Virology 504 (2017) 45–51

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

HTLV-1 Tax activates HIV-1 transcription in latency models

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ARTICLE INFO

Keywords:
HIV-1
Latency
HTLV-1
Tax
P-TEFb
Resting cells

ABSTRACT

HIV-1 latency is a major obstacle to HIV-1 eradication. Coinfection with HTLV-1 has been associated with faster progression to AIDS. HTLV-1 encodes the transactivator Tax which can activate both HTLV-1 and HIV-1 transcription. Here, we demonstrate that Tax activates HIV transcription in latent CD4$^+$ T cells. Tax promotes the activation of P-TEFb, releasing CDK9 and Cyclin T1 from inactive forms, promoting transcription elongation and reactivation of latent HIV-1. Tax mutants lacking interaction with the HIV-1-LTR promoter were not able to activate P-TEFb, with no subsequent activation of latent HIV. In HIV-infected primary resting CD4$^+$ T cells, Tax-1 reactivated HIV-1 transcription up to five fold, confirming these findings in an *ex vivo* latency model. Finally, our results confirms that HTLV-1/Tax hijacks cellular partners, promoting HIV-1 transcription, and this interaction should be further investigated in HIV-1 latency studies in patients with HIV/HTLV-1 co-infection.

1. Introduction

Despite major advances in antiretroviral therapy (ART), HIV-1 eradication is still a far off goal to. During early infection, HIV-1 can infect resting CD4$^+$ T cells whose lower metabolism and decreased levels of cellular factors assist virus transcription initiation and elongation. After virus integration, HIV-1 latency can be established in some cells by reduced transcription factors and activation of target cells, chromatin modification or Tat transactivator inactivity. Cells that harbor HIV-1 integrated genomes with no virus transcription are called latent cells, and are thought to contain the main viral reservoir. Current ART strategies do not eliminate latent cells, and virus replication can be re-activated in several tissues such as neurons, GALT (gut-associated lymphoid tissue) and blood. Proviral genomes remain dormant in these latent reservoirs, and after therapy interruption, detectable viral load is usually observed within two to three weeks (Geeraert et al., 2008).

Coinfection can modulate HIV replication and exacerbate the disease progression (Blanchard et al., 1997). The other major human retrovirus, Human T-lymphotropic virus type 1 (HTLV–1), also infects CD4$^+$ T cells and shares the same transmission routes as HIV-1. It is estimated that 5–10 million people are infected by HTLV 1, with the highest prevalence rates in southern Japan, The Caribbean islands, West Africa and parts of South America (Brites et al., 2009). Seroprevalence rates of HIV-1/HTLV-1 coinfection are high in some areas of the world, reaching 17% in some parts of Brazil (Brites et al., 2009; Etzel et al., 2001; Beilke, 2012; Proietti et al., 2005).

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia/lymphoma (ATLL) and the neurodegenerative disorder HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Matsuoka, 2005). HTLV-1 encodes the transactivator protein Tax which is critical for viral replication and gene regulation (Brauweiler et al., 1997; Boxus et al., 2008). Tax interacts with many different cellular partners including transcription factors (NF-κB, TFIIA, CBP), cyclins (such as Cyclin D1, D2, T1) and other proteins, regulating viral gene expression and influencing expression of various cellular genes, including different cytokines (Boxus et al., 2008). Similar to the HIV-1 transactivator Tat, HTLV-1 Tax also interacts with components of the positive transcription elongation factor b (P-TEFb) (Boxus et al., 2008; Cho et al., 2010). P-TEFb can be found in two distinct forms in the cells: a kinase-active P-TEFb, represented by a low molecular-weight (LMW) complex,

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http://dx.doi.org/10.1016/j.virol.2017.01.014
Received 10 October 2016; Received in revised form 17 January 2017; Accepted 19 January 2017
Available online 30 January 2017

composed only by cyclin-dependent kinase 9 (CDK9) and cyclin T1 (Cyc T1); and a kinase-inactive high molecular-weight (HMW) complex, composed by CDK9 and Cyc T1 associated with 7SK snRNP, HEXIM1-2 and other factors (Cho et al., 2010; Contreras et al., 2009). Active P-TEFb promotes transcriptional elongation through phosphorylation of serine 2 sites on the hepta-peptide of the C-terminal domain (CTD) of RNA polymerase II. Additionally, it has been described that Tax recruits active P-TEFb to the LTR region through a cyclin T1 interaction (Cho et al., 2010; Zhou et al., 2006).

In this study, we assessed whether HTLV-1 can reactivate HIV-1 from latency. We found that the expression of HTLV-1 proteins, more specifically Tax, was able to up regulate HIV-1 transcription in J-Lat cells harboring latent HIV-1 provirus and in HIV-1 latent resting T CD4+ primary cells. Similar to Tat, Tax from HTLV-1 activated P-TEFb and promoted reactivation of latent HIV-1. Our data lead us to speculate that HIV-1/HTLV-1 co-infection might induce diminishing of viral reservoir and higher HIV-1 viral load, influencing the outcome of disease. To address this question, it would be necessary to measure the size of HIV-1 reservoirs in HIV-1/HTLV-1 co-infected patients in a long-term survey.

2. Materials and methods

2.1. Plasmids and site directed mutagenesis

The Tax expression vectors (*pTax Wild Type* and *pTax M47*) were obtained from Addgene (cat number 23284 and 23286, respectively). The HTLV-1 proviral clone (pk30) was obtained from the NIH AIDS Reagent Program (Zhao et al., 1995). *pTax M47* encodes a Tax protein harboring an arginine and a serine at the positions 319 and 320 respectively, in place of two original leucines. *pTax M21* was obtained through site directed mutagenesis of *pTax Wild Type* to modify the 134 proline and 135 aspartate to alanine and serine, respectively (Smith and Greene, 1990; Béraud et al., 1994). The modifications were performed using Quick Change Lighting Multi Site Directed Mutagenesis (Stratagene), following the manufacturer's instructions. The primers M21 F (5′-CCA ACC CTG TCT TTT GCA AGC CCC GGA CTC CGG CC-3′) and M21 R (5′-GGC CGG AGT CCG GGG CTT GCA AAA GAC AGG GTT GG-3′) were used in the mutagenesis.

