AIDS Research and Human Retroviruses

DOI: 10.1089/AID.2018.0098

Influence of biological sex, age and HIV status in an *in vitro* primary cell model of HIV latency using a CXCR4 tropic virus

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Running Head: New insights in the T_{CM} model of HIV latency

Manuscript keywords: HIV, latency, reservoirs, primary cell models, biological sex, age

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Abstract

Primary cell models of HIV latency have become tools to both understand the mechanisms involved in the establishment of latency as well as to test pre-clinical strategies towards an HIV-1 cure. These models rely on the infection of CD4 T cells from healthy donors. As such, these models provide an opportunity to explore the role of biological sex, age and HIV status on the establishment and reactivation of latent HIV *in vitro*. We have used an established primary cell model of latency based on the generation of latently infected central memory CD4 T cells with the CXCR4-strain HIV-1_{NL4-3} to address whether these variables influence: i) HIV-1_{NL4-3} replication, ii) establishment of latency, and iii) latency reversal in CD4 T cells. Our results indicate that replication of HIV-1_{NL4-3}, but not the establishment of latency, is influenced by the age of female but not male donors. Moreover, the frequency of latently infected cells in this model is directly correlated with the levels of productive infection in both male and female donors independent of age. We did not find differences in the ability of five different latency-reversing agents (LRAs) to reactivate latent HIV-1_{NL4-3}. Finally, we have found that this model can be generated using cells from aviremic participants. In conclusion, we have further characterized the T_{CM} model of latency regarding biological sex and age and demonstrated that this model is suitable for use with cells isolated from aviremic participants, opening the opportunity to use this primary cell model to address cure approaches including 'shock and kill' in HIV-infected individuals.

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Introduction

Human immunodeficiency virus (HIV) has caused more than 35 million deaths worldwide. Management of the disease requires the daily administration of a combination of antiretroviral therapy (ART) drugs for the life of the infected individual. This is due to the presence of an intact and inducible latent reservoir of HIV that rebounds after discontinuation of ART therapy [1-3]. Elimination or reduction of this latent reservoir is crucial towards efforts to eradicate or control HIV. The frequency of the latent reservoir *in vivo* is limited (1-100 in a million resting CD4 T cells) [4-6]. The mechanisms by which HIV latency is established and maintained are not completely understood. In order to understand the mechanisms that control HIV latency, researchers have developed several latency models using either tumoral cell lines or primary cells isolated from HIV negative volunteers [7-22]. These models have led to the discovery of several mechanisms governing HIV latency, as well as the development of therapeutic strategies currently under clinical evaluation (Reviewed elsewhere [23-25]).

In this study, we used the cultured central memory T cell model (T_{CM} model) of HIV latency [19, 20] to investigate whether the donor's biological sex, age and HIV status influences the establishment of latency or its reactivation with latency reversing agents (LRAs) *in vitro*. We believe that these are important questions that have to be addressed as primary cell models of latency are becoming surrogates to both understanding the mechanisms of persistence as well as to test therapeutic strategies towards the latent reservoir. First, it is important to address whether any of the proposed strategies will be affected by the biological sex and age of future participants in a clinical trial. Interestingly, our study has found that viral replication of HIV-1_{NL4-3}, but not the establishment of latency, is influenced by the age of the donor cells only in female but not male donors. We further characterized the ability of five different LRAs to reactivate latent HIV-1_{NL4-3}. Our analysis suggests that the activity of these LRAs is independent of the biological sex. Furthermore, we demonstrate that this primary cell model can be performed in cells isolated from aviremic participants. This result suggest that the T_{CM} model can be used to specific evaluate LRAs in a patient-specific manner or to also evaluate "kill" strategies using syngeneic cytotoxic CD8 T cells or NK cells [26].

