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Compensatory reactions of B cells in response to chronic HIV-1 Tat exposure

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Abstract

Patients infected with human immunodeficiency virus-1 (HIV-1) have an increased incidence of B-cell lymphoma, even though HIV-1 does not infect B cells. The development of B-cell lymphomas appears to be related to the action of the HIV-1 transactivator protein (Tat), which is released from HIV-infected cells and penetrates uninfected B cells, affecting host cell gene expression. Upon chronic HIV-1 infection, Tat acts on the cells for a long time, probably allowing the cells to adapt to the presence of the viral protein. The aim of this work was to identify and study the mechanism of adaptation of cells to prolonged (chronic) exposure to HIV-1 Tat. We performed a comparative analysis of cells expressing Tat under the action of either an inducible promoter or a constitutive promoter, allowing us to model acute and chronic Tat effects, respectively. We found that the acute action of Tat leads to the suppression of cell proliferation, probably due to the downregulation of genes associated with replication and protein synthesis. In the case of chronic action of Tat, cell proliferation was restored and the expression of genes associated with the implementation of protective (antiviral) functions of the cell was increased. Analysis using proteasome inhibitors showed that in the case of chronic action, intense Tat proteolysis occurred, which could be the main mechanism of B-cell adaptation. Thus, B cells have a powerful mechanism to adapt to the entry of HIV-1 Tat, the efficiency of which may determine the frequency of lymphomagenesis in HIV-1-infected patients.

KEYWORDS
B cells, HIV-1, proteasome, Tat, transcriptome

1 | INTRODUCTION

Human immunodeficiency virus (HIV) infection leads to the development of acquired immunodeficiency syndrome (AIDS). Combination antiretroviral therapy (cART), developed and introduced into clinical practice in the 1990s, allows control of HIV progression and immunodeficiency, but even cART does not prevent the development of various HIV-associated diseases, including cancers. Particularly striking is the high incidence of several B-cell lymphomas (especially diffuse large B-cell lymphoma and Burkitt's lymphoma) (Hübel, 2020; Noy, 2020; Shmakova et al., 2020). The mechanisms of HIV-associated lymphomagenesis remain poorly understood. One of the

potential mechanisms involves the action of the HIV-1 transactivator of transcription (Tat). Indeed, expression of HIV-1 Tat alone in mice provokes the development of several neoplasms, including lymphomas, suggesting that the Tat protein is directly involved in oncogenesis (Altavilla et al., 1999; Corallini et al., 1993; Kundu et al., 1999; Vogel et al., 1988; Vogel et al., 1991).

Tat is a small, intrinsically disordered basic protein that plays multiple roles in the HIV-1 replication cycle, including promoting efficient viral RNA transcription (Ali et al., 2021); it also affects many processes within the host cell nucleus (Musinova et al., 2016). Tat protein affects the expression of many host genes in T cells (Reeder et al., 2015) and monocytes/macrophages (Carvallo et al., 2017) which modifies various cellular processes. Tat is recruited on a number of cellular gene promoters in HIV-1-infected T-cells (Dhamija et al., 2015) and Tat-expressing Jurkat cells (Reeder et al., 2015), indicating its possible involvement in the regulation of gene expression. Additionally, Tat is actively released from HIV-1-infected cells (Ensoli et al., 1990; Ensoli et al., 1993; Nath, 2015) and is detectable in the serum or the extracellular matrix of HIV-1-infected individuals (Cafaro et al., 2024; Germini et al., 2017; Mediouni et al., 2012; Poggi et al., 2004; Shmakova et al., 2024; Westendorp et al., 1995; Xiao et al., 2000). Exogenous HIV-1 Tat can enter uninfected cells, and, in particular, it was found within tumor cells of HIV-1-associated B-cell lymphomas (Alves de Souza Rios et al., 2021; Lazzi et al., 2002).

The exact mechanism of Tat protein-induced oncogenesis remains unclear, although Tat is known to affect gene localization (Germini et al., 2017), cell cycle and apoptosis (Chen, 2002; Colombrino et al., 2004), redox balance (El-Amine et al., 2018), and host gene expression (Akbay et al., 2021; Sall et al., 2019; Valyaeva et al., 2022) in B cells. The Tat increases the expression of interleukin-6 (IL-6) and 10 (IL-10) (Blazevic et al., 1996; Scala et al., 1994), leading to B cell activation. HIV-1 Tat also induces the overexpression of DNA Methyltransferase 1 (DNMT1), an enzyme that plays a key role in maintaining DNA methylation (Luzzi et al., 2014) therefore, Tat may participate in B cell lymphomagenesis through dysregulation of the epigenetic control of gene expression. Thus, the accumulated data suggest that HIV-1 Tat causes substantial changes in gene expression, some of which may contribute to lymphomagenesis.

The most convenient experimental approach to study the effect of Tat on cellular gene expression is to use Tat-expressing cell lines for subsequent high-throughput RNA-seq analysis. However, different studies report different (and sometimes adverse) effects of Tat on gene expression, which may be related to the experimental system used (for discussion see Valyaeva et al., 2022). For example, Reeder with coauthors used an inducible promoter for Tat expression (Reeder et al., 2015). This experimental system is more suitable for simulating an acute infection situation, but it does not allow the study of weak long-term (chronic) effects of the protein and compensatory reactions of the cell. The use of a constitutive promoter allows to simulate a prolonged systemic effect of the presence of Tat in the blood of chronically infected patients. In this case, the cell can at least partially compensate for the acute effects of Tat by activating protective cellular mechanisms. Thus, these two experimental systems allow to model and to compare fundamentally different phenomena - acute and chronic Tat effects.

Here, we established B cell lines with inducible and constitutive HIV-1 Tat expression to study the acute and chronic effects of Tat, respectively. Acute Tat effects, but not chronic Tat effects, inhibited cell proliferation; and RNA-seq analysis revealed robust inhibition of genes critical for cell proliferation (DNA replication, RNA synthesis and processing, and ribosome biogenesis) upon acute Tat exposure. However, in the case of chronic exposure, genes associated with innate immunity and the realization of anti-pathogenic responses were upregulated, and a substantial part of the deleterious effects of Tat was compensated by the cell. We also showed that chronic Tat exposure resulted in intense Tat proteolysis, which may be the main mechanism of B cell adaptation. Thus, Tat can significantly affect cellular processes in B cells, but the cells have effective mechanisms for their compensation, and Tat-induced lymphomagenesis may be due either to acute action of Tat or insufficient efficiency of the compensatory mechanisms.

2 | MATERIALS AND METHODS

2.1 | Cell culture

RPMI 8866 cells were grown at 37°C in the RPMI 1640-Gluta-Max medium (Gibco, cat. no. 61870-010) with the addition of 10% Tet System Approved FBS (Takara, cat. no. 631107) sodium pyruvate (PanEco, cat. no. F023), and an antibiotic and antimycotic solution (Gibco, cat. no. LS15240062). The induction was performed with doxycycline (Sigma, cat. no. D3447) in concentration 1 μg/ml for 12-24 h.

2.2 | Plasmids

Tat gene was inserted into pSBbi-GP (Addgene, #60511) and pSBtet-GP (Addgene, #60495) vectors. The pSBbi-GP and pSBtet-GP plasmids were a gift from Eric Kowarz (Kowarz et al., 2015). Transformation of cells with the resulting plasmids allows either stable (in the case of pSBbi-GP) or doxycycline-induced (in the case of pSBtet-GP) Tat protein expression. The generation of cell lines on RPMI 8866 cells with stable and doxycycline-induced Tat expression was described elsewhere (Gorbacheva et al., 2019).

