Original Article

Iranian Journal of Immunology

September 2024; 21(3):

JAK/STAT Signaling Pathway Mediates Anti-Tumor Immunity of CD8+ T Cells in Renal Cancer

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ABSTRACT

Background: $CD8^+$ T cells play a crucial role in immune responses, and have significant potential in tumor immunotherapy. The JAK/STAT pathway is essential for cytokine signal transduction and is linked to immune escape. However, its role in mediating $CD8^+$ T cell anti-tumor immunity in renal cancer is not fully understood.

Objective: To study the mechanisms underlying $CD8^+$ T cell-mediated anti-tumor immunity and propose new possibilities for immunotherapy in patients with renal cancer.

Methods: $CD8^+$ T cells from mouse spleens were sorted using immunomagnetic beads, and their purity was confirmed by flow cytometry. Proliferation was analyzed using CCK-8 and CFSE assays. Activation of $CD8^+$ T cells was assessed through ELISA and Western blotting. The malignant properties of Renca cells were evaluated through flow cytometry, Calcein-AM/PI staining, wound healing, Transwell, Western blot, and immunofluorescence. A subcutaneous tumor model in nude mice was used to examine the role of JAK1/STAT1 pathway *in vivo*.

Results: Inhibitors of JAK1 and STAT1 significantly reduced the proliferation and activation of $CD8^+$ T cell. Co-culture with $CD8^+$ T cells increased apoptosis and inhibited the proliferation, migration, and invasion of Renca cells. The effects were diminished by JAK1 and STAT1 inhibitors, confirming that $CD8^+$ T cells exert anti-tumor effects through the JAK1/STAT1 pathway. *In vivo,* inhibition of this pathway reduced the anti-tumor effects of $CD8^+$ T cells.

Conclusion: Inhibitors of JAK1 and STAT1 weakened the anti-tumor effects of $CD8^+$ T cells, suggesting that targeting this pathway could enhance $CD8^+$ T cell-mediated immunity in renal cancer.

Keywords: $CD8^+$T Cell, Immunity, JAK/STAT Pathway, Renal Cancer

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Cite this article as:
Shao J, Deng G, Wen G, Xie X. JAK/STAT Signaling Pathway Mediates Anti-Tumor Immunity of CD8+ T Cells in Renal Cancer. *Iran J Immunol.*
doi:

Received: 2024-08-12
Accepted: 2024-08-17

INTRODUCTION

Renal cancer, a prevalent malignant tumor, has varying incidence rates based on gender. It is the sixth most frequent cancer in men and the ninth most prevalent cancer in women, seriously affecting human health (1, 2). Kidney cancer rates are increasing globally by 2% annually, with over 100,000 deaths each year attributed to the disease (3, 4). More than half of the patients had their kidney cancer detected during an incidental examination, and nearly 1/3 of the patients were found to have metastases on their first visit to the doctor (5). In addition, renal cancer cells are extremely invasive and prone to form tumor thrombi after invading blood vessels, sometimes even migrating to the heart site (6). Although surgical resection is currently the most thorough treatment for kidney cancer, yet it comes with a high risk of recurrence, low survival rates, and a grim prognosis (7, 8). Consequently, it is essential to investigate new approaches for treating kidney cancer.

In recent years, immunotherapy has gained increasing attention in tumor treatment due to its specificity in effectively circumventing many of the side effects of current clinical treatment protocols (9, 10). At the occurrence of tumors, the body's immune cells can recognize the tumor antigen, causing a migration of diverse immune cells to the tumor location to kill and remove tumor cells (11). $CD8^+$ T cells are essential for defending against intracellular pathogens and monitoring for tumors within the body (12, 13). $CD8^+$ T cells that have been activated are capable of releasing cytokines such as interferon (IFN)-γ, in turn eliminating infected or cancerous cells, crucial for anti-tumor immunity (14, 15). Improved patient prognosis in renal cancer has been linked to $CD8^+$ T cells infiltration (16). Thus, in-depth exploration of the impact and action mechanism of $CD8^+$ T cell antitumor immunity on renal cancer progression can help provide new ideas and strategies for renal cancer immunotherapy.

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways are well-established mechanisms for the transmembrane transmission of cytokine signals and crucial in the development of various diseases (17, 18). Evidence suggests a strong association between the JAK/STAT pathway and tumors' resistance to drugs and evasion of the immune system (19). However, the JAK family is composed of four JAKs and the STAT family is composed of seven STATs, which contain multiple signaling pathways, and which JAK/STAT mediates $CD8^+$ T cells anti-tumor immunity in renal cancer remains unknown. Therefore, in this research, the impact of various JAK/STAT inhibitors on mouse spleen-derived $CD8^+$ T cells proliferation and activation was investigated. Building on this foundation, we delved deeper into the potential of blocking the JAK1/STAT1 signaling pathway to enhance $CD8^+$ T cell immunity and impede renal cancer advancement. This research aimed to clarify the potential mechanisms behind $CD8^+$ T cell anti-tumor immunity, with the goal of offering new insights for treating renal cancer in clinical practice.

MATERIALS AND METHODS

Cell Culture

Mouse renal cancer cells (Renca) were obtained from Pricella Biotechnology Co., Ltd. (Wuhan, Hubei, China). RPMI-1640 medium (Gibco, Grand Island, NY, USA) was enriched with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin double antibody (Gibco) served as the cell culture medium. The cell culture temperatures were 37°C with 5% CO_2. 1:3 digestion was passaged, and the solution was changed every 2-3 d.

$CD8^+$ T Cell Acquisition and Characterization

BALB/c nude mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Following the euthanization of the mice, their

spleens were extracted and spleen single cell suspensions were prepared. This experiment was approved by the Laboratory of Animal Ethics Committee of Hangzhou First People's Hospital. Mouse $CD8^+$ T cells were sorted in mouse spleen using the Mouse $CD8^+$ T Cell Sorting Kit (11417D, Invitrogen, Carlsbad, CA, USA). Approximately 1×10^6 $CD8^+$ T cells were collected in EP tubes, subsequently rinsed with PBS twice, then suspended again in PBS. We added PE-labeled CD8 and FITC-labeled CD3 antibodies and incubated them for 30 min at 4°C. Subsequently, the cells were rinsed twice with PBS buffer, and the purity of the sorted cells was assessed using flow cytometry.

Cell Transfection

Silencing plasmids were used to inhibit the expression of STAT2 and STAT4 inhibitors because they could not be purchased. RiboBio Co., Ltd. (Guangzhou, Guangdong, China) synthesized STAT2 silencing plasmid (si-STAT2), STAT4 silencing plasmid (si-STAT4), and their respective negative control (si-NC). The plasmids mentioned above were introduced into $CD8^+$ T cells via the Lipofectamine 3000 (Invitrogen), and the transfection method was described in the instruction manual.