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Materials and Methods

Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: Nelfinavir, Human rIL-2 from Dr. Maurice Gately, Hoffman-La Roche Inc.[27], Raltegravir (Cat # 11680) from Merck & Company, Inc and HIV-1_{NL4-3} from Dr. Malcolm Martin[28]. Raltegravir was also from Selleckchem (Houston, TX). rIL-2 was also obtained from the NCI Preclinical Repository. Pam3CKS4 was from InvivoGen (San Diego, CA), HODHBt was from AK Scientific, Inc (Union City, CA), Ingenol-3,20-dibenzoate and Bryostatin-1 were from Enzo Life Sciences (Farmingdale, NY) and SAHA was from Cayman Chemical (Ann Arbor, MI)

Generation of latently infected T_{CM} cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood from healthy donors. T_{CM} and latently infected T_{CM} were generated as previously described [19, 20, 29]. Briefly, naïve CD4 T cells were isolated from healthy donors using the EasySep™ Human Naïve CD4+ T cell isolation kit (Stemcell Technologies). After isolation, naïve cells were plated at a density of 0.5×10^6 cells per ml of RPMI (Supplemented with 10% FBS, L-glutamine and Penicillin/Streptomycin) and activated with 12.5 μl of αCD3/αCD28-coated beads (Human T-Activator CD3/CD28 for T Cell Expansion and Activation Dynabeads, Dynal/Invitrogen, Carlsbad, CA), in the presence of 10 ng/ml of TGF-β1, 2 μg/ml of anti-Human IL-12 and 1 μg/ml of anti-Human IL-4 (all of them from Peprotech, Rocky Hill, NJ). Activation was performed in 96-well round plates with 100 μl per well to ensure homogeneous activation. After activation, cells were resuspended and Dynabeads were removed using a Magnetic Particle Concentrator (Dynal MPC®-L, Invitrogen). Activated cells were kept at 1×10^6 cells per ml in complete medium with 30 IU/ml of IL-2. Media and IL-2 was replaced at day 4 and 5. To generate T_{CM}, media and IL-2 was replaced at day 7, 10 and 13. Cell density was maintained at 1×10^6. To generate latently infected T_{CM}, cells were infected at day 7 using NL4-3. One fifth of the culture was left uninfected as a control. One fifth of the culture was infected with and MOI of 0.3 by spinoculation at 2900 rpm (1,741 × g) for 2 h at 37 °C. After infection, cells were mixed with the other three fifths of uninfected cells at 1×10^6 cells per ml in complete medium with 30 IU/ml of IL-2. At day

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10, media and IL-2 was replaced and the cells were cultured in 96 well round plates at 1 × 10^6 cells per ml with 100 μl per well to ensure cell-to-cell transmission. At day 13, cells were transferred to flasks, media and IL-2 were replaced and 1 μM of Raltegravir and 0.5 μM of Nelfinavir was added to the cultures to stop viral replication. At day 17, CD4 positive cells were isolated using Dynabeads® CD4 Positive Isolation Kit (Invitrogen 11331D) as indicated by the manufactured. The amount of CD4 beads was increased three-fold to allow efficient recovery.

Reactivation assays

1 × 10^5 cells were treated for each condition. As controls, cells were left unstimulated, treated with 30 IU/ml of IL-2 or reactivated with αCD3/αCD28-coated beads (1 bead per cell). For LRA experiments, cells were incubated with either 1 μM of Pam3CSK4, 335 nM SAHA, 100 nM Ingenol-3,20-dibenzoate, 100 nM Bryostatin-1 or 100 μM of HODHBt in the presence of IL-2. Viral reactivation was measured by assessing surface CD4 and intracellular $p24^{Gag}$ expression.

Flow cytometry analysis

For the dual detection of CD4 and HIV-1 $p24^{Gag}$, cells were first stained with the viability dye (Fixable Viability Dye eFluor 450), followed by staining with CD4 antibody (S3.5), APC conjugate (Molecular Probes™). After staining, cells were fixed, permeabilized, and stained for HIV-1 $p24^{Gag}$ as previously described [20]. In all experiments, CD4 positive HIV-1 $p24^{Gag}$ negative staining regions were set with uninfected cells treated in parallel. Flow cytometry was performed with a BD FacsCanto II or BD LSRFortessa flow cytometer using FACSDiva acquisition software (Becton Dickinson, Mountain View, CA). Data were analyzed with Flow Jo (TreeStar Inc, Ashland, OR).

Participant involvement

University of Utah – Donors 18 years and older served as volunteer blood donors. Written informed consent was obtained from all donors. These studies are covered under the institutional review board (IRB) #67637 protocol approved by the University of Utah Institutional Review Board.

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Gulf Coast Regional Blood Center - Volunteers 17 years and older served as blood donors. White blood cell concentrate (buffy coat) prepared from a single unit of whole blood by centrifugation were purchased.

