Abrogation of USP7 is an alternative strategy to downregulate PD-L1 and sensitize gastric cancer cells to T cells killing

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Abstract
Targeting immune checkpoints such as programmed cell death protein 1 (PD-1) and programmed death ligand-1 (PD-L1) have been approved for treating melanoma, gastric cancer (GC) and bladder cancer with clinical benefit. Nevertheless, many patients failed to respond to anti-PD-1/PD-L1 treatment, so it is necessary to seek an alternative strategy for traditional PD-1/PD-L1 targeting immunotherapy. Here with the data from The Cancer Genome Atlas (TCGA) and our in-house tissue library, PD-L1 expression was found to be positively correlated with the expression of ubiquitin-specific processing protease 7 (USP7) in GC. Furthermore, USP7 directly interacted with PD-L1 in order to stabilize it,
1. Introduction

Gastric cancer (GC) is the sixth common malignant tumor all over the world\(^1\). The development of GC is a complicated, multistep process including various genetic and epigenetic alterations\(^6\). Treatment of GC depends on where the disease initiated and the extent of its metastasis throughout the body, and several types of standard treatment are used clinically, including surgery, chemotherapy and targeted therapy. Nevertheless, the 5-year overall survival of GC is 31.5% in the United States, and for patients with metastasis, 5-year overall survival rate is only 5.3%\(^3\). Immunotherapy is a rising therapy choice in a series of solid tumors, and has shown promising effect in patients with GC. As a part of immunotherapy, checkpoint blockades targeting the programmed cell death protein 1/programmed death ligand-1 (PD-1/PD-L1) axis shows a higher therapeutic efficacy. Patients with expression of PD-L1 on either cancer cells or on TILs prefer to respond to anti-PD-1 (e.g., pembrolizumab) therapy\(^11\).

Importantly, abrogation of USP7 not only sensitizes GC cells to T cell killing in vitro and in vivo. Besides, USP7 inhibitor suppressed GC cells proliferation by stabilizing P53 in vitro and in vivo. Collectively, our findings indicate that in addition to inhibiting cancer cells proliferation, USP7 inhibitor can also downregulate PD-L1 expression to enhance anti-tumor immune response simultaneously. Hence, these data posit USP7 inhibitor as an anti-proliferation agent as well as a novel therapeutic agent in PD-L1/PD-1 blockade strategy that can promote the immune response of the tumor.

2. Methods and materials

2.1. Cell lines and compounds

Human gastric cancer cell lines BGC-823, MGC-803, HGC-27 and MKN45 and HEK293 were obtained from Cell Bank of the Chinese Academy of Sciences, Shanghai, China. AGS, NCI-N87, SGC-7901 and MFC were obtained from Cell Bank of the Chinese Academy of Sciences, Beijing, China. Cells were grown in cO2 medium with 10% fetal bovine serum (FBS), at 37°C. Almac4 was a gift from Dr. Timothy Harrison. P5091 (P005091) was purchased from Meilun (Dalian, China).

2.2. Bioinformatics analysis

Two public web servers, Gene-Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn), and Kaplan-Meier Analysis (GEPIA, http://gepia.cancer-pku.cn), were used to comprehensively search the pathways regulating expression and stability of PD-L1.
plotter (KM-plotter, http://kmplot.com) were used to conduct bioinformatics analysis, respectively. In short, GEPIA was applied to calculate correlation coefficient of genes; KM-plotter was applied to explore cancer patient overall survival.

