NOVEL STRATEGIES TOWARDS TARGETING THE LONG-TERM HIV-1 LATENT RESERVOIR

by

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ABSTRACT

Thirty years after the isolation of HIV, despite significant advances in the understanding of the mechanisms of viral pathogenesis, a cure remains out of sight. Despite the effectiveness of combination antiretroviral therapy (ART) at preventing virus replication, a stable, transcriptionally silent viral reservoir within resting memory $CD4^+$ T cells persists and hampers HIV elimination. The current thinking in the field is that a combination of a hypothetical drug that reactivates latent viruses, with current antiretroviral therapy, will be an effective approach toward viral eradication.

Several vaccination regimens and pathogen infections have been shown to correlate with a transient increase in the levels of plasma RNA in HIV-1 infected patients even in the presence of ART. Hence, it is tempting to speculate that exposure to microbial products either by vaccination or infection may trigger reactivation of latent viruses. Pathogen infections are primarily sensed through pattern recognition receptors. Toll-like receptors, the most well investigated pattern recognition receptors, are important sentinels of innate host defense. These sensors recognize structural patterns from pathogens and induce an immune response through downstream signaling cascades that, among others, culminate in the activation of NF-kappaB and other transcription factors. Since the viral promoter contains two tandem NF-kappaB binding sites, it is possible to

speculate that toll-like receptor signaling toward reactivation of latent HIV has recently gained impetus. In this dissertation, I review the emerging body of literature on the correlation between NF-kappaB signaling, as triggered by toll-like receptor engagement, and the reactivation of latent HIV-1. Second, I present our findings that Pam3CSK4, a toll-like receptor-1/2 agonist, can reactivate latent HIV in primary $CD4^+$ T cells *in vitro* and in cells from aviremic patients. Finally, I described the ability of dynasore, a dynamin inhibitor, to activate latent HIV-1 provirus alone or in synergy with Pam3CSK4, and with well-known latency-reversing agents, such as SAHA, bryostatin-1 and JQ-1.

HIV latency is a complex phenomenon controlled by different molecular mechanisms. Although several latency-reversing agents have been described in the literature, the efficacy of these agents to eliminate the transcriptionally silent reservoir in past clinical trials has been limited. Toll-like receptor signaling represents a novel tool in our armamentarium against latent HIV, and therefore deserves further exploration. Throughout the body of this work, I define two novel approaches that could provide us with new therapeutic interventions towards the eradication of the latent reservoir and a cure for HIV/AIDS.

To those who encouraged me in this great adventure.

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The work presented in this dissertation would not have been completed without the great support that I have received from many people over the years.

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CHAPTER 1

TARGETING THE LONG-TERM HIV-1 RESERVOIR VIA TOLL-LIKE RECEPTOR SIGNALING

1.1 Introduction

HIV-1 infection remains incurable due to the persistence of long-lived latently HIV-1-infected T cells that constitute a stable latent viral reservoir [1-3]. This reservoir is the main reason why viremia is reestablished when ART is interrupted [4-7]. It is well known that the HIV-1 viral reservoir is established during the earliest stages of HIV infection, when immune responses against the virus are developing [8-10]. Among all T $CD4^+$ subsets, central memory T cells (T_{CM}) followed by transitional memory T cells (T_{TM}) are the major reservoirs for latent HIV [11, 12]. Quiescent memory $CD4^+$ T cells have a life span of months to years. In average, the half-life of these cells harboring a latent HIV provirus has been predicted to be 44 months [1]. The longevity of this pool has been attributed to its capacity of self-renewal by antigen-driven and homeostatic proliferation [11, 13-16]. Elimination of these cells has become a priority towards designing therapeutic strategies aimed to eradicate HIV-1 [17-19].

Maintenance of silent integrated HIV-1 in the host cell genome at the transcriptional level is mainly regulated at two levels. The first level of regulation is through chromatin modifications. Epigenetic marks affecting chromatin are histone acetylation, histone methylation and DNA methylation. In the case of histone acetylation, it has been shown that histone deacetylases (HDACs) repress LTR transcription by reducing acetylation of histones [20-22]. Low levels of histone acetylation are correlated with a closed chromatin conformation that blocks the recruitment of transcription factors [23]. Based on this notion, HDAC inhibitors are being studied as potential latency reversing agents (LRA). Several

HDAC inhibitors have been reported to reactivate transcriptionally silent HIV, including vorinostat (SAHA), valproic acid, romidepsin and panobinostat [24-30].

Histone methylation can induce or repress transcription depending on the target site [31]. Histone methyltransferases (HMTs), such as Suv39H1 and G9a, were found to silence HIV-1 transcription in association with H3K9 methylation of histones positioned at the LTR [32-34]. Accordingly, Chaetocin, a fungal metabolite and an inhibitor of Suv39H1, has been shown to induce latent HIV-1 expression in Jurkat T cells [35]. Furthermore, a specific inhibitor of G9a, BIX01294, reactivated latent HIV in ACH2 and OM10.1 cell lines [34]. Most importantly, both HMT inhibitors induced HIV-1 recovery from resting $CD4^+$ T cells isolated from HIV-1 aviremic patients [36].

DNA methylation has also been implicated in silencing of HIV-1 transcription [37]. DNA methylation of CpG islands promotes the formation of condensed heterochromatin leading to local gene silencing [38]. It has been shown that CpG islands flanking the HIV-1 transcription promoter are hypermethylated in latently infected Jurkat cells and primary $CD4^+$ T cells [39]. In this study, Kauder and collegues showed that Aza-CdR, a cytosine methylation inhibitor, reactivated HIV-1 in JLAT cells, a Jurkat model of HIV-1 latency [40]. However, another study by Fernandez and Zeichner showed that Aza-CdR antagonized the activation of HIV expression by TNF-alpha in JLAT 10.6, ACH2, U1 and J1.1 latently infected cell lines [41]. Blazkova and colleagues observed that resting $CD4^+$ T cells from ART-suppressed HIV infected patients carry very low levels of DNA methylation [42]. These results are in contrast with a previous

study which reported high levels of DNA methylation in HIV-1 chronically infected Jurkat cells, in *in vitro* infected primary $CD4^+$ T cells and in memory $CD4^+$ T cells from HIV aviremic patients [43]. The issue of whether methylation of the HIV-1 promoter is required for transcriptional suppression is still controversial, and will need further consideration.

The second level of regulation involves the scarcity of nuclear transcription factors in quiescent $CD4^+$ T cells that restrict HIV transcription initiation and elongation. Several transcription factors have been associated with HIV expression. Among those, NF-κB, NFAT, AP-1 and Sp1 have been shown to have a role in reactivating latent viruses [44-48]. In resting $CD4^+$ T cells, both NF-κB and NFAT are sequestered in the cytoplasm. NF-κB and NFAT translocation to the nucleus is regulated via I kappa-B interaction and the calcineurin/calmodulin axis, respectively [49, 50]. Nuclear translocation restriction of these transcription factors has been proposed to contribute to the latency state of HIV [51, 52].

Protein kinase C (PKC) activators are a wide group of LRAs that signal inducing NF-κB translocation to the nucleus, allowing HIV reactivation [53]. Including in this group are: phorbol-12-myristate-13-acetate (PMA), Prostatin, Bryostatin-1 and Ingenol. PMA, a T cell activator but a tumor-promoting compound, and prostatin, a nontumor-phorbol agent, reactivate HIV expression from aviremic patients [54]. Both phorbol esters were found to activate NF-κB [55]. Bryostatin-1, which has a different structure from other PKC activators and is characterized as an anti-tumor agent, also activates HIV expression [56].

Bryostatin-1 has been shown to induce increasing levels of intracellular HIV-1 mRNA in an *ex vivo* viral outgrowth assay [57]. Also, Ingenol, a natural compound from Euphorbia plant species and structurally similar to phorbol esters, drives NF-κB nuclear translocation through PKC activation. In addition, Ingenol derivatives reactivate HIV from latently infected cell lines (ACH2, U1 and J1.1), human PBMCs infected with HIV *in vitro* and $CD4^+$ T cells isolated from aviremic HIV infected patients under ART treatment [58, 59].

A major inducer of NF-κB activation is the family of toll-like receptors (TLR). In this review, we discuss how activation of TLR signaling can contribute toward HIV-1 reactivation strategies and viral clearance.

1.2 Review

1.2.1 Overview of the Toll-like receptor family

Toll-like receptors (TLRs) belong to the family of pattern recognition receptors and sense pathogen-associated molecular patterns (PAMPs) of bacteria, parasites, fungi and viruses. In addition, TLRs also recognize danger-associated molecular patterns (DAMPs), such as nuclear or cytosolic cell components that are released or mislocalized due to tissue damage [60].

TLRs are type I transmembrane glycoproteins composed of an extracellular leucine-rich-repeat (LRR) motif that mediates ligand recognition, a transmembrane domain, and a cytoplasmic Toll/IL-1R (TIR) responsible for signaling. To date, 10 and 12 TLR members have been identified in humans and in mice, respectively. TLRs-1, -2, -4, -5, -6 and -10 are expressed on the cell

surface, while TLRs-3, -7, -8, -9, -11, -12, -13 are found within endosomes. Cell surface TLRs interact with microbial cell wall components and intracellular TLRs interact with nucleic acids. TLR-2 forms homodimers and also heterodimers with TLR-1, TLR-6 and TLR-10, and these complexes recognize a broad variety of ligands such as lipoproteins, peptidoglycans, lipotheicoic acids and ligands from Listeria. TLR-3 recognizes double-stranded RNA (dsRNA). TLR-4 recognizes lipopolysaccharide (LPS), a major component of the outer wall of Gram-negative bacteria. TLR-5 recognizes flagellin, a constituent of bacterial flagella. TLR-7 and TLR-8 are highly homologous; their ligands are mainly single stranded RNA (ssRNA) and imidazoquinoline derivatives. TLR-9 recognizes unmethylated CpG-DNA [61, 62] (Figure 1.1). TLR-11, TLR-12 and TLR-13 are the mouse-specific TLRs. TLR-11 and -12 recognize profiling-like proteins from apicomplexa parasites, such as *Toxoplasma gondii* and *Plasmodium falciparum* [63, 64]. TLR-13 interacts with bacterial 23S rRNA [65-67]. TLRs are preferentially expressed in immune cell types, such as macrophages, dendritic cells, B cells, T cells, NK cells, mast cells, but also detected in fibroblasts and epithelial cells (reviewed in [68]).

1.2.2 Stimulation of the TLR pathway

The signaling cascades downstream of TLR activation are complex. Adaptor proteins such as myeloid differentiation primary-response protein 88 (MyD88), TIR-domain containing adaptor protein (TIRAP, also known as MAL), TIR-domain containing adaptor protein inducing IFN-betta (TRIF) and TRIF-

related adaptor molecule (TRAM) are recruited to the TIR domain after interaction with ligands. With the exception of TLR-3, all TLRs signal through the MyD88-dependent pathway. TLR-2 and 4 require TIRAP in order to recruit MyD88 to start signal transduction, while TLR-5, 7, 8 and 9 initiate signaling using uniquely MyD88 [69, 70]. After recruitment of MyD88, a complex is formed with IRAK1 and IRAK4. IRAK1 is phosphorylated and associates with TNF receptor-associated factor 6 (TRAF6), which activates TAK1. TAK1 activates, by phosphorylation, two routes: the IKK-NF-κB pathway and the mitogen-activated protein kinases (MAPK) pathway. In the first route, TAK-1 phosphorylates IκBalpha/beta. This phosphorylation leads to their degradation through the proteasome system and the release of NF-κB, which translocates to the nucleus, binds to DNA and initiates transcription. In the second route, TAK1 activates the MAPK members ERK, JNK and p38, which lead to activation of transcription factor AP-1 [61, 62]. In plasmocytoid dendritic cells (pDCs), TLR-7 and TLR-9 activates MyD88 signaling that phosphorylates interferon regulatory factors 7 (IRF7), which regulates expression of IFN-alpha [71] (Figure 1.1).

The alternative pathway, often called MyD88-independent pathway, is mediated via TRIF. TLR-3 interacts directly with TRIF to initiate signaling, while TLR-4, when endocytosed, requires the adaptor TRAM to facilitate association with TRIF [72, 73]. TRAF6 and TRAF3 are recruited by TRIF. While TRAF6 engages IKK and MAPK, leading to activation of NF-κB, AP-1 and IRF7, TRAF3 recruits TBK1/IKK-epsilon complex that activates IRF3, which culminates in IFN-beta expression [74, 75] (Figure 1.1).

1.2.3 Reactivation of the latent HIV-1 provirus through TLR signaling

It has been reported that HIV-1 patients exposed to vaccination regimens [76-79] or microbial infections [80-84] have increased HIV-1 RNA plasma levels when compared to control individuals. Furthermore, exposure to several pathogen-associated molecular patterns (PAMPs) and their corresponding microorganisms have been shown to directly transactivate the HIV-1 LTR. For example, it has been described that purified protein derivative (PPD) of *M. tuberculosis* increased viral mRNA expression in HIV infected monocytes [85]. Furthermore, monocytoid cell lines stimulated with live *M. tuberculosis* or lipomannan (LAM) increased p24 expression by 3-fold and enhanced HIV-1 LTR transcription [86]. Additionally, it has been shown that *M. tuberculosis* PPD from H37Ra strain and the mycobacterial major cell wall component ManLAM activated transcription of HIV-1 in the $CD4^+$ T cell line Jurkat. Man-LAM-induced HIV gene expression was mediated via protein kinases that culminated in NF-κB nuclear translocation. Mutations in the NF-κB binding sites in the HIV-1 LTR abolished the HIV expression increase driven by PPD [87, 88].

HIV-1 induction mediated by mycobacteria has been shown to be dependent on TLR-2 stimulation. Bhat *et al.* observed that the *M. tuberculosis* and *M. smegmatis* proline-proline-glutamic acid protein Rv1168c (PPE17) interacts with TLR-2, resulting in activation of NF-κB and HIV-1 transactivation [89]. Furthermore, Bafica and collegues crossed an HIV-1 transgenic mouse with either TLR-2-deficient or control mice to investigate the role of TLR-2 in the activation of HIV-1 expression. Culture filtrate proteins, phosphatidyl-inositol

mannoside from *M. tuberculosis* and the synthetic lipopeptdide Pam3CSK4, induced p24 expression in spleen cells from HIV-1 transgenic mouse expressing TLR-2. On contrary, p24 induction mediated by the above stimuli was completed impaired in spleen cells from TLR-2 deficient mice. Also, induction of HIV-1 by mycobacteria *in vivo* was 2-fold greater in control mice when compared with TLR-2-deficient mice [90].

Our group recently reported that Pam3CSK4, a potent activator of TLR-1/-2, reactivates latent HIV in an *in vitro* assay using latently infected primary T_{CM} $CD4^+$ cells and in *ex vivo* assays using resting $CD4^+$ T cells from aviremic patients. HIV reactivation through the TLR-1/2 pathway via Pam3CSK4 induces phosphorylation of CDK9, it is Tat-dependent and requires NF-κB, AP-1 and NFAT transcription factors. Also, Pam3CSK4 reactivates HIV in the absence of cell activation and/or proliferation [91]. Reactivation of HIV without global T cell activation is desirable as a potential therapy against latent HIV, thus Pam3CSK4 anti-latency efficacy should be highly considered.

Gonzalez *et al.* published that total extracts from *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, oral bacterial pathogens, induced significant HIV-1 promoter activation in latently infected monocytic leukemia cell lines (BF24 and THP89GFP). Also, neutralization of TLR-2 in these cells reduced HIV transcriptional enhancement by the bacterial extracts. Reactivation of HIV-1 promoter by *F. nucleatum* and *P. gingivalis* was mediated by NF-κB and Sp1 transcription factors [92].

Lipopolysaccharide (LPS), the major TLR-4 agonist, has been shown to

mediate HIV-1 activation in a chronically infected monocytic cell line [93]. Also, LPS induced the LTR promoter in human dermal endothelial cells (HMEC) transfected with an HIV-1 luciferase reporter via MyD88/TRAF6/NF-κB signaling [94]. It is important to point out that effect of LPS in reactivation of the HIV-1 promoter has been disputed by others groups [91, 92, 95]. Divergent results could be explained by the use of LPS that could be contaminated with other PAMPs, like bacterial lipopeptides. Other possible reasons for the discrepancy could be variation in the cell type used, the cell culture environment and/or disparities in TLR-4 binding affinity to LPS from different bacteria.

Flagellin, the structural protein in bacterial flagella, has been demonstrated to reactivate latent HIV in a T lymphocyte cell line chronically infected with HIV (JLAT and Jurkat clone E6-1) and in central memory T cells, previously infected with VSV-G pseudotyped NL43. Flagellin achieved this effect via TLR-5 stimulation. However, resting CD4+ T cells from aviremic patients, when challenged with flagellin, failed to elicit detectable levels of viral gene expression [96].

R-848, a TLR-7/-8 agonist, induced p24 expression in latently infected monocytic cell lines (U1 and OM10). Interestingly, TLR-7/-8 activation through ssRNA and R-848 (a guanosine derivative) interfered with the HIV replication cycle in lymphocyte cultures, potentially through the secretion of inhibitory soluble factors [97]. Therefore, TLR-7/-8 signaling may have two beneficial effects, namely stimulation of latent proviruses and, simultaneously, inhibiting viral spread.

Finally, DNA from *F. nucleatum* increased HIV-1 promoter activity through TLR-9 signaling [98]. CpG interaction with TLR-9 induced up-regulation of HIV p24 in the chronically infected monocytic cell line, U1, and viral gene expression in human epithelial kidney cells transfected with a HIV-luciferase reporter [95]. Furthermore, Scheller *et al.*, has reported that the TLR-9 agonist, cytosine-phosphodiester-guanine oligodeoxynucleotide (CpG ODN), reactivated HIV in the latently infected cell line ACH2 in an NF-κB-dependent manner [99].

1.2.4 Modulation of HIV-host immune responses by TLR stimulation

In order to purge the HIV-1 reservoir, reactivation of the latent provirus has to be followed by death of the infected cell through viral cytotoxic effects and/or host immune responses. It has been recently reported that the histone deacetylase (HDAC) inhibitor SAHA, shown to induce HIV expression in latently infected $CD4^+$ T cells, failed to reduce the size of the viral reservoir *in vitro* in cells isolated from aviremic patients [100]. In this study, Shan and colleagues demonstrated the importance of CTLs to clear the HIV-reservoir, emphasizing the necessity to enhance cellular immunity in order to achieve viral control [100]. In addition, elite controllers are able to spontaneously control HIV infection, mainly explained by their enhanced $CD8^+$ T cells, and still debated natural killer (NK), ability in suppressing replication of HIV [101, 102],

TLR stimulation can enhance CTL responses. For example, stimulation of $CD8^+$ T cells and NK anti-tumoral responses is enhanced by the polysaccharide krestin, a TLR-2 agonist [103]. Furthermore, murine $CD8^+$ T cell cytotoxic

responses were amplified by a TLR-3 ligand, bypassing the requirement for $CD4^+$ T cell help [104]. Also, TLR-3, TLR-7 and TLR-9 agonists were shown to enhance NK cell activity [105, 106].

Several studies have investigated the use of TLR signaling to modulate anti-HIV immune responses. The TLR-7/-8 agonist (3M-012, an analog of R-8480) has been given as vaccine adjuvant in combination with HIV-1 Gag antigen to nonhuman primates. The addition of 3M-012 to the Gag vaccine substantially enhanced Gag-specific T helper 1 and $CD8^+$ T cell responses compared to animals given the Gag protein alone [107]. In addition, PBMCs treated with TLR-7/-8 agonists (3M-002 or R-848) undergo activation of $CD8^+$ T cells and NK cells, as estimated by the appearance of the surface markers CD69 and CD107a, respectively [108].

A randomized controlled vaccine trial conducted with 95 HIV-infected subjects investigated the impact of TLR-9 agonist as an adjuvant for pneumococcal vaccine. The trial showed that the TLR-9 agonist, CpG ODN enhanced vaccine immunogenicity in the experimental group compared with the control group [109]. Post-hoc analyses of the vaccine trial confirmed that patients that received TLR-9 ligand as adjuvant expressed more CD107a and macrophage inflammatory protein 1β (MIP1β) markers in the $CD8^+$ T cell. In addition, a statistically significant reduction in proviral HIV-1 DNA was observed in the CpG-recipient HIV group compared with the control group [110]. While encouraging, TLR-9 agonist only reduced the latent reservoir in a minimal change of 12.6% in proviral load. After 1st and 2nd immunizations, p value was

calculated as nonsignificant; after 3rd immunization and total immunization change (data summarizing difference of proviral load between placebo and all three immunizations together), data showed p values of 0.056 and 0.023, respectively. Although this study did not present a dramatic depletion in the latent reservoir, the sum of this effect with the stimulation of CTL responses via TLR-9 agonist drives attention to the use of TLR agonists for clearance of the latent reservoir.

1.3 Conclusions

Toll-like receptors agonists, as proposed in the literature, can activate HIV transcription in latently infected cells. Eradication strategies point to the elimination of infected cells followed by reactivation as a crucial step. As reviewed, several TLR agonists efficiently activate CTLs, being a promising anti-HIV feature of TLR agonists. Understanding how TLR agonists modulate HIV expression and anti-viral immune responses may lead to therapeutic strategies toward viral clearance.

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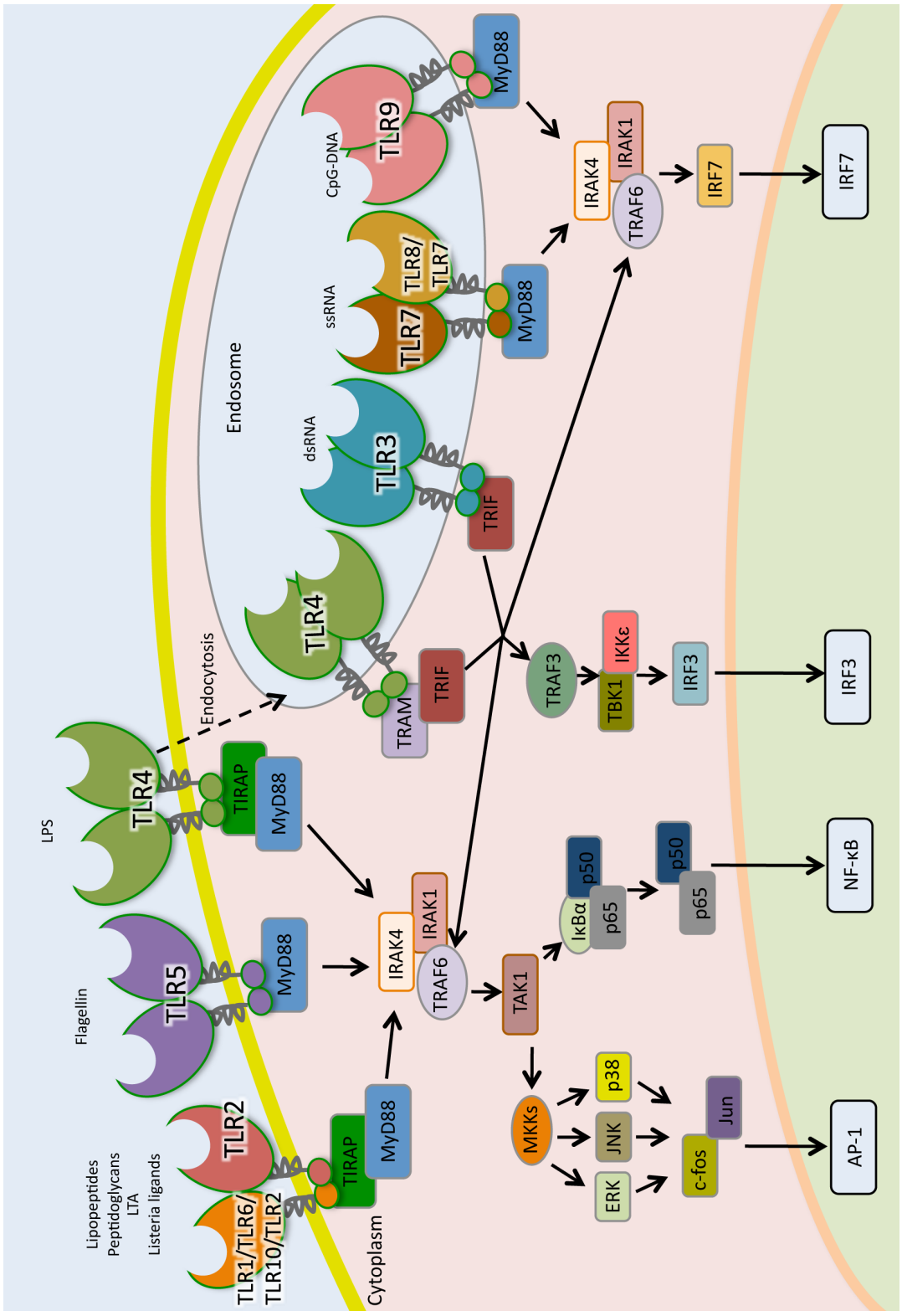
Figure 1.1: Human toll-like receptors signaling pathways.

