

RNA Sequencing Analyses and Validation of Immune-Related Genes in EBV-Specific Cytotoxic T Lymphocytes During Latent Infection

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Background: Epstein-Barr virus (EBV) infects more than 90% of the global population, but the immune mechanism underlying its infection remains incompletely understood.

Methods: In this study, transcriptomic profiling and validation of differentially expressed genes (DEGs) were performed between Epstein-Barr virus-specific cytotoxic T lymphocytes (EBV-CTLs) and nonspecific CTLs via RNA sequencing (RNA-seq). Bioinformatics analyses, including Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, functional molecular module construction, and key gene analysis, were conducted to explore the biological functions and potential regulatory mechanisms of the DEGs.

Results: A total of 1236 DEGs were identified in the EBV-CTL group compared with the nonspecific CTL group, including 645 upregulated genes and 591 downregulated genes. GO enrichment analysis revealed that these DEGs were localized mainly to cell membranes and MHC class II protein complexes and were involved in biological processes such as cellular defense, leukocyte activation, proliferation, differentiation, and chemotaxis. KEGG pathway enrichment analysis revealed that the JNK/p38 MAPK pathway was the most significantly enriched signaling pathway, with key DEGs including *p38*, *HSP72*, and components of the AP-1 transcription factor complex (mainly *JUN* and *FOS*). The functional molecular module construction revealed that the top-scoring modules were associated primarily with signal transduction, the inflammatory response, the immune response, and molecular interactions (eg, protein and receptor binding). Key gene analysis identified *JUN*, *FOS*, *TNF*, and *STAT1* as potential hub genes involved in the EBV-specific immune response.

Conclusion: Our transcriptomic analysis reveals the unique gene expression profile of EBV-CTLs and identifies the JNK/p38 MAPK pathway and hub genes (*JUN*, *FOS*, *TNF*, and *STAT1*) as critical regulators of the EBV-specific immune response. These findings provide novel insights into the molecular mechanism underlying EBV-specific immunity and potential targets for related therapeutic intervention.

Keywords: Epstein-Barr virus, cytotoxic T lymphocytes, RNA-seq sequencing, JNK/p38 MAPK pathway

Introduction

Epstein-Barr virus (EBV), a member of the Gammaherpesvirinae subfamily, is a ubiquitous human herpesvirus that infects more than 90% of the global population.¹ It possesses a ~172 kb double-stranded DNA genome. It has two replication modes: lytic replication (producing infectious progeny) and latent infection (expressing restricted latent antigens such as LMP1, LMP2, and EBNA1).² In contrast, lytic antigens (eg, BZLF1) are expressed during viral

replication and participate in immune stimulation. EBV primarily infects B lymphocytes and establishes lifelong latent infection, with host immune surveillance, primarily mediated by CD8⁺ cytotoxic T lymphocytes (CTLs), being pivotal for constraining EBV reactivation and associated pathogenesis.^{3,4} EBV-specific CTLs recognize latent antigens presented by MHC class I molecules to eliminate infected cells, but the virus has evolved immune escape strategies (eg, down-regulating MHC class I expression) to persist.^{5,6}

EBV is etiologically linked to multiple malignancies (eg, nasopharyngeal carcinoma, Hodgkin's lymphoma, post-transplant lymphoproliferative disorder [PTLD]) and nonmalignant diseases, where latent antigen-induced signalling dysregulation and immune evasion play key pathogenic roles.⁷ Adoptive immunotherapy with EBV-CTLs holds promise for treating these diseases, but its suboptimal efficacy is attributed to a limited understanding of EBV-CTL gene expression profiles and regulatory pathways.^{8,9} Additionally, no licensed EBV vaccines are available despite decades of research,¹⁰ highlighting the urgency of clarifying EBV-host immune interactions.

Despite extensive studies, the transcriptional profiles and core regulatory mechanisms governing EBV-specific CTL activation and cytotoxicity remain incompletely understood.³ To address this knowledge gap, we established EBV-CTL and nonspecific CTL models, performed RNA-seq to identify immune-related differentially expressed genes (DEGs), and conducted bioinformatics analyses to screen key pathways and hub genes. Our findings provide experimental evidence for understanding chronic EBV infection-related diseases and formulating diagnostic/therapeutic strategies.

Materials and Methods

Study Participants

Peripheral blood samples were collected from 3 healthy volunteers (1 male and 2 females, aged 25–30 years, mean age: 27.6 ± 2.2 years) from the 900th Hospital of People's Liberation Army (PLA) Joint Logistic Support Force, who tested negative for EBV DNA via blood tests. Samples were collected randomly from eligible volunteers to ensure the representativeness of the study population. The excluded groups included individuals with an immune deficiency, acute infections, or a history of EBV-related diseases. These EBV DNA-negative healthy volunteers were selected as donors for LCL establishment because EBV-naïve (EBV-negative) PBMCs can avoid interference from preexisting EBV-specific immune responses in the host, ensuring the successful transformation of B lymphocytes into LCLs by EBV infection. Informed consent was obtained from each participant included in the study. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (sixth revision, 2008), as reflected in a priori approval by the institution's human research committee. Ethical approval for this study was obtained from the Ethics Committee of the 900th Hospital of People's Liberation Army (PLA) Joint Logistic Support Force (No. 2020023).

LCL Cell Line Construction

Immortalized B lymphocytes (EBV-associated LCLs) were generated by infecting PBMCs from EBV-negative healthy donors (as described in the “Study participants” section, confirmed by blood EBV DNA testing) with EBV-containing supernatant (produced by B95-8 cells, Procell, CL-0476; American Type Culture Collection (ATCC) Cat. No. CRL-1612). B95-8 cells were cultured in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin) for two weeks, and the filtered supernatant (viral titer: 4×10^7 IU/mL EBV) was cocultured with 2×10^7 PBMCs in complete medium containing 1 µg/mL cyclosporine A for 3–4 weeks, and cell growth and clumping were monitored.