2.2. Transfections and HIV-1 latency reactivation

J-Lat cells clones 6.3 and 8.4 (cat. number 9846 and 9847, respectively) were obtained from the NIH AIDS Reagent Program and used as an HIV-1 latency model. These Jurkat derived T cells harbor a transcriptionally silent HIV-1-GFP proviral genome (Jordan et al., 2003). Transfections were performed using Neon Transfection System (Life Technologies) following the manufacturer's instructions. Flow cytometry analyses were performed using a FACScalibur (BD Biosciences) 48 h after all transfection events. As a positive control of HIV-1 latency reactivation, cells were treated with TNF-α (20 ng/ml) for 24 h. After this period, the cells were incubated for more 48 h in medium without TNF-α before flow cytometric analyses.

2.3. Immunoblotting assays

The cells were disrupted in lysis buffer NP-40 (NaCl 150 mM, NP-40 1%, Tris–HCl 50 mM pH 8.0, protease inhibitor 1%) on ice for 30 min. Cell lysates were centrifuged at 10,000×*g* for 10 min at 4 °C and supernatants were collected. The levels of protein were quantified and normalized by the Bradford method with BSA standard curve. Immunoblotting assays were performed using antibodies anti-CDK9 (1:1000), anti-Cyc T1 (1:1000), and anti HEXIM1 (Santa Cruz Biotechnologies, cat. number 484, 10750, and 81285 respectively), and anti-Tax of HTLV-1 (1:1000), anti RNA Pol II (1:1000) and anti RNA Pol II phospho S2 (1:1000) (Abcam, cat. number 26997,817 and 5095 respectively). Anti-tubulin (1:10000) (Abcam, cat. number 56676) was used to perform the loading controls. The secondary antibodies anti-mouse (1:2000) (GE Healthcare Life Sciences, cat number RPN4201) and anti-rabbit (1:2000) (GE Healthcare Life Sciences, cat number RPN4301) were used in immunoblotting assays to Tax/tubulin and CDK9/Cyc T1/HEXIM1, respectively.

2.4. RT-qPCR

Cells RNA was extracted using PureLink RNA Mini Kit (Thermo Fisher Scientific). RNA was quantified by a NanoVue spectrophotometer (GE Healthcare Lifesciences), treated with DNase (Thermo Fisher Sicentific) and reversed transcribed by High Capacity cDNA Reverse Transcription Ki (Thermo Fisher Scientific) according to the manufacturer's instruction. Quantitative PCR was performed as described elsewhere (Pasternak et al., 2008). Briefly, 200 ng of cDNA was quantified using Taqman Universal PCR Master Mix (Thermo Fisher Scientific) with 0.2 μM of each primer GAG1, GAG2 and GAG3 probe for unspliced RNA (Gag gene) and mf83, mf84 and ks2-tq probe for spliced RNA (Tat/Rev gene). GAPDH (Thermo Fisher Scientific, cat. number 4331182) was used as normalization gene. The PCR settings were as follows: 50 °C for 2 min, then 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.5. Coimmunoprecipitation assay

Cells (5×10^6) were disrupted as described for immunoblotting assays. 2 μg of anti Cyclin T1 antibody was added to 1 ml whole cell lysate and incubated overnight at 4 °C with rotation. Then, 50 μl of Protein A/G Plus-Agarose beads (Santa Cruz Biotechnologies, cat, number 2003) was added to lysate an incubated for 4 h at 4 °C with rotation. Bead-antibody conjugate was centrifuged at 1000×*g* for 30 s at 4 °C, and the pellet washed 5 times with PBS, centrifuging after each washing. Antibody-antigen complex was eluted in 3 subsequent elution using 50 μl 0.2 M glycine pH 2.6, neutralized with Tris pH 8.0 and washed 3 times with 150 μl lysis buffer. Then, the samples were resuspended in loading buffer and immunoblotted to investigate whether CDK9 and Hexim1 were co-immunoprecipitated with CycT1.

2.6. Chromatin immunoprecipitation (ChIP) assay

Cells (1×10^7) were pelleted by at 1200 RPM for 8 min and resuspended in PBS. Cross-linking was done using formaldehyde at 1% final concentration for 10 min at room temperature. The cross-linking was stopped by adding 125 mM glycine and incubating for 5 min at room temperature. Cells were washed 2 times with cold PBS and resuspended in ChIP lysis buffer (Santa Cruz Biotechnologies). Cells were then sonicated for 20 min on ice in Bioruptor Standard USD200 in high mode, 30 s on/off cycles. 25 ng of DNA was incubated with anti RNA Pol II phospho S 2 antibody and immunoprecipitated as described above. Then samples were washed once in ChIP Wash Buffer High Salt and once in ChIP Wash Buffer and then eluted in ChIP elution Buffer (Santa Cruz Biotechnologies), centrifuging for 2 min, 10.000×*g* at 4 °C after each step. Cross-linking was reversed by incubating the samples at 65 °C overnight. Samples were then treated with RNase A (10 mg/ml) and Proteinase K (20 mg/ml) at 45 °C for 2 h. DNA was then purified with PureLink PCR Micro Kit (Thermo Fisher Scientific) and submitted to quantitative PCR as described above.

