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JOURNAL	*Cell Host & Microbe*
ARTICLE TITLE	Dendritic cells potently purge latent HIV-1 in TCR-activated cells via the PI3K-Akt-mTOR pathway: implications for “shock and kill” strategies and reservoir analysis
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ARTICLE TYPE	Research Article
ABSTRACT	The latent HIV-1 reservoir in treated patients primarily consists of resting memory $CD4^{+}$ 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 demonstrate that TCR-stimulated effector T cells still frequently harbor latent HIV-1. Renewed TCR-stimulation or subsequent activation with latency reversing agents (LRAs) did not overcome latency. However, interaction of infected effector cells with dendritic cells (DCs) triggered further activation of latent HIV-1. When compared to TCR-stimulation only, $CD4^{+}$ 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 $CD4^{+}$ T cells. This insight could facilitate the development of a novel class of potent LRAs that purge latent HIV beyond levels reached by T-cell activation.

1 **Dendritic cells potently purge latent HIV-1 in TCR-activated cells via the PI3K-Akt-mTOR**
2 **pathway: implications for "shock and kill" strategies and reservoir analysis**

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27 **Abstract**

28 The latent HIV-1 reservoir in treated patients primarily consists of resting memory $CD4^+$ T cells.
29 Stimulating the T-cell receptor (TCR), which facilitates transition of resting into effector T cells, is the
30 most effective strategy to purge these latently infected cells. Here we demonstrate that TCR-
31 stimulated effector T cells still frequently harbor latent HIV-1. Renewed TCR-stimulation or
32 subsequent activation with latency reversing agents (LRAs) did not overcome latency. However,
33 interaction of infected effector cells with dendritic cells (DCs) triggered further activation of latent
34 HIV-1. When compared to TCR-stimulation only, $CD4^+$ T cells from aviremic patients receiving
35 TCR+DC-stimulation reversed latency more frequently. Such a "one-two punch" strategy seems ideal
36 for purging the reservoir. We determined that DC contact activates the PI3K-Akt-mTOR pathway in
37 $CD4^+$ T cells. This insight could facilitate the development of a novel class of potent LRAs that purge
38 latent HIV beyond levels reached by T-cell activation.

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40

41 **Introduction**

42 Early on in HIV infection, cellular reservoirs containing latent HIV-1 are formed (Bruner et al., 2016).
43 These cells contain a stably integrated and complete viral genome, but do not express sufficient
44 amounts of viral proteins to drive virus production and to be recognized by the immune system.
45 Resting memory $CD4^+$ T cells are the main cell type harboring latent HIV-1 in patients after prolonged
46 therapy (Perelson et al., 1996; Wei et al., 1995), but T cells with shorter half-lives, such as effector T
47 cells, can also harbor latent HIV-1 (Chavez et al., 2015; van der Sluis et al., 2013). Latency is
48 established and maintained through multiple mechanisms that act at transcriptional and post-
49 transcriptional levels (Darcis et al., 2017). At the transcriptional level, accessibility of the HIV-1 LTR
50 promoter could be blocked in repressive chromatin structures (which can be overcome with histone
51 deacetylase (HDAC) inhibitors) or by the sequestration of transcription initiation factors such as NF-
52 κB/NFAT/AP-1. Other blocks to HIV-1 transcription include inefficient elongation due to the lack of
53 elongation factors such as P-TEFb or the presence of negative elongation factors (NELFs). These
54 elongation factors influence the RNA polymerase complex and determine whether transcription is
55 prematurely aborted after synthesis of the trans-activation response (TAR) region or extended
56 towards the formation of full-length HIV-1 RNA transcripts. Yukl et al. recently described that HIV
57 latency at the transcriptional level occurs mainly due to inefficient RNA elongation accompanied by a
58 lack of splicing and polyadenylation factors rather than the absence of transcription initiation factors
59 (Yukl et al., 2018). Inefficient export of viral RNA from the nucleus may also contribute to HIV-1
60 latency, either due to low levels of Rev protein (Huang et al., 2007; Zolotukhin et al., 2003) or cellular
61 co-factors like Matrin-3 or PTB that assist in the nuclear RNA export (Kula et al., 2013; Yedavalli and
62 Jeang, 2011).

63 One of the proposed strategies to exhaust the reservoir is a “shock and kill” treatment in which
64 latency-reversing agents (LRAs) purge HIV-1 from latency, while uninfected cells are protected
65 against virus infection with antiretroviral therapy. Virus-induced cell death or cytotoxic T-cell killing

66 of virus-producing cells was proposed to eliminate the reactivated cells. Stimulation of the T-cell
67 receptor (TCR) to induce the transition of resting into effector T cells is currently the most effective
68 strategy to purge latent HIV. Ex vivo stimulation of the TCR with PHA or CD3-CD28 antibodies can
69 purge approximately 1 cell per million resting memory T cells (= 1 IUPM) as determined with the gold
70 standard quantitative viral outgrowth assay (qVOA) (Ho et al., 2014). Repeated TCR-stimulation can
71 increase this number 2-3 fold (Hosmane et al., 2017), but based on full-genome sequencing it has
72 been estimated that the intact HIV-1 reservoir size is around 30 cells per million resting T cells in
73 treated patients (Ho et al., 2014). This implies that T-cell activation can only purge a fraction of the
74 HIV reservoir and that additional stimuli are required to purge larger portions of latently infected
75 cells. We previously developed an HIV-1 latency assay for effector T cells and showed that
76 stimulation by DC contact efficiently purged latency (van der Sluis et al., 2013). We now show that DC
77 contact stimulates the PI3K-Akt-mTOR pathway in effector T cells. Importantly, we demonstrate that
78 stimulation of T cells from treated aviremic patients with PHA and DCs strongly increased the
79 frequency of HIV-1 release from latently infected cells (9 out of 9 patients) compared to PHA alone (4
80 out of 9). This “one-two punch” strategy (PHA + DCs) can purge the reservoir more potently than PHA
81 treatment alone (figure 1). These findings could direct the development of novel LRA treatments that
82 also strongly stimulate the PI3K-Akt-mTor pathway. The new insights could also be used for the
83 design of a new sensitive culture-based assay to measure the intact HIV-1 reservoir.

84

85 **Results**

86 **Dendritic cells purge residual latent HIV-1 from PHA-activated T cells**

87 To investigate the effectiveness of DCs or LRAs in reversing latency in effector T cells, we designed an
88 innovative primary T-cell model. Resting T cells from healthy donors were activated with PHA for 2
89 days. $CD8^+$ T cells were removed and $CD4^+$ T cells were cultured for three days before infection for 4

90 hours with HIV-1. New rounds of infection and virus replication were then blocked by addition of the
91 T1249 entry inhibitor. Cells were maintained in culture for 24 hours to allow completion of reverse
92 transcription and DNA integration was allowed to proceed for 24 hours. Subsequently, the infected
93 $CD4^+$ T cells were co-cultured for 48 hours with or without DCs and in the presence or absence of
94 LRAs (Figure 2A). As shown before, the percentage of virus-producing T cells increased from 7.5% to
95 28.2% by DC co-culturing (Figure 2B). Reversion of latency, expressed as "fold activation" by
96 calculating the percentages of CA-p24 positive DC-treated T cells divided by control untreated T cells
97 from three independent infections, was ~3.5-fold (Figure 2C). Importantly, this means that for each
98 virus-producing effector T cell, 2 to 3 additional HIV-infected cells could be purged from latency by
99 DCs. Thus, a bigger part of the reservoir could be purged using DCs as an extra stimulation. The
100 observed rate of reversion of HIV-1 latency by DCs is not due to increased viral transmission or
101 integration as extending the period before addition of DCs or use of the integrase inhibitor raltegravir
102 did not change the fold activation (van der Sluis et al., 2013).

103 Successive stimulation of the T-cell receptor (TCR) has been shown to increasingly revert the
104 level/efficiency of HIV latency in activated T cells (Hosmane et al., 2017). The heterologous DCs used
105 in this latency assay might also have non-specifically stimulated the TCR. Re-stimulating the TCR with
106 phytohaemaglutinin (PHA) or CD3/CD28 antibodies, however, did not purge latency, demonstrating
107 that DC-purging is not triggered via the TCR (Figure 2D). Other LRAs such as the HDAC inhibitor
108 romidepsin (Figure 2E), the PKC activator prostratin (Figure 2F), and the inflammatory cytokine TNFα
109 (Figure 2G) were also unable to revert additional latent HIV-1, illustrating that reversion of HIV-1
110 triggered by DCs is different from the LRAs tested.

111 **Tetraspanins are involved in DC-triggered HIV-1 activation in PHA-activated T cells**

112 DCs interact with T cells via immunological synapses where antigen is presented for TCR recognition.
113 Tetraspanins CD9, CD63, CD81, and CD151 play an important role in this process by stabilizing

114 recruited protein complexes in tetraspanin-enriched microdomains (TEMs) (Rocha-Perugini et al.,
115 2014, 2016). To investigate whether the tetraspanins are involved in DC-mediated HIV-1 latency
116 reversal in T cells, tetraspanin-specific antibodies (Ab) were added. Blocking of CD9 and CD63
117 abrogated HIV-1 latency reversal in effector T cells by DCs (Figure 3A). A modest reduction (by ~38 %)
118 in DC-mediated purging was observed when a CD81 Ab was used while the CD151 Ab had no effect
119 on DC-purging (Figure 3A). These data suggest that the RAS signaling pathway is targeted by DCs,
120 because the three responsive tetraspanins (CD9, CD63 and CD81) activate this pathway, whereas the
121 non-responsive CD151 tetraspanin inhibits it (Figure 3B, model adapted from Hemler et. al. (Hemler,
122 2005).