CCK-8 assay

$CD8^+$ T cells were inoculated in 24-well plates pre-coated with 1 μg/mL CD3 Antibody (14-0037-82, Invitrogen), and then 1 μg/mL CD28 Antibody (14-0289-82, Invitrogen) was added to stimulate $CD8^+$ T cell proliferation. Then, we added JAK1 inhibitor Solcitinib (100 nM, HY-16755, MedChemExpress, Monmouth Junction, NJ, USA), JAK2 inhibitor CEP-33779 (10 nM, HY-15343, MedChemExpress), JAK3 inhibitor Tofacitinib (10 nM, HY-40354, MedChemExpress), Tyk2 inhibitor Deucravacitinib (10 nM, HY-117287, MedChemExpress), STAT1 inhibitor Fludarabine (1 μM, HY- B0069, MedChemExpress), STAT3 inhibitor Niclosamide (100 nM, HY-B0497, MedChemExpress), STAT5 inhibitor AC-4-130 (1 μM, HY-124500, MedChemExpress), or STAT6 inhibitor AS1517499 (100 nM, HY-100614, MedChemExpress) and cultured them for 48 h. Each well added 10% CCK-8 reagent (C0038, Beyotime, Shanghai, China). After being incubated for 2 h at 37°C in an incubator protected from light, the OD_{450} value was measured utilizing a microplate reader.

CFSE Staining

$CD8^+$ T cells from different treatments were diluted in PBS, CFSE solution (C0051, Beyotime) was added to cell suspension (1×10^6 cells/mL), and stained for 10 min at 37°C. We added 5 times the volume of complete medium, mixed well, and terminated the staining. Then, we centrifuged it for 10 min at 250 g, discarded the supernatant, and subsequently rinsed with PBS twice to remove any unbound CFSE solution. We resuspended it with PBS buffer, took a small amount of drops and added it to the 96-well plate, and observed it under the fluorescence microscope to see if the labeling is successful. The CFSE pre-stained $CD8^+$ T cells were taken and added to the well plates pre-coated with CD3 and CD28, and after 48 h of culture, flow cytometry identified the $CD8^+$ T cells proliferation.

ELISA

$CD8^+$ T cells were inoculated into well plates pre-coated with CD3 antibody and CD28 antibody, to which different inhibitors were added for treatment, then the cell culture supernatants were collected 48 h later. IFN-γ ELISA Kit (PI508, Beyotime) was utilized to quantify the level of IFN-γ in supernatants. The supernatant was transferred to ELISA well plate and incubated for 2 h. Following three washes with PBS, IFN-γ antibody was added and incubated for 1 h. After being washed 3 times with PBS, Streptavidin was added and incubated for 20 min away from light, subsequently TMB solution was added and incubated for 30 min away from light. After

adding and thoroughly mixing termination solution, the OD_{450} value was measured and used to calculate the concentration.

Annexin V/PI Staining

After being stimulated by JAK inhibitors or STAT inhibitors for 48 h, $CD8^+$ T cells were transferred to the top chamber of Transwell and co-cultured with Renca cells in the bottom chamber. Renca cells were rinsed with PBS twice after being co-cultured for 48 h, and then gently mixed with Binding Buffer (500 μL). Following that, Annexin-V-FITC (5 μL, HY-K1073, MedChemExpress) and propidium iodide (5 μL, ST1569, Beyotime) were introduced and left to incubate for 15 min in the dark. Flow-specific super-sampling tubes were used to transfer the samples, and apoptosis was then identified through flow cytometry.

Calcein-AM/PI Staining

Cell survival was assessed using the Calcein-AM/PI Staining Kit (C2015M, Beyotime). We removed the Renca cell culture and washed it once with PBS. Then we dispensed Calcein AM/PI solution (100 μL), mixed it by blowing, and let it incubate for 30 min in the dark at 37°C. Following two PBS rinses, the cells were resuspended, examined for survival using fluorescence microscopy, and the survival rate was determined.

Immunofluorescence

Renca cells were inoculated in culture dishes after different treatments at 2×10^4/mL. Once the cells reached a density of 50% to 60%, the culture fluid in the dishes was aspirated, and exposed to 4% paraformaldehyde (P1110, Solarbio) for 20 min. The cells were covered with drops of 0.3% Tritonx-100 (Sigma-Aldrich, St. Louis, MO, USA) and permeabilized for 10 min, then covered with 5% bovine serum albumin (Sigma-Aldrich) for 30 min. Then, they were placed at 4°C for an overnight incubation with proliferating cell nuclear antigen (PCNA) primary antibody (ab29, 1:100, Abcam, Cambridge, MA, USA), Bax primary antibody (ab53154, 1:100, Abcam), Cleaved caspase-3 primary antibody (ab32024, 1:200, Abcam), matrix metalloproteinase-2 (MMP-2) primary antibody (ab97779, 1:200, Abcam) or MMP-9 primary antibody (ab76003, 1:300, Abcam). On the following day, after adding FITC-labeled goat anti-rabbit IgG (1:10,000), the mixture was kept in the dark and incubated for 1 h. Then we added DAPI staining solution (Solarbio) and incubated it for 10 min away from light, and we observed the development by fluorescence microscope within 1 h after sealing.

Wound Healing Assay

Renca cells were routinely digested and collected after different treatments, and 1 mL of cell suspension (1×10^6/mL) was aspirated with a sterile pipette and inoculated into 6-well plates that drew horizontal lines in advance. After the cells were completely adherent and grew to more than 80% density, a 20 μL sterile pipette was used to gently draw a vertical line perpendicularly to the horizontal line on the underside of the cell culture plate. Following, the floating cells were rinsed twice and resupplied with serum-free medium. The healing of the scratches was observed at 0 and 24 h after scratching and the cell migration rate was calculated.

Transwell

The Matrigel (Sigma-Aldrich) was thinned out using serum-free RPMI-1640 medium, and then 100 μL of the thinned gel was placed in each Transwell chamber (Corning, Tewksbury, MA, USA) and left in the incubator overnight. The following day, the remaining liquid in the chambers was aspirated and serum-free medium was added to hydrate the basement membrane. The top chamber was filled with cell suspension (200 μL, 1×10^5 cells/mL), followed by the addition of the right amount of RPMI-1640 medium added 10% fetal bovine serum in the lower section, and subsequently incubated for 24 h. The matrix gel and cells that had

not crossed the membrane were wiped away, then exposed to 4% paraformaldehyde for 30 min, dyed with 0.1% crystal violet (C0121, Beyotime) for 5 min, and rinsed twice with PBS. Randomly selected fields of view in each well were photographed with an inverted fluorescence microscope, and the amount of invasion cells was counted.

Renca cells were routinely digested, collected, and suspended in serum-free cultures from different treatments. The top section was filled with cell suspension (200 μL, 1×10^5 cells/mL), followed by the addition of the right amount of RPMI-1640 medium which added 10% fetal bovine serum in the lower section, and subsequently incubated for 24 h. The Transwell was taken out, rinsed twice with PBS, exposed to 4% paraformaldehyde, subsequently dyed with 0.1 % crystal violet. We carefully removed the unmigrated cells from the top part of the chambers using a cotton swab. A random field of view was selected, and the number of migratory cells was counted after being photographed under an inverted microscope.

In Vivo Hormonal Tumor Assay

Subcutaneous injections of Renca cell suspension (0.2 mL, 2×10^6 cells/mice) were administered to Balb/c nude mice, then randomly divided into the control, JAK1 inhibitor, and STAT1 inhibitor groups. The administration started on day 6 after inoculation, with 30 mg/kg Solcitinib injected intraperitoneally in the JAK1 inhibitor group, 0.8 mg/kg Fludarabine injected intraperitoneally in the STAT1 group, and an equal amount of substrate fluid injected in the control group. The drug was administered once a day for 7 d. Subcutaneous tumors were measured using a vernier caliper on days 6, 9, 12, 15, 18, and 21. The nude mice were then anesthetized and euthanized on day 21, and the tumors were removed and weighed. In addition, nude mouse tumor single-cell suspensions were generated to analyze $CD8^+$ T cells infiltration through flow cytometry.

