Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

The Modified Shuyu Pill Inhibits the Formation of Pre-Metastatic Niches in Triple-Negative Breast Cancer by Reducing the Number of Myeloid-Derived Suppressor Cells (MDSCs) via the JAK2/STAT3 Pathway

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Purpose: To investigate the effect of modified Shuyu pill on the timing of TNBC metastasis, MDSCs recruitment in tumor tissues and distant target organs, and the impact on the JAK2/STAT3 signaling pathway.

Methods: The fingerprint profile of the modified Shuyu pill was obtained using UPLC-Q-Exactive HRMS technology. A TNBC-bearing mouse model was established, and the mice were treated with modified Shuyu pill granules (low, medium, and high doses), paclitaxel (PTX), or saline for 25 days. Tumor growth was monitored during treatment, and small animal in vivo imaging was performed on days 1, 7, 14, 21, and 25. On day 21 of treatment, flow cytometry was used to assess the proportion of G-MDSCs and M-MDSCs in peripheral blood, spleen, lungs, and tumor tissues. Immunofluorescence was used to detect the number and distribution of MDSCs in the lungs and liver. Western blotting and RT-qPCR were used to detect the expression of proteins and genes related to the JAK2/STAT3 signaling pathway in tumor tissues.

Results: The modified Shuyu pill inhibited tumor growth and distant metastasis in 4T1 tumor-bearing mice, and its anti-tumor effect was enhanced when combined with paclitaxel. More importantly, modified Shuyu pill suppressed the recruitment of MDSCs in the peripheral blood, spleen, lungs, liver, and tumors of tumor-bearing mice, thereby inhibiting the formation of the pre-metastatic niche. Mechanistically, modified Shuyu pill inhibited the expression of proteins in the JAK2/STAT3 signaling pathway, including IL-6, JAK2, p-JAK2, STAT3, p-STAT3 (Tyr705), p-STAT3 (Ser727), S100A8, S100A9, NF-κB, MMP2, and MMP9, and this effect was more pronounced when combined with paclitaxel.

Conclusion: The modified Shuyu Pill can downregulate the JAK2/STAT3 signaling pathway, reduce the number of MDSCs in the tumor microenvironment and distant lung tissues of tumor-bearing mice, inhibit the formation of pre-metastatic niches, and ultimately suppress tumor growth and metastasis.

Keywords: modified Shuyu pill, triple-negative breast cancer, MDSCs, pre-metastatic ecological niches, JAK2/STAT3 signaling pathway

Received: 25 June 2025
Accepted: 26 November 2025
Published: 11 December 2025

Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer for approximately 15%-20% of all breast cancer cases with highly malignant, difficult to treat, and prone to metastasis.[1,2] The treatment of TNBC depends on the subtype and stage of the cancer, such as surgery, radiotherapy, chemotherapy, and immunotherapy. Although significant progress has been made in recent years, advanced or metastatic TNBC presents a poor prognosis, due to drug resistance and recurrences,[3,4] with a 5-year survival rate of less than 15%, significantly lower than the overall 5-year survival rate for breast cancer patients (31%).[5] Emerging evidence indicates that MDSCs also contribute to resistance to cancer treatments, particularly immunotherapies.[6] Therefore, exploring new strategies and drugs with low side effects to prevent TNBC metastasis is of great clinical value.

Recurrent metastases are the leading cause of death in TNBC patients. The formation of PMN both locally in the tumor and distant organs is critical to the success of tumor metastasis in a variety of primary tumors including breast cancer.[7] However, the complex molecular mechanisms involved in driving PMN formation are unknown. There is growing evidence that MDSCs are key determinants of PMN formation.[8] MDSCs derived from myeloid progenitor cells and immature myeloid cells have immunosuppressive properties. A variety of cytokines and chemokines secreted by activated MDSCs promote the proliferation and migration of tumor cells.[9,10] By suppressing immune cells, causing stromal, and encouraging angiogenesis, MDSCs in PMN facilitate the colonization of tumor cells.[11] Meanwhile, mice with homozygous breast cancer tumors had a large number of MDSCs in their lungs before any distant metastases formed, which is consistent with the development of lung metastases from breast cancer later on.[12] Researchers have suggested that the proliferation and activation of MDSCs are mediated by a "dual" pathway.[13,14] First, tumour-derived-associated factors trigger JAK2 and STAT3 phosphorylation and promote S100A8/A9 expression which further activates the NF-κB pathway and mediates MDSCs activation.[15,16] Studies have confirmed that S100A10 plays a core role. By reshaping the homeostatic imbalance of the pulmonary immune microenvironment, it provides a potential therapeutic target for blocking cancer metastasis to the lungs.[17] MDSCs assist PMN formation and circulating tumour cell.[18] Therefore, the JAK2/STAT3 pathway may be an essential route for MDSCs to form PMN.

At present, traditional Chinese medicine plays an increasingly obvious role in improving the prognosis of tumor patients.[19–21] Research indicates that the application of traditional Chinese medicine to tumor patients following surgery and radiation therapy is capable of preventing the tumors from growing and spreading, as well as increasing the patients' chances of survival.[22,23] Shuyu pill, a classic formula from Shang Han Lun, is renowned for its efficacy in regulating the imbalanced state of the body's immune function and restoring the body's immune homeostasis. Previous studies demonstrate the Shuyu pill can improve immune function during chemotherapy in TNBC patients and act as a potentiator and detoxifier;[24,25] the Shuyu pill reduced serum IL-2 levels and increased CD4+ and CD4+/CD8+ T cell levels to immune function in TNBC mice after chemotherapy.[26,27] Moreover, the Shuyu pill inhibits MDA-MB-231 invasive metastasis by down-regulating the PI3K/AKT/mTOR pathway.[28] In clinic practice, the prescription of the Shuyu pill is often modified by adding Chinese herbs with well-defined anti-tumour effects such as Curcuma longa and Hedyotis diffusa to increase its anti-tumour efficacy, which is known as modified Shuyu pill. However, the mechanism of action of the modified Shuyu pill specifically inhibiting distant metastasis of TNBC is not clear.

In this study, through mass spectrometry analysis, we identified the active components of modified Shuyu pill and explored its mechanism of action by observing the recruitment of MDSCs to the primary tumor and metastatic organs in an attempt to prevent TNBC metastasis, Figure 1.

Materials and Methods

Mass Spectrometry

Taken 0.5 g of modified Shuyu pill granules, added 50 mL of 50% methanol and shake gently, then ultrasonic for 30 min, and then filtered by using 0.22 μm microporous filter membrane, then the sample solution of modified Shuyu pill granules was obtained, for the detection and analysis by UPLC-Q-Exactive HRMS. The chromatographic column used was Thermo Fisher Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 μm) with a column temperature of 40°C. The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B), and gradient elution was