2.7. Glycerol gradient

Cells were disrupted in buffer A (20 mM of HEPES [pH 7.9], 0.3 M of KCl, 0.2 mM of EDTA, 0.1% NP-40) containing 0.1% of protease inhibitor (GE Healthcare) and 0.5% of RNase inhibitor (Life Technologies). The lysates were centrifuged at 10,000*g* for 10 min

and the supernatants (30 μg of total protein) were loaded in the top of the tubes containing the preformed glycerol gradients (10, 12, 14, 16, 18, 20, 22, 24, 26, 28 e 30% v/v). Protein complexes were fractionated by centrifugation in a SW 41Ti rotor (Beckman Instruments) at 38,000 rpm for 21 h. Thirteen fractions (1 ml) were collected from the top of the gradient, precipitated with trichloroacetic acid, and analyzed by immunoblotting using specific antibodies anti-CDK9, anti-Cyc T1 and anti-HEXIM1 (Contreras et al., 2009).

2.8. Isolation of HIV-1-infected resting CD4+ T primary cells

Buffy coat from different HIV-1 negative blood donors were obtained from Hemorio blood bank, Rio de Janeiro, Brazil. PBMCs were isolated using Ficoll Paque Plus (GE Lifesciences). Naïve $CD4^+$ T cells were purified using the Dynabeads® Untouched™ Human $CD4^+$ T Cells kit (Invitrogen) according to the manufacturer's protocol. $CD4^+$ T cells were maintained in RPMI, 10% FBS, 30 U/ml IL-2 at 37 °C with 5% CO_2 for 24 h as described elsewhere (Bartholomeeusen et al., 2013b; José et al., 2014). After this period, cells were activated using anti-CD3/anti-CD28 Dynabeads (Invitrogen) and maintained for 5 days in RPMI at the same conditions. Purity and activation levels were determined by FACS analysis using specific antibodies against CD4, CD38, CD69 and HLA-DR (BD Biosciences). Activated $CD4^+$ T cells were transduced by spin inoculation (1,200 g/2 h) using 200 ng of p24 of a pseudotyped HIV virus. This virus was produced in HEK-293T cells by co-transfection of *pNL4.3ΔenvΔNef-Luc* and *pEnvHIV* (at 1:3 proportion), that codify a HIV proviral clone with a deleted *env* gene and a Luciferase reporter gene in the Nef ORF; and HIV envelope proteins, respectively. Cells were reverted to a resting state by gradually decreasing the amount of IL-2 in the medium to 2 U/ml over the period of 18 days. Luciferase activity and activation markers (CD38, CD69 and HLA-DR) were measured over this period until a background plateau was reached. Luciferase activity was evaluated in the cell lysates using Luciferase Assay System (Promega) and the levels of activation markers were monitored by flow cytometer using specific antibodies anti-CD38, CD69 and HLA-DR. HIV-1 infected resting $CD4^+$ T cells were transfected with Tax expressing plasmids (*pTax Wild Type)* using Neon Transfection System (Life Technologies), according to the manufacturer's instructions. As a positive control of reactivation, cells were treated with the HDAC inhibitor suberanilohydroxamic acid (SAHA) (1 μM) for 48 h. HIV-1 activation was evaluated by luciferase reporter gene activity measured 48 h after transfections.

3. Results

3.1. Tax reactivates HIV-1 transcription in latency cell line models

In order to evaluate whether HTLV-1 coinfection was able to reactivate HIV-1 from latency, $CD4^+$ T cells harboring HIV-1 latent proviral genome (J-Lat cells clones 6.3 and 8.4) were transfected with the pK30 HTLV-1 proviral clone. The expression of HTLV-1 proteins in J-Lat cells promoted HIV-1 transcription activation in 12.5% and 7.8% of J-Lat 6.3 and 8.4 cells, respectively (Fig. 1A). TNF-α was used as activation control reaching up to 25% of cells expressing HIV-1 transcripts in both clones of J-Lat cells (Fig. 1A), validating the model of reactivation of latent virus. Additionally, HTLV-1 Tax expression was detected in the cells transfected with pK30 (Fig. 1B). To further investigate Tax transactivator as a candidate gene to promote HIV reactivation, we also transfected J-Lat cell clone 6.3 and 8.4 with Tax expressing vectors. Our results demonstrate that Tax expression by itself is responsible for HIV-1 reactivation from latency in 25 to 30% of both, 6.3 and 8.4 clones of J-Lat cells (Fig. 1C). These values were similar to TNF-α inducible activation.

Different Tax mutants were used to confirm the capacity of Tax to reactivate HIV-1. These mutants have different affinity levels for HIV-1 LTR and HTLV-1 LTR. Tax M47 has a higher affinity for HIV-1 LTR than Tax WT while Tax M21 has no affinity for HIV-1 LTR (Smith and Greene, 1990; Béraud et al., 1994). Our results show that Tax M47 reversed HIV-1 latency at higher levels than Tax wild type (up to 45%), confirming previous reports that this modification renders Tax M47 more specific for HIV-1-LTR (Fig. 1C). However, Tax M21 has no significant effect on HIV-1 latency reactivation, suggesting that HIV-1 latency reversal by Tax is dependent on Tax affinity to HIV-1-LTR region (Fig. 1C). Expression of WT Tax and mutants M47 and M21 was confirmed in both J-Lat clones by immunoblotting (Fig. 1D). To further confirms that Tax could reactivate HIV-1 transcription, we measured relative RNA levels of Tax transfected or mock transfected cells by RT-qPCR (Fig. 3A). Expression of both unspliced (Gag region, black bars) and spliced (Tat/Rev region, grey bars) HIV-1 RNA were confirmed in J-Lat 8.4 cells expressing wild-type Tax and M47 mutant, with higher levels of expression in cells harboring the M47 mutant, as previously observed by GFP reporter gene (Fig. 3A). However, we could not detect expression of both unspliced and spliced HIV RNA transcripts in cells expressing Tax M21 confirming the inability of this mutant to revert HIV latency..

