IL-2 and IL-27 synergistically promote growth and invasion of endometriotic stromal cells by maintaining the balance of IFN-γ and IL-10 in endometriosis

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Abstract

Immune cells and cytokines have important roles in the pathogenesis of endometriosis. However, the production and role of cytokines of T helper type 1 (Th1) and Th2 cells in the progress of endometriosis have remained to be fully elucidated. The present study reported that the interferon (IFN)−γ levels and the percentage of IFN-γ+CD4+ cells were significantly increased in the peritoneal fluid (PF) at the early stage and maintained at a higher level at the advanced stage of endometriosis; furthermore, interleukin (IL)-10 and IL-10+CD4+ cells were elevated in the advanced stage of endometriosis. In addition, IL-2 levels in the PF at the advanced stage of endometriosis were elevated and negatively associated with IFN-γ expression. In a co-culture system of ectopic endometrial stromal cells (ESCs) and macrophages, elevated IL-2 was observed, and treatment with cytokines IL-2 and transforming growth factor-β led to upregulation of the ratio of IL-2+ macrophages. IL-27-overexpressing ESCs and macrophages were able to induce a higher ratio of IL-10+CD4+ T cells. Blocking of IL-2 with anti-IL-2 neutralizing antibody led to upregulation of the ratio of IFN-γ+CD4+ T cells in the co-culture system in vitro. Recombinant human IL-10 and IFN-γ promoted the viability, invasiveness and transcription levels of matrix metalloproteinase (MMP)2, MMP9, and prostaglandin-endoperoxide synthase 2 of ESCs, particularly combined treatment with IL-10 and IFN-γ. These results suggest that IL-2 and IL-27 synergistically promote the growth and invasion of ESCs by modulating the balance of IFN-γ and IL-10 and contribute to the progress of endometriosis.

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Introduction

Endometriosis is the most common gynecologic disease in females of reproductive age and the feature symptom is the presence of endometrium-like tissue outside the uterine cavity, particularly in pelvic organs (Bulun 2009). While endometriosis has been studied for decades, the mechanism remains to be not fully understood. The most commonly accepted etiology of endometriosis is Sampson’s theory of retrograde menstruation (Sampson 1927). However, the majority of women have retrograde menstruation, and only 10% women develop endometriosis. The role of the peritoneal environment, including cytokines, on endometriosis has been investigated and several abnormalities have been detected (Gazvani & Templeton 2002). In women with endometriosis, immune dysfunction allows menstruation debris to implant and develop (Matarese et al. 2003).
Cytokines are key mediators and communicators of the immune system and may be divided into pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines, also known as Th1 helper cell responses, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, promote inflammation and exert immunoregulatory and pro-inflammatory activities. Anti-inflammatory cytokines, also known as Th2 cell responses, including IL-4 and IL-10, suppress pro-inflammatory cytokine production and inhibit inflammation. It is known that cytokines in peritoneal fluid (PF) of patients with endometriosis stimulate the progression of endometriosis (Podgaec et al. 2010). Aberrant cytokine levels in the serum and PF of patients with endometriosis are recognized as an important aspect of the pathogenesis of endometriosis (Pizzo et al. 2002). The responses of Th1 and Th2 cells in endometriosis are controversial. Certain studies reported on a Th2-cell bias in endometriosis despite the increase in the IFN-γ concentration, while others reported that Th1 and Th2 cells are diminished (Podgaec et al. 2007, Szymanowski et al. 2013, Zhou et al. 2019). Forkhead box (Fox)p3-expressing CD4+ regulatory T cells (Tregs) are an important subset of T lymphocytes with a crucial role in the maintenance of self-tolerance and immune homeostasis, and they are involved in various human diseases, including autoimmune diseases and cancer. The proportion of CD4+Foxp3+ Tregs is significantly increased in the peritoneal fluid of women with endometriosis. As the two key functional cytokines, IL-10 and transforming growth factor (TGF)-β are secreted by Tregs in ectopic lesions and PF, and they are significantly increased in patients with endometriosis (Hull et al. 2012, Li et al. 2014, Hanada et al. 2018).

