

Immunosuppressive macrophages induced by IDO1 promote the growth of endometrial stromal cells in endometriosis

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Abstract. It was previously demonstrated that anomalous expression of indoleamine 2,3-dioxygenase-1 (IDO1) in endometrial stromal cells (ESCs) stimulated an inflammatory response that subsequently initiated the activation of immunosuppressive macrophages in endometriosis. The aim of the present study was to clarify the effect of IDO1-induced macrophages on the growth of ESCs in endometriosis. Normal ESCs, ectopic ESCs and normal ESCs treated with plasmid pEGFP-N1-IDO1 or SD11-IDO1 short hairpin RNA were co-cultured with peripheral blood-derived monocyte (PBMC)-driven macrophages directly for 48 h. Compared with normal ESCs, the PBMC-driven macrophages that were co-cultured with ectopic ESCs displayed a lower phagocytic ability. pEGFP-N1-IDO1 transfection of normal ESCs also decreased the phagocytic ability of co-cultured macrophages. Additionally, pEGFP-N1-IDO1-transfected ESC-induced macrophages significantly increased the viability and proliferation of ESCs, while ESC apoptosis was decreased, compared with control ESCs. In conclusion, IDO1 educated-macrophages may facilitate the survival of retrograde endometrial tissues, and be involved in the pathogenesis of endometriosis.

Introduction

Endometriosis, which is defined by the presence of endometrial tissue outside of the uterus, occurs in ~10% women, and is associated with persistent pelvic pain and infertility. Despite extensive research, the etiology of endometriosis remains elusive. Inflammation of the surrounding pelvic

microenvironment is thought to have an important role in the initiation and progression of endometriosis, in addition to ovarian steroid hormones (1). The ectopic growth of 'lesions', consisting of endometrial cells outside the uterine cavity, stimulate an inflammatory response, which initiates the activation of macrophages and leads to increased concentrations of cytokines and growth factors in the peritoneal fluid (2,3).

Indoleamine 2,3-dioxygenase (IDO1) is an intracellular heme enzyme that catalyzes the initial and rate-limiting step in the metabolism of the essential amino acid tryptophan in the kynurenine pathway. In the last decade, numerous studies have demonstrated that IDO1 produces a marked tolerance effect in fetal rejection, organ transplantation, autoimmune disorders and cancer (4-6). We previously demonstrated that elevated IDO1 expression in eutopic and ectopic endometrial stromal cells (ESCs) promoted the expression of cyclooxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9), which further induced abnormal ESC growth, and initiated the invasion and implantation of the shed endometrium to the peritoneum (7,8).

Macrophages have a key role in regulating and executing the immune response under various conditions. They are considered to be involved in highly complex immunological gynecological processes, including endometriosis, preeclampsia and miscarriage (9,10). The activation and differentiation of macrophages is altered in these types of immune reactions. Higher levels of IDO1 in ectopic ESCs modulate adjacent macrophages through soluble factors, including interleukin (IL)-33, to generate a supportive microenvironment in endometriosis. Dysfunctional macrophages display reduced expression of Human Leukocyte Antigen-antigen D Related (HLA-DR) and CD11c, and increased secretion of modified cytokine IL-10 and transforming growth factor- β 1 (TGF- β 1) (11). Thus, the cross-talk between ESCs and macrophages within the peritoneal cavity remains unclear. The present study aimed to investigate the effect of IDO1-induced tolerant macrophages on the survival of ESCs involved in the pathogenesis of endometriosis.