Immunohistochemistry

Tumor tissues were exposed to 4% paraformaldehyde, routinely dehydrated, embedded and prepared as paraffin sections (4-5 μm in thickness). Xylene (Sigma-Aldrich) deparaffinization, gradient ethanol hydration, and antigen were repaired by microwave oven, and endogenous peroxidase inactivation by 3% H_2O_2. Following a 30-min blocking period, the blocking solution of bovine serum albumin (Sigma-Aldrich) was shaken off, and placed at 4°C for an overnight incubation with CD8 primary antibody (ab217344, 1:2000, Abcam), perforin primary antibody (1:100), or Granzyme B primary antibody (1:100). On the following day, the secondary antibody was left to incubate for 1 h before the color was developed in DAB solution (Solarbio), and the reaction was stopped in pure water. Mayer hematoxylin (Sigma-Aldrich) was used for re-staining, and the film was sealed with neutral balsam. It was observed under an inverted microscope and photographed.

TUNEL Staining

Paraffin sections underwent deparaffinization using xylene followed by hydration using ethanol gradient. DNase-free proteinase K (20 μg/mL, ST532, Beyotime) was slowly added, followed by a 30-min incubation and three washes with PBS. TUNEL assay solution (C1086, Beyotime) was carefully added dropwise and incubated for 1.5 h away from light. Then DAPI staining solution was added and incubated for 10 min. It was observed and photographed under inverted fluorescence microscopy.

Western Blot

$CD8^+$ T cells, Renca cells, or nude mice tumor tissues were placed in RIPA lysate (P0013B, Beyotime) to obtain the total proteins, and the protein content was assayed utilizing the BCA Kit (P0012, Beyotime). Following electrophoresis, the proteins were moved to PVDF membranes (Invitrogen), then closed with skimmed milk for 2 h. After rinsing the membranes, they were placed at

4°C for an overnight incubation with perforin primary antibody (ab16074, 1:1000, Abcam), Granzyme B primary antibody (MA1-80734, 1:1000, Invitrogen), PCNA primary antibody (ab29, 1:100, Abcam), Bax primary antibody (ab53154, 1:1000, Abcam), Cleaved caspase-3 primary antibody (ab32024, 1:500, Abcam), MMP-2 primary antibody (ab97779, 1:1000, Abcam), MMP-9 primary antibody (ab76003, 1:1000, Abcam), p-JAK1 primary antibody (44-422G, 1:1000, Invitrogen), JAK1 primary antibody (PA5-79546, 1:1000, Invitrogen), p-STAT1 primary antibody (33-3400, 1:100, Invitrogen), STAT1 primary antibody (AHO0832, 1:500, Invitrogen), or β-actin primary antibody (MA1-140, 1:5000, Invitrogen). On the next day, goat anti-rabbit IgG secondary antibody (31460, 1:10,000, Invitrogen) was incubated after washing the membrane 3 times, then it was developed, and exposed. After chemiluminescence color development, a gel imaging system was utilized to scan it. The gray value of each protein band was obtained after processing the image with Image J software, and the level of each target protein was evaluated with β-actin as the reference.

Statistical Analysis

Every experiment was conducted with a minimum of three times, with the outcomes noted as the mean value along with the respective standard deviation. SPSS 26.0 software (IBM SPSS Statistics 26) was utilized to process and analyze the data statistically. Student's T-test was utilized to evaluate the

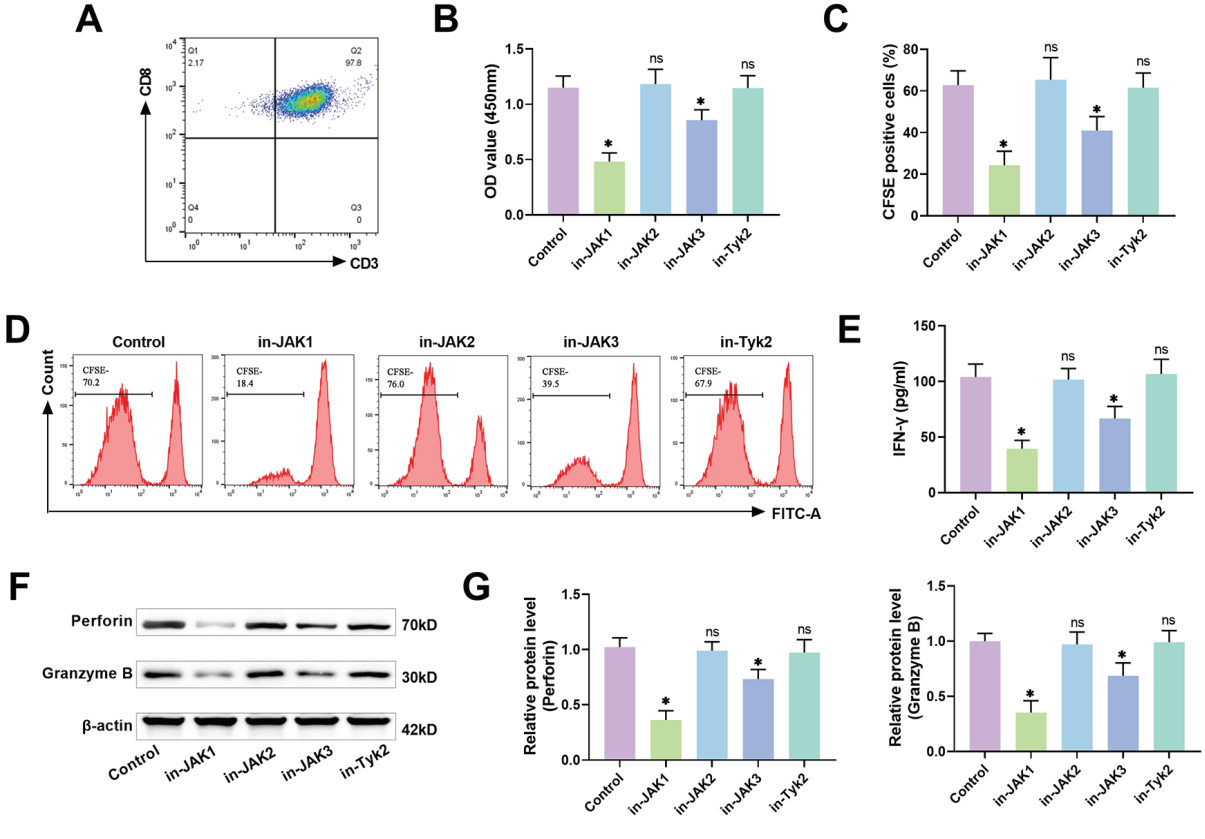

Fig. 1. Effect of JAKs on the proliferation and activation of CD8⁺ T cells. JAK: janus kinase; (A) Mouse spleen-derived CD8⁺ T cells were purified using immunomagnetic beads, and their purity was assessed by flow cytometry. (B) CD8⁺ T cells were inoculated into well plates previously coated with CD3 antibody and CD28 antibody, treated with JAKs inhibitor for 48 h, and cell viability was assessed utilizing CCK-8 assay. (C-D) CD8⁺ T cells were gathered post various treatments, labeled with CFSE, and their proliferation was measured using flow cytometry. (E) The IFN-γ content in the supernatants of CD8⁺ T cells post various treatments was measured using an IFN-γ ELISA Kit. (F-G) Measuring the levels of perforin and Granzyme B through Western blot analysis. (*p<0.05 vs control, ns p≥0.05 vs control)

distinctions between the two groups. To evaluate differences among multiple groups, we conducted a one-way analysis of variance. Prism software (Graphpad 9.0) was utilized for plotting. $P<0.05$ signified that there was a significant distinction.

