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# L-5-hydroxytryptophan promotes antitumor immunity by inhibiting PD-L1 inducible expression

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## ABSTRACT

**Background** The repression or downregulation of programmed death-ligand 1 (PD-L1) can release its inhibition of T cells and activate antitumor immune responses. Although PD-1 and PD-L1 antibodies are promising treatments for diverse tumor types, their inherent disadvantages and immune-related adverse events remain significant issues. The development of small molecule inhibitors targeting the interaction surface of PD-1 and PD-L1 has been reviving, yet many challenges remain. To address these issues, we aimed to find small molecules with durable efficacy and favorable biosafety that alter PD-L1 surface expression and can be developed into a promising alternative and complementary therapy for existing anti-PD-1/PD-L1 therapies.

**Methods** Cell-based screen of 200 metabolic molecules using a high-throughput flow cytometry assay of PD-L1 surface expression was conducted, and L-5-hydroxytryptophan (L-5-HTP) was found to suppress PD-L1 expression induced by interferon gamma (IFN-γ). Inhibition of PD-L1 induction and antitumor effect of L-5-HTP were evaluated in two syngeneic mouse tumor models. Flow cytometry was performed to investigate the change in the tumor microenvironment caused by L-5-HTP treatment.

**Results** We discovered that L-5-HTP suppressed IFN-γ-induced PD-L1 expression in tumor cells transcriptionally, and this effect was directly due to itself. Mechanistically, L-5-HTP inhibited IFN-γ-induced expression of RTK ligands and thus suppressed phosphorylation-mediated activation of RTK receptors and the downstream MEK/ERK/c-JUN signaling cascade, leading to decreased PD-L1 induction. In syngeneic mouse tumor models, treatment with 100 mg/kg L-5-HTP (intraperitoneal) inhibited PD-L1 expression and exhibited antitumor effect. L-5-HTP upregulated the ratio of granzyme B+ CD8+ activated cytotoxic T cells. An intact immune system and PD-L1 expression was critical for L-5-HTP to exert its antitumor effects. Furthermore, L-5-HTP acted synergistically with PD-1 antibody to improve anticancer effect.

**Conclusion** Our study illustrated L-5-HTP's inhibitory effect on PD-L1 induction stimulated by IFN-γ in tumor cells and also provided insight into repurposing L-5-HTP for use in tumor immunotherapy.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Antibodies blocking PD-1/PD-L1 interaction are promising immunotherapy for cancer treatment, yet inherent disadvantages and immune-related adverse events remain significant issues.

⇒ Finding small molecules to reduce PD-L1 surface expression can be an alternative and complementary therapy.

### WHAT THIS STUDY ADDS

⇒ L-5-HTP suppressed IFN-γ-induced PD-L1 expression, enhanced antitumor immunity, and synergized with PD-1 antibody to improve anticancer effect.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Role of L-5-HTP in promoting anti-tumor immunity is revealed and L-5-HTP can be repurposed for use in cancer immunotherapy.

## INTRODUCTION

The discovery of immune checkpoint molecules including cytotoxic T-lymphocyte-associated antigen-4, programmed death ligand-1 (PD-L1), and programmed death-1 (PD-1) has revealed that the interaction between tumor cells and the host immune system fosters cancer immune escape and ultimately accelerates cancer progression.[1–4] Among all immune checkpoints, the PD-1/PD-L1 axis stands out because of its value as a therapeutic target across a spectrum of different tumor types. PD-L1 expression is rarely observed in normal tissue,[5] while it is often rapidly enhanced by inflammatory cytokines, especially IFN-γ secreted by tumor-infiltrating lymphocytes.[1] IFN-γ-induced elevation of PD-L1 through the IFNAR-JAK1/2-STAT1/3-IRF1 axis has been found in various types of tumor cells, such as melanoma, gastric cancer, ovarian cancer, and breast cancer. Generally, PD-1, the receptor of PD-L1, is absent in resting T cells and is induced in activated T cells on T cell receptor engagement. When PD-1 on activated T cells interacts with its ligand PD-L1, T cell-activating signals are attenuated, and T cells subsequently exhibit anergy,

functional exhaustion and apoptosis.[6] Tumor cells often overexpress or induce PD-L1 expression in the tumor microenvironment to help evade immune attack. Hence, inhibiting the PD-1/PD-L1 signaling axis is a feasible strategy to normalize dysregulated immunosuppressive tumor microenvironment.

Existing treatment approaches that target immune checkpoint pathways are exclusively based on antibody therapies. While these antibodies have improved the survival of tumor patients, the benefits have come at the cost of serious side effects known as immune-related adverse events,[7–10] which result from the slow clearance of antibodies.[7] Moreover, the large molecular weight of antibodies and tumor distribution issues prevent antibody drugs from effectively penetrating into the tumor site, leading to an insufficient concentration and thus comprising therapeutic efficacy.[7 8] In turn, the significant advantages of small molecules may overcome the tumor penetration and uncontrollable immune-related biosafety problems of antibodies. Although great efforts have been made to develop small molecule inhibitors that disrupt the interaction of PD-1 and PD-L1, it is hard to design these types of small molecules because the binding surface of PD-1 and PD-L1 is typically flat and hydrophobic.[9] The development of small molecules with durable efficacy and favorable biosafety that inhibit PD-L1 expression thus represents a promising alternative and complementary therapy to anti-PD-1/PD-L1 therapies.

Endogenous metabolites are relatively safe and can be easily monitored for possible adverse effects; thus, both phenotype-oriented and target-oriented compound-screening strategies have used human endogenous metabolites as the starting point for new drug discovery.[10 11] Hence, in this study, we screened an in-house metabolite compound library to identify metabolic molecule downregulating PD-L1 expression. We found L-5-HTP suppressed the IFN-γ-triggered PD-L1 expression in tumor cells, revealed the underlying mechanism and evaluated its antitumor immunotherapeutic effects. Our study proved new idea to repurpose L-5-HTP for developing antitumor immunotherapies.

## MATERIALS AND METHODS

An expanded methods is available in the online supplemental material.

### Mice and *in vivo* tumor model

Female 6–8 week-old C57BL/6 mice, BALB/c mice, and BALB/c nude mice were purchased from Animal Center of Shanghai Institute of Materia Medica Chinese Academy of Sciences. All mice were fed a standard laboratory diet and provided with free access to water. The animals were housed under standard laboratory conditions (21°C±2°C, 12-hour light–dark cycle). CT26 ($3\times10^5$), MC38 ($3\times10^5$/$1\times10^6$), and MC38 $^{Pd\text{-}l1-/-}$ ($1\times10^6$) cells were subcutaneously inoculated into BALB/c mice or C57BL/6 mice. L-5-HTP (dissolved in 5% DMSO in PBS) was injected intraperitoneally (100 mg/kg) once per day. PD-1 monoclonal antibody (150 µg dissolved in PBS) (Bio X cell) was administered intraperitoneally at day 8, 12, and 16 alone or combined with L-5-HTP. Body weight and tumor volume were measured every 3 days. Tumor volume was calculated as $1/2\times(\text{length}\times\text{width}^2)$. Mice were sacrificed at the endpoint. All the procedures related to animal handling, care, and treatment were performed based on the guidelines approved by the Institutional Animal Care and Use Committee following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care. We used the ARRIVE1 reporting guidelines.[12]

### Immunoblotting

Total cell lysates were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Temecula, California, USA) and blotted with specific antibodies. The primary antibodies used are listed in online supplemental table S1. ImageJ is used for quantification analysis.

### RNA extraction and quantitative real-time PCR

Total RNA extraction reagent (Vazyme, China) was used to isolate the total RNA from cells, which was reverse transcribed to cDNA by HiScript II Q RT SuperMix (Vazyme, China). Gene expression was detected by quantitative RT-PCR on a Quant Studio 6 Flex Real-Time PCR System (Applied Biosystems). *GAPDH* /*Gapdh* was used as an internal control. Fold change in gene expression was calculated using of the ΔΔCt method. All samples were run in triplicate. Primer sequences are shown in online supplemental table S2.