2.3 | RNA extraction and RT-qPCR assays

Total RNA was isolated from RPMI 8866 cells using the RNeasy Mini Kit (Qiagen, cat. no. 74106). RNA concentration was measured using a NanoPhotometer (Implen). Reverse transcription was performed using an iScript Advanced cDNA Synthesis Kit (Bio-Rad, cat. no.

1725038) according to the manufacturer's instructions, and qPCR was performed in technical triplicates using a SYBR Green Kit (Syntol, cat. no. M-427) in a CFX96 Real-Time PCR Detection System (Bio-Rad). Melting curve analyses were performed to verify amplification specificity. The experiments were performed in biological triplicates. *HPRT*, *YWHAZ*, and *UBC* genes were used as references. Primers used for RT-qPCR analysis are listed in the Supporting Information: Table S1.

2.4 | RNA sequencing

RNA sequencing libraries were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina following the manufacturer's protocol. The sequencing was performed in single-end mode (55 nt) on an Illumina HiSeq 4000 instrument.

2.5 | RNA-seq data analysis

The RNA-seq reads quality was assessed using FastQC (version 0.11.7) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). BBDuk tool from BBTools suite (version 38.34) (c) was used with default parameters to remove Illumina adapters and to quality-trim sequence reads. Trimmed reads were then aligned against the human reference genome GRCh38.p10 using HISAT2 (version 2.0.5) (Kim et al., 2015). Expression matrix with gene counts was generated using htseq-count script from Python library HTSeq (version 0.12.4) (Anders et al., 2015) and GENCODE v26 gene annotation. The parameters of htseq-count run were the following: *-m intersection-strict –stranded=no -t gene*. Visualization of the RNA-seq count data (rlog-transformed) was performed using Principal Component Analysis (PCA).

Differential gene expression analysis was performed using DESeq2 R package (version 1.36.0) (Love et al., 2014). We declared the gene to be differentially expressed if padj (p-value adjusted by the Benjamini-Hochberg procedure) was smaller than 0.05 and the fold change was larger than 1.5 in any direction. clusterProfiler R package (version 4.4.4) was used to conduct gene set enrichment analysis (GSEA) on a pre-ranked list of genes ordered by the stat column of DESeq2 results. Gene sets of biological processes and pathways were obtained from the Gene Ontology resource (R package org.Hs.eg.db, version 3.15.0) (The Gene Ontology Consortium, 2017), and the KEGG database (release 104.0) (Kanehisa, 2000). An adjusted p-value cutoff of 0.1 was used to select statistically significant activated or suppressed pathways. GO, KEGG, and Reactome gene enrichment resulted in similar groups of terms and pathways, and we show only the most representational GSEA results obtained with KEGG. KEGG pathway maps were plotted using the Pathview R package (Luo & Brouwer, 2013). Heatmap visualization and sample clustering were performed with ComplexHeatmap R package (version 2.12.1) (Gu, 2022).

Raw RNA sequencing data and processed gene counts are deposited in the Gene Expression Omnibus (GEO) database with accession number GSE233499.

Code to reproduce the RNA-seq analysis and the figures is provided at https://github.com/Sheval-Lab/ind_Tat_in_B_cells.

2.6 | Analysis of the cell cycle

Cells were incubated in the presence of 1 μg/ml EdU for 15 min at 37°C, washed in PBS, fixed with 3.7% paraformaldehyde for 10 min, and permeabilized in 0.5% Triton X-100. EdU was labeled using a Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 555 dye (Thermo, cat. no. C10338), according to the manufacturer's instructions. DNA was stained with 1 μg/ml Hoechst 33342 (Thermo, cat. no. H3570). Cells were analyzed using a FACSAria SORP cell sorter (BD Biosciences). Detection parameters were as follows: Ex. 405 nm, Em. 450/50 BP for Hoechst 33342 and Ex. 561 nm, Em. 585/15 BP for EdU-Alexa Fluor 555.

2.7 | Analysis of apoptosis

Cell death was analyzed by flow cytometry using a FACSAria SORP instrument (BD Biosciences). Cells were simultaneously stained with 1 μg/ml Hoechst 33342, 100 nM TMRE (tetramethylrhodamine, ethyl ester, perchlorate, Thermo, cat. no. T669), and Annexin V-Alexa Fluor 647 (Thermo, cat. no. A23204) according to the manufacturer's instructions. Thus, DNA content, mitochondrial membrane potential, and phosphatidylserine externalization were analyzed together for each sample. Detection parameters were as follows: Ex. 405 nm, Em. 450/50 BP for Hoechst 33342; Ex. 561 nm, Em. 585/15 BP for TMRE; and Ex. 647 nm, Em. 640/14 BP for Annexin V-Alexa Fluor 647.

2.8 | Cell lysate preparation, SDS-PAGE, and Western blot analysis

For Western blot analysis, the cells were lysed in Laemmli sample buffer, boiled for 3 min, and resolved on a 10% SDS-polyacrylamide gel (TGX FastCast Acrylamide Kit, cat. no. 161-0173). The proteins were transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). These membranes were blocked in 1% bovine serum albumin (Merck, cat. no. A9647) and incubated with primary antibodies. anti-HIV-1 Tat antibody (Santa Cruz Biotechnology, cat. no. sc-65912) and β-tubulin (Sigma, cat. no. T0198) were used. The membranes were washed in PBS and then incubated with a secondary horseradish-peroxidase-conjugated antibody (Sigma, cat. no. A0168). The antibody-bound proteins were detected using the Pierce ECL Western Blotting Substrate (Thermo, cat. no. 32209), and then the membrane was developed using a ChemiDoc

Touch Imaging System (BioRad). Densitometry analyses were performed using ImageJ.

For analysis of proteasome activity, the cells were incubated 6 hours with the following proteasome inhibitors respectively: Bortezomib (MedChemExpress, cat. no. HY10227), Pevonedistat (MedChemExpress, cat. no. HY70062), NSC697923 (MedChem Express, cat. no. HY13811), and Thalidomide (Euromedex, cat. no. 50-35-1). For Western blot analysis, whole cell lysates were prepared by lysing the cells with 150 µL of ice-cold lysis buffer and different concentrations of proteasome inhibitors. The lysates were sonicated in a Vibra Cell sonicator (SONICS & MATERIALS, Inc.) for 10 s at 30% intensity, incubated on ice for 30 min and centrifuged at 4°C, 16000 g for 10 min. Protein concentrations of lysates were determined using BCA assay (ThermoFisher Scientific, cat. no. 23227). Whole cell lysates (20 µg) were resolved through NuPAGE™ 4-12% Bis-Tris SDS-PAGE gels (Invitrogen, cat. no. MP41G10, cat. no. MP41G12, cat. no. MP41G15) in MES SDS Running buffer (Invitrogen, cat. no. B0002). PageRuler Prestained Plus Protein Ladder (ThermoFisher Scientific, cat. no. 26616) was used as a molecular weight marker. After transferring the protein to the PVDF membrane (GE Healthcare), nonspecific binding was blocked with 5% nonfat dried milk in Tris-buffered saline with 0.01% Tween-20 (TBST) and was incubated overnight at 4°C with the following primary antibodies: mouse anti-β-actin (1:1000, Santa Cruz, cat. no sc-81178), mouse anti-GFP (1:1000, Roche, cat. no. 11814460001), mouse anti-Tat (1:200, Santa Cruz, cat. no. sc-65912). After washing with TBST, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies (Invitrogen) (in 1:2000 dilution) for 1 h at room temperature. Proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, cat. no. WBKLS0500) and Amersham ImageQuant 800 (Cytiva).

