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Original Research

5-Hydroxytryptamine1a receptors on tumour cells induce immune evasion in lung adenocarcinoma patients with depression via autophagy/pSTAT3

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KEYWORDS
5-HT1a receptor;
Depression;
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Abstract ***Background:*** Cancer patients frequently suffer from fatigue and depression. Dysregulation of the immune system, tumour recurrence and metastasis are more common in cancer patients with depression. 5-Hydroxytryptamine (5-HT), a neurotransmitter, contributes to immune evasion in lung adenocarcinoma patients by activating 5-HTRs, but the mechanism for this phenomenon is still unclear. In this study, we examined the function of 5-HT1a receptors (5-HT1aRs) in immune evasion in a mouse model and in samples from lung adenocarcinomas patients.
Experimental design: Sixty-four human lung adenocarcinoma patients with depression and 64 lung adenocarcinoma patients without depression were recruited for this study. The expression of 5-HT receptors on lung adenocarcinoma cells from tumour tissues were detected by using immunohistochemistry (IHC) and fluorescence-activated cell sorting (FACS). The depression models were established *in vitro* and *in vivo*. The effects of immunosuppression were evaluated by testing the function of cytotoxic lymphocyte (CTLs) and Tregs, measuring tumour weight or volume, assessing the survival of mice and staining of tissues by IHC. Changes in the expression of immunoregulatory factor genes were assessed to elucidate the mechanism of immune evasion induced by the 5- hydroxytryptamine receptor (HTRs).
Results: Higher levels of 5-HT, increased expression of 5-HT1Rs and decreased overall survival were observed in lung adenocarcinomas patients with depression compared with those without depression. Moreover, 5-HT1aR, a critical factor for increasing the number of $CD4^+CD25^+Foxp3^+$ Treg cells and decreasing the ratio of Th1/Th2 cells, which suggested

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immune system dysregulation. In addition, expression of 5-HT1aR on tumour cells was also negatively associated with CTL activity in both peripheral blood and tumour infiltrating lymphocytes. In a depressive state, 5-HT1aR activates p-signal transducer and activator of transcription 3 (STAT3) and autophagy, and programmed death ligand-1, a downstream gene of autophagy/p-STAT3 signalling, mediates an immunosuppressive environment. Moreover, in both the mouse model and lung adenocarcinoma patients, the activation of 5HT1aR and the elevated tumour autophagy/p-STAT3 axis were associated with reduced overall survival.

Conclusions: The 5-HT1aR/autophagy/p-STAT3 axis influences both tumour cells and immune cells, resulting in immunosuppression in lung adenocarcinomas patients with depression.

1. Introduction

Current studies report that the prevalence of cancer patients with major depression is 15–29%, which is approximately 3–5 times greater than in the general population [1,2]. Moreover, dysregulation of the immune system, tumour recurrence and metastasis are more frequent in cancer patients with depression [3,4]. Indeed, studies involving a model of repeated combination acoustics with restraint stress in BALB/c mice with implanted tumours, which models severe stress involving physical restraint stress presenting depression-like alterations, demonstrate that immunosuppression, lymphocyte apoptosis increasing and inflammatory cytokine bias [5,6]. Preclinical and clinical studies also indicate that depression as a chronic stress-related process may impact multiple pathways involved in immune regulation and cancer progression and invasion and metastasis [7,8].

One of the major theories for the pathogenesis of depression is the monoamine hypothesis, which proposes that high levels of brain monoamine neurotransmitters, especially 5-hydroxytryptamine (5-HT), can induce depression [9]. Under physical restraint stress, which causes depression, 5-HT may promote tumour growth through mediating the tumour microenvironment [6–8]. 5-HT has seven types of receptors, 5HT1-7, six of which belong to the G-protein coupled superfamily of receptors (5HT1, 5HT2, 5HT4, 5HT5, 5HT6 and 5HT7), and signal transducer and activator of transcription 3 (STAT3) is downstream of these receptors [10]. Furthermore, the 5-HT1a receptor (5-HT1aR) has been postulated to play an important role in patients with major depression as evidenced by 5-HT1aR knockout mice models [11,12]. 5-HT1aR not only is the most abundantly expressed of the 5-HT receptor subtypes in tumour cells [13] but also is related to human T cell activity in the periphery [14]. Although there is compelling evidence for 5HT participation in immunosuppression, its mechanism is still unclear.

Tumours evade immune surveillance through multiple methods, including the production of factors such as transforming growth factor-β (TGF-β) and interleukin (IL)-10, which impair tumour-specific T-cell immunity [15]. In addition to soluble factors, surface molecules on tumour cells are also involved in tumour evasion from immune surveillance. Programmed death ligand-1 (PD-L1) is mainly described as a negative regulatory molecule that is linked to T-cell–induced tolerance to tumours [16]. So far, no general pathway that controls PD-L1 expression has been identified. Depending on the stimulus and cell type, the expression of PD-L1 correlates with various signalling molecules: p44/42 and/or p38 MAPKs [17] or STAT-1, STAT-3 and IRF-1 [17].

STAT3 participates in tumour cell survival, proliferation, angiogenesis and metastasis and has been proven to be an important molecule involved in tumour progression and promotion of cancer cell death [18–24]. Moreover, autophagy is involved in the regulation of adaptive immune responses and tolerance induction [24]. In the context of immune escape, induction of pSTAT3 and activation of autophagy induce lung carcinoma cells to evade CTL-mediated lysis under hypoxia [18,23]. Furthermore, there is crosstalk between pSTAT3 and autophagy [24]. Although these studies highlight that autophagy and pSTAT3 are important factors in the regulation of cancer immunity, no studies have explored the relationship between autophagy/pSTAT3 signalling and the tumour immunosuppressive microenvironment during depression.

Increasing studies are attempting to elucidate the relationship between the immune response and the effects on tumours in response to depression [9,25,26]. In this study, we focused on exploring how higher expression of 5HTRs on tumour cells contributes to immune escape under depression conditions. The expression of different serotonin receptors and 5-HT in whole blood was compared in 64 lung adenocarcinoma patients without depression and 64 lung adenocarcinoma patients with depression. Immune imbalance, increased levels of 5-HT, elevated expression of 5-HT1aR and decreased overall survival were observed in lung adenocarcinoma patients with depression compared with those without depression. 5-HT1aR is a critical factor for creating an immunosuppressive environment

in lung adenocarcinoma patients with depression. Furthermore, the activation of 5-HT/5-HT1aR signalling induces autophagy to promote tumour cell resistance to CTL-mediated lysis, which is dependent on STAT3 phosphorylation. We also showed that PD-L1 is a downstream target gene of the autophagy/pSTAT3 axis in this process. The inhibition of autophagy, pSTAT3 and PD-L1 significantly inhibited tumour growth. In summary, 5HT/5-HT1aR trigger the autophagy/p-STAT3 axis to create an immunosuppressive environment, resulting in tumourigenesis and tumour growth under depression.

2. Materials and methods

2.1. Subjects

From July 1, 2009, to July 30, 2016, lung adenocarcinoma patients with grade IIIb or IV non-small cell lung cancer (NSCLC) who were diagnosed by mediastinoscopy or endobronchial ultrasound at XiangYang Central Hospital, Hubei University of Arts and Science, China, were considered for this study if they were diagnosed with major depression for the first time and had not previously used antidepressant medications. The diagnostic criteria we used are defined in the Diagnostic and Statistical Manual (DSM-IV), and a diagnosis of depression was made using the Structured Clinical Interview process from the DSM-IV. Patients were then screened for the inclusion criteria as follows: no concomitant psychiatric illness, no history of infection or other immune system disorders, no history of epilepsy or glaucoma, no liver- or kidney-related disorders, no history of alcohol dependence/abuse or drug addiction, no intake of alcohol or tobacco in the last 6 months, no immunomodulatory medications and females could not be pregnant or lactating. Sixty-four lung adenocarcinoma patients with a diagnosis of depression were recruited for the present study. The psychopathological status of patients was assessed by the Hamilton Depression Scale-24, and the scores were all higher than 18. For median follow-up, control subjects comprised 64 matched lung adenocarcinoma patients in good mental health, and data including age and gender of the patient and tumour stage were recorded. Patient characteristics are listed in Table 1. All participants provided written informed consent for participation. This study was approved by the Ethics Committee of Xiangyang Central Hospital, Hubei University of Arts and Science.

Table 1
Patient characteristics at baseline.

Controls	Major depression	Characteristics
Gender (male/female)	34/30	31/33
Age (years)	50.60 ± 2.18 (25–64)	51.13 ± 2.12 (22–67)
Classification	III b/IV a/IV b (13/25/26)	III b/IV a/IV b (15/24/25)
ECOG performance status		
0	29	27
1	35	37
Metastatic sites		
Lymph nodes	24	34
Liver	21	15
Lung	31	32
Bone	10	13
Brain	0	2
No. of metastatic sites		
0	18	20
1	17	18
2	18	21
≥3	11	5
Duration of episode at admission (months)	17.16 ± 4.60	
Subtypes		
Melancholic depression	15/12	
Atypical depression	12/10	
Catatonic depression	7/8	
HDRS	21.38 ± 1.61	

HDRS, Hamilton Depression Rating Scale; ECOG, Eastern Cooperative Oncology Group.
*No. of metastatic sites = parenchymal organ, besides lymph nodes and malignant pleural effusion.

2.2. Lung cancer patient blood samples

Blood was obtained from the lung adenocarcinoma patients (stages III_b-IV) according to a protocol approved by the Institutional Review Board of XiangYang Central Hospital, Hubei University of Arts and Science (XiangYang, China). Ulnar venous blood (23 ml) was withdrawn between 8 a.m. and 9 a.m. from each subject the day after the clinical assessment was completed. Twenty milliliters of venous blood from each patient was anticoagulated with heparin and stored at 4°C for lymphocyte isolation. Serum was separated from the remaining 3 ml venous blood and stored at −20°C.

2.3. High-performance liquid chromatography analysis of 5-HT in peripheral serum

To determine the concentration of 5-HT in the peripheral serum of lung adenocarcinoma patients and tumour-bearing mice, samples were analysed by high-performance liquid chromatography (HPLC) (LC-6A, Shimadzu Corporation, Japan) with a 250 mm × 4.6 mm C18 chromatographic column (particle size 5 μm). The mobile phase consisted of 20 mmol/L acetyltriethyl citrate (with 0.1 mmol/L Na− ethylene diamine tetra-acetic acid , pH = 4.50) and methyl cyanide and methanol (90:3:7). The flow rate was set up as 1.0 mmol/min at 35°C (voltage = 0.75 V, AUFS = 50 mV). The samples were detected with an external reference method, and a standard stock solution of 11.5 μg/ml 5-HT (Sigma, US) was used to produce standard curves.

