusions helping to grasp the essence of the issue of *in vivo* HIV latency.

Discussion

There are many differences between the described primary HIV latency models. This variability leaves space for a choice of which model to use based on several key aspects or components that the models have in common.

Cell type and preconditioning

The basic classification of latency models is based on cells used to establish latent infection. Primary CD4+ T-cells can be implemented directly (most authors) or *in vitro* differentiated to present a particular quiescent cell phenotype. Recent advances in cell isolation and culture techniques permit the isolation of naive, central memory, general CD4+ T-cells and other important subsets directly from PBMCs. Most authors choose to pre-treat the cells, either with cytokines CCL-19 or CCL-21 or global T-cell activators PHA or CD3/CD28 stimulation to boost infection rates. After infection, the activated cells are cultured under conditions that foster a return to the quiescent state; this transition after infection is consistent with the theory that infection of activated cells followed by their return to the resting state constitutes a valid mechanism of latency establishment *in vivo*[42]. However, there is partial evidence that direct infection of resting CD4+ T-cells occurs in infected individuals too[43]. Direct infection is possible, but comes with limited permissiveness for HIV entry and replication, resulting in low levels of latently infected cells. Other methods to precondition cells and better mimic the *in vivo* situation are based on co-culturing the CD4+ T-cells with dendritic cells before infection[17] or upon infection[21] to precondition or increase infection rates, respectively.

Upon infection, cells need to be kept in culture for a prolonged time in order to enable the establishment of latency and/or to enable a return to a quiescent state. Under standard conditions, CD4+ T-cells die quickly in culture. Therefore, specific strategies are followed to increase the viability of these cells. Some authors add cytokines (Il-2 or Il-7), or enable cytokine production from feeder cells. Alternatively, T-cells can be immortalized by transduction with bcl-2. Cytokines are the most commonly used, but these may induce partial reactivation of the CD4+ T-cells and induce latency reversal. To ensure cellular quiescence, determination of the activation status based on the expression of markers such as CD25, CD38, CD69 or HLA-DR is possible. These markers can be used either to enrich for resting cells in the early stages of the protocol, or to investigate the return to cellular quiescence in later phases.

Infection and viral strains

Viral infection is one of the key steps in every latency model. Early models utilized direct infection performed by mixing viral supernatants with target cells. Studies emphasizing the beneficial role of spinoculation propagated its role in viral entry among most of the recent models. The availability of many wild type and laboratory strains of HIV opens several possibilities influencing the further steps of the workflow. Laboratory HIV strains can be classified into replication-competent and replication-deficient. The former more closely resemble the *in vivo* setting, but the presence

of the full HIV genome results in more viral replication and more cell death due to the cytopathic effects of viral production. Additionally, the spreading infection needs to be stopped with antiretroviral (ART) treatment to detect the relatively inefficient latent infection in an environment with abundant productive infection. The use of replication-deficient vectors limits cell death, overcomes the need for ART in the culture, and permits the choice of envelopes used to complement the viral particles generated. Here, HIV or non-HIV Env glycoproteins can be used; the former increase the relevance, but may recombine and form replication competent virus. Non-HIV envelope proteins will not recombine and broaden the viral tropism, which can boost infection rates. Attenuated viruses carrying one or more deletions in accessory genes are another alternative; a deletion in *nef* is a common choice to decrease the pathogenicity of HIV *in vitro*[44].

Another decision, as far as the virus is concerned, is whether or not to choose strains with reporter genes. The presence of mCherry, EGFP, or Luciferase largely facilitates the tracking of infection rates during the experiments and at the final readout. The lack of reporters is more relevant as HIV strains in infected patients do not carry such genes, but creates the need for more laborious protocols to assess production of viral proteins.

Analysis of HIV latency

To assess the levels of latency in primary HIV models, most researchers use a reactivation step, upon which the level of HIV production is compared to a fraction of non-stimulated cells.

Reactivation of viral production can be performed with several LRAs. In most systems, TCR stimulation by CD3/CD28 antibodies or PHA is the most efficient and most frequently used method to determine maximal reactivation[39]. Other activation strategies include PMA or IL-7 treatment, but due to their suboptimal efficiency, these are not the first choice in most models.

In the case of infection with a replication-competent virus, integrase and/or protease inhibitor treatment before the cellular activation is routinely performed. Such strategy prevents new integrations upon activation, and is used to distinguish between the signal originating from already integrated proviruses and integrations that would take place due to the activation. However, the use of integrase inhibitors may cause the accumulation of episomal HIV DNA that recombines to 1- or 2LTR circles[45]. Recent data indicate that these HIV episomes can transcribe HIV RNA and produce viral proteins[46]. Hence, a possible accumulation of episomal HIV DNA should be assessed by quantifying 2LTR circles and the use of an additional ART, e.g. a nucleoside reverse transcriptase inhibitor can be introduced to block new infections and to form episomal HIV DNA. Antiretrovirals are not normally added in experiments exploring replication-deficient viruses, although sometimes this is an additional control[40]. Depletion of cells expressing reporter genes as a consequence of HIV infection is an optional step before the activation.

The most common methods to assess HIV production is performed by flow cytometry or by PCR-based quantification of HIV RNA. Flow cytometry enables the direct assessment of the levels of reporter genes (e.g. EGFP, mCherry) or intracellular p24 staining when no reporter genes are available. The readout is expressed in percentages of positive cells. Additionally, fluorescence intensity can be linked to the expression levels of proteins of interest. The PCR-based analysis of HIV RNA transcription is more labor-intensive and the interpretation of results is more difficult. Unless combined with flow cytometry, it is impossible to state whether HIV RNA is produced in high quantities by a single reactivated cell or in small amounts by a larger number of cells. The biggest advantage of PCR readout is its high sensitivity compared to flow cytometry.