IL-2 has a central role in Th2-cell differentiation (Cote-Sierra et al. 2004, Yamane et al. 2005). A previous study by our group indicated that IL-27 is able to induce the differentiation of IL-10+Th17 cells in the ectopic environment (Chang et al. 2017). However, it has remained elusive whether IL-2 and IL-27 have a role in Th1- and Th2-cell differentiation and the production of key cytokines (e.g. IFN-γ and IL-10) in the ectopic microenvironment. In the present study, the cytokine profiles in the PF of female patients with endometriosis were evaluated, and the phenotype and cytokine profiles of Th1 and Th2 cells at different stages were assessed. In addition, the role of the cytokines IFN-γ and IL-10 in regulating the growth and invasion of endometriotic stromal cells was explored in vitro.

Materials and methods

Subjects and sample collection

Research ethics was approved by the Research Ethics Committee in Obstetrics and Gynecology Hospital, Shanghai Medical School, Fudan University. Every patient signed the written informed consent. All patients were from the women attending Obstetrics and Gynecology Hospital of Fudan University (Shanghai, China), without any hormone therapy within 6 months prior to surgery, endocrine disorders, or autoimmune diseases. Ectopic endometrium was obtained from patients undergoing laparoscopic ovary endometriectomy and was then pathologically confirmed as endometriosis. Laparoscopic surgeries were performed in reproductive age women. Clinical suspicion of endometriosis was based on patient symptoms and included dysmenorrhea, deep dyspareunia, chronic pelvic pain, infertility, and cyclical alterations in bowel and urinary habits occurring only during menstruation. After physical examination, the patients were subjected to transvaginal ultrasound or MRI. According to the suspicion of the presence of deep infiltrating lesions or if the patient had persistent pain or infertility, a surgical laparoscopic procedure was indicated. Based on histopathology and medical records, patients with superficial peritoneal endometriosis (SPE), ovarian endometrioma (OMA), adenomyosis, or pelvic inflammatory disease (PID)-related infertility were excluded. Finally, a total of 28 patients (mean age 37.8 years; range 31–44 years) with endometriosis were grouped into rAFS stage I–II (early stage, n=12) and rAFS stage III–IV (late stage, n=16), according to the revised system of the American Society of Reproductive Medicine, 1996 (American Society for Reproductive Medicine, 1997). Normal endometrium was obtained at hysterectomy from patients with cervical intraepithelial neoplasia but without endometriosis and/or adenomyosis as healthy controls (n=12) (mean age 41.5 years; range 40–45 years). All samples were obtained in the proliferative phase of the cycle and were confirmed by histology according to established criteria. All samples were collected during surgery under sterile conditions and transported to the laboratory in Dulbecco’s modified Eagle’s medium (DMEM)/F12 for endometrial stromal cell (ESC) isolation and culture.

At the beginning of the laparoscopic surgery after the trocar insertion, PF was collected through the laparoscope from the pouch of Douglas from most patients with endometriosis (n=10 at the early stage; n=12 at the advanced stage) or without endometriosis (n=10, ovary teratoma and underwent laparoscopic surgery). Hemorrhagic fluids were excluded from the study only when the puncture site exhibited an obvious bleeding, PF samples were taken immediately to the laboratory and processed within 2 h.

Peripheral blood samples from healthy volunteers were drawn and collected in heparinized Hank’s buffer solution (Gibco; Thermo Fisher Scientific, Inc.) under sterile conditions. The samples were taken to the laboratory on ice for isolation of immune cells.

