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Thrombospondin-1 induces CD8$^+$ T cell exhaustion and immune suppression within the tumor microenvironment of ovarian cancer

Haiyan Liang[1] and Suwei Zhang[2*]

Abstract

Background Ovarian cancer (OC) progression is heavily influenced by the tumor microenvironment (TME), where immune suppression plays a critical role. This study explores the role of thrombospondin-1 (THBS1) in regulating tumor-associated macrophages (TAMs), T cell exhaustion, and immune checkpoint expression, as well as its transcriptional regulation by SNF2H.

Methods We analyzed THBS1 expression and its clinical significance using publicly available datasets (TCGA-OV, GSE14407) and tissue microarrays containing OC and adjacent normal tissues. In vitro functional studies were conducted using OC cell lines (SKOV3, A2780) and co-cultures with macrophages. Chromatin immunoprecipitation (ChIP) assays and RNA interference were employed to investigate SNF2H-mediated transcriptional regulation of THBS1. In vivo, the role of THBS1 in immune suppression was validated using mouse tumor models.

Results THBS1 was significantly overexpressed in OC tissues and associated with poor prognosis. High levels of THBS1 correlated with increased TAM infiltration, M2 macrophage polarization, and upregulation of immune checkpoints PD-L1 and GAL-3, which contribute to T cell exhaustion. Functional assays demonstrated that THBS1 promotes macrophage recruitment and induces M2 polarization through TGF-β1 and IL-4 signaling. Additionally, ChIP assays identified SNF2H as a transcriptional regulator of THBS1, contributing to its overexpression. In vitro targeting of THBS1 reduced TAM-mediated immune suppression and restored T cell cytotoxicity.

Conclusion This study positions THBS1 as a key regulator of the OC TME, linking TAM recruitment and polarization to CD8$^+$ T cell exhaustion via immune checkpoint modulation. By identifying SNF2H as a transcriptional regulator of THBS1, we offer new insights into its epigenetic dysregulation and suggest potential therapeutic strategies to reprogram the TME and improve the effectiveness of immunotherapy.

Keywords Ovarian cancer, Thrombospondin-1, Tumor microenvironment, Immune suppression, T cell exhaustion

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Background

Ovarian cancer (OC) is an aggressive malignancy and the leading cause of gynecological cancer-related deaths worldwide [1]. Its poor prognosis is primarily attributed to delayed diagnosis, high recurrence rates, and resistance to conventional therapies [2]. Recent research has underscored the critical role of the tumor microenvironment (TME) in driving tumor progression, metastasis, and immune evasion [3, 4]. The TME, a complex network of cancer cells, stromal cells, immune cells, and extracellular matrix components, creates a supportive niche for tumor growth and hinders effective immune responses [5, 6]. Among the immune cells within the TME, tumor-associated macrophages (TAMs) and T cells are crucial players in mediating cancer progression and immune suppression [7, 8].

TAMs, especially M2-polarized macrophages, are prevalent in the TME and exhibit pro-tumorigenic properties [9]. Unlike M1 macrophages, which promote inflammation and antitumor immunity, M2 macrophages contribute to immune evasion by secreting immunosuppressive cytokines, remodeling the extracellular matrix, and enhancing angiogenesis [10]. TAM infiltration correlates with advanced disease, metastasis, and unfavorable outcomes in OC [11]. Despite extensive research, the molecular mechanisms driving TAM recruitment and polarization in OC are not fully understood. Identifying factors that influence macrophage recruitment and polarization is crucial for developing therapeutic strategies targeting TAMs [12, 13].

T cell exhaustion, characterized by a progressive loss of effector functions and sustained expression of inhibitory receptors, is another key feature of immune suppression within the TME [14]. Exhausted T cells, often marked by high levels of PD-1, CTLA-4, and other immune checkpoints (ICPs), exhibit reduced proliferation and cytotoxicity [15]. ICPs, such as PD-L1 and GAL-3, are frequently upregulated in OC and contribute to T cell dysfunction by interacting with their receptors on T cells [16]. This immune evasion mechanism allows cancer cells to escape immune-mediated destruction and continue their progression [17]. The interplay between TAMs and T cell exhaustion exacerbates immunosuppression, creating a feedback loop that promotes tumor growth [18].

Thrombospondin-1 (THBS1), a multifunctional glycoprotein, has emerged as a critical regulator of the TME [19–21]. Known for its roles in cell-matrix interactions, angiogenesis, and immune modulation, THBS1 is frequently upregulated in several cancers, including OC [22–24]. Elevated THBS1 levels are associated with poor clinical outcomes, but the molecular mechanisms linking THBS1 to TAM recruitment, T cell exhaustion, and ICP regulation are poorly understood [25, 26]. Additionally, recent studies have suggested that transcriptional regulation of THBS1 by chromatin remodelers [27], such as SNF2H, may contribute to its dysregulation in cancer, yet this remains unexplored in OC.

This paper study aims to address these gaps by investigating THBS1's role in the OC TME. Specifically, we examined THBS1 expression and its relationship with clinical outcomes, TAM infiltration, and macrophage polarization. We further explored how THBS1 regulates ICPs, including PD-L1 and GAL-3, to mediate T cell exhaustion. Finally, we investigated the transcriptional control of THBS1 by SNF2H, providing insights into its dysregulation in OC. This work provides a comprehensive framework for understanding THBS1's role in the immunosuppressive TME and highlights its potential as a therapeutic target in OC.

Methods

Patient samples and tissue microarray analysis

Tissue microarrays containing tumor and adjacent normal tissues from 97 OC patients were commercially obtained, with ethical approval and informed consent secured by the supplier. Each tissue section was formalin-fixed and paraffin-embedded before being included in the microarray. Clinical data, including tumor stage, differentiation, and lymph node metastasis status, were collected from pathology reports and supplier annotations. For immunohistochemistry (IHC), tissue sections were dewaxed, rehydrated, and subjected to antigen retrieval in citrate buffer (pH 6.0) at 95 °C for 15 min. After blocking with 5% BSA, slides were incubated overnight at 4 °C with primary antibodies specific to THBS1, CD68, PD-L1, GAL-3, and SNF2H. Staining was visualized using HRP-conjugated secondary antibodies and DAB chromogen, and hematoxylin counterstaining was performed to highlight nuclei. Staining intensity was quantified using ImageJ software by two independent pathologists, who were blinded to the clinical data. Spearman and Pearson correlation coefficients were calculated to assess the relationships between protein expression and clinical parameters.

Bioinformatics analyses

Gene expression levels of THBS1 and SNF2H in OC and normal ovarian tissues were analyzed using publicly available datasets, including TCGA-OV, GTEx, and GSE14407. Raw expression data were downloaded from the GDC and GEO repositories. For TCGA-OV, normalized RNA-Seq data (FPKM values) were compared with GTEx normal ovarian tissue samples. In GSE14407, normalized microarray data were used to compare THBS1 expression between normal ovarian epithelial cells and OC cell lines. TIMER 2.0 was employed to evaluate the correlation between THBS1 expression and immune cell infiltration ($CD8^+$ T cells, dendritic cells,

and macrophages). Kaplan-Meier survival analysis was conducted to assess the association between THBS1 or SNF2H expression and overall survival, with statistical significance determined using the log-rank test.