2.4. In vitro experiments

Lung adenocarcinomas cell lines, A549 and LLC, were procured from the China Center for Type Culture Collection (CCTCC, Wuhan, China) and were derived and maintained in culture as previously described [25].

All agonist and antagonists were purchased from Tocris (Tocris Bioscience United Kingdom). The following selective antagonists were used: 5HT1aR agonist (S14506), 5HT1bR agonist (CP 93129) and 5HT1aR antagonist (S-WAY 100).

LLC mouse lung cancer cell lines were seeded into 24-well plates at a density of approximately 25%, corresponding to 2.5×10^4 cells per well, and allowed to adhere overnight before the medium was changed to the specified conditions, containing 100 mmol/L 5HT creatinine complex (Sigma Aldrich) and serotonin antagonists. Doses of 5HT and agonists or/and antagonists were determined using response curve experiments.

2.5. Enzyme-linked immunosorbent assay analysis of IL-2, IL-12, IL-10 and TGF-β1

The concentrations of IL-2, IL-12, IL-10 and TGF-β1 were measured in peripheral serum of patients and mice following the manufacturer's instructions for the human and mouse IL-2, IL-12, IL-10 and TGF-β1 enzyme-linked immunosorbent assay (ELISA) kits (Jingmei Biological Techniques, China). The optical density of cytokines was measured at 450 nm using a microplate reader (Perkin Elmer Company, US), and data were analysed using CurveExpert 1.3 software.

2.6. Flow cytometry

To isolate peripheral blood lymphocytes, each 20 ml serum sample was first separated into 5 ml and 15 ml tubes, from which the isolated lymphocytes would be used for Treg cell isolation separately. Lymphocytes were then isolated using the Ficoll-Hypaque density gradient procedure. Leucocytes in tumour tissues and the homogenised splenocytes were isolated using Lymphocyte Separation Medium (Cellgro). T-cells were isolated on a T cell enrichment column (R&D Systems). Leucocytes and homogenised splenocytes were stained with phycoerythrin-labelled anti-CD3, fluorescein isothiocyante (FITC)-labelled anti-CD4 and allophycocyanin-labelled anti-CD25 for Treg analysis by flow cytometry. All antibodies mentioned above and their isotype-matched monoclonal antibodies were purchased from eBioscience.

Solid tumour tissues were digested with collagenase and hyaluronidase for 1 h at 37°C and homogenised with semi-frosted slides. After the lysis of red blood cells, the dissociated cells were incubated on ice for 20 min, then centrifuged at 500 rpm for 2 min. This process was repeated once, resulting in predominantly epcam populations using anti-5HTRs-phycoerythrin and anti-EPCAM-FITC microbeads (Miltenyi Biotec, Auburn, CA). The cells were sorted to > 90% epithelial cell purity before use in subsequent experiments. The cells from lung cancer patients were sorted on a FACSAria II sorter (BD Biosciences, San Jose, CA).

2.7. CTL cytotoxicity

For cytolysis of LLC cells by CTL, splenocytes from LLC 5-HT1aR$^{-/-}$ cells or LLC Flag-5-HT1aR cell-bearing mice (10 days inoculation) were cocultured in 24-well plates with IL-2 (100 units/mL) and irradiated LLC cells (150 Gy) at a ratio of 12:1. Splenocytes from LLC cell−bearing mice (10 days inoculation) were cocultured in 24-well plates with IL-2 (100 units/mL), while irradiated LLC 5-HT1aR$^{-/-}$ cells or LLC Flag-5-HT1aR cells (150Gy) cocultured at a ratio of 12:1. Splenocytes from BECN1siRNA or ATG5siRNA or PD-L1siRNA was transfected into LLC cells−bearing mice (10 days inoculation), were cocultured in 24-well plates with IL-2 (100 units/mL) and irradiated LLC cells in the presence of 5HT and s14506 or not (150Gy) at a ratio of 12:1. Viable lymphocytes were harvested 10 days later as CTLs for cytotoxicity assays, as described in the figure legends.

2.8. Western blot

Western blot was performed as previously described [27]. B7-H1 (MAB1019) and ATG5 (MAB5294) were purchased from R&D Systems (Minneapolis, MN), BECN1 (PA5-67514) and P62 (PA5-27247) were purchased from eBiosciences (San Diego, CA) and p-STAT3 (9131L) and glyceraldehyde phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling (Beverly, MA).

2.9. Transfection with siRNA

We used HiPerFect transfection reagent for silencing BECN1 (beclin1), 5HT1aR, PD-L1 or ATG5 (Qiagen) or for expressing Luciferase (Invitrogen). Scrambled siRNA was used as a control for siRNA experiments. Cells were plated and allowed to adhere for 24 h. On the day of transfection, 12 μL transfection reagent was added to 25 nM siRNA in a final volume of 100 μL of culture medium.

2.10. Chronic stress and depressive-like behaviour murine model

LLC 5-HT1aR$^{-/-}$ cells were originally obtained from Jackson Laboratory. Wild-type C57BL/6 mice were obtained from the Experimental Animal Centre of the Chinese Academy of Sciences (Shanghai, China) for use in studies approved by the Animal Care and Use Committee of Tongji Medical College (Wuhan, China).

LLC 5-HT1aR$^{-/-}$ cells, LLC cells, LLC Flag-5-HT1aR cells, LLC 5-HT1bR$^{-/-}$ cells or LLC Flag-5-HT1bR cells were intraperitoneally injected into mice 7 days after the stress procedure began, and LLC cells were intraperitoneally injected into control mice (n = 8 mice per group). Mice were sacrificed, and tumours were dissected and weighed on the indicated day after inoculation. In some experiments, tumour incidence in mice and survival of tumour mice were recorded.

LLC 5-HT1aR$^{-/-}$ cells, LLC cells or LLC Flag-5-HT1aR cells were intraperitoneally injected into mice 7 days after the stress procedure began (n = 10 mice per group). Starting 4 days after tumour cell injection, mice were treated with inhibitors (PBS vehicle, 5HT reuptake inhibitor (escitalopram), S-WAY 100, 3MA, stattic or PD-L1 abrogated antibody (eBiosciences) every day, intraperitoneally) for the duration of the experiments. In some experiments, tumours were measured every other day with Vernier calipers, and tumour volume was calculated as the product of length × width × height × 0.5236. *In vivo* data are presented as the mean ± standard error of measurement (SEM). In some experiments, mice were depleted of specific lymphocytes ($CD4^+$, $CD8^+$ T-cells, or NK cells) during stress procedure treatments. The mice were sacrificed, and tumours were dissected and weighed on the indicated day after inoculation. In some experiments, tumour incidence in mice and survival of mice were recorded. At the end of the experiment, mice were sacrificed 24 h after the last administration of compound. Tumour samples were fixed in paraformaldehyde.

2.11. Oral fluoxetine administration

Fluoxetine (Sigma, St. Louis) was administered in the drinking water at a dose of 3 mg/kg/day (Sigma, St. Louis) beginning one day after injection of PBS or tumour cells. This dose of fluoxetine has previously been shown to inhibit depressive-like behaviour in mice without affecting the locomotor activity of healthy mice [28]. Water intake was monitored every other day. There was no difference in fluid intake during the first 2 weeks of tumour growth. During week 3, both tumour groups exhibited increased water intake, but there was no difference between water and fluoxetine intake in the tumour animals. Water bottles were changed weekly throughout the study. Fluoxetine diluted in water has previously been shown to be stable for 8 weeks at room temperature [28].

2.12. Voluntary wheel running activity

Fatigue-like behaviour was assessed using a voluntary wheel running activity (VWRA) [28]. Mice were singly housed and acclimated to a four inch diameter running wheel in the cage for one week, and baseline measures (week 0) of VWRA were recorded overnight prior to injection with tumour cells or phosphate buffer saline (PBS). Wheels were again placed in the home cages of all mice overnight (6 p.m.–8 a.m.) on day 8 (week 1), 14 (week 2) and 19 (week 3) of tumour growth, and the total number of turns each night was digitally recorded (Columbus Instruments, model 0297-004M).

2.13. Home cage locomotor activity

Home cage locomotor activity was monitored between 4:30–6 pm, before the beginning of the dark cycle when mice become more active and there is minimal disturbance in the vivarium. Mice were maintained in their home cages with a floor area of 26 × 20 cm, and activity was video recorded for 3 min. On the video records, cages were divided into six identical virtual rectangles, and the number of line crossings was quantified.

2.14. Depressive-like behaviour

Depressive-like behaviour was determined on day 13 using the forced swim test (FST) as previously described [28]. The FST was performed following the home cage locomotor test between 4:30–6 pm. Mice were placed in an inescapable cylinder (diameter 16 cm, height 30 cm) containing 15 cm of water, and behaviour was recorded for 5 min. The latency to become immobile and the duration of immobility were recorded.

2.15. Immunohistochemistry

Tumour tissues from the inoculation sites of treated mice or from lung cancer patients were surgically excised, embedded in paraffin, and stained with hematoxylin and eosin for histopathological evaluation. For indirect immunostaining, fresh tissues were embedded in an optimum cutting temperature solution and cut into 10-μm sections. The sections were fixed with acetone and incubated overnight at 4°C with rabbit antihuman or antimouse 5HT1aR, 5HT1bR, 5HT2aR, 5HT2bR, BECN1, pSTAT3 and PD-L1 monoclonal antibodies diluted 1:100, followed by incubation with horseradish peroxidase–linked secondary antibodies and development using a DAB kit (BD Bioscience) for optimal staining intensity.

2.16. Statistical analysis

Statistical analysis was performed using GraphPad (GraphPad Software). Data were analysed using 2-tailed Student t-test or analysis of variance (ANOVA). Survival curves were analysed by the Kaplan-Meier method, and statistical significance was determined by the log-rank test. Differences are considered statistically significant when $p < 0.05$.

3. Results

3.1. 5-HT receptor expression on tumour cells in patients with depression decreases overall survival

One hundred twenty-eight patients were enrolled in this study and divided into control and depression groups. Patient characteristics at baseline are summarised in Table 1, including age, gender and tumour stage. As shown in Fig. 1A, overall survival was significantly decreased in patients with major depression compared with control patients.