2.3. Immunohistochemical analysis (IHC)

Gastric carcinoma tissues were acquired from the First Affiliated Hospital of Zhengzhou University. Tissue was collected according to the clinical protocol approved by the ethics review board of the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China. Before taking written consents, patients were informed about the study. Collected specimens were anonymously controlled that supported the ethical and legal standards. Deparaffinization of the slides containing histological sample were performed by xylene and different concentrations of ethanol and washed with phosphate buffer saline (PBS) three times. Then the slides were incubated with 3% hydrogen peroxidase (H2O2) at room temperature for 20 min and 5% goat serum was applied to block the nonspecific binding of antibody. Anti-USP7 antibody was incubated at room temperature for 20 min and 5% goat serum was applied to block the nonspecific binding of antibody. Anti-USP7 antibody was used on the slides at 4 °C overnight. Following day, the slides were washed with PBS for three times and were probed with secondary antibody for 2 h at 37 °C. Subsequently, the slides were stained with DAB and hematoxylin. Finally, the slides were dehydrated and mounted. The slides were scanned and analyzed on Aperio AT2 (Leica, Wetzlar, Germany).

2.4. Protein extraction and Western blotting (WB)

To prepare samples for immunoblotting, protein was extracted from cells with Radioimmunoprecipitation (RIPA) lysis buffer containing protease inhibitor to protect protein from degradation. Cells were washed with PBS, mixed with lysis buffer and then centrifugation was applied at 12,000×g for 10 min at 4 °C. The supernatant was then collected and the protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit. The protein concentration was then measured by a bicinchoninic acid (BCA) protein assay kit. The supernatant was then collected and the protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit. The supernatant was then collected and the protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit. Supernatant was then collected and the protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit. Supernatant was then collected and the protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit. Supernatant was then collected and the protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit.

2.5. sgRNA-lentivirus and siRNA transfection and plasmids

Cells were seeded in 96-well plate at 20%–30% confluence for 24 h before sgRNA-lentivirus treatment. After transfection, the medium was replaced 12 h later. The following sequence was used: USP7 sgRNA1: GAGTGATGGACACAACCGG; USP7 sgRNA2 sequence: TCTTCAGCACTGCTTGTGCA; mUsp7 sgRNA: AGACCAACCCAAAAAGGT. The production and packaging of the lentivirus for USP7 knockout was done by Shanghai Genechen Co., Ltd., China. USP7 siRNA1: GCAUA-GUGAUAAACCUGUA; USP7 siRNA2: UAAGGACCCUG-CAAAUUAAU. The production of the siRNA for USP7 knockdown was done by GenePharma, Shanghai, China.

For plasmids, full-length expression cDNA of PD-L1 (Flag-PD-L1, HG10084-NF), HA-USP7 (HG11681-CY) and pCMV3-untagged negative control vector were purchased from Sino Biological Inc. (Beijing, China). pCDNA-HA-His-Ub was generated by GENEWIZ, Suzhou, China.

2.6. Detection of cell surface PD-L1

To analyze the cell surface PD-L1, 100 μL staining buffer containing human PE-PD-L1 antibody (catalog557924; BD Biosciences, Franklin Lakes, NJ, USA) was prepared and cells were suspended in it, and then incubated the mixture at room temperature for 30 min. Same procedure was followed for MFC cells with mouse PE-PD-L1 antibody (catalog 124308; BD Bioscience), after which, the cells were washed by the staining buffer and subjected to FACS analysis using BD FACSCanto flow cytometer (BD Biosciences).

2.7. Immunoprecipitation (IP)

For protein extraction, HEK293 cells were seeded into 60 mm plates and transiently transfected with 2.5 μg Flag-PD-L1 and 0, 0.625, 1.25, and 2.5 μg HA-USP7. At 48 h after transfection, cells were collected for protein extraction and WB analysis. Antibodies against Flag (catalog 14793s; Cell Signaling Technology), and HA (GenScript, Nanjing, China) were used.