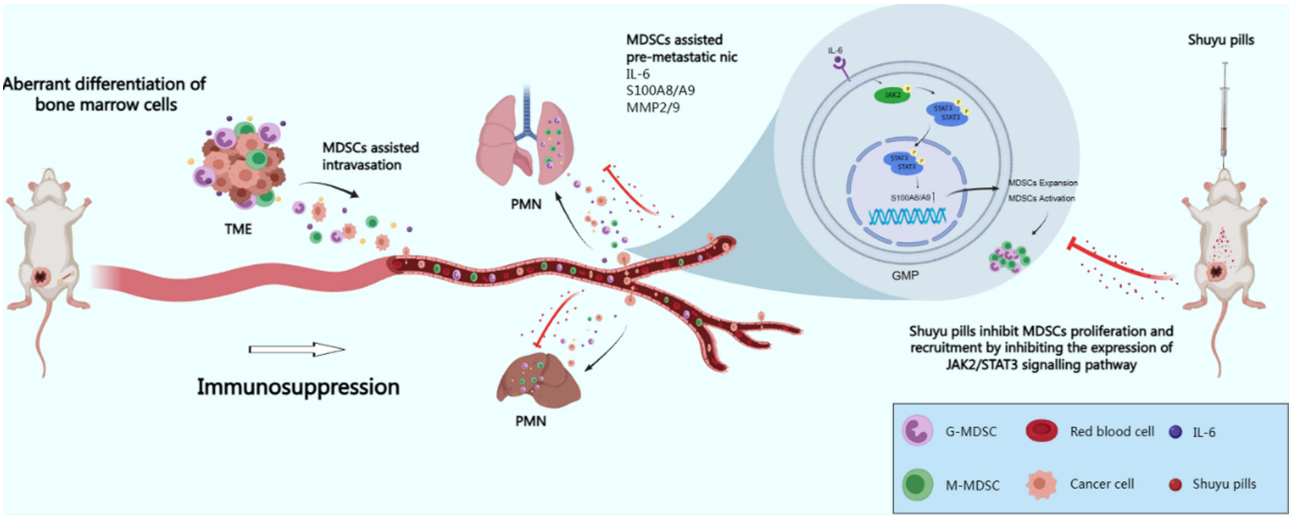

Figure 1 Schematic representation of the antitumor and anti-metastasis mechanisms of the modified Shuyu pill by suppressing MDSCs recruitment and regulating the JAK2/STAT3 signalling pathway.

performed as detailed in Table 1. The injection volume was 5 μL. The ion source was a heated electrospray ionization (HESI) source, and both positive and negative ion modes were monitored. The ion peaks in the chromatogram were compared with the spectra of reference compounds based on literature data to identify the chemical components.

Chinese Herbal Medicine

Based on the paste rate of each herb in the formula after the addition and subtraction of modified Shuyu pill, the granule dosage of the drinking tablets will be converted to 5.00g of Shuyu, 6.67g of Radix Angelicae Sinensis, 7.69g of Radix Rehmanniae Praeparata, 2.00g of Rhizoma Ligustici Chuanxiong, 3.00g of Colla Corii Asini, 1.09g of Cinnamomum Cassiae, 1.50g of Radix Bupleuri, 1.11g of Paeonia lactiflora, 3.85g of Atractylodes macrocarpa, 0.83g of Almonds, 0.48g of Divine Quartern, and 2.00g of Radix et rhizoma Ginseng, Platycodon grandiflorus 3.33g, Poria cocos 0.40g, Fenghuang 2.50g, Dried ginger 0.24g, Ampelopsis japonica 0.75g, Chasteberry 1.92g, Curcuma longa 1.88g, Jujubae 25.00g, Licorice 6.00g, Hedyotis diffusa 3.77 g (total 81.01g, one dose). The granules were provided by Guangdong Yi Fang Pharmaceutical Company. Paclitaxel injection (5 mL: 30 mg; Batch No. 21111811), purchased from Yangzijiang Pharmaceutical Group Co.

Cell Culture

Mouse-derived triple-negative breast cancer 4T1 cell line and fluorescently labelled 4T1-luc cell line were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co. The cells were cultured in complete medium (89% DMEM high glucose medium + 10% fetal bovine serum + 1% penicillin and streptomycin double antibody), placed in 37°C, 5% CO2 cell culture box, passaged and frozen.

Table 1 Gradient Elution Table

Flow Rate (mL/min)	Mobile Phase A(%)	Mobile Phase B(%)
0.3	99	1
0.3	99	1
0.3	1	99
0.3	99	1
0.3	99	1

https://doi.org/10.2147/BCTT.S549554

Mouse Feeding

SPF-grade BALB/c mice, female, 6 weeks old, body weight 20±2g, 36 in total, purchased from Beijing Viton Lihua Laboratory Animal Technology Co. Ltd, License No.: SCXK (Beijing) 2021–0006. The use of experimental animals was approved by the Ethics Committee for Animal Experiments of Guizhou Medical University (Animal Ethics Review No. 1900650). Throughout the experimental process, all operations involving animals strictly complied with the GB/T 35892–2018 Standard for Laboratory Animal Welfare.

Mouse Modelling

Cells in the logarithmic growth phase were harvested (4T1-luc for experiment 1, 4T1 for experiment 2), resuspended in PBS buffer, adjusted to a cell density of 1×107 cells/mL, and injected into the right groin of BALB/c mice. The blank group was injected with an equal amount of PBS buffer alone. Seven days after inoculation, the mice with successful modelling were randomly divided into five groups: the model group (Model), the modified Shuyu pill low-dose group (mSYW-L), the modified Shuyu pill medium-dose group (mSYW-M), the modified Shuyu pill high-dose group (mSYW-H), and the paclitaxel group (PTX), with six mice in each group.

Drug Delivery

TBased on the "Equivalent dose ratio between humans and animals according to body surface area", the daily gavage dose of modified Shuyu pill in mice was calculated as the daily dose of modified Shuyu pill in adults × the equivalence coefficient in mice (0.0026)=10.04 g/kg/d. Mice in the mSYW-L, mSYW-M and mSYW-H groups were given the pellet drug of modified Shuyu pill in low, medium and high doses (equivalent to 10.53 g/kg/d, 21.06 g/kg/d and 42.12 g/kg/d) once a day. The mice in mSYW-L, mSYW-M, and mSYW-H groups were given low, medium, and high doses of modified Shuyu pill by gavage of granules (equivalent to 10.04 g/kg/d, 20.08 g/kg/d, and 40.16 g/kg/d) once a day. Mice in the PTX group were injected with 10 mg/kg of PTX solution once every 3 days. The blank and model groups were gavaged with equal amounts of saline daily.

Tumor Growth Monitoring

Starting from the 1st day of treatment, the feeding, activity and tumour growth of the hormonal mice were observed every day, the body weight of the mice was measured once a day, the length and diameter of the tumours of the mice were measured every 3 days, and the subcutaneous tumour volume of the mice was calculated (Volume = 1/2×Width2×Length), and the tumour growth curves of the hormonal mice were plotted using the Graphpad Primer software.

Small Animal Live Imaging

After successful 4T1-luc homozygous modelling in each group of mice, small animal live imaging was performed on the 1st, 7th, 14th, 21st and 25th days of treatment, respectively. The mice were anesthetized with 1L/min oxygen flow mixed with isoflurane, and D-luciferin potassium solution (150 mg/kg) was injected intraperitoneally. After 5–10 minutes, the mice were placed in the anesthesia chamber for inhalation anesthesia for 3–5 minutes. The anesthetized tumor-bearing mice were placed in a supine position on the imaging platform. The IVIS Lumina XRMS system (USA) was used for bioluminescent imaging to monitor tumor growth and metastasis.

Collect Material

Mice were anaesthetised by ether inhalation and peripheral blood was obtained. After death, the chest and abdominal cavities of the mice were exposed to check for any obvious metastatic tumors in the organs. Tumor tissue from the right inguinal region was excised, and the surrounding skin, connective tissue, and scabbed areas were removed. The tumor weight was recorded, and spleen, lung, and liver tissues were collected.

Flow Cytometry

Single cell suspensions of peripheral blood and lung, spleen, liver and tumour tissues were prepared with erythrocyte lysate and enzymatic solution (1 mg/mL type IV collagenase+0.5 mg/mL DNAase + DMEM high sugar medium), respectively, and the immune cells in the tissues were separated and collected by Percoll. The antibody-labelled cells were stained with fluorescently labelled CD45, CD11b, Ly6C, Ly6G, CD45, CD11b, Ly6C, Ly6G antibodies, respectively, and incubated on ice for 30 min. After pre-cooled PBS washing of the cells, the antibody-labelled cells were analysed using flow cytometry. Data were analysed using FlowJo v10 software (BD Biosciences), and CD11b+Ly6C +Ly6 cells were considered MDSCs and CD11b+Ly6C-Ly6G+ cells were considered G-MDSCs.

