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

Qing Feng, Sili He, Jianfa Jiang

Department of Gynecology, The Third Xiangya Hospital, Central South University, Changsha, Hunan, China

Submitted: 24 January 2022; **Accepted:** 8 May 2022
Online publication: 13 May 2022

Arch Med Sci
DOI: https://doi.org/10.5114/aoms/149886

Corresponding author:
Jianfa Jiang
Department of Gynecology
The Third Xiangya Hospital
Central South University
138 Tongzipo Rd
Yuelu District, Changsha
Hunan, China, 410013
E-mail: jjfxy3yy@163.com

Abstract

Introduction: In this study, we investigated the use of anti-interleukin-6 (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 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.

Key words: endometriosis, IL-6, Th17, Treg, macrophages.

Introduction

Endometriosis (EMS) is a common inflammatory gynecological disease [1]. It is 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 is ectopic endometrial periodicity. Bleeding, periodic tissue fibrosis, and ectopic nodule formation can cause chronic pelvic pain, dysmenorrhea or infertility [3]. The pathogenesis of EMS involves the regulation of multiple cytokines and the coordination and transduction of multiple signal pathways [4]. A large number of basic and clinical studies have been performed in recent years to explore the etiology and mechanism of EMS. The exact origin and pathogenesis of the development of EMS have remained controversial [5, 6]. More and more studies have proved

that EMS is 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, its clinical diagnosis and treatment still lack 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 identify 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]. A recent study 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 expressing 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 T (Treg) cells respectively express specific transcription factors retinoic acid-related orphan nuclear receptor γt (ROR-γt) 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 not only lose IL-6Rα 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 [18]. Women with EMS show increased expression of angiogenic factor (VEGF) and a higher incidence of autoimmune diseases [19]. Women with EMS usually show increased activation of peritoneal macrophages and related inflammatory cytokines [20]. IL-6 interfered with different components of the immune response (cytotoxic T cells, Treg cells, myeloid cells), which 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 they are 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 suggest 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. 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

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% humidity. Four rats were kept in each cage in a 14 h light/10 h dark cycle. They took 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 the vaginal smear method, which was generally 4–5 days. The treatment of animals during the experiment 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).

Endometriosis model

Twenty-four female rats were divided into 4 groups, with 6 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 solution. 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 solution, 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 the 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].

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 was added for digestion. Digestion was carried out in a CO_2 incubator supplied with 5% CO_2 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 CO_2 incubator supplied with 5% CO_2 at 37°C.The culture medium was first changed after 24 h and subsequently refreshed every 2 days.

Enzyme-linked immunosorbent assay (ELISA)

All samples were repeated three times according to the ELISA kit manufacturer's instructions (csb-e04640r, csb-e04727r, csb-e0510r, csb-e0745r and csb-e04595r). The standard hole and the sample hole were tested. 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 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 450 nm wavelength within 5 minutes after the reaction was terminated.

Hematoxylin-eosin (HE) staining

The sections were dewaxed with dewaxing solution 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, and the plate was finally sealed and labeled. We used a microscope to observe the results.

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, followed by PBS wash 3 times, 5 min 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.

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 are shown in Table I. With 2 µg cDNA as template, the relative quantitative method ($2^{-\Delta\Delta Ct}$ method) was used to calculate the relative transcription level of the target gene: $\Delta\Delta Ct = \Delta$ experimental group $- \Delta$ control group, $\Delta Ct = Ct$ (target gene) $- Ct$ (β-actin). The experiment was repeated three times.

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 (600 µg/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.

Table I. Primer sequences

Gene	Sequences (5′-3′)
ERβ	F: ACTGGTGCACATGATTGGCT
	R: CCTCATCCCTGTCCAGAACG
ERα	F: AAGACGCTCTTGAACCAGCA
	R: CGAGTTACAGACTGGCTCCC
IL-6	F: GACTTCCATCCAGTTGCCTT
	R: ATGTGTAATTAAGCCTCCGACT
ROR-γt	F: AACTGCCCCATTGACCGAAC
	R: CGGCCAAACTTGACAGCATCTCG
FOXP3	F: CTCCAATCCCTGCCCTTGACC
	R: ACATCATCGCCCGGTTTCCA
CD86	F: ACGCAAGCTTATTTCAATGGG
	R: GTTGTTCCTGTCAAAGCTCGT
VEGF	F: GAACCAGACCTCTCACCGGAA
	R: ACCCAAAGTGCTCCTCGAAG
Bcl-2	F: GGACCGCGTATCAGAGCTTT
	R: CAGTGCCCCGCCAAAGGA
Bax	F: TGAAGACAGGGGCCTTTTTG
	R: AATTCGCCGGAGACACTCG
β-actin	F: ACATCCGTAAAGACCTCTATGCC
	R: TACTCCTGCTTGCTGATCCAC

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. 50 µl of endogenous avidin was added to each sample to block liquid A, then it was incubated at room temperature for 20 min. 50 µl of endogenous biotin sealed B solution was added to each sample, then it was 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 the sample, added neutral gum and cover glass, and finally observed and photographed it under a light microscope.

Flow cytometry

Cells were collected, cleaned once with 1 ml of PBS buffer, and all were added into a 15 ml tube. After centrifugation at 3 800 rpm for 5 minutes, the supernatant was removed, 5 mL of PBS buffer was added to re-suspend the cells, the supernatant was discarded again, this was repeated twice, and finally cells were re-suspended in 0.5 mL of PBS. Cells were resuspended with 0.4 ml of PBS. We added 5 µl of 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 of PBS and detected on the computer.

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. For two sets of data conforming to the normal distribution

we used the unpaired *t*-test. For multiple sets of data conforming to the normal distribution we adopted one-way analysis of variance, and then performed Tukey's post-hoc test. At $P < 0.05$ we considered the difference to be statistically significant.

Results

Construction of endometriosis 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 1 A). ELISA was performed to measure the levels of pro-inflammatory factors IL-6 and TGF-β in the serum of rats. The results showed 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 1 B). In general, the results obtained indicated that the induction was successful.