Aviremic participants – Cells from aviremic participants were obtained through the Reservoir Characterization Section of the BELIEVE collaborative. Secondary use of the samples was approved through George Washington University Institutional Review Boards. All subjects were adults and gave informed consent.

Statistics

For intra-sex analysis, two-tailed paired-samples nonparametric t-test analysis (Wilcoxon matched-pairs signed rank tests) was used to calculate p-values. For inter-sex analysis, unpaired two-tailed nonparametric t-test analysis (Mann-Whitney test) was used to calculate p-values. Pearson correlation coefficients and two tailed p-values were calculated for correlations. All statistics were calculated using Prism 7 for Mac OS X software (GraphPad Software, Inc., La Jolla, CA).

Results

Biological sex and age as variables in HIV-1 replication

We first wanted to address whether biological sex and age were intrinsic variables impacting HIV-1_{NL4-3} replication in CD4 T cells. To address this question, we used the primary cell model of HIV infection and latency based on the generation of T_{CM} cells [19, 20]. To that end, naïve CD4 T cells isolated from 16 females and 15 males ranging from ages 17.2-65.7 were activated under conditions that generate central memory CD4 T cells (T_{CM}) [19, 30]. After activation, cells were infected with the replication-competent HIV-1_{NL4-3} and subjected to the protocol previously described (Figure 1A) [20, 31]. First, we wanted to address whether biological sex and age influence viral replication in T_{CM} *in vitro*. To that end, cells infected with replication-competent HIV-1_{NL4-3} were stained with fluorescently conjugated anti-CD4 antibodies, followed by an intracellular staining against $p24^{Gag}$ (Figure 1B, Day 10). At day 10, cells were crowded to allow cell-to-cell transmission for additional 3 days and stained for CD4 and $p24^{Gag}$ (Figure 1B, Day 13). As shown in Figure 2A, the percentage of infected cells increased in all donors regardless of sex. To assess whether there was a difference in viral replication at the cellular level, we calculated the replication

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ratio as the % of $p24^{Gag}$ positive CD4 negative cells at day 13 divided by those at day 10. This ratio allowed us to normalize viral replication to the initial spinoculation. There were no differences in viral replication in female versus male donors (Figure 2B). Interestingly, there is a negative correlation between the ability of HIV to replicate in female donors and the age of the donor. This correlation was not observed in experiments using cells from male donors.

Biological sex and age as variables in the establishment of HIV-1 latency

From day 13, a combination of 1 μM Raltegravir and 500 nM Nelfinavir was introduced in the cultures to block further viral replication (Figure 1A, 1B). At day 17, cells that remained CD4 positive (containing both latently infected and uninfected cells) were sorted based on CD4 expression (Figure 1B, Day 17 Post-sort). After sorting, cells were left unstimulated for 48 hours or stimulated with either IL-2 or αCD3/αCD28, and viral reactivation was measured by flow cytometry (Figure 1B). IL-2 induced a small degree of viral reactivation relative to unstimulated controls in both male and female donors (Figure 3A). Stimulation with αCD3/αCD28 reactivated latent HIV-1_{NL4-3} in all the donors tested (Figure 3A). No differences were observed between female and male donors with any of these reactivation conditions (Figure 3A).

In this model, there is a direct correlation between the levels of infection before ART at day 13, the levels of integrated provirus in resting T_{CM} at day 17, and the levels of cells that can reactivate latent HIV-1_{NL4-3} with αCD3/αCD28 at day 17 [20, 31]. We thus compared the levels of viral infection at day 13 (productive infection) with the levels of viral reactivation with αCD3/αCD28 (latent infection) in both female and male donors. There was a positive correlation between both productive and latent infection for both male and female donors (Figure 3B). To compare whether biological sex had an influence on the establishment of latency, we calculated the latency ratio as the percentage of latent infection divided by the percentage of productive infection. We did not observe a statistically significant difference between female and male donors (Figure 3C). In contrast with the replication ratio, there was not a correlation between the age of the donor and the latency ratio (Figure 3D).