Production of Nonspecific CTLs and EBV-CTLs

Nonspecific CTL Cell Line Construction

PBMCs isolated from the same 3 healthy volunteers (as described in the “Study participants” section) were stimulated with a CD3/CD28 monoclonal antibody. The cells were cultured in complete medium supplemented with 100 U/mL IL-2 for 5 days. After monoclonal antibody removal, the cultures were maintained in IL-2-containing medium for an additional 3 to 4 weeks. The nonspecific CTL line was also derived from the PBMCs of the same healthy volunteers and authenticated by flow cytometry to ensure the purity of the CD3⁺/CD8⁺ T lymphocytes. The resulting nonspecific

CTLs exhibited a typical activated T-cell morphology (small round cells with clear boundaries) under light microscopy, and flow cytometry revealed that the CD3⁺/CD8⁺ purity was consistently above 85%, indicating successful construction of functional nonspecific CTLs.

EBV-CTL Cell Line Construction

LCLs in the logarithmic growth stage were treated with mitomycin C at a concentration of 50 µg/mL for 2 hours to induce inactivation. The inactivated LCLs were then cocultured with PBMCs from the same healthy volunteer donor at a ratio of 1:20 for 1 week in medium containing 100 U/mL IL-2, after which the inactivated LCLs were replenished weekly at a PBMC:LCL ratio of 5:1 for a total of 3–4 weeks. The constructed EBV-CTL cell line was derived from the PBMCs of healthy volunteers and was authenticated by flow cytometry (CD3⁺/CD8⁺ ratio >85%) before subsequent experiments. The EBV-CTLs clearly clustered under light microscopy (consistent with the morphology of antigen-specific activated CTLs), and flow cytometry confirmed their high viability (>90%), indicating that the EBV-CTLs were functionally active and could be used for subsequent transcriptomic analysis.

Flow Cytometry Staining and Analysis

LCLs and CTLs were stained with the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit for 10 minutes at 4°C to exclude dead cells. Then, the LCLs were stained with an anti-CD19 antibody for 15 min at 4°C and identified as CD19⁺ cells. CTLs (including both EBV-CTLs and nonspecific CTLs constructed in this study) were stained with anti-CD3 and anti-CD8 antibodies and identified as CD3⁺/CD8⁺ subsets. After staining, the cells were resuspended in 300 µL of 1× phosphate-buffered saline (PBS) and used for flow cytometry.

RNA Sequencing and Quantitative Real-Time Polymerase Chain Reaction

RNA Sequencing

Total RNA was extracted from EBV-CTLs and nonspecific CTLs via TRIzol reagent (Beijing Quanshijin Biotechnology Co., Ltd.) according to the manufacturer's instructions. RNA purity and integrity were detected via a NanoQ™ microspectrophotometer (CapitalBio Technology Co., Ltd.) and agarose gel electrophoresis, respectively. Only RNA samples with OD260/OD280 values between 1.8 and 2.0 and intact 18S/28S rRNA bands were used for subsequent experiments. Library construction was performed via a commercial cDNA library construction kit, and sequencing was carried out on the Illumina NovaSeq 6000 platform with paired-end 150 bp reads. Three biological replicates were set for each group (EBV-CTLs and nonspecific CTLs), corresponding to the 3 healthy volunteers, and no technical replicates were required owing to the high reproducibility of the Illumina NovaSeq 6000 platform. The raw sequencing data were filtered to remove low-quality reads, adapter sequences and contaminating sequences via standard bioinformatics software. Clean reads were aligned to the human reference genome via HISAT2 (v2.0.5) software. Gene expression levels were quantified as fragments per kilobase of transcript per million mapped reads (FPKM) values via featureCounts (1.5.0-p3) software. For differential expression analysis, RNA-seq was performed on samples from each subject individually first; subsequently, the transcriptomic data of all 3 subjects were combined, and standard bioinformatics methods were used to identify genes that were differentially expressed between EBV-CTLs and nonspecific CTLs. Differentially expressed genes (DEGs) were identified via DESeq2 software (1.20.0) with the criteria of $\text{padj} \leq 0.05$ and $|\log_2\text{FoldChange}| \geq 1.0$. GO and KEGG enrichment analyses were conducted via the DAVID database ($\text{padj} \leq 0.05$) to annotate the biological functions and signalling pathways of the DEGs. Protein-protein interaction (PPI) network analysis of DEGs was performed for molecular module screening via the Molecular Complex Detection (MCODE) plugin in Cytoscape 3.9.1. The PPI network was constructed by importing DEGs into the STRING database (confidence ≥ 0.7) and visualized via Cytoscape 3.9.1; the MCODE plugin was used with the following parameters: degree cutoff=2, node score cutoff=0.3, K-core=2, and max depth=100 to screen the core module.

Quantitative Real-Time Polymerase Chain Reaction

RNA extraction was performed via an RNA extraction kit (AMRESCO Inc.), and cDNA was synthesized via a reverse transcription kit (Shanghai Yuanpei Biotechnology Co., Ltd.) according to the manufacturer's instructions. Quantitative

Table 1 Primer Information for the Genes Used for qRT-PCR

Gene	Primer Sequence
JUN	F: AAGTCATGAACCACGTTAACAG R: AAATGTTTGCAACTGCTGC
FOS	F: GTGGAACAGGAGACAGACC R: TTTCCTTCTCCTTCAGCAGG
TNF	F: CTGCACTTTGGAGTGATCG R: GAAGATGATCTGACTGCCTG
STAT 1	F: TAACCTCGACAGTCTTGGC R: TACCACTGAGACATCCTGC
p38	F: ACCAGACAGTTGATATTTGGTC R: GGATACGGTCTGTACCAGG
HSP72	F: TGGTGCTGACGAAGATGAAG R: AGGTCGAAGATGAGCACGTT

Abbreviations: F, forward primer; R, reverse primer.

