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## Dendritic cells potently purge latent HIV-1 beyond TCR-stimulation, activating the PI3K-Akt-mTOR pathway

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### ABSTRACT

**Background:** The latent HIV-1 reservoir in treated patients primarily consists of resting memory CD4<sup>+</sup> T cells. Stimulating the T-cell receptor (TCR), which facilitates transition of resting into effector T cells, is the most effective strategy to purge these latently infected cells. Here we supply evidence that TCR-stimulated effector T cells still frequently harbor latent HIV-1.

**Methods:** Primary HIV-1 infected cells were used in a latency assay with or without dendritic cells (DCs) and reversion of HIV-1 latency was determined, in the presence or absence of specific pathway inhibitors.

**Findings:** Renewed TCR-stimulation or subsequent activation with latency reversing agents (LRAs) did not overcome latency. However, interaction of infected effector cells with DCs triggered further activation of latent HIV-1. When compared to TCR-stimulation only, CD4<sup>+</sup> T cells from aviremic patients receiving TCR + DC-stimulation reversed latency more frequently. Such a “one-two punch” strategy seems ideal for purging the reservoir. We determined that DC contact activates the PI3K-Akt-mTOR pathway in CD4<sup>+</sup> T cells.

**Interpretation:** This insight could facilitate the development of a novel class of potent LRAs that purge latent HIV beyond levels reached by T-cell activation.

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### 1. Introduction

Early on in HIV infection, cellular reservoirs containing latent HIV-1 are formed [1]. These cells contain a stably integrated and complete viral genome, but do not express sufficient amounts of viral proteins to drive virus production and to be recognized by the immune system. Resting memory CD4<sup>+</sup> T cells are the main cell type harboring latent HIV-1 in patients after prolonged therapy [2,3], but T cells with shorter half-lives, such as effector T cells, can also harbor latent HIV-1 [4,5]. Latency is established and maintained through multiple mechanisms that act at transcriptional and post-transcriptional levels [6]. At the transcriptional level, accessibility of the HIV-1 LTR promoter could be blocked in

repressive chromatin structures (which can be overcome with histone deacetylase (HDAC) inhibitors) or by the sequestration of transcription initiation factors such as NF-κB/NFAT/AP-1. Other blocks to HIV-1 transcription include inefficient elongation due to the lack of elongation factors such as P-TEFb or the presence of negative elongation factors (NELFs). These elongation factors influence the RNA polymerase complex and determine whether transcription is prematurely aborted after synthesis of the trans-activation response (TAR) region or extended towards the formation of full-length HIV-1 RNA transcripts. Yukl et al. recently described that HIV latency at the transcriptional level occurs mainly due to inefficient RNA elongation accompanied by a lack of splicing and polyadenylation factors rather than the absence of transcription initiation factors [7]. Inefficient export of viral RNA from the nucleus may also contribute to HIV-1 latency, either due to low levels of Rev protein [8,9] or cellular co-factors like MatrIn-3 or PTB that assist in nuclear RNA export [10,11].

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## Research in context

### Evidence before this study

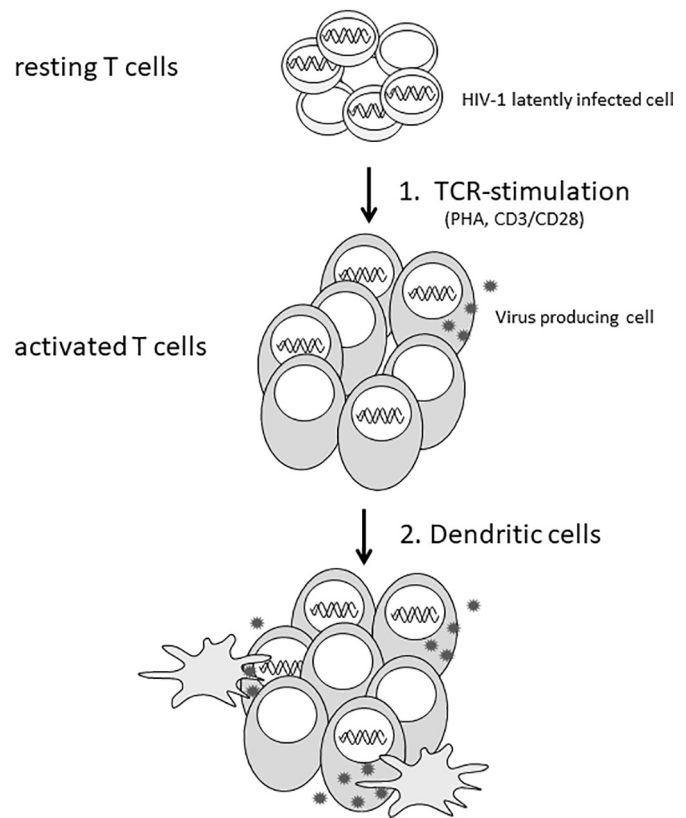
Management of HIV has significantly improved over the past decades, due to combinations of antiretroviral drugs preventing viral replication. However, the virus cannot be eradicated because of the so-called latent reservoir, primarily consisting of resting memory CD4<sup>+</sup> T cells. Several strategies to target this reservoir have been tested, but none are satisfactory. Stimulating the T-cell receptor (TCR), facilitating transition of resting into effector T cells, is currently the most effective strategy to purge these latently infected cells.

### Added value of this study

Here we demonstrated that TCR-stimulated effector T cells can still contain latent HIV-1. Renewed TCR-stimulation or activation of such effector cells with latency reversing agents (LRAs) did not overcome latency. We decided to concentrate on alternative methods of activation next. We found that the interaction of infected effector cells with dendritic cells (DCs) could further activate latent HIV-1. Using such a “one-two punch” strategy might thus be ideal for purging the bodily latent reservoir. Indeed, CD4<sup>+</sup> T cells taken from aviremic patients, which received our DC-stimulation on top of TCR-stimulation, more frequently reversed latency. Our experiments also showed that latency reversal upon DC contact is due to the activation of the PI3K-Akt-mTOR pathway in the target CD4<sup>+</sup> T cells.

### Implications of all the available evidence

These findings might aid the development of novel classes of potent LRAs as drugs used to purge latent HIV beyond the current levels reached by T-cell activation.



**Fig. 1.** Purging latent HIV-1 according to the “one-two punch” strategy. First, resting T cells are activated by stimulating the T cell receptor. Activated effector T cells are subsequently brought into contact with DCs to increase the purging efficiency of latent HIV-1.

Here we want to stress the difference between latent HIV-1 infection reversal and cell activation and the fact that current strategies towards human reversal and cell activation however, is relatively inefficient in also activating latent HIV-1 expression. Our observations offer insights to guide new approaches to increasing the efficiency of latent HIV-1 reversion. We show that the PI3K-Akt-mTOR pathway has to be triggered to activate

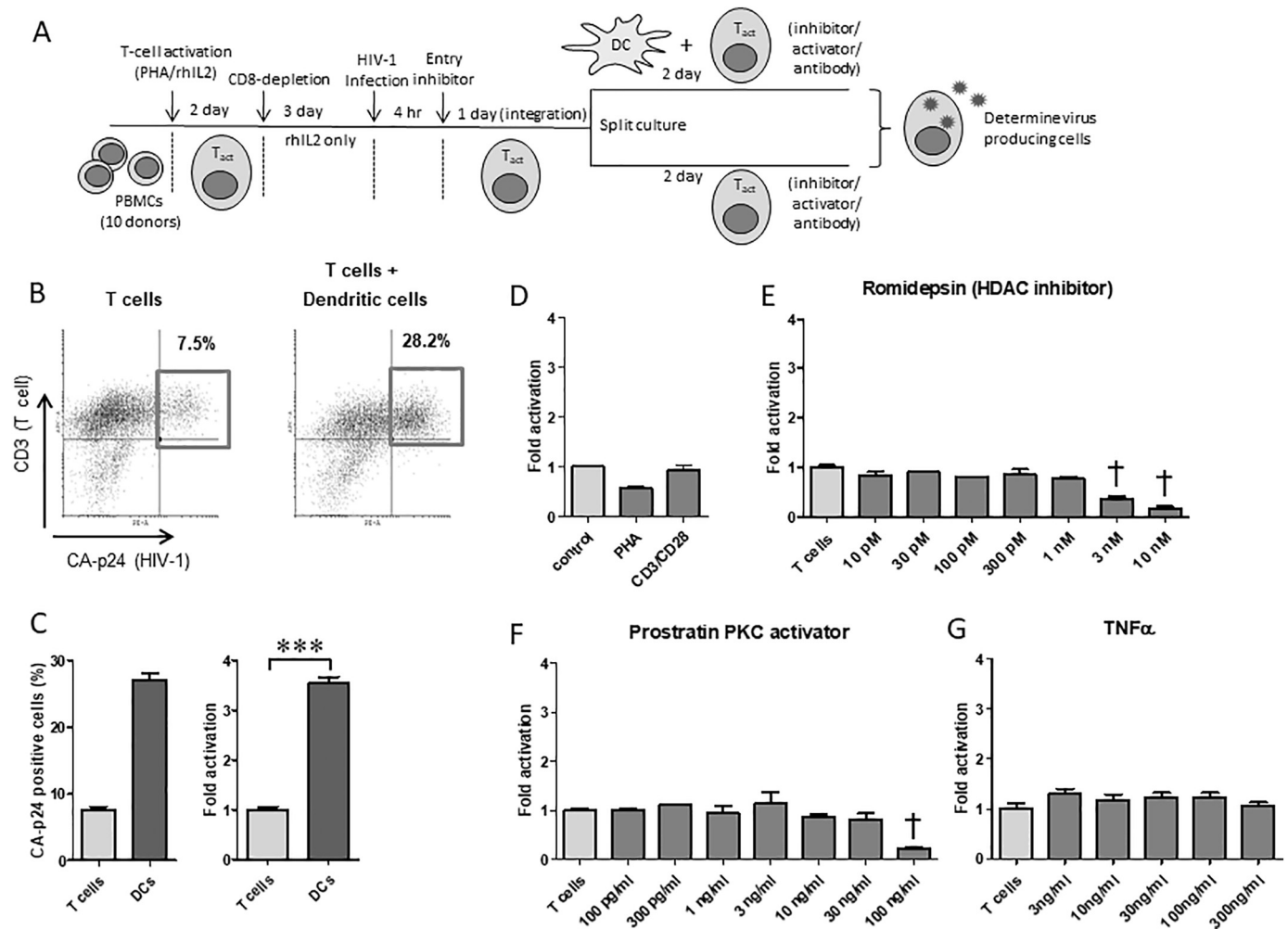
**Table 1**  
HIV-1 patient history.

