The Presence of HIV-1 Tat Second Exon Delays Fas-Mediated Apoptosis in CD4+ T lymphocytes: a Potential Mechanism for Persistent Viral Production*

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*Running title: HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

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Key words: HIV-1, Tat, FasL pathway, NF-κB, mitochondrial apoptotic pathway.

Background: HIV-infected T cells are quite resistant to apoptosis.
Results: Intracellular expression of HIV-1 Tat in T cells stabilized the mitochondrial membrane and reduced caspase activation mainly through NF-κB activation.
Conclusion: Intracellular Tat induced resistance to FasL-mediated apoptosis in T cells mainly through the second exon.
Significance: Tat-mediated protection against apoptosis may be a mechanism for HIV-1 persistence.

SUMMARY
HIV-1 replication is efficiently controlled by the regulator protein Tat (101aa), codified by two exons, although the first exon (1-72aa) is sufficient for this process. Tat can be released to the extracellular medium, acting as a soluble pro-apoptotic factor in neighboring cells. However, HIV-1 infected CD4+ T lymphocytes show a higher resistance to apoptosis. We observed that the intracellular expression of Tat delayed FasL-mediated apoptosis in both PBLs and Jurkat cells, being that an essential pathway to control T-cell homeostasis during immune activation. Jurkat-Tat cells showed impairment in the activation of caspase-8, deficient release of mitochondrial cytochrome c, and delayed activation of both caspase-9 and -3. This protection was due to a profound deregulation of proteins that stabilized the mitochondrial membrane integrity, such as heat shock proteins, prohibitin or nucleophosmin, as well as to the up-regulation of NF-κB-dependent anti-apoptotic proteins, such as BCL2, c-FLIPS, XIAP and C-IAP2. These effects were observed in Jurkat expressing full-length Tat (Jurkat-Tat101) but not in Jurkat expressing the first exon of Tat (Jurkat-Tat72), proving that the second exon – and particularly the NF-κB-related motif ESKKKVE - was necessary for Tat-mediated protection against FasL apoptosis. Accordingly, the protection exerted by Tat was independent of its function as a regulator of both viral transcription and elongation. Moreover, these data proved that HIV-1 could have developed strategies to delay FasL-mediated apoptosis in infected CD4+ T lymphocytes through the expression of Tat, thus favoring the persistent replication of HIV-1 in infected T cells.

The human immunodeficiency virus type 1 (HIV-1) infection is characterized by a continuous viral replication in CD4+ T lymphocytes and macrophages (1), leading
ultimately to the development of the acquired immunodeficiency syndrome (AIDS). This is caused by a progressive depletion of CD4+ T lymphocytes through different mechanisms such as apoptosis, cellular syncytia, plasma membrane disruption by viral budding, or cytolysis by soluble viral proteins as Tat, Nef and gp120 (2-4). Vpr may also have a cytotoxic effect on bystander or infected CD4+ T cells by increasing the mitochondrial membrane permeabilization (5-8), although it has also been described as an anti-apoptotic factor (9, 10). The programmed cell death or apoptosis of CD4+ T cells during HIV-1 infection is a complex process that affects both infected and uninfected cells. Apoptosis occurs mainly in bystander non-infected cells whereas productively HIV-1 infected cells have evolved strategies to prevent or delay apoptosis in the context of immune activation (11-14).

HIV-1 regulator Tat induces apoptosis of bystander cells when it is released to the extracellular medium as a soluble form (15). However, when Tat is expressed intracellularly, it produces the efficient elongation of the viral transcripts through the recruitment of the RNA polymerase II (RNAPII) complex. Tat binds to a stem-loop RNA termed Tat response element (TAR), located at the 5' end of the nascent viral transcripts (16), and activates the recruitment of cellular elongation factors such as P-TEFb, increasing the processivity of RNAPII (17). Tat is a 101-residue long protein codified by two exons: first exon codifies the amino acids 1-72, forming a transcriptionally active protein - Tat72 -; and second exon codifies the amino acids 73-101 and overlaps with the env gene (18, 19). Tat first exon (1-72aa) contains the minimal functional domain to generate a protein competent in HIV-1 replication through a TAR-dependent activation of the transcription whereas the second exon (73-101aa) has been described as dispensable for Tat activity (20). However, the expression of Tat second exon is conserved in all lentivirus, suggesting a biological importance. In fact, the second exon is essential for Tat-mediated cell genome deregulation, thereby indicating that it may control the transcription of non-viral genes (TAR-independent activation) (21-23), probably through binding to canonical enhancer sequences of cellular transcription factors such as NF-κB or Sp1 (24-26). This would indirectly affect the expression of several genes related to cellular functions such as T-cell activation or apoptosis (15, 23, 27, 28). Moreover, the important contribution of Tat second exon to HIV-1 in vivo replication was demonstrated by the accidental infection of three laboratory workers with the HIV-1 HXB2 isolate, which shows a premature stop codon at the residue 89 (29, 30). In one of the infected patients, the HXB2 virus with first-exon Tat reverted to two-exon Tat (30), changing the until then mild course of the infection to a steep decline in CD4+ T cell count and a rapid progression to AIDS within 1 year (29). This unfortunate situation provided a conclusive evidence for the biological requirement of Tat second exon for HIV-1 replication and pathogenesis in vivo.

The role of Tat in apoptosis of bystander cells is controversial because, although soluble Tat has been described as an inductor of apoptosis (15, 31, 32), it has also been proved to be a protector against apoptosis when is expressed inside the host cell (28, 33). Our group demonstrated previously that intracellular Tat profoundly deregulates cellular gene expression, modifying the expression of genes involved in apoptosis (23). This deregulation was mainly due to the presence of the second exon, proving that although the first exon is sufficient for activating viral replication, full-length Tat should exert further control on HIV-1 pathogenesis by protecting the host cells against apoptosis. Apoptosis is essential to control T-cell homeostasis, especially during the contraction of the immune response (34). As the antigen wanes, the number of T cells is appropriately reduced through the induction of apoptosis by Fas (CD95 or Apo-1), a member of the tumor necrosis factor receptor (TNFR) superfamily (35). Engagement of Fas receptor/CD95 with Fas ligand (FcαL)/CD178 or Fas-activating antibodies (anti-CD95) recruits procaspase-8 to death-inducing signaling complex (DISC) through FADD adapter protein. Within DISC, procaspase-8 is activated by dimerization and auto-cleavage (36-38). After that, Fas may activate apoptosis through two different pathways that distinguish type I and type II cells. Most cell types are classified as type I, where caspase-8 directly activates caspase-3, the main effector of the morphological and biochemical changes characteristic of
apoptosis (36, 37). In type II cells, Fas receptors are excluded from lipid rafts and assemble DISC inefficiently upon activation of death receptors (39). Consequently, only a small fraction of procaspase-8 is activated and therefore, they require a subsequent amplification step through the mitochondrial cell death pathway. The mitochondria are highly involved in the induction of apoptosis in mammalian cells as they release to the cytosol many important factors that induce caspase activation and chromosome fragmentation after the permeabilization of the mitochondrial outer membrane (40, 41). This mechanism is controlled by pro- and anti-apoptotic members of BCL2 family such as the “BH3-domain only” protein Bid (42). Caspase-8-mediated cleavage of Bid initiates the mitochondrial cell death pathway (43-45) as truncated Bid (tBid) translocates to the mitochondrial membrane and triggers the release of cytochrome c to the cytosol (46). Cytochrome c participates then in the assembly of the apoptosome, a multiprotein complex required for caspase-9 activation that subsequently activates caspase-3 (47, 48).

Apoptosis is tightly controlled by many cellular proteins at different levels and the final cell death occurs by imbalance between pro- and anti-apoptotic factors. One central regulator of cell survival and apoptosis is the transcription factor NF-κB that regulates the expression of anti-apoptotic genes as BCL2, c-IAP, XIAP, as well as the cellular FLICE-inhibitory protein (c-FLIP) (49). Upstream in the apoptotic pathway, c-FLIP interferes with FasL-mediated activation through the binding to FADD and/or caspase-8/10 in a ligand-dependent manner, preventing DISC formation (50). There are three functional c-FLIP splice variants: short form (c-FLIPs, 26kDa) (51); intermediated or Raji form (c-FLIPs, 43kDa) (52); and long form (c-FLIPL, 55kDa) (53). c-FLIPs is exclusively a caspase-8 inhibitor, whereas c-FLIPL has dual function as caspase-8 inhibitor or activator, depending on the different ratio of c-FLIPL:caspase-8 (54). The mechanisms by which intracellular Tat interferes with apoptosis are not well-known, although it has been described that Tat may enhance the expression of anti-apoptotic factors such as c-FLIP (33) or BCL2 (55, 56). The role of second exon in the ability of Tat to protect against apoptosis is completely unknown.

