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Original Article

Chronic stress promotes tumor immune evasion via the suppression of MHC-I expression and the upregulation of PD-L1

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Abstract: Chronic stress induces cancer initiation and progression via regulation of diverse cancer risk factors including immune evasion. Our previous research demonstrated that β-adrenergic blockade with propranolol almost completely reversed the accelerated tumor growth induced by chronic restraint stress, but the underlying mechanism of immune escape remains largely unknown. In the present study, a chronic restraint stress paradigm was applied to the H22 hepatocellular carcinoma (HCC) bearing mice to mimic the psychological stress. We observed that chronic restraint stress significantly promoted HCC growth and tumor escape from T cell surveillance. Chronic restraint stress reduced intratumor MHC-I expression and enhanced PD-L1 expression, whereas propranolol rectified the changes of MHC-I and PD-L1. Under chronic stress, the activated MAPK pathway suppressed MHC-I production by inactivating STAT1/IRF1 signaling pathway, and promoted PD-L1 translation by elevating eIF2α phosphorylation. These findings support the crucial role of β-adrenergic signaling cascade in the tumor escape from T cell surveillance under chronic restraint stress.

Keywords: Chronic restraint stress, immune evasion, β-adrenergic receptor, MHC-I, PD-L1, T cells

Introduction

Stress is inevitable in our modern daily life. Acute stress tends to be beneficial to our health, helping us respond quickly to danger but chronic stress is seriously deleterious to body and promotes the development of various diseases, especially cancer [1-4]. There is now growing evidence that chronic psychological stress activates the sympathetic nervous system (SNS) or the hypothalamic-pituitary-adrenocortical (HPA) axis, ultimately affecting the release of catecholamine neurotransmitters [3, 5, 6]. In response to neurosensory signals, the hypothalamus secretes corticotrophin-releasing factor (CRF) which activates the pituitary gland to produce hormones such as adrenocorticotropic hormone (ACTH). Circulating ACTH can stimulate the synthesis of corticosteroids from the adrenal cortex. In addition, stress signals can also activate the peripheral nervous system by cortical region, causing post-ganglion neurons to release epinephrine and norepinephrine [7]. Through the activation of hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS), these catecholamine stress hormones extensively activate β-adrenergic receptor (β-AR) in the body, directly affecting tumor microenvironment and promoting tumor malignant properties [8, 9]. However, tumor cells can also emit neuronal growth factors that increase sympathetic innervation of the tumor. This creates a feed-forward loop that further promotes cancer progression [10, 11]. Therefore, a full understanding of the molecular mechanism by which chronic stress promotes tumor growth would provide a better understanding of the etiology of cancer and guide the appropriate use of drugs.

Researchers have found that chronic stress promotes tumor growth in part because chronic stress weakens the body's immune surveillance and immune clearance, thereby facili-

tating immune escape of tumors [12, 13]. β-adrenergic signaling can stimulate the transcription of pro-inflammatory cytokines such as IL-6 and IL-8 by myeloid lineage immune cells and promote the expression of several genes related to tumor progression such as TGF-β, VEGF and PTGS2 in macrophages [14-16]. Additionally, β-adrenergic signaling may also enhance the density of tumor associated macrophages by stimulating myelopoietic development of precursor monocytes and markedly enhance macrophage recruitment into the tumor parenchyma by stimulating tumor cells' production of chemokines such as CSF1 and CCL2 [13, 17]. Increased stress hormone level significantly suppresses the cytotoxic function of T lymphocytes and NK cells [13, 18, 19]. Activation of T cells is a key event in the adaptive immune response. T cell antigen receptors (TCRs) on the surface of T cells are recognized by peptide bound major histocompatibility complexes (MHC) presented on the surface of antigen-presenting cells, leading to activation of TCR signaling transduction and further stimulation of synergistic signaling. As a result, fully activated T cells directly kill tumor cells and activate humoral immunity [20-22]. Upon TCR stimulation, T cells express programmed death-1 (PD-1) which prevents the overactivation of T cells and avoids autoimmune diseases [23, 24]. However, engagement of PD-1 by programmed death ligand-1 (PD-L1) on the surface of tumor cells negatively attenuates tumor-specific T-cell responses [25, 26].

Recently, studies on the inhibition of T-cell function by chronic stress have attracted widespread attention. Stress can inhibit the proliferative capacity of T lymphocytes in lymph nodes and spleen as well as suppress the secretion of tumor-killing cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) and reduce T cell infiltration at tumor sites [27, 28]. It has been shown that the suppression of T cell function is significantly correlated to activation of β-adrenergic receptors [29, 30]. In our previous work, we found that β-adrenergic signaling but not steroids plays a crucial role in tumor progression induced by chronic restraint stress and CXCL5-CXCR2-Erk activation enhances the immunosuppressive function and mobilization of myeloid-derived suppressor cells (MDSCs) [31]. In addition to instigating an immunosuppressive tumor microenvironment, tumor cells evade T cell surveillance by avoiding immune recognition. In the present study, a chronic restraint stress paradigm was applied to mouse hepatocellular carcinoma model to explore the role of β-adrenergic signaling in promoting immune evasion. We demonstrated that the stress-induced β-adrenergic signaling protects tumor cells from T cell surveillance via the suppression of MHC-I expression and the upregulation of PD-L1. These findings highlight that β-adrenergic signaling antagonism might be a beneficial strategy for cancer therapy.

Material and methods

Chemical and reagents

Propranolol was purchased from TargetMol (Target Molecule Corp., Beijing, China), prepared freshly, and dissolved in 5% ethanol + 5% polyoxyethylated castor oil + 90% normal saline prior to treatment. Antibodies against p38 MAPK (Cat. No. 9212), p-p38 MAPK (Cat. No. 9215), eIF2α (Cat. No. 5324), p-eIF2α (Cat. No. 3398), LMP7 (Cat. No. 13635), TAP1 (Cat. No. 12341), STAT1 (Cat. No. 9172), and p-STAT1 (Cat. No. 9167) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against PKA (Cat. No. sc-365615) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against IRF1 (Cat. No. A7692) was obtained from ABclonal Biotechnology (Beijing, China). Antibodies against PD-L1 (Cat. No. ab269253), LMP2 (Cat. No. ab184172) and TAP2 (Cat. No. ab235110) were purchased from Abcam (Cambridge, UK). All antibodies were diluted as recommended by the manufacturer's instructions unless otherwise specified.

