Indoleamine 2,3-dioxygenase suppresses the cytotoxicity of NK cells in response to ectopic endometrial stromal cells in endometriosis

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Abstract

It has been reported that the impaired cytotoxicity of natural killer (NK) cells and abnormal cytokines that are changed by the interaction between ectopic endometrial cells and immune cells is indispensable for the initiation and development of endometriosis (EMS). However, the mechanism of NK cells dysfunction in EMS remains largely unclear. Here, we found that NK cells in peritoneal fluid from women with EMS highly expressed indoleamine 2,3-dioxygenase (IDO). Furthermore, IDO⁺NK cells possessed lower Nkp46 and NKG2D but higher IL-10 than that of IDO⁻NK. Co-culture with endometrial stromal cells (nESCs) from healthy control or ectopic ESCs (eESCs) from women with EMS led to a significant increase in the IDO level in NK cells from peripheral blood, particularly eESCs, and an anti-TGF- β neutralizing antibody suppressed these effects *in vitro*. NK cells co-cultured with ESC more preferentially inhibited the viability of nESCs than eESCs did, and pretreating with 1-methyl-tryptophan (1-MT), an IDO inhibitor, reversed the inhibitory effect of NK cells on eESC viability. These data suggest that ESCs induce IDO⁺NK cells differentiation partly by TGF- β and that IDO further restricts the cytotoxicity of NK cells in response to eESCs, which provides a potential therapeutic strategy for EMS patients, particularly those with a high number of impaired cytotoxic IDO⁺NK cells.

Reproduction (2018) 156 397–404

Introduction

Endometriosis (EMS), characterized by the implantation of functional endometrium tissue outside the uterine cavity, is estimated to affect up to 15% of reproductive-aged women (Han & O'Malley 2014). The main clinical symptoms, including chronic pelvic pain, pain during intercourse and infertility, can seriously influence the quality of a patient's life because of the high recurrence rates (Young *et al.* 2017). However, there are no treatments thus far due to the controversial pathogenesis of the disease. Despite anatomical, genetic, endocrine, environmental and dietary factors (Simpson *et al.* 1980, Missmer *et al.* 2004, Bulun 2009, Vannuccini *et al.* 2016), accumulating evidence have showed that an aberrant immune microenvironment in the abdominal cavity plays a significant role in the initiation and progression

of EMS by preventing retrograde menstruation from immune clearance, as well as allowing the attachment, implantation and angiogenesis of endometrial cells (Matarese *et al.* 2003, Jeung *et al.* 2016, Yu *et al.* 2016, Yang *et al.* 2017).

Human NK cells are crucial parts of the innate immune system with the capability to produce cytokines and to lyse target cells without prior sensitization (Vivier *et al.* 2008). NK cells compose 15% of all circulating lymphocytes that mediate early protection against viral infections and tumor development and play an important role in immune regulatory functions (Sun & Lanier 2009). Furthermore, together macrophages, lymphocytes, eosinophils, mesothelial cells and mast cells, NK cells are one of the most important cellular components of peritoneal fluid (PF) (Gazvani & Templeton 2002). To date, it is well documented that