Materials and methods

Sample collection and cell culture. Patients (age, 23-40 years) that underwent laparoscopy and additional curettage for

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treatment of endometriosis (n=16) or ovary dermoid cyst (n=14) were originally enrolled in this study. Patients that were later considered to be negative for endometriosis (n=4) or ovary dermoid cyst (n=2) following laparoscopy and histological diagnosis were excluded from the study. In total, 12 endometrial and 12 endometriotic samples were obtained from patients who underwent laparoscopy for treatment of endometriosis or ovary dermoid cyst. Inclusion criteria were as follows: Reproductive age (23-40 years old); in the secretory phase of the menstrual cycle; absence of systemic pathologies; and no drug therapy in the past 6 months. Diagnosis was confirmed visually by laparoscopy and histological analysis. All of the women with endometriosis were classified as stage III/IV, according to the revised America Fertility Society classification of endometriosis (12). Peripheral blood samples (15 ml) were collected sterily in women with dermoid cyst (n=12; mean \pm SD: 33.6 \pm 6.2 years old) as controls prior to the administration of general anesthesia with tracheal intubation in heparinized Hank's buffer solution (Gibco; Thermo Fisher Scientific, Inc.; Waltham, MA, USA). Endometriotic cyst wall tissue (ectopic endometrium) was obtained from ovarian endometriosis patients (n=12; mean \pm SD: 30.2 \pm 8.1 years old) during surgery, and normal endometrial samples were collected from control groups. The protocol was approved by the Research Ethics Committee of Nanjing Drum Tower Hospital (Nanjing, China) and informed written consent was obtained from all participants. All tissue samples, which were \geq 200 mg, were collected under sterile conditions and transported to the laboratory on ice in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco; Thermo Fisher Scientific, Inc.). ESCs were purified as described previously (13). Immunocytochemistry identified >95% vimentin-positive and cytokeratin-negative ESCs (13).

IDO1 overexpression or short hairpin RNA (shRNA) plasmid transfection. Normal ESCs were cultured in DMEM/F-12 with 10% fetal bovine serum in a 6-well plate (FBS; Gibco; Thermo Fisher Scientific, Inc.). When cells had reached confluency, 10 μ l Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), 2 ml OPTI-MEM[™] (Gibco, Thermo Fisher Scientific, Inc.) and 4 μ g plasmid pEGFP-N1-IDO1 (Gene Chem Co., Ltd., Shanghai, China) or 4 μ g SD11-IDO1 shRNA (Gene Chem Co., Ltd.) were mixed and incubated at room temperature for 20 min, and then added to the cells at room temperature according to manufacturer's protocol. The vector-only plasmid pEGFP-N1 and SD11 (Gene Chem Co., Ltd.) were used as negative controls, respectively. After 6 h incubation, cells were cultured in DMEM/F-12 containing 10% FBS in 5% CO₂ at 37°C.

Generation of human macrophages. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by Ficoll-Hypaque density gradient centrifugation (11). CD14⁺ cells were obtained through positive selection by CD14⁺ micromagnetic beads according to the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell purity was identified as >95% by flow cytometry identification using a fluorescein isothiocyanate (FITC)-anti CD14 monoclonal antibody (catalogue no. 555397; BD Biosciences, Franklin Lakes, NJ, USA). CD14⁺ cells were harvested at a final concentration of 2 \times 10⁷ cells/ml and washed in cold

PBS. Then, 5 μ l CD14 monoclonal antibody was added into each 100 μ l of cell suspension for 15 min in the dark at room temperature. Following staining, cells were washed twice with cold PBS and then analyzed by FACS Calibur BD flow cytometry (BD Biosciences). Data were acquired in the list mode, and the relative proportions of cells within different areas of the fluorescence profile were quantified using the FlowJo 7.6 software program (FlowJo, LLC., Ashland, OR, USA). Monocytes were subsequently cultured with granulocyte macrophage colony-stimulating factor (GM-CSF; 5 ng/ml; catalogue no. 300-03-100, PeproTech, Rocky Hill, NJ, USA) and macrophage colony-stimulating factor (M-CSF; 20 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 2 mM L-glutamine for up to 6 days. The medium containing GM-CSF and M-CSF was changed every 3 days.