RNA isolation and-quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted from ovarian epithelial cells (IOSE) and OC cell lines (SKOV3, A2780, and OVCAR3) using TRIzol reagent (Thermo Fisher Scientific). RNA purity and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using a reverse transcription kit (Takara Bio) with 1 μg of total RNA. qPCR was performed using SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 6 Real-Time PCR System. Primer sequences for THBS1, SNF2H, and GAPDH (internal control) are provided in the supplementary materials. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

IHC

Paraffin-embedded tissue sections from patient samples and mouse models were sectioned at 4 μm thickness. Sections were dewaxed in xylene, rehydrated through graded alcohols, and subjected to heat-induced antigen retrieval using a pressure cooker in citrate buffer (pH 6.0) for 20 min. After blocking endogenous peroxidase with 3% H2O2 and nonspecific binding with 5% goat serum, slides were probed overnight at 4 °C with primary antibodies (dilution ratios and sources listed in the supplementary materials). Subsequently, the slides were probed with an HRP-conjugated secondary antibody and DAB chromogen, followed by hematoxylin counterstaining. Images were captured using a Nikon light microscope, and staining intensities were quantified with ImageJ software. At least five high-power fields (400 ×) were analyzed per sample. The H-score (Histoscore) was used as a semi-quantitative measure of THBS1 expression levels, with the median value of the H-score (H-score = 170.97) serving as the grouping threshold. The H-score was calculated as the sum of (staining intensity × percentage of positive cells). Staining intensity was categorized as 0 (none), 1 (weak), 2 (moderate), and 3 (strong), while the percentage of positive cells was counted from 0 to 100%. The final score range was 0-300.

Cells and treatment

The human OC cell lines SKOV3 (#HTB-77, ATCC) and A2780 (#152706, Ximbio) were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C with 5% CO_2. The human monocyte macrophage cell line THP-1 (#TIB-202, ATCC) and $CD8^+$ cytotoxic T cells (#PCS-800-017) were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% P/S.

To investigate the role of THBS1 in OC cells, SKOV3 and A2780 cell lines with stable THBS1 overexpression were constructed. First, the human THBS1 gene sequence was obtained from the NCBI database, and the full-length THBS1 cDNA was synthesized by Genepharma and cloned into the pLVX-Puro lentiviral vector (THBS1), while an empty vector control group (Empty) was also constructed. Lentivirus was packaged in 293T cells using the second-generation lentiviral packaging system (psPAX2 and pMD2.G). After 48 h of viral harvest, the virus supernatant was filtered using a 0.45 μm membrane filter, and SKOV3 and A2780 cells were infected at a multiplicity of infection of 10. Polybrene (8 μg/mL) was added during infection to improve the infection efficiency. After 24 h of infection, fresh culture medium was replaced, and 2 μg/mL puromycin was used to select stable clones 48 h post-infection. Selection was continued for 7 days. After selection, qPCR and Western blot (WB) analyses were performed to detect THBS1 mRNA and protein levels to ensure high expression of THBS1 in the stable clones. The successfully constructed stable cell lines were used in subsequent experiments, including co-culture, chemotaxis assays, and immune regulation functional studies.

In the study of THBS1's effect on macrophage polarization, THBS1 overexpressing or control cells were co-cultured with phorbol-12-myristate- 13-acetate (PMA)-induced THP-1 macrophages at a 1:1 ratio for 48 h. The macrophages were then collected for flow cytometry analysis of $CD86^+$ M1 and $CD206^+$ M2 phenotypes. In the chemotaxis assay, the culture medium from THBS1 overexpressing or control cells was used in the lower chamber of a Transwell chamber to attract PMA-treated THP-1 cells to migrate to the lower chamber. After incubation at 37 °C for 24 h, the migrated cells were fixed, stained, and counted to assess the impact of THBS1 on macrophage migration.

In the T-cell function assay, $CD8^+$ T cells were co-cultured with OC cells stably overexpressing THBS1. The secretion of $CD8^+$ T-cell activation markers and cytotoxic factors was measured. Additionally, a co-culture lysis assay was performed to detect the cytotoxicity of CD8 + T cells against cancer cells.

Immunofluorescence staining

For immunofluorescence analysis, cells or tissue sections were fixed for 15 min with 4% paraformaldehyde, washed with PBS, and permeabilized for 10 min with 0.1% Triton X-100. After blocking with 1% BSA, samples were mixed overnight at 4 °C with primary antibodies against CD206, CD8, CTLA4, or VISTA. Alexa Fluor 488- or 594-conjugated secondary antibodies were applied for 1 h at

ambient temperature. Nuclei were counterstained with DAPI, and slides were mounted with antifade reagent (Thermo Fisher Scientific). Images were acquired using a Leica SP8 confocal microscope, and fluorescence intensity was quantified.

Flow cytometry

For macrophage polarization and T cell exhaustion analysis, cells were rinsed with PBS, resuspended in FACS buffer (PBS with 2% FBS), and stained with fluorescently conjugated antibodies against CD69, TCF7, PFN, VISTA, CD206, PD-1, and CTLA4. After incubation for 30 min at 4 °C, cells were analyzed on a BD LSRFortessa flow cytometer. Data were processed and analyzed using FlowJo software (BD Biosciences). For macrophage polarization, $F4/80^+CD86^+$ (M1) or $F4/80^+CD206^+$ (M2) populations were quantified. T cell activation and exhaustion markers were assessed in $CD8^+$ populations.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in culture supernatants were measured with ELISA kits for TGF-β1 (#DB100C), CCL1 (#DY272), IL-4 (#D4050), TNF-α (#DTA00D), IL-1β (#DY201), IL-6 (#D6050B), PFN (#QK8011), GZMB (#DGZB00), IFN-γ (#QK285), and IL-2 (#QK202) (R&D Systems). Supernatants were harvested after 48 h of culture, centrifuged at 1,500 rpm for 10 min to remove debris, and stored at − 80 °C until analysis. Absorbance was read at 450 nm on a BioTek microplate reader. Cytokine concentrations were normalized to cell counts.

Chemotaxis assay

Macrophage chemotaxis was assessed using 24-well Transwell chambers with 8 μm pore inserts (Corning). OC cells (SKOV3 or A2780) with or without THBS1 overexpression were seeded in the lower chamber. PMA-treated THP-1 cells were paved in the upper chamber. After 24 h, macrophages migrating to the lower chamber were fixed in methanol, dyed with 0.1% crystal violet, and imaged under a microscope. Migrated cells were quantified by counting five random fields per membrane.

