IL-6 could regulate Th17/Treg cells to restore the local microenvironment of endometriosis

Keywords
IL-6, Treg, Th17, Estrogen, Endometriosis, Macrophages

Abstract
Introduction
In this study, we studied anti-IL-6 antibodies to treat endometriosis (EMS) by balancing Th17/Treg cells, providing a new basis for EMS research and treatment.

Material and methods
The rat EMS model was constructed to study the effect of anti-IL-6 on EMS. HE staining was performed to observe the lesions of the endometrium in rats. We have used anti-IL-6 to treat EMS rats. Then ELISA was used to measure the inflammatory factors IL-6, TGF-β and hormone estrogen levels in the rat serum. qRT-PCR and Western blot were used to measure estrogen receptor protein and apoptosis protein levels. Th17/Treg and CD163+/CD86+ ratios were determined by flow cytometry. CD34+ and CD163+ were measured by immunohistochemistry. TUNEL was performed to detect apoptosis in EMS lesions.

Results
The elevated levels of IL-6, TGF-β and estrogen in EMS rats indicated successful modeling. The ratio of Th17/Treg was imbalanced and raised. The ratio of Th17/Treg in anti-IL-6 rats recovered. The expression of FOXP3 in anti-IL-6 rats increased, and ROR-γt expression was suppressed. Anti-IL-6 could increase the apoptosis of the cells in the lesion. The size of the lesion was reduced. The expression of VEGF in the microenvironment of the lesions of EMS rats decreased.

Conclusions
IL-6 regulated Th17/Treg cells to restore the local microenvironment of EMS. Anti-IL-6 could regulate the immune microenvironment of EMS rats to alleviate the progression of EMS disease.

Explanation letter
Dear Reviewer,

Thank you for your comments concerning our manuscript entitled “IL-6 could regulate Th17/Treg cells to restore the local microenvironment of endometriosis”. Your comments are all very valuable and helpful in the revision and improvements of our paper, and they also have very important guiding significance to our research. We have studied your comments carefully and made corrections. All the changes in the manuscript were highlighted in red. We do hope this could help achieve a better understanding of our work.

Review 1:
This study investigated the effect of anti-IL-6 antibodies in treatment of endometriosis (EMS). The results suggest that blocking IL-6 protects the local tissue environment of EMS by restoring the balance of Th17/Treg cells.
1. What is the rational for the duration of treatment? How long is the period cycle in rats?
Response: Thanks a lot for raising your concern. The duration of treatment is usually determined by
the course of treatment during administration in order to determine when to check the results of treatment. Our method is written to feed the drug up to 14 days. The estrus cycle of each female rat was measured by vaginal smear method, which was generally 4-5 days. Based on the rat's menstrual cycle of about 4 days, which corresponds to three cycles, this can be used as a course of treatment. This is one of our bases for the duration of treatment. There are reports of surgery based on three estrus periods, with results observed within 28 days after surgery.

The report described “The growth, invasion and adhesion of the transplanted endometrium were observed 28 days after operation. Ectopic endometrial growth (5 × 5 mm for transplanted tissue), tissue edema (as a sign of inflammation), and vesicle formation were observed macroscopically. The model was considered successful if the transplanted endometrium had grown at least 2-fold”. We have cited the reference 21 in method #2.2.

Please check the revised manuscript (page 7-8, line112-126).

2. The interaction of humanized antibody to rat IL6 needs to be approved.
Response: We are sorry for causing the confusion. We wrote humanized antibody due to an oversight. We have added source information about anti-IL-6 in method #2.2. “rat anti-IL-6 (AF506, R&D cystems, USA,)”. Please check the revised manuscript (page 7, line 113-114).

3. The effect of anti-IL6 in tissues in Fig. 5 and 6 was examined at only time-point. It would be more convincing to add a middle time-point, such as at 5 days after treatment.
Response: Thanks a lot for your advice. At that time, we did not sacrifice the rats to observe the tissue about 5 days after the operation. The estimated therapeutic effect has not been reflected because of the short time. At present, due to the cost and conditions, it is impossible to reconstruct the rat model and remove the tissues. We will make further exploration if the later experimental conditions were permitted. Thank you for the time and efforts in reviewing our manuscript. These suggestions enable us to improve our future work.

Review 2:
The manuscript entitled "IL-6 could regulate Th17/Treg cells to restore the local microenvironment of endometriosis " seems interesting and fits the profile of the magazine. The described retrospective study may be applicable in everyday clinical practice. I recommend this manuscript for acceptance.
Response: Thank you for your time and efforts in reviewing our manuscript. We will continue to work hard and deliver our achievements to the field.