Mice and cell lines

Male Balb/c mice were acquired from Beijing Weitong Lihua Animal Center (Beijing, China). Animal experiments were performed according to the procedures approved by the Institutional Animal Care and Use Committee at Peking Union Medical College (Beijing, China). All mice were housed at four to five mice per cage at room temperature (24°C) in a specific pathogen-free circumstance with a 12-hour light/12-hour dark cycle and free access to food and water. Murine hepatocellular carcinoma cell line H22 (RRID: CVCL_H613) was a generous

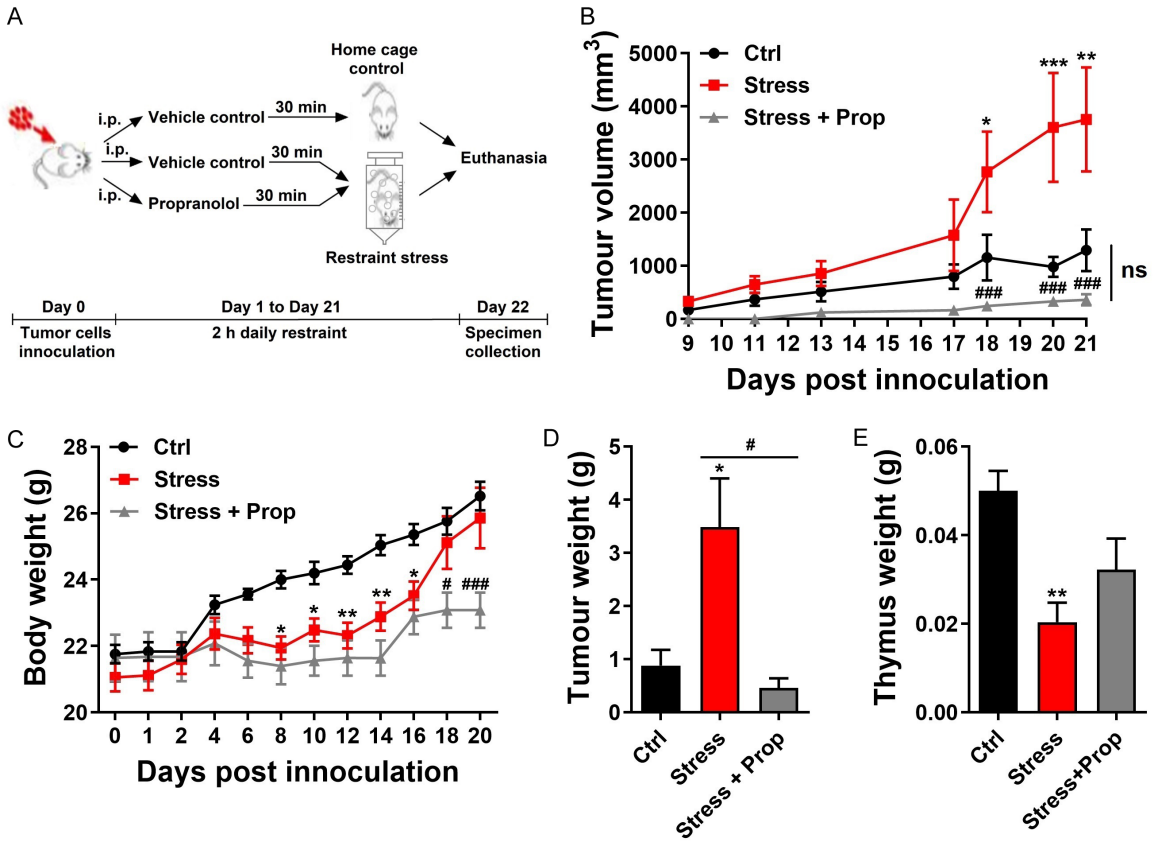

Figure 1. Blockade of β-adrenergic receptors by propranolol inhibits tumor growth under chronic restraint stress. A. Schematic of the animal experiment. B. Volume of tumor mass. Propranolol attenuated tumor growth under chronic restraint stress. (n = 6. One mouse each died in the control group and the stress + propranolol group respectively at the end of the restraint progress on day 18. A single outlier in the control group from Day 18 was rejected by Dixon's Q test at 95% confidence). C. Changes in body weight of mice during the animal experiment procedure. D. Wet weight of final dissected tumor mass. E. Wet weight of final dissected thymus. All data are shown as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs. control group; $^{\#}P < .05$, $^{\#\#\#}P < .001$ vs. stress group.

gift from Dr. Yuying Liu at Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and cultured in RPMI-1640 medium (Macgene, Beijing, China) supplemented with 10% (v/v) fetal bovine serum (PAN-Biotech GmbH, Aidenbach, Germany) and proper antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in a 37°C humidified atmosphere containing 5% CO_2. H22 cells used in our research were mycoplasma-free as tested by MycAway™-Color One-Step Mycoplasma Detection Kit (Yeasen, Shanghai, China).

Chronic restraint stress procedure and blockade of β-adrenergic receptors

Male Balb/c mice (6 weeks old) were habituated to feeding conditions for 1 week before the experiment. The chronic restraint stress model was established based on previous studies [31]. Briefly, 1 × 10^5 H22 cells were subcutaneously injected into the right flank of each mouse. On the following day, the mice were randomly assigned to home cage control condition or 2 hours/day restraint stress. For the stress groups, the mice were randomly divided to receive vehicle control (5% ethanol + 5% polyoxyethylated castor oil + 90% normal saline) and propranolol (10 mg/kg) via intraperitoneal injection 30 minutes prior to episode of restraint stress. Stressed mice were then restrained in well-ventilated 50-ml syringes that prevented them from moving freely. Each restraint session was carried out between 9 a.m. and 11 a.m. to avoid effects associated with the circadian rhythm, and the stress pattern was kept on 21 days (**Figure 1A**). Mice in the control group were administrated with vehicle control by intraperitoneal injection and deprived of food and water in their home cages simultaneously. The tumor volume was measured every other day with a digital caliper

(tumor volume = (length) × (width)^2/2). At the end of the experiment, all the mice were euthanized by cervical dislocation.