RESULTS

Impact of JAKs on the Proliferation and Activation of CD8$^+$ T Cells

CD8$^+$ T cells were extracted from mouse spleen tissue using immunomagnetic beads, followed by verification of the purity of cells through flow cytometry. The findings indicated that the purity of CD8$^+$ T cells could reach 97%, which satisfied the requirements for subsequent experiments (Fig. 1A). Western blot results revealed that CD8$^+$ T cells treatment with JAK1, JAK2, JAK3, and Tyk2 inhibitors all led to a notable decline in the phosphorylation levels of the corresponding JAKs, providing a basis for subsequent experiments (Figs. S1A-S1D). CD8$^+$ T cell viability was assessed utilizing CCK-8 assay to determine the impact of different JAK inhibitors. The findings demonstrated that CD8$^+$ T cell viability notably decreased by JAK1 and JAK3 inhibitors, whereas JAK2 and Tyk2 inhibitors did not have a notable impact on CD8$^+$ T cell viability (Fig. 1B). CFSE staining findings indicated a notable reduction in the proliferative ability of CD8$^+$ T cells with the use of JAK1 and JAK3 inhibitors, while JAK2 and Tyk2 inhibitors did not exhibit a notable impact on cell proliferation (Figs. 1C-1D). Not only that, ELISA results revealed that the secretion of activation marker IFN-γ was significantly reduced by JAK1 and JAK3 inhibitors, while JAK2 and Tyk2 inhibitors did not impact CD8$^+$ T cells (Fig. 1E). The tumor-killing ability of CD8$^+$ T cells relies heavily on the activity of perforin and Granzyme B (20). Additionally, JAK1 and JAK3 inhibitor treatments significantly decreased perforin, Granzyme B levels, while treatment with JAK2 and Tyk2 inhibitors did not result in any noticeable change in the levels of perforin and Granzyme B (Figs. 1F-1G). The above results indicated that JAK1 and JAK3 inhibitors hindered CD8$^+$ T cells proliferation and activation, and JAK1 inhibitor had the most significant effect.

Effect of STATs on the Proliferation and Activation of CD8$^+$ T Cells

Next, we investigated the effects of different STAT inhibitors on CD8$^+$ T cell proliferation and activation. Western blot findings demonstrated that the phosphorylation levels of different STATs notably decreased in CD8$^+$ T cells following treatment with corresponding STATs inhibitors, and silencing of STAT2 or STAT4 significantly down-regulated the levels of the corresponding STATs, which met the requirements of the subsequent experiments (Figs. S1E-S1J). By CCK-8 and CFSE assay, the viability and proliferation of CD8$^+$ T cells were significantly reduced by inhibitors of STAT1 and STAT5, while inhibitors of STAT3 had the opposite effect, whereas STAT6 inhibitors and silencing STAT2 or STAT4 did not lead to a significant change (Figs. 2A-2G). ELISA results indicated that IFN-γ level in CD8$^+$ T cells was notably reduced by STAT1 and STAT5 inhibitor treatments, and STAT3 inhibitor treatment notably elevated IFN-γ level, whereas STAT6 inhibitor and silencing STAT2 or STAT4 did not produce a notable impact (Figs. 2H-2J). Not only that, Western blot findings revealed a substantial decrease in perforin and Granzyme B levels following treatment with STAT1 and STAT5 inhibitors, while inhibitors of STAT3 had the opposite effect, and STAT6 inhibitor and silencing STAT2 or STAT4 had no significant effect (Figs. 2K-2N). The above results indicated that STAT1 and STAT5 inhibitor treatment could hinder CD8$^+$ T cells proliferation and activation, and the STAT1 inhibitor had the most notable effect. Therefore, to delve deeper into the action mechanism of CD8$^+$ T cells, we selected JAK1 inhibitors and STAT1 inhibitors for the subsequent experiments.

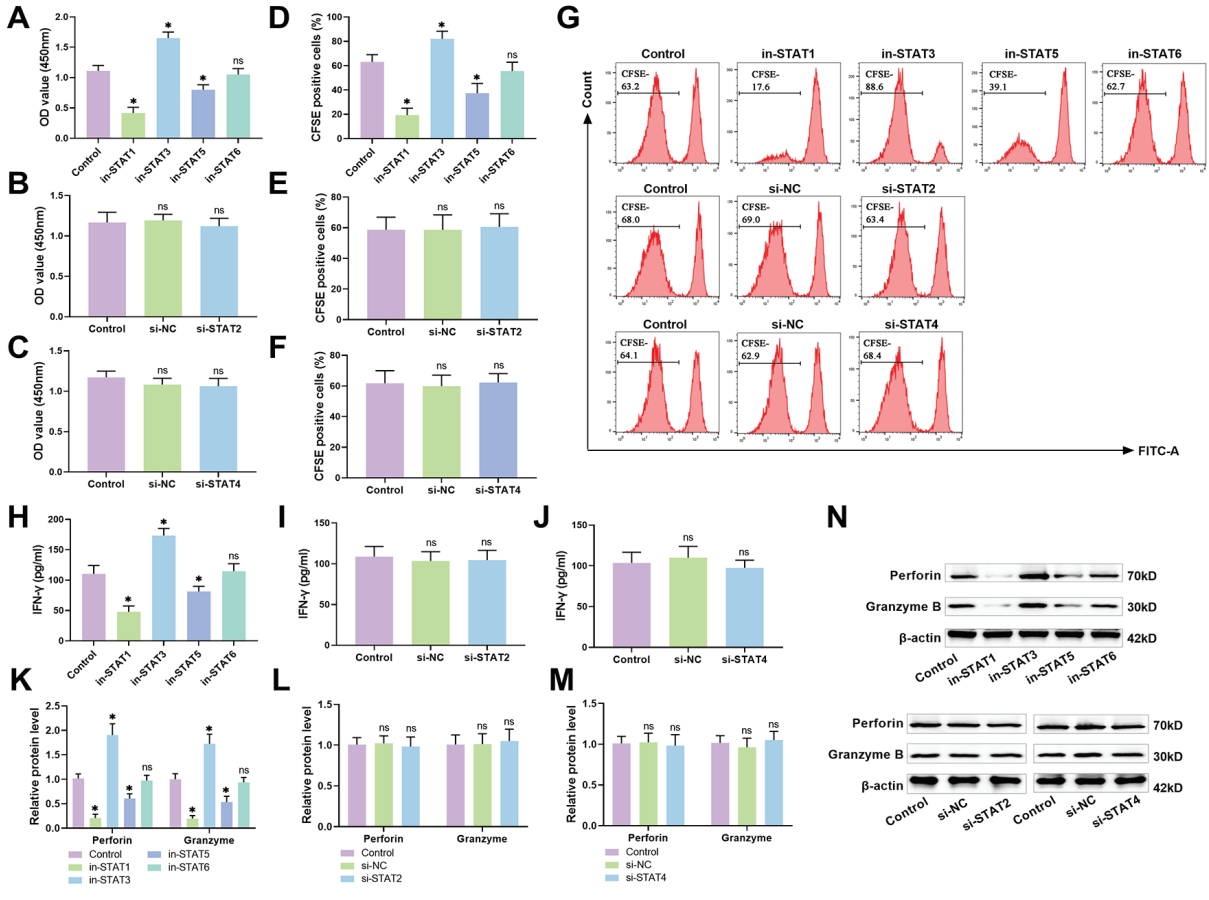

Fig. 2. Effect of STATs on proliferation and activation of CD8⁺ T cells. (A-C) CD8⁺ T cells were treated with STATs inhibitors for 48 h, STAT2 or STAT4 was knocked down, and CD8⁺ T cells viability was measured utilizing CCK-8 assay. (D-G) CD8⁺ T cells were gathered post various treatments, labeled with CFSE, and their proliferation was measured using flow cytometry. (H-J) The IFN-γ content in the supernatants of CD8⁺ T cells post various treatments was measured using an IFN-γ ELISA Kit. (K-N) Measuring the levels of perforin and Granzyme B through Western blot analysis. (*p<0.05 vs control, ns p≥0.05 vs control). STAT: signal transducer and activator of transcription