For immunoprecipitation, HEK293 cells were transfected with the indicated plasmids and collected at 48 h after transfection. The cells were lysed in IP lysis buffer (26147, Thermo Fisher Scientific) and were incubated overnight at 4 °C. The mixture was then incubated with the indicated primary antibody diluted in PBST at 4 °C overnight. Followed by, membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 2 h at room temperature. After the application of first and second antibody, the membranes were washed for 4 times with 5 min in every time. Finally, the membranes were autorgraphed to X-ray film by chemiluminescence kit from Thermo Fisher Scientific, Waltham, MA, USA. The relative expression ratio for the control and experimental groups were analyzed on the basis of density by Image J software and the GAPDH signal as a reference. Antibodies were used against USP7 (catalog ab10893; Abcam, Cambridge, UK), human PD-L1 (catalog 13684s; Cell Signaling Technology, Danvers, MA, US), mouse PD-L1 (catalog ab213480; Abcam), MDM2 (catalog 86934s; Cell Signaling Technology), P53 (catalog 9282T; Cell Signaling Technology), P21 (catalog 2946T; Cell Signaling Technology), GAPDH (GoodHere No. AB-M-M 001, Hangzhou, China).

2.8. In vivo ubiquitination assay

HEK293 cells were transfected with 10 μg His-ub, 10 μg Flag-PD-L1, and 10 μg HA-USP7 by H4000 (Engreen Biosystem Co., Ltd., Beijing, China) according to the instruction. The cells were treated with 10 μmol/L MG132 for 6 h at 48 h post-transfection and then lysed for immunoprecipitated using anti-Flag-M2 affinity gel.
2.9. Protein half-life assay

Initially, cell transfection with sgRNA lentivirus or treated with Almac4 (5 μmol/L) were done under indicated parameters. After cycloheximide (CHX, 20 μmol/L) was applied to the medium and at indicated time points, cells were collected and immunoblotting was performed to evaluate the corresponding protein levels.

2.10. Quantitative reverse transcription (qRT) PCR assay

Quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed to analyze the expression level of mRNA. Total RNA extraction was performed with the TaKaRa MiniBEST Universal RNA Extraction Kit (Code No. 9767, TaKaRa, Shiga, Japan). To measure the expression level of mRNA, cDNA was synthesized from 2 μg purified total RNA using Revert Aid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific) based on the manufacturer’s instructions. qPCR was done in a real-time PCR machine Quant Studio 6 Flex (Life Technologies, Rockville, IN, US) using the following primers: human PD-L1, 5'-TCACCTGTAATTCTGGGAGC-3' (forward) and 5'-GAGTTTGTATCTTGGATGCC-3' (reverse) β-actin, 5'-GCAAAGCCTGTACGGCAATG-3' (forward) and 5'-TGATCTCTGTCGGAATG-3' (reverse). All data was analyzed by the comparative Ct method. Internal control β-actin mRNA was used to normalize the results.

2.11. PD-L1/PD-1 binding assay

Cells were treated with 20 μg/mL recombinant human PD-1 Fc protein (Sino Biological) at 25 °C for 2 h. Then cells were washed with staining buffer and stained with anti-human Alexa Fluor 488 dye conjugated antibody (A32731, Thermo Fisher Scientific) at 25 °C for 30 min. Next cells were washed by staining buffer and stained with DAPI (Bioskarp, Hefei, China) at 25 °C for 10 min and were examined by Nikon C2 Plus confocal microscope (Nikon, Tokyo, Japan).

2.12. T cell killing assay and IL-2 expression measurement

MGC-803 Con and USP7 KO cells were planted in a 24-well plate. Jurkat T cells were incubated with Dynabeads Human T-Activator CD3/CD28 beads ( Gibco, NewYork, NY, USA) for 12 h to activate Jurkat T cells activity. Then MGC-803 Con and USP7 KO cells were incubated with activated Jurkat T cells for 84 h, after which, the cells were fixed. After washing, the cells were stained with (4',6-diamidino-2-phenylindole) DAPI for 10 min. The cell counts were measured and analyzed by high content screening system (ArrayScan XTI, Thermo Fisher Scientific).