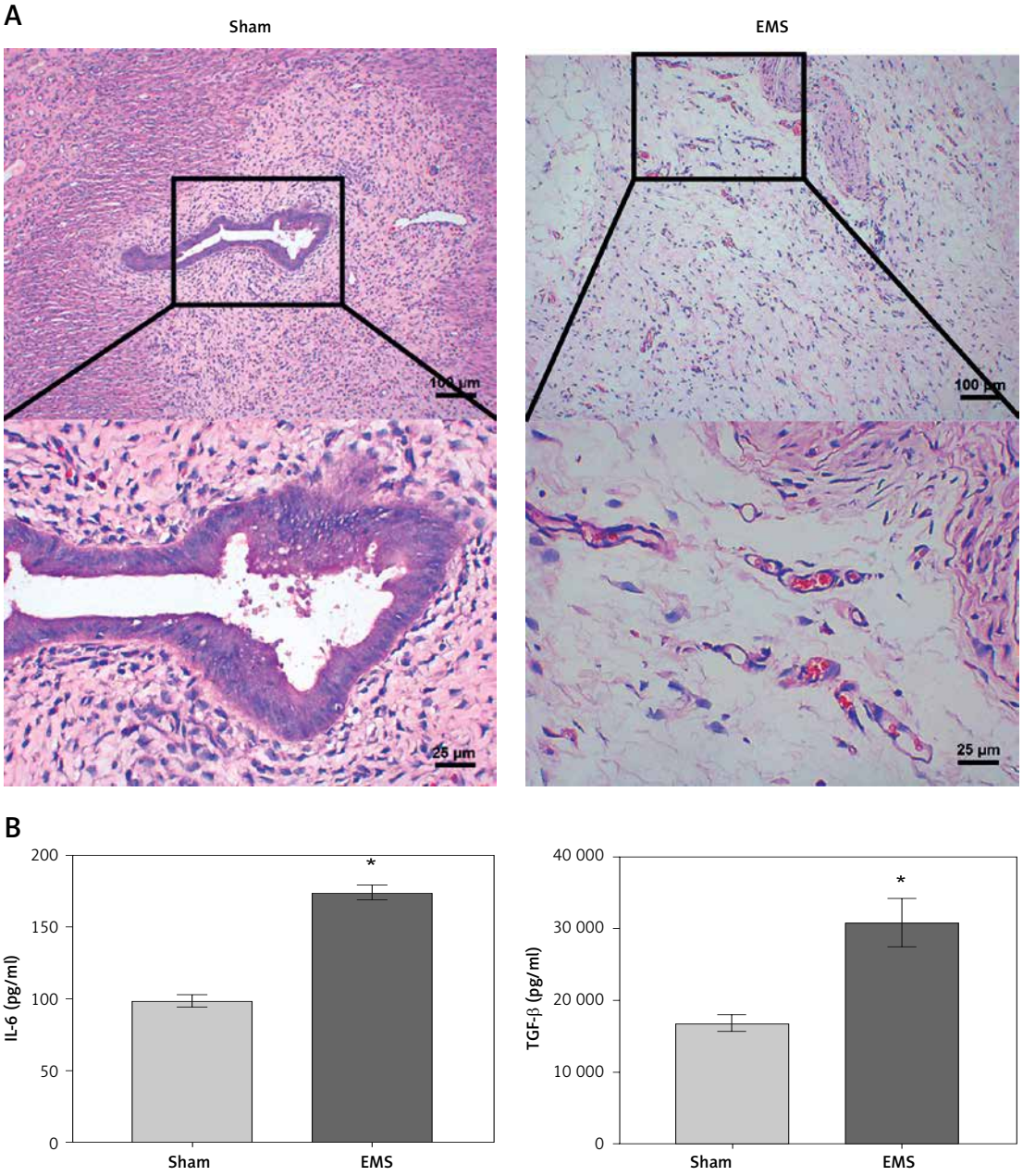

Figure 1. Content of IL-6 and TGF-β in EMS. **A** – HE staining of the endometrium (scale bar, top: 100 µm, bottom: 25 µm). **B** – Level of IL-6 and TGF-β in the EMS group. *$P < 0.05$ compared with the Sham group. $n = 6$

Estrogen in endometriosis

From the above results, we found that the content of pro-inflammatory factors was 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 2 A–C). In conclu-