First, we focused on 5-HT. The concentration of 5-HT in peripheral blood from 128 patients was measured using HPLC. The concentrations in lung adenocarcinoma patients with depression (0.801 ± 0.760 μg/ml) were significantly higher than in control patients (0.249 ± 0.080 μg/ml) ($p < 0.01$) (Fig. 1B). Next, the expression of 5-HT1aR, 5-HT1bR, 5-HT2aR and 5-HT2bR was compared between the patients with

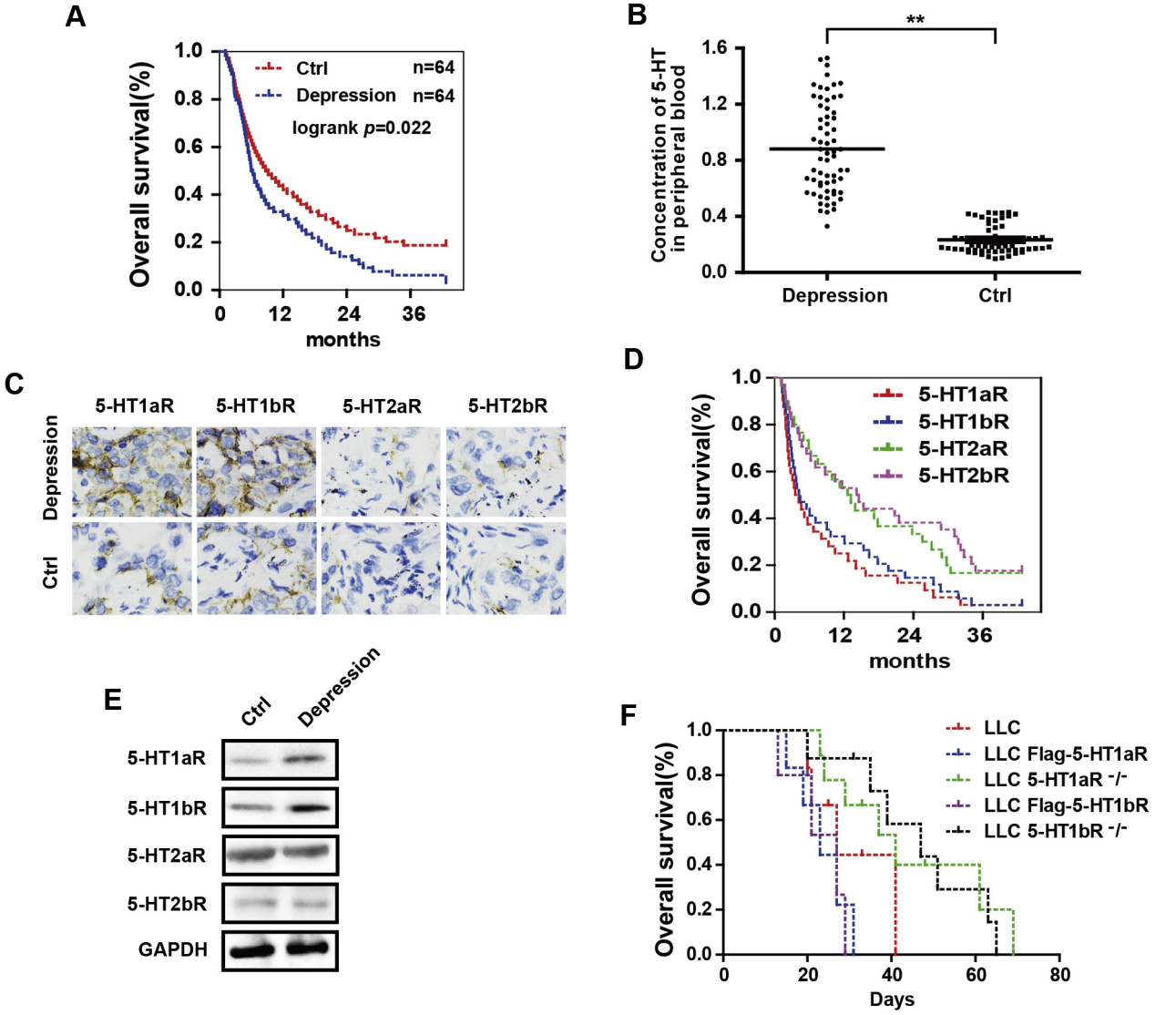

Fig. 1. **Concentration of 5-HT and expression of 5HTRs in tumour cells of lung adenocarcinomas patients with depression.** (A) Kaplan-Meier estimates of overall survival rate of lung carcinomas patients with depression compared with lung carcinomas control patients. (B) The level of 5-HT was detected in peripheral serum of the patients. (compared with control group. **p<0.01) (C) Representative IHC of four 5HT receptors (5-HT1aR, 1bR, 2aR and 2bR) in lung adenocarcinomas tissue of patients (40 × magnification). (D) Kaplan-Meier estimates of overall survival rate of lung adenocarcinomas patients with higher 5HTRs expression. (E) The expression of 5-HTRs was detected in tumour tissues of tumour-bearing mice with depressive-like behaviour. Total cell lysates were subjected to Western blot analysis with specific antibodies to 5-HTRs. (F) The survival period of LLC 5-HT1aR$^{-/-}$ cells and LLC 5-HT1bR$^{-/-}$ cells groups were significantly prolonged compared with LLC Flag-5-HT1aR cells, LLC Flag-5-HT1bR cells and LLC cells group ($n = 8$, $p < 0.01$; Kaplan-Meier analysis). 5HT, 5-hydroxytryptamine; IHC, immunohistochemistry; 5-HT1aR, 5-HT1a receptor.

depression and the control patients (Fig. 1C). As shown in Table 2, there were no differences in the expression of 5-HT2aR or 5-HT2bR between control and depression groups. In contrast, the expression of 5-HT1aR and 5-HT1bR was significantly elevated in lung adenocarcinoma patients with depression (Table 2). Moreover, increased expression of 5-HT1aR and 5-HT1bR was significantly associated with decreased overall survival compared with the high expression of 5-HT2aR and 5-HT2bR (Fig. 1D), while reduced expression of 5-HTRs was not significantly different with respect to overall survival (Fig. S1). Furthermore, we investigated the prognostic value of 5-HTR mRNA expression in 1171 lung cancer patients using the Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service&cancer=lung) [15]. As shown in Fig. S2, the expression of 5-HT1aR and 5-HT1bR mRNA was negatively correlated with overall survival in lung cancer patients. These results indicate that 5-HTRs, especially 5-HT1aR and 5-HT1bR, might have a previously unrecognised biological function in lung carcinoma patients with depression.

$EpCAM^+$ cells were identified for quantification by sorting from patient tumour tissue (Fig. S3A). As shown in Fig. S3B, the expression of 5-HT1aR and 5-HT1bR on $Epcam^+$ cells was significantly higher than on $EpCAM^-$ cells, revealing that 5-HT1aR and 5-HT1bR are primarily expressed on adenocarcinomas cells.

To confirm these findings, we used a mouse model with well-characterised depressive-like behaviour that exhibits higher 5-HT levels than normal mice (Fig. S4). Mice inoculated with LLC cells were subjected to daily restraint stress. The expression of 5-HT1aR and 5-HT1bR in the depression group was significantly higher than in the control group, but there was no significant difference between the depression and control groups in terms of the expression of 5-HT2aR or 5-HT2bR (Fig. 1E). Subsequently, 5-HT1aR or 5-HT1bR genes were knocked out or overexpressed in LLC cells (Fig. S5). Mice inoculated with LLC 5-$HT1aR^{-/-}$ cells, LLC Flag-5-HT1aR cells, LLC 5-$HT1bR^{-/-}$ cells, LLC Flag-5-HT1bR cells or LLC cells were subjected to daily restraint stress. As shown in Fig. 1F, survival of the LLC Flag-5-HT1aR and LLC Flag-5-HT1bR groups was reduced compared with that of the LLC group and was significantly decreased compared with those of the LLC 5-$HT1aR^{-/-}$ and LLC 5-$HT1bR^{-/-}$ groups. Taken together, the increased expression of 5-HT1aR and 5-HT1bR on tumour cells in patients with depression leads to reduced overall survival.

Table 2
5HT-receptor expression in lung carcinomas tissue number of 5-HT receptor expression.

5-HT1aR			
	3	2	1
Depression (n = 64)	22	14	28
Control (n = 64)	2	4	58
5-HT1bR			
	3	2	1
Depression (n = 64)	18	13	33
Control (n = 64)	2	5	57
5-HT2aR			
	3	2	1
Depression (n = 64)	8	18	38
Control (n = 64)	11	17	36
5-HT2bR			
	3	2	1
Depression (n = 64)	13	16	35
Control (n = 64)	8	23	33

ps: 3 = high, 2 = middle, 1 = low; 3 and 2 mean positive, 1 mean negative.
5HT, 5-hydroxytryptamine; 5-HT1aR, 5-HT1a receptor.

3.2. 5-HT1aR is critical in the immunosuppression of lung adenocarcinoma in patients with depression

Depression, a chronic form of stress, has long been identified as a determinant of immune function [26,28]. Levels of IL-2, IL-12, IL-10 and TGF-β1 were measured in serum from lung adenocarcinoma patients to elucidate the status of Th1 and Th2 cells (Table 3) (All four cytokines were detected in each sample). The expression levels of IL-2 (82.845 ± 12.292 ng/ml versus 184.681 ± 8.472 ng/ml $p < 0.01$; Table 3) and IL-12 (94.017 ± 11.992 ng/ml versus 205.384 ± 9.575 ng/ml; $p < 0.01$; Table 3) were lower in patients with major depression compared with controls, respectively (94.017 ± 11.992 ng/ml versus 205.384 ± 9.575 ng/ml; $p < 0.01$; Table 3). However, IL-10 (9.593 ± 0.921 ng/ml versus 6.765 ± 0.611 ng/ml $p < 0.01$; Table 3) and TGF-β1 (20.981 ± 3.98 pg/ml versus 14.042 ± 2.17 pg/ml; $p < 0.01$; Table 3) levels were higher in patients with major depression than in controls, respectively. In addition, lymphocytes were isolated and quantified by FACS, revealing no significant differences between depression ($3.055 \pm 0.483 \times 10^6$/ml) and control patient groups ($3.797 \pm 0.756 \times 10^6$/ml) ($p > 0.05$). However, the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the depression patient group was significantly higher than in the control group ($17.483 \pm 2.942 \times 10^4$/ml versus $7.834 \pm 0.252 \times 10^4$/ml, respectively, Fig. 2A, $p < 0.01$). These results indicate that patients with lung adenocarcinoma suffering from depression are immunosuppressed.