It was determined that the intracellular expression of full-length Tat was able to delay Fas-mediated apoptosis in both PBLs and Jurkat and that this effect was due to the presence of the second exon. The mechanism of protection was based, first, on the deregulation of several NF-κB-dependent proteins, including the overexpression of BCL2 and c-FLIPs, and second, on the preservation of the mitochondrial outer membrane integrity by several anti-apoptotic factors, delaying the release of cytochrome c and subsequent activation of caspase-9 and caspase-3. Getting further insight on this mechanism of protection against apoptosis in CD4+ T cells mediated by intracellular full-length Tat would provide a better understanding of the role of Tat in the ability of HIV-1 to create a persistent infection in the host.

**EXPERIMENTAL PROCEDURES**

**Cells** – Peripheral blood lymphocytes (PBLs) were isolated from blood of healthy donors by centrifugation through a Ficoll-Hypaque gradient (Pharmacia Corporation, North Peapack, NJ). Jurkat E6-1 cells were obtained from the NIH AIDS Reagent Program (57). Jurkat-Tat72 and Jurkat-Tat101 stably express, respectively, HIV-1 Tat first exon (1-72aa) or full-length Tat (1-101aa) by using a TetOff system BD Biosciences Clontech (Mountain View, CA). Jurkat TetOff cells transfected with empty vector pTRE2hyg were used as negative control. Jurkat-Tat72 and Jurkat-Tat101 are not clones but mixed populations in which more than 50% of the cells express high amounts of intracellular Tat101 or Tat72 protein. It was determined that the expression of Tat in Jurkat-Tat101 and Jurkat-Tat72 was very similar to a real infection performed in MT-2 cells infected with the NL4.3wt strain (23). Both PBLs and Jurkat were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2mM L-glutamine, 100µg/ml streptomycin, 100U/ml penicillin (Biowhittaker, Walkersville, MD). In Jurkat-Tat cells, the culture medium was supplemented with 300µg/ml of geneticin (Sigma-Aldrich, St. Louis, MO) and 300 mg/ml hygromycin B (BD Biosciences Clontech).

**Reagents and antibodies** – A monoclonal antibody against HIV-1 Tat (aa 2–9) was
obtained from Advanced Biotechnologies Inc. (Columbia, MD). A monoclonal antibody against human Fas receptor (MBL International, Woburn, MA; clone SY-001) was used at 50 or 500ng/ml during 4 or 18h at 37°C for inducing cell death in Jurkat or PBLs, respectively, and for quantifying the amount of Fas receptor on the cell surface by flow cytometry. A polyclonal antibody against procaspase 3 (p32) and active subunits p17 and p20 (clone H-277) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against procaspase 8 (p55) (clone 90A992) was also obtained from Santa Cruz Biotechnology. A monoclonal antibody against procaspase 9 (p46) and active p35/p37 (clone 5B4) was obtained from Abcam (Cambridge, UK). Antibodies against BCL2 (clone C-2), Bid (clone SC9), poly (ADP-ribose) polymerase (PARP-1) (clone 194C1439), c-Flip (clone G-11), and p65/RelA (clone C-20) were obtained from Calbiochem (Merck Chemicals Ltd., Nottingham, UK). A monoclonal antibody against cytochrome c was obtained from Santa Cruz Biotechnology. The monoclonal antibody against β-actin (clone AC-15) was obtained from Sigma-Aldrich. Propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (Dapi) were also obtained from Sigma-Aldrich. MitoTracker Red CMxRos Mitochondrial Probe was obtained from Lonza. Secondary antibodies conjugated to Alexa 488 and Alexa 546 were purchased from Molecular Probes (Eugene, OR). Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from GE Healthcare (Milwaukee, WI). Secondary IgG/IgM antibody fluorescein-conjugated (FITC) used in flow cytometry assays was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Transient transfection and vectors**

Resting PBLs and Jurkat cells were transiently transfected with an Easyjet Plus Electroporator (Equibio, Middlesex, UK). In brief, 20x10⁶ PBLs or 10⁷ Jurkat were collected in 350µl of RPMI without supplement and mixed with 1µg/10⁶ cells of plasmid DNA. Cells were transfected in a cuvette with 4mm electrode gap (EquiBio), at 340V for PBLs and 280V for Jurkat, 1500µF and maximum resistance.

pCMV-Tat72-tag or pCMV-Tat101-tag expression vectors were obtained by cloning Tat72 and Tat101, obtained from pCMV-Tat101 vector (58), in pCMV-tag (Stratagene, Agilent Technologies, Madrid, Spain), in NotI/BamHI. pNL4.3-TatM1I, which contains a point mutation in the start codon of the tat gene, was obtained from pNL4.3 wild-type (wt) vector (kindly provided by Dr M.A. Martin (59) by site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), using the following oligonucleotide:

5’-TCGACAGAGGAGAGCAAGAATTGAGCCAGTAGAT-3’, which introduced a point mutation (underlined) in the start codon, changing a methionine by an isoleucine (Supplemental data, Figure 1). The presence of the selected mutation was confirmed by automatic sequencing. pNL4.3-TatM1I vector was co-transfected along with pCMV-Tat72 or pCMV-Tat101 in a proportion 2:1. pEYFP-C1 vector (BD Biosciences Clontech) was co-transfected as control of transfection efficiency and measured by flow cytometry.

**Quantitative PCR**

Total RNA was isolated with RNeasy Mini kit (Qiagen Iberia, Madrid, Spain) and cDNA was synthesized by using the GoScript Reverse Transcription System (Promega), according to manufacturers’ instructions. HIV-1 transcription was determined by qPCR analysis of all viral mRNAs using the following primers directed against env/nef genes:

- P3 (5’-TTGCTCAATGCCACAGCCAT-3’) and P4 (5’-TTTGACCACTTGCCACCCCAT-3’) (60). The expression of β-actin was used as housekeeping gene to calculate the relative expression of env/nef genes. Primers for amplifying the human β-actin gene were β-actin-s (5’-AGGCCCAAGCAAGAGGGAAGCA-3’) and β-actin-as (5’-CGCAGCTCATTGAGAGGATGTGTTGTT-3’). SYBR Green PCR Master Mix (Applied Biosystems) was used according to manufacturer’s instructions.

**Analysis of apoptosis**

Cells were stained with 1.5µM PI or Annexin-V conjugated with fluorescein isothiocyanate (FITC) (Immunostep, Salamanca, Spain) and fluorescence was measured by FACSealibur Flow Cytometer (BD Biosciences Clontech).
Data were analyzed by CellQuest software. Cell viability was also determined by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega), following the manufacturer’s instructions. Briefly, 1x10⁵ cells were harvested by centrifugation, washed twice with PBS1X and resuspended in lysis buffer. After incubation for 10min at room temperature to stabilize the luminescent signal, cell lysates were deposited in an opaque-walled multiwell plate and analyzed in an Orion Microplate Luminometer with Simplicity software (Berthold Detection Systems, Oak Ridge, TN).

**Immunoblotting assays** – Nuclear and cytosolic protein fractions were obtained as described previously (61). Protein extracts were fractionated by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and transferred onto a Hybond-ECL nitrocellulose paper (GE Healthcare). After blocking and incubation with primary and secondary antibodies, proteins were detected by chemiluminescence with SuperSignal West Pico/Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

Densitometry was performed in a Gel Doc 2000 System (Bio-Rad, Madrid, Spain) by using Quantity One software. Gel bands were quantified and background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated regarding the internal control (β-actin) per each lane.

**Immunofluorescence assays** – Living cells treated or not with anti-CD95 for 4h were stained with MitoTracker and then adhered on PolyPrep slides (Sigma-Aldrich) and fixed with 2% paraformaldehyde (PFA) in PBS1x. Immunofluorescence assays were then performed as previously described (23). Images were obtained with a Leica DMI 4000B Inverted Microscope (Leica Microsistemas, Barcelona, Spain).

**Measurement of caspase activity** – Activity of caspase-3/-7, -8 and -9 was measured with CaspaseGlo®3/7, CaspaseGlo®8 and CaspaseGlo®9 systems (Promega Biotech Iberica, Madrid, Spain), respectively. The luminescent signal (relative light units, RLUs), which was directly proportional to caspase activation, was measured in an Orion Microplate Luminometer with Simplicity software (Berthold Detection Systems, Oak Ridge, TN) and data were normalized according to protein concentration in each sample.

**Mitochondrial cytochrome c depletion** – The release of cytochrome c from the mitochondria intermembrane space to the cytosol was measured by using InnoCyte Flow Cytometric Cytochrome c Release Kit (Calbiochem). Briefly, 5x10⁶ cells treated or not with FasL were permeabilized and then fixed with PFA. After staining with a primary monoclonal antibody against cytochrome c and a secondary antibody conjugated with FITC, the measurement of cytochrome c release was performed by flow cytometry.

**Mitochondria membrane potential gradient (ΔΨm)** – ΔΨm was measured by flow cytometry using MitoProbe JC-1 Assay Kit (Molecular Probes), accordingly to manufacturer’s instructions. Briefly, 2x10⁶ cells/ml were incubated in JC-1 staining solution and both green and red fluorescence were measured in FL1 and FL2 channels, respectively, by flow cytometry. The mitochondrial depolarization was calculated by measuring the decrease in the red/green fluorescence intensity ratio.

