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Curcumol inhibits endometrial cell invasion through the NF-κB pathway in rats with endometriosis

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Abstract: Curcumol (Cur) has been found to be useful in improving Endometriosis (EMS), however its mechanism of action is still not completely understood. Based on this, the current study was carried out to investigate the mechanism of Cur on the invasion and metastasis of endothelium cells in rats with EMS, with the goal of supporting the development of new Chinese medicinal formulations for EMS. The Endometrial stromal cells (ESCs) were separated from the EMS rat model. Effects of Cur on the viability, migration and invasive activity of ESC were tested by CCK-8, LDH release assay, Transwell, and Western blot assays, respectively. The effects of Cur on inflammation and immunological function in rats were assessed by ELISA and flow cytometry. Meanwhile, Cur's effect on ESC biological behavior and *in vivo* function was studied by treating with PMA and analyzing the potential mechanism. Cur inhibited ESC proliferation in a concentration-dependent manner, with an inhibition rate of approximately 50% at 0.1μmol/L. ESC's invasive and migratory forces decreased considerably following treatment with 0.1μmol/L Cur, which was effectively reversed by adding PMA. Cur gavage administration reduced blood levels of inflammatory factors, elevated the $CD3^+$, $CD4^+/CD8^+$ immune index in rats. PMA gavage therapy weakened Cur's ability to suppress inflammation and improve immunological function in EMS rats. Cur inhibits endothelial cell invasion and metastasis, attenuates inflammatory response and improves immune function in EMS rats via modulating the NF-κB pathway.

Keywords: Endometriosis, endometrial stromal cells, curcumol, NF-κB pathway, invasion, metastasis.

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INTRODUCTION

Endometriosis (EMS) is a gynecological illness characterized by the metastasis and invasion of endometrial tissue in women, causing it to grow beyond the uterine cavity (Bulun *et al.*, 2019; Zondervan *et al.*, 2020). This has a serious impact on a woman's fertility, social life, and normal emotions and her quality of life suffers significantly (Horne and Missmer, 2022). Surgery, nonsteroidal anti-inflammatories (NSAIDs), and hormonal medications (e.g., GnRH-a, progesterone, DNG) are now used in the treatment of EMS (Capezzuoli *et al.*, 2022). Compound oral contraceptives and progesterone are considered to be the first-line treatment for EMS. However, tiny lesions that cannot be removed by surgery will continue to grow after surgery, increasing the recurrence rate and the likelihood of patients requiring reoperation by up to 15-20%. The efficacy of medication therapy is difficult to maintain after drug withdrawal, and the recurrence rate of EMS is significant. Hormonal medicines, in particular, are very easy to cause unpleasant responses and have evident side effects (Taylor *et al.*, 2021), resulting in an unsatisfactory clinical therapeutic effect of EMS. As a result, it is critical to develop novel treatment strategies and medications to increase the effectiveness of EMS.

Curcumol (Cur) is the main active ingredient of the Chinese herb *Curcuma zedoary*, which has the efficacy of moving qi and breaking up blood (promoting blood circulation), eliminating accumulation and relieving pain (aiding digestion, anti-inflammatory, and analgesic) and is used to treat symptoms such as menstrual occlusion of bruises and blood, as well as distension and pain caused by food accumulation (Qin *et al.*, 2022). Cur has been found in modern study to have antitumor and antiviral properties (Wei *et al.*, 2019). Bian et al. (Bian *et al.*, 2023) showed that Cur disrupted the process of epithelial mesenchymal transition by stimulating the degradation of HIF-1α, and inhibited the invasion and migration of colorectal cancer cells by suppressing Gls1-mediated glutamine catabolism. Cur was able to promote the secretion of more IFN-β proteins from encephalomyocarditis virus-infected HEK-293 T cells and inhibit encephalomyocarditis virus-mediated degradation of TANK proteins, promote the expression of MDA5 and P-IRF3, and exert antiviral activities (Zheng *et al.*, 2021). Cur has been demonstrated to decrease endometrial

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stromal cell (ESC) proliferation, migration, and inflammatory cytokine secretion via the JAK2/STAT3 signaling pathway, as well as the formation of ectopic foci in rats with EMS (Wang *et al.*, 2022). However, there have been fewer research on Cur in EMS, and it is unclear whether Cur can affect the inflammation and malignant progression of endometrial cells through other pathways. Furthermore, no studies have examined the influence of Cur on immunological function during the development of EMS.

Inflammatory response and aberrant immune function cause pain, surrounding tissue remodeling, fibrosis, adhesion formation, and infertility and are strongly linked to the initiation and progression of EMS (Riccio *et al.*, 2018). The inflammatory reaction is accompanied by the degranulation and activation of local mast cells and macrophages, which produce substantial amounts of pro-inflammatory cytokines and chemokines. Cytokines carry information between cells, govern inflammatory responses, and disturb the body's immunity, whereas chemokines are a special class of cytokines that chemotactic inflammatory cells and drive them to move in a directed manner (Zhang *et al.*, 2023). Abnormalities in the expression and regulatory mechanisms of immune and inflammation-related cytokines cause a decrease in the body's immune surveillance and defense functions, and ectopic lesions produce immune tolerance or immune escape, allowing the lesions to survive and grow and eventually develop into EMS (Wang *et al.*, 2020). To treat EMS, it is critical to control the inflammatory response and reduce immune function impairment.

Endometrial cells include endometrial glandular epithelial cells and embryonic stem cells (ESCs). During EMS creation, glandular epithelial cells are engaged in the growth of ectopic foci, whereas ESCs are involved in their attachment, with ESC adhesion being the first step in EMS formation (Ochoa Bernal and Fazleabas, 2024). In this experiment, the effect of Cur on the proliferative viability, migration, and invasive activity of ESCs, which can represent endometrial cells, was investigated. Rat serum was extracted, and the ameliorative effects of Cur on inflammation and immune function in rats were analyzed by determining the percentage of inflammatory factors and T-lymphokine subtypes and exploring their mechanisms. The results of the study provide data support for the development of clinical EMS agents.

MATERIALS AND METHOD

EMS rat model construction

30 healthy, unmated SD female rats (7 weeks old, 260~280 g) were acquired from Beijing Institute of biological products Co. Ltd (License No.: SYXK (Beijing) 2016-0051). The First Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine Animal Ethics Committee accepted the study (approval number: TCM-LAEC2019-05). Rats were acclimatized and fed freely for 7 days at 22±2°C, 55% humidity, and 12 h/12 h light/darkness. After acclimation, the EMS rat model was established: rats were anesthetized with an intraperitoneal injection of 2% sodium pentobarbital at a dose of 0.25 mL/100 g. Rats were secured in the head and limbs, with the abdomen exposed. An abdominal incision was made and the uterus was examined. The right uterus was isolated and exposed, and its cervix was ligated around 1 cm from the ovary. After ligation, around 2 cm of middle uterine tissue was removed and deposited in a sterile petri plate with 0.9% saline. Excess fat and connective tissue were removed from the tissue in the petri dish. The tissue was cut longitudinally along the tethered area into 4 mm×4 mm endometrial tissue fragments. These were sutured to the bifurcation of the uterus, the vicinity of the left ovary, and the left peritoneal wall, respectively. The abdominal cavity was cleaned with saline and closed with a 4-0 silk suture. Four weeks following modeling, the transplanted endometrium was dissected for examination. When the endometrium grew in size, was hyaline cystic or nodular in shape, had fluid accumulation inside it, and had connective tissue coverings and vascularization on the surface, a small piece of endometrial tissue was removed for pathological evaluation. Examination results indicating the presence of endometrial glands or epithelial and mesenchymal cell production demonstrated successful modeling (Bashir *et al.*, 2023). In this work, we successfully produced 27 model rats, with a 90.00% success rate.