Flow cytometry

Fresh spleens were collected at the end of the animal experiment and lysed with red blood cell lysis buffer (Solarbio, Beijing, China) in ice bath for 4 minutes. The tumor tissues were collected, cut into small pieces, and digested in serum-free RPMI-1640 medium with 0.1 mg/ml DNase I (Dingguo Changsheng Biotechnology, Beijing, China) and 1.5 mg/ml collagenase (Dingguo Changsheng Biotechnology) at 37°C for 2 hours, and then ground and filtered through a 70-μm cell strainer (Corning, Manassas, VA) to obtain single-cell suspension. Both tumor cells and splenocytes were stained with the fluorescent antibodies in phosphate-buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin according to the manufacturer's instructions. The following monoclonal antibodies were used in the present study: FITC-labeled anti-H2-K^d/H2-D^d (Cat. No. 114706), APC-labeled anti-CD274 (Cat. No. 124306), FITC-labeled anti-CD45 (Cat. No. 103107), FITC-labeled anti-CD4 (Cat. No. 100509), PE-labeled anti-CD8 (Cat. No. 100707), FITC-labeled anti-CD11b (Cat. No. 101206) and APC-labeled anti-CD11c (Cat. No. 117310). All antibodies mentioned and their isotype-matched control were purchased from BioLegend (San Diego, CA, USA).

RNA sequencing (RNA-seq) analysis

Total RNA was isolated from tissue samples using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and genomic DNA was removed using DNase I (Takara Biomedical Technology, Beijing, China). Then RNA quality was determined by Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA, USA) and quantified using NanoDrop® ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only high-quality RNA sample ($OD_{260/280}$ = 1.8~2.2, $OD_{260/230} \geq$ 2.0, RIN ≥ 6.5, 28S:18S ≥ 1.0, and > 2 μg) was used to construct sequencing library. RNA purification, reverse transcription, library construction, and sequencing were performed by Shanghai Majorbio Bio-Pharm Biotechnology Co., Ltd. (Shanghai, China). Data analysis was performed on a cloud-based bioinformatics workflow platform provided by Shanghai Majorbio Bio-Pharm Biotechnology. To identify differential expression genes (DEGs) between two different samples, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method using RSEM (http://deweylab.biostat.wisc.edu/rsem/). R statistical package software edgeR (Empirical analysis of Digital Gene Expression in R, http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) was utilized for differential expression analysis. In addition, functional-enrichment analysis including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed to identify which DEGs were significantly enriched with Benjamini-Hochberg correction.

Immunohistochemistry

First, tumor tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Then, the tumor section was deparaffinized and rehydrated. After antigen retrieval, tumor section stained with anti-CD274 antibody or anti-H2-K^d antibody overnight at 4°C and horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB-BIO, Beijing, China) at 37°C for 1 hr. The expression of CD274 or H2-K^d in tumors were visualized with diaminobenzidine staining.

Western blotting and real-time reverse transcription (RT-PCR) analyses

Both western blotting and real-time RT-PCR analyses were performed as we previously described [32]. For western blotting analysis, briefly proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membranes that were probed using antibodies against different proteins and visualization by Tanon 5800 Luminescent Imaging Workstation (Tanon Science & Technology, Shanghai, China) with enhanced chemiluminescence. For real-time RT-PCR analysis, total mRNAs were reverse transcribed to cDNA using All-In-One RT MasterMix Kit followed by real-time PCR using StepOnePlus Systems with primers shown as follows: GAPDH (forward, 5'-GCCGCCTGGAGAAACCTGCCAAGT-3', reverse, 5'-TATTCAAGAGAGTAGGGAGGGCTC-3'); LMP2 (forward, 5'-ATGCTGCGGGCAGGAGCACCTACCGC-3', reverse, 5'-TCACTCATCGTAGAATTTTGGCAGCTC-3'); LMP7 (forward, 5'-CTTGGCCGCAGAGCTATTGCTTAT-3', reverse, 5'-ACATCGGAA-

CTCTCCACTTTCACC-3'); TAP1 (forward, 5'-TCTCTACCAGCTTCAGTTCACCCA-3', reverse, 5'-AAGGCCTTTCATGTTTGAGGGTGC-3'); TAP2 (forward, 5'-GTCCTACCTGAGGCCCTGGGTC-3', reverse, 5'-GCAGCTATGAGGAAAGGCAGGTC-3'); IRF1 (forward, 5'-ACCCTGGCTAGAGATGCAGA-3', reverse, 5'-GCTTTGTATCGGCCTGTGTG-3'). GAPDH was used as the internal control. The relative mRNA levels were determined using $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Mouse Interferon-γ EILSA kit (Cat. No. ab100690, Abcam) was used for the quantitative determination of IFN-γ in tumor tissues according to the manufacturer's instructions. In brief, tumor tissue was lyzed in cold PBS. 100 μl of each sample and standards were placed into an antibody-coated microplate and incubated at room temperature for 2.5 h followed by incubating with biotinylated anti-mouse IFN-γ antibody for 1 h at room temperature and further incubating with horseradish peroxidase-conjugated streptavidin for 45 min at room temperature. After complete washing, the amount of removal of IFN-γ was quantified by the peroxidase retained in the immunocomplex with TMB as a substrate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (San Diego, CA, USA). Data were presented as mean ± standard deviation. Statistical differences between two groups were compared using the Student's *t*-test. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by *post-hoc* pairwise comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Blockade of β-adrenergic receptors by propranolol attenuates tumor immune evasion under chronic restraint stress via MHC class I downregulation and PD-L1 upregulation

To investigate whether chronic stress influences tumor evasion from T cell surveillance via activation of β-adrenergic stress hormone systems, the canonical chronic restraint stress model was employed to assess the effects of propranolol, a β-adrenergic receptor antagonist, on tumor progression and immune evasion. The H22 tumor bearing mice were exposed to daily restraint stress for 21 consecutive days from the next day of inoculating H22 cells (**Figure 1A**). We observed significant tumor growth and weight loss in the stressed-mice while propranolol treatment significantly attenuated chronic restraint stress-induced tumor progression (**Figure 1B**, **1C**). Enhanced malignant tumor growth in the stressed mice was also confirmed by weight measurement at the end of animal experiment, and propranolol treatment significantly reduced tumor growth (**Figure 1D**). The above findings illustrated that stress hormone signaling through β-adrenergic receptors promotes tumor growth and progression under chronic restraint stress. Furthermore, we observed a significant weight loss in thymus in the stressed mice compared to the controls, suggesting that chronic stress may induce a systemic immune change in the H22 tumor-bearing mice (**Figure 1E**).