Western Blot (WB)

Tissue cells were lysed using tissue lysate to obtain proteins from lung and tumour tissues. Protein concentration was determined according to BCA protein quantification. The samples were electrophoresed with 10% and 12% polyacrylamide gels at 220 V. The samples were electrotransferred to PVDF membranes and incubated with antibodies against IL-6, JAK2, p-JAK2, STAT3, p-STAT3(705), p-STAT3(727), S100A8, S100A9, NF-κB, MMP2, and MMP9. Exposure was performed on a chemiluminescent imager and the bands were analysed for grey values using Image J software.

RT-qPCR

Total RNA was extracted from the samples using Trizol reagent, and the RNA concentration and purity were measured using the NanoDrop 2000 software. cDNA was synthesized using a two-step method with a reverse transcription kit. Primers for target genes were designed using Primer 6.0 software (synthesized by Shanghai Shenggong Biotechnology Co., Ltd.) based on sequences obtained from PubMed. The RT-qPCR reaction mixture was prepared according to the instructions of the Hieff UNICON Universal Blue qPCR SYBR Green Master Mix (11184ES08, Yeasen).

Statistical Methods

SPSS 25.0 statistical software was used to process and analyse the data, and the data involved in this study were all measured data, expressed as mean ± standard deviation ($\bar{x}$±s). The data were first tested for normality, and the test of variance chi-square was performed when it met normality. When the variances are homogeneous, the independent samples *t*-test is used for comparing the means between two groups, and One-Way ANOVA (Analysis of Variance) is applied for comparing the means among three or more groups. For multiple comparisons between multiple groups, the Bonferroni method is adopted. For skewed data, the Mann–Whitney *U*-test is used for comparisons between two groups, and the Kruskal–Wallis *H*-test is employed for comparisons among three or more groups. For skewed data, a non-parametric rank sum test was used. $P<0.05$ was considered statistically significant.

Result

Main Components in the Modified Shuyu Pill

By comparing the mass spectrometry data information of each chromatographic peak in positive and negative modes, the cleavage pattern of the constituents of the modified Shuyu pill was further analyzed and in combination with the characteristic neutral loss of fragment ions, the constituents of the modified Shuyu pill were structurally identified. Through local databases, PubMed databases and literature, a total of 67 components were identified in the modified Shuyu pill by UPLC-Q-Exactive HRMS (Table S1). For example, Trigonelline,[29] Citric acid,[30] Gallic acid,[31] Salidroside,[32] Chlorogenic acid,[33] Apigetrin,[34] Ginsenoside Re,[35] trans-Cinnamaldehy[36] decertain compounds can inhibit the migration and invasion of solid tumors such as breast cancer, lung cancer, and colorectal cancer[37–39] The positive and negative ion chromatograms obtained are shown in Figure 2.

Anti-Tumor and Anti-Metastasis Effect of Modified Shuyu Pill in vivo

We first observed the effect of the modified Shuyu pill on distant metastasis in tumor-bearing mice (Figure 3A). The body weight of mice in the mSYW-M and mSYW-H groups increased while the body weight of the PTX group and Model

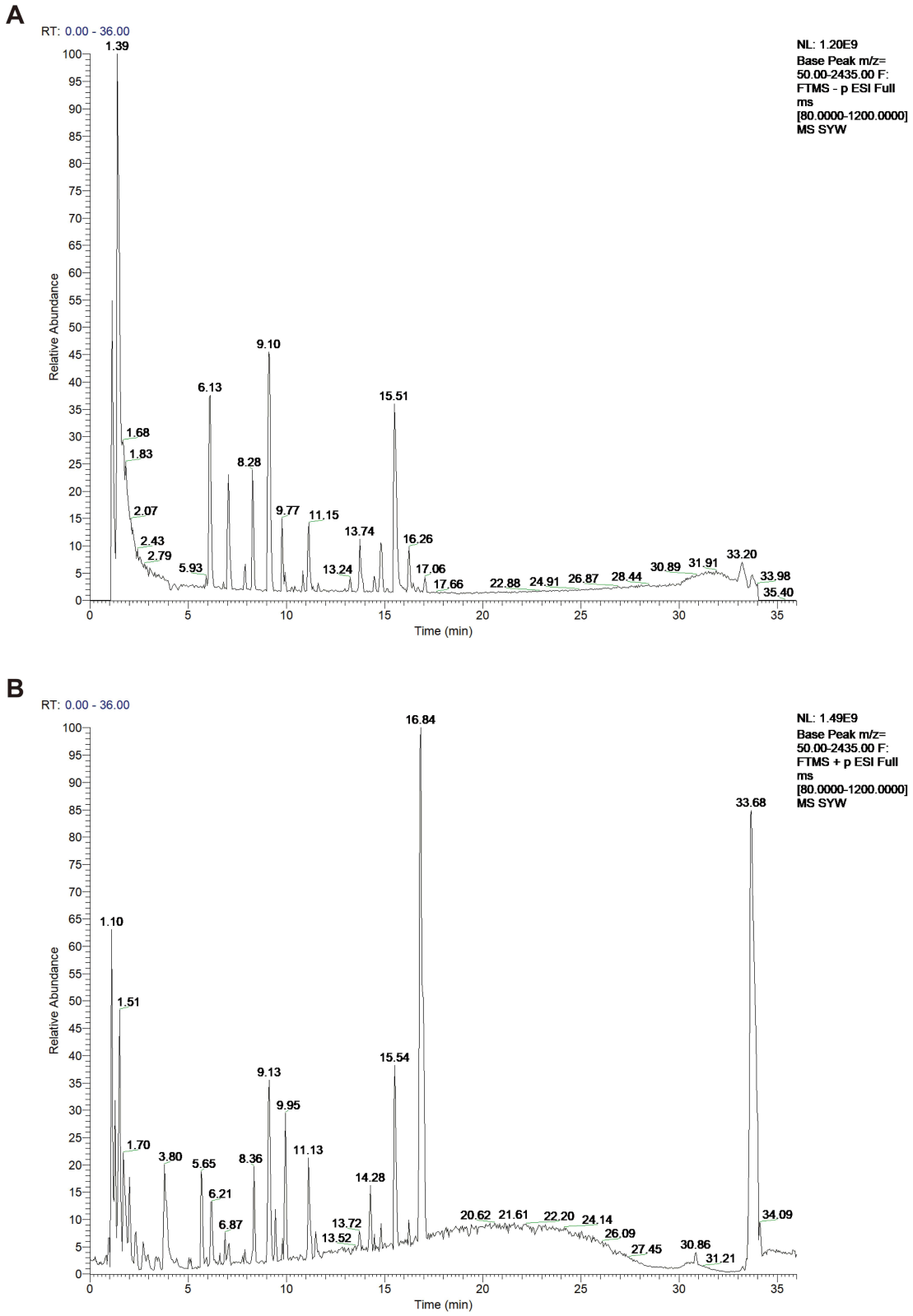

Figure 2 The total ion chromatogram of modified Shuyu pill was analyzed in both negative-ion mode (**A**) and positive-ion mode (**B**) by UPLC-Q-Exactive HRMS.