HE staining for pathological examination

A tiny amount of fixed endothelium tissue was treated to ethanol gradient dehydration, xylene clearing, and wax dipping before being sliced into 5 μm-thick paraffin sections with a slicer. The paraffin sections were baked at 65°C for 2 h, then deparaffinized with xylene and rehydrated using an alcohol gradient. Sections were stained with hematoxylin (C0107, Beyotime, Shanghai, China) for 15 min, then differentiated with 1% acidic alcohol (containing 70% hydrochloric acid) for 30 seconds, rinsed with running water, and finally stained with 0.5% eosin (G1100, Solarbio, Beijing, China) for three min. Following staining, the sections were dehydrated using an alcohol gradient, then cleared with xylene and sealed with neutral gum (G8590, Solarbio). Finally, the tissue and cellular morphology of the slices were examined using an inverted microscope (to see if there were endometrial glands or epithelial and mesenchymal cells) to ensure that the EMS rat model had been effectively constructed.

Cultivation and passage of endometrial cells

Four rats were randomly chosen from successful modeling rats, sedated with 2% sodium pentobarbital intraperitoneally and the abdomen was opened under aseptic conditions to separate endometrial tissues. Tissues

were cleaned and cuted before being digested with three times the volume of trypsin at 37ºC for 40 min. The digestion was then terminated by adding DMEM/F12 medium containing 20% FBS (whole medium). The supernatant was removed by centrifugation, and the cells were resuspended in whole medium. Endometrial cells were grown in 25 cm^2 flasks at 1×10^5 cells/mL at 37ºC in a 5% CO_2 incubator. The cell passaging was started when the cell apposition reached 85%, and the cells were passaged at 3 d intervals.

Separation and purification of ESC

After trypsin digestion, take the second generation endometrial cell suspension, filter it to remove mucus and undigested tissue, add 10 mL of complete medium, mix well, and leave for 30 min. Then, take 8 mL of top cell suspension. Centrifuge to extract the cell precipitate, then resuspend the whole medium and allow it to stand. Take 8 mL of the top cell suspension and filter the residual adenoepithelial cell clusters through a sieve to yield ESC. ESC were cultivated and passaged similarly to procedure 2.2, with the fluid replaced every two days. An inverted microscope was used to investigate cell morphology.

Immunofluorescence assay

The immunofluorescence technique was utilized to determine the purity of ESC. Purified 4th generation ESC was injected into 6-well plates, and immunofluorescence was used to identify cells when around 80% of them were adherent to the wall. ESC were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then closed with 2% BSA. ESC were then treated with rabbit anti-Vimentin (ab92547, 1:500, Abcam, Waltham, MA, USA) and Cytokeratin (ab53280, 1:500) at 4℃ overnight. The cells were rinsed with PBS and then treated with FITC-labeled sheep anti-rabbit IgG (ab205718, 1:2000) for 1 h at 37℃. The nuclei were labeled with DAPI for 5 min, rinsed with PBS, and then examined using a fluorescence inverted microscope with the addition of anti-fluorescence quencher to assess the purity of the cells.

Reagent interventions and grouping

Cur powder (batch no. 170524, purity ≥90%) was procured from China National Institute for the Control of Pharmaceutical and Biological Products, whereas NF-κB activator PMA was obtained from Shanghai Acmec Biochemical Co., Ltd. Cur powder (100 g) was weighed into a 15 mL centrifuge tube, and 10 mL of anhydrous ethanol (Sigma, MO, USA) was added to form a Cur master batch at a mass concentration of 1μmol/L. This was then diluted with cell culture medium to form Cur solutions at mass concentrations of 0, 0.05, 0.1, 0.2, and 0.4 μmol/L. DMSO (Solarbio, Beijing, China) was used to prepare PMA.

The experimental rats were assigned at random into five groups: Sham, Model, PMA, Cur, and Cur+PMA. In the Sham group, just surgical suture was performed, but in the Model group, autograft and surgical suture were conducted. The model rats in the PMA group were given 5 mg/kg PMA via gavage, while the model rats in the Cur group was given 20mg/kg Cur via gavage. The Cur+PMA group received 20mg/kg Cur and 5mg/kg PMA by gavage.

ESC were separated into four groups: Control, PMA, Cur, and Cur+PMA. The Control group received ESC isolated from Sham group rats, whereas the PMA group received ESC 1μmol/L PMA therapy, the Cur group received ESC 0.1μmol/L Cur treatment and the Cur+PMA group received both ESC 0.1μmol/L Cur and 1μmol/L PMA.

CCK-8 detection

ESCs in logarithmic growth phase were inoculated into 96-well plates at a density of 1×10^4 cells/well. 200μL of culture medium was added to each well, and 0.05, 0.1, 0.2, and 0.4μmol/L Cur were added to treat the ESCs for 48 h. The control group received 0μmol/L Cur. The control group was treated with 0μmol/L Cur for 48 h. After adding 20μL of CCK-8 reagent solution (HY-K0301, MedChem Express, Monmouth Junction, NJ, USA) to each well and mixing, the culture was allowed to continue for 2 h. The cell proliferation viability (OD value at the 450 nm wavelength) in each well was determined using a DR-200Bc enzyme labeling device (Diatek, Jiangsu, China). The proliferative inhibition rate was determined. The proliferation inhibition rate was determined as "(1 - OD value of treatment group/OD value of control group) × 100%" and a Cur concentration close to half of the inhibition rate was chosen.

LDH level detection

LDH levels in cell culture supernatants were measured with the LDH Cytotoxicity Assay Kit (C0016, Beyotime). The amount of LDH released from damaged cell can be used to determine the number of dead cells. ESC were injected into 96-well enzyme-labeled plates following the directions. Plates were stimulated with 0.05, 0.1, 0.2 and 0.4μmol/L Cur for 48 h, then centrifuged and aspirated the supernatant. After a 10-fold dilution with PBS, add the LDH release reagent, mix thoroughly, and incubate for 1h. After centrifugation, 120μL of supernatant was aspirated from each well and moved to a new plate. An enzyme labeler detected the absorbance value at 450 nm, and the LDH release level of ESCs at various Cur treatment conditions was determined using the LDH standard curve.