Patient	Age (years)	Load copies/ml	Current treatment	CD4	Duration UVL (years)
1	37	<40	TDF/FTC/EFV	ND	4.2
2	81	<40	TDF/FTC/EFV	0.46	5.2
3	48	<40	DTG/3TC/ABC	0.38	2.4
4	48	<40	EVG/c/ TAF/FTC	ND	1.2
5	54	<40	NVP/TDF/FTC	ND	12.9
6	42	<40	EVG/c/ TAF/FTC	0.90	3.9
7	23	<40	EFV/3TC/ABC	ND	≥4
8	50	<40	DTG/3TC/ABC	0.80	5.9
9	56	<40	LPV/r/TDF/FTC	ND	12.7
10	52	<40	NVP/3TC/ABC	0.49	13.2
11	61	<40	RAL/DRV/r/TDF/FTC	0.38	8.6
12	50	<40	EVG/c/ TDF/FTC	ND	12.9
13	55	<40	TDF/FTC/EFV	0.73	2.3
14	69	<40	NVP/TDF/FTC	ND	11.5
15	24	<40	ATZ/r/3TC/ABC	ND	≥1
16	41	<40	NVP/TDF/FTC	0.92	13.1
35	48	<40	s/r/TDF/FTC	ND	≤14.2
36	62	<40	NVP/TDF/FTC	ND	≤9.9
37	51	<40	DRV/r/RAL	520	≥1.6

ND = Not determined.

TDF: tenofovir disoproxil fumarate; FTC: emtricitabine; EFV: efavirenz; DTG: dolutegravir; 3TC: lamivudine; ABC: abacavir; EVG/c: elvitegravir cobicistat; TAF: tenofovir alafenamide; NVP: Nevirapine; LPV: lopinavir; r: ritonavir; RAL: raltegravir; DRV: darunavir; ATZ: atazanavir ritonavir; s: Saquinavir.

One of the proposed strategies to exhaust the reservoir is a “shock and kill” treatment in which latency-reversing agents (LRAs) purge HIV-1 from latency, while uninfected cells are protected against virus infection with antiretroviral therapy. Virus-induced cell death or cytotoxic T-cell killing of virus-producing cells was proposed to eliminate the reactivated cells. Stimulation of the T-cell receptor (TCR) to induce the transition of resting into effector T cells is currently the most effective strategy to purge latent HIV. Ex vivo stimulation of the TCR with PHA or CD3-CD28 antibodies can purge approximately 1 cell per million resting memory T cells (= 1 IUPM), as determined with the gold standard quantitative viral outgrowth assay (qVOA) [12]. Based on full-genome sequencing, however, it has been estimated that the intact HIV-1 reservoir size is around 30 cells per million resting T cells in treated patients [12]. This implies that T-cell activation can only purge a fraction of the HIV reservoir and that additional stimuli are required to purge larger portions of latently infected cells. We previously developed an HIV-1 latency assay for activated effector T cells as opposed to quiescent resting T cells [5]. Stimulation of these effector T cells with DCs purged HIV-1 latency efficiently (~3-fold), whereas stimulation with other LRAs had no impact on latency reversal. This in vitro HIV-1 latency model shows that many HIV-1 infected T cells, although fully activated, keep their latent state. This finding is corroborated with data from an ex vivo study [13], using TCR stimulation of quiescent resting patient T cells. Although the T cells were fully activated, a second or third TCR-stimulation could further increase latency reversal 2–3 fold, implying that more is needed to purge activated latently infected cells.



**Fig. 2.** Purging of latent HIV-1 in vitro infected primary activated T cells with classical LRAs or DCs. A) Time-line of the HIV-1 latency assay. Pooled primary PBMCS from 10 healthy individuals were activated with PHA for 2 days. CD8 T cells were removed and CD4-enriched PBMCS were expanded for three days before infecting them in vitro with HIV-1 for 4 h. Cell-free virus was washed away and residual virus cell-fusion events and replication were blocked with the T1249 entry inhibitor. Proviral integration was allowed for 24 h prior to applying various stimuli for another 48 h. B) Representative dot plot of CA-p24 and CD3 stained CD4-enriched T cells stimulated with or without DCs. C) Results are shown as percentage of virus producing cells and fold activation. Shown data are the average of three independent experiments with standard error of the mean. D) Activation of latent HIV-1 with T cell receptor agonist phytohemagglutinin (PHA) and CD3/CD28 antibodies. E) Using different concentrations of the HDAC inhibitor romidepsin. F) Using different concentrations of the PKC activator prostratin or TNF $\alpha$  (G). Representative data are shown with standard error of the mean of at least three independent experiments. Drug toxicity is indicated with †.

virus production in PHA pre-activated T cells, which is achieved upon DC contact. Importantly, we show that reversal of HIV-1 latency in T cells isolated from treated aviremic patients (See Table 1), even when activated with PHA, is further stimulated by DCs. DC coculture increases the frequency of HIV-1 release (all 9 patients) compared to PHA alone (4 out of 9). Using an adapted quantitative virological outgrowth assay (qVOA), the golden standard to measure reversal of HIV-1 latency, we found replicating virus in 8 out of 12 patients (starting at a baseline of 2 out of 12 patients upon PHA stimulation only) upon DC treatment. Collectively, this “one-two punch” strategy (PHA + DCs) can purge the reservoir more potently than PHA treatment alone (Fig. 1). These findings could direct the development of novel LRA treatments that also strongly stimulate the PI3K-Akt-mTOR pathway. The new insights could also be used for the design of a new sensitive culture-based assay to measure the intact HIV-1 reservoir.

## 2. Material and methods

### 2.1. Reagents

The fusion inhibitor T1249 was obtained from Pepsan (Therapeutics BV, Lelystad, The Netherlands) and used at a final concentration of

100 ng/ml. Antibodies against CD9, CD63, CD81, CD83, CD86, CD151, CD45RO, CD45RA, were purchased from Biolegend (San Diego, CA, USA). Other antibodies are directed against CA-p24 (clone KC-57RD1) (Beckman Coulter, Brea, CA, USA) and CD3-APC (BD Pharmingen, Breda, the Netherlands).

### 2.2. Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Central Laboratory Blood Bank, Amsterdam, The Netherlands) by use of a Ficoll gradient and frozen in multiple vials. When required, PBMCs were thawed, activated with phytohemagglutinin (PHA, Remel, 2  $\mu$ g/ml) and cultured in RPMI medium supplemented with 10% FCS and recombinant human IL-2 (rhIL-2, Novartis, 100 U/ml). On day 2 of culture, CD8<sup>+</sup> T lymphocytes were depleted using CD8 immunomagnetic beads (Dyna, Invitrogen, Carlsbad, CA, USA) and the CD4<sup>+</sup>-enriched T lymphocytes were cultured for 3 more days before use in the in vitro latency assay.

Monocytes were isolated from PBMCs with CD14 magnetic beads from Miltenyi Biotec (GmbH, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Purified monocytes were cultured in RPMI 1640 medium containing 10% FCS and differentiated into



monocyte-derived DCs by stimulation with 45 ng/ml interleukin-4 (rIL-4; Biosource, Nivelles, Belgium) and 500 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Brussels, Belgium) on day 0 and 2, and used on day 6 [14].

HEK 293 T cells were grown as a monolayer in Dulbecco's minimal essential medium (Gibco, BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (FCS), 40 U/ml penicillin, 40 µg/ml streptomycin at 37 °C and 5% CO<sub>2</sub>.

SupT1 CCR5 expressing T cells, a kind gift of prof. J. Hoxie [15] were cultured in RPMI 1640 medium containing 10% FCS.