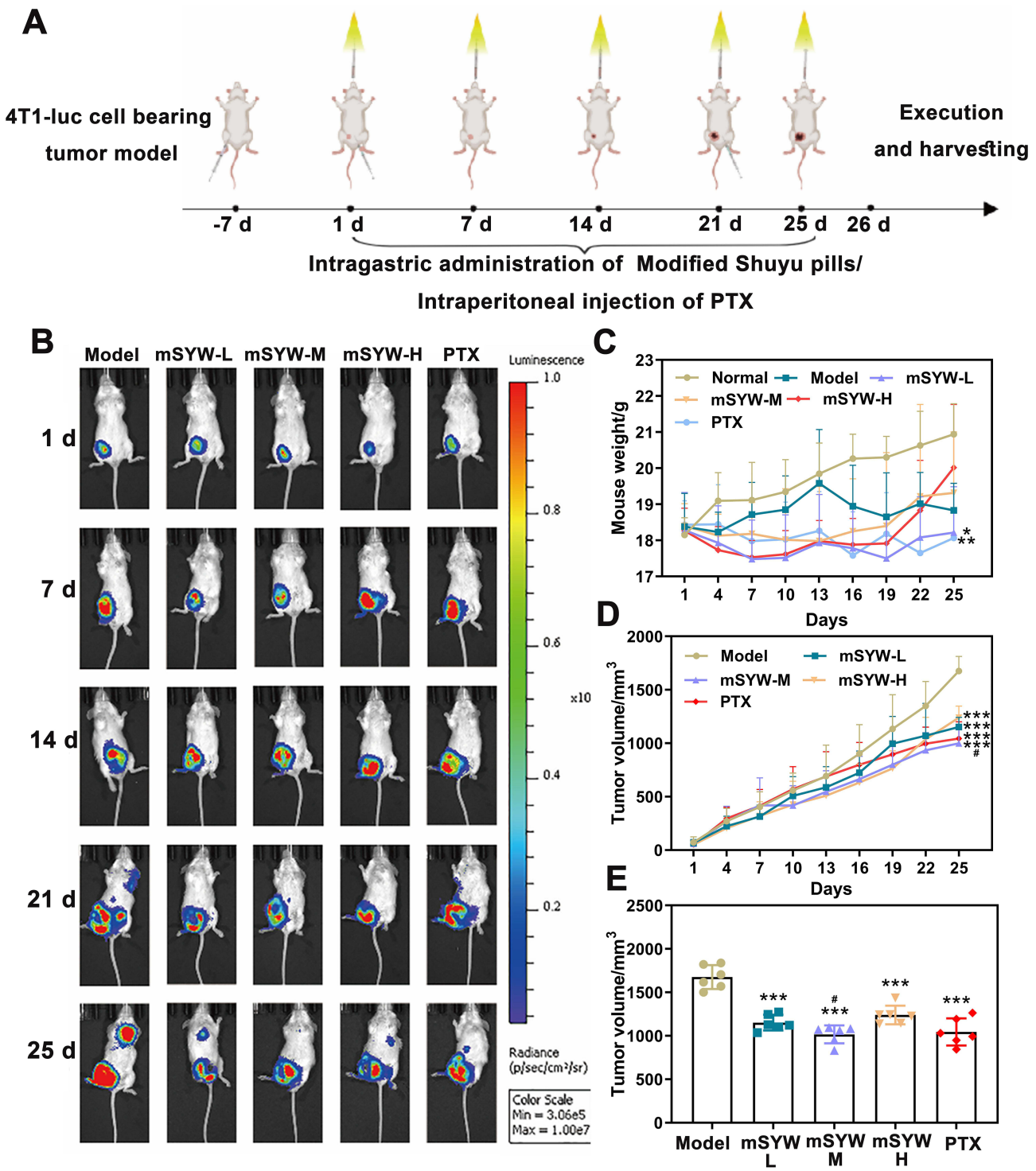

Figure 3 The modified Shuyu pill inhibited tumor growth and metastasis in tumor-bearing mice. (**A**) Flow chart of the animal experiment. (**B**) Live imaging of tumor-bearing mice on days 1, 7, 14, 21, and 25 after treatment. (**C**) Body weight of the mice during the treatment period. (**D**) Tumor growth curves of the mice during the treatment period. (**E**) Tumor volume of the mice in each group at the end of the treatment period. "#" stands for $P<0.05$ compared to the model group, "*" stands for $P<0.05$, "**" stands for $P<0.01$ and "***" stands for $P<0.001$, compared to the normal group. All experiments were repeated three times.

group decreased significantly (Figure 3C). All treatment groups suppressed the tumor volume effectively compared with the Model group (Figure 3D and E). The mSYW-M group had the best tumor suppression effect.

As shown in Figure 3B, the modified Shuyu pill inhibited tumor growth and distant metastasis dynamically and notably on days 1, 7, 14, 21 and 25 in live imaging of tumor-bearing mice. Mice in the Model group had metastasized to

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distant lungs on the 22nd day of treatment. Whereas on the 25th day of treatment, all groups showed different degrees of distant metastatic signals, except for the mSYW-M group. Considering the increase in tumor volume and the restriction of mouse activity, the experiment was stopped after 25 days of treatment and the mice were put to death on the premise that the experiment was following animal ethics (the tumor volume of the loaded mice did not exceed 2000 mm^3). Investigations demonstrated that the modified Shuyu pill could prevent tumor growth and lung metastasis and the medium-dose modified Shuyu pill had the greatest tumor-suppressive impact. Thus, we determined that the 4T1-luc tumour-bearing mice formed PMN within 3 weeks of treatment, and the medium dose of modified Shuyu tablets was the ideal dosage.

Tumor Suppression of Modified Shuyu Pill Combined with Paclitaxel

To further clarify the tumour-inhibitory effect of the synergistic group, we treated the tumour-bearing mice with mSYW, PTX, and mSYW+PTX for 21 days (Figure 4A and B), respectively. During the treatment period, each treatment group was able to suppress tumor volume in mice compared with the model group (Figure 4C). Comparing the tumor suppression effect of each group, the synergistic group had the highest tumor suppression rate, and compared with the model group, the tumor volume was reduced in tumor-bearing mice of the modified Shuyu pill group, the Paclitaxel group and the synergistic group which the tumor of the mice in the synergistic group was smaller (Figure 4D and E). In terms of spleen volume and weight, it was observed that the spleens in the administered group were smaller than those in the model group (Figure 4F and G).

Modified Shuyu Pill Influenced the Percentage of MDSCs Typed in Tumor-Bearing Mice

Aggregation of MDSCs is one of the initiators of tumor metastasis. In Figure 5, we used flow cytometry to detect the proportions of G-MDSCs and M-MDSCs in peripheral blood, spleen, lungs and tumor tissues of tumor-bearing mice. After 21 days of treatment, the proportions of G-MDSCs and M-MDSCs increased in the peripheral blood, spleen and lung of tumor-bearing mice, and G-MDSCs and M-MDSCs proportions declined in the mSYW, PTX, and mSYW+PTX when compared to the Mo additionally, the mSYW+PTX exhibited a decrease in these proportions when compared to the PTX. G-MDSCs and M-MDSCs proportions in tumor tissues declined in each treatment group as compared to the model, while M-MDSCs proportions fell in the mSYW+PTX when compared to the PTX.

Inhibition of the Recruitment Distribution of MDSCs by Modified Shuyu Pill in vivo

To visualise the effect of modified Shuyu pill on the distribution of MDSCs recruitment in tumor-bearing mice in vivo, we used the immunofluorescence technique to detect the number and distribution of MDSCs in the lung and liver tissues of mice. In Figure 6A and B, the number of MDSCs cells in the lung tissues in tumor-bearing mice was significantly higher than in the normal group. In comparison to the model group, the modified Shuyu pill group, the Paclitaxel group, and the synergistic group all had fewer MDSCs cells. The synergistic group's number of MDSCs cells decreased, but the difference was not statistically significant in contrast to the Paclitaxel group.

Moreover, MDSCs appeared in greater quantity in the tumor-bearing group in the liver tissues when compared to the normal group. In contrast, the number of MDSCs in the modified Shuyu pill group and synergistic group was lower than that in the model group and the number of MDSCs in the synergistic group was lower than that in the Paclitaxel group (Figure 6A and C).