abnormal differentiation and the decreased cytotoxicity of NK cells in the peritoneal cavity is involved in the pathogenesis of EMS. Specifically, reductions in NK cell cytotoxicity, such as low levels of granzyme B, perforin, TRAIL and CD107a, were observed in the PF of patients with EMS (Oosterlynck *et al.* 1991, Funamizu *et al.* 2014). Our previous study had revealed that crosstalk between ESCs and macrophages impairs the cytotoxicity of NK cells by secreting IL-10 and transforming growth factor (TGF)- β in EMS (Yang *et al.* 2017), and ESCs derived IL-15 inhibits the killing activity of NK cells in EMS (Yu *et al.* 2016). That is, cytokines change the endometrial milieu in the peritoneal cavity of patients with EMS and thus diminish the cytotoxic activity of NK cells, although the detailed intracellular mechanisms for NK cells remain unknown.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that catabolizes tryptophan to N-formylkynurenine, which is further converted into a series of extracellular messengers collectively known as kynurenines (Frumento *et al.* 2002). IDO is expressed by a large variety of cells, including lymphocytes (macrophages, dendritic cells and B cells), human tumor-cell lines, endothelial cells and stromal cells (Taylor & Feng 1991). IDO modifies the immune responses in two main ways by degrading tryptophan to trigger the amino acid-sensing signal transduction and by producing toxic kynurenines to affect lymphocytes. In our previous studies, we found that IDO expression was much higher in ectopic ESCs (ESCs from the ectopic lesions from women with EMS) and eutopic ESCs (ESCs from the eutopic endometrium lesions from women with EMS) than that in normal ESCs (ESCs from the endometrium from women without EMS) and that IDO enhances the survival, proliferation and invasion of ESCs via the c-Jun N-terminal kinase (JNK) signaling pathway (Mei *et al.* 2013), as well as upregulation of cyclooxygenase (COX)-2 and matrix metalloproteinase (MMP)-9 (Mei *et al.* 2012). In addition, the increased expression of IDO in ectopic ESCs mediates macrophages tolerance in EMS through the activation of IL-33 (Mei *et al.* 2014). However, the expression of IDO in NK and its modulatory mechanism are relatively unknown. Therefore, this study was performed to investigate whether IDO is expressed in NK cells and how the mechanism by which it affects NK cell activity during crosstalk with ESCs *in vitro*.

Materials and methods

Subjects and sample collection

Ethical approval for this study was obtained from the Research Ethics Committee in Obstetrics and Gynecology Hospital, Shanghai Medical School, Fudan University. All patients gave written informed consent. Subjects of this study were reproductive age women (21–45 years) attending the Obstetrics and Gynecology Hospital of Fudan University between May

2017 and September 2017. None of the patients had any hormonal therapy within 6 months before surgery, and patients who suffered from any endocrine disorders or autoimmune diseases were excluded. The ectopic endometrium samples were obtained from patients who underwent laparoscopic ovary endometrial cystectomy and were later pathologically diagnosed as EMS ($n=23$). According to the revised American Society for Reproductive Medicine staging (1997), 15 of 23 cases were classified as early stage (stage I+II), whereas the other eight cases were advanced stage (stage III+IV). In the vast majority of cases, the primary location of EMS was in the ovaries ($n=17$), followed by the pouch of Douglas ($n=5$) and the lateral pelvic wall ($n=1$). The endometrial tissues used as healthy controls were obtained from patients with leiomyoma ($n=22$). All samples were obtained in the proliferative phase of the cycle, which was confirmed histologically according to established criteria. All samples were collected during surgery under sterile conditions and were transported to the laboratory on ice in DMEM/F-12 (Gibco) within 2 h for isolating and culturing ESCs.

PF was aspirated through the laparoscope from the pouch of Douglas at the beginning of the laparoscopic surgery after insertion of the trocar. Samples were obtained from patients with EMS ($n=12$; aged 25–35 years) or without EMS ($n=10$; aged 29–41 years). The hemorrhagic fluids were excluded from the study only when the puncture site had an obvious bleeding. PF was transported immediately to the laboratory and processed within 2 h.

Peripheral blood samples from 53 healthy volunteers were sterilely obtained in heparinized Hank's buffer solution (Gibco). The samples were transported to a laboratory on ice to isolate the NK cells.