### RNA sequencing and bioinformatic analysis

BXPC3 cells were treated with one of four treatments—DMSO, IFN-γ, L-5-HTP (10 µM), and IFN-γ+L-5-HTP (10 µM)—for 24 hours. Three biological replicates were performed. Total RNA was extracted to prepare cDNA libraries, which were sequenced on the Illumina HiSeq 2000 platform using the paired-end approach. The sequencing reads were mapped to hg19 using STAR 2.5, and gene expression was quantified using the featureCounts software. Differential gene expression analysis was performed using the DESeq2 R package, and genes with significant changes in expression were identified as those with a p value<0.05 and a $\log_2$ (fold change) >2. Differentially expressed genes were subjected to enrichment analysis in KOBAS.[13] An FDR value of 0.05 was used as a cut-off.

### Small interfering RNA transfection assay

BXPC3 cells were plated in 12-well culture plate to allow cell attachment overnight, and then Lipofectamine RNAiMax (Invitrogen) was used for transfection. Small interfering RNAs (siRNAs) were transfected into each well according to the manufacturer's recommended procedure. Sequences of si *c-JUN* are provided in online supplemental table S3.

### Intratumoral immune cell analysis

Tumors were excised at the endpoint of the experiment, cut into small pieces, and digested with 0.1% collagenase, 0.001% hyaluronidase, and 0.002% DNase for 60 min in a 37°C shaking incubator. Cell suspensions were filtered through a 70 µm cell strainer. Single immune cell suspensions were prepared using lymphocyte separation medium (DKW-33-R0100) following the manufacturer's instructions. Then the single-cell suspensions were blocked with anti-CD16/32 antibody (Biolegend, Cat:101320) and stained with the indicated surface antibodies for 30 min on ice. Intracellular antibody was added after fixation and permeabilization following the manufacturer's instructions (BD Cytofix/Cytoperm, Cat: 554722). Antibodies used are listed in online supplemental table S4. Samples were analyzed on an AglientNovoCyte 3130 or iQue Screener PLUS (IntelliCyt). The gating strategies for flow cytometry are shown in online supplemental figure S5A.

### Immunohistochemistry (IHC)

Mice tumor tissues were obtained from sacrificed mice at the endpoint of the experiment for IHC analysis. Tumor tissues were fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and stained with specific primary antibodies. Then the slides were further processed using the appropriate secondary antibodies, followed by counter-staining with hematoxylin. The primary antibodies used are listed in online supplemental table S5. Slides were scanned by a KFBIO KF-PRO-120 digital pathology slide scanner. For immune cell quantification, photographs of random fields were blindly taken. Positively stained cells were analyzed by Image-Pro Plus 6.0, and representative views are displayed.

### Statistical analysis

Data analysis was performed using GraphPad Prism V.7.0 software. Unpaired Student's t-test was used for normally distributed data, and repeated measures analysis of variance was used to compare time-dependent tumor growth. P values less than 0.05 were considered statistically significant. Data are shown as mean±SD; not significant (ns); $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$; and ****$p<0.0001$.

## RESULTS

### L-5-HTP blocks PD-L1 induction in cancer cells

Aiming to find metabolic molecules that modulate PD-L1 expression in tumor cells, we established a high-throughput flow cytometry screening assay based on detection of inducible PD-L1 surface expression on BXPC3 tumor cells (figure 1A). BXPC3 cells were seeded in 96-well plate, stimulated by IFN-γ, and treated with each candidate molecule. An in-house library of 200 metabolic molecules was screened, and we found that both 5-hydroxy-*L*-tryptophan (L-5-HTP) and 5-hydroxy-*D*-tryptophan (D-5-HTP) significantly decreased the extent of IFN-γ-induced PD-L1 surface expression on BXPC3 cells (figure 1B; online supplemental figure S1A). 5-hydroxytryptophan (5-HTP) is the precursor of 5-hydroxytryptamine (5-HT), which is an aromatic amino acid produced from the essential amino acid L-tryptophan by tryptophan hydroxylase (TPH).[14] Since the naturally occurring stereoisomer of 5-HTP is L-5-HTP,[15] we used L-5-HTP in the following study.

We also detected an inhibitory effect of L-5-HTP against IFN-γ-induced PD-L1 surface expression in two other human cancer cell lines: the non-small cell lung carcinoma cell line A549 and the ovarian cancer cell line SKOV3. All of these cancer cells had elevated PD-L1 levels in response IFN-γ, and treatment with L-5-HTP suppressed the IFN-γ induced elevation of PD-L1 surface expression, doing so in a dose-dependent manner (figure 1C). Immunoblotting showed that inducible PD-L1 levels in whole cell lysates from BXPC3, A549, and SKOV3 cells were consistently decreased by L-5-HTP (figure 1D). Interestingly, we noticed that L-5-HTP had little influence on constitutive PD-L1 expression in BXPC3, A549, and SKOV3 cancer cells (online supplemental figure S1B–D), which implied that L-5-HTP suppresses IFN-γ inducible rather than constitutive PD-L1 expression in cancer cells. Collectively, these data demonstrated that L-5-HTP decreases IFN-γ-induced PD-L1 expression in a variety of cancer cell lines.

### L-5-HTP transcriptionally suppresses inducible PD-L1 expression directly rather than via transformation to related metabolites

Next, we investigated the mechanism by which L-5-HTP regulates IFN-γ-induced PD-L1 surface expression. qPCR analysis showed that L-5-HTP did significantly inhibit *PD-L1* mRNA expression induced by IFN-γ in BXPC3, A549, SKOV3, and SW48 cells, as well as in the mouse cancer cell lines MC38 and CT26 (figure 2A; online supplemental figure S2A), suggesting that L-5-HTP suppresses IFN-γ-induced PD-L1 surface expression at the transcription level. We also examined whether L-5-HTP regulates PD-L1 stability. The addition of the proteasome inhibitor MG132 did not affect L-5-HTP's suppression of IFN-γ induced PD-L1 expression (online supplemental figure S2B). These data indicated that L-5-HTP regulates IFN-γ induced transcription of PD-L1, rather than its protein stability.

It is well established that over 95% of tryptophan is degraded by the kynurenine (Kyn) pathway, which generates metabolites including Kyn and kynurenic acid as products[16] (figure 2B). The remaining fraction of tryptophan is used for protein synthesis and the synthesis of neurotransmitters such as serotonin (5-HT) and melatonin (figure 2B). In 5-HT biosynthesis, L-5-HTP is first hydroxylated from tryptophan by TPH and is subsequently decarboxylated by aromatic *L*-amino-acid decarboxylase (AADC) to generate 5-HT[17] (figure 2B). Previous studies have demonstrated that L-5-HTP can be converted to 5-methoxytryptophan by hydroxyindole-O-methyltransferase (HIOMT) (figure 2B), yet HIOMT is specifically expressed in mesenchymal cells and