### 2.3. Virus

Plasmid DNA encoding the CXCR4-using HIV-1 LAI primary isolate was transiently transfected in HEK293T cells with the lipofectamin 2000 reagent according to manufacturer's protocol (Fisher Scientific, Landsmeer, NL). Virus supernatant was harvested 2 days after transfection, passed through a 0.2 µm filter and stored in aliquots at -80 °C. The concentration of the virus stocks was determined by CA-p24 ELISA.

### 2.4. In vitro HIV-1 latency assay

HIV-1 infected cells were used in the latency assay as described previously [16, 19, 20]. In short, PHA-activated CD4<sup>+</sup> T lymphocytes (1.5 × 10<sup>6</sup> or 2.0 × 10<sup>6</sup> cells) were infected with HIV-1 for 4 h (20 ng CA-p24). To establish infection over a 4 h window, excess virus was diluted with three washes and cells were incubated with 100 ng/ml T1249 fusion inhibitor to prevent late fusion events and new rounds of virus replication. Cells were incubated for 24 h to allow HIV-1 proviral

integration. Subsequently, cells were treated with or without DCs (ratio DC:T 1:3), with or without 20 µg/ml CD9/CD63/CD81/CD151 antibody, or in the presence or absence of drugs for 48 h in the continued presence of T1249. Single cells were analysed for the presence of the CD3 T cell receptor and expression of intracellular CA-p24 by flow cytometry [5,16]. The percentage of CA-p24 positive CD3-positive cells in the treated culture was divided by the percentage of CA-p24 cells in the mock treated culture and reported as fold activation (a measure of HIV-1 latency reversion).

### 2.5. Intracellular CA-p24 analysis

Cells were fixed in 4% formaldehyde at room temperature for 20 min and subsequently washed with 2% FACS buffer (PBS supplemented with 2% FCS). The cells were permeabilised with BD Perm/Wash™ buffer (BD Pharmingen) and antibody staining was performed in BD Perm/Wash™ at room temperature for 1 h. HIV-1 was intracellularly stained with an CA-p24 antibody and T lymphocytes were discriminated from DCs using the T cell receptor CD3 antibody. Unbound antibody was removed and the cells were analysed on a BD Canto II flow cytometer with BD FACSDiva Software v6.1.2 (BD biosciences, San Jose, CA) in FACS buffer. The T lymphocyte population was defined based on forward/sideward scatter and CD3 expression. Virus production on the gated T lymphocyte population was determined by measuring the intracellular viral CA-p24 protein level. Dendritic cells and T cell doublets were removed from the analyses by gating.

### 2.6. Human phospho kinase array

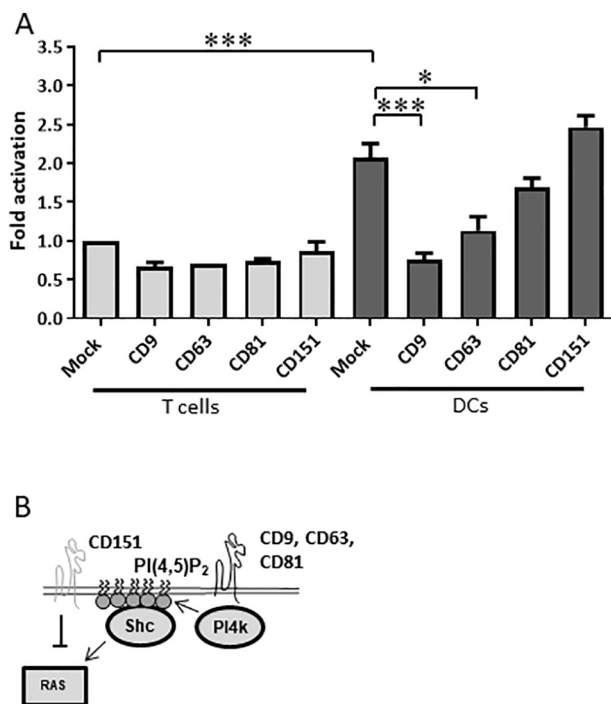
PHA-activated CD4<sup>+</sup> T lymphocytes were infected according to the in vitro HIV-1 latency assay protocol. Infected PBMCs (15 × 10<sup>6</sup>) were incubated with 7 × 10<sup>6</sup> DCs or without DCs for 2.5 h. To disrupt the DC-T cell interaction, which was observed after 1 h incubation, cell cultures were resuspended and washed twice in cold PBS supplemented with 2 mM EDTA and 0.5% FCS. CD4<sup>+</sup> T lymphocytes were isolated at 4 °C using the human CD4<sup>+</sup> T lymphocytes isolation kit from Miltenyi Biotec according to the manufacturer's protocol. Approximately 10 × 10<sup>6</sup> CD4<sup>+</sup> T lymphocytes were obtained after the isolation protocol. Cells were pelleted and treated according to the manufacturer's protocol from the human phospho-kinase antibody array kit (R&D systems).

### 2.7. Ethical approval

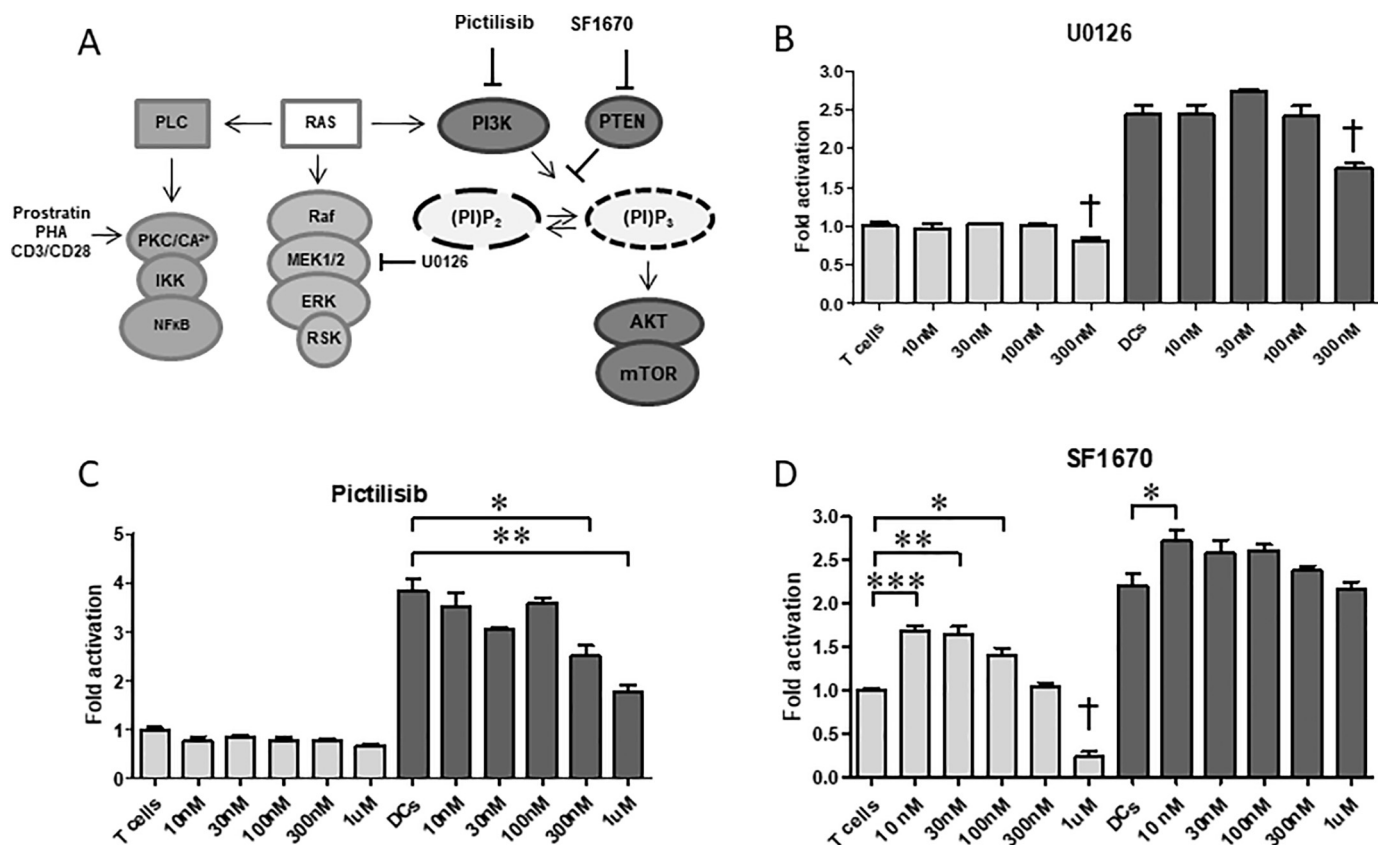
Informed written consent was obtained from all participants of this study, and the study was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

### 2.8. Quantification of HIV-1 release into culture supernatant

Patient-derived CD4<sup>+</sup> T cells were isolated using the human CD4<sup>+</sup> T-lymphocyte isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Between 1.4 and 2.0 × 10<sup>6</sup> cells were used per condition and treated with 2 µg/ml PHA, heterologous monocyte-derived DCs in a 1:3 ratio (DC:T), PHA combined with DCs (1:3 ratio) or left untreated for 72 h in RPMI1640 medium containing rhIL-2 and 80 ng/ml saquinavir. Cell-free supernatant was collected by centrifugation for 5 min at 500 rcf. Extracellular HIV-1 RNA was isolated from 300 µl of the supernatant using the Boom isolation method [17] with the addition of 2 µg of carrier RNA (poly A RNA, Qiagen, Venlo, The Netherlands) and processed for reverse transcription and first PCR using QIAGEN® OneStep RT-PCR kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The primers used were TAR-F (5'-GGGTCTCTCTG GTTAGACCAG-3') and HIV-FOR [18]. The PCR settings were as follows:



**Fig. 3.** Reversion of latency depends on specific tetraspanin enriched microdomains formed between DCs and T cells. A) Fold activation from latency of infected primary T cells cultured with or without DCs in the presence of 20 µg/ml CD9, CD63, CD81 and CD151 antibodies targeting specific tetraspanin enriched microdomains (TEMs). B) Schematic overview, adapted from Hemler et al. [22], regarding the role of the tetraspanins targeted in activating the RAS signaling cascade. Phosphatidylinositol 4-kinase is recruited to the membrane in CD9, CD63, CD81 TEMs and phosphoinositides, such as phosphatidylinositol-4,5- bisphosphate (PI(4,5)P<sub>2</sub>) are produced. This causes activation of Shc, resulting in the activation of the RAS signaling pathway. CD151 negatively influences the RAS activation pathway [22]. Representative data are plotted as the average fold activation with standard error of the mean of at least three independent experiments.



**Fig. 4.** Purging of latent HIV-1 mediated by DCs depends on PI3K, not MEK-ERK signaling. A) Schematic overview of inhibitors targeting specific signaling pathways. B) Infected primary T cells were cultured with or without DCs in the presence of the MEK inhibitor U0126, or C) the PI3K inhibitor pictilisib, or D) the PTEN inhibitor SF1670. Drug toxicity is indicated with †.

94 °C for 3 min, followed by 15 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min. Two μl of the first PCR product were subsequently used as a template in the second, semi-nested, real-time, PCR amplification, performed using the primers and a TaqMan probe described by Malnati et al. [18].

### 2.9. qVOA with 1) PHA, 2) DC one-two punch

Isolated CD4<sup>+</sup> T cells from aviremic patients were activated with 2 μg/ml PHA for 72 h in RPMI1640 medium containing rhIL-2 and 100 ng/ml entry inhibitor T1249. Cells were washed three times and cultured in 96-well plate with a cell density of  $2.0 \times 10^5$  CD4 T cells/well with or without  $8.3 \times 10^4$  DCs (1:3 DC:T cell ratio) in fresh RPMI1640 containing rhIL-2. HIV-1 susceptible SupT1 CCR5 cells ( $5.0 \times 10^4$ ) were added to the microcultures to disseminate and sustain HIV-1 replication [19]. After 7, 14 and 21 days of culturing half of the culture was used to determine HIV-1 CA-p24 by ELISA [5] and fresh RPMI 1640 media containing  $5.0 \times 10^4$  SupT1 CCR5 cells was added to sustain T cell growth and virus replication. The number of infectious units per million (IUPM) cells was determined on the number of HIV-1 CA-p24 positive microcultures on day 21 for each condition per million patient-derived CD4 T cells.

### 2.10. Statistical analysis

Significance of differences between groups or pairs was determined by one-way ANOVA and Student's *t*-tests. For patient-derived samples, Fisher's exact tests were used to compare the detectability of HIV-1 extracellular RNA between different conditions. Significance levels: \*:  $p < .05$ ; \*\*:  $p < .01$ ; \*\*\*:  $p < .001$ . All tests were two-sided and were performed using GraphPad Prism, version 7.

## 3. Results

### 3.1. Dendritic cells purge residual latent HIV-1 from PHA-activated T cells

To investigate the effectiveness of DCs or LRAs in reversing latency in effector T cells, we designed an innovative primary T-cell model. Resting T cells from healthy donors were activated with PHA for 2 days. CD8<sup>+</sup> T cells were removed and CD4<sup>+</sup> T cells were cultured for three days before infection for 4 h with HIV-1. New rounds of infection and virus replication were then blocked by addition of the T1249 entry inhibitor. Cells were maintained in culture for 24 h to allow completion of reverse transcription and DNA integration was allowed to proceed for 24 h. Subsequently, the infected CD4<sup>+</sup> T cells were co-cultured for 48 h with or without DCs and in the presence or absence of LRAs (Fig. 2A). As shown before, the percentage of virus-producing T cells increased from 7.5% to 28.2% by DC co-culturing (Fig. 2B). Reversion of latency, expressed as “fold activation” by calculating the percentages of CA-p24 positive DC-treated T cells divided by control untreated T cells from three independent infections, was ~3.5-fold (Fig. 2C). Importantly, this means that for each virus-producing effector T cell, 2 to 3 additional HIV-infected cells could be purged from latency by DCs. Thus, a bigger part of the reservoir could be purged using DCs as an extra stimulation. The observed rate of reversion of HIV-1 latency by DCs is not due to increased viral transmission or integration as extending the period before addition of DCs or use of the integrase inhibitor raltegravir did not change the fold activation. Moreover, the infected activated effector T cells showed heightened expression levels of CD25, CD69, CD45RO and CCR7 and the additional DC stimulation did not change expression levels [5].

Successive stimulation of the T-cell receptor (TCR) has been shown to increasingly revert the level/efficiency of HIV latency in activated T

cells [13]. The heterologous DCs used in this latency assay might also have non-specifically stimulated the TCR. Re-stimulating the TCR again with phytohaemagglutinin (PHA) or CD3/CD28 antibodies, however, did not purge latency, demonstrating that DC-purging is not triggered via the TCR (Fig. 2D). Other LRAs such as the HDAC inhibitor romidepsin (Fig. 2E), the PKC activator prostratin (Fig. 2F), and the inflammatory cytokine TNF $\alpha$  (Fig. 2G) were also unable to revert additional latent HIV-1, illustrating that reversion of HIV-1 triggered by DCs is different from the LRAs tested and does not depend on T cell receptor activation.

### 3.2. Tetraspanins are involved in DC-triggered HIV-1 activation in PHA-activated T cells

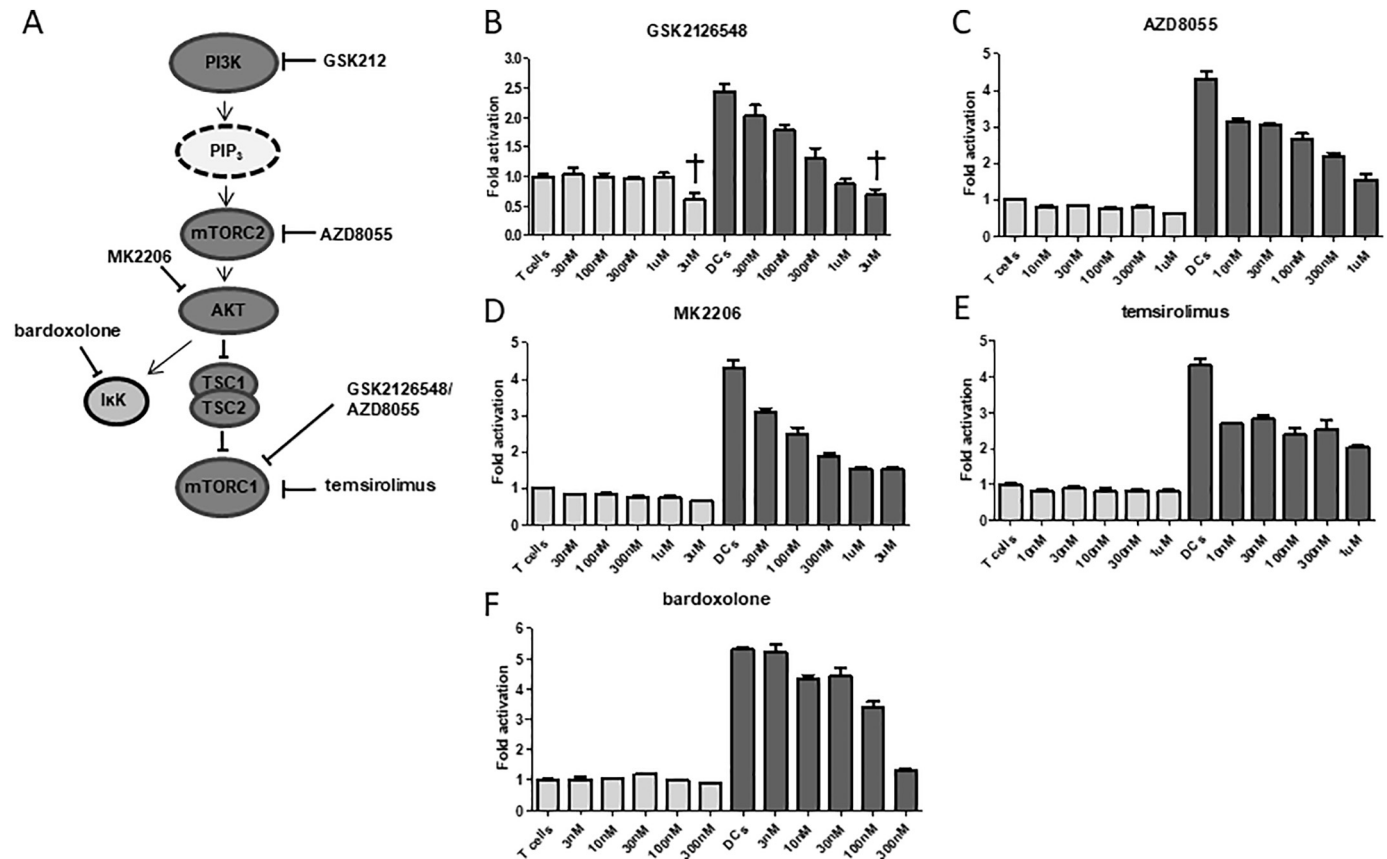
DCs interact with T cells via immunological synapses where antigen is presented for TCR recognition. Tetraspanins CD9, CD63, CD81, and CD151 play an important role in this process by stabilizing recruited protein complexes in tetraspanin-enriched microdomains (TEMs) [20,21]. To investigate whether the tetraspanins are involved in DC-mediated HIV-1 latency reversal in T cells, tetraspanin-specific antibodies (Ab) were added. Blocking of CD9 abrogated HIV-1 latency reversal in effector T cells by DCs, beyond mock-incubated T cell levels. CD9 also showed a negative effect on activation in T cells in the absence of DCs (Fig. 3A), implying that latency is induced. CD63 also blocked DC-mediated reversal of latency and modestly induced latency in non-DC treated T cells. A reduction of ~38% in DC-mediated purging was observed when a CD81 Ab was used while the CD151 Ab had no effect