3 | RESULTS

3.1 | Acute Tat exposure inhibits B cell growth

To study the acute and chronic effects of HIV-1 Tat on B cells, we generated RPMI 8866 lymphoblastoid cell lines co-expressing untagged HIV-1 Tat and EGFP (the latter was necessary for cell selection by FACS and to control cell line homogeneity during the work) (Gorbacheva et al., 2019). Tat expression was regulated by either an inducible tetracycline promoter (RPMI^{iTat} cells) or a constitutive EF-1α promoter (RPMI^{Tat} cells). RPMI^{iTat} cells were used to model acute Tat exposure in B cells, and RPMI^{Tat} cells were used to study chronic Tat exposure. RPMI 8866 cells and RPMI^{iTat} cells before doxycycline treatment (dox-) were used as negative controls for RPMI^{Tat} and doxycycline treated (dox+) RPMI^{iTat} cells, respectively. Before experiments, Tat expression levels were assessed by RT-qPCR (Figure 1a). In RPMI$^{iTat\ dox+}$ cells, Tat expression was detected within 1 h after the addition of doxycycline.

We first compared the effects of acute and chronic Tat exposure on cell growth and found a significantly lower proportion of RPMI$^{iTat\ dox+}$ cells in S and G2/M phases, and a higher proportion of cells in G1/G0 compared to RPMI 8866, RPMI^{Tat}, RPMI$^{iTat\ dox-}$ cells (Figure 1b, c). To confirm this effect, we incubated cells in the presence of EdU to detect cells in S-phase. We observed significantly lower amounts of S-phase cells in RPMI$^{iTat\ dox+}$ compared to cells without Tat expression (RPMI 8866 and RPMI$^{iTat\ dox-}$) or with stable Tat expression (RPMI^{Tat}) (Figure 1d).

Since HIV-1 Tat could affect apoptosis in cells (Chen, 2002), we next stained RPMI^{Tat}, RPMI^{iTat} and the control cells for Annexin V. We did not observe any significant differences between the samples (Supporting Information: Figure S1). As RPMI^{Tat} and RPMI^{iTat} cells might have different resistance to apoptosis induction, we additionally analyzed apoptotic cells after treatment with several apoptosis-inducing drugs with different mechanisms of action (cisplatin, nocodazole and ICRF-187). Although these drugs increased apoptosis, no difference was observed between the cell lines used (Supporting Information: Figure S2).

Thus, acute Tat exposure might affect cell growth by suppression of cell cycle progression, but, importantly, this effect was compensated by the cell upon chronic Tat exposure.

3.2 | Acute Tat exposure downregulated different metabolic pathways

To identify possible mechanisms of cell growth suppression during the acute Tat effect and ways to compensate for this effect in the case of chronic Tat exposure, we analyzed the transcriptomes of RPMI$^{iTat\ dox+}$, RPMI$^{iTat\ dox-}$, RPMI^{Tat}, and RPMI cells. Three biological replicates were sequenced for each cell line, and ~61% of the reads obtained were unambiguously mapped to the GENCODE gene annotation (Supporting Information: Figure S3A). A principal component plot showed that the biological replicates were tightly clustered and at the same time, the four cell lines were clearly separated from each other (Figure 2a). Notably, the controls (RPMI and RPMI$^{iTat\ dox-}$) clustered relatively close to each other, while samples with stable and induced HIV-1 Tat expression were spread out across the principal component 1. The Spearman correlation coefficients of normalized expression profiles confirmed the expected clustering of the sample replicates (Supporting Information: Figure S3B).

A gene was considered as differentially expressed (DEGs) if it had an adjusted p-value of less than 0.05 and fold change greater than 1.5 in any direction (Supporting Information: Table S2). A significant difference in the number of DEGs was observed between the acute and chronic situation: 1465 genes were deregulated between RPMI$^{iTat\ dox+}$ and RPMI$^{iTat\ dox-}$ cells (acute Tat effect), whereas only 362 genes were differentially regulated between RPMI^{Tat} stably expressing Tat and the control RPMI 8866 cells (chronic Tat effect) (Figure 2b). This suggests that the acute effect of Tat on cells is more pronounced than the chronic effect. Below we will consider the deregulated genes and pathways in detail.

To study the acute effects of Tat, we used RPMI^{iTat} cells. To confirm the feasibility of using RPMI$^{iTat\ dox-}$ cells as a negative