123 **DC-mediated HIV-1 latency reversal depends on the PI3K-Akt-mTOR pathway**

124 Active GTP-bound RAS can activate the extracellular signal-regulated kinase (Raf-MEK/ERK),
125 phosphoinositide kinase 3 (PI3K-Akt-mTOR) and the phospholipase C (PLC)/PKC pathway (Figure 4A).
126 As shown above, stimulation of the TCR or the PKC pathway with prostratin did not purge latent HIV-
127 1 in effector T cells, illustrating that the PLC-PKC pathway is not involved in DC-purging (Figure 2D
128 and 2F). Inhibiting the Raf-MEK/ERK pathway with increasing concentrations of the MEK1/2 inhibitor
129 U0126 (up to 100 nM) also did not block HIV-1 purging (Figure 4B) and cell death was observed at
130 300 nM. In contrast, inhibition of the PI3K route with 100 nM pictilisib (GDC-0941) potently inhibited
131 DC purging (Figure 4C).

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

137 **Analysis of DC-mediated signaling downstream of the Akt pathway**

138 To confirm involvement of the PI3K-Akt-mTOR pathway, we tested additional inhibitors that block
139 specific steps of this pathway (Figure 5A). The inhibitor GSK2126548 that blocks the activation of
140 PI3K, mTORC1 and mTORC2, the mTOR-inhibitor AZD8055 that blocks activation of mTORC1 and
141 mTORC2, and the Akt-inhibitor MK2206 all inhibited DC-mediated HIV-purging in a dose-dependent
142 manner (Figure 5B-D). These inhibitors had no effect on HIV-1 production in T-cell only cultures. We
143 used the inhibitor temsirolimus to specifically inhibit mTORC1, which inhibited DC-mediated
144 reversion of latent HIV-1 by 70% at concentrations as low as 10 nM, without any cell toxicity (Figure
145 5E). Inhibiting the IκB kinase (IκK) complex, another signaling branch downstream of Akt with
146 bardoxolone also blocked HIV-1 reactivation, but only at high concentrations above 100 nM (Figure
147 5F). In summary, although the PLC, PKC, MEK/ERK and PI3K/Akt pathways are activated in PHA-
148 activated T cells, a further stimulation of the PI3K/Akt pathway by DC contact purged latent HIV-1
149 beyond the level of TCR activation.

150

151 **DC contact does not increase availability of nuclear NF-κB or cJun/cFos (hetero/homo) dimers**

152 The IκK pathway is involved in activation of the transcription factor NF-κB. To explore whether the
153 purging effect mediated by DCs is dependent on increased transcription initiation, we blocked
154 nuclear translocation of NF-κB with JSH-23 (Shin et al., 2004), which marginally reduced DC-induced
155 HIV-1 production by ~33% at 300 nM (Figure 6A). Blocking c-Jun (Figure 6B) or c-Fos (Figure 6C),
156 transcription factors that bind to AP-1 and SP1 sites in the HIV-1 LTR promoter, did not affect DC-
157 mediated purging. Thus, the availability of transcription factors that are important for HIV-1 gene
158 expression in activated effector T cells is sufficient and is not changed upon DC addition.

159 **DCs trigger (de)phosphorylation of proteins downstream of the Akt signaling pathway**

160 To explore which pathways become activated by DCs in effector T cells, we also studied the
161 phosphorylation status of 43 proteins from different signaling cascades. Seven proteins that belong

162 to the Akt signaling pathway became phosphorylated upon 2.5 hr DC contact (p38alpha, JNK1/2/3,
163 GSK-3AB, p53, CREB, c-Jun and WNK-1) (Figure 7A and 7C). Surprisingly, two other proteins that are
164 also associated with Akt signaling, p27 and p70SK6, became dephosphorylated. It could be that these
165 kinases were dephosphorylated to prevent over-activation of the mTOR pathway via negative
166 feedback loops. Apart from the over-all activation of the Akt pathway, we also observed STAT5/6
167 proteins being less frequently phosphorylated after DC contact. We also analyzed the
168 phosphorylation status after an extended (21 hrs) DC contact. Three proteins (CREB, WNK-1 and c-
169 Jun) downstream the Akt pathway still showed an increased phosphorylation status (Figure 7B).
170 Thus, part of the PI3K-Akt-mTOR pathway in T cells remains active upon prolonged DC contact.
171 Overall, these results demonstrate that DCs activate the PI3K-Akt-mTOR pathway in T cells, thus
172 purging latent HIV-1 proviruses.

173

174 **DCs purge latent HIV-1 in TCR activated CD4 cells from aviremic patients**

175 Since DCs activate the PI3K-AKt-mTor pathway and purge latent HIV-1 in activated T cells, we
176 examined whether they can also purge latent HIV-1 beyond PHA-mediated activation in $CD4^+$ T cells
177 from aviremic HIV-infected patients. $CD4^+$ T cells were isolated from 9 aviremic ART-treated patients
178 and stimulated with PHA or mock-treated in the absence or presence of heterologous DCs for 72 hrs.
179 Purging of HIV-1 latency was assessed by the detection of extracellular HIV-1 virion RNA released into
180 the cell culture supernatant. In mock-stimulated $CD4^+$ T cells, HIV-1 RNA could be detected for 4 out
181 of 9 patients (Figure 8A). PHA treatment did not increase the detectability of HIV-1 RNA compared to
182 mock-stimulated $CD4^+$ T cells, while DC contact slightly decreased the HIV+ scores. However,
183 importantly, a combined PHA + DC treatment made HIV-1 RNA detectable for 9 out of 9 patients. The
184 analyses showed the purging efficiency to be significantly higher in the double treatment (when
185 compared to PHA alone) (Figure 8B).

186 Next, we tested the "double treatment" with the quantitative virological outgrowth assay (qVOA),
187 the golden standard to measure reversion of latency. Again the combination proved superior. With a
188 limited number of patient cells available for this assay (7 patients in total), we could detect an
189 increased number of replicating infectious HIV-1 units per million (IUPM) CD4 T cells upon DC
190 addition for patient 11 and 12. Moreover, for patient 10 and 15 detectable IUPMs were *only*
191 observed after DC-stimulation (Figure 8C and 8D). Collectively, we conclude that DCs can purge HIV-1
192 latency beyond levels achieved by PHA activation.

193

194 **Discussion**

195 The latent HIV-1 reservoir in patients under suppressive ART consists predominantly of long-lived
196 resting memory CD4$^+$ T cells (Zhang et al., 1999). A major proportion of the reservoir contains
197 replication-incompetent proviral DNA that lacks gene fragments, is hypermutated, or is epigenetically
198 silenced (Kieffer et al., 2005) (Sanchez et al., 1997). However, based on sequencing, approximately 30
199 resting T cells per million still contain infectious replication-competent HIV-1 genomes (Ho et al.,
200 2014). TCR activation that converts resting T cells into effector T cells can only purge HIV-1 in
201 approximately 1 resting T cell per million (Bullen et al., 2014; Ho et al., 2014). Sequential stimulation
202 with several TCR-based LRAs can increase the purging efficiency 2-3 fold (Hosmane et al., 2017).
203 Thus, full T-cell activation allows latency to persist in most cells. To study if more complete purging
204 can be achieved, we developed an HIV-1 latency model with (TCR pre-stimulated) primary effector T
205 cells. We demonstrated that DC contact can efficiently purge latent HIV in these T cells, something
206 not achieved by a second round of T-cell activation or treatment with other LRAs (Figure 2). Thus, the
207 one-two punch consisting of TCR activation and subsequent stimulus with DCs can reach a
208 significantly larger portion of the reservoir than other purging methods.

209 Using CD4$^+$ T cells isolated from ART-treated aviremic patients, we confirmed results obtained with
210 our in vitro latency model by showing that *ex vivo* TCR-stimulation combined with DC contact
211 induced the release of HIV-1 RNA into the supernatant in a significantly larger proportion of patients
212 compared with TCR stimulation alone, which is indicative for purging of latent HIV-1 (Laird et al.,
213 2015). Although RNA could be detected for all patients with the double treatment, the levels of RNA
214 released were not increased. This could be caused by efficient capture and degradation of free HIV-1
215 by DCs which will limit the amount of HIV-1 RNA to be detected in supernatant (van Montfort et al.,
216 2015). In agreement with this hypothesis, treatment of patient cells with DCs alone without PHA
217 activation also decreased the amount of RNA released in supernatant compared to untreated cells.
218 To understand why DCs increase the purging efficiency we dissected the molecular mechanisms

219 induced in T cells pretreated with PHA. Our data illustrate that formation of tetraspanin-enriched
220 microdomains (TEMs) between DCs and T cells is important for the purging ability of DCs (Figure 3A)
221 (Berditchevski et al., 1997; Lin and Fan, 2016; Mittelbrunn et al., 2002; Rocha-Perugini et al., 2016).
222 These TEMs recruit PI4K to the membrane to locally produce phosphoinositols, such as
223 phosphatidylinositol-4,5 bisphosphate (PtdIns(4,5)P_2), which activate RAS via Shc proteins (Hemler,
224 2005; Pawson and Scott, 1997). Active RAS can activate the MEK-ERK, PLC-PKC and the PI3K-Akt-
225 mTOR pathway. The DC-mediated HIV-1 purging in T cells, however was predominantly caused by
226 activation of the PI3K-Akt-mTOR pathway, which is associated with improved RNA elongation,
227 ribosomal activity, protein synthesis and cell survival (Darcis et al., 2015). Thus, activation of the
228 PI3K-Akt-mTOR probably overcomes post-transcriptional blocks to form infectious viral particles.
229 Whether activation of this pathway is strictly regulated via RAS activation or via other DC-T cell
230 mediated interactions needs to be explored in future research.