Cell isolation and culture

ESCs from control and ectopic endometrium were isolated and cultured as previously described (Mei et al. 2014). In brief, endometrial tissues were cut into <2 mm sections and then digested by stirring in a DMEM/F12 medium with collagenase type IV (0.1%; Sigma-Aldrich; Merck KGaA)
at 37°C for 40–70 min according to the amount of the tissue. The resulting suspension was filtered in turn through sterile gauze pads (pore diameter size: 100, 200, and 400 mesh) to remove any undigested tissue debris and epithelial cells and then centrifuged at 800 g for 9 min at 4°C to remove the leukocytes and erythrocytes prior to discarding the supernatant. Finally, ESCs were resuspended in DMEM/F12 containing 10% fetal bovine serum (HyClone) with 100 U/mL penicillin and 100 mg/mL streptomycin and seeded in culture flasks that were incubated at 37°C in a humidified atmosphere with 5% CO₂. The culture medium was replaced every 2–3 days. Flow cytometric (FCM) analysis was performed to verify the purity of vimentin⁺ ESCs (>98%).

Isolation and culture of immune cells

Immune cells were isolated and purified as previously described (Chang et al. 2017). Peripheral blood mononuclear cells were isolated from peripheral blood samples of the healthy controls by Ficoll-Hypaque density gradient centrifugation. Naïve CD4⁺ T cells and CD14⁺ monocytes (Mo)/macrophages were obtained using magnetic beads according to the manufacturer's protocol (Miltenyi Biotec).

Co-culture system of ESCs, Mo and T cells

The IL-27 overexpression (IL-27over) and negative control (Mock) plasmids were constructed by GeneChem Co., Ltd (Shanghai, China). Mock or IL-27over ESCs were co-cultured with monocytes from peripheral blood (n=10) for 48 h and naïve T cells were then added to the co-culture system that was further treated with or without anti-human IL-2-neutralizing antibody (Ab) (αIL-2; 1 µg/mL; R&D Systems) for 5 days. Subsequently, IL-10 and IFN-γ levels in CD4⁺ T cells were detected by FCM. Prior to co-culture, naïve T cells were activated with anti-CD3 (5 µg/mL; eBioScience), anti-CD28 (1 µg/mL; eBioScience), and recombinant human (rh)IL-2 (10 ng/mL; R&D Systems) for 2 days.

Flow cytometry analysis (FCM)

Antibodies (Abs) for FCM were purchased from BioLegend (USA), including fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (300506, BioLegend), phycoerythrin-cyanine 7 (PE-Cy7)-conjugated IL-10 (510421, BioLegend), and PE-conjugated IFN-γ monoclonal (502509, BioLegend), FITC anti-human CD14 (301804), and allophycocyanin (APC) anti-human IL-2 (500310, BioLegend). To identify and evaluate the Th1 and Th2 cells, the mononuclear cells from peritoneal fluid were stained with anti-CD4 Ab, followed by intracellular staining of IL-2, IL-10, and IFN-γ according to the manufacturer's instructions. In addition, flow cytometry was performed to analyze the percentage of IL-10 and IFN-γ levels in CD4⁺ T cells, the expression of IL-2 in monocytes/macrophages, and the IL-2, IL-10, and IFN-γ levels in PF, using isotypic IgG antibodies such as FITC Mouse IgG1, κ Isotype Ctrl (FC) Antibody (400109, BioLegend), PE/Cy7 Rat IgG1, κ Isotype Ctrl Antibody (400415, BioLegend), PE Mouse IgG1, κ Isotype Ctrl (ICFC) Antibody (400139, BioLegend), FITC Mouse IgG2a, κ Isotype Ctrl Antibody (400207, BioLegend), and APC Rat IgG2a, κ Isotype Ctrl Antibody (400511, BioLegend) as isotope controls. Human Trustain FcX (422301, BioLegend) was used to block Fc receptors first prior to the application of others Abs. Unstained, single stains, fluorescence Minus One (FMOs) controls were used for setting compensation and gates. The samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and Cellquest software (Becton Dickinson). The levels of cytokines in PF were detected by CBA assay (BD, San Jose, CA, USA).

