

Expression of V-domain Ig suppressor of T cell activation in ovarian cancer and its regulation of follicular regulatory T-cell function via the AKT/MAPK pathway

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Received April 7, 2026; Accepted August 12, 2026

DOI: 10.3892/or.2026.9206

Abstract. The high mortality associated with ovarian cancer is closely linked to immune remodeling within the tumor microenvironment (TME). V-domain Ig suppressor of T cell activation (VISTA), a novel immune checkpoint, has attracted increasing attention in tumor immunology; however, its effects on follicular regulatory T (Tfr) cell function remain unclear. The present study examined VISTA expression in ovarian cancer and explored the correlation between VISTA and alterations in the AKT/MAPK pathway in Tfr-like cells. Ovarian cancer tissues from different clinical stages and non-malignant control tissues were analyzed by immunofluorescence, reverse transcription-quantitative PCR and western blotting to assess VISTA expression and its association with FOXP3⁺CXCR5⁺ Tfr-like cell infiltration. Naive CD4⁺ T cells were differentiated into Tfr-like cells *in vitro*, and VISTA overexpression and silencing models were used to evaluate cell

proliferation, cytokine secretion, and AKT/MAPK pathway activation. VISTA expression increased with ovarian cancer progression and was positively associated with Tfr-like cell infiltration. *In vitro*, VISTA promoted Tfr-like cell proliferation and the secretion of the anti-inflammatory cytokines interleukin-10 and transforming growth factor beta 1 while suppressing the immune effector molecules interleukin-21 and granzyme B, accompanied by reduced AKT and extracellular signal-regulated kinase 1/2 phosphorylation. Collectively, VISTA is upregulated in ovarian cancer and changes in VISTA expression are associated with enhanced immunosuppressive function of Tfr-like cells as well as altered AKT/MAPK phosphorylation levels. These findings provided new insights into the regulation of the ovarian cancer TME and support further investigation of VISTA-targeted immunotherapy.

Introduction

Tumor immune escape is a major contributor to treatment failure and disease progression in ovarian cancer (1). Aberrant immune checkpoint expression and infiltration by immunosuppressive cells are central to this process (2). Immune checkpoint molecules negatively regulate T cell activation, proliferation, and cytokine secretion, thereby dampening antitumor immune responses (3-5). To date, immune checkpoint inhibitors targeting programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated protein 4 have demonstrated substantial clinical activity in multiple solid tumors. However, the objective response rate in ovarian cancer remains 10-15%, suggesting that additional, incompletely characterized immunoregulatory mechanisms operate in the tumor microenvironment (TME) (6,7). V-domain Ig suppressor of T cell activation (VISTA), a novel B7 family immune checkpoint molecule, has emerged as an immune checkpoint of increasing interest (8). VISTA directly inhibits T cell activation and proliferation by engaging receptors on T cells and also modulates the function of antigen-presenting cells such as dendritic cells and macrophages, thereby broadly contributing to immune tolerance and tumor immune escape (9-11). Previous studies have confirmed that VISTA is highly expressed in various solid

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Abbreviations: AKT, protein kinase B; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase 1/2; FIGO, International Federation of Gynecology and Obstetrics; FOXP3, forkhead box P3; IL, interleukin; MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; qPCR, quantitative PCR; Tfr, follicular regulatory T cell; TGF- β 1, transforming growth factor beta 1; Treg, regulatory T cell; VISTA, V-domain Ig suppressor of T cell activation

Key words: V-domain Ig suppressor of T cell activation, follicular regulatory T cells, ovarian cancer, AKT/MAPK signaling pathway, tumor microenvironment, immunotherapy, immune checkpoint

tumors, including lung cancer, melanoma and gastric cancer and its expression level is closely associated with tumor stage, lymph node metastasis and patient prognosis; most studies indicate that high VISTA expression predicts poorer clinical outcomes (12,13). In ovarian cancer, accumulating evidence confirms VISTA expression in tumor tissues, although its prognostic significance remains inconsistent. Some studies associate high VISTA expression with unfavorable outcomes in patients with ovarian cancer (14-16), while others report that high VISTA expression is associated with improved survival in high-grade serous ovarian carcinoma (17). This discrepancy may partly reflect the predominantly bulk-tissue assessment of VISTA in previous studies, which has not resolved its distribution across specific cell populations or the cellular mechanisms through which it remodels the ovarian cancer immune microenvironment. Whether VISTA exerts immunosuppressive effects through specific immune cell subsets in ovarian cancer, and how its expression changes across disease stages, remain poorly characterized.

Among immunosuppressive cells in the TME, follicular regulatory T (Tfr) cells are increasingly recognized for their distinct immunoregulatory functions. As a specialized subset of regulatory T cells (Tregs), Tfr-like cells co-express the transcription factor forkhead box P3 (FOXP3), which mediates immunosuppressive function, and the chemokine receptor CXCR5, which drives homing to lymphoid follicles; these features enable their localization to secondary lymphoid follicles and tumor-associated lymphoid structures. Under physiological conditions, Tfr-like cells maintain humoral immune homeostasis by restraining excessive activation of follicular helper T cells, thereby regulating B-cell proliferation, antibody secretion, and germinal-center reactions (18-20). In the TME, however, Tfr-like cell function may become dysregulated. Increased Tfr-like cell infiltration has been reported in breast cancer, colorectal cancer, and other malignancies, in which these cells may reinforce immunosuppression through secretion of anti-inflammatory cytokines such as IL-10 and TGF- β 1 and thereby promote tumor progression (21-23). However, the infiltration patterns, functional properties, and regulatory mechanisms of Tfr-like cells in ovarian cancer remain poorly defined. The regulatory interaction between VISTA and Tfr-like cells in ovarian cancer, as well as the clinical significance of VISTA-positive Tfr-like cells across ovarian cancer stages, therefore remains to be established (24,25).

Aberrant activation of intracellular signaling pathways is a common molecular feature of dysfunction in both tumor and immune cells (26). The protein kinase B (AKT) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways, which coordinate cell proliferation, differentiation, and immune function, are important regulators of immune-cell activity (27,28). Previous studies suggest that AKT phosphorylation can impair the immunosuppressive function of Treg cells, whereas ERK1/2 inhibition may enhance Treg cell stability by increasing FOXP3 expression (29,30). AKT phosphorylation has been reported to correlate negatively with the abundance of CXCR5⁺FOXP3⁺ cells, a characteristic phenotype of Tfr-like cells, suggesting that the AKT/MAPK pathway is involved in the functional regulation of Tfr-like cells. However, whether VISTA modulates Tfr-like cell

proliferation, cytokine secretion and immunosuppressive function via the AKT/MAPK pathway and thereby contributes to immune escape in ovarian cancer, has not been experimentally established.

To address these questions, the present study characterized the VISTA-Tfr-like cell axis in ovarian cancer, focusing on VISTA expression in Tfr-like cells, its stage-associated accumulation, and downstream AKT/MAPK signaling. The findings may clarify how VISTA contributes to immune microenvironment remodeling in ovarian cancer and provides a basis for further evaluation of VISTA-targeted immunotherapeutic strategies.

Materials and methods

Clinical samples. Surgically resected ovarian cancer tissues and non-malignant control ovarian tissues were consecutively collected at the Affiliated Cancer Hospital of Xinjiang Medical University between September 2024 and August 2025. A total of 71 female subjects were enrolled in this study, and the age of patients with ovarian cancer ranged from 45-65 years old. The ovarian cancer cohort (n=51) was stratified by FIGO stage into stage I/II (n=21), stage III (n=21) and stage IV (n=9). Non-malignant controls (n=20) were histologically non-malignant ovarian tissues from patients with benign gynecological diseases. The final sample size was based on the availability of eligible surgical specimens. The small stage IV subgroup reflects clinical practice, in which primary surgical resection is uncommon for stage IV ovarian cancer. A post hoc power analysis for the primary endpoint (the difference in VISTA protein expression between advanced ovarian cancer and non-malignant controls) indicated that the sample size provided >80% statistical power at $\alpha=0.05$. Baseline clinical characteristics are provided in Table SI. The present study was approved by Affiliated Tumor Hospital of Xinjiang Medical University ethics committee (approval No. K-2025116), and all participants provided written informed consent. Eligible participants had pathologically confirmed ovarian cancer without prior antitumor therapy; controls had no history of malignancy or autoimmune disease. Participants with other malignancies, severe infection, or incomplete clinical data were excluded.