Subsequently, we evaluated whether 5-HTRs play a role in immunosuppression during depression. The results demonstrated that the levels of IL-2 and IL-12 were significantly reduced, while the levels of TGF-β1 and IL-10 were significantly elevated, in the 5-HT1a receptor group compared with other 5-HTR groups in

Table 3
Concentrations of IL-2, IL-12, IL-10 and TGF-β1 in peripheral serum.

	N	IL-2 (ng/ml)	IL-12 (ng/ml)	IL-10 (ng/ml)	TGF-β1 (pg/ml)
Major depression	64	82.845 ± 12.292**	94.017 ± 11.992**	9.953 ± 0.921**	20.981 ± 3.980**
control	64	184.681 ± 8.472	205.384 ± 9.575	6.765 ± 0.611	14.042 ± 2.170

IL, interleukin; TGF-β, tumour growth factor-β.
Compared with control group. **p < 0.01.

lung adenocarcinoma patients with depression (Fig. 2B, p < 0.05). Furthermore, the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the group with increased expression of 5-HT1aR ($19.053 \pm 0.071 \times 10^4$/ml) was significantly higher than in the 5-HT1bR ($11.792 \pm 0.714 \times 10^4$/ml), 5-HT2aR ($7.321 \pm 0.208 \times 10^4$/ml) and 5-HT2bR groups ($6.757 \pm 0.186 \times 10^4$/ml) in lung adenocarcinoma patients with depression (Fig. 2C, p < 0.05). In addition, the number of tumour-specific T-cells in the group with higher 5-HT1aR expression (41 ± 8) was lower than in the 5-HT1bR (162 ± 18), 5-HT2aR (158 ± 15) and 5-HT2bR groups (173 ± 17) (tumour-specific T-cells per million splenocytes; p < 0.05; Fig. 2D). In addition,

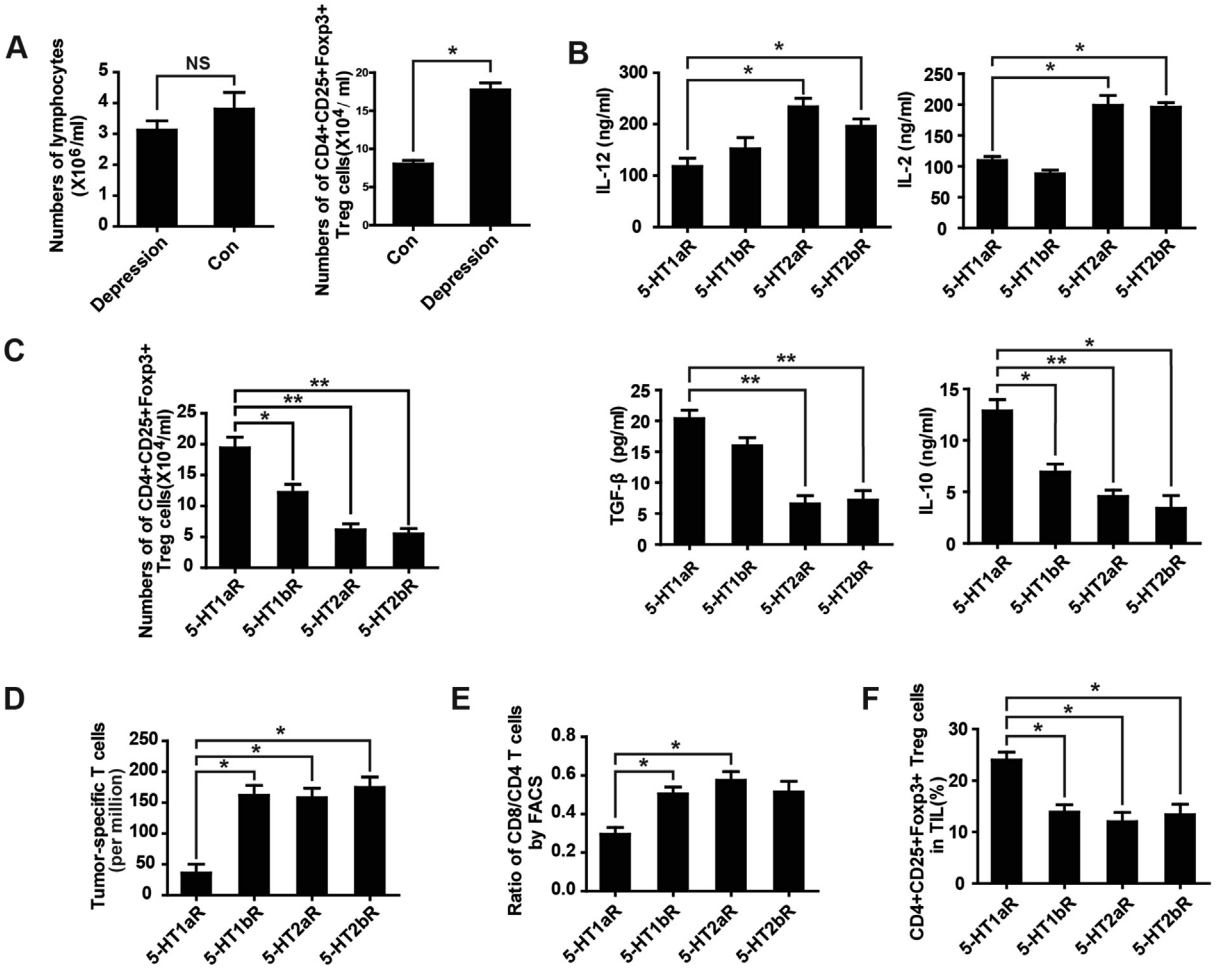

Fig. 2. **Higher 5HT1aR expression in tumour cell contributes to lung adenocarcinomas patients with depression-mediated immune suppression.** (A) The percentages of lymphocytes showed prominent increase in lung adenocarcinomas patients with depression compared to that in controls. (B) Concentration of interleukin-2 (IL-2), IL-12, IL-10 and transforming growth factor-β (TGF-β) were compared with the different 5HTR groups in lung carcinomas patients by ELISA. The results are expressed as the mean ± SE from three independent experiments. (C) The percentages of $CD4^+CD25^+Foxp3^+$ Treg cells showed in 5HT1aR of tumour cell for lung adenocarcinomas patients compared to that in other 5HTR of tumour cell for lung adenocarcinomas patients. (D) The number of tumour-specific T-cells per million splenocytesin in the different 5HTR groups. (E) The ratio of $CD8^+/CD4^+$ T-cells in TIL from tumour cell in the different 5HTR groups (n = 4 per group). (F) the percentage of regulatory T-cells (Treg; $CD4^+CD25^+Foxp3^+$) among total TIL of tumour cell in the different 5HTR groups (n = 4 per group). All results are expressed as the mean ± SE from three independent experiments. *p < 0.05, **p < 0.01. 5HT, 5-hydroxytryptamine;TIL, tumour infiltrating lymphocyte; 5-HT1aR, 5-HT1a receptor; SE, standard error; ELISA, enzyme-linked immunosorbent assay.

there was a significantly decreased ratio of CD8/CD4 T-cells in the group with higher expression of the 5-HT1a receptor ($p < 0.05$; Fig. 2E). However, the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells among total tumour infiltrating lymphocytes (TILs) was higher in groups with higher expression of 5-HT1aR ($p < 0.05$; Fig. 2F). These data suggest that 5-HT1aR is a key driver for building an immunosuppressive environment in lung adenocarcinoma patients with depression.

3.3. 5-HT1aR is critical in immunosuppression in mice with depressive-like behaviour

As shown in Fig. 3A, the expression of IL-2 and IL-12 was reduced, while expression of TGF-β and IL-10 was elevated, in the mouse model of depression compared with control mice. In addition, the expression of IL-2 and IL-12 was significantly reduced, and the expression of TGF-β and IL-10 was significantly higher, in tumour-bearing mice under depression stress than in tumour-bearing mice without depression. Furthermore, the 5HT re-uptake inhibitors affected immunosuppression in both depression and tumour-bearing mice. These results suggest that depression leads to immunosuppression *in vivo*, and the presence of tumours enhances this immunosuppression.

Subsequently, a mouse model with depressive-like behaviour inoculated with LLC 5-HT1aR$^{-/-}$ or LLC Flag-5-HT1aR cells was subjected to daily restraint stress for 3 weeks. The expression of IL-2 and IL-12 was significantly reduced, and the expression of TGF-β and IL-10 was significantly elevated in the Flag LLC 5-HT1aR group compared with the LLC 5-HT1aR$^{-/-}$ group (Fig. 3B, $p < 0.05$). Systemically, the percentages of $CD4^+$ (28.1 ± 2.5% versus 36.6 ± 2.7%; $p = 0.04$) and $CD8^+$ T-cells (5.5 ± 0.6% versus 9.6 ± 0.8%, $p = 0.02$) among total splenocytes, as well as the number of tumour-specific T-cells (37 ± 13 versus 160 ± 18 tumour-specific T-cells per million splenocytes; $p = 0.03$) in the LLC Flag-5-HT1aR group was lower than in the LLC 5-HT1aR$^{-/-}$ group (Fig. 3C). In addition, the ratio of $CD8^+/CD4^+$ T-cells in the LLC Flag-5-HT1aR group was lower than that in the LLC 5-HT1aR$^{-/-}$ group (Fig. 3D). The percentage of $CD4^+CD25^+Foxp3^+$regulatory T-cells among total TILs increased from 5.73 ± 0.60% in the LLC 5-HT1aR$^{-/-}$ group to 15.02 ± 0.53% in the LLC Flag-5-HT1aR group ($p = 0.01$; Fig. 3E). Finally, we examined CTL-mediated cell lysis. The splenic cells from the different groups of tumour-bearing mice were cocultured with irradiated LLC cells for CTL evaluation. The cytolysis of tumour cells by CTLs was measured using the ratio of 7-AAD^+CFSE^+/total $CFSE^+$ LLC cells. As expected, the LLC Flag-5-HT1aR group exhibited significantly reduced tumour cell lysis compared with the LLC 5-HT1aR$^{-/-}$ group (15.23 ± 2.68% versus 41.64 ± 3.18%, respectively, $p < 0.01$, Fig. 3F). Interestingly, the splenic cells from LLC-bearing mice were cocultured with irradiated LLC Flag-5-HT1aR or LLC 5-HT1aR$^{-/-}$ cells for the evaluation of CTL lysis. The ratio of LLCs in the Flag-5-HT1aR cells was significantly lower than in the LLC 5-HT1aR$^{-/-}$ cells (13.54 ± 2.39% versus 35.13 ± 2.54%, respectively, $p < 0.01$, Fig. 3G). These results suggest that 5-HT1aR is critical not only for creating an immunosuppressive environment but also for mediating immune escape of tumour cells in individuals suffering from depression.