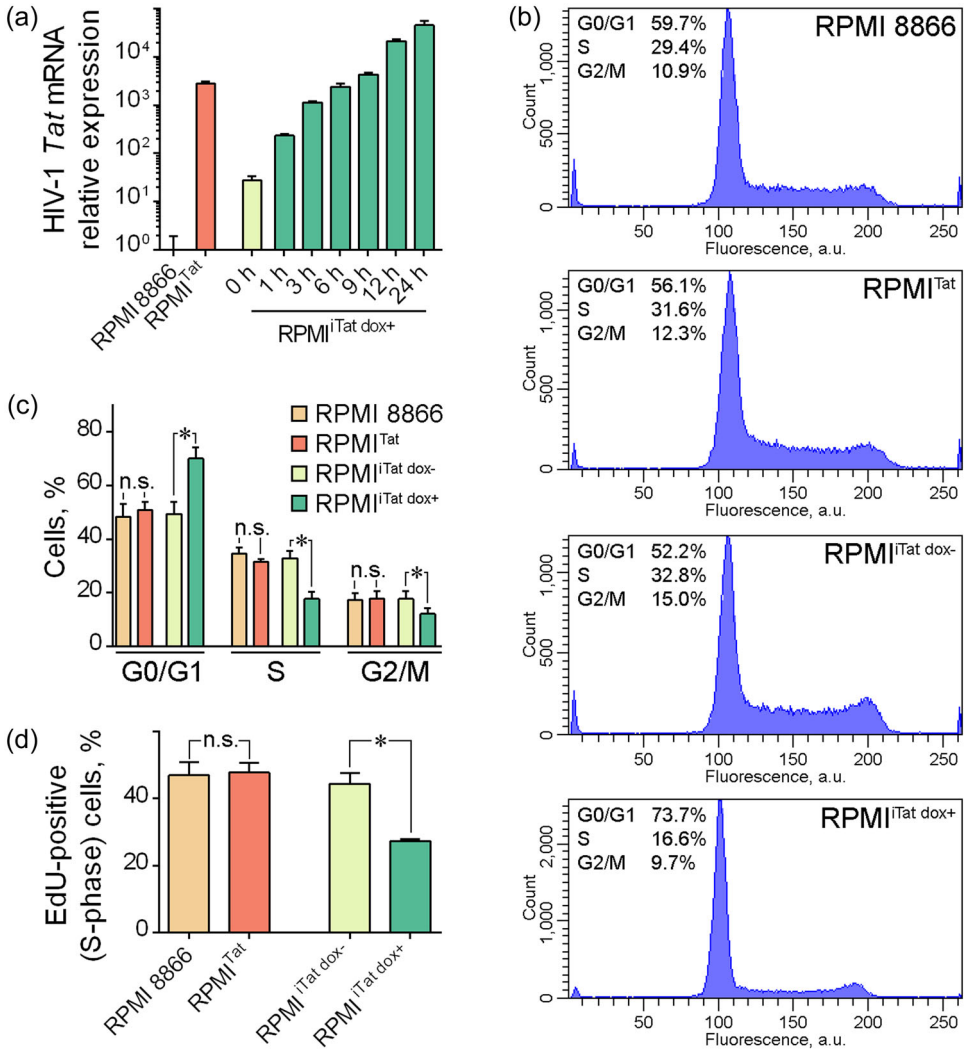

FIGURE 1 Acute Tat action influences cell cycle progression in cultured B cells (RPMI 8866). (a) RT-qPCR estimation of Tat mRNA in cells used in this study. Tat expression was regulated either by an inducible promoter (RPMI^{iTat} cells, acute Tat exposure) or by a constitutive promoter (RPMI^{Tat} cells, chronic Tat exposure). In RPMI^{iTat} cells, Tat expression was detected already after 1 h of doxycycline treatment. (b) Acute but not chronic Tat action led to accumulation of cells in G0/G1 phase of cell cycle (flow cytometry, a representative experiment is shown). (c) Accumulation of cells in G0/G, and depletion of cells in S and G2/M phases upon acute Tat action (mean ± SD; Mann-Whitney U test, $p > 0.05$; n = 5). (d) Estimation of S-phase cells using incorporation of synthetic nucleotides (EdU) (mean ± SD, Mann-Whitney U test, $p > 0.05$; n = 5).

control, we compared the transcriptomic profiles of the RPMI$^{iTat\ dox-}$ cells and control RPMI 8866 cells. The comparison of these cell lines revealed only 88 differentially expressed genes (41 upregulated and 47 downregulated) (Figure 2b), but this difference might be due to the slight promoter leakage of the tetracycline promoter. Nevertheless, we considered the effect of the leakage negligible, and used RPMI$^{iTat\ dox-}$ samples as the negative control.

The comparison between RPMI$^{iTat\ dox+}$ and RPMI$^{iTat\ dox-}$ samples, which we expected to reveal transcriptomic changes induced by the acute effect of Tat, resulted in more than 1400 DEGs, which is significantly higher than in RPMI^{Tat} cells vs. RPMI 8866 cells (Figure 2b). Notably, the number of upregulated DEGs was higher than that of downregulated DEGs – 978 and 487 genes, respectively. We validated the expression of several DEGs using RT-qPCR, thus confirming the reliability of RNA-seq (Figure 2c). To identify the biological pathways and processes affected by the acute Tat exposure, we conducted KEGG gene set enrichment analysis (GSEA) on the rank-ordered list of expressed genes (Figure 2d). The transcriptomic changes caused by inducible Tat expression negatively affected several important cellular processes: the *DNA replication* (hsa03030, p. adjust = 1.5×10^{-2}), RNA synthesis and processing (*RNA polymerase*, hsa03020, p. adjust = 3.0×10^{-2}; *Spliceosome*, hsa03040, p. adjust = 2.0×10^{-4}) and ribosome biogenesis processes (*Ribosome*, hsa03010, p. adjust = 2.1×10^{-4}; *Ribosome biogenesis in eukaryotes*, hsa03008, p. adjust = 5.6×10^{-5}). The genes with elevated expression were involved in the pathways associated with cell proliferation and survival (*Ras signaling pathway*, hsa04014, p. adjust = 3.9×10^{-3},

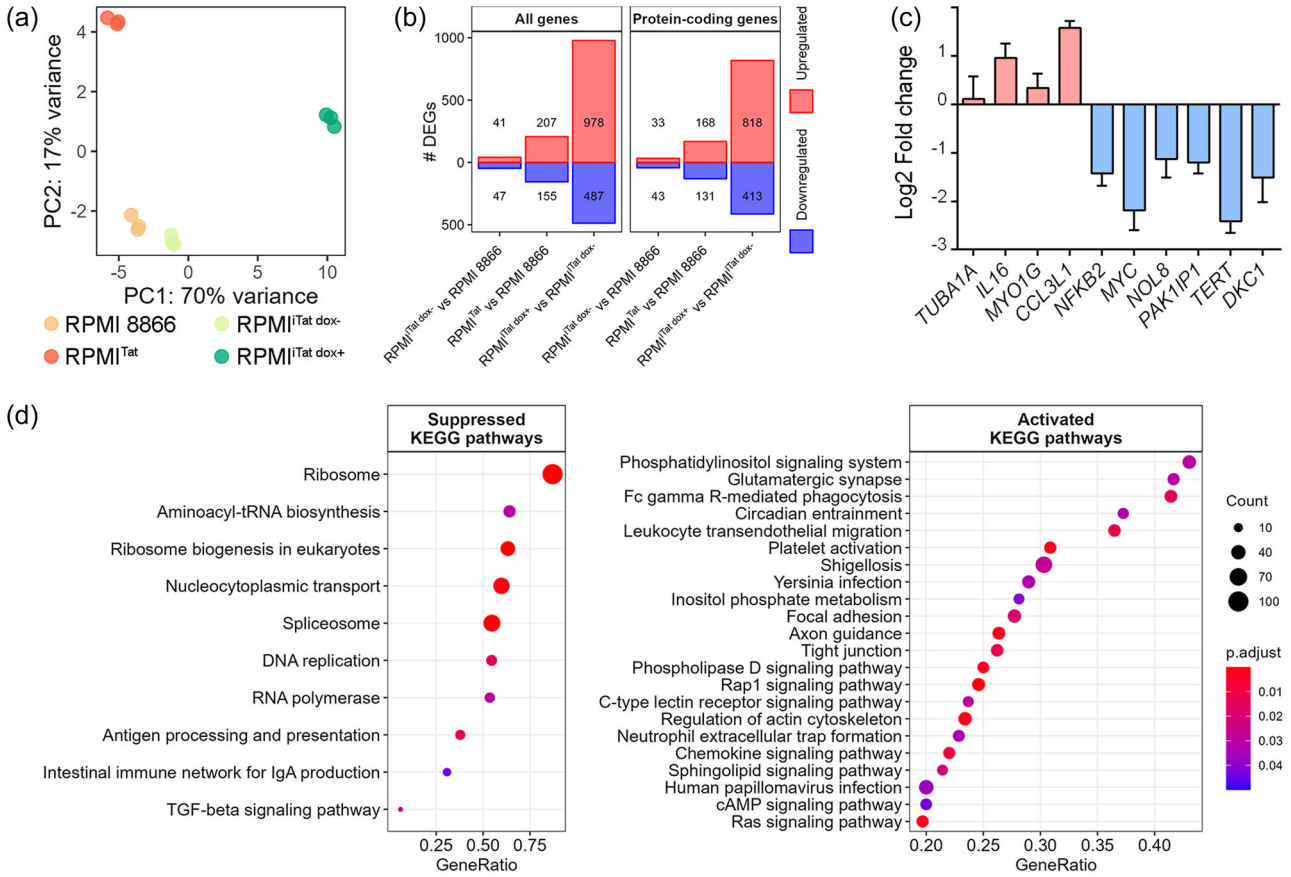

FIGURE 2 Differentially expressed genes (DEGs) in RPMI cells expressing HIV-1 Tat (RPMI^{Tat} cells). (a) Quality control of the RNA-seq replicates (PCA performed on regularized log-transformed filtered count data). (b) The number of all DEGs (left) and protein-coding DEGs (right) found in three comparisons: RPMI$^{iTat\ dox-}$ vs RPMI 8866, RPMI^{Tat} vs RPMI 8866, and RPMI$^{iTat\ dox+}$ vs RPMI$^{iTat\ dox-}$. (c) Validation of the RNA-seq data set using RT-qPCR on the indicated upregulated and downregulated genes (mean ± SEM; n = 3). (d) Downregulated (left) and upregulated (right) KEGG pathways affected by acute Tat action (RPMI$^{iTat\ dox+}$ vs RPMI$^{iTat\ dox-}$ comparison). In case of upregulated terms only the top 20 pathways ranked by gene count are shown.