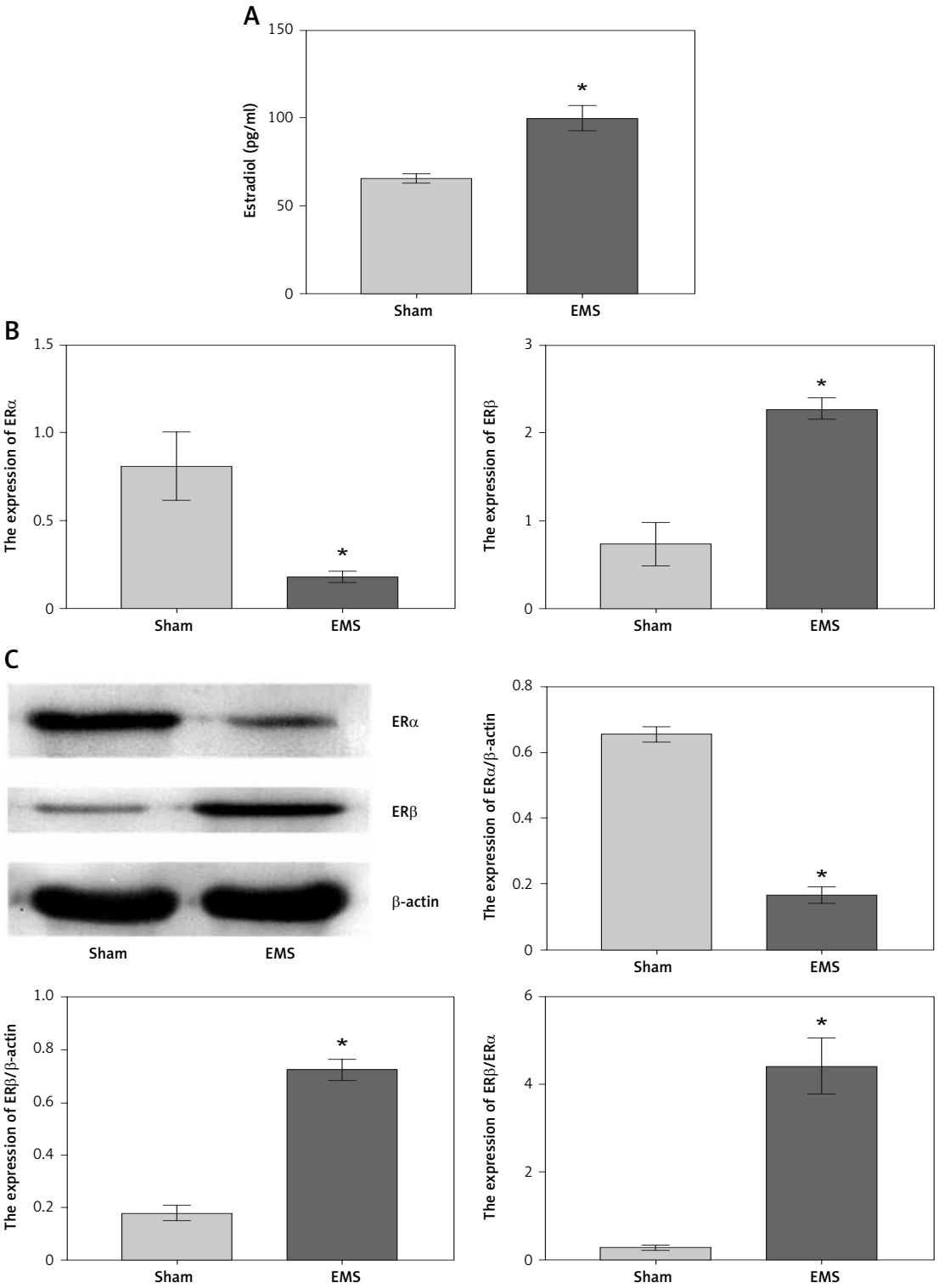

Figure 2. Estrogen receptor (ERα, ERβ) level was abnormal. **A** – ELISA was utilized to test the content of estrogen in plasma. **B** – mRNA expression levels of ERα and ERβ. **C** – Western blot was used to detect the levels of ERα and ERβ. *$P < 0.05$ compared with the Sham group. $n = 6$

sion, the levels of estrogen, ERα and ERβ in EMS rats were abnormal.

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 Treg. We next investigate whether changes in IL-6 would 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 in the anti-IL-6 group (Figure 3 A). 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 3 B). 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 3 C). The above results indicated that anti-IL-6 could activate Treg secretion factor channels.

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 the 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 4 A). 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 higher, and the expression of CD86 was significantly lower. Compared with the Oil group, the expression of CD163 in the anti-IL-6 group was significantly lower, and the expression of CD86 was significantly higher (Figure 4 B). Similarly, IHC staining analysis showed that the CD163 staining intensity of macrophages in the anti-IL-6 group was significantly reduced (Figure 4 C–D).

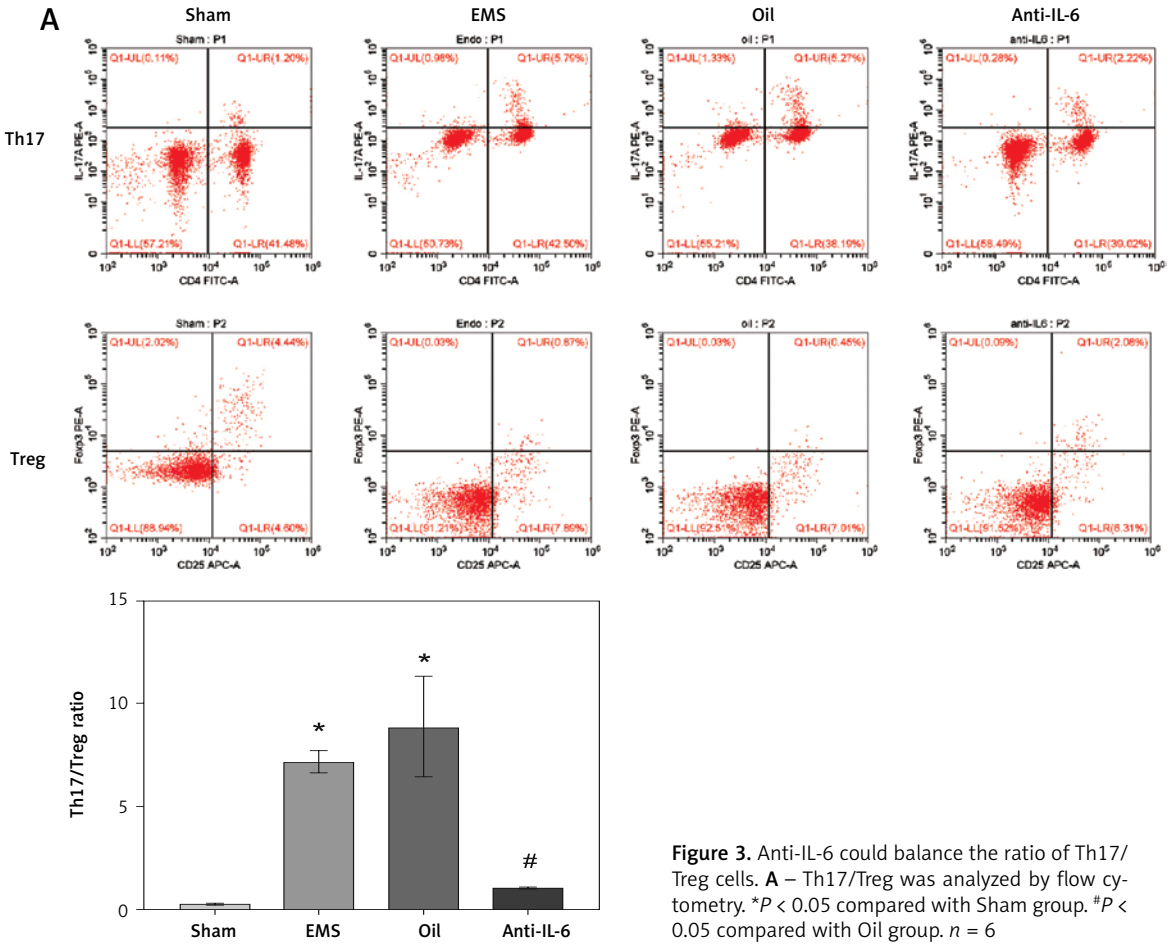

Figure 3. Anti-IL-6 could balance the ratio of Th17/Treg cells. **A** – Th17/Treg was analyzed by flow cytometry. *$P < 0.05$ compared with Sham group. #$P < 0.05$ compared with Oil group. $n = 6$