**NF-κB activation assays** – The ability of NF-κB to bind DNA was measured in nuclear proteins extracts by DNA affinity immunoblotting (DAI) assay, as described previously (23, 62). The quantity of NF-κB bound to DNA was detected by immunoblotting with a polyclonal antibody against p65/RelA (Santa Cruz Biotechnology). As internal control, an input of nuclear protein for each sample was analyzed by using an antibody against β-actin (Sigma-Aldrich).

NF-κB transactivation activity was measured by transient tranfection with p3κB-LUC vector, which contains a luciferase gene under the control of three -κB consensus sites from the immunoglobulin κ-chain promoter, as described before (23, 58). After incubation for 18h, cell were treated or not with 50ng/ml anti-CD95 for 4h and then the luciferase activity was measured by using Luciferase Assay System (Promega) in a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN), according to manufacturer’s instructions. The RLUs were normalized with protein concentration in each sample and with the percentage of efficiently transfected cells
by using pEYFP-C1 vector as transfection internal control.

*Tat101 site-directed mutagenesis* – Site-specific mutagenesis of the 85ESKKKVE91 sequence necessary for Tat-mediated NF-κB activity, located in Tat second exon, was performed in the pTRE2hyg-Tat101 vector (28) by using QuikChange Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Santa Clara, CA), according to manufacturer’s instructions. The following oligonucleotide was used to introduce selected mutations (underlined) in the 85ESKKKVE91 motif to generate the motif 85ESRVNVE91: 5'- AGACGGAATCGAGGGAACGTGGTGAGAGACAGAGACAGAT-3'. The presence of the selected mutations was confirmed by automatic sequencing.

*Proteome profiling* – Two hundred micrograms of protein extracts were subjected to reduction and alkylation, and then digested with trypsin (Promega) in 50mM ammonium bicarbonate, pH 8.8. The resulting tryptic peptides were loaded into the liquid chromatography/tandem mass spectrometry (LC-MS/MS) system and analyzed by using a C-18 reversed phase nano-column (75mm I.D. x 25cm, 3mm particle size, Acclaim PepMap 100 C18, Thermo-Fisher) in a continuous acetonitrile gradient consisting of 0-30% B in 145 min, 30-43% A in 5 min and 43-90% B in 1 min (A= 0.5% formic acid; B=95% acetonitrile, 0.5% formic acid). A flow rate of ca. 300 nL/min was used to elute peptides from the reverse phase nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on an orbital ion trap mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific, San Jose, CA) (63). An enhanced resolution spectrum (resolution=60,000) followed by the MS/MS spectra from the five most intense parent ions were analyzed during the chromatographic run (180min). Dynamic exclusion was set at 0.5min. For peptide identification, all spectra were analyzed with Proteome Discoverer (version 1.2, Thermo Fisher Scientific), using both Sequest (Thermo Fisher Scientific; version 1.0.43.2) (64) and Mascot search engines (www.matrixscience.com). For database searching, a Uniprot database was interrogated selecting the following parameters: trypsin digestion with 2 maximum missed cleavage sites, precursor mass tolerance of 20ppm, fragment mass tolerance of 1200mmu, carbamidomethyl cysteine as fixed modification, and methionine oxidation, asparagine deamidation and serine and threonine phosphorylation as dynamic modifications. For peptide and protein identification validation, results were loaded into Scaffold 3.0 software (http://www.proteomsoftware.com/) (Proteome Software Inc., Portland, OR), adding X!Tandem (http://www.thegpm.org/tandem/) as additional search engine. Sequest search engine facilitates the “XCorr score” -values above 2.0-, which measures how close the spectrum detected in the sample fits to the ideal spectrum. Differential expression in samples was determined using the quantity values of normalized spectra for each peptide, as determined by Scaffold software in a triplicate experiment. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (65). Only those proteins involved in apoptotic pathways, cell survival and mitochondrial function were selected for further analysis.

*Antibody-based apoptosis microarray* – RayBio Human Apoptosis Antibody Array Kit (Raybiotech Inc., Norcross, GA) detects the relative level of 43 apoptosis related proteins in cell lysates by using an array of antibodies spotted on a glass chip. Procedure was performed according to manufacturer’s instructions. All microarrays were scanned under the same conditions in a ScanArray Express HT microarray scanner (PerkinElmer, Waltham, MA) using Alexa 555 specific filters. Images were quantified by Quanarray analysis software and a fixed-circle segmentation algorithm. Data were filtered to discard those data points that were not considered positive respect to the local background and the local negative controls. A second filtering round was done removing from further analyses those proteins with less than three passing data points. Data were normalized against the positive controls of each array and then were mean aggregated to result in a single value for each analyzed protein. Normalized data points where then logarithmically transformed for data compression and a better visualization of the results. Finally, logarithmic ratios (Log2
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ratios) against the reference data obtained from control cells were generated. Only proteins that were deregulated in Jurkat-Tat101 and Jurkat-Tat72 vs. Jurkat TetOff cells were represented.

Apoptosis-related proteins networks – All proteins related to apoptosis that were deregulated were subjected to analysis with STRING 9.0 (http://string-db.org/; (66)). Deregulated proteins were integrated in interconnected networks built by inputting the list of protein symbols, selecting Homo sapiens as organism and medium confidence, which does not consider interactions with combined scored under 0.4.

Statistical analysis – Statistical analysis was performed using Graph Pad Prism 5.0 (Graph Pad Software Inc., San Diego, CA). Corporations between control and FasL-treatment groups were made using two-way analysis of variance (ANOVA) with Bonferroni post-test analysis to describe the statistical differences among groups. The p-values (p) <0.05 were considered statistically significant in all comparisons and were represented as *, ** or *** for p<0.05, p<0.01 or p<0.001, respectively.

RESULTS

Apoptosis induced by FasL was delayed in PBLs transiently transfected with Tat101 alone or in the context of HIV-1 genome – PBLs from healthy donors were transfected with pCMV-Tat101 or pCMV-Tat72 expression vectors. Transfection with pcDNA3 was used as negative control and pEYFP-C1 was co-transfected in all cases as a control of transfection efficiency. Viability after transfection was 40-45% (data not shown) and transfection efficiency was 20-25%, depending on the batch of PBLs. Transfected PBLs were cultured for three days and then treated with FasL for 18 hours. Viability was measured by chemiluminescence. A media of three different, independent experiments was represented in Figure 1a. It was observed that the intracellular expression of Tat101 in PBLs was able to exert 2.0-fold more protection against FasL-mediated apoptosis than control cells (p<0.01), whereas Tat72 exerted lower protection. The efficient expression of Tat72 and Tat101 was assessed in EYFP+ cells by immunofluorescence (Fig. 1b), using Dapi to stain the nucleus, where most Tat was localized. In order to evaluate whether the resistance to apoptosis occurred mainly on CD4+ T cells, the transfected PBLs challenged with FasL for 18 hours were stained with an antibody against CD4 conjugated with phycoerythrin (PE) and then analyzed by flow cytometry. PI- cells were gated and EYFP+/CD4+ cells were quantified within this group. It was determined that Tat101 alone was able to induce 2-fold more protection against FasL-mediated apoptosis than Tat72 or empty vector pcDNA3 in CD4+ T cells (Fig. 1c).

The role of Tat in the protection against FasL apoptosis was also evaluated in the context of viral replication. PBLs were transfected with the Tat-defective viral genome pNL4.3-TatM1I (described in Supplemental data, Fig. 1) along with pCMV-Tat101 or pCMV-Tat72 expression vectors. Viability was measured by chemiluminescence and it was determined that Tat101 produced 1.7-fold more protection against apoptosis induced by FasL than control cells (p<0.01), whereas Tat72 exerted lower protection (Fig. 2a). HIV-1 replication was assessed by quantitative RT-PCR (Fig. 2b). It was also evaluated whether the cells showing higher protection against apoptosis were CD4+ T cells. PBLs were then transfected with pCMV-Tat101 or pCMV-Tat72 expression vectors alone or along with pNL4.3-TatM1I, using pEYFP-C1 as control of transfection efficiency. As Tat is not known to be packaged inside the virions (67) and the viral genome NL4.3-TatM1I of the new generated virions did not produce Tat, only transfected cells would express the viral proteins encoded in the defective viral genome. After 3 days in culture, cells were challenged with FasL for 18 hours and then stained with anti-CD4. As described above, the quantification of apoptosis was performed in EYFP+/CD4+ cells after selecting living PI- cells (Fig. 2c). The results obtained were quite similar to those when only Tat101 or Tat72 were expressed, proving that Tat was a significant viral protein in the induction of resistance to FasL-mediated apoptosis in infected CD4+ T cells, despite the presence of other viral proteins as Nef, Vpr or gp120.