Rap1 signaling pathway, hsa04015, p. adjust = 1.1×10^{-3}, *Phospholipase D signaling pathway*, hsa04072, p. adjust = 4.5×10^{-3}, *Phosphatidylinositol signaling system*, hsa04070, p. adjust = 3.1×10^{-2}), cell adhesion (*Focal adhesion*, hsa04510, p. adjust = 1.8×10^{-2}; *Tight junction*, hsa04530, p. adjust = 1.3×10^{-2};), as well as responses to various pathogenic infections (*Fc gamma R-mediated phagocytosis*, hsa04666, p. adjust = 1.5×10^{-2}; *Shigellosis*, hsa05131, p. adjust = 2.7×10^{-2}; *Yersinia infection*, hsa05135, p. adjust = 3.2×10^{-2}; *Amoebiasis*, hsa05146, p. adjust = 3.3×10^{-2}; *C-type lectin receptor signaling pathway*, hsa04625, p. adjust = 3.0×10^{-2}; *Human papillomavirus infection*, hsa05165, p. adjust = 3.7×10^{-2}) (Figure 2d).

Additionally, we compared our results with the list of genes affected by inducibly expressed Tat protein in the Jurkat T cell line (Reeder et al., 2015). We found only a small overlap between the sets of differentially expressed genes (Supporting Information: Figure S4). Notably, a number of genes showed opposite signs of expression fold change in B and T cells. Thus, it appears that the observed changes in gene expression are specific to B cells.

3.3 | Chronic Tat exposure induces weak and predominantly protective responses in B cells

Detailed comparison of RPMI^{Tat} samples against the RPMI 8866 control revealed only 207 upregulated and 155 downregulated genes (Figure 2b). Downregulated genes, as shown by GSEA, were associated with pathways *Pancreatic secretion* (hsa04972, p. adjust = 6.9×10^{-2}), *Cardiac muscle contraction* (hsa04260, p. adjust = 6.9×10^{-2}), and *Adrenergic signaling in cardiomyocytes* (hsa04261, p. adjust = 7.9×10^{-2}), and were generally implicated in calcium-dependent processes (*ASPH*, *ATP2A*, *CACNA1D*), cation transport (*ATP1A3*, *ATP1B1*), and G-protein signal transduction (*ADCY6*, *PIK3R6*, *PLCB3*) (Figure 3a). Activation of KEGG pathways was associated with anti-pathogenic and innate immunity, such as *Toll-like receptor signaling pathway* (hsa04620, p. adjust = 9.8×10^{-2}), *Influenza A* (hsa05164, p. adjust = 6.9×10^{-2}), *Measles* (hsa05162, p. adjust = 6.9×10^{-2}), and *Yersinia infection* (hsa05135, p. adjust = 9.8×10^{-2}) (Figure 3b). Activated pathways were interconnected and shared common upregulated genes of the JAK-STAT signaling pathway (*JAK1*, *STAT1*, and *STAT2*).

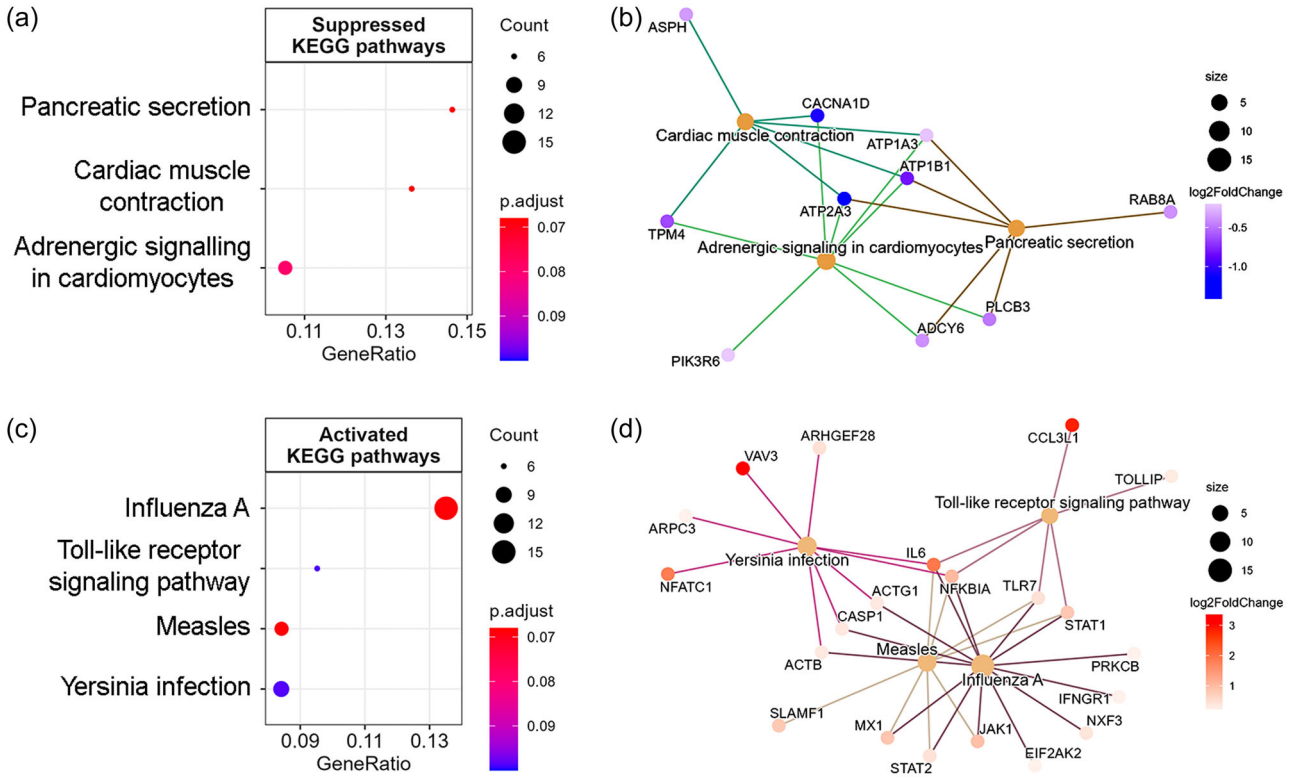

FIGURE 3 Enrichment analysis of Tat-affected genes (chronic Tat exposure, RPMI^{Tat} vs RPMI 8866 comparison). (a) KEGG pathways are negatively regulated by Tat, identified by gene set enrichment analysis (GSEA). (b) Suppressed KEGG pathways and associated genes (after filtering by adjusted *p*-value < 0.05). (c) KEGG pathways are positively regulated by Tat, identified by GSEA. (d) Activated KEGG pathways and associated genes (after filtering by adjusted *p*-value < 0.05).

3.4 | Identification of compensatory cellular responses in RNA-seq data

While acute exposure of Tat resulted in relatively strong effects that could significantly alter cellular physiology, the chronic effect was much more moderate, most likely due to the ability of the cell to compensate for it. In this case, one would expect opposite effects on the same metabolic and signaling pathways in RPMI$^{iTat\ dox+}$ and RPMI^{Tat} cells. To determine the direction of the changes in the various signaling pathways altered by acute Tat, we additionally compared transcriptomes of RPMI^{Tat} and RPMI$^{iTat\ dox+}$ cells (Figure 4a). To illustrate possible compensation events, we generated a diagram in which we have simultaneously plotted the changes in the case of acute Tat exposure (RPMI$^{iTat\ dox+}$ vs. RPMI$^{iTat\ dox-}$ - the direct action of Tat), and for the same categories the changes in the comparison RPMI^{Tat} vs. RPMI$^{iTat\ dox+}$ (the direction of compensation if there was) (Figure 4b-d).