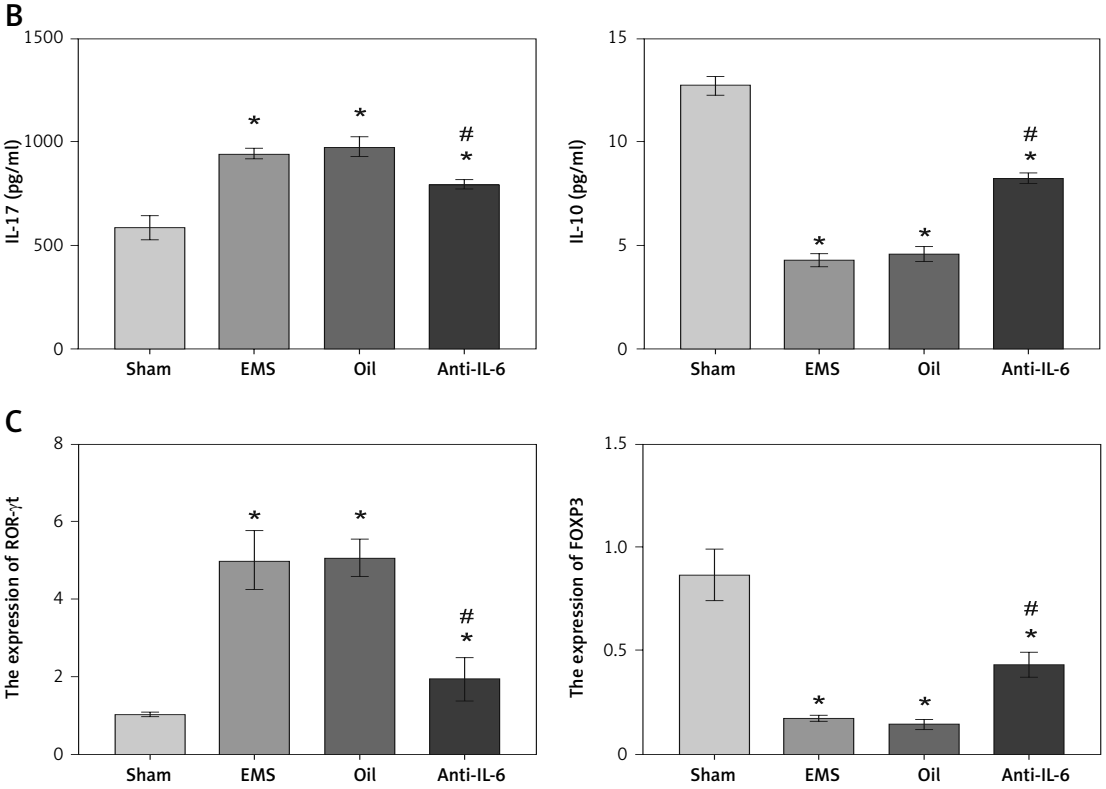

Figure 3. Cont. **B** – Levels of cytokines IL-17 and IL-10 secreted by Th17/Treg were detected by ELISA. **C** – QRT-PCR was used to measure the expression of transcription factors FOXP3 and ROR-γt of Th17/Treg. $^{*}P < 0.05$ compared with Sham group. $^{\#}P < 0.05$ compared with Oil group. $n = 6$

The above results indicated that anti-IL-6 reduced the M2 polarization of macrophages in EMS.

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

After the successful modeling of EMS, we observed the size of the lesion. Figure 5 A shows 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 lower (Figure 5 B), and the microvessel density was lower (Figure 5 C). The positive conditions of VEGF, a gene related to angiogenesis, were analyzed by immunohistochemistry. The data showed that compared with the EMS group and the Oil group, the anti-IL-6 group had a lower positive VEGF (Figure 5 D). The apoptosis of cells in the lesions was further evaluated. The expression levels 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 expression levels of VEGF and Bcl-2 in the anti-IL-6 group were significantly lower, while the expression of Bax was significantly higher (Figure 5 E). Cell apoptosis at the lesion was tested by TUNEL. The result showed that anti-IL-6 promoted cell apoptosis (Figure 5 F). In conclusion, anti-IL-6 could increase the apoptosis of the cells in the lesion.

Discussion

The development of EMS is the result of the combined effects of immunity, estrogen, genetics, blood vessels and other factors [30]. The pro-inflammatory cytokine IL-6 plays 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 with 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 has pro-inflammatory and anti-inflammatory properties [32]. Neutrophils derived IL-6 transduction signals, which are important for recruiting monocytes, stimulating integrin induction, cell adhesion, actin polymerization, chemotaxis, migration and proliferation [33]. Once IL-6 has been 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 were higher and the levels of estrogen, ERα and ERβ in EMS rats were abnormal. This suggests 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 has 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 immuno-inflammatory 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 are 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 adjustment of the Treg level should be to control the function and quantity of Treg instead of clearing Treg [37]. Our results show