Review 3:
Dear Authors,
I read the article entitled ‘IL-6 could regulate Th17/Treg cells to restore the local microenvironment of endometriosis’. There are many spelling and grammatical errors. Please write the explanation of the abbreviations where they are used for the first time.
Response: We thank the reviewer for pointing out this question. We have checked and defined the abbreviations, such as “estrogen receptor alpha (ERα) and beta (ERβ)”, “T helper cell 17 (Th17)” and “classically activated (M1) and alternatively activated (M2)”. All changes were marked in red. Please check the revised manuscript.

Abstract
1. Please give the result part as statistical comparisons of both groups. Please specify as numeric value.
Key Words: Please delete Estrogen in Key words.
Response: We appreciate your suggestion. We have provided p value and specific data while describing our results part as statistical comparisons of both groups.
“The results predicted that compared with the Sham group, the levels of IL-6 (p < 0.001) and TGF-β (p < 0.001) in the EMS group increased (Figure 1B).”

“We tested the Estrogen content in rats. The data displayed that compared with the Sham group, the EMS group had higher Estrogen (Estradiol) content (p < 0.001), increased ERβ expression (p < 0.001) and decreased ERα expression (p < 0.001), and the ERβ/ERα ratio (p < 0.001) increased sharply (Figure 2A-2C).

Introduction
1. Please add some sentences about the scientific and clinical significance of the relationship between IL6 and Treg.
Response: We appreciate your suggestion. We have added some sentences about the scientific and clinical significance of the relationship between IL6 and Treg.

“The mode of IL-6 sensing by Treg cells is under debate. Some investigators have suggested that Treg cells not only lose IL-6Rα but also downregulate gp130 in highly inflammatory milieus to become resistant any type of IL-6 signaling (1). However, overall the IL-6 signaling pathway has clearly been identified as a major pathway to dismantle Treg cell identity (2).”

“IL -6 interfered with different components of the immune response (cytotoxic T cells, Treg cells, myeloid cells) may provide an idea for cancer therapy (3).”

Material Methods
1. Please add reference for The treatment of animals during the experiment was conformed to the standard of Guiding Opinions on treating experimental animals issued by the Ministry of science and technology in 2006.
Response: Many thanks for your suggestion. We have added reference 22.

“The treatment of animals during the experiment was conformed to the standard of Guiding Opinions on treating experimental animals issued by the Ministry of science and technology in 2006 (6).”

2. Please add reference and references for EMS model.
Response: Thanks a lot for your advice. We have cited the reference 23 for EMS model. Please check the revised manuscript (page 8, line126).

3. The protein concentration was determined using the BCA method. Please add reference.
Response: Many thanks for your advice. We have added the reference 24 for BCA method.

“Total protein was extracted from endometrium cells using the Ripa Kit (r0010, Solarbio, China). The protein concentration was determined using the BCA method (7).”

Results:
1. Please delete the sentence ‘We constructed a rat EMS model to view the pathology of EMS’.
Response: Thank you for your advice. We have deleted “We constructed a rat EMS model to view the pathology of EMS”. Please check the revised manuscript.
2. Why do you measure proinflammatory factors in serum of rats?
Response: We are grateful for the question you pointed out. Endometriosis forms a specific hormonal environment characterized by high concentrations of estrogens and androgens, whose levels are several times greater than those found in patients' peripheral blood. Consequently, different phenomena are triggered, including cell proliferation, the release of various immunological and inflammatory factors, such as TNF, IL-1, IL-6, IL-8, IL-10, TGF-β1 cytokines, lymphocytic infiltration, or eicosanoid and metalloproteinase activation. So we have measured proinflammatory factors in serum of rats.

3. Please delete the sentence “The interaction between IL-6 pathway and Estrogen pathway is of great significance in disease treatment (17).”
Response: Thanks a lot for your advice. We have deleted “The interaction between IL-6 pathway and Estrogen pathway is of great significance in disease treatment (17)”. Please check the revised manuscript.

4. Please delete “Two T cell subgroups play an important role in immune function (21).”
Response: Your suggestion is greatly appreciated. We have deleted the sentence “Two T cell subgroups play an important role in immune function (21)”. Please check the revised manuscript.

5. Please delete M2 macrophages express the specific marker CD163 (22).
Response: We appreciate your suggestion. We have deleted the sentence “M2 macrophages express the specific marker CD163 (22)”. Please check the revised manuscript.

Discussion
1. The Discussion section is complex and disorganized. Please rewritten this section.
Response: Thank you for the suggestions you kindly offered us. We have rewritten the Discussion section. All changes have been marked in red.
For example, “Based on this phenomenon, the aim of tumor treatment with interfered Treg level should be to control the function and quantity of Treg instead of clearing Treg (8). Our results show that anti-IL-6 could favor Treg differentiation and inhibit Th17 production. Under the intervention of anti-IL-6, the content of pro-inflammatory factor IL-17 and the expression of ROR-γt in EMS rats decreased, while the content of anti-inflammatory factor IL-10 and the expression of FOXP3 increased”. Please check the revised manuscript (page 15-16, line 283-317).

