Received: 27 July 2021 | Revised: 24 November 2021 | Accepted: 25 November 2021

DOI: 10.1111/cas.15237

ORIGINAL ARTICLE

Cancer Science WILEY

Thrombospondin-1 induced programmed death-ligand 1-mediated immunosuppression by activating the STAT3 pathway in osteosarcoma

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Funding information
National Nature Science Foundation of China, Grant/Award Number: 81802840, 82072957 and 81702661; Shanghai Sailing Program, Grant/Award Number: 21YF1426100; Science and Technology Commission of Shanghai Municipality, Grant/Award Number: 20ZR1434000; Climbing Talents Program of Shanghai Tenth People's Hospital, Grant/Award Number: 04.01.20041

Abstract

Thrombospondin-1 (TSP1) is generally assumed to suppress the growth of osteosarcoma through inhibiting angiogenesis; however, it is unclear whether TSP1 could affect the antitumor immunity against osteosarcoma. We aimed to explore the immune-related tumor-promoting effects of TSP1 and decipher its underlying mechanism. First, we identified that TSP1 regulated programmed death-ligand 1 (PD-L1) expression, which was related to the $CD8^+$ T cells anergy in osteosarcoma cells. The exact role of PD-L1 in the immunosuppressive effect of TSP1 was then further confirmed by the addition of the PD-L1 neutralizing Ab. With the addition of PD-L1 neutralizing Abs during cocultivation, the inhibition of $CD8^+$ T cells was abolished to a certain extent. Further mechanistic investigations showed that TSP1-induced PD-L1 upregulation was achieved by activation of the signal transducer and activator of transcription 3 (STAT3) pathway. In vivo experiments also indicated that TSP1 overexpression could promote the growth of primary lesions, whereas TSP1 knockdown effectively inhibits the growth of the primary lesion as well as lung metastasis by restoring the antitumor immunity. Thrombospondin-1 knockdown combined with PD-L1 neutralizing Ab achieved a more pronounced antitumor effect. Taken together, our study showed that TSP1 upregulates PD-L1 by activating the STAT3 pathway and, therefore, impairs the antitumor immunity against osteosarcoma.

KEYWORDS
immunosuppression, osteosarcoma, PD-L1, STAT3, thrombospondin-1

Abbreviations: APC, allophycocyanin; DC, dendritic cell; IFN-γ, interferon-γ; IL, interleukin; PD-1, programmed death receptor 1; PD-L1, programmed death-ligand 1; PE, phycoerythrin; STAT3, signal transducer and activator of transcription 3; TIL, tumor-infiltrating lymphocyte; TNF-α, tumor necrosis factor-α; TSP1, thrombospondin 1.

Zhuochao Liu, Junxiang Wen, and Fangqiong Hu contributed equally to this work.

1 | INTRODUCTION

Osteosarcoma is the most common primary high-grade bone malignancy among children and adolescents.[1] The application of chemotherapy combined with surgery significantly increased the 5-year survival rates of localized osteosarcoma patients from 20% to 60%.[2,3] However, the survival rates of patients with relapse or metastasis remain poor and have not improved over the last 30 years because of the deficiency of novel and effective medicines. Osteosarcoma is a genomically unstable sarcoma, which makes efforts to develop effective drugs against it generally disappointing.[4,5] But this genomic characteristic and the associated high mutational burden could also produce tumor neoantigens to activate antitumor immunity, given that osteosarcomas should be suitable for immunotherapy.[6]

Thrombospondin-1 is a class of proteins closely related to tumorigenesis. Our previous study showed that TSP1 derived from osteosarcoma cells was highly expressed in lung metastases and was significantly associated with the Enneking III stage osteosarcoma.[7] Thrombospondin -1 also induced immature T cells to differentiate into $CD4^+$ $Foxp3^+$ T cells by combining with CD47 on immune cells, thus negatively regulating antitumor immunity.[8,9] In addition, TSP1, released from DCs during the early activation stage, could also reduce the secretion of TNF-α, IL-10, and IL-12 by interacting with CD47.[10] In the extracellular adenosine triphosphate-induced immunosuppressive microenvironment, DCs also inhibited the proliferation of T cells by upregulating indoleamine 2,3-dioxygenase and TSP1.[11] These results indicate that TSP1 plays an important role in the regulation of immune responses by directly affecting DCs or lymphocytes. Whether the protumor effect of TSP1 in osteosarcoma is related to its role in the regulation of antitumor immunity is still uncertain.

Programmed death-ligand 1, also denoted as B7-H1, is a cell surface protein of the B7 family.[12] Programmed death-ligand 1 is the ligand for PD-1, and the ligation of PD-L1 expressed on tumor cells to PD-1 expressed on T cells suppresses the activation and induces apoptosis of T cells.[13] Recent studies have shown that there is a subtype of osteosarcoma with high expression of PD-L1 and the expression of PD-L1 is positively correlated with TILs, implying that osteosarcoma patients might benefit from T cell-based immunotherapy.[14-16] As mentioned above, PD-L1 and TSP1 are highly expressed in osteosarcoma, and both of them negatively regulate antitumor immunity. However, it is not clear whether TSP1 is involved in the regulation of PD-L1 expression in osteosarcoma.

In this study, we showed that TSP1 was involved in the regulation of PD-L1 in osteosarcoma, as TSP1 could upregulate PD-L1, whereas TSP1 knockdown induced the downregulation of PD-L1. Thrombospondin-1 also played an essential role in the antitumor immunity impairment of $CD8^+$ T cells induced by osteosarcoma cells, as TSP1 knockdown could restore the proliferation and debilitate apoptosis of $CD8^+$ T cells. The immunosuppressive effects of TSP1 were achieved by upregulating PD-L1 by activating the STAT3 pathway. With further in vivo experiments, we found that TSP1 overexpression could stably upregulate PD-L1 on osteosarcoma cells, thereby decreasing the proportion of $CD4^+$ and $CD8^+$ T cells as well the ratio of CTLs and promoting the growth of primary lesions. Knockdown of TSP1 could restore antitumor immunity and inhibit the growth and metastasis of osteosarcoma. Therefore, TSP1 could serve as a therapeutic target to restore antitumor immunity against osteosarcoma and further improve the survival rate of osteosarcoma patients.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Antibodies for immunohistochemistry were anti-PD-L1 (10084-MB55; Sino Biological), anti-Ki-67 (ab16667; Abcam). Antibodies for western blot analyses were anti-PD-L1 (ab213524; Abcam), anti-phospho-STAT3 (9145; Cell Signaling Technology), anti-STAT3 (9139; Cell Signaling Technology), and anti-GAPDH (5174; Cell Signaling Technology). Antibodies for flow cytometry were APC-anti-human PD-L1 (563741; BD Biosciences), BV421-anti-mouse PD-L1 (564716; BD Biosciences), APC-anti-human CD3 (555342; BD Biosciences), PE-anti-human CD8 (561949; BD Biosciences), APC-anti-mouse CD3 (553059; BD Biosciences), PE-anti-mouse CD4 (557308; BD Biosciences), APC-CY7-anti-mouse CD8a (561967; BD Biosciences), PE-CY7-anti-mouse CD8a (561097; BD Biosciences). Lymphoprep density gradient centrifugation was purchased from Axis-Shield (1114546; Axis-Shield). Neutralizing Abs were anti-human PD-L1 neutralizing Ab (16-5983-82; eBioscience) and anti-mouse PD-L1 neutralizing Ab (16-5982-85; eBioscience). STATTIC was purchased from Selleck (S7024). Human recombinant TSP1 (3074-TH-050; R&D Systems), mouse recombinant TSP1 (7859-TH-050; R&D Systems), human IFN-γ (C005; Novoprotein), human IL-6 (C009; Novoprotein), and IL-10 (CX04; Novoprotein) were used to stimulate osteosarcoma cells.

2.2 | Cell culture

Human osteosarcoma cell line WELL5 was established by our laboratory. The mouse osteosarcoma cell line K7 was kindly gifted from Zhengdong Cai from the Osteosarcoma Research Institute, Shanghai, China. Osteosarcoma cells were cultured in high glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37℃.