Cell isolate and culture

Endometrial stromal cells (ESCs) from normal and ectopic endometrium tissues were purified as previously described (Mei *et al.* 2012). The endometrial tissues were cut into <2 mm thick sections and digested in DMEM/F12 (HyClone, Logan, UT, USA) containing collagenase type IV (0.1%; Sigma) with constant agitation at 37°C for 40–70 min according to the amount of the tissue. The resulting suspension was filtered through sterile gauze pads (pore diameter sizes: 100, 200 and 400 mesh) in turn to remove undigested tissue debris and epithelial cells. After the filtrate was centrifuged at 800g for 9 min at 4°C to remove the leukocytes and erythrocytes, the supernatant was discarded. Finally, ESCs were re-suspended in DMEM/F-12 containing 10% fetal bovine serum (FBS; HyClone) in the presence of 100 U/mL penicillin and 100 mg/mL streptomycin and plated in culture flasks at 37°C under 5% CO₂. The culture medium was replaced every 2–3 days. Flow cytometry (FCM) analysis was used to verify the purity of vimentin⁺ cytokeratin (CK)7[−] ESCs (>98%).

Isolation of human NK cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood samples of healthy fertile women ($n=53$) by Ficoll-Hypaque density gradient centrifugation.

NK cells were obtained through negative selection using an NK cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). The purity of the cell population was assessed by FCM, measuring the membrane expression of CD3-FITC (2.5 μ L; eBioscience, San Diego, CA, USA) and CD56-PE (2.5 μ L; eBioscience). Only cell preparations with purity >95% were used. NK cells were cultured with in RPMI-1640 medium (HyClone) in the presence of recombinant human IL-2 protein (10 ng/mL, R&D system) before co-culture.

Co-culture system of ESCs and NK cells

Normal ESCs (nESCs) and ectopic ESCs (eESCs) were cultured in 12-well plates (Corning, Steuben County, NY, USA) at a density of 2×10^5 cells/well. NK cells were subsequently directly added to the wells at the same density as the ESCs. Then, these cells were incubated with mouse anti-human TGF- β neutralizing antibody (anti-TGF- β , 5 μ g/mL, R&D system, USA) or mouse IgG antibody (control, 5 μ g/mL, R&D system). ESCs or NK cells at 2×10^5 cells/well were cultured alone as controls. After 5 days of co-culture for ESCs and NK cells, some NK cells were collected to detect IDO expression by FCM. To further investigate the effect of IDO activity in NK cells on ESCs viability, the other NK cells (ESC-educated NK cells) were pre-treated with 1-MT (0.5 mM; Sigma) for 24 h and co-cultured with fresh ESCs for another 24 h. Then, these ESCs were collected and their viability was analyzed by cell counting kit-8 (CCK-8) assay.

FCM analysis

All collected PF samples were centrifuged at 400 *g* at 4°C for 10 min, and then the supernatant was discarded. After incubation with allophycocyanin (APC)/cyanine 7 (Cy7)-conjugated anti-human CD45, fluorescein isothiocyanate (FITC)-CD3, Brilliant Violet 421 (BV421)-CD56, APC-NKG2D, BV510-NKp46 and phycoerythrin-cyanine 7 (PE-Cy7)-NKp44, cells were fixed and permeabilized using Fixation/Permeabilization Solution and Perm/Wash Buffer (BD, San Diego, CA, USA) for further intracellular staining, including PE-IDO, BV510-IFN- γ , PE/Cy7-IL-10 and APC-IL-22 (all antibodies were purchased from eBioscience). Staining was performed at room temperature for 30 min. Finally, these cells were washed with PBS and analyzed by FCM (Becton Dickinson). NK cells cultured with ESCs for 5 days were collected and analyzed for the expression of IDO by FCM according to the above protocol. For intracellular cytokine analysis, brefeldin A (10 μ g/mL, BioLegend), phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, BioLegend) and ionomycin (1 μ g/mL, BioLegend) were added 4 h before flow cytometry.

CCK-8 assay

After co-culturing with NK cells, the viability of ESCs was evaluated by the CCK-8 assay. The cell medium was replaced with 100 μ L fresh RPMI-1640 medium (HyClone) containing 10 μ L CCK-8 solution and incubated for 1–4 h. The absorbance (optical density) at 450 nm was measured and used to represent the viability of cells. Each experiment was performed in six parallel wells and repeated three times.