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Biological sex as a variable in the reactivation of HIV-1 latency

A panel of five LRAs with different mechanisms of action were tested for their ability to reactivate latent HIV-1_{NL4-3} in this model (Figure 4). First, we have tested two protein kinase C (PKC) agonists, Ingenol-3,20-dibenzoate and Bryostatin-1 (Figure 4A and 4B). The main mechanism of action of these two agonists is the activation of NFκB in a PKC-dependent manner [32, 33]. In this primary cell model, Ingenol-3,20-dibenzoate was able to reactivate latent HIV-1_{NL4-3} in both male and female donors at similar levels that were superior to αCD3/αCD28 (average of 140.2%) (Figure 4A). This viral reactivation was independent of biological sex. As for Ingenol-3,20-dibenzoate, Bryostatin-1 was able to reactivate latent HIV in both male and female donors at similar levels (average of 79.8%) (Figure 4B). We then tested HODHBt, a novel inhibitor of STAT5 SUMOylation that reactivates latent HIV by increasing STAT5 transcriptional activity within the HIV LTR [34]. HODHBt also reactivated latent HIV in both male and female donors at similar levels (19.2% relative to αCD3/αCD28). Next, we tested the TLR-1/2 agonist Pam3CSK4 which reactivates latent HIV-1_{NL4-3} through the activation of NFκB in a MyD88-mediated manner [35, 36]. This TLR-2 agonist reactivated an average of 7.3% relative to αCD3/αCD28 and no differences were observed between female and male donors (Figure 4D). Finally, we tested the histone deacetylase inhibitor (HDACi) vorinostat (SAHA) [37, 38]. Our data indicated that the ability of SAHA to reactivate latent HIV in this primary cell model is modest, and an average of 4.2% relative to αCD3/αCD28, and no differences were observed between female and male donors (Figure 4E). In conclusion, biological sex of the donors did not influence the activity of the five LRAs tested in this model.

Generating latently infected cells in cells isolated from aviremic participants.

The T_{CM} model is based on the generation of latently infected cells in cells isolated from HIV negative donors. We wanted to explore whether this model could be adapted to *ex vivo* infect cells from aviremic HIV participants. This will allow better evaluation of 'shock-and-kill' strategies in cells isolated from aviremic HIV participants or to test the activity of LRAs prior to administration in clinical trials. To that end, naïve CD4 T cells from 5 male aviremic participants were isolated, activated and infected *ex vivo* to generate

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latently infected cells as previously described [19, 20]. We compared the ability of HIV-1_{NL4-3} to replicate and establish latency with that of male HIV negative donors. As shown in Figure 5A, levels of infection were similar between participants and their male donors counterparts. No statistically significance differences were observed in the ability of HIV_{NL4-3} to replicate (Figure 5B). Similar levels of latently infected cells were generated and able to reactivate in cells from aviremic participants and HIV negative donors (Figure 5C and 5D). Of interest, we were unable to detect any p24 positive cells in the *ex vivo* uninfected controls of cells isolated from aviremic participants (data not shown and [26]). These results indicate that it is possible to generate latently infected T_{CM} from aviremic participants and opens up the possibility to use the T_{CM} model to evaluate different cure strategies towards the latent reservoir.

Discussion

The search for an HIV cure is an uphill battle. Any strategy designed to target the latent reservoir must go through a series of validations in different models from cell culture, to animal models and, eventually, clinical trials. The more complex a model is, the fewer strategies that can be tested before reaching clinical trials (Figure 6). The path towards finding a cure is complex and primary cell models of latency can serve as reliable tools to help achieve this holistic goal.

In this work, we have further characterized the T_{CM} model of latency and answered two important questions relevant to the HIV cure field. First, we have characterized whether biological sex and age of the donor can influence the establishment or reactivation of the latent reservoir using the CXCR4-tropic strain HIV_{NL4-3}. We did not observe any significant difference between female and male donors in the ability of HIV_{NL4-3} to establish latency or to reactivate with 7 different stimuli: IL-2, αCD3/αCD28, Ingenol-3,20-dibenzoate, Bryostatin-1, HODHBt, Pam3CSK4, or SAHA. We did observe a negative correlation between the replication of HIV_{NL4-3} and age, and this correlation was specific to female donors. It is worth noting that this work does not take into account any extrinsic factor that may affect viral replication and latency. For example, sex hormones have been shown to influence HIV infection and immune responses in general [39, 40]. Whether and how 17 β-oestradiol (E2), progesterone (P4) or the androgens dihydrotestosterone and