MGC-803 Con and USP7 KO cells were planted in a 96-well plate and put into Xcell Lignence RTCA eSight (Agilent, San Diego, CA, USA). Human peripheral blood mononuclear cells (PBMC, derived from health donor) were incubated with Dynabeads Human T-Activator CD3/CD28 beads and then cocultured with MGC-803 Con and USP7 KO cells at 0.1:1, 1:1, 5:1, 10:1 ratio (PBMC:tumor cell). After 84 h, the cells were fixed. After washing, the cells were stained with DAPI for 10 min. The cell counts were measured and analyzed by high content screening system. The level of IL-2 secreted by the PBMC cells was detected according to the manufacturer instructions (human IL-2 ELISA kits, Thermo Scientific).

2.13. Animals and tumor xenograft model

All animal experiments were performed based on the guidelines of the Institutional Animal Care and Use Committee of Zhengzhou University, Zhengzhou, China. Female BALB/c mice (4-5 week-old, weighing 18-21 g) were obtained from Hunan Slack Scene of Laboratory Animal Company Ltd. (Hunan, China). Optimum food and water were supplied to every animal and the nursing was done under sterilized condition. The right scapular region of the mice was selected and the cancer cells (5 × 10⁶) were injected. The weight of mice and the tumors were measured every 3 days. At the 10th day, the tumors were obtained from mice and weighted. The size of tumor was measured using vernier caliper measurements.

2.14. Tumor-infiltrating lymphocyte profiling analysis

MFC tumors were stripped and the TIL cells were collected after digestion with collagenase type 4 (LS004188, Worthington, Columbus, OH, USA)/hyaluronidase (Sigma—Aldrich). TIL were stained with APC-conjugated anti-mouse CD8α (catalog 100714, BioLegend, San Diego, CA, USA)/FITC-conjugated with anti-mouse CD3 (catalog 100204, BioLegend) and analyzed by FACS.

2.15. Statistical analysis

Data were represented as the mean ± standard deviation (SD). The statistical significance of the difference between two groups was measured with Student’s t-test. The real-time PCR data was represented by analyzing with the 2⁻ΔΔCT method. P < 0.05 and P < 0.01 were considered statistically significant.

3. Results

3.1. USP7 and PD-L1 were positively correlated in GC tissues

To overcome the clinical response problem, it is crucial to find the upstream regulator that may modulate the amount of PD-L1 in GC. Hence, deubiquitinase attracted our eyes as there have been three deubiquitinases reported to stabilize PD-L1, including COP9 signalosome 5 (CSN5), ubiquitin specific peptidase 9 X-linked (USP9X) and ubiquitin specific peptidase 22 (USP22) 33–35. Nevertheless, correlation analysis using GEPIA (http://gepia.cancer-pku.cn) 36 indicated that PD-L1 is not correlated with CSN5, USP9X and USP22 in GC (Fig. 1A), indicating that all these three deubiquitinases may not be the upstream regulator of PD-L1 in GC. Hence, the correlation coefficients between PD-L1 and other different 66 deubiquitinases were analyzed (Supporting Information Table S1), and the results indicate that PD-L1 is correlated with USP7 (Fig. 1B), ubiquitin specific peptidase 18 (USP18, Supporting Information Fig. S1A) and ubiquitin specific peptidase 38 (USP38, Fig. S1A) in GC. In addition, Kaplan-Meier analysis from Kmploi reveals that the poor differentiated patients with high USP7 expression indicated worse overall survival time than those with low levels of USP7 (P = 0.05, Fig. 1C, bottom), but not all patients (P = 0.32, Fig. 1C, top) and well differentiated patients (P = 0.45, Fig. 1C, middle) 37. Besides, the expression of USP18 and USP38 had no correlation with overall survival in GC (Fig. S1B). Based on the above data, USP7 was supposed to be the upstream regulator of PD-L1. To explore the expression of USP7 in diverse tumor samples and paired normal tissues, Fig. S1C show that USP7 is...
overexpressed in breast cancer (BRCA), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), prostate cancer (PRAD) and GC. In order to further verify this result, expression of USP7 in 286 human GC tissues and paired adjacent normal tissues was examined with our in-house tissue library using IHC. The IHC results also show that USP7 is overexpressed in GC patient tumors compared with adjacent normal tissues significantly (Fig. 1D) and the expression levels of USP7 in tumors are higher than adjacent normal tissues no matter the stage of differentiation (Fig. 1D). Besides, clinical relevance of USP7 in the progression of GC was also investigated. Table 1 shows that USP7 expression is correlated with the stage of differentiation ($P = 0.033 < 0.05$), but not age, gender and metastasis of GC.
patients. To further validate this finding in human GC patient samples, the correlation between PD-L1 and USP7 expressions in GC patient samples was analyzed by WB. The WB results indicate that PD-L1 expression levels are highly correlated with USP7 expression ($r = 0.44, P < 0.01$, Fig. 1E and Supporting Information Table S2). These results suggest that USP7 is correlated with PD-L1 expression in GC.

