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Chronic HIV-1 Tat action induces HLA-DR downregulation in B cells: A mechanism for lymphoma immune escape in people living with HIV

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Abstract

Despite the success of combination antiretroviral therapy, people living with human immunodeficiency virus (HIV) still have an increased risk of Epstein–Barr virus (EBV)-associated B cell malignancies. In the HIV setting, B cell physiology is altered by coexistence with HIV-infected cells and the chronic action of secreted viral proteins, for example, HIV-1 Tat that, once released, efficiently penetrates noninfected cells. We modeled the chronic action of HIV-1 Tat on B cells by ectopically expressing Tat or TatC22G mutant in two lymphoblastoid B cell lines. The RNA-sequencing analysis revealed that Tat deregulated the expression of hundreds of genes in B cells, including the downregulation of a subset of major histocompatibility complex (MHC) class II-related genes. Tat-induced downregulation of *HLA-DRB1* and *HLA-DRB5* genes led to a decrease in HLA-DR surface expression; this effect was reproduced by coculturing B cells with Tat-expressing T cells. Chronic Tat presence decreased the NF- κ B pathway activity in B cells; this downregulated NF- κ B-dependent transcriptional targets, including MHC class II genes. Notably, *HLA-DRB1* and surface HLA-DR expression was also decreased in B cells from people with HIV. Tat-induced HLA-DR downregulation in B cells impaired EBV-specific CD4+ T cell response, which contributed to the escape from immune surveillance and could eventually promote B cell lymphomagenesis in people with HIV.

Abbreviations: ANOVA, analysis of variance; BL, Burkitt's lymphoma; cART, combination antiretroviral therapy; CFSE, carboxyfluorescein diacetate succinimidyl ester; CHIP-qPCR, chromatin immunoprecipitation quantitative PCR; DAPI, 4',6-diamidino-2-phenylindole; DEGs, differentially expressed genes; E/T, effector/target; EBNA-1, Epstein–Barr virus nuclear antigen 1; EBV, Epstein–Barr virus; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GO, gene ontology; GO BP, gene ontology biological process; gp42, glycoprotein 42; HIV, human immunodeficiency virus; IFN γ , interferon γ ; IGH, immunoglobulin heavy chain; IKK, I κ B kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LTR, long terminal repeat; MHC, major histocompatibility complex; ORA, overrepresentation analysis; PBMC, peripheral blood mononuclear cells; PMA, phorbol-12-myristate-13-acetate; P-TEFb, positive transcription elongation factor b; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean; shRNA, small hairpin RNA; Tat, transactivator of transcription; TF, transcription factor.

Coline Hugot, and Yana Kozhevnikova contributed equally to this work.

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KEYWORDS

B cells, CD4+ T cells, EBV, HIV-1, MHC class II, NF- κ B pathway, RNA-sequencing, Tat

1 | INTRODUCTION

Over 38 million people worldwide are infected with the human immunodeficiency virus (HIV) (<http://www.who.int/hiv/en/>). People living with HIV experience marked improvements in terms of mortality and quality of life since the introduction of combination antiretroviral therapy (cART) that blocks HIV replication and restores CD4+ T cell counts.¹ Nevertheless, the high prevalence of certain types of malignancies in the cART era, such as B cell lymphomas,² suggests a possible role of factors other than immune suppression in the pathogenesis of HIV-associated cancers.

Compared to the general population, people living with HIV are 12 times more likely to be diagnosed with B cell non-Hodgkin lymphomas, including diffuse large B-cell lymphoma and Burkitt's lymphoma (BL), and eight times more likely to be diagnosed with Hodgkin lymphoma.³ The molecular mechanisms of these lymphomas are quite different, which implies that there might be a common B cell lymphoma-predisposing factor in people with HIV. It was recently shown that even defective HIV proviruses (>95% of proviruses in the peripheral blood of cART-treated people living with HIV are defective, i.e., incapable of virus production) still produce viral proteins.⁴ Among them, the HIV transactivator of transcription (Tat) protein is particularly interesting, since it is actively released by infected cells,⁵ can be found in the bloodstream,⁶ and in the cerebrospinal fluid⁷ in people with HIV under cART. Due to its cell-penetration capacities, Tat efficiently penetrates neighboring cells.⁵ Tat presence was detected in HIV-associated B cell lymphomas.⁸

In infected cells, Tat activates HIV transcription through the release of a positive transcription elongation factor b (P-TEFb) from its inactive state bound to the inhibitory 7SK small nuclear ribonucleoprotein complex and recruitment of active P-TEFb to the HIV trans-activation response RNA element at the 5' end of stalled nascent HIV-1 transcripts.^{9,10} This transactivation activity of Tat depends on cysteine residue at position 22.¹⁰ The underlying mechanisms of Tat effects in noninfected cells, including B cells, are much less studied, although it is estimated that up to 2/3 of Tat is exported from infected cells and is efficiently uptaken by neighboring cells via endocytosis.^{5,11} We have recently shown that the acute action of Tat induced oxidative stress and DNA damage in B cells within the first 6 h and promoted genetic instability and chromosomal aberrations¹² as well as *MYC* relocalization next to the immunoglobulin heavy chain *IGH* locus (potential translocation partners, characteristic for BL).⁶ The long-term effects of Tat action on B cells remain elusive.

In the current study, we unraveled the effects of chronic HIV-1 Tat action on B cells. We found that Tat downregulated the expression of HLA-DR by inhibiting the NF- κ B pathway activity,

which led to a decrease in CD4+ T cells recognition of Epstein-Barr virus (EBV)-transformed B cells, thus leading to the acquisition of the cancer hallmark of immune evasion and B cell lymphoma predisposition in people living with HIV.

2 | MATERIALS AND METHODS

2.1 | Blood collection, peripheral blood mononuclear cells (PBMCs), and primary B cell isolation

Blood samples from HIV-positive individuals with written informed consent were collected at Hôpital Saint-Louis, following French legislation. The first patient cohort was analyzed for *HLA-DRB1* and *HLA-DRB5* mRNA expression in B cells (agreement 2016-A01316-45).^{6,12} The second patient cohort was analyzed for HLA-DR surface expression in B cells (agreement VIHEBV). Clinicopathological details for these cohorts are in Tables 1 and 2. Tat serum concentrations were determined using dot blotting and enzyme-linked immunosorbent assay. Blood samples from anonymous, age-, and sex-matched healthy donors were obtained from Etablissement Français du Sang, Hôpital Saint-Louis, following French legislation (agreement number 14/EFS/033). Primary human B cells were isolated by negative selection from total PBMCs.

2.2 | Plasmids and cloning

The long terminal repeat (LTR)-TurboRFP plasmid harbored the HIV-1 3' LTR fragment and a promoterless TurboRFP gene within the pTurboRFP-PRL plasmid (Table S1). For the NF- κ B-mCherry reporter plasmid, five NF- κ B response element repeats and a minimal promoter (Table S2) were incorporated in the mCherry2-C1 plasmid. Small hairpin RNA (shRNA) targeting the human *CIITA* gene and a scrambled shRNA sequence (Table S2) were cloned into the pLKO.1 plasmid as previously described.¹³

2.3 | Cell lines

Human EBV-transformed B lymphoblastoid cell line RPMI8866, freshly EBV-transformed B lymphoblastoid cell line from healthy donor AS (BLAS), immortalized T cell line Jurkat, Jurkat that stably expresses HIV-1 Tat (Jurkat-Tat), EBV-negative BL cell line Ramos, monocytic THP-1 cell line, neuroblastoma SH-SY5Y cell line, human embryonic kidney 293T cell line and their derivatives were used in

TABLE 1 The main clinicopathological features of people living with HIV from the first cohort investigated in this study.

#	Age	Sex	CD4+ T cells ($\times 10^6/L$)	Plasma HIV RNA (\log_{10} copies/mL)	cART	cART regimen	Effective cART duration (months)	Serum Tat concentration (ng/mL)	HLA-DRB1 relative expression in B cells	HLA-DRB5 relative expression in B cells
1	69	M	20	6.37	No		0	0.34	0.01	0.00
2	48	M	247	4.83	No		0	5.72	1.07	0.95
3	51	F	556	<1.3	Yes	3TC-ABC-NVP	102	0.21	0.10	0.73
4	49	M	683	<1.3	Yes	3TC-ABC-DRV-RTV	19	0.67	0.44	1.42
5	64	M	737	<1.3	Yes	3TC-ABC-DTG	203	0.78	0.81	0.54
6	59	M	525	<1.3	Yes	FTC-TDF-NVP	66	1.14	1.20	0.79
7	48	F	535	<1.3	Yes	3TC-ABC-DTG	59	1.55	0.82	1.67
8	56	F	502	<1.3	Yes	RPV-FTC-TDF	100	2.83	0.76	1.06
9	62	F	536	<1.3	Yes	3TC-ABC-DTG	73	9.02	0.60	2.20
10	66	M	527	1.78	Yes	3TC-ABC-ATV	98	1.7	0.55	1.63

Abbreviations: 3TC, lamivudine; ABC, abacavir; ATV, atazanavir; cART, combination antiretroviral therapy; DRV, darunavir; DTG, dolutegravir; FTC, emtricitabine; NVP, nevirapine; RPV, rilpivirine; RTV, ritonavir; TDF, tenofovir disoproxil fumarate.

TABLE 2 The main clinicopathological features of people living with HIV from the second cohort investigated in this study.

#	Age	Sex	CD4+ T cells ($\times 10^6/L$)	Plasma HIV RNA (copies/mL)	Whole blood EBV PCR (log)	cART start date	cART regimen	Serum Tat concentration (ng/mL)	Relative HLA-DR surface expression
1	69	M	656	108	3.2	1997	BIC-FTC-TAF	6.04	0.75
2	47	M	816	<20	<2.18	2015	FTC-EVG-COBI-TAF	6.29	0.80
3	48	M	727	<20	3.07	2012	DTG-RPV	4.63	0.39
4	69	F	391	<20	<2.18	2015	3TC-ABC-DTG	<2.5	0.62
5	29	F	563	<20	<2.18	2020	BIC-FTC-TAF	<2.5	1.01
6	70	F	757	<20	<2.18	2008	3TC-ABC-DTG	<2.5	0.91
7	47	F	644	<20	2.61	2010	BIC-FTC-TAF	<2.5	0.86
8	57	M	336	51	3.18	2014	3TC-ABC-DRV-RTV	<2.5	0.79
9	69	M	366	<20	<2.26	1995	3TC-ABC-NVP	<2.5	1.31
10	77	M	374	<20	<2.26	2003	3TC-ABC-DTG	<2.5	1.08
11	59	M	499	23	2.33	1995	3TC-ABC-DTG	<2.5	0.83
12	46	F	475	<20	<2.26	2008	FTC-EVG-COBI-TAF	4.02	0.68
13	61	M	359	<20	2.86	2011	FTC-EVG-COBI-TAF	<2.5	0.75
14	59	M	905	<20	3.73	2021	3TC-ABC-DTG	<2.5	0.75
15	63	M	500	<20	2.61	2000	FTC-EVG-COBI-TAF	<2.5	0.71
16	62	F	1428	41	<2.26	1993	BIC-FTC-TAF	<2.5	1.02
17	55	M	468	76100	<2.26	1997	FTC-EVG-COBI-TAF	<2.5	0.82

Abbreviations: 3TC, lamivudine; ABC, abacavir; BIC, bictegravir; cART, combination antiretroviral therapy; COBI, cobicistat; DRV, darunavir; DTG, dolutegravir; EBV, Epstein-Barr virus; EVG, elvitegravir; FTC, emtricitabine; HIV, human immunodeficiency virus; NVP, nevirapine; RPV, rilpivirine; RTV, ritonavir; TAF, tenofovir alafenamid.

the study (Table S1). Cell lines that stably expressed enhanced green fluorescent protein (EGFP), EGFP-Tat or EGFP-TatC22G were produced through lentiviral transduction as described previously.¹⁴ Cells were handled, treated, and stained as described in Supporting Information. Cells were electrotransfected as previously described,¹⁵ with the specific parameters outlined in Table S3.