Cell co-culture unit. Normal, ectopic and transfected normal ESCs (ESCs transfected with pEGFP-N1-IDO1 plasmid or SD11-IDO1 shRNA) were cultured in 24-well plates (Corning Incorporated, Corning, NY, USA) at a density of 2 \times 10⁵ cells/well. After ESCs had reached confluency, the monocyte-generated macrophages were subsequently added to the wells directly at the same density as ESCs. After 48 h, following gentle scattering, the macrophages were collected. Some were immediately analyzed by flow cytometry for the phagocytosis assay; others were further co-cultured directly with normal ESCs for another 36 h, and the normal ESCs were subsequently analyzed in cell viability, cell proliferation and Annexin V/propidium iodide (PI) apoptosis assays.

Phagocytosis assay. A total of 2 \times 10⁶ ESC-pretreated macrophages were mixed with fluoresbrite carboxy NYO-labeled beads in a ratio of 10:1 (1 μ m-diameter microspheres; Polysciences Inc., Warrington, PA, USA) for 30 min with shaking at 37°C. The unbound beads were washed away by cold PBS (Corning Incorporation) twice and cells were then resuspended in 2% bovine serum albumin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Then, the cell suspension was added to the upper layer of Ficoll-hypaque solution (GE Healthcare, Sunnyvale, CA, USA) at a ratio of 1:1, and centrifuged at 300 \times g at room temperature for 10 min. Using density-gradient centrifugation, the macrophages were separated in the Ficoll-hypaque solution layer. Following the resuspension of macrophages in PBS, the ratio of macrophages that ingested fluorescent beads was directly determined using FACS Calibur BD flow cytometry (BD Biosciences) and analyzed using the FlowJo 7.6 software program (FlowJo, LLC., Ashland, OR, USA).

Cell viability assay. To detect cell viability, an MTT (Sigma-Aldrich, Merck KGaA) assay was used. Normal ESCs (2 \times 10⁴ cell/per well in 96-well plate) were co-cultured with ESC-pretreated macrophages for 36 h. Normal ESCs were subsequently incubated with 2.5 mg/ml MTT for 4 h, and 100 μ l dimethyl sulfoxide (Sigma-Aldrich; Merck, KGaA) was added. Absorbance (450 nm) was determined using the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria). These values were normalized to the measurement in normal ESCs co-cultured with macrophages that had not been

previously cultures with ESCs, in which the absorbance was set at 1.

Cell proliferation assay. Normal ESCs co-cultured with pretreated-macrophages were separated and detection by BrdU (5-bromo-2-deoxyuridine) Cell Proliferation Kit (Merck Millipore, Billerica, MA, USA) was performed for cell proliferation according to the manufacturer's instructions. The absorbance values (at 450 nm) were detected by the DigiScan Microplate Reader (ASYS Hitech GmbH) and represent the rate of DNA synthesis, which corresponds to the number of proliferating cells. The values were normalized to the absorbance of normal ESCs co-cultured with untreated macrophages, which was set at 1.

Measurement of apoptosis. The rate of apoptosis of co-cultured normal ESCs was analyzed by flow cytometry with Cell Apoptosis kit with Annexin V-FITC and propidium iodide for flow cytometry according to the manufacturer's protocol (Invitrogen, Thermo Fisher Scientific, Inc.). The relative proportions of cells within different areas of the fluorescence profile were quantified using the FlowJo 7.6 software program (FlowJo, LLC).

Statistical analysis. One-way analysis of variance with the S-N-K method post hoc test was used for multiple comparisons, using SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IDO1 in ectopic ESCs modulates macrophage phagocytosis. Our previous study demonstrated that IDO1 expression was higher in ectopic endometrial tissues compared with normal ones (7). To investigate the effect of IDO1 in ESCs on PBMC-derived macrophages, macrophages were pretreated with normal ESCs, ectopic ESCs, normal ESCs transfected with plasmid pEGFP-N1-IDO1, SD11-IDO1 shRNA, or vector-only plasmids. The aim of the current study was to determine whether IDO1 expression in ESCs is responsible for the phagocytic ability of macrophages. Compared with normal ESCs that had not been transfected (blank controls), pEGFP-N1 and SD11 vector-transfected ESCs (negative controls) had the same effect on macrophage phagocytosis ($P > 0.05$; data not shown). IDO1 overexpression in normal ESCs significantly reduced the phagocytosis of co-cultured macrophages, compared with normal ESCs ($P < 0.05$; group c vs. group b; Fig. 1), whereas IDO1 interference demonstrated the opposite effects ($P < 0.01$; group d vs. group b; Fig. 1). Ectopic ESCs significantly inhibited the phagocytic capacity of co-cultured macrophages, compared with normal ESCs ($P < 0.01$; group e vs. group b; Fig. 1).