3.2. Tax translocates CDK9 and Cyc T1 from inactive to active P-TEFb complex in order to activate HIV-1 transcription

As previously described, Tax interacts with P-TEFb components and releases this factor from the large inactive complex, inducing an effective transcriptional elongation process (Cho et al., 2010). We investigated if the same occured during reactivation of HIV-1 latency promoted by Tax. P-TEFb activation status was evaluated in the lysates of J-Lat 6.3 cells expressing WT, M47 and M21 versions of HTLV-1 Tax submitted to glycerol gradients ranging from 10% to 30%. As expected, the levels of CDK9, Cyc T1 and HEXIM1 were equally distributed in the active (LMW, fractions 1–6) and inactive (HMW, fractions 8–13) forms of P-TEFb in mock cells with no Tax transfection (Fig. 2A, B and C). The expression of wild type and M47 versions of Tax clearly induced CDK9 displacement to active forms (LMW, fractions 1–6) comparing with inactive forms (HMW, fractions 8–13) (Fig. 2A). Similar results were observed with Cyc T1 (Fig. 2B). These results suggest that Tax is able to activate P-TEFb through releasing CDK9 and Cyc T1 from inactive hard molecular weight complex in HIV-1 latent cells, promoting HIV-1 transcription elongation. This was confirmed by the results in J-lat cells expressing Tax M21, which was not able to reverse HIV-1 latency. In this case, CDK9 and Cyc T1 are enriched in the inactive high molecular weight portion (HMW) of P-TEFb (Fig. 2A and B, fractions 9 and 10) suggesting that Tax M21 does not recruit the active form of P-TEFb due to lack of interaction with HIV1-LTR. Similar results could be observed with the repressor protein HEXIM1 (Fig. 2C). Comparing Tax WT and M47, cells transfected with Tax M21 had higher levels of the HMW portion (Fig. 2C, fractions 8 and 11). This suggests that in this case there was a higher quantity of inactive P-TEFb complex (CDK9 and Cyc T1 conjugated with HEXIM1/2, 7skRNP and other proteins) compared with Tax WT and M47. Tax expression levels were very similar for all versions of this protein, as confirmed by immunoblotting of the input in all conditions (Fig. 2D).

We confirmed that Tax promotes P-TEFb release from inactive complex through co-immunoprecipitation assay by pulling down CycT1. In this case, CDK9 was immunoprecipitated with CycT1 in J-Lat 8.4 cells expressing WT and M47 versions of Tax from HTLV-1 (Fig. 3B). However, the levels of the repressor HEXIM1 associated to CycT1/CDK9 complexes were lower in the same cells expressing Tax WT and M47 when compared with mock controls and cells expressing the inactive mutant M21 (Fig. 3B Co-IP panel). Active P-TEFb is responsible for phosphorylate CTD domain of RNA Pol II, leading to productive elongation of RNA transcripts. To further investigate if phosphorylation of CTD domain was affected by Tax overexpression, we evaluated phosphorylation of RNA pol II using specific antibodies against serine 2 from CTD domain in the same J-Lat 8.4 cells harboring