Enzyme-linked immunosorbent assay

Normal ESC, ectopic ESCs, and/or monocytes (Mo) from peripheral blood were cultured for 48 h, and then the IL-2 secretion level in the supernatant was detected by the Human IL-2 ELISA Kit (Cat. No. 431807, LEGEND MAX, BioLegend, San Diego, CA, USA), in accordance with the manufacturer's instructions. A standard curve was generated by using serial diluted standards. For each plate, an independent standard curve was run for reference. The IL-2 concentration was calculated by using a generated standard curve. The minimum detectable level of the ELISA kit was 4 pg/mL, and the kit had no cross-reactivity with any other human cytokines.

Cell counting kit-8 (CCK-8) assay

The CCK8 kit was purchased from DOJINDO (Shanghai, China). Ectopic ESCs in the logarithmic growth phase were collected and counted by using a hemocytometer counting plate and then seeded onto a 96-well plate at 2000 cells in a 100 µL culture medium per well, and each experimental condition was set up in three multiple wells. rhIFN-γ (0, 10, or 100 ng/mL; Peprotech), and/or rhIL-10 (0, 10, or 100 ng/mL; Peprotech), or vehicle (1% PBS as the control) were added after cell adherence and cultured at 37°C with 5% CO₂ in a cell incubator for 0 or 24 h. At the end of the incubation period, 10 µL CCK8 solution was added to each well and incubation was continued as previously described for 2 h. The absorption value was measured at 450 nm to determine the number of viable cells.

Immunofluorescence staining

After stimulation with rhIFN-γ and/or rhIL-10 for 24 h, the expression of Ki67 in ectopic ESCs were detected by immunofluorescence staining. Specifically, cell slides were soaked with 0.01 M PBS (pH 7.2–7.4) for 5 min and washed twice. The cells were then infiltrated with 0.1% Triton X-100 for 10 min, followed by washing with PBS three times. Excess liquid was removed by applying absorbent paper to the sides of the slides and, subsequently, the samples were covered in drops of immunized sheep serum for blocking at room temperature for 30 min. The blocking solution was poured away without washing, and drops of diluted Rabbit Anti-Ki67 antibody (1 µg/ml, ab15580 Abcam) were added onto the cells, followed by incubation in a wet box at the temperature of 4°C overnight. The antibody solution was then poured away and
the sample was washed with PBS for three times. The sample was then covered with drops of Alexa Fluor 488-conjugated goat anti-human IgG (1:1000 dilution; Beyotime Institute of Biotechnology) secondary antibody, followed by incubation in a light-proof wet box at room temperature for 2 h. The antibody solution was then discarded and the sample was washed with PBS once. DAPI (D9542; Sigma-Aldrich; Merck KGaA) was added, followed by incubation for 2 min, and the solution was discarded, followed by three washes with PBS. Anti-quenching tablets were used for sealing, and the samples were observed under a fluorescence microscope.

**Matrigel invasion assay**

Matrigel was diluted to a final concentration of 1 mg/mL in serum-free medium pre-cooled to 4°C and kept on ice. The filters of each of the upper compartments of a Transwell chamber were vertically filled with 100 µL diluted Matrigel, followed by incubation at 37°C for 4–5 h to solidify into a gel. All cell culture reagents and the Transwell chamber were incubated in a thermostat at 37°C. Cells in the logarithmic growth phase were collected and the concentration was adjusted to 1 x 10⁶/mL after counting. A total of 600 µL medium containing 20% serum was added to each of the lower chambers (bottom of a 24-well plate). The upper chambers were filled with 150 µL cell suspension each, followed by culture at 37°C with 5% CO₂ for 24 h. The chambers were then carefully removed with tweezers, the liquid in the upper chambers was aspirated, and the membranes were fixed with methanol at room temperature for 30 min. The liquid was then discarded and the upper chambers were dried and stained with crystal violet dye at room temperature for 15–30 min. The liquid was aspirated and the cells on the surface of the Matrigel and polycarbonate film were gently wiped away with swabs soaked with distilled water. The cells on the surface of the membrane were carefully uncovered with small tweezers and the bottom sides of the filters were dried and transferred onto a slide with neutral resin. For each sample, five randomly selected visual fields were counted under a microscope and the quantitative results were obtained.