Review 4:
The paper is interesting and well written. I suggest to add a reference paper by Murdaca et al concerning TH17 in chronic inflammatory diseases.
Response: Thank you for the time and efforts in reviewing our manuscript.
We have added the reference paper by Murdaca et al concerning TH17 in chronic inflammatory diseases.
“Th17 cells are considered as a distinct T helper lymphocyte subset. Development of Th17 cells can be divided into three stages: differentiation, driven by transforming growth factor-b1 (TGF-b), IL-1b and IL-6, autocrine proliferation triggered by IL-21, and amplification maintained by IL-23 (9)”. Please check the revised manuscript (page 4, line 58-61).

Review 5:
1. Please describe in detail what exactly the anti-IL-6 is How is it made or where is it purchased.
Response: Thank you very much for your careful review. We have added source information about anti-IL-6 in method #2.2. “rat anti-IL-6 (AF506, R&D systems, USA,)”.
Please check the revised manuscript (page 7, line 113-114).
2. Please improve the writing in English. There are several grammar problems.
Response: We are thankful for your suggestion. We have modified the language throughout the text.
At the same time, the manuscript has been edited by an English-native speaker. We hope that our
language is now easier to follow with this new version.

1. O’Connor RA, Floess S, Huehn J, Jones SA, Anderton SM. Foxp3⁺ Treg cells in the inflamed CNS
expression of phosphatase PTPN2 promotes pathogenic conversion of Tregs in autoimmunity. J Clin
3. Korn T, Hillensperger M. Role of IL-6 in the commitment of T cell subsets. Cytokine.
2021;146:155654.
4. Fujisaka S. The role of adipose tissue M1/M2 macrophages in type 2 diabetes mellitus. Diabetol
macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res.
Inflammation and Aggravate Spinal Cord Injury Through the TLR4/MyD88 Signaling Pathway. Front
Nutr. 2021;8:702659.
8. Theil A, Tuve S. Adoptive transfer of allogeneic regulatory T cells into patients with chronic grafted-
9. Murdaca G, Colombo BM, Puppo F. The role of Th17 lymphocytes in the autoimmune and chronic

Response.docx
IL-6 could regulate Th17/Treg cells to restore the local microenvironment of endometriosis

Running title: IL-6 could regulate microenvironment of EMS
Abstract

Introduction: In this study, we studied anti-IL-6 antibodies to treat endometriosis (EMS) by balancing Th17/Treg cells, providing a new basis for EMS research and treatment.

Material and methods: The rat EMS model was constructed to study the effect of anti-IL-6 on EMS. HE staining was performed to observe the lesions of the endometrium in rats. We have used anti-IL-6 to treat EMS rats. Then ELISA was used to measure the inflammatory factors IL-6, TGF-β and hormone estrogen levels in the rat serum. qRT-PCR and Western blot were used to measure estrogen receptor protein and apoptosis protein levels. Th17/Treg and CD163+/CD86+ ratios were determined by flow cytometry. CD34+ and CD163+ were measured by immunohistochemistry. TUNEL was performed to detect apoptosis in EMS lesions.

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Conclusions: IL-6 regulated Th17/Treg cells to restore the local microenvironment of EMS. Anti-IL-6 could regulate the immune microenvironment of EMS rats to alleviate the progression of EMS disease.

Keywords: Endometriosis, IL-6, Th17, Treg, Macrophages
1. Introduction

Endometriosis (EMS) is a common inflammatory gynecological disease (1). It was characterized by endometrioid lesions located outside the uterus, causing chronic pelvic pain and infertility, affecting approximately 5-10% of women of childbearing age (2). The main pathological change of EMS was ectopic endometrial periodicity. Bleeding, periodic tissue fibrosis, and ectopic nodule formation could cause chronic pelvic pain, dysmenorrhea or infertility (3). The pathogenesis of EMS involved the regulation of multiple cytokines and the coordination and transduction of multiple signal pathways (4). There have been a large number of basic and clinical studies in recent years to explore the etiology and mechanism of EMS. The exact origin and pathogenesis of the development of EMS were still controversial (5, 6). More and more studies have proved that EMs was not a single disease, but a chronic disease caused by multiple factors, including genetic factors, epigenetics, hormones, immune response, microenvironment and environmental factors (2, 7, 8). Despite the increasing research on EMs, the clinical diagnosis and treatment of EMs still lacks sufficiently sensitive and specific signs, symptoms, and blood tests (9). Currently, the gold standard for diagnosing EMs is still laparoscopy (10). Therefore, it is of great significance to find out the regulatory factors and pathways involved in the pathogenesis of EMS.