JAK1/STAT1 Signaling Pathway Mediates CD8⁺ T Cell Killing of Renca Cells

To investigate whether CD8⁺ T cells exert killing effects on Renca cells via the JAK1/STAT1 pathway, we co-cultured Renca cells with CD8⁺ T cells after 48 h of stimulation by JAK1 inhibitor or STAT1 inhibitor. By flow cytometry, co-culture with CD8⁺ T cells led to a markedly higher rate of apoptosis in Renca cells, significantly ameliorated by treatment with JAK1 or STAT1 inhibitors (Figs. 3A and 3C). In addition, Calcein-AM/PI staining results indicated a marked decrease in the viability of Renca cells when co-cultured with CD8⁺ T cells, whereas cell survival significantly increased after treatment with JAK1 or STAT1 inhibitors (Figs. 3B and 3D). Western blot results demonstrated a notable decline in proliferative protein PCNA level and a notable increase in apoptotic proteins Bax and Cleaved caspase-3 levels in Renca cells following treating with CD8⁺ T cells, whereas JAK1 or STAT1 inhibitors treatment attenuated the impact of CD8⁺ T cells (Figs. 3E-3F). Not only that, immunohistochemical results showed that co-culture with CD8⁺ T cells markedly reduced PCNA level and notably increased Bax and Cleaved caspase-3 level, whereas JAK1 or STAT1 inhibitors attenuated the effect of CD8⁺ T cells (Fig. 3G). These findings further indicate that CD8⁺ T cells exert killing effects on Renca cells through the JAK1/STAT1 pathway.

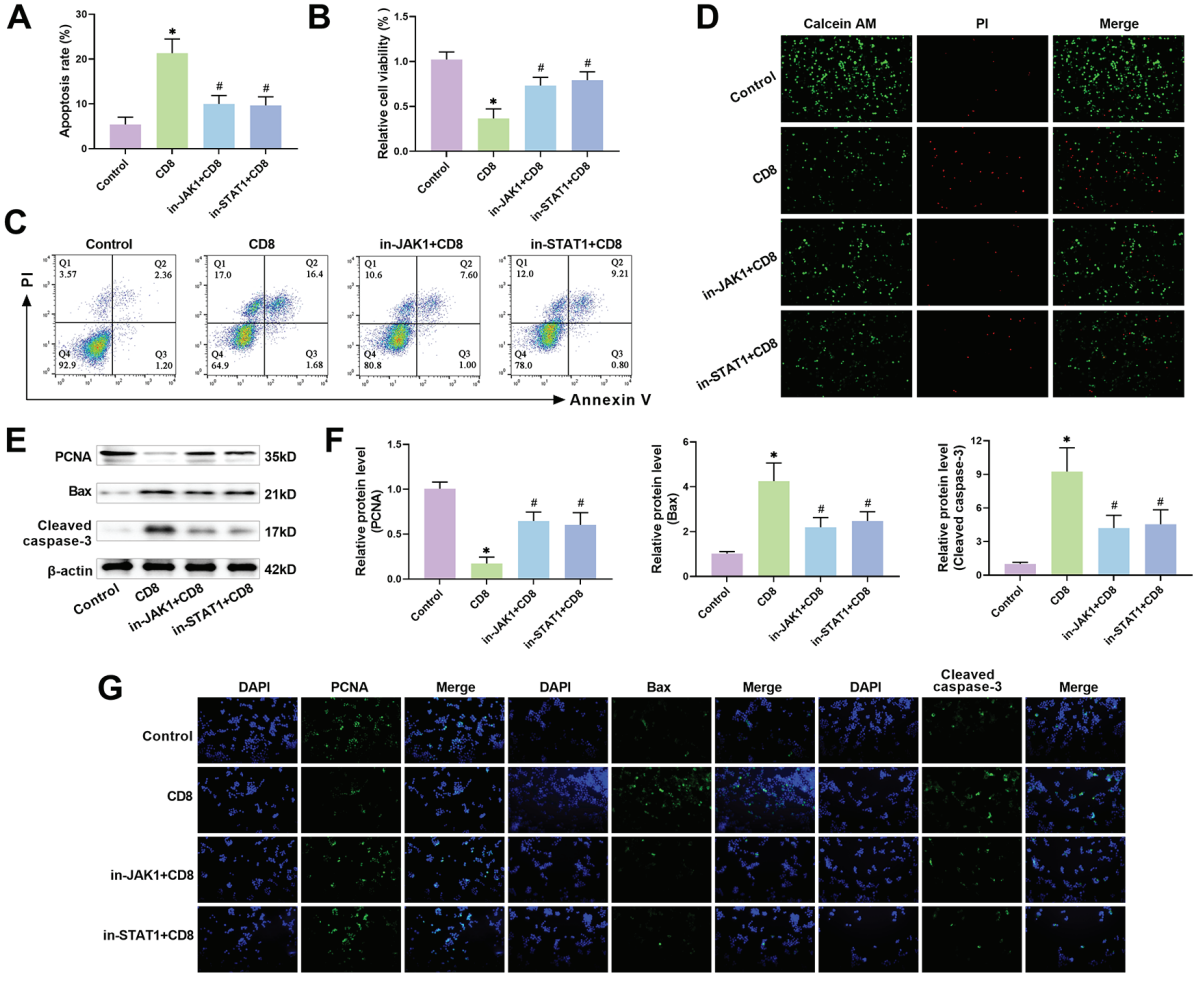

Fig. 3. JAK1/STAT1 signaling pathway mediates CD8⁺ T cell killing of Renca cells. (A) CD8⁺ T cells stimulated by JAK1 inhibitor or STAT1 inhibitor were co-cultured with Renca cells, and the apoptosis rate of Renca cells was determined utilizing flow cytometry. (B) The survival of Renca cells post-treatment with various inhibitors was determined utilizing the Calcein AM/PI method. (C) Flow cytometry images of the Annexin V/PI method. (D) Calcein AM/PI fluorescence staining images. (E-F) Examining the proliferative protein PCNA, apoptotic protein Bax, and Cleaved caspase-3 level in Renca cells through Western blot. (G) Immunofluorescence detection of localized expression of PCNA, Bax and Cleaved caspase-3 in Renca cells. (*p<0.05 vs control, #p<0.05 vs CD8). JAK: janus kinase, STAT: signal transducer and activator of transcription, PCNA: proliferating cell nuclear antigen