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testosterone may affect our model of HIV infection is currently unknown. For example, androgens can repress the activity of NFκB in T cells [41]. NFκB is one of the principal transcription factors involved in HIV transcription [42]. Inhibiting NFκB by androgens may increase the establishment of latency and/or block the reactivation of latent HIV. In fact, 17β-estradiol has been shown to inhibit HIV replication through inhibition of HIV transcription in *in vitro* infected PBMCs [43]. Further investigation is warranted to determine how sex hormones influence HIV infection in this primary cell model of latency. Second, we have shown that this model can be performed in cells isolated from aviremic participants. This has wide implications for cure research and opens the way to test novel cure strategies besides latency-reversing agents. For example, this model can be used to directly test "shock-and-kill" strategies using syngeneic cytotoxic cells such CD8 T or NK cells It provides the opportunity to study relevant "shock-and-kill" strategies that use syngeneic CD8 or NK cells; or evaluate LRAs, combinations, or chimeric antigen receptors in a patient-specific, completely MHC-matched system. For example, we have recently shown that latently infected T_{CM} cells can be recognized by syngeneic Nef-specific CD8+ T cells [26]. Furthermore, addition of the Bcl-2 antagonist ABT-199 increased reservoir reduction when cells were reactivated with Bryostatin-1.

Primary cell models of HIV latency have made several important contributions to the field. In particular, the T_{CM} model help with the identification of two novel pathways to target the latent reservoir. First, we found that TLR-2 agonists can directly reactivate latent HIV in resting CD4 T cells from both the T_{CM} model as well as cells isolated from aviremic participants [35]. This is in line with multiple studies showing that TLR agonists can reactivate the latent reservoir and has contributed to the designing of clinical trials to assess the effects of these ligands on the latent reservoir [44-51]. The second pathway identified involves targeting SUMOylation of STAT5 [34]. We found that HODHBt and derivatives reactivate latent HIV by inhibiting SUMOylation of STAT5. Interestingly, this LRA displays no activity in cell lines, therefore its activity had most likely been overlooked in previous efforts made to identify LRAs in tumoral cell models. Besides identifying novel LRAS, the T_{CM} model has been also useful to characterize mechanisms involved in HIV persistence. We have previously shown that IL-7 can promote cell division in the absence

of viral reactivation, involving homeostatic proliferation as a mechanism of persistence [52]. This mechanism of persistence was first proposed by Chomont et al and has been recently proposed to be one of the mechanisms involved in clonal expansion of latently infected cells [53-55]. As clonal expansion of latently infected cells is becoming more apparent as a mechanism of persistence, it is important to understand the mechanisms that regulate this process [56, 57]. We have previously shown that the T_{CM} model of latency supports the generation of clonally expanded clones [58]. Therefore, this model represents an ideal tool to also understand clonal expansion.

In spite of its advantages, the T_{CM} model of latency has some caveats that will need further exploration. First, we are expanding the repertoire of molecular clones of HIV used beyond HIV_{NL4-3}. We are in the process of optimizing this model to use founder viruses and viruses obtained from the latent reservoir to better represent *in vivo* infections. Second, this model only generates central memory CD4 T cells. As latent viruses are found in other subsets of memory CD4 T cells, it will be important to address whether the same mechanisms found in T_{CM} are found in other subsets. We had previously shown that latently infected cells can be generated when cells are polarized in T_H1 and T_H2 [19]. The laboratory of Jonathan Karn has used a similar model to understand latency in T_H17 cells [17]. Whether the mechanisms controlling latency and reactivation in these subsets are shared with T_{CM} warrants further investigation.

In summary, we have further characterized the T_{CM} model of latency and address the influence of biological sex and age in this model. Additionally, we demonstrate that this model can be generated using cells from aviremic participants. Our work expands the multiple uses of this primary cell model of latency towards exploring both the mechanisms involved in HIV persistence, as well as to investigate therapeutic treatments to eradicate HIV.

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Competing interests

The authors declare no conflict of interest.