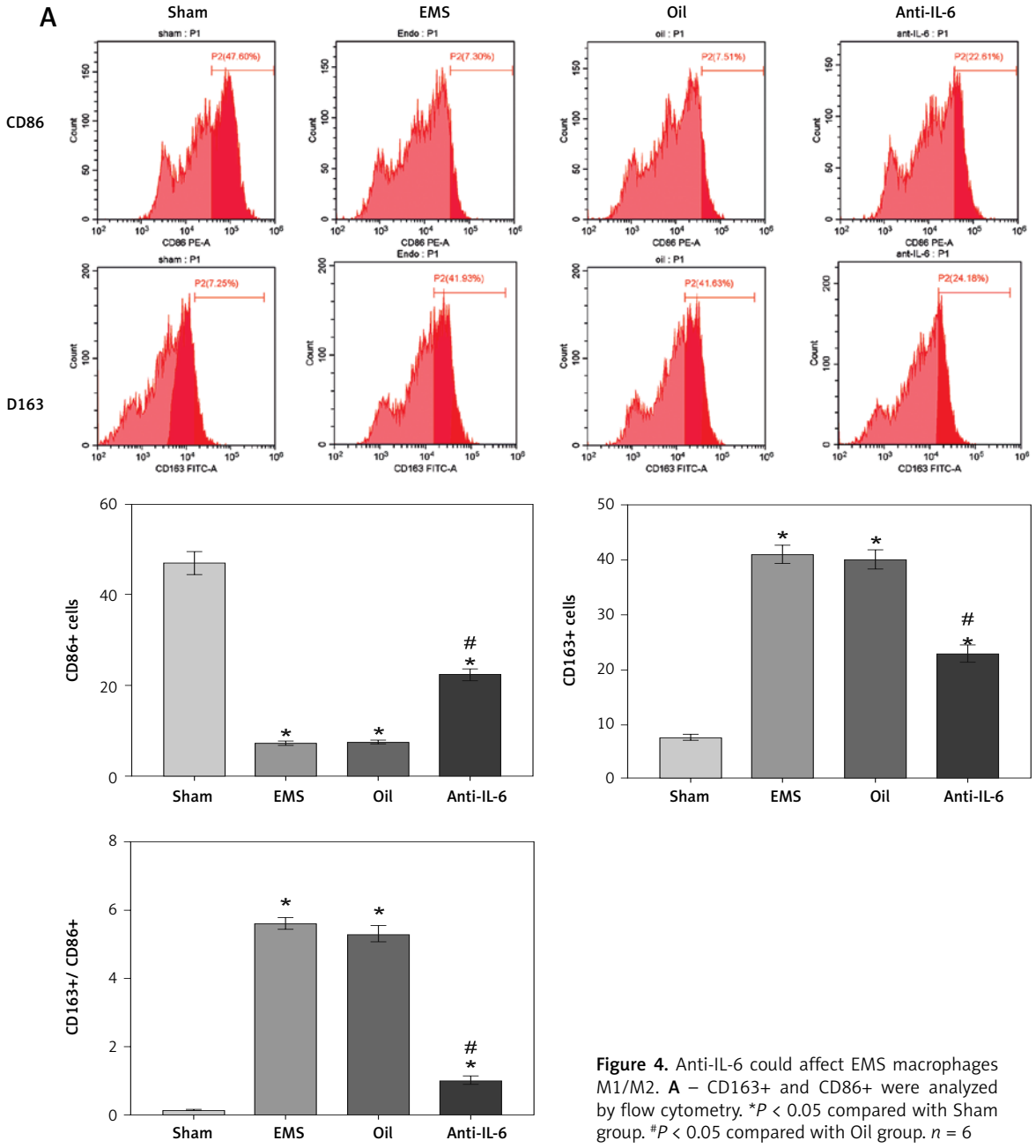

Figure 4. Anti-IL-6 could affect EMS macrophages M1/M2. **A** – CD163+ and CD86+ were analyzed by flow cytometry. *$P < 0.05$ compared with Sham group. #$P < 0.05$ compared with Oil group. $n = 6$

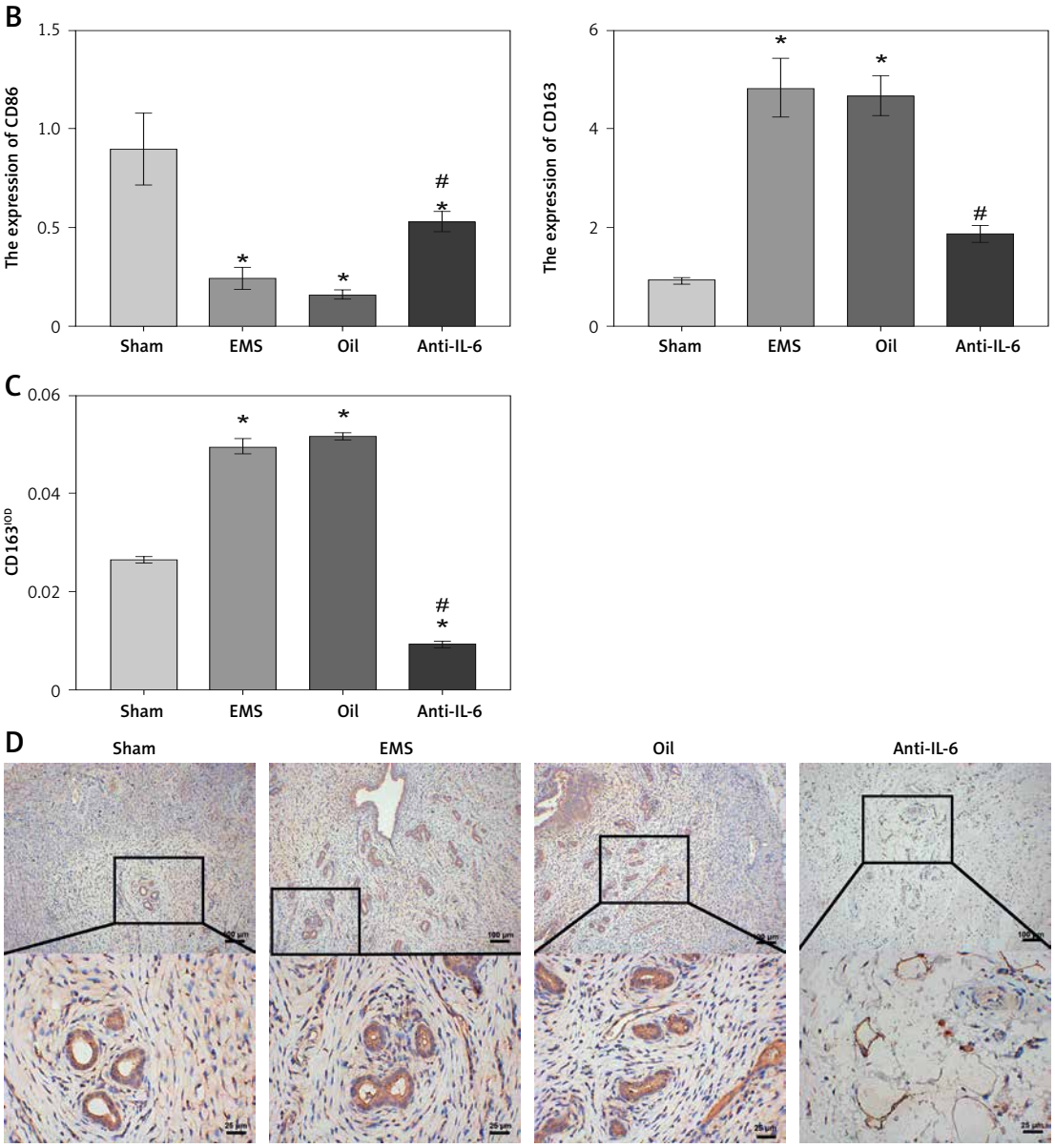

Figure 4. Cont. **B** – Expression of CD163 and CD86 was tested by qRT-PCR. **C**, **D** – The positive rate of CD163 was evaluated by IHC staining (scale bar, top: 100 μm, bottom: 25 μm). *$P < 0.05$ compared with Sham group. #$P < 0.05$ compared with Oil group. $n = 6$

that anti-IL-6 could favor Treg differentiation and inhibit Th17 production. Under the intervention of anti-IL-6, the content of the 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 the 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 are phagocytes in the immune system, distributed in different tissues, and play 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