FasL-mediated apoptosis was delayed in Jurkat with transient or stable expression of Tat101 or Tat72 – As a model of CD4+ T cells was necessary to dissect the mechanism of action of Tat to exert protection against FasL-
mediated apoptosis, the potential use of Jurkat cells for this purpose was evaluated. Jurkat E6-1 cells were transiently transfected with the Tat-defective viral genome pNL4.3-TatM1I along with pCMV-Tat101 or pCMV-Tat72 expression vectors. Transfection with pcDNA3 was used as negative control and pEYFP-C1 was co-transfected as control of transfection efficiency, which was 20-25% in all cases. HIV-1 replication was assessed by quantitative RT-PCR. Efficient expression of Tat72 and Tat101 was assessed by immunofluorescence (data not shown). Transfected Jurkat were cultured for three days and then treated with FasL for 4 hours. Cell viability was measured by luminometric assay and a media of three different, independent experiments was represented as a ratio of the apoptosis induced in FasL-treated PBLs regarding untreated cells (Fig. 3a, first bar diagram). It was observed that the intracellular expression of Tat101 in Jurkat produced 1.8-fold more protection against FasL-mediated apoptosis than control cells, whereas Tat72 exerted lower protection ($p<0.05$).

In order to dissect the mechanism underlying intracellular Tat-mediated protection against apoptosis by FasL in T cells, the use of Jurkat-Tat101 and Jurkat-Tat72 stable cell lines was analyzed. Jurkat-Tat101 and Jurkat-Tat72 were treated with FasL at different times and early apoptosis was measured by Annexin-V-FITC staining (Fig. 3b). Flow cytometry analysis showed that the percentage of pro-apoptotic cells was 1.8-fold lower in average in Jurkat Tat101 than in control cells, whereas this percentage in Jurkat Tat72 was 1.2-fold lower in average than control. Measurement of apoptosis by PI staining in Jurkat-Tat72 and Jurkat-Tat101 treated with FasL for 4h showed that Tat101 delayed 4.6-fold the induction apoptosis, whereas Tat72 delayed apoptosis 1.7-fold (Fig. 3c).

The expression of Fas receptors/CD95 on the cell surface was increased in 20% by Tat101 but Tat 72 did not produced significant changes (Fig. 3d).


- **FasL-mediated activation of caspase-8 and -3 was decreased in Jurkat-Tat101** – Cleavage of procaspase-3 was analyzed by immunoblotting in protein extracts obtained from Jurkat-Tat101, Jurkat-Tat72 and control cells treated with FasL for 4 or 18h. As shown Fig. 4a, the active subunits p17 and p11 were rapidly generated in Jurkat-Tat72 and control cells after treatment with FasL for 4h. After 18h of treatment, the cleavage of caspase-3 was also initiated in Jurkat-Tat101, although 2-fold more high quantity of unprocessed precursor p32 could also be observed in these cells.

To further evaluate the activation of caspase-3 in Jurkat-Tat cells treated with FasL, the cleavage of the caspase-3 substrate PARP-1 was analyzed by immunoblotting. PARP-1 cleaved form p89 was abundantly detected in the cytoplasm of control cells treated with FasL for 4h, but it was only weakly produced in Jurkat-Tat101 even after treatment for 18h (Fig. 4b). In Jurkat-Tat72, the levels of cytosolic p89 were halfway between those detected in Jurkat-Tat101 and control cells.

Measurement of caspase-3/-7 activity showed that the activation of these caspases in response to FasL was delayed in Jurkat-Tat101 regarding Jurkat-Tat72 and control cells ($p<0.05$) (Fig. 4c). Measurement of the activation of caspase-8 showed 1.6-fold reduction in Jurkat-Tat101 ($p<0.001$), whereas the difference between Jurkat-Tat72 and control cells was lower (Fig. 4d). The cleavage of caspase-8 by immunoblotting showed in Jurkat-Tat101 a great delay in the cleavage of procaspase-8 (p55) after treatment with FasL for 18 hours. This was not observed in Jurkat-Tat72.

Release of cytochrome c and activation of caspase-9 was impaired in Jurkat-Tat101 cells due to high stability of the mitochondrial inner-membrane electrochemical potential – Analysis by flow cytometry showed that the release of cytochrome c to the cytoplasm from the mitochondrial intermembrane space in response to treatment with FasL for 4 or 18h was reduced nearly 2-fold in Jurkat-Tat101 cells, whereas no significant difference with control cells was observed in Jurkat-Tat72 cells (Fig. 5a). These results were confirmed by confocal microscopy through the analysis of the subcellular localization of cytochrome c (Fig. 5b). Consistent with data obtained by flow cytometry, the mitochondria of Jurkat cells with stable expression of Tat101 were more resilient to release cytochrome c in response to FasL. Besides, the mitochondria of Jurkat-Tat101 cells showed a more diffuse and less compact distribution than those in Jurkat-Tat72 and control cells.
Cytosolic protein extracts for Jurkat-Tat101, Jurkat-Tat72 and control cells treated with FasL for 4 or 18h were analyzed by immunoblotting using an antibody against precursor p46 and active forms p37/p35 of caspase-9. As shown Fig. 6a, p37/p35 were only detected in control cells and weakly in Jurkat-Tat72 cells after treatment with FasL for 4h, but no band corresponding to the activation of caspase-9 was observed in Jurkat-Tat101. After 18h, p37/p35 were detected in the cytosol of Jurkat-Tat101 but there was 3.5-fold more unprocessed precaspase-9 than in control cells, proving that the impaired release of cytochrome c in these cells resulted in a delayed activation of caspase-9. This deficient caspase-9 activation was confirmed by measuring the activity of caspase-9 by chemiluminescence, which was 1.7-fold lower in Jurkat-Tat101 cells (p<0.001) (Fig. 6b).

The mitochondrial membrane potential gradient ($\Delta \Psi_m$) was measured by using the cationic, lipophilic dye JC-1 (68). The dispersion of green JC-1 monomers from the mitochondria throughout the entire cell, which correlates with the induction of apoptosis, was measured by flow cytometry after treatment with FasL for 4 or 18h (Fig. 6c). The population of Jurkat-Tat101 cells committed to apoptosis – low $\Delta \Psi_m$ higher monomeric JC-1 with green fluorescence – was smaller than in Jurkat-Tat72 and control cells after treatment with FasL. This difference was mainly observed after 18h of treatment, where nearly 30% of Jurkat-Tat101 cell population showed high resistance to lose the mitochondrial membrane potential, regarding control cells. In accordance, the population of living cells with higher $\Delta \Psi_m$ - where JC-1 formed aggregates with red fluorescence - was also 2.8-fold greater in Jurkat-Tat101. Jurkat-Tat72 cells showed an intermediate profile of mitochondrial membrane integrity loss with 1.8-fold higher $\Delta \Psi_m$ than control cells. As a result, the mitochondrial depolarization, measured by the decrease in the red/green fluorescence intensity ratio, was delayed by the intracellular expression of Tat101 and, with less intensity, by Tat72.

Analysis by immunoblotting of BCL2 showed that the expression was enhanced in Jurkat-Tat101 in basal conditions and its quantity was not significantly reduced even after treatment with FasL during 18h (Fig. 6d). The expression of Bid (p22) diminishes as caspase-8 cleaves it to generate the truncated form tBid/p15 that tranlocates to the mitochondria and directly induces the outer-membrane permeabilization (43). The expression of Bid/p22 was significantly reduced in Jurkat-Tat72 and control cells after treatment with FasL, suggesting that tBid was being synthesized and translocated to the mitochondrial membrane. However, Bid/p22 remained uncleaved in Jurkat-Tat101, proving that the activation of caspase-8 was deficient in these cells.

Tat deregulated the expression of cellular proteins related to apoptosis and to the maintenance of mitochondrial outer-membrane integrity – The whole proteome of Jurkat-Tat101, Jurkat-Tat72 and control cells was analyzed by LC-MS/MS in basal conditions. The analysis showed that Tat mostly induced the up-regulation of proteins related to cell cycle and proliferation such as PHB, HEBP2/SOUL, NUDC, MCM2/3, PCNA and NASP (Table 1). The expression of most of these proteins was more enhanced by the expression of Tat101 than Tat72. Besides, proteins related to apoptosis and stress response were also up-regulated by Tat, such as 14-3-3 $\zeta/\delta$, Bid, DJ-1/PARK7 and several heat shock proteins. More than 50% of apoptotic proteins deregulated were related to the mitochondrial function, including ATP synthase $\alpha$ and $\beta$ subunits, which were mainly enhanced by the expression of Tat101. Most of these proteins belong to interconnected cellular pathways and in some cases their activation was related to caspase-3 activity. This was observed after analyzing the predicted protein interactions by STRING 9.0 database. As shown Fig. 7, caspase-3 was directly connected to Bid, HSPE1, HSPD1, YWHAZ and PCNA. In turn, these proteins were connected with the others which expression was modulated by Tat, forming an intertwined network that included mostly all deregulated proteins.