### 3.2. Inhibition of USP7 decreased PD-L1 expression in GC cells

Due to the identification of the positive correlation between USP7 and PD-L1, there may be inter-regulation between USP7 and PD-L1. As PD-L1 is a transmembrane protein and USP7 distributes from nucleus to the cytoplasm as a deubiquitinase, USP7 was supposed as an upstream regulator of PD-L1. To study the regulation of USP7 on PD-L1, expressions of USP7 and PD-L1 were evaluated in eight gastric cell lines. As shown in Fig. 2A, USP7 was ubiquitously expressed in all tested cell lines and six GC cell lines express high amount of USP7 comparing with the cell line of normal gastric epithelium or GES-1. In addition, BGC-823, MGC-803, NCI-N87 and SGC-7901 cells expressed higher level of PD-L1 than other GC cell lines, while PD-L1 was absent in MKN45 cells. Among these cell lines, MGC-803 cell line was chosen to explore the effect of endogenous USP7 on regulating PD-L1 expression due to its high expression of PD-L1 and USP7. Firstly, USP7 inhibitor Almac4 was applied to MGC-803 and mouse gastric cancer cell MFC. As indicated in Fig. 2B, when cells were treated with Almac4 to inhibit USP7 activity, expression of PD-L1 was decreased in both of the two cell lines in a time-dependent manner (Fig. 2B). Moreover, to further verify these results, USP7 was knocked down in MGC-803 and MFC cell lines, and PD-L1 expression was significantly reduced compared to control cells (Fig. 2C). Subsequently, BGC-823, MGC-803 and SGC-7901 cells were incubated with Almac4, and results in Fig. 2D suggest that Almac4 treatment decreased the total amount of PD-L1 in all these three cell lines in a dose-dependent way. Moreover, treatment of MFC with Almac4 also dramatically decreased mPD-L1 expression either (Fig. 2D). As PD-L1 on membrane of cancer cells exhibits immunosuppressive effect through binding to PD-1 on activated T cells, whether USP7 positively regulates membrane PD-L1 remains unclear. Results in Fig. 2E reveal that the amount of membrane PD-L1 was lower than that in parental cells for MGC-803 cells when USP7 was abrogated genetically or pharmacologically in the presence of Almac4, respectively (Fig. 2E). Likewise, in the presence of Almac4, amount of membrane PD-L1 was decreased in SGC-7901 and MFC cells (Fig. 2E). Meanwhile, in the presence of P5091, another USP7 inhibitor, amount of membrane PD-L1 also decreased in MGC-803 and BGC-823 cells (Supporting Information Fig. S2). All these results indicate that USP7 abrogation can decrease the expression level of PD-L1 in GC cells.