Transwell detection

Transwells were inserted in 6-well plates and 100μL of pre-cooled Matrigel matrix gel was added to the chambers in advance for the invasiveness assay (this step was skipped for the migration assay). The chambers were then

allowed to stand for 5 h at 37ºC in a 5% CO_2 incubator. After adding 200μL of 1×10^5 cells/mL ESC suspension and 0.1μmol/L Cur to the tiny chambers, 600μL of medium with 10% FBS was added to each well of the lower chamber. The plates were then placed in a 37ºC, 5% CO_2 incubator for 48 h. The plates were removed, and the lower chamber's culture fluid was discarded. They were then rinsed with PBS, fixed with 1mL 4% paraformaldehyde for 30 min, and dried. The cells were stained with 1 mL 1% crystal violet for 10 min, rinsed with PBS for 3 min, and photographed under a high magnification microscope. The number of invaded and migrating cells was determined using Image J software.

Cell scratching assay

Control and 0.1μmol/L Cur-treated ESCs were digested with trypsin, resuspended in PBS, and injected at 5×10^5 cells/well in 6-well plates with labeled lines. When the cell growth reached more than 90% confluence, a 200 μL sterile pipette tip was used to draw a line perpendicular to the bottom of the well plate uniformly and gently. The detached cells were washed with PBS and 2 mL of FBS-free media was added to the culture. The cells were inspected and photographed under an inverted microscope at 0 and 48 h. The breadth of the scratch was measured with Image J software and the cell migration rate was determined.

ELISA assay

In Sham, Model, PMA, Cur and Cur + PMA groups, the abdominal aorta of rats by drawing 2 mL of blood and leaving it at room temperature for 30 min. The serum was separated after being centrifuged for 15 min at 4ºC. The serum was tested using ELISA (enzyme-linked immunosorbent assay). IL-8, IL-6, and TNF-α concentrations were measured using purchased from eBioscience kits (160528, 180704, and 201206) as per instructions. Sample analysis buffer (100μL) was added to the plate and incubated at 37ºC for 2h. Next, 100μL of biotinylated antibody was added to the plate and incubated at 37ºC for 1.5h. To begin, add 100μL of affinity hormone-horseradish peroxidase marker and incubate at 37ºC for 20 min. Next, add 100μL of TMB color development solution and incubate for 20 min away from light. Finally, add 50μL of termination solution to end the reaction. The absorbance of the samples at 450 nm was measured with an enzyme marker. A standard curve was created by using the absorbance value as the vertical coordinate and the concentration of the standard as the horizontal coordinate. The concentrations of IL-8, IL-6 and TNF-α were then determined based on the standard curve.

T-lymphocyte subpopulation assay

T-lymphocyte subsets $CD3^+$, $CD4^+$ and $CD8^+$ were identified using flow cytometry (FACS Calibur, BD, NJ, USA). All T-lymphocytes have the CD3 receptor on their surface, hence $CD3^+$ represents the total number of T-lymphocytes (Wu *et al.*, 2022). CD4 and CD8 are markers of helper T cells that are linked to T cell activity. $CD4^+/CD8^+$ ratios typically range from 1.4 to 2.5. A ratio greater than 2.5 indicates an overactive immune system, with immune cells attacking normal cells, leading to autoimmune diseases. A ratio less than 1.4 indicates low immune function (less than 1.0 indicates significant immunosuppression). Sera were obtained from the Sham, Model, PMA, Cur and Cur+PMA groups and operated according to the 8920012 kit (Agilent, CA, USA) and instrument instructions. SSC and CD3-FITC-FL1, CD3-FITC-FL1 and CD4-APC-FL4, CD3-FITC-FL1 and CD8-PE-FL2 were used as two-parameters to measure the proportion of fluorescence of $CD3^+$, $CD4^+$ and $CD8^+$ cells, respectively, and $CD4^+/CD8^+$ values were computed.

Western blot

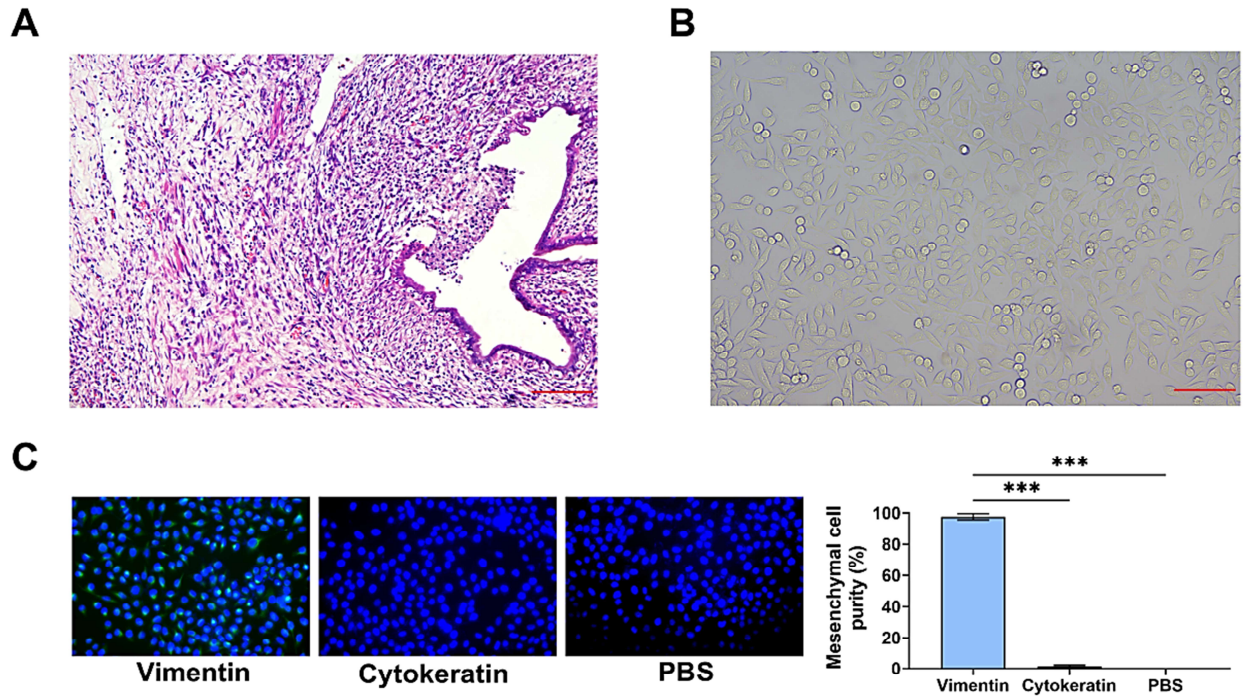
ESC cells and endothelial tissues of rats in Model, PMA, Cur and Cur+PMA groups were collected after various treatments, and total protein was extracted using RIPA lysis buffer. Protein concentration was measured using a BCA kit (R21250, Yuanye, Shanghai, China). The proteins were then separated using SDS-PAGE electrophoresis and transferred to a PVDF membrane. The membrane was closed at room temperature for 2 h with 5% skimmed milk powder and then washed with TBST. Primary antibodies (1:1000) were used overnight at 4°C against rabbit anti-MMP-2 (ab92536), MMP-9 (ab76003), NF-κB p65 (ab32536), p-NF-κB p65 (ab76302), IκBα (ab32518), p-IκBα (ab92700), PRL-3 (ab50276), and the endogenous reference antibody GAPDH (ab22555). Following that, the protein was washed three times with TBST and incubated with sheep anti-rabbit IgG (170418, 1:2000) at room temperature for 2 h. Antibodies were bought from Abcam (Waltham, Massachusetts, USA). The bands were developed with ECL (P0018S, Beyotime) chemiluminescent solution, exposed and shot with a gel imager (5200, Tanon, Shanghai, China), and analyzed with Image J for the gray value of the protein bands and the relative expression amount.