After rinsing with physiological saline, the surgically resected tissues were divided into three aliquots for sectioning and immunofluorescence staining, reverse transcription-quantitative (RT-q) PCR and western blot analysis. All samples were processed within 30 min of collection and either frozen at -80°C or fixed in 4% paraformaldehyde at room temperature for 24 h. Patient information, including age, disease stage, and histologic type, was recorded. All samples were coded and managed under blinded conditions while maintaining sample traceability.

Cell culture. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples obtained from three independent healthy adult donors (two men and one woman; aged 25-35 years) with no history of malignancy, autoimmune disease, or acute or chronic infection. Written informed consent was obtained from all donors, and the protocol was approved by the Ethics Committee of the Affiliated Cancer

Hospital of Xinjiang Medical University. All datasets reported as $n=3$ comprised three biological replicates, each derived from a different donor.

Human peripheral blood lymphocyte separation medium (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. P8610) was used for PBMC isolation. Fresh anticoagulated whole blood was diluted 1:1 with RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11875101). Diluted blood (6 ml) was gently layered over 3 ml of separation medium in a 15-ml centrifuge tube (ExCell Bio; cat. no. CS015-0001). After centrifugation at $400 \times g$ (Shanghai Feige; DK-80) for 20 min at room temperature, cells from the intermediate buffy-coat layer were collected, washed twice with 5 ml PBS ($300 \times g$, 5 min, room temperature), and resuspended in RPMI 1640 medium for subsequent use.

Naive $CD4^+$ T cells were isolated using the Naive $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec GmbH; cat. no. 130-094-131). The cells were resuspended in 10 μ l of PBMCs and 40 μ l of isolation buffer. The biotin antibody mixture (10 μ l) and magnetic beads (20 μ l) were added sequentially, and the suspension was incubated for 5 min at room temperature. LS Columns (Miltenyi Biotec GmbH; cat. no. 130-042-401) were placed in an OctoMACS™ Separator (Miltenyi Biotec GmbH; cat. no. 130-042-108), rinsed with 3 ml of separation buffer, and the cell suspension was then added. Unlabeled cells were collected and rinsed with 3 ml of separation buffer. The retained cells were treated with 1 ml of Bead Release Reagent and subsequently washed with PBS for later use. Naive $CD4^+$ T cells were differentiated into FOXP3 $^+$ CXCR5 $^+$ T cells by culture in RPMI 1640 medium supplemented with fetal bovine serum (Excell Bio; cat. no. FND500), penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 15070-063), CD3 monoclonal antibody [MultiSciences (Lianke Biotech); cat. no. F1100300], CD28 antibody (Santa Cruz Biotechnology, Inc.; cat. no. SC-53876; 0.5 μ g/ml), IL-2 (Proteintech Group, Inc.; HZ-1015, 5 ng/ml), TGF- β 1 (Proteintech Group, Inc.; HZ-1011, 5 ng/ml), and recombinant IL-23 (Proteintech Group, Inc.; cat. no. HZ-1254, 25 ng/ml), for 5 days. For flow cytometric (BD FACSCanto II; BD Biosciences) sorting of Tfr-like cells, cells were suspended in serum-free medium at 1×10^7 cells/ml and first stained with Zombie NIR fixable viability dye (BioLegend, Inc.; cat. no. 423106) for dead cell exclusion, followed by surface staining with CD25 (BD Biosciences; cat. no. 557753), CD127 (BD Biosciences; cat. no. 557938), CXCR5 (Thermo Fisher Scientific, Inc.; cat. no. 17-9185-42), and VISTA (Thermo Fisher Scientific, Inc.; cat. no. 25-1088-42) antibodies. The cells were incubated at 4°C in the dark for 30 min. Compensation controls were prepared using UltraComp eBeads (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. 01-2222-42) stained with each individual fluorochrome, and compensation matrices were calculated using BD FACSuite software v1.6 (BD Biosciences) prior to each experiment. Fluorescence-minus-one controls were set up for each fluorochrome to define accurate gating boundaries, particularly for the VISTA channel with relatively dim expression. The sequential gating strategy was applied as follows: Lymphocytes were first identified by forward scatter area (FSC-A) vs. side scatter area (SSC-A); single cells were then discriminated using FSC-height (FSC-H) vs. FSC-A; dead cells were excluded based on viability dye

negativity; CD25 $^+$ CD127 $^+$ CXCR5 $^+$ Tfr-like cells were further gated and subdivided into VISTA $^+$ and VISTA $^-$ subsets for sorting. CD25 $^+$ CD127 $^+$ CXCR5 $^+$ VISTA $^+$ and CD25 $^+$ CD127 $^+$ CXCR5 $^+$ VISTA $^-$ cells were sorted using a flow cytometer (BD FACSCanto II; BD Biosciences). Post-sort purity was verified by re-analyzing an aliquot of the sorted cell suspension and consistently exceeded 95% across all experiments. The gating strategy and post-sort purity results are shown in Fig. S1.

Construction of Tfr-like cells with VISTA overexpression or silencing. Lentivirus particles were packaged via a third-generation lentiviral vector system and 293T packaging cells (ATCC; cat. no. CRL-3216) were used for viral production. The lentiviral vectors used for VISTA overexpression and silencing were purchased from Shanghai GeneChem Co., Ltd. Then three groups were set up: Group M (conventional medium), Group A (supplemented with HiTransG A), and Group P (supplemented with HiTransG P), to compare the effects of different infection conditions. For lentiviral transduction, a cell suspension at 5×10^4 cells/ml was seeded into a 96-well plate at 100 μ l per well (including three control wells), and cultured at 37°C for 24 h until the reported confluence reached 30%. The negative control virus was diluted to concentrations of 2×10^7 , 1×10^7 and 1×10^6 TU/ml, and added to the corresponding systems at multiplicities of infection (MOIs) of 1, 10, and 20, respectively (Group M: 90 μ l medium + 10 μ l virus; Groups A/P: 86 μ l medium + 10 μ l virus + 4 μ l infection solution). After 12 h of culture, the medium was replaced with normal medium. At 72 h, cells were examined microscopically and the condition and MOI yielding ~80% infection efficiency with acceptable cell morphology were selected for subsequent experiments. For establishment of stably transduced Tfr-like cell lines, puromycin at a final concentration of 2 μ g/ml was used for continuous screening over 7 days; the stable cell pool was maintained in complete culture medium supplemented with 0.5 μ g/ml puromycin during routine passaging. The targeting sequences of short hairpin (sh)RNA used in the present study were: sh-VISTA-1: CCCTGACTC TCCAAACTTTGA; sh-VISTA-2: CCGTATTCCTGTAT GTCTGT; sh-NC: TTCTCCGAACGTGTACGT. Only the gene-targeting fragment sequences are presented.