real-time PCR was performed to estimate the expression of *JUN*, *FOS*, *TNF*, *STAT1*, *p38*, and *HSP72*. The primers used are shown in [Table 1](#). The RT-PCR system was prepared as follows: 4 μL of RNase-free water, 0.25 μL of forward primer (target gene), 0.25 μL of reverse primer (target gene), 0.5 μL of cDNA template, and 5 μL of SYBR Select Master Mix (2×, Applied Biosystems, USA). Each sample had 3 replicate wells, and GAPDH was used as the internal reference gene. Quantitative real-time PCR was carried out on an Applied Biosystems ABI real-time fluorescent quantitative PCR instrument with the following program: 95°C predenaturation for 10 min; 45 cycles of denaturation at 95°C for 15s, annealing for 20s, and extension at 72°C for 30s. Relative quantification of genes was performed via the $2^{-\Delta\Delta C_t}$ (2 to the power of the negative delta delta Ct) method, a widely used relative quantitative method for RT-qPCR that calculates the relative expression level of target genes normalized to the internal reference gene (GAPDH) and compared to the control group (nonspecific CTLs).

Results

LCL and CTL Construction

LCLs were successfully established from the PBMCs of 3 healthy volunteers, and the validation results revealed that the cell viability and CD19+ B lymphocyte proportion both exceeded 95% and that the LMP1/LMP2 genes were stably amplified in the cultured cells ([Figure 1A–C](#)).

EBV-CTLs were generated by coculturing inactivated LCLs with PBMCs, and nonspecific CTLs were induced via CD3/CD28 monoclonal antibody stimulation. The established CTLs showed high viability (>90%), and CD3+CD8+ T lymphocytes accounted for more than 85% of the total cell population ([Figure 1D](#) and [E](#)). These results confirmed the successful construction of functional EBV-CTL and nonspecific CTL models, with CD3+CD8+ T lymphocytes as the dominant population for subsequent transcriptomic analysis.

RNA Sequencing Analysis of Differentially Expressed Genes and Signalling Pathways

To determine the differentially expressed genes and associated signalling pathways in EBV-specific cytotoxic T lymphocytes compared with nonspecific cytotoxic T lymphocytes. RNA sequencing analysis was subsequently performed. A total of 1236 DEGs were identified, of which 645 genes were upregulated, and 591 genes were down-regulated ($\text{padj} \leq 0.05$ $|\log_2\text{FoldChange}| \geq 1.0$) ([Figure 2A](#)). To further investigate the heterogeneity between the two groups with different CTL activation modes, hierarchical clustering analysis was performed on the identified DEGs. The results revealed high consistency in gene expression among biologically replicated samples within each group, while significant differences were detected between EBV+ and EBV- CTLs ([Figure 2B](#)). Gene Ontology (GO) functional enrichment analysis revealed that the DEGs were involved mainly in biological processes such as cysteine-type