Suppression of JAK2/STAT3 Pathway Expression in the Local Tumor Microenvironment

As mentioned earlier, the JAK2/STAT3 pathway may be an important route for PMN construction by MDSCs. In order to better explore the underlying mechanism of the modified Shuyu pill for preventing PMN development, we identified the expression of proteins associated with the JAK2/STAT3 signaling pathway in tumor tissues by RT-qPCR and Western blotting. In the tumour tissues, IL-6, JAK2, p-JAK2, STAT3, p-STAT3 (Tyr705), p-STAT3 (Ser727), S100A8, S100A9 and the downstream NF-κB pathway-related proteins NF-κB were discovered in the modified Shuyu pill group, the

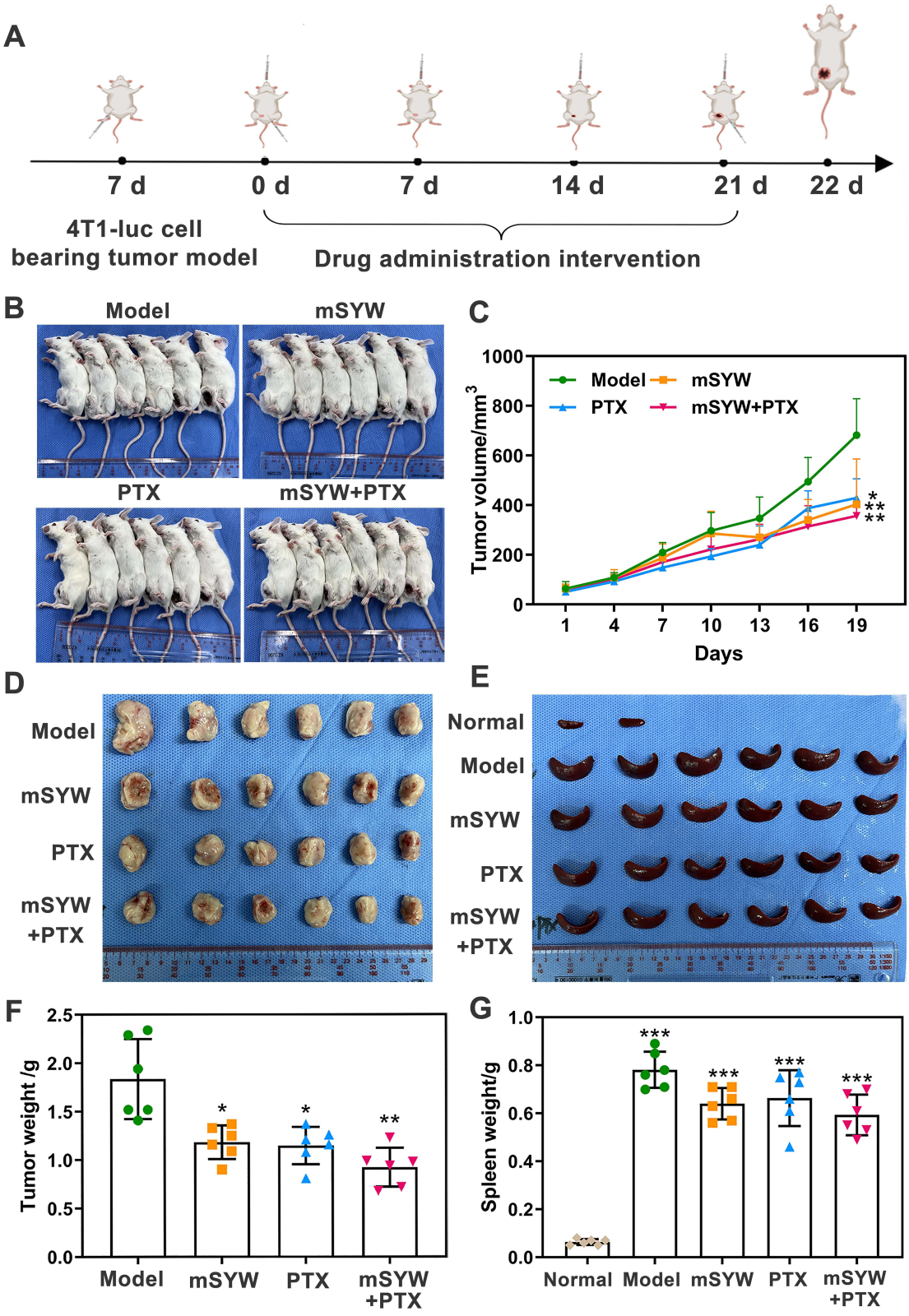

Figure 4 The antitumor effect of the modified Shuyu pill combined with paclitaxel. (**A**) Experimental flow chart. (**B**) Pictures of tumor-bearing mice in each group after 21 days of treatment. (**C**–**E**) Comparison of tumour volume and weight of mice in the model group, the modified Shuyu pill group, paclitaxel group and synergistic group. (**F** and **G**) Spleen weight of mice in the normal group, model group, the modified Shuyu pills group, Paclitaxel group and synergistic group. "*" stands for $P<0.05$, "**" stands for $P<0.01$, "***" stands for $P<0.001$, compared to the normal group.

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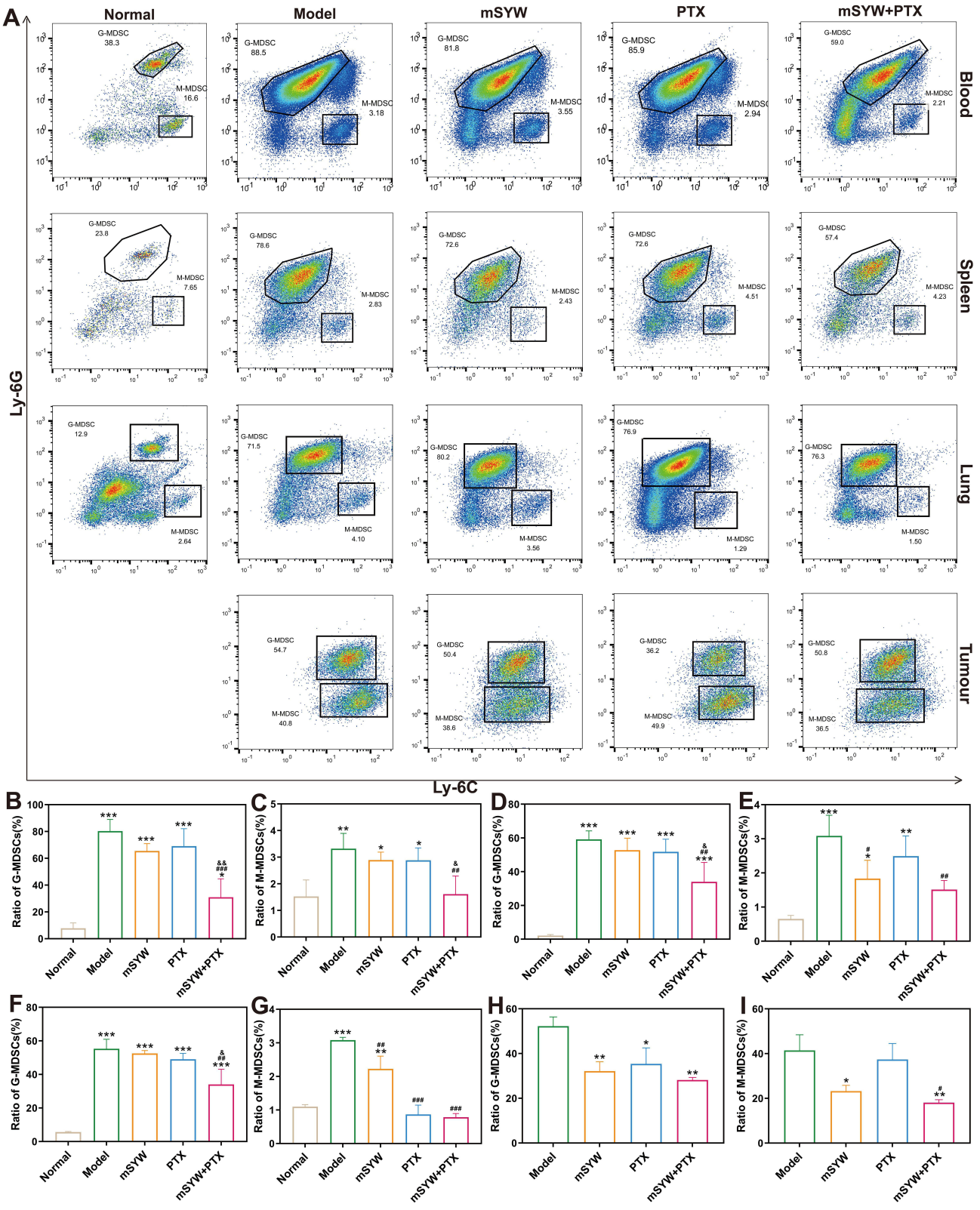