ETHICAL APPROVAL

The First Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine Animal Ethics Committee accepted the study (approval No.TCM-LAEC2019-05).

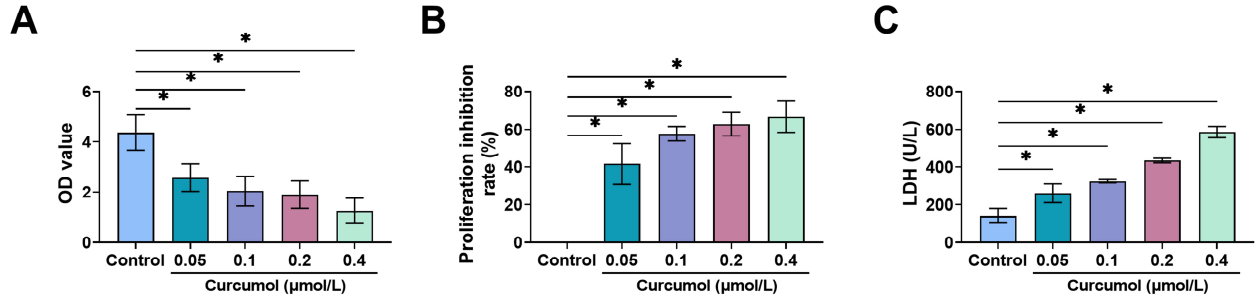
STATISTICAL ANALYSIS

The data was analyzed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA). The measurement data of normal distribution were shown as mean ± standard error ($\bar{x}$ ±s). The unpaired *t*-test and One-way ANOVA test were used to compare groups, with Turkey post hoc analysis included. $P<0.05$ indicates a significant difference.



A: The EMS rat model was created via autologous transplantation as well as surgical suture. A tiny sample of endothelium tissue was collected for HE staining histology. (20×, bar=100 μm).
B: Use an inverted microscope to examine the sectioned tissue and ESC morphology. (40×, bar=50 μm).
C: The ESC marker protein Vimentin was detected, and the purity of ESC was determined by positive green fluorescence in the cells. (40×, bar=50 μm).

Fig. 1: Building an EMS rat model and determining the purity of ESCs.



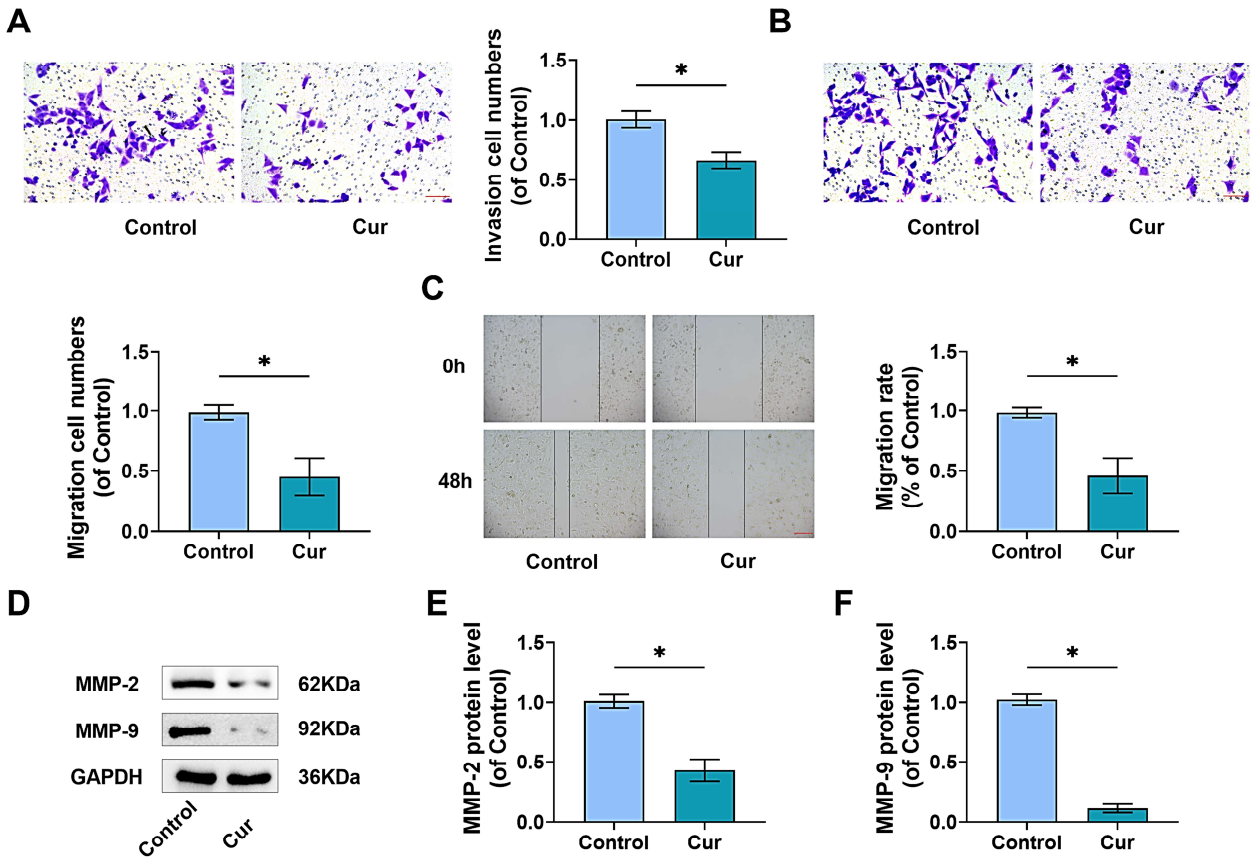
A-B: ESC were treated with 0, 0.05, 0.1, 0.2, and 0.4 μmol/L of Cur for 48 h. ESC proliferative activity was measured using the CCK-8 test, and the proliferation inhibition rate was determined.
C: The cytotoxic effect of Cur on ESCs was determined by measuring the LDH release level of ESCs under various Cur treatment concentrations using the LDH kit.

Fig. 2: Cur reduces ESC proliferation

RESULTS

EMS rat model and ESC purity characterisation

A tiny sample of endometrial tissue from the EMS rat model was used for HE staining. Under the inverted microscope, fig. 1A shows that the ectopic endometrium is thicker, with more glands, epithelial cells, and ESCs, a dense structure, and a rich blood supply, indicating that the model creation was successful. Furthermore, it was discovered that the ESCs showed irregular protrusions similar to fibroblasts, with distinct borders, tight organization, large nuclei, full cytoplasm, and tight growth in clusters after adhesion to the wall (fig. 1B). Specific flag molecules on the surface of various cells vary; the ESC marker protein is Vimentin, whereas the adenoepithelial cell marker protein is Cytokeratin. Immunofluorescence analysis revealed that green luminous cells of the ESC marker protein accounted for more than 95% of the cells, which proved that ESCs were predominantly found in the endometrial cells (fig. 1C). As a result, we picked ESC as the study object and then examined the influence of Cur on ESC's biological function.