3.4. 5HT/5-HT1aR activation in lung adenocarcinoma cells mediates autophagy/p-STAT3 signalling to create an immunosuppressive environment

Next, we wanted to explore how 5-HT1aR mediates biological activity in tumour cells. Evasion of immune attack by cancer cells occurs through activation of STAT3, and autophagy regulates p-STAT3 activation [29]. 5HT1aR induces autophagy in retinal pigment epithelium [30], inducing STAT3 and resulting in neurite outgrowth and neuronal survival [31]. We hypothesised that autophagy/p-STAT3 signalling is involved in the process of 5HT1aR-mediated induction of immunosuppression and immune escape. To test this hypothesis, we established an *in vitro* model of LLC or LLC 5-HT1aR$^{-/-}$ cells incubated with 5-HT, 5HT and S14506 (specific agonist for 5-HT1aR) or 5-HT and S-WAY100 (specific antagonists for 5-HT1aR). First, we observed elevated expression of ATG5 and BECN1 in LLC cells incubated with S14506 and 5-HT, but no significant change occurred in LLC 5-HT1aR$^{-/-}$ cells incubated with S14506 and 5-HT (Fig. 4A). ATG5 or BECN1 can be used as markers for autophagy [7]. Next, we investigated activation of p-STAT3 and its upstream gene p62 using Western blot. The p62, also called sequestosome 1 (SQSTM1), can also be used as a marker of autophagy. Levels of p62 increase in response to inhibition of autophagy, leading to degradation of p-STAT3 and restoration of CTL-mediated lysis [5,19]. We found that p62 expression was reduced, while p-STAT3 was activated, in LLC cells incubated with S14506 and 5-HT but not in LLC 5-HT1aR$^{-/-}$ cells (Fig. 4B). Similar results were obtained in the A549 cancer cell line (data not shown). Moreover, the inhibition of autophagy using siRNA molecules against ATG5 or BECN1 inhibited the activation of p-STAT3 and restored tumour cell sensitivity to CTL-mediated lysis (Fig. 4C and D). Furthermore, the use of stattic, a p-STAT3 inhibitor, also restored tumour cell sensitivity to CTL-mediated lysis but did not alter expression of ATG5 or BECN1 in LLC cells incubated with S14506 and 5-HT (data not shown), indicating that p-STAT3 is a downstream gene of the autophagy pathway in this process. These findings suggest that 5HT-5HT1aR activates the autophagy/p-STAT3 signalling pathway to induce tumour cell resistance to CTL-mediated lysis.

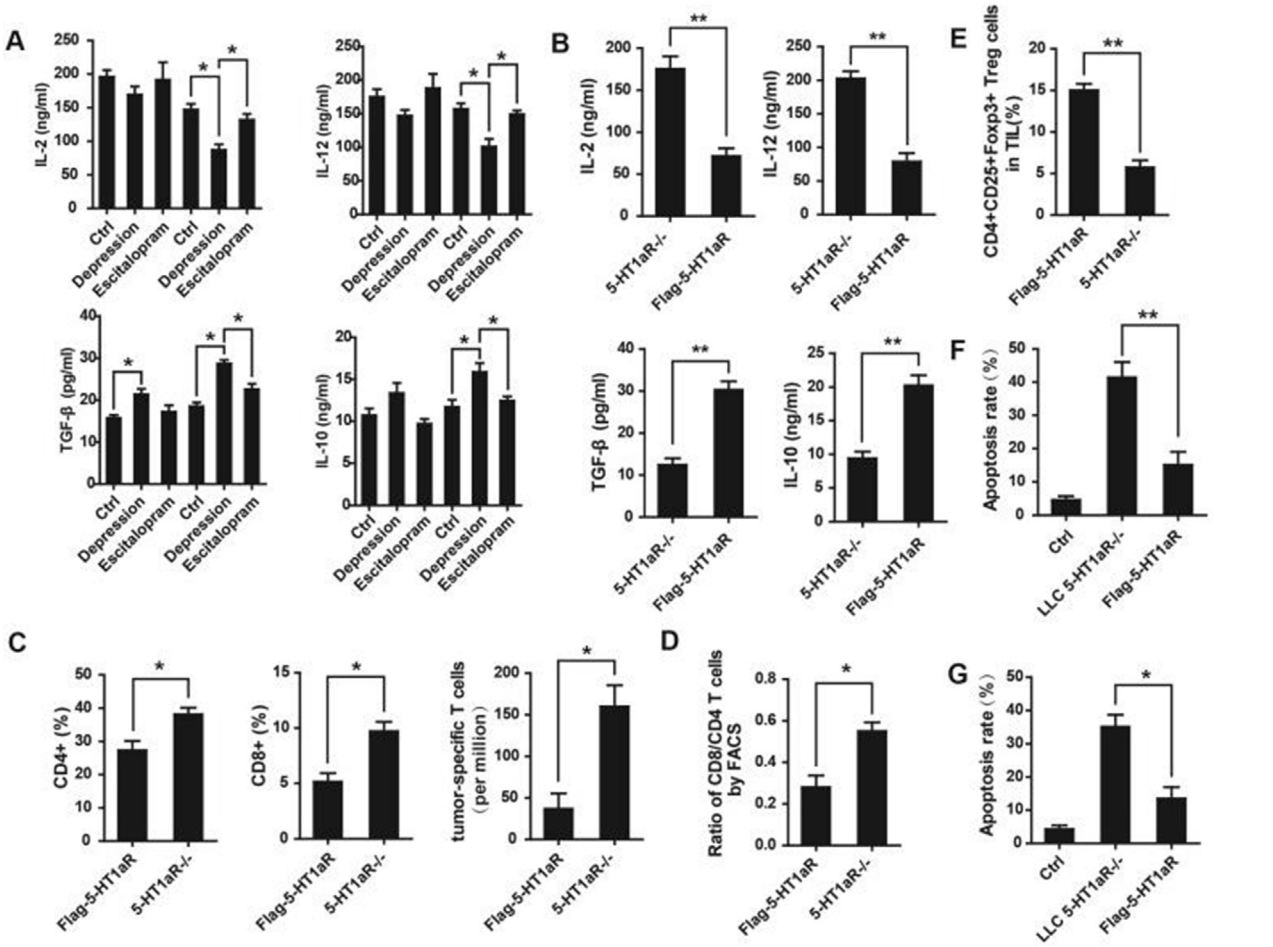

Fig. 3. **5HT1aR contributes to tumour-bearing mice with depressive-like behaviour-mediated immune suppression.** (A) Concentration of interleukin-2 (IL-2), IL-12, IL-10 and transforming growth factor-β (TGF-β) were compared the mice with depression or the tumour-bearing mice with depression to the different control mice by ELISA. The results are expressed as the mean ± SE from three independent experiments. (B) Concentration of IL-2, IL-12, IL-10 and TGF-β compared LLC 5-HT1aR$^{-/-}$ cells group to LLC Flag-5-HT1aR cells group by ELISA. (C) The ratio of CD8$^+$ and CD4$^+$ T-cells shown in splenocytes from LLC 5-HT1aR$^{-/-}$ cells group or LLC Flag-5-HT1aR cells group (n = 4 per group). (D, E) The number of tumour-specific T-cells and the percentages of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells showed in TIL from LLC 5-HT1aR$^{-/-}$ cells group or LLC Flag-5-HT1aR cells group (n = 4 per group). All results are expressed as the mean ± SE from three independent experiments. (F, G) Tumour cells were labelled with CFSE and used as targets for CTL at an effector/target ratio of 12:1 (F) Splenocytes from LLC 5-HT1aR$^{-/-}$ cells or LLC Flag-5-HT1aR cells bearing mice (10 days inoculation) were cocultured in 24-well plates with IL-2 (100 units/mL) and irradiated LLC cells (150Gy), (G) splenocytes from LLC cells bearing mice with irradiated LLC Flag-5-HT1aR or LLC 5-HT1aR$^{-/-}$ cells (150Gy). The data presented were generated from three independent experiments (*$p < 0.05$, Student t-test). *$p < 0.05$, **$p < 0.01$. 5HT, 5-hydroxytryptamine;TIL, tumour infiltrating lymphocyte; 5-HT1aR, 5-HT1a receptor; SE, standard error; ELISA, enzyme-linked immunosorbent assay; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester.

Subsequently, the mouse model with depressive-like behaviour was inoculated with LLC Flag-5-HT1aR or LLC 5-HT1aR$^{-/-}$ cells and was subjected to daily restraint stress for 3 weeks. For LLC Flag-5-HT1aR or LLC 5-HT1aR$^{-/-}$ cells, approximately 5 days after tumour implantation, the mice were randomised into three groups and treated i.p. with 5 mg/kg stattic twice a week, 30 mg/kg 3MA (autophagy inhibitor) twice a week or the same volume of saline for 4 weeks. IL-2 and IL-12 expressions in LLC Flag-5-HT1aR mice after 3MA or stattic treatment were significantly higher than in LLC 5-HT1aR$^{-/-}$ mice, while TGF-β and IL-10 expressions in LLC Flag-5-HT1aR mice after 3MA or stattic treatment were significantly lower than in LLC 5-HT1aR$^{-/-}$ mice (Fig. 4E). The percentage of CD4$^+$CD25$^+$Foxp3$^+$regulatory T-cells among the total TILs was significantly decreased by 3MA or stattic treatment in LLC Flag-5-HT1aR mice (Fig. 4F). Taken together, 5HT/5-HT1aR activation in lung adenocarcinoma cells mediates autophagy/STAT3 signalling, resulting in immunosuppression.

