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Overexpression of SLAP2 inhibits triple-negative breast cancer progression by promoting macrophage M1-type polarization

Shun Wu[1], Fang Guo[2], Manxiu Li[1], Wei Chen[1] & Liting Jin[1]✉

Breast cancer is the most common malignant tumor in women, and triple-negative breast cancer (TNBC) is a specific subtype of breast cancer characterized by high invasiveness, high metastatic potential, ease of recurrence, and poor prognosis. Src-like adaptor protein 2 (SLAP2), which can be involved in the regulation of multiple signaling pathways, may be a key target for TNBC. The aim of this study was to investigate the effect of overexpression of SLAP2 on TNBC and to explore the underlying mechanisms. First, we constructed and transfected SLAP2 overexpressing lentivirus based on MDA-MB-231 human TNBC cell line, screened for differential downstream target genes in combination with mRNA high-throughput sequencing (RNA-Seq), and predicted their functions and enriched pathways in conjunction with bioinformatics analysis. The effects of SLAP2 overexpression on macrophage polarization, as well as on tumor proliferation and apoptosis, were assessed by tail vein injection of a stable transfection line of 4T1 cells transfected with SLAP2 overexpressing lentivirus. The effect of SLAP2 on macrophage polarization was assessed by inducing M1/M2 polarization and transfecting SLAP2 overexpressing lentivirus. Meanwhile, a transwell co-culture system was constructed between differently treated macrophages and 4T1 cells to assess the effect of SLAP2 overexpression on the malignant behavior of the cells via macrophage polarization. Overexpression of SLAP2 revealed 179 genes up-regulated and 74 genes down-regulated by mRNA high-throughput sequencing, and the enriched functions and pathways of differential genes were mainly related to immunity response. In vivo experiments revealed that overexpression of SLAP2 inhibited the growth of tumor in nude mice, decreased the expression of ki67 in tumor tissues, and increased the rate of apoptosis in tumor tissues. Meanwhile, we found that overexpression of SLAP2 promoted macrophage polarization toward M1 type and inhibited M2 type polarization in tumors. In vitro experiments further verified its effect on M1/M2 polarization by transfecting SLAP2 overexpressing lentivirus. By transwell co-culture system, we further demonstrated that overexpression of SLAP2 inhibits cell proliferation and invasion, promotes apoptosis, up-regulates the expression of Bax in cells, and down-regulates the expression of Bcl-2 in cells by promoting macrophage M1-type polarization. Overexpression of SLAP2 inhibits TNBC progression by promoting macrophage M1-type polarization.

Keywords SLAP2, Triple-negative breast cancer, Macrophage, Proliferation, Apoptosis

Breast cancer is the most common malignant tumor in women. Triple-negative breast cancer (TNBC) is a specific subtype of breast cancer that is negative for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2), and is characterized by high invasiveness, high metastatic ability, ease of recurrence, and poor prognosis[1,2]. TNBC is a highly heterogeneous cancer with specific mutations and aberrant activation of signaling pathways[3]. Conventional treatments for TNBC include pharmacologic chemotherapy, surgical excision, adjuvant radiotherapy, or adjuvant radiotherapy after breast-conserving surgery. Currently, drug therapy is still the most effective means of treatment, but chemotherapeutic drugs cause more damage to normal tissues, which seriously weakens the therapeutic effect. In addition, because TNBC

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tumors lack expression of ER, PR, and HER2, they are insensitive to endocrine therapy or HER2 therapy, and standardized treatment regimens for TNBC are still lacking[1]. Therefore, it is important to excavate suitable targets for TNBC therapy.

SRC-like adaptor protein 2 (SLAP2, also named SLA2) is a junction protein encoded by the SLA2 gene that is structurally similar to the Src family of protein kinases, containing an SH3 and an SH2 structural domain, but the kinase domains have been replaced by a unique C-terminal region[4]. SLAP2 is involved in the regulation of multiple signaling pathways[5–7], may be a key target for TNBC. We have found in our previous studies that SLAP2 is closely associated with the survival of breast cancer patients and significantly decreased in clinical TNBC patient specimens. Overexpression of SLAP2 in MDA-MB-231 cells inhibits cell proliferation, invasion, and migration and promotes apoptosis, whereas interference exhibits the opposite effect[8]. It is hypothesized that SLAP2 may be a potential target for breast cancer therapy, but the mechanism of its action in influencing breast cancer development is currently unclear.

Tumor-associated macrophages (TAMs) are functionally diverse and highly plastic, and are important immune cells in the breast cancer tumor microenvironment[9], which indicate an important direction in anti-cancer therapy. Generally, M1 macrophages exert immunoprotective effects by secreting pro-inflammatory cytokines, whereas M2 macrophages have anti-inflammatory properties that contribute to tissue remodeling and tumor progression[10]. Based on mRNA high-throughput sequencing, we found that the enriched functions and pathways of differential genes were mainly related to immunity response while overexpressing SLAP2. We hypothesized that SLAP2 might also affect breast cancer progression by mediating the polarization of TAMs, which has not yet been reported in any study.

Therefore, we constructed SLAP2 overexpressing lentivirus and co-validated the effect of overexpressing SLAP2 on the growth of breast cancer tumors and on the polarization of TAMs by ex vivo and in vivo experiments. This will elucidate the mechanism of SLAP2 in breast cancer and provide some theoretical basis for its application in targeted therapy.

Materials and methods

Cell culture

MDA-MB-231 cells, 4T1 cells and RAW264.7 cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences. Cells were incubated with DMEM containing 10% fetal bovine serum and 1% dual antibody (penicillin-streptomycin) in an incubator at 37 °C with 5% CO_2 saturated humidity, and logarithmic growth phase cells were taken for experiments.

RNA-Seq

According to the sequence of SLAP2 (GI No. 16797891), SLAP2 overexpression viral vector was constructed and transfected into MDA-MB-231 cells, respectively, and then the cells were collected, and the total cellular RNA was extracted, and the transcriptome was subjected to high-throughput sequencing and analysis. Sequencing results were done by Lianchuan Bio (Hangzhou, China). We then analyzed the sequencing results for raw confidence. A P value of < 0.05 was used as the differential gene screening condition to compare the differentially expressed genes in the NC and OV-SLAP2 groups. Volcano plots were drawn for all genes in the differential expression analysis using log2(fc) as the horizontal coordinate and -log10(qvalue) as the vertical coordinate. The top-ranked KEGG pathways with significant Q-values were utilized for mapping, with the vertical coordinate being the pathway name and the horizontal coordinate being the number of significantly different genes S analyzed by enrichment of this GO entry[11,12]. Finally, an enrichment pathway was screened for SLAP2-targetable regulation in triple-negative breast cancer.

qRT-PCR

Cells in each group were collected, and total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), which was tested for purity and then reverse transcribed into cDNA and amplified for detection. Reaction conditions: pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s, for a total of 40 cycles. The relative expression of target gene was calculated by $2^{-\Delta\Delta CT}$. PCR primers were synthesized by Wuhan Tianyi Huayu Gene Technology Co. The primer sequences are shown in Table 1.