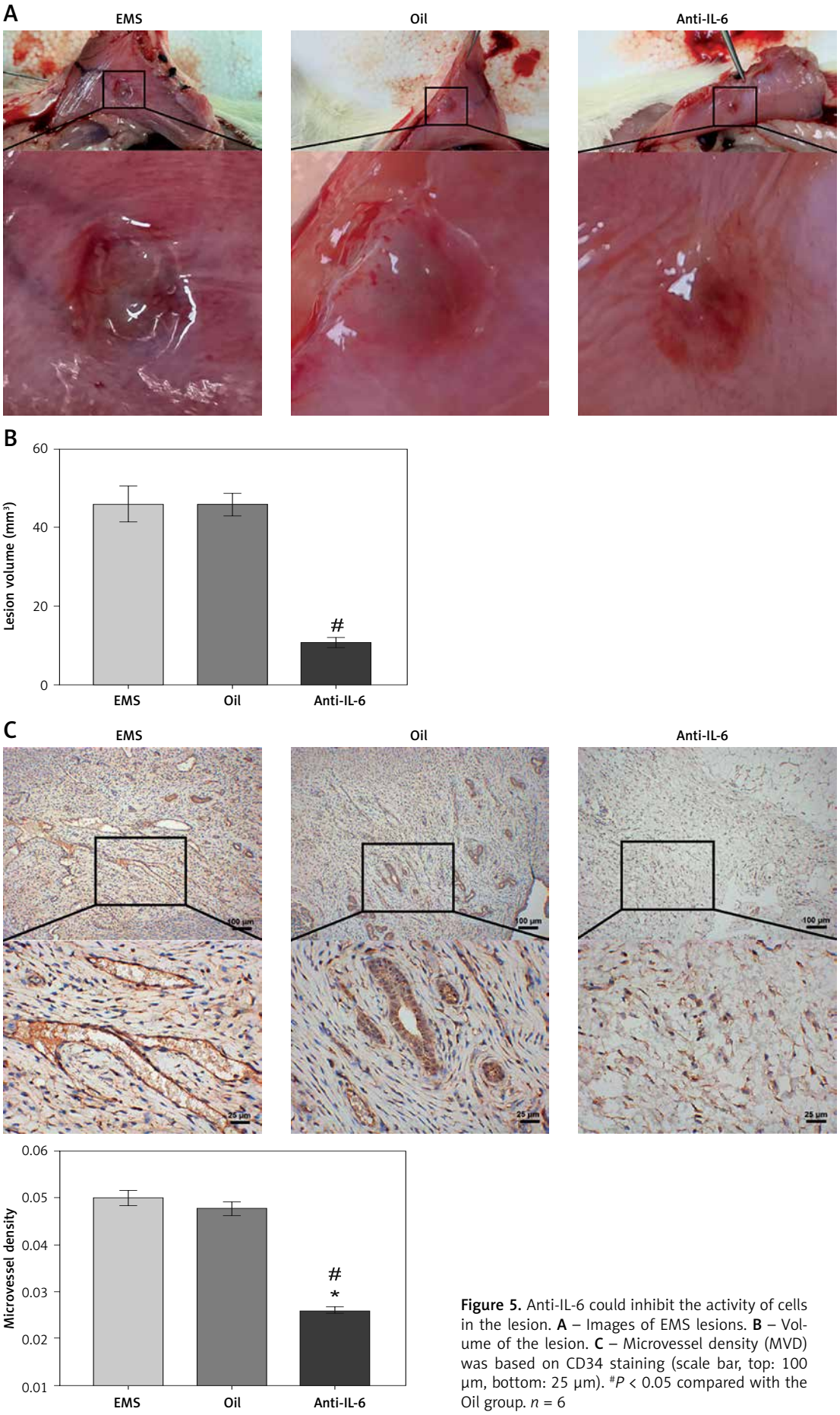

Figure 5. Anti-IL-6 could inhibit the activity of cells in the lesion. **A** – Images of EMS lesions. **B** – Volume of the lesion. **C** – Microvessel density (MVD) was based on CD34 staining (scale bar, top: 100 µm, bottom: 25 µm). $^{\#}P < 0.05$ compared with the Oil group. $n = 6$