2.4 | Generation of EBV-specific CD4+ T cell line and the analysis of their reactivity

A human EBV-specific CD4+ T cell line was generated from healthy donor AS PBMCs. PBMCs, depleted for CD8+ T cells, were cocultured with autologous irradiated BLAS cells, with subsequent restimulations and interleukin-2 supplementation, resulting in a CD4+ T cell line. For interferon γ (IFN γ) production analysis, the CD4+ T cell line or PBMCs from the same donor were cocultured with various target cells at 40:1 effector/target (E/T) ratio, and CD4 and IFN γ staining were performed. Cytotoxicity assays employed a nonradioactive carboxyfluorescein diacetate succinimyl ester (CFSE)-based method, mixing sensitive BLAS target cells and control RPMI8866 target cells with effector CD4+ T cells to calculate specific lysis percentages (Supporting Information S6: Figure 1). The details are outlined in Supporting Information.

2.5 | Immunofluorescent staining and microscopy

For immunofluorescent staining, suspension cells adhered to poly-D-lysine-covered coverslips were fixed in 4% paraformaldehyde and permeabilized with 2% Triton X-100 for intracellular staining. Cells were stained with primary antibodies, followed by AF546 or AF647 secondary antibodies. Coverslips were mounted with 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium. Fluorescent images were acquired using Leica TCS SP8 Multiphoton Microscope (Leica Microsystems). Image analysis is detailed in Supporting Information.

2.6 | Western blot analysis

Western blot analysis with whole cell extracts was performed as previously described.¹⁶ For cytoplasmic and nuclear extracts, NE-PER nuclear and cytoplasmic extraction reagents were employed following the manufacturer's instructions. Proteins were resolved on 4%–12% polyacrylamide gels, transferred onto a polyvinylidene fluoride membrane, blocked and probed with the primary antibodies. Secondary peroxidase-conjugated antibodies were used to reveal bands. Densitometric analysis was carried out using ImageJ. The original uncropped western blot analysis images are presented Supporting Information S6: Figure 2.

2.7 | RNA extraction, sequencing, data analysis, reverse transcription, and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from cells using the NucleoSpin RNA kit. For BLAS cell lines, RNA-sequencing experiments were conducted as part of this study (GSE212499). For RPMI8866 cell lines, raw sequencing data was obtained from GSE182538. Reads were mapped to the human genome (GRCh38.p10) and counted using HISAT2 and featureCounts, respectively. Differential expression analysis was performed with DESeq2, and genes meeting the criteria of adjusted p -value < 0.05 and $|\log_2$ fold change| > 0.5 as compared to the initial cell lines (RPMI8866 or BLAS) were considered differentially expressed (see Table S4 for the gene list). Overrepresentation analysis (ORA) was carried out using clusterProfiler, and redundant terms were removed. Gene ontology biological process (GO BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used for annotation. Transcription factor (TF)-target enrichment analysis utilized the TRRUST TF-target database (2019) via the Enrichr server with a p -value cutoff of 0.05.

For qPCR, reverse transcription of total RNA was performed, and qPCR was conducted using Powerup SYBR Green Mastermix on a StepOnePlus Real-Time PCR System machine. Human cDNA primers were used (Table S5). The Pfaffl method with *RPL32* and *GAPDH* reference genes was employed for relative transcript level calculation.

2.8 | Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR)

Cells were cross-linked using formaldehyde, and the chromatin was sonicated to achieve 200–1000 bp fragments. ChIP was carried out overnight with magnetic beads and anti-RelA antibody or normal rabbit IgG. Beads were washed with different buffers, and DNA was eluted and purified. qPCR was performed to measure RelA binding to *HLA-DRB1* and *HLA-DRB5* promoter sequences using specific primers (Table S5). Data were normalized to input DNA and reported as % of input. Primers for promoter sequences were selected based on RelA binding data from the ReMap 2022 database (Supporting Information S6: Figure 3).

2.9 | Statistical analysis

Data were analyzed using GraphPad Prism 9 software (GraphPad Software Inc.). Student's unpaired t -tests were used to compare data between two groups. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test was used to determine differences between more than two groups that involved one factor. Two-way ANOVA followed by Tukey's post hoc test was used to determine differences between two or more groups that involved two factors.

Data are presented as a mean \pm standard error of the mean. The level of significance was set at $p < 0.05$.

3 | RESULTS

3.1 | HIV-1 Tat deregulated hundreds of genes in cultured B cells

To analyze the effects of chronic HIV-1 Tat presence on B cells, we used an RPMI8866 lymphoblastoid B cell line ectopically expressing either Tat or TatC22G (Tat deprived of transactivation capacity), RPMI8866^{Tat} or RPMI8866^{TatC22G}, respectively; both proteins were coupled to EGFP (hereafter referred to as GFP).¹⁷ We also used a new lymphoblastoid B cell line from a healthy donor (BLAS), established by immortalization of mature B cells with EBV. We constructed BLAS cells expressing Tat or TatC22G, hereafter referred to as BLAS^{Tat} or BLAS^{TatC22G}. Cells expressing GFP (RPMI8866^{GFP} or BLAS^{GFP}) and initial cell lines (RPMI8866 or BLAS) were used as controls (Figure 1A). The expression of Tat and GFP in these cell lines was confirmed by western blot analysis (Figure 1B,C). The transactivation assay with 3'-LTR-TurboRFP reporter plasmid confirmed that Tat substantially increased TurboRFP fluorescence compared to TatC22G, GFP, or the control, confirming that fusion with GFP did not perturb the transactivation functions of wild-type Tat, while the transactivation function of TatC22G was decreased (Supporting Information S6: Figure 4).

To detect possible changes in B cell physiology caused by chronic HIV-1 Tat presence, we performed an RNA-sequencing of BLAS cell lines and compared the results with the previously published transcriptomes of RPMI8866 cell lines (GSE182538).¹⁷ As expected, we confirmed that GFP expression did not alter significantly the transcriptome profile of RPMI8866 cells: only 20 upregulated and 60 downregulated genes were identified. Thus, the effect of GFP on gene expression in B cells could be neglected, therefore, for the BLAS line the RNA-sequencing of BLAS^{Tat}, BLAS^{TatC22G}, and BLAS cells was performed (Figure 1D). Our analysis revealed that 1372 genes (578 upregulated and 794 downregulated) were differentially expressed in RPMI8866^{Tat} versus RPMI8866 cells, and 4424 genes (2121 upregulated and 2303 downregulated) were differentially expressed in BLAS^{Tat} versus BLAS cells (Figure 1D). Comparable results were observed in cells expressing TatC22G: 1198 genes (526 upregulated and 672 downregulated) were differentially expressed in RPMI8866^{TatC22G} versus RPMI8866 cells, and 3486 genes (1542 upregulated and 1944 downregulated) were differentially expressed in BLAS^{TatC22G} versus BLAS cells (Figure 1D). A slightly higher proportion of genes were downregulated in the presence of either Tat or TatC22G. Among these genes, the majority were protein-coding genes but differentially expressed long noncoding RNAs were also identified (Supporting Information S6: Figure 5A,B). A considerably higher number of differentially expressed genes (DEGs) in BLAS cells can be explained by the fact that these cells were freshly immortalized and carried a normal genome of primary B cells, and

thus were more sensitive to any changes in their state, including viral protein expression. In culture, BLAS cells proliferated considerably slower than RPMI8866 cells and were more sensitive to culture conditions (data not shown).

To explore whether genes regulated by Tat action in B cells have any common biological functions, we next performed ORA and searched for activated and suppressed functional categories defined by GO BP and KEGG databases. GO BP terms significantly enriched in the set of 84 upregulated DEGs overlapped between RPMI8866^{Tat} versus RPMI8866 and BLAS^{Tat} versus BLAS comparisons (Figure 1E) included: regulation of neuron projection development and positive regulation of cell projection organization (p adjust = 0.011). No significantly enriched KEGG pathways were revealed for the same gene set. GO BP terms significantly enriched in the set of 146 downregulated DEGs overlapped between RPMI8866^{Tat} versus RPMI8866 and BLAS^{Tat} versus BLAS comparisons (Figure 1F) included: leukocyte cell-cell adhesion, positive regulation of T cell activation, T cell receptor signaling pathway, peptide antigen assembly with the major histocompatibility complex (MHC) class II protein complex, antigen processing, and presentation of exogenous peptide antigen via MHC class II (Figure 1G,H). The same gene set was significantly enriched in the following KEGG pathways: cell adhesion molecules, intestinal immune network for IgA production, hematopoietic cell lineage, viral myocarditis, and TNF signaling pathway (Figure 1I,J). To conclude, both in RPMI8866 and BLAS cells, Tat downregulated a subset of MHC class II genes (*HLA-DRB1*, *HLA-DRB5*, *HLA-DQA1*, *HLA-DQB1*) and thus Tat could potentially decrease CD4+ T cell activation in response to EBV-infected cells. As *HLA-DRB1* and *HLA-DRB5* were more markedly downregulated by Tat (log₂ fold changes -0.92 and -1.08, respectively in BLAS^{Tat} cells) compared to *HLA-DQA1* and *HLA-DQB1* (log₂ fold changes -0.73 and -0.85, respectively in BLAS^{Tat} cells), we further focused on HLA-DR expression regulation.