2.3 | Lentiviral transfection

Lentiviral vectors for TSP1 or STAT3 knockdown and TSP1 overexpression were constructed by GeneChem, to target the following cDNA sequences: human shTSP1, 5′-GCGUGUUUGACAUCUUUGATT-3′

and negative control, 5′-TTCTCCGAACGTGTCACGT-3′; mouse shTSP1, 5′-TGGAAAGATTTCACTGCAT-3′ and negative control, 5′-TTCTCCGAACGTGTCACGT-3′; and STAT3si, 5′-GCAGCAGCTG AACAACATG-3′ and negative control, 5′-TTCTCCGAACGTGT CACGT-3′.

Seventy-two hours after transfection, osteosarcoma cells were treated with puromycin (GeneChem) at 5 μg/mL to construct TSP1 stable knockdown cell lines (shTSP1).

2.4 | Quantitative RT-PCR analysis

Total RNA from osteosarcoma cells was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was carried out to amplify the cDNA using the SYBR Premix Ex Tag kit (TaKaRa) and an ABI 7500 Sequencing Detection System (Applied Biosystems). The expression of target genes was calculated using $2^{-\Delta\Delta C_t}$. Primer sequences are listed in Table 1.

2.5 | Western blot analysis

Osteosarcoma cells were lysed with prechilled RIPA (50 mmol/L Tris/HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate) on ice for 10 minutes. The lysates were centrifuged at 12 000 *g* for 10 minutes at 4°C and the supernatants were collected for protein concentration determination. The total proteins were separated on 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). The blot was incubated with appropriate primary Abs at 4°C overnight. Protein amounts were determined by densitometric analysis and normalized to GAPDH.

2.6 | Immunohistochemistry

Immunohistochemical staining of PD-L1 and Ki-67 was undertaken using the Super Sensitive IHC Detection System Kit (BD5001; Bioworld) according to the manufacturer's instruction. Slides were incubated with Abs against PD-L1 and Ki-67 overnight at 4°C. Slides were evaluated by three independent investigators who were blinded to the identity of each slide.

2.7 | Flow cytometry

Osteosarcoma cells were harvested and incubated with APC-anti-human PD-L1 or BV421-anti-mouse PD-L1 or isotype Abs for 30 minutes at room temperature. Cells were then washed with PBS buffer and analyzed with CytoFlex S (Beckman); the gate was set according to the fluorescence intensity of the isotype group.

2.8 | $CD8^+$ T cell proliferation assay

The $CD8^+$ T cell proliferation assay was carried out as described previously.[17] Before cocultivation, osteosarcoma cells were pretreated with recombinant TSP1 protein (5 μg/mL) for 24 hours. Human anti-PD-L1 neutralizing Ab or mouse anti-PD-L1 neutralizing Ab was added to the medium at 5 μg/mL. After cocultivation, $CD8^+$ T cells ($CD3^+$ and $CD8^+$ T cells) were selected to detect their proliferation by the CytoFlex S (Beckman).

2.9 | $CD8^+$ T cell apoptosis assay

An Annexin V Apoptosis Detection Kit FITC (BMS500FI-300; eBioscience) was used to detect the apoptosis of $CD8^+$ T cells. The detailed procedure was the same as described previously.[17] The pretreatment of osteosarcoma cells and the usage of anti-PD-L1 neutralizing Abs were the same as mentioned above. $CD8^+$ T cells apoptosis was calculated as the percentage of annexin V^+ cells in a gated $CD8^+$ population.

2.10 | Subcutaneous osteosarcoma models

mouse osteosarcoma cells K7 (with or without TSP1 knockdown, and with or without TSP1 overexpression) (2×10^6 cells) were inoculated into 6-week-old male Balb/c mice subcutaneously as previously described.[18] Tumor volume was measured every 4 days and calculated using the equation (length × width^2)/2. Mice were killed 4 weeks after implantation. Both the primary tumors and lung metastases were dissected and digested using 1 mg/mL collagenase IV (Sigma-Aldrich) and 0.2 mg/mL DNase I (Sigma-Aldrich) for 60 minutes at 37°C to obtain single-cell suspensions. The single-cell suspensions

TABLE 1 Primer sequences used in this study

Gene	Primer sequence (5′-3′)	
TSP1	Forward:	CTCAGGACCCATCTATGATAAAACC
(human)	Reverse:	AAGAAGGAAGCCAAGGAGAAGTG
TSP1	Forward:	GAAAGACGCCTGCCCAATTAAT
(mouse)	Reverse:	ACTTGATTTTCTGTCACATCGC
PD-L1	Forward:	GCTGCACTAATTGTCTATTGGG
(human)	Reverse:	CACAGTAATTCGCTTGTAGTCG
PD-L1	Forward:	AAGCCTCAGCACAGCAACTTCAG
(mouse)	Reverse:	TGTAGTCCGCACCACCGTAGC
STAT3	Forward:	TCGGCTAGAAAACTGGATAACG
(human)	Reverse:	TGCAACTCCTCCAGTTTCTTAA
GAPDH	Forward:	GGACCTGACCTGCCGTCTAG
(human)	Reverse:	GTAGCCCAGGATGCCCTTGA
GAPDH	Forward:	AAGAAGGTGGTGAAGCAGGCATC
(mouse)	Reverse:	CGGCATCGAAGGTGGAAGAGTG

were incubated with Abs against CD31 and CD45 (to remove the pan-lymphocyte and endothelial cells) and PD-L1 (to analyze PD-L1 expression on K7 cells).

2.11 | Lung metastatic osteosarcoma models

mouse osteosarcoma cells (K7 with or without TSP1 knockdown) (2×10^6 cells) were inoculated into 6-week-old male Balb/c mice through the tail vein as previously reported.[19] Mice were killed 8 weeks after injection. The lung metastasis was dissected and digested using a Lung Dissociation Kit (130-095-927; Miltenyi) to obtain single-cell suspensions. The single-cell suspensions were incubated with Abs against CD31 and CD45 (to remove the pan-lymphocyte and endothelial cells), PD-L1 (to analyze PD-L1 expression on K7 cells), and CD3, CD4, and CD8 to examine the population of T cells. An IFN-γ staining kit (eBioscience) was used to examine CTLs according to the manufacturer's instructions.

2.12 | Ethics statement

All the animal experiments were approved by the Ethics Committee of Ruijin Hospital, affiliated with Shanghai Jiaotong University School of Medicine.

2.13 | Statistical analysis

All data from at least three independent experiments are expressed as mean ± SD. Statistical differences between each group were estimated using Student's *t* test or one-way ANOVA. Statistical analyses were undertaken using GraphPad Prism 5.0.

3 | RESULTS

3.1 | Thrombospondin-1 regulated the expression of PD-L1 in osteosarcoma cells

To investigate whether PD-L1 was regulated by TSP1 in osteosarcoma, TSP1 knockdown osteosarcoma cell lines (shTSP1) were constructed by lentiviral transfection (Figure 1A-C). First, as shown in Figure 1B-G, the shTSP1 group showed a relatively lower total expression of PD-L1 compared to the control group. In addition, the surface expression of PD-L1 in the shTSP1 group was reduced compared to the control group (Figure 1E-G).

To further verify the relationship between TSP1 and PD-L1, we added recombinant TSP1 protein to the medium. As shown in Figure 1H-L, TSP1 induced dose-dependent increments of total PD-L1 expression in WELL5 and K7 cells. The change of PD-L1 on the surface was consistent with that in total (Figure 1K,L).

Overall, our results suggest that TSP1 does regulate PD-L1 expression in osteosarcoma cells. However, the exact role of TSP1 in antitumor immunity still needs further exploration.