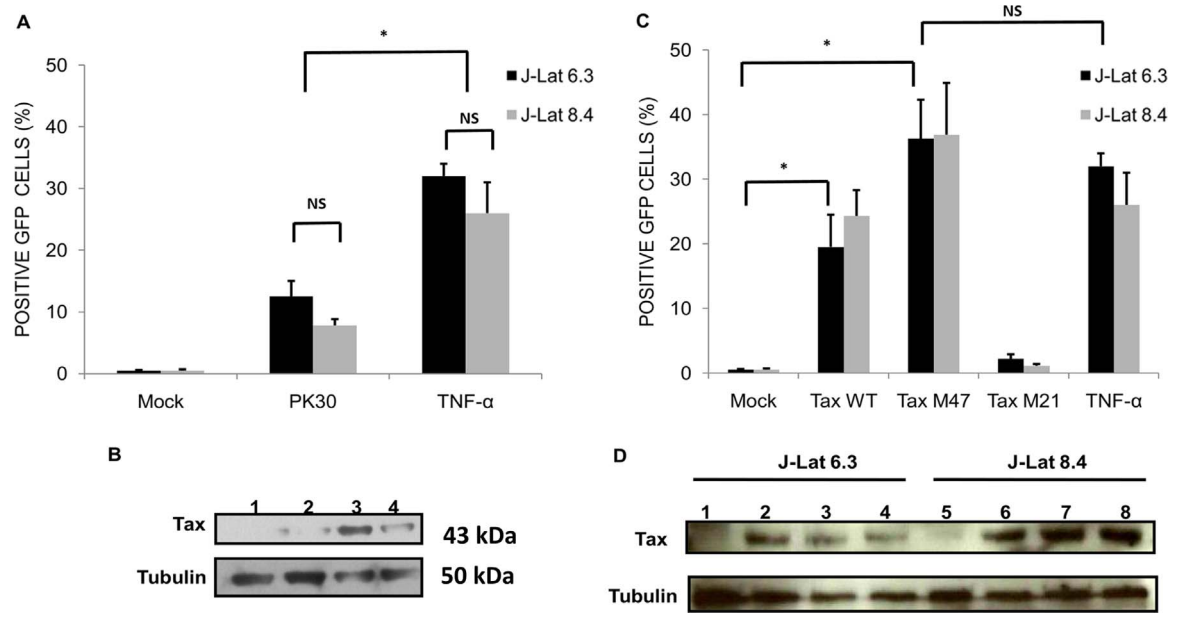

Fig. 1. HTLV-1 Tax protein promotes reactivation of latent HIV-1. J-Lat cells clones 6.3 (black bars) and 8.4 (grey bars) were used as a model of HIV-1 latency. TNF-α (20 ng/ml) was used as positive control of activation. **(A)** J-Lat cells were transfected with HTLV-1 pK30 proviral clone and HIV-1 latency reactivation was evaluated by GFP expression 48 h after transfections. Error bars represent the standard deviation of triplicate experiments. Statistical analyses were performed using Tukey test. NS – non-significant, *– $p < 0,05$. **(B)** Tax expression was identified by immunobloting using specific antibodies anti-Tax in cell lysates from J-Lat clones 6.3 and 8.4 transfected with pK30 plasmid (48 h post-transfection). Lines 1 and 2: mock not-transfected J-Lat clones 6.3 and 8.4, respectively; lines 3 and 4: pK30 transfected J-Lat 6.3 and 8.4, respectively. Tubulin was used as loading control (lower panel). **(C)** HTLV-1 Tax expression activates HIV-1 transcription in J-lat cells, clones 6.3 and 8.4. Cells were transfected with wide type Tax (Tax WT), M47 and M21 mutants that have different HIV-LTR affinities. HIV-1 latency reactivation was evaluated by GFP expression 48 h after transfections. Error bars represent the standard deviation of triplicate experiments. Statistical analyses were performed using Tukey test. NS – non-significant, *– $p < 0,05$. **(D)** Tax expression was identified by immunobloting using specific antibodies anti-Tax in cell lysates from J-Lat clones 6.3 (1–4) and J-Lat 8.4 (5–8). Lines 1 and 5: mock non-transfected cells; lines 2 and 6: Tax WT transfected cells; lines 3 and 7: Tax M47 transfected cells; lines 4 and 8: Tax M21 transfected cells. Tubulin was used as loading control (lower panel).

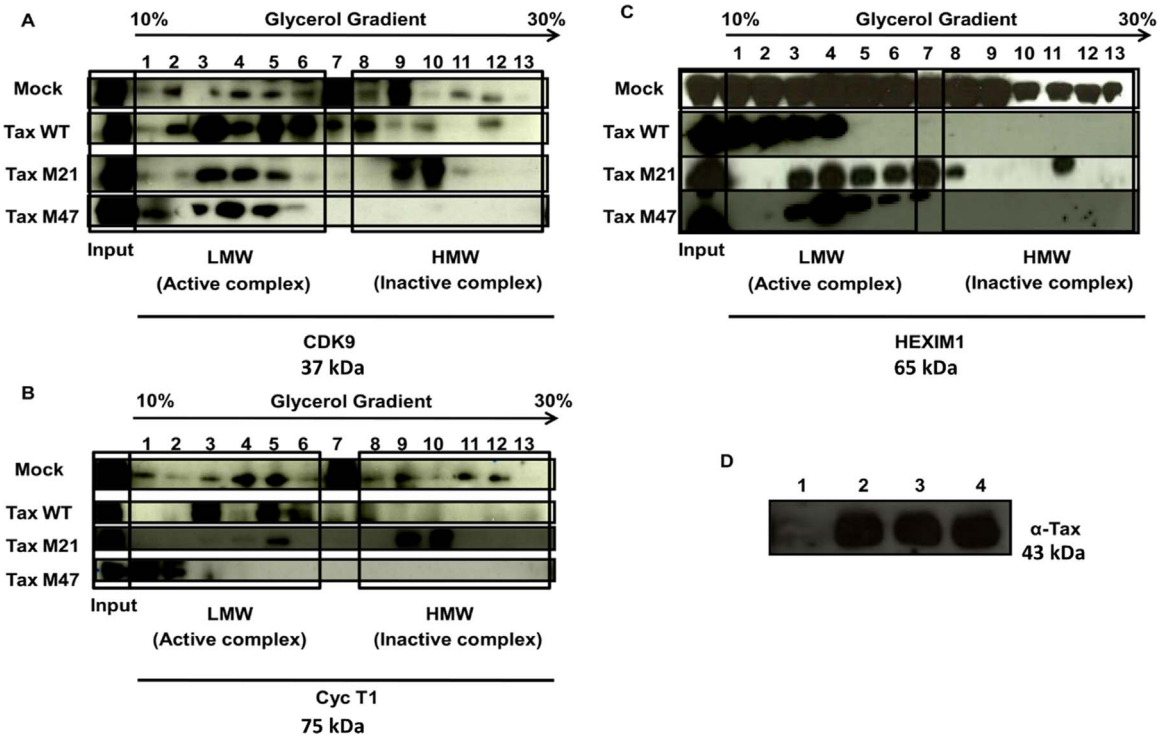

Fig. 2. Tax releases P-TEFb components from inactive forms in HIV-1 latent cells. J-Lat 6.3 cells were transfected with Tax WT, M47 and M21 separately. CDK9 and Cyc T1 components were identified in active (LMW) and inactive (HMW) forms of P-TEFb through ultracentrifugation in glycerol gradients (10–30%). The same glycerol gradient was performed to the repressor protein HEXIM1. Input panels stands for 10% of proteins volume before ultracentrifugation. **(A)** Immunobloting for CDK9 in the glycerol gradient fractions. **(B)** Glycerol gradient fractions corresponding to Cyc T1. **(C)** Glycerol gradient fractions corresponding to HEXIM1. **(D)** Immunobloting to identify Tax proteins in all transfections conditions: 1, mock non-transfected cells; 2: Tax WT transfected cells; 3: Tax M21 transfected cells and 4: Tax M47 transfected cells. Mock panels stand for non-transfected cells The data are representative of three independent experiments.