**Real-time PCR**

Total RNA was extracted from ESCs using the RNeasy Mini kit according to the manufacturer’s protocol (Qiagen). The transcription levels of matrix metalloproteinase (MMP) 2, MMP9, and prostaglandin-endoperoxide synthase 2 (PTGS2) in ESCs were analyzed by real-time PCR, which was performed using an ABI PRISM™ 7900 Sequence Detector (Applied Biosystems). The primer sequences were designed and synthesized by TaKaRa Biotechnology Co., Ltd as described in the following (5’–3’): MMP2: forward: GGCCTCTCTGACATTGACCTTG, reverse: CACCCAGGATCCTGAGCAGATGC; MMP9: forward: TCTCGTGCTCCTGGTGCTG, reverse: CTGCCTGTGGGAGATTGTTGC; PTGS2: forward: TCTCTGGCTCCTGGTGCTG, reverse: CACCACCGATGAGCAGATGC. The expression levels in the samples were presented as arbitrary units determined by the 2^−ΔΔCT method. All the measurements were performed in triplicate. The specificity of the product was assessed by melting curve analysis.

**Statistical analysis**

Continuous variables are expressed as the mean ± s.d. After the analysis of normal distribution and variance homogeneity, continuous variables were analyzed using a Student’s t-test for two groups and a one-way ANOVA with post hoc test for multiple groups. A correlation analysis was also performed. All analyses were performed using SPSS 19.0 Statistical Package for the Social Sciences software. The results were considered statistically significant at P < 0.05.

**Results**

**IFN-γ and IL-10 are increased in PF from patients at the advanced stage of endometriosis**

To investigate the immune status in the PF of patients with endometriosis, FCM was performed to determine the level of IFN-γ and IL-10 and the percentage of CD4⁺ IL-10 T cells and CD4⁺IL-10 T cells in the PF. Compared with that in the control group, IFN-γ expression was much higher at both early and advanced stages in patients with endometriosis, and there was no difference between the early and advanced stages, suggesting that a pro-inflammatory environment exists among the whole progress of endometriosis; IL-10 was higher at the advanced stage of endometriosis only, and there was no difference between the control and the early stage of endometriosis, suggesting that an anti-inflammatory environment established in advanced stage of endometriosis (Fig. 1A). Consistent with the expression of IFN-γ and IL-10 in the PF, the percentage of CD4⁺IFN-γ⁺ T cells in the PF were much higher at both the early and advanced stages; while CD4⁺IL-10⁺ T cells in the PF were higher at the advanced stage only (Fig. 1B and C).

**Increased IL-2 is negatively associated with IFN-γ in the PF of patients with endometriosis**

It has been reported that IL-2 has an important role in stimulating naive CD4⁺ T cells to generate a Th2-inducing cytokine environment (Hidehiro et al. 2005). In the present study, IL-2 production was much higher in the PF at advanced stage of patients with endometriosis than the control group and early stage of patients with endometriosis (Fig. 2A). In addition, the level of IL-2 was negatively associated with IFN-γ in PF of patients with endometriosis (Fig. 2B), suggesting that the high level of IL-2 in advanced stage of endometriosis may contribute to the inhibition of IFN-γ production. Furthermore, IL-27 production in the PF at early stage of patients with endometriosis was higher, and it was much higher at the
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advanced stage of patients with endometriosis (Fig. 2C). In addition, the level of IL-27 was positively associated with IL-10 in the PF of patients with endometriosis (Fig. 2D), suggesting the high level of IL-27 in endometriosis may contribute to the increase of IL-10 production.

Co-culture of ectopic ESCs and macrophages increases the production of IL-2

In order to determine the origin of IL-2 in the endometriotic microenvironment, an *in vitro* co-culture system of ESCs and macrophages was constructed. Compared with normal ESCs, ectopic ESCs secreted more IL-2, particularly following co-culture with macrophages (Fig. 3A). However, there was no difference in IL-2 secretion between normal ESCs and the normal ESCs-macrophage co-culture groups (Fig. 3A). Treatment with recombinant human IL-2 (rhIL-2), rhTGF-β, and rhIL-27 led to an increase in the percentage of IL-2⁺ macrophages, while treatment with rhIL-6 did not (Fig. 3B). These results indicate that co-culture of ectopic ESCs and macrophages produce high levels of IL-2 and that the cytokines IL-2 and TGF-β may be involved in this process.