Western-blot

Cells of each group were collected and protein extraction was performed with RIPA lysate (containing PMSF, proteinase inhibitor cocktail, and phostop), plus sample buffer, and boiled at 100 °C for 15 min. Proteins were separated by 10% SDS-PAGE electrophoresis and then transferred to PVDF membranes, which were closed at room temperature for 1 h in a solution containing 5% skimmed milk prepared with TBST, and then the corresponding primary antibodies SLAP2 (1:1000, bioswamp), Bcl-2 (1:1000, bioswamp), Bax (1:1000, bioswamp), and GAPDH (1:1000, bioswamp) were added, respectively, at 4 °C overnight. bioswamp), Bcl-2 (1:1000, bioswamp), Bax (1:1000, bioswamp) and GAPDH (1:1000, bioswamp), overnight at 4 °C. On the following day, the cells were washed three times with TBST, and the secondary antibody Goat anti-Rabbit IgG (1:20,000, bioswamp) was added and incubated for 1 h at room temperature. TBST was washed 3 times, color development was fixed, and GAPDH was used as an internal reference control to detect the protein expression of different transfection groups.

Primer name	Sequences	Size (bp)
TNF-α-F	ACTTCGGGGTGATCGGT	98
TNF-α-R	TTGGTGGTTTGTGAGTGTGA	
SLAP2-F	GAAGTTTGTCCAGCAGAGGG	204
SLAP2-R	CTGTCCACCAATCTCCATCC	
IL-1β-F	TAACCTGCTGGTGTGTGA	300
IL-1β-R	TTCTTCTTTGGGTATTGC	
IL-10-F	TTTCAAACAAAGGACCAGC	237
IL-10-R	TTCACAGGGGAGAAATCG	
TGF-β-F	AATGGTGGACCGCAACAAC	98
TGF-β-R	GCACTGCTTCCCGAATGTC	
SLAP2-F	GAAGTTTGTCCAGCAGAGGG	204
SLAP2-R	CTGTCCACCAATCTCCATCC	
GAPDH-F	CCTTCCGTGTTCCTAC	152
GAPDH-R	GACAACCTGGTCCTCA	

Table 1. Primer sequences.

CCK8

Cell viability was detected by CCK8 assay. 2×10^3 cells of each group were taken and inoculated in 96-well plates. After 24 h of adherence culture, 10 μL of CCK-8 solution was added to each well. The plates were incubated in an incubator for 4 h and the absorbance value at 450 nm was determined using an enzyme meter.

Subcutaneous tumor formation in nude mice

Twelve, 6-week-old, female, BALB/c nude mice were selected and housed under specific pathogen free (SPF) conditions. All animals originated from Hunan Slaughter Kingda. The experiment was divided into 2 groups: control group (injection of 4T1 cells: 1×10^7, injection volume: 100 μL) and OV-SLAP2 group (construction of SLAP2 overexpression lentivirus transfected into 4T1 cells to obtain a stable transfected cell strain, by subcutaneous injection into nude mice, the amount of cells injected: 1×10^7, injection volume: 100 μL). There were 6 mice in each group. At the end of the experiment, mice were anesthetized using 1% pentobarbital sodium, photographed, and blood was collected from the eyes; tumors were taken, photographed, and weighed; half of the tumors were fixed and half were frozen.

Immunohistochemistry

After routine paraffin embedding and sectioning (4 μm) of nude mouse tumor tissues, they were routinely deparaffinized with xylene, hydrated with gradient alcohol, and inactivated with endogenous peroxidase by 3% H_2O_2 occlusion for 10 min. Microwave repair was performed by applying 0.01 mol/L sodium citrate buffer (pH = 6.0, 15 min), which was closed by 5% bovine serum albumin (BSA) for 20 min and then titrated with primary antibodies Ki-67 (1:600) and SLAP2 (1:200) at 4 °C overnight. On the next day, goat, anti-rabbit secondary antibody was added dropwise, incubated at room temperature for 20 min, washed with PBS and then color developed by DAB. After hematoxylin re-staining, dehydrated and transparent, sealed film microscopy.

TUNEL

Paraffin-embedded tumor tissue sections were routinely deparaffinized and hydrated. Rinse with PBS for 2 times, add proteinase K working solution and react at 37℃ for 15 min. PBS was rinsed twice, immersed in the sealing solution, and closed for 10 min at room temperature. PBS was rinsed twice and the area around the sample was blotted with absorbent paper. 50 μL of TdT enzyme reaction solution was added dropwise to each sample, and the reaction was humidified with a coverslip for 60 min at 37 °C protected from light. PBS was rinsed 3 times, and after staining the nuclei with DAPI, the nuclei were rinsed 3 times with PBS, quencher was added, the film was sealed, protected from light, and examined and photographed in a Leica (DM2500) fluorescence microscope.

Immunofluorescence

The expression of F4/80 + CD86+ (M1 macrophages), F4/80 + CD206+ (M2 macrophages) in the tumor was detected by immunofluorescence double labeling. Formaldehyde-fixed tumor tissues were subjected to sucrose gradient dehydration and frozen sections, washed in 0.01 mol/L PBS solution, incubated at room temperature for 2 h by adding 5% sheep serum containment solution, and then added with primary antibody diluted with 2.5% sheep serum antibody diluent at 4℃ overnight. The specific impediments are as follows: F4/80 (1:100, bioswamp), CD86 (1:200, NOVUS) and CD206 (1:400, santa). The slides were washed with 0.01 mol/L PBS and then 0.01 mol/L PBS diluted secondary antibody was added and incubated for 2 h at room temperature away from light. The specific secondary antibodies were as follows: Affinipure Goat Anti-Mouse (1:50, triple eagle) and Goat Anti-Rabbit (1:50, triple eagle). They were washed with 0.01 mol/L PBS under light-avoiding conditions, observed under a fluorescence microscope and photographed.

ELISA
Expression of M1-type cytokines TNF-a and IL-1β and M2-type cytokines IL-10 and TGF-β in serum and cells were detected by ELISA. All ELISA kits were purchased from bioswamp (Wuhan, China). The experimental steps were performed strictly according to the kit instructions.