Studies have shown that both estrogen receptor alpha (ERα) and beta (ERβ) mRNA were expressed in the normal uterus, but the expression of ERα mRNA was dominant. In EMS, the high level of ERβ in EMS can inhibit ERα, resulting in a very high ratio of ERβ to ERα (11). Inflammatory factors (IL-6, IL-10, IL-13, TNF-α) could be used as an essential reference index for the diagnosis of EMS with infertility (12). Recent study has reported that overexpression of
miR-98-5p promoted balance of Treg/Th17 cells via directly targeting IL-6 to downregulate expression of IL-6 (13). However, although they play a role in EMS, the molecular mechanism of the role of IL-6 in the progression of EMS and EMS-related endometrial dysfunction is not fully understood. We use anti-IL-6 for in vivo experiments to observe the dynamics of the immune microenvironment in rats.

The initial CD4\(^+\) T cells were activated and differentiated into different T cell subgroups under the action of antigen antibodies and costimulatory molecules (14), including Th1, Th2, Th17, Treg and so on. Different CD4\(^+\) T cell subsets express different specific transcription factors, such as Th1 cells and Th2 cells respectively express specific transcription factors T-box expressed in T cells (T-bet) and GATA Protein 3 (GATA-3). T helper cell 17 (Th17) cells are considered as a distinct T helper lymphocyte subset. Development of Th17 cells can be divided into three stages: differentiation, driven by transforming growth factor-b1 (TGF-b), IL-1b and IL-6, autocrine proliferation triggered by IL-21, and amplification maintained by IL-23 (15). Th17 cells and Regulatory (Treg) cells respectively express specific transcription factors retinoic acid-related orphan nuclear receptor \(\gamma\)t (ROR-\(\gamma\)t) and Forkhead box protein P3 (FOXP3). The mode of IL-6 sensing by Treg cells is under debate and some investigators have suggested that Treg cells do not only lose IL-6R\(\alpha\) but also downregulate gp130 in highly inflammatory milieus to become resistant to any type of IL-6 signaling (16). However, overall the IL-6 signaling pathway has clearly been identified as a major pathway to dismantle Treg cell identity (17). These transcription factors act on specific target genes and play different roles in the immune response\(\text{(18)}\). Women with EMS show increased expression of angiogenic factor (VEGF) and a higher incidence of autoimmune diseases \(\text{(19)}\). Women with EMS usually show increased activation of peritoneal macrophages and related inflammatory cytokines \(\text{(20)}\). IL-6 interfered
with different components of the immune response (cytotoxic T cells, Treg cells, myeloid cells) may provide an idea for cancer therapy (21). It is important to understand how Th17/Treg cell balance regulates the progression of inflammation under different pathological conditions, because it may be a therapeutic target for EMS.

The abnormal distribution of immune cells in the abdominal cavity of EMS patients has been confirmed (22). More and more studies have shown that macrophages in the peritoneum show high activity in EMS patients and were believed to regulate and promote inflammation and disease progression (23). Originally, the concept of classically activated (M1) and alternatively activated (M2) macrophages was derived from in vitro studies. M1 macrophages are mainly induced by Th1 signaling, involving factors such as lipopolysaccharide (LPS) and IFNγ, and express high levels of inflammatory cytokines (24). M2 macrophages, on the other hand, are induced by Th2 signaling, involving factors such as IL-4 and IL-13, and are associated with anti-inflammatory reactions (25). M1 macrophages express specific biomarkers CD40, CD80, CD86 and human leukocyte antigen-antigen D-related (HLA-DR), which were used to eliminate invading microorganisms and secrete IL-1β, IL-6, IL-12, TNF-α and other effective effector cells of pro-inflammatory cytokines. In contrast, M2 macrophages express specific markers CD163 and CD206, improving inflammation and produce many anti-inflammatory factors, such as IL-10, TGF-β and IL-1α (26). We will further explore whether IL-6 affects the local microenvironment of EMS by adjusting the ratio of M1 and M2.

Although the results of previous studies support that Estrogen interferes with the immune system of EMS. IL-6 could affect the progress of EMS. However, whether IL-6 could affect the immune microenvironment of EMS by changing the ratio of Th17/Treg cells has not been studied. The research on this subject could clarify the abnormal level of Estrogen in EMS rats. The secretion
of IL-6 is affected. We could balance the Th17/Treg cell ratio by treating EMS rats with anti-IL-6.

6. M1 is polarized to M2 to improve the immune microenvironment in EMS rats. Anti-IL-6 could be used as a new idea and basis for the research and treatment of EMS.
Material and methods

2.1 Animals

We purchased 24 SD female rats from Hunan Slake Jingda experimental animal Co., Ltd. Female rats, aged 2-3 months, weighed 280 ± 20 g. The feeding conditions of all rats were 20-25 °C and 45-60%, respectively. Four rats were kept in each cage in a 14 h light / 10 h dark cycle. They take sterile feed and water at will. The rats were fed adaptively for 2-3 days before the experiment. The estrus cycle of each female rat was measured by vaginal smear method, which was generally 4-5 days. The treatment of animals during the experiment was conformed to the standard of Guiding Opinions on treating experimental animals issued by the Ministry of science and technology in 2006 (27). This animal protocol was approved by the Animal Care and Use Committee of Central South University (approval number: 2018-S146).