Statistical analysis

The continuous variable is shown as the mean $\pm$ S.E.M. Continuous variables are analyzed using a Student's *t*-test for two groups and a one-way ANOVA for multiple groups. All analyses were conducted using the SPSS 19.0 Statistical Package for the Social Sciences software. The results were considered statistically significant at $P < 0.05$.

Results

NK cells in PF from women with EMS highly express IDO

To investigate the expression of IDO in NK cells in PF from women with or without EMS, FCM was performed to analyze the expression of IDO in CD45⁺CD3⁺CD56⁺NK cells (Fig. 1A). As shown, the mean fluorescence intensity

Figure 1 NK cells in PF from women with EMS highly express IDO. (A and B) After collecting PF from patient with EMS ($n = 12$) or without EMS ($n = 10$), the mean fluorescent index (MFI) of IDO expression in CD45⁺CD3⁺CD56⁺NK cells was determined by FCM. Ctrl, PF from patients without EMS; EMS, PF from patients with EMS. Data were presented as the mean $\pm$ S.E.M. ** $P < 0.01$ compared to the control group.

(MFI) of IDO expression in NK cells of PF from patients with EMS is significantly higher than that of the control group (Fig. 1B) ($P < 0.01$), suggesting that an abnormally high level of IDO may be associated with the phenotype and function of NK cells in the microenvironment of ectopic lesions.

IDO⁺NK cells present lower levels of Nkp46 and NKG2D

To further investigate the phenotype and functional difference between IDO⁺NK cells and IDO⁻NK cells, NK cell cytotoxicity-associated receptors and cytokines from IDO⁺NK and IDO⁻NK in PF from women with or without EMS was detected by FCM. As shown in Fig. 2, lower levels of Nkp46 and NKG2D were present in IDO⁺NK cells of the PF from both women with or without EMS (Fig. 2A and B) ($P < 0.01$ or $P < 0.0001$). Additionally, a higher level of IL-10 was only observed in IDO⁺NK from the PF of women with EMS (Fig. 2A and B) ($P < 0.05$). However, the level of other molecules associated with NK cells killing activity, including Nkp44, IFN- γ and IL-22, showed no significant difference between IDO⁺NK and IDO⁻NK in the PF from both women with or without EMS (Fig. 2A and B) ($P > 0.05$). In addition, there was no significant difference for the expression of IDO in NK cells or the expression of these functional molecules in IDO⁺NK cells between mild endometriosis and severe endometriosis (data not shown). These data suggest that IDO⁺NK cells express lower level of Nkp46 and NKG2D.

Ectopic ESCs significantly upregulate IDO levels in NK cells

To determine the effect of nESCs and eESCs on the expression of IDO in NK cells, freshly isolated NK cells from PBMCs were co-cultured with or without nESCs or eESCs in the presence of recombinant human IL-2 protein for 5 days. As shown, both nESCs and eESCs upregulated the level of IDO in NK cells in co-culture system (Fig. 3A and B) ($P < 0.01$ or $P < 0.001$). Compared to the nESCs-NK cells co-culture unit, the eESCs-NK cell co-culture unit led to a significantly higher level of IDO expression (Fig. 3A and B) ($P < 0.01$). These data reveal that ESCs from ectopic lesions may promote the expression of IDO in NK cells, and these results were consistent with the highly expressed IDO of NK cells in the PF from women with EMS.

The stimulatory effect of ectopic ESCs on IDO in NK cells is partly dependent on TGF- β