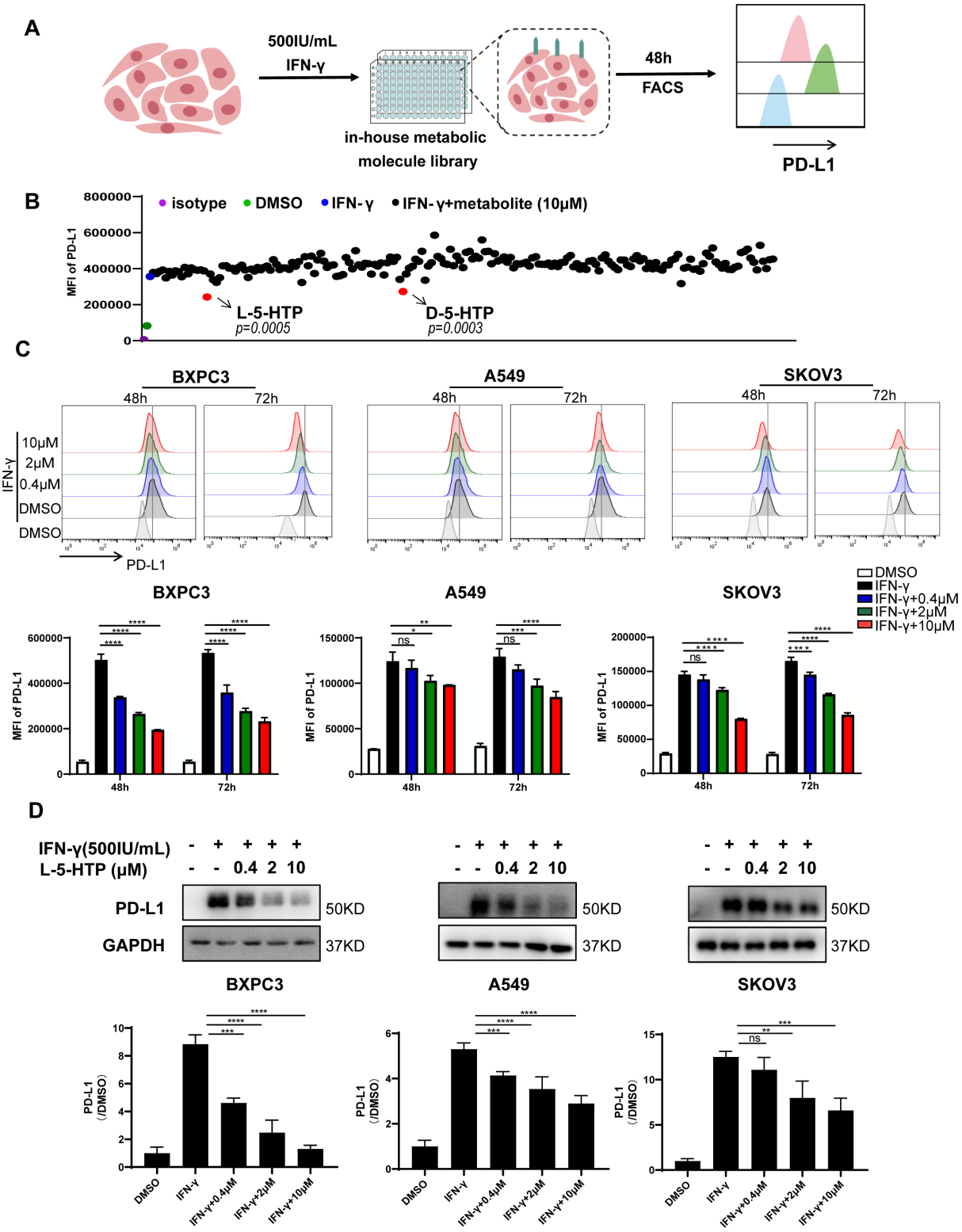


**Figure 1** L-5-HTP blocks PD-L1 induction in cancer cells. (A) Flow chart of the high-throughput flow cytometry screening assay for identification of metabolic molecules regulating inducible PD-L1 expression on the surface of cancer cells. (B) Results of the screen for metabolic molecules modulating PD-L1 induction in BXPC3 cells are shown in a scatter plot. PD-L1 expression is shown as mean fluorescent intensity (MFI). L-5-HTP and D-5-HTP are highlighted in red. P values for L-5-HTP and D-5-HTP compared with the IFN-γ group are shown. (C) PD-L1 expression on the BXPC3, A549, and SKOV3 cell surfaces was detected by flow cytometry with different concentrations and time of L-5-HTP treatment as indicated. (D) PD-L1 expression in whole cell lysates of BXPC3, A549, and SKOV3 cells was detected by immunoblotting. Quantification of western blot results for PD-L1 are shown (lower panel). Relative fold change of PD-L1 expression was normalized to the DMSO group (n=3 independent experiments). Data are shown as mean±SD; ns; p>0.05; *p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001. D-5-HTP, 5-hydroxy-*D*-tryptophan; L-5-HTP, 5-hydroxy-*L*-tryptophan; ns, not significant; PD-L1, programmed death ligand-1.

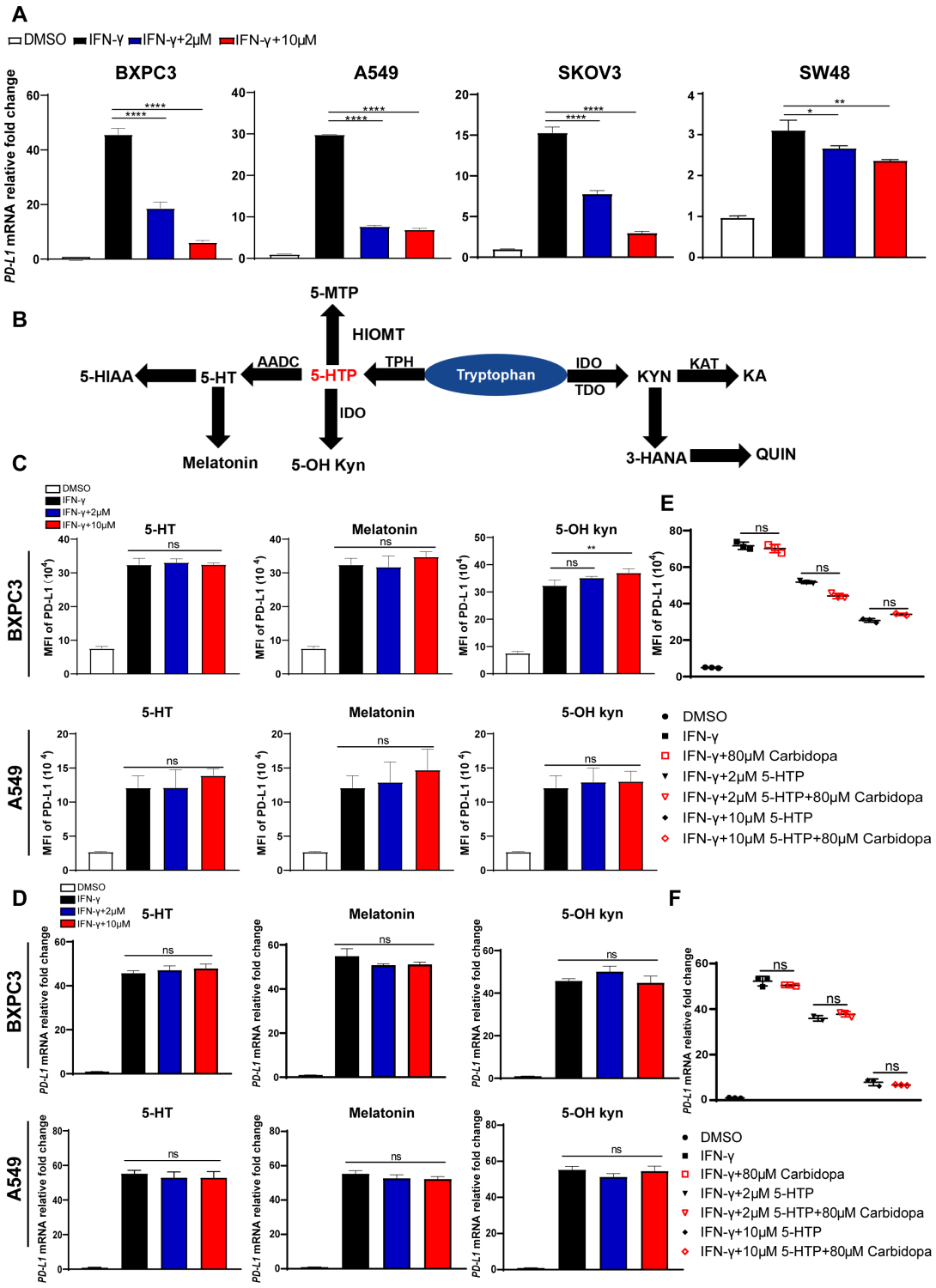


**Figure 2** L-5-HTP transcriptionally suppresses inducible PD-L1 expression directly rather than via transformation to related metabolites. (A) qPCR analysis of the effects of L-5-HTP treatment (48 hours) on *PD-L1* expression in IFN-γ-stimulated BXPC3, A549, SKOV3, and SW48 cells. (B) Schematic representation of tryptophan metabolism pathways. (C) PD-L1 surface expression on IFN-γ challenged BXPC3 and A549 cells after 5-HT, melatonin, or 5-OH Kyn treatment was detected by flow cytometry. (D) *PD-L1* transcript levels in IFN-γ-challenged BXPC3 and A549 cells after 5-HT, melatonin, or 5-OH Kyn treatment were analyzed by qPCR. BXPC3 cells were treated with IFN-γ and L-5-HTP for 48 hours with or without the AADC inhibitor carbidopa. PD-L1 surface expression and *PD-L1* mRNA expression were determined by flow cytometry (E) and qPCR (F), respectively. (n=3 independent experiments). Data are shown as mean±SD; ns; p>0.05; *p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001. 3-HANA, 3-hydroxykynurenine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; 5-HTP, 5-hydroxytryptophan; 5-OH Kyn, 5-hydroxykynurenamine; AADC, L-amino-acid decarboxylase; IDO, indoleamine 2,3-dioxygenase; KA, kynurenic acid; KAT, kynurenine aminotransferase; KYN, kynurenine; L-5-HTP, 5-hydroxy-*L*-tryptophan; ns, not significant; PD-L1, programmed death ligand-1; QUIN, quinolinic acid; TDO, tryptophan-2,3-dioxygenase; TPH, tryptophan hydroxylase.

fibroblasts and not in tumor cells.[18–20] L-5-HTP can also be transformed into 5-hydroxykynurenamine (5-OH Kyn) by indole-2,3-dioxygenase[21] (figure 2B). Therefore, it is possible that the L-5-HTP added to tumor cells could be converted to 5-HT and/or melatonin or to 5-OH-Kyn. We investigated whether the observed inhibition of IFN-γ induced PD-L1 surface expression results directly from L-5-HTP *per se* or whether this function is mediated after its transformation to a related metabolite(s).