2.2 EMS model

Twenty-four female rats were divided into 4 groups. Six in each group: Sham (sham operation group), EMS (EMS rats), Oil (abdominal fat transplantation in EMS rats) and anti-IL-6 (EMS rats were injected intravenously with rat anti-IL-6 (AF506, R&D systems, USA, (500 μg i.v., day 0; 250 μg i.p., days 1, 3, 5, 7, 10, and 14)). In the EMS group, the left uterus was found. The two ends of the uterus were ligated near the end of the ovary. The middle segment was cut off about 2 cm and quickly put into 0.9% sodium chloride injection. After the other tissues were trimmed, they were cut longitudinally, and the intimal surface was attached to the inner surface of the abdominal wall 2 cm away from the incision on the right side of the mouse, and diagonally sutured to fix the inner wall of the abdominal cavity. The abdominal cavity was irrigated with Gentamicin sulfate injection, and the abdomen was closed. A longitudinal abdominal incision was made in the sham operation group and then sutured. The transplanted endometrium's growth,
invasion, and adhesion were observed 28 days after operation. Ectopic endometrial growth (5 × 5 mm for transplanted tissue), tissue edema (as a sign of inflammation), and vesicle formation were observed macroscopically. The model was considered successful if the transplanted endometrium had grown at least 2-fold (28).

2.3 Isolation of primary endometrial stromal cells

The cells were washed in FBS, and the endometrial tissues were cut into pieces (<1 mm); 0.8 mg/ml collagenase I type (volume ratio: 1: 5) was added for digestion. Digestion was carried out in a CO₂ incubator supplied with 5% CO₂ for 60 min at 37°C, with oscillations at every 15 min interval. DMEM supplemented with 10% FBS was added to terminate digestion, and the cells were filtered through a 100-mesh (150 μm aperture) screen. The endometrial cell suspension was divided into two equal parts and centrifuged (50×g×1 min). The supernatant was filtered through a 400 mesh screen and centrifuged to collect the endometrial cells. The cells were cultured in a CO₂ incubator supplied with 5% CO₂ at 37°C. The culture medium was first changed after 24 h and subsequently refreshed every 2 days.

2.4 Enzyme-linked immunosorbent assay (ELISA)

All samples were repeated three times according to the manufacturer's instructions of ELISA kits (csb-e04640r, csb-e04727r, csb-e0510r, csb-e0745r and csb-e04595r). The standard hole and the sample hole was tested respectively. 100 μL standard samples were added to each hole. The sample was covered with a board sticker and placed at 37°C for 2 hours. 100 μL of horseradish peroxidase-labeled avidin working solution was added to the standard wells, covered with a new plate and incubated at 37°C for 1 hour. We have added 90 μL of the substrate solution to each well and developed the color at 37°C for 15-30 minutes in the dark. We used a microplate reader
to measure each well's optical density (OD value) at 450nm wavelength within 5 minutes after the reaction was terminated.

### 2.5 Hematoxylin-eosin (HE) staining

The sections were dewaxed with dewaxing solution for 3 times, 5 min to 10 min each time, and then dewaxed with alcohol from high to low concentration (anhydrous ethanol, 95% ethanol, 80% ethanol and 70% ethanol) and distilled water. The dyeing operation was carried out and hematoxylin violet solution was applied for 3 min to 5 min. After washing with 1% hydrochloric acid or 1% acidic solution and soaking for 15 min, the nuclei and ribosomes should be blue and purple. Eosin dye was added to make the cytoplasm reddish or reddish, and the plate was finally sealed and labeled. We used a microscope to observe the results.

### 2.6 Immunohistochemical (IHC) staining

The sections were placed in xylene for 20 min. Then the slices were placed in 100%, 95%, 85%, and 75% ethanol in sequence for 5 min at each level. We dropped appropriately diluted primary antibodies (CD163 (600 μg/ml, 1:200, 16646-1-AP, Proteintech), CD34 (550 μg/ml, 1:200, 14486-1-AP, Proteintech), VEGF (600 μg/ml, 1:200, 19003-1-AP, Proteintech)) on the slices and kept them at 4°C overnight. PBS wash 3 times, 5min per time. We added rabbit-IgG antibody-HRP polymer dropwise to the slices and incubated them at 37°C for 30 min. We then dripped 50-100 μL of the pre-made DAB working solution on the slices and then incubated them at room temperature for 1 to 5 minutes. We used hematoxylin to counterstain the sections for 5-10 minutes. Finally, we used a microscope to observe the staining results.
2.7 Quantitative real-time PCR (qRT-PCR)

Total RNA from endometrium cells was extracted using TRIzol (15596026, Thermo Fisher Scientific, Waltham, MA, USA). The reaction conditions were denaturation at 95°C for 10 min, denaturation at 94°C for 15 s, annealing at 60°C for 30 s, for 40 cycles. The primer internal reference was β-actin. The primer sequences were shown in Table 1. With 2 μg cDNA as template, the relative quantitative method (2^−ΔΔCt method) was used to calculate the relative transcription level of the target gene: ΔΔCt=Δ experimental group -Δ Control group, ΔCt= Ct (target gene)-Ct (β-actin). The experiment was repeated three times.