Assessment of cell proliferation. The induced Tfr-like cells were sorted by flow cytometry to obtain VISTA $^+$ Tfr-like cells and VISTA $^-$ Tfr-like cells. For VISTA $^-$ Tfr-like cells, three groups were set up: blank group, OE negative control group (transduced with negative control virus), and VISTA overexpression group (transduced with LV-VISTA overexpression lentivirus). For VISTA $^+$ Tfr-like cells, three groups were set up: blank group, shRNA negative control group (transduced with negative control virus), and VISTA-silencing group (transduced with VISTA-RNAi). Prior to lentiviral transduction, cells in each group were stained with the CellTrace™ 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) cell proliferation kit (Thermo Fisher Scientific, Inc.; cat. no. C34570) at 0.5 μ M with a cell density of 1×10^6 cells/ml, and the staining reaction was incubated at 37°C for 20 min in the dark. After lentiviral transduction, cells in each group were cultured individually. At the end of culture, cells in each group

were collected, and cell viability was evaluated by trypan blue exclusion to determine whether cell death confounded the proliferation findings. Total cell apoptosis rate was calculated as the sum percentage of early apoptotic cells and late apoptotic cells. No statistically significant difference in cell viability was observed among the groups. Subsequently, cells were washed once with PBS, resuspended in 500 μ l PBS, and CFSE fluorescence intensity was measured by flow cytometry using a BD FACSCanto II flow cytometer (BD Biosciences). Three independent experiments were performed, and all replicates were included in the statistical analysis. Flow cytometric raw data were acquired and analyzed using BD FACSDiva software v8.0 (BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA). Induced VISTA⁺ Tfr-like cells and VISTA⁻ Tfr-like cells were sorted by flow cytometry and assigned to blank control, negative control, and target intervention groups as aforementioned. After lentiviral transduction, cells in each group were cultured separately. At the end of culture, the culture supernatant from each group was collected, and cytokine concentrations were measured by ELISA using a microplate reader (Bio-Rad Laboratories, Inc.; xMark™). For each monoculture group, IL-10, TGF- β 1, IL-21 and granzyme B were detected (detection kits from Multi Sciences; cat. nos. EK110, EK981, EK121 and EK158), and the assays were performed according to the manufacturers' instructions. Three independent experiments were performed, and all replicates were included in the statistical analysis.

Western blotting. For the extraction of total protein from ovarian cancer tissues, RIPA lysis buffer (Wuhan Boster Biological Technology, Ltd.; cat. no. AR0105) containing a protease inhibitor (Wuhan Boster Biological Technology, Ltd.; cat. no. AR1178) was used. The tissues were ground in liquid nitrogen and mixed with prechilled lysis buffer. The mixture was incubated at 4°C for 60 min, followed by centrifugation at 13,500 $\times$ g, 4°C (Shanghai Lishen Scientific Equipment Co., Ltd.; Neofuge) for 15 min to collect the supernatant. Protein concentration was determined using the Easy II Protein Quantitative Kit (TransGen Biotech Co., Ltd.; cat. no. DQ111-01), and total protein quantification was performed via the bicinchoninic acid (BCA) colorimetric assay. The absorbance was measured at 562 nm with a microplate reader (Bio-Rad Laboratories, Inc.; xMark), and the protein concentration was calculated accordingly. Protein samples were mixed with SDS-PAGE loading buffer (containing β -mercaptoethanol; Amresco, LLC; cat. no. 0482), denatured at 100°C for 5 min, and centrifuged at 13,500 $\times$ g, 4°C for 5 min. Then, 50 μ g of protein per well and a prestained protein marker (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. PR1910) were loaded onto 10% separating SDS-PAGE gel with 4% stacking gel. Following electrophoresis, proteins were transferred to a 0.45 μ m PVDF membrane (MilliporeSigma; cat. no. IPVH00010) using a Mini-PROTEAN Tetra System transfer apparatus (Bio-Rad Laboratories, Inc.). The membrane was blocked with 5% non-fat milk blocking solution at room temperature for 1 h. All TBST washing buffer contained 0.05% (v/v) Tween-20. After rinsing with TBST, the membrane was incubated with primary antibodies separately: anti- β -actin (SinoBiological, cat. no. 100166-MM10, 1:1,000 dilution) and

anti-VISTA (Abcam; cat. no. ab300042, 1:1,000 dilution) at 4°C overnight. After rinsing with TBST, the corresponding secondary antibodies were added: Goat anti-mouse IgG H&L (HRP) (Abcam; cat. no. ab205719, 1:10,000 dilution) and goat anti-rabbit IgG H&L (HRP) (Abcam; cat. no. ab205718, 1:5,000 dilution), followed by incubation at room temperature for 1 h. After rinsing with TBST, the membrane was developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.; cat. no. 34580), and imaged using a ChemiScope 3000 chemiluminescence imaging system (Clinx Science Instruments Co., Ltd.). For quantitative analysis, Band intensities were measured by manually drawing rectangular regions of interest around each target band in ImageJ, band gray value quantification was completed using ImageJ software v1.54f (National Institutes of Health); total protein levels were normalized to β -actin as the loading control; relative phosphorylation levels of AKT and ERK1/2 were calculated as the ratio of phosphorylated (p-) protein to the corresponding total protein (p-AKT/total AKT, p-ERK1/2/total ERK1/2). Three independent experiments were performed, and all replicates were included in the statistical analysis.

Reverse transcription-quantitative (RT-q) PCR. Total RNA extraction from human ovarian tissues was performed using TRIzol® Reagent (Thermo Fisher Scientific, Inc.; cat. no. 15596026), and all procedures for RNA isolation, cDNA reverse transcription and quantitative real-time PCR strictly followed the standard protocols provided by each reagent manufacturer. TRIzol® Reagent was used as the dedicated lysis/extraction buffer for total RNA isolation, which contains phenol, guanidinium isothiocyanate and other proprietary components for simultaneous RNA, DNA and protein separation. Tissue (50 mg) was ground in liquid nitrogen, then 1 ml of TRIzol was added. The mixture was incubated at room temperature for more than 15 min, followed by addition of 200 μ l of chloroform, thorough mixing, and incubation for 5 min. Centrifugation was conducted at 13,500 $\times$ g, 4°C (Thermo Fisher Scientific, Inc.; Heraeus Multifuge X1R) for 15 min. An equal volume of isopropanol was added to the supernatant, and the mixture was placed at -20°C for 60 min, followed by centrifugation at 13,500 $\times$ g, 4°C for 15 min. The precipitate was washed twice with 75% ethanol (centrifugation at 13,500 $\times$ g, 4°C for 15 min each time) then dissolved in RNase-free water. RNA concentration and purity were measured using a micro-spectrophotometer (Hangzhou Allsheng Instrument Co., Ltd.; Nano-100), with an A260/A280 ratio of 1.8-2.1 considered acceptable. RNA integrity was assessed by agarose gel electrophoresis (agarose: Sangon Biotech Co., Ltd.; cat. no. A610013-0250; electrophoresis power supply: Beijing Baygene Biotech Co., Ltd.; BG-Power600i; electrophoresis tank: Beijing Liuyi Biotechnology; cat. no. DY CZ-21) combined with a gel imaging system (Tanon Science and Technology Co., Ltd.; 2500), with clear 28S and 18S bands considered acceptable. First-strand cDNA synthesis was performed using 5X All-In-One RT MasterMix (Applied Biological Materials; cat. no. G492). The reaction system contained 1,000 ng of total RNA, 4 μ l of 5X RT MasterMix, and RNase-free water to make up the total volume to 20 μ l. The reaction was carried out in a PCR instrument (Bio-Rad

Laboratories, Inc.; MyCycler Thermal Cycler) under the following conditions: 37°C for 15 min, 60°C for 10 min, and 95°C for 3 min. The synthesized cDNA was stored at -80°C and diluted 1:1 before use. For qPCR, EvaGreen Express 2XqPCR MasterMix-Low Rox (Applied Biological Materials; cat. no. G891) was used. The primers were *VSIR-F* (TGTAACCAT CCTCCTCCAG), *VSIR-R* (GAATGTTGCTGTCCATCC GC), *GAPDH-F* (TGTTGCCATCAATGACCCCTT), and *GAPDH-R* (CTCCACGACGTACTCAGCG). The qPCR reaction system contained 10 μ l of 2XMasterMix, 0.5 μ l of each forward and reverse primer, 2 μ l of cDNA, and RNase-free water to adjust the total volume to 20 μ l. The reaction was run on a Real-Time PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.; 7500 Fast) with the following program: Pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta C_q}$ method (31). Results were obtained from three independent experiments, with three technical replicates for each gene.