TLRs are the sentinels of host defense. The homodimers TLR5, TLR4 and TLR2 and the heterodimers TLR2-TLR1, TLR2-TLR6 and TLR2-TLR10 bind to their specific ligand at the cell surface, whereas TLR3, TLR7, TLR7-TLR8 and TLR9 localize to the endosomes, where they interact to their ligands. TLR4 following microbial detection is endocytosed into the endosome. When TLRs are activated by interaction with their ligands, adaptor molecules are recruited to stimulate downstream signaling pathways.


Lipopeptides
Peptidoglycans
LTA
Listeria ligands
TLR1/TLR6/
TLR10/TLR2
TLR2
TIRAP
MyD88
Flagellin
TLR5
MyD88
LPS
TLR4
TIRAP
MyD88
Endocytosis
Endosome
CpG-DNA
TLR9
MyD88
ssRNA
TLR7
TLR8/
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TLR4
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IRAK4
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TRAF6
TAK1
MKKs
p38
JNK
ERK
c-fos
Jun
AP-1
IκBα
p50
p65
p50
p65
NF-κB
TRAF3
TBK1
IKKε
IRF3
IRF3
IRAK4
IRAK1
TRAF6
IRF7
IRF7

CHAPTER 2

REACTIVATION OF LATENT HIV-1 IN CENTRAL MEMORY CD4+ T CELLS THROUGH TLR-1/2 STIMULATION

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http://www.retrovirology.com/content/10/1/119

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Reactivation of latent HIV-1 in central memory CD4$^+$ T cells through TLR-1/2 stimulation

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Abstract

Background: Toll-like receptors (TLRs) are crucial for recognition of pathogen-associated molecular patterns by cells of the innate immune system. TLRs are present and functional in CD4$^+$ T cells. Memory CD4$^+$ T cells, predominantly central memory cells (T_{CM}), constitute the main reservoir of latent HIV-1. However, how TLR ligands affect the quiescence of latent HIV within central memory CD4$^+$ T cells has not been studied.

Results: We evaluated the ability of a broad panel of TLR agonists to reactivate latent HIV-1. The TLR-1/2 agonist Pam3CSK4 leads to viral reactivation of quiescent HIV in a model of latency based on cultured T_{CM} and in resting CD4$^+$ T cells isolated from aviremic patients. In addition, we investigated the signaling pathway associated with Pam3CSK4 involved in HIV-1 reactivation. We show that the transcription factors NFκB, NFAT and AP-1 cooperate to induce viral reactivation downstream of TLR-1/2 stimulation. Furthermore, increasing levels of cyclin T1 is not required for TLR-mediated viral reactivation, but induction of viral expression requires activated pTEFb. Finally, Pam3CSK4 reactivates latent HIV-1 in the absence of T cell activation or proliferation, in contrast to antigen stimulation.

Conclusions: Our findings suggest that the signaling through TLR-1/2 pathway via Pam3CSK4 or other reagents should be explored as an anti-latency strategy either alone or in combination with other anti-latency drugs.

Background

The existence of latent reservoirs of HIV-infected cells constitutes the major impediment towards viral eradication. Latent infection is associated with undetectable levels of viral gene expression and appears to be non-cytopathic. However, upon reactivation, latent viruses enter an active mode of replication in which they are fully competent for spread and induction of disease [1-3]. The main latent reservoir is known to reside within the subset of CD4$^+$ memory T cells [1-5]. The current thinking in the field is that a combination of agents that disrupt latency ("anti-latency" drugs), when given with continuous antiretroviral therapy (ART), may be an effective approach toward viral eradication [6-8].

Transient bursts or "blips" of HIV-1 replication occur even in patients whose virus is well suppressed by antiretroviral therapy (ART) [9]. The origin of viral "blips" is not known. Several factors can contribute to these viral blips, such as selection of drug resistant variants, antigen-driven target cell activation, vaccination, opportunistic infections or random variation of test measurements [10]. Interestingly, several vaccination regimens [11-14] and pathogen infections [15-19] have been shown to transiently increase the levels of plasma RNA in HIV-1 infected patients even in the presence of ART. Therefore, it is tempting to speculate that exposure to microbial products may trigger reactivation of latent viruses and thus influence the size of the latent reservoir.

Pathogen infections are primarily sensed by the innate immune system through the interaction of conserved molecular structures named pathogen-associated molecular patterns (PAMPs) via host-encoded pattern recognition receptors (PRRs) [20,21]. PRRs are germline-encoded receptors that recognize several classes of molecules typical of pathogens, such as proteins, lipids, carbohydrates and nucleic acids [21]. Among PRRs, Toll-like receptors (TLRs) are the most widely studied. TLR-1, 2, 4, 5, 6 and 10 are present on the cell surface and recognize PAMPs derived from bacteria, fungi and protozoa. Whereas, TLR-3, 7,

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8 and 9 are present in endosomal compartments and recognize mainly nucleic acids derived from bacteria and viruses [21,22]. TLRs have been detected on cells of both the innate and adaptive immune system (such us dendritic cells, macrophages, granulocytes, T cells, B cells, NK cells and mast cells) as well as endothelial and epithelial cells [23].

However, little is known about whether and how TLR ligands affect the latent reservoir of HIV infection in central memory $CD4^+$ T cells. We have analyzed the potential ability of TLR agonists to transactivate the HIV-1 LTR using a previously described method for the generation of latently infected central memory T cells (T_{CM}) [24,25]. We demonstrate that Pam3CSK4, a TLR-1/2 agonist, is able to reactivate latent HIV-1 in this *in vitro* model and in cells isolated from aviremic patients. This reactivation is NFκB, NFAT and AP-1-mediated and require pTEFb activity. This pathway differs from that initiated by T cell receptor engagement, which was shown to be mediated, in the same latency model, primarily by NFAT [24].

Importantly from the standpoint of therapeutic applications, Pam3CSK4-induced viral reactivation is achieved in the absence of T cell activation and proliferation. Therefore, the signaling pathway activated by Pam3CSK4 appears to be selective for latent, integrated viruses and represents an attractive therapeutic target that can be exploited in eradication strategies.

Results

Pam3CSK4, a TLR-1/2 agonist, reactivates latent HIV-1 in cultured T_{CM} cells

We explored whether TLR agonists could reactivate latent HIV-1 using cultured T_{CM} as model of latency [24,25]. We used representative agonists for the different TLR receptors such as triacylated synthetic lipopeptide Pam3CSK4, a TLR-1/2 agonist; diacylated synthetic lipopeptide (FSL-1), a known TLR-2/6 agonist; the synthetic analog of double-stranded RNA (Poly(I:C)), recognized by TLR-3; lipopolysaccharide (LPS), the principal component of Gram negative bacteria that activates TLR-4; flagellin, a potent stimulator of TLR-5; imiquimod, an analog to guanosine that specifically activates TLR-7; ssRNA40, a GU-rich single-stranded RNA oligonucleotide also known as R-1075, recognized by TLR-7/8 [26] and ODN2006, an unmethylated CpG dinucleotide and activator of TLR-9. As shown in Figure 1A, the triacylated lipopeptide and TLR-1/2 agonist Pam3CSK4 was able to efficiently reactivate latent HIV in latently infected cultured T_{CM} generated from 5 different donors. No other TLR agonist tested had activity above background. In order to verify that the above agonists were biologically active at the concentrations used, we performed four additional tests. First, we tested the above TLR agonists in their ability to reactivate latent HIV-1 in three other models of latency. Second, we tested their ability to induce IL-8 production in the promonocytic cell line THP-1 [27]. As shown in Additional file 1: Figure S1A, Flagellin was able to reactivate latent HIV-1 in the J-Lat clone 10.6 as previously described [28]. Even though the TLR-2/6 agonist Pam2CSK4 and the TLR-4 agonist LPS were unable to reactivate latent HIV-1 in cultured T_{CM}, both were able to induce a strong IL-8 response in THP-1 cells (Additional file 1: Figure S1B). The TLR7 agonist imiquimod was able to reactivate latent HIV-1 in the T cell line ACH2 (Additional file 1: Figure S1D). Finally, the TLR-9 agonist ODN2006 was able to reactivate latent HIV-1 in the cell lines U1 and ACH2 (Additional file 1: Figure S1C and D) [29]. Neither the TLR-3 nor the TLR-8 agonist had activity in any of our experimental systems. We do not disregard the possibility that these TLRs may reactivate latent viruses.

TLR-2 recognizes a variety of molecular patterns from viruses, bacteria, fungi and protozoa [21]. The specificity of PAMP recognition by TLR-2 is somewhat broad because TLR-2 can form functional homodimers, as well as heterodimers with TLR-1, TLR-6, TLR-10 or Dectin-1 [30-33]. To test which TLR-2 homo or heterodimers are capable of reactivating latent viruses in cultured T_{CM} cells, we incubated latently infected cells with ligands for TLR-2 that use different co-receptors. We tested the synthetic diacylated lipopeptide Pam2CSK4 and the N-terminal part of the diacylated lipoprotein derived from *Mycoplasma salivarium*, FSL-1, both of which have been shown to induce signaling through a TLR-2/6 complex [34], although some of these diacylated lipopeptides can also induce signaling in the absence of TLR-6 [35]. We also tested zymosan, a β-glucan present in yeast cell wall, which uses the Dectin-1/TLR-2 complex as receptor [33]. Finally, we tested lipoarabidomannan of *Mycobacterium smegmatis* (LAM-MS) and polysaccharide A of *Bacteroides fragilis* (PSA). These last two have been shown to induce signaling mainly through TLR-2 alone [36,37]. As shown in Figure 1B, the TLR-1/2 agonist Pam3CSK4 was the only TLR-2 agonist able to induce reactivation of latent HIV-1 in cultured T_{CM}. We confirmed the activities of all TLR-2 agonists to induce IL-8 production in THP-1 cells (Additional file 2: Figure S2B). Furthermore, Zymosan behaved as a strong inductor of HIV-1 expression in the cell line J-LAT 10.6 (Additional file 2: Figure S2A). Finally, Pam3CSK4, Pam2CSK4 and Zymosan were able to reactivate latent HIV-1 in the U1 cell line (Additional file 2: Figure S2C). These results suggest that signaling through TLR-2/TLR-1 complexes, but not other TLR-2-containing complexes, are able to reactivate latent HIV-1 in cultured T_{CM}. Thus, we conclude that signals that use TLR-2 alone or TLR-2 in combination with TLR-6 or Dectin-1 are not sufficient to reactivate latent HIV-1 in cultured T_{CM}.

We next decided to analyze the expression levels of TLR-1 and TLR-2 in cultured T_{CM} and in *ex vivo*

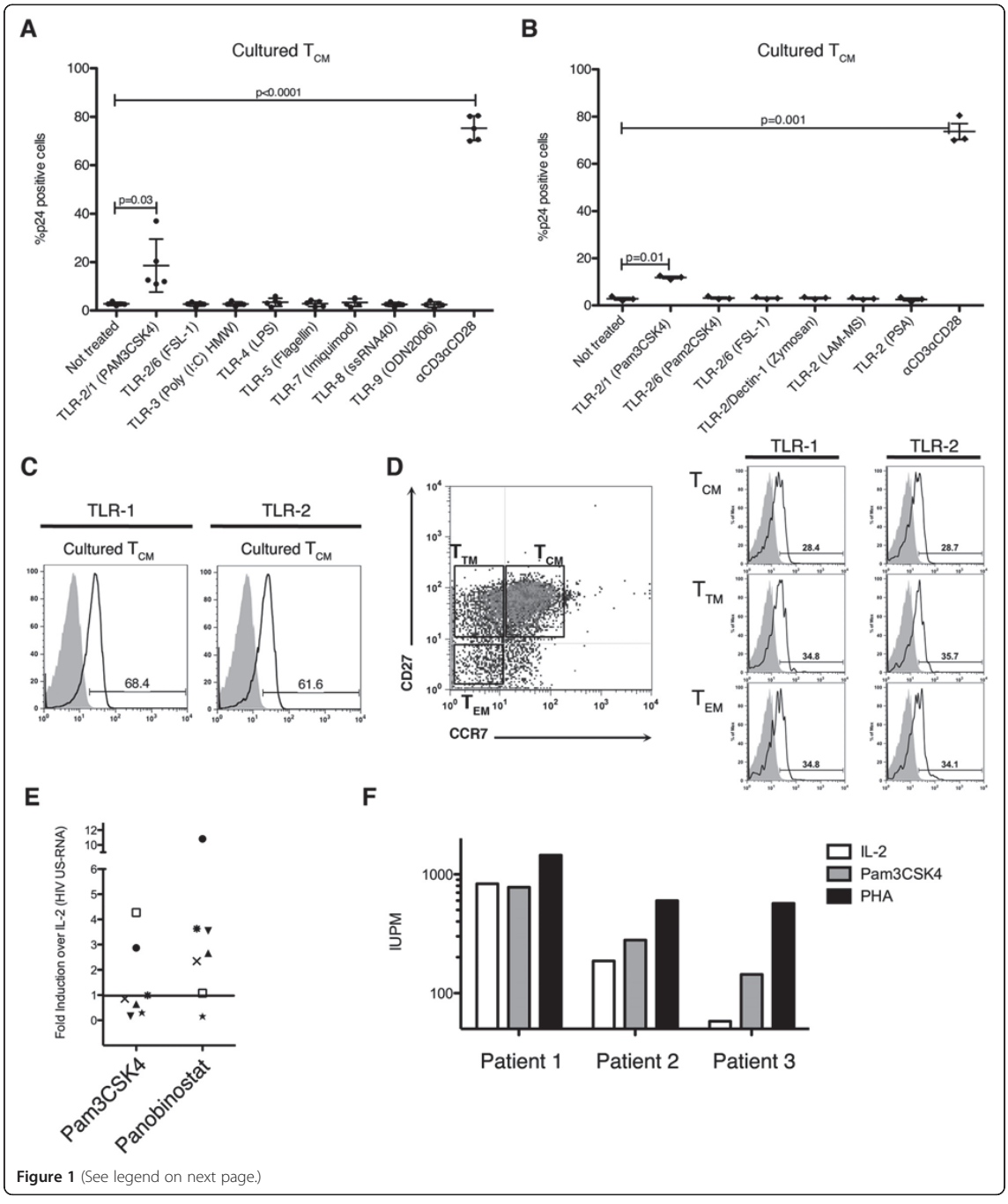

Figure 1 (See legend on next page.)

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Figure 1 Reactivation of latent HIV-1 through toll-like receptors agonists. (A) Cultured T_{CM} were treated with the toll-like receptors agonists indicated between parentheses for different TLRs or costimulated with αCD3/αCD28 and assessed for intracellular p24Gag expression by flow cytometry. Experiments were done in 5 different donors. Each dot represents a different donor and mean and SD are indicated with horizontal lines. Significance was calculated by 2-tailed paired samples *t* test analysis (P vales provided). **(B)** Cultured T_{CM} were treated with different TLR-2 agonists indicated between parentheses or costimulated with αCD3/αCD28 and assessed for intracellular p24Gag expression by flow cytometry. Experiments were done in 3 donors from A. Significance was calculated by 2-tailed paired samples *t* test analysis (P vales provided). **(C)** Cultured T_{CM} were stained with specific antibodies against TLR-1 and TLR-2 (open black histogram) and analyzed by flow cytometry. Isotype controls were used as control (closed grey histogram). The percentage of TLR-1 and TLR-2 positive cells is indicated in each panel. **(D)** *Ex vivo* isolated memory $CD4^+$ T cells were stained with specific antibodies against CCR7, CD27, TLR-1 and TLR-2 and analyzed by flow cytometry. Expression of TLR-1 and TLR-2 was analyzed in each subset of memory $CD4^+$ T cells (open black histogram). Isotype controls were used as control (closed grey histogram). The percentage of TLR-1 and TLR-2 positive cells in each subset is indicated in each panel. **(E)** Cells isolated from seven aviremic patients were treated with Pam3CSK4 or Panobinostat and the levels of HIV-1 US RNA were measured three days later. Each symbol corresponds to a different patient. Data is represented as fold induction over the IL-2 treatment control **(F)** Cells isolated from three aviremic patients were treated with IL-2, Pam3CSK4 or PHA for 24 hours and supernatants were subject to the Q-VOA assay.

isolated memory $CD4^+$ T cells from healthy donors. As shown in Figure 1C, we detected surface expression of both, TLR-1 and TLR-2, in cultured T_{CM}. Also, both receptors were expressed comparably in the three main subsets of memory $CD4^+$ T cells, namely T_{CM}, T_{TM} and T_{EM} (Figure 1D).

To further confirm whether Pam3CSK4 was able to reactivate latent HIV-1 *ex vivo*, we performed two different assays using resting $CD4^+$ T cells from aviremic patients. In the first one, cells were treated with IL-2, IL-2 plus Pam3CSK4 or IL-2 plus panobinostat, an HDAC inhibitor that has been shown to reactivate latent HIV-1 [38]. Three days after treatment, intracellular HIV-1 unspliced mRNA was quantified by RT-PCR. As shown in Figure 1E, Pam3CSK4 increased the levels of US-RNA over IL-2 treatment control in 2 of the 7 patients compared with 5 of 7 for panobinostat. In one of the patients, neither Pam3CSK4 nor panobinostat was able to reactivate latent HIV over IL-2 control (Figure 1E, closed start symbol). In the second assay, cells from 4 aviremic patients were subjected to the quantitative viral outgrowth assay (Q-VOA) assay [39,40]. In this case, exposure to Pam3CSK4 allowed recovery of replication competent virus from resting $CD4^+$ T cells of 2 ART-suppressed patients, although, as is generally seen with HDAC inhibitors, the frequency of induction of HIV outgrowth was greater after cells were fully activated by mitogen (Figure 1F). In the fourth patient, the frequency of latent infection was very low (less than 1 infected cell in 5 million resting $CD4^+$ T cells), and the activity of Pam3CSK4 could not be assessed.

Pam3CSK4 induces viral reactivation through the activation of NFκB, NFAT and AP-1 transcription factors

We decided to investigate the signaling pathway that leads to viral reactivation mediated by Pam3CSK4 in cultured T_{CM}. First, we analyzed the cis-acting elements in the viral long-terminal repeat (LTR) required for viral reactivation induced by Pam3CSK4 and compared them with those required for αCD3/αCD28. This was accomplished by generating defective (*env-)* HIV mutants with nucleotide substitutions in the binding sites for NFκB/NFAT, Sp1 or NF-IL6 as previously described [24]. As shown in Figure 2A, mutation of the three binding sites for Sp1 abrogated viral reactivation mediated by Pam3CSK4 and by αCD3/αCD28. Mutation of both NFκB binding elements impaired the reactivation by Pam3CSK4 by 80% on average. Interestingly, in one of the three donors, mutation of NFκB binding sites did not disrupt viral reactivation by αCD3/αCD28. This result suggests that other transcription factor binding sites may bypass the presence of intact NFκB/NFAT binding sites in viral reactivation mediated by αCD3/αCD28, as it has been previously described [41]. When cells were infected with a virus containing mutations in the NFκB/NFAT and Sp1 binding sites, viral reactivation induced by both stimuli was also almost completely abrogated. As a control, we mutated both NF-IL6 binding sites and showed that mutation in these cis-acting elements had almost no effect on viral reactivation driven by Pam3CSK4 and αCD3/αCD28. These results indicate that efficient Pam3CSK4-induced viral reactivation requires the presence of intact NFκB/NFAT and/or Sp1 binding sites on the LTR.