Figure 5 Shuyu pill reduced the percentage of G-MDSCs and M-MDSCs fractions in peripheral blood, spleen, lungs and tumors in tumor-bearing mice. The gating expression of G-MDSCs and M-MDSCs in the blood, spleen, liver, and tumors of mice in each group (**A**) Streaming trapdoor results for four. Percentage of G-MDSCs and M-MDSCs in the blood (**B** and **C**), spleen (**D** and **E**), lungs (**F** and **G**), tumors (**H** and **I**) of mice in each group. "*" stands for $P<0.05$, "**" stands for $P<0.01$, "***" stands for $P<0.001$, compared to the normal group, "#" stands for $P<0.05$, "##" stands for $P<0.01$, compared to the model group, "###"stands for $P<0.001$. "&"stands for $P<0.05$, "&&"stands for $P<0.01$, compared to the PTX group, all experiments were repeated three times.

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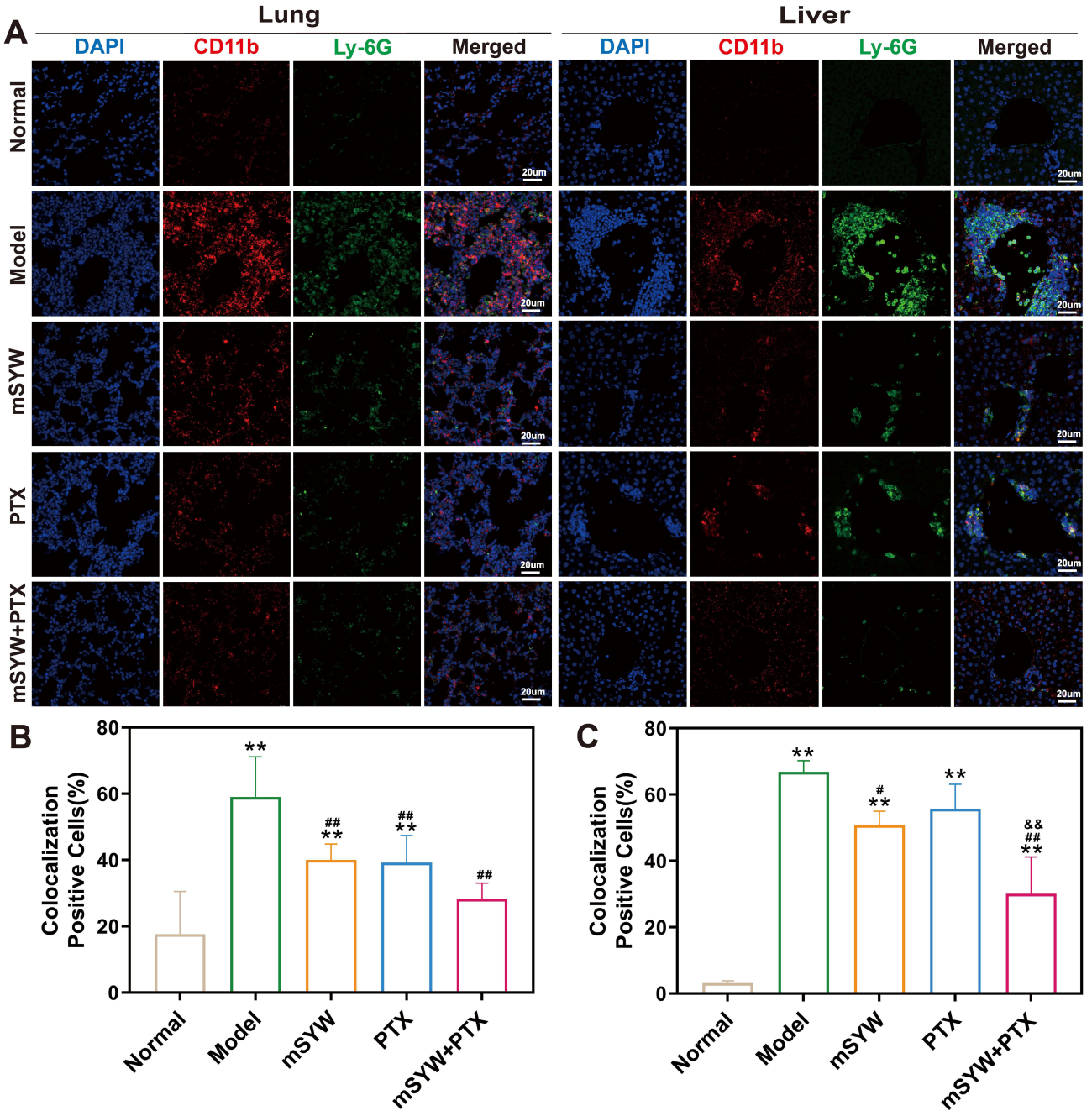

Figure 6 Inhibition of the recruitment distribution of MDSCs by modified Shuyu pill in vivo. (**A** and **B**) Immunofluorescence analysis to detect the recruitment of MDSCs in the lung (**A** and **C**) Immunofluorescence analysis to detect the recruitment of MDSCs in the liver. "**"stands for $P<0.01$ compared to the normal group, "#"stands for $P<0.05$, "##"stands for $P<0.01$ compared to the model group, "&&" stands for $P<0.01$ compared to the PTX group.

Paclitaxel group and the synergistic group. In Figure 7, in comparison with the model group, MMP2, and MMP9 were reduced in both mRNA and protein expression. When compared to the paclitaxel group, the modified Shuyu pill combined with the paclitaxel group displayed reduced expression of JAK2/STAT3 signaling pathway-related proteins. There was a statistically significant difference in the decreased levels of IL-6, JAK2, p-STAT3 (Ser727), S100A9, NF-κB, MMP2, and MMP9. This establishes that one of the key mechanisms for PMN conformation in MDSCs is, in fact, the JAK2/STAT3 signaling pathway.