on DC-purging (Fig. 3A). These data suggest that the RAS signaling pathway is targeted by DCs, because the three responsive tetraspanins (CD9, CD63 and CD81) activate this pathway, whereas the non-responsive CD151 tetraspanin inhibits it (Fig. 3B, model adapted from Hemler et al. [22]).

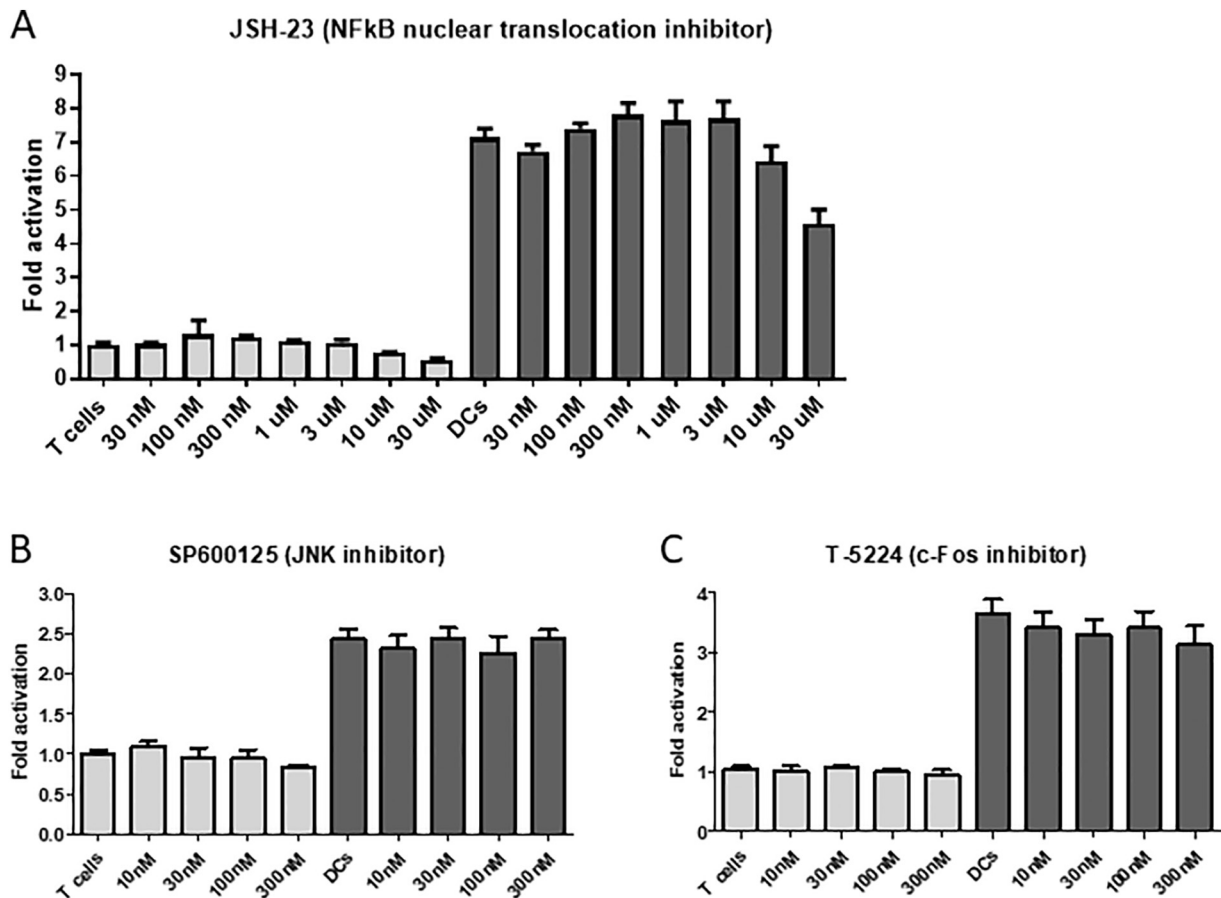
### 3.3. DC-mediated HIV-1 latency reversal depends on the PI3K-Akt-mTOR pathway

Active GTP-bound RAS can activate the extracellular signal-regulated kinase (Raf-MEK/ERK), phosphoinositide kinase 3 (PI3K-Akt-mTOR) and the phospholipase C (PLC)/PKC pathway (Fig. 4A). As shown above, stimulation of the TCR or the PKC pathway with prostratin did not purge latent HIV-1 in effector T cells, illustrating that the PLC-PKC pathway is not involved in DC-purging (Fig. 2D and F). Inhibiting the Raf-MEK/ERK pathway with increasing concentrations of the MEK1/2 inhibitor U0126 (up to 100 nM) also did not block HIV-1 purging (Fig. 4B) and cell death was observed at 300 nM. In contrast, inhibition of the PI3K route with 100 nM pictilisib (GDC-0941) potentially inhibited DC purging (Fig. 4C).

To confirm the involvement of the PI3K pathway, we indirectly activated this pathway by inhibiting PTEN with SF1670 (Fig. 4A and D). In accordance with the suggested role of the PI3K-Akt-mTOR pathway in latency reversal, inhibition of PTEN in the T-cell culture reversed HIV-1 latency by 1.7-fold. In contrast, inhibition of PTEN increased the DC-purging activity only minimally (and only at high concentrations of SF1670), suggesting that the PI3K-Akt pathway was already active.



**Fig. 5.** Effects of targeting the PI3K-Akt-mTOR signaling transduction cascade on latency reversal. A) Schematic overview of inhibitors targeting the PI3K-Akt-mTOR signaling pathway. B) Fold activation from latency of infected primary T cells cultured with or without DCs in the presence of the PI3K/mTORC2/mTORC1 inhibitor GSK2126548, C) PI3K/mTORC1 inhibitor AZD8055, D) Akt inhibitor MK2206, E) mTORC1 inhibitor temsirolimus, or F) inhibitor of nuclear factor kappa-B kinase (IKK) antagonist bardoxolone. Representative data are plotted as the average fold activation with standard error of the mean at the different indicated concentrations of inhibitor from three independent cultures. Drug toxicity is indicated with †.



**Fig. 6.** Transcription factors binding NF-κB and SP1 target sites in the LTR promoter do not seem to be involved in DC-dependent latency reversal. A) Fold activation of infected primary T cells cultured with or without DCs in the presence of JSH-23, inhibiting nuclear translocation of the transcription factor NF-κB, B) in the presence of SP600125 inhibiting dimerization of c-Jun transcription factors binding SP1 motifs, or C) the inhibitor T5224 inhibiting c-Fos-c-Jun dimerization. Representative data are plotted as the average fold activation with standard error of the mean at the different indicated concentrations of inhibitor from three independent cultures. Of note, blocking activation of transcription factors beyond the indicated concentrations of JSH-23 and SP600125 was toxic for T cells regardless of whether they were co-cultured with DCs (results not shown).

### 3.4. Analysis of DC-mediated signaling downstream of the Akt pathway

To confirm involvement of the PI3K-Akt-mTOR pathway, we tested additional inhibitors that block specific steps of this pathway (Fig. 5A). The inhibitor GSK2126548 that blocks the activation of PI3K, mTORC1 and mTORC2, the mTOR-inhibitor AZD8055 that blocks activation of mTORC1 and mTORC2, and the Akt-inhibitor MK2206 all inhibited DC-mediated HIV-purging in a dose-dependent manner (Fig. 5B-D). These inhibitors had no significant effect on HIV-1 production in T-cell only cultures, but a negative purging trend seemed to occur. The results again illustrate that an active mTOR pathway in activated T cells is involved in the induction of virus production [23]. We used the inhibitor temsirolimus to specifically inhibit mTORC1, which inhibited DC-mediated reversion of latent HIV-1 by 70% at concentrations as low as 10 nM, without any cell toxicity (Fig. 5E). Inhibiting the IκK complex, another signaling branch downstream of Akt with bardoxolone also blocked HIV-1 reactivation, but only at high concentrations above 100 nM (Fig. 5F). In summary, although the PLC, PKC, MEK/ERK and PI3K/Akt pathways are activated in PHA-activated T cells, a further stimulation of the PI3K/Akt pathway by DC contact purged latent HIV-1 beyond the level of TCR activation.