3.2 | Thrombospondon-1 induced apoptosis and inhibited proliferation of $CD8^+$ T cells in vitro

To explore the exact role of TSP1 in antitumor immunity, we first detected the apoptosis of $CD8^+$ T cells after coculturing with osteosarcoma cells. As shown in Figure 2A,B, in the cocultivation system, K7 could induce the apoptosis of $CD8^+$ T cells. After the pretreatment of osteosarcoma cells with recombinant TSP1 protein, its pro-apoptotic effect on $CD8^+$ T cells was further exacerbated. To determine whether the enhanced pro-apoptotic effect of osteosarcoma cells was related to the TSP1-induced PD-L1 upregulation, we added anti-PD-L1 neutralizing Ab during cocultivation in vitro. We observed that the apoptosis of $CD8^+$ T cells was partially impeded in the presence of anti-PD-L1 neutralizing Ab, but was still lower than that of $CD8^+$ T cells cultured alone (Figure 2A,B). Similar results were also observed in WELL5 cells (Figure 2C,D).

We further examined the proliferation of $CD8^+$ T cells after coculturing with osteosarcoma cells. As shown in Figure 3A,B, the proliferation of $CD8^+$ T cells could be activated by nonspecific stimuli (CD3 and CD28). However, the proliferation of $CD8^+$ T cells was inhibited when cocultured with K7 cells (Figure 3A,B). Moreover, the inhibitory effect of osteosarcoma cells on the proliferation of $CD8^+$ T cells further deteriorated after pretreatment with TSP1. We observed that the proliferation of $CD8^+$ T cells was partially restored following the addition of anti-PD-L1 neutralizing Ab during cocultivation, which proved that PD-L1 was closely related to the inhibition of $CD8^+$ T cell proliferation caused by TSP1 (Figure 3A,B). The same phenomenon was also observed in WELL5 cells (Figure 3C,D).

These results indicate that TSP1 can not only aggravate the apoptosis of $CD8^+$ T cells induced by osteosarcoma cells, but also inhibit the proliferation of $CD8^+$ T cells, and the PD-L1/PD-1 pathway is partially involved in the immunosuppressive effect of TSP1.

3.3 | Thrombospondin-1 regulated PD-L1 expression through the STAT3 pathway

To investigate the underlying mechanism by which TSP1 modulated the expression of PD-L1, we undertook the following experiments using WELL5 cells. As shown in Figure 4A, the phosphorylation level of STAT3 was decreased in the shTSP1 group compared to the control group. Furthermore, the phosphorylation level of STAT3 increased gradually as the concentration of recombinant TSP1 increased (Figure 4B). But when we added STATTIC, the specific STAT3 inhibitor, together with recombinant TSP-1, the upregulation of PD-L1 caused by TSP1 stimulation was partially reduced (Figure 4C,D). Additionally, PD-L1 expression on the surface in WELL5 cells was also reduced by the addition of STATTIC (Figure 4E).

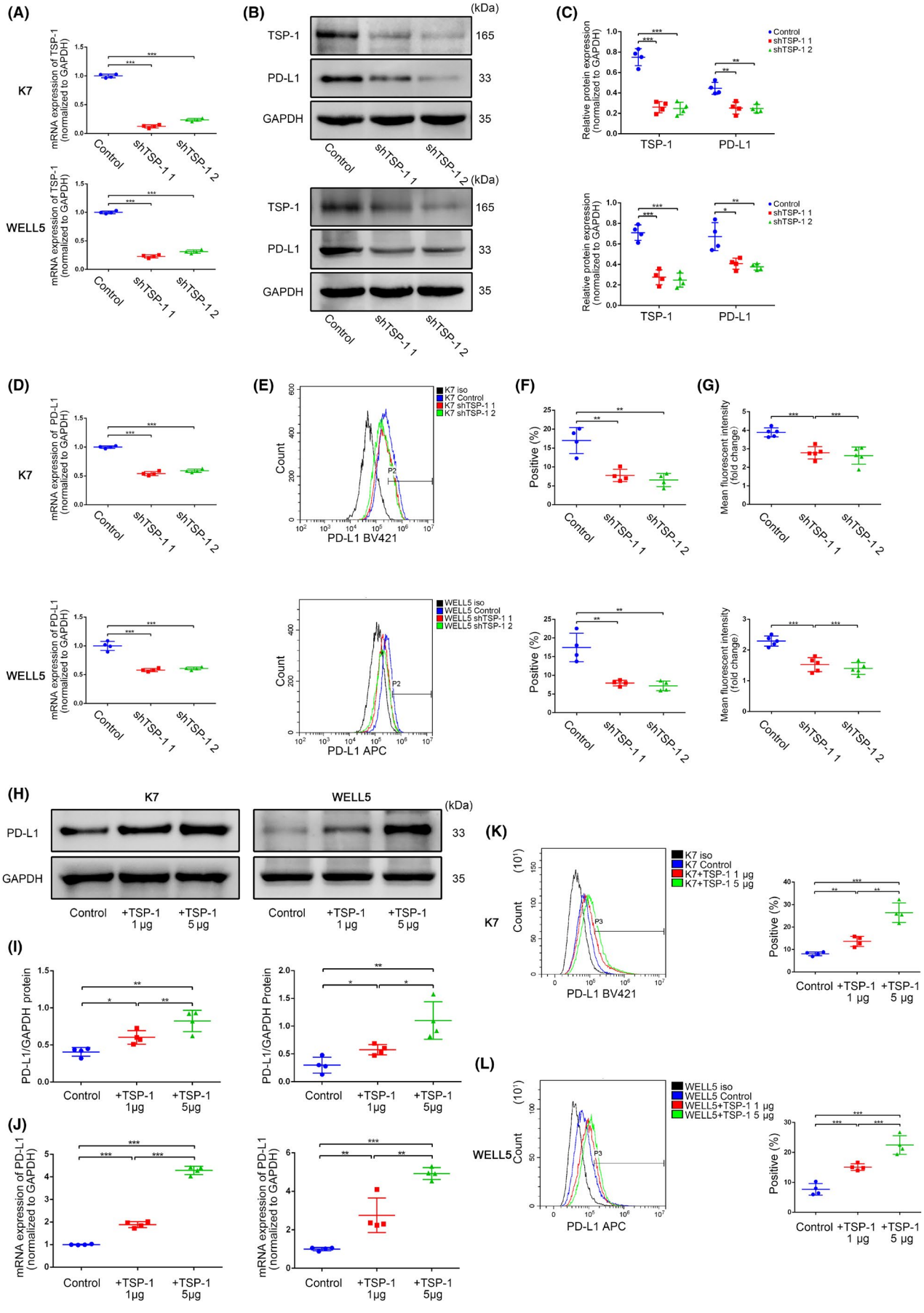
(A)
K7
WELL5
mRNA expression of TSP-1 (normalized to GAPDH)
Control
shTSP-1 1
shTSP-1 2
(B)
(kDa)
TSP-1
PD-L1
GAPDH
165
33
35
(C)
Relative protein expression (normalized to GAPDH)
Control
shTSP-1 1
shTSP-1 2
TSP-1
PD-L1
(D)
mRNA expression of PD-L1 (normalized to GAPDH)
(E)
K7 iso
K7 Control
K7 shTSP-1 1
K7 shTSP-1 2
Count
PD-L1 BV421
WELL5 iso
WELL5 Control
WELL5 shTSP-1 1
WELL5 shTSP-1 2
PD-L1 APC
(F)
Positive (%)
(G)
Mean fluorescent intensity (fold change)
(H)
K7
WELL5
PD-L1
GAPDH
Control
+TSP-1 1 μg
+TSP-1 5 μg
(I)
PD-L1/GAPDH protein
PD-L1/GAPDH Protein
(J)
mRNA expression of PD-L1 (normalized to GAPDH)
(K)
K7 iso
K7 Control
K7+TSP-1 1 μg
K7+TSP-1 5 μg
(L)
WELL5 iso
WELL5 Control
WELL5+TSP-1 1 μg
WELL5+TSP-1 5 μg