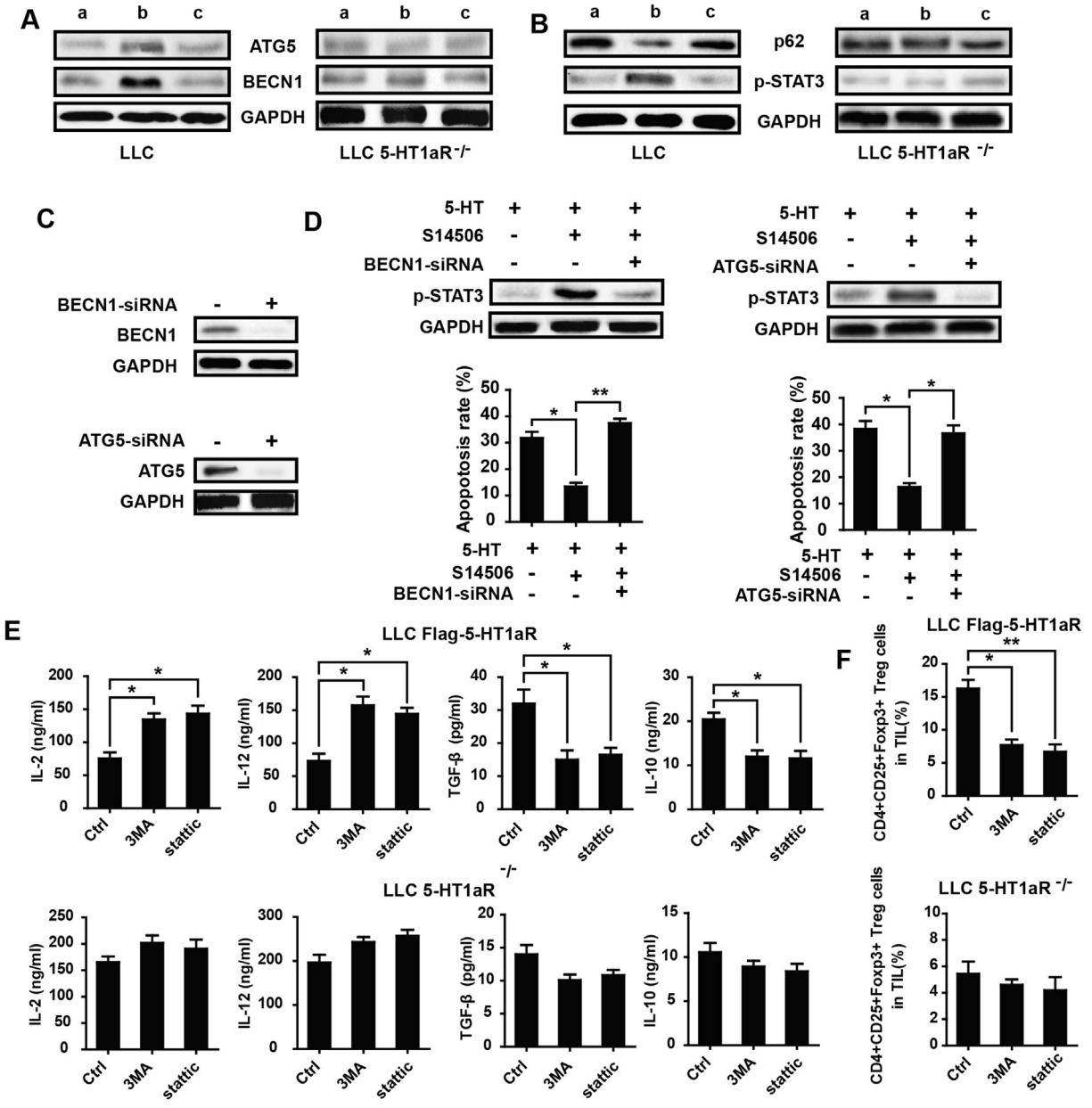

Fig. 4. **5-HT1aR create immunological suppression tumour environment though autophagy/pSTAT3 signal.** (A, B) The expression of autophagy signing and pSTAT3 on LLC cells or LLC 5-HT1aR^{-}/$^{-}$ cells. a: 5HT (100 mmol/L) for 4 h, b: 5HT (100 mmol/L)+S14506 (100 mmol/L) for 4 h, c: 5HT (100 mmol/L) + S-WAY100 (100 mmol/L) for 4 h. (A) Representative immunoblots of autophagy-related proteins ATG5 and BECN1. (B) Representative immunoblots of autophagy-related proteins p62, phosphorylated STAT3. (C) BECN1 siRNA or ATG5 siRNA function in LLC cells, representative immunoblots of autophagy-related proteins ATG5 and BECN1. (D) BECN1 siRNA or ATG5 siRNA abrogated 5HT+S14506 inducing LLC cells phosphorylation of STAT3 and escape from CTL cytotoxicity. LLC cells were labelled with CFSE and used as targets for CTL at an effector/target ratio of 12:1. The data presented were generated from three independent experiments (Student t-test). (E) Concentration of IL-2, IL-12, IL-10 and TGF-β on 10 day in LLC Flag-5-HT1aR cells group or LLC 5-HT1aR^{-}/$^{-}$ cells group in 3MA or stattic treatment after tumour inoculation were assayed by ELISA. Data represent five mice in each treatment group compared with control group (ANOVA). (F) The percentages of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells in TIL from LLC 5-HT1aR^{-}/$^{-}$ cells group or LLC Flag-5-HT1aR cells group after 3MA or stattic treatment. Data represent five mice in each treatment group compared with control group (ANOVA). *$p < 0.05$, **$p < 0.01$. 5HT, 5-hydroxytryptamine;TIL, tumour infiltrating lymphocyte; 5-HT1aR, 5-HT1a receptor; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

3.5. PD-L1 participates in 5-HT1aR/autophagy/p-STAT3 signalling in immunosuppression of tumour-bearing mice with depression

B7 costimulatory family members are involved in promoting an immunosuppressive environment, especially with respect to the cytotoxicity of CTLs [17,32]. In addition, STAT3 mediates PD-L1 in chronic lymphocytic leukaemia [32]. We examined whether 5-HT1aR regulates PD-L1 in lung adenocarcinoma patients with depression. As shown in Fig. 5A, 5HT and S14506 upregulated expression of PD-L1. In contrast, 5-HT1aR knockdown using siRNA nearly abrogated the effect on 5HT and S14506 (Fig. 5B). Similar results were obtained in an A549 cancer cell line (data not shown).

Next, we explored the relationship between PD-L1 and autophagy/STAT3 signalling during 5-HT1aR activation. Treatment with 3MA or stattic decreased the expression of PD-L1 in LLC cells treated with 5HT and S14506 (Fig. 5C). In addition, siRNAs against PD-L1 restored tumour cell sensitivity to CTL-mediated lysis (Fig. 5D). *In vivo*, the expressions of IL-2 and IL-12 in LLC Flag-5-HT1aR mice after PD-L1 blocking antibody treatment were significantly higher than in LLC 5-HT1aR$^{-/-}$ mice, while the expressions of TGF-β and IL-10 in LLC Flag-5-HT1aR mice after PD-L1 blocking antibody treatment were significantly lower than in LLC 5-HT1aR$^{-/-}$ mice (Fig. 5E). The percentage of CD4^{+}CD25^{+}Foxp3^{+}regulatory T-cells among total TILs was significantly decreased in response to PD-L1 blocking antibody in LLC Flag-5-HT1aR mice (Fig. 5F). There were no significant differences in the levels of these cytokines and the regulatory T-cell numbers between the control and PD-L1 blocking antibody groups in LLC 5-HT1aR$^{-/-}$ mice (Fig. 5E and F). These data suggest that PD-L1 acts downstream of 5-HT1aR/autophagy/p-STAT3 signalling in the immunosuppression of lung carcinoma patients with depression.

3.6. Blockade of 5-HT1aR/autophagy/p-STAT3 signalling prolongs survival in tumour-bearing mice with depression

We used an LLC s.c. *in vivo* tumour model to validate the aforementioned results. As shown in Fig. 6A, the survival of the S-WAY 100, 3MA or stattic groups was increased compared with the control group. Additionally, the S-WAY 100, 3MA or stattic treatments resulted in marked reductions in abdominal tumour burden compared with the control group (Fig. 6B). Moreover, after 3 weeks, the expression of IL-2 and IL-12 was significantly increased, while expression of TGF-β and IL-10 was significantly decreased in S-WAY 100, 3MA and stattic groups compared with controls (Fig. 6C). After the last tumour measurement, mice were sacrificed, and tumour masses were isolated and analysed by IHC. BECN1, pSTAT3 and PD-L1 levels were significantly increased in the controls compared with the S-WAY 100, 3MA and stattic groups (Fig. 6D).

Next, we explored the effects of different immune subsets in 5-HT1aR knockout tumour-bearing mice. After the selective depletion of CD4^{+} or CD8^{+} T-cells or NK cells, mice were inoculated with LLC cells or LLC 5-HT1aR$^{-/-}$ cells and were subsequently subjected to daily restraint stress. As shown in Fig. 6E, the average tumour volume from the LLC group with depleted CD8^{+} T-cells was significantly higher than those from the LLC 5-HT1aR$^{-/-}$ tumours with depleted CD8^{+} T-cells. In contrast, the tumour volume was unaffected in response to CD4^{+} depletion.

Overall, these findings demonstrate that 5-HT1aR is a critical factor in tumour progression during depression, which acts by mediating autophagy/p-STAT3 signalling.

3.7. The activated 5-HT1aR/autophagy/p-STAT3 axis is associated with decreased survival in lung adenocarcinoma patients with depression

To confirm the above findings, we assessed expression of p-STAT3, BECN1 and PD-L1 by IHC in tumour tissues from 128 patients. All the results were divided into one of three groups: high, middle or low. In the 5HT1aR high samples, for p-STAT3, BECN1 and PD-L1, the high level was greater than 50%, while the low levels of p-STAT3 and BECN1 were below 20%. In the 5HT1aR low samples, results were reversed: for p-STAT3, BECN1 and PD-L1, the high level was approximately 20%, while the low level was 50% (Fig. 7A). In 128 patients, protein expression of 5-HT1aR was negatively correlated with overall survival ($p = 0.0069$, Fig. 7B). Using 5-HT1aR, p-STAT3 and BECN1 at same time, simultaneous high protein expression of these three indicators was significantly associated with decreased overall survival ($p = 0.0005$, Fig. 7C). Together, these results demonstrate the 5-HT1aR/autophagy/p-STAT3 axis is a potential therapeutic target in lung cancer patients with depression.