### 3.5. DC contact does not increase availability of nuclear NF-κB or c-Jun/cFos (hetero/homo) dimers

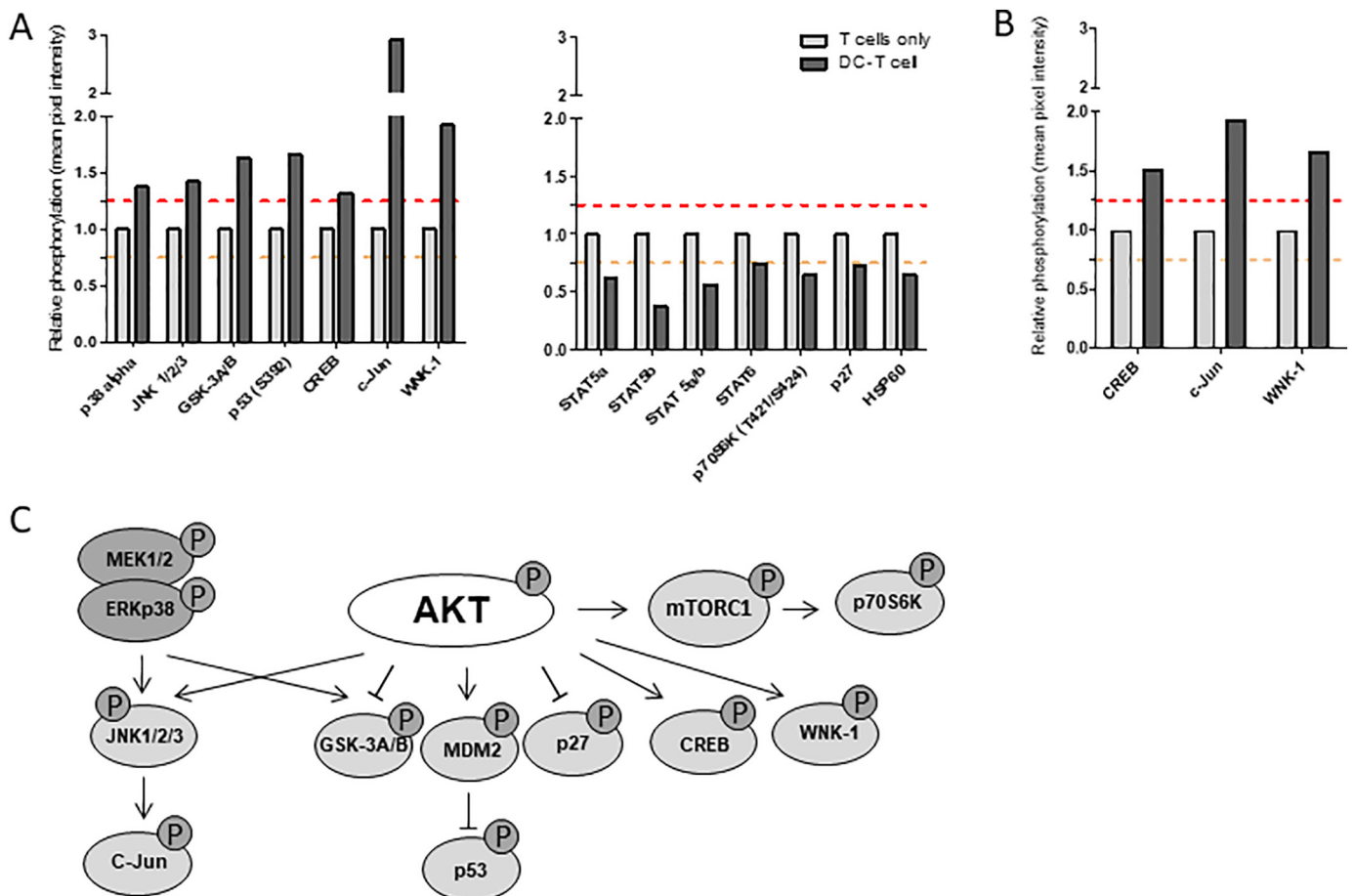
The IκK pathway is involved in activation of the transcription factor NF-κB. To explore whether the purging effect mediated by DCs is dependent on increased transcription initiation, we blocked nuclear

translocation of NF-κB with JSH-23 [24], which did not inhibit DC-mediated reversal of latency up to 3 μM (Fig. 6A). Higher concentrations of JSH-23 partially inhibited the DC purging effect, but these high concentrations also induced latency in the absence of DCs. This result illustrates that latency can be induced by blocking nuclear translocation of the NF-κB, but the purging ability observed with DCs is not dependent on increased production or translocation of NF-κB. Blocking c-Jun (Fig. 6B) or c-Fos (Fig. 6C), transcription factors that bind to AP-1 and SP1 sites in the HIV-1 LTR promoter, also did not affect DC-mediated purging. Thus, the availability of transcription factors that are important for HIV-1 gene expression in activated effector T cells is sufficient and is not changed upon DC addition.

### 3.6. DCs trigger (de)phosphorylation of proteins downstream of the Akt signaling pathway

To explore which pathways become activated by DCs in effector T cells, we also studied the phosphorylation status of 43 proteins from different signaling cascades. Seven proteins that belong to the Akt signaling pathway became phosphorylated upon 2.5 h DC contact (p38alpha, JNK1/2/3, GSK-3AB, p53, CREB, c-Jun and WNK-1) (Fig. 7A and C). Surprisingly, two other proteins that are also associated with Akt signaling, p27 and p70SK6, became dephosphorylated. It could be that these kinases were dephosphorylated to prevent over-activation of the mTOR pathway via negative feedback loops. Apart from the over-all activation of the Akt pathway, we also observed STAT5/6 proteins being less frequently phosphorylated after DC contact. We then analysed the phosphorylation status after an extended (21h) DC





**Fig. 7.** Characterization of phosphorylated protein kinases in infected primary CD4<sup>+</sup> T cells upon coculturing with DCs. PHA-activated CD4<sup>+</sup> T cells are infected according to the in vitro latency assay and cocultured with DCs, for A) 2 h or B) 21 h. DC treated and untreated CD4<sup>+</sup> T cells are isolated with the CD4<sup>+</sup> isolation kit (Miltenyi), pelleted and lysed at 4 degrees. Of the 43 kinases analysed using the human-phospho kinase array from R&D systems, only differentially phosphorylated kinases with >25% up- or downregulation (indicated with red and orange dotted line) compared to untreated cells are shown. C) Activating and inhibitory signaling cascades with corresponding phosphorylation status of kinases for the Akt, MEK-ERK signaling pathway are shown schematically [56,57]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contact. Three proteins (CREB, WNK-1 and c-Jun) downstream the Akt pathway still showed an increased phosphorylation status (Fig. 7B). Thus, part of the PI3K-Akt-mTOR pathway in T cells remains active upon prolonged DC contact. Overall, these results provide evidence that DCs activate the PI3K-Akt-mTOR pathway in T cells, thus purging latent HIV-1 proviruses.

### 3.7. DCs purge latent HIV-1 in TCR activated CD4 cells from aviremic patients

Since DCs activate the PI3K-Akt-mTOR pathway and purge latent HIV-1 in activated T cells, we examined whether they can also purge latent HIV-1 beyond PHA-mediated activation in CD4<sup>+</sup> T cells from aviremic HIV-infected patients. CD4<sup>+</sup> T cells were isolated from 9 aviremic ART-treated patients and stimulated with PHA or mock-treated in the absence or presence of heterologous DCs for 72 h. Purging of HIV-1 latency was assessed by the detection of extracellular HIV-1 virion RNA released into the cell culture supernatant. In mock-stimulated CD4<sup>+</sup> T cells, HIV-1 RNA could be detected for 4 out of 9 patients (Fig. 8A). Treatment with PHA, however, also did not increase the detectability of HIV-1 RNA when compared to mock-stimulated CD4<sup>+</sup> T cells and DC contact even slightly decreased the HIV+ scores, possibly due to consumption of free virus in the supernatant [25,26]. Importantly, a combined PHA + DC treatment made HIV-1 RNA detectable for 9 out of 9 patients, implying that non-responding patients cells