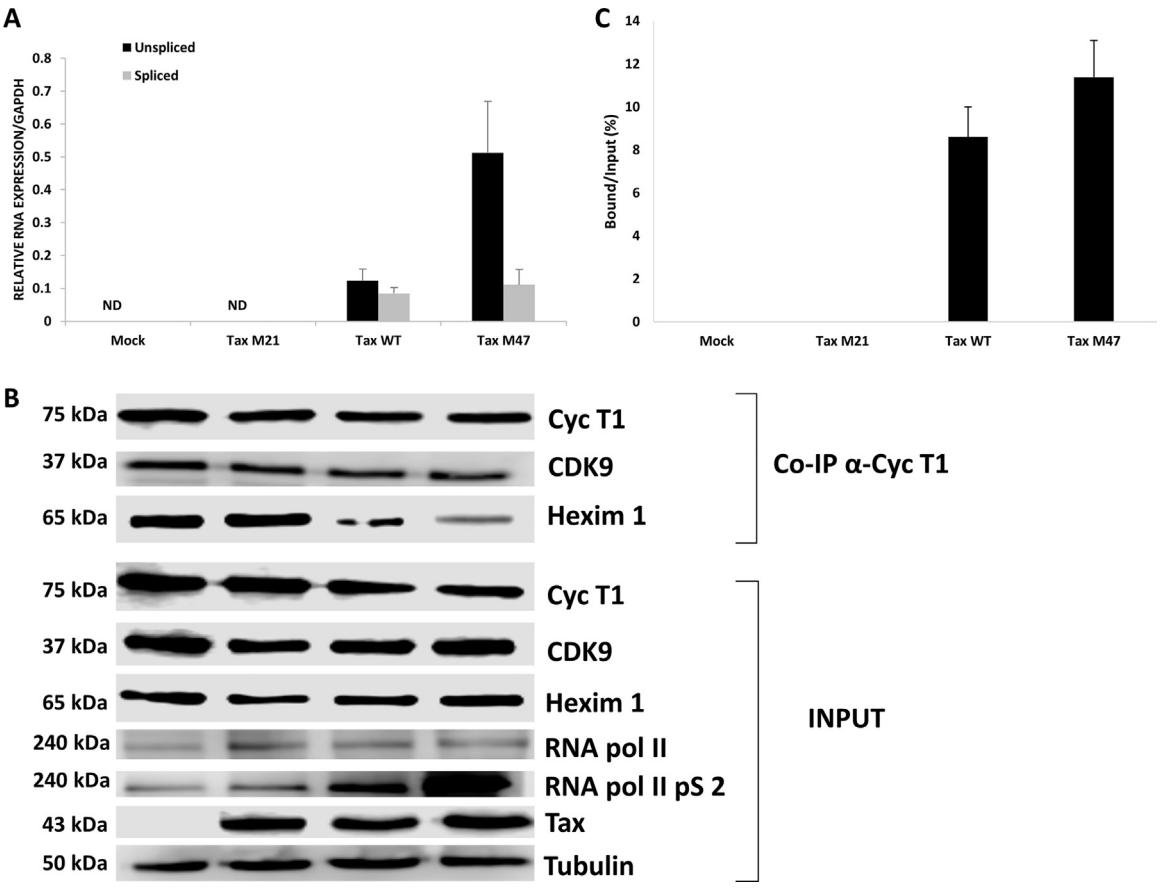

Fig. 3. Tax promotes P-TEFb activation and increasing in RNA Pol II phosphorylation. (A) RT-qPCR for unspliced GAG region (black bars) and spliced Tat/Rev region in J-Lat 8.4 cells expressing Tax. Data was normalized to GAPDH levels (set as 1). Error bars represents standard deviation for 2 replicates from 3 independent experiments. ND – not detected. **(B)** Co-immunoprecipitation using anti Cyclin T1 antibody in J-Lat 8.4 cells expressing Tax. Input represents cell lysates before immunoprecipitation. 40 μg was used for immunoblotting assay. The data are representative of three independent experiments. **(C)** Chromatin immunoprecipitation using anti RNA Pol II phospho S 2 antibody in J-Lat 8.4 cells 48 h after tax transfection. Black bars represents relative GAG DNA bound after precipitation compared to total GAG DNA (input). Error bars represents standard deviation of 3 replicates from 2 independent experiments.

HIV latent virus (Fig. 3B, input panel). Only wild-type Tax and positive mutant M47 induced RNA Pol II phosphorylation, while levels of non-phosphorylated RNA pol II did not change in any condition. Our results suggest that Tax is able to activate P-TEFb excluding HEXIM1 from the CDK9/CycT1 complexes that in turn phosphorylates the CTD domain from RNA pol II activating general HIV transcription. These results are corroborated by the higher levels of unspliced and spliced forms of HIV RNA in J-Lat 8.4 cells expressing WT and M47 versions of Tax from HTLV-1 (Fig. 3A).

To confirm that Tax induced transcription elongation, we immunoprecipitated phosphorylated forms of RNA pol II to investigate the association with HIV-1 DNA. Our Chip-qPCR assay for Gag region from J-Lat 8.4 cells expressing Tax showed the enrichment detection of HIV genomic DNA associated with phosphorylated RNA pol II only in HIV latent cells expressing the wild-type and M47 versions of Tax from HTLV-1 (8.2% and 11.11%, respectively) (Fig. 3C). Altogether, these results suggest that HIV-1 transcription activation observed in our latent model expressing Tax from HTLV-1 was a consequence of increased transcriptional elongation of HIV-1 proviral DNA integrated in J-Lat cells.

3.3. *Tax reactivates HIV-1 transcription in* ex-vivo *latent model*

To expand these results of HTLV-1 Tax reactivation into human primary models of HIV-1 latent infected cells, $CD4^+$ T cells from HIV negative blood donors were isolated, activated with anti-CD3/CD28 and transduced with a pseudotyped HIV-1 virus harboring a luciferase reporter gene. This virus is defective in the envelope gene and restricted to a single cycle of replication, allowing the virus to integrate in the cellular genome establishing the HIV-1 latency. After transduction, the cells were allowed to return to the resting state by gradually decreasing IL-2 concentration (30 U/ml to 2 U/ml) over the course of 18 days. The entire experiment is depicted in Fig. 4A. The resting state of the cells was verified by detection of activator markers (CD38, CD69 and HLA-DR) by FACS and virus latency evaluated by measuring the luciferase activity during all IL-2 reduction points (Fig. 4B). The resting status of purified $CD4^+$ T cells was confirmed by the progressive reduction of CD38, CD69 and HLA-DR levels in all replicates (Fig. 4B, lines). HIV-1 latency was established in all replicates as confirmed by the low levels of luciferase expression (Fig. 4B, white bars). At this point, cells were transfected with Tax WT or treated with 1 μM of SAHA (positive control of reactivation) and reactivation levels were measured 48 h post-transfection or post-treatment. HIV-1 latency reactivation occurred in all donors expressing Tax WT protein (up to 6.2 fold induction compared with not-transfected cells), confirming that Tax was able to reactivate HIV-1 latency in resting primary cells (Fig. 4C). The positive control with SAHA reactivated latent HIV about 2 fold compared with untreated cells from all three donors (Fig. 4C). Our results suggest that the co-infection with HTLV-1 is able to reactivate HIV-1 virus from latent reservoirs and this effect is mediated by Tax ability to reactivate P-TEFb in this context.