In cancer cells, adaptive T-cell-mediated immune responses against malignant cells are actively suppressed via immune tolerance. For this, we further evaluated the proportions of T lymphocytes in spleen by flow cytometry at the end of animal experiment. Our results showed that splenic $CD4^+$ T cells and $CD8^+$ T cells were significantly decreased in the stressed mice compared to the controls while propranolol restored T cell populations in spleen ($P < 0.05$ for $CD4^+$ T cells) (**Figure 2A**, **2B**). Immunohistochemistry staining also confirmed the loss of T lymphocytes in tumor tissues under chronic stress (**Figure 2C**). The critical event in the activation of T lymphocytes is their interaction with a molecular complex consisting of a peptide bound to MHC class I or class II molecule on the surface of an antigen-presenting cell [33]. However, tumor cells evade T cell-mediated immunosurveillance via the interaction between PD-L1 on tumor cells and PD-1 on T cells [34]. Given that propranolol failed to restore splenic dendritic cells under repeated restrain stress, we further evaluated the expression levels of MHC-I and PD-L1 in tumor (Figure S1). As evident from **Figure 2C-E**, both loss of MHC-I expression and upregulation of PD-L1 were observed in stressed-mice and blockade of β-adrenergic receptors by propranolol reconstituted MHC-I presentation competence. From the above findings, we suggest

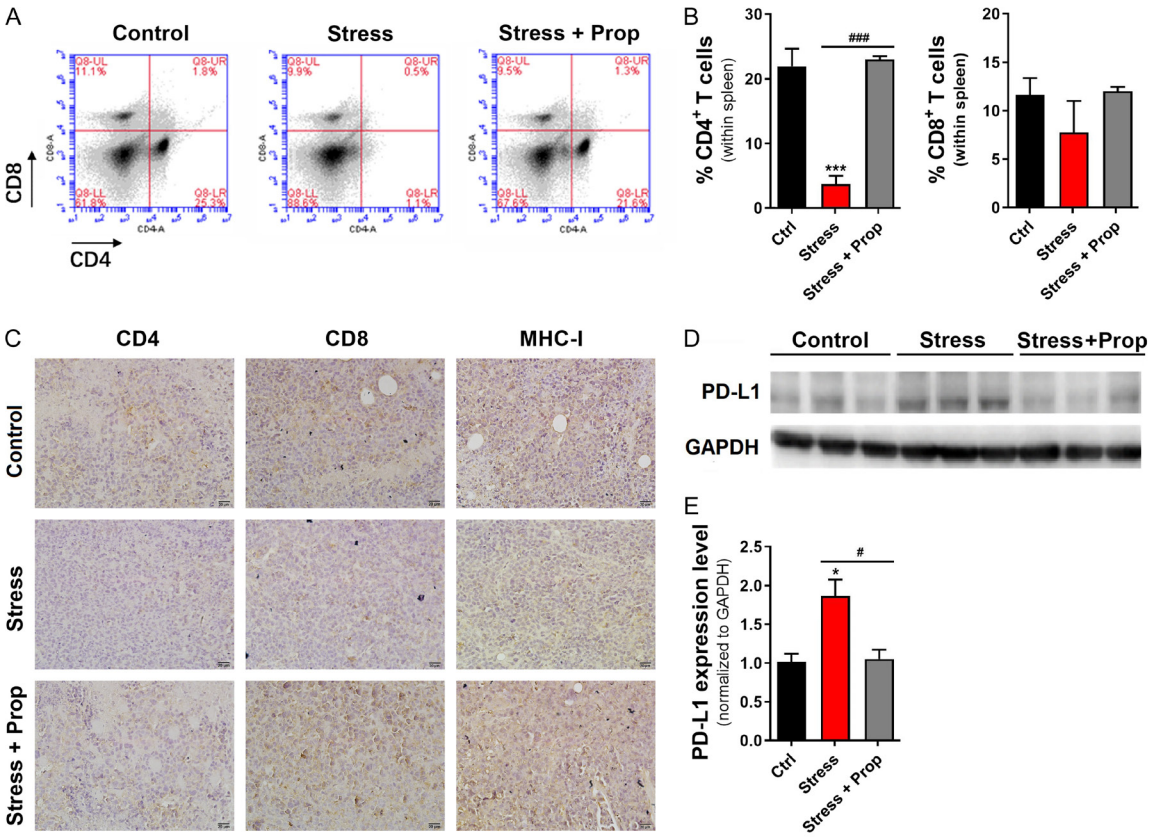

Figure 2. Blockade of β-adrenergic receptors by propranolol inhibits tumor immune evasion under chronic restraint stress via MHC class I downregulation and PD-L1 upregulation. A. At the end of animal experiment, three mice of each group were randomly selected and subjected to flow cytometric analysis of $CD4^+$ and $CD8^+$ T lymphocytes in spleens. Shown is a representative of flow cytometry data. B. Bar charts show quantification of flow cytometry data of three independent experiments. C. Immunohistochemistry staining of H22 tumor tissues for the expression of MHC-I as well as $CD4^+$ and $CD8^+$ T lymphocytes in tumor tissues. D. Protein expression of PD-L1 in tumor tissues obtained from control, stress, and stress + propranolol groups. GAPDH was used as a loading control. E. The PD-L1 expression level was quantified by the intensity of bands using ImageJ software and normalized against the internal control, GAPDH. All data are shown as mean ± SEM. $^*P < .05$, $^{***}P < .001$ vs. control group; $^{\#}P < .05$, $^{\#\#\#}P < .001$ vs. stress group.

that chronic restraint stress suppresses tumor-specific T-cell effector functions via activation of β-adrenergic stress hormone systems.

Transcriptome changes induced in tumor tissues of H22-bearing mice under chronic restraint stress

To disclose the molecular mechanism of MHC-I downregulation and PD-L1 upregulation under chronic stress, we carried out RNA-seq analysis on the tumor tissues obtained from the control group and stress group. Using the Illumina platform, a total of 320,041,150 raw reads (155,251,716 raw reads from the control and 164,789,434 raw reads from the stress group) were identified. The statistical significance of any difference in expression levels between control and stress samples was assessed using the edgeR software, applying $P < 0.05$ cut-off values to select differentially expressed genes (DEGs). Compared with the control group, there were 1,414 DEGs in the stress group, including 260 upregulated genes and 1,154 downregulated genes ($P < 0.05$) (**Figure 3A**). Cluster analysis of DEGs produced volcano plot (**Figure 3B**). Heat map of DEGs was also shown in **Figure 3C**. These data indicated that there were significant DEG patterns between the control and stress groups. To characterize the functions and subcellular locations of the DEGs in response to chronic stress, significant DEGs were mapped to terms in the Gene Ontology (GO) database with three GO classifications including biological process, cellular component, and molecular function. Among all the