FIGURE 1 Thrombospondin-1 (TSP1) upregulated programmed death-ligand 1 (PD-L1) in osteosarcoma cells. A, Expression of TSP1 in K7 and WELL5 cells was evaluated by quantitative RT-PCR (qRT-PCR) after shTSP1 transfection. B, C, Expression of TSP1 and PD-L1 in K7 and WELL5 cells was evaluated by western blot analysis after shTSP1 transfection. D, Total expression of PD-L1 in K7 and WELL5 cells was evaluated by qRT-PCR after shTSP1 transfection. E-G, Surface expression of PD-L1 in K7 cells was evaluated by flow cytometry after shTSP1 transfection. H, I, Total expression of PD-L1 in WELL5 and K7 cells was evaluated by western blot analysis after stimulation by different concentrations of recombinant TSP1. J, Total expression of PD-L1 in K7 and WELL5 cells was evaluated by qRT-PCR after stimulation by different concentrations of recombinant TSP1. K, Surface expression of PD-L1 in K7 was evaluated by flow cytometry after stimulation by different concentrations of recombinant TSP1. L, Surface expression of PD-L1 in WELL5 cells was evaluated by flow cytometry after stimulation by different concentrations of recombinant TSP1. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

To further confirm the role of the STAT3 pathway in TSP1-induced PD-L1 upregulation, we silenced the expression of STAT3 by siRNA transfection in WELL5 cells (Figure 4F). As shown in Figure 4G, the knockdown of STAT3 achieved the same effect as adding STATTIC, both of which attenuated the upregulation of PD-L1 induced by TSP1 stimulation. Immunofluorescence staining in Figure 4H further

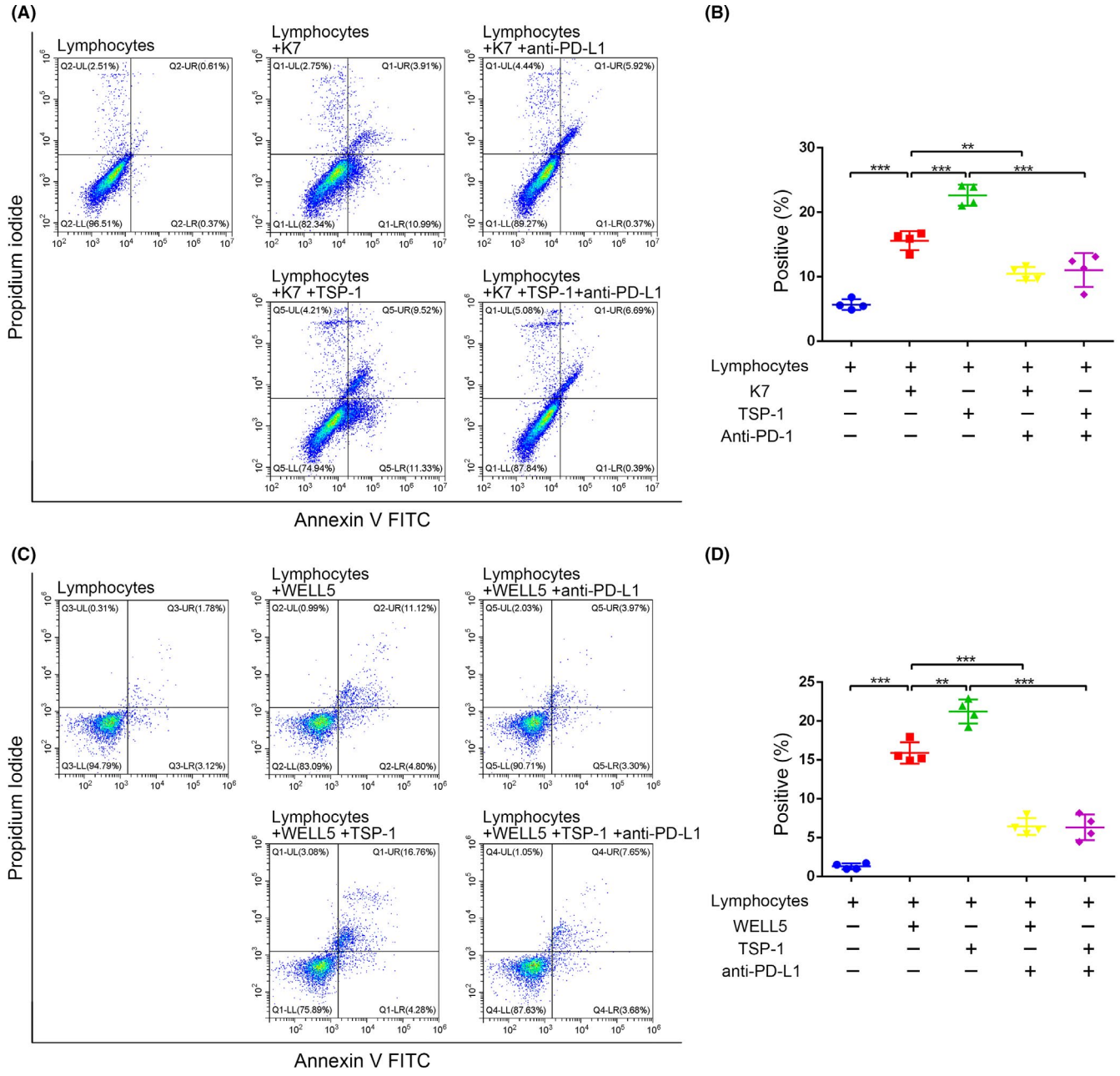

FIGURE 2 Thrombospondin-1 (TSP1) promoted the apoptosis of $CD8^{+}$ T cells through upregulating programmed death-ligand 1 (PD-L1). A, B, Apoptosis of $CD8^{+}$ T cells after coculture with K7 TSP1 control or K7 shTSP1. C, D, Apoptosis of $CD8^{+}$ T cells after coculture with WELL5 TSP1 control or WELL5 shTSP1. $^{**}P < .01$, $^{***}P < .001$