231 We hypothesize that phosphoinositols, especially the availability of PtdIns(4,5)P_2, determines HIV-1
232 latency at various levels (Figure 9). First, transcription factors such as NF-κB or c-Jun/c-Fos are
233 required for HIV-1 transcription initiation (Bullen et al., 2014; Darcis et al., 2015; Laird et al., 2015).
234 These factors are upregulated via the MEK-ERK and PLC-PKC pathway, where PtdIns(4,5)P_2 is
235 converted by PLC into IP_3 and DIAG (Mendoza et al., 2011). This can be achieved by TCR stimulation
236 or PKC activators. Second, PI3K-Akt-mTor pathway activation is required to allow transcriptional
237 elongation and protein synthesis. DCs can initiate this pathway by PtdIns(4,5)P_2 production in TEMs
238 via PI4K activation and subsequent phosphorylation into PtdIns(3,4,5)P_3 by PI3K. Since the PI3K-Akt-
239 mTOR and PLC-PKC pathway either phosphorylate or dephoshorylate PtdIns(4,5)P_2, strong activation
240 of either one of these pathways will directly inhibit the activation of the other pathway. Thus, a fine
241 balance between these pathways should be pursued to purge latent HIV-1, or both pathways should
242 be activated sequentially as achieved for instance with our "one-two punch" strategy, in which, after
243 transcription initiation, RNA elongation and protein translation should follow.

244 In this light it is not surprising that TCR stimulation, which can trigger sub-optimally the PLC-PKC and
245 PI3K-Akt-mTOR pathway, has some HIV-1 purging ability, leading to outgrowth of infectious particles,
246 whereas solely activating the transcription initiation route does not (Bullen et al., 2014).
247 Interestingly, a recent study discovered the importance of the mTOR pathway in purging latent HIV-1
248 in resting cells (Besnard et al., 2016). Specifically blocking the mTOR pathway after TCR stimulation
249 negated reversal of latency in patient cells, illustrating that TCR-stimulation can sub-optimally
250 activate this pathway. Our results extend those data by showing that DCs activate this PI3K-Akt-
251 mTOR pathway stronger than TCR stimulation, leading to increased purging efficiency.

252 Blocking multiple targets in the PI3K-Akt-mTOR pathway, for instance with GSK2126458, inhibited
253 DC-specific activation of the PI3K-AKt-mTOR pathway better than the mTORC1 inhibitor
254 temsirolimus. This is in agreement with a study from Martin *et al.*, who reported that the mTORC1
255 inhibitor rapamycin (Calne et al., 1989; Ernst et al., 1989) did not block purging of latency HIV-1, but
256 could reduce the toxic side effect of CD3/CD28 T cell activation without affecting CTL-mediated killing
257 (Martin et al., 2017).

258 DC contact may influence HIV-1 reactivation in T cells by additional means. Ren *et al.* showed that
259 mature DCs are capable of releasing TNFα upon contact with infected Jurkat T cells, leading to
260 reversion of latency in this cell line (Ren et al., 2017).

261 Since DCs strongly revert HIV-1 latency, it would be an option to use DCs as a natural cell source to
262 revert HIV-1 latency in HIV-infected patients. We previously observed that primary DC homing to
263 different lymphoid organs have substantially different latency-purging capacities. Monocyte-derived
264 DCs cultured under conditions towards a gut DC phenotype efficiently purged latent HIV-1, whereas
265 no, or only modest, reversal of latency was achieved with DCs representing the subtype homing to
266 the genital tract. Myeloid DCs that have been matured with different toll-like receptor agonists,
267 representing lymph node derived DCs, efficiently purge HIV-1 from latency (van der Sluis et al., 2015).

268 The fact that monocyte-derived DCs can purge HIV-1 from patient-derived latently infected cells
269 suggests that DCs might control the size of the viral reservoir in patients in particular tissues. This
270 could happen especially when T cells are activated due to secondary infection, or in the acute phase
271 of disease when T cells are activated to fight the HIV-1 infection (van der Sluis et al., 2013). DC-
272 mediated HIV-1 reversion however, is not to be expected in peripheral blood (van der Sluis et al.,
273 2015), as the frequency of myeloid DCs is ~7000 per million CD4 T cells in healthy donors, with even
274 lower numbers in HIV-1 infected individuals (~2300 per million) (Donaghy et al., 2012). But, DC:T
275 contact might be more efficient in tissues, which could result in the observed low levels of ongoing
276 virus production, even in the presence of therapy (Lorenzo-Redondo et al., 2016; Pasternak et al.,
277 2012; Sigal et al., 2011).

278 Interestingly, Gramatica and colleagues already raised the possibility that latency reversal might be
279 more effective in lymphoid tissues where contact of T cells with DCs is more prominent than in blood
280 (Gramatica, A; Greene, WC; Montano, 2015). The lack of efficacy of clinical trials using LRAs aimed at
281 decreasing the HIV-1 reservoir size might therefore be caused by the fact that the reservoir was
282 analysed only in the peripheral blood and not in tissues, where preferential DC-mediated purging
283 could occur (Archin et al., 2012; Gutiérrez et al., 2016; Rasmussen et al., 2018; Søgaard et al., 2015;
284 Spivak et al., 2014). One approach to use DCs to increase the LRA purging efficiency in patients might
285 be the combination of an LRA with a specific TLR agonists that will direct DCs to tissues, where latent
286 reservoirs are maintained. The use of DCs as a natural mechanism to revert HIV-1 latency has limited
287 side effects (Palucka and Banchereau, 2013), as compared to T cell receptor agonists (Prins et al.,
288 1999).

289 Our results have important implications. Firstly, the potent capacity of DCs to reactivate HIV-1
290 proviruses which were not purged despite PHA stimulation could be used to design a more sensitive
291 culture-based assay to measure the replication-competent reservoir, for instance by adapting the
292 promising TZM-bl based assay (Sanyal et al., 2017). Secondly, the role of DCs in HIV-1 persistence

293 highlights the need to look at tissue reservoirs when possible, since cell-to-cell contact appears to be
294 a major factor controlling HIV-1 latency. Finally, our results further support the importance of the
295 PI3K-Akt-mTOR pathway in HIV-1 latency. These novel insights could be used to develop more
296 efficient LRA strategies in the quest to get rid of the latent HIV-1 reservoir.

297

298 **Materials and methods**

299 **Reagents.** The fusion inhibitor T1249 was obtained from Pepscan (Therapeutics BV, Lelystad, The
300 Netherlands) and used at a final concentration of 0.1 µg/ml. Antibodies against CD9, CD63, CD81,
301 CD83, CD86, CD151, CD45RO, CD45RA, were purchased from Biolegend (San Diego, CA, USA). Other
302 antibodies are directed against CA-p24 (clone KC-57RD1) (Beckman Coulter, Brea, CA, USA) and CD3-
303 APC (BD Pharmingen, Breda, the Netherlands).

304 **Cells**. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Central
305 Laboratory Blood Bank, Amsterdam, The Netherlands) by use of a Ficoll gradient and frozen in
306 multiple vials. When required, PBMCs were thawed, activated with phytohaemagglutinin (PHA,
307 Remel, 2 µg/ml) and cultured in RPMI medium supplemented with 10% FCS and recombinant human
308 IL-2 (rhIL-2, Novartis, 100 U/ml). On day 2 of culture, $CD8^+$ T lymphocytes were depleted using CD8
309 immunomagnetic beads (Dynal, Invitrogen, Carlsbad, CA, USA) and the $CD4^+$-enriched T lymphocytes
310 were cultured for 3 more days. SupT1 CCR5 expressing T cells, a kind gift of prof. J. Hoxie (Del Prete
311 et al., 2009), were cultured in RPMI 1640 medium containing 10% FCS.

312 Monocytes were isolated from PBMCs with CD14 magnetic beads from Miltenyi Biotec (GmbH,
313 Bergisch Gladbach, Germany) according to the manufacturer's protocol. Purified monocytes were
314 cultured in RPMI 1640 medium containing 10% FCS and differentiated into monocyte-derived DCs by
315 stimulation with 45 ng/ml interleukin-4 (rIL-4; Biosource, Nivelles, Belgium) and 500 U/ml

316 granulocyte macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Brussels, Belgium) on
317 day 0 and 2, and used on day 6 (van Montfort et al., 2008).

318 HEK 293T cells were grown as a monolayer in Dulbecco's minimal essential medium (Gibco, BRL,
319 Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (FCS), 40 U/ml penicillin, 40 µg/ml
320 streptomycin at 37°C and 5% CO_2.

321 SupT1 CCR5 expressing T cells, a kind gift of prof. J. Hoxie (Means et al., 2001), were cultured in RPMI
322 1640 medium containing 10% FCS.