Cell co-culture
Macrophages and breast cancer cells in logarithmic phase were made into cell suspension, macrophages of different treatments were spread in the upper chamber of Transwell with 0.5 μm pore size at 6×10^5/well, and breast cancer cells were spread in the bottom of a 6-well plate in the lower chamber of Transwell at 3×10^5/well, and the appropriate amount of complete medium was added to build up a co-culture system, and cultured for 24 h, and the cells in the lower chamber were collected for the subsequent detection. Cells were grouped as follows: control group (without any treatment), LPS + IFN-γ group (M1 polarization was induced by treatment with 20 ng/mL IFN-γ + 100 ng/mL LPS for 24 h), LPS + IFN-γ + ov-SLAP2 group (M1 polarization was induced by treatment with 20 ng/mL IFN-γ + 100 ng/mL LPS for 24 h, and at the same time, the cells were transfected with ov-SLAP2), IL-4 group (treated with 20 ng/mL IL-4 for 24 h to induce M2 polarization) and IL-4 + ov-SLAP2 group (treated with 20 ng/mL IL-4 for 24 h to induce M2 polarization while transfecting ov-SLAP2).

Flow cytometry
Take the samples of each group, 1×10^6 cells, add 2 mL PBS to wash once, 4℃ 400 g, centrifuge for 5 min, discard the supernatant. Add 1 mL of 4% paraformaldehyde to fix for 30 min at room temperature. Wash twice with 1mL PBS for 3 min each time and discard PBS. Add 200 μL of antibodies CD86 and CD206 diluted with PBS 1:100 and incubate for 1 h at room temperature; wash twice with 1 mL of PBS for 5 min each time and discard PBS. Add secondary antibody dilution: 200 μL of secondary antibody diluted with PBS 1:200, 37℃, incubate for 1 h. Wash twice with 1mL PBS for 5 min each time and discard PBS. The cells were resuspended in 400 μL of PBS, stored at 4 °C, protected from light, and assayed on the machine. Experimental results were analyzed using NovoCyte analysis software.

Cellular scarring
Cells were collected from each group and inoculated into 6-well plates at a density of 1×10^5/mL. 2 mL of cell suspension was used in each well, and three parallel wells were set up for each group. Placed in 37 ℃, 5% CO_2 and 95% humidity constant temperature environment culture, after the cell spread to 90%, using 200 μL pipette gun tip in the middle of each well plate from top to bottom vertical scratches, PBS rinsed the cell plate for 3 times, after the removal of exfoliated cells. Photographs were recorded with a light microscope in the same field of view at 0 and 24 h after scratching, respectively.

Statistical analysis
SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA) was applied to analyze the data of the measurement information, and the data of the measurement information were expressed as the mean ± standard deviation ($x\pm s$), t-test was used for the comparison of two groups, and analysis of variance (ANOVA) was used for the comparison of the means between multiple groups. Differences were considered statistically significant at $P<0.05$.

Results
Bioinformatics analysis of differential genes and enrichment pathways after overexpression of SLAP2
First, SLAP2 overexpressing lentivirus was constructed and transfected into MDA-MB-231 cells. SLAP2 expression was significantly increased in the cells of the OV- SLAP2 group compared with the NC group (Fig. 1A). Next, we screened for differential gene expression after overexpression of SLAP2 by mRNA high-throughput sequencing. We found that a total of 179 genes were up-regulated and 74 genes were down-regulated in the OV- SLAP2 group (Fig. 1B). Its differential gene volcano map is shown in Fig. 1C. The top-ranked GO items and KEGG pathways with significant Q values were utilized for mapping. We found that the functions and pathways enriched for differential genes after overexpression of SLAP2 were mainly associated with immunity response, Fig. 1D-E.

SLAP2 overexpression and identification of stably transduced strains
Based on the sequence of mouse SLAP2, we constructed a SLAP2 overexpressing lentivirus and transfected it into 4T1 cells to obtain a stable transfected cell strain. Compared with the NC group, the expression of SLAP2 in the cells of the OV-SLAP2 group was significantly increased, and the cell proliferation ability was significantly reduced (Fig. 2A-B). This shows that the SLAP2 overexpression lentivirus was successfully constructed. SLAP2 overexpression lentivirus was then transfected into 4T1 cells. Compared with the control group, the mRNA and protein expression of SLAP2 in cells in the OV-SLAP2 group increased significantly (Fig. 2C-D). We thus obtained a stable transgenic strain that stably expresses SLAP2.

SLAP2 overexpression promotes tumor apoptosis and inhibits tumor growth
Then we injected the stably transduced strain into nude mice subcutaneously. Photographs of tumors in nude mice in vivo and in ex vivo are shown in Fig. 3A-B. On day 4 of injection, we began to calculate changes in tumor volume. Beginning on day 8, compared with the control group, the tumor volume of nude mice in the OV-SLAP2 group was significantly reduced (Fig. 3C). At the end of the experiment, we counted the tumor weight and found that the tumor mass was significantly reduced after overexpression of SLAP2 (Fig. 3D). Immunohistochemistry