Animal experiments

ID8 mouse OC cells were injected subcutaneously into the flanks of female C57BL/6 mice (6–8 weeks old). When tumors reached ~ 5 mm in diameter, the mouse tumor organoids (MTOs) were excised and transplanted into wild-type (WT) or THBS1 knockout (KO) mice. Tumor size was gauged every 3 days using calipers, and volume was computed as (length × width^2) / 2. Mice were euthanized when tumor size reached ethical limits. Tumor tissues were utilized for IHC and immunofluorescence analysis of immune infiltration, angiogenesis markers (VEGFA), and immune evasion markers (PD-L1, GAL-3). Animal protocols were approved by the Institutional Animal Care and Use Committee.

Chromatin Immunoprecipitation (ChIP)-qPCR assays

A ChIP assay was conducted to assess SNF2H binding to the THBS1 promoter. SKOV3 and A2780 cells were crosslinked for 10 min with 1% formaldehyde and quenched with 125 mM glycine. Subsequently, cells were lysed, and chromatin was sheared into 200–500 bp fragments using a Bioruptor sonicator (Diagenode). Immunoprecipitation was performed using an anti-SNF2H antibody or IgG control, with Dynabeads Protein A/G (Invitrogen). After reverse crosslinking, DNA was purified and analyzed by qPCR using primers targeting the THBS1 promoter. The data were normalized to input DNA.

Recombinant protein and T cell analysis

$CD3^+$ T cells were isolated from splenocytes of C57BL/6 mice utilizing magnetic beads (Miltenyi Biotec) and stimulated with anti-CD3/CD28 antibodies (BioLegend) in RPMI-1640 medium supplemented with 10% FBS. Recombinant THBS1 protein (R&D Systems) was added to the culture at varying concentrations (0–100 ng/mL). After 72 h, cells were harvested for flow cytometry analysis of activation (CD69, TCF7, PFN) and exhaustion (PD-1, CTLA4) markers. Cytokines in the supernatant were quantified by ELISA.

Statistical analysis

Data analysis was made in GraphPad Prism v8.0 (GraphPad, CA, USA). Data are presented as violin plots, with each point representing an experimental replicate or patient sample. Statistical significance was determined using Student's *t*-test, or by the one or two-way analysis of variance (ANOVA), as appropriate. Tukey's multiple comparison test was performed after ANOVA.

Results

THBS1 is upregulated in OC and correlates with poor survival

We first identified a significant upregulation of THBS1 in OC patients from the TCGA-OV database (Fig. 1A). Consistent findings were observed in the GSE14407 dataset, where THBS1 expression was notably elevated in OC cell lines (Fig. 1B). Further survival analysis revealed a strong link between high THBS1 expression and unfavorable prognoses, with shorter survival observed in patients with elevated THBS1 levels (Fig. 1C). To validate these findings, we analyzed tumor and adjacent normal tissue microarrays from 97 OC patients and observed markedly stronger THBS1 staining in tumor tissues (Fig. 1D). Elevated THBS1 levels positively correlated with advanced tumor stage, poor differentiation, and lymph node

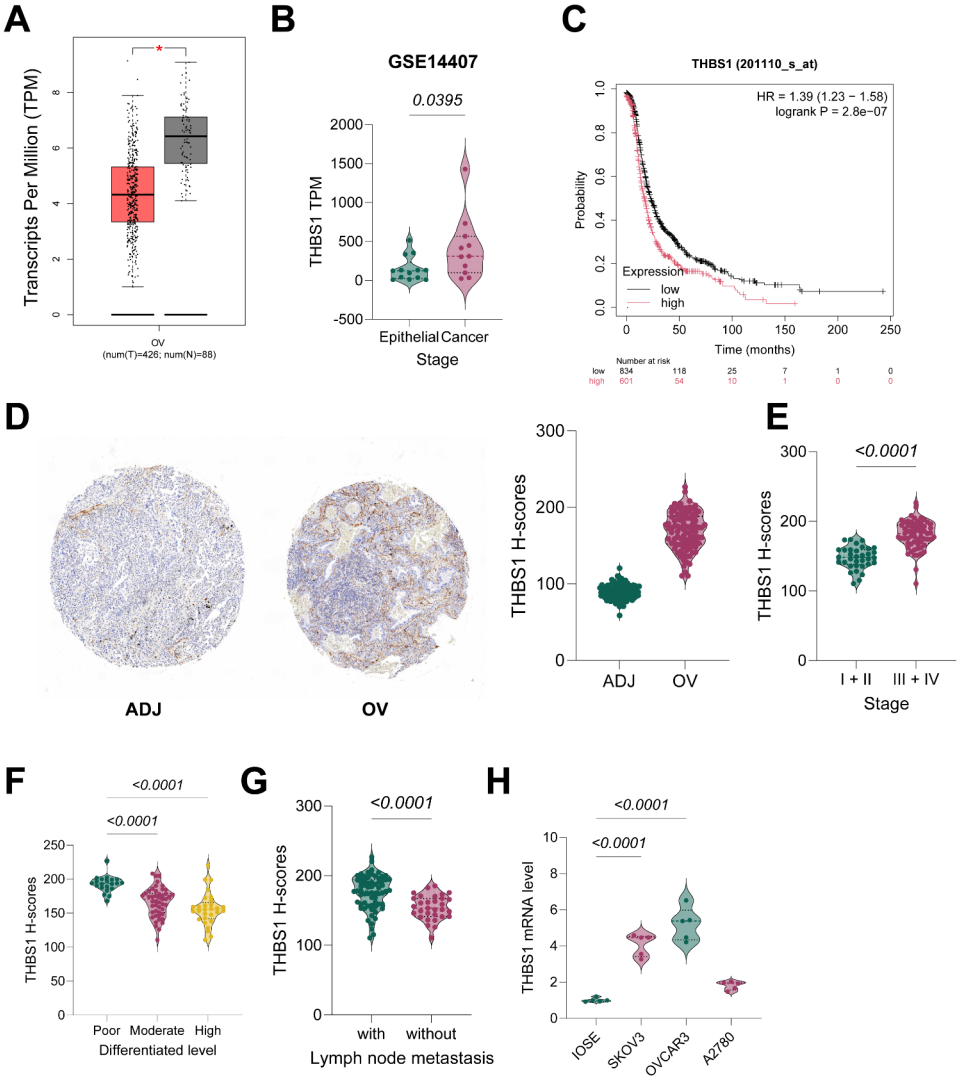

Fig. 1 THBS1 is significantly overexpressed in OC and correlates with poor prognosis. **(A)** Comparison of THBS1 expression levels between OC tissues (TCGA-OV dataset) and normal ovarian tissues (GTEx dataset). **(B)** THBS1 expression levels in normal ovarian epithelial cells versus OC cell lines (GSE14407 dataset). **(C)** Association between THBS1 expression levels and poor prognosis in OC patients (TCGA-OV dataset). **(D)** IHC analysis of THBS1 staining intensity in tumor and adjacent normal tissues from 97 OC patients. (**E**–**G**) Correlation of THBS1 staining intensity with clinical stage, tumor differentiation, and lymph node metastasis in 97 OC patients. **(H)** qPCR analysis of THBS1 mRNA expression levels in the ovarian epithelial cell line IOSE and OC cell lines SKOV3, A2780, and OVCAR3. Data are presented as violin plots, with each point representing an experimental replicate or patient sample. Statistical significance was determined using Student's t-test or one-way ANOVA, followed by Tukey's multiple comparison test ($P < 0.05$)

metastasis (Fig. 1E–G). Additionally, OC cell lines exhibited significantly higher THBS1 expression than normal ovarian epithelial cells (Fig. 1H–I).