IL-2 and IL-27 synergistically induce the balance of IFN-γ and IL-10 of CD4⁺ T cells in co-culture system

To clarify whether IL-27 and IL-2 participate in the differentiation of IFN-γ⁺CD4⁺ T cells and IL-10⁺CD4⁺ T cells, IL-27-overexpressed ESCs (IL-27*overESC*) and anti-IL-2 neutralizing antibody (aIL-2) were used...
to investigate the effect on the populations of IFN-γ+CD4+ T cells and IL-10+CD4+ T cells in an ESC+macrophage+naive T cell (E+M+T) co-culture system (Fig. 4A). Compared with the naive T cells alone (Ctrl), co-culturing with ESCs and macrophages (E + M) promoted the differentiation of IFN-γ+CD4+ T cells and IL-10+CD4+ T cells (Fig. 4B and C). In addition, IL-27overESC further enhanced this effect of E + M on IL-10+CD4+ T cells (Fig. 4B and C). Whereas αIL-2 was able to increase the stimulatory effect of E + M on IFN-γ+CD4+ T cells (Fig. 4B and C). These results suggest that high levels of IL-2 and IL-27 in the endometriotic milieu maintain the balance of IFN-γ and IL-10 in CD4+ T cells.

IFN-γ and IL-10 synergistically promote the growth and invasion of ectopic ESCs in vitro

To determine the effect of IFN-γ and IL-10 on the biological behavior of ectopic ESCs, ectopic ESCs were treated with rhIFN-γ and/or rhIL-10 in vitro. The results indicated that rhIFN-γ and rhIL-10 increased the number of cell viability (Fig. 5A), the expression of Ki67

**Figure 3** Co-culture of ectopic ESCs and macrophages enhances the production of IL-2. (A) Normal ESC (nESC), ectopic ESCs (eESC), or co-culture with monocytes (Mo) from peripheral blood for 48 h, and supernatant was then collected for the determination of IL-2 levels by ELISA. (B) Monocytes were cultured with different recombinant human (rh) IL-27, IL-6, TGF-β, or IL-2 at a concentration of 10 ng/mL for 48 h, and the population of IL-2+ macrophage cells was then analyzed by FCM. Data were presented as the mean ± s.d. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the control group (one-way ANOVA for A; Student’s t-test for B).

**Figure 4** IL-2 and IL-27 synergistically induce the balance of IFN-γ and IL-10 in CD4+ T cells in co-culture system. (A, B, and C) Negative Control ESCs (Mock E) or IL-27-overexpressed ESC (IL-27over E) were co-cultured with monocytes from peripheral blood (n = 6) for 48 h and, subsequently, naive T cells were added to the co-culture system, followed by further treatment with or without anti-human IL-2 neutralizing antibody (αIL-2, 1 µg/mL) for 5 days. Populations of IFN-γ+CD4+ T cells, IL-10+CD4+ T cells were detected by FCM. Prior to co-culture, naive T cells were activated with anti-CD3 (5 µg/mL), anti-CD28 (1 µg/mL), and rhIL-2 (10 ng/mL) for 2 days. Mock E+M: Negative Control ESCs were co-cultured with monocytes from peripheral blood; IL-27over E+M: IL-27-overexpressed ESCs were co-cultured with monocytes from peripheral blood. Data were presented as the mean ± s.d. *P < 0.05, **P < 0.01, and ***P < 0.001 (one-way ANOVA).
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Endometriosis is an inflammatory disease and immunological aspects have a vital role in its development (Králičková & Vetvicka 2015, Jiang et al. 2016, Riccio et al. 2018). Cytokine profiles in the serum and PF of infertile women with endometriosis have recently attracted the attention of researchers (Jørgensen et al. 2017, Jaeger et al. 2018, Tarokh et al. 2019). Different levels of inflammatory cytokines have been detected in serum and PF from patients with endometriosis at different stages (Fan et al. 2018). IFN-γ, the only known type-II IFN, is one of the signatures of the Th1 phenotype. IFN-γ is an important factor, stimulating the adhesion of ectopic endometrial cells to the peritoneum through the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) and inhibiting the apoptosis of ectopic endometrial cells. The present study showed that IFN-γ was first increased at early stage of patients with endometriosis, suggesting that a pro-inflammatory environment is dominant at this stage.