A: 0.1μmol/L Cur was used to treat ESCs and the amount of ESCs invading the lower compartment was measured using the Transwell assay. (20×, bar=100μm).
B: ESCs were treated with 0.1μmol/L Cur and the number of ESCs migrating to the bottom compartment was determined by Transwell assay. (20×, bar=100μm).
C: Scratch tests were conducted to determine the cell spacing of ESC at 0 and 48h of culture, respectively, in order to quantify their migration rates. (10×, bar=200μm).
D-F: Western blot analysis to determine the effect of Cur therapy on the expression of ESC invasive migration-related proteins MMP-2 and MMP-9.

Fig. 3: Cur prevents ESC invasion and metastases

Cur prevents ESC proliferation

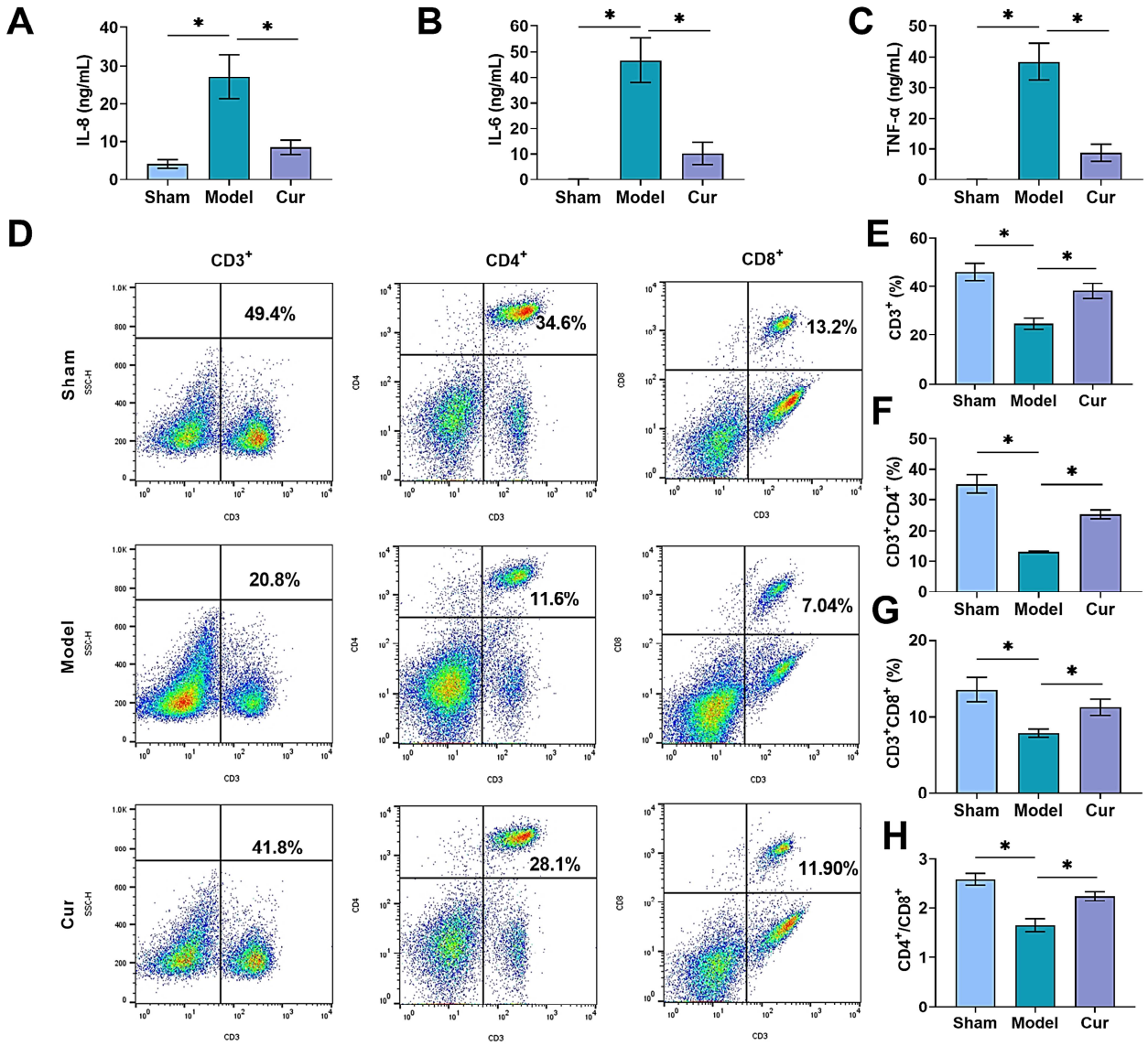
The study included a control group (0μmol/L Cur) and experimental groups with varying concentrations of Cur (0.05, 0.1, 0.2, 0.4μmol/L Cur). The CCK-8 assay revealed that the OD value of ESC cells decreased significantly with the increase of Cur concentration within 48 hours (fig. 2A), but the inhibition rate of cell proliferation increased significantly (fig. 2B), indicating that Cur inhibited the proliferation of ESCs in a concentration-dependent manner. The amount of LDH released from injured ESCs was also enhanced, as shown in fig. 2C, showing that the frequency of ESC fatalities was growing. The experimental results showed that Cur significantly inhibited ESC growth. At the same time, Cur at 0.1μmol/L significantly reduced cell growth by nearly 50%, so this concentration was selected for subsequent experiments.

Cur prevents ESC invasion and metastases

After screening, Cur at 0.1μmol/L was chosen for invasion and migration tests. The Cur group had considerably fewer number of ESCs invading and migrating to the lower chamber than the control group (fig. 3A-3B). Cur treatment resulted with considerably decreased ESC migration rates (fig. 3C). Western blot analysis revealed that Cur treated dramatically reduced the levels of the ESC invasive migration-related proteins MMP-2 and MMP-9 (fig. 3D-3F). Therefore, 0.1μmol/L Cur could significantly inhibit the invasion and migration ability of ESCs.