3.2 | HIV-1 Tat decreased HLA-DR expression in cultured B cells

We next confirmed the Tat-induced downregulation of HLA-DR genes by qPCR: *HLA-DRB1* mRNA expression was significantly decreased both in RPMI8866^{Tat} and RPMI8866^{TatC22G} as compared to RPMI8866 ($p = 0.0004$ and $p = 0.0111$, respectively, Figure 2A). *HLA-DRB5* mRNA expression was also significantly decreased both in RPMI8866^{Tat} and RPMI8866^{TatC22G} as compared to RPMI8866 ($p = 0.0001$ and $p = 0.0021$, respectively, Figure 2B). Accordingly, *HLA-DRB1* mRNA expression was significantly decreased both in BLAS^{Tat} and BLAS^{TatC22G} as compared to BLAS ($p = 0.0008$ and $p < 0.0001$, Figure 2A). *HLA-DRB5* mRNA expression was significantly decreased both in BLAS^{Tat} and BLAS^{TatC22G} as compared to BLAS ($p = 0.0247$ and $p = 0.0366$, Figure 2B). Both *HLA-DRB1* and *HLA-DRB5* mRNAs were upregulated in RPMI8866^{GFP} versus RPMI8866 ($p = 0.0006$ and $p = 0.0002$, Figure 2A,B). This result was further confirmed by the analysis of HLA-DR surface expression by flow cytometry: we observed that HLA-DR surface expression was

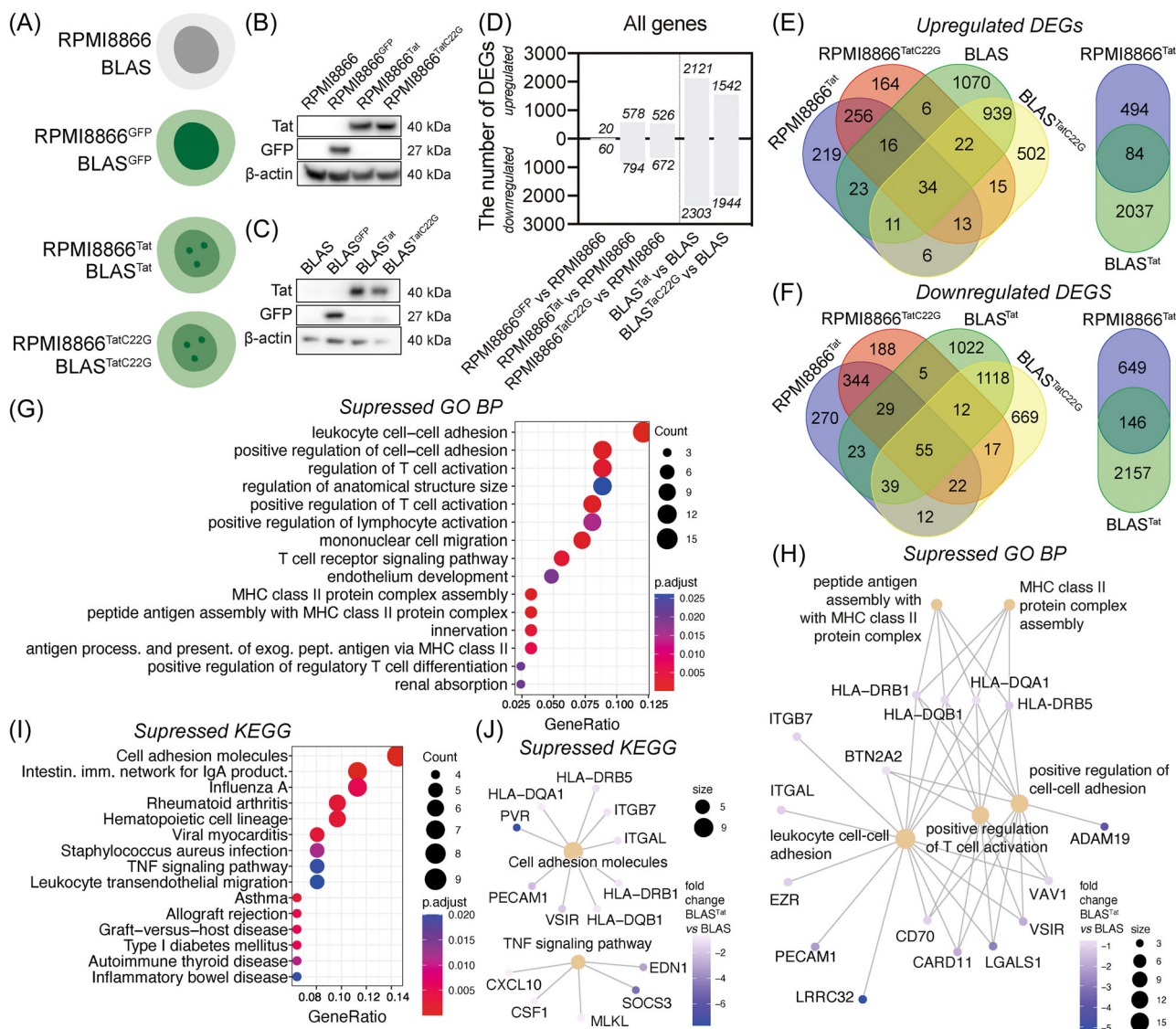


FIGURE 1 HIV-1 Tat protein presence affects the gene expression profile of lymphoblastoid B cells. (A) Cell lines that were used to analyze the action of HIV-1 Tat on cultured B cells: human EBV-transformed B lymphoblastoid cell line RPMI8866 and freshly EBV-transformed B lymphoblastoid cell line from healthy donor AS (BLAS, established by EBV [B95-8] immortalization of mature B cells), which were used as a negative control; cells expressing EGFP (RPMI8866^{GFP} and BLAS^{GFP}), which were used as a control of the possible effects of vector integration and the fluorescent tag; cells expressing HIV-1 Tat fused with EGFP (RPMI8866^{Tat} and BLAS^{Tat}); and cells expressing mutated HIV-1 TatC22G deprived of transactivation activity fused with EGFP (RPMI8866^{TatC22G} and BLAS^{TatC22G}). (B) Western blot analysis of Tat and GFP expression in BLAS cell lines. β -Actin was used as a loading control. (C) Western blot analysis of Tat and GFP expression in RPMI8866 cell lines. β -Actin was used as a loading control. (D) The number of all differentially expressed genes (DEGs) found in comparisons: RPMI8866^{GFP} versus RPMI8866, RPMI8866^{Tat} versus RPMI8866, RPMI8866^{TatC22G} versus RPMI8866, BLAS^{Tat} versus BLAS, BLAS^{TatC22G} versus BLAS. The RPMI8866^{GFP} versus RPMI8866 comparison revealed only small amount of DEGs, implying that the wild type cell lines (RPMI8866 and BLAS) could be used as control cell lines without taking into account the effect of GFP on gene expression. DEGs were defined as genes with p . adjust < 0.05 and $|\log_2$ fold change > 0.5 as compared to the initial cell lines (RPMI8866 or BLAS). (E) Venn diagrams demonstrating the overlap of upregulated DEGs between RPMI8866^{Tat} versus RPMI8866, RPMI8866^{TatC22G} versus RPMI8866, BLAS^{Tat} versus BLAS, and BLAS^{TatC22G} versus BLAS (left panel) and separately between RPMI8866^{Tat} versus RPMI8866 and BLAS^{Tat} versus BLAS (right panel). (F) Venn diagrams demonstrating the overlap of downregulated DEGs between RPMI8866^{Tat} versus RPMI8866, RPMI8866^{TatC22G} versus RPMI8866, BLAS^{Tat} versus BLAS, and BLAS^{TatC22G} versus BLAS (left panel) and separately between RPMI8866^{Tat} versus RPMI8866 and BLAS^{Tat} versus BLAS (right panel). (G) Overrepresentation analysis (ORA) of Tat-downregulated DEGs (overlap between RPMI8866^{Tat} versus RPMI8866 and BLAS^{Tat} versus BLAS), top 15 significantly enriched and nonredundant GO BP terms are shown. (H) Selected suppressed GO BP terms pathways and associated DEGs. (I) ORA of Tat-downregulated DEGs (overlap between RPMI8866^{Tat} versus RPMI8866 and BLAS^{Tat} versus BLAS), top 15 significantly enriched KEGG pathways are shown. (J) Selected suppressed KEGG pathways (hsa04514 and hsa04668) and associated DEGs. DEGs, differentially expressed genes; EBV, Epstein-Barr virus; GFP, green fluorescent protein; GO BP, gene ontology biological processes; KEGG, Kyoto encyclopedia of genes and genomes; ORA, overrepresentation analysis.