To further characterize the signaling pathway that leads to viral reactivation induced by Pam3CSK4 in cultured T_{CM}, we used chemical inhibitors of known signaling pathways activated by TLRs. It is known that TLR-1/2 activates NFκB *via* MyD88 and the subsequent formation of a complex with IRAK1/IRAK4 and TRAF6. This complex leads to the activation of the IKK complex through the kinase TAK1 and the phosphorylation and degradation of IκBα leading to the activation and translocation to the nucleus of NFκB [22]. To assess the role of NFκB in viral reactivation induced by Pam3CSK4, we incubated cells with BAY 11–7082, an inhibitor of IκBα phosphorylation. As shown in Figure 2B, BAY 11–7082 blocked an average of 60% of viral reactivation induced by Pam3CSK4. However, BAY 11–7082 had a minor inhibitory effect (less than 20% inhibition on average) on viral reactivation induced

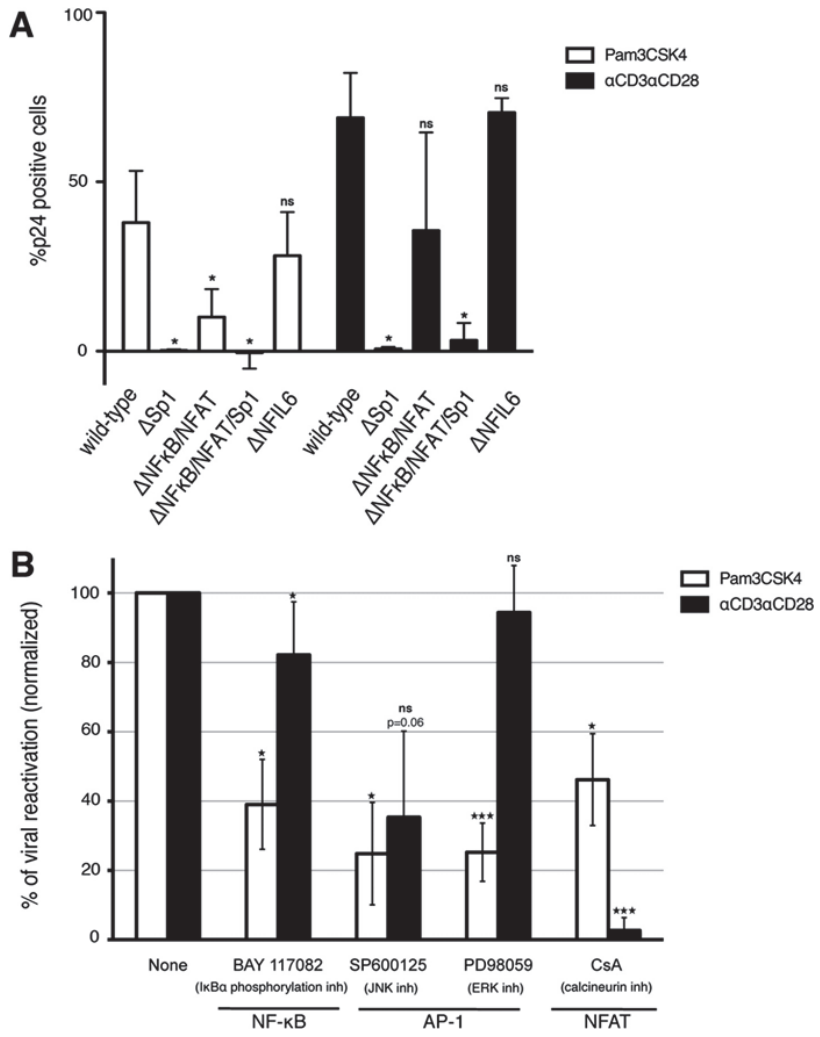

Figure 2 Pam3CSK4 requires NFAT, AP-1 and NF-κB to induce viral reactivation in cultured T_{CM}. (A) Cultured T_{CM} cells were infected with wild-type DHIV or with different LTR mutants. 9 days after infection, cells were treated with Pam3CSK4 or costimulated with antibodies to CD3 and CD28 (αCD3/αCD28) for 3 days and assessed for intracellular p24Gag expression by flow cytometry. Percentage of reactivation was normalized to that of wild-type DHIV for each treatment. Bar graph corresponds to mean and SD of experiments performed with three donors. **(B)** Cultured T_{CM} cells were infected with wild-type DHIV and treated with Pam3CSK4 or stimulated with αCD3/αCD28 in the presence of the indicated inhibitor for the protein or transcription factor indicated and assessed for intracellular p24 Gag expression by flow cytometry. Bar graph corresponds to mean and SD of experiments performed with four different donors. Significance was calculated by 2-tailed paired samples *t* test analysis (* < 0.05, ** < 0.01, *** < 0.001).

by αCD3/αCD28 (Figure 2B). This result is in agreement with our previous studies indicating an unexpected lack of requirement for NFκB toward viral reactivation after stimulation with αCD3/αCD28 [24]. However, in a signaling pathway other than αCD3/αCD28, we showed that NFκB was active and required for reactivating latent viruses in a stimulus-dependent manner in cultured T_{CM}.

MyD88 activation also leads to the activation of the MAP kinase cascade, in particular JNK and ERK-1/2, which leads to the activation of the transcription factor AP-1. Cooperation between these transcription factors, NFκB and AP-1, has been shown to transactivate the HIV-1 LTR in cells lines [42,43]. To investigate the role of AP-1 in viral reactivation induced by Pam3CSK4, we incubated the cells with SP600125 or PD98059, known

inhibitors of JNK and ERK-1/2 respectively [44,45]. As shown in Figure 2B, both inhibitors significantly abrogated viral reactivation by Pam3CSK4.

We have previously shown that NFAT is required for viral reactivation mediated by αCD3/αCD28 in cultured T_{CM} [24]. In agreement with that finding, Cyclosporine A (CsA), a potent NFAT inhibitor, blocked viral reactivation induced with αCD3/αCD28 (Figure 2B). Interestingly, CsA was able to impair viral reactivation mediated by Pam3CSK4 by 55% on average. These data suggest NFAT is also required for viral reactivation downstream of Pam3CSK4.

In an effort to validate the experiments performed with chemical inhibitors, we investigated whether Pam3CSK4 could induce nuclear translocation of NFκB, NFAT and AP-1 in cultured T_{CM} in a time-dependent manner. To that end, we isolated cytoplasmic and nuclear fractions after stimulating with IL-2 alone (baseline condition), Pam3CSK4 or αCD3/αCD28. We isolated fractions of cultured T_{CM} that were unstimulated; after 30 minutes of stimulation; or 3 hours of stimulation. The translocation of the different transcription factors was analyzed by Western-blot. α-tubulin and histone H3 proteins were used as controls for purity of the fractionation and β-actin was used as a loading control. As shown in Figure 3A, p50 was detected in the nucleus after 30 minutes of incubation with Pam3CSK4 (compare lanes 8 and 11) but not with IL-2 alone (compare lane 8 with 9 and 10) or αCD3/αCD28 (compare lane 8 with 13 and 14) but decreased to basal level by 3 hours of stimulation. In the case of p65, increased nuclear levels could be detected after treatment of the cells with Pam3CSK4 at 30 min and 3 hours post-reactivation (lanes 11 and 12 compared with lane 8). Interestingly, both Pam3CSK4 and αCD3/αCD28 induced the nuclear translocation of NFATc1, which was detectable at 30 min and 3 hours (Figure 3A, lanes 11 to 14 compared with lane 8). We also detected a strong nuclear translocation of NFATc2 when cells were reactivated with αCD3/αCD28 (lanes 13 and 14 compared with lane 8). Nuclear translocation of NFATc2 was also detected after treatment of the cells with Pam3CSK4 but at a much smaller magnitude (lanes 11 and 12 compared with lane 8).

We have previously shown that JNK and ERK inhibitors blocked viral reactivation mediated by Pam3CSK4 (Figure 2B). JNK and ERK are involved in the activation respectively of cJun and cFos, components of the transcription factor AP-1. As shown in Figure 3B, we detected nuclear translocation of cJun at 3 hours with either IL-2, Pam3CSK4 or αCD3/αCD28 stimulation (lanes 10, 12 and 14 compared with lane 8). The levels of nuclear translocation were slightly higher with Pam3CSK4 than with IL-2 alone (Figure 3B, compare lanes 10 and 12). Surprisingly, we were unable to detect nuclear translocation of cFos at any time with either IL-2 or Pam3CSK4 (Figure 3B, lanes 9 to 12). The inability to detect efficient nuclear translocation of cFos and cJun with Pam3CSK4 may be due to the low sensitivity of the assay. For this reason, we decided to analyze whether Pam3CSK4 could activate JNK and ERK. As shown in Figure 3C, treatment with Pam3CSK4 resulted in increased JNK and ERK phosphorylation (lane 5) above what was observed with IL-2 alone (lane 2). These results suggest than Pam3CSK4 can efficiently induce NFκB and NFAT translocation into the nucleus. Furthermore, Pam3CSK4 induces activation of the JNK and ERK pathways required for AP-1 activation.

Pam3CSK4 but not other TLR2 agonist triggers intracellular Ca^{2+} influx

We found that NFAT was translocated into the nucleus after challenging cultured T_{CM} with Pam3CSK4. NFAT activation is induced after dephosphorylation mediated by the Ca^{2+}/calmodulin-dependent serine phosphatase calcineurin, which is activated after an increase in the levels of intracellular Ca^{2+}. Therefore, we inferred that NFAT activation by Pam3CSK4 is a consequence of an increase in the intracellular levels of Ca^{2+}. To test this idea, cultured T_{CM} were loaded with Fluo3-AM, a fluorescent indicator of intracellular Ca^{2+}, and changes in intracellular levels were measured by flow cytometry. We incubated cells with the ionophore ionomycin (positive control), or the TLR-2 agonists Pam3CSK4, Pam2CSK4 or LAM-MS. As expected, ionomycin induced an increase in the intracellular levels of Ca^{2+} (Figure 4). When cells were treated with Pam3CSK4, we observed an increase in the intracellular levels of Ca^{2+} in a dose dependent manner. However, this increase in calcium levels was not observed after treatment with the non-HIV reactivating agents Pam2CSK4 or LAM-MS.

Taken together, these data suggest that Pam3CSK4 increases intracellular Ca^{2+} flux, which is consistent with NFAT nuclear translocation and its role on HIV-1 reactivation mediated by Pam3CSK4. Furthermore, the inability of Pam2CSK4 and LAM-MS to increase intracellular Ca^{2+} may explain their failure to transactivate the HIV-1 LTR (Figure 1B).

Pam3CSK4 reactivates HIV-1 in a tat-dependent manner but in the absence of upregulation of cyclin T1

Tat is a viral transactivator necessary for the HIV-1 promoter to achieve maximal levels of activity. We therefore examined whether reactivation by Pam3CSK4 is Tat dependent. Tat recruits the positive transcription elongation factor pTEFb, a protein kinase complex that consists of Cyclin T1 and CDK9, to the TAR (trans acting response element) RNA located at the 5′ end of viral transcripts [46]. pTEFb is responsible for hyperphosphorylation of the C-terminal domain of RNA Pol II and eviction of negative elongation factors, which culminates in transcription activation [47-49]. We used Flavopiridol, a

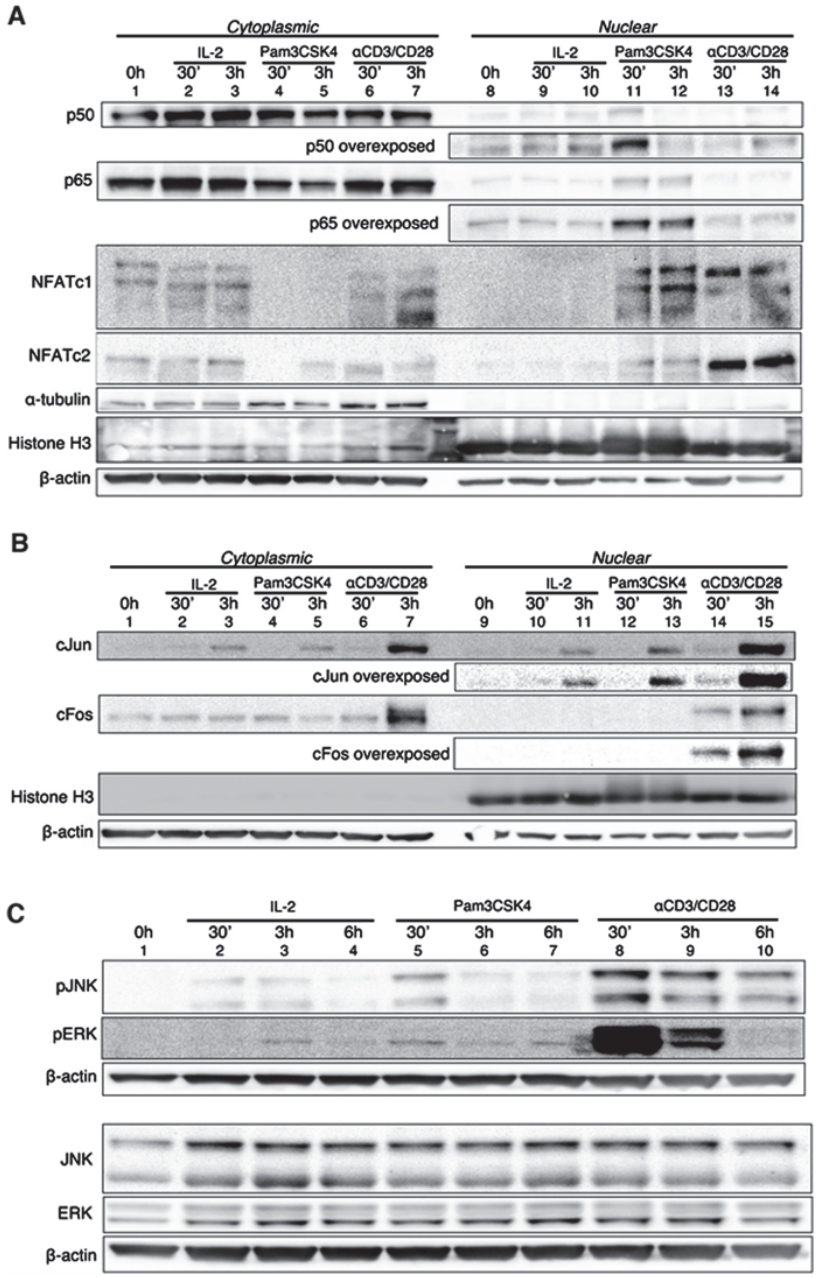

Figure 3 Signaling triggered by Pam3CSK4 in cultured T_{CM}. Culture T_{CM} were either left unstimulated (0 h) or stimulated with IL-2, Pam3CSK4 and αCD3/αCD28 after 30 minutes or 3 hours. Nuclear and cytoplasmic fractions were isolated as indicated in the experimental procedure section. Proteins were loaded on a SDS-polyacrylamide gel, transferred to a membrane and western-blotted against p50, p65, NFATc1, NFATc2, α-tubulin, histone H3 and β-actin **(A)** or against cJun, cFos, histone H3 and β-actin **(B)**. Data is representative of two donors. **(C)** Culture T_{CM} were left unstimulated (0 h) or stimulated with IL-2, Pam3CSK4 and αCD3/αCD28 after 30 minutes or 3 hours. Whole cell extracts were isolated as indicated in the experimental procedure section. Proteins were loaded on a SDS-polyacrylamide gel, transferred to a membrane and western-blotted against JNK, phospho-JNK, ERK, phospho-ERK and β-actin. Data is representative of two donors.

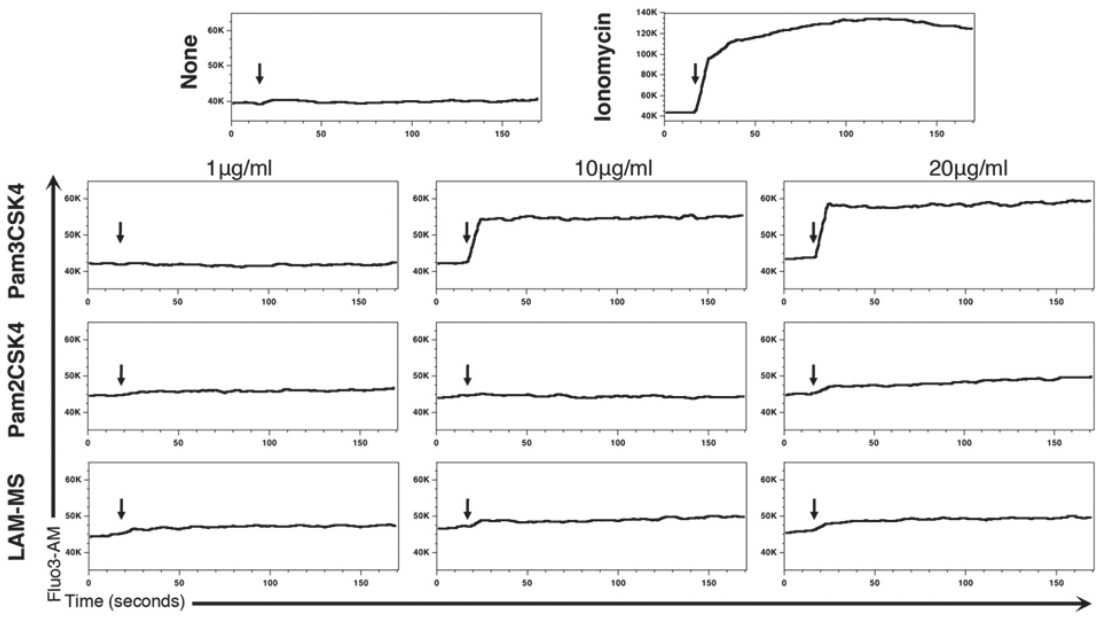

Figure 4 Pam3CSK4 triggers intracellular Ca^{2+} influx. Cultured T_{CM} were loaded with the Ca^{2+} dye Fluo3-AM and cells were tested for their ability to increase intracellular calcium levels after treatment with ionomycin, or with increasing concentrations of Pam3CSK4, Pam2CSK4 or LAM-MS by flow cytometry. The arrows indicate when the stimuli were added. Data is representative of two donors.

selective pTEFb inhibitor, to analyze whether pTEFb was involved in viral reactivation mediated by Pam3CSK4. As shown in Figure 5A, flavopiridol blocked viral reactivation mediated by Pam3CSK4 and by αCD3/αCD28 in a dose dependent manner (Figure 5A).

Low levels of the pTEFb main components, cyclin T1 and pCDK9, have been proposed to limit the ability of the LTR to efficiently drive transcription in resting cells [50]. We analyzed whether Pam3CSK4 induced an increase in the levels of cyclin T1 or in the phosphorylation levels of CDK9 in cultured T_{CM}. As shown in Figure 5B, levels of cyclin T1 remained constant when cells were incubated with IL-2 alone or in the presence of Pam3CSK4 (lanes 1 to 7). In contrast, incubation with αCD3/αCD28 increased the total levels of cyclin T1 in culture T_{CM} as previously described (lines 8 to 10) [50]. We then analyzed the levels of CDK9 and pCDK9. It has been previously shown that CDK9 exists in two isoforms generated from two different promoters [51]. Both isoforms can be found as part of pTEFb complexes [51]. As shown in Figure 5B, cultured T_{CM} express both isoforms and the total levels of each isoform of CDK9 ($CDK9_{55}$ and $CDK9_{42}$ as indicated in Figure 5B) did not change with any of the treatments. Levels of $pCDK9_{55}$ were drastically increased when cells were incubated with αCD3/αCD28 (Compare lanes 1 to 8 and 9). Pam3SCK4 was able to increase levels of $pCDK9_{55}$ at 12 h when compare with IL-2 treatment alone (Figure 5B, lines 2 and 5). These results indicate that Pam3CSK4 reactivates latent HIV in a pTEFb dependent manner but in the absence of cyclinT1 upregulation.

Pam3CSK4 reactivates latent HIV-1 in the absence of T cell activation and/or proliferation

We have shown that stimulation through CD3 and CD28 or Pam3CSK4 leads to viral reactivation through the activation of different transcription factors. It is well known that activation through CD3 and CD28 leads to global T cell activation encompassing release of cytokines and chemokines, massive T cell proliferation and can lead to durable T cell depletion in HIV-infected patients [52,53]. To test whether Pam3CSK4 triggers global T cell activation in cultured T_{CM}, we assessed the induction of CD69 and CD25. As shown in Figure 6A, Pam3CSK4 failed to induce up regulation of CD69 when compared with stimulation with IL-2 alone (baseline condition). As expected, αCD3/αCD28 stimulation strongly induced up-regulation of the activation marker. We have previously shown that cultured T_{CM} and *ex vivo* isolated T_{CM} show low levels of CD25 protein expression on the surface and the expression is up-regulated after TCR engagement [54]. As shown in Figure 6B, Pam3CSK4 did not up-regulate the expression levels of CD25 when compared with stimulation with IL-2 alone (baseline condition). However, treatment of cultured

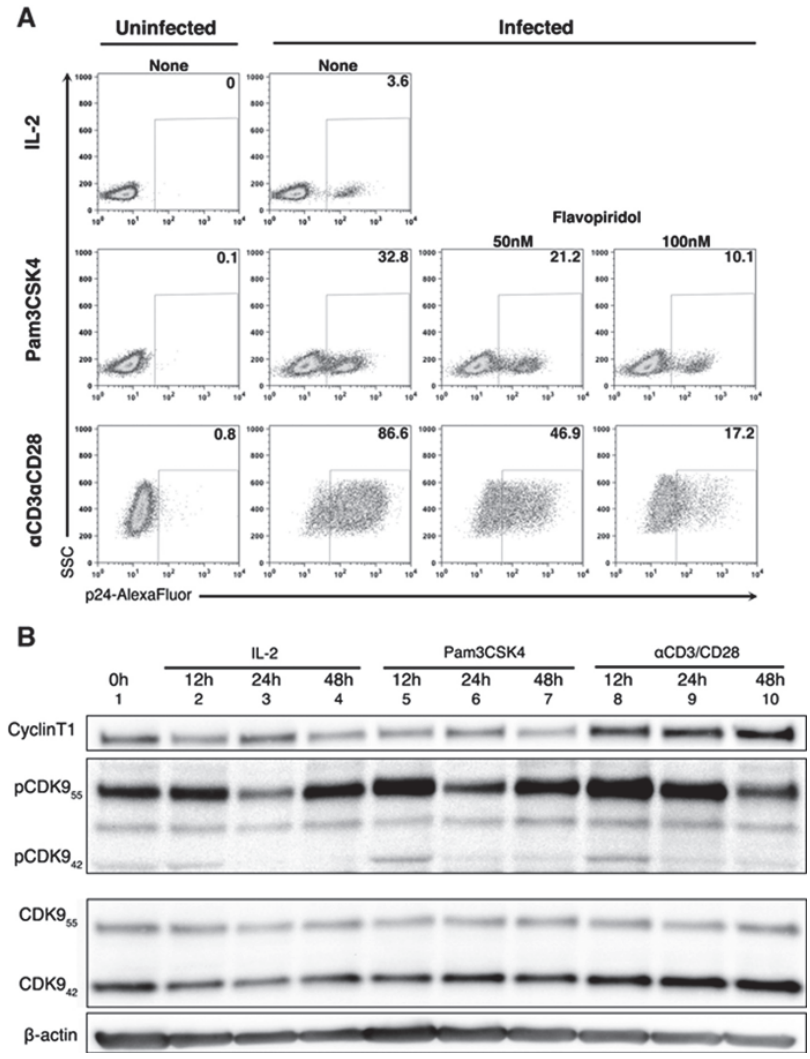

Figure 5 Pam3CSK4 induces viral reactivation in a Tat-dependent manner. (A) Cultured T_{CM} cells were infected with wild-type DHIV and treated with Pam3CSK4 or stimulated with αCD3/αCD28 in the presence of 50 nM and 100 nM of Flavopiridol and assessed for intracellular p24 Gag expression by flow cytometry. Data is representative of two donors. **(B)** Culture T_{CM} were either left unstimulated (0 h) or stimulated with IL-2, Pam3CSK4 and αCD3/αCD28 for 12 h, 24 h or 48 h. Whole cell extracts were isolated as indicated in the experimental procedure section. Proteins were loaded on a SDS-polyacrylamide gel, transferred to a membrane and western-blotted against CyclinT1, phospho-CDK9, CDK9, and β-actin. Data is representative of two donors.

T_{CM} with αCD3/αCD28 led to a dramatic increase in this activation marker (63 times increased MFI compared with IL-2 treatment alone).