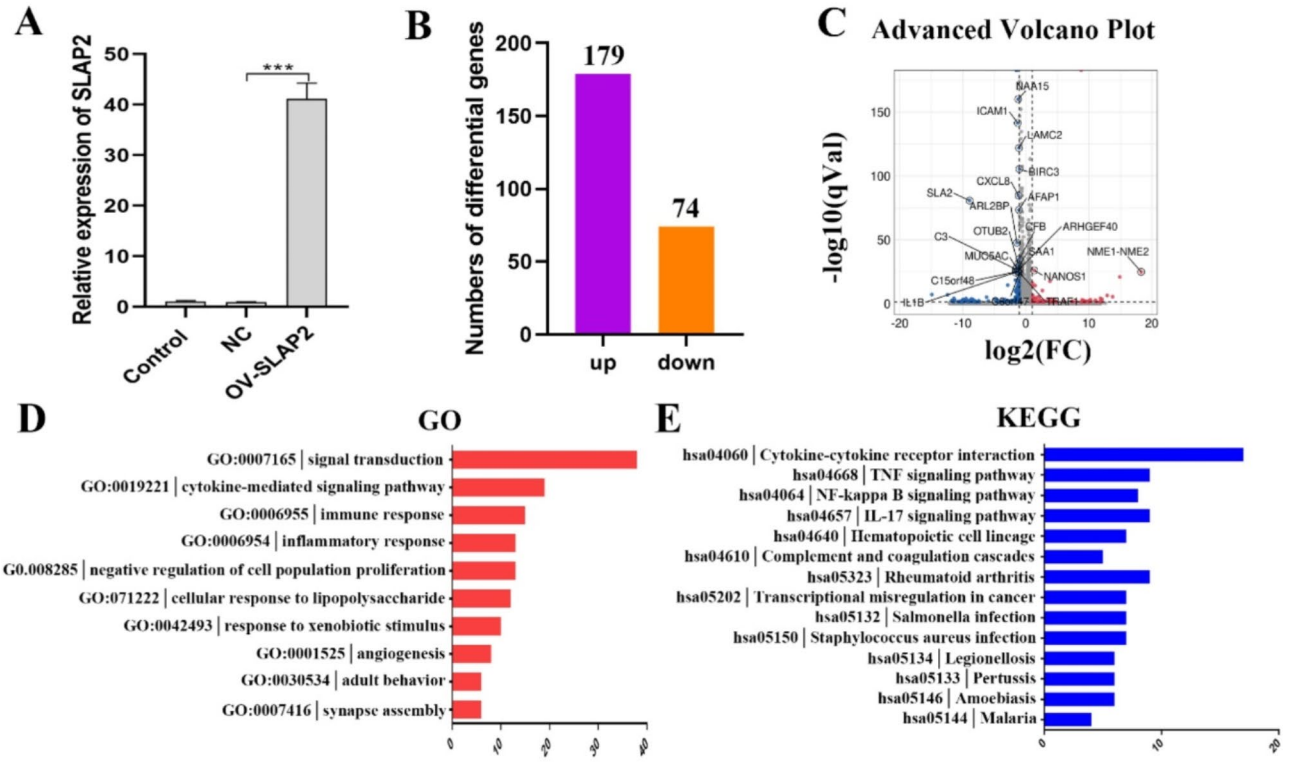

Fig. 1. Bioinformatics analysis of differential genes and enriched pathways after overexpression of SLAP2. (**A**) qRT-PCR detects the transfection efficiency of SLAP2 overexpression. (**B**) The number of differential genes between the SLAP2 overexpression group and the NC group. (**C**) Volcano plot of differentially expressed genes between the SLAP2 overexpression group and the NC group. (**D**)-(**E**) GO and KEGG functional annotation of differentially expressed genes between the SLAP2 overexpression group and the NC group.

and qRT-PCR detection revealed that the expression of SLAP2 in tumor tissues increased significantly after overexpression of SLAP2 (Fig. 3E-F). At the same time, compared with the control group, the expression of ki67 in the tumor tissues of the OV-SLAP2 group was significantly reduced (Fig. 3G). TUNEL detection revealed that apoptosis increased in tumor tissues after overexpression of SLAP2 (Fig. 3H). The Western-blot method showed that compared with the control group, the expression of Bcl-2 in the tumor tissue of the OV-SLAP2 group was significantly down-regulated, and the expression of Bax was significantly up-regulated (Fig. 3I).

SLAP2 overexpression promotes macrophage M1 polarization in tumors

Through immunofluorescence detection, it was found that compared with the control group, the fluorescence intensity of CD86 in the tumor tissue of the OV-SLAP2 group was stronger and the fluorescence intensity of CD206 was weaker (Fig. 4A-B). The expression of M1 type cytokines TNF-a, IL-1β and M2 type cytokines IL-10 and TGF-β in serum was detected by ELISA. Compared with the control group, the expression of TNF-a and IL-1β in tumor tissue of the OV-SLAP2 group increased, while the expression of IL-10 and TGF-β decreased (Fig. 4C). This result shows that overexpression of SLAP2 can promote the polarization of macrophages in tumor tissue toward the M1 type.

In vitro experiments verify that SLAP2 overexpression promotes M1 polarization of macrophages

We further verified the effect of overexpression of SLAP2 on macrophage polarization through in vitro experiments. Compared with the control group, the expression of CD86 in cells in the LPS + IFN-γ group was significantly increased, and the expression of CD206 in the IL-4 group was significantly increased. Compared with the LPS + IFN-γ group, the expression of CD86 in the cells of the LPS + IFN-γ + ov-SLAP2 group was significantly increased. Compared with the IL-4 group, the expression of CD86 in the cells of the IL-4 + ov-SLAP2 group was significantly increased, and the expression of CD206 was significantly decreased (Fig. **5**A-B). Through qRT-PCR and ELISA tests, it was found that compared with the control group, the expressions of TNF-α and IL-1β in the cells of the LPS + IFN-γ group were significantly increased, and the expressions of IL-10 and TGF-β were significantly decreased; the expressions of TNF-α and IL-1β in cells in the IL-4 group were significantly reduced, while the expressions of IL-10 and TGF-β were significantly increased. Compared with the LPS + IFN-γ group, the expressions of TNF-α and IL-1β in cells in the LPS + IFN-γ + ov-SLAP2 group were significantly increased, and the expressions of IL-10 and TGF-β were significantly decreased. Compared with the IL-4 group, the expressions of TNF-α and IL-1β in the cells of the IL-4 + ov-SLAP2 group were significantly