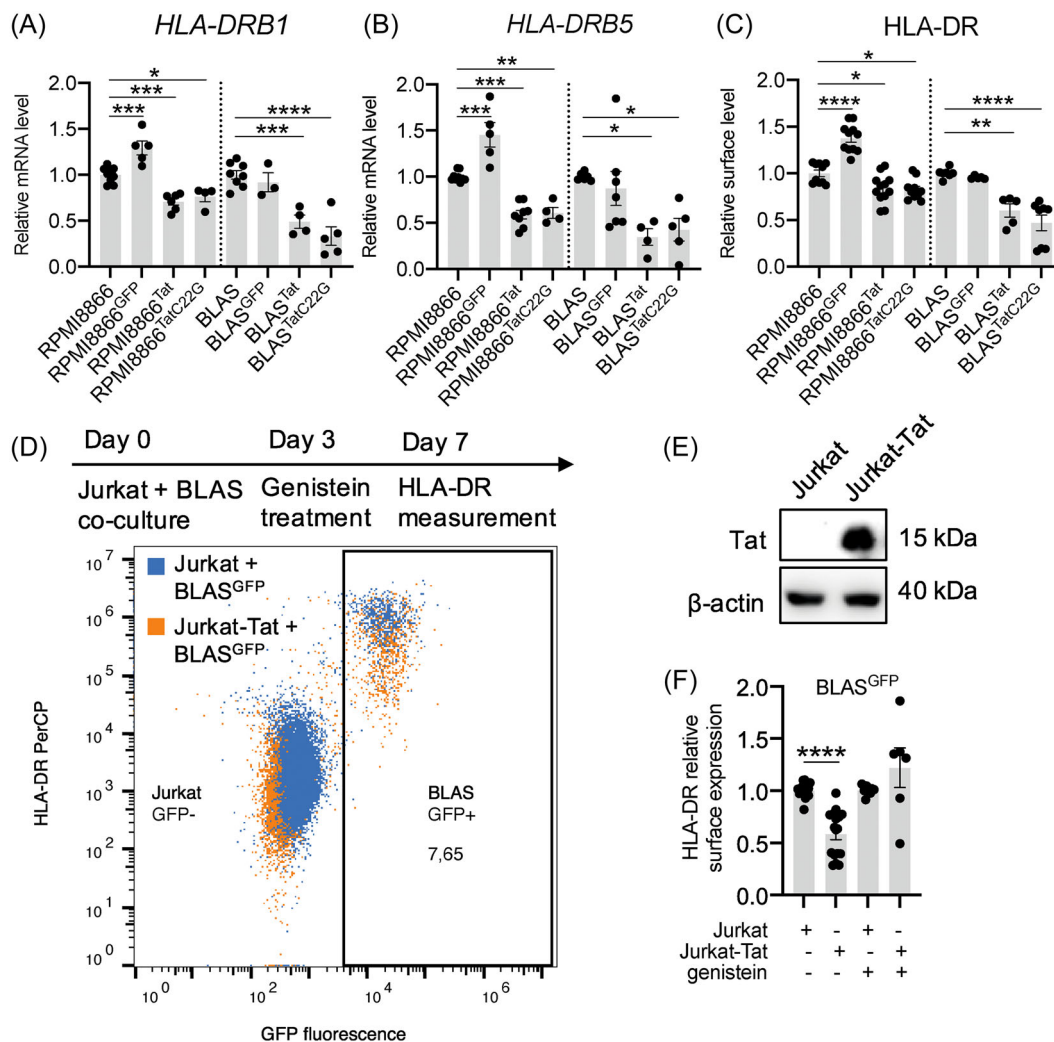


FIGURE 2 HIV-1 Tat decreases HLA-DR expression in cultured B cells. (A) The level of *HLA-DRB1* mRNA expression in studied cell lines (RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, RPMI8866^{TatC22G} and BLAS, BLAS^{GFP}, BLAS^{Tat}, BLAS^{TatC22G} cells). (B) The level of *HLA-DRB5* mRNA expression in studied cell lines (RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, RPMI8866^{TatC22G} and BLAS, BLAS^{GFP}, BLAS^{Tat}, BLAS^{TatC22G} cells). (A, B) The mRNA level of *HLA-DRB1* or *HLA-DRB5* was normalized to housekeeping gene expression; the normalization was done assuming the mean level of transcript in wild type cells (RPMI8866 or BLAS) to be 1. (C) The level of HLA-DR surface expression in studied cell lines (RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, RPMI8866^{TatC22G} and BLAS, BLAS^{GFP}, BLAS^{Tat}, BLAS^{TatC22G}) as analyzed by flow cytometry. HLA-DR surface relative expression was calculated as the median fluorescence intensity in the sample normalized relative to the median fluorescence intensity in the control (RPMI8866 or BLAS). (D) An experimental setup for the experiment of BLAS^{GFP} coculture with Jurkat or Jurkat expressing Tat (Jurkat-Tat) cells. Briefly, BLAS^{GFP} cells were mixed with Jurkat or Jurkat-Tat cells and cocultivated for 7 days, HLA-DR expression was measured in GFP+ cells. As a control, at Day 3 an aliquot of cocultivated cells was taken and 100 μ M genistein, an inhibitor of endocytosis, was added to the culture medium. A representative dot-plot for flow cytometry analysis of HLA-DR surface staining in BLAS^{GFP} cells (GFP+), cocultured with Jurkat (blue) or Jurkat-Tat (orange) cells. (E) Western blot analysis of Tat expression in Jurkat and Jurkat-Tat cell lines. β -Actin was used as a loading control. (F) The level of HLA-DR surface expression in BLAS^{GFP} cells cocultured with Jurkat or Jurkat expressing Tat (Jurkat-Tat) cells as analyzed by flow cytometry. HLA-DR surface relative expression was calculated as the median fluorescence intensity in the sample normalized relative to the median fluorescence intensity in the respective control. Data are presented as individual values, mean \pm SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, ANOVA, Tukey post hoc. ANOVA, analysis of variance; GFP, green fluorescent protein; SEM, standard error of mean.

decreased both in RPMI8866^{Tat} and RPMI8866^{TatC22G} compared to RPMI8866 ($p = 0.032$ and $p = 0.0295$, Figure 2C, Supporting Information S6: Figure 5C) and in BLAS^{Tat} and BLAS^{TatC22G} compared to BLAS ($p = 0.0024$ and $p < 0.0001$, respectively, Figure 2D, Supporting Information S6: Figure 5C). Thus, HIV-1 Tat presence decreased HLA-DR expression in B cells.

As a control, we also established a BL cell line Ramos and a monocytic cell line THP-1 that ectopically expressed HIV-1 Tat (Supporting Information S6: Figure 6). To ensure that the effect of Tat on HLA-DR surface expression was not limited to cells infected with EBV, we analyzed HLA-DR surface expression in EBV-negative Ramos cells (Supporting Information S6: Figure 6A,B) that stably

expressed Tat (Supporting Information S6: Figure 6C) and found that *HLA-DRB5* mRNA level, HLA-DR surface, and total expression were significantly decreased in Ramos^{Tat} cells compared to Ramos (Supporting Information S6: Figure 6E-I). On the contrary, Tat expression had no effect on the HLA-DR surface expression level in the monocytic THP-1 cells, where the overall HLA-DR expression level was low (Supporting Information S6: Figure 6D,L). Tat, however, increased the *HLA-DRB5* mRNA level and the percentage of HLA-DR-positive THP-1 cells (Supporting Information S6: Figure 6J,K,M). This indicates that HIV-1 Tat-dependent decrease in HLA-DR expression was specific to B cells and was not dependent on EBV infection.

Thus, HIV-1 Tat released from infected T cells could potentially act on B cells and decrease HLA-DR expression. We decided to confirm this by cocultivating BLAS cells with CD4+ T cells that expressed Tat (Jurkat-Tat) and a respective control (Jurkat) (Figure 2D,E). Jurkat-Tat cells were previously shown to efficiently secrete Tat protein.⁵ Briefly, BLAS^{GFP} cells were mixed with the Jurkat cells and cocultured for 7 days to allow for long-term effects of Tat to manifest, HLA-DR expression was then measured in BLAS^{GFP} cells (Figure 2D). The proposed approach to evaluate the long-term effects of Tat on B cells allows to overcome the difficulties related to the use of recombinant Tat protein (susceptibility to oxidation, quick degradation). We found that HLA-DR expression in BLAS^{GFP} cells cocultured with Jurkat-Tat cells decreased significantly as compared to BLAS^{GFP} cells cocultured with Jurkat cells ($p < 0.0001$, Figure 2F).

Tat is taken up by cells mainly through endocytosis.^{11,18} To confirm this, a fluorescently labeled Tat-derived cell-penetrating peptide (Tat CPP-Cy5) was used. Cells were first pretreated or not (control) for 1 h with genistein, an inhibitor of endocytosis,¹⁹ and then Tat CPP-Cy5 was added to cell medium for 30 min followed by extensive cell wash and subsequent analysis of Tat CPP-Cy5 cellular and nuclear penetration (Supporting Information S6: Figure 7). The addition of genistein drastically inhibited Tat CPP-Cy5 penetration in cells (Supporting Information S6: Figure 7). We next treated an aliquot of cocultivated cells with genistein as a control that HLA-DR downregulation was induced by Tat penetration into B cells in our coculture experiments (Figure 2K). When Tat entry in B cells was blocked by genistein, no decrease in HLA-DR expression in BLAS^{GFP} cells cocultured with Jurkat-Tat cells as compared to BLAS^{GFP} cells cocultured with Jurkat cells was observed (Figure 2M). These results confirm our RNA-sequencing data and demonstrate for the first time that chronic HIV-1 Tat secretion from infected CD4+ T cells can induce HLA-DR downregulation in B cells.

3.3 | HLA-DR expression is regulated by the NF- κ B pathway in cultured B cells

HIV-1 Tat reprograms host gene expression both directly through chromatin binding and transactivation/repression by controlling RNA polymerase II recruitment to target genes and indirectly, for example, through protein-protein interaction with host TFs.^{20,21} The mutation

of C22 in the transactivation domain largely disrupts Tat interaction with chromatin and direct regulation of gene expression (both stimulation and downregulation), favouring the indirect effects of Tat.²⁰ Our results demonstrated that in B cells, a high proportion of the genes were regulated by Tat indirectly, sharing a substantial overlap with TatC22G-regulated genes (Figure 1G,H, Supporting Information S6: Figure 9). This included *HLA-DR* genes, downregulated by both Tat and TatC22G (Figure 2). Consequently, we assumed that *HLA-DR* downregulation could be caused by Tat interaction with the TFs regulating MHC class II gene expression. HIV-1 Tat was previously shown to inhibit class II, MHC complex, transactivator (CIITA), a transcriptional regulator of MHC class II genes, by competing for the binding to cyclin T1, a component of P-TEFb complex.²² The interaction between HIV-1 Tat and human cyclin T1, however, requires C22 residue and TatC22G mutant can not bind cyclin T1,²³ and thus can not compete with CIITA. Our results indicated that both Tat and TatC22G decreased the expression of *HLA-DR* genes in B cells (Figure 2). Moreover, when we knocked down the expression of *CIITA* in RPMI8866 cells via shRNA, Tat was still able to downregulate *HLA-DR* expression as compared to GFP ($p < 0.0001$, ANOVA, Tukey's post hoc test) (Supporting Information S6: Figure 8). This indicates that the downregulation of *HLA-DR* expression by Tat in B cells is not exclusively dependent on CIITA and might involve interaction with other TFs.

To test this hypothesis, we performed TF-target enrichment analysis to predict potential TFs that could regulate the expression of a subset of downregulated genes common between RPMI8866^{Tat} and BLAS^{Tat} (Figure 3). We found that the highest overlap with the downregulated target gene set was for RelA and NF κ B1 TFs of the NF- κ B pathway (Figure 3A). Since indirect gene regulation in Tat-expressing cells is not dependent on C22 residue, we also performed the enrichment for DEGs common between Tat- and TatC22G-expressing RPMI8866 and BLAS cells. Again, RelA and NF κ B1 were significantly enriched with the highest overlap for the target gene set when considering the union (Figure 3B) and intersection (Figure 3C) for two cell lines. Thus, the gene downregulation in response to Tat or its mutant was potentially linked to a decrease in NF- κ B pathway activity, since an enrichment for NF- κ B-regulated targets was observed among downregulated genes.

We next decided to confirm that *HLA-DR* expression was regulated by the NF- κ B pathway in B lymphoblastoid cells. We treated RPMI8866 and BLAS cells with the inhibitors of NF- κ B activation: the inhibitor of I κ B kinase (IKK) Bay 11-7082, which inhibited both NF- κ B1 (classical) and NF- κ B2 (alternative) pathways, proteasome inhibitor MG132 that prevented pl κ B and p100 degradation and thus also inhibited both NF- κ B1 (classical) and NF- κ B2 (alternative) pathways, and inhibitor of RelB:p52 nuclear import SN52, which specifically inhibited NF- κ B2 (alternative) pathway. We then analyzed surface and total *HLA-DR* expression by flow cytometry and western blot analysis. We found that treatment with Bay 11-7082, MG132, or SN52 decreased ~twofold both surface and total *HLA-DR* expression in RPMI8866 B lymphoblastoid cells (Figure 3D-G, Supporting Information S6: Figure 10A-H). The same