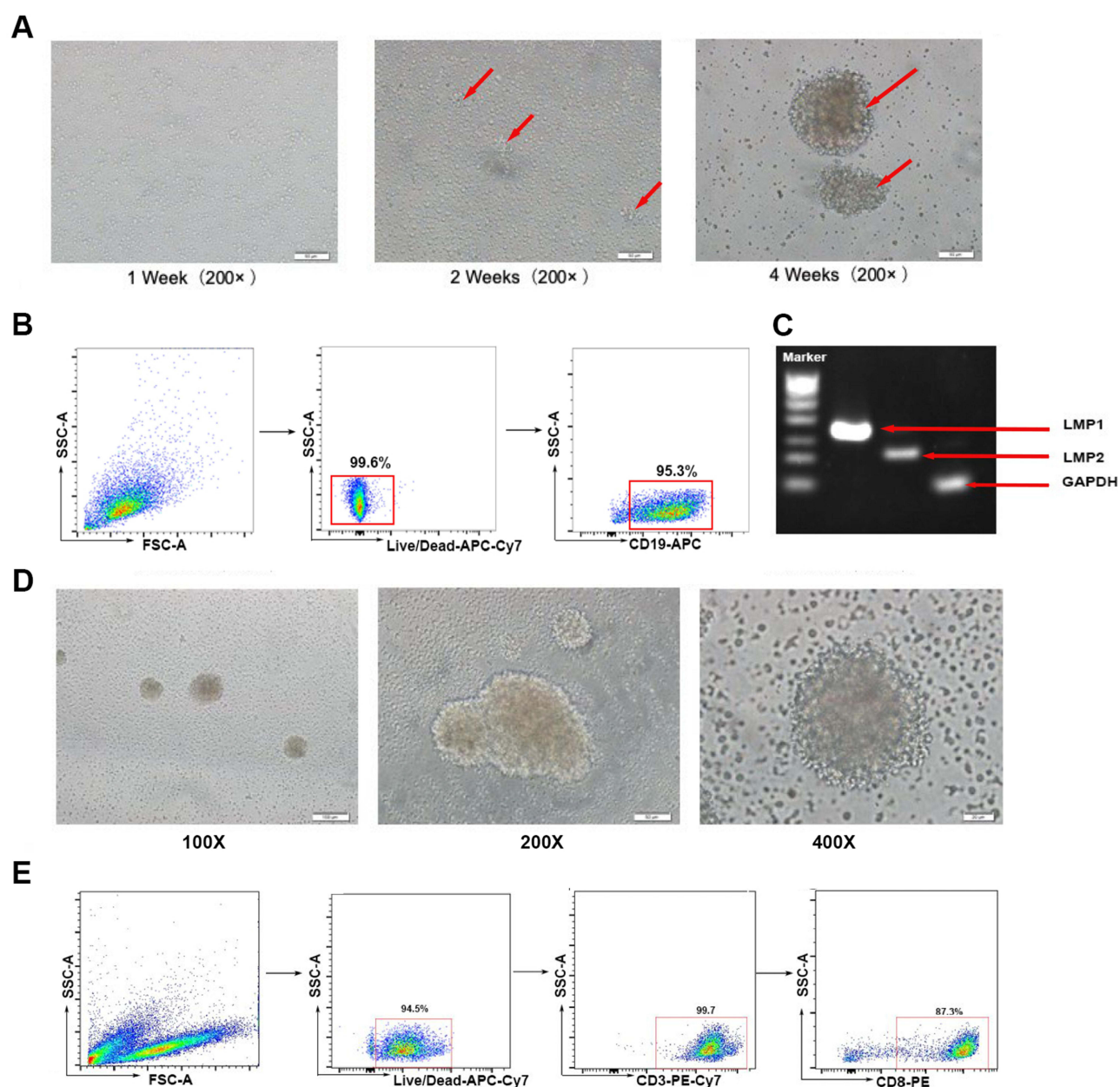


Figure 1 Construction and identification of LCLs and CTLs.

Notes: (A) PBMCs cocultured with B95-8 cells for 1–4 weeks (200× magnification). Red arrows indicate the formation of initial cell clusters, which represent the early stages of LCL transformation. Scale bar: 50 μ m; (B) Flow cytometry analysis of CD19 expression in cells cocultured with B95-8 cells for 4 weeks. The first red square box (left) indicates the live cell gate, used to exclude dead cells and debris (99.6% viable cells). The second red square box (right) indicates the CD19-positive B-cell gate, representing the purity of transformed LCLs (95.3% CD19+ cells); (C) RT-PCR analysis of EBV LMP1 and LMP2 mRNA expression in cells cocultured with B95-8 cells for 4 weeks, with GAPDH as an internal control; (D) Morphology of EBV-CTLs at 3 weeks under different magnifications (100×, 200×, 400×). Scale bar: 100 μ m (100×); 50 μ m (200×); 20 μ m (400×); (E) Flow cytometry analysis of EBV-CTLs at 3 weeks. The red square boxes represent sequential gating steps: the first red square box shows the live cell gate (94.5% viable cells), the second shows the CD3-positive T-cell gate (99.7% CD3+ cells), and the third shows the CD8-positive cytotoxic T-cell gate (87.3% CD8+ cells within the CD3+ population).

endopeptidase inhibitor activity, C-C (C-C motif) chemokine activity, and transcription factor activity (Figure 2C). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed 282 enriched pathways (padj, adjusted *P*-value < 0.05) and the 20 most significant KEGG pathways, including pathways associated with graft-versus-host disease, natural killer cell-mediated cytotoxicity, antigen processing and presentation, cytokine-cytokine receptor interactions, and allograft rejection (Figure 2D). To better investigate the roles that the enrichment pathways play in organisms, we summarized the affiliation of each enrichment pathway and drew a summary map of the secondary