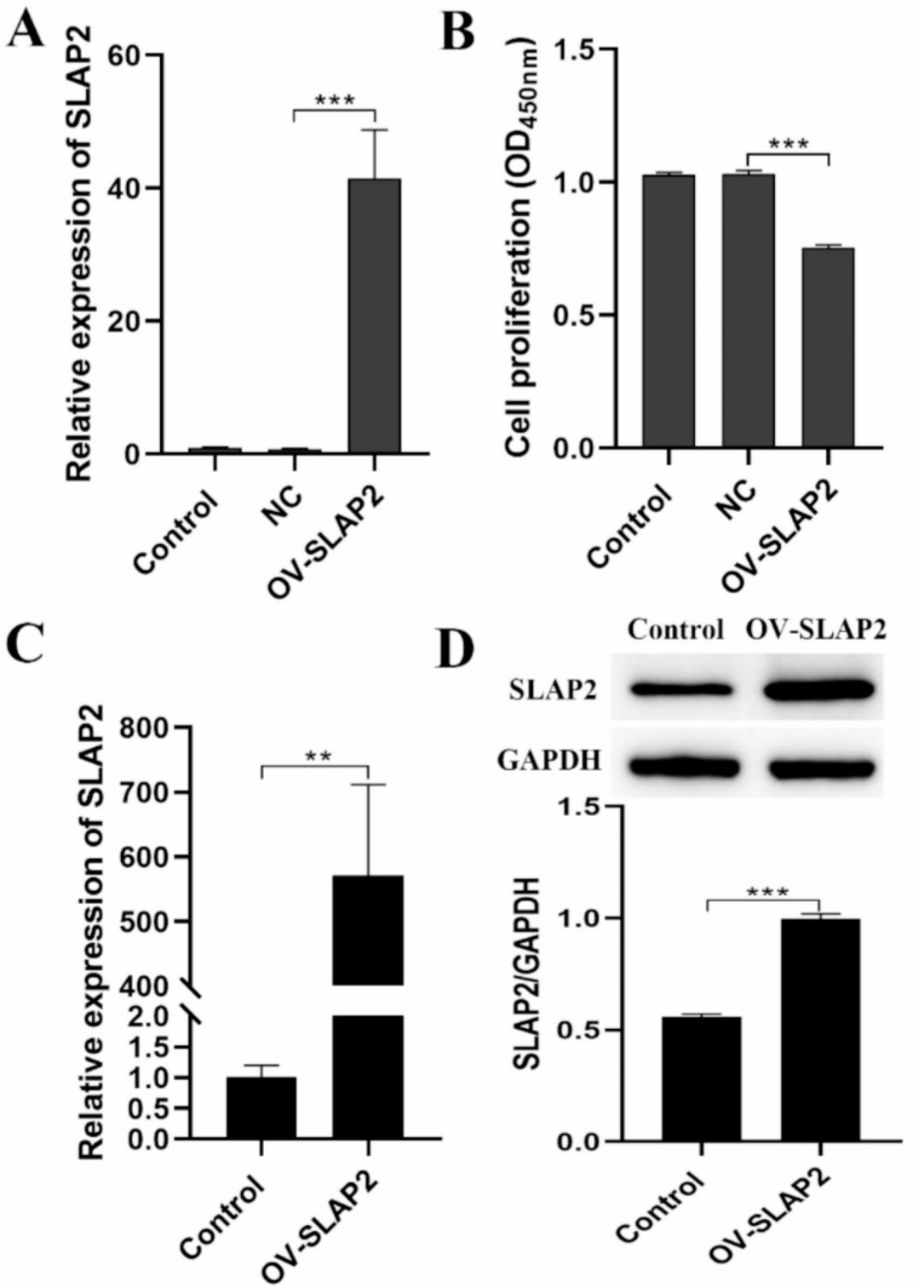

Fig. 2. SLAP2 overexpression and identification of stably transduced strains. (**A**) qRT-PCR detection of SLAP2 overexpression infection efficiency ($n=3$). (**B**) CCK8 detects cell proliferation after SLAP2 overexpression ($n=3$). (**C**) Screening of SLAP2 overexpression stable transfectants by qRT-PCR ($n=3$). (**D**) Western-blot was used to verify the expression of SLAP2 in the stably transformed strain ($n=3$).

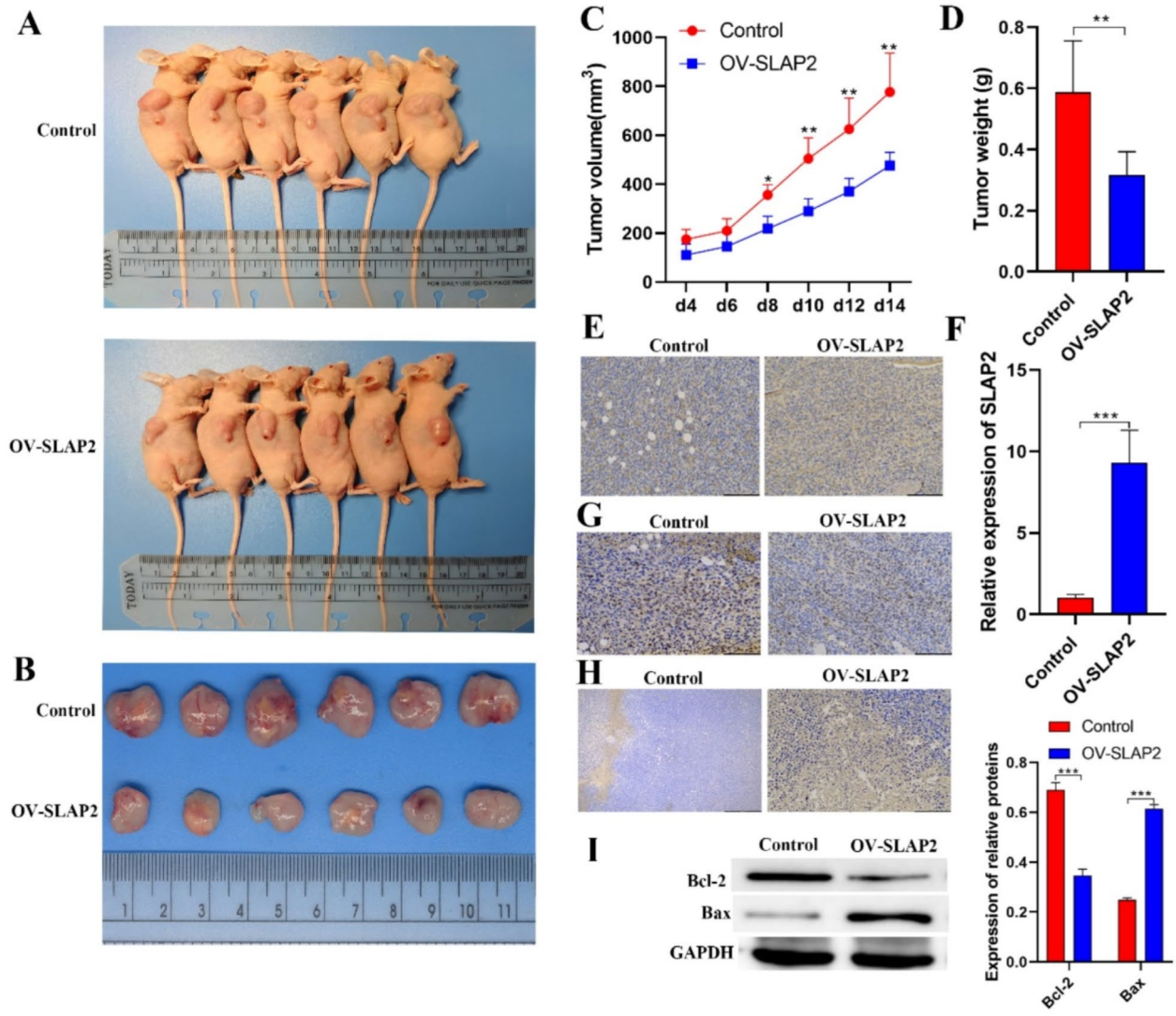

Fig. 3. SLAP2 overexpression promotes tumor apoptosis and inhibits tumor growth. (**A**) Tumor display in vivo ($n=6$). (**B**) Tumor display in ex vivo ($n=6$). (**C**) Changes in tumor volume in nude mice ($n=6$). (**D**) Change in tumor weight at the end of the experiment ($n=6$). (**E**) Immunohistochemistry detects the expression of SLAP2 in tumors in each group ($n=3$). (**F**) qRT-PCR detects the expression of SLAP2 in tumors in each group. (**G**) Detection of ki67 expression by immunohistochemistry ($n=3$). (**H**) Tumor apoptosis was assessed by TUNEL detection ($n=3$). (**I**) Detect the protein expression of Bcl-2 and Bax in tumors by western-blot method ($n=3$).

increased, and the expressions of IL-10 and TGF-β were significantly decreased (Fig. 5C-D). The expression of SLAP2 in cells was evaluated by qRT-PCR detection. It was found that the expression of SLAP2 increased in cells after inducing M1 polarization and decreased in cells that induced M2 polarization (Fig. 5E).