Using this *in vitro* model, we have previously shown that IL-7 plus IL-2 can induce a low degree of viral reactivation in the presence of cellular proliferation [54]. Cellular proliferation in the absence of viral reactivation has been proposed as a mechanism for maintenance of the latent reservoir [5,54]. To address whether Pam3CSK4 was able to induce cellular proliferation, we stained cells with the cell proliferation dye CPD eFluor647. After staining, cells were incubated with IL-2 alone, Pam3CSK4 or αCD3/αCD28 and cellular proliferation and viral reactivation was measured 3 days later. As shown in Figure 6C, Pam3CSK4 induced viral reactivation in the absence of cellular proliferation when compared with our control treatment of IL-2 alone. However, reactivation through CD3 and CD28 led to massive cellular proliferation as well as viral reactivation

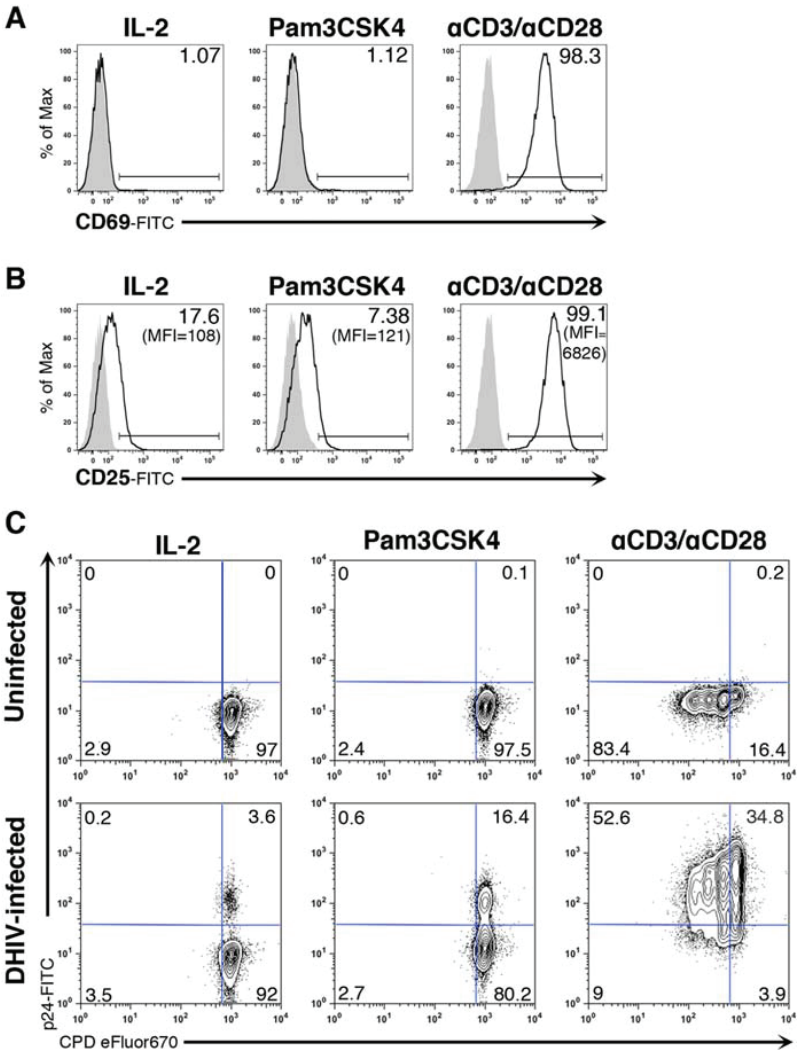

Figure 6 Pam3CSK4 induces viral reactivation in the absence of T cell activation or T cell proliferation. Cultured T_{CM} cells were treated with IL-2, Pam3CSK4 or costimulated with antibodies to CD3 and CD28 (αCD3/αCD28) for 3 days and assessed for the induction of CD69 **(A)** and CD25 **(B)** by flow cytometry (open black histograms). Isotype control was used as control (closed gray histogram). The percentage of CD69 and CD25 positive cells is indicated in each panel and the mean fluorescence intensity (MFI) is indicated between parentheses for CD25. Data is representative of three donors. **(C)** Cultured T_{CM} cells were infected with wild-type DHIV or left uninfected. At 9 days after infection, cells were stained with the cell-proliferation dye CPD eFluor670. Stained cells were treated with IL-2, Pam3CSK4 or costimulated with antibodies to CD3 and CD28 (αCD3/αCD28) for 3 days and assessed for intracellular p24Gag expression and CPD eFluor670 staining by flow cytometry. Numbers indicate percentage of cells. Data is representative of three donors.

from latency. These results indicate that Pam3CSK4 can reactivate latent HIV-1 in the absence of T cell activation or proliferation.

Discussion

In this study, we have found that Pam3CSK4, a TLR-1/2 agonist, is able to reactivate HIV-1 from latency in primary cultured T_{CM} cells. We have also tested the ability of Pam3CSK4 to reactivate latent HIV-1 in two *ex vivo* models. In this case, Pam3CSK4 is able to reactivate latent HIV-1 in a fraction of the patients. Several polymorphisms have been described to affect TLR-1/2 signaling [55-57]. It will be interesting to address whether these polymorphisms are associated with the ability of

Pam3CSK4 to reactivate latent HIV-1 in memory $CD4^+$ T cells.

Several PAMPs and their corresponding microorganisms have been shown to directly transactivate the HIV-1 LTR. Live mycobacteria as well as some of their components induce HIV-1 expression in human monocytes, lymphocytes, or cell lines *in vitro* [58-62]. This induction has been shown to be dependent on TLR-2 [62,63]. Flagellin, a TLR-5 agonist, reactivates latent HIV-1 in the cell line J-Lat [28]. Furthermore, the TLR-7/8 agonist, R-848, is able to reactivate latent HIV-1 from myeloid-monocytic cells lines [64]. Finally, the TLR-9 agonist, CpG oligodeoxynucleotide, has been shown to reactivate latent HIV-1 in the cell line ACH-2 [29]. In an independent study, performed while this work was in progress, Dr. Jonathan Karn and colleagues have found that TLR-5 agonist flagellin leads to viral reactivation from latency in microglial cells, and that the TLR-3 and TLR-9 agonist, poly (I:C) and ODN2006 respectively, weakly reactivate latent HIV-1 in a primary cell model of T_H17 cells (manuscript in preparation).

TLR-2 recognizes a wide range of ligands because it functions in conjunction with other receptors [65]. Thus, TLR-2 can form heterodimers with TLR-1 or TLR-6. TLR-1/2 complexes recognize triacylated lipopeptides whereas TLR-2/6 complexes recognize diacylated lipopeptides [34,66]. In addition, TLR-2 has been shown to cooperate with TLR-10 [32] and with the c-type lectin, Dectin-1 [33]. We have shown that the triacylated lipopeptide Pam3CSK4 is the only TLR-2 agonist tested able to induce viral reactivation. Interestingly, Pam3CSK4 was able to induce an increase in intracellular calcium and subsequent activation of the transcription factor NFAT. We have shown that, in addition to the canonical pathway of NFκB and AP1 activation mediated by TLR-1/2, NFAT activation is also required for viral reactivation. Our result is in agreement with a published report showing that Pam3CSK4 stimulates release of Ca^{2+} from intracellular stores in lung fibroblast [67] and with several reports showing activation of NFAT after stimulation of bone marrow-derived macrophages with Pam3CSK4, zymosan and other TLR agonists [68,69]. To our knowledge, this is the first time that it has been reported that Pam3CSK4 can activate NFAT in human primary $CD4^+$ T cells.

We have previously demonstrated that NFAT but not NFκB plays a major role in viral reactivation through αCD3/αCD28 in cultured T_{CM} (this report and [24]). On the other hand, stimulation with the TLR-1/2 agonist Pam3CSK4 leads to the activation of both NFκB and NFAT and both transcription factors are involved in viral reactivation mediated by Pam3CSK4. These results suggest that HIV-1 has evolved to use different transcription factors to increase viral transcription and that the ability of NFAT or NFκB to induce viral transcription is not determined by the viral LTR but by the stimulus.

It is well known that stimulation through CD3 and CD28 leads to large-scale T cell activation, release of cytokines and chemokines, massive T cell proliferation and ultimately leads to profound T cell depletion in humans [52,53]. However, we show here that Pam3CSK4 is able to activate the HIV-1 promoter in a NFκB and NFAT-dependent manner; but it does so in the absence of overt signs of T cell activation, specifically, CD69, CD25 and T cell proliferation. We have identified several differences between the signaling pathways activated by αCD3/αCD28 and Pam3CSK4 that can account for the difference in T cell activation. First, αCD3/αCD28 induced a stronger nuclear translocation of the constitutive NFAT isoform NFATc2 and a better activation of the transcription factor AP-1. Both, AP-1 and NFAT form a quaternary complex during T-cell activation [70]. Second, Pam3CSK4 is a more potent inductor of NFκB relative to αCD3/αCD28. Third, Pam3CSK4 does not increase the levels of cyclin T1 and induces low levels of pCDK9 whereas αCD3/αCD28 induce a strong increase of both, cyclin T1 and pCDK9. These results demonstrate that viral reactivation and cellular activation can be effectively decoupled. Also, these differences can have implications in the search for molecules that efficiently and safely reactivate latent HIV-1 virus in the absence of massive T cell activation. We hypothesized that those molecules that specifically activate NFκB, NFATc1, a weak AP-1 and do not upregulate cyclin T1 will be more desirable as anti-latency drugs than substances that induce a strong NFATc2, AP-1, cyclin T1 and pCDK9 responses.

Conclusion

Our results show that Pam3CSK4 has the potential to reactivate latent HIV-1 in T_{CM}. These findings raise a series of important questions for further study: can Pam3CSK4 or other TLR agonists reactivate latent HIV in other primary cell models; are NFAT, NFκB and AP-1 recruited to the LTR following Pam3CSK4 signaling; why is the response to Pam3CSK4 heterogeneous in cells isolated from patients? As agents that can induce the expression of latent HIV without mediating global T cell activation are uncommon but highly valuable as potential drugs to attack the latent HIV reservoir, further study and testing of Pam3CSK4 and its signaling pathway is a high priority.

Although PAMPs are normally associated with infectious agents, their ability to enhance immune responses was documented in the context of cancer therapy a century ago, when William Coley used bacterial components named “Coley’s toxins” to treat cancer patients [71]. Since then, several TLR agonists have been investigated for the use in treatment of cancer; viral or bacterial infections; allergy; asthma and autoimmunity (reviewed in [72]). In particular the triacylated lipopeptide outer surface protein A

(OspA) of *Borrelia burgdorferi*, which is a TLR-1/2 agonist, has been previously clinically used as a vaccine against Lyme disease with minor side effects (reviewed in [73]). We suggest that triacylated lipopeptides and/or the TLR-1/2 signaling pathway can be targeted toward future development of anti-latency strategies, either alone or in combination with others anti-latency drugs.

Methods

Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: Human rIL-2 from Dr. Maurice Gately, Hoffman-La Roche Inc. [74]; Monoclonal Antibody to HIV-1 p24 (AG3.0) from Dr. Jonathan Allan [75]; and Flavopiridol.

Generation of cultured T_{CM} cells and their latent infection

Naïve $CD4^+$ T cells were isolated via negative selection from peripheral blood mononuclear cells (PBMC) from healthy unidentified donors 18 years and older. Written informed consent was obtained from all donors. These studies are covered under the IRB #392 protocol approved by the University of Utah Institutional Review Board. Cultured T_{CM} were generated and infected as previously described [24].

Stimulation of cells

2.5×10^5 cells were left untreated or stimulated for three days with the PRR agonists or beads coated with αCD3 and αCD28 (1 bead per cell, Dynal/Invitrogen, Carlsbad, CA). PRR agonists were obtained from Invivogen (San Diego, CA) and used at the concentration indicated: Pam3CSK4 (10 μg/ml), Pam2CSK4 (200 ng/ml), FSL-1 (10 μg/ml), Poly (I:C) HMW (10 μg/ml), LPS (10 μg/ml), flagellin (10 μg/ml), imiquimod (10 μg/ml), ssRNA40 (5 μg/ml), ODN2006 (5 μM), zymosan (200 μg/ml) and lipoarabinomannan from *Mycobacterium smegmatis* (LAM-MS) (10 μg/ml). TLR-2 agonist polysaccharide-A (PSA) was kindly provided by June Round (Pathology Department, University of Utah).

For inhibitors studies, cells were pre-incubated with the indicated inhibitors for 2 hours before stimulation. The inhibitors used were 1 μg/ml Cyclosporine-A (Sigma-Aldrich, Saint Louis, MO); 0.8 μM BAY 11-7082 and 50 μM PD98059 (Calbiochem, San Diego, CA); 25 μM SP600125 (A.G. Scientific Int., San Diego, CA); 50 nM and 100nM Flavopiridol (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID).

Ex-vivo HIV-1 RNA reactivation assay

Frozen cells from 7 HIV-infected patients receiving HAART with plasma viral load <50 copies/ml for at least 6 months and CD4 count of >350 ul^{-1} were used for the *ex-vivo* HIV-1 RNA reactivation assay. A median of 50×10^6 frozen PBMCs were used to isolate $CD4^+$ T cells using $CD4^+$ T cell isolation kit (Milteny Biotec). $CD4^+$ T cells were incubated in media containing 10 ug/ml Pam3CSK4 and 30 IU/ml IL-2, or 1uM Panobinostat and 30 U/ml IL-2. A background control well was set up with media supplemented with 30 U/ml IL-2. After 72 hours, total RNA was extracted and cDNA synthesized. Unspliced HIV-1 RNA was quantified by real-time PCR, using primers and probes previously described [76]. Data were normalized to the expression of the housekeeping gene Actb (encoding b-actin). Results were plotted as a fold change induction of HIV-1 RNA expression between the test well and the background control well.

Quantitative viral outgrowth assay (Q-VOA)

Outgrowth assays were performed, as described previously [39]. Briefly, PBMC were obtained by continuous-flow leukapheresis from HIV-infected volunteers receiving stable ART with plasma HIV-1 RNA less than 50 copies/ml and a $CD4^+$ T cell count of more than 300 cells/ml. Resting $CD4^+$ T cells were isolated by negative selection from PBMC and incubated in limiting dilutions with Pam3CSK4 or IL-2 for 24–48 hours or maximally stimulated with PHA-L, allogeneic irradiated PBMC from a sero-negative donor, and rIL-2. Cultures were fed twice with CD8-depleted PBMC, collected from a CCR5 high sero-negative donor. Supernatant was collected on days 15 and 19 and HIV p24 Gag antigen was measured by ELISA. Cultures that maintained an equivalent or greater level of p24 antigen on day 19 as on day 15 were scored as positive. A maximum likelihood method was used to calculate the infectious unit per million resting $CD4^+$ T cells.

Flow cytometry analysis

Intracellular p24 Gag expression was analyzed as previously described [24].

Surface expression was determined using anti-human CD281-PE (TLR-1, clone GD2.F4, eBioscience, San Diego, CA), anti-human CD282-PE (TLR-2, clone TL2.1, eBioscience, San Diego, CA), anti-human CD25-FITC (Molecular Probes, Eugene, OR), anti-human CD69-FITC (Molecular Probes, Eugene, OR), anti-human CCR7-APC (R&D Systems, Minneapolis, MN) and anti-human CD27-FITC (Molecular Probes, Eugene, OR)

To analyze cell division with Cell Proliferation Dye eFluor 670 (eBioscience, San Diego, CA), cells were stained as indicated by the manufacturer.

Flow cytometry was performed with a BD FacsCanto II flow cytometer using the FACSDiva software (Becton Dickinson, Mountain View, CA). Data was analyzed with FlowJo (TreeStar Inc, Ashland, OR).

Intracellular calcium flux

Cells were loaded using Fluo-3 AM (Molecular Probes, Eugene, OR) following manufacturer's protocol and analyzed by flow cytometry. Cells were left at room temperature in the dark, for 15 minutes. A 20 seconds baseline was recorded prior to addition of the stimulus. After addition of the stimulus cells were vortexed and analysis was performed during additional 150 seconds. 20 ng/ml of Ionomycin was used as a positive control.

Western blotting

To analyze phosphorylation, five million cultured T_{CM} were lysed using a lysis buffer containing 50 mM Tris–HCl [pH 8], 150 mM NaCl, 1% NP-40, and 0.1% protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN) for 30 minutes at 4°C. Lysates were cleared by centrifugation at 12000 rpm for 20 min at 4C.

To analyze nuclear translocation, five million cultured T_{CM} cells were washed in PBS and incubated with a cell lysis buffer containing 5 nM PIPES [pH8], 85 mM KCl, 0.5% NP-40 and 0.1% protease and phosphatase inhibitors for 30 min. After incubation, nuclei were pelleted by centrifugation at 5000 rpm during 20 min at 4°C. Supernatants were collected as cytoplasmic fractions. Nuclei were washed once with PBS containing 0.1% protease and phosphatase inhibitors and pelleted by centrifugation at 5000 rpm during 20 min at 4C. Nuclei were lysed with a buffer containing 50 mM Tris [pH 8.1], 10 nM EDTA, 1% SDS and 0.1% protease and phosphatase inhibitors and boiled for 10 min at 100°C. Nuclear extracts were cleared by centrifugation at 12000 rpm for 10 min at RT.

Proteins were separated on SDS-PAGE electrophoresis. Western blotting was performed according to the standard protocols. The following antibodies were used: p50, p65, NFATc1, NFATc2, cJun, cFos and cyclin-T1 (Santa Cruz Biotechnology, Santa Cruz, CA); total JNK, pJNK, total ERK, pERK, total CDK9 and pCDK9 (Cell Signaling, Danvers, MA); anti-β-actin antibody (Sigma-Aldrich, Saint Louis, MO); anti-α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-histone H3 (Biolegend, San Diego, CA).

Additional methods provided in Additional file 3.

Additional files

Additional file 1: Figure S1. Effects of TLR agonists in four cellular models. (A) J-Lat 10.6 cells were treated with the toll-like receptors agonists indicated between parentheses or PMA and assessed for GFP expression by flow cytometry. (B) THP-1 cells were treated with the toll-like receptors agonists indicated between parentheses or PMA and assessed for IL-8 expression by flow cytometry. (C) U1 cells were treated with the toll-like receptors agonists indicated between parentheses or PMA and assessed for intracellular p24 expression by flow cytometry. (D) ACH-2 cells were treated with the toll-like receptors agonists indicated between parentheses or PMA and assessed for intracellular p24 expression by flow cytometry.

Additional file 2: Figure S2. Effects of TLR-2 agonists in four cellular models. (A) J-Lat 10.6 cells were treated with the TLR-2 agonists indicated between parentheses or PMA and assessed for GFP expression by flow cytometry. (B) THP-1 cells were treated with the TLR-2 agonists indicated between parentheses or PMA and assessed for IL-8 expression by flow cytometry. (C) U1 cells were treated with the TLR-2 agonists indicated between parentheses or PMA and assessed for intracellular p24 expression by flow cytometry. (D) ACH-2 cells were treated with the TLR-2 agonists indicated between parentheses or PMA and assessed for intracellular p24 expression by flow cytometry.

Additional file 3: Supplemental Methods.

Competing interests

AB and VP have a patent application related to the method for the generation of latently infected cultured T_{CM}. The authors declare that they have no other competing interest.

Authors' contributions

AB conceived this study, designed and analyzed experiments. CLN designed, performed and analyzed experiments. MJB, ML, NMA and DMM performed and analyzed *ex vivo* experiments. JLR and EV provided reagents. CLN, VP, DMM and AB wrote the manuscript. All authors read and approved the final manuscript.

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CHAPTER 3

ABILITY OF DYNASORE TO REACTIVATE LATENT HIV-1 SYNERGISTICALLY WITH OTHER KNOWN LATENCY-REVERSING AGENTS

3.1 Abstract

Viral persistence during therapy is the major barrier to eradicate HIV-1. Reactivation of the viral reservoir followed by viral clearance through cellular immunity and/or cytopathic effects has been proposed as a strategy towards a cure. Several latency-reversing agents have been developed to date. However, the magnitude of HIV expression induced by these single compounds has been modest and likely insufficient to purge the latent reservoir. The general thought in the field is that a combination of compounds may be more effective to reactivate and eliminate all the latent HIV-1 viruses in patients. We report here the ability of dynasore to reactivate latent HIV alone and in synergy with known LRAs, such as Pam3CSK4, SAHA, bryostatin-1 and JQ-1. Of note, this reactivation is achieved independently of the ability of dynasore to inhibit dynamin.

3.2 Introduction

Combinatory antiretroviral therapy (ART) introduced in the mid-1990s reduced mortality and morbidity of HIV patients by suppressing viral loads to undetectable levels [1]. However, despite the ability of ART to suppress HIV-1 replication, a small population of latent but replication-competent viruses persists within the $CD4^+$ memory T cell compartment [2-6].

Several strategies have been proposed towards the elimination of this latent reservoir. Among those, the “shock and kill” strategy to reverse HIV-1 latency is a promising candidate for a global therapy towards HIV-1 eradication. This strategy is based on two key steps [7]: First, eliciting transcription of HIV-1

genes from latently infected cells with a “shock” mediated by a latency-reversing agent (LRA); second, cell death of the reactivated cell through viral cytopathic effects or by immune effector mechanisms as CTLs or NK cells.

HIV latency is a complex phenomenon controlled at different molecular levels. Due to its complexity, efficient latent reactivation may require a combination of multiple drugs. Combinatorial action of different LRAs has been previously described [8-12]. In this study, we demonstrate the ability of dynasore, a dynamin inhibitor, to reactivate HIV-1 alone or in synergy with other LRAs, such as Pam3CSK4, SAHA, bryostatin-1 and JQ-1 in JLAT. This reactivation is independent of dynasore’s ability to inhibit dynamin and it represents a novel pathway towards HIV-1 reactivation.

3.3 Results

3.3.1 Dynasore reactivates HIV-1 in latency cell line models

We have previously reported that Pam3CSK4, a TLR-2 agonist, can reactivate latent HIV-1 in cultured T_{CM} and in cells isolated from aviremic patients [13]. In order to further investigate the signaling pathway that leads to viral reactivation mediated by triacylated lipopeptides, we engineered the tumoral cell line JLAT 10.6 [14] to express TLR-2 on the surface. To that end, we engineered the lentiviral vector pFIN-EF1-GFP-2A-mCherry-WPRE [15] to express TLR-2 in place of GFP. This reconstructed lentiviral vector encodes a single polyprotein consisting of a fusion of TLR-2 and mCherry, whose expression is driven by the elongation factor 1 (EF1) promoter (Figure 3.1A). The presence of the 2A protein

from porcine teschovirus-1 leads to ribosomal skipping and equimolar production of TLR-2 and mCherry [15]. We also constructed a vector that only encodes the mCherry protein, as a control. JLAT 10.6 cells were infected with the lentiviral vectors, and then mCherry expressing cells were sorted by FACS. Sorted cells infected with the lentiviral vector encoding TLR-2 showed over 30 times more surface expression of TLR-2 than cells infected with the lentiviral vector expressing only mCherry (Figure 3.1A, histograms). Most importantly, this modification rendered JLAT sensitive to the TLR-2 agonist Pam3CSK4 in a dose-dependent manner (Figure 3.1B). It is known that TLR-1/2 activates NF-κB via MyD88 and the subsequent formation of a complex with IRAK1/IRAK4 and TRAF6. This complex leads to the activation of the IKK complex through the kinase TAK1 and the phosphorylation and degradation of IκBα, leading to the activation and nuclear translocation of NF-κB [16]. Moreover, one of the mechanisms proposed by which triacylated lipopeptides can activate NF-κB is by internalization of TLR-2 to endosomal compartments in a process mediated by dynamin [17]. To test whether TLR-2 internalization has a role in viral reactivation mediated by Pam3CSK4, JLAT-mCherry or JLAT-TLR2-mCherry were treated with the dynamin inhibitor dynasore in the presence or absence of Pam3CSK4. As shown in Figure 3.2A, right panel, dynasore not only failed to inhibit reactivation by Pam3CSK4, but it induced viral reactivation on its own and enhanced the activity of Pam3CSK4. The reactivation effect of dynasore was independent of the presence of TLR-2 because it also occurred, to a similar degree, in cells not expressing TLR-2. Furthermore, dynasore alone had the

ability to reactivate latent HIV-1 in JLAT-mCherry in a dose-dependent manner (Figure 3.2B, black bars).