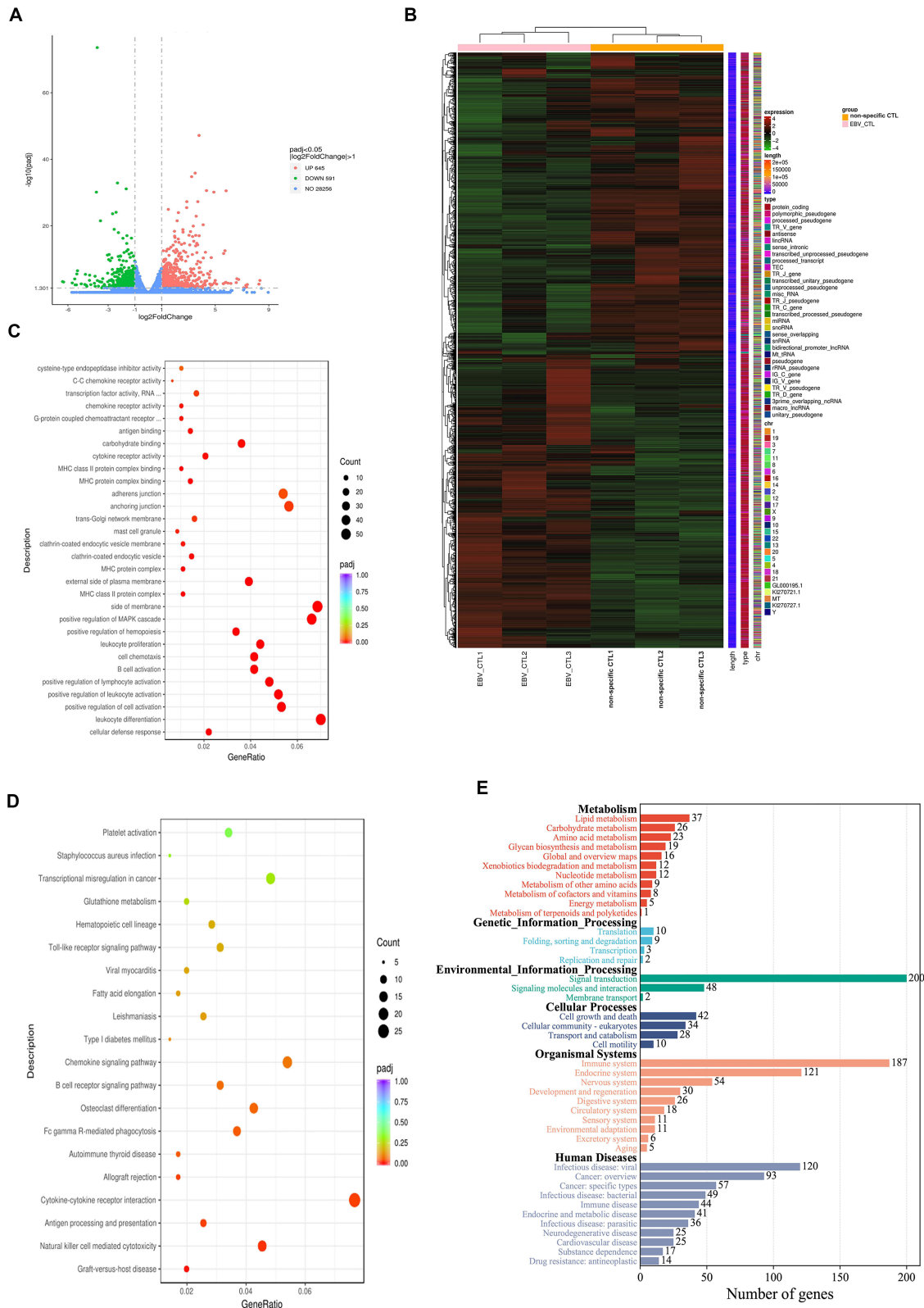


Figure 2 RNA sequencing analysis of EBV-CTLs and nonspecific CTLs. **Notes:** (A) Volcano plot of DEGs of EBV-CTL group and non-specific CTL group; (B) Heatmap showing hierarchical clustering of DEGs; (C) GO annotation analysis of DEGs; (D) KEGG pathway enrichment analysis of DEGs; (E) Summary chart of secondary classification of KEGG pathway enrichment.

classification of the KEGG pathway enrichment results (Figure 2E). The results in the graph revealed that the DEGs were involved mainly in the signalling process of cellular environmental information processing, in which the most significant enrichment was in the MAPK (mitogen-activated protein kinase) signalling pathway and the PI3K-AKT (phosphoinositide 3-kinase-protein kinase B) signalling pathway, especially in the JNK/p38 MAPK (c-Jun N-terminal kinase/p38 mitogen-activated protein kinase) signalling pathway. In addition, DEGs are involved in human diseases, such as viral infectious diseases and cancer, which are associated with the body's immune and endocrine systems. Specifically, the identified DEGs (eg, JUN, FOS, and TNF) regulate immune cell activation and inflammatory responses, which are closely related to the pathogenesis of EBV-associated viral infectious diseases and malignancies; aberrant expression of these DEGs may promote viral persistence and tumor progression.

PPI Network Construction and Module Analysis

Protein-protein interaction (PPI) network analysis of the DEGs was further performed for molecular module screening via the Molecular Complex Detection (MCODE) plugin in Cytoscape 3.9.1 (Figure 3A). With the set parameters, the top-ranked core module with the highest MCODE score (11.513), which contained 120 nodes and 685 edges and represented the most functionally relevant gene cluster in the EBV-CTLs, was identified (Figure 3B). GO and KEGG enrichment analyses of the DEGs within this core module were conducted via the DAVID database (<https://david.niaid.nih.gov>), and the results demonstrated that this module was associated primarily with signal transduction, the inflammatory response, and the immune response, which are biological processes that are central to EBV-specific T-cell immune regulation (Figure 3C and D). To identify the key regulatory genes in EBV-CTLs, the constructed PPI network was analyzed via the CytoHubba plugin with four topological analysis methods (MCC, maximal clique centrality; MNC, maximum neighborhood component; Degree, degree centrality; EPC, edge percolated component). This analysis identified the top 20 hub nodes with the strongest connectivity, and four overlapping core hub genes (JUN, FOS, TNF, and STAT1) were finally determined to be the key regulatory genes of EBV-CTL-mediated immune responses (Figure 3E). Notably, these core hub genes were consistent with the key genes screened from the DEG and pathway enrichment analyses, further confirming their critical roles in EBV-CTL function.

Validation of Hub Gene Expression via RT-qPCR

To confirm the reliability and accuracy of the RNA-seq results, we performed quantitative real-time polymerase chain reaction (RT-qPCR) to validate the expression levels of the identified hub genes and key pathway-related genes. We validated the mRNA expression of the hub genes via RT-qPCR. Specifically, we assessed the expression of *HSP72*, a gene associated with the JNK/p38 MAPK pathway; *FOS* and *JUN*, members of the AP1 transcriptional complex; and key genes, including *FOS*, *TNF*, and *STAT 1*, through the above screening. The results revealed significant differences in the expression of these genes between the EBV-CTL group and the nonspecific CTL group. *JUN*, *FOS*, *TNF*, *HSP72* and *p38 mRNA expression* was significantly upregulated in the EBV-CTL group, and *STAT 1* was downregulated, which is consistent with our previous bioinformatics analysis (RNA-seq-based differential expression analysis, GO/KEGG enrichment analysis, and protein-protein interaction (PPI) network analysis) (Figure 4A–F). These results suggest that the above genes may play important roles in the activation, proliferation and functional regulation of CTLs following EBV infection.

Discussion

EBV, a member of the Gammaherpesvirinae subfamily within the human herpesvirus family, is distinct from other herpesviruses because of its unique tropism and long-term persistence in hosts. Unlike alphaherpesviruses (eg, herpes simplex virus), which often cause acute mucosal lesions, EBVs exhibit broad host tropism but primarily target B lymphocytes, establishing lifelong latent infection in more than 90% of the global population.¹ In immunocompetent individuals, EBVs remain dormant in B cells and are controlled predominantly by cytotoxic T lymphocytes (CTLs). However, under immunosuppressive conditions (eg, posttransplantation, immunodeficiency disorders), the virus reactivates, driving aberrant B-cell proliferation and transformation into Epstein-Barr virus-transformed lymphoblastoid cell lines (LCLs), which may induce systemic pathological manifestations.² Notably, EBV rarely infects T or NK cells in