Ectopic ESC-pretreated macrophages increase the survival of ESCs via higher IDO1 expression in ESCs. To determine the effect of ESC-treated macrophages on viability and proliferation of ESCs, ESC-pretreated macrophages were separated and co-cultured with normal ESCs. pEGFP-N1 and SD11 vector-transfected ESC-educated macrophage

(negative controls) had the same effect on ESC survival as normal ESCs (blank controls; data not shown). The viability and proliferation index of ESCs co-cultured with ectopic ESC-educated macrophages were 1.9- and 2.2-fold of the control, respectively ($P < 0.01$; ectopic ESC vs. normal ESC; Fig. 2). Significant increases in the viability and proliferation index of ESCs were observed when co-cultured with pEGFP-N1-IDO1-transfected ESC-induced macrophages compared with normal ones ($P < 0.05$; IDO1 overexpression vs. normal ESC; Fig. 2). However, the viability and proliferation index of ESCs co-cultured with pEGFP-N1-IDO1-transfected ESC-induced macrophage were significantly lower compared with those cultured with ectopic ESC-induced macrophages ($P = 0.017$, IDO1 overexpression vs. ectopic ESC in Fig. 2A; $P = 0.021$, IDO1 overexpression vs. ectopic ESC in Fig. 2B) which indicated that ectopic ESC-educated macrophages may promote the growth of ESCs through a mediator other than IDO1.

Effect of IDO1-induced tolerant macrophages on the apoptosis of ESCs. ESCs co-cultured with ectopic ESC or pEGFP-N1-IDO1 transfected ESC-pretreated macrophages exhibited a significantly lower apoptosis rate compared with ESCs co-cultured with normal ESC-pretreated macrophages ($P < 0.01$; group e vs. group b and group c vs. group b; Fig. 3), and the apoptosis of ESCs increased when co-cultured with macrophages that were pretreated with SD11-IDO1 shRNA-transfected ESCs ($P < 0.01$; group d vs. group b; Fig. 3).

Discussion

Endometriosis-associated inflammation is chronic and long lasting (14). An increasing number of studies have focused on the importance of immunological imbalances in women with endometriosis. It has been confirmed that, a permissive peritoneal environment may be associated with the initiation and development of endometriosis (1-3,11). Rather than effectively removing the retrograde endometrial fragments in the pelvic cavity, the tolerant environment facilitates the implantation, neo-angiogenesis and proliferation of ectopic endometrial tissue (15,16). Conditions in the tolerant environment may include elevated levels of activated peritoneal macrophages, reduced natural killer cell activity, an abnormal T lymphocyte response and an increased number of regulatory T cells in endometriotic tissue and peritoneal fluid (17-20).

Cells of the monocyte-macrophage lineage are characterized by diversity and plasticity, which respond to environmental stimuli by acquiring diverse phenotypes. In response to external cues, M1 macrophage activation occurs with microbicidal and tumoricidal features. Alternatively, the M2 pathway predominantly participates in parasite containment, tissue remodeling and immunomodulation (21). The activated peritoneal macrophages have an important role in the onset and development of endometriosis (22). Impaired macrophages cannot effectively clear the ectopic endometrial cells in endometriosis patients (23), and inversely secrete various inflammatory mediators that may contribute to the progression of endometriosis. However, the understanding of the contribution of macrophages to the endometriotic environment remains inadequate.