Acknowledgements

We would like to specially thank Dr. Mathilde Krim and The Foundation for AIDS Research (amfAR) for their support to A.B. in 2009 with a Mathilde Krim Fellowship (Grant ID 107402) in Basic Biomedical Research to study HIV latency and further develop the primary cell model used in this work in V.P. laboratory. We would like to thank Thomas Zaikos for the insightful comments during the preparation of the manuscript. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R21/R33 AI116212 and R01 AI124722 to A.B and BELIEVE (NIH grant 1UM1AI26617) to D.N.. M.A.S. was supported with a fellowship by the National Institutes of Health under Ruth L. Kirschtein National Research Service Award NIH 5T32 DK007115-40 from the National Institute of Diabetes and Digestive and Kidney Diseases. This research has been facilitated by the services and resources provided by the District of Columbia Center for AIDS Research, an NIH funded program (AI117970), which is supported by the following NIH Co-Funding and Participating Institutes and Centers: NIAID, NCI, NICHD, NHLBI, NIDA, NIMH, NIA, FIC, NIGMS, NIDDK, and OAR. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure Legends

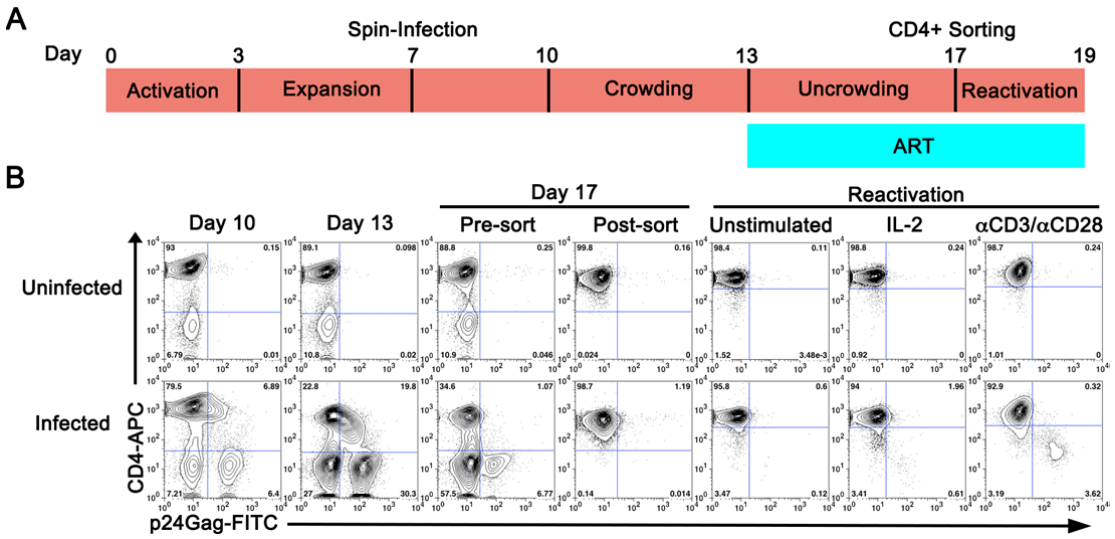

Figure 1. Outline of the T_{CM} model of HIV latency. (**A**) Time line of the assay. (**B**) Surface CD4 expression and intracellular $p24^{Gag}$ was measured at different time points after infection and after reactivation of latent HIV-1_{NL4-3}. Analysis is representative of the experiments presented in this work.

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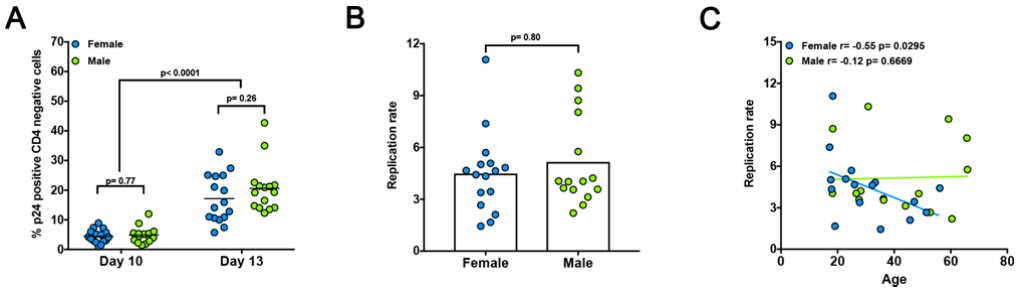

Figure 2. Analysis of viral replication in the T_{CM} model. (**A**) Percentage of infected cells measured as $p24^{Gag}$ positive CD4 negative at day 10 and day 13 measured in CD4 T cells from 16 female and 15 male donors following the protocol in Figure 1. Replication ratio (**B**) was calculated from values in (**A**). (**C**) Correlation between the replication ratio and the age of the donors stratified by biological sex. Two-tailed paired-samples nonparametric *t*-test analysis was used to calculate *p*-values between days. Unpaired two-tailed nonparametric t-test analysis (Mann-Whitney test) was used to calculate p-values between biological sex. Correlations were determined using the Pearson correlation coefficient.