IFN-γ induced PD-L1 expression in BXPC3 and A549 cells was not abolished on the addition of 5-HT or melatonin at both protein and transcription levels (figure 2C,D). Furthermore, the addition of the AADC inhibitor carbidopa (which blocks the conversion of L-5-HTP to 5-HT) did not abolish L-5-HTP's capacity to suppress IFN-γ-induced PD-L1 surface expression and PD-L1 mRNA expression (figure 2E,F). Moreover, the addition of 5-OH Kyn did not affect IFN-γ induced PD-L1 expression in BXPC3 and A549 cells at the protein or transcription level (figure 2C,D). These data indicate that L-5-HTP's capacity to suppress IFN-γ-induced PD-L1 expression at the transcription level does not obviously involve metabolites produced from L-5-HTP.

### The inhibitory effects of L-5-HTP on IFN-γ stimulated PD-L1 induction are mediated by reduced expression of RTK ligands and suppression of the downstream RTK receptor/MEK/ERK/c-JUN signaling cascade

We next tried to unravel the underlying mechanism by which L-5-HTP suppresses IFN-γ induced *PD-L1* mRNA expression. The JAK1/JAK2-STAT1/STAT2/STAT3-IRF1 axis is the canonical pathway that functions in IFN-γ exposure-mediated PD-L1 induction.[22] Cotreatment with L-5-HTP did not affect IFN-γ induced activation of JAK1-STAT1 (online supplemental figure S3A). A slight decrease of *PD-L1* mRNA was only observed after 6 hours of treatment with L-5-HTP, and the inhibitory effects reached a maximum at 24 hours (figure 3A). Hence, we hypothesized that L-5-HTP might inhibit IFN-γ induced mediators that somehow function in transcriptional activation of *PD-L1*. To identify which mediators of PD-L1 expression are affected by L-5-HTP treatment and to clarify the mechanism underlying reduced PD-L1 induction, RNA sequencing was performed on BXPC3 cells treated with one of the following conditions for 24 hours: DMSO, L-5-HTP, IFN-γ, and IFN-γ+L-5-HTP. The sole treatment of L-5-HTP did not trigger any significant differential expression genes in comparison with DMSO group (online supplemental figure S3B). When comparing the DMSO group and the IFN-γ group, 2261 genes were upregulated in the IFN-γ group. Among these upregulated genes, the expression of 672 genes subsequently decreased with the addition of L-5-HTP (figure 3B). KEGG pathway analysis was performed on these 672 genes. The top 20 enriched KEGG pathways are shown in figure 3B. Of note, MAPK signaling and NF-κB signaling were found to be relevant to PD-L1 regulation. Immunoblotting showed that L-5-HTP had little effect on NF-κB signaling (online supplemental figure S3C), but it did significantly dampen the IFN-γ induced activation of the MEK/ERK/c-JUN signaling cascade (online supplemental figure S3D). Consistent with this, L-5-HTP treatment decreased the phosphorylation levels of ERK, c-JUN, and PD-L1, doing so in a time-dependent manner (figure 3C,D).

MEK/ERK/c-JUN signaling has been reported to activate *PD-L1* transcription,[23 24] so we next determined whether L-5-HTP's suppression of IFN-γ induced *PD-L1* transcriptional activation is mediated by this pathway. Specifically, we knocked down *c-JUN* using siRNA and confirmed efficient knockdown with immunoblotting. The extent of the IFN-γ induced *PD-L1* transcriptional activation was significantly decreased when *c-JUN* was knocked down (online supplemental figure S3E). Importantly, upon *c-JUN* knockdown, L-5-HTP treatment conferred no further reduction in *PD-L1* transcriptional activation, supporting the notion that c-JUN mediates L-5-HTP's suppression of *PD-L1* transcriptional activation (online supplemental figure S3E).

We next investigated how MEK/ERK/c-JUN signaling is regulated by L-5-HTP. Six of the top 20 most downregulated genes in the MAPK/ERK/c-JUN pathway are upstream RTK ligands, namely *HGF*, *VEGFC*, *ANGPT4*, *AREG*, *VEGFA*, and *TGFA* (figure 3E). BXPC3 cells were treated with IFN-γ with or without L-5-HTP for 24 hours. qPCR and ELISA analysis confirmed that the mRNA and supernatant protein levels of these RTK ligands were increased by IFN-γ and that this induction was suppressed by L-5-HTP treatment (figure 3E, online supplemental figure S3F). We further examined the effect of L-5-HTP on IFN-γ induced activation of the upstream RTK receptors c-MET (receptor of HGF) and EGFR (receptor of AREG). L-5-HTP treatment significantly suppressed the phosphorylation-mediated activation of c-MET and EGFR (online supplemental figure S3G), suggesting that L-5-HTP inhibited the expression of RTK ligands and accordingly suppressed the activation of the related RTK receptors. The presence of HGF or AREG partially rescued the L-5-HTP-mediated suppression of PD-L1 transcriptional activation and MEK/ERK/c-JUN signaling cascade activation induced by IFN-γ (figure 3F–H), supporting a functional impact from HGF and AREG. Collectively, these results indicated that L-5-HTP somehow inhibits the IFN-γ-induced expression of RTK ligands and thereby blocks the downstream activation of the RTK receptors/MEK/ERK/c-JUN signaling cascade that controls *PD-L1* transcriptional activation.

### L-5-HTP reduces PD-L1 expression and tumor growth *in vivo*

We next investigated the potential immunotherapeutic effects of L-5-HTP. First, we determined that L-5-HTP does not have any significant deleterious effects on the proliferation of OVA-activated primary T cells isolated from an OT-I mouse (online supplemental figure S4A). We then tested for any *in vivo* antitumor effects of L-5-HTP in immunocompetent mouse models. Two immunogenic