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

328

329 **HIV-1 latency assay.** HIV-1 infected cells were used in the latency assay as described previously[16, 19,
330 20]. In short, PHA-activated $CD4^+$ T lymphocytes (1.5×10^6 or 2.0×10^6 cells) were infected with HIV-1
331 for 4 hours (20 ng CA-p24). To establish infection over a 4 hr window, excess virus was diluted with
332 three washes and cells were incubated with 100 ng/ml T1249 fusion inhibitor to prevent late fusion
333 events and new rounds of virus replication. Cells were incubated for 24 hr to allow HIV-1 proviral
334 integration. Subsequently, cells were treated with or without DCs (ratio 1:3), with or without 20
335 µg/ml CD9/CD63/CD81/CD151 antibody, or in the presence or absence of drugs for 48 hr in the
336 continued presence of T1249. Single cells were analysed for the presence of the CD3 T cell receptor
337 and expression of intracellular CA-p24 by flow cytometry (van der Sluis et al., 2013, 2015). The
338 percentage of CA-p24 positive CD3-positive cells in the treated culture was divided by the percentage

339 of CA-p24 cells in the mock treated culture and reported as fold activation (a measure of HIV-1
340 latency reversion).

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

351 **Human phospho kinase array.** PHA-activated $CD4^+$ T lymphocytes (3.0×10^7) were infected according
352 to the HIV-1 latency assay protocol. Infected PBMCs were incubated with 7.0×10^6 DCs or without
353 DCs for 2.5 hr. To disrupt the DC-T cell interaction, which was observed after 1 hr incubation, cell
354 cultures were resuspended and washed twice in cold PBS supplemented with 2 mM EDTA and 0.5%
355 FCS. $CD4^+$ T lymphocytes were isolated at 4°C using the human $CD4^+$ T lymphocytes isolation kit from
356 Miltenyi Biotec according to the manufacturer's protocol. Approximately 1.0×10^7 $CD4^+$ T
357 lymphocytes were obtained after the isolation protocol. Cells were pelleted and treated according to
358 the manufacturer's protocol from the human phospho-kinase antibody array kit (R&D systems).

359 **Patient material.** PBMCs were isolated from aviremic ART-treated HIV-infected patients. Informed
360 written consent was obtained from all participants of this study, and the study was approved by the
361 Medical Ethical Committee of the Academic Medical Center, Amsterdam. All procedures followed
362 were in accordance with the ethical standards of the responsible committee on human

363 experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in

364 2000.

365

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

366

367 Table I: HIV-1 patient history

368 ND = Not determined, UDV = undetectable viral load

369 TDF: tenofovir disoproxil fumarate; FTC: emtricitabine; EFV: efavirenz; DTG: dolutegravir; 3TC: lamivudine; ABC: abacavir;

370 EVG/c: elvitegravir cobicistat; TAF: tenofovir alafenamide; NVP: Nevirapine; LPV/r: lopinavir ritonavir; RAL: raltegravir;

371 DRV/r: darunavir ritonavir, ATZ/r: atazanavir ritonavir

372 **Quantification of HIV-1 release into culture supernatant.** Patient-derived $CD4^+$ T cells were isolated

373 using the human $CD4^+$ T-lymphocyte isolation kit (Miltenyi Biotec) according to the manufacturer's

374 protocol. ~1.4-2.0 x 10^6 cells were treated with 2 µg/ml PHA, heterologous monocyte-derived DCs in
375 a 1:3 ratio (DC:T), PHA combined with DCs (1:3 ratio) or left untreated for 72 hrs in RPMI1640
376 medium containing rhIL-2 and 80 ng/ml sanquinavir. Cell-free supernatant was collected by
377 centrifugation for 5 min at 500 rcf. Extracellular HIV-1 RNA was isolated from 300 microliters of the
378 supernatant using the Boom isolation method (Boom et al., 1990) with the addition of 2 micrograms
379 of carrier RNA (poly A RNA, Qiagen, Venlo, The Netherlands) and processed for reverse transcription
380 and first PCR using QIAGEN® OneStep RT-PCR kit (Qiagen, Venlo, The Netherlands) according to the
381 manufacturer's instructions. The primers used were TAR-F (5'-GGGTCTCTCTGGTTAGACCAG-3') and
382 HIV-FOR (Malnati et al., 2008). The PCR settings were as follows: 94°C for 3 min, followed by 15
383 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 microliters
384 of the first PCR product were subsequently used as a template in the second, semi-nested, real-time,
385 PCR amplification, performed using the primers and a TaqMan probe described by Malnati et al.
386 (Malnati et al., 2008).

387

388 **qVOA with 1) PHA, 2) DC one-two punch**

389 Isolated CD4 T cells from aviremic patients were activated with 2 µg/ml PHA for 72 hrs in RPMI1640
390 medium containing rhIL-2 and 100 ng/ml entry inhibitor T1249. Cells were washed three times and
391 cultured in 96-well plate with a cell density of 2.0 x 10^5 CD4 T cells/well with or without 8.3 x 10^4 DCs
392 (1:3 DC:T cell ratio) in fresh RPMI1640 containing rhIL-2. HIV-1 susceptible SupT1 CCR5 cells (5.0 x
393 10^4) were added to the microcultures to disseminate and sustain HIV-1 replication (Fun et al., 2017).
394 After 7, 14 and 21 days of culturing half of the culture was used to determine HIV-1 CA-p24 by ELISA
395 (van der Sluis et al., 2013) and fresh RPMI 1640 media was added to sustain T cell growth and virus
396 replication. The number of infectious units per million (IUPM) was determined on the number of HIV-
397 1 CA-p24 positive microcultures for each condition per million patient-derived CD4 T cells on day 21.

398

399 **Statistical analysis.** Significance of differences between groups or pairs was determined by one-way

400 ANOVA and Student's t-tests. For patient-derived samples, Fisher's exact tests were used to compare

401 the detectability of HIV-1 extracellular RNA between different conditions. Significance levels: *:

402 $p<0.05$; ** : $p<0.01$; *** : $p<0.001$. All tests were two-sided and were performed using GraphPad

403 Prism, version 7.

404

405 **Acknowledgments**

406 We would like to thank George Leslie and Jim A. Hoxie for providing us with the SupT1 CCR5 T cell

407 line (Del Prete et al., 2009).

408 **Funding**

409 RvdS, TvM, AOP, BB and REJ were supported by the Dutch Aidsfonds (Project numbers 2007028,

410 2008014, 2011020, 2012025, 2013021 and P-22602; http://www.aidsfonds.nl/about/organisation).

411

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565

566 **Figure Legends**

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

570

571 **Figure 2: Purging of latent HIV-1 in *in vitro* infected primary activated T cells with classical LRAs or**
572 **DCs.**

573 **A**) Time-line of the HIV-1 latency assay. Primary PHA-activated T cells are infected *in vitro* with HIV-1
574 for 4 hr. Residual virus cell-fusion events and replication are blocked with the T1249 entry inhibitor.
575 Proviral integration is allowed for 24 hr prior to applying various stimuli for another 48 hr. **B**)
576 Representative dot plot of CA-p24 and CD3 stained CD4-enriched T cells stimulated with or without
577 DCs. **C**) Results shown as percentage of virus producing cells and fold activation. Data shown as
578 average of three independent experiments with standard error of the mean. **D**) Activation of latent
579 HIV-1 with T cell receptor agonist phytohaemagglutinin (PHA) and CD3/CD28 antibodies. **E**) Using

580 different concentrations of the HDAC inhibitor romidepsin. F) Using different concentrations of the
581 PKC activator prostratin or TNFα (**G**). Representative data are shown with standard error of the mean
582 of at least three independent experiments. Drug toxicity is indicated with ✞.

583 **Figure 3: Reversion of latency depends on specific tetraspanin enriched microdomains formed**
584 **between DCs and T cells.**

585 **A**) Fold activation from latency of infected primary T cells cultured with or without DCs in the
586 presence of CD9, CD63, CD81 and CD151 antibodies targeting specific tetraspanin enriched
587 microdomains (TEMs). **B**) Inhibition of DC-mediated HIV-1 reversion with antibodies against the co-
588 stimulatory molecule CD86 and DC-activation marker CD83**. C)** Schematic overview, adapted from
589 Hemler et. al. (Hemler, 2005), regarding the role of the tetraspanins targeted in activating the RAS
590 signaling cascade. Phosphatidylinositol 4-kinase is recruited to the membrane in CD9, CD63, CD81
591 TEMs and phosphoinositides, such as phosphatidylinositol-4,5- bisphosphate ($PI(4,5)P_2$) are
592 produced. This causes activation of Shc, resulting in the activation of the RAS signaling pathway.
593 CD151 negatively influences the RAS activation pathway (Hemler, 2005). Representative data are
594 plotted as the average fold activation with standard error of the mean of at least three independent
595 experiments.

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

600 **Figure 5: Effects of targeting the PI3K-Akt-mTOR signaling transduction cascade on latency reversal.**

601 **A**) Schematic overview of inhibitors targeting the PI3K-Akt-mTOR signaling pathway. **B**) Fold
602 activation from latency of infected primary T cells cultured with or without DCs in the presence of the
603 PI3K/mTORC2/mTORC1 inhibitor GSK2126548, **C**) PI3K/mTORC1 inhibitor AZD8055, **D**) Akt inhibitor

604 MK2206, **E**) mTorc1 inhibitor temsirolimus, or **F**) inhibitor of nuclear factor kappa-B kinase (IKK)
605 antagonist bardoxolone. Representative data are plotted as the average fold activation with standard
606 error of the mean at the different indicated concentrations of inhibitor from three independent
607 cultures. Drug toxicity is indicated with ✞.