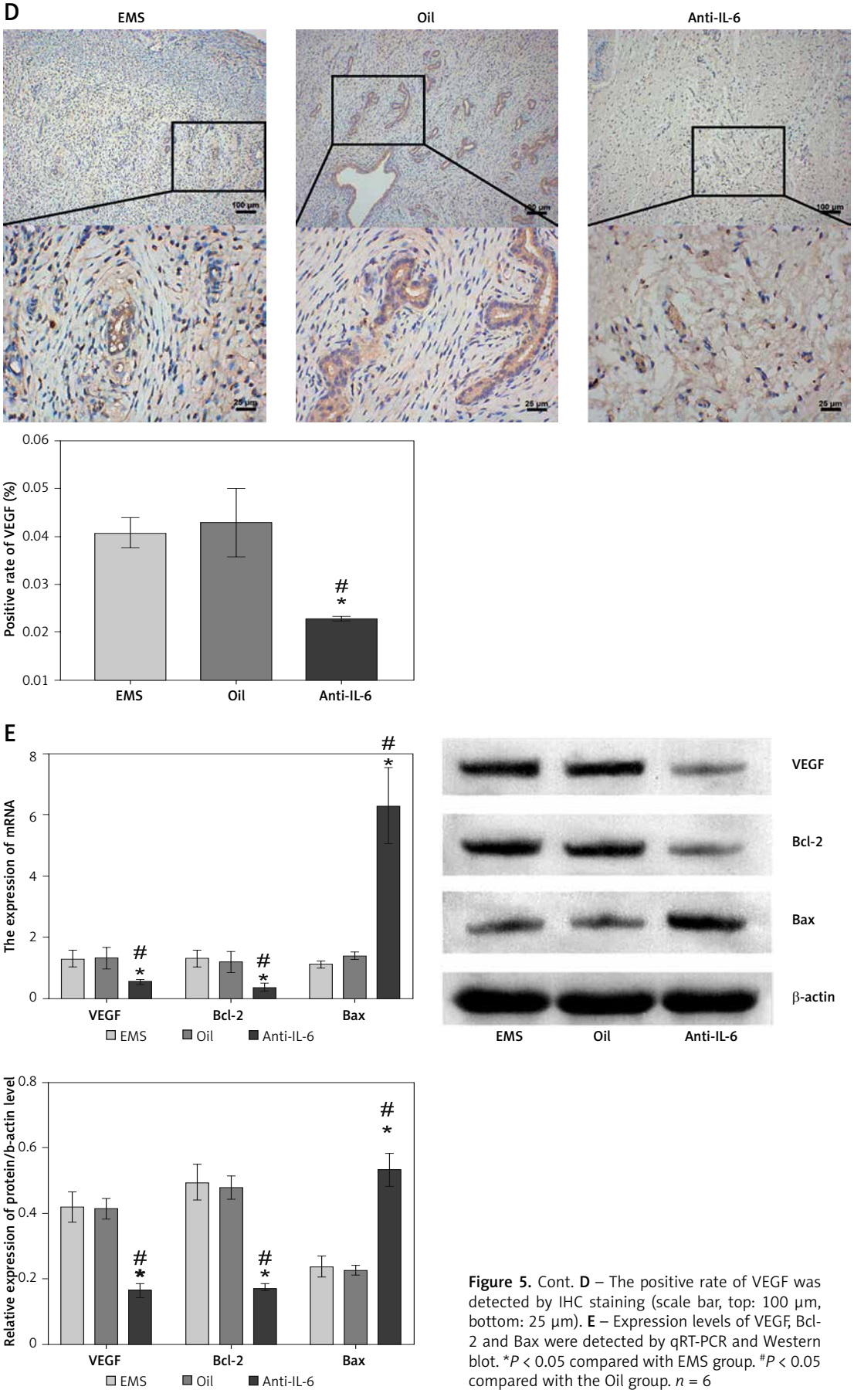

Figure 5. Cont. **D** – The positive rate of VEGF was detected by IHC staining (scale bar, top: 100 μm, bottom: 25 μm). **E** – Expression levels of VEGF, Bcl-2 and Bax were detected by qRT-PCR and Western blot. $^{*}P < 0.05$ compared with EMS group. $^{\#}P < 0.05$ compared with the Oil group. $n = 6$

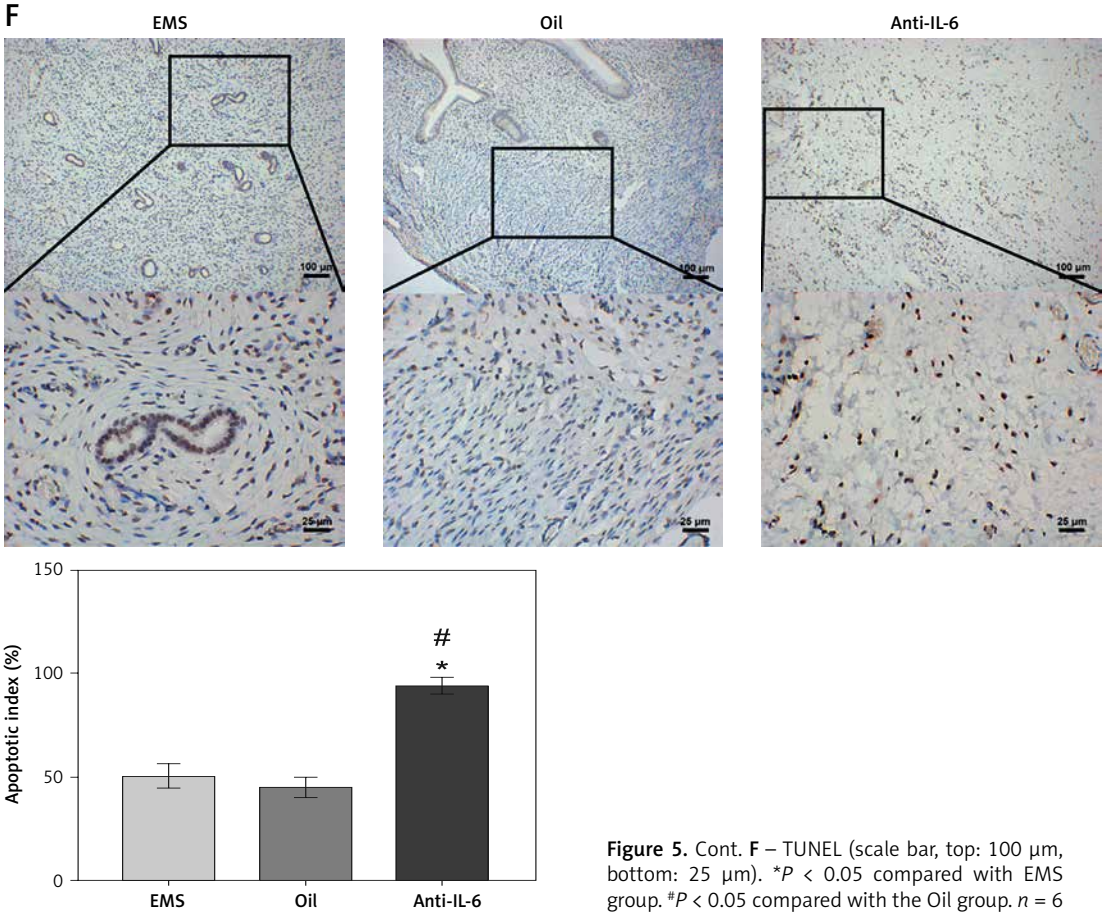

Figure 5. Cont. **F** – TUNEL (scale bar, top: 100 µm, bottom: 25 µm). $^{*}P < 0.05$ compared with EMS group. $^{\#}P < 0.05$ compared with the Oil group. $n = 6$

production of the M1 marker CD86 and inhibit the level of the 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 has potential value as a treatment for EMS.

Funding source

This work was supported by the Wisdom Accumulation and Talent Cultivation Project of the Third Xiangya Hospital of Central South University (No. YX202112).

Conflict of interest

The authors declare no potential conflicts of interest.

References

1. Xiang D, Zhao M, Cai X, Wang Y, Zhang L, Yao H, et al. Betulinic acid inhibits endometriosis through suppression of estrogen receptor β signaling pathway. Front Endocrinol (Lausanne) 2020; 11: 604648.
2. Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN, Viganò P. Endometriosis. Nat Rev Dis Primers 2018; 4: 9.
3. Berlac JF, Hartwell D, Wessel Skovlund Ch, Langhoff-Roos J, Lidegaard Ø. Endometriosis increases the risk of obstetrical and neonatal complications. Acta Obstet Gynecol Scand 2017; 96: 751-60.
4. Hou XX, Zhou WJ, Wang XQ, Li DJ. Fractalkine/CX3CR1 is involved in the pathogenesis of endometriosis by regulating endometrial stromal cell proliferation and invasion. Am J Reprod Immunol 2016; 76: 318-25.
5. Koninckx PR, Ussia A, Adamyan L, et al. The epidemiology of endometriosis is poorly known as the pathophysiology and diagnosis are unclear. Best Pract Res Clin Obstet Gynaecol 2020; 71: 14-26.
6. Lin YH, Yang YC, Chen SF, Hsu CY, Shen YC. Risk of systemic lupus erythematosus in patients with endometriosis: A nationwide population-based cohort study. Arch Gynecol Obstet 2020; 302: 1197-203.
7. Chen H, Malentacchi F, Fambrini M, Harrath AH, Huang H, Petraglia F. Epigenetics of estrogen and progesterone receptors in endometriosis. Reprod Sci 2020; 27: 1967-74.
8. Jeljeli M, Riccio LGC, Chouzenoux S, et al. Macrophage immune memory controls endometriosis in mice and humans. Cell Rep 2020; 33: 108325.
9. Calagna G, Corte LD, Giampaolino P, Maranto M, Perino A. Endometriosis and strategies of fertility preservation: a systematic review of the literature. Eur J Obstet Gynecol Reprod Biol 2020; 254: 218-25.