SLAP2 overexpression promotes apoptosis by promoting macrophage M1 polarization

We further validated the role of SLAP2 overexpression on macrophage polarization by constructing a transwell co-culture system. The expression of SLAP2 was significantly increased in the cells of the LPS + IFN-γ group and significantly decreased in the cells of the IL-4 group compared with the control group. SLAP2 expression was significantly increased in the cells of the LPS + IFN-γ + ov-SLAP2 group compared with the LPS + IFN-γ group. The expression of SLAP2 was significantly increased in cells in the IL-4 + ov-SLAP2 group compared with the IL-4 group (Fig. 6A). Meanwhile, compared with the control group, the proliferation ability and invasion rate of cells in the LPS + IFN-γ group were significantly reduced, and the apoptosis rate was significantly increased; the proliferation ability and invasion rate of cells in the IL-4 group were significantly increased, and the apoptosis rate was significantly reduced. Compared with the LPS + IFN-γ group, the proliferation ability and invasion rate of cells in the LPS + IFN-γ + ov-SLAP2 group were significantly reduced, and the apoptosis rate was significantly increased. Compared with the IL-4 group, the proliferation ability and invasion rate of cells in the IL-4 + ov-SLAP2 group were significantly reduced, and the apoptosis rate was significantly increased

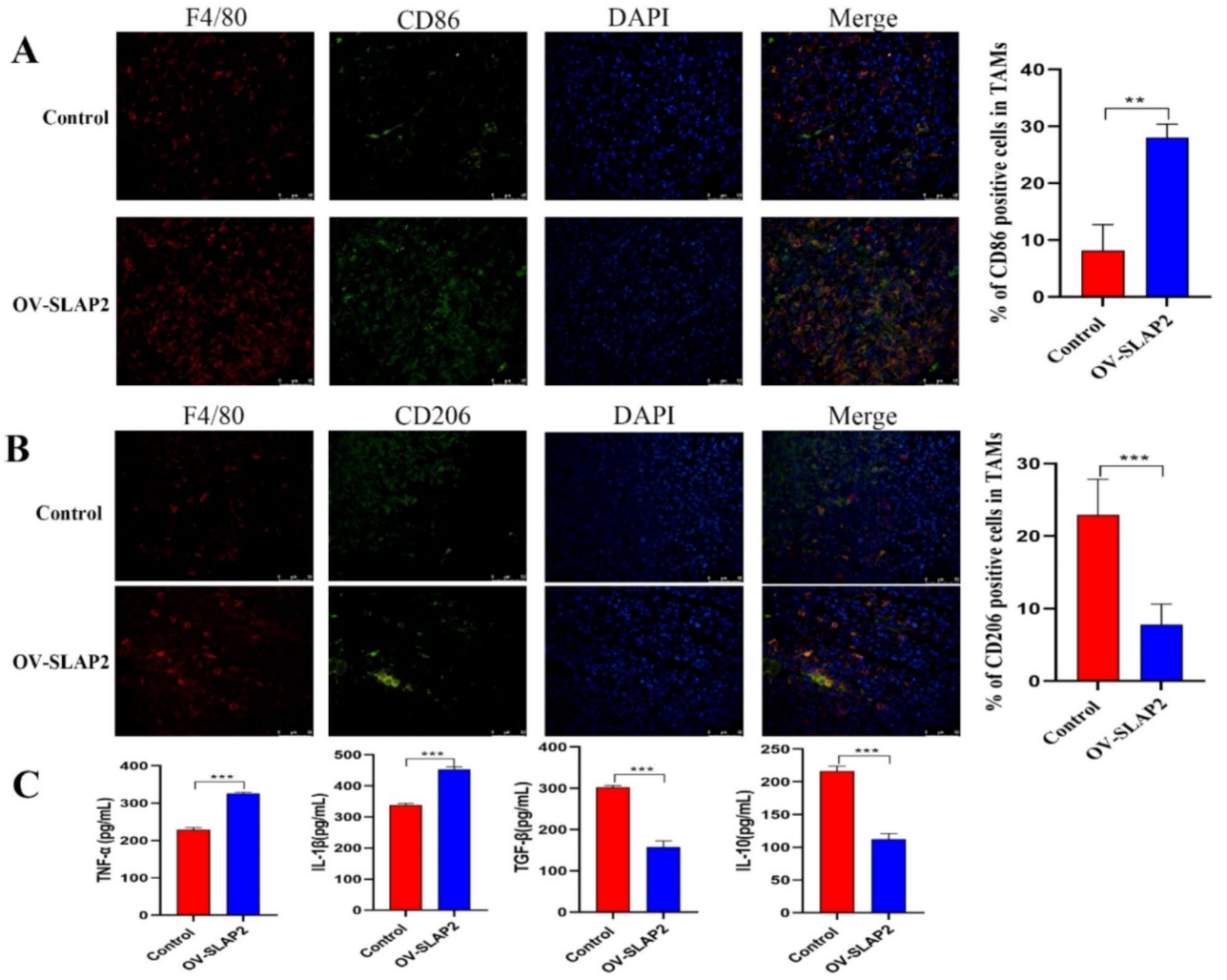

Fig. 4. SLAP2 overexpression promotes M1 polarization of macrophages. (**A**)-(**B**) Immunofluorescence double labeling detects the expression of F4/80 + CD86 + and F4/80 + CD206 + in tumors ($n = 3$). (**C**) The expression of M1 type cytokines TNF-a, IL-1β and M2 type cytokines IL-10 and TGF-β in serum was detected by ELISA ($n = 3$).

(Fig. 6B-F). Meanwhile, compared with the control group, the expression of Bcl-2 in the cells of the LPS + IFN-γ group was significantly reduced, and the expression of Bax was significantly increased; the expression of Bcl-2 in the cells of the IL-4 group was significantly increased, and the expression of Bax was significantly reduced. And overexpression of SLAP2 in both induced M1 macrophages and M2 macrophages was able to downregulate the expression of Bcl-2 and upregulate the expression of Bax (Fig. 6G). In addition, overexpression of SLAP2 significantly increased the expression of the M1-type markers TNF-α and IL-1β and decreased the expression of the M2-type markers IL-10 and TGF-β1(Fig. 6H).

Discussion

Treatment options for TNBC are limited and more effective treatments are urgently needed. In this study, we found that 179 genes were up-regulated and 74 genes were down-regulated after overexpression of SLAP2 by mRNA high-throughput sequencing, and that the enriched pathways of differential genes were mainly related to immunity response. In addition, it was jointly verified by in vivo and in vitro experiments that overexpression of SLAP2 significantly inhibited tumor growth and cell proliferation and invasion in nude mice, as well as promoted macrophage polarization toward M1 type and inhibited M2 type polarization.