Because of this unexpected result, we further evaluated whether dynasore alone had the ability to reactivate latent HIV-1 in different models of latency. We first assessed the activity of this compound in two other JLAT clones (10.6 and 6.3) [14]. In JLAT 6.3, dynasore induced modest HIV reactivation at concentrations ranging from 12.5μM to 100 μM (Figure 3.3A). The maximal dynasore activity reached in clone 6.3 was about 16% of the activity of the positive control phorbol-myristate-acetate (PMA). In JLAT 10.6, PMA activated ~75% of the cells and dynasore activated up to ~13%, reaching a plateau at 12.5μM concentrations (Figure 3.3B). The JLAT clone 5A8 [12] was obtained via selection for increased responsiveness to anti-CD3 and anti-CD28 antibodies. PMA, in 5A8 cells, induced reactivation of ~33% of the cells. Under dynasore treatment, 5A8 cell line expressed GFP in increasing levels up to 100μM of dynasore (Figure 3.3C). The 2D10 cell line [18] carries a provirus with a hypomorphic mutation in Tat (H13L), which promotes viral entry into latency, still allowing HIV transcription to be inducible. The provirus in 2D10 cells can be efficiently induced by stimulation with PMA, resulting in ~95% of reactivation. As shown in Figure 3.3D, we observed induction of latently infected 2D10 cells by stimulation with dynasore in a dose-dependent manner, where 12.5 μM and 25 μM reactivated ~75% of the cells,

These results indicate that dynasore has the ability to reactivate latent HIV-1 alone in several transformed cell models of latency and also enhancing the effect of Pam3CSK4 in JLAT-TLR2-mCherry cells.

3.3.2 Dynasore synergize with other known latency reversing agents

Due to the ability of dynasore to synergize with Pam3CSK4, we tested whether dynasore had also the ability to synergize with other LRAs. To that end, we selected three compounds belonging to three different classes of LRAs. First, we used suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor known to reactivate latent HIV-1 in cell lines and in cells isolated from aviremic patients [19-22]. Inhibition of HDACs allows histone lysine acetylation in nucleosomes positioned at the HIV-1 long terminal repeat (LTR) [23]. As shown in Figure 3.4A, SAHA in combination with dynasore triggered 20% of cells to express GFP, while in the presence of the solvent DMSO alone, SAHA only induced ~6% of GFP expressing cells at 100nM concentration. At lower concentrations, SAHA reactivated to a higher degree when combined with dynasore. We also tested bryostatin-1, a protein kinase C (PKC) activator that targets HIV transcription through the AMP-activated protein kinase (AMPK) pathway [24]. Bryostatin-1 activates latent HIV in cell lines, primary $CD4^+$ T cell models and patient cell outgrowth assays [24-26]. Dynasore enhanced the activity of bryostatin-1 by about 3-fold (Figure 3.4B). Furthermore, we tested the small molecule JQ1, an inhibitor of bromodomain. Bromodomain containing 4 (BRD4) competes with Tat for interaction to P-TEFb at the HIV promoter. JQ1

increased HIV-1 transcription in cell line models of HIV latency and virus outgrowth from cultured $CD4^+$ T cells [27, 28]. Dynasore enhanced JQ1 reactivation signal from 2% of cells expressing GFP to 6.5% in the lower JQ1 concentration, 50nM (Figure 3.4C).

To evaluate whether dynasore acts synergistically with the above drugs, we used the Bliss independence drug interaction analysis [29]. The Bliss model is based on probability theory and assumes that when two drugs are independent, the expected (F_{EXP}) combinatorial effect should be the sum of the two fractional responses minus their product [$(F_A+F_B)-(F_A*F_B)$]. The interaction of each combination is described by the difference between the observed and the expected response ($\Delta F=F_{OBS}-F_{EXP}$). Bliss independence analysis yields synergistic ($\Delta F > 0$), independent ($\Delta F = 0$) or antagonistic ($\Delta F < 0$) combinatorial interactions. According to the Bliss model, synergy was found for the combination of bryostatin-1 (33nM, 100nM or 330nM) + dynasore (12.5nM), for which the observed combined drug effect was 2.4-, 1.8- and 1.7-fold higher, respectively, than expected in the absence of synergy. In addition, SAHA was found in synergy with dynasore at two concentrations (330nM and 1μM). The bromodomain inhibitor JQ1 synergized with dynasore at all three concentrations tested. The analysis of combinatorial drug interaction is summarized in Table 3.1.

3.3.3 Dynamins do not participate in reactivation of latent HIV

Dynasore is a noncompetitive inhibitor of dynamin 1, dynamin 2 and dynamin–related protein 1 (Drp1), the mitochondrial dynamin. Discovered in a

high-throughput screen of ~16,000 compounds, dynasore inhibits GTP hydrolysis without interfering with the binding of GTP to dynamin [30].

Dynamin belongs to the GTPase family and its main function is to excise newly formed vesicles during endocytosis [31]. There are three dynamin genes in the mammalian genome, which share domain organization but are differentially expressed. Dynamin 1 is predominantly expressed in neurons (pre-synapses), dynamin 2 is ubiquitous and dynamin 3 is found mostly in testis, brain and lungs [32, 33]. Their structure comprises a large GTPase domain (~300 amino acids) that binds and hydrolyzes GTP, a middle and a GTPase effector domain (GED) involved in oligomerization and increase of GTPase activity, a pleckstrin-homology (PH) domain that binds to phospholipids therefore important for interaction with lipid membranes and a proline-rich domain (PRD) at the carboxy-terminus that allows interaction with Src-homology-3 (SH3) domain proteins [31] (Figure 3.5A). Dynamin-related protein 1 (drp1) lacks the proline-rich domain and it is involved in mitochondrial division [34, 35].

There is a vast array of published dynamin modulators, which have different target domains, mechanisms of action and potencies. In an effort to validate the role of dynamin in the reactivation of HIV by dynasore, we tested several inhibitors for their ability to reactivate latent HIV-1. We tested the GTPase inhibitors Iminodyn-22 and Sertraline hydrochloride [36, 37], the phospholipid binding (PH) inhibitors MiTMAB and RTIL-13 [38, 39] and the dual GTPase and PH inhibitor Pyrimidyn-7 [40]. None of these inhibitors showed any activity towards reactivating latent HIV-1 (Figure 3.5B, C). We also tested Mdivi-1, a

selective inhibitor of Drp1 assembly on mitochondria [41]. Mdivi-1 did not induce any significant HIV activation in JLAT 10.6 (Figure 3.5D). Finally, we tested the dynasore analogue, dyngo 4a. Dyngo 4a inhibits dynamin and endocytosis with 37-fold higher potency when compared with dynasore [42]. Dyngo 4a showed modest but higher activity than dynasore at concentrations below 5μM, but plateaued at 2.5% reactivation activity, whereas dynasore's effect increased gradually to reach a plateau at 12% (Figure 3.5E). In summary, as shown in Figure 3.5, none of the dynamin inhibitors, other than dynasore, efficiently reactivated HIV-1 in JLAT.

Data shown in Figure 3.5 indicate that dynasore can reactivate latent HIV by a mechanism independent of its role against dynamin. To further characterize or rule out the role of dynamin in HIV latency, we knockdown dynamin 2, the ubiquitously expressed member, and drp1, the mitochondrial fission protein, in JLAT cells by nucleofection using siRNAs. Cells nucleofected with siRNA against DNM2 or DRP1 reduced dynamin 2 or drp1 expression compared with cells nucleofected with siRNA Control (Figure 3.6A). As depicted in Figure 3.6B, knockdown of dynamin 2 and drp1 was not sufficient to induce HIV-1 expression in JLAT cells. Finally, neither dynasore nor PMA reactivation was altered by dynamin 2 or drp1 knockdown (Figure 3.6C).

Taken together, these results suggest that the latency reversing effect of dynasore utilizes a mechanism that is independent of dynamin inhibition. This result is in agreement with previously published data, which showed that dynasore has off-target effects [43].

3.3.4 Dynasore requires NF-κB, JNK and Calcium influx to trigger viral reactivation

We next addressed the molecular mechanisms by which dynasore induces HIV reactivation. Nuclear factors such as NF-κB, NFAT, SP1 and AP1 have binding sites on the 5' LTR of HIV and have been shown to be required for HIV-1 transcription [44-48]. First we tested the role of NF-κB in viral reactivation mediated by dynasore. To that end, we incubated cells with the chemical inhibitor of NF-κB, BAY 11-7092 (IκBα phosphorylation inhibitor). As shown in Figure 3.7A**,** BAY 11-7092 (1μM) blocked an average of 40% of viral reactivation induced by dynasore. Known LRAs, prostatin, bryostatin and ingenol, antagonize HIV latency through PKC-mediated activation of NF-κB [24, 49, 50]. Bisindolylmaleimide (BIM) is a global inhibitor of PKC. To assess the potential role of PKC, cells were incubated with BIM and then challenged with dynasore or PMA. BIM abrogated HIV reactivation mediated by PMA in a dose-dependent manner. In contrast, PKC inhibition did not affect dynasore-induced reactivation. Unexpectedly, 5μM concentration of BIM increased viral reactivation in both dynasore and DMSO control treated cells (Figure 3.7B).

Dynasore has been shown to induce JNK phosphorylation in human pleural mesothelial cells [51]. JNK phosphorylation activates c-Jun, a member of the AP-1 transcription factor (reviewed in [52]). Therefore, we decided to explore whether JNK is required for dynasore-induced viral reactivation. To that end, we used SP600125, a selective inhibitor of JNK. As shown in Figure 3.7C, SP600125 decreased viral expression induced by dynasore about 36%, while it had no effect on PMA-induced viral expression.

NFAT is considered an important regulator of HIV transcription [45, 53]. Intracellular calcium activates calcineurin, which dephosphorylates NFAT, enabling it to translocate to the nucleus. It has been recently reported that calcium/calcineurin axis can also activate HIV latently infected T cells through NF-κB [12]. Interestingly, the calcium chelator BAPTA-AM was able to reduce viral reactivation induced by dynasore while the calcineurin inhibitors (FK506 and cyclosporine A) did not have a significant effect (Figure 3.7D and data not shown). These data, taken together, suggest that dynasore mediates viral reactivation in an NF-κB-dependent manner by a mechanism independent of PKC but dependent on the calcium/calcineurin axis. Further studies are required to delineate the role of NF-κB in dynasore-mediated reactivation.

Finally, dynasore but not PMA-mediated viral reactivation was abrogated by Rapamycin (Figure 3.7E). Rapamycin is a pharmacological inhibitor of mTOR, an important kinase regulator of ribosome biogenesis, protein synthesis and cell cycle progression (reviewed in [54]). How mTOR regulates reactivation from latency is currently unknown and further experimentation will be needed to address the specific role of mTOR in this pathway.

3.4 Conclusion

A growing number of agents capable of reactivating HIV-1 from latency have been described. Clinical trials using latency-reversing agents are ongoing and efforts to understand the complex machinery behind efficiently activating silent HIV-1 proviruses continue. Reports showing that single agents *in vitro* are

not powerful enough to purge completely the latent viral reservoir [55-57] led to increased interest in testing drug combinations.

Drug combinations are extensively used to treat diseases, such as cancer and AIDS, because multiple drugs affect numerous targets, resulting in an efficient treatment. Additionally, synergy is designated as the combined action of two drugs in a manner that produces a greater effect than the sum of the effects of the drugs alone. Our results reveal that dynasore has the ability to reactivate latent HIV-1, and this effect is greater when dynasore synergizes with SAHA, bryostatin and JQ-1. Dynasore requires NF-κB, JNK and calcium influx to activate transcriptionally silent HIV. However, common targets of dynasore, dynamin and drp1 are not necessary for proviral reactivation. Further investigation of dynasore off-target ability in HIV reactivation could lead us to uncovering a novel mechanism for reactivating HIV.

3.5 Materials and Methods

3.5.1 Cell lines

2D10 cells were provided by Dr. Jonathan Karn. The 2D10 cells contain a HIV-1 genome with GFP inserted in the place of nef and carry the H13L mutation in Tat which helps to promote proviral entry into latency but still allows HIV transcription [18]. Jurkat cells stably infected with LTR-Tat-IRES-GFP vector (A7) and LTR-IRES-GFP (A72) were acquired from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. JLat 10.6 and JLat 6.3 cells were provided by Dr. Eric Verdin and contain a full-length HIV-1 genome with a frameshift in env that

restricts the insert from producing env or nef [14]. JLAT 5A8 were provided by Dr. Jonathan Karn and were selected to be responsive to aCD3aCD28. All cell lines were cultured in RPMI 1640 with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine (Invitrogen). Cell cultures were maintained at 37°C under 5% CO_2.

3.5.2 Stimulation of cells

$0.3x10^6$ cells were left untreated or treated with DMSO (Fischer Scientific), dynasore (Santa Cruz Biotechnology), Pam3CSK4 (Invivogen, San Diego, CA), phorbol-myristate-acetate (PMA), SAHA (Sigma-Aldrich, Saint Louis, MO), bryostatin (Enzo Life Science, Farmingdale, NY) and JQ1 (Cayman Chemicals, Ann Arbor, MI).

For inhibitors studies, cells were pre-incubated with the indicated inhibitors for 2 hours before stimulation. The inhibitors used were BAY11-7082, bisindolylmaleimide II (BIM) (Calbiochem, San Diego, CA), Rapamycin from *S. hygroscopicus* (Sigma-Aldrich, Saint Louis, MO), SP600125 (A.G. Scientific Int., San Diego, CA), BAPTA-AM (Enzo Life Science, Farmingdale, NY), MiTMAB, Midivi-1 (Tocris, Bristol, UK), Iminodyn-22trade, Sertraline hydrochloride, RTIL-13 and Pyrimidin-7 (Abcam, Cambridge, MA).

3.5.3 Flow cytometry analysis

GFP and mCherry fluorescence were measured in a BD FACSCanto II analyzer and cells were sorted in a BD FACSAria II. TLR-2 surface expression

was determined using anti-human CD282-APC and isotype control (Biolegend, San Diego, CA) measured in a BD FACSCanto II flow cytometer. Data were analyzed using FlowJo (TreeStar Inc, Ashland, OR).

3.5.4 Transfection of siRNAs

JLAT 10.6 cells were transfected with 200nM siRNA corresponding to the DNM2 mRNA, DRP1 or nontargeting control siRNA (siGENOME, Dharmacon) by electroporation twice within 24 hours using Amaxa Nucleofector Kit V and program C-16 (Amaxa Biosystems). 24 hours after second nucleofection, cells in each siRNA condition were collected for flow cytometry analysis and lysed for western analysis.

3.5.5 Western blotting

To analyze gene knockdown, cells were lysed with whole-cell extract buffer containing 20mM Tris HCl [ph 6.8], 2% SDS and 1mM EDTA for 15 minutes at 100^{o}C. Lysates were cleared by centrifugation at 1200 rpm for 10 minutes at 4^{o}C.

Proteins were separated on SDS-PAGE electrophoresis. Western blotting was performed according to the standard protocols. The following antibodies were used: anti-dynamin2 (Abcam, Cambridge, MA), anti-drp1 (Santa Cruz Biotechnology, Santa Cruz, CA) anti-β-actin antibody (Sigma-Aldrich, Sain Louis, MO).

3.5.6 Drug interaction analysis

Bliss independence is derived from a probability theory and is used to explain the combined effect of two drugs [29] . This model is defined by the equation: $F_{EXP}=(F_A+F_B)-(F_A*F_B)$, for $0<E<1$ and where F_{EXP} is the additive expected effect of drugs A and B. The difference between the observed (F_{OBS} = percentage of cells expressing GFP after treatment with drug A (F_A) or drug B (F_B) and the expected response ($\Delta F=F_{OBS}-F_{EXP}$) determines the interaction. Bliss independence analysis describes synergistic ($\Delta F > 0$), independent ($\Delta F = 0$) or antagonistic ($\Delta F < 0$) interactions for each combination of drugs.

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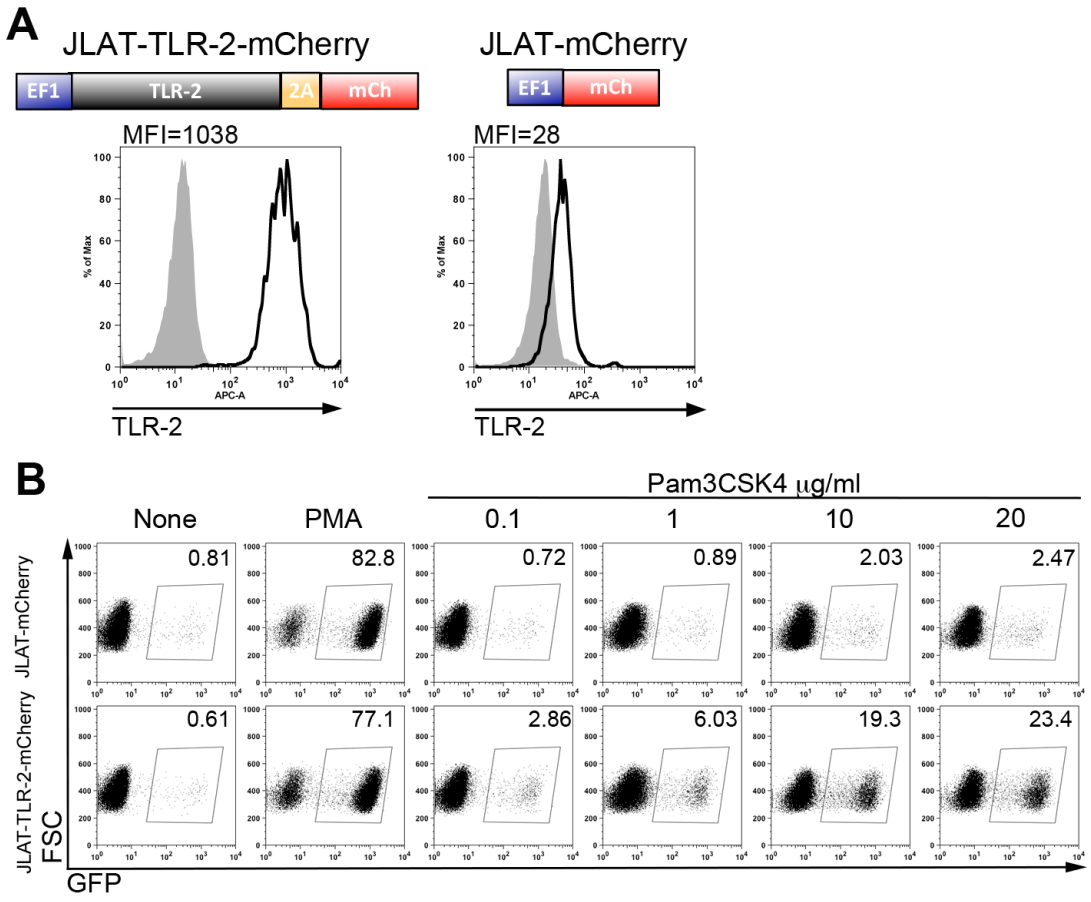


Figure 3.1: TLR-2 overexpression rendered JLAT highly sensitive to HIV-1 reactivation mediated by Pam3CSK4.

(A) Lentiviral vector structure diagram. TLR-2 surface expression (black lines) in JLAT cells after infection with lentiviral vectors and sorting of mCherry expressing cells (JLAT-TLR2-mCherry and JLAT-mCherry). Grey histograms are the isotype control. **(B)** Percentage of GFP expressing cells in JLAT-TLR2-mCherry and JLAT-mCherry after incubation with increasing concentrations of Pam3CSK4 and PMA. All data analyzed by flow cytometry. MFI = mean fluorescence intensity.

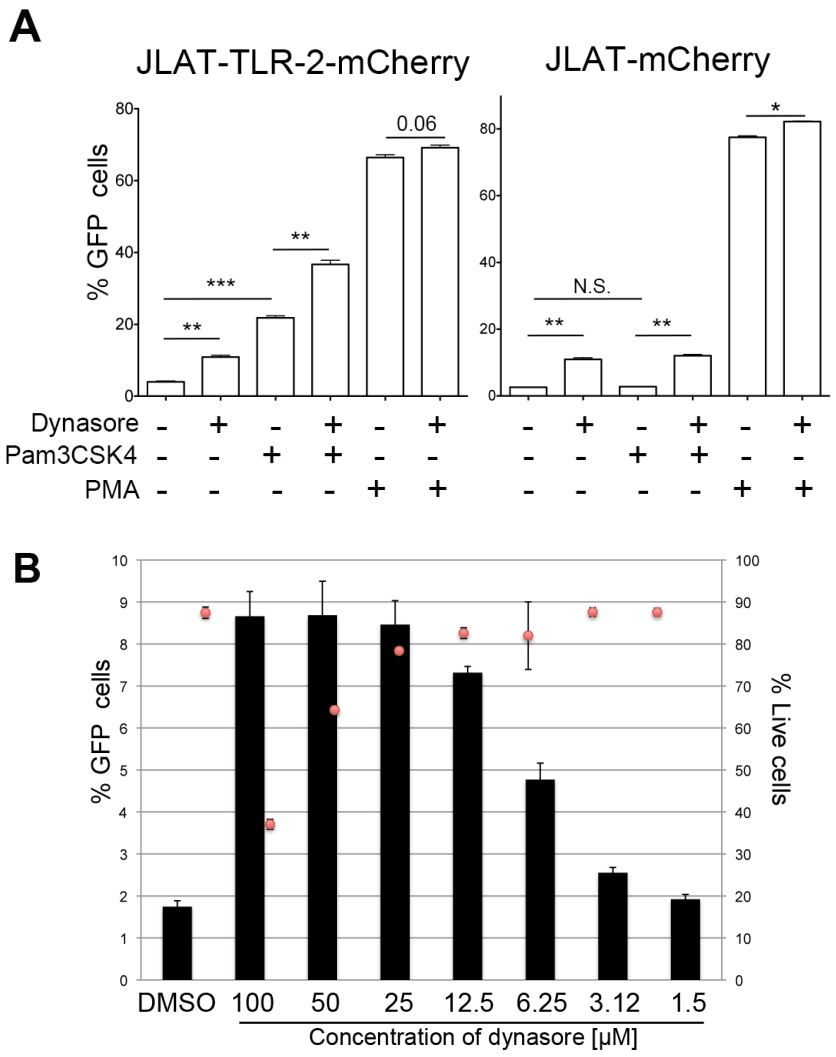


Figure 3.2: Dynasore reactivates latent HIV-1 alone and in combination with Pam3CSK4.