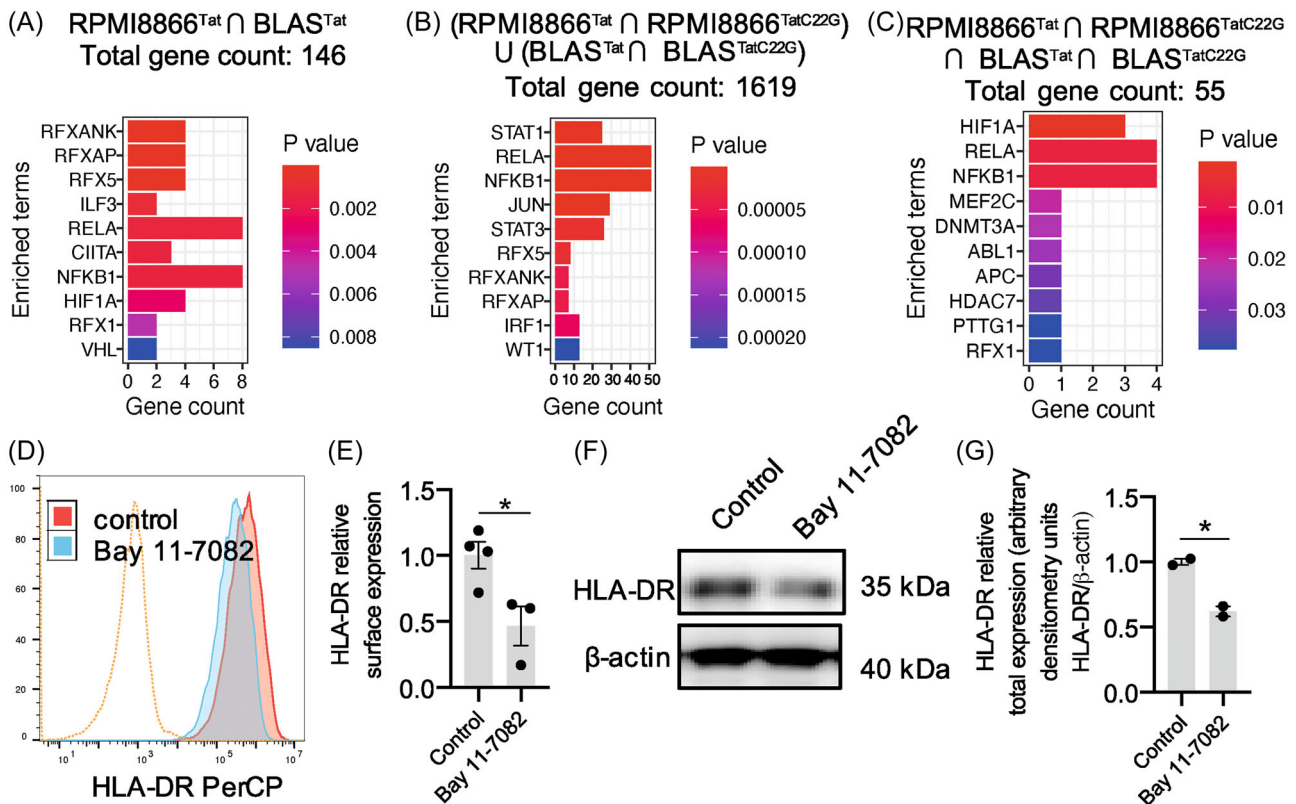


FIGURE 3 NF-κB pathway regulates HLA-DR expression in B cells. (A) Barplot visualizing the top 10 significantly enriched (p . adjust < 0.05) transcription factors (TF) potentially regulating downregulated genes, common between RPMI8866^{Tat} and BLAS^{Tat}, based on TRRUST TF-target database (2019). (B) Barplot visualizing the top 10 significantly enriched (p . adjust < 0.05) TFs potentially regulating downregulated genes, common between RPMI8866^{Tat} and RPMI8866^{TatC22G}, or BLAS^{Tat} and BLAS^{TatC22G}, based on TRRUST TF-target database (2019). (C) Barplot visualizing the top 10 significantly enriched (p . adjust < 0.05) TFs potentially regulating downregulated genes, common between RPMI8866^{Tat}, RPMI8866^{TatC22G}, BLAS^{Tat}, and BLAS^{TatC22G}, based on TRRUST TF-target database (2019). (D–G) RPMI8866 cells were treated for 24 h with 1 μM Bay 11-7082 or left untreated (control), HLA-DR expression was then analyzed by flow cytometry analysis and western blot analysis. (D) A representative histogram for flow cytometry analysis of HLA-DR surface staining in control RPMI8866 cells (red) and cells, treated with 1 μM Bay 11-7082 (blue). A negative control sample is shown for comparison (orange dotted line). (E) HLA-DR surface relative expression was calculated as the median fluorescence intensity in the sample normalized relative to the median fluorescence intensity in the control, averaged data from at least three biologically independent experiments are shown. (F) Western blot analysis of HLA-DR content in RPMI8866 cells. β-Actin was used as a loading control. A reproducible result is presented. (G) Densitometry analysis of HLA-DR content normalized to β-Actin. Data are presented as individual values, mean ± SEM, * p < 0.05, t -test. SEM, standard error of mean.

results were observed in BLAS cells (Supporting Information S6: Figure 10I–T). Thus, HLA-DR was regulated by the NF-κB pathway in B lymphoblastoid cells.

3.4 | Chronic HIV-1 Tat decreased classical NF-κB activation in B cells

We next tested whether NF-κB activation decreased in lymphoblastoid cells expressing Tat. To measure NF-κB activation, we analyzed the cytosolic and nuclear content of key proteins of the NF-κB signaling pathway: inhibitory protein IκBα and its phosphorylated form p-IκBα, transactivation-competent components RelA and RelB, and their partners p50 (NFKB1) and p52 (NFKB2) as well as their precursors (p105 and p100) (Figure 4A, Supporting Information S6: Figure 11). When NF-κB is activated, IκBα in the cytoplasm is

phosphorylated and degraded, which permits RelA/p50 (classical) or RelB/p52 (alternative) dimer translocation from the cytoplasm into the nucleus where they regulate gene expression.²⁴ The presence of Tat or TatC22G in cells led to an increased IκBα phosphorylation and a decrease in total IκBα content in the cytoplasm of RPMI8866 cells (Figure 4A,B, Supporting Information S6: Figure 11A,B) while RelA accumulated in the cytoplasm of Tat- or TatC22G-expressing cells and decreased in the nuclear compartment, which confirmed our *in silico* predicted decrease in the NF-κB activity (Figure 4A,C). No significant changes in RelB, NFKB1 and NFKB2 cytoplasmic/nuclear presence were detected (Supporting Information S6: Figure 11A–D, Figure 4A,D) while an increase in nuclear p-IκBα in Tat expressing cells was observed (Supporting Information S6: Figure 11A,B).

To corroborate the observed results, we also performed immunofluorescent staining of RelA and NFKB1 in RPMI8866 cells (Figure 4E,F). While in RPMI8866 and RPMI8866^{GFP} cells RelA

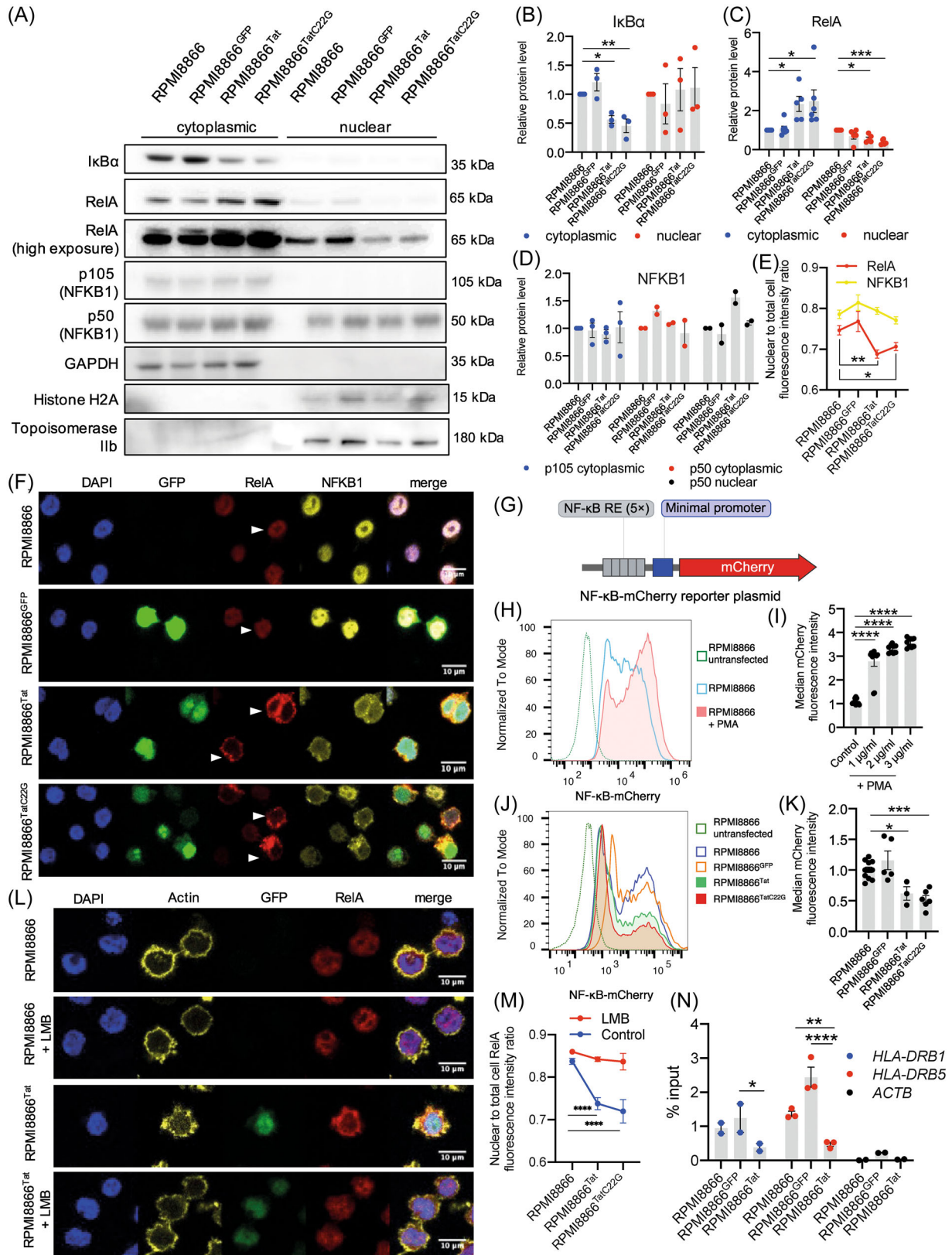


FIGURE 4 (See caption on next page).

showed mostly nuclear staining, in RPMI8866^{Tat} and RPMI8866-^{TatC22G} cells, RelA was sequestered in the cytoplasm (Figure 4F), which indicated lower classical NF- κ B activation in Tat-expressing B cells. This was further confirmed when we calculated the ratio of nuclear to total cellular RelA fluorescence in individual cells: the mean ratio of RelA was significantly lower in RPMI8866^{Tat} and RPMI8866-^{TatC22G} cells as compared to RPMI8866 and RPMI8866^{GFP} cells ($p < 0.01$ and $p < 0.05$ compared to RPMI8866 cells, Figure 4E). No significant changes in NF κ B1 nuclear to total ratio were observed between studied cell lines, which was in accordance with western blot analysis results (Figure 4E). Additionally, no significant changes in RelB distribution were observed between studied cell lines (Supporting Information S6: Figure 12). In BLAS^{Tat} and BLAS^{TatC22G} cells, RelA was also more present in the cytoplasm when compared to BLAS and BLAS^{GFP} cells (Supporting Information S6: Figure 13), which confirmed lower classical NF- κ B activation in Tat-expressing B cells.