Next, we will consider three situations that have occurred with gene expression of different signaling and metabolic pathways.

(i) As described above, pathways related to *DNA replication* (hsa03030), ribosome biogenesis (*Ribosome*, hsa03010; *Ribosome biogenesis in eukaryotes*, hsa03008), RNA synthesis and processing (*RNA polymerase*, hsa03020; *Spliceosome*, hsa03040), were suppressed by the acute Tat exposure, but were found to be restored to baseline gene expression level upon chronic exposure to Tat (Figure 4b). Importantly, these processes were not detected as dysregulated when we compared RPMI^{Tat} and the control cell line, thus they were downregulated by inducible Tat expression and then their activity was recovered during chronic Tat exposure. Interestingly, *TFAP4*, a transcriptional repressor of HIV-1 (Imai & Okamoto, 2006), was found among downregulated genes in RPMI$^{iTat\ dox+}$ cells, but its expression was restored in RPMI^{Tat} cells.

(ii) Gene categories upregulated by acute Tat exposure included highly interconnected signaling pathways (*Ras signaling pathway* (hsa04014), *Rap1 signaling pathway* (hsa04015), *Phospholipase D signaling pathway* (hsa04072), *Phosphatidylinositol signaling system* (hsa04070), *Focal adhesion* (hsa04510)), involved in many important cellular processes, such as cell proliferation and survival, cytoskeletal rearrangement, and cell motility. Comparison of the transcriptomic profiles of RPMI^{Tat} and RPMI$^{iTat\ dox+}$ showed that prolonged chronic Tat action suppressed the categories activated by the acute Tat action (Figure 4c). The same pattern was observed for pathways related to intercellular junctions, and cell adhesion (*Tight junction*, hsa04530; *Gap junction*, hsa04540; *Focal adhesion*, hsa04510), cytoskeletal organization (*Regulation of actin cytoskeleton*, hsa04810) and *Leukocyte*

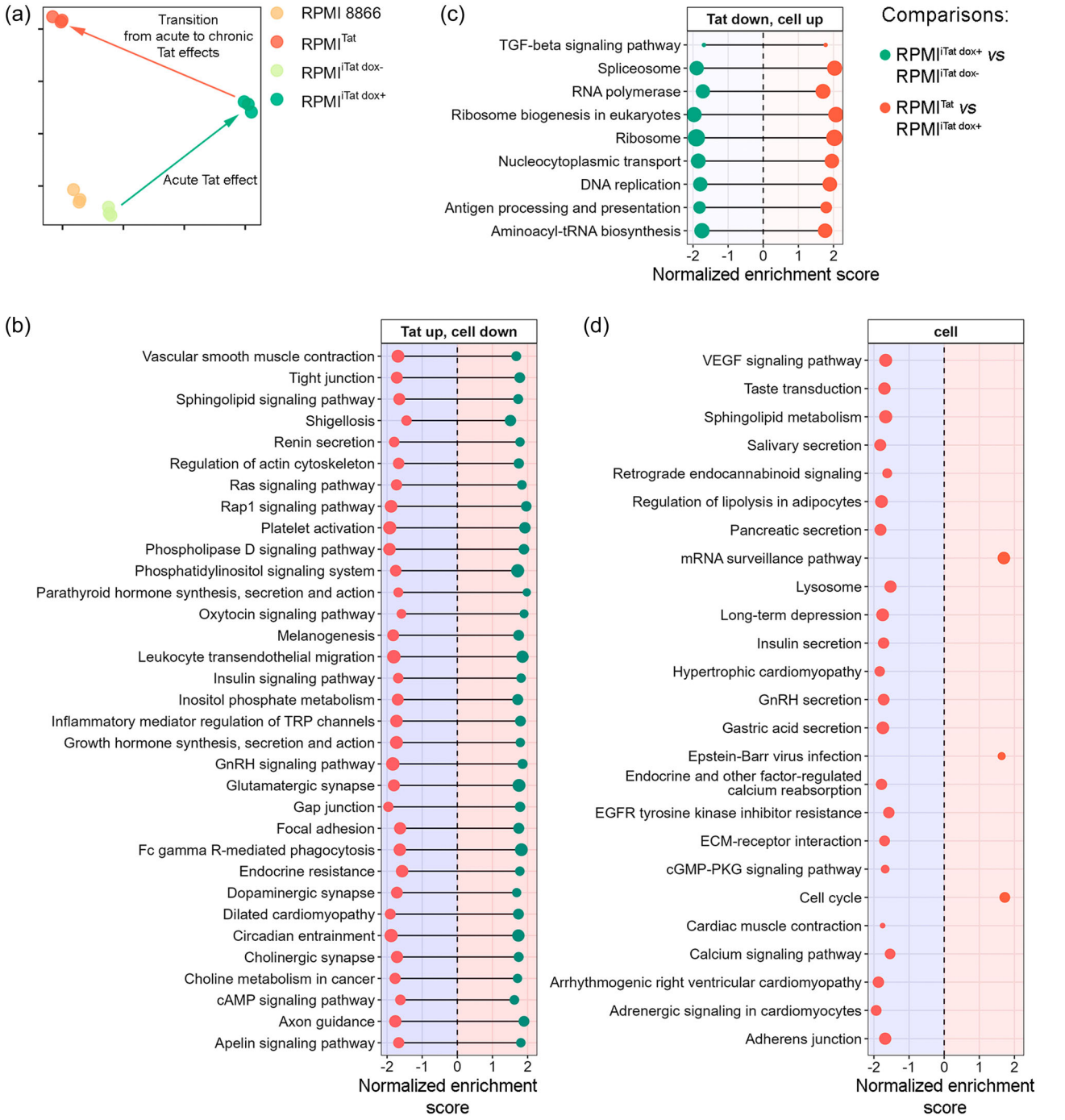

FIGURE 4 Effects of the acute and chronic Tat exposure. (a) Schema of the conducted comparisons in the principal components space. Green arrow represents RPMI$^{iTat\ dox+}$ vs RPMI$^{iTat\ dox-}$ comparison, red arrow - RPMI^{Tat} vs RPMI$^{iTat\ dox+}$ comparison. (b-d) Comparison of possible Tat effects and cellular reactions on the Tat presens (comparisons RPMI$^{iTat\ dox+}$ vs RPMI$^{iTat\ dox-}$ and RPMI^{Tat} vs RPMI$^{iTat\ dox+}$). Three situations are possible: Tat upregulates KEGG pathways, cell compensate (downregulate) them (b); Tat downregulates KEGG pathways, cell compensate (upregulate) them (c); chronic Tat exposure disregulates KEGG pathways that were not affected by acute Tat action (d).

transendothelial migration (hsa04670). We also noted several activated proteins whose expression was suppressed by chronic exposure. For example, expression of the transcription factor GLI1, which mediates sonic hedgehog signaling, regulates tight junction protein expression, and has been implicated in Tat-associated blood-brain barrier disruption (Khan et al., 2022), was also upregulated by acute Tat exposure and downregulated by chronic Tat exposure of B cells. The expression of genes associated with Tat uptake by bystander cells (genes of heparan sulfate proteoglycans, integrins and *LRP1*) was deregulated in the same manner. Interestingly, the expression of the caveolin 1 gene (*CAV1*), essential for caveolin-mediated Tat internalization

(Ferrari et al., 2003; Szewczyk-Roszczenko et al., 2023)(Ferrari et al., 2003), was downregulated in RPMI$^{iTat\ dox+}$ cells and upregulated in RPMI^{Tat} cells.