### 3.3. USP7 directly interacted with PD-L1 and promoted its deubiquitination and stabilization

As USP7 expression was positively correlated with PD-L1 expression in human GC tissues and abrogation of USP7 decreased the expression of PD-L1 in different GC cell lines, we next asked how USP7 regulates the PD-L1 expression. First, PD-L1 mRNA levels in MGC-803 cells treated with or without Almac4 were examined. Nevertheless, no significant alteration was discovered for the mRNA of PD-L1 after Almac4 treatment with different concentrations or times (Fig. 3A). As USP7 abrogation can decrease the expression of PD-L1 without impacting on its mRNA level, and PD-L1 was reported to be ubiquitinated and subjected to proteasome mediated degradation, whether USP7 erases the ubiquitination of PD-L1 needs to be answered.

When Flag-PD-L1 and HA-USP7 were co-transfected in HEK293 cells, results in Fig. 3B suggest that they can interact with each other. To further validate these results, endogenous PD-L1 from MGC-803 and BGC-823 cells was enriched with immunoprecipitation and the endogenous USP7 can be detected (Fig. 3C). Moreover, USP7 can promote the amount of PD-L1 in a dose-dependent manner (Fig. 3D). Then, to further verify that USP7 can deubiquitinate PD-L1, Flag-PD-L1 was co-transfected with HA-His-ub in the presence of HA-USP7 or not in HEK293 cells. Results in Fig. 3E suggest that PD-L1 was strongly ubiquitinated (lane 2, Fig. 3E) in the presence of MG132 but without HA-USP7, while HA-USP7 abolished PD-L1 ubiquitination (lane 3, Fig. 3E). Similar results in Fig. 3F show that PD-L1 ubiquitination was significantly increased when USP7 was absent in MGC-803 cells in the presence of MG132, while absence of MG132 led to the sharply decreasing of PD-L1 expression when USP7 was abrogated. To test whether endogenous PD-L1 was also subjected to similar regulation by USP7, USP7 was knocked out genetically (Fig. 3G and Supporting Information Fig. S3A) or pharmacologically (Fig. 3H and Fig. S3B) in MGC-803 cells, and results suggest that endogenous PD-L1 became unstable and degraded rapidly. Similarly, treatment of MFC cells with mUSP7-sgRNA-lentivirus (Fig. 3C) or Almac4 (Fig. 3D) also led to similar results which further confirm the results derived from Fig. 3G and H. All these results indicate that USP7 interacts with PD-L1 and deubiquitinates PD-L1 to promote the stability of PD-L1.

### 3.4. Blockade of USP7 sensitized cancer cells to T cell killing

Based on the above results, whether USP7 abrogation induced decreasing expression of PD-L1 may affect the binding between PD-L1 and PD-1 and sensitivity of tumor cells to T cell-mediated killing remains unclear. Hence, MGC-803 cells were treated with USP7 sgRNA-lentivirus or Almac4, respectively, followed by incubation with recombinant human PD-1 Fc chimera protein
that enabled fluorescent labeling and visualization. As expected, the binding of PD-1 on the cell surface decreased as a result of abrogation of USP7 both genetically and pharmacologically (Fig. 4A). Moreover, the question regarding whether USP7 KO-mediated PD-L1 downregulation may affect T-cell function remains to be answered. Hence, T cell killing assay was performed by co-culturing activated Jurkat cells with MGC-803 cells in the presence of USP7 or not. This mechanistic assay reveals that knockout of USP7 uninterruptedly activates the cytotoxic T cell towards tumor cells (Fig. 4B). Moreover, another T cell
killing assay using activated human PBMCs also confirmed that abrogation of USP7 sensitized MGC-803 cells to T cell in a time- and T cell number-dependent manner (Fig. 4C, D, and Supporting Information Videos S1–S10). Interleukin 2 (IL-2) was one of the first cytokines to be discovered and produced by activated T cells and promoted CD8⁺ T cell cytotoxicity activity⁴⁵. USP7 knockout enhances IL-2 expression in PBMC cells (Fig. 4E). These results demonstrate that abrogation of USP7 can attenuate the PD-L1/PD-1 interaction and sensitize gastric cancer cells to T cell killing.