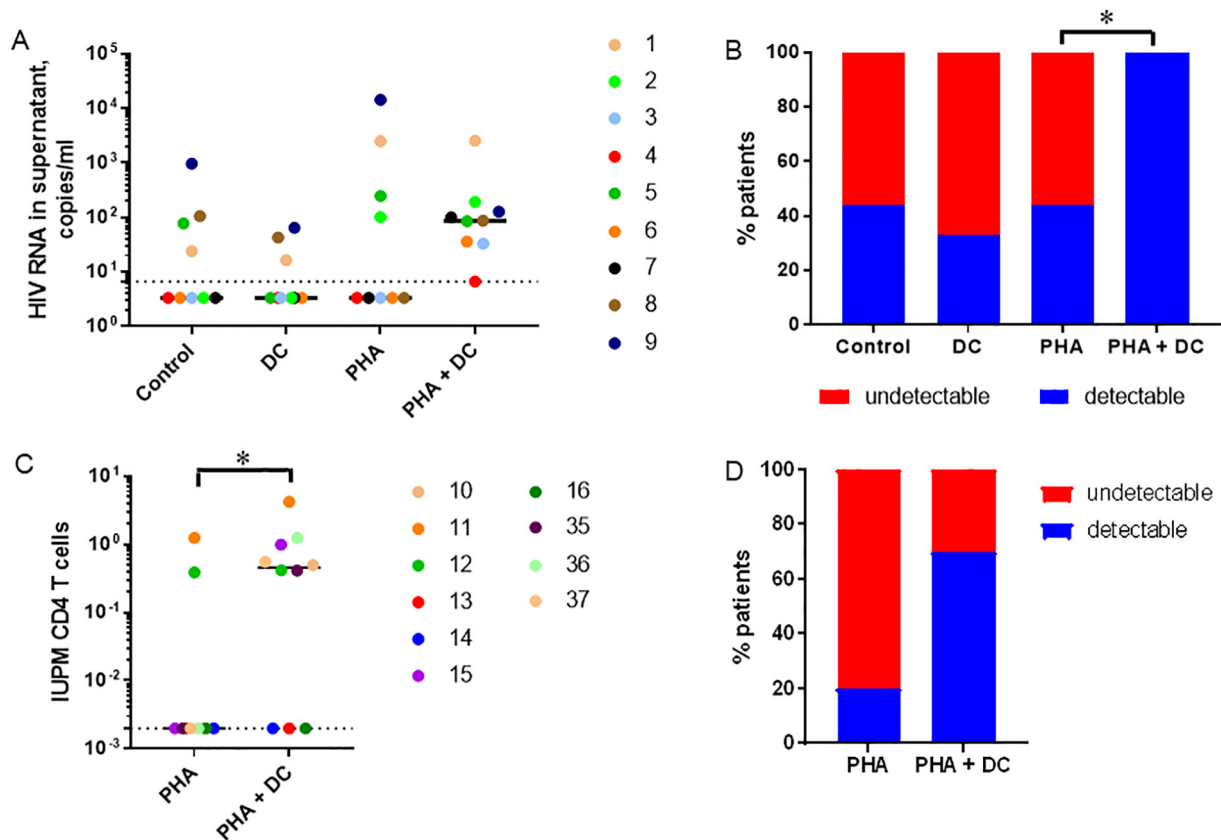
(with PHA only), responded with virus production upon the DC stimulus (Fig. 8B). The analyses however showed that the mean amount of RNA produced upon combined treatment was not increased compared to a single PHA treatment, which again could be due to the capture of free virus by DCs.

Next, we tested reversion of latency from single cells with the quantitative virological outgrowth assay (qVOA) on CD4<sup>+</sup> T cells from 10 aviremic patients. Due to a limited number of patient cells, obtained from 15 ml of blood, we performed the qVOA with a cell density of 200.000 T cells per culture. With this number of T cells, if all cultures are positive, a maximal IUPM of 5 can be reached. An increased number of IUPM cells was observed for the PHA + DC treatment compared to a single PHA shock. For 4 patients replicating virus only grew out with the PHA + DC treatment. For two patients (10 and 12) an increased IUPM level was observed with the double treatment and no virus outgrowth occurred upon PHA or PHA + DC treatment for 3 patients (Fig. 8C and D). Therefore, we conclude that DCs can purge HIV-1 latency beyond levels achieved by PHA activation.

## 4. Discussion

The latent HIV-1 reservoir in patients under suppressive ART consists predominantly of long-lived resting memory CD4<sup>+</sup> T cells [27]. A major proportion of the reservoir contains replication-incompetent proviral DNA that lacks gene fragments, is hypermutated, or is





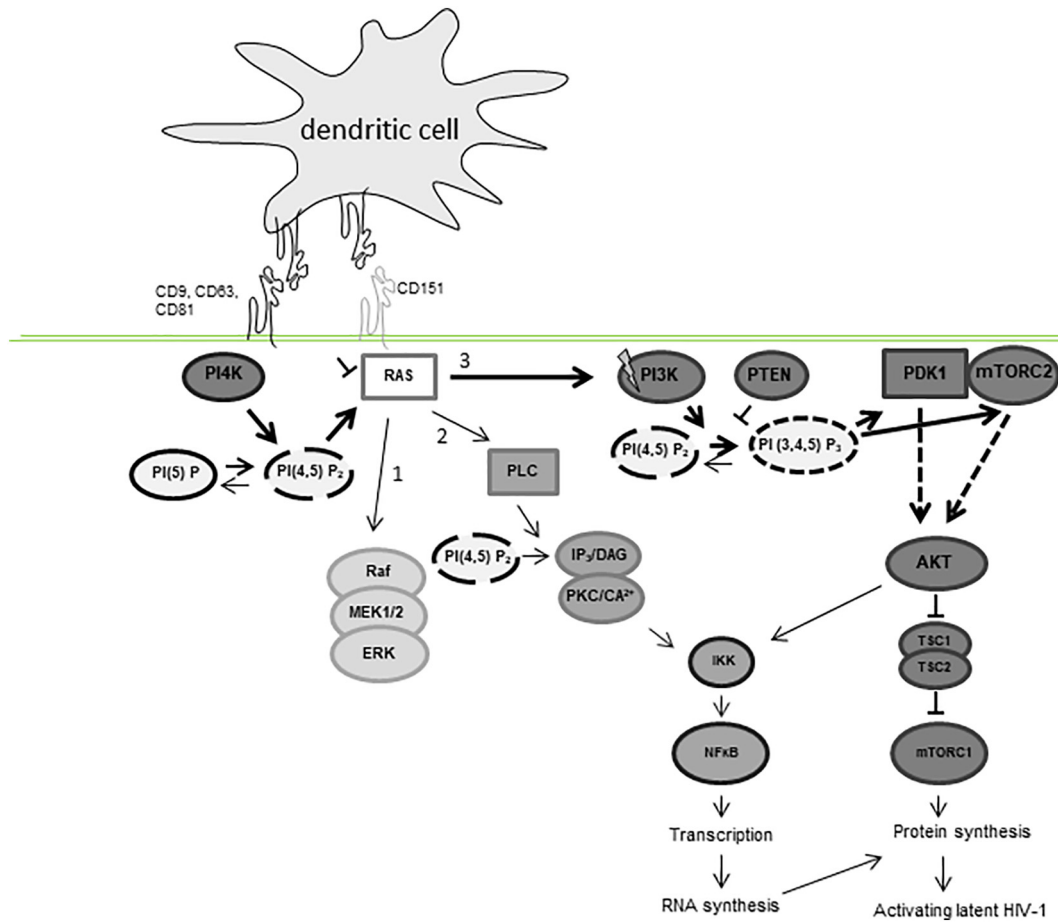
**Fig. 8.** Purging of latent HIV-1 from patient-derived resting primary T cells with DCs. Isolated CD4<sup>+</sup> cells ( $1.4\text{--}2.0 \times 10^6$ ) from aviremic HIV-1 patients were cultured with or without PHA in presence or absence of DCs (ratio 1:3 DC:T cell) for 72 h. A) Copy numbers of HIV-1 extracellular RNA released into cell culture supernatant, as determined by RT-qPCR. Median values are represented by horizontal lines. The dotted line represents the detection limit of the assay. The undetectable values were plotted as 50% of the detection limit. B) Percentage plot of detectable versus undetectable HIV-1 RNA. C) Outgrowth of replication competent HIV-1 measured at day 21 from 10 aviremic patient-derived CD4<sup>+</sup> T cells activated with PHA or PHA + DCs. Reversal of latency is depicted as infectious units per million cells (IUPM) based on the number of CA-p24 negative and positive microcultures. Median values are represented by horizontal lines. D) Percentage plot of detectable versus undetectable replication competent HIV-1 after differential stimulation for these patient samples.

epigenetically silenced [28] [29]. However, based on sequencing, approximately 30 resting T cells per million still contain infectious replication-competent HIV-1 genomes [12]. TCR activation that converts resting T cells into effector T cells can only purge HIV-1 in approximately 1 resting T cell per million [12,30]. Even though a full T-cell activation status is reached, sequential stimulation with a second TCR-based stimulus can increase the purging efficiency 2–3 fold [13], suggesting that latency persisted in most activated T cells. To study if more complete purging is feasible, we developed an HIV-1 latency model with (TCR pre-stimulated) primary effector T cells [5]. We provide evidence implicating DC contact in efficiently purging latent HIV in these T cells, something not achieved by a second round of T-cell activation or treatment with other LRAs (Fig. 2). Thus, the one-two punch consisting of TCR activation and subsequent stimulus with DCs can reach a significantly larger portion of the reservoir than other purging methods.