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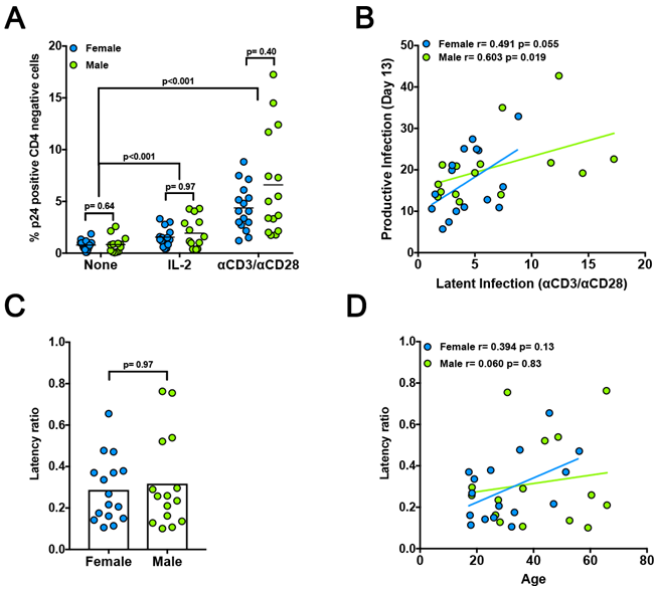

Figure 3. Influence of biological sex and age in HIV-1_{NL4-3} latency and reactivation in T_{CM}. (**A**) Percentage of reactivated cells measured as $p24^{Gag}$ positive CD4 negative after treatment for 48 hours with IL-2 or αCD3/αCD28 compared with unstimulated cells from latently infected cells generated in Figure 1. (**B**) Correlation between the percentage of $p24^{Gag}$ positive CD4 negative cells during productive infection (day 13) with those during latent infection (αCD3/αCD28) stratified by biological sex. Latency ratio (**C**) was calculated from values in (**B**). (**D**) Correlation between the latency ratio and the age of the donors stratified by biological sex. Two-tailed paired-samples nonparametric *t*-test analysis was used to calculate *p*-values between stimuli. Unpaired two-tailed nonparametric t-test analysis (Mann-Whitney test) was used to calculate p-values between sex. Correlations were determined using the Pearson correlation coefficient.

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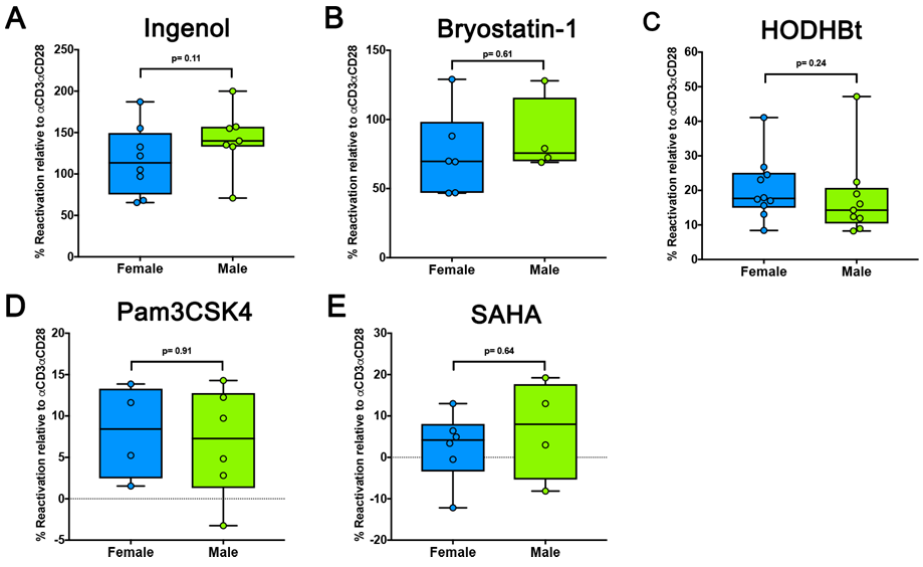