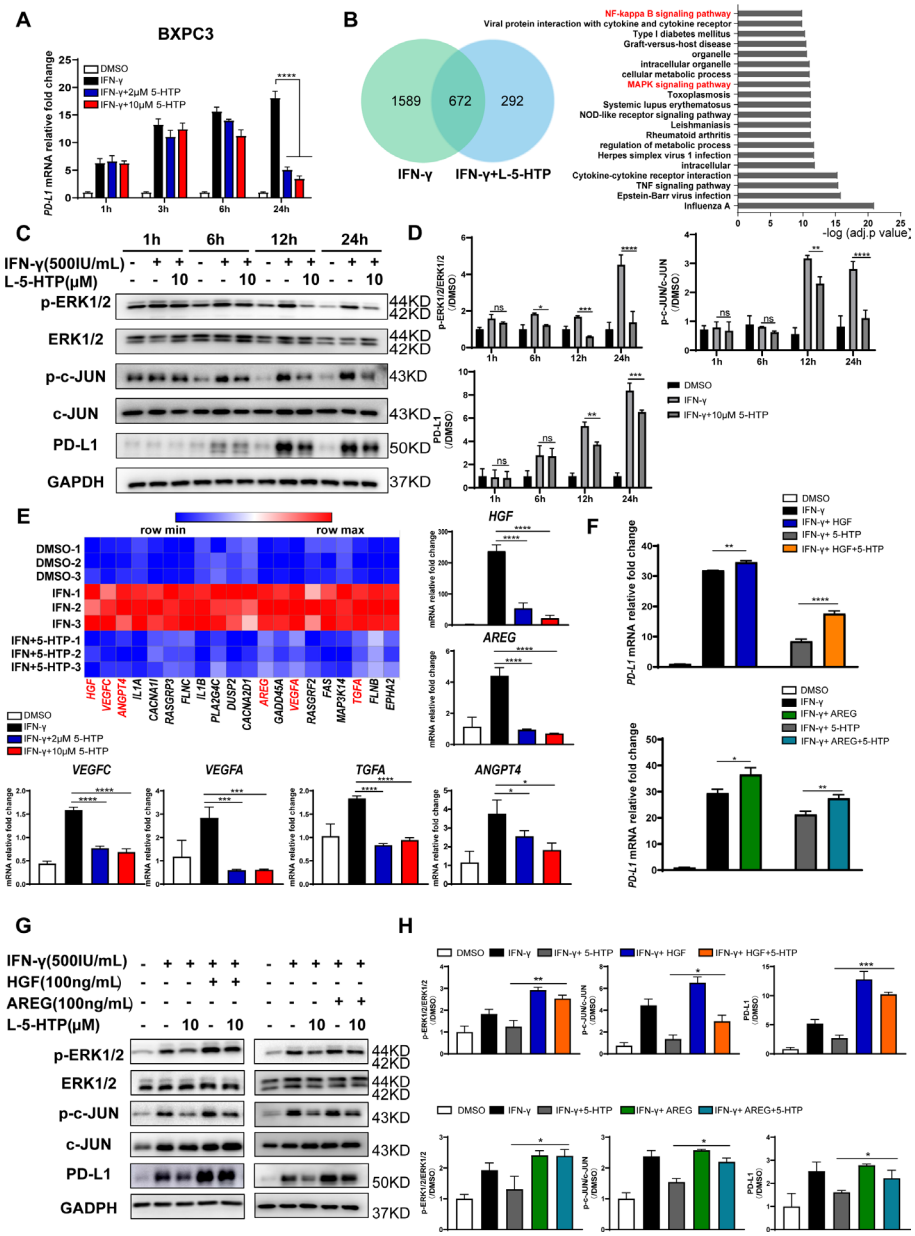


**Figure 3** The inhibitory effects of L-5-HTP on IFN-γ stimulated PD-L1 induction are mediated by reduced expression of RTK ligands and suppression of the downstream RTK receptors/MEK/ERK/c-JUN signaling cascade. (A) *PD-L1* mRNA expression level was determined by qPCR analysis of IFN-γ stimulated BXPC3 cells treated with different concentrations L-5-HTP for different time as indicated. (B) Green: gene set of IFN-γ induced genes (upregulated compared with the DMSO group). Blue: genes downregulated in the IFN-γ+5 HTP group compared with IFN-γ group. Venn diagram showing that among the IFN-γ induced upregulated genes, 672 genes were subsequently downregulated on treatment with L-5-HTP. These 672 genes were subjected to KEGG pathway analysis, and the top 20 most significantly enriched pathways are shown. (C) BXPC3 cells were treated with IFN-γ and L-5-HTP for different time (1 hour, 6 hours, 12 hours, and 24 hours), and then ERK1/2, p-ERK1/2, c-JUN, p-c-JUN, PD-L1, and GAPDH expression was analyzed by western blot. (D) Quantification of western blot results for p-ERK1/2/ERK1/2, p-c-JUN/c-JUN, and PD-L1 shown in figure part C. Relative fold change in expression levels of p-ERK1/2/ERK1/2, p-c-JUN/c-JUN, and PD-L1 were normalized to the DMSO group. (E) Heat map showing the 20 most significantly downregulated genes in the MAPK pathway. RTK ligands are highlighted in red. qPCR analysis was conducted to validate the repression of RTK ligands. BXPC3 cells were treated with IFN-γ and different concentrations of L-5-HTP for 24 hours. (F) BXPC3 cells were treated with DMSO, IFN-γ (500 IU/mL), IFN-γ+L-5-HTP (10 μM), IFN-γ+HGF (100 ng/mL), IFN-γ+HGF+L-5-HTP, IFN-γ+AREG (100 ng/mL), or IFN-γ+AREG + L-5-HTP for 24 hours. *PD-L1* mRNA expression under the indicated treatments was determined by qPCR analysis. (G) ERK1/2, p-ERK1/2, c-JUN, p-c-JUN, PD-L1, and GAPDH levels under the indicated treatments for 24h were determined by western blot analysis. (H) Quantification of western blot results for p-ERK1/2/ERK1/2, p-c-JUN/c-JUN, and PD-L1 shown in figure part G. Relative fold changes in expression levels of p-ERK1/2/ERK1/2, p-c-JUN/c-JUN, and PD-L1 were normalized to the DMSO group (n=3 independent experiments). Data are shown as mean±SD; ns; p>0.05; *p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001 versus IFN-γ group. L-5-HTP, 5-hydroxy-*L*-tryptophan; ns, not significant; PD-L1, programmed death ligand-1.

mouse colon cancer cell lines (MC38 and CT26) were subcutaneously implanted into immunocompetent mice, which were given daily intraperitoneal injections of 100 mg/kg L-5-HTP. Compared with the vehicle control, L-5-HTP treatment significantly delayed the growth of both MC38 and CT26 tumors and reduced tumor weight (figure 4A,B). No significance in body weight were detected between the vehicle group and the treated group (figure 4C), suggesting that L-5-HTP was well tolerated. Moreover, the L-5-HTP treated group showed significantly better survival over the vehicle control group in both MC38 and CT26 tumor models (figure 4D). We also confirmed the antitumor therapeutic effects of L-5-HTP by showing that delaying the start of treatment until tumors reached 5 mm×5 mm (online supplemental figure S4B–D) resulted in similar suppression of tumor growth.

The induction of PD-L1 within the tumor is mainly mediated by inflammatory cytokines, especially IFN-γ secreted by tumor infiltrating lymphocytes. We measured PD-L1 expression on tumor cells dissected from MC38 and CT26 tumor tissues with vehicle or 100 mg/kg L-5-HTP treatment (intraperitoneally) at the endpoint of experiments. Consistent with the *in vitro* results, the L-5-HTP-treated group had reduced PD-L1 expression in tumor cells as tested by flow cytometry and IHC analysis (figure 4E,F). PD-L1 expression on CD11c+MHC-II+DCs and CD11b+ F4/80+ macrophages within MC38 tumors was also reduced by L-5-HTP treatment (online supplemental figure S4E). Since PD-L2 can compensate for PD-L1 and has been hypothesized as a possible cause of resistance to existing monoimmunetherapy,[25] we investigated PD-L2 expression within MC38 tumors. Interestingly, *Pd-l2* mRNA expression in tumors was downregulated on L-5-HTP administration (online supplemental figure S4F). We further validated the inhibitory effect of L-5-HTP on *PD-L2* induction in the human cancer cell lines BXPC3 and A549 (online supplemental figure S4G). Our results collectively suggest that L-5-HTP treatment can suppress PD-L1/PD-L2 expression and confer a well-tolerated antitumor effect.

### L-5-HTP enhances antitumor immune responses

L-5-HTP inhibited PD-L1 expression in tumors, and we noticed the presence of greater numbers of CD45+ cells within tumor tissues treated with L-5-HTP (online supplemental figure S4H). Since PD-L1 is employed by tumor cells to avoid T cell attack, we collected tumors at the endpoint of the *in vivo* experiment and isolated single-cell suspensions to study T cell immunity. Flow cytometry analysis indicated that compared with vehicle controls, L-5-HTP treatment increased the number of CD3+ T cells in MC38 tumors (figure 5A,B). Importantly, increased number of intratumoral CD8+ cytotoxic T cells and augmented ratio of granzyme B+ CD8+ activated cytotoxic T cells was observed in L-5-HTP-treated MC38 tumors, as detected by flow cytometry and IHC staining (figure 5C–H), indicating that L-5-HTP can boost antitumor immunity in mice. CD8 and granzyme B IHC staining of CT26 tumors were also performed, and consistent results demonstrating increased number of CD8+ cytotoxic T cells, and granzyme B+ CD8+ activated cytotoxic T cells within tumors on L-5-HTP treatment were obtained (online supplemental figure S5B,C). Since a previous study discovered that intratumor injection of L-5-HTP (100 µg per mouse) induces the expression of inhibitory receptors in CD8+ T cells,[26] we investigated PD-1 expression in the intratumoral CD8+ T cells used in our study on injection with 100 mg/kg L-5-HTP (intraperitoneal). PD-1 expression in CD8+ T cells remained similar compared with that of the vehicle group (online supplemental figure S5D). In OVA-activated primary T cells isolated from OT-I mice, 2 µM/10 µM of L-5-HTP also did not influence PD-1 expression *in vitro* (online supplemental figure S5E). Together, our results suggest that L-5-HTP promotes T cell-mediated antitumor immunity *in vivo*.