High THBS1 expression positively correlates with TAM infiltration

Building on the observed overexpression of THBS1 in OC, we investigated its association with immune cell infiltration. Correlation analysis revealed that THBS1 expression was negatively related to CD8 + T cell infiltration but positively correlated with dendritic cell (DC) and macrophage infiltration (Fig. 2A). IHC analysis of tumor microarrays showed increased CD68 (a macrophage marker) staining intensity in tumor tissues, which correlated positively with THBS1 expression (Fig. 2B–C). Based on the median H-score of THBS1 (170.97) detected in tumor tissues, the samples were allocated with High THBS1 (H-score > 170.97) and low THBS1 (H-score ≤ 170.97) groups. Since TAMs contribute to immune evasion, we analyzed immune evasion markers PD-L1 and GAL-3. Both markers showed significantly stronger staining in patients with high THBS1 expression (Fig. 2D–E), and their levels were positively correlated with THBS1, CD68, and other immune evasion markers in the TCGA-OV dataset (Fig. 2F).

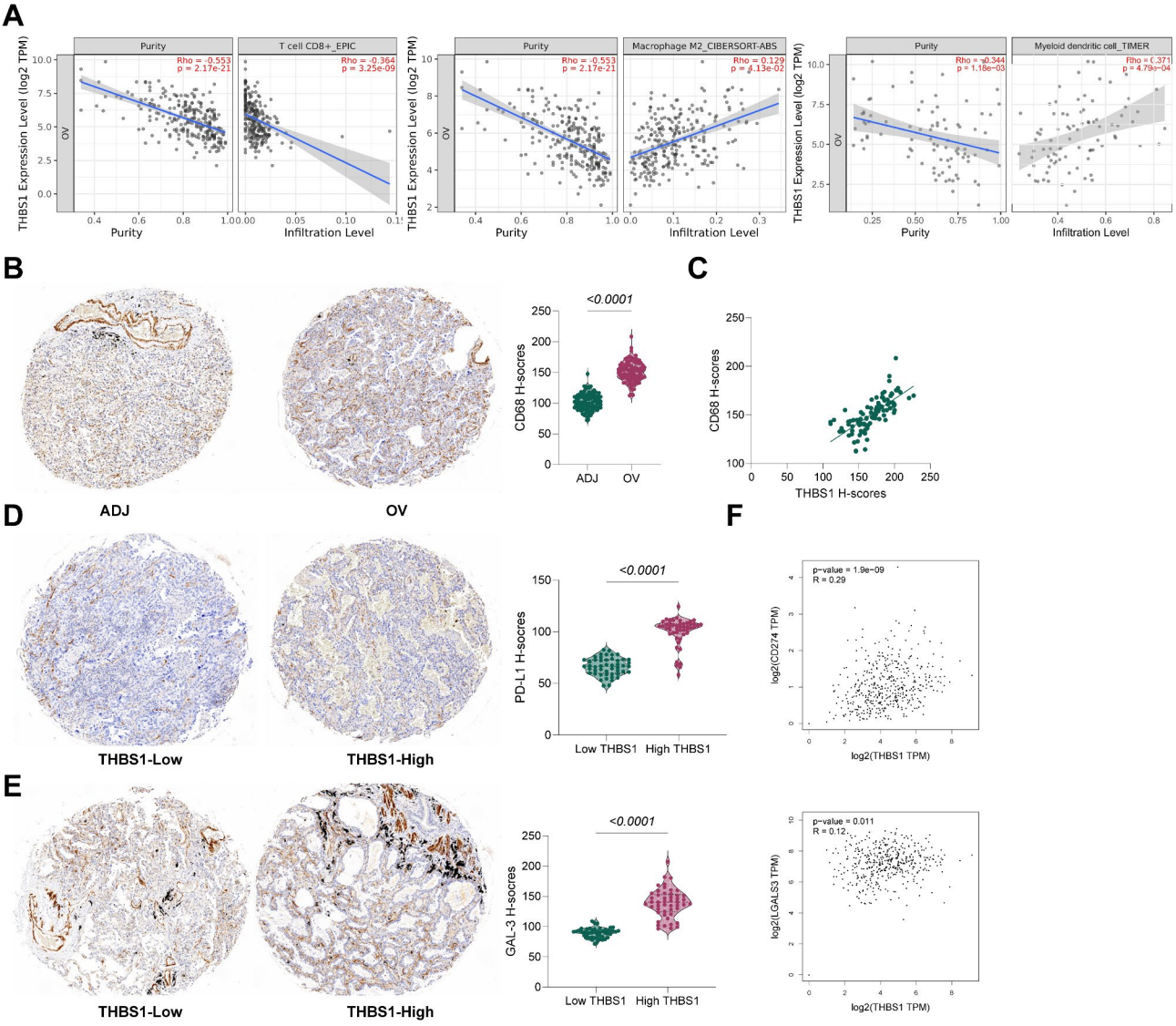

Fig. 2 High THBS1 expression positively correlates with tumor-associated macrophage infiltration. **(A)** Correlation between THBS1 expression and infiltration of CD8 + T cells, dendritic cells (DCs), and macrophages, analyzed using TIMER 2.0. **(B)** IHC analysis of CD68 (a macrophage marker) staining intensity in tumor and adjacent normal tissues from 97 OC patients. **(C)** Spearman correlation analysis between THBS1 and CD68 staining intensities. (**D**–**E**) IHC analysis of PD-L1 and GAL-3 staining intensities in tumor tissues from 97 OC patients. **(F)** Pearson correlation analysis of THBS1 with PD-L1, GAL-3, and CD68 expression levels in the TCGA-OV dataset. Data are presented as violin plots, with each point representing an experimental replicate or patient sample. Statistical significance was determined using Student's t-test or one-way ANOVA, followed by Tukey's multiple comparison test ($P < 0.05$)

THBS1 overexpression increases M2 polarization and chemotaxis of macrophage

To investigate THBS1's role in macrophage polarization, we overexpressed THBS1 in SKOV3 and A2780 cells and co-cultured them with PMA-treated THP-1 cells (Fig. 3A–B). Flow cytometry analysis after 48 h of co-culture revealed considerably enhanced F4/80 + CD206 + M2 macrophages (Fig. 3C). Immunofluorescence confirmed that THBS1-overexpressing OC cells strongly promote M2 polarization (Fig. 3D). Cytokine profiling of culture supernatants showed elevated levels of M2-associated cytokines (TGF-β1, CCL1, and IL-4) and decreased levels of M1-associated cytokines (TNF-α, IL-1β, and IL-6) (Fig. 3E). Chemotaxis assays demonstrated that THBS1 overexpression significantly enhanced macrophage migration (Fig. 3F). Additionally, co-cultured OC cells exhibited increased expression of immune evasion markers PD-L1 and GAL-3, with more pronounced effects in THBS1-overexpressing cells (Fig. 3G).