Supporting video related to this article can be found at https://doi.org/10.1016/j.apsb.2020.11.005
3.5. Abrogation of USP7 suppressed GC cell proliferation and cell cycle progression

As the above results confirmed that USP7 knockout enhanced the sensibility to T cell killing, it was also reported that abrogation of USP7 can suppress tumor growth. Next, whether the same function exits in GC needs to be elucidated. To explore whether abrogation of USP7 can suppress GC cell proliferation, knockout of USP7 in MGC-803 was applied. As indicated in Fig. 5A, when USP7 was genetically inactivated in MGC-803 cells by two...
different sgRNA-lentivirus, the colony formation efficiency of MGC-803 was inhibited (Fig. 5B). Because MDM2 and P53 are the substrates of USP7\textsuperscript{41,42}, expression of MDM2 and P53 in MGC-803 cells were investigated, and the results show that MDM2 was reduced and P53 was accumulated upon USP7 knockout (Fig. 5C). As P53 controls the G2/M checkpoint of cell cycle and regulates growth arrest\textsuperscript{43}, the cell cycle analysis was performed when cells were exposed to Almac4 and results in Fig. 5D indicate that Almac4 can induce G2/M phase arrest (42.22%). Besides, Almac4 can also reduce the colony formation activity in MGC-803 cells in a dose-dependently manner (Fig. 5E). Moreover, Almac4 induced the expression of P53 and P21 in MGC-803 cells in a dose-dependent manner (Fig. 5F) and accelerated endogenous MDM2 turnover with CHX (20 \( \mu \text{mol/L} \), Fig. 5G). Hence, abrogation of USP7 inhibits proliferation of GC cells through promoting the stability of P53.

### 3.6. Inhibition of USP7 decreased PD-L1 expression in vivo

The results from \textit{in vitro} analysis prompted us to make a hypothesis that knockdown of USP7 can affect mPD-L1 expression \textit{in vivo}, we assessed the ability of USP7-depleted cells to impair tumor growth in BALB/c mice bearing MFC Con and USP7 KD cells. Results in Fig. 6A show that knockdown of USP7 in MFC reduces tumor size compared to control group, and mice bearing MFC USP7 KD cells had lower mPD-L1 expression in tumors compared to control mice (Fig. 6B). Moreover, abrogation of USP7 decreased the tumor volume and weight in USP7 KD group compared with the control group (Fig. 6C and D). As cytotoxic CD8\textsuperscript{+} T cells play a critical role in mediating the antitumor effect of anti-PD-L1 treatment\textsuperscript{44} and the tumor-infiltrated activated CD8\textsuperscript{+} T-cells were quantified, result in Fig. 6E suggests that the tumor-infiltrated activated CD8\textsuperscript{+} T-cell
population was significantly increased in mice bearing MFC USP7 KD cells (Fig. 6E). Because the expression of Ki-67, which is widely used in routine pathological investigation as a proliferation marker, has strong association with tumor cell proliferation and growth, Ki-67 of each tumor was examined. Result in Fig. 6F shows that Ki-67 positive tumor cells in MFC USP7 KD significantly decreased compared with MFC Con group. Moreover, it is commonly known that P53 is a powerful tumor suppressor and can be functionally activated to eradicate tumors. However, its function was effectively inhibited through direct interaction with MDM2. Since both P53 and MDM2 are substrates of USP7, expression of P53 and MDM2 in each tumor was tested, and result in Fig. 6G reveals that MDM2 expression decreased in MFC USP7 KD compared with MFC Con group. At the same time, expression of P53 accumulated in MFC USP7 KD compared with MFC Con group. Together, abrogation of USP7 decreased the expression of mPD-L1 and suppressed the tumor growth.