### An intact immune system and PD-L1 axis are essential for L-5-HTP to reduce tumor growth

Tumor and serum concentrations of L-5-HTP before and after intraperitoneal treatment with 100 mg/kg L-5-HTP were determined by ultra-high-pressure liquid chromatography-mass spectrometry/mass spectrometry (UHPLC-MS/MS) (online supplemental figure S6A,B). We examined the cytotoxicity of L-5-HTP on tumor cells using the concentrations that close to or much higher than that detected in serum after L-5-HTP treatment. 10 µM, 50 µM, and 100 µM of L-5-HTP did not inhibit the proliferation of MC38 or CT26 cells, indicating that L-5-HTP has no obvious cytotoxic effects on tumor cells (online supplemental figure S6C,D). We tested whether the immune system defects would compromise the antitumor effects of L-5-HTP. At a dose of 100 mg/kg, L-5-HTP exerted a significant antitumor effect in the immune competent model (wild-type C57BL/6 mice) (figure 6A; online supplemental figure S6E,F). However, the same dosage of L-5-HTP in an immune compromised (nude mice) model did not result in the same antitumor effect as that in the immune competent background (figure 6A; online supplemental figure S6E,F), revealing that an intact immune system plays an indispensable role in the efficacy of L-5-HTP. We next tested whether the antitumor effect of L-5-HTP depends on its inhibition of PD-L1 expression. An MC38 $^{Pd\text{-}l1-/-}$ cell line was verified (figure 6B). The MC38 $^{Pd\text{-}l1-/-}$ cell line showed similar growth characteristics to those of the wild-type cell line *in vitro* as well as *in vivo* in nude mice (online supplemental figure S6G,H). Then, we implanted the same number of MC38 $^{WT}$ cells and MC38 $^{Pd\text{-}l1-/-}$ cells in wild-type C57BL/6 mice and compared tumor growth in response to L-5-HTP treatment. MC38 $^{Pd\text{-}l1-/-}$ tumors grew significantly slower than MC38 $^{WT}$ tumors, which potentially is due to improved T cell control in the absence of PD-L1 expression (figure 6C). Notably, L-5-HTP impeded MC38 $^{WT}$ tumor growth while it had no effect on MC38 $^{Pd\text{-}l1-/-}$ tumors (figure 6C). These results demonstrated

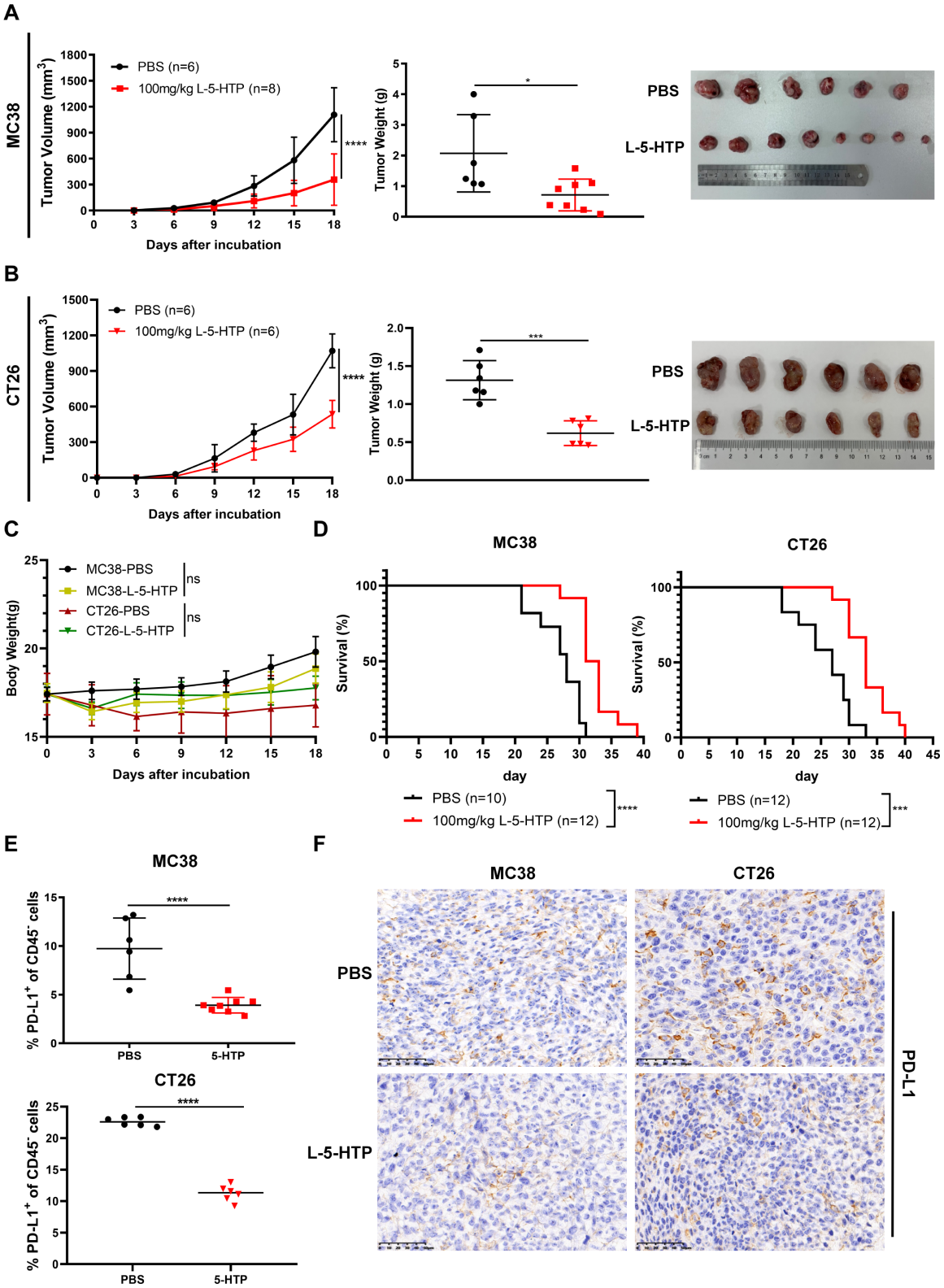


**Figure 4** L-5-HTP reduces PD-L1 expression and tumor growth in vivo. L-5-HTP was administered (intraperitoneally) the day after implantation of MC38 tumor cells (A) and CT26 tumor cells (B). Tumor volume and body weight of wild-type C57BL/6 mice (A, left) and wild-type BALB/c mice (B, left) were recorded once every 3 days. Weights of MC38 tumors (A, middle) and CT26 tumors (B, middle) and representative images of MC38 tumors (A, right) and CT26 tumors (B right) are shown. Body weight of mice in figure parts A and B are shown in figure part C. (D) MC38 tumor-related survival and CT26 tumor-related survival are shown. (E) Percentages of tumor cells expressing PD-L1 (gated on CD45– cells) isolated from MC38 tumor tissues (top) and CT26 tumor tissues (bottom) were determined by flow cytometry. (F) Representative images of MC38 and CT26 tumors immunohistochemically stained for PD-L1. Scale bar=50 µm. Data are shown as mean±SD; ns; p>0.05; *p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001. L-5-HTP, L-5-hydroxytryptophan; ns, not significant; PD-L1, programmed death-ligand 1.