Next to HIV RNA measurement, integrated HIV DNA can be quantified to investigate the frequencies of cells carrying proviral DNA. However, not all integrated HIV provirus is replication competent[4]. Some earlier models took advantage of ELISA to quantify the levels of cellular or cell-free p24 protein; immunoenzymatic methods can be also used to measure the levels of Luciferase encoded by some viral strains. However, as discussed before, the representation of the results as a total score rather than expression per cell is a major drawback.

Conclusion

Despite the vast differences between primary latency models described, they all share common features. The basic components of the models are primary T-cells and HIV strains capable of at least one round of infection. Upon latency establishment, activation is performed and the results are read out with one of several available techniques. The minimum requirements for primary *in vitro* HIV latency models are assessed in figure 2 and outlined in figure 3. According to these scores, there is still room for further optimisation in some of the described primary latency models.

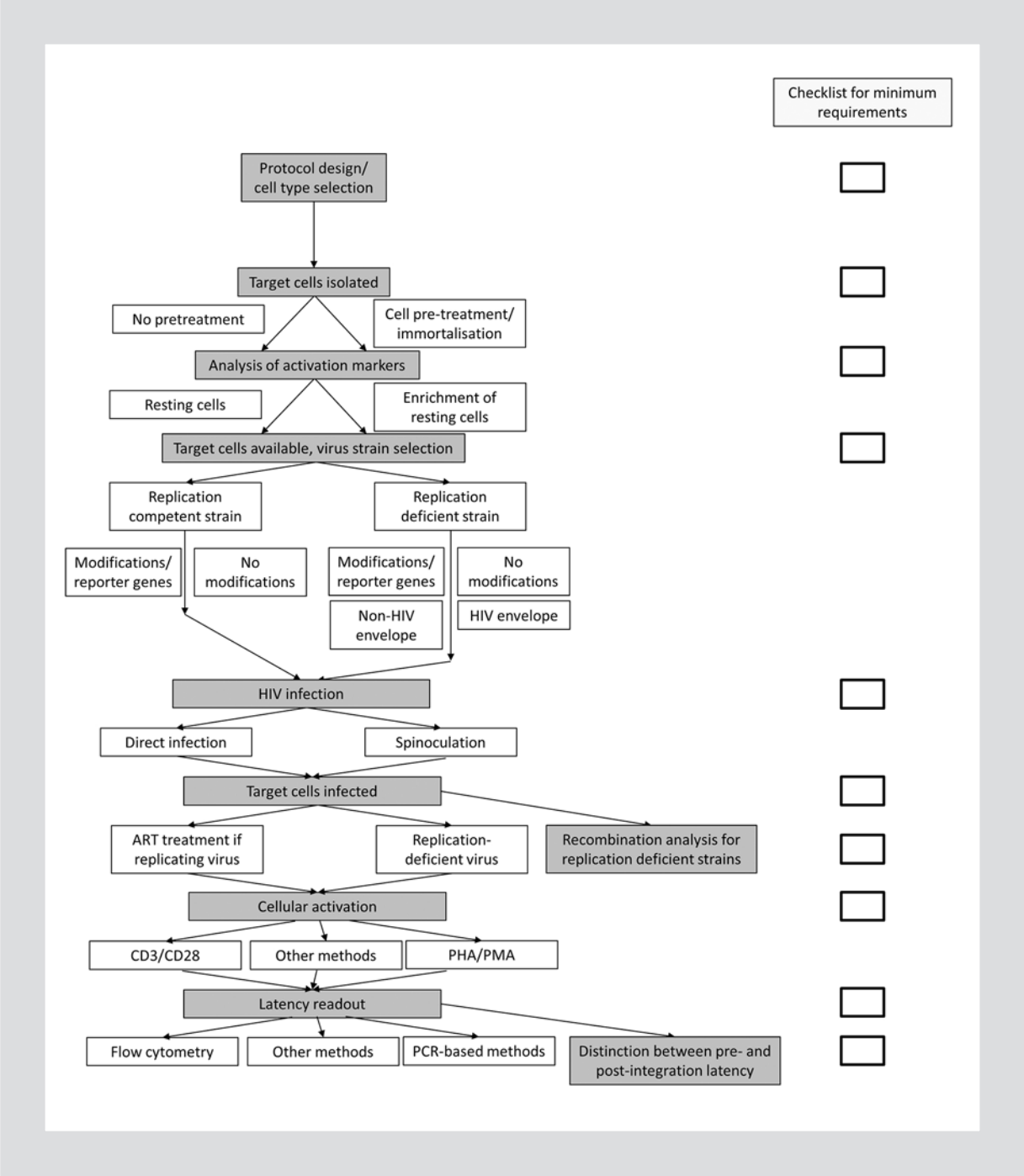

Figure 3. *Minimal requirements for primary HIV latency models with a checklist. The graph presents the common features of the described latency models and their basic components: primary T-cells and HIV strains capable of at least one round of infection, HIV infection, cellular activation and latency readout with one of several available techniques. ART: antiretroviral therapy; LRA: latency reversing agent; PHA: phytohemagglutinin; PMA: phorbol myristate acetate; PCR: polymerase chain reaction.*

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