Finally, to confirm that the observed RelA sequestration in the cytoplasm of B cells was associated with the decrease in NF- κ B-mediated gene transcription, we generated an NF- κ B-mCherry reporter plasmid (Figure 4G). RPMI8888 cell transfection with this plasmid led to a detectable expression of mCherry2, which was significantly higher upon NF- κ B activation with phorbol-12-

myristate-13-acetate (PMA) (Figure 4H,I). mCherry expression was significantly lower in RPMI8866^{Tat} and RPMI8866^{TatC22G} cells when compared to RPMI8866 and RPMI8866^{GFP} cells ($p = 0.0409$ and $p = 0.0007$ relative to RPMI8866, respectively, Figure 4J,K). No significant differences in mCherry fluorescence level were found between RPMI8866^{GFP} and RPMI8866. Additionally, the NF- κ B reporter activity was decreased by ~10% in BLAS^{GFP} cells cocultured with Jurkat-Tat cells for 7 days ($p = 0.0001$, *t*-test, Supporting Information S6: Figure 14A,B), while the NF- κ B reporter activity in Jurkat-Tat cells was higher than in Jurkat cells ($p < 0.0001$, Supporting Information S6: Figure 14C), which is in agreement with previous reports.²⁵ These results confirmed that Tat presence induced a decrease in classical NF- κ B activation in lymphoblastoid B cells.

We proceeded to verify whether the Tat-induced RelA sequestration within the cytoplasm was dependent on the functionality of the nuclear export by treating cells with the nuclear export inhibitor leptomycin B (LMB) (Figure 4L). Our observations revealed that the inhibition of nuclear export canceled the effect of Tat and TatC22G on RelA subcellular localization (Figure 4L,M). As a result of nuclear export inhibition, RelA in RPMI8866^{Tat} and RPMI8866^{TatC22G} cells transitioned from cytoplasmic to primarily nuclear localization similar to what was observed in RPMI8866 cells. These findings

FIGURE 4 HIV-1 Tat expression reduced classical NF- κ B activation in B cells. (A) Western blot analysis of I κ B α , RelA, NF κ B1 (p105 and p50) cytoplasmic and nuclear content in RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, and RPMI8866^{TatC22G} cells. GAPDH was used as a loading control for cytoplasmic extracts; histone H2A and topoisomerase II β were used as loading controls for nuclear extracts. A reproducible result is presented. (B–D) Densitometry analysis of I κ B α (B), RelA (C), NF κ B1 (D), cytoplasmic and nuclear content normalized to respective cytosolic or nuclear control of protein load. (E, F) Immunofluorescent staining with antibodies to RelA and to NF κ B1 (p105 and p50) in RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, and RPMI8866^{TatC22G} cells. (E) The ratio of total fluorescence intensity of RelA (red) or NF κ B1 (yellow) in the nucleus divided by the respective total cellular fluorescence intensity for each cell. (F) A representative confocal image of RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, and RPMI8866^{TatC22G} cells, stained with antibodies to RelA (red) and to NF κ B1 (p105 and p50, yellow). Nuclei were counterstained with DAPI (blue) and GFP fluorescence in cells expressing GFP, Tat-GFP or TatC22G-GFP was detected (green). White arrows indicate RelA sequestration in the nucleus or cytoplasm. Scale bar 10 μ m. (G) Schematic illustration of NF- κ B-mCherry reporter plasmid that consisted of five repeats of the NF- κ B response elements, a minimal promoter and red fluorescent protein mCherry2 as a reporter gene. (H) A representative histogram for flow cytometry analysis of RPMI8866 cells transfected with the NF- κ B-mCherry reporter plasmid and treated (red) or not (blue) with 1 μ g/mL PMA for 24 h. Untransfected RPMI8866 cells served as negative control (green dotted line). (I) The effect of different concentrations of PMA on mCherry expression in RPMI8866 cells after transfection with the NF- κ B-mCherry reporter plasmid calculated as the median fluorescence intensity in the sample normalized relative to the median fluorescence intensity in the control (untreated RPMI8866 cells after transfection with the NF- κ B-mCherry reporter plasmid), averaged data from at least three biologically independent experiments are shown. (J) A representative histogram for flow cytometry analysis of RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, and RPMI8866^{TatC22G} cells transfected with the NF- κ B-mCherry reporter plasmid. Untransfected RPMI8866 cells served as negative control (green dotted line). (K) The effect of HIV-1 Tat on the activity of the NF- κ B pathway analyzed by the reporter assay. RPMI8866, RPMI8866^{GFP}, RPMI8866^{Tat}, and RPMI8866^{TatC22G} cells were transfected with the NF- κ B-mCherry reporter plasmid and mCherry expression level was calculated as the median fluorescence intensity in the sample normalized relative to the median fluorescence intensity in the control RPMI8866 cells, averaged data from at least three biologically independent experiments are shown. (L, M) Immunofluorescent staining with antibodies to RelA and to actin (cytoplasm marker) in RPMI8866, RPMI8866^{Tat}, and RPMI8866^{TatC22G} cells treated or not with 5 ng/mL Leptomycin B (LMB), an inhibitor of nuclear export. (L) A representative confocal image of RPMI8866, and RPMI8866^{Tat} cells (treated or not with LMB), stained with antibodies to RelA (red) and to actin (yellow). Nuclei were counterstained with DAPI (blue) and GFP fluorescence in cells expressing Tat-GFP was detected (green). Scale bar 10 μ m. (M) The ratio of total fluorescence intensity of RelA in the nucleus divided by the respective total cellular fluorescence intensity for each cell in cells treated with LMB (red) or not (blue). (N) ChIP-qPCR analysis for RelA binding to the indicated promoters calculated as a percentage of the input. ChIP was performed with anti-p65 antibody in RPMI8866, RPMI8866^{GFP}, and RPMI8866^{Tat} cells. qPCR was performed with primers specific for the indicated promoters. Data are presented as individual values, mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way (B, C, D, E, I, K) or two-way ANOVA (M, N), Tukey's post hoc test. ANOVA, analysis of variance; ChIP-qPCR, chromatin immunoprecipitation quantitative polymerase chain reaction; DAPI, 4',6-diamidino-2-phenylindole; HIV, human immunodeficiency virus; LMB, leptomycin B; PMA, phorbol-12-myristate-13-acetate; qPCR, quantitative polymerase chain reaction; SEM, standard error of mean.

demonstrate that Tat promotes the nuclear export of RelA (rather than inhibits RelA nuclear import), as evidenced by the restoration of RelA accumulation within the nucleus upon nuclear export inhibition.

To further substantiate the implications of these findings for MHC class II gene regulation, we conducted ChIP-qPCR assay. We performed ChIP using antibodies targeting RelA and analyzed RelA binding to the promoters of the *HLA-DRB1* and *HLA-DRB5* genes. Our results demonstrated a decrease in the binding of RelA to the promoters of both *HLA-DRB1* and *HLA-DRB5* genes in Tat-expressing cells compared to the control cells (Figure 4N). No significant differences for the RelA binding to the control *ACTB* promoter in RPMI8866, RPMI8866^{GFP}, and RPMI8866^{Tat} cells were found. This reduction in NF- κ B binding to the promoters of MHC class II genes corroborates our earlier observations of Tat-induced cytoplasmic sequestration of RelA and provides mechanistic insights into the downregulation of HLA-DR expression.

3.5 | HIV-1 Tat presence in B cells reduced EBV-specific CD4+ T cell response

The expression of cell surface HLA-DR is downregulated during B cell lymphomagenesis decreasing the efficacy of functional immune responses and worsening the prognosis.²⁶ MHC class II-restricted CD4+ T cell response is also responsible for the control of EBV infection and EBV-induced B cell proliferation.²⁷ To confirm that Tat-induced HLA-DR downregulation could impair CD4+ T cell response and contribute to the escape from the immune surveillance, we generated a BLAS-reactive EBV-specific CD4+ T cell line. Briefly, PBMCs from healthy donor AS were depleted in CD8+ T cells and stimulated with autologous irradiated BLAS cells which caused their *in vitro* expansion (Figure 5A). After seven restimulation cycles, the T cell line was >90% CD4+ by flow cytometric analysis (Figure 5B).

To study EBV-specific CD4+ T cell responses, we cocultured BLAS cells with autologous PBMCs or the generated CD4+ T cell line and analyzed the percentage of activated IFN γ + /CD4+ cells in response to antigen stimulation (Figure 5C). As expected, a small proportion of PBMCs were responsive to BLAS without any preceding stimulation (Figure 5D). On the contrary, the coculture of the CD4+ T cell line with BLAS led to a significant increase in the percentage of activated IFN γ + /CD4+ cells (Figure 5D). Notably, this percentage was significantly lower when CD4+ T cell line was cocultured with BLAS^{Tat} or BLAS^{TatC22G} compared to BLAS or BLAS^{GFP} ($p < 0.0001$, Figure 5D), confirming the functional significance of Tat-downregulated HLA-DR expression in lymphoblastoid cells.

EBV-specific CD4+ T cells are also capable of inducing lymphoblastoid cell cytotoxic lysis that necessitates MHC class II-restricted recognition²⁸; therefore we decided to measure the cytotoxic activity of the generated BLAS-reactive CD4+ T cell line. The cytotoxicity was assessed as the percentage of specific lysis. Briefly, sensitive target lymphoblastoid cells (BLAS) and control target lymphoblastoid cells (RPMI8866) were labeled with CFSE at two different

concentrations to allow their differentiation between each other and with CD4+ T cells (Supporting Information S6: Figure 15A). The effector (E, CD4+ T cell line) and target cells (T, BLAS or RPMI8866) were mixed at various E/T ratios and incubated for 4 h; the percentage of specific lysis was derived from the decrease in the sensitive target population (Supporting Information S6: Figures 1, 15B). We found that BLAS^{Tat} or BLAS^{TatC22G} cells were less susceptible to cytotoxic cell lysis by CD4+ T cells as compared to BLAS cells ($p < 0.0001$, Figure 5E). Taken together, we provide compelling evidence that Tat-induced HLA-DR expression downregulation contributes to the immune escape of EBV-infected lymphoblastoid B cells.

3.6 | HIV-1 Tat presence in the serum of people living with HIV was linked to decreased HLA-DR surface expression in B cells

HLA-DR surface expression is decreased in B cells from people with HIV²⁹; this can be attributed to the chronic secretion of Tat protein from infected cells. To support this hypothesis, we decided to test whether HLA-DR expression was decreased on the mRNA level in people living with HIV. We analyzed *HLA-DRB1* and *HLA-DRB5* gene expression in B cells isolated from the peripheral blood of 11 HIV-negative healthy individuals (control) and 10 people living with HIV; their clinicopathological characteristics are summarized in Table 1. We found that the expression of *HLA-DRB1* was indeed decreased ($p = 0.0463$, Figure 6A), while *HLA-DRB5* expression remained unchanged ($p = 0.6889$, Figure 6B); this might indicate differential transcriptional regulation of these genes. Thus, the decrease in HLA-DR surface expression in people with HIV could result from a transcriptional downregulation of *HLA-DRB1* and could be due to the action of Tat protein secreted from infected CD4+ T cells.