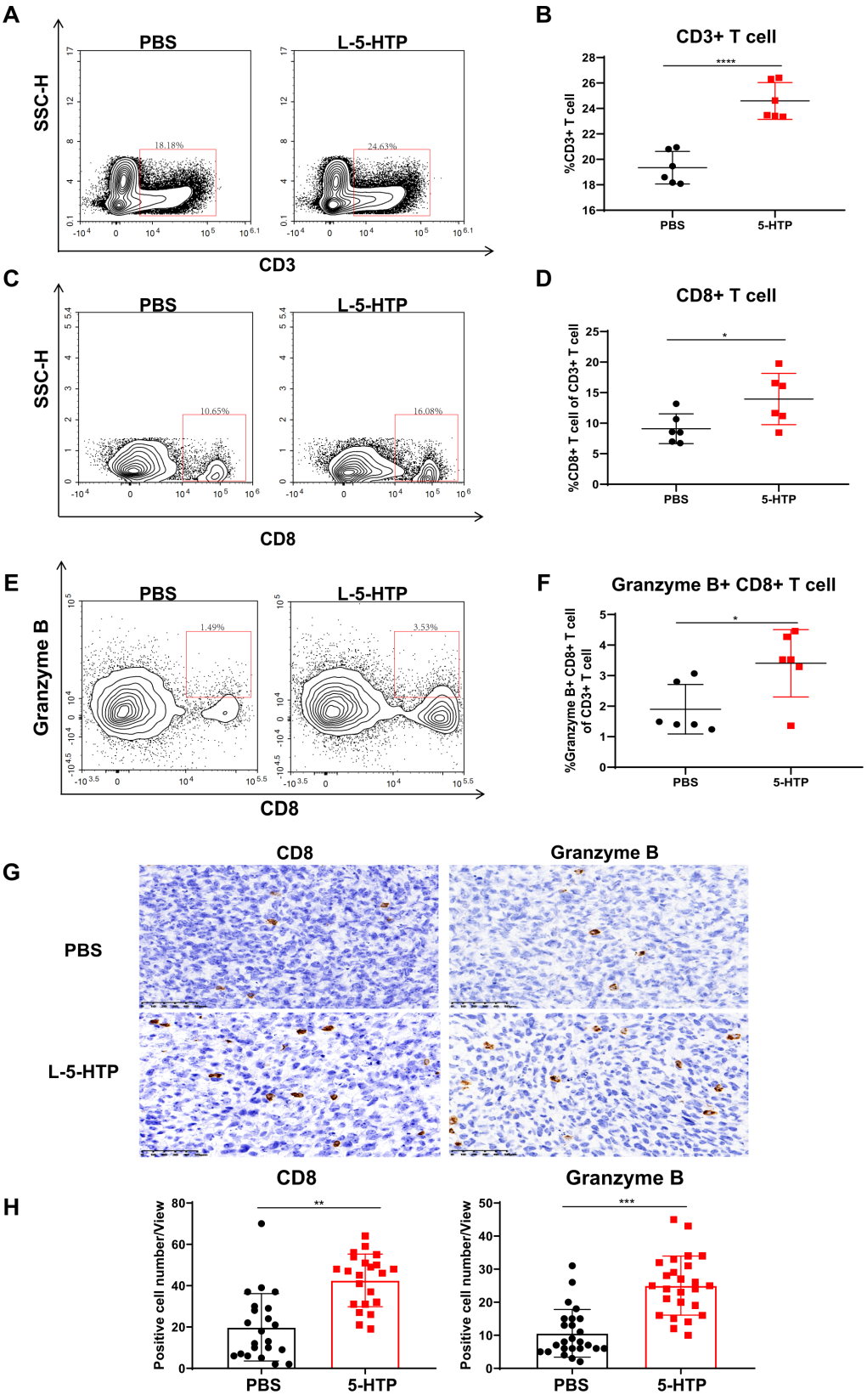


**Figure 5** L-5-HTP enhances the antitumor immunity response. MC38 tumors were harvested at day 18, and the immune profiles of these tumors were analyzed by flow cytometry. Representative plots and data are shown. Flow cytometry analysis of CD3+ total T cells (A and B), CD8+ cytotoxic T cells (C and D), and granzyme B+ CD8+ activated cytotoxic T cells (E and F) from the primary tumors. IHC staining of CD8 and granzyme B was performed using the resected tumors at day 18. Representative images of staining are shown in (G).The number of CD8 and granzyme B IHC-positively stained cells in each view are shown in (H). Data are shown as mean±SD; ns; p>0.05; *p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001. Scale bar=50 µm. IHC, immunohistochemistry; L-5-HTP, L-5-hydroxytryptophan; ns, not significant.

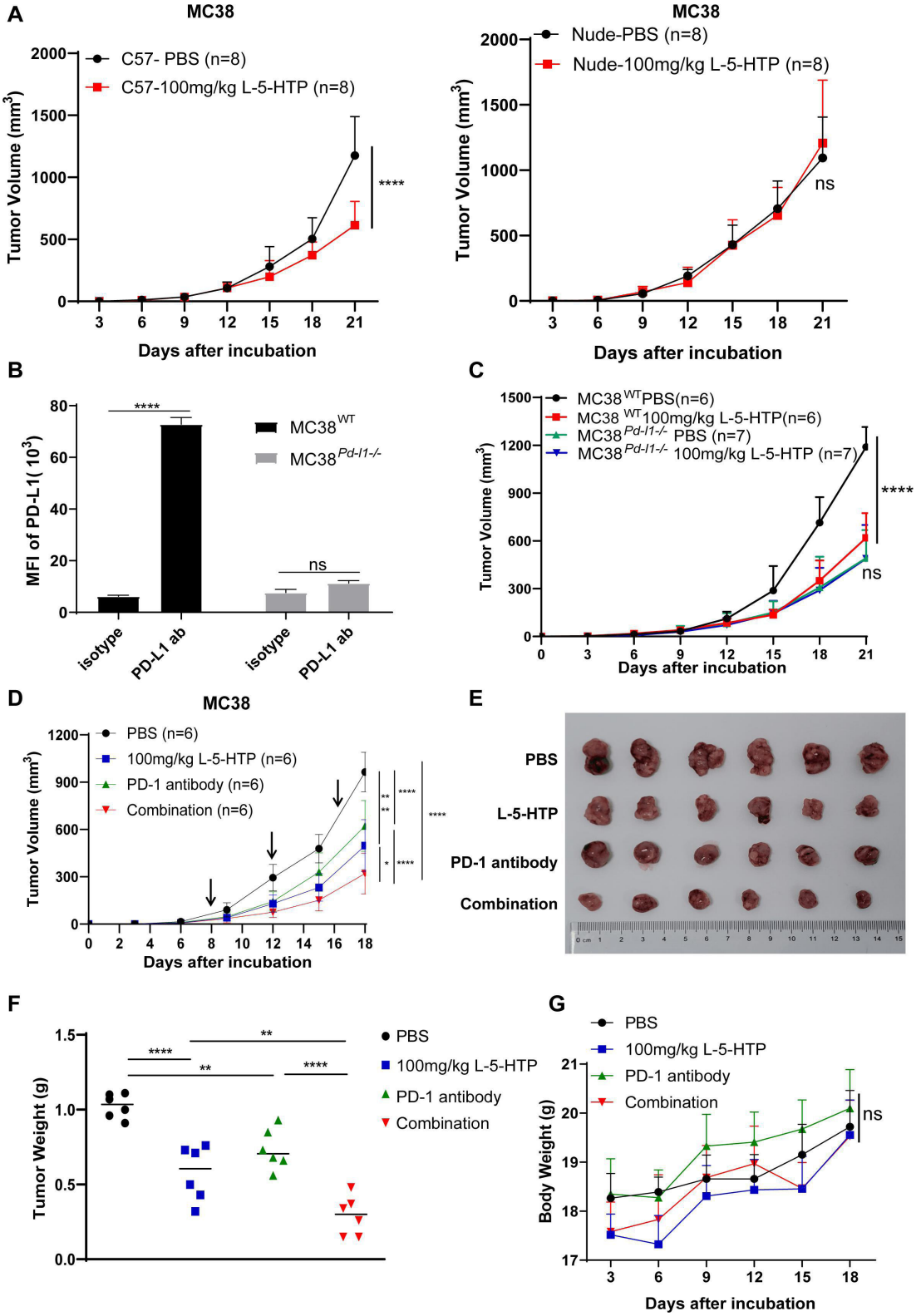


**Figure 6** An intact immune system and PD-L1 axis are essential for L-5-HTP to reduce tumor growth. (A) Wild-type (WT) C57BL/6 mice (immune competent) and nude mice (immunocompromised) were incubated with MC38 tumors. Mice were treated with 100 mg/kg L-5-HTP (intraperitoneally) or PBS (intraperitoneally). Median tumor volume was recorded once every 3 days. (B) Validation of the MC38 $^{Pd\text{-}l1-/-}$ cell line by flow cytometry. (C) MC38 $^{WT}$ and MC38 $^{Pd\text{-}l1-/-}$ cells were implanted in WT C57BL/6 mice. Median tumor volume was recorded once every 3 days. (D) Growth curves of MC38 tumors in L-5-HTP and/or PD-1 antibody-treated C57BL/6 mice. Median tumor volume and body weight were recorded once every 3 days. Representative images of tumors and tumor weight are shown in figure parts E and F, respectively. Body weight is shown in figure part G. Data are shown as mean±SD; ns; p>0.05; *p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001. L-5-HTP, L-5-hydroxytryptophan; ns, not significant; PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

that L-5-HTP acts via PD-L1 to confer its antitumor effect. Taken together, an intact immune system and PD-L1 expression in tumor cells are required for L-5-HTP to exert its antitumor effect.