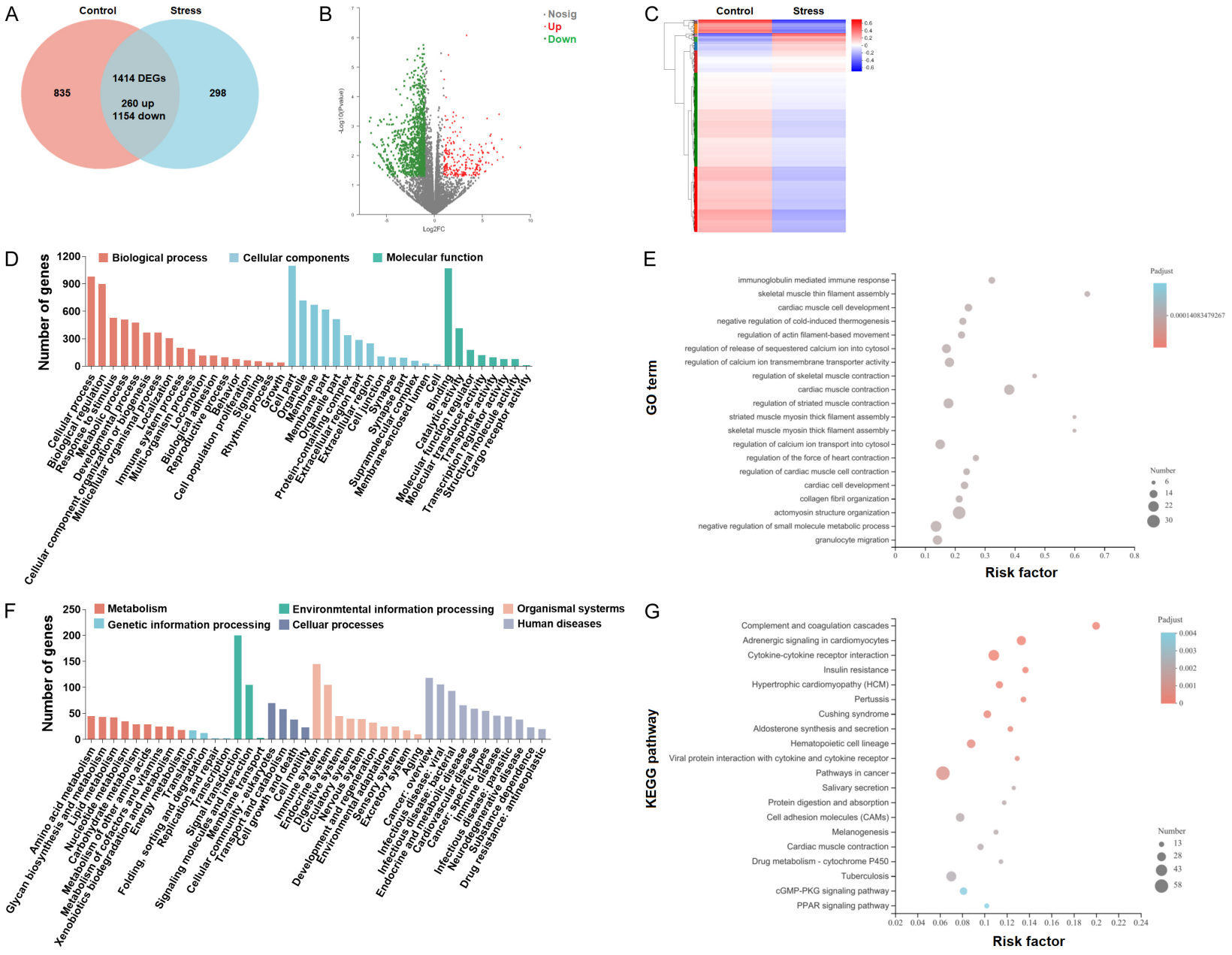
A
Control
Stress
835
1414 DEGs
260 up
1154 down
298
B
Nosig
Up
Down
-Log10(Pvalue)
Log2FC
C
Control
Stress
D
Number of genes
Biological process
Cellular components
Molecular function
Cellular process
Biological regulation
Response to stimulus
Metabolic process
Developmental process
Cellular component organization or biogenesis
Multicellular organismal process
Localization
Immune system process
Multi-organism process
Locomotion
Biological adhesion
Reproductive process
Behavior
Cell population proliferation
Signaling
Rhythmic process
Growth
Cell part
Organelle
Membrane
Membrane part
Organelle part
Protein-containing complex
Extracellular region part
Extracellular region
Cell junction
Synapse
Synapse part
Supramolecular complex
Membrane-enclosed lumen
Cell
Binding
Catalytic activity
Molecular function regulator
Molecular transducer activity
Transcription regulator activity
Structural molecule activity
Cargo receptor activity
E
GO term
immunoglobulin mediated immune response
skeletal muscle thin filament assembly
cardiac muscle cell development
negative regulation of cold-induced thermogenesis
regulation of actin filament-based movement
regulation of release of sequestered calcium ion into cytosol
regulation of calcium ion transmembrane transporter activity
regulation of skeletal muscle contraction
cardiac muscle contraction
regulation of striated muscle contraction
striated muscle myosin thick filament assembly
skeletal muscle myosin thick filament assembly
regulation of calcium ion transport into cytosol
regulation of the force of heart contraction
regulation of cardiac muscle cell contraction
cardiac cell development
collagen fibril organization
actomyosin structure organization
negative regulation of small molecule metabolic process
granulocyte migration
Padjust
Number
Risk factor
F
Number of genes
Metabolism
Genetic information processing
Environmental information processing
Cellular processes
Organismal systems
Human diseases
Amino acid metabolism
Glycan biosynthesis and metabolism
Lipid metabolism
Nucleotide metabolism
Carbohydrate metabolism
Metabolism of other amino acids
Metabolism of cofactors and vitamins
Xenobiotics biodegradation and metabolism
Energy metabolism
Folding, sorting and degradation
Translation
Replication and repair
Transcription
Signal transduction
Signaling molecules and interaction
Membrane transport
Cellular community - eukaryotes
Transport and catabolism
Cell growth and death
Cell motility
Immune system
Endocrine system
Digestive system
Circulatory system
Nervous system
Development and regeneration
Environmental adaptation
Sensory system
Excretory system
Aging
Cancer: overview
Infectious disease: viral
Infectious disease: bacterial
Endocrine and metabolic disease
Cardiovascular disease
Cancer: specific types
Immune disease
Infectious disease: parasitic
Neurodegenerative disease
Substance dependence
Drug resistance: antineoplastic
G
KEGG pathway
Complement and coagulation cascades
Adrenergic signaling in cardiomyocytes
Cytokine-cytokine receptor interaction
Insulin resistance
Hypertrophic cardiomyopathy (HCM)
Pertussis
Cushing syndrome
Aldosterone synthesis and secretion
Hematopoietic cell lineage
Viral protein interaction with cytokine and cytokine receptor
Pathways in cancer
Salivary secretion
Protein digestion and absorption
Cell adhesion molecules (CAMs)
Melanogenesis
Cardiac muscle contraction
Drug metabolism - cytochrome P450
Tuberculosis
cGMP-PKG signaling pathway
PPAR signaling pathway
Padjust
Number
Risk factor