(A) Reactivation of latent HIV-1 in JLAT-TLR2-mCherry or JLAT-mCherry by combinations of Dynasore, Pam3CSK4 and PMA. **(B)** Percentage analysis of GFP expressing and live JLAT 10.6 cells after incubation with solvent DMSO or increasing concentrations of dynasore. All data analyzed by flow cytometry. Bar graph corresponds to mean and standard deviation of experiments performed in triplicates. Significance was calculated by 2-tailed paired samples t test analysis (*<0.05, **<0.01, ***<0.001, ns = not significant).

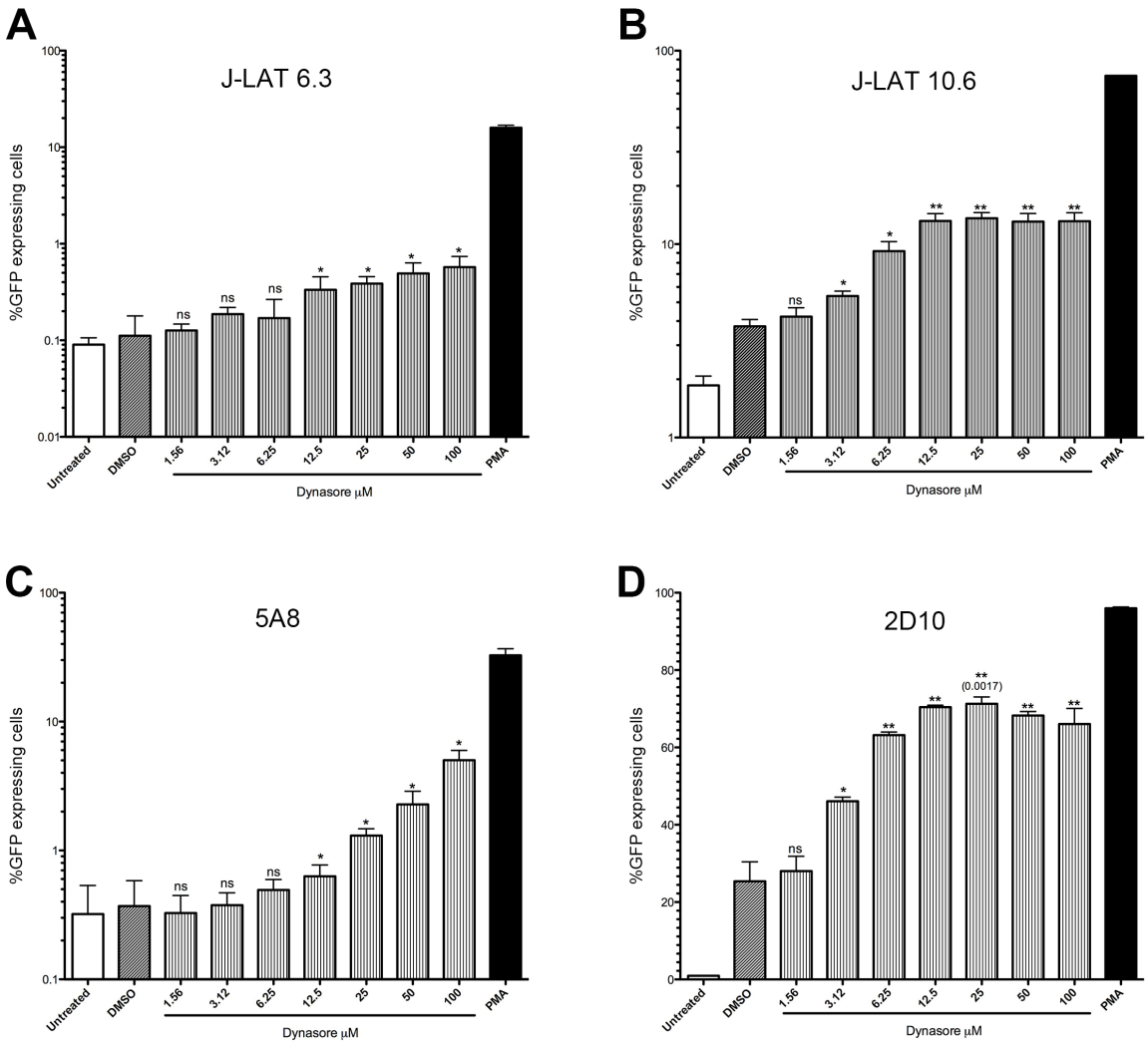


Figure 3.3: HIV activation by dynasore and PMA in four cell models of latency.

(A) J-LAT 6.3, **(B)** J-LAT 10.6, **(C)** 5A8 and **(D)** 2D10 cell lines were treated with increasing concentrations of dynasore and with PMA. % of cells expressing GFP expression was assessed by flow cytometry. Bar graph corresponds to mean and standard deviation of experiments performed in triplicates. Significance was calculated by 2-tailed paired samples t test analysis (*<0.05, **<0.01, ***<0.001, ns = not significant).

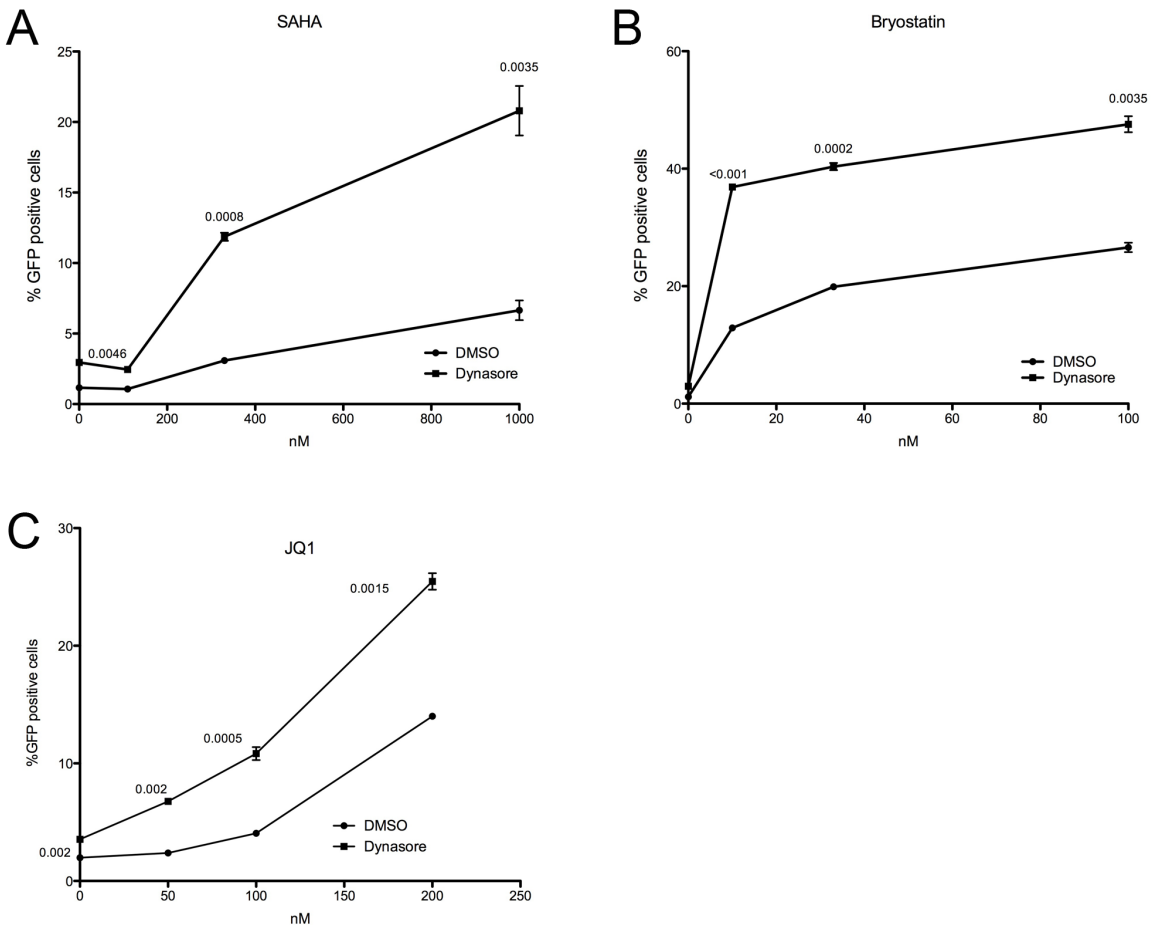


Figure 3.4: Reactivation of latent HIV-1 by treatment with dynasore in combination with SAHA, bryostatin and JQ-1.

(A) Effect in HIV-1 reactivation after treatment with increasing concentrations of SAHA, **(B)** bryostatin and **(C)** JQ1 with solvent or with dynasore (12.5μM) assessed by flow cytometry. Bar graph corresponds to mean and standard deviation of experiments performed in triplicates. Significance was calculated by 2-tailed paired samples t test analysis.

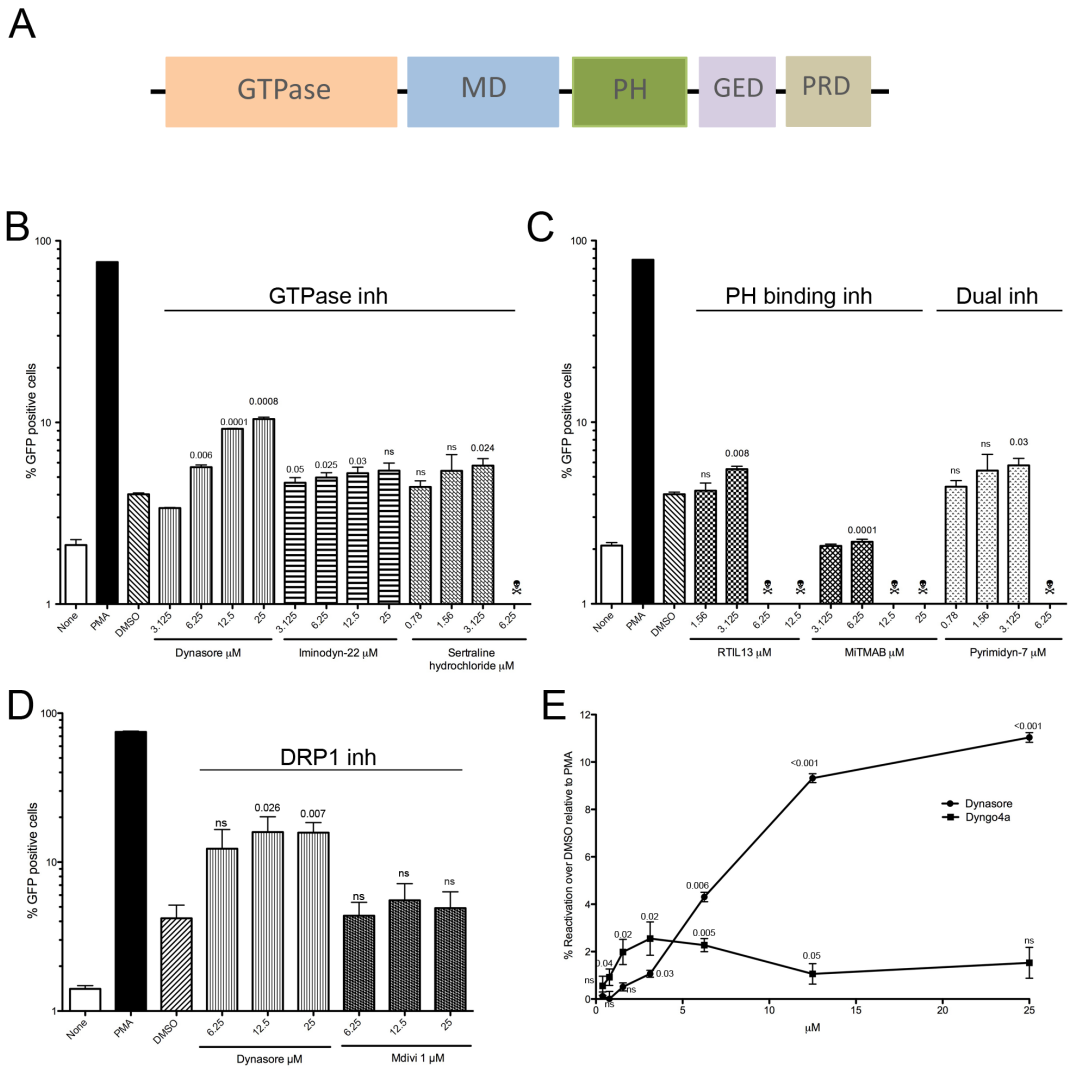
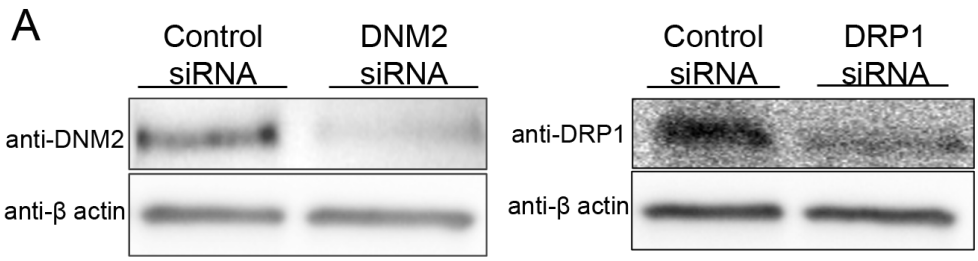


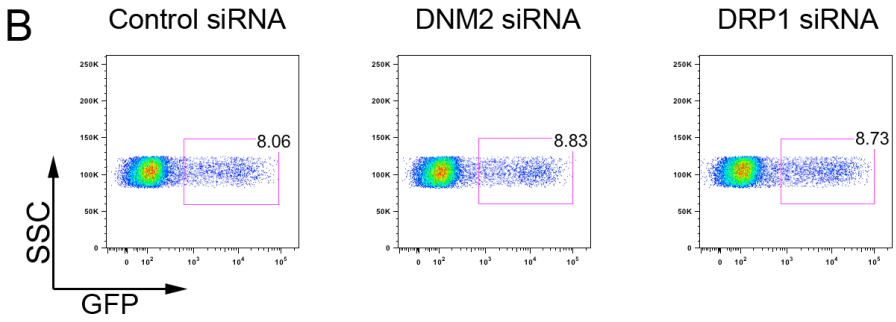
Figure 3.5: Effects of dynamin-domain inhibitors, drp1 inhibitor and a dynasore analog in the reactivation of latent HIV-1.

(A) Domain architecture of dynamin. **(B)** JLAT 10.6 cells were treated with dynasore, GTPase inhibitors, **(C)** lipid binding pleckstrin-homology (PH) domain inhibitor, dual (GTPase and PH domain) inhibitors and (D) drp1 specific inhibitor; percentage of cells expressing GFP was assessed by flow cytometry. Untreated cells (white bar), PMA (black bar) and DMSO (diagonal hatched bar) data were also assessed. **(E)** Dynasore and dyngo 4a (dynasore analog) effect on HIV reactivation in JLAT 10.6 cells was assessed by flow cytometry. Graphic plots show percentage of GFP expressing cells over DMSO and relative to PMA. Bar graph corresponds to mean and standard deviation of experiments performed in triplicates. Significance was calculated by 2-tailed paired samples t test analysis; ns = not significant.

Figure 3.6: HIV expression is not modified by the knockdown of DNM2 or DRP1.

(A) Immunoblot analysis of dynamin-2 (DNM2) and drp1 knockdowns in JLAT 10.6 cell line. Negative control siRNA and siRNA against DNM2 and DRP1 were introduced into JLAT cell by Amaxa nucleofection twice. After 24 hours, cells were either lysed for western blot analysis, **(B)** analyzed by flow cytometry **(C)** or incubated with/out DMSO, dynasore and PMA for 24 hours, and collected for flow cytometry analysis. Data are representative of two experiments.


A
Control siRNA
DNM2 siRNA
anti-DNM2
anti-β actin
Control siRNA
DRP1 siRNA
anti-DRP1
anti-β actin


B
Control siRNA
DNM2 siRNA
DRP1 siRNA
8.06
8.83
8.73
SSC
GFP

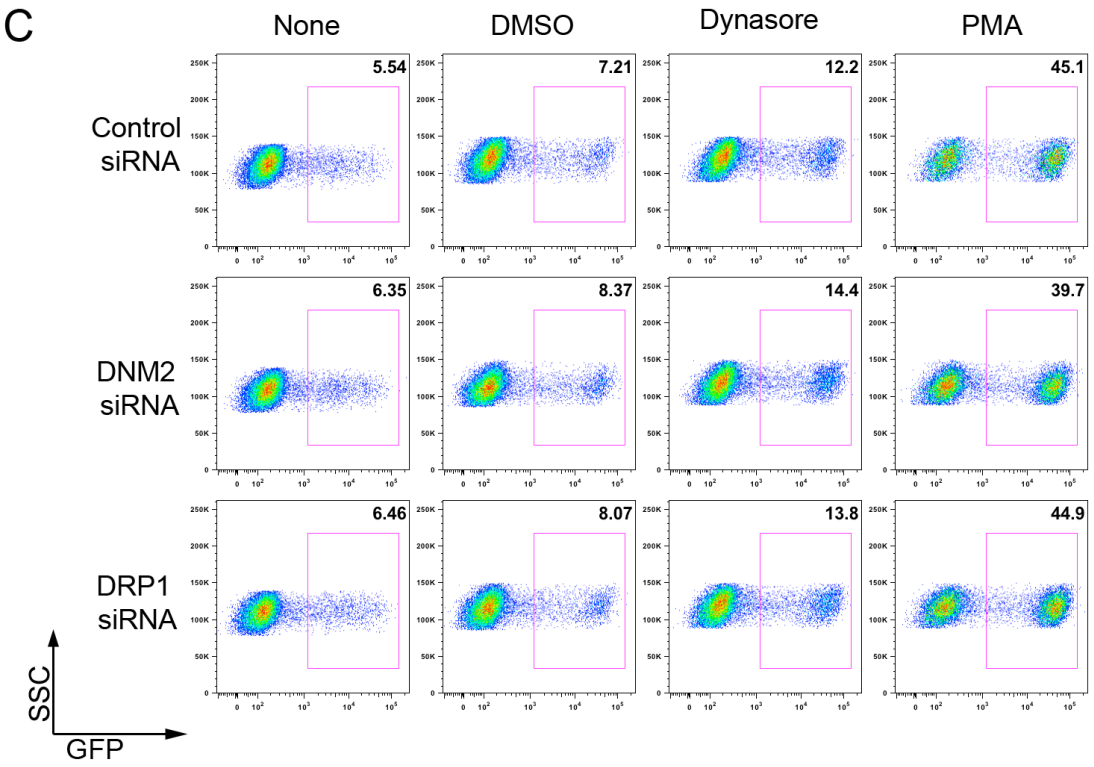

C
None
DMSO
Dynasore
PMA
Control siRNA
DNM2 siRNA
DRP1 siRNA
5.54
7.21
12.2
45.1
6.35
8.37
14.4
39.7
6.46
8.07
13.8
44.9
SSC
GFP

Figure 3.7: Characterization of dynasore signaling associated with HIV-1 reactivation from latency.

(A) 2D10 cells were pre-incubated with increasing concentrations of BAY11-7082, **(B)** BIM, **(C)** SP600125, **(D)** BAPTA-AM and **(E)** Rapamycin for 2h before stimulation with/out DMSO, dynasore and PMA. Percentage of GFP expressing cells was analyzed by flow cytometry. Bar graph corresponds to mean and standard deviation of experiments performed in triplicates. Significance was calculated by 2-tailed paired samples t test analysis (*<0.05, **<0.01, ***<0.001, ns = not significant).

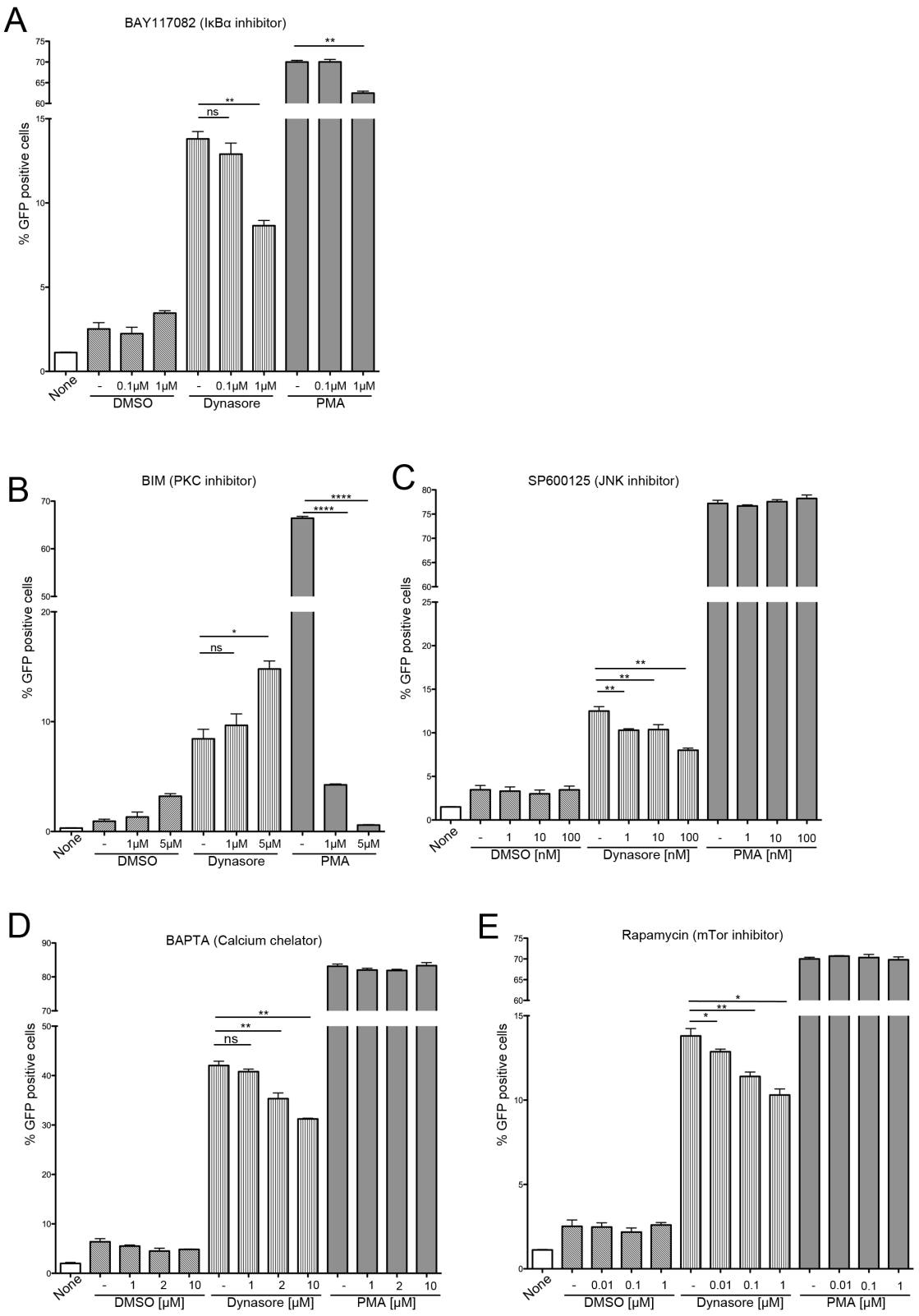

A
BAY117082 (IκBα inhibitor)
% GFP positive cells
None
DMSO
Dynasore
PMA
B
BIM (PKC inhibitor)
C
SP600125 (JNK inhibitor)
DMSO [nM]
Dynasore [nM]
PMA [nM]
D
BAPTA (Calcium chelator)
DMSO [μM]
Dynasore [μM]
PMA [μM]
E
Rapamycin (mTor inhibitor)

Table 3.1: Estimated synergy interaction based on Bliss Independence.