TGF- β plays a key role in decreasing immune cells activity and promoting the implantation, growth and angiogenesis of endometrial tissue in the peritoneal

cavity (Hull *et al.* 2012). Although a high level of TGF- β is reported in the serum, PF and ectopic endometrium of women with EMS (Oosterlynck *et al.* 1994, Pizzo *et al.* 2002, Young *et al.* 2014), our recent study confirmed that TGF- β could impair the cytotoxicity of NK cells (Yang *et al.* 2017). In addition, it was reported that IDO expression is negatively correlated with the suppressor of cytokine signaling 3 (SOCS3) and positively correlated with TGF- β (Liu *et al.* 2017). TGF- β can also induce IDO expression and enhance IDO activity (Pallotta *et al.* 2011), and anti-TGF- β was added into a co-culture unit of ESCs and NK cells to clarify the role of TGF- β in the regulation of IDO expression in NK. The results of FCM showed that anti-TGF- β suppressed the IDO expression of NK cells induced by nESCs (Fig. 4A) ($P < 0.05$) or eESCs (Fig. 4B) ($P < 0.05$). Overall, these data suggest that ESCs upregulate the expression of IDO in NK cells partly by secreting TGF- β .

Figure 2 IDO⁺NK cells present lower levels of Nkp46 and NKG2D. (A and B) The MFI of Nkp44, Nkp46, NKG2D, IL-10, IL-22, and IFN- γ expression of IDO⁺NK and IDO⁻NK cells in PF from patient with EMS ($n = 12$) or without EMS ($n = 10$) was analyzed by FCM. Data were presented as the mean $\pm$ s.e.m. NS, no significant difference; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$ compared to the IDO⁺NK cell group.

Figure 3 Ectopic ESCs significantly upregulate IDO levels in NK cells. (A) The purity of vimentin⁺CK7⁻ ESCs isolated from normal control (nESCs) and ectopic endometrium (eESCs) were identified by flow cytometry. (B and C) NK cells isolated from PBMCs (pNK, $n = 6$) were co-cultured with or without nESCs or eESCs for 5 days and the MFI of IDO in the NK cells of each group were analyzed by FCM. Data were presented as the mean $\pm$ S.E.M. $**P < 0.01$ and $***P < 0.001$.

IDO may inhibit the cytotoxicity of NK cells to ectopic ESCs

To further reveal the role of IDO in regulating the NK cells response to ESCs, we established a co-culture unit to mimic the microenvironment of ectopic lesions in the peritoneal cavity. Specifically, after co-culture with nESCs or eESCs, NK cells were collected and pre-treated with 1-MT (a specific inhibitor of IDO activity) for 24h. These NK cells were named 1-MT pre-treated ESC-educated NK cells, and further co-cultured with fresh nESCs or eESCs for another 24h. As shown, NK cells co-cultured ESCs suppressed the viability of ESCs, particularly for nESCs (Fig. 5A and B) ($P < 0.05$ or $P < 0.01$). Notably, pretreatment with 1-MT resulted in the enhancement of NK cell restriction on the viability of eESCs (Fig. 5B) ($P < 0.05$), but not nESC (Fig. 5A) ($P > 0.05$), in the co-culture unit. These data demonstrate that a high level of IDO induced by eESCs may impair NK cytotoxicity in response to eESCs.

Discussion

The IDO-induced reduction in tryptophan concentration was originally recognized as an innate immune mechanism of host defense against infection in the inflammatory environment (Yoshida & Hayaishi 1978). Since then, Munn *et al.* made a groundbreaking discovery that the toxic production of IDO together with decreased tryptophan plays an important role in mediating immune privilege and preventing T cell-driven rejection of allogeneic fetuses during pregnancy, which established a foundation to further determine the immunosuppressive potential of IDO in allergy, autoimmunity, transplantation and cancer (Munn *et al.* 1998, Puccetti & Grohmann 2007). There is a strong link between IDO and NK cells according to known

reports. For example, a metabolite mixture (kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid) prevents NK cell accumulation and contributes to NK cells death in a time-dependent manner (Terness *et al.* 2002). L-Kynurenine also induces NK cells apoptosis through a reactive oxygen species (ROS)-mediated pathway (Song *et al.* 2011). Moreover, tryptophan-derived L-kynurenine affects NK cells function by suppressing the surface expression of activating receptors NKP46 and NKG2D (Della Chiesa *et al.* 2006). Interestingly, Kai *et al.* first confirmed IDO expression in NK cells at the mRNA level and showed that it could be enhanced by IFN- γ . Endogenous IDO in NK cells is reported to be a necessary enzyme for maintaining killing activity against tumor cells because inhibition of IDO reduced the NK activity in a dose-dependent manner (Kai *et al.* 2004). Here, we found that the presence of ectopic endometrial lesions in the abdominal cavity results in the insufficiency of NK cells by upregulating IDO expression in NK cells. In an