THBS1 suppresses immunosurveillance in an MTO model

To assess the in vivo effects of THBS1 on OC growth, we transplanted mouse ID8 cell-derived MTOs into WT or THBS1-KO mice (Fig. 4A). THBS1 knockout in

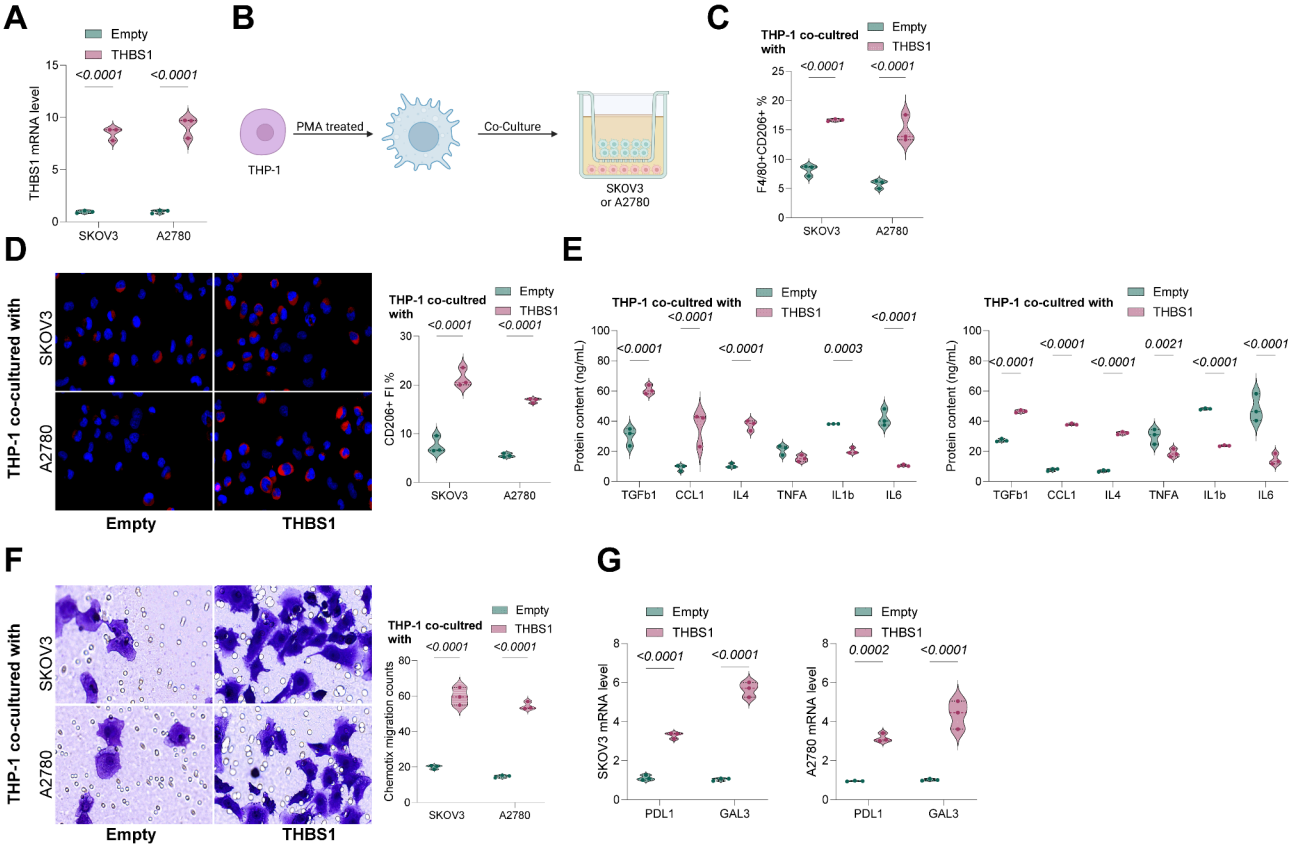

Fig. 3 High THBS1 expression enhances the chemotactic recruitment of M2 macrophages. **(A)** qPCR analysis of THBS1 mRNA expression after overexpression in SKOV3 and A2780 cells. **(B)** Schematic representation of co-culture experiments between OC cells and PMA-treated THP-1 macrophages. **(C)** Flow cytometry analysis of F4/80 + CD206 + macrophage populations after co-culture. **(D)** Immunofluorescence analysis of CD206 intensity in macrophages post-co-culture. **(E)** ELISA quantification of M2 cytokines (TGF-β1, CCL1, IL-4) and M1 cytokines (TNF-α, IL-1β, IL-6) in culture supernatants. **(F)** Crystal violet staining to assess chemotactic recruitment of macrophages by OC cells. **(G)** qPCR analysis of PD-L1 and GAL-3 mRNA levels in SKOV3 and A2780 cells post-co-culture. Data are presented as violin plots, with each point representing an experimental replicate. Statistical significance was determined using two-way ANOVA, followed by Tukey's multiple comparison test ($P < 0.05$)

mice led to a significant decrease in the positive staining of the proliferation marker Ki67 while an increase in staining of the apoptosis marker C-Cas-3 (Fig. 4B), indicating an inhibition in tumor growth. Consistent with prior reports of THBS1's anti-angiogenic effects, VEGFA staining intensity was significantly reduced in tumors from THBS1 KO mice (Fig. 4C). Furthermore, THBS1 KO tumors exhibited decreased $CD206^+$ M2 macrophage infiltration and increased $CD8^+$ T cell numbers without significant changes in $CD4^+$ T cell levels (Fig. 4D–F). Immunofluorescence staining also revealed reduced PD-L1 and GAL-3 expression in THBS1 KO tumors (Fig. 4G–H). These findings suggest that THBS1 modulates the tumor microenvironment to facilitate OC growth.

THBS1 knockdown reduces $CD8^+$ T cell exhaustion

Given the enhanced $CD8^+$ T cell infiltration and lowered PD-L1 and GAL-3 levels in THBS1 KO tumors, we hypothesized that THBS1 inhibition restores CD8 + T cell activity. We observed reduced levels of the exhaustion marker CTLA4 and elevated levels of the active killing marker VISTA in CD8 + T cells from THBS1 KO tumors (Fig. 5A). In vitro, recombinant THBS1 protein blocked CD8 + T cell activation in a dose-dependent fashion, reducing the proportion of CD69+, TCF7+, PFN+, and VISTA + cells while increasing PD-1 + and CTLA4 + exhausted cells (Fig. 5B–C). Furthermore, THBS1 suppressed the secretion of cytotoxic mediators (PFN, GZMB, IFN-γ, and IL-2) (Fig. 5D) and impaired the cytolytic activity of CD8 + T cells against ID8 cells in co-culture (Fig. 5E).