Immunofluorescence staining. Human ovarian tissue sections were incubated at 65°C in a constant-temperature incubator (Shanghai Jinghong; Model DNP-9272) for 1.5-2 h. All ovarian tissue specimens were fixed in 4% paraformaldehyde at room temperature for 24 h before paraffin embedding and sectioning. Subsequently, dewaxing was performed with xylene twice, followed by hydration through absolute ethanol I and II, 95, 90, 80 and 70% ethanol and distilled water. The sections were placed in 0.01 M citrate buffer (pH 6.0) and heated in a pressure cooker; heating was discontinued after steam release. The sections were soaked for 10 min, then the lid was opened to cool to room temperature. PBS was used as the uniform washing reagent for all subsequent washing steps throughout the immunofluorescence staining procedure. After washing with PBS, the tissue boundaries were marked with a hydrophobic barrier pen (Fuzhou Maxim; cat. no. PEN-0002). Then, 3% H₂O₂ was added dropwise to cover the tissue and the sections were incubated at room temperature in the dark for 25 min to inactivate endogenous peroxidase. After washing with PBS, excess buffer was removed and 3% goat serum (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1209) was added dropwise for blocking. Diluted primary antibodies were applied sequentially: anti-VISTA antibody (Abcam; cat. no. ab300042; 1:2,000 dilution), anti-CD4 antibody (Abcam; cat. no. ab133616; 1:2,000 dilution), CXCR5 antibody (Abcam; cat. no. ab254415; 1:2,000 dilution), and FOXP3 antibody (Santa Cruz Biotechnology, Inc.; cat. no. sc-53876; 1:1,000 dilution). The sections were incubated overnight at 4°C in a humidified chamber, then washed with PBS. HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies were applied as appropriate, followed by incubation at room temperature for 50 min. The sections were then washed with PBS. Corresponding tyramide conjugates (100 μ l each) were added dropwise: iF440-Tyramide (Wuhan Servicebio Technology Co., Ltd. cat. no. G1250), iF546-Tyramide (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1251), iF488-Tyramide (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1231), and iF647-Tyramide (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1232)

(all at 1:500 dilution). The sections were incubated at room temperature in the dark for 10 min, then washed with PBS. DAPI staining solution (Wuhan Boster Biological Technology, Ltd.; cat. no. AR1176) was added dropwise, and the sections were incubated at room temperature in the dark for 10 min, followed by washing with PBS. Antifade mounting medium (Wuhan Boster Biological Technology, Ltd.; cat. no. AR0036) was added dropwise for mounting. Images were acquired using a laser confocal microscope (Nikon Corporation; Model AIRHD25).

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software (IBM Corp.). Unless otherwise specified, *in vitro* data were derived from three independent experiments. The Shapiro-Wilk test was used to assess data normality, and Levene's test was used to assess homogeneity of variance across groups. Normally distributed continuous data were summarized as the mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) was used for overall comparisons among multiple groups, followed by pairwise comparisons using Tukey's multiple comparisons test (for homogeneous variance) or Dunnett's T3 test (for heterogeneous variance). Non-normally distributed continuous data were summarized as the median (interquartile range) [M (P₂₅-P₇₅)]. The Kruskal-Wallis H test was used for overall comparisons among multiple groups, and pairwise post-hoc comparisons were performed using Dunn's test with false discovery rate correction for multiple testing, which is consistent with the analytical workflow implemented in GraphPad Prism software (Dotmatics). Spearman rank correlation was used to assess the association between VISTA expression and Tfr-like cell infiltration density. Exact P-values are reported for all statistical comparisons. P<0.05 was considered to indicate a statistically significant difference (Tables SII-SIX). Graphs were generated using GraphPad Prism 9.0 software (Dotmatics).

Results

VISTA expression increased with ovarian cancer progression. Ovarian tissue sections from non-malignant controls and patients with different pathological stages (Stage I/II, III, and IV) of ovarian cancer were collected, and CD4, FOXP3, CXCR5, and VISTA expression was assessed by immunofluorescence staining (Fig. 1A). CD4 protein (red fluorescence, primarily localized to the cell membrane) showed the highest mean fluorescence intensity in the non-malignant controls group, which gradually decreased with advancing ovarian cancer stage (Stage I/II $\rightarrow$ III $\rightarrow$ IV). By contrast, the expression of FOXP3 (yellow fluorescence, nuclear localization), CXCR5 (green fluorescence, membrane localization) and VISTA (cyan fluorescence, membrane localization) showed the opposite pattern, with low expression in non-malignant controls and progressive increases across disease stages. A small population of VISTA-positive Tfr-like cells coexpressing all four proteins was also observed in ovarian tissues, and their apparent abundance and fluorescence intensity increased with advancing tumor stage. Compared with the non-malignant controls group, CD4 expression was markedly decreased in all stages of ovarian cancer, while the other markers were markedly increased (Fig. 1B-F).