4. Discussion

In this study, we observed that levels of 5-HT in plasma and the levels of certain serotonin receptors in tumour tissues were elevated in lung adenocarcinoma patients with depression. Multiple serotonin receptors, especially 5-HT1aR, were always activated in this pathological state. In addition, the increased CD4^{+}CD25^{+}Foxp3^{+} Treg cell numbers and the decreased Th1/Th2 ratio suggested existing immune system dysregulation in lung adenocarcinoma patients with major depression, which was positively correlated with 5-HT1aR activation. In

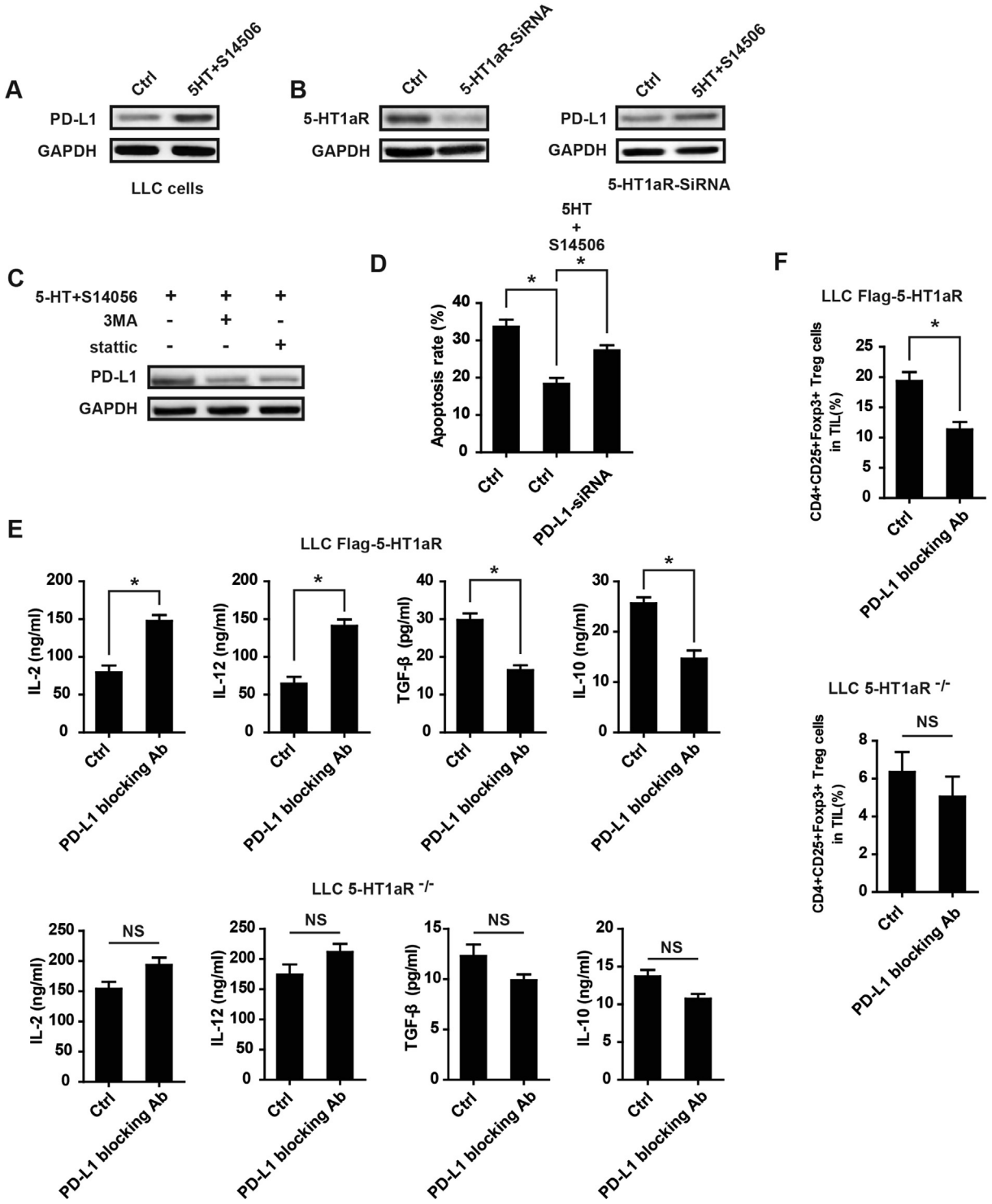

Fig. 5. **PD-L1 is critical downstream gene in 5HT/5-HT1aR activates autophagy/pSTAT3 signal in tumour-bearing mice with depressive like behaviour.** (A) The expression of PD-L1 on LLC cells after 5HT+S14506 (100 mmol/L) treatment for 4 h, representative immunoblots of PD-L1. (B) 5HT1aR siRNA function in LLC cells after 5HT+S14506 (100 mmol/L) treatment for 4 h, representative immunoblots of 5HT1aR and PD-L1. (C) The expression of PD-L1 on LLC cells after 5HT+S14506 or/and 3MA or/and stattic treatment, representative immunoblots of PD-L1. (D) PD-L1 siRNA abrogated 5HT+S14506 inducing LLC cells escape from CTL cytotoxicity. LLC cells were labelled with CFSE and used as targets for CTL at an effector/target ratio of 12:1. The data presented were generated from three independent experiments compared with control group (ANOVA). (E) Concentration of IL-2, IL-12, IL-10 and TGF-β on 10day in LLC Flag-5-HT1aR cells group or LLC 5-HT1aR$^{-/-}$ cells group in PD-L1 blocking antibody treatment after tumour inoculation were assayed by ELISA. Data represent five mice in each treatment group (Student t-test). (F) The percentages of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells in TIL from LLC 5-HT1aR$^{-/-}$ cells group in PD-L1 blocking antibody treated. Data represent five mice in each treatment group ($p < 0.05$, Student t-test). *$p < 0.05$. 5HT, 5-hydroxytryptamine;TIL, tumour infiltrating lymphocyte; 5-HT1aR, 5-HT1a receptor; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; PD-L1, programmed death ligand-1.

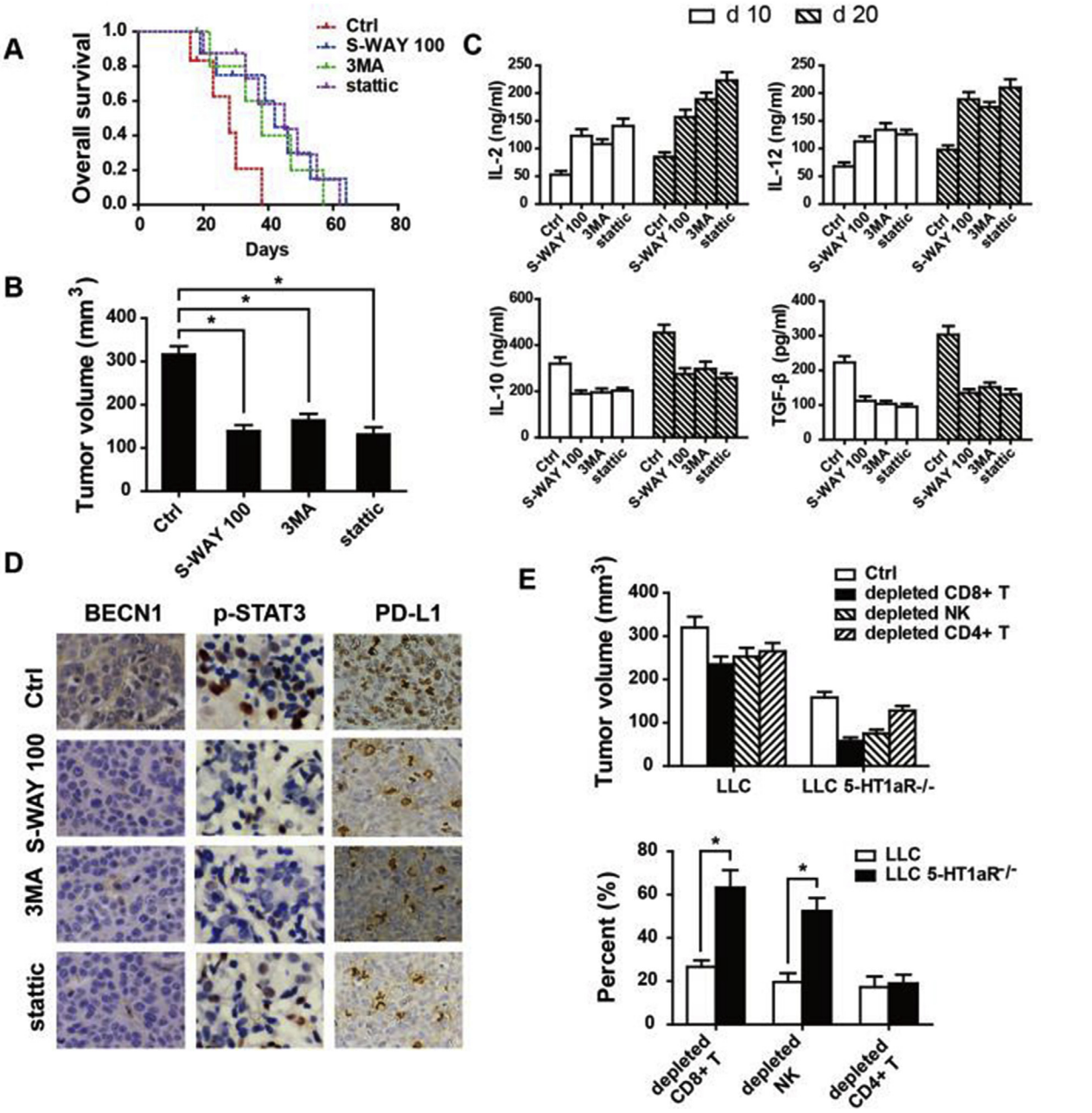

Fig. 6. **Blockade of 5HT/5H1aR signalling prolongs survival of tumour bearing mice.** (A, B, C, D) C57BL/6 mice inoculated with LLC cells were subjected to daily restraint stress. (A) Long-term survival of tumour-bearing mice. The survival period of S-WAY 100, or 3MA or stattic treatment groups were significantly prolonged compared with the control ($n = 8$, $P < 0.01$; Kaplan−Meier analysis). (B) Blockade 5HT/5H1aR signalling inhibits tumour growth. The typical size of tumours on day 16 is shown. (C) Concentration of interleukin (IL)-2, IL-12, IL-10 and tumour growth factor-β on days 10 and 20 in S-WAY 100, or 3MA or stattic treatment groups after tumour inoculation were assayed by ELISA. Data represent five mice in each treatment group compared with control group (ANOVA). (D) Expression of PD-L1, BECN1 and pSTAT3 in tumour tissues of control, S-WAY 100, 3MA or stattic treatment group. Data represent five mice in each treatment group. (E) Evaluation the $CD4^+T$, $CD8^+T$ or NK on LLC cells or LLC 5-$HT1aR^{-/-}$ cells implanted in C57BL/6 mice were subjected to daily restraint stress. The typical size of tumours on day 16 is shown. Data represent five mice in each treatment group. *$p < 0.05$, analysis of variance (ANOVA). *$p < 0.05$. 5HT, 5-hydroxytryptamine; 5-HT1aR, 5-HT1a receptor; ELISA, enzyme-linked immunosorbent assay; PD-L1, programmed death ligand-1.