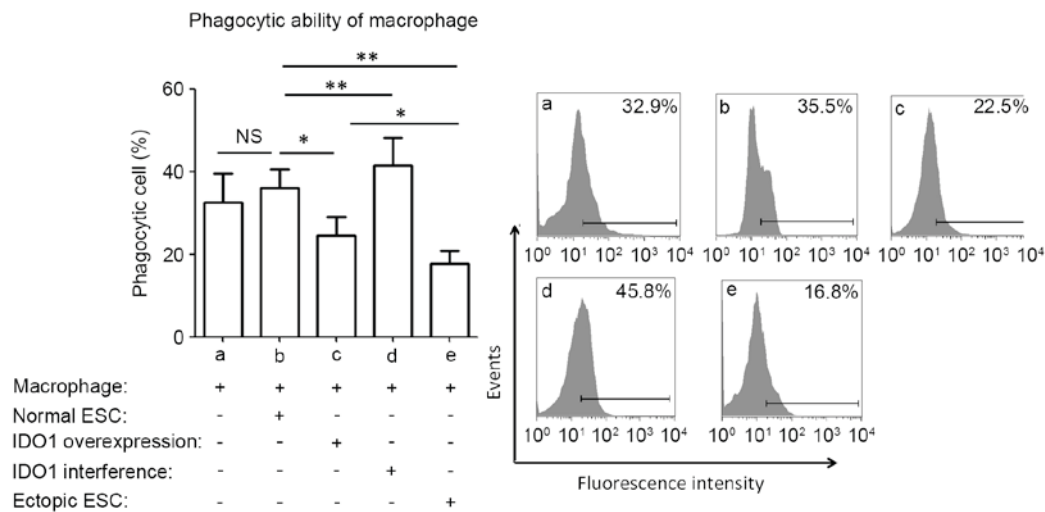


Figure 1. Phagocytic ability of macrophages in various ESC-macrophage co-culture groups. Normal ESCs, ectopic ESCs and normal ESCs treated with pEGFP-N1-IDO1 or SD11-IDO1 short hairpin RNA were co-cultured with human macrophages directly. The phagocytosis of macrophages was detected by flow cytometry. Phagocytic ability was significantly lower when macrophages were cultured with ectopic ESCs compared with normal ESCs. IDO1 overexpression in normal ESCs significantly decreased the phagocytosis of macrophages in the normal ESC-macrophage co-culture group, while IDO1 interference significantly increased it, compared with normal ESC-treated macrophages. Results are presented as the ratio of macrophages that ingested fluorescent beads, as the mean + standard deviation of 12 different experiments ($P<0.05$ and $^{**}P<0.01$). ESC, endometrial stromal cells; IDO1, indoleamine 2,3-dioxygenase-1; NS, not significant; normal ESC, ESCs from patients without endometriosis; IDO1 overexpression, normal ESCs transfected with pEGFP-N1-IDO1; IDO1 interference, normal ESCs transfected with SD11-IDO1 short hairpin RNA; ectopic ESC, ESC from endometriosis-derived endometriotic tissue.