THBS1 expression is transcriptionally regulated by SNF2H

Using the JASPAR database, we identified a conserved SNF2H-binding motif near the THBS1 transcription start site (TSS) (Fig. 6A). Analysis of TCGA-OV data revealed that SNF2H is notably overexpressed in OC and correlated with dismal survival (Fig. 6B–C). IHC analysis confirmed elevated SNF2H levels in tumor tissues, which

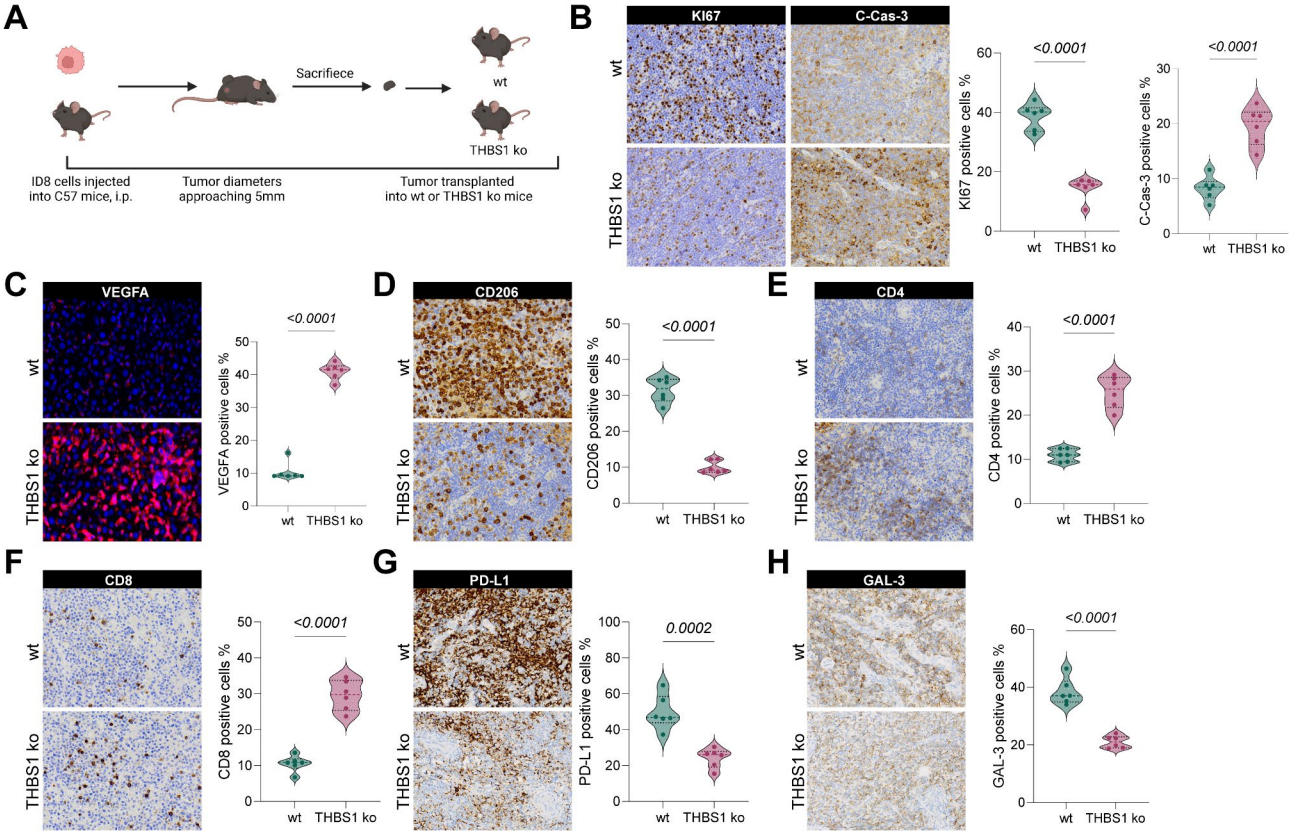

Fig. 4 THBS1 suppresses inflammation and immunosurveillance in an OC MTO mouse model. **(A)** Workflow illustrating the transplantation of ID8 OC cells into wild-type (WT) and THBS1 knockout (KO) mice. **(B)** IHC analysis of Ki67 and cleaved caspase-3 (C-Cas-3) staining intensities in tumor tissues. **(C)** Immunofluorescence analysis of VEGFA expression in tumor tissues. **(D)** IHC analysis of CD206 staining intensity in tumor tissues. (**E**–**F**) IHC analysis of CD4 + and CD8 + T cell infiltration in tumor tissues. (**G**–**H**) IHC analysis of PD-L1 and GAL-3 staining intensities in tumor tissues. Data are presented as violin plots, with each point representing a single mouse. Statistical significance was determined using two-way ANOVA, followed by Tukey's multiple comparison test ($P < 0.05$)

positively correlated with THBS1 expression (Fig. 6D–E). Similar correlations were noted in the TCGA-OV database (Fig. 6F). Knockdown of SNF2H in SKOV3 and A2780 cells significantly reduced THBS1 expression (Fig. 6G). ChIP assays demonstrated that SNF2H directly binds to the THBS1 promoter (Fig. 6H), confirming SNF2H as a transcriptional regulator of THBS1.

Discussion

This paper investigates the role of THBS1 in the OC TME, with a particular focus on its effects on TAMs, $CD8^+$ T cell exhaustion, and ICP regulation. Our findings reveal that THBS1 is significantly overexpressed in OC and functions as a key driver of immunosuppression by promoting TAM recruitment, inducing M2 macrophage polarization, and upregulating ICPs such as PD-L1 and GAL-3. Additionally, we identified SNF2H as a transcriptional regulator of THBS1, offering new insights into its epigenetic regulation.

T cell exhaustion is a state of T cell dysfunction induced by chronic antigen stimulation in the TME, characterized by the upregulation of inhibitory ICPs such as PD-1 and CTLA-4, along with a loss of cytotoxic effector functions [28, 29]. ICPs like PD-L1 and GAL-3 play a crucial role in maintaining this dysfunctional state by binding to inhibitory receptors on T cells, thereby preventing their activation [30, 31]. Our findings show that THBS1 upregulates PD-L1 and GAL-3, establishing a direct link between THBS1 overexpression and T cell exhaustion in OC. This connection suggests that THBS1's immune-modulatory functions go beyond TAM polarization and contribute significantly to $CD8^+$ T cell dysfunction.