2.8 Western blot

Total protein was extracted from endometrium cells using the Ripa Kit (r0010, Solarbio, China). The protein concentration was determined using the BCA method (29). Quantitative analysis was performed in accordance with the different concentrations. For primary antibodies, we used rabbit anti-ERα (600μg/ml, 1:1000, 21244-1-AP, Proteintech), rabbit anti-IL-6 (0.442 mg/ml, 1:500, ab233706, abcam), rabbit anti-ERβ (2 μg/mL, 1:2000, MAB7106, RD Systems), rabbit anti-p-VEGF (600 μg/ml, 1:1000, 19003-1-AP, Proteintech), rabbit anti-Bcl-2 (1.288 mg/ml, 1:2000, ab182858, abcam) and rabbit anti-Bax (0.118 mg/ml, 1:5000, ab32503, abcam). This was followed by exposure to horseradish peroxidase-conjugated goat anti-mouse IgG (0.2 mg/mL, 1:5000, sa00001-1, Proteintech). For luminescence development, the membrane was immersed in Supernal Plus (k-12045-d50, Advansta, USA). The experiment was repeated three times.
2.9 TdT-mediated dUTP Nick-End Labeling (TUNEL) assay

Paraffin sections at 60°C were deparaffinized according to conventional methods. The slices were immersed into the sealing solution and sealed at room temperature (15-25°C) for 12 min. Proteinase K working fluid was prepared. Each sample was added with 50 μL endogenous avidin to block liquid A and incubated at room temperature for 20 min. Each sample was added with 50 μL of endogenous biotin sealed B solution and incubated at room temperature for 20 min. Streptavidin-HRP labeling solution was prepared. We used hematoxylin (Wellbio, Changsha, China) for dyeing. We dried and sliced, added neutral gum and cover glass, and finally observed and photographed under a light microscope.

2.10 Flow cytometry

Cells were collected, cleaned once with 1 mL PBS buffer, and all were added into a 15mL tube. Centrifugation at 3,800 rpm for 5 minutes, supernatant was removed, 5mL PBS buffer was added to re-suspend the cells, supernatant was discarded again, repeated twice, and finally cells were re-suspended in 0.5mL PBS. Cells were resuspended with 0.4 mL PBS. We added 5 μL RNase (10 mg/ml) and digested it for 1 h at 37°C. Finally, propidium iodide (PI) with a 50 mg/mL final concentration was added. The cells were resuspended with 150 μL PBS and detected on the computer.

2.11 Statistical analyses

All data were expressed by Mean ± standard deviation (SD). GraphPad Prism 8.0 software (La Jolla, CA, USA) was used for data analysis. The two sets of data conforming to the normal distribution were used unpaired T test. The multiple sets of data conforming to the normal distribution adopt one-way analysis of variance, and then perform Tukey's post-hoc test. P <0.05 considered the difference to be statistically significant.
3. Results

3.1 Construction of EMS Model

In order to observe whether the EMS model was successful, we performed HE staining on the rat endometrium. There were a large number of glands in the endometrial epithelial cells of rats in the Sham group. The glandular epithelial cells were mostly columnar with vacuoles and apical secretions. Endometrial epithelial cells in the EMS group displayed low columnar, circular or serrated growth, local necrosis and inflammatory cell infiltration, thinning of the endometrial interstitial layer, and varying degrees of fibrosis (Figure 1A). ELISA was performed to measure the levels of pro-inflammatory factors IL-6 and TGF-β in the serum of rats. The results predicted that compared with the Sham group, the levels of IL-6 ($p < 0.001$) and TGF-β ($p < 0.001$) in the EMS group increased (Figure 1B). In general, the results obtained indicated that the induction was successful.

3.2 Estrogen in EMS

From the above results, we found that the content of pro-inflammatory factors is higher in EMS rats. Studies have shown that Estrogen receptor (ERα, ERβ) and IL-6 gene mutations were closely related to diseases. We tested the Estrogen content in rats. The data displayed that compared with the Sham group, the EMS group had higher Estrogen (Estradiol) content ($p < 0.001$), increased ERβ expression ($p < 0.001$) and decreased ERα expression ($p < 0.001$), and the ERβ/ERα ratio ($p < 0.001$) increased sharply (Figure 2A-2C). In conclusion, the levels of estrogen, ERα and ERβ in EMS rats were abnormal.