10. Bulun SE, Yilmaz BD, Sison Ch, et al. Endometriosis. Endocr Rev 2019; 40: 1048-79.
11. Simmen RCM, Kelley AS. Reversal of fortune: estrogen receptor-β in endometriosis. J Mol Endocrinol 2016; 57: F23-7.
12. Wang XM, Ma ZY, Song N. Inflammatory cytokines IL-6, IL-10, IL-13, TNF-α and peritoneal fluid flora were associated with infertility in patients with endometriosis. Eur Rev Med Pharmacol Sci 2018; 22: 2513-8.
13. Xu QF, Peng HP, Lu XR, Hu Y, Xu ZH, Xu JK. Oleanolic acid regulates the Treg/Th17 imbalance in gastric cancer by targeting IL-6 with miR-98-5p. Cytokine 2021; 148.
14. Afzali B, Mitchell PJ, Edozie FC, et al. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. Eur J Immunol 2013; 43: 2043-54.
15. Murdaca G, Colombo BM, Puppo F. The role of Th17 lymphocytes in the autoimmune and chronic inflammatory diseases. Intern Emerg Med 2011; 6: 487-95.
16. O'Connor RA, Floess S, Huehn J, Jones SA, Anderton SM. Foxp3$^+$ Treg cells in the inflamed CNS are insensitive to IL-6-driven IL-17 production. Eur J Immunol 2012; 42: 1174-9.
17. Svensson MN, Doody KM, Schmiedel BJ, et al. Reduced expression of phosphatase PTPN2 promotes pathogenic conversion of Tregs in autoimmunity. J Clin Invest 2019; 129: 1193-210.
18. Jung MK, Kwak JE, Shin EC. IL-17A-producing Foxp3 + regulatory T cells and human diseases. Immune Netw 2017; 17: 276-86.
19. Jia L, Wu R, Han N, et al. Porphyromonas gingivalis and Lactobacillus rhamnosus GG regulate the Th17/Treg balance in colitis via TLR4 and TLR2. Clin Transl Immunology 2020; 9(11).
20. Burns KA, Thomas SY, Hamilton KJ, Young SL, Cook DN, Korach KS. Early endometriosis in females is directed by immune-mediated estrogen receptor α and IL-6 crosstalk. Endocrinology 2018; 159: 103-18.
21. Korn T, Hiltensperger M. Role of IL-6 in the commitment of T cell subsets. Cytokine 2021; 146: 155654.
22. Lu C, Liu Y, Wang X, Jiang H, Liu Z. Tumor necrosis factor receptor type 1-associated death domain (TRADD) regulates epithelial-mesenchymal transition (EMT), M1/M2 macrophage polarization and ectopic endometrial cysts formation in endometriosis. Ann Transl Med 2021; 9: 148.
23. Takebayashi A, Kimura F, Kishi Y, et al. Subpopulations of macrophages within eutopic endometrium of endometriosis patients. Am J Reprod Immunol 2015; 73: 221-31.
24. Fujisaka S. The role of adipose tissue M1/M2 macrophages in type 2 diabetes mellitus. Diabetol Int 2021; 12: 74-9.
25. Cinti S, Mitchell G, Barbatelli G, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 2005; 46: 2347-55.
26. Rőszer T. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. Mediators Inflamm 2015; 2015: 816460.
27. Rong Z, Huang Y, Cai H, et al. Gut microbiota disorders promote inflammation and aggravate spinal cord injury through the TLR4/MyD88 signaling pathway. Front Nutr 2021; 8: 702659.
28. Cai H, Zhu X, Li Z, Zhu Y, Lang J. lncRNA/mRNA profiling of endometriosis rat uterine tissues during the implantation window. Int J Mol Med 2019; 44: 2145-60.
29. Di J, Yang M, Zhou H, Li M, Zhao J. MicroRNA-21-containing microvesicles from tubular epithelial cells promote cardiomyocyte hypertrophy. Ren Fail 2021; 43: 391-400.
30. Dodds KN, Beckett EAH, Evans SF, Hutchinson MR. Lesion development is modulated by the natural estrous cycle and mouse strain in a minimally invasive model of endometriosis. Biol Reprod 2017; 97: 810-21.
31. El-Zayadi AA, Mohamed SA, Arafa M, et al. Anti-IL-6 receptor monoclonal antibody as a new treatment of endometriosis. Immunol Res 2020; 68: 389-97.
32. Hirano T. IL-6 in inflammation, autoimmunity and cancer. Int Immunol 2021; 33: 127-48.
33. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011; 1813: 878-88.
34. Stanic AK, Kim M, Styer AK, Rueda BR. Dendritic cells attenuate the early establishment of endometriosis-like lesions in a murine model. Reprod Sci 2014; 21: 1228-36.
35. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. Eur J Immunol 2010; 40: 1830-5.
36. Luo J, Ming B, Zhang C, et al. IL-2 inhibition of Th17 generation rather than induction of treg cells is impaired in primary Sjögren's syndrome patients. Front Immunol 2018; 9: 1755.
37. Theil A, Tuve S, Oelschlägel U, et al. Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. Cytotherapy 2015; 17: 473-86.
38. Yin Z, Ma T, Lin Y, et al. IL-6/STAT3 pathway intermediates M1/M2 macrophage polarization during the development of hepatocellular carcinoma. J Cell Biochem 2018; 119: 9419-32.
39. Nie MF, Xie Q, Wu Yh, et al. Serum and ectopic endometrium from women with endometriosis modulate macrophage M1/M2 polarization via the Smad2/Smad3 pathway. J Immunol Res 2018; 2018: 6285813.
40. Sekulovski N, Whorton AE, Tanaka T, et al. Niclosamide suppresses macrophage-induced inflammation in endometriosis. Biol Reprod 2020; 102: 1011-9.
41. Symons LK, Miller JE, Kay VR, et al. The immunopathophysiology of endometriosis. Trends Mol Med 2018; 24: 748-62.