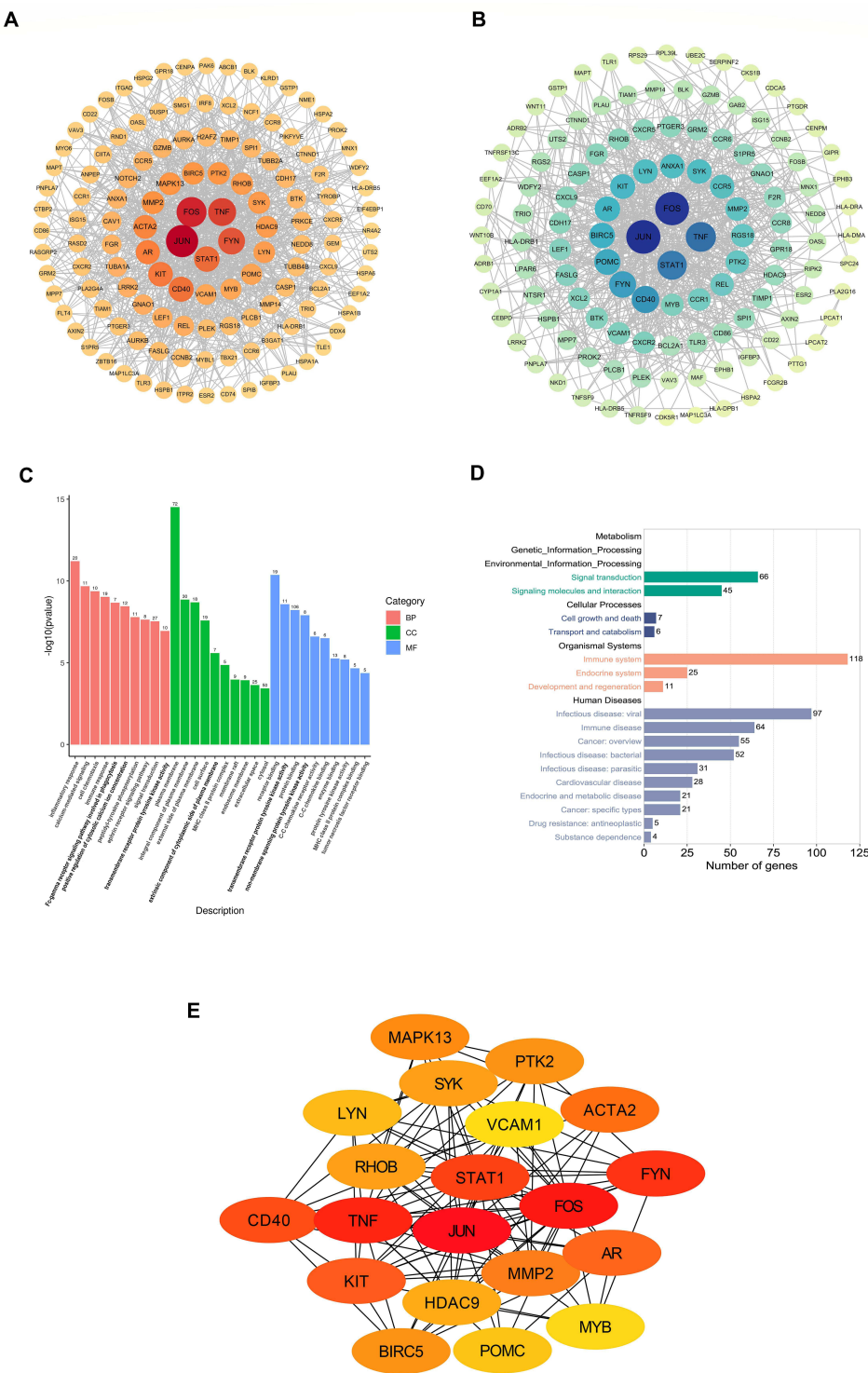


Figure 3 Identification of core regulatory genes and functional modules in EBV-CTLs via protein-protein interaction (PPI) network analysis.

Notes: (A) Whole PPI network constructed from DEGs ($\text{padj} \leq 0.05$, $|\log_2\text{FoldChange}| \geq 1.0$) via Cytoscape 3.9.1. Node size represents gene connectivity and edge thickness indicates the strength of protein-protein interactions; (B) The top-ranked functional module screened by the MCODE plugin (parameters: degree cutoff=2, node score cutoff=0.3, k-core=2, max depth=100) with the highest MCODE score (11.513), containing 120 nodes and 685 edges; (C) GO functional enrichment analysis of the core module, showing the top 15 significantly enriched terms in biological processes (BP, red), cellular components (CC, green), and molecular functions (MF, blue); (D) Secondary classification of the KEGG pathway enrichment analysis of the core module, highlighting significant enrichment in immune-related signaling pathways (eg, immune system, 118 genes) and the MAPK signaling pathway (45 genes), with the bar length indicating the number of genes in each pathway; (E) Top 20 hub genes identified by CytoHubba via four topological analysis methods (MCC, MNC, Degree, EPC), with four overlapping core hub genes (JUN, FOS, TNF, and STAT1) highlighted in red, with darker node color indicating higher node importance and stronger interaction intensity.