JAK1/STAT1 Signaling Pathway Mediates CD8⁺ T Cell Inhibition of Renca Cell Migration, Invasion

Then, we further explored whether CD8⁺ T cells inhibit the malignant biological behavior of Renca cells by regulating the JAK1/STAT1 signaling pathway. Wound healing assay findings demonstrated a marked decrease in the migration rate of Renca cells after treatment with CD8⁺ T cells, whereas treatment with JAK1 or STAT1 inhibitors notably enhanced migration ability (Figs. 4A and 4G). Transwell assay results indicated that co-culture with CD8⁺ T cells notably declined in terms of the number of migrated (Figs. 4B and 4H) and invaded (Figs. 4C and 4I) Renca cells, while the inhibitory impacts of CD8⁺ T cells on cell migration and invasion were attenuated by JAK1 or STAT1 inhibitor treatment. Additionally, MMP-2 and MMP-9 level in Renca cells markedly reduced after treatment with CD8⁺ T cells, whereas treatment with JAK1 or STAT1 inhibitors significantly increased both MMP-2 and MMP-9 level (Figs. 4D-4F). Immunofluorescence results also indicated a notable decrease in MMP-2 and MMP-9 level in Renca cells when co-cultured with

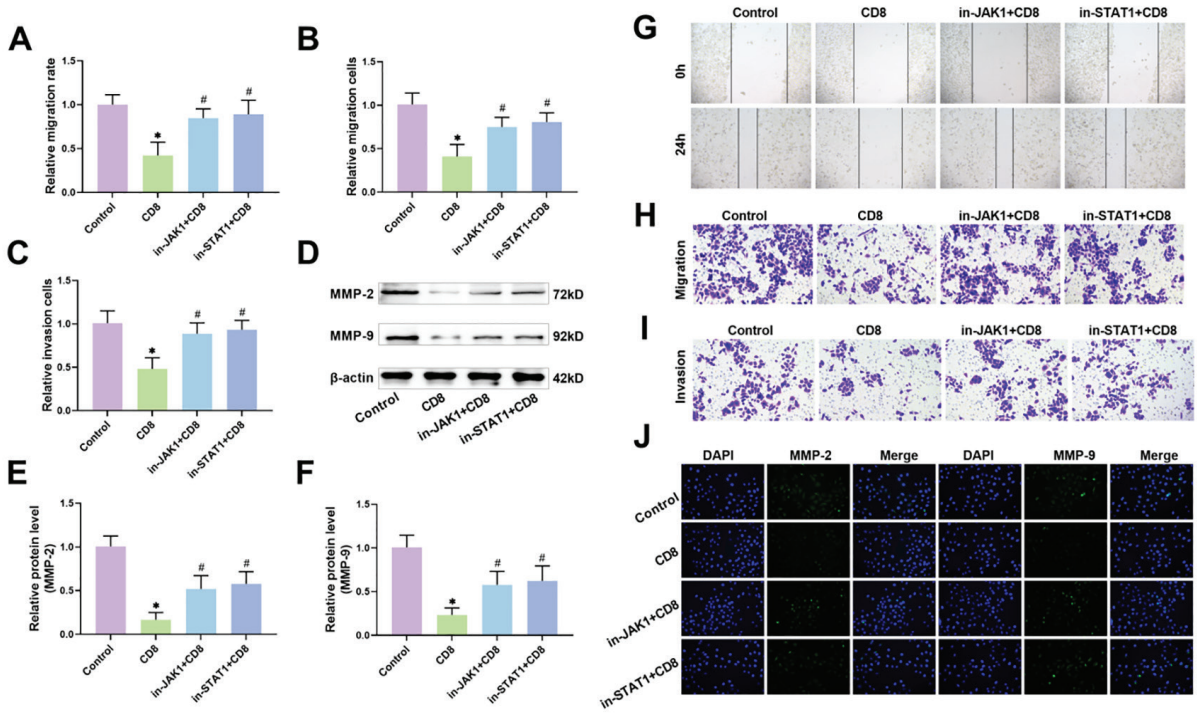

Fig. 4. JAK1/STAT1 signaling pathway mediates CD8+ T cell inhibition of Renca cell migration, invasion. (A) After co-cultured with CD8+ T cells, Renca cells migration rate was measured utilizing wound healing assay. (B-C) The number of migrating cells (B) and invading cells (C) of Renca cells after different treatments were detected by Transwell. (D-F) Examining the MMP-2 (E) and MMP-9 (F) protein level in Renca cells through Western blot. (G) Wound healing assay cell migration images. (H) Transwell cell migration images. (I) Transwell cell invasion images. (J) Immunofluorescence detection of localized expression of MMP-2 and MMP-9 in Renca cells. (*p<0.05 vs control, #p<0.05 vs CD8). JAK: janus kinase, STAT: signal transducer and activator of transcription

CD8+ T cells, whereas treatment with JAK1 or STAT1 inhibitors attenuated this impact, consistent with the Western blot results (Fig. 4J). These findings confirmed that CD8+ T cells hindered the migration and invasion of Renca cells via the JAK1/STAT1 pathway.

JAK1/STAT1 Signaling Pathway In Vivo Mediates Anti-tumor Immunity of CD8+ T Cells

Finally, we established a subcutaneous graft tumor model according to the flowchart shown in Fig. 5A, to explore whether CD8+ T cells exert anti-tumor immunity via the JAK1/STAT1 pathway *in vivo*. The JAK1 inhibitor treatment markedly declined phosphorylated JAK1 and STAT1 levels, while the STAT1 inhibitor treatment decreased the phosphorylation of STAT1, it did not show a significant impact on JAK1, further confirming that STAT1 is located downstream of JAK1 (Figs. 5B-5C). Flow cytometry results indicated that either JAK1 or STAT1 inhibitor treatment markedly reduced CD8+ T cells infiltration in tumor tissues (Figs. 5D-5E). Not only that, immunohistochemical results showed that either JAK1 or STAT1 inhibitor treatment significantly reduced CD8, perforin and Granzyme B levels in tumor tissues (Fig. 5F). Intervention with JAK1 or STAT1 inhibitors resulted in significantly higher tumor volume and weight in mice *in vivo* (Figs. 5G-5I). By TUNEL staining, we observed that either JAK1 or STAT1 inhibitor intervention significantly inhibited apoptosis in tumor cells (Figs. 5J-5K). The above results indicated that inhibiting the JAK1/STAT1 pathway reduced the antitumor impacts of CD8+ T cells, suggesting that CD8+ T cells exert antitumor immunity *in vivo* through the JAK1/STAT1 pathway.