Figure 3. Different expression profiles of RNA-seq data between control and stress groups. A. Venn diagram of differentially expressed genes (DEGs) between control and stress groups. B. Volcano plots of DEGs. Red or blue indicates either higher or lower expression levels of DEGs. C. The hierarchical cluster heat map of DEGs in tumor tissues of control and stressed mice. D. The biological process (BP) terms, cellular component (CC) terms and molecular function (MF) terms in the Gene Ontology (GO) annotation analysis. E. GO enrichment analysis of DEGs. F. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation analysis of DEGs in tumor tissues of control and stressed mice. G. The top 20 enriched KEGG pathway terms in the KEGG enrichment analysis.

DEGs, 23 GO terms-biological process, 17 GO terms-cellular component and 14 GO terms-molecular function were statistically significantly enriched in the stress group. For the biological process classification in GO annotation, the altered genes in stressed mice were predominantly involved in cellular process, biological regulation, and response to stimulus (**Figure 3D**). The top three ranks of the cellular components were cell part, organelle, and membrane (**Figure 3D**). The altered genes involved in binding, catalytic activity, and molecular function regulator were the main proportions of genes enriched by molecular function analysis (**Figure 3D**). In the GO enrichment analysis, the most enriched GO terms included immunoglobulin mediated immune response, skeletal muscle thin filament assembly, and cardiac muscle cell development (**Figure 3E**). We then focused specifically on the annotation terms linked to KEGG pathways. In the present study, the classified pathways enriched in the tumor tissues of stressed mice were shown in **Figure 3F**. Further KEGG enrichment analysis provided insight into the cellular pathways associated with complement and coagulation cascades, cytokine-cytokine receptor interaction pathway, and adrenergic signaling pathway (**Figure 3G**). These data, along with the results of animal experiment prompt us to investigate the in-depth mechanism whereby chronic restraint stress protects tumor escape from immune system via the suppression of MHC-I expression and the upregulation of PD-L1.

Chronic restraint stress inhibits MHC class I antigen processing and presentation via STAT1/IRF1 signaling pathway

The expression of MHC-I is dynamically controlled by hormonal/cytokine-mediated signals and resulting transcriptional regulation from tissue-specific transcription factors. It has been shown that activation of signal transducer and activator of transcription 1 (STAT1) by IFN-γ can induce interferon regulatory factor 1 (IRF1) expression, which in turn promotes MHC-I expression [35-37]. In this study, we observed a significant decline in intratumor IFN-γ level after chronic restraint stress (**Figure 4A**). IFN-γ is a critical cytokine which mediates tumor cell clearance. Upon binding to IFN receptors, IFN activates STAT1 signaling via phosphorylation of STAT1 in cells, leading to the transcription of its downstream, IRF-1 and the subsequent expression of multiple genes that are related to MHC class I antigen processing and presentation. Our results showed that the protein levels of p-STAT1 and STAT1 were decreased in tumor tissues of the stressed mice compared to controls, but calculation of the ratio of p-STAT1 to total STAT1 showed no significant difference between control mice and stressed mice, which suggests that activation of STAT1 by phosphorylation may decrease concomitantly with STAT1 expression (**Figure 4B**, **4C**). The transcriptional repression of STAT1 was then documented by real-time PCR analysis (**Figure 4D**). As a result, decrease in STAT1 led to a significant decrease in IRF1 mRNA and protein upon chronic restraint stress, and *in-vivo* β-adrenergic blockade by propranolol restored restraint stress-induced suppression of STAT1/IRF1 signaling pathway (**Figure 4B-D**). The above findings suggest chronic restraint stress suppresses MHC class I antigen processing and presentation via STAT1/IRF1 signaling pathway. We further examined MHC-I antigen presentation-associated gene expression and found that LMP2/7 and TAP1/2 mRNA and protein levels were reduced in tumor tissues of the stressed mice (**Figure 4E-G**). These genes have been shown to be actively involved in antigen presentation and cells deleted for LMP2/7 or TAP1/2 have very low levels of surface class I molecules [38-40]. Moreover, blockade of β-adrenergic receptors by propranolol restored LMP7 expression, suggesting the role of β-adrenergic signaling in suppressing antigen presentation via LMP7 under repetitive restraint stress.