3.3 Anti-IL-6 could regulate Th17/Treg cells

The above results indicated that Estrogen disorder in EMS rats affected the level of IL-6. IL-6 plays a very important role in regulating the balance between Th17 cells that produce IL-17 and
We next investigate whether changes in IL-6 will affect the ratio of Th17/Treg cells. The results of flow cytometry detection of Th17 and Treg cells showed that compared with the Sham group, the Th17/Treg ratio of the EMS group was significantly increased. Compared with the oil group, the Th17/Treg ratio was significantly reduced after the Anti-IL-6 group (Figure 3A). ELISA was performed to test the IL-17 secreted by Th17 and the IL-10 factor secreted by Treg. We found that the content of IL-17 in the anti-IL-6 group was significantly reduced, and the content of IL-10 was significantly increased (Figure 3B). Next, the Th17-specific transcription factor ROR-γt and the Treg-specific transcription factor FOXP3 in the intimal tissue were tested, and the results showed that anti-IL-6 could promote the expression of FOXP3 and inhibit the expression of ROR-γt (Figure 3C). The above results indicated that anti-IL-6 could activate Treg secretion factor channels.

3.4 Anti-IL-6 could affect the ratio of M1/M2

Studies have shown that the development of EMS may be caused by inflammation and polarization of macrophages M1 and M2 in the peritoneal fluid. M1 macrophages express a specific biomarker CD86. Compared with the Sham group, the CD163+ ratio in the EMS group was higher, and the CD86+ ratio was lower. Compared with the Oil group, the ratio of CD163+ in the anti-IL-6 group was lower, and the ratio of CD86+ was higher (Figure 4A). We further tested the mRNA expression of CD163 and CD86. Compared with the Sham group, the expression of CD163 in the EMS group was significantly increased, and the expression of CD86 was significantly decreased. Compared with the Oil group, the expression of CD163 in the anti-IL-6 group was significantly decreased, and the expression of CD86 was significantly increased (Figure 4B). Similarly, IHC staining analysis showed that the CD163 staining intensity of...
macrophages in the anti-IL-6 group was significantly reduced (Figure 4C and 4D). The above results indicated that anti-IL-6 reduced the M2 polarization of macrophages in EMS.

3.5 Anti-IL-6 could promote cell apoptosis in the lesion

After the successful modeling of EMS, we observed the size of the lesion. Figure 5A displayed that there were significant differences in the size of the lesions in the three groups. Compared with the EMS group and the Oil group, the lesion volume in the anti-IL-6 group was significantly reduced (Figure 5B), and the microvessel density decreased (Figure 5C). The positive conditions of VEGF, a gene related to angiogenesis, were analyzed by immunohistochemistry. The data displayed that compared with the EMS group and the Oil group, the anti-IL-6 group had a lower positive VEGF (Figure 5D). The apoptosis of cells in the lesions was further evaluated. The expressions of angiogenic gene VEGF, pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 were evaluated by qRT-PCR and Western blot. Compared with the Oil group, the expressions of VEGF and Bcl-2 in the anti-IL-6 group were significantly decreased, while the expression of Bax was significantly increased (Figure 5E). Cell apoptosis at the lesion was tested by TUNEL. The result showed that anti-IL-6 promoted cell apoptosis (Figure 5F). In conclusion, anti-IL-6 could increase the apoptosis of the cells in the lesion.
The development of EMS was the result of the combined effects of immunity, estrogen, genetics, blood vessels and other factors (30). The pro-inflammatory cytokine IL-6 played a key role in the pathogenesis of EMS (31). In this study, we found abnormalities in IL-6 and Estrogen in EMS rats. Estrogen interfered the expression of IL-6. We observed the effect of anti-IL-6 on the expression of Estrogen receptors (ERα, ERβ) in rats. Anti-IL-6 affected the ratio of Th17/Treg and macrophages. Finally, we detected the size of the endometrial lesion and the apoptosis of the lesion. IL-6 regulated the local microenvironment of EMS by affecting immune cells.

IL-6 had pro-inflammatory and anti-inflammatory properties (32). Neutrophils drived IL-6 transduction signals, which were important for recruiting monocytes, stimulating integrin induction, cell adhesion, actin polymerization, chemotaxis, migration and proliferation (33). Once IL-6 was recruited, monocytes can differentiate into macrophages. Macrophages can express ERα. Cell migration and adhesion may be associated with E2, as ERα/E2 regulates the IL-6 promoter (34). Therefore, the role of IL-6 in the pathogenesis of EMS needs further detailed investigation. Our results indicated that compared with the Sham group, the levels of IL-6 and TGF-β in the EMS group increased and the levels of estrogen, ERα and ERβ in EMS rats were abnormal. This can be considered that estrogen disorders with abnormal serum IL-6 concentrations may be a cause of EMS development.