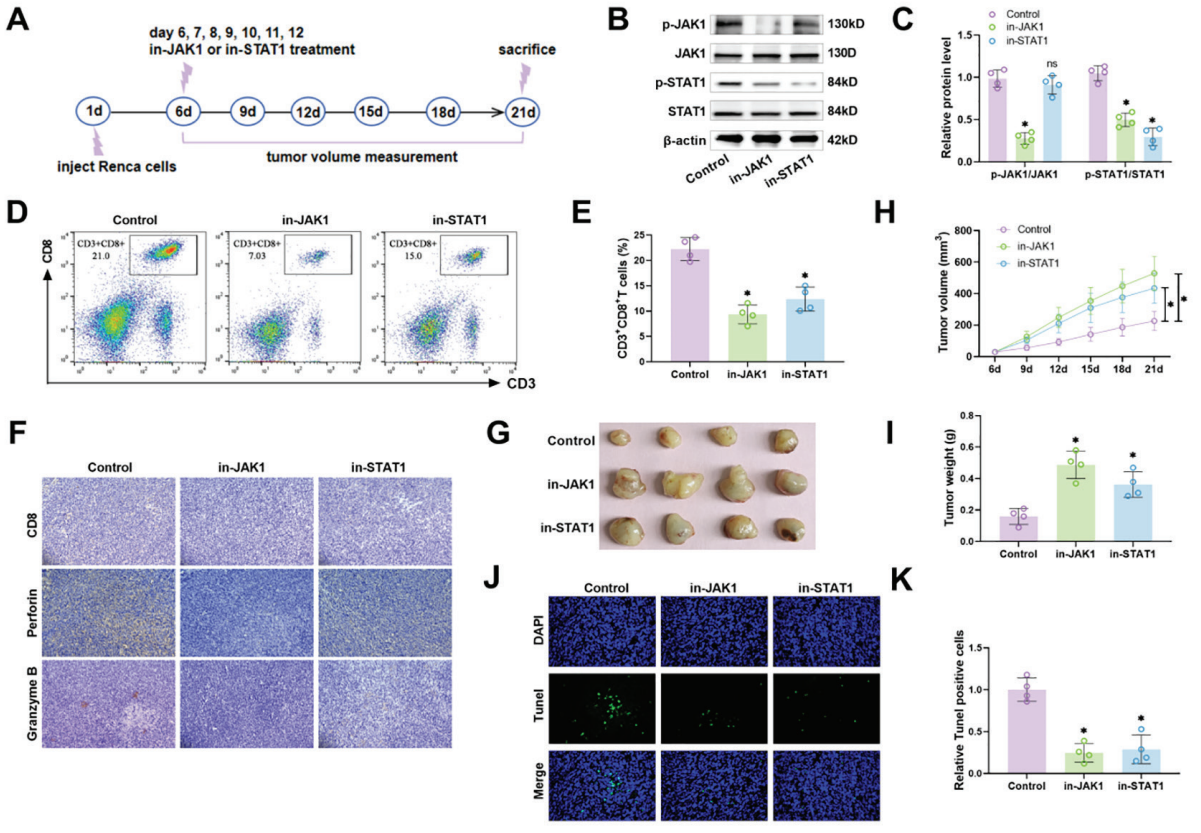

Fig. 5. JAK1/STAT1 signaling pathway *in vivo* mediates anti-tumor immunity of CD8⁺ T cells. (A) Flowchart of the construction of subcutaneous transplantation tumor model in nude mice. After subcutaneous injection of Renca cell suspension in nude mice, they were divided into JAK1 inhibitor, STAT1 inhibitor, and the control group. The administration started on day 6 after inoculation, with 30 mg/kg Solcitinib injected intraperitoneally in the JAK1 inhibitor group, 0.8 mg/kg Fludarabine injected intraperitoneally in the STAT1 group, and an equal amount of substrate fluid injected in the control group. Administration of the drug once a day for 7 days. (B-C) Examining the JAK1/STAT1 pathway protein level in tumor tissues after treatment with different inhibitors through Western blot. (D-E) Preparation of single-cell suspensions of tumor tissues, and CD8⁺ T cells infiltration in the tumor was examined utilizing flow cytometry. (F) Immunohistochemical detection of CD8⁺ T cell infiltration and localized expression of perforin and Granzyme B in renal cancer tissues. Nude mice were executed after anesthesia on d 21, tumor tissues were excised, photographed (G) and weighed (I). (H) The subcutaneous tumor dimensions were assessed on days 6, 9, 12, 15, 18, and 21 using a vernier caliper to determine the tumor volume. (J-K) The rate of apoptosis in tumor tissues was detected and quantified by TUNEL staining. (*p<0.05 vs control). JAK: janus kinase, STAT: signal transducer and activator of transcription

DISCUSSION

In recent years, immunotherapy has developed into the fourth anti-tumor treatment after surgery, chemotherapy and radiotherapy (21). Immunotherapy mainly refers to the treatment of tumors through the regulation and enhancement of the body's immune function (22). During tumorigenesis and progression, T cells, the primary anti-tumor immune cells, have demonstrated superior effectiveness in enhancing the survival outcomes of patients with melanoma (23), breast cancer (24), colorectal cancer (25), and other tumors. CD8⁺ T cells, an essential group within T cells, are responsible for releasing killing factors including perforin and Granzyme B. They also secrete a variety of cytotoxic factors in the vicinity of target cells, promoting immune effector functions (26, 27). CD8⁺ T cells can secrete IFN-γ, a major cytokine that directly contributes to killing tumor cell (28). In addition to promoting senescence and autophagy-mediated apoptosis of tumor cells,

IFN-γ further heightens the killing capacity of activated $CD8^+$ T cells, and is a key mediator of anti-tumor immunity and a marker of $CD8^+$ T cell activation (29). Isolation of highly pure $CD8^+$ T cells is a prerequisite for their in-depth study. In this study, $CD8^+$ T cells from mouse spleens were separated using an immunomagnetic bead method, achieving a purity of 97% and showing high levels of IFN-γ, perforin, and Granzyme B, which meets the experimental requirements.

In the immune response of T cells, the JAK/STAT pathway is crucial for detecting tumor cells and their ability to evade immune monitoring (30, 31). Ravindran et al. showed that epigallocatechin gallate inhibits JAK/STAT signaling, which in turn activates T cells and enhances anti-tumor immunity (32). Pan et al. discovered that EHBP1L1 has the ability to shield JAK1 from degradation, activates JAK1/STAT1/PD-L1 pathway, and thus inhibits immune escape in renal cancer (33). In this research, we treated $CD8^+$ T cells with different JAK and STAT inhibitors, and found that JAK1 and STAT1 inhibitors significantly hindered the proliferation and activation of $CD8^+$ T cells and decreased the level of IFN-γ, perforin, and Granzyme B. This indicates the crucial role of JAK1/STAT1 in $CD8^+$ T cells anti-tumor immunity.

An increasing number of studies indicate that the tumor microenvironment (TME) is comprised of stromal cells, immune cells, and various cytokines that have infiltrated the area surrounding the tumors, providing a relatively suitable environment for the development, growth and metastasis of tumor (34, 35). TME limits $CD8^+$ T cells immune infiltration, which results in tumor cells evading immune surveillance, thus hindering the anti-tumor immunity of $CD8^+$ T cells (36). Tumor-infiltrating $CD8^+$ T cells are an important part of blocking tumor progression, and to simulate this infiltration in renal cancer, we conducted a co-culture experiment with $CD8^+$ T and Renca cells. The research indicates that MMP-2 and MMP-9 are crucial players in degrading the extracellular matrix, facilitating cell migration and invasion (37). We found that after co-culture with $CD8^+$ T cells, Renca cells showed significantly higher apoptosis rate, significantly down-regulated MMP-2 and MMP-9 levels, and significantly declined survival, migration and invasion ability. Notably, inhibiting the JAK1/STAT1 pathway attenuated the inhibitory impact of $CD8^+$ T cells on the malignant biological behaviors of Renca cells, suggesting $CD8^+$ T cells exert anti-tumor immune impact via the JAK1/STAT1 pathway. Additionally, we found that inhibiting the JAK1/STAT1 pathway attenuated $CD8^+$ T cells infiltration *in vivo* and promoted the malignant progression of renal cancer.