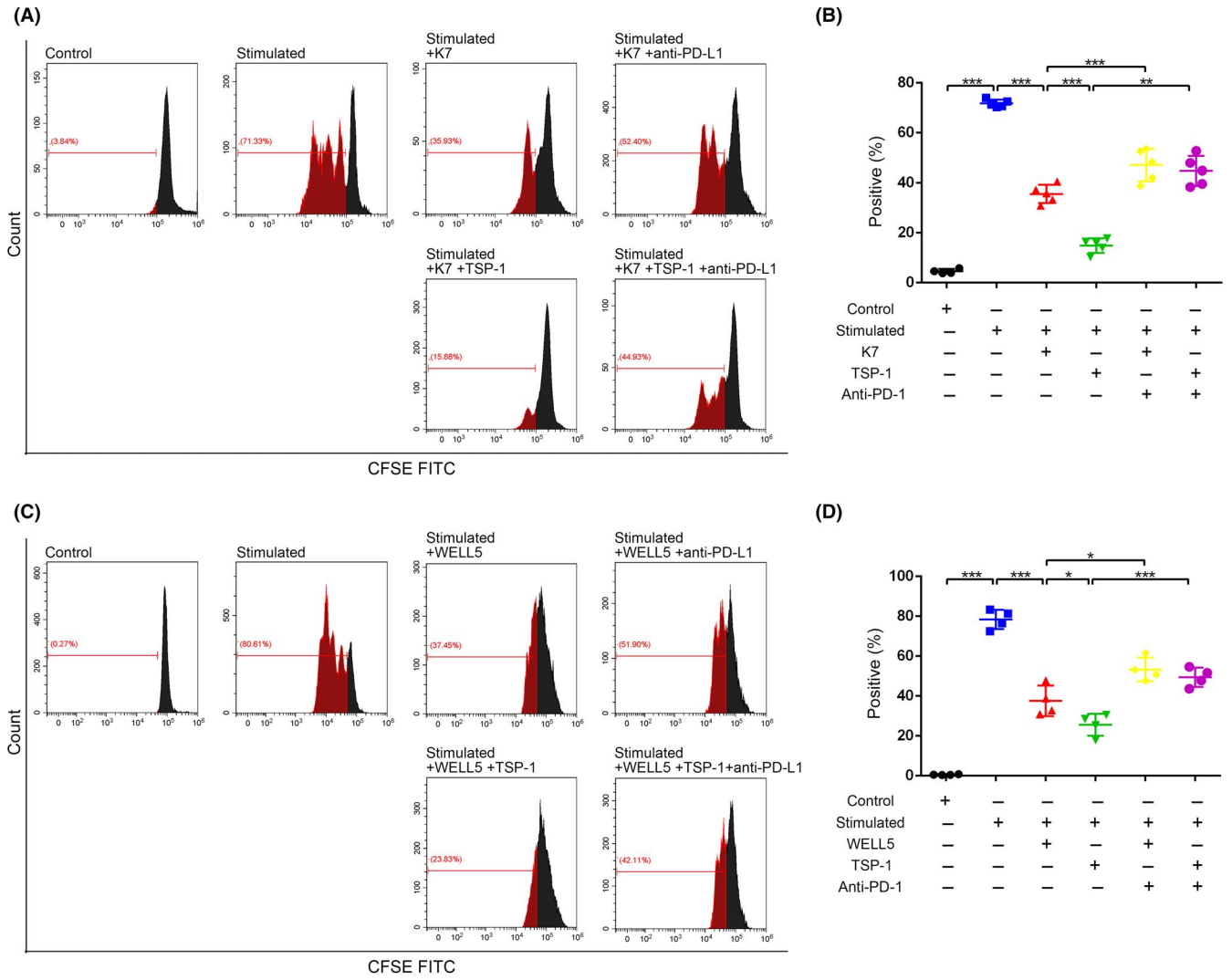

FIGURE 3 Thrombospondin-1 (TSP1) inhibited the proliferation of $CD8^+$ T cells by upregulating programmed death-ligand 1 (PD-L1). A,B, Examine the proliferation of $CD8^+$ T cells after co-cultured with K7 TSP1 control or K7 shTSP1 separately. C,D, Examine the proliferation of $CD8^+$ T cells after co-cultured with WELL5 TSP1 control or WELL5 shTSP1 separately. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

confirmed that the activation of STAT3 pathway played an important role in the TSP1-induced PD-L1 upregulation.

Thus, the results mentioned above indicate that the STAT3 pathway is involved in the regulation of PD-L1 in osteosarcoma cells, and blocking STAT3 pathway can mitigate the TSP1-induced PD-L1 upregulation.

3.4 | Thrombospondin-1 promoted the growth of osteosarcoma by inhibiting antitumor immunity in vivo

Previously, we reported that TSP1 knockdown in osteosarcoma did not affect the growth of primary lesions in the absence of the immune system. This time, we explored the exact role of TSP1 in tumorigenesis under an immunocompetent state. First, TSP1 overexpression osteosarcoma cell line (TSP1 OE) was constructed by lentiviral transfection and implanted subcutaneously. Thrombospondin-1 significantly promoted the growth of osteosarcoma compared to the control group (Figure 5A,B) and could stably upregulate PD-L1 in vivo (Figure 5C,D). The positive rate of Ki-67 in the TSP1 OE group also showed consistent modifications (Figure 5E). We then detected the content of intratumoral $CD8^+$ T cells. As shown in Figure 5F, the proportion of $CD8^+$ T cells in the TSP1 OE group was lower than that of the control group. After the application of the anti-PD-L1 neutralizing Ab, the tumor-promoting effect of TSP1 was attenuated to some extent (Figure 5A-E), and the intratumoral proportion of $CD8^+$ T cells was increased as well (Figure 5F). However, with the application of anti-PD-L1 neutralizing Ab, the abundance of intratumoral $CD8^+$ T cells in the control group was still higher than that of TSP1 OE group (9.95% vs 7.09%, $P = .0022$).

To further verify the exact role of TSP1 in tumorigenesis, we also inoculated shTSP1 osteosarcoma cells subcutaneously. As shown in Figure 6A,B, TSP1 knockdown in K7 cells significantly inhibited the growth of osteosarcoma as the increment of tumor volume

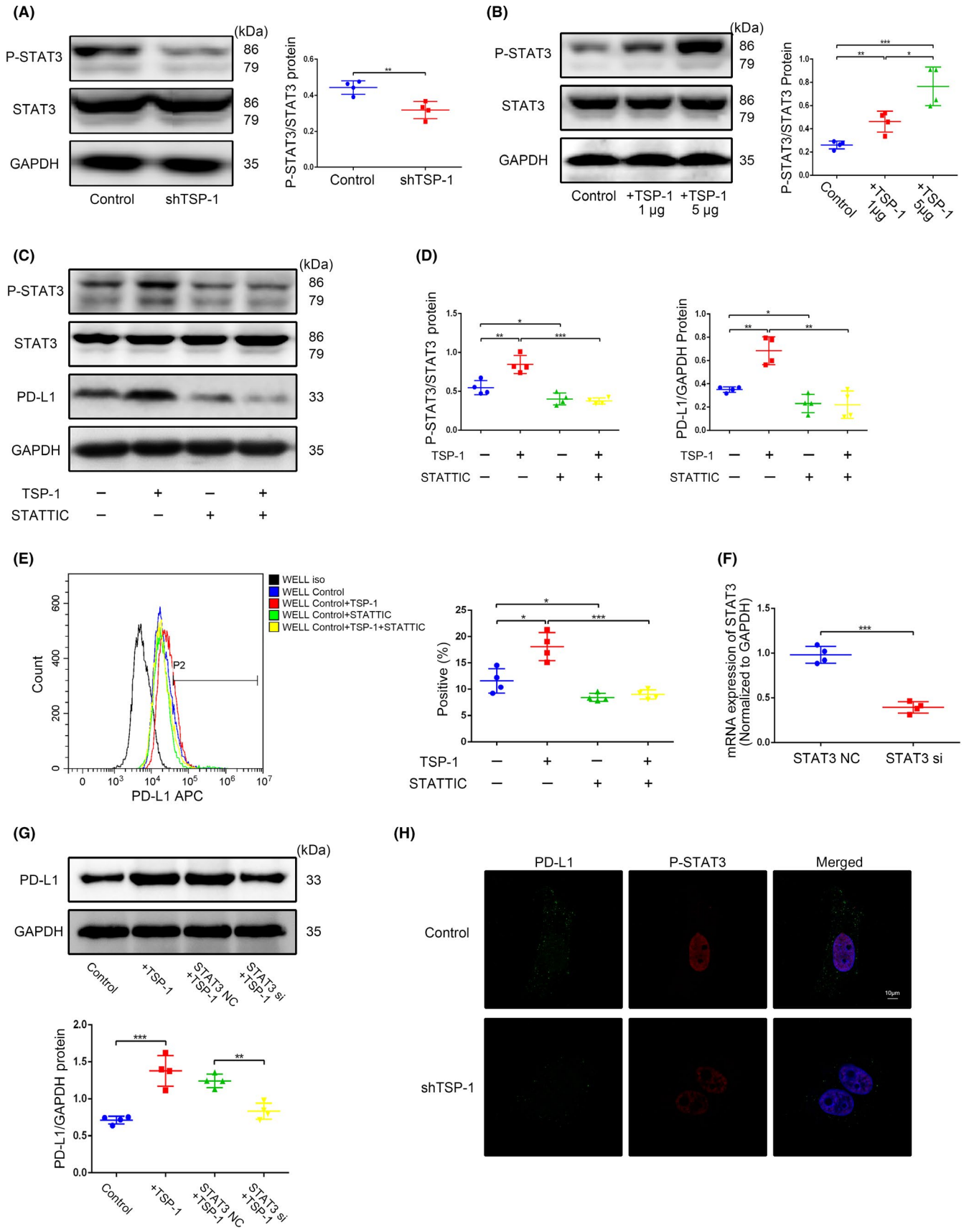

FIGURE 4 Legend on next page

FIGURE 4 Thrombospondin-1 (TSP1) upregulated programmed death-ligand 1 (PD-L1) in osteosarcoma cells by activating the signal transducer and activator of transcription 3 (STAT3) pathway. A, Expression of P-STAT3 and STAT3 in WELL5 cells was evaluated by western blot analysis after shTSP1 transfection. B, Expression of P-STAT3 and STAT3 in WELL5 cells was evaluated by western blot analysis after stimulation by different concentrations of recombinant TSP1. C, D, Expression of P-STAT3, STAT3, and PD-L1 in WELL5 cells was evaluated by western blot analysis after stimulation by recombinant TSP1 (5 μg/mL for 24 h) with or without specific STAT3 inhibitor STATTIC (10 μmol/L for 24 h). E, Surface expression of PD-L1 in WELL5 cells was evaluated by flow cytometry after stimulation by recombinant TSP1 (5 μg/mL for 24 h) with or without specific STAT3 inhibitor STATTIC (10 μmol/L for 24 h). F, Expression of STAT3 in WELL5 cells was evaluated by quantitative RT-PCR after STAT3 siRNA transfection. G, Total expression of PD-L1 in WELL5 cells was evaluated by western blot analysis after shTSP1 transfection. H, Immunofluorescence staining of PD-L1 and P-STAT3 expression after STAT3 siRNA transfection. Scale bar, 10 μm. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