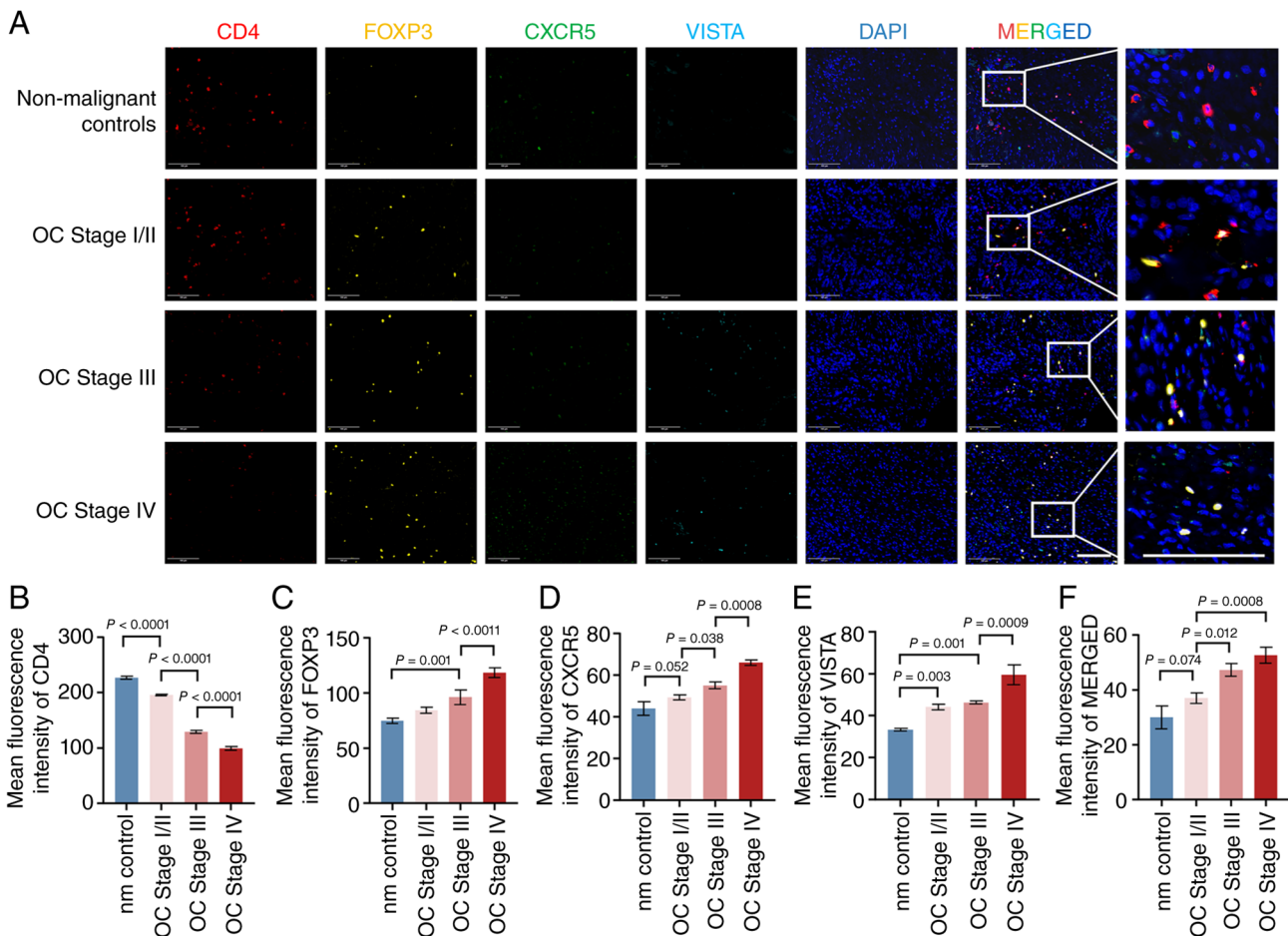


Figure 1. Expression of VISTA in ovarian cancer tissues. (A) Immunofluorescence staining images of ovarian tissue sections from the non-malignant control group and patients with ovarian cancer at different FIGO stages (Stage I/II, Stage III and Stage IV). CD4 was visualized in red, FOXP3 in yellow, CXCR5 in green, VISTA in cyan, and nuclei were stained with DAPI in blue. Scale bar, 100 μ m. Quantitative bar graphs of the fluorescence intensity of each protein: (B) CD4, (C) FOXP3, (D) CXCR5, (E) VISTA and (F) merged signals (n=3). Data are presented as the mean $\pm$ SD. VISTA, V-domain Ig suppressor of T cell activation; FIGO, International Federation of Gynecology and Obstetrics; CD4, cluster of differentiation 4; FOXP3, forkhead box P3; CXCR5, C-X-C motif chemokine receptor 5; DAPI, 4',6-diamidino-2-phenylindole; NM, non-malignant control; OC, ovarian cancer; SD, standard deviation.

Differences between adjacent stages were also statistically significant, suggesting that changes in these four markers and VISTA⁺ Tfr-like cells may be associated with ovarian cancer progression.

Association of VISTA mRNA and protein expression with ovarian cancer stage. qPCR and western blotting were used to examine VISTA expression across the clinical groups. VISTA mRNA expression was low in non-malignant control tissues (Fig. 2A). Across ovarian cancer stages, VISTA mRNA expression showed an overall upward trend from stage I/II to stages III and IV. However, one-way ANOVA indicated that the between-group differences were not statistically significant. Nevertheless, some individual stage III/IV samples showed mRNA levels approximately threefold higher than those in non-malignant controls, indicating substantial interindividual variability. By contrast, changes in VISTA protein levels were more pronounced. VISTA protein expression differed markedly among the stage I/II, stage III, and stage IV groups and associated positively with FIGO stage. These findings indicate that upregulation of VISTA protein expression may be associated with ovarian cancer progression (Fig. 2B and C).

In vitro induction and sorting of Tfr-like cells and validation of VISTA modulation. Naive CD4⁺ T cells were isolated using the Naive CD4⁺ T Cell Isolation Kit II according to the manufacturer's instructions. The isolated naive CD4⁺ T cells were stimulated with CD3 monoclonal antibody, CD28 monoclonal antibody, recombinant IL-2, recombinant TGF- β 1, and recombinant IL-23 for 5 days to induce differentiation (Fig. 3A). After induction, phenotypic characterization and sorting were performed using flow cytometry. CD25⁺CD127⁺ cells were selected, and the Tfr-like subset was further identified by CXCR5 expression. VISTA⁺ Tfr-like cells (CD25⁺CD127⁺CXCR5⁺VISTA⁺) and VISTA⁻ Tfr-like cells (CD25⁺CD127⁺CXCR5⁺VISTA⁻) were sorted (Fig. 3B). To investigate the functional role of VISTA, VISTA expression was manipulated by lentiviral transduction: VISTA⁺ Tfr-like cells were transduced with VISTA-shRNA lentivirus (knockdown group), and VISTA⁻ Tfr-like cells were transduced with LV-VISTA overexpression lentivirus (overexpression group). Blank control and negative control virus groups were also included. qPCR results showed that VISTA mRNA levels were decreased in the knockdown group and increased in the overexpression group compared with the control groups.

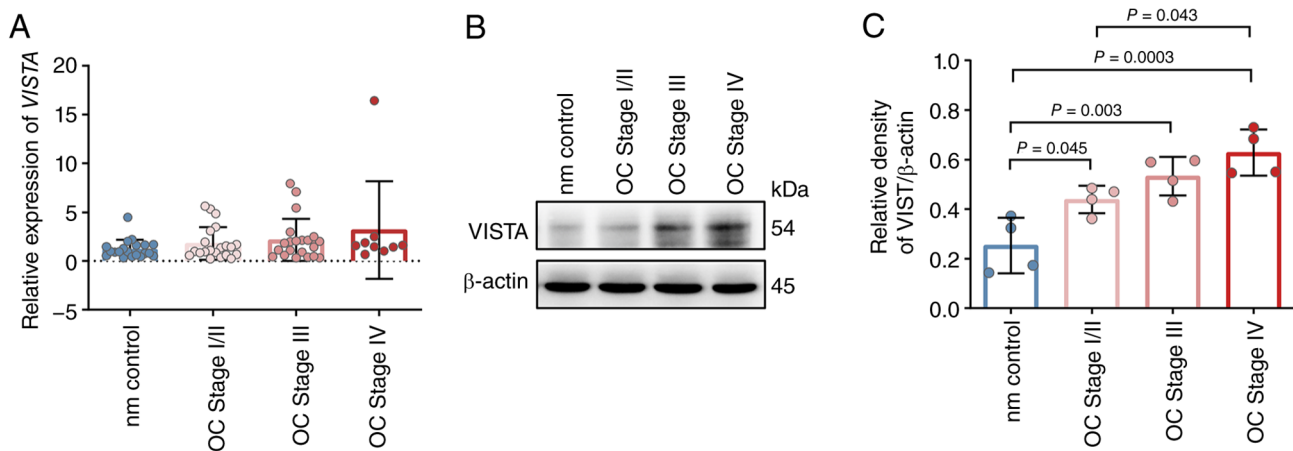


Figure 2. VISTA mRNA and protein expression in non-malignant control and ovarian cancer tissues across pathological stages. (A) Quantification of VISTA mRNA expression in tissues from the non-malignant controls group (n=20), ovarian cancer Stage I/II (n=21), Stage III (n=21), and Stage IV (n=9), measured by qPCR. (B) Western blot images of VISTA protein in ovarian tissues from the non-malignant controls group and ovarian cancer tissues with different pathological stages. (C) Quantitative analysis of VISTA protein expression was performed using ImageJ, and the results were normalized to the expression level of β -actin (n=4). Data are presented as the mean $\pm$ SD. VISTA, V-domain Ig suppressor of T cell activation; mRNA, messenger RNA; qPCR, quantitative real-time polymerase chain reaction; NM, non-malignant control; OC, ovarian cancer; SD, standard deviation.

(Fig. 3C and F). Western blot analysis confirmed corresponding changes at the protein level, supporting effective modulation of VISTA expression (Fig. 3D, E, G and H).

Effects of VISTA on Tfr-like cell proliferation and cytokine secretion. CFSE fluorescence decreases by approximately half with each cell division, allowing flow-cytometric assessment of proliferation under 488-nm excitation. Proliferation assays showed that the CFSE fluorescence decay ratio was markedly higher in VISTA⁺ Tfr-like cells than in VISTA⁻ Tfr-like cells, indicating greater proliferative activity in the VISTA⁺ subset (Fig. 4A and B). Following VISTA overexpression in VISTA⁻ Tfr-like cells, the CFSE fluorescence decay ratio was markedly higher than in the blank control and OE negative control groups (Fig. 4C). Conversely, silencing VISTA in VISTA⁺ Tfr-like cells resulted in a markedly lower ratio than in the blank control and shRNA negative control groups (Fig. 4D). These findings indicate that VISTA promotes Tfr-like cell proliferation, whereas VISTA silencing reduces proliferation. ELISA results revealed that the levels of IL-10 and TGF- β 1 in the supernatant of VISTA⁺ Tfr-like cells were markedly higher than those in VISTA⁻ Tfr-like cells, whereas the levels of IL-21 and granzyme B were markedly lower (Fig. S2A-D). After VISTA overexpression in VISTA⁻ Tfr-like cells, IL-10 and TGF- β 1 levels markedly increased, while IL-21 and granzyme B levels markedly decreased (Fig. 4E). By contrast, silencing VISTA in VISTA⁺ Tfr-like cells led to markedly reduced IL-10 and TGF- β 1 levels and markedly elevated IL-21 and granzyme B levels (Fig. 4F). These findings indicate that VISTA promotes anti-inflammatory cytokine secretion and suppresses immune effector molecules, producing a profile consistent with enhanced immunosuppressive function in Tfr-like cells.