IL-6 is a pleiotropic cytokine involved in the physiology of virtually every organ system. Recent studies have demonstrated that IL-6 had a very important role in regulating the balance between Th17 cells and Treg (35). The decrease in Treg levels led to an increase in T cells with immunoinflammatory effects, which caused an inflammatory response. Th17 is another type of helper T cells, and its specific transcription factor is ROR-γt. In contrast, FOXP3+ Treg cells were
essential for mediating immune tolerance, and the lack of Treg cells is usually related to autoimmunity (36). The elimination of Treg cells in the body greatly enhanced the anti-tumor immune function of tumor patients. However, the number of Treg in the body of patients would recover or increase in a short time, seriously affecting the clinical efficacy. Based on this phenomenon, the aim of tumor treatment with interfered Treg level should be to control the function and quantity of Treg instead of clearing Treg (37). Our results show that anti-IL-6 could favor Treg differentiation and inhibit Th17 production. Under the intervention of anti-IL-6, the content of pro-inflammatory factor IL-17 and the expression of ROR-γt in EMS rats decreased, while the content of anti-inflammatory factor IL-10 and the expression of FOXP3 increased.

The inhibition of IL-6/STAT3 signaling pathway mediated by anti-IL6 was shown to significantly enhance the effects of M1-type macrophages on HCC cells and rescue HCC cells from co-culture with M2-type macrophages (38). Macrophages were phagocytes in the immune system, distributed in different tissues, and played an important role in inflammation, tumors, and other diseases (39). The differentiation of macrophages to M2 type requires the activation of inducers such as IL-4, IL-10 or TGF-β to promote EMS angiogenesis and tissue adhesion (40). M1-type macrophages can improve the level of inflammation in the body and maintain the balance of the local inflammatory microenvironment of EMS. Macrophages maintain the balance of the inflammatory microenvironment in EMS model mice, promote the polarization of macrophages to M1 type, enhance the immune response and reduce the volume of ectopic endometrial lesions (41). Our results further support the high proportion of M2 macrophages in EMS. Anti-IL-6 can inhibit the polarization of M1 to M2. Anti-IL-6 could promote the production of M1 marker CD86 and inhibit the level of M2 marker CD163.
In conclusion, we have verified in vivo that Anti-IL-6 could balance the ratio of Th17/Treg cells. Anti-IL-6 could inhibit the polarization of M1 to M2 in EMS rats. Thereby it can improve the immune microenvironment of the EMS rat. Anti-IL-6 can inhibit the expression of VEGF related to cell angiogenesis in the lesion. Anti-IL-6 can inhibit cell proliferation and promote cell apoptosis. IL-6 had potential value as a treatment for EMS.

Conflict of interest

The authors declare no potential conflicts of interest.
References


Table. 1 Primer sequences
Figure Legends

**Figure 1. The content of IL-6 and TGF-β in EMS.** A. HE staining of the endometrium (Scale bar, up: 100 µm, down: 25 µm). B. The level of IL-6 and TGF-β in the EMS group. *, P < 0.05 compared with the Sham group. n=6

**Figure 2. Estrogen receptor (ERα, ERβ) level was abnormal.** A. ELISA was utilized to test the content of estrogen in plasma. B. The mRNA expression levels of ERα and ERβ. C. Western blot was used to detect the protein of ERα and ERβ. *P < 0.05 compared with the Sham group. n=6

**Figure 3. Anti-IL-6 could balance the ratio of Th17/Treg cells.** A. Th17/Treg was analyzed by flow cytometry. B. The levels of cytokines IL-17 and IL-10 was secreted by Th17/Treg were detected by ELISA. C. QRT-PCR was used to measure the expression of transcription factors FOXP3 and ROR-γt of Th17/Treg. *P < 0.05 compared with Sham group. #P < 0.05 compared with Oil group. n=6

**Figure 4. Anti-IL-6 could affect EMS macrophages M1/M2.** A. CD163+ and CD86+ were analyzed by flow cytometry. B. The expression of CD163 and CD86 was tested by qRT-PCR. C&D. The positive rate of CD163 was evaluated by IHC staining (Scale bar, up:100 µm, down:25 µm). *P < 0.05 compared with Sham group. #P < 0.05 compared with Oil group. n=6
Figure 5. Anti-IL-6 could inhibit the activity of the cells in the lesion. A. Images of EMS lesions. B. The volume of the lesion. C. Microvessel density (MVD) was based on CD34 staining (Scale bar, up: 100 µm, down: 25 µm). D. The positive rate of VEGF was detected by IHC staining (Scale bar, up: 100 µm, down: 25 µm). E. The expressions of VEGF, Bcl-2 and Bax were detected by qRT-PCR and Western blot. F. TUNEL (Scale bar, up: 100 µm, down: 25 µm). *P < 0.05 compared with EMS group. #P < 0.05 compared with the Oil group. n=6.
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<tr>
<th>Gene</th>
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