Studies in other cancers have suggested a role for THBS1 in immune suppression [32, 33], but its connection to T cell exhaustion remains underexplored. For example, in glioblastoma [34], THBS1 promotes an immunosuppressive TME by recruiting macrophages and facilitating their M2 polarization. Similarly, in pancreatic and breast cancers, THBS1 has been implicated in TAM-mediated immune suppression and extracellular matrix remodeling, though its direct effects on T cell function were not investigated [25, 35]. Our research expands on

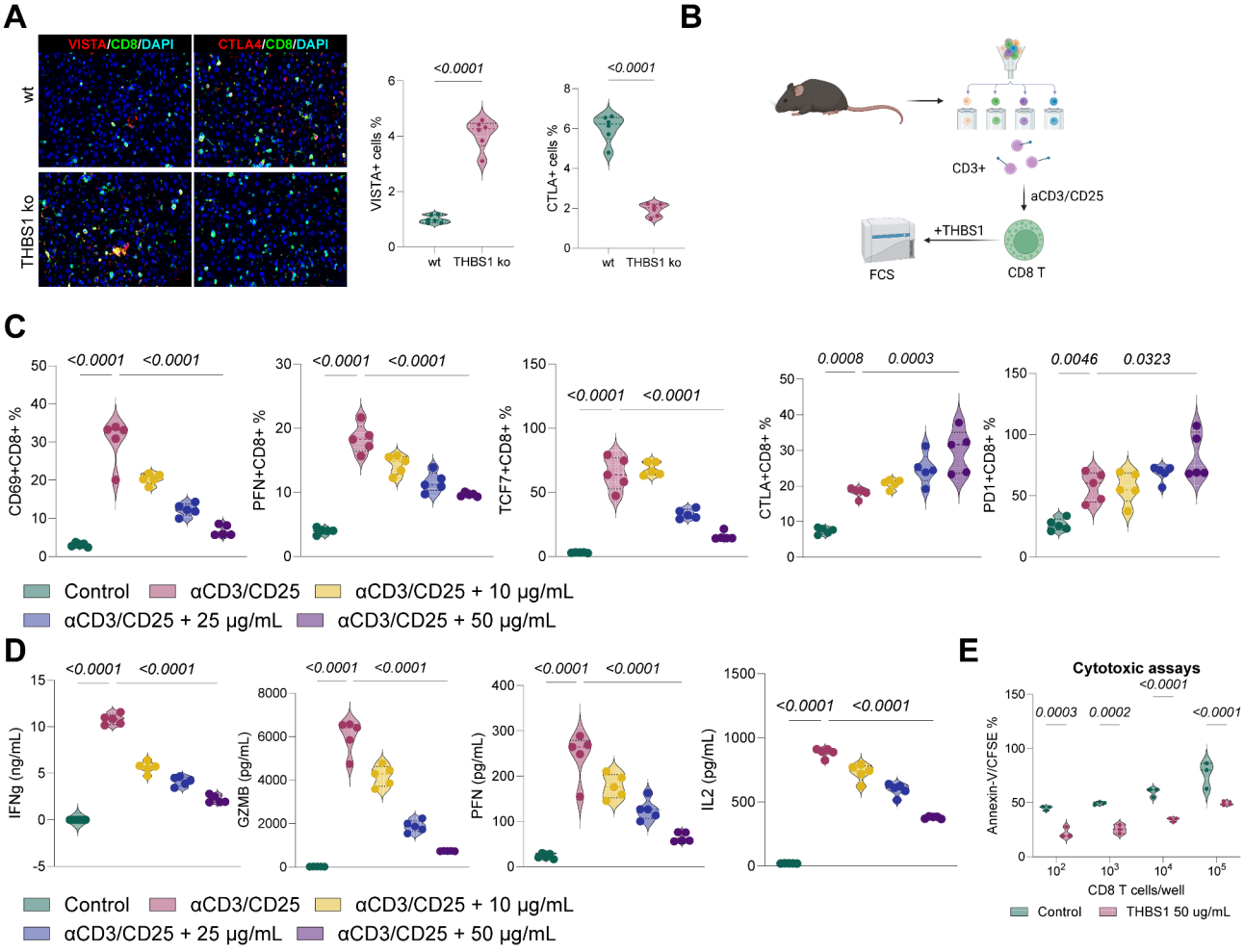

Fig. 5 THBS1 knockdown (KD) alleviates T cell exhaustion. **(A)** Immunofluorescence analysis of CD8+/CTLA4 + and CD8+/VISTA + T cell populations in tumor tissues. **(B)** CD3 + T cells isolated from C57 mice were stimulated with anti-CD3/CD28 to induce polarization into CD8 + T cells, with or without recombinant THBS1 protein. **(C)** Flow cytometry analysis of CD69+, TCF7+, PFN+, VISTA+, PD-1+, and CTLA4 + CD8 + T cells. **(D)** ELISA quantification of PFN, GZMB, IFN-γ, and IL-2 levels in culture supernatants. **(E)** Annexin-V/CFSE staining to evaluate the cytotoxic effect of T cells on ID8 OC cells. Data are presented as violin plots, with each point representing a single mouse. Statistical significance was determined using two-way ANOVA, followed by Tukey's multiple comparison test ($P < 0.05$)

these findings by identifying THBS1 as a key upstream regulator of ICPs, linking TAM-driven immunosuppression with T cell exhaustion. This dual role underscores the multifaceted nature of THBS1 in promoting immune evasion in OC.

THBS1's role in linking macrophage behavior and T cell exhaustion likely involves a feedback loop within the TME [36]. TAMs are known to secrete cytokines such as IL-10 and TGF-β1, which reinforce their M2 phenotype and promote ICP expression on both tumor and immune cells [37, 38]. THBS1 appears to amplify this process by inducing M2 polarization, marked by increased levels of TGF-β1, CCL1, and IL-4, while decreasing levels of TNF-α, IL-1β, and IL-6. This polarization results in the increased expression of PD-L1 and GAL-3 in the co-cultured OC cells. Recently, bispecific antibodies targeting TGF-β/PD-L1 like YM101 and BiTP have been developed [39, 40], providing new options for solid cancer treatment, including OC. GAL-3 is unique in inducing T cell apoptosis and impairing $CD8^+$ T cell functionality by binding to glycosylated receptors on their surface [41]. The upregulation of GAL-3 by THBS1 adds another layer of complexity to its role in T cell exhaustion, providing a mechanistic link between its immune-modulatory functions and T cell dysfunction.