slowed down. The positive rate of PD-L1 in the control group was higher than that of the shTSP1 group. The positive rate of Ki-67 in the shTSP1 group was also lower than that of the control group (Figure 6D). We then detected the content of intratumoral $CD8^+$ T cells. As shown in Figure 6E,F, the proportion of $CD8^+$ T cells in the control group was lower than that of the shTSP1 group. After the application of the anti-PD-L1 neutralizing Ab, we confirmed that shTSP1 combined with an anti-PD-L1 neutralizing Ab could achieve a more pronounced antitumor effect because not only the tumor volume but also the intratumorally positive rate of Ki-67 decreased significantly after the addition of the anti-PD-L1 neutralizing Ab (Figure 6A-D). It was also confirmed by the detection of the intratumoral proportion of $CD8^+$ T cells as both the control and shTSP1 groups had a higher level of $CD8^+$ T cells after anti-PD-L1 neutralizing Ab treatment (Figure 6E,F).

Therefore, we suggested that TSP1 was closely related to the growth of osteosarcoma as TSP1 knockdown suppressed, whereas TSP1 overexpression promoted, the growth of osteosarcoma in vivo. Moreover, the role of TSP1 in regulating the growth of osteosarcoma is closely related to its inhibitory effect on antitumor immunity.

3.5 | Thrombospondin-1 knockdown inhibited lung metastasis of osteosarcoma by restoring antitumor immunity in vivo

Our previous research also indicated that, in the absence of the immune system, TSP1 knockdown in osteosarcoma could reduce lung metastasis. In order to explore whether TSP1 has the same effect in the presence of the immune system, we carried out the following experiments. First, TSP1 knockdown considerably attenuated tumor burden at metastatic sites of osteosarcoma cells, reflected by the number of metastases and the significantly decreased weight of the lung (Figure 7A-C). Immunohistochemistry staining of PD-L1 suggested that shTSP1 group had a stable low expression of PD-L1, even in the lung metastases (Figure 7D). Furthermore, both the content of $CD4^+$ and $CD8^+$ T cells in the shTSP1 group increased dramatically compared to the control group (Figure 7E-G). The proportion of IFN-γ^+ CTLs was significantly higher in the shTSP1 group compared to the control group (Figure 7H,I).

Overall, the knockdown of TSP1 can also inhibit the lung metastasis of osteosarcoma by increasing the abundance of lymphocytes and the function of $CD8^+$ T cells in vivo.

Taken together, these findings indicate that TSP1 might induce PD-L1-mediated immunosuppression by activating the STAT3 pathway in osteosarcoma, and TSP1 knockdown inhibits the growth and metastasis of osteosarcoma both in vitro and in vivo. Combined with an anti-PD-L1 neutralizing Ab, shTSP1 could be a promising combined approach in the treatment of osteosarcoma.

4 | DISCUSSION

In this study, we investigated the potential role of TSP1 in the immunomodulation of osteosarcoma. First, we found that TSP1 was closely related to the expression of PD-L1 in osteosarcoma, as TSP1 knockdown could induce simultaneous downregulation of PD-L1, and conversely, with the stimulation of recombinant TSP1 protein, there was a synchronous upregulation of PD-L1. We then showed that TSP1 knockdown in osteosarcoma cells could partially restore the impairment of antitumor immunity of $CD8^+$ T cells induced by osteosarcoma cells, as the proliferation and apoptosis of $CD8^+$ T cells were alleviated. The further mechanistic investigations indicated that the upregulation of PD-L1 induced by TSP1 was partially related to activation of the STAT3 pathway. Further in vivo experiments also indicated that TSP1 overexpression could promote the growth of the primary lesion whereas TSP1 knockdown effectively inhibits the growth of the primary lesion as well as lung metastasis by restoring the antitumor immunity. Thrombospondin-1 knockdown combined with PD-L1 neutralizing Ab achieved a more pronounced antitumor effect. Taken together, we propose that TSP1 can upregulate PD-L1 by activating the STAT3 pathway and thereby inducing the immunosuppressive microenvironment in osteosarcoma. Thrombospondin-1 knockdown can restore the antitumor immunity of $CD8^+$ T cells to a certain extent and could serve as a potential therapeutic target.

Thrombospondin-1 is known to be involved in tumorigenesis by affecting angiogenesis and therefore suppressing tumor formation.[20] In these studies, the function of TSP1 in tumor progression was detected in a xenograft mouse model and the exact role of TSP1 in immune regulation has not been further explored. With the deepening of relevant research, Weng et al[21] found that TSP1 knockdown through shRNA exerted pronounced antitumor effects in the MBT-2 bladder tumor-bearing syngeneic C3H/HeN mouse model and the LL2 lung tumor-bearing C57BL/6 mouse model. Saito et al also reported that anti-TSP1 mAbs could increase the proportion of tumor-specific TILs and activate the systemic immune response,