To confirm the observed role of HIV-1 Tat in the regulation of HLA-DR expression in B cells and EBV-specific immunity, we analyzed HLA-DR surface expression in people living with HIV from an independent cohort. PBMC from six HIV-negative healthy individuals (control) and 17 people living with HIV, whose clinicopathological characteristics are summarized in Table 2, were immunophenotyped and analyzed by flow cytometry. B cells in people with HIV exhibited a tendency to have lower HLA-DR expression compared to HIV-uninfected controls ($p = 0.0677$, Figure 6C). This observation was consistent across both memory ($p = 0.0352$, Figure 6D) and naïve B cells ($p = 0.0451$, Figure 6E). Furthermore, among people with HIV, those with serum Tat concentrations exceeding 2.5 ng/mL demonstrated significantly diminished HLA-DR surface expression compared to PLWH with lower Tat concentrations ($p = 0.0478$, Figure 6F). Moreover, EBV reactivation as detected by PCR EBV DNA levels surpassing 2.26 log had a tendency to be associated with reduced HLA-DR expression in B cells ($p = 0.0543$, Figure 6G). Collectively, these findings underscore a link between HIV-1 Tat presence and decreased HLA-DR

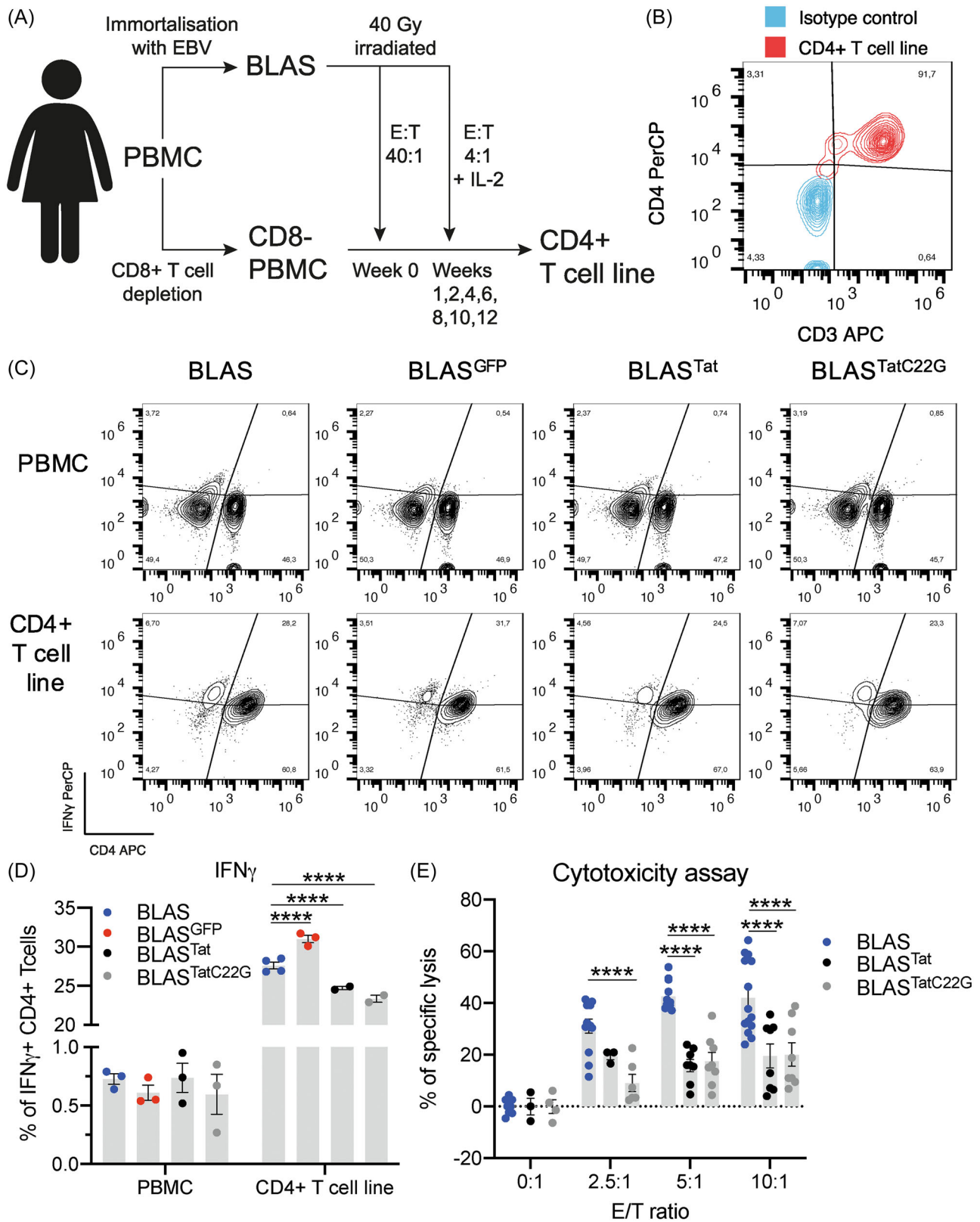


FIGURE 5 (See caption on next page).

expression on B cells, highlighting potential implications for EBV-specific immune response modulation in people living with HIV.

4 | DISCUSSION

cART has reshaped HIV infection from an almost inevitably lethal disease to a manageable chronic infection for the majority of infected individuals. Nevertheless, HIV infection remains incurable and, despite suppression of viral replication and immune restoration, people living with HIV still have an increased risk of B cell malignancies.² In the current study, we identified some mechanisms that contributed to B cell lymphomagenesis in this population. B cells are not infected by HIV, but they can be affected by HIV indirectly, for example, through coexistence with HIV-infected cells and the chronic action of secreted viral proteins produced by the cells bearing both normal and defective HIV proviruses.⁴ Likewise, HIV-1 Tat, a crucial regulator of HIV transcription, is efficiently exported from infected cells and uptaken by neighboring noninfected cells.^{5,11} The chronic presence of HIV-1 Tat in people with HIV is estimated to alter the physiology of multiple cells not-infected by HIV and contribute to neurocognitive, cardiovascular disorders, and adipose tissue senescence.^{30,31} To study the effects of chronic HIV-1 Tat presence on B cells, we used two EBV-immortalized lymphoblastoid B cell lines (RPMI88666 and a freshly immortalized BLAS cell line) that ectopically expressed HIV-1 Tat or its mutant TatC22G, deprived of transactivation capacity. The proposed approach to evaluate the long-term effects of Tat on B cells allows to overcome the difficulties related to the use of recombinant Tat protein (susceptibility to oxidation, quick ex vivo degradation).

We performed an RNA-sequencing analysis of these cell lines and revealed that Tat deregulated the expression of hundreds of genes (Figure 1). Specifically, Tat downregulated a subset of genes related to MHC class II (including *HLA-DRB1* and *HLA-DRB5*) and thus could potentially decrease CD4+ T cell activation in response to EBV-infected B cells.

EBV is a γ -herpesvirus that infects more than 90% of the human adult population.³² Compared to the general population, people living with HIV are more likely to be seropositive to EBV and more often present EBV reactivation.³³ B cell lymphomas in people living with HIV are more frequently associated with EBV.³⁴ Failing EBV control and EBV reactivation before the oncological disease are important

steps for oncogenesis and poor prognosis in people with HIV regardless of their immune status.^{35,36} Notably, HIV-positive people progressing to B cell subtype primary central nervous system lymphoma were recently found to lack EBV-specific CD4+ T cells irrespective of their absolute CD4+ T cell counts.³⁷ A critical role for EBV-specific CD4+ T cells in coordinating an immune response to BL was previously shown: unlike EBV nuclear antigen 1 (EBNA-1)-specific CD8+ T cells, EBNA-1 specific CD4+ T cells were necessary and sufficient for tumor growth inhibition in mice.³⁸

Our results suggest that HIV-1 Tat protein can contribute to B cell immune escape by transcriptionally downregulating *HLA-DRB1* and *HLA-DRB5*, which led to a decrease in the surface HLA-DR expression (Figure 2). HLA-DR surface expression was previously shown to be decreased in B cells from people with HIV.²⁹ Here we confirmed that both memory and naïve B cells from HIV-positive individuals exhibited consistently lower HLA-DR surface expression compared to the controls (Figure 6). Remarkably, people with HIV with higher serum Tat concentrations displayed a significantly decreased HLA-DR (Figure 6F). In an independent cohort, we demonstrated that *HLA-DRB1* expression in B cells from people with HIV was decreased on the mRNA level (Figure 6A), which can be attributed to the chronic secretion of Tat protein from infected cells. Additionally, prolonged coculture of lymphoblastoid cells with CD4+ T cells that express Tat led to a significant decrease in HLA-DR expression in lymphoblastoid cells; this effect was blocked when Tat entry in cells was inhibited by genistein, an inhibitor of endocytosis (Figure 2D–F).

The observed effect of HIV-1 Tat on HLA-DR expression in lymphoblastoid B cells was not dependent on Tat C22 residue (Figure 2), which suggests that the mechanism of Tat action on HLA-DR expression was indirect, probably through protein-protein interaction with host proteins. The mutation of C22 in the transactivation domain largely disrupts Tat interaction with chromatin and prevents direct regulation of gene expression (both stimulation and downregulation), favouring the indirect effects of Tat.²⁰ The effect of Tat on HLA-DR expression in B cells was also not dependent on CIITA (Supporting Information S6: Figure 8). Tat might inhibit other TFs that normally upregulate their targets, leading to the gene downregulation. We found that RelA and NFKB1 TFs of NF- κ B pathway were the most likely TFs that regulate the expression of downregulated genes (Figure 3). Using a set of inhibitors of NF- κ B activation, we confirmed that HLA-DR expression was indeed

FIGURE 5 HIV-1 Tat expression in B cells reduces EBV-specific CD4+ T cell response. (A) An overview of the experimental setup for the generation of BLAS-reactive EBV-specific CD4+ T cell line. (B) Representative flow cytometry contour plots showing CD3 and CD4 and respective isotype control antibody staining of BLAS-reactive EBV-specific CD4+ T cell line. (C) Representative flow cytometry contour plots showing CD4 and IFN γ intracellular staining in non-stimulated PBMC population and BLAS-reactive CD4+ T cell line, cocultured for 18 h with different BLAS cells at E/T ratio 40:1. Outliers are shown as dots. (D) The percentage of IFN γ + /CD4+ cells in the population of non-stimulated PBMC or BLAS-reactive CD4+ T cell line cells, cocultured for 18 h with different BLAS cells at E/T ratio 40:1. (E) Percentage of specific lysis of different BLAS cells after cocultivation with BLAS-reactive EBV-specific CD4+ T cell line at various E/T ratios for 4 h. Data are presented as individual values, mean \pm SEM, **** p < 0.0001, two-way ANOVA, Tukey's post hoc test. EBV, Epstein–Barr virus; E/T, effector/target; HIV, human immunodeficiency virus; IFN, interferon; PBMC, peripheral blood mononuclear cells; SEM, standard error of the mean.