### L-5-HTP acts synergistically with PD-1 antibody and alleviates depression-like behaviors in a chronic social defeat stress (CSDS)-conditioned tumor mouse model

We next tested its ability to sensitize tumors to anti-PD-1 therapy by further blocking the PD-L1/PD-1 axis. We treated mice bearing MC38 tumors with L-5-HTP alone (100 mg/kg), PD-1 antibody (150 µg) alone, or a combination of L-5-HTP and PD-1 antibody. The combination treatment reduced tumor growth (figure 6D,E) and decreased tumor weight (figure 6F) to a greater extent than observed for vehicle control mice and for mice that received either of the monotherapies. Moreover, the body weight of mice in the combination group was comparable with that of other groups (figure 6G). These data suggest the potential therapeutic benefits of combining L-5-HTP with PD-1 antibody.

Finally, we tested the effect of L-5-HTP on tumor growth and depression in CSDS-conditioned tumor mice (online supplemental figure S7A). Administration of L-5-HTP significantly impeded tumor growth on CSDS-conditioned tumor mice (online supplemental figure S7B). In depressive behavior tests, tumor-PBS-CSDS mice exhibited depressive behaviors, which were relieved by the treatment of L-5-HTP: L-5-HTP decreased immobility time in the tail suspension test (TST) and improved sucrose preference in sucrose preference test (SPT) (online supplemental figureS7C,D). Thus, L-5-HTP delayed tumor progression and relieved depression in tumor-bearing mice and in CSDS-conditioned tumor mice.

## DISCUSSION

In this study, we report that L-5-HTP functions as a potent inhibitor of IFN-γ induced PD-L1 expression at the transcription level by inhibiting IFN-γ induced expression of RTK ligands and suppressing phosphorylation-mediated activation of RTK receptors and the downstream MEK/ERK/c-JUN signaling cascade. L-5-HTP treatment significantly suppressed tumor growth and improved survival in MC38 and CT26 murine tumor models; these effects depend on the presence of an intact immune system and PD-L1 expression in tumor cells. L-5-HTP inhibited PD-L1 expression *in vivo* and ameliorated PD-L1 related immunosuppression by enhancing the ratio of granzyme B+ CD8+ activated cytotoxic T cells. Critically, L-5-HTP strengthened the therapeutic effect of the PD-1 antibody.

At present, blocking of immune checkpoints is a promising approach to significantly improve treatments for a number of diverse tumor types. However, response failures remain significant issues, which could be addressed by employing optimized combination therapies. Related to this concern, we found when combining PD-1 antibody with L-5-HTP, PD-1 antibody and L-5-HTP demonstrated significant antitumor effect and showed well tolerance, suggesting that this combination may be valuable clinically to enhance the therapeutic effect of the PD-1 antibody. Although L-5-HTP has been reported to have an immunological function, relieving allergy-induced lung inflammation and arthritis by decreasing the production of proinflammatory elements,[27 28] its role in antitumor immunity and the tumor environment was unclear. We found L-5-HTP inhibited PD-L1 expression *in vivo* and ameliorated PD-L1 related immunosuppression by enhancing the ratio of granzyme B+ CD8+ activated cytotoxic T cells. A recent study reported that L-5-HTP had inhibitory effect on CD8+ T cells and intratumoral injection of 100 µg L-5-HTP per mouse upregulated inhibitory receptors on the CD8+ T cells.[26] In our study, we treated mice with 100 mg/kg L-5-HTP by intraperitoneal injection. As detected by UHPLC-MS/MS, nearly 15 nmol/g L-5-HTP (about 3 µg per mouse) was found in tumor tissues and 14 µM was found in serum. Under these conditions, L-5-HTP treatment had little effect on PD-1 expression in intratumoral CD8+ T cells *in vivo*, and it did not have obvious deleterious effects on primary T cells *in vitro*. Moreover, L-5-HTP treatment also reduced *PD-L2/Pd-l2* expression *in vitro* and *in vivo*. PD-L2 has been reported to be able to compensate for PD-L1, and elevated expression of PD-L2 is associated with worse outcomes across several cancer types.[25] Thus, L-5-HTP has the potential to target both PD-L1 and PD-L2, and when used in combination with anti-PD therapies may help overcome the resistance caused by PD-L2 upregulation and compensation.

Intriguingly, we found that none of the metabolic molecules that are possibly produced from L-5-HTP transformation had the same effect on PD-L1 downregulation as L-5-HTP, demonstrating that the transcriptional suppression of inducible PD-L1 expression was caused by L-5-HTP itself. A recently published article by Schneider *et al*[29] reported that 100 µM 5-HT upregulated constitutive PD-L1 expression through serotonylation in tumor cells. In our study, we found that 2 µM/10 µM L-5-HTP blocked IFN-γ induced PD-L1 induction by inhibiting the RTK ligands/RTK receptors/MEK/ERK/c-JUN signaling cascade, while it had little effect on constitutive PD-L1 expression. Since metabolites are also chemicals with inherent reactivities,[30] 5-HT and L-5-HTP might have different functions independent of tryptophan metabolic pathway. It has been reported that IFN-γ activates the JAK1-STAT1 cascade to induce expression of PD-L1, yet we showed that L-5-HTP did not inhibit JAK-STAT phosphorylation activation. Instead, we found that L-5-HTP inhibited IFN-γ induced RTK ligands expression, which led to significant suppression of phosphorylation activation of RTK receptors and the downstream MEK/ERK/c-JUN signaling cascade. Further investigation is still needed to identify the direct cellular target through which L-5-HTP inhibits transcription of RTK ligands.

Depression is common among patients with cancer[31] and has been indicated to decline in the quality of life

and even prompt an increase in suicidality among patients with cancer.[32] L-5-HTP has been marketed as a nutritional supplement for treating depression for over 30 years. We showed that in our CSDS-conditioned mouse model, L-5-HTP supplementation impeded tumor growth and improved depressive-like behaviors. Depression has been shown to negatively affect immune function,[33–37] and it is possible that L-5-HTP might regulate some stress hormones, which may have contributed to the enhancement of antitumor immune responses and suppression of tumor growth. However, based on the findings that PD-L1 KO in tumor cells compromised the antitumor effects of L-5-HTP, we suggest that the antitumor effects of L-5-HTP largely depend on its suppression of PD-L1 expression. Moreover, because L-5-HTP has been reported to easily cross the blood–brain barrier, we hypothesize that L-5-HTP might be used as potential adjunct to block PD-L1 induction in the brain, thereby promoting antitumor immunity when used with current antitumor therapies for brain tumors. Chemical modifications or the design of prodrugs would help to increase L-5-HTP's metabolic stability and enhance its enrichment in the brain or its targeting to tumors in the future.

Taken together, we identified a safe, well-tolerated molecule, L-5-HTP, that could effectively decrease IFN-γ triggered PD-L1 induction in tumor cells. Coordinated administration of L-5-HTP with PD-1 antibody potently inhibited tumor growth. T cell immunity was enhanced on L-5-HTP treatment, and this antitumor response was dependent on an intact immune system and PD-L1 expression in tumor cells. Collectively, our findings illustrate the immunological role of L-5-HTP in tumor cells and offer an opportunity for repurposing L-5-HTP for use in cancer immunotherapy to improve wider tumor-related outcomes.

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**Contributors** YZ, YD and CL conceived and designed the study. HJ and KC supervised the study. JHua, XW, and BL performed most of the experiments. JHua and JHu analyzed the RNA-sequencing data. RW established the knockout cell line. HT performed qPCR experiments. YJ supervised the CSDS tumor model establishment, and SS and XW performed behavior tests. JHua and YZ wrote and edited the manuscript. All authors read and approved the final manuscript. YZ and JH are responsible for the overall content as the guarantor.

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