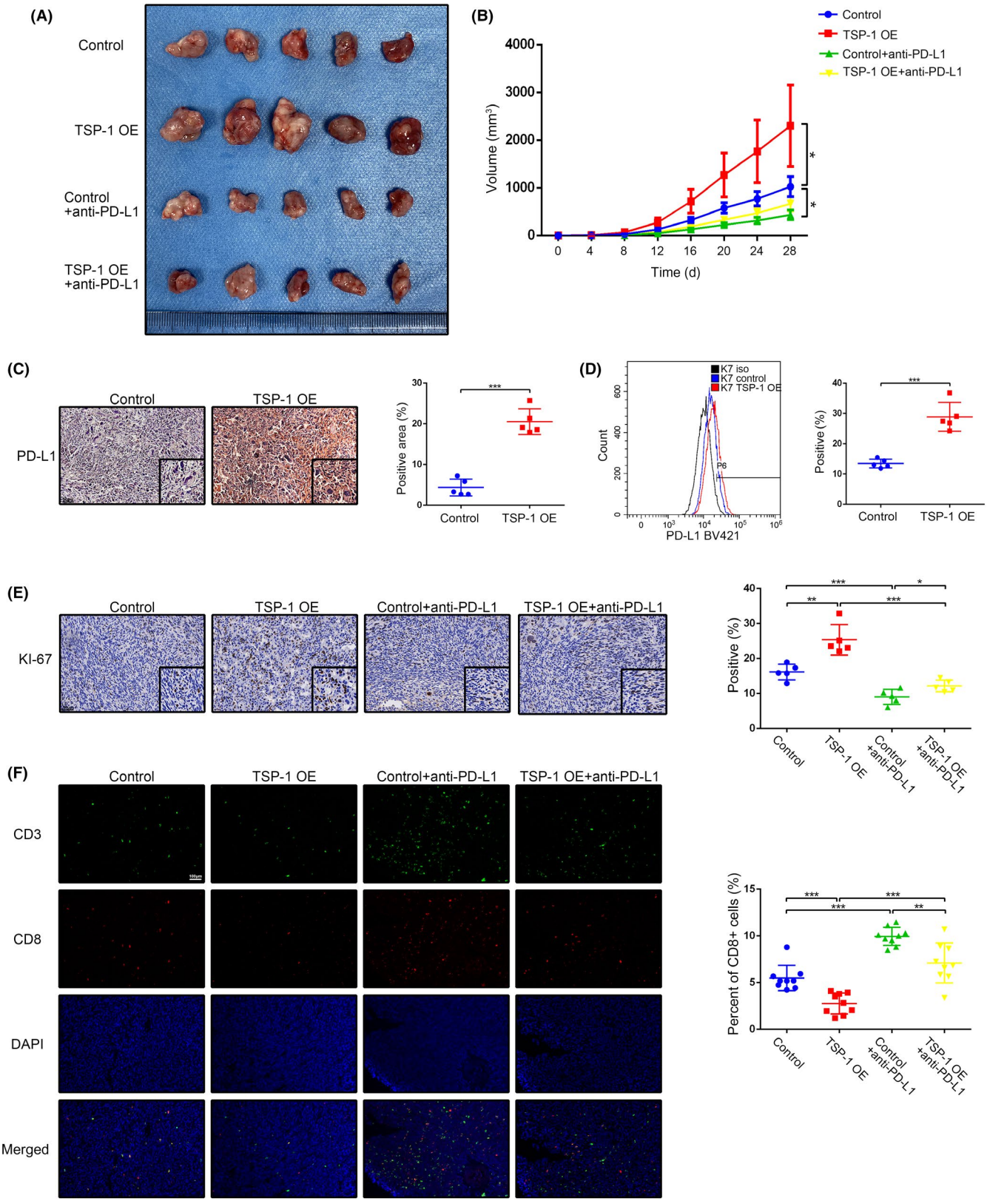

FIGURE 5 Thrombospondin-1 (TSP1) promoted the growth of osteosarcoma through impairing antitumor immunity in vivo. A, TSP1 promoted the growth of osteosarcoma by upregulating programmed death-ligand 1 (PD-L1). B, Quantification of tumor volume. C, Immunohistochemical staining and analysis of Ki-67 expression in tumor tissues. Scale bar, 50 μm. D, Surface expression of PD-L1 in each group was evaluated by flow cytometry in vivo. E, Immunohistochemical staining and analysis of Ki-67 expression in tumor tissues. Scale bar, 50 μm. F, Immunofluorescence staining and analysis of $CD8^+$ cells in tumor tissues. Scale bar, 100 μm. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. OE, overexpression

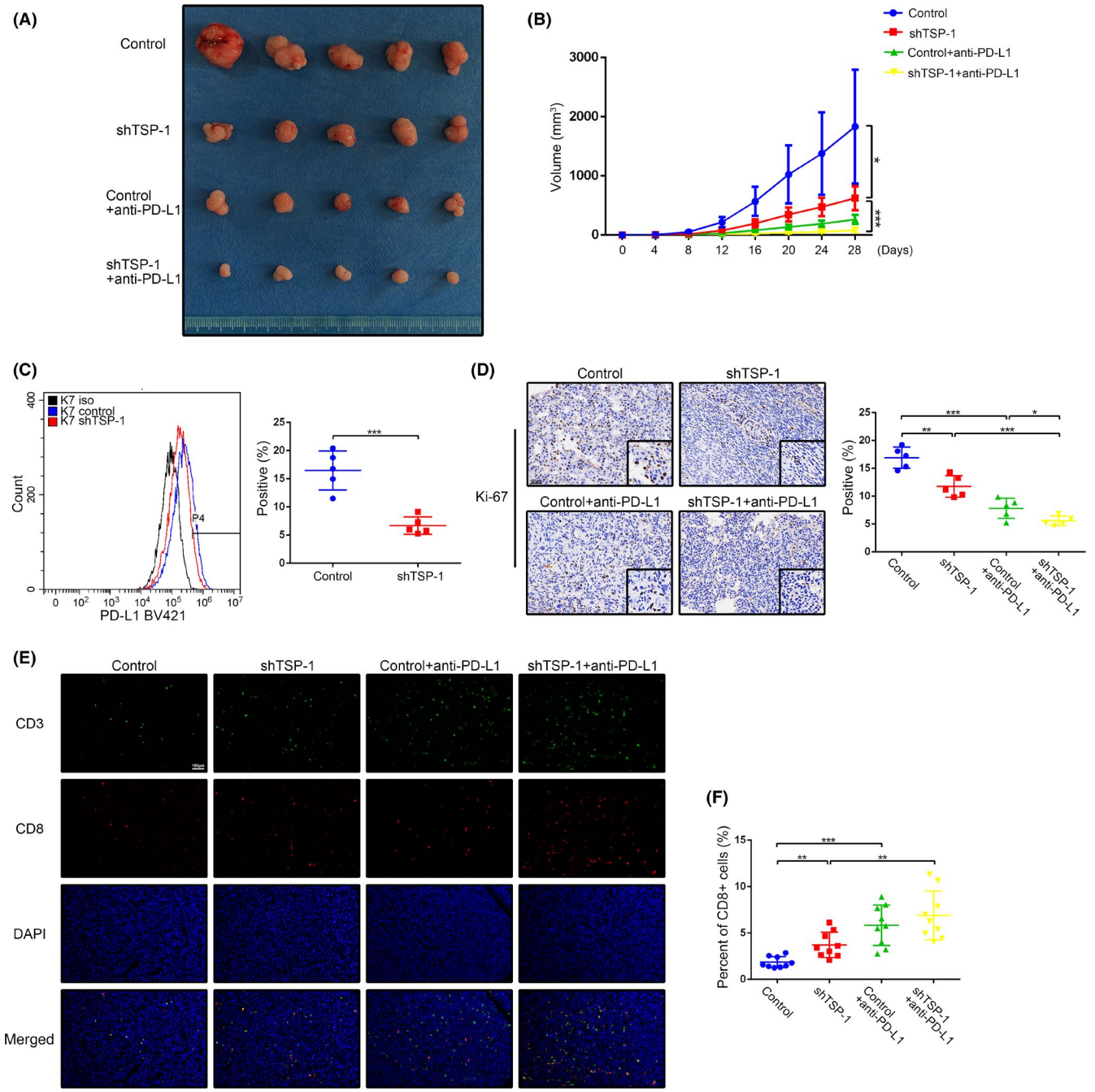

FIGURE 6 Thrombospondin-1 (TSP1) knockdown inhibits the growth of osteosarcoma by recovering antitumor immunity in vivo. A, TSP1 knockdown inhibits the growth of osteosarcoma by downregulating programmed death-ligand 1 (PD-L1). B, Quantification of tumor volume. C, Surface expression of PD-L1 in each group was evaluated by flow cytometry in vivo. D, Immunohistochemical staining and analysis of Ki-67 expression in tumor tissues. Scale bar, 50 μm. E, F, Immunofluorescence staining and analysis of $CD8^+$ cells in tumor tissues. Scale bar, 100 μm. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

thereby suppressing tumor growth and metastasis.[22] In this study, we constructed stable TSP1 overexpression or TSP1 knockdown osteosarcoma cells through lentiviral transfection and observed the same phenomenon that Weng and Satio reported previously, that the growth of primary lesion, as well as lung metastasis of osteosarcoma, were suppressed by TSP1 knockdown and promoted by TSP1 overexpression in the presence of the immune system. These results strongly indicated that TSP1 had multifaceted effects on the tumor progression, as it could not only promote angiogenesis but also modulate antitumor immunity, which has rarely been reported in previous research.