	Fraction Efficacy of Monotherapy	**% Efficacy of combination with Dynasore**				**Fold difference between Exp and Obs Effects**
$F_{Dynaso(A)}$	0.030	Observed	Expected ($=F_A+F_B-F_A*F_B$)	$\Delta F=F_{OBS}-F_{EXP}$	**Bliss Interaction**	
$F_{Bryo-10(B)}$	0.129	36.867	15.475	0.21	S($\Delta F>0$)	2.4
$F_{Bryo-33(B)}$	0.199	40.367	22.268	0.18	S($\Delta F>0$)	1.8
$F_{Bryo-100(B)}$	0.266	47.567	28.770	0.19	S($\Delta F>0$)	1.7
	Fraction Efficacy of Monotherapy	**% Efficacy of combination with Dynasore**				**Fold difference between Exp and Obs Effects**
$F_{Dynaso(A)}$	0.030	Observed	Expected ($=F_A+F_B-F_A*F_B$)	$\Delta F=F_{OBS}-F_{EXP}$	**Bliss Interaction**	
$F_{SAHA-100(B)}$	0.011	2.450	3.992	-0.02	A($\Delta F<0$)	0.6
$F_{SAHA-330(B)}$	0.031	11.867	5.952	0.06	S($\Delta F>0$)	2.0
$F_{SAHA-1000(B)}$	0.067	20.800	9.413	0.11	S($\Delta F>0$)	2.2
	Fraction Efficacy of Monotherapy	**% Efficacy of combination with Dynasore**				**Fold difference between Exp and Obs Effects**
$F_{Dynaso(A)}$	0.035	Observed	Expected ($=F_A+F_B-F_A*F_B$)	$\Delta F=F_{OBS}-F_{EXP}$	**Bliss Interaction**	
$F_{JQ1-50(B)}$	0.024	6.764	5.836	0.01	S($\Delta F>0$)	1.2
$F_{JQ1-100(B)}$	0.040	10.834	7.450	0.03	S($\Delta F>0$)	1.5
$F_{JQ1-200(B)}$	0.140	25.467	17.045	0.08	S($\Delta F>0$)	1.5

CHAPTER 4

DISCUSSION AND SUMMARY

Life expectancy of HIV infected patients has dramatically improved with the introduction of antiretroviral therapy (ART). However, ART alone is not able to eradicate the virus, which persists dormant within reservoirs [1-3] from where it will re-emerge after treatment interruption [4-7]. Currently, there are no available therapies that target the latent form of the virus, thus depletion strategies have been proposed. It is speculated that reversing the latency state of the virus will induce clearance of virus-infected cells through cell cytotoxicity or host immune effector responses [8].

The research presented in this dissertation provides insight into emerging strategies to purge the HIV reservoir, specifically in regards to the reactivation of transcriptionally silent viruses.

In Chapter 1, the contribution of toll-like receptor (TLR) agonists toward HIV-1 latency eradication is reviewed. First, the two major mechanisms of maintenance of latency by quiescent $CD4^+$ T cells are explained. Epigenetic markers, such as histone acetylation, histone methylation and DNA methylation, are defined and the compounds reported to enable reversing of these epigenetics modifications are described. The lack of transcription factors is the second main contributor to HIV latency. Several protein kinase C (PKC) activators were reported to induce HIV transcription, through activation of the transcription factor NF-κB. Furthermore, the TLR-mediated NF-κB signaling capacity to perform activation of HIV in latently infected cells is described, in addition to an overview of TLR receptors, agonists and signaling pathways. Finally, in Chapter 1, a literature review is presented, elucidating the agonists of

TLRs capable of HIV reactivation and HIV-host immune responses regulation. Chapter 2 consists of a report from our group, in which the TLR-2 agonist, Pam3CSK4, was reported as an activator of transcriptionally latent HIV[9]. Pam3CSK4 signaling involved in HIV reactivation requires NF-κB, AP-1 and NFAT transcription factors, and it depends on Tat protein. In addition, Pam3CSK4 triggers phosphorylation of CDK9, in the presence of constant concentration of Cyclin T1. Most significantly, Pam3CSK4 reactivates HIV in the absence of cell activation and/or proliferation [9].

Another important point to mention is that TLR agonists have been investigated for the treatment of cancer, allergies, and viral infections and as vaccines adjuvants. Several clinical trials with TLR agonists already resulted in drugs being FDA approved [10, 11].

Latency is driven by a multifactor and complex mechanisms. Purging therapies targeting multiples targets required for maintenance of the silent provirus will likely be more successful than single target approaches. Chapter 3 unveils a novel activator of HIV, dynasore. Dynasore reactivates HIV in cell line models of latency and requires NF-κB, JNK and calcium influx. A synergistic effect on reactivation from latency is observed when dynasore is combined with known latency-reversing agents, such as Pam3CSK4, SAHA, Bryostatin-1 and JQ1. Exploration of dynasore anti-latency effect in clinical research and a more detailed investigation of dynasore reactivation signal should be evaluated.

The content of this dissertation presented two novel mechanisms that trigger transcription of the latent HIV provirus (Chapter 2 and 3). A review of the

literature about the association between TLR agonists and HIV latency (Chapter 1) has never been published, which will bring the attention to a greater number of researchers. Unraveling new anti-latency compounds and understanding their mechanisms of action in reactivation of latent HIV-1 and modulation of cellular immunity will likely increase chances to achieve efficient therapeutic strategies.

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APPENDIX

MODELING HIV-1 LATENCY IN PRIMARY T CELLS USING

A REPLICATION-COMPETENT VIRUS

Submitted Article: Martins L, Bonczkowski P, Spivak A, Spiegelaere WD, Novis CL, DePaula-Silva AB, Bosque A, Vanderkerckhove L, Planelles V: Modeling HIV-1 Latency in Primary T Cells Using a Replication-Competent Virus. *Retrovirology*.

A.1 Abstract

HIV-1 latently infected cells *in vivo* can be found in extremely low frequencies. Therefore, *in vitro* cell culture models have been used extensively for the study of HIV-1 latency. Often, these *in vitro* systems utilize defective viruses. Defective viruses are appealing choices as they allow for synchronized infections and circumvent the use of antiretrovirals. In addition, replication-defective viruses cause minimal cytopathicity because they fail to spread and usually do not encode *env* or accessory genes. On the other hand, replication-competent viruses encode all or most viral genes and better recapitulate the nuances of the viral replication cycle. The study of latency with replication-competent viruses requires the use of antiretroviral drugs in culture, and this mirrors the use of ART *in vivo*.

We describe a model that utilizes cultured central memory CD4+ T cells and replication-competent HIV-1. Viral replication is blocked by antiretroviral drugs present in the culture medium prior to, during and after viral reactivation. This method generates latently infected cells that can be reactivated using latency reversing agents in the presence of antiretroviral drugs. We also describe a method for removal of productively infected cells prior to viral reactivation, which takes advantage of the down-regulation of CD4 by HIV-1.

The *in vitro* HIV-1 latency model described here provides added relevance to *in vivo* latent HIV-1 through the use of replication-competent HIV-1, which is suppressed by antiretroviral drugs. This method is suitable for the study of

factors that influence the establishment of latency as well as those required for reversing latency.

A.2 Introduction

The existence of cellular reservoirs where HIV-1 resides in a latent state constitutes a formidable barrier towards eradication of viral infection despite the ability of combination antiretroviral therapy (ART) to durably suppress viral replication and restore the circulating CD4+ T cell population [1-3]. One of the major known cellular reservoirs is established in quiescent central memory CD4+ T cells [4, 5]. Reactivation of latent viruses followed by killing of the infected cells has been proposed as a possible strategy ("shock and kill") to purge the latent reservoir [6]. The interest in discovering signals that will induce latent proviruses through the introduction of latency-reversing agents (LRAs) has prompted the development of *in vitro* cellular models for the study of viral latency and reactivation from latency [7-19].

In an effort to recapitulate latency in the CD4+ central memory T cell subset (T_{CM}), we developed a latency model [13, 20] in which naïve cells from the peripheral blood of healthy donors are activated and polarized *in vitro* to direct differentiation into T_{CM}. *In vitro* culture of these cells in the presence of IL-2 leads to the acquisition of a quiescent phenotype [20]. We initially utilized an envelope-defective proviral construct, which was pseudotyped with a second plasmid encoding a full-length HIV-1 envelope glycoprotein gene. This system was designed to circumvent the use of antiretrovirals because the virus was

engineered to be replication-defective. However, we have identified two reasons that prompted us to explore the use of replication-competent viruses. First, we wanted to create an *in vitro* model that more closely resembles that of the *in vivo* environment in which replication-competent virions with the entire HIV-1 genome are present, and in which latency is maintained in the presence of ART. This will allow for more accurate predictions of the efficacy of candidate LRAs to support future HIV-1 eradication clinical trials. Second, as we recently reported [21], we have documented a recombination event between the proviral construct and the envelope glycoprotein construct, leading to the production of an unexpected replication-competent virus in culture.

To address these goals, we here describe two unique modifications of a previously published latency model [13].

A.3 Results and Discussion

A.3.1 Infection of cultured T_{CM} cells with HIV-1_{NL4-3} results in latently infected cells that can be reactivated

In order to characterize the effects of replication-competent HIV-1 and ART on the establishment of latent HIV-1 and on reactivation of latently infected cells, we developed an *in vitro* cell culture method based on infection of cultured T_{CM} cells with replication-competent viruses. We selected HIV-1_{NL4-3}, an X4-tropic virus that encodes a complete HIV-1 genome [22]. We decided to used an X4-tropic virus due to the lack of CCR5 expression in cultured T_{CM} cells [13]. Cultured T_{CM} cells were generated as previously described [13, 20, 23]. Briefly, naïve CD4 T cells were activated for three days, and then cultured for an

additional four days in medium containing 30 IU/mL of IL-2 to ensure maintenance of cell viability. Cells were infected with HIV-1_{NL4-3} by spinoculation at day 7 (Figure A.1) [13, 20, 23]. Following inoculation of the culture with HIV-1_{NL4-3}, viral spread to uninfected cells was allowed to continue for 6 days. Because cell-to-cell transmission of HIV-1 is highly efficient *in vitro* [24], we incorporated a "cell crowding" step into the culture protocol as described in the Materials and Methods section. In this method, cells are cultured in round-bottom wells, which allow them to be highly concentrated by gravity, in contrast to culture in flasks, in which cells are not confined to a small surface area. We estimate, based on surface area, that culturing in a round-bottom 96-well plate with a density 10^5 cells/100 mL/well relative to a 25 cm^2 culture flask results in an increase in number of cells/cm^2 of between 15- and 20-fold. To test utility of crowding cells, we used HIV-1_{NL4-3}-infected cells from five blood donors, and crowded cells between days 10 and 13 (Figure A.1A, protocol B) or cultured the cells in standard culture flasks (Figure A.1A, protocol A). Culturing under crowded conditions (protocol B) resulted in a 65 +/- 8% increase in the frequency of productively infected cells at day 13 (Figure A.1B). However, we also found that after continued culturing for an additional 6 days, cells in the crowded condition (protocol B) contained 36 +/- 20% fewer viable cells than those cultured in standard culture flasks (protocol A; Figure A.2).

ART was introduced to the cultures, starting at day 13 and maintained for the remainder of the experiment. ART consisted of either 1 mM Raltegravir and 0.5 mM Nelfinavir; or 1 mM Nelfinavir. We assessed the effectiveness of ART

treatment to block new infections by using MT2 cells [25], a highly permissive cell type to X4-tropic HIV-1, as indicators. Culture supernatants from HIV-1_{NL4-3} infected (or uninfected control) cells at day 17 were isolated and incubated with fresh MT2 cells, cultured for 48 hours, and then evaluated for intracellular p24 (ICp24) by flow cytometry. As shown in Figure A.3, supernatants from primary cell cultures containing ART did not result in intracellular ICp24 levels that were higher than background detection levels (< 0.05% ICp24+ cells) from uninfected cell supernatants. In contrast, culture supernatants from ART-negative cultures resulted in very high levels (63%) of intracellular ICp24+ MT2 cells. We, therefore, conclude that concentrations of ART in our cultures are sufficient to block additional infections.

To evaluate latent infection in these cultures, we applied strong reactivating conditions (aCD3/aCD28 treatment) during days 17 and 18. Following this 48 hour treatment, cells were analyzed for ICp24. We compared the percentages of ICp24+ cells in the absence of reactivation conditions (IL-2 alone) with those in the presence of reactivation (IL-2 + aCD3/aCD28). Because virus spread was effectively suppressed, we conclude that any increase in the frequency of ICp24+ cells after aCD3/aCD28 treatment would be solely due to reactivation of latently infected cells and not to viral spread. We found that cells that were crowded (protocol B) produced the highest frequency of latently infected cells, as evidenced by the 3.8-fold increase in levels of viral reactivation (Figure A.1C). Furthermore, the frequency of cells that reactivate latent virus following stimulation with aCD3/aCD28 is directly proportional with the frequency

of productive infection measured at day 13, and inversely proportional to cell viability (data not shown).

A.3.2 Removal of productively infected cells using magnetic bead isolation

HIV-1_{NL4-3}–infected cultures that were not stimulated with aCD3/aCD28 contained significant numbers of productively infected cells (i.e., ICp24+) in addition to latently infected cells. Therefore, the actual number of cells containing reactivated viruses should be calculated by subtracting the number of cells that were productively infected prior to reactivation from the number of cells that were productively infected after reactivation. However, for the purpose of characterizing genomic, transcriptional or proteomic features of latently infected cells, it would be ideal if productively infected cells could be removed from the cultures. To that end, we adopted a previously described magnetic isolation method to remove productively infected cells (“P”) [26], based on the ability of HIV-1 to down-regulate CD4 [27-29]. We, therefore, isolated cells expressing high levels of cell surface CD4, which would contain both uninfected (“U”) and latently infected (“L”) cells (Figure A.4A). This procedure rendered a purity of 98.5 +/- 1.5% of CD4+ICp24(-) cells (Figure A.4B) for a representative donor of nine donors. Latently infected cells and uninfected cells constitute the positive fraction (UL) binding to the magnetic beads as they express high levels of CD4, whereas productively infected cells (“P”) express extremely low levels of CD4 (Figure A.4A).

We then treated cells in the UL fraction with aCD3/aCD28 beads + IL-2 or with IL-2 alone (baseline) for 48 hours. After reactivation, cells were collected and analyzed for ICp24 expression (Figure A.4C) and cell associated (CA) HIV-1 RNA (Figure A.4D). Upon stimulation with aCD3/aCD28, ICp24+ cells consistently increased relative to IL-2 alone (Figure A.4C). The increase in the protein levels was concomitant with increased levels of cell-associated HIV-1 RNA (Figure A.4D).

Viral reactivation with aCD3/aCD28 in the absence of ART led to viral spread in the culture (Figure A.5). Levels of ICp24+ cells (Figure A.5A) and cell-associated HIV-1 RNA (Figure A.5B) increased with aCD3/aCD28 treatment with levels that are dramatically higher than that observed for samples cultured in ART.

A.3.4 Infection of cultured T_{CM} cells with a replication-competent GFP variant of HIV-1_{NL4-3} results in latently infected cells that can be reactivated

In order to facilitate drug discovery efforts, for which higher cell numbers and more rapid assessment of latency reversal is desired, we modified the above model as follows. We use the HIV-1_{NL4-3}–derived construct, HIV-1 NLENG1-IRES [30], in which the EGFP coding sequence followed by an IRES element were inserted between the *env* and *nef* genes (Figure A.6A). Cells infected with HIV-1 NLENG1-IRES were crowded for the entire 6-day viral spread period to accommodate slower replication of this virus (Figure A.6B). On day 13, 2 mM Raltegravir was added to cultures. On day 14, cells were treated with IL-2, IL-2 +

aCD3/aCD28 beads or IL-2 + PHA for 48 hours and EGFP expression was measured by flow cytometry (Figure A.6C). Treatment with aCD3/aCD28 beads resulted in increased production of EGFP+ cells in all 9 samples. PHA treatment also generated increased production of EGPF+ cells for five out of the nine blood donor samples tested.

A.4 Conclusions

We describe here a primary T cell *in vitro* model for studying HIV-1 latency using replication-competent virus that is suppressed by the addition of ART. In contrast to previous models used for the study of HIV-1 latency that employ pseudotyped virions engineered to undergo a single round of replication, this assay permits the use HIV-1 that expresses the entire HIV-1 genome. It is unclear whether HIV-1 accessory genes influence the establishment of latency or the reactivation of latent proviruses. However, it is known that upon reactivation, HIV-1 accessory genes are predicted to downregulate cell surface markers, such as CD4, tetherin, MHC I, NTBA, CCR7 and CD1d, [27-29, 31-35]. Since it is now clear that reactivation of latent HIV-1 is not always followed by recognition by immune surveillance mechanisms [36], the actions of accessory proteins during the process of reactivation must be taken in consideration when testing for CTL and NK killing.

The use of a replication-competent virus allows us to achieve high viral titers within our cultures because multiple rounds of replication and infection of new cells occur during the six days infection/spread period. New infections are

facilitated during the second half of the infection by crowding the cells, which would facilitate cell-to-cell spread of HIV-1 throughout the culture. This condition is reminiscent of cell crowding that occurs in lymph nodes. The increase in productive infection that we observe is proportional to the number of latently infected cells that can be reactivated. However, we also see a proportional decrease in cell viability as a result of increased viral titers.

To facilitate drug discovery efforts, we describe the use of a replication-competent HIV-1 that expresses EGFP. Detection of HIV-1 reactivation with this virus does not require cell fixation or staining, but simply direct flow cytometric analysis. Therefore, the use of the HIV-1 NLENG1-IRES virus would be ideal for medium- or high-throughput screens.

One disadvantage of using replication-competent viruses for the study of latency is that, as observed in our system, there is a background of productively infected cells that complicates the study of the latent infections. We show that this can be overcome by the removal of CD4(-) cells via magnetic bed isolation. The result is a population containing both uninfected and latently infected cells, but mostly devoid of productively infected ones. Therefore, studies aimed at documenting the presence or absence of transcription factors and co-activators at the HIV LTR, as well as studies on the cellular transcription profiles of latently infected cells, can be undertaken with minimal contamination from productively infected cells.

A.5 Methods

A.5.1 Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Nelfinavir, Raltegravir (Cat # 11680) from Merck & Company, Inc., Human rIL-2 from Dr. Maurice Gately Hoffman-La Roche Inc [37], HIV-1_{NL4-3} from Dr. Malcolm Martin [22], MT-2 cells from Dr. Douglas Richman [38, 39] and ACH-2 cells from Dr. Thomas Folks [7, 40]. HIV-1 NLENG1-IRES was a kind gift from Dr. David Levy [30]. The VQA plasmid was a kind gift from Dr. Greg Laird and Dr. Robert Siliciano.

A.5.2 Generation of infected cultured T_{CM} cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors following protocols outlined in IRB #392 (University of Utah Institutional Review Board approved). Naïve cells were isolated and cultured T_{CM} cells were generated as previously described [13, 20, 23]. HIV-1_{NL4-3} and HIV-1 NLENG1-IRES viruses were generated in HEK293FT cells using calcium phosphate transfection as previously described [13, 20]. For infection of cultured T_{CM} cells with HIV-1_{NL4-3} or HIV-1 NLENG1-IRES, cells were infected by spinoculation at a multiplicity of infection (MOI) of 0.1 as previously described [20]. Prior to infection of cells with HIV-1_{NL4-3}, cells were cultured in standard tissue culture flasks. Following infection of cells with HIV-1_{NL4-3}, cells were either cultured in standard tissue culture flasks at a cell density of 10^6 cells/mL or in 96-well round bottom

plates using a density of 10^5 cells/100 mL/well. Prior to infection of cells with HIV-1 NLENG1-IRES, cells were cultured in 96-well flat bottom plates using a density of 2 x 10^5 cells/200 mL/well. After spinoculation, cells were cultured in this same condition.

A.5.3 Removal of productively infected cells using CD4 positive isolation

Magnetic isolation of CD4 positive cells was achieved using Dynabeads® CD4 positive isolation kit as described by the manufacturer (Life Technologies) with the exception that 75 mL of the aCD4 magnetic bead suspension were added per 10^7 cells instead of 25 mL.

A.5.4 Viral reactivation

1-3x10^5 cells were left untreated, stimulated with Dynabeads® Human T-Activator CD3/CD28 (1 bead/cell, Life Technologies, cat. No 111.32D) or 1.2 mg/mL PHA (Thermo Scientific, cat. No. R30852801) for 48 hours.

A.5.5 Flow cytometry analysis

For analysis of HIV-1_{NL4-3} infected cells, samples were first stained with cell viability dye (Fixable Viability Dye eFluor® 450, affymetrix, eBioscience, San Diego, CA.) at 0.1 mL/1-3x10^5 cells for 15 minutes at 4^{o}C and then stained, intracellularly, with a conjugated ICp24-FITC antibody (KC57, Coulter) as previously described [13]. For detection of surface CD4 expression, cells were stained with mouse anti-human CD4-APC (clone S3.5, Invitrogen). Flow

cytometry was performed with a BD FacsCanto II flow cytometer using the FACSDiva acquisition software (Becton Dickinson, Mountain View, CA.). Data were analyzed with Flow Jo (TreeStar Inc, Ashland, OR).

A.5.6 Assay for infection of indicator cells

On day 17, 100 mL aliquots of cell culture supernatants were added to 400 mL of MT2 cells (2.5×10^5). MT-2 culture ART concentrations were matched with those of the inoculating cell culture supernatants. Cells were centrifuged for 2 hours at 2900 RPM and at 37^{o}C. Following spinoculation, MT-2 cells were cultured for an additional 48 hours in 500 mL of fresh RPMI. ICp24 was measured by flow cytometry.

A.5.7 PCR analysis

Quantitative polymerase chain reaction (qPCR) for cell-associated HIV-1 mRNA was carried out according to a recently published protocol [41]. Briefly, cultured cells were counted and pelleted by centrifugation. Aliquots of 10^5 cells underwent RNA extraction and purification using a commercial viral RNA isolation kit according to manufacturer's protocol (Zymo Research). DNase treatment was performed (Quanta Biosciences) followed by cDNA synthesis using qScript cDNA Supermix containing oligo-dT primers and random hexamers according to manufacturer's protocol (Quanta Biosciences). RNA aliquots that did not contain reverse transcriptase (no RT controls) were run in parallel for every sample. Real-time quantitative PCR was subsequently performed in

triplicate on cDNA and RNA (no RT control) samples using TaqMan Universal Master Mix II (Applied Biosystems) on a Roche LC480 Real-Time PCR instrument. Primers and probe used were as follows: forward primer (5' to 3') CAGATCCTGCATATAAGCAGCTG, reverse primer (5' to 3') TTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC and probe (5' to 3') FAM-CCTGTACTGGGTCTCTCTGG-BHQ1. Cycling conditions were as follows: 50°C for 2 minutes followed by 95°C for 10 minutes for polymerase activation, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Serial 10-fold dilutions of a plasmid containing the HIV-1 3'LTR (VQA plasmid; obtained from Greg Laird and Robert Siliciano) from 10^6 to 1 copy per well were amplified in triplicate along with unknowns in order to provide a standard curve and quantify cell-associated viral mRNA.