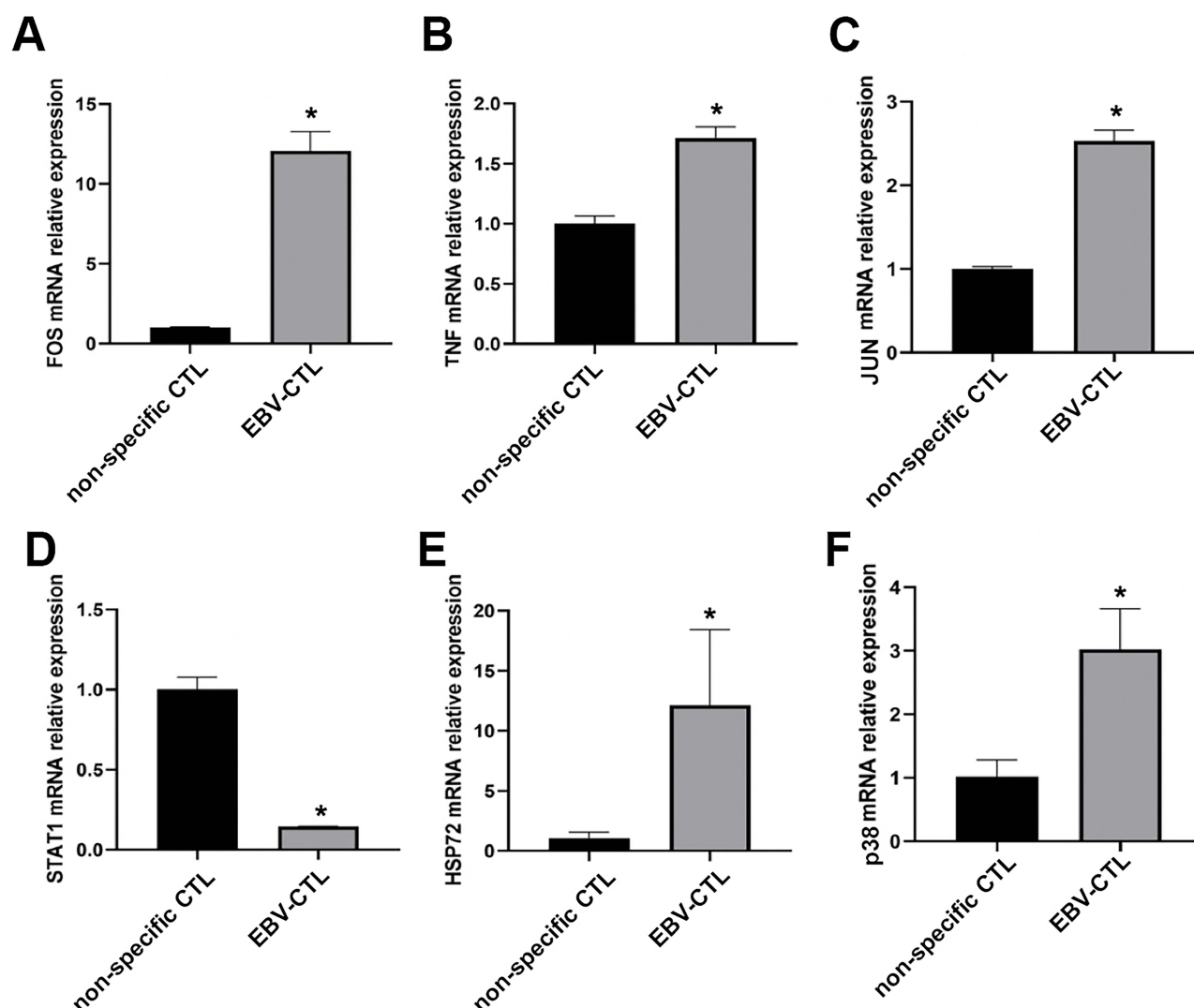


Figure 4 Validation of hub gene expression via RT-qPCR.

Notes: (A–F) RT-qPCR validation of FOS, TNF, JUN, STAT1, HSP72, and p38 mRNA expression. *P < 0.05 indicates a statistically significant difference between EBV-CTL group and non-specific CTL group.

healthy individuals, but once this cellular tropism barrier is breached, it can trigger T/NK-cell lymphoproliferative disorders (EBV-TNKLPD), a group of aggressive diseases with limited therapeutic options.¹¹ Clarifying the molecular mechanisms underlying EBV-specific CTL (EBV-CTL) activation and function is therefore critical for improving the treatment of EBV-associated diseases and addressing the unmet clinical need for effective vaccines.⁹

In this study, we established an in vitro EBV-CTL model by coculturing peripheral blood mononuclear cells (PBMCs) with inactivated LCLs, with nonspecific CTLs (stimulated with CD3/CD28 monoclonal antibodies) used as controls. By combining transcriptome sequencing (RNA-seq) and bioinformatics analyses, we systematically identified differentially expressed genes (DEGs) and key signalling pathways involved in EBV-induced T-cell modulation. The results revealed a total of 1236 DEGs between EBV-CTLs and nonspecific CTLs, including 645 upregulated genes (eg, JUN, FOS, TNF, HSP72, and p38) and 591 downregulated genes (eg, STAT1). KEGG pathway enrichment analysis highlighted the JNK/p38 MAPK pathway as the most significantly enriched signalling cascade, whereas protein-protein interaction (PPI) network analysis identified JUN, FOS, TNF, and STAT1 as core hub genes. These findings collectively suggest that EBV reprograms T-cell function through a coordinated regulatory network involving the JNK/p38 MAPK pathway and these

four hub genes—aligning with known mechanisms of T-cell responses to EBV, where antigen-specific T-cell activation relies on precise signalling cascades.¹²

Tumor necrosis factor- α (TNF- α), encoded by the TNF gene (one of our identified hub genes), is a pleiotropic proinflammatory cytokine pivotal for T-cell-mediated cytotoxicity and immune regulation. Upon binding to its receptors (TNFR1/TNFR2), TNF- α activates NF- κ B and MAPK signalling cascades, which are known to increase CTL proliferation, cytokine secretion, and target cell killing—the key functional features of EBV-CTLs in combating EBV-associated lymphoproliferative disorders (eg, posttransplant lymphoproliferative disorder [PTLD] and EBV-TNKLPD).^{13,14} The significant upregulation of TNF- α in EBV-CTLs (vs. nonspecific CTLs) observed in our study indicates that EBV-specific antigen stimulation reprograms TNF- α expression, thereby potentiating the effector function of EBV-CTLs in targeting EBV-infected cells. This finding aligns with the clinical application of EBV-CTLs, as increased TNF- α signalling may strengthen their ability to constrain EBV-driven lymphoproliferation, whereas dysregulation of this pathway could contribute to the pathogenesis of EBV-TNKLPD when EBV-CTL function is impaired.⁸