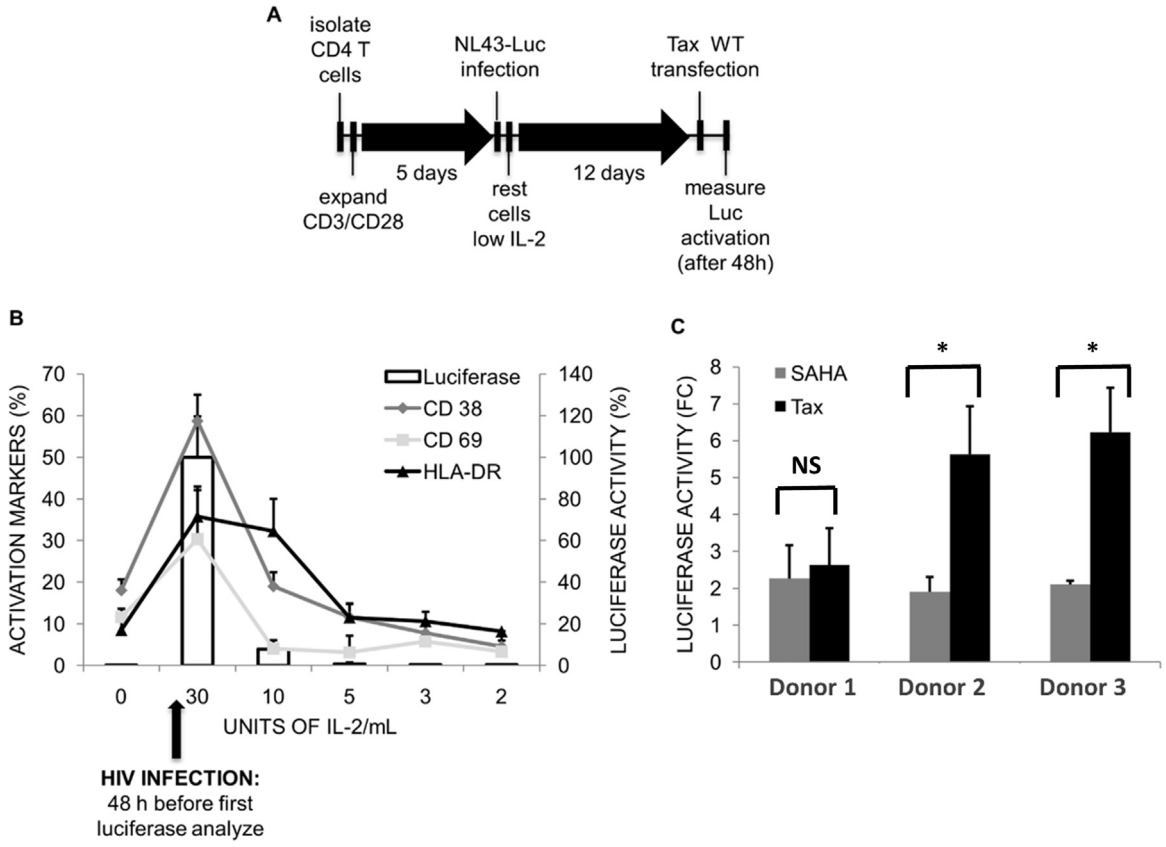

Fig. 4. Tax reactivates virus transcription in *ex vivo* HIV-1 latency model. Naïve CD4+ T cells were isolated from three different HIV-1 negative blood donors following by activation with anti-CD3/CD28, infection with a pseudotyped HIV-Luciferase and returned to resting state through IL-2 reduction in the medium. Level of CD38, CD69 and HLA-DR activation markers were evaluated during the whole experiment. The establishment of viral latency was evaluated through luciferase activity in all IL-2 reducing points. Induction of resting state was performed gradually decreasing IL-2 levels (30 U/ml to 2 U/ml). **(A)** Chronological representation of the entire experiment. **(B)** Average levels of CD38, CD69, HLA-DR and luciferase activity during the establishment of HIV-1 latency. In the graph, average of CD38 is represented by grey line, CD69 by light grey line, HLA-DR by black line and luciferase activity by white columns. Black bars represent standard deviations. Black arrow below the graph indicates the point that HIV-1 spinoculation was performed. **(C)** Levels of latent HIV-1 reactivation in the primary resting CD4+ T cells expressing HTLV-1 Tax protein (black columns). The treatment with SAHA was used as a positive control of reactivation (grey columns). All of three donors are presented in the graph (Donor 1, Donor 2 and Donor 3). Luciferase activity was analyzed 48 h after transfections with Tax WT or treatment with SAHA in triplicates and is presented in fold change (FC) relative to cells transfected with empty vector or DMSO treated, respectively. Error bars represent standard deviations; *– $p < 0,05$; NS – non-significant.

4. Discussion

Different studies of humans co-infected with HIV-1 and HTLV-1 have led to disparate conclusions. Some of these studies showed that co-infection is associated with higher HIV-1 viral load (Rockwood et al., 2015) and faster disease progression (Brites et al., 2009); however, other studies failed to find such association (Beilke, 2012; Oo et al., 2015). Several factors can influence this phenomenon: (I) if cells are co-infected; (II) whether the individual was first infected by HTLV-1 or HIV-1; (III) HTLV-1 immune activation might restrict HIV-1 replication; (IV) direct interaction of virus components between these two retroviruses in co-infected cells. Our results show that, in the context of co-infected cells, HTLV-1 Tax promotes transcription and activation of HIV-1 in primary resting $CD4^+$ T cells harboring latent virus.