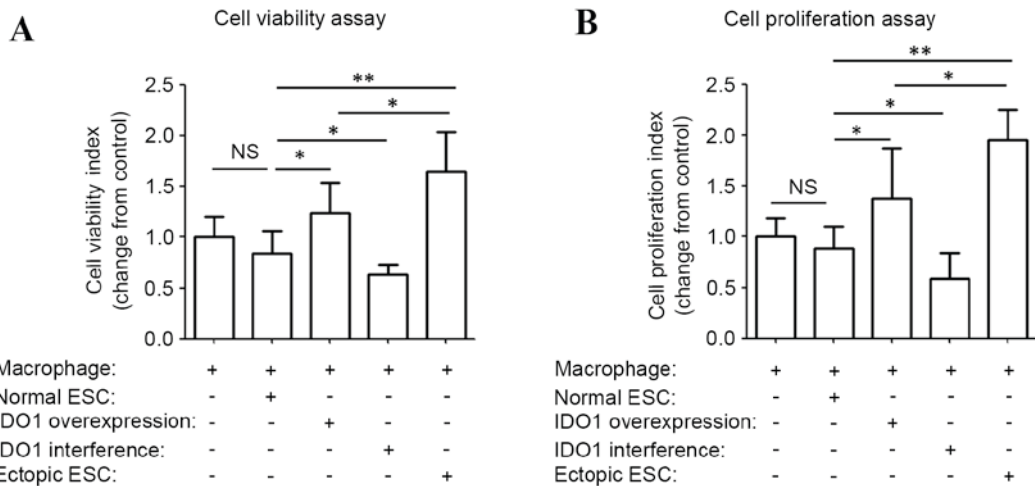


Figure 2. ESC-pretreated macrophages increase the survival of ESCs by IDO1. Peripheral blood mononuclear cell-derived macrophages were pretreated with normal ESCs, ectopic ESCs, IDO1-overexpressing and IDO1-deficient ESCs for 48 h, and subsequently co-cultured with normal ESCs (2×10^4 cells/well in a 96-well plate) for 36 h. (A) MTT assay and (B) 5-bromo-2-deoxyuridine ELISA assay were subsequently performed to analyze the viability and proliferation of normal ESCs, respectively. Results are presented as the mean \pm standard deviation of 12 different experiments ($P<0.05$ and $^{**}P<0.01$). ESC, endometrial stromal cells; IDO1, indoleamine 2,3-dioxygenase-1; NS, not significant; normal ESC, ESCs from patients without endometriosis; IDO1 overexpression, normal ESCs transfected with pEGFP-N1-IDO1; IDO1 interference, normal ESCs transfected with SD11-IDO1 short hairpin RNA; ectopic ESC, ESCs from endometriosis-derived endometriotic tissue.

As it was previously demonstrated that the phenotype of PBMCs did not differ in women with and without endometriosis (24), PBMCs were obtained from control women in the present study. The current study co-cultured ESCs with macrophages to determine if IDO1 in ESCs had any effect on the immune function of macrophages. Following incubation with IDO1-overexpressing ESCs, macrophages exhibited a decreased phagocytic ability. Wu *et al* (25) and Chuang *et al* (26) demonstrated that the master regulator of the peritoneal microenvironment, prostaglandin E2 (PGE2), suppressed at least two aspects of the scavenger function of

macrophages, including decreased secretion and activation of MMP-9, and reduced class B scavenger receptor (CD36) expression in peritoneal macrophages. Furthermore, PGE2 also inhibited annexin A2 expression, which led to a reduced immunological response by peritoneal macrophages (23). The present study subsequently investigated how the tolerant macrophages affected the growth of ESCs. The results demonstrated that the viability and proliferation of ESCs were significantly increased, and apoptosis index decreased, when co-cultured with macrophages pretreated with IDO1-overexpressing ESCs compared with macrophages treated with normal