Cur reduces inflammation and boosts immunity in EMS rats

EMS is an inflammatory disease and we evaluated the expression of IL-8, IL-6 and TNF-α in rat serum using the Elisa method. Fig. 4A-4C shows that rats in the model group had significantly higher levels of three inflammatory factors in their serum compared to the

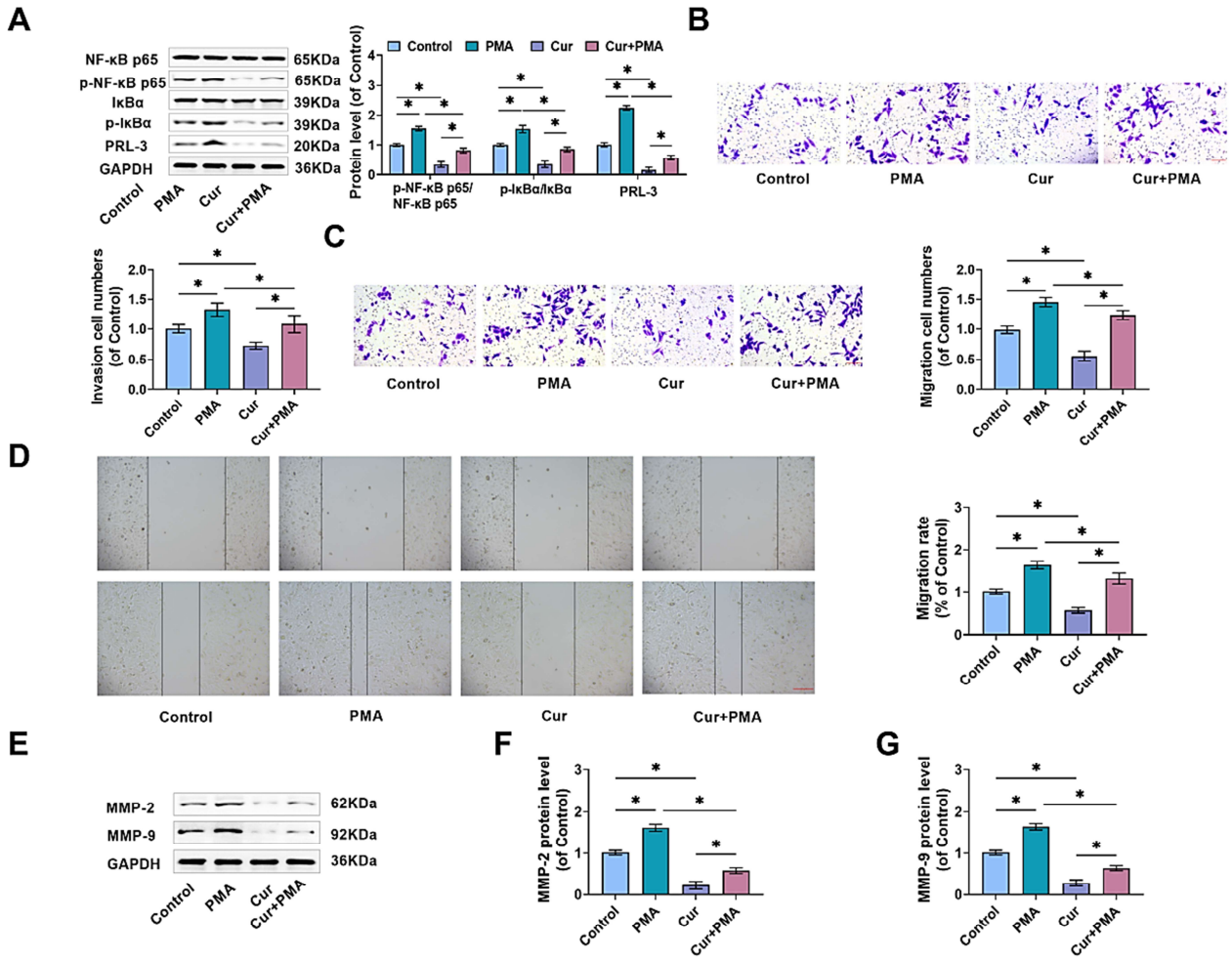


A-C: ELISA kits were used to measure serum levels of IL-8, IL-6 and TNF-α in rats from Sham, Model, and Cur groups.
D-H: A flow cytometric test was used to determine the percentage of T-lymphocyte subpopulations $CD3^+$, $CD4^+$ and $CD8^+$ in the serum of rats in each group, and the $CD4^+/CD8^+$ ratio was computed.

Fig. 4: Cur reduces inflammation and enhances immunological function in EMS rats

control group. However, treatment with 20 mg/kg Cur gavage significantly reduced the expression of IL-8, IL-6, and TNF-α. This suggests that Cur successfully controlled the inflammatory response in the model rats and helped to alleviate tissue damage. In addition, an overactive inflammatory response causes immunological dysfunction. As a result, using the clinical immunity assay, we measured T-lymphocyte subpopulations ($CD3^+$, $CD4^+$, $CD8^+$) in the rats' serum to determine whether the ratio of $CD4^+$ to $CD8^+$ was within the normal range, allowing us to assess whether the experimental rats' immune function was abnormal.

Fig. 4D-4G show the results from flow cytometry analysis. Following Cur gavage administration, the model rats' $CD3^+$ content grew considerably, while the proportions of $CD4^+$ and $CD8^+$ increased and decreased, respectively. By calculation, the $CD4^+/CD8^+$ value in the serum of the Sham group was highest and the rats' immune function was normal, the ratio of the model group was reduced to lowest, indicating that the rats were in a state of severe immunosuppression *in vivo* at this time. And the rats' immune function returned to normal after Cur gavage (the $CD4^+/CD8^+$ value was higher than the model group, fig. 4H). The study found that 20 mg/kg Cur gavage therapy significantly improved the immunological function of model rats.