608 **Figure 6: Transcription factors binding NF-κB and SP1 target sites in the LTR promoter do not seem**
609 **to be involved in DC-dependent latency reversal.** **A**) Fold activation of infected primary T cells
610 cultured with or without DCs in the presence of JSH-23, inhibiting activation of the transcription
611 factor NF-κB, **B**) in the presence of AP-1 JNK inhibitor JNKV **C**) in the presence of SP600125 inhibiting
612 dimerization of c-Jun transcription factors binding SP1 motifs, or **D**) the inhibitor T5224 inhibiting c-
613 Fos-c-Jun dimerization. Representative data are plotted as the average fold activation with standard
614 error of the mean at the different indicated concentrations of inhibitor from three independent
615 cultures. Of note, blocking activation of transcription factors beyond the indicated concentrations of
616 JSH-23 and SP600125 was toxic for T cells regardless of whether they were co-cultured with DCs
617 (results not shown)

618 **Figure 7: Characterization of phosphorylated protein kinases in infected primary $CD4^+$ T cells upon**
619 **coculturing with DCs.** Phosphorylation of a selected panel of kinase proteins (as indicated) using the
620 human-phospho kinase array from R&D systems was determined in isolated $CD4^+$ T cells cocultured
621 with DCs, for **A**) 2 hr or **B**) 21 hr. Of the 43 kinases analyzed, only differentially phosphorylated
622 kinases with more than 25% up- or downregulation compared to untreated cells are shown. **C)**
623 Activating and inhibitory signaling cascades with corresponding phosphorylation status of kinases for
624 the Akt, MEK-ERK signaling pathway are shown schematically (Bunney and Katan, 2010; Downward,
625 2003).

626

627 **Figure 8: Purging of latent HIV-1 from patient-derived resting primary T cells with DCs.**

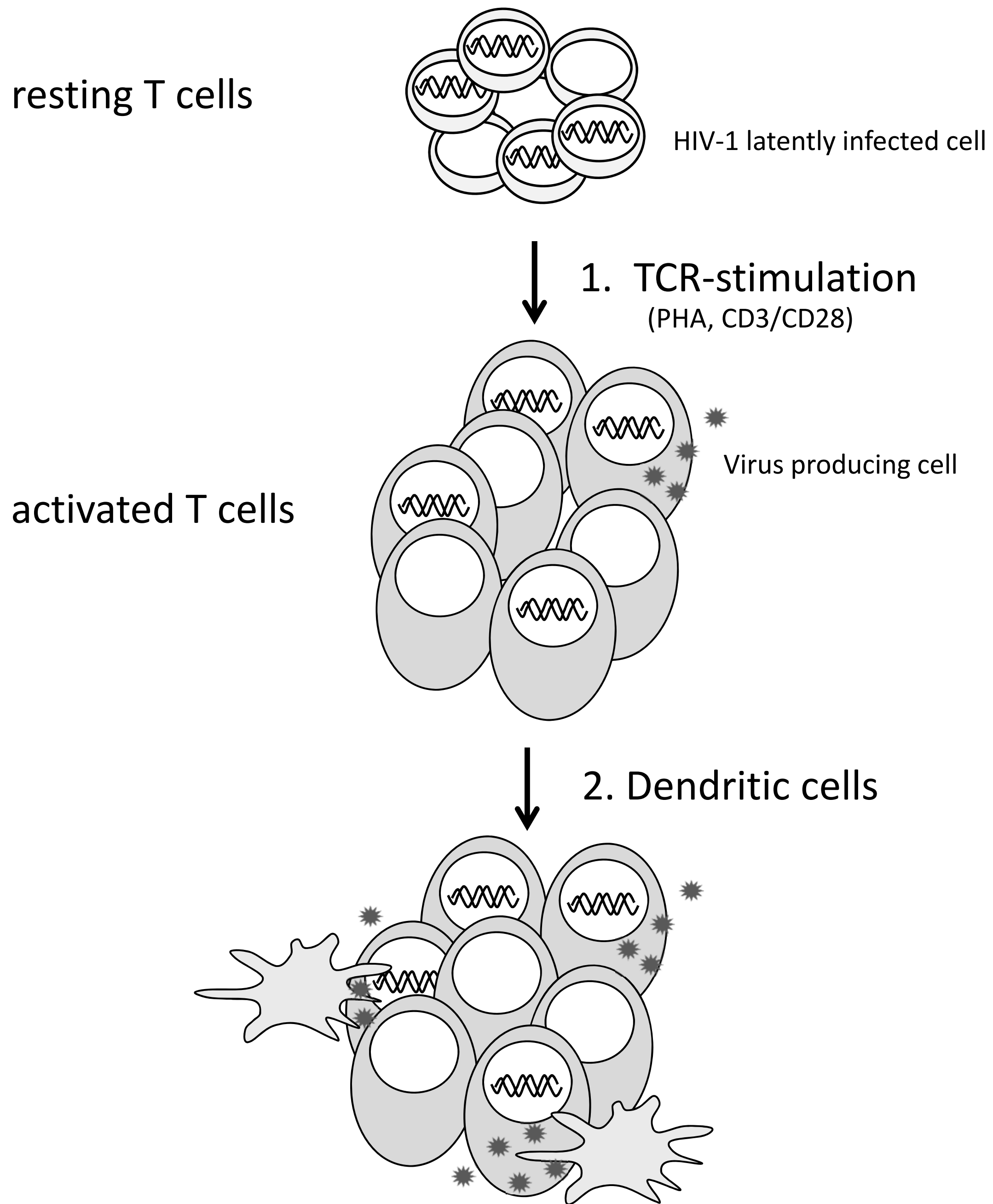
628 Isolated $CD4^+$ cells (1.4-2.0 x 10^6) from aviremic HIV-1 patients were cultured with or without PHA in
629 presence or absence of DCs (ratio 1:3 DC:T cell) for 72 hrs. **A)** Copy numbers of HIV-1 extracellular
630 RNA released into cell culture supernatant, as determined by RT-qPCR. Median values are
631 represented by horizontal lines. The dotted line represents the detection limit of the assay. The
632 undetectable values were plotted as 50% of the detection limit. **B)** Percentage plot of detectable
633 versus undetectable HIV-1 RNA. **C)** Outgrowth of replication competent HIV-1 from 7 patient-derived
634 $CD4^+$ T cells activated with PHA followed by addition of DCs on day 3. Reversal of latency is depicted
635 as infectious units per million cells (IUPM) based on the number of CA-p24 negative and positive
636 microcultures. Median values are represented by horizontal lines. **D)** Percentage plot of detectable
637 versus undetectable replication competent HIV-1 after differential stimulation for these patient
638 samples.

639 **Figure 9: Reversion of HIV-1 latency regulated by DCs.** Binding of DCs to T cells is facilitated by
640 tetraspanin membrane molecules that recruit interacting DC-T cell proteins in tetraspanin enriched
641 microdomains (TEMs) to induce multiple signaling events. Recruitment of PI4K to CD9/CD63/CD81
642 TEM leads to local production of PI(4,5)P_2. DC-dependent activation of PI3K further phosphorylates
643 PI(4,5)P_2 into PI(3,4,5)P_3, which activates PDK1 to induce recruitment of Akt to the membrane. Akt
644 docking to PI(3,4,5)P_3 triggers activation followed by activation of downstream signaling cascades
645 such as mTORC1 or IKK that promote transcription and protein synthesis reactiving latent HIV-1 in
646 active proliferating cells. The signaling cascade that is responsible for DC-mediated reversion of
647 latent HIV-1 independent of T cell receptor activation is indicated with bold arrows.

648

649 **Author contributions**:

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



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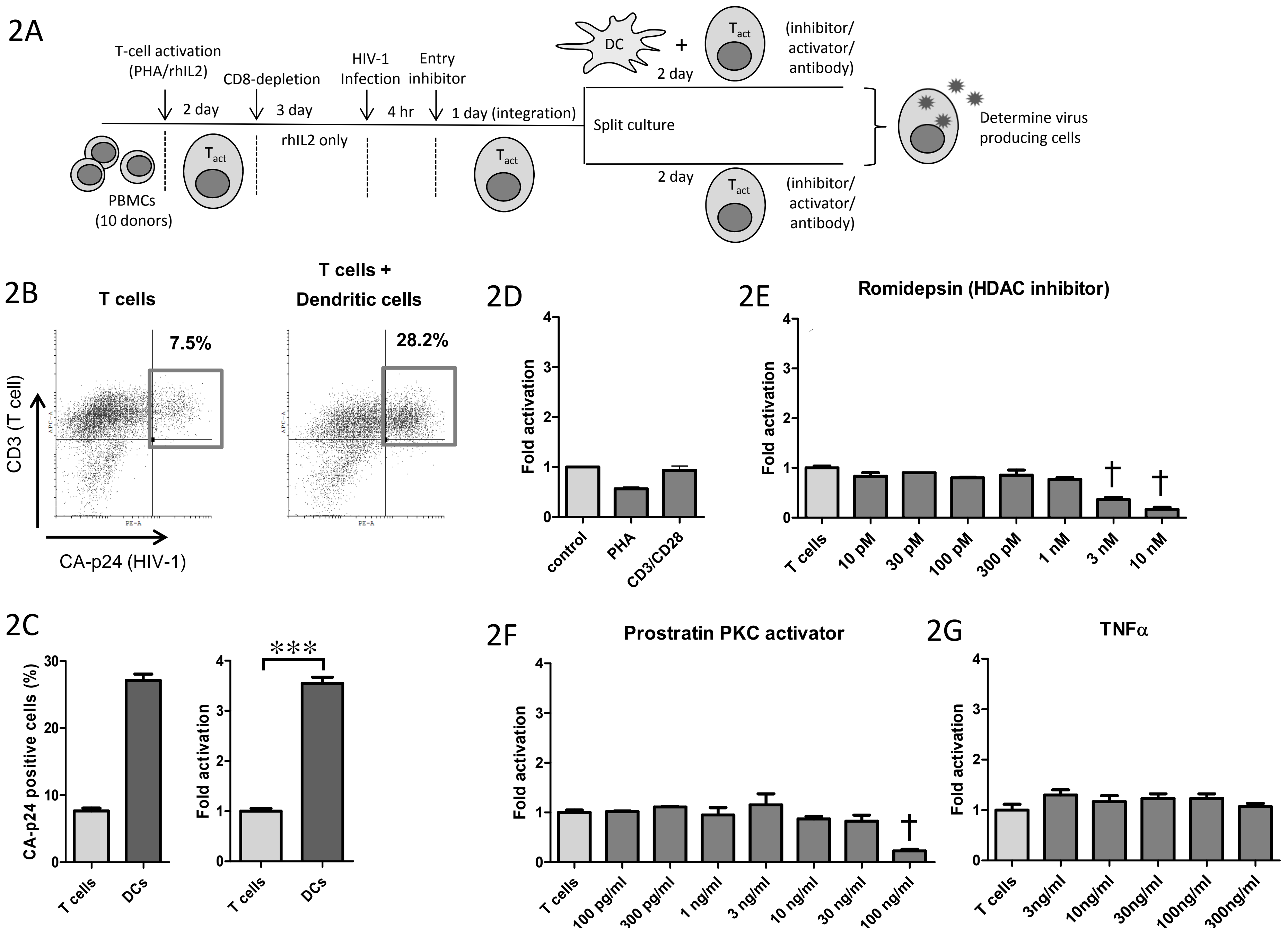
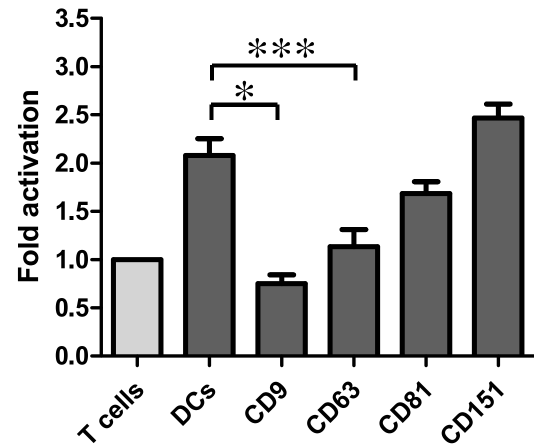


Figure 2

3A



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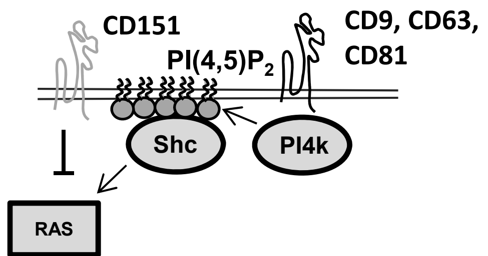

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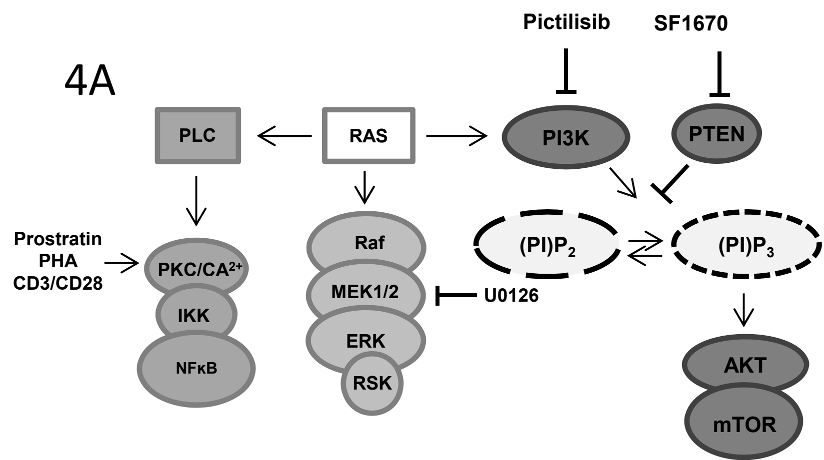

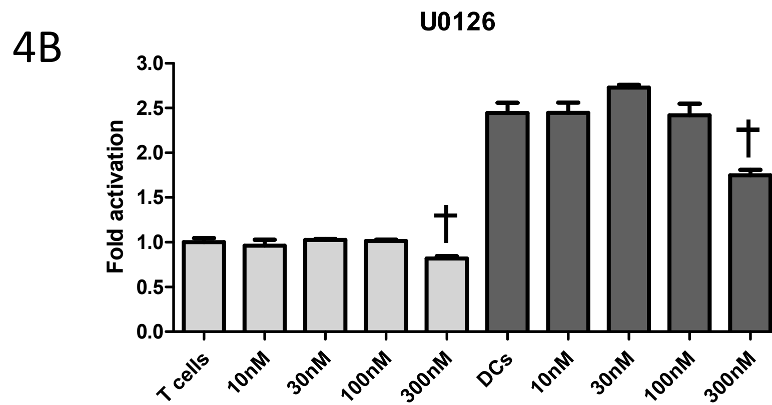

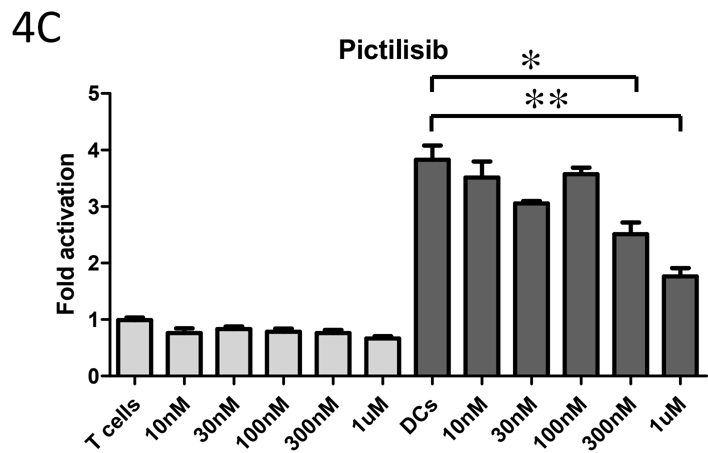

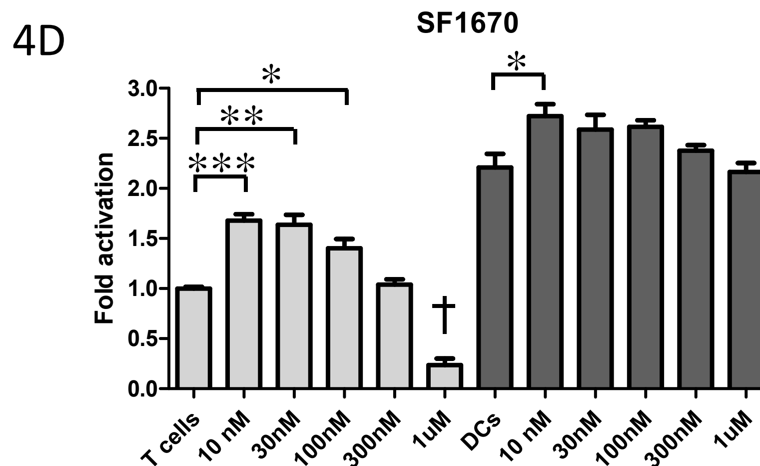

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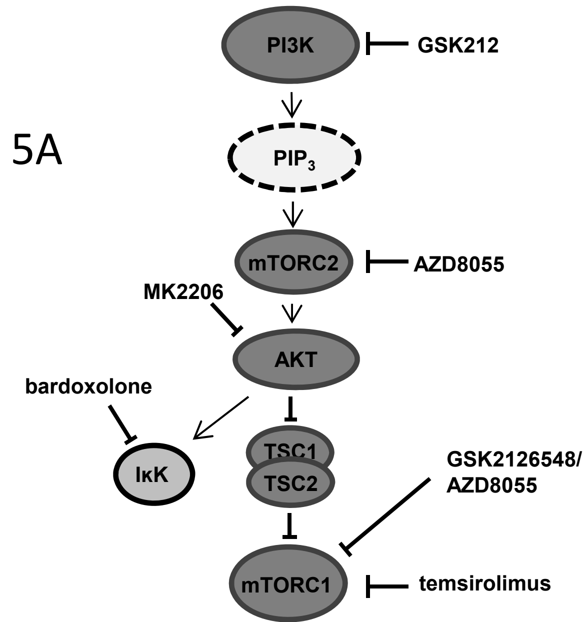