4. Discussion

USP7 was primarily discovered to be associated with viral proteins such as infected cell protein (ICP0) and Epstein-Barr nuclear antigen 1 (EBNA1). Besides, a lot of research work reported that USP7 promotes the stability of several other proteins, including MDM2 and forkhead box O4 (FOXO4) indicating that USP7 plays as an oncogene in tumorigenesis. Here, we reported that USP7 was upregulated in clinical gastric cancer tissues, and poor differentiation patients with high USP7 expression show worse overall survival rate compared to the patients with low
levels of USP7. Further, we found that USP7 inhibitor inhibited tumor proliferation via promoting the stability of P53 in GC and arrested the cell cycle at G2–M phase. Hence, we demonstrate that abrogation of USP7 can suppress GC cells proliferation via stabilizing P53 in vitro and in vivo.

Recently, immunotherapies, such as PD-1/PD-L1 targeting therapy and chimeric antigen receptor (CAR) T-cell therapy, have been proved as powerful treatments in clinical oncology. However, PD-L1 inhibitor pembrolizumab only achieved 22% response clinically52. To crack this problem, multifactorial biomarkers, including tumor mutational load, infiltrating CD8+ T cell intensity, and PD-L1 expression levels, have been proposed as bona fide biomarkers of therapeutic response to anti-PD-L1 therapies53. However, the overall response rate was 30% in gastric and gastroesophageal junction (GEJ) cancers irrespective of PD-L1 status54. Moreover, changes in the expression of PD-L1 and tumor mutational burden are independent biomarkers in most cancers55. As PD-L1 expression status is insufficient in determining which patient may be benefit from PD-1/PD-L1 targeting therapy56, findings of new drug or combinational therapy approaches that modulate the tumor microenvironment to promote antitumor immunity are in urgent6,57. In this study, abrogation of USP7 reduced the expression of PD-L1 in a dose- and time-dependent manner in GC cells and results in Fig. 2B show that it took 48 h to decrease expression of PD-L1. We supposed that USP7 may stabilize PD-L1 in an indirect manner although USP7 can interact with PD-L1 directly. These results demonstrate that abrogation of USP7 increased PD-L1 polyubiquitination, decreased the PD-1/PD-L1 interaction and sensitized gastric cancer cells to T cell-mediated killing. All these data suggest that USP7 acted as an upstream deubiquitinase of PD-L1 in GC and abrogation of USP7 suppressed tumor growth via downregulating PD-L1-associated immunosuppression, and thus stabilizing P53 and subsequent cell cycle arrest simultaneously.

As PD-L1 can be regulated at the transcriptional level, post-transcriptional, post-translational and extracellular levels58, several post-translational modifications (PTMs) of PD-L1, including phosphorylation30,59–62, ubiquitination33,39,63,64, glycosylation32,33,68 and palmitoylation69,70, have been reported to regulate PD-L1 stability. In the current study, we show that USP7 deubiquitinates PD-L1 to cause cancer immune resistance to promote cancer growth.

5. Conclusions

In summary, stabilization of PD-L1 by USP7 supports a role for USP7 in antitumor immunity and provides insight into the mechanism how USP7 inhibitor elicits its immunomodulatory effect. Our data support that small molecule USP7 inhibitors may be used as novel promoter of the tumor immune response. The application of USP7 inhibitors to pharmacologically promote antitumor immunity gives a novel insight for novel drug combinations with checkpoint inhibitor agents that can broaden the population of patients that respond to PD-L1/PD-1-targeted therapies.

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Author contributions

Zhiru Wang and Wenting Kang designed this study and finalized the manuscript. Wenting Kang and Fengyu Qi performed the USP7 immunohistochemistry and analyzed the data. Zhiru Wang and Ouwen Li conducted most of experiments and collected and analyzed the data. Junwei Wang and Yinghua You performed the mice experiment. Pingxing He did the vector construction. Zhenhe Suo critically revised the manuscript for important intellectual content. Yichao Zheng supervised the whole study. Hongmin Liu sincerely provided his time to obtain funding and gave valuable suggestions to improve the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors of this manuscript clearly declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.11.005.

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