Our study also highlights the transcriptional regulation of THBS1 by SNF2H, a chromatin remodeler of the ISWI family [42]. While SNF2H has been previously implicated in regulating oncogenes and maintaining chromatin structure in cancers such as gastric and lung cancer [43], its role in regulating immune-related genes has not been fully explored. We demonstrate that SNF2H directly binds to the THBS1 promoter, driving its overexpression in OC. This regulatory axis likely contributes

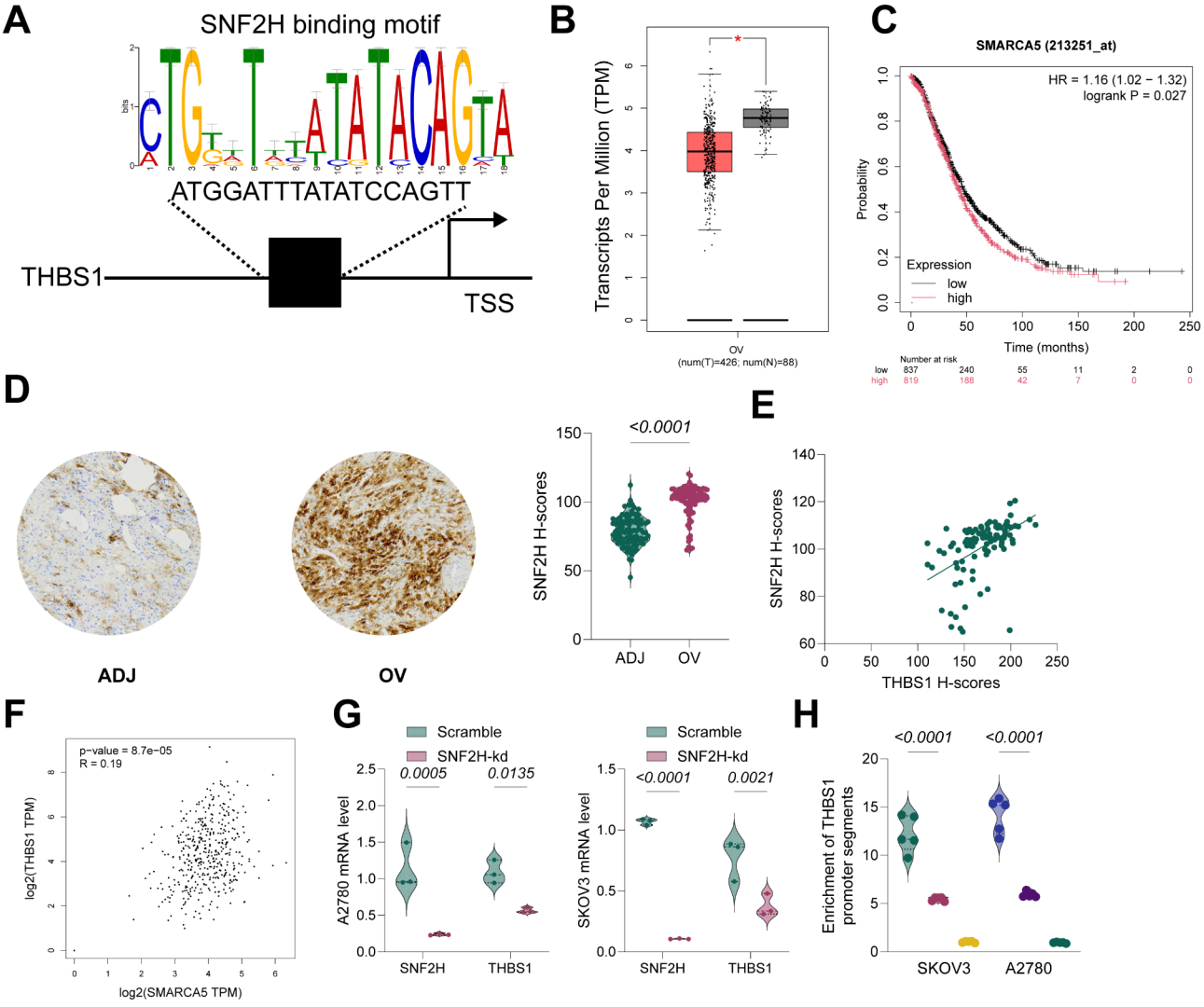

Fig. 6 THBS1 expression is transcriptionally regulated by SNF2H. **(A)** Prediction of an SNF2H-binding motif near the THBS1 transcription start site using the JASPAR database. **(B)** Comparison of SNF2H expression levels between OC tissues (TCGA-OV dataset) and normal ovarian tissues (GTEx dataset). **(C)** Association of SNF2H expression with poor prognosis in OC patients (TCGA-OV dataset). **(D)** IHC analysis of SNF2H staining intensity in tumor and adjacent normal tissues from 97 OC patients. **(E)** Spearman correlation analysis of THBS1 and SNF2H staining intensities. **(F)** Pearson correlation analysis of THBS1 and SNF2H expression levels in the TCGA-OV dataset. **(G)** qPCR analysis of SNF2H and THBS1 mRNA expression after SNF2H knockdown in SKOV3 and A2780 cells. **(H)** ChIP analysis with anti-SNF2H showing enrichment of THBS1 promoter fragments. Data are presented as violin plots, with each point representing an experimental replicate or patient sample. Statistical significance was determined using Student's t-test or one-way ANOVA, followed by Tukey's multiple comparison test ($P < 0.05$)

to the establishment of an immunosuppressive TME, as elevated THBS1 levels promote TAM recruitment, macrophage polarization, and modulation of ICPs. These findings suggest that targeting SNF2H could indirectly alleviate THBS1-mediated T cell exhaustion and help restore immune function in OC.

In comparison, while other studies have highlighted THBS1's role in TAM-mediated immune suppression, its direct contribution to T cell exhaustion through ICP modulation is relatively novel. For instance, previous research in colorectal cancer identified THBS1 as a promoter of TGF-β1 signaling, which indirectly contributes to T cell dysfunction [36, 44]. Our findings advance this understanding by establishing a direct link between THBS1 overexpression and the upregulation of PD-L1 and GAL-3, providing a clearer mechanism through which THBS1 influences T cell exhaustion in OC. This underscores the importance of targeting THBS1, not only to disrupt TAM-driven immunosuppression but also to alleviate T cell dysfunction and reinvigorate antitumor immunity.

Conclusions

In conclusion, this paper establishes THBS1 as a pivotal regulator of immune evasion in OC, mediating TAM recruitment, M2 polarization, and T cell exhaustion through ICP modulation. By identifying SNF2H as a key transcriptional regulator of THBS1, we provide novel insights into its dysregulation and uncover potential therapeutic opportunities for targeting the THBS1-driven immunosuppressive axis. These findings enhance our understanding of the complex interplay between TAMs, ICPs, and T cells in the TME, offering new strategies to improve the efficacy of cancer immunotherapy.

Supplementary Information

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Supplementary Material 1

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Author contributions
Haiyan Liang conceived and designed the study, supervised the research, and contributed to data interpretation and manuscript preparation. Suwei Zhang performed experiments, conducted data analysis, and drafted the manuscript. Both authors reviewed and approved the final manuscript.

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Data availability
The corresponding author can provide the date used to support the results of this study upon reasonable request.

Declarations

Ethics approval
The Helsinki Declaration's ethical criteria were followed in the conduct of this investigation. The research protocol was approved by Ethics Committee of the First Affiliated Hospital of Shantou University Medical College And informed consent was obtained from all patients. All animal experiments were ratified by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Shantou University Medical College.

Consent to participate
Not applicable.

Consent for publication
All listed authors have reviewed and approved the manuscript for publication.

Conflict of interest
The authors claim to have no conflicting interests.

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