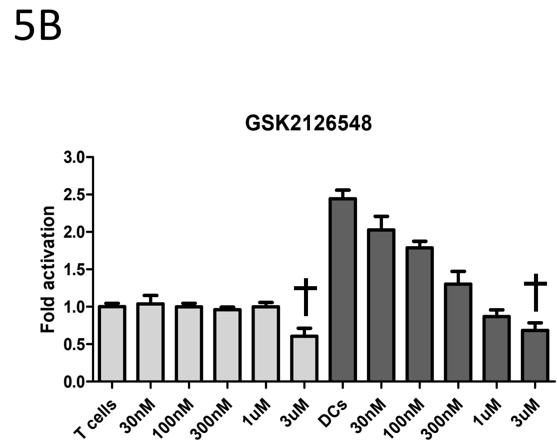

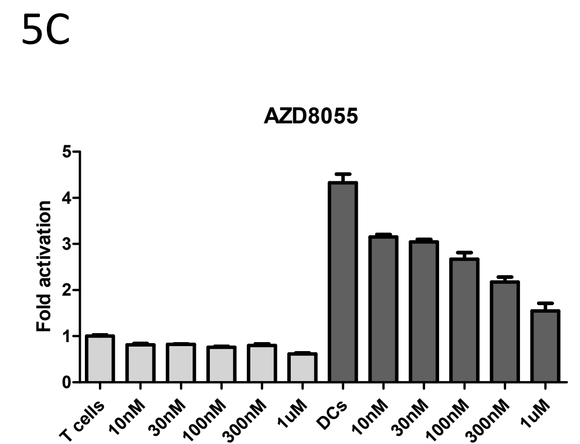

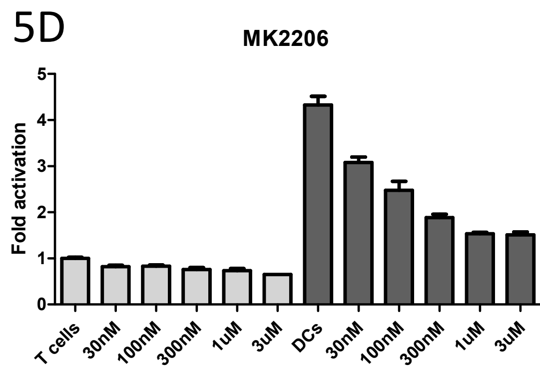

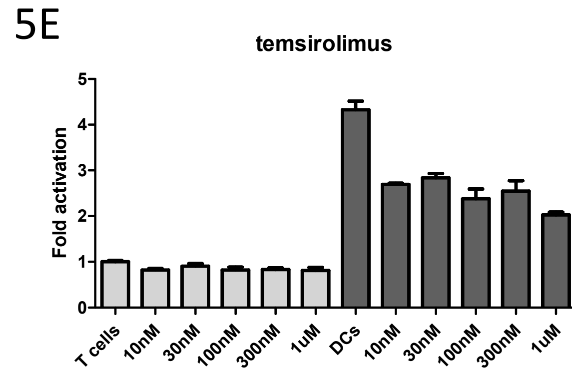

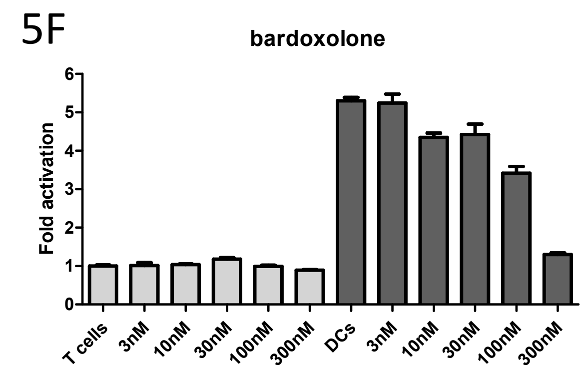

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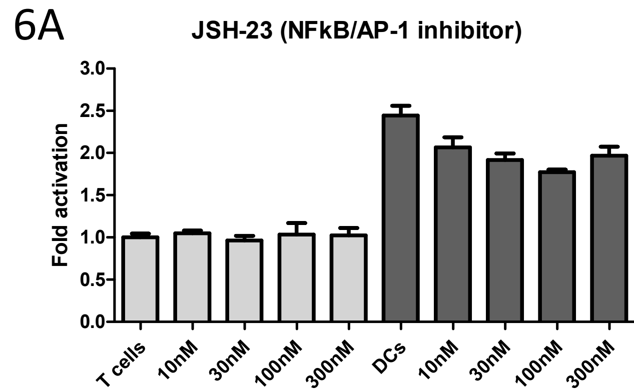

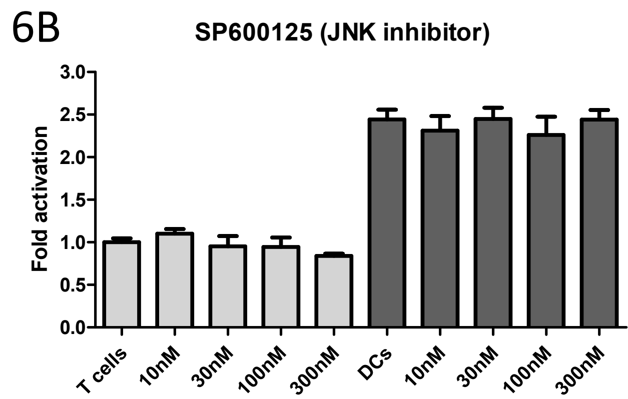

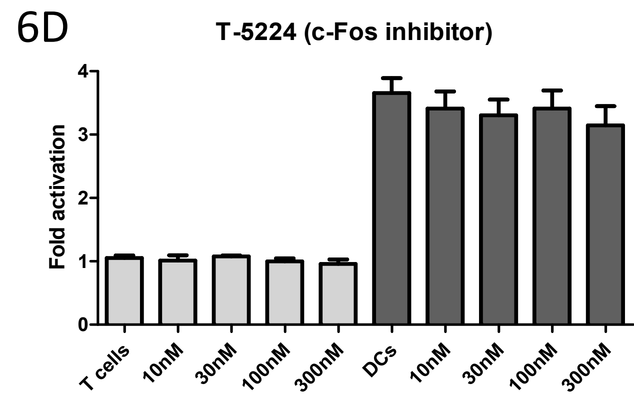

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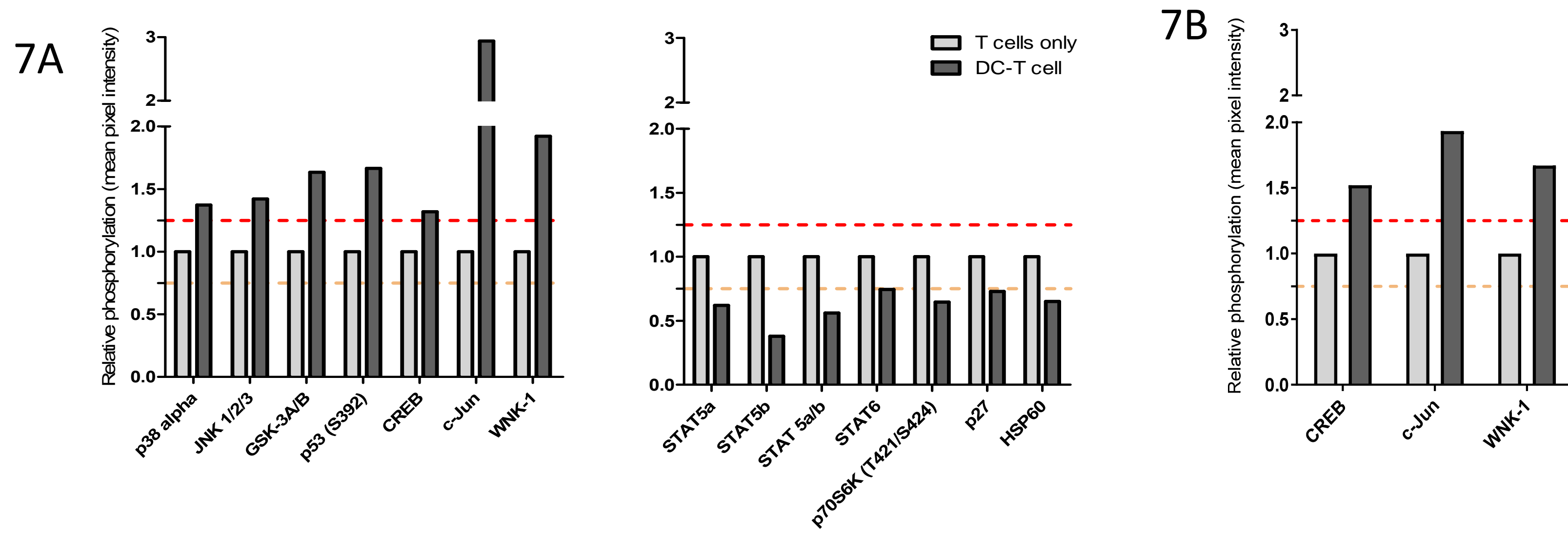