addition, higher expression of 5-HT1aR in tumour cells was also associated with reduced CTL activity in both peripheral blood and TILs. Indeed, some studies report that tumour growth is associated with reduced tumour-specific T-cell activity in TILs and a decreased percentage of tumour-specific T-cells in tumour ascites [33,34]. Moreover, analysis of 128 patients in our study and a large number of lung adenocarcinoma by using an external database revealed that 5HT1aR is negatively correlated with survival in lung adenocarcinoma

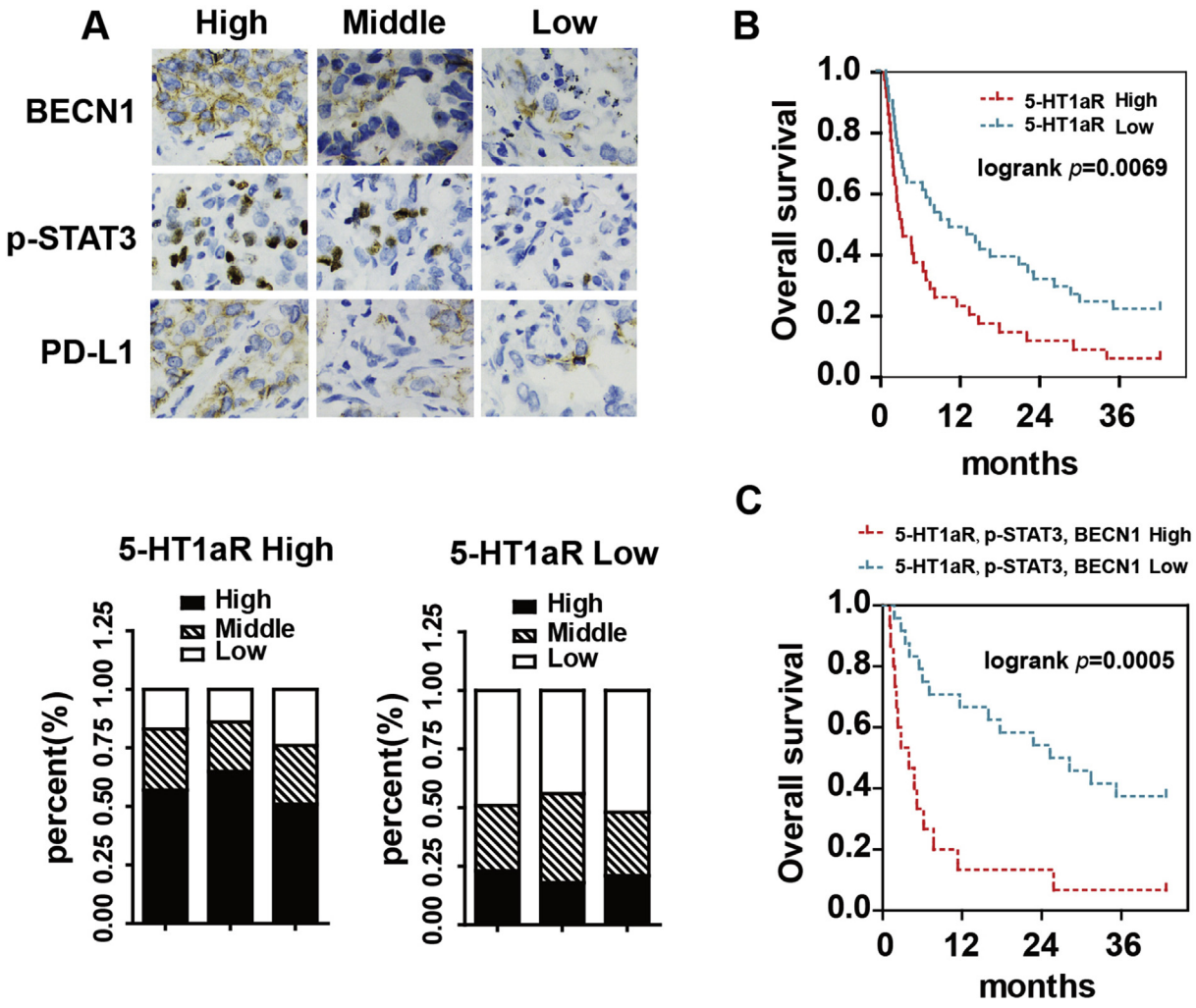

Fig. 7. **Activated 5-HT1aR/autophagy/p-STAT3 axis is associated with decreased survival in lung adenocarcinomas patients with depression.** (A) Representative IHC of PD-L1, BECN1, p-STAT3 in lung adenocarcinomas tissue of patients (40 × magnification). (B) Kaplan-Meier estimates overall survival rate of lung carcinomas patients with high expression of 5HT1aR compared with lung adenocarcinomas patients with low expression of 5HT1aR. (C) Kaplan-Meier estimates overall survival rate of lung carcinomas patients with high expression of 5HT1aR, BECN1 and p-STAT3 compared with lung adenocarcinomas patients with low expression of 5HT1aR, BECN1 and p-STAT3. 5HT, 5-hydroxytryptamine; 5-HT1aR, 5-HT1a receptor; PD-L1, programmed death ligand-1.

patients. These findings all suggest that 5-HT1aR might participate in remodelling of the tumour immune microenvironment to promote tumour development in lung adenocarcinoma patients with depression.

Immune cells can inhibit or promote tumour growth in different tumour microenvironments. Immune escape is considered a key factor in tumour cell survival, growth, recurrence and drug resistance, being a multifactorial and complex process that includes many factors and cell types. For example, the down-regulation of the costimulatory ligand B7-1 with concomitant the upregulation of B7-H1 (PD-L1) and the ligand for the programmed cell death receptor (PD-1) leads to T-cell inactivation [35]. CTLs are the one type of T-cell that uses ligands from the tumour necrosis factor superfamily on their cell surface to eliminate tumour cells [17,22]. In the study, elevated expression of PD-L1 on lung adenocarcinomas cells enabled tumour cells to deliver an inhibitory signal to CTLs, actively suppressing their function, and increased expression of 5HT1aR in tumour cells induced both tumour cells and T-cells to create an immunosuppressive environment under depression stress. 5HT1aR activation significantly upregulated PD-L1 both *in vitro* and *in vivo*, which is the mechanism for 5HT1aR-induced tumour cell escape from CTL attack. Furthermore, the blockade of 5HT1aR using either antagonists or tumour-specific knockout promoted the recognition of tumour cells by the host immune system. These findings provide evidence for the 5HT-5HT1aR axis inducing tumour cells to circumvent endogenous immune surveillance.

To account for this specific effect on PD-L1 signalling, we postulated a STAT3-mediated mechanism. Several studies have shown that activated STAT3 promotes tumour cell survival, proliferation and

angiogenesis/metastasis, and STAT3 is an important molecule involved in tumour escape from immune surveillance [20,36]. In the context of immune escape, crosstalk between tumour cells and several immune cells can promote immunosuppression by both innate and adaptive immune mechanisms after STAT3 activation in tumour cells [20,37]. In this study, we observed a significant increase in the levels of p-STAT3 in lung adenocarcinoma cells in response to 5HT-5HT1aR activation both *in vivo* and *in vitro*. These findings, together with reports of p-STAT3 regulating the expression of PD-L1 in T-cell lymphoma and acute myeloid leukaemia, as well as in tolerogenic antigen-presenting cells [32], led us to conclude that the inhibition of p-STAT3 decreases the expression of PD-L1 and restores CTL-mediated lysis. In addition, our data demonstrated that 5-HT-5HT1aR activation stimulates the expression of ATG5 and BECN1, suggesting depression stress-induced autophagy. Indeed, autophagy is an evolutionarily conserved, dynamic and lysosome-mediated process involving sequestration and delivery of cytoplasmic material to the lysosome, where it is degraded and recycled [7, 38]. Blocking autophagy increased susceptibility to CTL-mediated lysis under depression stress. Meanwhile, we found that siRNAs against ATG5 or BECN1 decreased expression p-STAT3 under depression stress. The interplay between autophagy and pSTAT3 is mainly mediated by the adaptor protein p62/SQSTM1, which plays a cargo role for targeting proteins to proteasomal degradation and autophagy [7]. In this regard, it has been recently reported that excess p62 inhibits the clearance of ubiquitinated proteins destined for proteasomal degradation by delaying their delivery [7]. Inconsistent with this mechanism, the degradation of p62, which is negatively correlated with autophagy and promotes p-STAT3, was increased under depressive stress. Although additional work is needed to clarify this discrepancy, one possible reason for this observation could be that the degradation of p62 in 5HT/5HT1aR activated tumour cells may represent a feedback mechanism to restrict further autophagy and excessive destruction of proteins. In fact, such a feedback mechanism has been recently described [29]. Moreover, blocking either autophagy or p-STAT3 abrogated 5HT-5HT1aR-induced PD-L1 expression and tumour cell resistance to CTL attack. These results suggest that autophagy/p-STAT3 signalling is a key axis for tumour immune escape in tumour-bearing depressive-like mice though the 5HT-5HT1aR pathway.

Using 128 tumour tissue samples, we observed evidence supporting these conclusions. Samples with high expression of 5HT1aR were always accompanied by high and middle expression of BECN1 and p-STAT3, while exhibiting low expression of p62. In addition, simultaneously enhanced protein expression of 5-HT1aR, p-STAT3 and BECN1 was significantly associated with decreased overall survival.

Our data demonstrate that sustained depression signalling could create an immune-depressed environment. Under this condition, protumour cytokines are secreted, T-cell phenotypes transition from T1 to T2 and cancer cells become resistant to CTL attack. These phenomena were all confirmed by 5HT-5HT1aR/autophagy/p-STAT3 axis activation, providing a novel mechanism for sustained depression signalling-induced tumour growth. In addition, our data suggest that 5HT1bR is a key gene in lung adenocarcinoma patients with depression. However, 5HT1bR did not affect immune status. These findings may facilitate our understanding of depression's role in cancer, prompting follow-up studies.

Acknowledgements

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Conflict of interest statement

1. The Author(s) declare that the paper is being submitted for consideration for publication in seminars in oncology, that the content has not been published or submitted for publication elsewhere.
2. **Disclosure** The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.03.017.

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