Figure 4 The stimulatory effect of ectopic ESCs on IDO in NK cells is partly dependent on TGF- β . (A and B) After adding 5 μ g/mL anti-TGF- β -neutralizing antibody (anti-TGF- β) to the co-culture unit of ESCs and NK cells, the MFI of IDO of NK cells in nESCs group (A, $n = 12$) and eESCs (B, $n = 14$) group was determined by FCM. Data were presented as the mean $\pm$ S.E.M. $*P < 0.05$, $**P < 0.01$.

Figure 5 IDO inhibit the cytotoxicity of NK cells to ectopic ESCs. (A and B) After co-cultured with nESCs or eESCs for 5 days, NK cells derived from PBMC were collected and treated with or without 1-MT (0.5 mM) or not for 24 h, and these NK cells ($n=13$) were collected and further co-cultured with fresh nESCs or eESCs. The viability of nESCs (A) and eESCs (B), which indirectly reflected NK cell's cytotoxicity, was tested by CCK8 assay, and was presented as the fold change from the control group (ESCs only). Data were presented as the mean $\pm$ S.E.M. NS, no significant difference; * $P<0.05$; ** $P<0.01$.

in vitro assay, the higher expression of IDO in NK cells from NK-eESCs co-culture had a lower killing activity for eESCs. Furthermore, the decreased killing activity of NK cells could be restored by 1-MT, a direct IDO inhibitor. Although the expression of IDO in NK cells from the NK-nESCs co-culture unit was slightly upregulated, the killing activity of NK cells showed no difference after pretreatment with 1-MT. These phenomena appear contradictory but could be reconciled by considering IDO as a pleiotropic regulatory factor in response

Figure 6 Schematic roles of IDO in NK cell in response to ESCs. NK cells in the peritoneal fluid from women with EMS highly express IDO. Additionally, IDO⁺NK cells possess lower NKp46 and NKG2D but higher IL-10 than that of IDO⁻NK, which indicates that IDO⁺NK cells present lower killing activity. Ectopic ESCs upregulate IDO levels in NK cells, dependent on TGF- β . Thus, IDO⁺NK cells may have a low cytotoxicity in response to eESCs, which is beneficial to the immune escape of eESCs in the pelvic cavity and the development of endometriosis. Moreover, 1-MT may partly restore impaired NK cells killing activity mediated by IDO.

to diverse immune microenvironment induced by nESCs or eESCs rather than a sole suppressor of cell proliferation. Then further investigation was conducted to determine the mechanism by which different immune microenvironment changed by nESCs or eESCs mediates the effects on IDO in NK cells.

NK cell receptors recognize cells and enable target cell lysis by recruiting various phosphatases due to their immunoreceptor tyrosine-based motifs. The function of NK cells requires cooperative engagement of activating or inhibiting cell-surface molecules. It was reported that the phenotypes of NK cells in the peritoneal cavity are altered in women with endometriosis. Specifically, markers of NK cell cytotoxicity or activation are downregulated, such as NKG2D, CD16, NKp44, NKp46 and CD107a (Jeung *et al.* 2016). By contrast, killer cell inhibitory receptors containing Ig domains (KIR2DL1, KIR2DL2, KIR3DL1 and KIR3DL2) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs) are upregulated, which suppresses the immune response via binding with MHC-1 on target cells. In this study, we found that NKG2D and NKp46 expression on IDO⁺NK cells are distinctly lower than that on IDO⁻NK cells both in PF from women with or without EMS, whereas IDO⁺NK cells are more abundant in the PF from women with EMS, which indicates that IDO may regulate NK cells function via NK cells activating receptors.