VISTA-associated suppression of AKT and ERK1/2 phosphorylation in Tfr-like cells. Following VISTA overexpression in VISTA⁻ Tfr-like cells, the levels of p-AKT and p-ERK1/2 were markedly lower than in the blank control and negative control

groups. Conversely, silencing VISTA in VISTA⁺ Tfr-like cells using VISTA shRNA resulted in markedly higher p-AKT and p-ERK1/2 levels than in the blank and negative control groups (Figs. 5A-I, S2E-H). By contrast, total AKT and total ERK1/2 protein levels showed no significant differences across groups, suggesting that VISTA alters pathway phosphorylation without affecting total AKT or ERK1/2 abundance.

Previous studies suggest that AKT phosphorylation can inhibit Treg function³¹ and may correlate negatively with the abundance of CXCR5⁺FOXP3⁺ cells, a characteristic Tfr-like phenotype, in a model of acute colitis (32). In addition, ERK inhibition can promote FOXP3 expression (33) and FOXP3 is a key transcriptional regulator of Treg and Tfr-like cell function. Together, these observations are consistent with an inverse relationship between AKT/ERK1/2 phosphorylation and Tfr-like cell proliferation and immunosuppressive activity. These data revealed that altered VISTA expression coincided with changes in AKT and ERK1/2 phosphorylation alongside shifts in Tfr-like cell proliferation and immunosuppressive activity. These observations identified correlative changes between VISTA levels, AKT/ERK1/2 phosphorylation and Tfr-like cell functional phenotypes.

Discussion

The present study characterized VISTA expression in ovarian cancer tissues and examined how VISTA may regulate Tfr-like cell function in the context of tumor immune escape. To the best of the authors' knowledge, the findings identified a previously underexplored role for the VISTA-Tfr-like cell axis in ovarian cancer progression. The findings provided experimental evidence relevant to remodeling of the ovarian cancer immune microenvironment and may inform further development of immunotherapeutic strategies.

The high mortality rate of ovarian cancer is closely associated with its complex tumor immune escape mechanisms and the abnormal expression of immune checkpoint molecules is

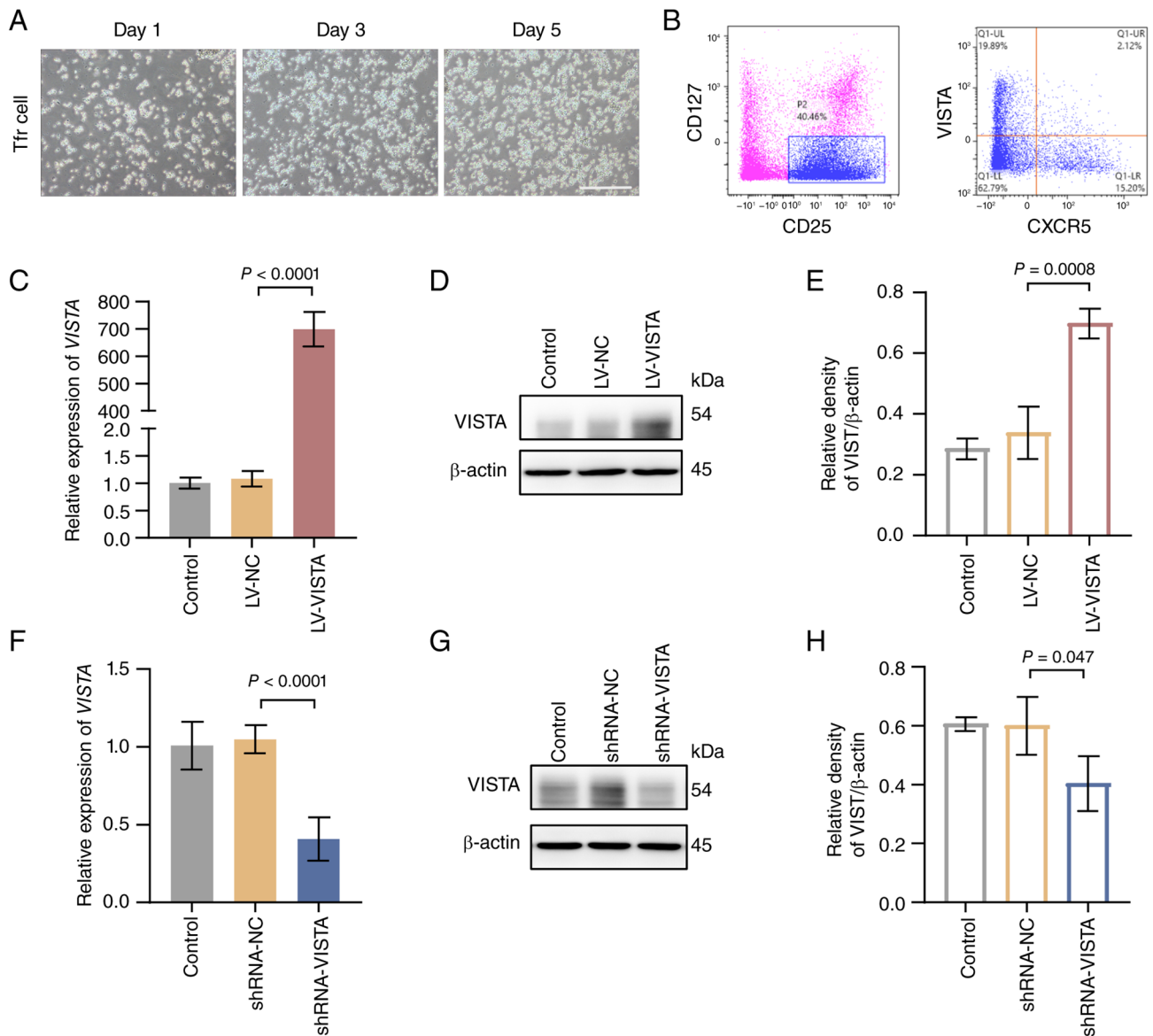


Figure 3. Tfr-like cell differentiation and validation of VISTA overexpression and silencing models. (A) Microscopic morphology of naive CD4⁺ T cells after induction for 1 day, 3 days, and 5 days. Naive CD4⁺ T cells were treated with a CD3 monoclonal antibody, a CD28 monoclonal antibody, recombinant IL-2, recombinant TGF- β 1, and recombinant IL-23. Scale bar, 100 μ m. (B) VISTA⁺ Tfr-like cells (CD25⁺CD127⁺CXCR5⁺VISTA⁺) and VISTA⁺ Tfr-like cells (CD25⁺CD127⁺CXCR5⁺VISTA⁺) were sorted by flow cytometry. VISTA knockdown was performed in VISTA⁺ Tfr-like cells, and VISTA overexpression was performed in VISTA⁺ Tfr-like cells by lentiviral transduction. (C) VISTA mRNA expression in VISTA⁺ Tfr-like cells after lentivirus-mediated overexpression, measured by qPCR. Western blot analysis of VISTA protein expression in VISTA⁺ Tfr-like cells after lentivirus-mediated overexpression: (D) Representative western blot image and (E) relative expression. (F) VISTA mRNA expression in VISTA⁺ Tfr-like cells after lentivirus-mediated overexpression, measured by qPCR. Western blot analysis of VISTA protein expression in VISTA⁺ Tfr-like cells after lentivirus-mediated overexpression: (G) Representative western blot image and (H) relative expression. Data are presented as the mean $\pm$ SD. Tfr, follicular regulatory T cell; VISTA, V-domain Ig suppressor of T cell activation; CD4, cluster of differentiation 4; CD3, cluster of differentiation 3; CD28, cluster of differentiation 28; IL, interleukin; CD25, cluster of differentiation 25; CD127, cluster of differentiation 127; CXCR5, C-X-C motif chemokine receptor 5; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; SD, standard deviation; TGF- β 1, transforming growth factor β 1; LV-VISTA, VISTA-overexpression lentiviral vector; sh, short interfering.