Intracellular Tat101 deregulated several NF-$\kappa$B-dependent proteins involved in the control of apoptosis – The expression of c-FLIP$_{L/S}$ was analyzed by immunoblotting (Fig. 8a). c-FLIP$_{S}$ - rather than c-FLIP$_{L}$ -, which is the one that confers resistance to human T-cells against Fas-mediated apoptosis (69), was 2.1- and 2.8-fold enhanced in
Jurkat-Tat72 and Jurkat-Tat101 in basal conditions. Treatment with FasL for 18h reduced the expression of c-FLIP\text{L} similarly in all cell types but the expression of c-FLIP\text{S} was very different depending on the expression of Tat or the treatment with FasL. Jurkat-Tat101 and Jurkat-Tat72 showed 10.5 and 3.8-fold more c-FLIP\text{S} expression than control cells, respectively, after treatment with FasL for 18h. On the other hand, the expression of c-FLIP\text{R}/p43 was enhanced 22.5- and 12.5-fold more in Jurkat-Tat101 and Jurkat-Tat72, respectively, than in control cells, in basal conditions. After treatment with FasL, the cleavage of c-FLIP\text{L} to c-FLIP\text{R}/p43 was increased 30-fold more in both Jurkat-Tat101 and Jurkat-Tat72 (see Supplemental data, Figure 2).

As the expression of BCL2 and c-FLIP depends on NF-\kappa B activity, the ability of the main NF-\kappa B subunit p65/RelA to bind DNA was analyzed by DAI assay in Jurkat-Tat101 and Jurkat-Tat72 cells in basal conditions, regarding control cells. As described previously, NF-\kappa B binding capacity was higher in Jurkat-Tat101 than in Jurkat-Tat72 and in turn, higher in Jurkat-Tat72 than in control cells (Fig. 8b) (23, 70). This was reflected in the enhancement of the expression of T-cell activation markers as CD69, which was 3.3-fold increased in Jurkat-Tat101 (p<0.01) and 1.5-fold in Jurkat-Tat72 cells, regarding control cells (Fig. 8c). Treatment with FasL did not reduce significantly the expression of CD69 on the cell surface (p<0.05). NF-\kappa B transcriptional activity was also measured by transfecting p3\kappa B-LUC vector (Fig. 8d). Jurkat-Tat101 cells showed 20-fold more NF-\kappa B activity than control cells (p<0.01) and 4-fold more than Jurkat-Tat72 (p<0.01), in basal conditions. The treatment with FasL for 4h reduced the NF-\kappa B activity in all cell types but the differences with control cells were maintained (p<0.01).

In order to identify other regulators of apoptosis related to NF-\kappa B which expression could be modified by Tat, whole protein extracts from non-stimulated cells were hybridized in an antibody-based array which simultaneously detected 35 human apoptosis-related proteins, most of them related directly or indirectly to NF-\kappa B activity. The expression of 11 proteins was deregulated in Jurkat-Tat101 and Jurkat-Tat72, regarding the expression levels detected in control cells (Table 2). Both C-IAP2 and XIAP were enhanced in Jurkat-Tat101 whereas only XIAP was enhanced in Jurkat-Tat72 cells. Heat shock protein 70kDa (HSPA1A/1B) and 60kDa (HSPD1) were also upregulated in both Jurkat-Tat101 and Jurkat-Tat72 cells. Surprisingly, Jurkat-Tat101 also showed great quantities of pro-apoptotic factors such as lymphotoxin \alpha (LTA) or Bax, which were not detected in Jurkat-Tat72. On the contrary, the expression of BCL2 interacting mediator of cell death (BIM/BCL2L11) was diminished 2-fold in Jurkat-Tat101. The analysis of the predicted interactions between these proteins by STRING 9.0 database showed a connection between all of them and that p65/RelA had a central position in the center of this complex network (Fig. 8e).

**Tat-mediated activation of NF-\kappa B through the ESKKKVE motif was greatly involved in the induction of resistance to Fas-mediated apoptosis** – The motif \text{ESKKKVE}\text{\textsuperscript{52}} located in Tat second exon has been described as critical for Tat-mediated NF-\kappa B transactivation (70). A mutagenesis was performed in this motif in order to render a protein that should not be able to induce NF-\kappa B activity. Both pTRE2hyg-Tat101 ESKKKVE (wild-type) and pTRE2hyg-Tat101 ESRVVV (mutated) were transiently co-transfected in Jurkat-TetOff cells along with p3\kappa B-LUC vector. As shown Fig. 8f, Tat ESRVVV induced a 1.9-fold lower activation of NF-\kappa B activity than Tat ESKKKVE (p<0.001). The treatment of these transfected cells with FasL for 4h showed that Tat ESKKKVE was more efficient than Tat ESRVVV to protect them against FasL-induced apoptosis (p<0.01 and p<0.05, respectively).

**DISCUSSION**

Activation of death signals is part of the antiviral immune response in the infected cell and represents a major threat to virus (71, 72). Many viruses have evolved mechanisms to inhibit apoptosis in order to extent the survival of infected cells, enhance the production of viral progeny, and permit the establishment of viral persistence. DNA viruses, such as adenovirus, Epstein-Barr or African swine fever virus, encode viral anti-
apoptotic proteins similar to cellular proteins, such as D-type cyclin, BCL2 and c-FLIP (73), whereas the herpes simplex virus type 1 (HSV-1) maintains the cellular BCL2 levels through different mechanisms, including the activation of p38 MAPK (74). It is not well-known whether the RNA viruses have similar mechanisms, but HIV-1 infected cells appear to have developed certain resistance to apoptosis in order to prolong the viral production (13, 14). The molecular mechanisms underlying this protection are not fully comprehended, but the intracellular expression of viral regulatory proteins such as Vpr or Tat during the first step of the viral replication may be involved. Interestingly, both proteins seem to overlap their function, suggesting that preventing apoptosis during the first steps of the HIV-1 replication is a crucial step for the viral cycle and its pathogenesis. Vpr exerts a dual role on regulating apoptosis during HIV-1 infection because whereas extracellular or high intracellular levels of Vpr induces cellular death through the alteration of mitochondrial membrane permeabilization (75, 76), an intracellular low concentration of Vpr seems to protect against FasL-mediated apoptosis in Jurkat cells (9). Similarly, we and others have previously described that the intracellular expression of HIV-1 Tat regulator protein appears to have an anti-apoptotic effect (23, 28, 77), but the mechanisms underlying this function of Tat has not been described yet. In this work, we show that the expression of Tat second exon within the full-length Tat protein (Tat101) during HIV-1 replication enhances cellular survival in T lymphocytes during the first 72 hours post-infection and describe the mechanisms involved. Furthermore, we demonstrated that PBLs -specifically, CD4+ T cells- and Jurkat cells expressing intracellular full-length Tat (Tat101) were more resistant to Fas-induced apoptosis than those expressing only the first exon (Tat72) or Tat-free control cells, and the molecular mechanisms involved in this process are described. Our data suggest that the protection against apoptosis mediated by full-length Tat occurred through two major mechanisms: first, a higher maintenance of the mitochondrial membrane potential and subsequent impairment in caspase-3 activation; and second, activation of NF-kB, which in turn induces the expression of survival proteins such as c-FLIP and BCL2. In these mechanisms, the presence of the second exon within full-length Tat appeared to be of paramount importance.

Resistance to apoptosis in HIV-1 persistently-infected T cells is related to the mitochondrial apoptotic pathway (78, 79). In Jurkat cells, the induction of Fas-mediated apoptosis requires an amplification step through the mitochondrial cell death pathway in order to activate caspase-3 (39, 45). This is achieved through the translocation of tBid to the mitochondrial membrane and subsequent release of cytochrome c (45, 47). The intracellular expression of Tat101 was able to maintain the mitochondrial outer-membrane integrity by different mechanisms, delaying the release of cytochrome c and causing an inefficient activation of caspase-9 and -3. Tat72 was less competent than Tat101 to exert this protection. Despite the quantification of caspase-3 activity by chemiluminescence did not correlate well with the cleavage of procaspase-3 to p17/p11 active forms in Jurkat-Tat72 cells, the lesser inhibitory effect of Tat72 on the activation of caspase-3 was confirmed through the analysis of PARP-1 cleavage, which was undoubtedly less effective in Jurkat-Tat101 than in Jurkat-Tat72.

The measure of ΔΨm showed that the mitochondrial depolarization was lower in Jurkat-Tat101 in response to FasL, likely due to the control exerted by c-FLIPs over the activity of caspase-8 and a lower cleavage of Bid, but also due to the overexpression of other proteins such as BCL2 that may counteract this mechanism and stabilize the mitochondrial membrane (80, 81). BCL2 was described to be over-expressed by full-length Tat (55, 56), inducing protection against mitochondrial-mediated apoptosis in Jurkat (82). BCL2 expression did not decrease in Jurkat-Tat101 even after long treatment with FasL. As the cleavage of Bid and BCL2 levels were quite similar in Jurkat-Tat72 than in control cells, this would explain the higher mitochondrial depolarization observed in these cells, reinforcing the assumption that the ability of Tat to protect against apoptosis mostly resided in the second exon.