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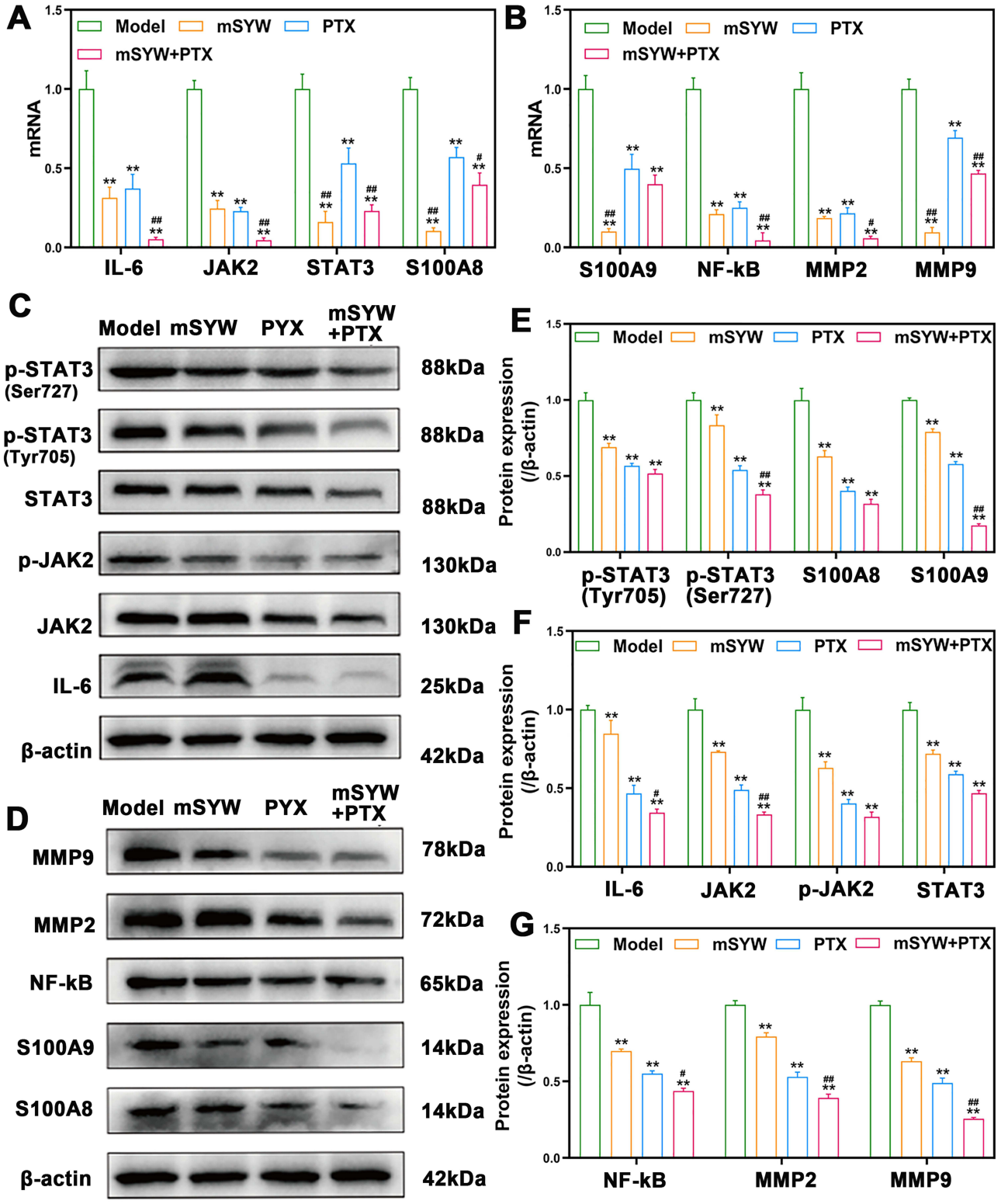

Figure 7 RT-qPCR and Western Blot analysis of JAK2/STAT3 signalling pathway-related protein expression in tumor tissues of different groups. (**A** and **B**) RT-qPCR statistical results. (**C** and **D**) Western Blot bands. (**E**–**G**) Statistical results of Western Blot. "**"stands for $P<0.01$ compared to the normal group, "#"stands for $P<0.05$, "##"stands for $P<0.01$ compared to the model group. All experiments were repeated three times.

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Inhibitions of JAK2/STAT3 Pathway Expression via Modified Shuyu Pill in Pre-Metastatic Lung Ecotopes

One of the most common metastatic sites of breast cancer is the lung. We therefore further examined the expression of the JAK2/STAT3 pathway in the lung tissues of tumour-bearing mice to observe the regulatory effect of the modified Shuyu pill on the JAK2/STAT3 pathway in the PMN. In Figure 8, compared with the normal group, mRNA and protein expression of JAK2/STAT3 pathway-related proteins were increased in the lung tissues of mice in the tumour-bearing group, mRNA and protein expression of JAK2/STAT3 pathway-related proteins were decreased in the modified Shuyu pill group, Paclitaxel group and synergistic group as compared to the model group. Compared with the Paclitaxel group, IL-6, JAK2, p-STAT3 (Ser727), S100A9, NF-κB, MMP2, and MMP9 proteins were significantly reduced. Interestingly, the modified Shuyu pill combined with the paclitaxel group was able to reduce IL-6, JAK2, p-STAT3(Ser727), S100A9, NF-κB, MMP2 and MMP9 protein expression efficiently in both tumour localities and lungs which indicated that modified Shuyu pill combined with paclitaxel inhibited pre-metastatic tumour ecotone formation better than the two alone.

Discussion

The occurrence, progression, invasion, and metastasis of malignant tumors are determined by a series of complex factors. The pre-metastatic niche (PMN) is not merely a passive bystander in the metastasis process but an active participant.[16] PMN is a key determinant of the successful metastasis of tumors to secondary organs or sites, forming in advance at the target location.[12]

PMN consists of resident cells, bone marrow-derived cells (BMDCs), soluble factors,[40] and extracellular vesicles[41] among which BMDCs are indispensable for PMN formation. Myeloid-derived suppressor cells (MDSCs) are the most crucial BMDCs involved in PMN formation, and their generation is strictly regulated by various cytokines and transcription factors.[42] Upon activation, MDSCs secrete a series of cytokines and chemokines that promote tumor cell proliferation and migration, leading to tumor invasion and metastasis.[11] Within the PMN, MDSCs facilitate tumor cell escape and metastasis by inhibiting immune cells, inducing stromal remodeling, and promoting angiogenesis. G-MDSCs initiate PMN formation in target organs by producing high levels of matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), thereby aiding the penetration and colonization of circulating tumor cells (CTCs).[43] By intervening to reduce MDSC numbers and inhibit their function, immune suppression of tumor cells can be restored, malignant tumor progression can be inhibited, and the survival of tumor-bearing mice can be prolonged.[44]

With the deepening of cancer research, it has become evident that merely excising solid tumors does not effectively prevent metastasis. Before metastasis occurs, CTCs often remain in a dormant state for an extended period. When a suitable PMN forms, CTCs are awakened from dormancy and disseminate, leading to tumor metastasis. Enhancing immune function, remodeling the PMN, and improving the overall systemic environment are equally important components of comprehensive therapy. The traditional Chinese medicine concept of "treating pre-disease" emphasizes enhancing the body's vital energy to intervene in the impact of primary tumors on distant organs, thereby inhibiting metastasis. Triple-negative breast cancer (TNBC) patients often exhibit deficiencies in qi, blood, yin, and yang after surgery and chemotherapy, with residual cancer toxins and drug toxins leading to cancer recurrence. Based on this pathophysiological understanding, the modified Shuyu Pill was selected to regulate the balance of qi, blood, and body fluids, alleviate cancer toxins, and ultimately prevent postoperative tumor metastasis.

To verify the regulatory effect of the modified Shuyu Pill on the PMN, this study primarily employed tumor-bearing mouse models and established a spontaneous lung metastasis model of TNBC. In the first phase of the experiment, small animal in vivo imaging technology confirmed that distant lung metastases appeared in TNBC tumor-bearing mice after 21 days of treatment. Furthermore, the optimal dose for inhibiting tumor metastasis was determined to be 20.08 g/kg/d. The second phase results indicated that the modified Shuyu Pill reduced the number of MDSCs in the peripheral blood, spleen, lungs, liver, and tumor tissues of tumor-bearing mice, inhibiting tumor growth and distant metastasis. Additionally, when used in combination with paclitaxel, the inhibitory effect was more pronounced.