(iii) In some cases, the KEGG pathways changed only in RPMI$^{iTat\ dox+}$ cells. For example, several categories associated with anti-pathogenic cellular response (*Yersinia infection* (hsa05135), *Neutrophil extracellular trap formation* (hsa04613), *Human papillomavirus infection* (hsa05165), *C-type lectin receptor signaling pathway* (hsa04625), *Amoebiasis* (hsa05146)) were activated by acute Tat exposure, and they were maintained by chronic Tat action (Figure 3d). This seems logical as the anti-pathogenic reactions induced by acute Tat exposure were not suppressed later. On the contrary, as described above, the number of activated anti-pathogenic pathways increased upon chronic Tat exposure.

3.5 | Proteolysis reduces the HIV-1 Tat content in RPMI 8866 cells and the pro-viral effects of Tat

Transcriptomic data suggest the ability of B cells to compensate for acute effects, apparently by activating anti-pathogenic mechanisms and the innate immune system. We have previously observed that Tat-EGFP protein expressed in RPMI 8866 cells was partially degraded (Valyaeva et al., 2022). Therefore, we focused on changes in the expression of genes related to proteasomal ubiquitin-dependent protein degradation.

To investigate the possible deregulation of ubiquitin-mediated protein degradation pathways induced by acute or chronic Tat exposure, we examined genes associated with KEGG pathway hsa04120 (*Ubiquitin mediated proteolysis*). Although this gene category was not highlighted by GSEA, the suppression of even a few genes can lead to the inhibition of the whole metabolic pathway. *Ubiquitin mediated proteolysis* pathway included genes with elevated and suppressed expression levels in RPMI$^{iTat\ dox+}$ cells (Supporting Information: Figure S5). The set of ubiquitin-conjugating (E2) enzyme genes were mostly downregulated, while HECT type E3 genes were upregulated by acute Tat exposure. Conversely, almost no gene associated with ubiquitin mediated proteolysis exhibited altered expression level in RPMI^{Tat} compared to the control cell line, which might indicate the compensatory reactions during chronic Tat exposure.

Thus, data of transcriptomic analysis verified by RT-qPCR indicate that Tat degradation may be suppressed in case of acute Tat exposure, and normalized or even activated in case of chronic Tat exposure. To ascertain the supposition that Tat degradation is activated in RPMI^{Tat} cells, we performed a comparative study of *Tat* mRNA and Tat protein levels in RPMI^{Tat} and RPMI$^{iTat\ dox+}$ cells. *Tat* mRNA level at 24 h of doxycycline treatment was substantially higher in RPMI$^{iTat\ dox+}$ cells than in RPMI^{Tat} cells (Figure 1a), therefore, we used the 12 h doxycycline incubation for this analysis. We measured the relative *Tat* mRNA and Tat protein levels by RT-qPCR and Western blot analysis, respectively. RPMI^{Tat} cells contained slightly more *Tat* mRNA compared to RPMI$^{iTat\ dox+}$ cells (Figure 5a). However, Tat levels were significantly higher in RPMI$^{iTat\ dox+}$ cells (Figure 5b, C; Supporting Information: Figure S6). This indirectly supported the suggestion that Tat was strongly degraded in RPMI^{Tat} cells compared to RPMI$^{iTat\ dox+}$ cells.

To test whether proteasomes are involved in Tat degradation, we incubated cells in the presence of the proteasome inhibitor Bortezomib. The presence of Bortezomib resulted in a significant increase in Tat protein levels in RPMI^{Tat} cells at 100 nM inhibitor concentration, indicating that proteasome degradation of HIV-1 Tat was indeed increased in the case of RPMI^{Tat} cells (Figure 5d). In addition, we used several inhibitors of different ubiquitin-activating enzymes: Pevonedistat, NSC697923, and Thalidomide (Supporting Information: Figure S7). All these inhibitors resulted in a significant increase in HIV-1 Tat levels in RPMI^{Tat} cells after 6 hours of incubation (Figure 5e-g; Supporting Information: Figure S8), indicating that HIV-1 Tat in RPMI^{Tat} cells was indeed substantially degraded via the ubiquitin-dependent proteasome pathway. Intriguingly, higher doses of proteasome inhibitors had no effect on Tat degradation. This may be accounted for by the fact that in some cells, high concentrations of bortezomib (and potentially other proteasome inhibitors) induce resynthesis of some proteasomal genes, e.g. PSMB5 (Oerlemans et al., 2008); this may account for an increased Tat degradation.

4 | DISCUSSION

We previously noted that HIV-1 Tat stably expressed in RPMI 8866 cells did not show a robust effect on cell proliferation or programmed death, and speculated that the effect of HIV-1 Tat might be compensated by cells (Valyaeva et al., 2022). If this assumption is correct, the changes in gene expression will be different in the case of acute and chronic action of Tat on cells. To test this hypothesis, here we compared the transcriptomes of cells in which Tat expression was dependent on either the inducible tetracycline promoter (RPMI^{iTat} cells) or the constitutive EF-1α promoter (RPMI^{Tat} cells); these two cell lines allowed us to study the effects of acute and chronic Tat exposure on cells, respectively. In case of inducible expression, RPMI^{iTat} cells did not have time for realization of antiviral programs, and thus allowed to distinguish Tat effects per se (acute effects). Therefore, we used a relatively short doxycycline treatment (16 h in RNA-seq experiments or 24 h in other experiments) for the analysis of acute Tat effects. Cells with stable Tat expression (RPMI^{Tat}) were used to study chronic Tat effects, i.e. the results of cellular compensation of Tat effects.

We first found that acute Tat exposure led to the suppression of cell growth by inhibiting cell cycle progression and accumulation of cells in the G1/G0 phase of the cell cycle. Changes in the cell transcriptome explain this perturbation. The expression of a higher number of genes was altered with inducible Tat expression compared to stable Tat expression. Acute Tat exposure induced suppression of genes associated with replication, splicing, ribosome biogenesis, etc. Thus, Tat suppressed genes involved in key metabolic processes required for cell proliferation. On the contrary, in the case of chronic