However, BCL2 was not the only protein responsible for delaying apoptosis as other proteins overexpressed by Tat101 and Tat72 were also able to stabilize the mitochondrial membrane potential such as several....

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mitochondrial heat shock proteins (HSPD1, HSPE1, HSPA1), nucleophosmin (NPM1) and BCL2-associated transcription factor 1 (BCLAF1). Overexpression of HSPs has been related to an increase in BCL2, mitochondrial membrane potential stabilization, impairment of cytochrome c release, caspase-3 inhibition, and suppression of PARP cleavage (83-85). HSPE1 was acetylated at K99 in Jurkat-Tat101, which has been related to a higher gene expression regulation (86). BCLAF1 was also overexpressed by Tat101 and Tat72 and was heavily phosphorylated at S177 and S512 mainly in Jurkat-Tat101. Recent studies proved that BCLAF1 has a major role in T-cell activation (87, 88). On the other hand, nucleolar NPM1 suppresses apoptosis by inhibiting caspase-mediated activation of DNase, favoring genomic stability and DNA repair (89), as well as by blocking p53 mitochondrial localization (90). NPM1 is known to interact with HIV-1 Rev (91) and Tat, being critical for Tat nuclear localization and Tat-mediated transcription (92). Although NPM1 was overexpressed in both Jurkat-Tat101 and Jurkat-Tat72, it was deamidated at N210 in Jurkat-Tat72 and therefore, it was not fully functional in these cells. NPM1 also enhances the expression of pro-apoptotic protein Bax and acts as a chaperone, binding only to activated conformationally altered Bax (93). Bax was actually over-expressed in Jurkat-Tat101, as well as the “BH3-domain only” SOUL. SOUL has a similar function to Bax, inducing the mitochondrial membrane permeabilization and subsequent release of cytochrome c. The high expression of Bax in Jurkat-Tat101 could indicate a higher sensitivity to apoptosis. However, the pro-apoptotic function of Bax should be activated through the interaction with other factors as Bid or Bim, promoting a critical conformational change in Bax necessary for its death capacity (94, 95). In Jurkat-Tat101 cells, Bid was not efficiently cleaved to tBid and the expression of Bim was reduced nearly 3-fold, indicating that the pro-apoptotic function of Bax was likely impaired by deficient activation. Moreover, Bim can be inhibited through its interaction with YWHAZ, a member of the 14.3.3 protein family that antagonizes the activity of pro-apoptotic proteins such as Bad, Bim and Bax (96, 97). YWHAZ was enhanced in both Jurkat-Tat101 and Jurkat-Tat72, although it was heavily deamidated in Jurkat-Tat72, likely rendering a not fully functional protein, unable to control the pro-apoptotic proteins. Other anti-apoptotic factors such as prohibitin (PHB) contributed to the higher protection against apoptosis provided by Tat101 but not Tat72. PHB is a potent survival factor that inhibits the release of cytochrome c and caspase-3 activation (98, 99) and it was increased 70-fold in Jurkat-Tat101, which was 2-fold higher than in Jurkat-Tat72.

Overall, these results indicated that the higher stability of the mitochondrial membrane integrity in Jurkat-Tat101 was due to a group of stabilizing proteins acting together to prevent the release of cytochrome c. A direct consequence of this inhibition was the inefficient activation of caspase-3, which could hinder the activation of other cellular pathways as the cell cycle control. In fact, DNA replication factors as MCM2 and MCM3 were overexpressed in Jurkat-Tat101. MCM3 is a substrate for caspase-3 cleavage and the truncated forms contribute to initiate apoptosis (100), inactivating the MCM complex and preventing DNA replication (101). The low caspase-3 activity detected in Jurkat-Tat101 would contribute to MCM3 stability and therefore, to the maintenance of MCM complex, avoiding apoptosis. Other factors related to cell cycle progression, cytokinesis and cell proliferation were also overexpressed in Jurkat-Tat101, such as NUDC, NASP and PCNA (102-105). The overexpression of somatic NASP (sNASP) has been related to changes in NF-κB activity (106), which is known to be enhanced by Tat (23, 70). NF-κB may in turn activate the expression of several potent anti-apoptotic factors as those belonging to IAP family. The expression of XIAP was enhanced in both Jurkat-Tat101 and Jurkat-Tat72 but C-IAP2 was only overexpressed in Jurkat-Tat101, nearly 40-times. XIAP is a potent inhibitor of initiator caspases - caspase-9 - (107), as well as execution caspases - caspases-3 and -7 - (108), preventing both CD95- and Bax-induced apoptosis (109, 110). This would explain the high quantities of procaspase-3, -8 and -9 that were detected in Jurkat-Tat101 even after treatment with FasL. C-IAP2 is a component of the tumor necrosis factor receptor 2 (TNFR2) complex that inhibit cell death by direct repression of caspase activity (111) and also targets for ubiquitin degradation several times.
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pro-apoptotic components of TNF-α signaling pathway (112). Moreover, there should be a
deregulation in TNF signaling pathway because the expression of several components
such as TNFRSF1A, Fas receptor and TNF-
β/LTA showed an anomalous expression.
Likely, the higher release of TNF cytokines by Jurkat-Tat cells was able to activate NF-κB via
autocrine feedback, keeping a sustained
activation of this transcription factor (113). As Tat72 lacks of the motif
ESKKKVE92 - located in the second exon -, which has been
described as critical for NF-κB transactivation
(70), the lower NF-κB basal activity detected
in Jurkat-Tat72 regarding Jurkat-Tat101
should be related to this motif. Accordingly,
Tat101 with mutated ESRVNVV92 motif was
less efficient than wild-type Tat101 to activate
NF-κB and protect Jurkat against FasL-
mediated apoptosis.

In summary, intracellular HIV-1 Tat
deregulated the expression of several proteins
related to apoptosis, rendering an anti-
apoptotic general effect able to protect PBLs
and Jurkat from FasL-induced apoptosis. This
was achieved through a high stability of the
mitochondrial membrane integrity because
Tat101 increased the expression of several
proteins - HSPs, NPM1, PHB - committed to
avoid mitochondrial depolarization, stabilize
the cell cycle, and counteract pro-apoptotic
factors such as Bid, Bim or Bax. This effect
was also achieved through the ability of
Tat101 to activate NF-κB, an essential
transactivating factor that promotes the cell
survival through different mechanisms, such
as the up-regulation of C-IAP2, XIAP, BCL2
and c-FLIPs. As Tat72 lacks of the essential
motif ESKKKVE for activating NF-κB, this
could be the most important mechanism
through which Tat101 exerted its protective
function. A scheme of the most important
factors interfering with the apoptotic pathway
in T lymphocytes which expression was
modified by Tat is depicted in Fig. 9.

In conclusion, Tat101-mediated
protection against apoptosis would allow a
prolonged HIV-1 production and spread and
might explain why apoptosis mostly occurs in
bystander uninfected cells. This viral strategy
would be mainly achieved through the second
exon. A better understanding of the molecular
mechanisms responsible for the resistance to
apoptosis in HIV-infected T cells is essential
to fully characterize the ability of HIV-1 to
establish a long-term infection.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. FasL-mediated apoptosis was reduced in PBLs expressing intracellular Tat101. A. Resting PBLs were transiently transfected with pCMV-Tat72 or pCMV-Tat101 expression vectors or with pcDNA3 as negative control. pEYFP-C1 was used as control of transfection efficiency. PBLs were maintained for 3 days without stimulus and then were treated with FasL for 18h. Viability was measured by chemiluminiscence. The bar diagram represents the media of three independent experiments and lines on top of the bars correspond to the standard error of the mean (SEM). Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis and ** indicates \( p<0.01 \). B. Tat expression and nuclear localization were confirmed by immunofluorescence. Dapi was used for nuclear staining. C. Resting PBLs were transiently transfected with pCMV-Tat72 or pCMV-Tat101 expression vectors or with pcDNA3 as negative control. pEYFP-C1 was used as control of transfection efficiency. PBLs were maintained for 3 days without stimulus and then were treated with FasL for 18h. Cells were stained with a monoclonal antibody against CD4 conjugated with PE and then with IP. Signals corresponding to EYFP, PE and PI were analyzed by flow cytometry in FL1, FL2 and FL3 channels, respectively. SSC/FSC dot plot was used to select living (PI-) cells (region R1): red events correspond to PI-, living cells; magenta events correspond to PI+, dead cells; black events correspond to cellular debris. The numbers in the PI-/EYFP+/CD4+ dot plots show the percentage of CD4+/EYFP+ cells within the PI- region (region R3). The bar diagram represents the media of three independent experiments and the lines on top of the bars correspond to the standard deviation (SD).