IL-10 is a key immune-suppressive cytokine, which is produced by Tregs and Th cells. IL-10 was first described as a Th2-secreted cytokine, inhibiting IL-2 and IFN-γ synthesis. IL-10 is also secreted by numerous cell and tissue types and is recognized as an inhibitory cytokine on inflammation and immune response that may regulate the growth and differentiation of cells. In the present study, cytokines (e.g. IFN-γ and IL-10) in the PF of patients with endometriosis were analyzed. At the advanced stage, IFN-γ and IL-10 were increased, suggesting that a high and balanced pro-inflammatory and anti-inflammatory environment may be associated with the progression of endometriosis.

The polarization of CD4+T cells toward a Th1, Th2, Th17, and Tregs phenotype is an underlying mechanism of protective and pathologic immune responses (Maluțan et al. 2015). Th1, Th2, and Th17 interleukin pathways are involved in infertile patients with minimal/mild endometriosis (Andreoli et al. 2011). Th1 cells secrete IL-2 and IFN-γ, directing cell-mediated immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13, driving humoral immunity and allergic reactions. Th17 cells are characterized by the production of IL-17A. The cytokines IFN-γ and TNF-α are typical for the Th1 cell response,
while cytokines IL-4, IL-5, and IL-10 are characteristic for Th2-type responses. In the present study, consistent with the IFN-γ and IL-10 level in the PF, IFN-γ+CD4+ T cells from patients with endometriosis at early stage expressed higher levels, suggesting a Th1 environment; at advanced stage, IFN-γ+CD4+ T cells and IL-10+CD4+ T cells were increased, suggesting that a Th1 and Th2 environment co-existed and was maintained at a balanced and high level. Although endometriosis is multifactorial, Th2 cytokines are highly associated with the disease, particularly IL-4, IL-10, IL-5, and IL-13 (Oral & Arici 1996). In recent years, it has been indicated that anti-inflammatory factors IL-4 and IL-10 are increased in endometriosis; it has been suggested that the local microenvironment features coexistent pro-inflammatory and tolerance factors in women with endometriosis (Harada et al. 2001, Zhou et al. 2019). In the initial stage of endometriosis, the dominant status is pro-inflammatory, however, the environment tends toward tolerance during the advanced stage (Zhou et al. 2019). The process that controls the initiation of such responses may have significant healthcare potential.

A new subset of induced immunoregulatory T cells (iTregs) that produced IFN-γ has been discovered (Daniel et al. 2013). CD4+CD25+Foxp3+IFN-γ+ Tregs are associated with good long-term graft outcome in renal transplant recipients and inhibit allogeneic T-cell responses antigen-unspecically as well as antigen-specially. It has been reported that there is an increased number and function of Treg in the PF from patients with endometriosis (Li et al. 2014). Therefore, IFN-γ may also be secreted by Tregs in the microenvironment of ectopic lesions. By secreting IFN-γ and traditional functional molecules, including IL-10 and TGF-β, Tregs may contribute to the creation of a microenvironment in which inflammation and anti-inflammatory cytokines coexist in ectopic lesions, so as to have a role in the development of endometriosis. This possibility may be worthy of further research.