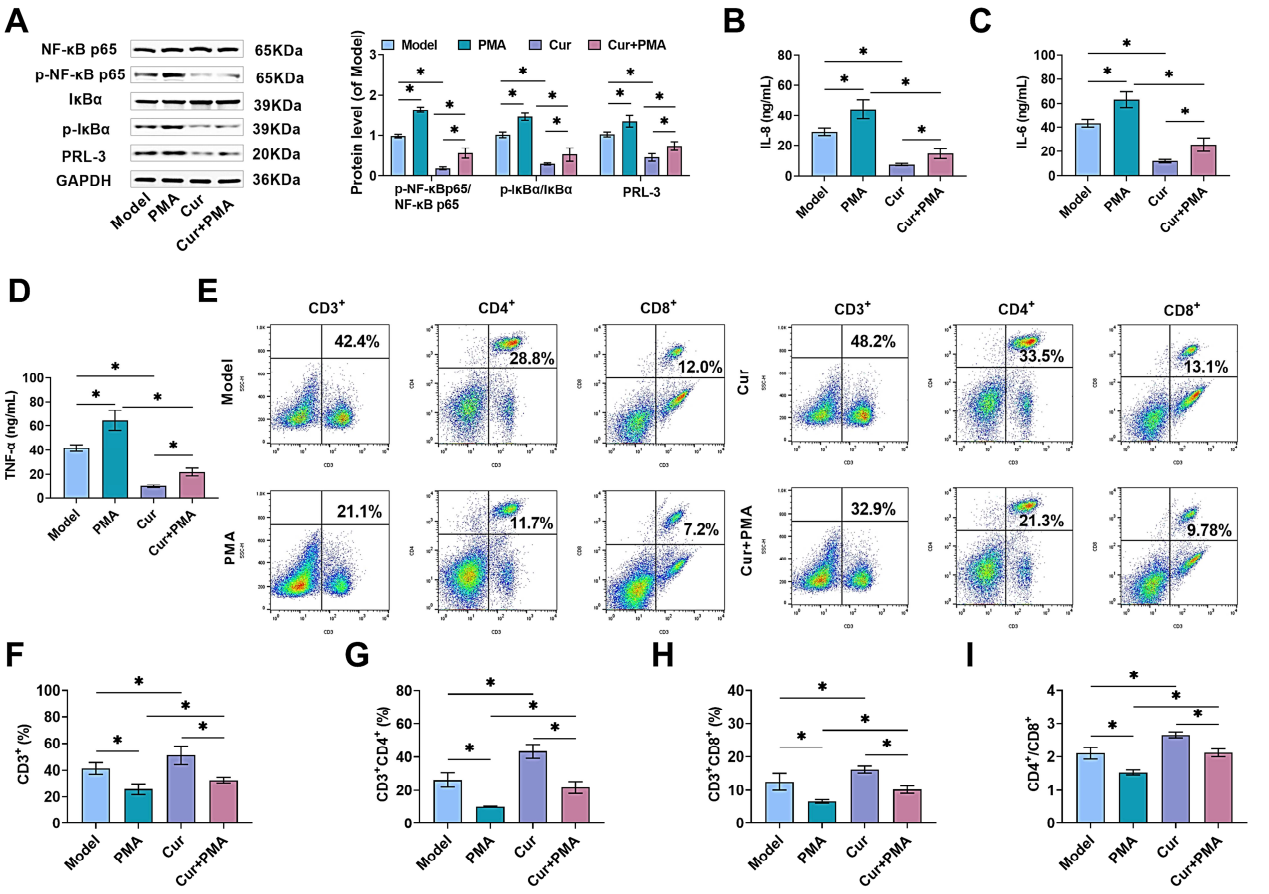


A: After PMA treatment of ESCs, Western blots were used to identify the expression levels of NF-κB signaling pathway proteins NF-κB p65, p-NF-κB p65, IκBα, p-IκBα, and PRL-3 in each group.
B: Following the selection of Cur and PMA to treat ESCs, the number of ESCs entering the lower chamber was determined using the Transwell test. (40×, bar=50 μm). (20×, bar=100 μm).
C: Following the selection of Cur and PMA to treat ESCs, the number of ESCs migrating to the lower chamber was determined using the Transwell assay.
D: A scratch test was used to determine the cell spacing of PMA-treated ESCs at 0 and 48 h, respectively, in order to compute migration rates. (10×, bar=200 μm).
E-G: A western blot was used to detect changes in the expression of ESC invasive migration-related proteins MMP-2 and MMP-9 following PMA treatment of ESCs.

Fig. 5: Cur suppresses ESC invasion and metastasis by regulating the NF-κB signaling pathway

Cur suppresses ESC invasion and metastasis via targeting the NF-κB pathway

The NF-κB pathway plays a role in various physiological and pathological processes in the body, such as inflammation, innate immunity, cell proliferation and self-destructive apoptosis. Inhibiting the NF-κB signaling pathway may be a possible target for non-hormonal treatment of EMS, as it plays a critical role in the pathophysiology of EMS ectopic cells, including inflammation, proliferation, survival, invasion and metastasis (Li *et al.*, 2019). Therefore, we investigated whether Cur mediates the NF-κB pathway and its potential role. ESCs were randomly assigned to four groups: Control, PMA, Cur and Cur+PMA groups. fig. 5A shows that administering 1μmol/L PMA and 0.1μmol/L Cur alone resulted in significant up-regulation and down-regulation of p-NF-κB p65, p-IκB and PRL-3 proteins in ESCs, indicating activation and inhibition of the NF-κB pathway, respectively. Adding 1μmol/L PMA dramatically reduced the impact of 0.1μmol/L Cur, while upregulating p-NF-κB p65, p-IκB and PRL-3 transcripts. The transwell and cell scratch experiments revealed the same pattern: PMA and Cur enhanced and prevented ESC invasion and migration, respectively, whereas ESC migration rate rose and reduced, as evaluated quantitatively by Image J. Combining the two treatments partially negated the effect of Cur (fig. 5B-5D), indicating that Cur regulates ESC invasion and migration via the NF-κB pathway. Western



A: After PMA gavage, Western blot revealed the expression levels of NF-κB signaling pathway proteins NF-κB p65, p-NF-κB p65, IκBα, p-IκBα, and PRL-3 in the endothelium tissues of rats in each group.
B-D: ELISA kits were used to measure serum levels of IL-8, IL-6, and TNF-α in rats in each group.
E-I: Flow cytometric assay to determine the percentage of T-lymphocyte subpopulations $CD3^+$, $CD4^+$, and $CD8^+$ in the serum of rats in each group and calculate $CD4^+/CD8^+$ ratios.

Fig. 6: Cur enhances immunological function in EMS rats by regulating the NF-κB signaling pathway

blot analysis revealed that activating the NF-κB pathway (with PMA) and inhibiting the NF-κB pathway (with Cur) resulted in significant increases and decreases in the invasive migration-associated proteins MMP-2 and MMP-9. Synergistic PMA effectively reversed the inhibitory effect of Cur on the expression of MMP-2 and MMP-9 (fig. 5E-5G), confirming that Cur inhibited ESC invasion and metastasis.

Cur improves immunological function in EMS rats via blocking the NF-κB pathway

Next, we used PMA gavage treatment on model rats to study Cur's mechanism of modulating inflammation and immunological function *in vivo* using rescue studies. *In vivo*, 5 mg/kg PMA and 20 mg/kg Cur gavage treatment resulted in increased and decreased protein and phosphorylation levels of the NF-κB pathway, respectively. Co-gavage treatment effectively reversed Cur's inhibitory effect on the pathway (fig. 6A). Cur alone significantly reduced serum levels of inflammatory factors IL-8, IL-6 and TNF-α in rats. However, when combined with PMA, the inhibitory effect of Cur was effectively reversed and IL-8, IL-6 and TNF-α were up-regulated again (fig. 6B-6D), indicating that Cur reduces inflammatory factors in the model rats by inhibiting the NF-κB pathway, thus reducing the inflammatory response. fig. 6E-6H depicts the results of T-lymphocyte subtypes in serum: both $CD3^+$ and $CD3^+CD4^+$ ratios were decreased or raised after PMA or Cur gavage, respectively and PMA was able to counteract Cur's effect; however, $CD3^+CD8^+$ ratios showed the opposite trend. According to calculations, the $CD4^+/CD8^+$ values in the serum of the model and PMA groups were 0.88 and 0.37, respectively, which were less than one, indicating that the rats' immune function was compromised and their immune response was inhibited. The $CD4^+/CD8^+$ value increased to 1.93 after Cur gavage and the rats' immune function was restored to normal. The $CD4^+/CD8^+$ value declined to 1.09 after combined PMA gavage, resulting in the rats' low immune function and weakening the effect of Cur (fig. 6I). The *in vivo* results showed that Cur reduced the NF-κB pathway, preventing inflammation and improving immunological function in EMS rats.