Our results expand the findings showing that HTLV-1 supernatants and more specifically Tax protein or HTLV-I Env glycoprotein also induced expression of HIV-1 transcripts (Moriuchi and Moriuchi, 2000). Here, we show that Tax expression in HIV-1 latent cells promotes CDK9 and Cyc T1 release from inactive forms of P-TEFb, inducing transcriptional elongation and virus activation. Tax protein is able to complex with P-TEFb and competes for Brd4 and 7SK snRNP/HEXIM1 binding promoting transcription elongation by releasing CDK9 and Cyc T1 from inactive forms of P-TEFb (Cho et al., 2010). We also show that HTLV-1 Tax is able to activate P-TEFb and promotes virus activation even in HIV-1 latent cells, which have lower levels of P-TEFb components, such as Cyc T1. In primary resting cells, P-TEFb levels are lower due to actions of specific miRNAs and NF-90, which block the translation of Cyc T1 mRNA (Bartholomeeusen et al., 2013a; Chiang et al., 2012). Both CDK9 and Cyc T1 were still associated to inactive forms of P-TEFb (HMW) in HIV-1 latent cells expressing Tax M21 that lacks the ability to interact with HIV-1 LTR (Smith and Greene, 1990). It could explain the lower activation levels found with this protein. Nevertheless, it is important to emphasize that Tax interacts with NF-κB and other host transcriptional regulators such as chromatin remodeling complexes (SWI / SNF), HATs and HDACs (Boxus et al., 2008). Tax M21 is also ineffective to interact with NF-κB sub-units, which reinforces the idea of its incapacity to promote HIV transcription (Boxus et al., 2008). Tax M47 binds to HIV-1 LTR with higher affinity than it does to HTLV-1 LTR, possibly explaining higher levels of HIV-1 reactivation by Tax M47 when compared to Tax WT (Smith and Greene, 1990).

Considering that co-infection of the same cell with HIV-1 and other microbial pathogen is probably uncommon *in vivo* (Bertram et al., 1996), Tax still can stimulate HIV-1 reactivation from latent cells in a paracrine manner, where Tax can be secreted (Alefantis et al., 2005). Tax can act on HIV-1 expression directly at molecular levels, as shown here or indirectly by modulating the expression of immune host factors. Tax in extracellular compartments promotes overstimulation of the immune system that in turns could reverse HIV-1 latency.

Recently, it was demonstrated that Tax could interact with TRAF3 and IKK-related complexes, promoting activation of IFN-β promoter *in vitro (*Diani et al., 2015*)*. Interestingly, Tax retained the ability to activate NF-κB promoter in this model. Another group has shown that Tax interacts with RIP1 kinase, blocking the interaction with IRF-7, therefore hampering mainly the production of IFN-α by RIG-I/MDA5 signaling pathway (Hyun et al., 2015). Such results make it tempting to speculate that Tax stimulates reactivation of latent HIV-1 provirus through NF-κB-dependent and P-TEFb-dependent pathways whilst partially downmodulating type-I IFN innate immune responses, increasing HIV-1 viral load and ultimately contributing to disease progression.

Tax also induces the expression of different cytokines, such as interleukin-2 (IL-2) and tumor necrosis factor (TNF), as observed in primary human glial cells (Banerjee et al., 2007), and it might contribute to reactivation of HIV-1 in co-infected individuals. Additionally, the expression of TNF-α and IL-1β induced by Tax efficiently activate HIV-1 transcription through a NF-κB-dependent pathway (Siebenlist et al., 1994). This state of cell activation was observed among HTLV-1/HIV-1 co-infected patients and could influence the faster HIV-1 disease progression (Brites et al., 2009; Beilke, 2012). However, the impact of HIV-1/HTLV-1 co-infection on HIV-1 disease progression is still a matter of debate (Beilke, 2012).

Finally, we speculate that the synergistic effects of both Tat and Tax to stimulate HIV-1 transcription in HIV-1/HTLV-1 co-infected cells could promote virus transcription and higher HIV-1 viral load, contributing to faster progression to AIDS. Some reports showed a higher mortality and shortened survival rates in HTLV-1/HIV-1-coinfected patients compared to HIV-1-monoinfected patients (Brites et al., 2009; Pedroso et al., 2011). We suggest that new strategies to inhibit Tax/Tat transactivation should be addressed in these patients, which combined with current HAART could minimize the pathogenesis of these two relevant immunodeficiency viruses. Tat inhibitors, such as Cortistatin A (CA) selectively inhibits Tat-mediated transactivation of the integrated HIV-1 provirus. CA binds specifically to the TAR-binding domain of Tat and as a consequence reduces cell-associated HIV-1 viral RNA and capsid p24 antigen production in acutely and chronically infected cultured and primary cells (Mousseau et al., 2012). Furthermore, our findings demonstrate that HTLV-1 co-infection also should be addressed in HIV-1 latency reversal strategies that aim to eradicate virus from patients by combining HIV-1 transcription activating drugs with HAART.

Author disclosure statement

The authors declare that they have no competing interests.

Acknowledgements

We thank Rodrigo Delvecchio da Cunha and Dr. Koen Bartholomeeusen for the support in the experiments. We also thank Dr. Kotaro Shirakawa to gently provide us the plasmid pEnvHIV and Dr. José Marcello Salabert de Campos for the statistical analyses. This work was supported by research grants from Brazilian agencies CNPq (Grant no. 405283/2013-0) and FAPERJ (Grant no. E-26/111.267/2013) and Victor Emmanuel Viana Geddes was supported by CAPES (Grant no. 88881.130757/2016-01) fellowship.

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