a central component of this process (34-37). VISTA protein expression increased across FIGO stages and associated positively with the infiltration density of FOXP3⁺CXCR5⁺ Tfr-like cells, suggesting that VISTA may be a candidate stage-associated marker in ovarian cancer. This finding was consistent with reports linking high VISTA expression to more aggressive disease in several solid tumors (17,29). The present data further suggested that accumulation of VISTA⁺ Tfr-like cells may contribute to the association between VISTA expression and ovarian cancer stage (17). Notably, the expression

trends of VISTA at the mRNA and protein levels were not completely consistent: VISTA protein expression showed a clearer association with stage, whereas mRNA expression varied substantially among individuals (38). This discordance may reflect post-translational modification of VISTA, such as glycosylation, or regulation of protein stability (39,40). VISTA activity in ovarian cancer may therefore be influenced by post-transcriptional or post-translational regulation rather than transcription alone, a possibility that warrants further investigation (41).

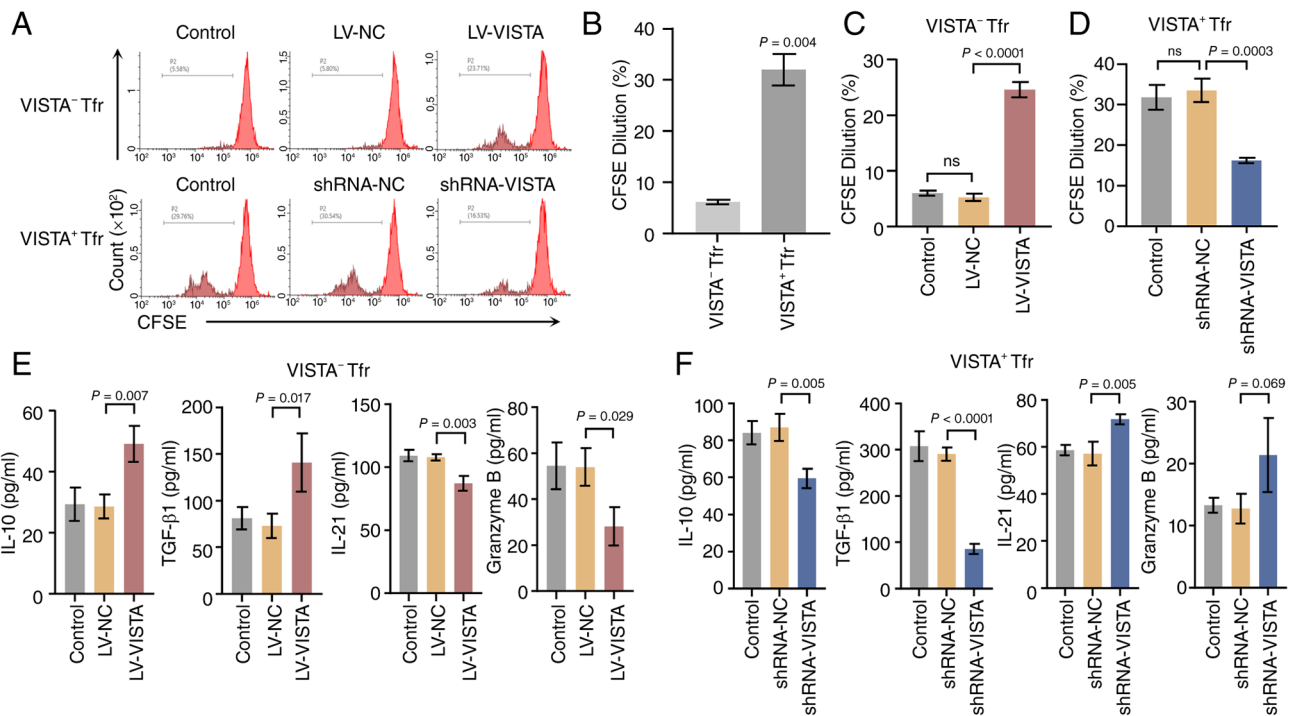


Figure 4. Effects of VISTA on Tfr-like cell proliferation and cytokine secretion. (A) Representative flow-cytometry histograms from the CFSE proliferation assay. The x-axis represents CFSE fluorescence intensity, and the y-axis represents cell count (n=3). (B) Quantification of CFSE fluorescence intensity in VISTA⁻ Tfr-like cells and VISTA⁺ Tfr-like cells. (C) Quantification of CFSE fluorescence intensity in VISTA⁻ Tfr-like cells after VISTA overexpression. (D) Quantification of CFSE fluorescence intensity in VISTA⁺ Tfr-like cells after VISTA silencing (VISTA-shRNA). (E and F) Levels of IL-10, TGF-β1, IL-21, and granzyme B in the culture supernatants of VISTA⁻ Tfr-like cells (E) and VISTA⁺ Tfr-like cells (F), detected by ELISA (n=3). Data are presented as the mean ± SD. VISTA, V-domain Ig suppressor of T cell activation; Tfr, follicular regulatory T cell; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; TGF-β1, transforming growth factor β1; IL, interleukin; LV-VISTA, VISTA-overexpression lentiviral vector; SD, standard deviation; shRNA, short hairpin RNA.

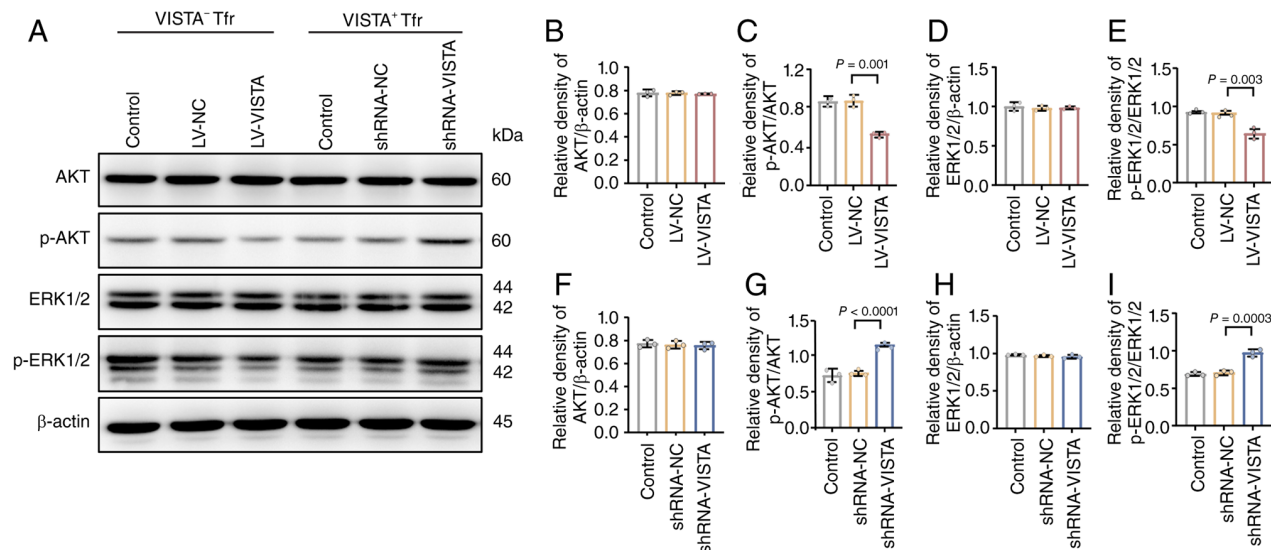


Figure 5. Effects of VISTA expression on AKT and ERK1/2 pathway proteins in Tfr-like cells. (A) Western blot images showing the protein expression of AKT, p-AKT, ERK1/2 and p-ERK1/2 in VISTA⁻ Tfr-like cells, VISTA⁺ Tfr-like cells, VISTA⁻ Tfr-like cells with VISTA overexpression and VISTA⁺ Tfr-like cells with VISTA silencing. (B-I) Quantitative analysis of protein expression levels was performed using ImageJ. Total AKT and total ERK1/2 protein levels were normalized to β-actin, while the relative phosphorylation levels of p-AKT and p-ERK1/2 were calculated as the ratio of phosphorylated protein to the corresponding total protein (n=3). Data are presented as the mean ± SD. VISTA, V-domain Ig suppressor of T cell activation; AKT, protein kinase B; ERK1/2, extracellular signal-regulated kinase 1/2; p-, phosphorylated; SD, standard deviation; Tfr, follicular regulatory T cell.