Signal transducer and activator of transcription 1 (STAT1), a key mediator in the JAK-STAT signaling pathway, is regulated by dual phosphorylation at tyrosine (Y701) and serine (S727) residues.¹⁵ Upon activation, STAT1 forms homo or heterodimers that translocate to the nucleus, where they drive the transcription of interferon-stimulated genes (ISGs) and play pivotal roles in antiviral defense and tumor suppression.¹⁶ Intriguingly, the expression of STAT1 (a key antiviral transcription factor) is significantly downregulated in EBV-CTLs, potentially due to its inhibitory effect on the IKK/NF- κ B signaling pathway—a cascade critical for CTL activation and cytotoxicity.¹¹ Prior studies have demonstrated that STAT1 deficiency leads to T-cell hyperactivation and immune evasion,¹⁷ suggesting that EBV-specific antigen stimulation may suppress STAT1 to balance the effector function and viral clearance of EBV-CTL, while excessive suppression could promote viral persistence and contribute to EBV-TNKLPD pathogenesis.³

As core components of the AP-1 transcription factor complex, JUN and FOS are key hub genes identified in our PPI network analysis. These two genes are significantly upregulated in EBV-CTLs and closely associated with the activation of the JNK/p38 MAPK pathway. AP-1 integrates upstream signals from the JNK and p38 MAPKs to modulate T-cell proliferation, differentiation, and apoptosis—processes essential for EBV-CTL-mediated immune responses. The coordinated upregulation of JUN, FOS, and JNK/p38 MAPK pathway components (p38, HSP72) in EBV-CTLs suggests that EBV exploits this signaling axis to increase T-cell activation and sustain the cytotoxic phenotype of EBV-TNKLPD.⁵ Specifically, JUN and FOS dimers form dimers that bind to the promoters of target genes involved in immune cell activation and inflammatory responses, amplifying the effector function of EBV-CTLs against EBV-infected cells.¹⁸ This regulatory mechanism may also contribute to the pathogenesis of EBV-TNKLPD, as dysregulated activation of JUN/FOS-AP-1 could lead to uncontrolled T-cell proliferation and lymphoproliferative disorders.¹¹

Further experimental validation confirmed that EBV reprograms T-cell functionality via activation of the JNK/p38 MAPK pathway. This pathway, a key branch of the MAPK family, is responsive to inflammation, oxidative stress, and pathogen invasion and transduces signals through a hierarchical MAP3K-MAP2K-MAPK phosphorylation cascade.⁵ Notably, the regulatory effects of EBVs are often cell type-specific—Mendelian randomization analysis has ruled out a causal association between EBV infection and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), suggesting that EBV-mediated immune dysregulation is more specifically targeted to lymphoid and epithelial cells than to central nervous system cells.¹⁹ This cell type-specific regulatory pattern may be partially mediated by the JNK/p38 MAPK pathway, which has distinct downstream effects on different cell lineages. In our study, abnormal activation of the JNK/p38 pathway was closely related to the upregulation of HSP72, a molecular chaperone that assists in protein folding and cellular repair.¹⁸ Recent studies have shown that HSP72 stabilizes mitochondrial function by inhibiting apoptosis signal-regulating kinase 1 (ASK1), thereby playing a protective role in virus-mediated cellular stress.²⁰ These findings collectively suggest that EBV reprograms T-cell function through a coordinated regulatory network involving the JNK/p38 MAPK pathway and these four hub genes, which is critical for enhancing the effector function of EBV-CTLs in targeting EBV-infected cells and combating EBV-associated lymphoproliferative disorders, which is consistent with the clinical application of EBV-CTLs.

Consistent with our identification of JUN, FOS, TNF, and the JNK/p38 MAPK pathway as critical regulators of EBV-CTL function, immunoinformatics studies targeting EBV envelope glycoproteins (gB, gH, gM) have highlighted the

importance of T-cell epitope-driven immune responses in vaccine development.²¹ The overlap between our identified immune regulatory genes and potential vaccine targets underscores the clinical relevance of our transcriptomic findings, providing a foundation for integrating EBV-CTL regulatory networks into novel immunotherapeutic or prophylactic strategies. For example, targeting the JNK/p38 MAPK pathway or hub genes (JUN, FOS, TNF) could increase the efficacy of EBV-CTL-based adoptive immunotherapy for EBV-associated malignancies, whereas modulating STAT1 expression may improve viral clearance and reduce the risk of EBV reactivation.

Notably, this study has certain limitations. First, the in vitro EBV-CTL model does not fully recapitulate in vivo complexity, including T-cell subset cross-talk and the tumor microenvironment. Second, posttranslational modifications (eg, p38, JUN/FOS phosphorylation) require further validation by Western blotting or flow cytometry. Third, the sample size was limited to 3 healthy volunteers, and larger cohorts with clinical samples are needed to confirm the generalizability of the findings. Future research will use conditional knockout models, single-cell sequencing, and JNK/p38 pathway inhibitor experiments to address these gaps and explore EBV-induced T-cell signaling networks.

Conclusion

In conclusion, this study revealed significant differences in gene expression between Epstein-Barr virus-specific cytotoxic T lymphocytes (EBV-CTLs) and normal human cytotoxic T lymphocytes (CTLs). The key differentially expressed genes (DEGs) identified were JUN, FOS, TNF, and STAT1. Bioinformatics analysis revealed that these DEGs were enriched mainly in the cell membrane and MHC class II protein complexes, and the most significantly enriched signalling pathway was the JNK/p38 MAPK pathway. The identified hub genes and the JNK/p38 MAPK pathway may serve as potential therapeutic targets for EBV-associated diseases, laying a foundation for the development of novel immunotherapeutic strategies.

Data Sharing Statement

The datasets used and analyzed during the current study are available from Professor Dongliang Li (corresponding author) upon reasonable request.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the 900th Hospital of the PLA Joint Logistic Support Force (No. 2020023). Informed consent was obtained from all participants in this study.

Author Contributions

All the authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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