SLAP is an articulated protein that is aberrantly expressed in various cancer types. SLAP is expressed in a variety of tissues and controls downstream signaling by binding to multiple receptors[7]. It has been found that reduced SLAP expression may enhance malignant progression of intrahepatic cholangiocarcinoma through activation of Wnt signaling[13]. Mansha et al.[14] found that SLAP expression was reduced in acute lymphoblastic leukemia. In this study, we constructed an overexpression SLAP2 stable transducer strain by subcutaneous injection into nude mice, and found that overexpression of SLAP2 promoted apoptosis, inhibited tumor growth, and promoted macrophage to M1 phenotype in tumor tissues.

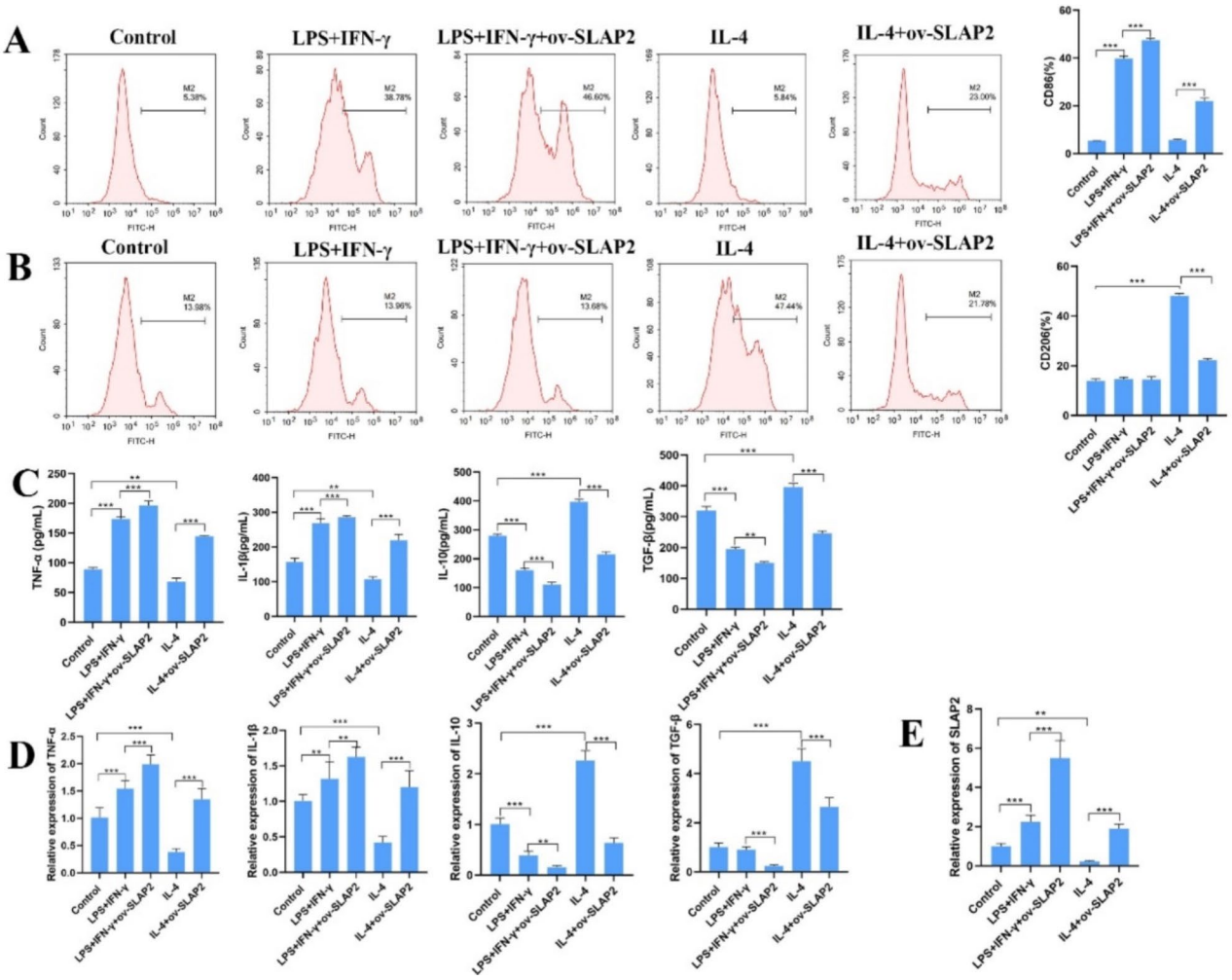

Fig. 5. In vitro experiments to verify that SLAP2 overexpression promotes macrophage M1-type polarization. (**A**)-(**B**) Expression of CD86 in M1-type macrophages and CD206 in M2-type macrophages was detected by flow cytometry ($n=3$). (**C**) Levels of TNF-α, IL-1β, IL-10, and TGF-β were detected in cell supernatants by ELISA. (**D**) Expression of the M1 marker (TNF-α, IL-1β), M2 marker (IL-10, TGF-β1) expression in cells. (**E**) SLAP2 expression in cells detected by qRT-PCR ($n=3$).

Tumor macrophages are one of the major populations of tumor-infiltrating leukocytes in breast cancer and are associated with poor clinical outcomes[15]. It has been demonstrated that TAMs are a major component of the tumor microenvironment and directly influence tumor cell growth and immunosuppression[16]. M2 macrophages promote tumor progression and M1 macrophages inhibit tumor progression[17]. To further validate the role of overexpression of SLAP2 on TNBC macrophage polarization, we assessed the effect of SLAP2 on macrophage polarization by inducing M1/M2 polarization and transfecting SLAP2 overexpressing lentivirus. The results revealed that overexpression of SLAP2 was able to further up-regulate the expression of CD86 to promote the polarization of M1-type macrophages and down-regulate the expression of CD206 to inhibit the polarization of M2-type macrophages. In addition, we constructed a transwell co-culture system of differently treated macrophages with 4T1 cells, and found that overexpression of SLAP2 was able to inhibit breast cancer cell proliferation and invasion and promote apoptosis by mediating macrophage polarization. Macrophages have been shown to be directly or indirectly involved in several key features of malignancy, including angiogenesis, invasiveness, metastasis and regulation of the tumor microenvironment[18]. It has been found that Nrf2 plays a protective role in sepsis-induced lung injury and inflammation by regulating macrophage polarization[19]. Astragaloside IV (AS-IV) inhibits the proliferation, invasion and migration ability of hepatocellular carcinoma by inhibiting macrophage M2 polarization, and its mechanism may be related to the TLR4/NF-κB/STAT3 signaling pathway[20]. Chen et al.[21] demonstrated that Alisol B23 acetate (AB23A) inhibited lung cancer progression by promoting macrophage polarization toward M1 through targeted regulation of CD11b/CD18. Thus, the role of macrophage polarization in different diseases has been demonstrated. In our study, it was demonstrated that overexpression of SLAP2 could inhibit breast cancer development by promoting macrophage M1 polarization. In fact, other types of immune cells, including innate immune cells such as natural killer cells, dendritic cells (dc), and adaptive immune cells including effector T cells ($CD4^+$ and $CD8^+$ T cells) or regulatory T cells (Tregs) have