TGF- β s are inflammatory growth factors consisting of TGF- β 1, TGF- β 2 and TGF- β 3, with TGF- β 1 as the prevalent form expressed in the immune system. The TGF- β signal transducers Smad3, pSmad3, Smad4 as well as the inhibitory Smad7 proteins were simultaneously observed in the endometrial stromal and epithelial cells (Luo *et al.* 2003, Young *et al.* 2017). Additionally, TGF- β 1 was more strongly expressed in the epithelial cells of endometriotic cysts (Tamura *et al.* 1999) and endometriotic nerve fibers, which is related to dysmenorrhea compared with the normal endometrial tissue (Tamburro *et al.* 2003). These observations indicate that TGF- β s are involved in the normal function of the human endometrium and pathophysiology of endometriosis at the same time. Mizumoto *et al.* reported that increased TGF- β concentrations in the peritoneal cavity from women with endometriosis contribute to the decreased NK cells activity, which has an adverse effect on mouse embryos development (Mizumoto 1996). Previous research demonstrated that TGF- β suppresses NK cells cytolytic activity through inhibiting the expression of the NKp30 and NKG2D receptor and NKG2D ligand MICA (Castriconi *et al.* 2003). Our previous study also demonstrated that TGF- β from an ESC macrophage co-culture unit impairs NK cells killing activity (Yang *et al.* 2017). Here, we demonstrated that an anti-TGF- β neutralizing antibody partly reverse the IDO expression in NK cells; however, the relationship between the IDO-induced low cytotoxicity of NK cells and TGF- β remains unknown.

Given that SHP-2 was reported to negatively regulate NK cell function (Purdy & Campbell 2009), and TGF- β 1 was reported to induce the phosphorylation of ITIMs in IDO and the expression of SHP-1 and SHP-2 via Smad and PI3(K)-dependent pathways (Pallotta *et al.* 2011), we hypothesized that ITIMs in IDO may inhibit NK cells-mediated cytolytic activity through SHP-2 induced by TGF- β signaling. This hypothesis remains to be confirmed by further research. In addition, whether TGF- β regulates the expression of IDO in endometrial NK cells remains to be further studied.

It was reported that IDO normally has low basal expression but is rapidly induced by IFN- γ alone or synergized with other proinflammatory stimuli including IL-1 β and TNF- α and lipopolysaccharide (LPS) in specific cell types (Babcock & Carlin 2000, Fujigaki *et al.* 2001). These proinflammatory cytokines were also highly expressed in ectopic lesion and PF from women with EMS (Kráľčková & Vetvicka 2015). Therefore, these proinflammatory cytokines may also contribute to the high level of IDO⁺NK cells in EMS, which remains to be assessed.

In conclusion, as shown in Fig. 6, our present study suggests that eESC led to a high level of IDO in NK cells partly by secreting a high level of TGF- β and that IDO may further impair the cytotoxicity of NK cells in response to eESCs possibly by suppressing IFN- γ , NKG2D and Nkp46, resulting in the poor clearance of eESCs and finally contributing to the formation of ectopic endometrial lesions. Together with our previous findings (Li *et al.* 2014, Wei *et al.* 2016), anti-TGF- β and 1-MT represent potential treatment strategies for EMS.

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.

Funding

This study was supported by the Major Research Program of National Natural Science Foundation of China (NSFC) 91542108, the NSFC 81471513, the NSFC 31671200, the Shanghai Rising-Star Program (16QA1400800), the Development Fund of Shanghai Talents (201557), the Innovation-oriented Science and Technology Grant from NPFPC Key Laboratory of Reproduction Regulation (CX2017-2), the Program for Zhuoxue of Fudan University (all to M-Q Li) and the Research Program of Changzhou Health Bureau ZD201403 (to Z-F Zhang).

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Received 1 March 2018

First decision 16 April 2018

Revised manuscript received 31 July 2018

Accepted 7 August 2018