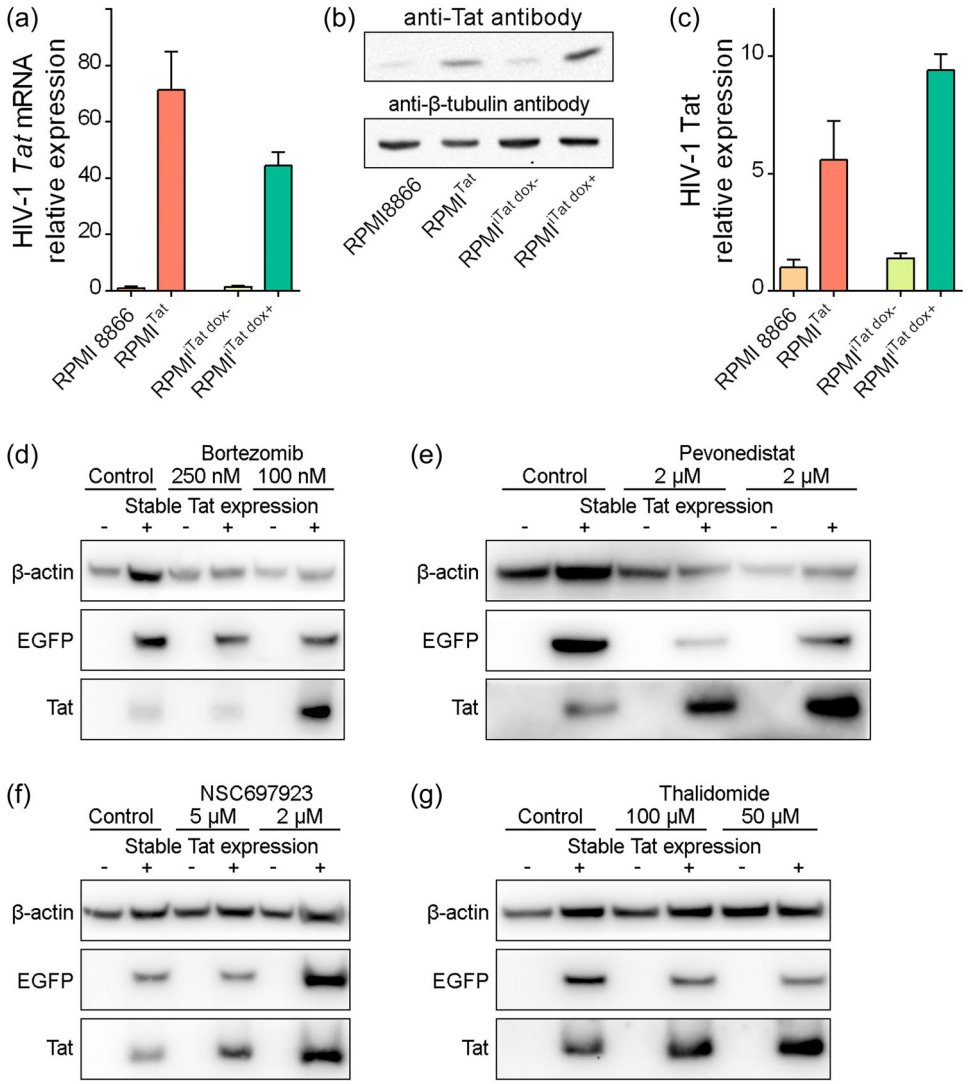

FIGURE 5 HIV-1 Tat is partially proteolyzed during chronic expression. (a) RT-qPCR analysis of *Tat* mRNA levels in cells with acute and chronic Tat expression. After 12 h incubation, *Tat* mRNA levels in RPMI$^{iTat\ dox+}$ cells were lower than in RPMI^{Tat} cells. (b) At the protein level, Tat content was higher in RPMI$^{iTat\ dox+}$ cells compared to RPMI^{Tat} cells (representative western blot). The background labeling is visible in RPMI8866 and RPMI$^{iTat\ dox-}$ cells. (c) Tat content in RPMI$^{iTat\ dox+}$ cells was higher than in RPMI^{Tat} cells. (d) The proteasome inhibitor Bortezomib inhibits the degradation of HIV-1 Tat. (e-g) Effects of inhibitors of different ubiquitin-activating enzymes: Pevonedistat (e), NSC697923 (f), and Thalidomide (g) (see also Supporting Information: Figure S7). The presence of inhibitors of proteasomes or ubiquitin-activating enzymes led to a substantial increase in Tat protein content in RPMI^{Tat} cells.

Tat action, the main event was the upregulation of genes involved in innate immunity and regulation of various cellular antipathogenic (including antiviral) responses.

The comparison of cells with induced and stable expression of HIV-1 Tat (RPMI$^{iTat\ dox+}$ vs. RPMI^{Tat} cells) demonstrated that the observed effect of stable Tat protein expression might actually represent the response of a cell to Tat protein. A substantial part of the KEGG pathways that were either suppressed or activated by Tat RPMI$^{iTat\ dox+}$ cells were, on the contrary, activated or suppressed in cells with stable Tat expression (RPMI^{Tat}). These processes were not detected as dysregulated when we compared RPMI^{Tat} and control cells, thus their activity was dysregulated upon acute Tat exposure and restored during prolonged chronic Tat exposure.

Next, we sought to identify possible molecular mechanisms by which cells adapt to the presence of Tat. The compensation of Tat activity may be due to several mechanisms, but the activation of ubiquitin-dependent proteolysis appears to be the key one. The experimental data obtained suggested that proteasomes were quite active in digesting Tat protein as treatment with proteasome inhibitors increased the amounts of Tat in cells; this was particularly evident in RPMI^{Tat} cells (chronic Tat exposure), where the presence of the protein was very difficult to detect at the protein level. In fact, the cell degraded Tat, thereby compensating for the acute effects of Tat protein. With apparent simplicity, this method of compensation seems very appropriate, because in this case the cell not only eliminates the symptoms of the disease but also destroys, as far as possible, its cause.

According to published data, treatment with the proteasome inhibitors increased the efficiency of HIV-1 infection, leading to the hypothesis that the proteasome degrades incoming virions as part of the intracellular antiviral defense (Butler et al., 2002; Schwartz et al., 1998; Wei et al., 2005). However, some data obtained suggest that a proteasome inhibitor (MG-132) may also promote transduction by inducing G2/M cell cycle arrest (Dueck & Guatelli, 2007; Groschel & Bushman, 2005). However, the situation in the case of expression of a single viral protein seems to be much simpler compared to a viral infection, and here even a simple mechanism, proteasomal degradation of the protein, can work quite effectively. It is also important to consider that the efficiency of proteasome degradation may vary in different cell types. In particular, it was shown that the proteasome inhibitor MG-132 could significantly prevent HIV-1 Tat protein degradation in Tat-overexpressing HeLa cells, but had only a moderate effect in preventing Tat protein degradation in Jurkat T cells (Xu & Zhang, 2022). It should be also noted that a large number of transcription factors are regulated by ubiquitin-proteasome system (Bathish et al., 2022; Fujinaga et al., 2023; Lee et al., 2023; Thomas & Tyers, 2000). The precise mechanism by which HIV-1 Tat exerts its influence on proteasomal protein degradation remains unclear. Published data suggest that this effect may involve a direct interaction between HIV-1 Tat and proteasomes. Indeed, HIV-1 Tat strongly inhibits the peptidase activity of the 20S proteasome (Seeger et al., 1997) and alters proteasome composition and enzymatic activity in T cells (Gavioli et al., 2004). This issue requires further investigation.

Thus, our data show that the Tat protein can have a rather strong effect on a cell, which is expressed as a change in the expression of genes involved in the realization of key cellular processes. Cells are able to compensate for these effects, but the development of pathological processes, including B-cell lymphoma, in HIV-infected patients directly indicates that this compensation does not completely eliminate the effects of the protein. Lymphomagenesis could be due to imperfection of antipathogenic mechanisms. It seems that therapeutic intervention on cellular antiviral mechanisms may be one of the ways to prevent cancer induced by chronic Tat action.

AUTHOR CONTRIBUTIONS

Yegor S. Vassetzky and Eugene V. Sheval conceived and designed the study. Anna A. Valyaeva, Maria A. Tikhomirova, Junyi Feng, Anastasia A. Zharikova, Daria M. Potashnikova, Yana R. Musinova, Andrey A. Mironov, Yegor S. Vassetzky and Eugene V. Sheval contributed to the acquisition or interpretation of data. Anna A. Valyaeva, Yegor S. Vassetzky and Eugene V. Sheval drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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