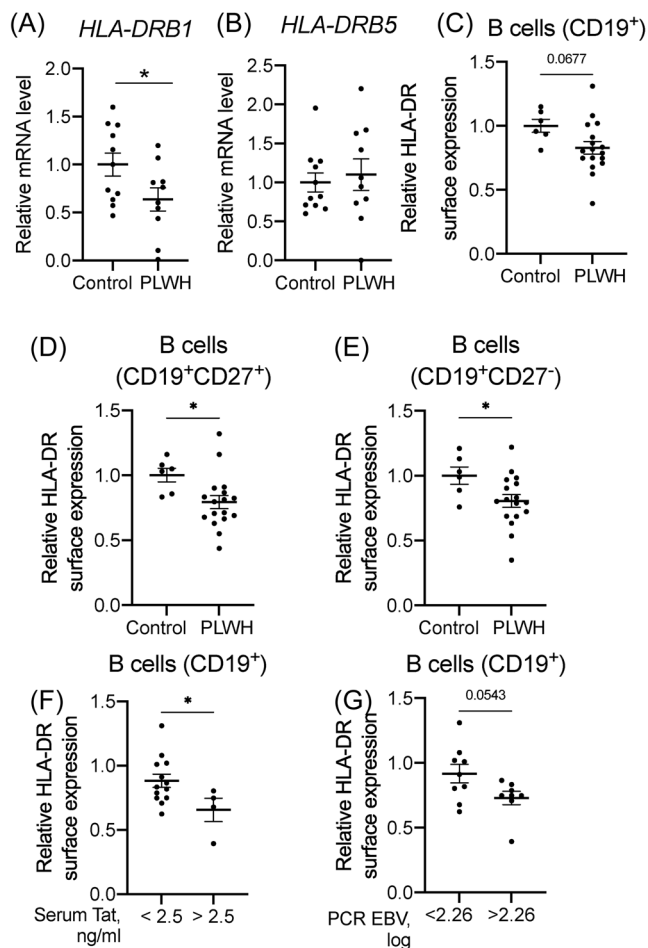


FIGURE 6 The analysis of HLA-DR expression in B cells from people living with HIV and HIV-uninfected controls. (A, B) The level of *HLA-DRB1* (A) and *HLA-DRB5* (B) mRNA expression in B cells isolated from peripheral blood of people living with HIV (PLWH) and HIV-uninfected control individuals (control). The mRNA level of *HLA-DRB1* or *HLA-DRB5* was normalized to the expression as housekeeping genes; the normalization was done assuming the mean level of transcript in control to be 1. (C) The level of HLA-DR surface expression in B cells (gated as CD19⁺) as analyzed by flow cytometry. (D) The level of HLA-DR surface expression in memory B cells (gated as CD19⁺CD27⁺) as analyzed by flow cytometry. (E) The level of HLA-DR surface expression in naïve B cells (gated as CD19⁺CD27⁻) as analyzed by flow cytometry. (F) The level of HLA-DR surface expression in B cells (gated as CD19⁺) as analyzed by flow cytometry in people living with HIV with serum Tat concentration below and above 2.5 ng/mL cut-off. (G) The level of HLA-DR surface expression in B cells (gated as CD19⁺) as analyzed by flow cytometry in people living with HIV with PCR EBV DNA in whole blood below and above 2.26 log cut-off. Data are presented as individual values, mean \pm SEM, * $p < 0.05$, t -test. EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; PLWH, people living with HIV; SEM, standard error of the mean.

regulated by the NF- κ B pathway in B lymphoblastoid cells (Figure 3, Supporting Information S6: Figure 10), and as predicted in silico, we found that Tat or TatC22G chronic presence in B cells led to increased RelA sequestration in the cytoplasm, indicating reduced

NF- κ B activity (Figure 4). RelA sequestration in the cytoplasm, induced by HIV-1 Tat, relied on nuclear export and was accompanied by a decrease in NF- κ B-mediated transcription and decreased RelA association with the promoters of *HLA-DRB1* and *HLA-DRB5* genes (Figure 4). Thus, HLA-DR downregulation in response to Tat or its mutant TatC22G was linked to a decrease in NF- κ B pathway activity. Notably, an NF- κ B inhibitory effect was previously shown for another viral protein: EBV Zta,³⁹ a protein that has similar activities as HIV-1 Tat (transcriptional regulation, host chromatin binding, cell penetration).⁴⁰

It was previously shown that the acute action of Tat (treatment with recombinant protein or expression induction) was, in fact, associated with the activation of the NF- κ B pathway.^{12,25} The mechanism of NF- κ B activation was related to Tat interaction with the inhibitory protein I κ B α and Tat-induced I κ B α degradation; this was demonstrated in Jurkat T cells.²⁵ We found that Tat presence led to an increased I κ B α phosphorylation and a decrease in total I κ B α cytoplasmic content in lymphoblastoid B cells (Figure 4A,B, Supporting Information S6: Figure 11A,B), which is in agreement with previous results. However, we also found that the phosphorylated I κ B α was accumulated in the nucleus of Tat-expressing B cells (Supporting Information S6: Figure 11A,B). The presence of nuclear I κ B promotes NF- κ B nuclear export and decreases the NF- κ B-dependent gene transcription, including a decrease in Tat-induced NF- κ B-dependent LTR activation.^{41,42} In our case, the transactivation-active component of NF- κ B signaling, RelA, was more present in the cytoplasm and decreased in the nuclear compartment in Tat-expressing cells. This might be associated with an increase in nuclear p-I κ B α in Tat-expressing cells. To our knowledge, little is known about the role of the phosphorylated form of I κ B α in the nucleus (although both I κ B α and its kinases IKK are present in the nucleus), but chronic action of Tat may lead to an increased NF- κ B nuclear export through p-I κ B α accumulation/stabilization in the nucleus, which should be further explored. The inhibition of nuclear export restored the accumulation of RelA in the nuclei of Tat-expressing B cells (Figure 4L,M), reinforcing the proposed model of Tat's role in promoting the nuclear export of RelA. Apart from I κ B, other proteins that interact with RelA and shuttle between the nucleus and cytoplasm (e.g., p28) may also promote RelA export from the nucleus.⁴³ HIV-1 Tat directly interacts with both RelA and NFKB1 components of the NF- κ B pathway^{21,25} and is able to shuttle between the nucleus and cytoplasm,⁴⁴ which may constitute another potential mechanism of Tat-induced NF- κ B pathway inactivation in B cells.

MHC class II-restricted CD4⁺ T cell response controls EBV infection and EBV-induced B cell proliferation²⁷ and the loss of cell surface HLA-DR promotes B cell lymphomagenesis by decreasing the efficacy of functional immune response.²⁶ Of note, a recent large genome-wide association study found that anti-EBV antibody response presents a strong association with *HLA-DRA*, *HLA-DQA1*, and *HLA-DQB1* loci,³³ which confirms the importance of MHC class II-restricted response for the immune control of EBV. We demonstrated that EBV-infected B cells that expressed Tat were less

responsive to EBV-specific CD4+ T cell-mediated cytotoxic lysis compared to control cells and to a smaller degree stimulated EBV-specific CD4+ T cell activation as evidenced by IFN γ production (Figure 5). Consequently, Tat-induced HLA-DR downregulation could impair CD4+ T cell response and contribute to the escape from immune surveillance of EBV-infected B cells. Notably, we observed a trend linking EBV reactivation, denoted by elevated EBV DNA levels, with reduced HLA-DR expression in people living with HIV (Figure 6G).

Besides antigen presentation, MHC class II is a signaling molecule that regulates B cell activation, proliferation, differentiation, and promotes antibody production during cognate CD4+ T cell-B cell interaction.⁴⁵ B cell activation is triggered by the antigen binding to the B cell receptor, which initiates a cascade of intracellular signaling, including NF- κ B activation, and stimulates B cell differentiation into antibody-producing B cells.⁴⁶ A potential consequence of reduced NF- κ B pathway activity and MHC class II expression, induced by HIV-1 Tat in B cells, is impaired B cell activation, leading to a compromised ability to initiate an effective immune response against pathogens, including HIV itself. It is known that despite its circulation in the blood and effective cell penetration, HIV-1 Tat is able to elicit an antibody-mediated immune response only in ~20% of people living with HIV.⁴⁷ HIV-1 Tat-mediated downregulation of MHC class II genes in B cells might contribute to the defects in B cell activation and antibody response.

MHC class II also serves as a coreceptor for EBV entry by interacting with EBV membrane glycoprotein 42 (gp42) during B cell infection.⁴⁸ Upon EBV reactivation, the interaction of newly produced gp42 with the MHC class II complex targets gp42 for degradation, which leads to the release of gp42-depleted viral particles that preferentially target epithelial cells.⁴⁹ This results in the switch of EBV tropism from B cells to epithelial cells and may limit the direct EBV spread from reactivating B cells to other B cells.⁴⁹ In general, people living with HIV tend to have higher levels of EBV DNA in PBMCs as compared to HIV-negative individuals.⁵⁰ Our data demonstrate that despite the effective control of HIV infection exerted by cART for the majority of HIV-positive individuals in the studied cohort, 8/17 of them (47%) had high levels of EBV DNA in the whole blood (Table 2). The expansion of the pool of EBV-infected B cells in people living with HIV (and hence higher EBV load) might partly arise from HIV-1 Tat penetration in EBV-infected cells, which decreases MHC class II expression and might increase the production of gp42-enriched viral particles that would target B cells, which requires further studies.

In conclusion, HIV-1 Tat chronic presence can contribute to immune evasion of B cells via HLA-DR downregulation, thus leading to the acquisition of a cancer hallmark and B cell lymphoma predisposition in people living with HIV. Targeting HIV-1 Tat secretion by infected cells or its penetration in noninfected cells is a promising therapeutic strategy.

AUTHOR CONTRIBUTIONS

Anna Shmakova designed and performed research, analyzed data, and wrote the paper. Coline Hugot, Yana Kozhevnikova, Anna Schwager (Karpukhina), Ivan Tsimailo, Olga Szewczyk-

Roszczenko, Piotr Roszczenko, Kamila Buzun, and Diego Germini performed research and analyzed data. Eugene V. Sheval performed research and contributed new reagents. Laurence Gérard, David Boutboul, and Eric Oksenhendler provided samples from patients and clinical data. Yegor Vassetzky designed research, analyzed data, and wrote the paper. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data set supporting the conclusions of this article (raw RNA-sequencing data as well as processed data (gene counts)) is available in the GEO repository, accession number GSE212499, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE212499>. The data that support the findings of this study are openly available in GEO at <https://www.ncbi.nlm.nih.gov/geo/>, reference number GSE212499.

ETHICS STATEMENT

Blood samples from HIV-positive individuals who signed a written informed consent for the study and were treated at the Department of Clinical Immunology, Hôpital Saint-Louis, Paris, France, were collected in accordance with the French legislation (agreements 2016-A01316-45, VIHEBV). Consent for publication has been obtained from the patients.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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