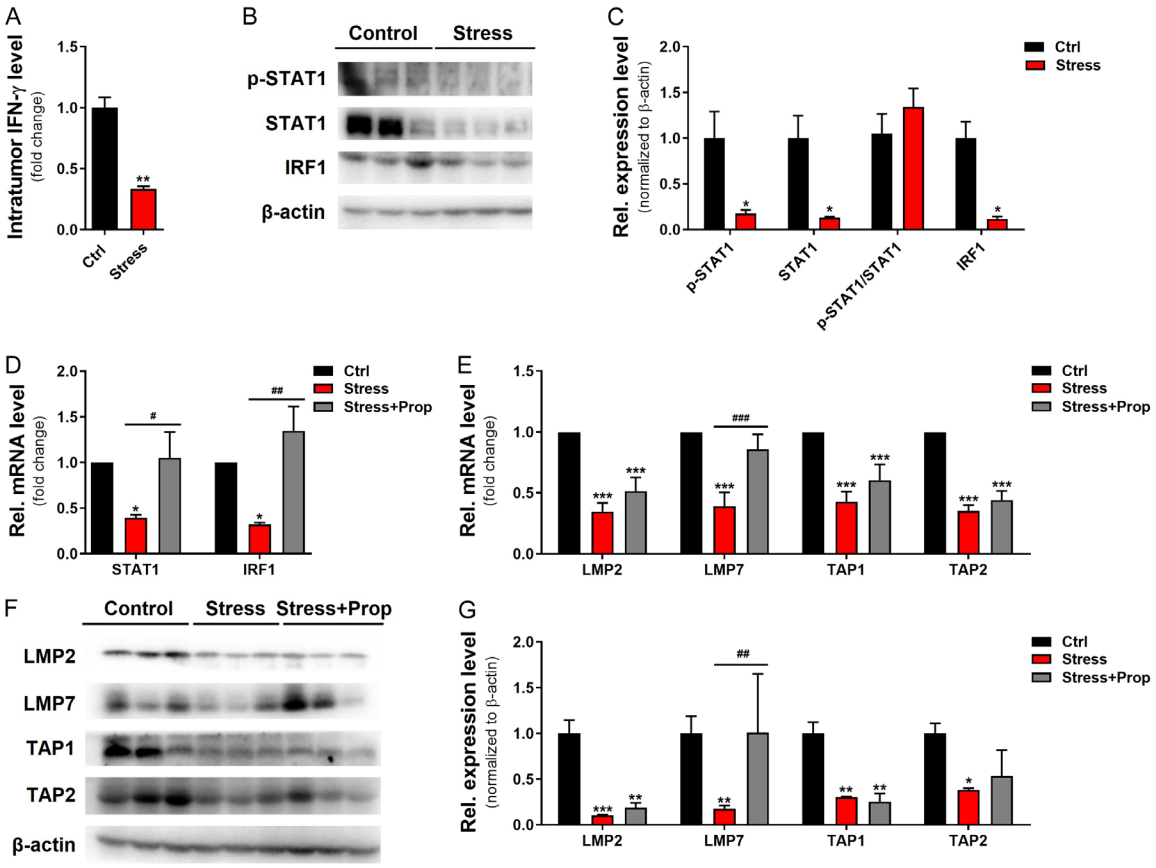

Figure 4. Chronic restraint stress inhibits MHC class I antigen processing and presentation via STAT1/IRF1 signaling pathway. A. At the end of animal experiment, three mice of each group were randomly selected and subjected to analysis of intratumor IFN-γ levels using ELISA assay. B. Protein expression of p-STAT1, STAT1 and IRF1 in tumor tissues obtained from control and stress groups. β-actin was used as a loading control. C. The protein expression levels were quantified by the intensity of bands using ImageJ software and normalized against the internal control, β-actin. D. The mRNA expression levels of STAT1 and IRF1 in tumor tissues obtained from control, stress, and stress + propranolol groups. E. The mRNA expression levels of MHC-I antigen presentation-associated genes in tumor tissues obtained from control, stress, and stress + propranolol groups. F. Protein expression of LMP2/7 and TAP1/2 in tumor tissues obtained from control, stress, and stress + propranolol groups. G. The relative protein levels were quantified by the intensity of bands using ImageJ software and normalized against the internal control, β-actin. For Western blots, only one representative loading control is shown. All data are shown as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs. control group; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs. stress group.

Chronic restraint stress enhances the expression of PD-L1 through phosphorylation of eIF2α and activation of p38 MAPK

Overexpressed PD-L1 on tumor cells binds to PD-1 receptors on the activated T cells, which leads to the inhibition of the cytotoxic T cells [25]. To investigate the underlying mechanism that β-adrenergic activation upregulated PD-L1 expression on tumor cells under chronic stress, real-time qRT-PCR was performed in tumor tissues and the PD-L1 mRNA level was observed to remain unchanged (**Figure 5A**). Since no significant change was found in PD-L1 mRNA level under chronic restrain stress, we hypothesized that the mechanism of β-adrenergic-activated PD-L1 upregulation would be a post-transcriptional process and examined the possibility that chronic stress promotes the translation of PD-L1. The eukaryotic initiation factor eIF2α has been shown to enhance the expression of PD-L1 by promoting the translation of its mRNA [41, 42]. Our results showed that the phosphorylation of eIF2α was significantly elevated after stress (**Figure 5B**). The alpha subunit of polypeptide chain initiation factor eIF2 can be phosphorylated by several related protein kinases which are activated in response to cellu-

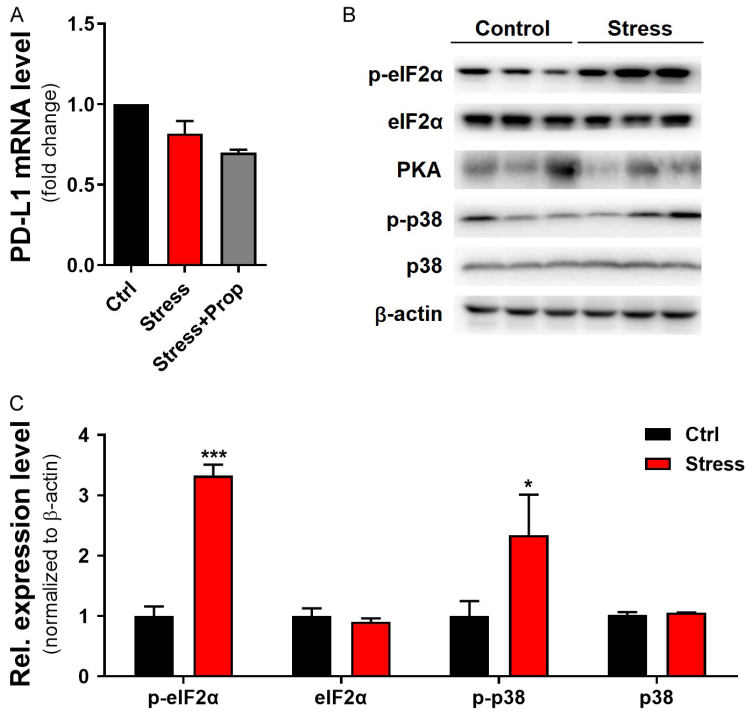

Figure 5. Chronic restraint stress enhances the expression of PD-L1 through phosphorylation of eIF2α and activation of p38 MAPK. A. At the end of animal experiment, three mice of each group were randomly selected and subject to tumor specimen collection followed by qRT-PCR analysis of PD-L1 mRNA expression. B. Protein expression of p-eIF2α, eIF2α, PKA, p-p38 and p38 in tumor tissues obtained from control and stress groups. β-actin was used as a loading control. C. The protein expression levels were quantified by the intensity of bands using ImageJ software and normalized against the internal control, β-actin. For Western blots, only one representative loading control is shown. All data are shown as mean ± SEM. $^{*}P < .05$, $^{***}P < .001$ vs. control group.