It has been reported that IL-2 has an important role on Th2 differentiation (Cote-Sierra et al. 2004). It has been reported that in vivo and in vitro IL-2 neutralization inhibits not only IL-4 production but also other Th2 cytokines. In the present study, patients with endometriosis had higher IL-2 concentrations in the PF at the late stage of disease, suggesting that a potential role of IL-2 on the progress of endometriosis. Functionally, macrophages have a critical role in endometriotic lesion development and inflammation. In particular, peritoneal macrophages from patients with endometriosis possess reduced phagocytic capacity. Macrophages are the major source of pro-inflammatory and chemotactic cytokines in the PF, including TNF-α, IL-8, vascular endothelial growth factor, and monocyte chemoattractant protein-1 (Gazvani & Templeton 2002, Mei et al. 2014, Yang et al. 2017). A previous study by our group indicated that the crosstalk between ESCs and macrophages impairs cytotoxicity of NK cells in endometriosis by secreting IL-10 and TGF-β (Yang et al. 2017). In the present study, ectopic ESC and macrophages produced more IL-2. Blocking of IL-2 increased IFN-γ+CD4+ T cells differentiation, suggesting that IL-2 controls the continuous increase of IFN-γ at the advanced stage of endometriosis. Of note, co-culture of ectopic ESCs and macrophage, as well as cytokines IL-2 and transforming growth factor-beta (TGF-β) led to an upregulated production and secretion of IL-2. TGF-β was reported to participate in the pathophysiology of peritoneal endometriosis (Tamburro et al. 2003, Young et al. 2017, Sikora et al. 2018). TGF-β activity is increased in the PF from women with endometriosis (Oosterlynck et al. 1994). These results indicate that TGF-β is involved in the regulation of ectopic ESCs and macrophage on IL-2. However, further verification is required.
IL-27 is mainly secreted by antigen-presenting cell (APC) following stimulation by microbial products or other immune stimuli. IL-27 may modulate autoimmunity and induce proliferation of naive CD4+ T cells (Pflanz et al. 2002, Meka et al. 2015). A previous study by our group reported that the interaction between ESCs and macrophages in ectopic lesions result in a high level of IL-27 (Chang et al. 2017). The accumulation of IL-27 from ectopic lesions reflects the tissue and cell specificities of IL-27 expression. In the present study, it was observed that treatment with IL-27 led to up-regulation of IL-2 expression in macrophages, and co-culture with IL-27+ESCs led to up-regulation of IL-10 expression in CD4+T cells. This result was in line with previous reports, for example, IL-27 and IL-6 were indicated to induce STAT3-mediated production of IL-10 by T cells (Stumhofer et al. 2007).

In the vitro experiment, IL-10 and IFN-γ promoted cell viability, proliferation, and invasion of ectopic ESCs and may contribute to the progression of endometriosis. The invasion-associated genes MMP 2, MMP9, and PTGS2 were also increased after exposure to IFN-γ and IL-10, particularly following treatment with IFN-γ and IL-10 combined, suggesting their synergistic effect on ESCs. However, the specific molecular mechanisms remain to be further studied.

In conclusion, the present study indicated that at the early stage of the disease, IFN-γ was elevated in the PF of patients with endometriosis. Co-culture of ectopic macrophages and ESCs led to the secretion of an increased amount of IL-27 to enhance IL-10 expression, as well as IL-2 to control the continuous increase of IFN-γ production by T cells, which is expected to contribute to the high levels of IFN-γ and IL-10 at the advanced stage of endometriosis. The high levels of IFN-γ and IL-10 cooperatively promote ESCs growth and invasion to accelerate the progression of endometriosis, possibly through the up-regulation of MMP2, MMP9, and PTGS2 (Fig. 6). Strategies intended to block IL-2 and IL-27 signaling may have clinical implications for the treatment of endometriosis.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
X M Q, Z Z L, and S Y H performed all experiments and drafted the manuscript; H L Y, L B L, Y W, J W S, and L Y R assisted with the sample collection and FCM assay; J F Y and J N W participated in data analysis; Q F helped with manuscript writing; M Q L, K K C, and X F Y designed the study, supervised the project, and edited the manuscript. All the authors were involved in writing the manuscript. All authors read and approved the final manuscript.

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