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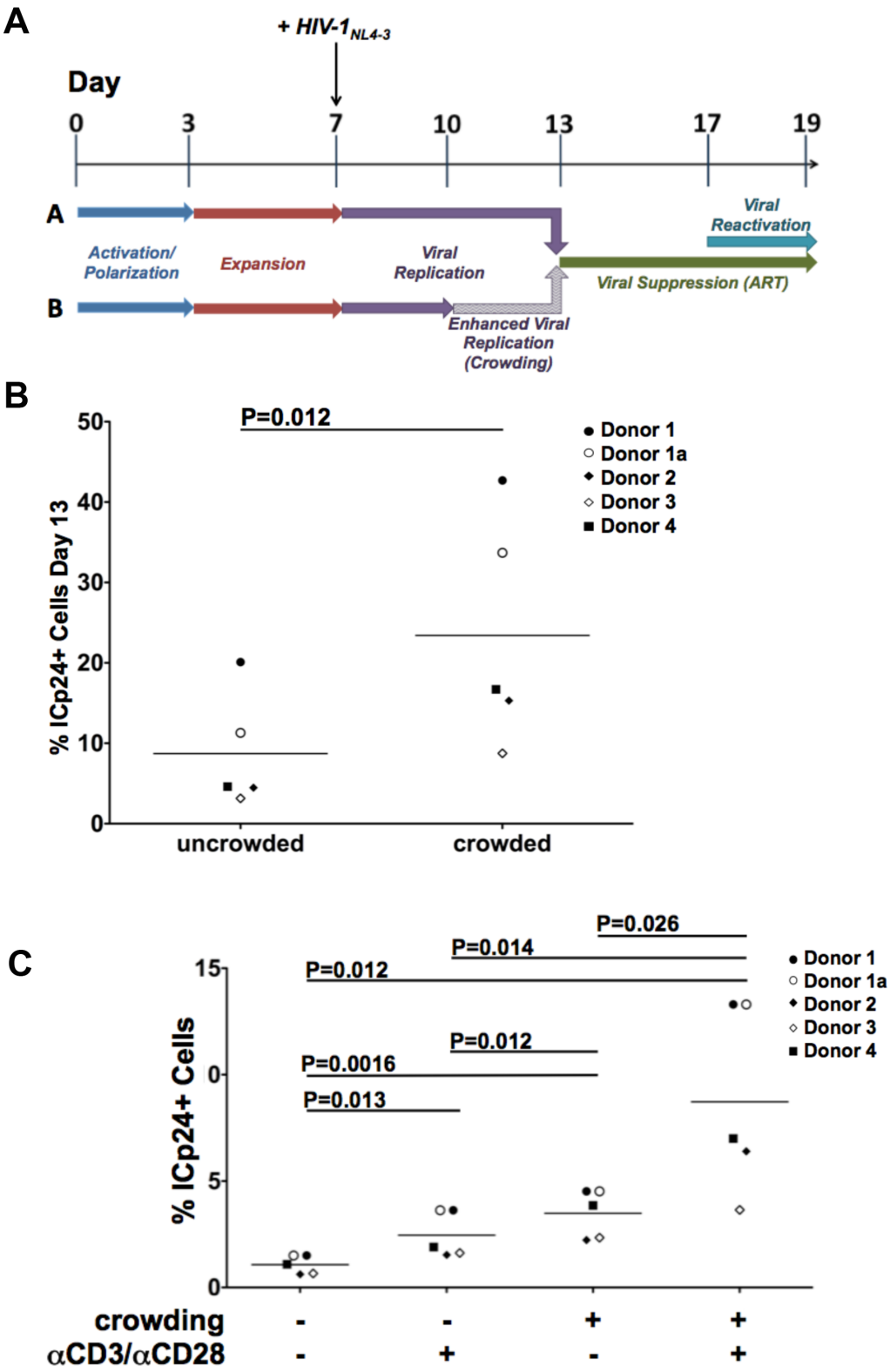
Figure A.1: Generation of cultured T_{CM} cells latently infected with HIV-1_{NL4-3} and reactivation of latent HIV-1.

(A) Two protocols were investigated for generation of latently infected cultured T_{CM} cells using HIV-1_{NL4-3} (protocols A and B) **(B)** Intracellular ICp24 was measured using flow cytometry to assess levels of productive infection on day 13 using five samples from four blood donors (Naïve CD4+ cells from Donor 1 were used in two separate expermiments) following protocol B. Each symbol represents a different sample. The mean is represented with a horizontal line. Significance was calculated using a 2-tailed paired t test analysis (P values provided) **(C)** HIV-1 infected cell cultures were generated using protocols A (no cell crowding) or B (cells were crowded between days 10 and 13) from the same blood donors in Figure 1B. Cells were treated with IL-2 alone or IL-2 + aCD3/aCD28 on Day 17. Intracellular ICp24 was measured using flow cytometry to assess reactivation of latent HIV-1 on Day 19. The mean is represented with a horizontal line. Significance was calculated using a 2-tailed paired t test analysis (p values provided)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Day
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3
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17
19
A
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Activation/ Polarization
Expansion
Viral Replication
Viral Reactivation
Viral Suppression (ART)
Enhanced Viral Replication (Crowding)
B
P=0.012
% ICp24+ Cells Day 13
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uncrowded
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Donor 1
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P=0.012
P=0.012
P=0.0016
P=0.013
% ICp24+ Cells
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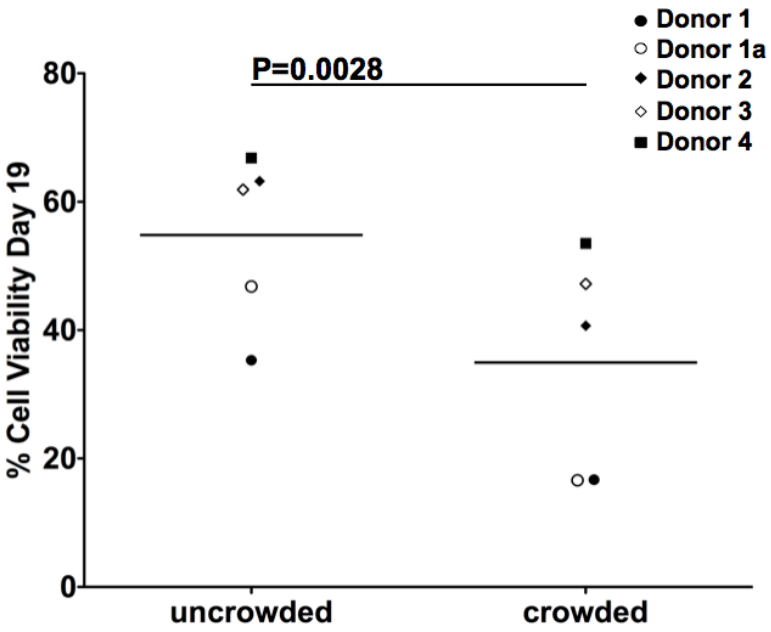


Figure A.2: Comparison of cell viability between two methodologies.

Cultured T_{CM} cells latently infected with HIV-1_{NL-43} were generated using protocol A (uncrowded) or protocol B (crowded). Cell viability was measured using flow cytometry. Each symbol represents a different sample. The mean is represented with a horizontal line. Significance was calculated using a 2-tailed paired t test analysis (p value provided).

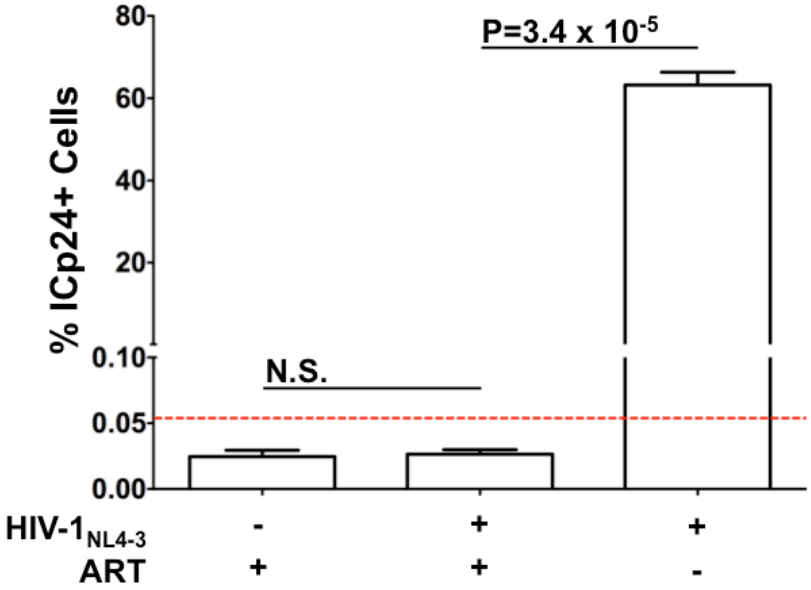
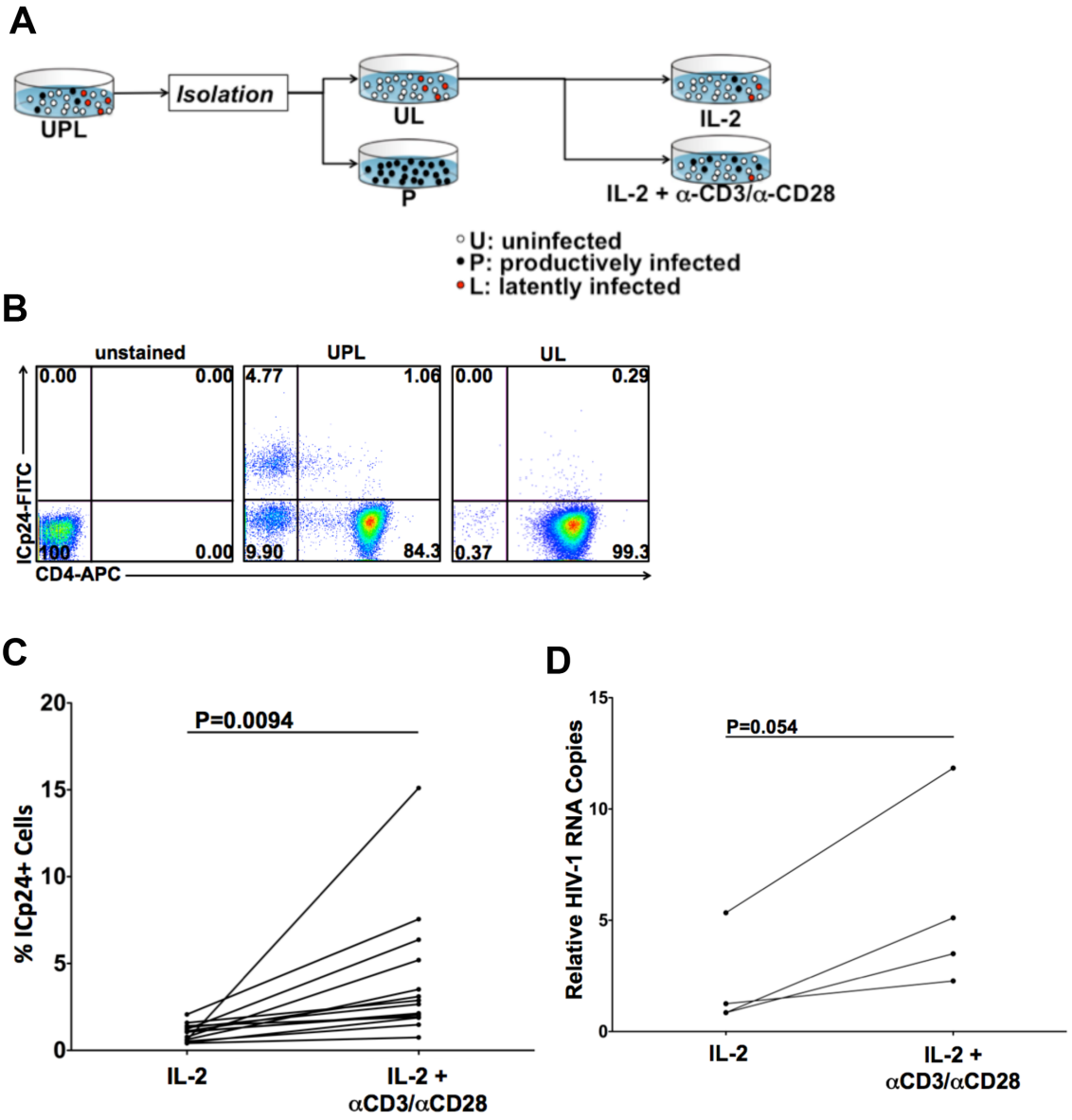


Figure A.3: Antiretroviral is sufficient to impair viral infections.

Cultured T_{CM} cells from a single blood donor (Donor 6) were latently infected with HIV-1_{NL-43} using protocol B. On day 17, MT2 cells were spinoculated with cell culture supernatents in the presence or absence of 1 mM Nelfinavir as indicated. After 48 hours in culture, ICp24 was measured using flow cytometry. Significance was calculated using a 2-tailed paired t test analysis (p value provided).

Figure A.4: Removal of productively infected cells from culture

(A) Cultured T_{CM} cells latently infected with HIV-1_{NL4-3} were generated using protocol B. On day 17 HIV-1 infected cells containing uninfected, productively infected and latently infected cells (UPL) were subjected to magnetic isolation based on cell-surface CD4 expression. CD4+ cells contain uninfected and latently infected cells (UL) and CD4- cells contain productively infected cells (P). The UL fraction was treated with either IL-2 alone or IL-2 + aCD3/aCD28 for 48 hours. **(B)** Cells from a single blood donor (Donor 5) were cultured and infected with HIV-1_{NL4-3} following protocol B. On day 17, CD4+ cells were isolated using positive magnetic selection. Cells before isolation are denoted UPL and purified cells are denoted UL. Cells were stained with a cell viability dye followed by cell-surface staining with a CD4-APC antibody then stained intracellularly with a p24-FITC antibody. Dot plots of the viable fraction are shown. **(C)** Fourteen CD4+ purified samples were treated with IL-2 alone or IL-2 + aCD3/aCD28 for 48 hours. ICp24 was analysed by flow cytometry. Significance was calculated using a 2-tailed paired t test analysis (P values provided). **(D)** Four CD4+ purified samples were treated with IL-2 alone or IL-2 + aCD3/aCD28 for 48 hours. CA HIV-1 RNA copies were measured by qPCR in triplicate samples. RNA copies were divided by cell number. Mean values are ploted and error bars denote standard deviations. In most measurments, the standard deviation is smaller than the size of the symbol.


A
UPL
Isolation
UL
P
IL-2
IL-2 + α-CD3/α-CD28
U: uninfected
P: productively infected
L: latently infected
B
unstained
UPL
UL
0.00
0.00
0.00
100
4.77
1.06
9.90
84.3
0.00
0.29
0.37
99.3
ICp24-FITC
CD4-APC
C
P=0.0094
% ICp24+ Cells
20
15
10
5
0
IL-2
IL-2 +
αCD3/αCD28
D
P=0.054
Relative HIV-1 RNA Copies
15
10
5
0
IL-2
IL-2 +
αCD3/αCD28

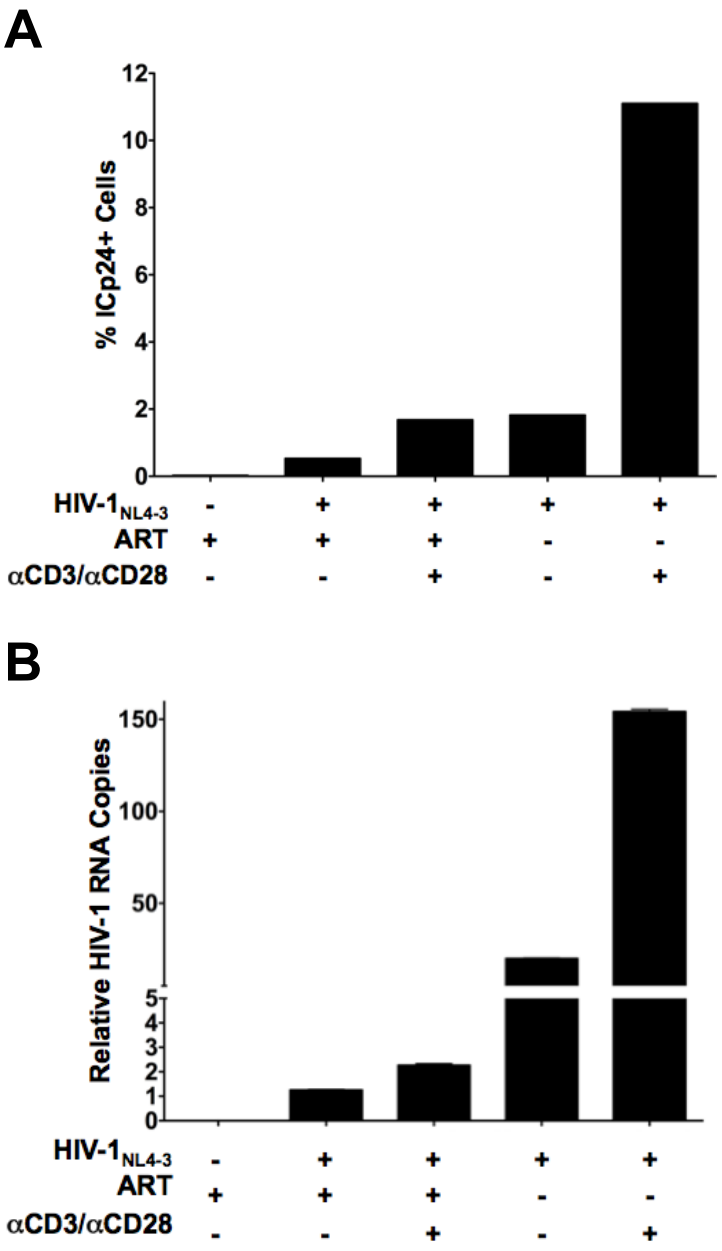


Figure A.5: Viral spread after treatment with aCD3/aCD28 in the absence of ART.

Cultured T_{CM} cells from a single blood donor were latently infected with HIV-1_{NL-43} using protocol B with the exception that one sample was cultured in the absence of ART (1 mM Nelfinavir). On day 17, cells were treated with IL-2 alone or with IL-2 + aCD3/aCD28 as indicated. **(A)** After 48 hours, ICp24 was measured using flow cytometry. **(B)** After 48 hours treatment, HIV-1 RNA was measured.

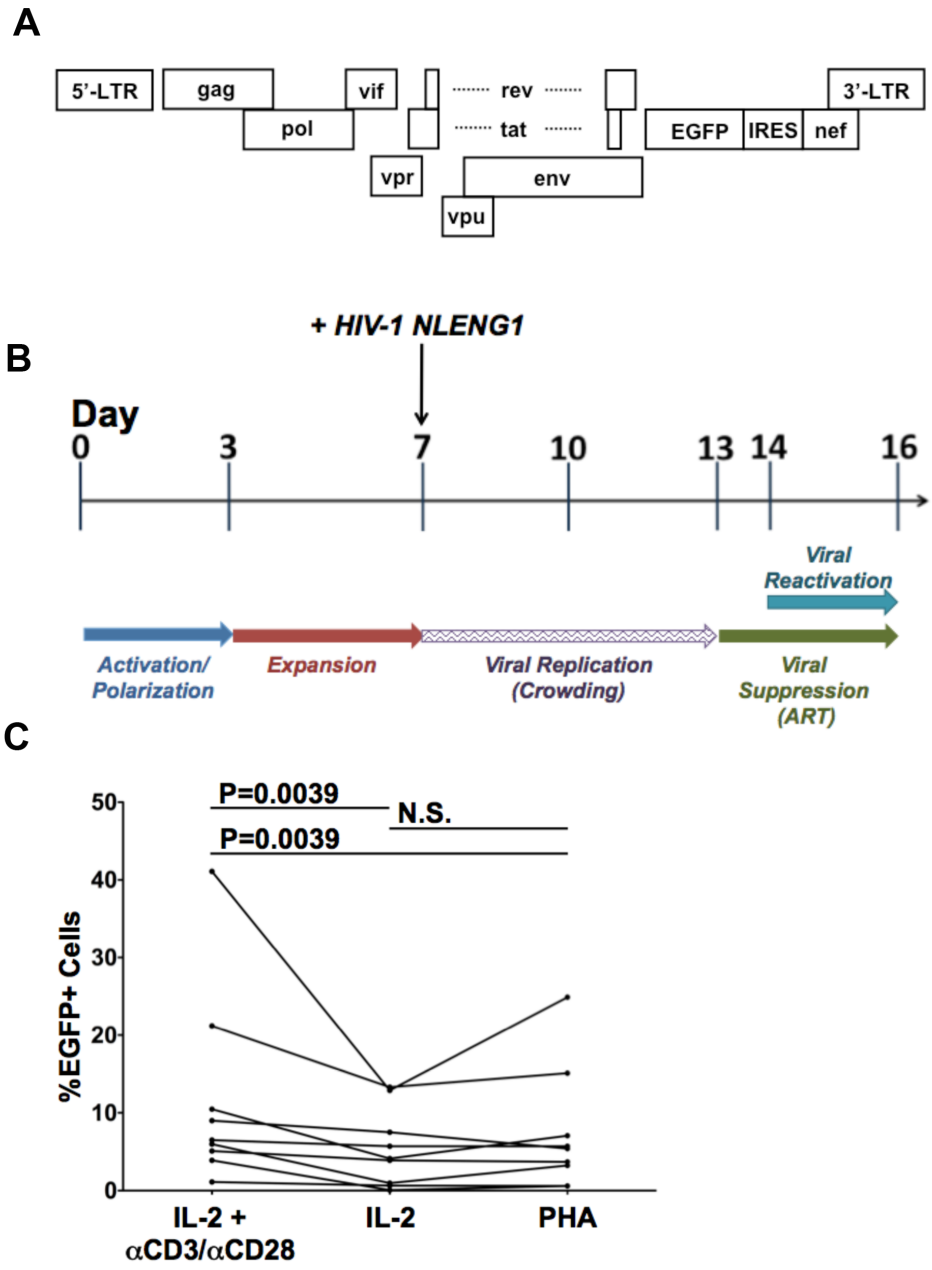


Figure A.6: Generation of cultured T_{CM} cells latently infected with HIV-1 NLENG1-IRES and reactivation of latent HIV-1.

(A) Plasmid used for the generation of latently infected cells with an EGFP reporter. **(B)** Protocol for generation of cultiured T_{CM} cells latently infected with HIV NLENG1-IRES **(C)** Cells were cultured and infected with HIV-1 NLENG1-IRES. On day 14, cultures were treated with IL-2 alone or IL-2 + aCD3/aCD28 or IL-2 + PHA. EGFP expression was measured using flow cytometry on day 16. Significance was calculated using a Wilcoxon matched-pairs signed rank test (p values provided)