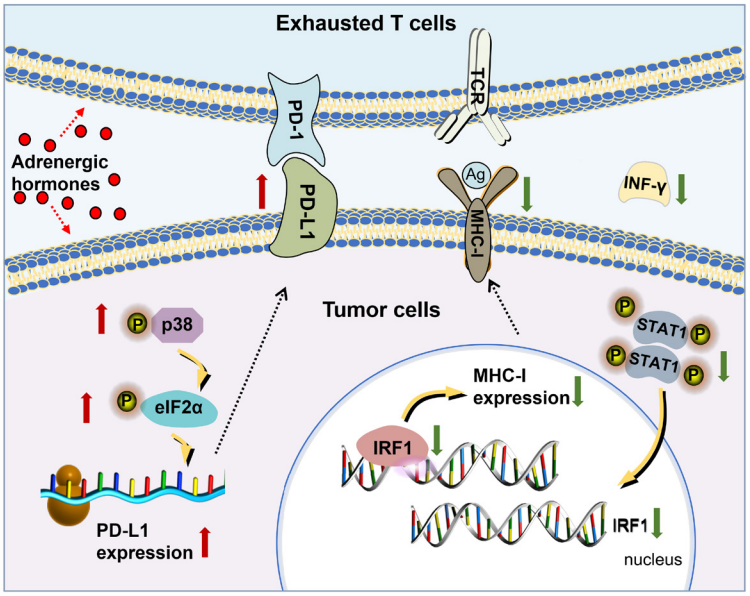

Figure 6. Chronic stress promotes tumor immune evasion via the suppression of MHC-I expression and the upregulation of PD-L1.

lar stresses [43]. Intriguingly, β-adrenergic stimulation has been shown to cause activation of p38 mitogen-activated protein kinase (p38 MAPK). In this study, we observed an increased level of p-p38, implying that chronic restraint stress enhances the expression of PD-L1 through phosphorylation of eIF2α and activation of p38 MAPK (**Figure 5C**).

Discussion

As shown in **Figure 6**, the present study provides evidence that β-adrenergic stress hormone systems promote tumor evasion from T cell surveillance under chronic restrain stress via MHC-I downregulation and PD-L1 upregulation, linking psychological stress with the enhanced tumor progression. Chronic stress inhibits MHC class I antigen processing and presentation via β-adrenergic signaling and STAT1/IRF1 pathway; on the other hand, activation of β-adrenergic receptors by chronic stress induces p38 MAPK activation, leading to PD-L1 upregulation.

Cancer research has mostly focused on the investigations on gene mutations and signal transduction pathways in tumor cells, whereas the potential role of psychological stress has been not yet been fully understood. The wide distribution of stress hormone receptors in periphery appears to be important in remodeling the tumor microenvironment. A variety of cancer cells such as liver, breast, and ovarian cancers express β-adrenergic receptors. Owing to the function of β-adrenergic receptors as G-protein-coupled cell membrane receptors, the release of catecholamines stimulated by

psychological stress triggers the formation of cAMP and activation of PKA, leading to phosphorylation of the transcription factor cAMP response element binding protein (CREB). In stress responses, phosphorylation of eIF2α protects cells by reducing the general rate of protein synthesis and biases the cell's translation initiation machinery towards translation of the mRNAs of genes and it's consistent with our RNA sequencing data that most differentially expressed genes in the stress group were downregulated [43, 44]. It was noted that eIF2α phosphorylation promotes specific expression of PD-L1 [41, 42]. Since no significant change was found in PD-L1 mRNA level under chronic restrain stress, we speculate that the activation of β-adrenergic stress hormone systems may induce phosphorylation of eIF2α indirectly via p38 MAPK. Eventually, upregulation of PD-L1 helps tumor cells escape from immune surveillance. Furthermore, concurrent downregulation of MHC class I antigen processing and presentation occurs under chronic restrain stress. In addition to cancer cells, most immune cells express β-adrenergic receptors, which raise the basis for chronic stress impairing antitumor immunity. We observed a decrease in $CD4^+$ and $CD8^+$ T lymphocytes within the spleen and tumor, and suppression of T lymphocyte activation signals after chronic stress. This may be due to β-adrenergic activation of the p38 MAPK pathway and the enhancement of T cell negative selection in the thymus, thereby impairing the proliferative capacity of T cells [45-47]. In this study, transcriptional repression of STAT1 led to a significant decline in the expression of its downstream target gene IRF1 and multiple genes that are related to MHC class I antigen processing and presentation.

It is noteworthy that the stress response is mediated by a complex and interconnected infrastructure which is in both the central nervous system and the periphery. In this research topic, we tried to design in-vitro systems to simulate the in-vivo tumor growth under chronic stress. Unfortunately, exposure to catecholamine hormones failed to mimic the features of in-vivo pathological conditions because cell-based research may not reflect the complex process existed in the body. More conclusive evidence and powerful findings would benefit from more studies in diverse animal models in the future.

In conclusion, our results demonstrate that β-adrenergic signaling protects tumor cells from T cell surveillance under chronic restraint stress via the suppression of MHC-I expression and the upregulation of PD-L1. Deeply deciphering the role of β-adrenergic signaling in the tumorigenesis and progression under stress condition will ultimately benefit the development of new strategies to improve the treatment of hepatocellular carcinoma, especially in patients who are subjected to stress.

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Disclosure of conflict of interest

None.

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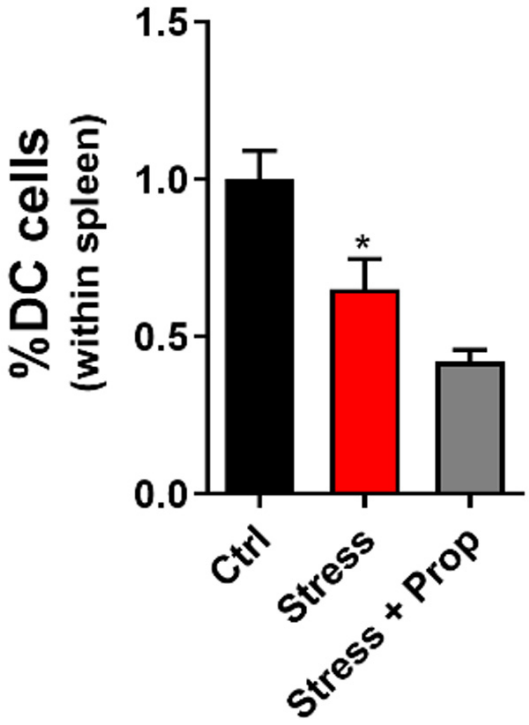

Figure S1. Changes of proportion of dendritic cells in spleen. *n* = 4 for control and stress group respectively and *n* = 5 for stress + propranolol group.