DISCUSSIONS

EMS has both benign illness and malignant tumor characteristics, and its pathogenesis is linked to uncontrolled cell proliferation, as well as local and distant migration and invasion, with the ability to migrate and invade being critical to EMS pathogenesis (Hung *et al.*, 2021; Lu *et al.*, 2020). According to reports, ESC migration, invasion, and survival may be the primary cause of EMS growth (Nezhat *et al.*, 2008). The ability to effectively and actively prevent the invasion and migration of ESCs has become critical to preventing EMS, and inhibiting their invasive and migratory abilities may potentially aid in inhibiting the formation and growth of EMS foci, promoting the shrinkage, atrophy, or even disappearance of ectopic foci and slowing the development of EMS. In this study, we chose ESC as the research object and investigated the alterations of ESC invasion and migration capacity using Transwell, cell scratch and Western blot assays following the intervention of 0.1μmol/L Cur. The results showed that the number of ESCs invading and migrating to the lower compartment was significantly reduced, as was the calculated migration rate, and the expression of invasion transfer proteins MMP-2 and MMP-9 (Begum *et al.*, 2021) was significantly suppressed, indicating that Cur significantly inhibited ESC invasion and migration, which could be an important reason for Cur's inhibition of EMS development.

ESC immune function is critical to the development of EMS (Ahn *et al.*, 2015). When endometrial tissue fragments appear outside the uterine cavity surface, the body's immune system recognizes them and launches an immunological response, stimulating the production of a high number of macrophages to phagocytose and eliminate them. The immune system releases cytokines such IL-8, IL-6 and TNF-α. TNF-α, a multifunctional cytokine, triggers the release of inflammatory molecules such IL-6, IL-8 and chemokines. This promotes the infiltration of inflammatory cells and neovascularization, exacerbating local inflammation (Leenen *et al.*, 2021). These cytokines play important roles in the initiation, development, and regulation of the immune-inflammatory response, among other things, and they also stimulate T and B lymphocyte responses, which eventually lead to the formation of localized adhesions and promote the onset and progression of EMS (Zhou *et al.*, 2019). T lymphocytes are among of the most significant adaptive immune response cells in the immune system (Szukiewicz, 2022). T lymphocyte subsets include $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes. $CD3^+$ is expressed on all T cells and $CD3^+$ T cell co-receptors help activate $CD4^+$ and $CD8^+$ T cells (Bafor *et al.*, 2022). In EMS, $CD4^+$ T cells include Th1 and Th2, which work together to provide cellular immunity. When Th1 and Th2 are out of balance, the body's immune activity is suppressed, which causes local pelvic adhesions, fibrosis, and changes in the immunological milieu, as well as the formation and development of ectopic lesions (Scioscia *et al.*, 2011). The primary role of $CD8^+$ T lymphocytes is to identify endogenous antigens and kill infected cells. $CD4^+$ and $CD8^+$ numbers are in equilibrium in healthy organisms, but they fluctuate in response to infection, autoimmune illness, or malignancy (Brummelman *et al.*, 2018). $CD4^+/CD8^+$ is an important predictor of clinical outcome, immunological dysfunction and viral reservoir size; this ratio shows the strength of the patient's immune system and helps predict the possibility of EMS in patients (Laidlaw *et al.*, 2016). In this study, we found that serum levels of IL-8, IL-6 and TNF-α decreased, the ratios of $CD3^+$ and $CD3^+CD4^+$ increased significantly, the ratio of $CD3^+CD8^+$ decreased significantly, and the value of $CD4^+/CD8^+$ was elevated to the normal value in the model rats after gavage treatment with 20 mg/kg Cur. This indicated that Cur effectively alleviated the inflammatory response and impaired immune function in the model rats *in vivo*.

Relevant research have demonstrated that the NF-kB signaling pathway regulates the inflammatory response, cell migration, and metastasis of EMS, among other things, and plays a significant role in the pathophysiology of EMS (Li *et al.*, 2023). NF-κB p65 normally binds to IκB, preventing it from entering the nucleus. In endometriotic cells, IL-8, IL-6, TNF-α and cytokines secreted by T cells activate the IKK complex, which phosphorylates the IκB protein, leading to its ubiquitination and proteasomal degradation. This releases NF-κB p65 into the nucleus and participates in transcriptional regulation of relevant genes and cytokines, affecting the degree and extent of inflammation (Laczko *et al.*, 2020; Liu *et al.*, 2022). PRL-3, a protein complex phosphatase, regulates p65 phosphorylation, which activates NF-κB signaling (Zhang *et al.*, 2016) and then play its biological role. Some investigations found that PRL-3 expression was considerably higher in endometrial cells of EMS patients, perhaps promoting lesion spread (Ren *et al.*, 2017), suggested that the NF-κB signaling pathway may promote invasive metastasis in EMS endometrial cells. Our study found that ESC treated with 0.1μmol/L Cur significantly reduced the expression levels of the pathway proteins p-NF-κB p65/NF-κB p65, p-IκB/IκB and PRL-3. However, up-regulation of the pathway proteins occurred when combined with the 1 μmol/L pathway activator, PMA, indicating that the inhibitory effect of Cur on the NF-κB pathway was impaired. The results of Transwell, cell scratch, and Western blot showed the same trend: PMA effectively reversed the inhibitory effect of Cur on the expression of ESC invasion, migration and related proteins (MMP-2, MMP-9), proving that Cur inhibited ESC invasion and migration by inhibiting the NF-κB pathway. Model rats were given 20 mg/kg Cur and 5 mg/kg PMA via gavage.

Western blot analysis was used to assess changes in the expression of NF-κB pathway proteins in endothelial tissues. Compared with Cur alone, Cur+PMA significantly lowered the expression levels of p-NF-κB p65/NF-κB p65, p-IκB/IκB, and PRL-3 in rat endothelial tissues, indicating that PMA can diminish Cur's inhibitory action on the NF-κB pathway *in vivo*. Meanwhile, after PMA gavage, serum levels of IL-8, IL-6 and TNF-α, as well as $CD4^+/CD8^+$ values, significantly increased, weakening Cur's inhibitory effect on inflammatory response and immune impairment in rats, proving that Cur alleviated the inflammatory response and improved immune function by inhibiting the NF-κB pathway in rats.

Taken together, compared with previous studies (Wang et al., 2022), this study confirmed that Cur can also reduce the invasion and metastasis of endothelial cells in EMS mice by inhibiting the NF-κB signaling pathway, reduce the inflammatory response *in vivo* and also improve immunological function.

CONCLUSION

Cur was shown in this work to decrease ESC migration and invasio *in vitro* cellular assays, as well as to attenuate the inflammatory response and immune function impairment in *in vivo* rat model experiments, implying that Cur may be used to treat EMS in the clinic. Activating the NF-κB signaling pathway increased ESC migration and invasion, indicating that Cur may suppress EMS through this mechanism. In the future, NF-κB can be used as a target to delay the deterioration of EMS by inhibiting the activation of the NF-κB pathway. This study presents an effective approach for EMS treatment and supports the data for further “precision therapy”. In the future, we can investigate Cur's influence on the growth of ectopic lesions *in vivo*, as well as its additional effect when combined with other medications. Based on clinical trials, we may rationally construct Cur's dosage and ratio to promote the development of effective EMS medications. Furthermore, we need seek collaboration from the domains of engineering and materials science in order to select appropriate Cur delivery systems and carriers for EMS patients, taking into account Cur bioavailability.

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