Using CD4<sup>+</sup> T cells isolated from ART-treated aviremic patients, we confirmed results obtained with our in vitro latency model by showing that ex vivo TCR-stimulation combined with DC contact induced the release of HIV-1 RNA into the supernatant in a significantly larger proportion of patients compared with TCR stimulation alone, which is indicative for purging of latent HIV-1 [31]. Although RNA could be detected for all patients with the double treatment, the levels of RNA released were not increased. This could be caused by efficient capture and degradation of free HIV-1 by DCs [25,26], which will strongly limit the amount of HIV-1 RNA to be detected in supernatant. In agreement with this hypothesis, treatment of patient cells with DCs alone without PHA activation also decreased the amount of RNA released in supernatant compared to untreated cells. To understand why DCs increase the

purging efficiency we dissected the molecular mechanisms induced in T cells pretreated with PHA. Our data illustrate that formation of tetraspanin-enriched microdomains (TEMs) between DCs and T cells is important for the purging ability of DCs (Fig. 3A) [20,32–34]. These TEMs recruit PI4K to the membrane to locally produce phosphoinositols, such as phosphatidylinositol-4,5 bisphosphate (PtdIns(4,5)P<sub>2</sub>), which activate RAS via Shc proteins [22,35]. GTP-bound RAS can activate the MEK-ERK, PLC-PKC and the PI3K-Akt-mTOR pathway. The MEK-ERK and PLC-PKC pathways are strongly activated upon TCR stimulation, achieved with the first PHA stimulus, which promotes HIV-1 transcription by increasing the levels of activated transcription factors such as NF- $\kappa$ B [36,37]. Restimulating the TCR with CD3/CD28 Abs or PHA again did not increase HIV-1 latency purging, indicating that both these pathways are still fully activated. Stimulation with DCs, however, purged latency efficiently. This was predominantly caused by activation of the PI3K-Akt-mTOR pathway. Activation of this pathway is associated with improved RNA elongation, ribosomal activity, protein synthesis and cell survival [38]. Thus, activation of the PI3K-Akt-mTOR probably overcomes post-transcriptional blocks to form infectious viral particles. Whether activation of this pathway is strictly regulated via RAS activation or via other DC-T cell mediated interactions needs to be explored in future research.

We hypothesize that phosphoinositols, especially the availability of PtdIns(4,5)P<sub>2</sub>, determines HIV-1 latency at various levels (Fig. 9). First, transcription factors such as NF- $\kappa$ B or c-Jun/c-Fos are required for HIV-1 transcription initiation [30,31,38]. These factors are upregulated via TCR stimulation, where PtdIns(4,5)P<sub>2</sub> is converted by PLC into IP<sub>3</sub> and DIAG in the MEK-ERK and PLC-PKC pathway [39]. Second, PI3K-Akt-mTOR pathway activation is required to allow transcriptional



**Fig. 9.** Reversion of HIV-1 latency regulated by DCs. Binding of DCs to T cells is facilitated by tetraspanin membrane molecules that recruit interacting DC-T cell proteins in tetraspanin enriched microdomains (TEMs) to induce multiple signaling events. Recruitment of PI4K to CD9/CD63/CD81 TEM leads to local production of PI(4,5)P<sub>2</sub>. DC-dependent activation of PI3K further phosphorylates PI(4,5)P<sub>2</sub> into PI(3,4,5)P<sub>3</sub>, which activates PDK1 to induce recruitment of Akt to the membrane. Akt docking to PI(3,4,5)P<sub>3</sub> triggers activation followed by activation of downstream signaling cascades such as mTORC1 or IKK that promote transcription and protein synthesis reactivating latent HIV-1 in active proliferating cells. The signaling cascade that is responsible for DC-mediated reversion of latent HIV-1 independent of T cell receptor activation is indicated with bold arrows.

elongation and protein synthesis. DCs can initiate this pathway by PtdIns(4,5)P<sub>2</sub> production in TEMs via PI4K activation and subsequent phosphorylation into PtdIns(3,4,5)P<sub>3</sub> by PI3K. Since the PI3K-Akt-mTOR and PLC-PKC pathway either phosphorylate or dephosphorylate PtdIns(4,5)P<sub>2</sub>, strong activation of either one of these pathways will directly inhibit the activation of the other pathway. Thus, a fine balance between these pathways should be pursued to purge latent HIV-1, or both pathways should be activated sequentially as achieved for instance with our “one-two punch” strategy, in which, after transcription initiation, RNA elongation and protein translation should follow.

In this light it is not surprising that TCR stimulation, which can trigger sub-optimally the PLC-PKC and PI3K-Akt-mTOR pathway, has some HIV-1 purging ability, leading to outgrowth of infectious particles. Solely activating the transcription initiation route using a PKC agonist, however, does not [30]. Interestingly, a recent study discovered the importance of the mTOR pathway in purging latent HIV-1 in resting cells [23]. Specifically blocking the mTOR pathway after TCR stimulation negated reversal of latency in patient cells, illustrating that TCR-stimulation can sub-optimally activate this pathway. Our results extend those data by showing that activation of the PI3K-Akt-mTOR pathway by DCs, is stronger than stimulating the TCR in isolation, leading to increased purging efficiency.

Targeting multiple intermediates in the PI3K-Akt-mTOR pathway, e.g. using small molecule inhibitors, we dissected their contributions in reverting latency. For instance using GSK2126458, we found that it inhibited DC-specific activation of the PI3K-Akt-mTOR pathway better than the mTORC1 inhibitor temsirolimus. This is in agreement with a

study from Martin et al., who reported that the mTORC1 inhibitor rapamycin [40,41] did not block purging of latent HIV-1, but could reduce the toxic side effect of CD3/CD28 T cell activation without affecting CTL-mediated killing [42]. The use of small molecule inhibitors can have off-targets effects as well as general toxicities, which can distort results. In as far as possible our controls excluded these as a source of error. Our findings regarding the activation of the PI3K-Akt-mTOR pathway upon DC contact were further corroborated by phosphate modification readouts. This again argues against aspecific effects of the small molecule inhibitors.

DC contact may influence HIV-1 reactivation in T cells by additional means. Ren et al. showed that mature DCs are capable of releasing TNFα upon contact with infected Jurkat T cells, leading to reversion of latency in this cell line [43].

Since DCs strongly revert HIV-1 latency, it would be an option to use DCs as a natural cell source to revert HIV-1 latency in HIV-infected patients. We previously observed that primary DCs homing to different lymphoid organs have substantially different latency-purging capacities. Monocyte-derived DCs cultured under conditions towards a gut DC phenotype efficiently purged latent HIV-1, whereas no, or only modest, reversal of latency was achieved with DCs representing the subtype homing to the genital tract. Myeloid DCs that have been matured with different toll-like receptor agonists, representing lymph node derived DCs, efficiently purge HIV-1 from latency [16]. The fact that monocyte-derived DCs can purge HIV-1 from patient-derived latently infected cells suggests that DCs might control the size of the viral reservoir in patients in particular tissues. This could happen especially when T cells are

activated due to secondary infection, or in the acute phase of disease when T cells are activated to fight the HIV-1 infection [5]. DC-mediated HIV-1 reversion however, is not to be expected in peripheral blood [16], as the frequency of myeloid DCs is ~7000 per million CD4 T cells in healthy donors, with even lower numbers in HIV-1 infected individuals (~2300 per million) [44]. But, DC:T contact might be more efficient in tissues, which could result in the observed low levels of ongoing virus production, even in the presence of therapy [45,46].

Interestingly, Gramatica and colleagues already raised the possibility that latency reversal might be more effective in lymphoid tissues where contact of T cells with DCs is more prominent than in blood [47]. The lack of efficacy of clinical trials using LRAs aimed at decreasing the HIV-1 reservoir size might therefore be caused by the fact that the reservoir was analysed only in the peripheral blood and not in tissues, where preferential DC-mediated purging could occur [48–52]. One approach to use DCs to increase the LRA purging efficiency in patients might be the combination of an LRA with specific TLR agonists that will direct DCs to tissues, where latent reservoirs are maintained. The use of DCs as a natural mechanism to revert HIV-1 latency has caveats and limitations, due to difficulties inherent in specific targeting of this interaction. However, if feasible this approach will have limited side effects [53] compared to using T cell receptor agonists [54].

Our results could have important implications. Firstly, the potent capacity of DCs to reactivate HIV-1 proviruses which were not purged despite PHA stimulation could be used to design a more sensitive culture-based assay to measure the replication-competent reservoir, for instance by adapting the promising TZM-bl based assay [55]. Secondly, the role of DCs in HIV-1 persistence highlights the need to look at tissue reservoirs when possible, since cell-to-cell contact appears to be a major factor controlling HIV-1 latency. Finally, our results further support the importance of the PI3K-Akt-mTOR pathway in HIV-1 latency. These novel insights could be used to develop more efficient LRA strategies in the quest to get rid of the latent HIV-1 reservoir.

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## Declaration of interest

The authors declare no competing interests.

## Author contributions

TvM, wrote the original manuscript draft; TvM, GJD, RvdS, MV, DB, KG, AOP and BvdP carried out the investigation; MB, SJ took care of the project administration; GP, EMW, MH and JP provided resources; RJ, GD, AOP, AAMT, DS and BB helped with writing, reviewing and editing.

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