As regulators linking cellular and humoral immunity, Tfr-like cells may contribute to tumor immune escape when dysregulated (18,42,43). The present findings indicated that

VISTA may enhance the immunosuppressive function of Tfr-like cells by promoting proliferation and secretion of the anti-inflammatory cytokines IL-10 and TGF-β1 while reducing

release of the immune effector molecules IL-21 and granzyme B. This finding was consistent with breast cancer studies in which Tfr-like cells have been implicated in tumor progression through anti-inflammatory cytokine secretion (21). The present study extended those observations by implicating VISTA as a regulator of this process. At the mechanistic level, the findings suggested that VISTA-associated changes in Tfr-like cell function are accompanied by reduced AKT and ERK1/2 phosphorylation. Previous studies have confirmed that AKT phosphorylation can inhibit the immunosuppressive function of Treg cells, while ERK1/2 inhibitors can enhance Treg cell stability by upregulating FOXP3 expression (32-34). In the present study, after VISTA silencing, the levels of p-AKT and p-ERK1/2 increased, accompanied by decreased Tfr-like cell proliferation and reduced secretion of anti-inflammatory factors. These observations suggested that VISTA may support Tfr-like cell activity by attenuating AKT/MAPK pathway phosphorylation. This proposed mechanism expands the signaling framework for VISTA in immune cells and offers a perspective on molecular regulation of Tfr-like cell function. However, the present study focused on verifying the involvement of AKT and ERK1/2 core kinases in this regulatory process, while the upstream initiating molecules and downstream transcriptional targets of this pathway require investigation in future studies.

The observed discordance between VISTA mRNA and protein expression trends indicates that post-translational regulation may contribute to the upregulation of VISTA protein during ovarian cancer progression. Ubiquitin-proteasome-mediated degradation is a recognized regulator of VISTA protein homeostasis. VISTA undergoes constitutive K48-linked polyubiquitination in T cells, contributing to a short protein half-life. Protective modifications such as TRIM25-mediated K63-linked ubiquitination may counteract this degradation. TRIM25 knockdown increases K48-linked VISTA ubiquitination and accelerates protein turnover, providing mechanistic evidence that post-translational regulation can dissociate VISTA protein abundance from mRNA expression (44,45). Glycosylation is another post-translational modification of B7-family immune checkpoints that can influence protein stability, membrane localization and receptor-binding affinity (45). Studies in clear cell renal cell carcinoma have shown that B7-H5 (VISTA) is highly glycosylated and that mutations at specific N-glycosylation sites alter its abundance and subcellular distribution (46-48). The UniProt entry for VISTA also lists experimentally validated N-glycosylation sites, supporting the presence of this modification. On this basis, aberrant glycosylation could partly explain the discrepancy between VISTA mRNA and protein expression in ovarian cancer. However, this hypothesis has not been experimentally validated in the current study and the specific regulatory mechanism warrants dedicated investigation in future work. Coexpression of VISTA with other immune checkpoints, such as PD-L1, also warrants investigation. Multiple immune checkpoints can be coordinately upregulated in solid tumors, contributing to an immunosuppressive microenvironment. Concurrent VISTA and PD-L1 upregulation in ovarian cancer could intensify Tfr-like cell-mediated immunosuppression (49-51). This

possibility provides a rationale for evaluating combined VISTA and PD-1/PD-L1 targeting. Future studies should assess coexpression in clinical samples and evaluate combined intervention in animal models.

Certain limitations of the present study should be acknowledged. First, this was a single-center exploratory study with potential selection bias and no independent external validation cohort. The small sample size of the Stage IV subgroup (n=9) may limit the statistical robustness of stage-stratified subgroup analyses; non-parametric tests were therefore applied for all small-subgroup comparisons to reduce the risk of false-positive results. In addition, control tissues were non-malignant ovarian samples from patients with benign gynecological diseases rather than from healthy individuals, and long-term follow-up survival data were unavailable, so the prognostic value of VISTA and VISTA⁺ Tfr-like cells could not be evaluated. Second, functional assays were limited to *in vitro* cell culture with induced Tfr-like cells, which were identified only by a limited marker panel (CD4⁺FOXP3⁺CXCR5⁺). Additional Tfr lineage markers such as BCL6, PD-1 and ICOS were not assessed, and thus the cells should be interpreted as Tfr-like cells rather than definitively confirmed bona fide Tfr cells; *in vivo* models are further required to evaluate the role of the VISTA-Tfr-like axis in ovarian cancer growth and immune microenvironment remodeling. Third, the molecular mechanism underlying the discordance between VISTA mRNA and protein expression in ovarian cancer was not experimentally verified; the glycosylation-based hypothesis was derived from prior literature and requires further functional validation. Fourth, the mechanistic investigation of the present study focused on the core kinases AKT and ERK1/2; upstream regulators and downstream transcriptional effectors including FOXP3 and Blimp-1 were not examined. Notably, formal pathway rescue experiments were not been performed to establish a definitive causal link between VISTA-mediated AKT/MAPK modulation and functional changes in Tfr-like cells, nor to exclude potential off-target effects of lentiviral intervention. Despite these limitations, the findings characterized VISTA expression in ovarian cancer and its association with Tfr-like cell function, providing a basis for further mechanistic and translational studies.

In summary, the present study characterized VISTA expression in ovarian cancer and its potential regulation of Tfr-like cell function. VISTA expression increased with advancing tumor stage and was positively associated with Tfr-like cell infiltration. *In vitro*, VISTA promoted Tfr-like cell proliferation and anti-inflammatory cytokine secretion while reducing immune effector molecule release; these functional changes were accompanied by reduced AKT and ERK1/2 phosphorylation. These findings only revealed correlative changes among VISTA levels, AKT/MAPK phosphorylation status and the immunosuppressive phenotype of Tfr-like cells. Overall, the results highlighted a potential role for the VISTA-Tfr-like cell axis in regulating the ovarian cancer immune microenvironment and provide a basis for further evaluation of VISTA-targeted immunotherapeutic strategies.

Acknowledgements

Not applicable.

Funding

This work was supported by National Natural Science Foundation of China-Regional Project (project name: Study on the Role and Mechanism of Vista-Mediated Follicular Regulatory T Cell Activation in Ovarian Cancer Immune Evasion, project number: 82160302); Key Research and Development Project of Xinjiang Uygur Autonomous Region (project name: Research on the Construction of an Intelligent and Precise Early Screening and Treatment System for Ovarian Cancer in Xinjiang Women, project number: 2024B03037).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LL conceptualized the study and designed the research protocol. CY and HW acquired the experimental data. YM and YX analyzed the data, drafted the initial manuscript and contributed to manuscript preparation. LL critically revised the manuscript for important intellectual content. YM and YX confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work, ensuring that questions concerning the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Affiliated Tumor Hospital of the Third Clinical Medical College, Xinjiang Medical University (approval no: K-2025116). Written informed consent was obtained from all participants (patients with ovarian cancer for tumor tissues, individuals with benign diseases for non-malignant ovarian tissues, and healthy donors for peripheral blood) prior to sample collection. Patient information was de-identified and managed confidentially to protect privacy.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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