Figure 4. Influence of biological sex in latency reversal with 5 different LRAs. Latently infected cells were generated from healthy donors and the ability of (**A**) 100 nM Ingenol-3,20-dibenzoate; (**B**) 100 nM Bryostatin-1; (**C**) 100 μM HODHBt; (**D**) 1 μM Pam3CSK4; and (**E**) 335 nM SAHA to reactivate latent HIV-1_{NL4-3} was assessed. Viral reactivation over IL-2 alone for each treatment was normalized to that of αCD3/αCD28. Unpaired two-tailed nonparametric t-test analysis (Mann-Whitney test) was used to calculate p-values between sex.

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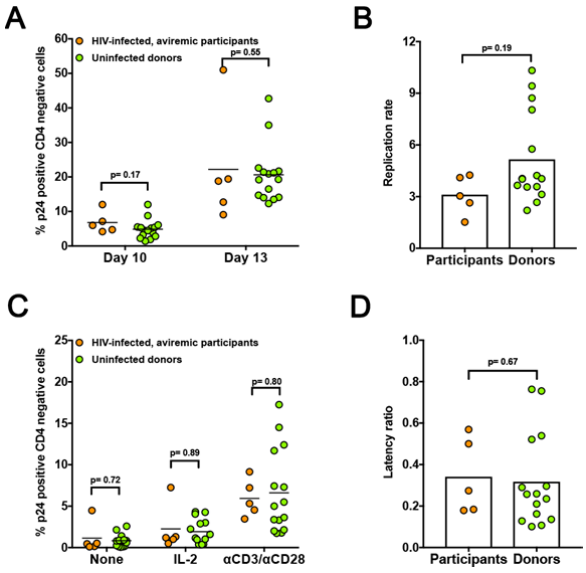

Figure 5. Generation of latently infected cells in cells isolated from aviremic participants. (**A**) Percentage of infected cells measured as $p24^{Gag}$ positive CD4 negative at day 10 and day 13 measured in CD4 T cells from 5 male participants and 15 male donors following the protocol in Figure 1. Data from HIV negative donors is the same as in Figure 1. Replication ratio (**B**) was calculated from values in (**A**). (**C**) Percentage of reactivated cells measured as $p24^{Gag}$ positive CD4 negative after treatment for 48 hours with IL-2 or αCD3/αCD28 compared with unstimulated cells form latently infected cells generated in (**A**). Latency ratio (**D**) was calculated from values in (**C**). Unpaired two-tailed nonparametric t-test analysis (Mann-Whitney test) was used to calculate p-values between sex.

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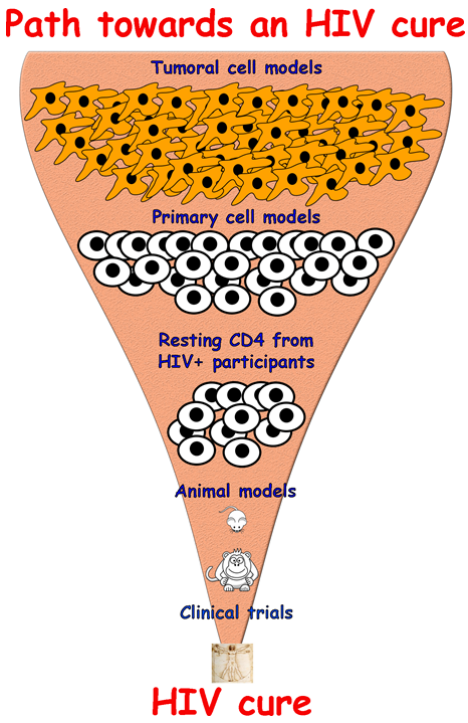

Figure 6. Path towards an HIV cure. Therapeutic strategies towards an HIV cure go through a series of validations in different models before reaching clinical trials. These models include tumoral cells, primary cells, cells isolated from aviremic participants and animal models. The increase complexity of these models reduces the range of therapeutic strategies that can be tested in each one.