FIGURE 2. Tat101 reduced FasL-mediated apoptosis in HIV-1 transfected PBLs. A. PBLs were transfected with pNL4.3-TatM1I and pCMV-pTat72 or pCMV-Tat101 expression vectors. pcDNA3 was used as negative control. pEYFP-C1 was used as control of transfection efficiency. HIV-1 replication was allowed for 3 days before treatment with FasL for 18h. Viability was measured by chemiluminiscence. The bar diagram represents the media of three independent experiments and lines on top of the bars correspond to SEM. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis (* and ** indicates \( p<0.05 \) and \( p<0.01 \), respectively). B. The expression of HIV-1 genes was monitored by quantifying the expression of the env/nef genes by qRT-PCR using \( \beta\)-actin as internal control. C. Resting PBLs were transiently transfected with pNL4.3-TatM1I and pCMV-pTat72 or pCMV-Tat101 expression vectors. pcDNA3 was used as negative control. pEYFP-C1 was used as control of transfection efficiency. PBLs were maintained for 3 days without stimulus and then were treated with FasL for 18h. Cells were stained with a monoclonal antibody against CD4 conjugated with PE and then with IP. Signals corresponding to EYFP, PE and PI were analyzed by flow cytometry in FL1, FL2 and FL3 channels, respectively. SSC/FSC dot plot was used to select living (PI-) cells (region R1): red events correspond to PI-, living cells; magenta events correspond to PI+, dead cells; black events correspond to cellular debris. The numbers in the PI-/EYFP+/CD4+ dot plots show the percentage of CD4+/EYFP+ cells within the PI- region (region R3). The bar diagram represents the media of three independent experiments and the lines on top of the bars correspond to SD.

FIGURE 3. Tat101 reduced FasL-mediated apoptosis in HIV-1 transfected Jurkat cells and in Jurkat cells with stable expression of intracellular Tat101. A. Jurkat E6-1 cells were transfected with pNL4.3-TatM1I together with pCMV-Tat72 or pCMV-Tat101 expression vectors. pcDNA3 was used as negative control. pEYFP-C1 was used as control of transfection efficiency. After 3 days in culture, cells were treated with FasL for 4h. Viability was measured by chemiluminiscence. The bar diagram represents the media of three independent experiments and the lines on top of the bars correspond to SD.
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and lines on top of the bars correspond to SEM. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis (* indicates \( p<0.05 \)). HIV-1 replication was monitored by quantifying the expression of the \textit{env/nef} genes by qRT-PCR using \( \beta\)-actin as internal control. B. Jurkat-Tat101, Jurkat-Tat72, and control cells were treated with FasL for 2, 4, 6 and 18h and then stained with Annexin-V-FITC in order to measure by flow cytometry the percentage of apoptotic cells. C. Jurkat-Tat101, Jurkat-Tat72 and control cells were treated with FasL during 4h and then stained with PI. The induction of apoptosis was measured by flow cytometry. Region R1 was used to select the living PI- cells in the groups of untreated cells, displayed as SSC/FSC dot plots. The percentage of apoptotic cells (PI+) was determined within R1 in treated cells and then represented as histograms where the basal cell death (continuous line) was compared to the apoptosis induced after treatment with FasL (discontinuous line). The numbers shown correspond to the percentage of apoptotic cells within R1 after subtracting the basal death. D. Jurkat-Tat101, Jurkat-Tat72 and control cells were stained with a monoclonal antibody against Fas receptor (FasR/CD95) and a secondary antibody conjugated with FITC. The percentage of cells expressing Fas was measured by flow cytometry. The histograms compare the isotype control (discontinuous line) with the expression of FasR (continuous line) on the cell surface. Numbers shown are the percentage of cells expressing CD95 after subtracting the isotype control. All data shown are media or representative of three independent experiments.

FIGURE 4. Activation of caspase-3 and -8 was impaired in Jurkat-Tat101 treated with FasL. A. Procaspase-3 cleavage was analyzed by immunoblotting using an antibody against procaspase-3 (p32) and active fragments p17/p11 in protein extracts obtained from Jurkat-Tat101, Jurkat-Tat72 and control cells treated or not with FasL for 4 or 18h. \( \beta\)-actin was used as internal loading control. Gel bands were quantified by densitometry and the background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated regarding the internal control (\( \beta\)-actin) per each lane. B. Cleavage of PARP-1 was analyzed by using a monoclonal antibody against the cleaved fragment p89. \( \beta\)-actin was used as loading control. C. Caspase-3/-7 activation was measured by chemiluminescence in cells treated or not with FasL. D. Caspase-8 activation was measured by chemiluminescence in cells treated or not with FasL. Levels of procaspase-8 were analyzed by immunoblotting using an antibody against procaspase-8 (p55) in protein extracts obtained from Jurkat-Tat101, Jurkat-Tat72 and control cells treated with FasL for 18h. \( \beta\)-actin was used as internal loading control. The bar diagrams show the media of relative RLUs fold from three independent experiments and the lines on the top of the bars represent the SD. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis. Symbols * and *** indicates \( p<0.05 \) or \( p<0.001 \) respectively.

FIGURE 5. The release of cytochrome c induced by FasL was reduced in Jurkat-Tat101. A. The release of cytochrome c from the mitochondria was quantified by flow cytometry in Jurkat-Tat101, Jurkat-Tat72 and control cells treated or not with FasL during 4 or 18h. The histograms correspond to one representative experiment out of three and compare the percentage of cytochrome c that was retained in the mitochondrial intermembrane space (continuous line; marker M1) vs. the cytochrome c released after treatment with FasL (discontinuous line; marker M2). B. The release of cytochrome c was analyzed by immunofluorescence. After staining with the Mitotracker probe, the cells were fixed and stained with a monoclonal antibody against cytochrome c and a secondary antibody conjugated with Alexa 488. The nucleus was stained with Dapi.

FIGURE 6. Jurkat-Tat101 showed a reduced cleavage of Bid and procaspase-9, overexpression of BCL2 and higher stability of the mitochondrial inner-membrane potential. A. Cleavage of procaspase-9 was analyzed by immunoblotting in cytosolic protein extracts obtained from Jurkat-Tat101, Jurkat-Tat72 and control cells treated or not with FasL during 4 or 18h, using an antibody against the pro-caspase-9 (p46) and active caspase-9 (p37/p35) fragments. \( \beta\)-actin was
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

used as loading control. Gel bands were quantified by densitometry and the background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated regarding the internal control (β-actin) per each lane. B. Caspase-9 activity was measured by chemiluminescence. The bar diagram shows the media of relative RLU's fold from three independent experiments and the lines on the top of the bars represent the SD. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis and * , ** and *** correspond to \( p<0.05, p<0.01 \) or \( p<0.001 \), respectively. C. The mitochondrial membrane potential gradient (\( \Delta \Psi_m \)) was measured by flow cytometry using the lipophilic dye JC-1. Red fluorescent aggregates in the undamaged mitochondria were represented in a dot plot vs. the green fluorescence monomers dispersed through the cytosol. The numbers up left on the diagrams represents the cells with stable mitochondrial membrane integrity; the numbers down right represent the loss of mitochondrial membrane integrity. A representative experiment out of three is shown. D. The expression of BCL2 and Bid (p22) was analyzed by immunoblotting and β-actin was used as loading control.

FIGURE 7. Network of predicted interactions between proteins related to apoptosis and mitochondrial membrane integrity -specified in Table1-, which expression was modified in Jurkat-Tat101 and Jurkat-Tat72 vs control cells. Medium confidence score level was 0.400. Caspase-3 was included to evaluate its central point in the network. Data supporting protein-protein interactions derived from experimental studies (dark purple lines), homology (light purple lines), databases (light blue lines), text mining (light green lines), concurrence (dark blue lines) and co-expression (black lines). Node colour is arbitrary.

FIGURE 8. NF-κB-dependent proteins involved in the control of apoptosis were up-regulated in Jurkat-Tat101. A. The expression of c-FLIP\(_L\) (55kDa), c-FLIP\(_R\) (43kDa) and c-FLIP\(_S\) (28kDa) isoforms was analyzed by immunoblotting in cytosolic protein extracts from Jurkat-Tat101, Jurkat-Tat72 and control cells treated or not with FasL for 4 or 18h. β-actin was used as loading control. B. NF-κB activity was analyzed in basal conditions by using DAI assay. Gel bands were quantified by densitometry and the background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated regarding the internal control (β-actin) per each lane. C. Jurkat-Tat72, Jurkat-Tat101 and control cells were treated with FasL during 4h and then stained with an antibody against CD69 conjugated with PE. The percentage of cells expressing CD69 on the cell surface was measured by flow cytometry. The bar diagram represents the percentage of cells expressing CD69 after subtracting the isotope control. D. NF-κB-dependent transactivation activity was measured by transient transfection of vector p3κB-LUC. Fold mean of RLU's corresponding to three independent experiments is represented and lines on the top of the bars correspond to SD. E. Network of proteins related to apoptosis and identified by antibody-based microarray (see Table 2), which expression was modified in Jurkat-Tat101 and Jurkat-Tat72 vs control cells. Predicted protein-protein interaction was obtained using a medium confidence score level (0.400). Data supporting protein-protein interactions derived from experimental studies (reddish purple lines), homology (light purple lines), databases (light blue lines), text mining (light green lines) and co-expression (black lines). The colour of the nodes is arbitrary. F. Jurkat TetOff cells were transiently co-transfected with p3κB-LUC vector and pTRE2hyg-Tat101 ESKKKVE (wild-type), pTRE2hyg-Tat101 ESRVNVV (mutated), or pTRE2hyg empty vector as negative control. After 18h, NF-κB activity was measured by chemiluminescence. Fold of relative RLU's regarding to control cells are represented (upper bar diagram). Cells were then treated with FasL for 4h and apoptosis was measured by PI staining and analyzed by flow cytometry (lower bar diagram). The bar diagrams show the media obtained from three independent experiments and lines on the top of the bars represent the SD. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis. The symbols *, ** and *** indicates \( p<0.05, p<0.01 \) or \( p<0.001 \), respectively.
FIGURE 9. Model summarizing the steps in Fas apoptotic pathway that were thwarted by the intracellular expression of Tat. See Table 1, Table 2 and text for further explanation.
**HIV-1 Tat101 delays Fas-mediated apoptosis in T cells**

Table 1. Selected proteins differently expressed, detected by mass spectrometry in protein extracts from Jurtkat-Tat101 and Jurkat-Tat72 cells, in comparison with TetOff cells.