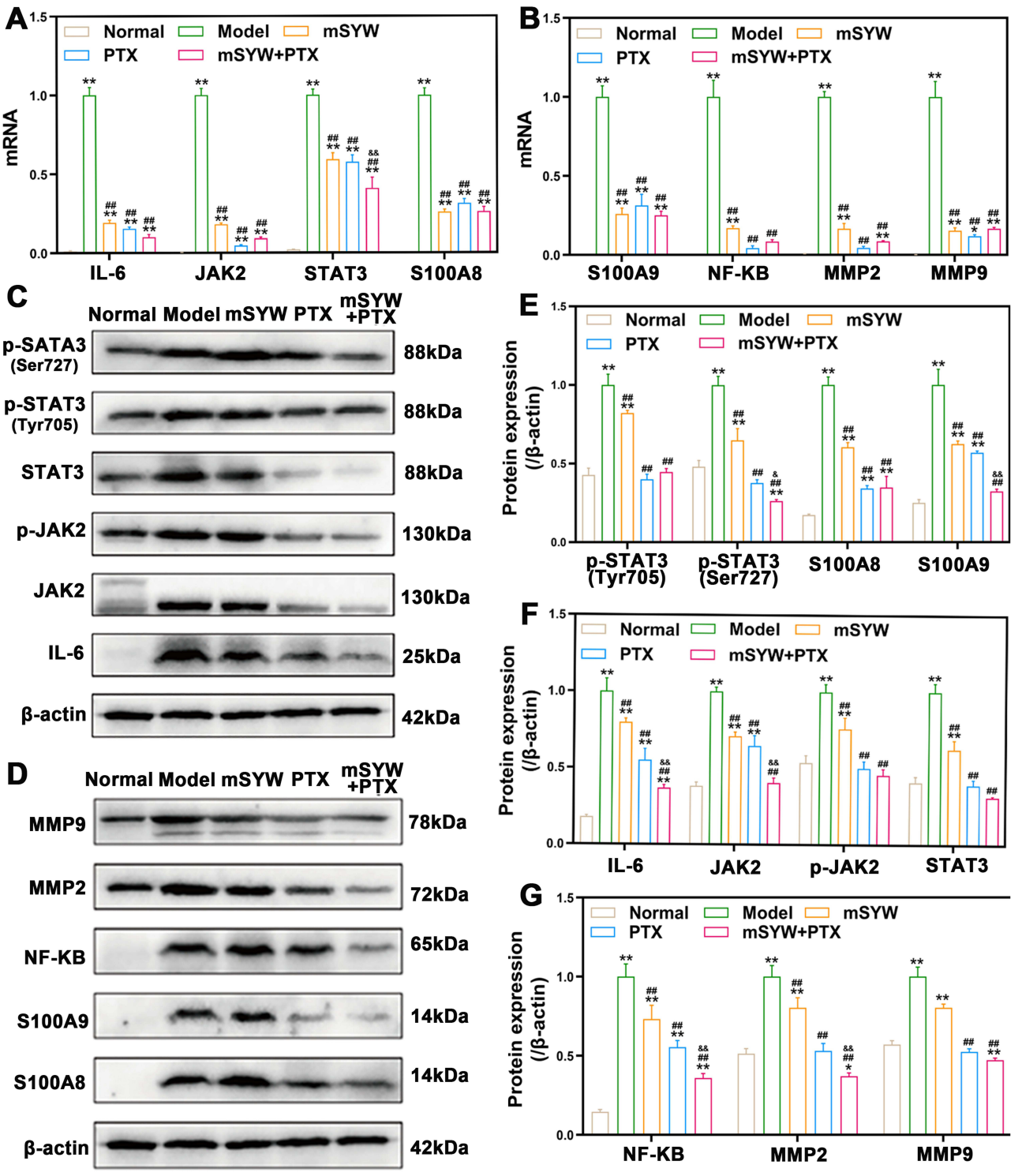

Figure 8 RT-qPCR and Western Blot analysis of JAK2/STAT3 pathway-related protein expression in lung tissues of normal, model, the modified Shuyu pill, Paclitaxel, and synergistic groups. (**A** and **B**) RT-qPCR statistical results. (**C** and **D**) Western Blot bands. (**E**–**G**) Western Blot statistical results. "*"stands for P<0.05, "**"stands for P<0.01 compared to the normal group, "##"stands for P<0.01 compared to the model group, "&"stands for *P*<0.05, "&&" stands for *P*<0.01 compared to the PTX group. All experiments were repeated three times.

Subsequently, we explored the mechanisms by which the modified Shuyu Pill inhibits the recruitment of MDSCs in local tumors and the PMN of lung metastases Experimental results showed that the modified Shuyu Pill reduced the expression of IL-6, JAK2, p-JAK2, STAT3, p-STAT3 (Tyr705), p-STAT3 (Ser727), S100A8, S100A9, NF-κB, MMP2,

and MMP9 proteins in tumor and lung tissues. The JAK2/STAT3 signaling pathway and its downstream NF-κB signaling pathway jointly mediate the proliferation and activation of MDSCs.[45] Various cytokines, such as IL-6, TGF-β, and VEGF, can trigger JAK2 phosphorylation, and p-JAK2 promotes STAT3 phosphorylation. Phosphorylation is a crucial post-translational modification process in organisms. By altering the charge state, spatial conformation, or intermolecular interaction ability of target molecules, it enables precise regulation of key physiological functions such as cell proliferation, signal transduction, and metabolic regulation, thereby ensuring the orderly progression of life activities.[15,46] The two key phosphorylation sites, Tyr705 and Ser727, in the STAT3 transcription activation domain play a crucial role in this process.[47] Tyr705 phosphorylation promotes STAT3 dimerization, while Ser727 phosphorylation further enhances STAT3 transcriptional activation of downstream target genes.

Notably, p-STAT3 induces the overexpression of calcium-binding proteins S100A8/A9, which belong to the damage-associated molecular pattern molecules. Studies have confirmed that S100A8/A9 play a central role in MDSC recruitment and migration, and blocking S100A8/A9 can remodel the tumor immune microenvironment and inhibit tumor progression[48] In breast cancer tumor-bearing mice,[49,50] S100A8/A9 derived from bone marrow and tumor cells bind to RAGE receptors on MDSCs, promoting MDSC migration and aggregation.

Subsequently, S100A8/A9 drive the NF-κB signaling pathway to mediate MDSC activation.[16,51] Once activated, MDSCs inhibit T cell function through direct cell-cell interactions and the release of soluble mediators. Meanwhile, MDSCs secrete MMPs, inducing vascular permeability, disrupting the extracellular matrix, and compromising the integrity of the basement membrane, thereby facilitating PMN formation and the colonization of CTCs.[22]

In summary, this study suggests that the deep mechanism by which the modified Shuyu Pill inhibits PMN formation is through the downregulation of JAK2/STAT3 signaling pathway phosphorylation, thereby suppressing the expression of key downstream molecules S100A8/A9, reducing MDSC migration and aggregation, and ultimately reshaping the tumor immune microenvironment. When used in combination with paclitaxel, this inhibitory effect is more pronounced. These findings indicate that combining the modified Shuyu Pill with chemotherapeutic agents can effectively suppress tumor recurrence and metastasis.

Conclusion

The modified Shuyu Pill can downregulate the JAK2/STAT3 signaling pathway, reduce the number of MDSCs in the tumor microenvironment and distant lung tissues of tumor-bearing mice, inhibit the formation of pre-metastatic niches, and ultimately suppress tumor growth and metastasis. Furthermore, surprisingly, the combination of Shuyu Pills with paclitaxel does not reduce the tumor-inhibitory effect of paclitaxel; instead, it enhances this effect.

Data Sharing Statement

The datasets generated and analyzed in this study are not publicly available temporarily due to involving unpublished original experimental records, but can be obtained from the first author or corresponding author upon reasonable request. For data access, please contact YaZhen Huang (E-mail: 2981174694@ qq.com) and Dr. Su Xie (E-mail: xiesutcm@163.com).

Funding

This study was supported by the National Natural Science Foundation of China (Grant 82460883), Ling Xiangli National Famous Traditional Chinese Medicine Practitioner Inheritance Workshop Construction Project (State Office of Traditional Chinese Medicine Human Education Letter [2022] No. 245), Project of Guizhou Provincial Administration of Traditional Chinese Medicine, Scientific and Technological Research Project of Ethnic Medicine (QZYY-2023-001), Guizhou Provincial Science and Technology Programme Project (Qiankehe Foundation - ZK[2022] General 442), Guizhou Provincial Science and Technology Programme Project (Qiankeheji - ZK[2024] General 204).

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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