7C

MEK1/2

ERKp38

AKT

mTORC1

p70S6K

JNK1/2/3

GSK-3A/B

MDM2

p27

CREB

WNK-1

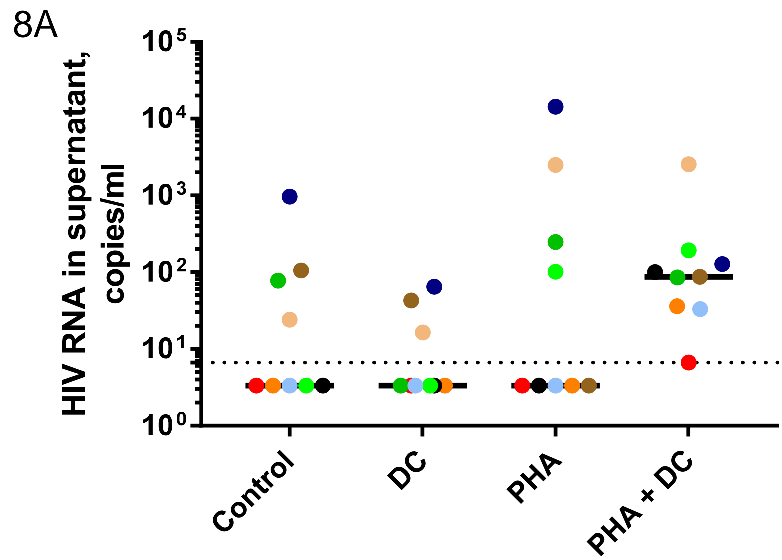
C-Jun

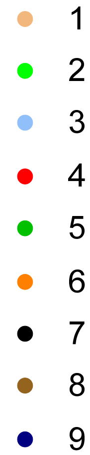
p53

P

Figure 7

8A





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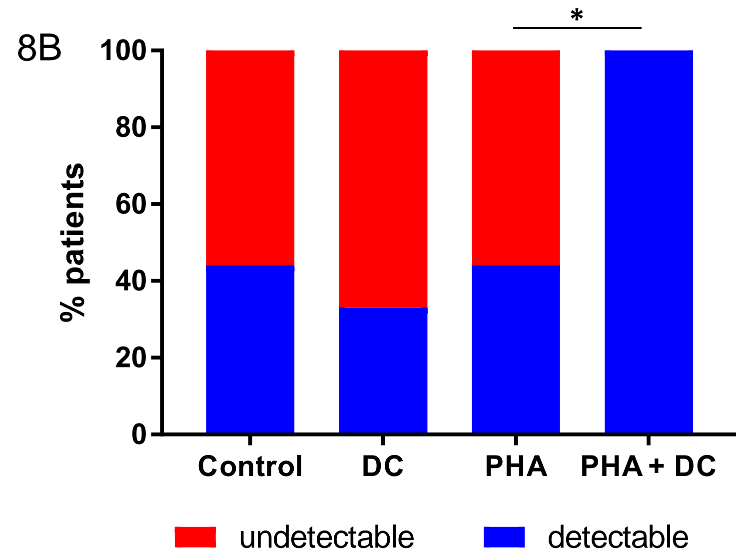

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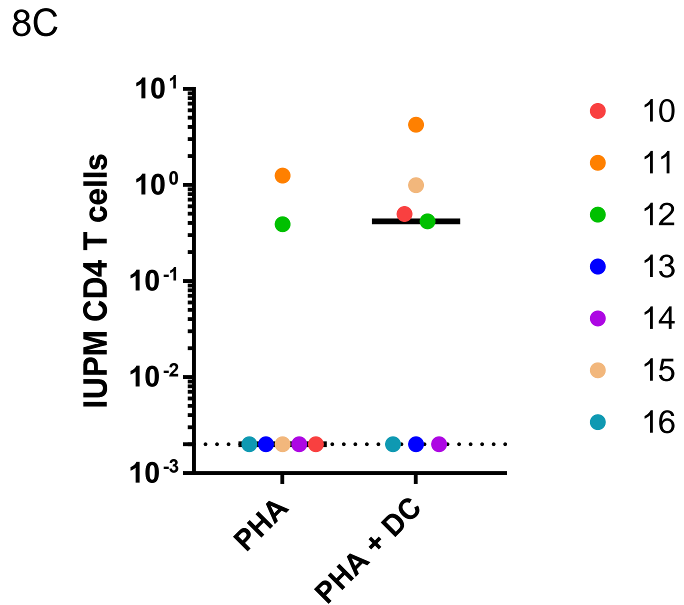

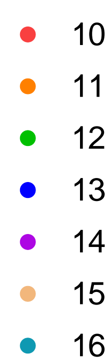

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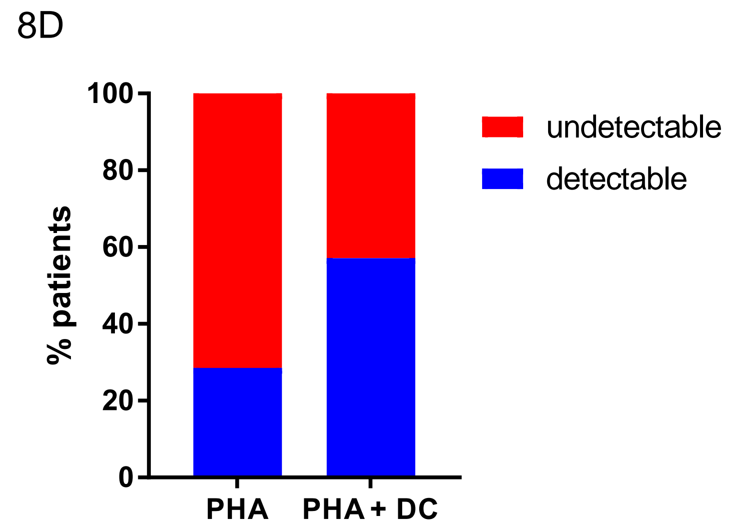

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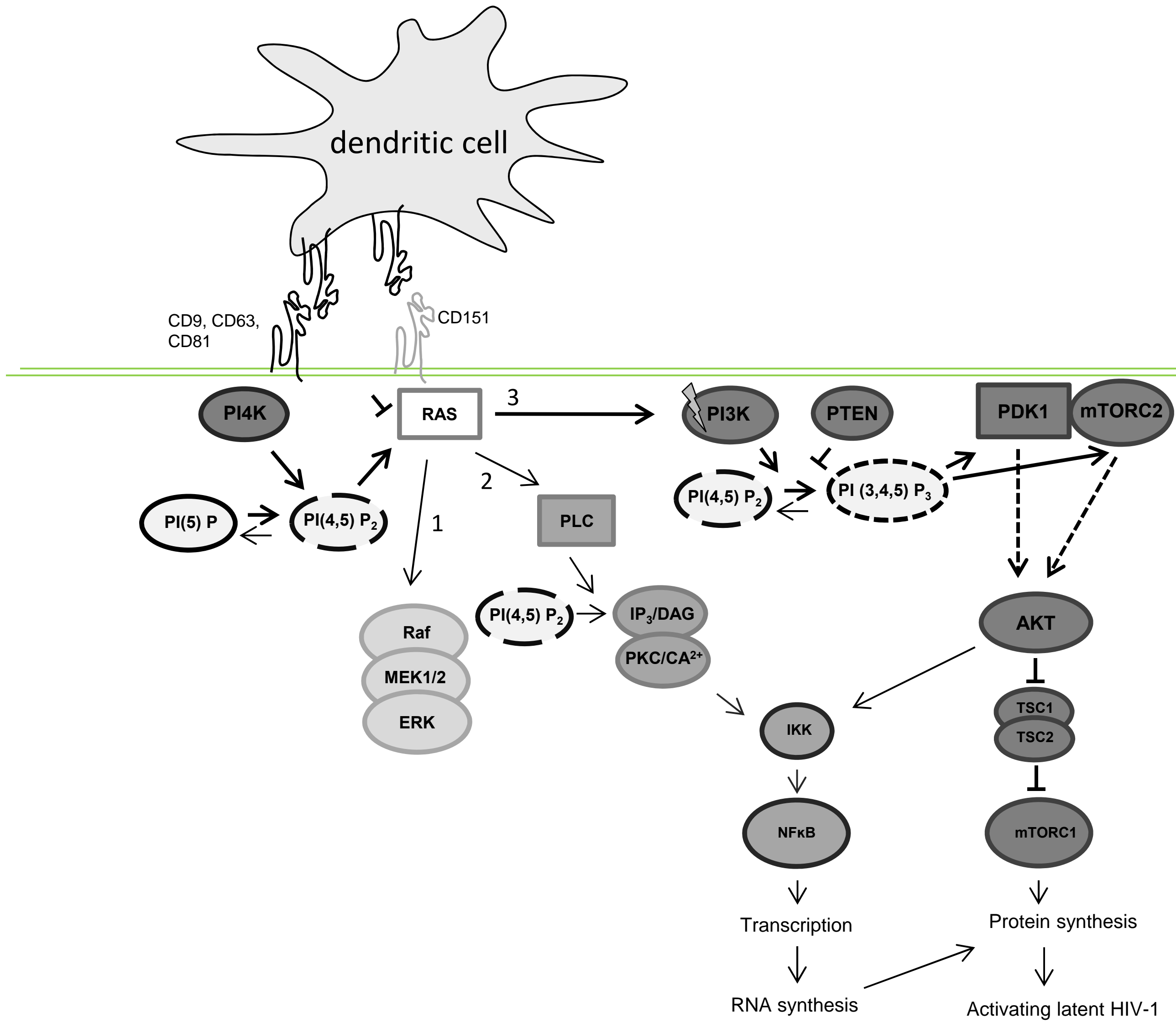

Figure 9