<table>
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<tr>
<th>Accession code</th>
<th>Name</th>
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<th>JJ Tat101 vs JJ TetOff</th>
<th>Biological process</th>
<th>Subcellular localization</th>
<th>Peptide Sequence &amp; PTM</th>
<th>Protein probability</th>
<th>XCorr score</th>
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<tr>
<td>gi</td>
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<td>BCL2-associated transcription factor 1 (BCLAF1)</td>
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<td>1.48</td>
<td>Pro-apoptosis</td>
<td>Cytoplasm Nucleus</td>
<td>LKDLFDyqPPLHK KAEGEPQEgPLSK (p) (p)</td>
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<tr>
<td>gi</td>
<td>31543380</td>
<td>Protein DJ-1 (PARK7)</td>
<td>-1.22</td>
<td>1.58</td>
<td>Stress response Autophagia</td>
<td>Cytoplasm Mitochondrion Nucleus</td>
<td>DGLILTSR GAEEnETVIPVDvmR (a)</td>
<td>99.80% 99.80%</td>
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<td>gi</td>
<td>10835063</td>
<td>Nucleophosmin (NPM1)</td>
<td>2.10</td>
<td>1.98</td>
<td>Host-virus interaction</td>
<td>Cytoplasm Nucleus</td>
<td>SNQnGKDKSKPSTTPR MSVQPTVSLGGFETTPPVLR (d) (d)</td>
<td>100% 100%</td>
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<td>ATP synthase subunit beta (ATP5B)</td>
<td>1.50</td>
<td>2.00</td>
<td>ATP synthesis</td>
<td>Mitochondrion</td>
<td>TIamDGTEGVLVR VVDDLAAPAk (a) (o)</td>
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<td>gi</td>
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<td>Glycolysis</td>
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<td>GADFLVTEVErGOSGLS (d)</td>
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<td>Cytoplasm Mitochondrion</td>
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<td>Cytoplasm Nucleus</td>
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Aminoacidic residues with post-translational modifications (PTMs) are underlined and in lower case. PTMs detected were: (p) phosphorylation; (a) acetylation; (d) deamidation; and (o) oxidation.
### Table 1 (continue).

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Aminoacidic residues with post-translational modifications (PTMs) are underlined and in lower case. PTMs detected were: (p) phosphorylation; (a) acetylation; (d) deamidation; and (o) oxidation.
Table 2. Proteins differentially expressed, detected by human apoptosis antibody array in protein extracts from Jurkat-Tat72 and Jurkat-Tat101 in comparison with TetOff cells.

<table>
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<th>Ratio JJ Tat72 vs JJ TetOff</th>
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*HIV-1 Tat101 delays Fas-mediated apoptosis in T cells*
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

Figure 1.
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

Figure 2.
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

Figure 3.
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

Figure 4.
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

Figure 5.

A

B

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HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

Figure 6.
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells
HIV-1 Tat101 delays Fas-mediated apoptosis in T cells

Figure 8.
Figure 9.
**Supplemental figure legends**

**Fig. 1.** Obtaining the HIV-1 Tat-defective clone NL4.3TatM11. A. The start codon of the tat gene (methionine, M) in the infectious clone pNL4.3-wt was changed into isoleucine (I) by introducing a missense point mutation through site-directed mutagenesis (TatM1I). This strategy did not affect the splicing regulator elements located near the start codon of the tat gene in HIV-1 genome (ESE2: exonic splicing enhancer 2; ESS2: exonic splicing silencer 2). B. HEK293T cells were transfected with pNL4.3-wt or pNL4.3-TatM11 vectors and then MT-2 cells were infected with the resultant culture supernatant for 5 days. HIV-1 replication was assessed by quantifying the concentration of p24 (ng/ml) in MT-2 culture supernatant. C. Syncytia formation was observed in MT-2 cells infected with NL4.3-wt or NL4.3-TatM11 for 5 days by light microscopy. Syncytia are indicated with white arrows. D. Jurkat cells were co-transfected with pNL4.3-wt or pNL4.3-TatM11 vectors, together with pCMV-Tat101 expression vector. pEYFP-C1 vector was used as a control of transfection efficiency. The expression of Tat and its nuclear localization was analyzed by immunofluorescence 48h after transfection using a monoclonal antibody directed against Tat and a secondary antibody conjugated with Alexa-546. Nuclei were stained with Dapi. E. Jurkat-Tat72 and Jurkat-Tat101 cells were transfected with pNL4.3-TatM11, using Jurkat TetOff as negative control. Efficient transcription of HIV-1 genome from Tat-defective NL4.3-TatM11 genome was determined by qRT-PCR using specific primers against the env/nef gene. β-actin was used as housekeeping gene. Data shown correspond to the relative HIV-1 gene expression regarding control cells (left bar diagram). The concentration of p24 (ng/ml) was assessed in culture supernatants by ELISA (right bar diagram). The virions produced by Jurkat-Tat72 and Jurkat-Tat101 were infectious, as was assessed by the subsequent infection of MT-2 cells (data not shown).

**Fig. 2.** Pattern of expression of c-FLIP in Jurkat-Tat72 and Jurkat-Tat101 cells. Changes in the pattern of expression of c-FLIP were analyzed by immunoblotting in Jurkat-Tat72 or Jurkat-Tat101, treated or not with FasL for 4 or 18 hours. Different exposures allowed the detection of all forms of c-FLIP (c-FLIP\(_S\) (p28), c-FLIP\(_R\) (p43) and c-FLIP\(_L\) (p55)). Non saturated bands were used for quantifying the expression of the different forms by densitometry using the signal detected in untreated control cells as the basal reference. * indicates a non-specific band.
Supplemental material and methods

**HIV-1 infection**- Infectious supernatants were obtained from calcium phosphate transfection of HEK293T cells with plasmids pNL4.3-wt or pNL4.3-TatM1l. Culture supernatants were used to infect MT-2 cells for 2 hours at gently rotation, room temperature. Cells were then centrifuged at 600xg for 30min at 25°C. After extensive washing with PBS1x, cells were left in culture for 5 days. Supernatants were collected and p24 antigen was measured by using an enzyme-like immunoassay (InnotestTM HIV Ag mAb; Innogenetics, Barcelona, Spain).

**Cells**- HEK293T cells were grown in DMEM (Dulbecco’s modified Eagle Medium, Gibco) with 10% fetal bovine serum (Gibco) supplemented with penicillin/streptomycin (100U and 0.1 mg/ml, respectively) and 4mM L-glutamine (Gibco).
Figure 1 Supplemental data

A

AGGAGAGCAAGAAATGGAGCCAGTAGATCCTAGACTAGAGCCCTG

B

![p24 levels](#)

C

![Representative images](#)

D

![Bright field and fluorescence images](#)

E

![Graphs of HIV-1 relative gene expression and p24 levels](#)
**Figure 2 Supplemental data**

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<tr>
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**IB: αFLIP**

- Exposure 30’
  - 55kDa cFLIP<sub>L</sub>
  - 43kDa cFLIP<sub>R</sub>
  - 28kDa c-FLIP<sub>S</sub>

- Exposure 10’
  - 43kDa cFLIP<sub>R</sub>
  - 28kDa c-FLIP<sub>S</sub>

- Exposure 1’
  - 43kDa cFLIP<sub>R</sub>
  - 28kDa c-FLIP<sub>S</sub>
Molecular Bases of Disease: The Presence of HIV-1 Tat Second Exon Delays Fas-Mediated Apoptosis in CD4+ T lymphocytes: a Potential Mechanism for Persistent Viral Production

María Rosa López-Huertas, Elena Mateos, María Sánchez del Cojo, Francisco Gómez-Esquer, Gema Díaz-Gil, Sara Rodríguez-Mora, Juan Antonio López, Enrique Calvo, Guillermo López-Campos, José Alcamí and Mayte Coiras

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