In this research, and by treating $CD8^+$ T cells with different JAK/STAT inhibitors, we found that JAK1 and STAT1 inhibitors inhibited the proliferation and activation of

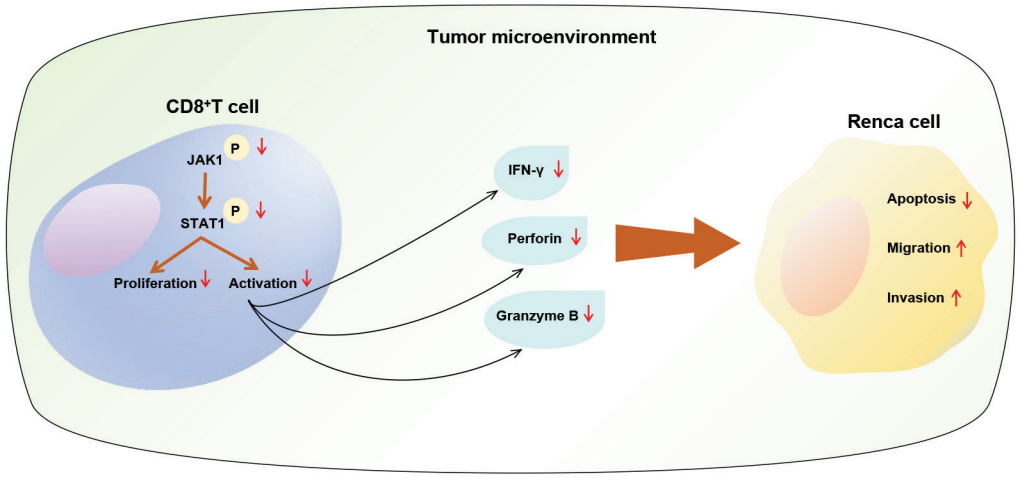

Fig. 6. Mechanism diagram

$CD8^+$ T cells, and decreased the level of IFN-γ, perforin and Granzyme B. Furthermore, the malignant biological behavior of Renca cells was suppressed following the co-culture of $CD8^+$ T cells with Renca cells, whereas inhibiting the JAK1/STAT1 pathway attenuated $CD8^+$ T cells anti-tumor impact (Fig. 6). These results offer theoretical support for tumor immunotherapy by targeting the JAK1/STAT1 pathway to strengthen the anti-tumor effectiveness of $CD8^+$ T cells.

JAK1 and STAT1 inhibitors inhibited the proliferation and activation of $CD8^+$ T cells, and decreased IFN-γ, perforin and Granzyme B levels. The malignant biological behavior of Renca cells was suppressed following the co-culture of $CD8^+$ T cells with Renca cells, whereas inhibiting the JAK1/STAT1 pathway attenuated $CD8^+$ T cells anti-tumor impact.

CONCLUSION

In conclusion, the JAK/STAT signaling pathway is crucial for regulating anti-tumor immunity response of $CD8^+$ T cells in renal cancer. Importantly, JAK1 and STAT1 inhibitors attenuate the antitumor effects of $CD8^+$ T cells, indicating that JAK1 and STAT1 are crucial players in regulating immune response. This study reveals the potential action mechanism of the JAK1/STAT1 pathway in modulating the anti-tumor immunity of $CD8^+$ T cells, offering a valuable insight for the immunotherapy in renal cancer. Nevertheless, there were some constraints in this study. The regulatory network of signaling pathways in cancer cells is very complex, thus, further researches are warranted to examine the interplay between the JAK/STAT pathway and other signaling pathways.

ACKNOWLEDGMENT

This experiment was approved by the Laboratory Animal Ethics Committee at Hangzhou First People's Hospital. It is part of the 2021 Zhejiang Medical and Health Science and Technology Program Project 2021KY230, titled "Research on the Coordination Mechanism of Immune Microenvironment Related Genes in Prostate Specific Antigen (PSA) Molecular Evolution" and Hangzhou Medical and Health Science and Technology (Project Contract, A20200066). Additionally it is funded by the Hangzhou Medical and Health Science and Technology Project Contract, (No.B20210391).

AUTHOR CONTRIBUTION

JS conducted and designed the research, carried out experiments, and analyzed findings. Edited and refined the manuscript with a focus on critical intellectual contributions. GD participated in collecting, assessing, and interpreting the data. Made significant contributions to data interpretation and manuscript preparation. GW and XX: Provided substantial intellectual input during the drafting and revision of the manuscript. The final version of the manuscript has been reviewed and approved by all authors.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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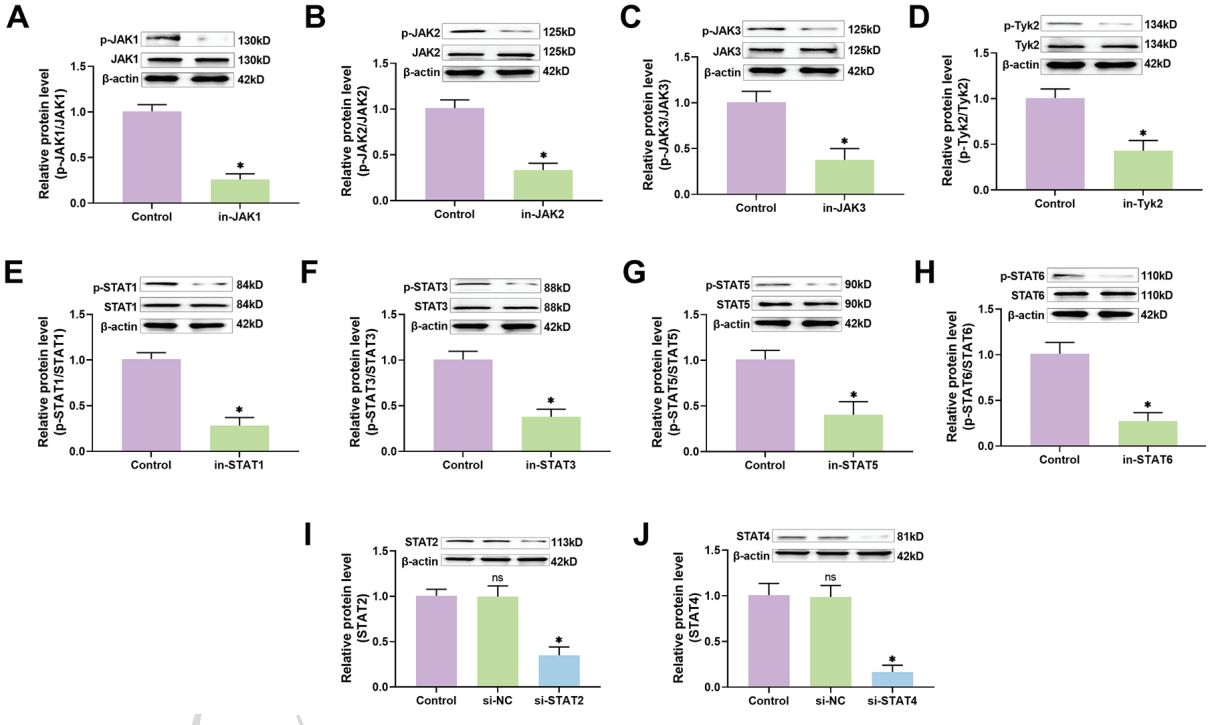

Fig. S1. JAK/STAT signaling pathway protein expression. JAK: Janus kinase, STAT: Signal transducer and activator of transcription