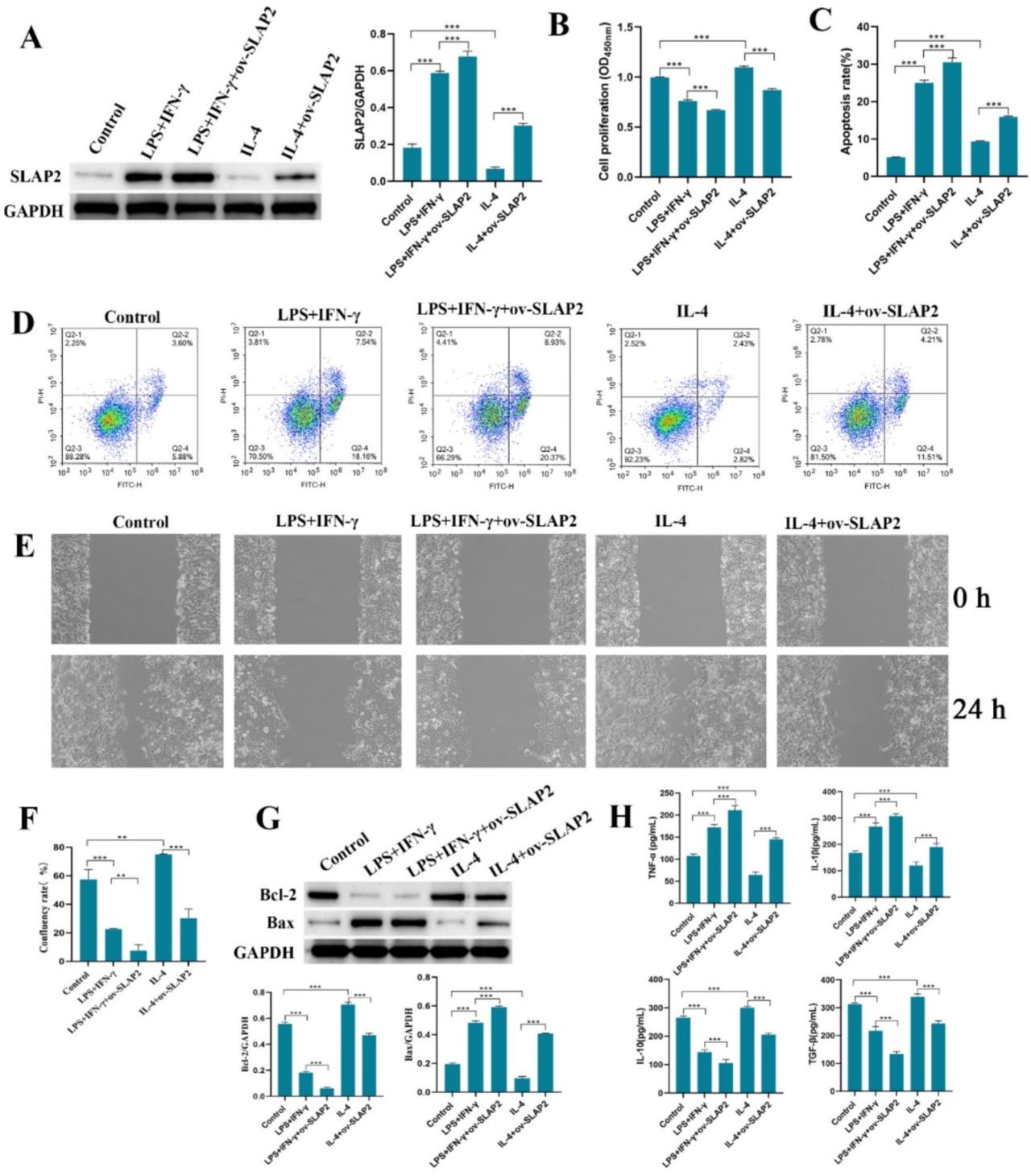

Fig. 6. Cell co-culture validation that SLAP2 overexpression promotes macrophage M1 polarization. (**A**) SLAP2 expression in lower compartment cells detected by western-blot assay ($n=3$). (**B**) Cell activity detected by CCK8 ($n=3$). (**C**)-(**D**) Apoptosis detected by flow cytometry ($n=3$). (**E**) Cell invasion detected by cellular scratch assay ($n=3$). (**F**) Confluency rate calculated as percentage of scratch closure in respect to initial scratch area ($n=3$). (**G**) Detection of Protein expression of Bcl-2 and Bax in lower compartment cells ($n=3$). (**H**) Detection of TNF-α, IL-1β, IL-10, TGF-β in lower compartment cells by ELISA ($n=3$).

also been shown to be involved in tumor microenvironment (TME) infiltration of solid organ cancers[22]. Besides, increased granulocyte-colony stimulating factor (G-CSF) may be an important factor involved in reshaping the immune response[23]. Therefore, further research is needed to be elucidate the immune mechanisms by which SLAP2 is involved in TNBC progression.

Conclusion

In conclusion, we first found that the enrichment pathways of differential genes after overexpression of SLAP2 were mainly associated with inflammation (TNF-a, NF-kB, etc.) and immunity (IL17, TLR, etc.) by mRNA high-throughput sequencing. It was jointly verified by in vivo and in vitro experiments that overexpression of SLAP2 inhibited tumor growth, suppressed tumor cell proliferation and invasion, and promoted apoptosis in nude mice. Exploration of the mechanism revealed that it may be related to the promotion of macrophage M1 polarization. This provides some theoretical basis for the application of SLAP2 to targeted therapy of breast cancer. Due to time and financial constraints, this study still has some limitations, and we will explore the pathway of action of SLAP2 on breast cancer in future studies.

Data availability

All data from this study can be requested directly from the corresponding author upon reasonable request.

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Author contributions

Shun Wu: Writing and revising articles; Fang Guo: Experimental verification; Manxiu Li: data analysis; Wei Chen: Collating data; Liting Jin: Conceptual Design.

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Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Wuhan Myhalic Biotechnology Co., Ltd. All methods

were performed in accordance with the relevant guidelines and regulations.

Competing interests
The authors declare no competing interests.

Ethical standard
The study is reported in accordance with ARRIVE guidelines.

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