Programmed death-ligand 1, an important immune checkpoint, plays an indispensable role in immunosuppression by interacting with PD-1 on immune cells.[23,24] The expression of PD-L1 in osteosarcoma is regulated by several inflammatory factors, such as IL-6, IL-10, and IFN-γ (Figure S1)

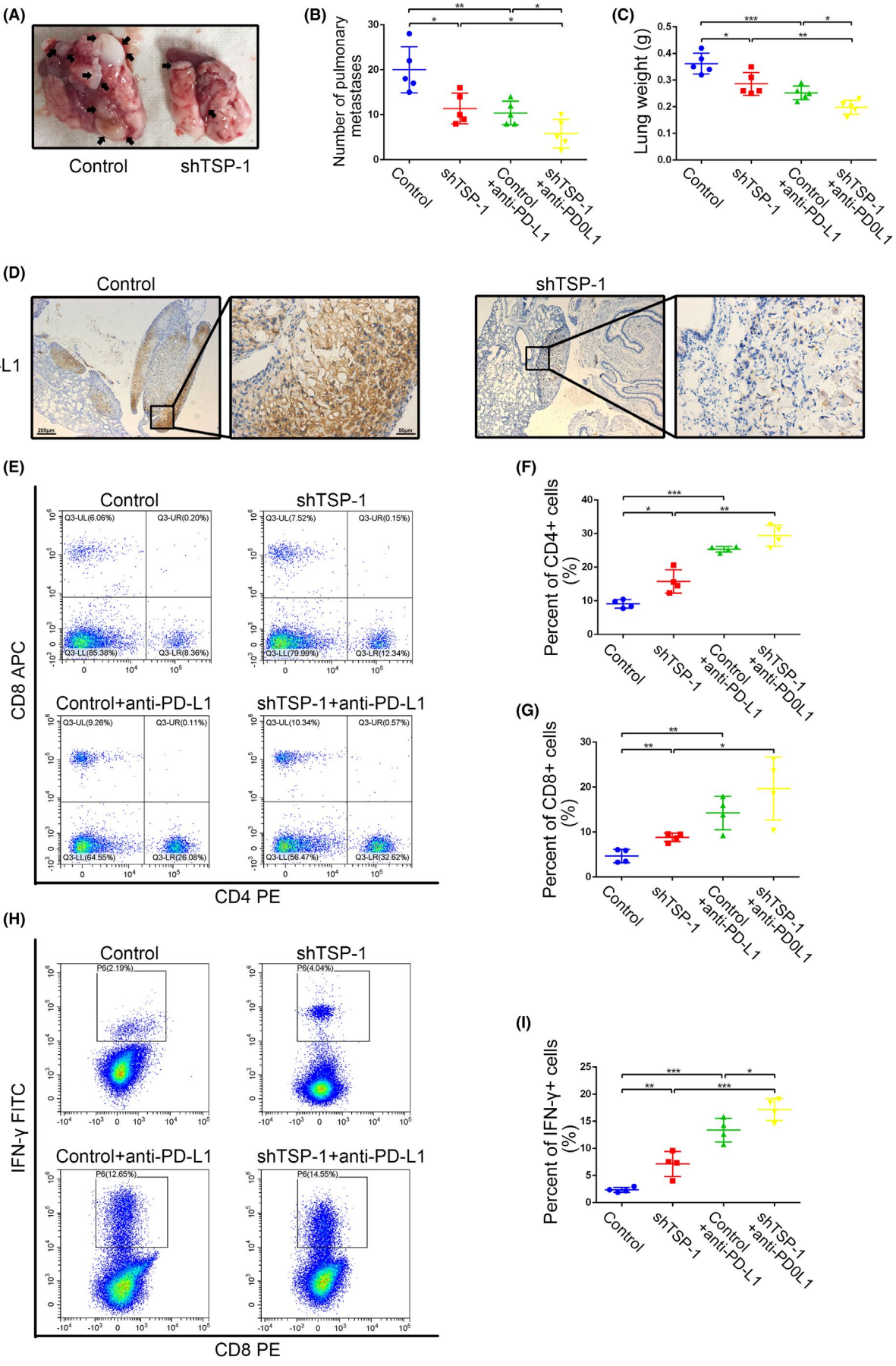

FIGURE 7 Legend on next page

FIGURE 7 Thrombospondin-1 (TSP1) promoted the lung metastasis of osteosarcoma by impairing antitumor immunity in vivo. A-C, TSP1 promoted the lung metastasis of osteosarcoma by upregulating programmed death-ligand 1 (PD-L1) (black arrows indicate lung metastasis). D, Immunohistochemical staining of PD-L1 expression in lung metastasis. Scale bars, 200 or 50 μm. E-G, The proportion of tumor infiltrating lymphocytes was evaluated by flow cytometry. H, I, The proportion of interferon-γ (IFN-γ)$^+$ cells was evaluated by flow cytometry. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

. In this study, we proposed that PD-L1 could be also modulated by TSP1 in osteosarcoma, as TSP1 overexpression could upregulate PD-L1 whereas TSP1 knockdown could downregulate PD-L1 on osteosarcoma cells. As CD8$^+$ T cells are the key components of antitumor immunity,[25,26] TILs, especially CD8$^+$ T cells, are considered to be good prognostic factors of many kinds of tumors.[27-30] Interactions between PD-L1 and PD-1 inhibit the proliferation of CD8$^+$ T cells and affect the function of TILs.[31,32] Several studies have reported that the inhibition of TSP1 could significantly increase intratumoral CD4$^+$ and CD8$^+$ T cells.[21,22] We found the same phenomenon, that the abundance of tumor-infiltrating CD4$^+$ and CD8$^+$ T cells in the shTSP1 group was elevated compared to that of the control group both in the primary and metastatic lesions. It has been reported that the interaction between TSP1 and its receptors (CD36 and CD47) could negatively regulate the secretory function of T cells, resulting in the reduction of the secretion of IL-12, TNF-α, and IL-10.[10,33] We proposed that TSP1-induced PD-L1 upregulation was partially related to the interaction with CD47, as the blockade of the binding of TSP1 and CD47 could partially debilitate the upregulation of PD-L1 (Figure S2) . This also gives us a more comprehensive understanding of the mechanism by which TSP1 inhibits antitumor immunity.

In order to verify the role of the PD-L1/PD-1 axis in TSP1-induced immunosuppression, we used anti-PD-L1 neutralizing Ab to block this combination and found that, with the addition of anti-PD-L1 neutralizing Abs, the immunosuppressive effects of osteosarcoma cells on CD8$^+$ T cells was partially abolished. However, the proportion of CD8$^+$ T cells in the control + anti-PD-L1 group was still higher than that of the TSP1 OE + anti-PD-L1 group, suggesting that in addition to PD-L1, TSP1 can also affect antitumor immunity through other ways, which requires further exploration.

Recently, some studies reported that the regulation of PD-L1 was closely related to aberrant activation of the STAT3 pathway.[34-36] Silencing the STAT3 pathway could trigger the systemic immune response in leukemia.[37] In this study, we found that, with the stimulation of recombinant TSP1 protein, there was a consistent activation of the STAT3 pathway as well as the upregulation of PD-L1 on osteosarcoma cells. The addition of STATTIC, a specific STAT3 inhibitor, could attenuate the upregulation of PD-L1 induced by recombinant TSP1 protein. Thus, our data suggested that TSP1 induced PD-L1 upregulation was associated with the activation of the STAT3 pathway, and this might be another mechanism by which TSP1 induced an immunosuppressive microenvironment and promoted tumorigenesis.

Based on our previous research, we suggest that in osteosarcoma, TSP1 can weaken antitumor immunity while promoting angiogenesis, thereby building a microenvironment conducive to osteosarcoma growth. Knockdown of TSP1 showed a pronounced antitumor effect against osteosarcoma. Therefore, TSP1 could be a potential therapeutic target for the treatment of osteosarcoma.

ACKNOWLEDGMENTS

Thanks to Dr Zhengdong Cai for generously donating osteosarcoma cell line K7. This work was supported by the National Nature Science Foundation of China (No. 81802840; 82072957; 81702661), Shanghai Sailing Program (21YF1426100), Science and Technology Commission of Shanghai Municipality (20ZR1434000), and Climbing Talents Program of Shanghai Tenth People's Hospital (No. 04.01.20041).

DISCLOSURE

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

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How to cite this article: Liu Z, Wen J, Hu F, Wang J, Hu C, Zhang W. Thrombospondin-1 induced programmed death-ligand 1-mediated immunosuppression by activating the STAT3 pathway in osteosarcoma. *Cancer Sci*. 2022;113:432–445. doi:10.1111/cas.15237