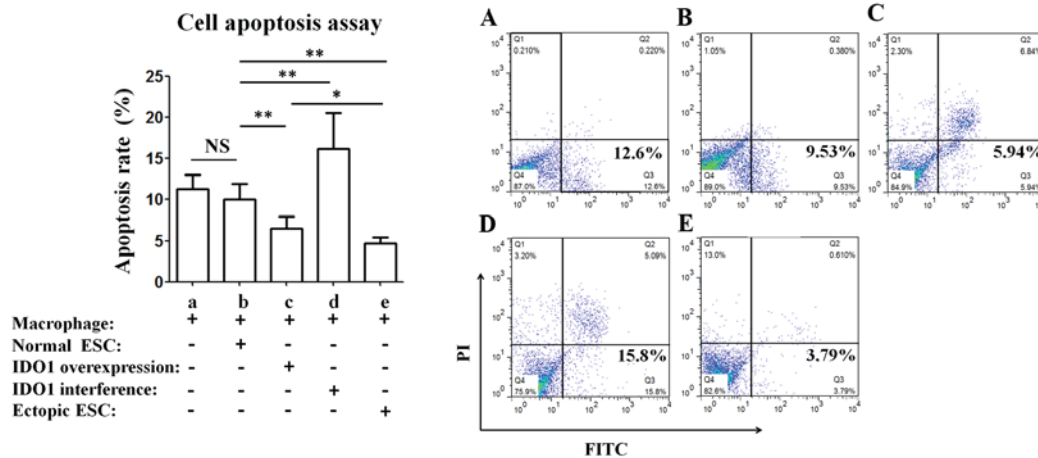


Figure 3. IDO1-induced tolerant macrophages inhibit the apoptosis of ESCs. Normal ESCs were co-cultured with peripheral blood mononuclear cell-derived macrophages, which were pretreated with normal ESCs, ectopic ESCs, normal ESCs transfected with plasmid pEGFP-N1-IDO1 or SD11-IDO1 short hairpin RNA. Subsequently, ESCs were collected and apoptosis was evaluated by flow cytometry. Results are presented as the mean + standard deviation of 12 different experiments (* $P < 0.05$, ** $P < 0.01$). ESC, endometrial stromal cells; IDO1, indoleamine 2,3-dioxygenase-1; NS, not significant; PI, propidium iodide; normal ESC, ESCs from patients without endometriosis; IDO1 overexpression, normal ESCs transfected with pEGFP-N1-IDO1; IDO1 interference, normal ESCs transfected with SD11-IDO1 short hairpin RNA; ectopic ESC, ESCs from endometriosis-derived endometriotic tissue.

ESCs. The results indicate that high levels of IDO1 expressed in the ectopic environment may induce the formation of tolerant macrophages, which in turn may promote ectopic ESC growth in the progression of endometriosis. There are various soluble cytokines in the peritoneal microenvironment that are secreted by macrophages and may affect the survival of endometrial tissue and the development of endometriosis. Shi *et al* (27,28) demonstrated that estradiol and 2,3,7,8-tetrachlorodibenzo-p-dioxin coordinated to the excessive growth of endometriotic cells *in vitro* by stimulating the secretion of pro-inflammatory cytokines, IL-8 and chemokine (C-C motif) receptor 8 (CCR8), by macrophages, which led to persistent and severe inflammation. Furthermore, increased RANTES in eutopic and ectopic ESCs recruited more macrophages into the environment, and also induced tolerance in macrophages, which inhibited the apoptosis and promoted the proliferation of ESCs in the endometriotic environment (3). Additionally, Khan *et al* (29) demonstrated that an inflammatory reaction in the intrauterine environment stimulated the expression of the stress reaction marker, human heat shock protein 70 (HSP70), which further stimulated the production of IL-6 and tumor necrosis factor α by macrophages, and promoted the proliferation of ESCs. However, these effects of HSP70 were more prominent in cells derived from women with endometriosis compared with normal ones. It may be inferred that macrophages from control women were less responsive to the inflammatory surrounding. This may be due to variations in cytokine secretion and the receptor-ligand binding affinity in macrophages of control women. The evidence discussed indicates that ESC-educated macrophages may regulate the growth of ESCs by secreting soluble cytokines. Further studies investigating the mechanism of the cross-talk between endometrial tissues and macrophages in the pelvis of women with endometriosis are required to strengthen the results of the present study.

In conclusion, the progression of endometriosis may be recognized as the product of evolving cross-talk between

ectopic ESCs and macrophages within the peritoneal cavity. Increased IDO1 protein expression in eutopic and ectopic endometria of women with endometriosis is of biological importance. It may directly promote the proliferation and invasion of endometrial tissue by regulating the expression of COX-2 and MMPs. Additionally, it may also modulate adjacent macrophages to generate a supportive microenvironment. Dysfunctional macrophages exhibit impaired phagocytic abilities, which may lead to decreased clearance of endometriotic tissue outside the uterus. Furthermore, the altered polarization of macrophages may promote endometrial tissue growth and suppress its apoptosis by releasing a modified profile of cytokines, including IL-10 and TGF- β 1. Due to the potential role of IDO1 in endometriosis and its application in clinical trials of cancer therapy (30), further investigation is required to identify potential IDO1-based therapies for endometriosis.

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