The crosstalk between endometrial stromal cells and macrophages impairs cytotoxicity of NK cells in endometriosis by secreting IL-10 and TGF-β

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Abstract

The dysfunction of NK cells in women with endometriosis (EMS) contributes to the immune escape of menstrual endometrial fragments refluxed into the peritoneal cavity. The reciprocal communications between endometrial stromal cells (ESCs) and lymphocytes facilitate the development of EMS. However, the mechanism of these communications on cytotoxicity of natural killer (NK) cells in endometriotic milieu is still largely unknown. To imitate the local immune microenvironment, the co-culture systems of ESCs from patients with EMS and monocyte-derived macrophages or of ESCs, macrophages and NK cells were constructed. The cytokine levels in the co-culture unit were evaluated by ELISA. The expression of functional molecules in NK cells was detected by in vitro flow cytometry. The NK cell behaviors were analyzed by cell counting kit-8 and cytotoxic activation assays. After incubation with ESCs and macrophages, the expression of CD16, NKG2D, perforin and IFN-γ, viability and cytotoxicity of NK cells could partly reverse these effects of ESCs and macrophages on NK cells. These results suggest that the interaction between macrophages and ESCs regulates cytotoxicity of NK cells possibly by stimulating the secretion of IL-10 and TGF-β, and may further trigger the immune escape of ectopic fragments and promote the occurrence and the development of EMS.

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Introduction

Endometriosis (EMS) is a chronic, inflammatory, estrogen-dependent disease that affects 6–10% of reproductive-aged women. It is characterized by the growth of endometrial tissues outside the uterine cavity, causing pelvic pain, dyspareunia and infertility (Shi et al. 2013, Miller et al. 2017). The causes of this gynecological disease are complicated and not thoroughly clear yet. The theory of retrograde menstruation is widely accepted. As known, 76–90% of women have been shown to experience retrograde menstruation; however, only 6–10% of women develop EMS (Halme et al. 1984). To date, it has been suggested that the women with EMS have biochemical, genetic or immunological dysfunction that prevents the removal of the tissues from the peritoneal cavity and rather facilitates tissue adhesion to extra uterine structures (Ahn et al. 2015). Indeed, accumulating studies have revealed that the dysfunction of immune cells in the microenvironment of peritoneal cavity, including neutrophils, macrophages, dendritic cells, natural killer (NK) cells, T helper cells and B cells, contributes to the pathogenesis and progression of EMS via improving the proliferation, adhesion and invasion of the endometrial cells, as well as enhancing angiogenesis of endometriotic tissues (Kwak et al. 2002, Hever et al. 2007, Schulke et al. 2009, Shao et al. 2016, Yu et al. 2016, Chang et al. 2017).

In healthy women, ectopic fragments that are shed into the peritoneal cavity by retrograde menstruation...
and were largely unclear. Macrophages, the monocyte-derived immune cells, originate from the progenitor in the bone marrow and migrate to the tissue associated with their differentiation into distinct population depending on the anatomical location and the microenvironment of the lesion (Sprangers et al. 2016). Infiltrating macrophages are a consistent feature of inflammatory endometriotic milieus. There have been many studies demonstrating the abnormal distribution of macrophages within the ectopic lesion of patients with EMS (Scheerer et al. 2016, Wickstrom et al. 2017). Compared with healthy control, EMS patients exhibit an increasing volume of PF and elevated numbers of peritoneal macrophages (Itoh et al. 2013). Numerous cytokines derived from macrophages, such as monocyte chemoattractant protein (MCP)-1, interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β increase in PF from EMS patients, leading to a positive feedback loop and the further propagation of a peritoneal inflammatory environment (Wieser et al. 2005, Capobianco & Rovere-Querini 2013). It has been well accepted that reciprocal communication between macrophages and endometrial stromal cells (ESCs) exists and the intercellular dialogue may contribute to the establishment of EMS (Wang et al. 2014, Shao et al. 2016). The abnormal endometriotic milieu is beneficial to the proliferation and invasiveness of ESCs by inducing more M2 polarization of macrophages (Wang et al. 2014).

Therefore, this study was undertaken to clarify whether and how macrophages co-cultured with ESCs from endometriotic lesions alter the immunosuppressive effect on NK cells in vitro.

Materials and methods

Tissue collection

This study was approved by the Ethical Committee of the Obstetrics and Gynecology Hospital, Fudan University. All tissue samples were obtained with the consent of the patients. The study’s subjects were women of reproductive age (25–49 years old) attending the Obstetrics and Gynecology Hospital of Fudan University between October 2016 and August 2017. The eutopic endometrium (n=6) and ectopic endometrial tissues (n=49) were obtained from fertile women with EMS (n=49). The endometrial tissues were obtained from patients with leiomyoma by a laparoscopic hysterectomy (n=6) and were used as controls. None of the patients took any medications or received hormonal therapy within 6 months before surgery. All the samples were collected in the proliferative phase of the menstrual cycle. All the samples were confirmed histologically according to the established criteria and were transported to the laboratory on ice in Dulbecco’s modified Eagle’s medium (DMEM/F-12) (Gibco) for isolating and culturing ESCs and for using for in vitro assays.

Cell isolation and culture

For isolation of ESCs according to our previous work (Shao et al. 2016, Yu et al. 2016), the ectopic endometrial tissues were minced into 2 mm pieces and then digested with 10% collagenase type IV (0.1%; Sigma, USA) for 40 min at 37°C with constant agitation. To remove cellular debris and separate ESCs from epithelial cells, the tissue pieces were filtered through sterile gauze pads (pore diameter sizes: 100, 200 and 400 mesh). The filtrate was then centrifuged at 150 g for 8 min to further remove the leukocytes and erythrocytes and was washed with phosphate-buffered saline (PBS). After centrifugation, the supernatant was discarded, and the cells were resuspended in DMEM/F-12 containing 10% fetal bovine serum (PBS; Hyclone, Logan, UT, USA), plated on culture flasks and incubated in a humidified incubator with 5% CO2 at 37°C. The ESCs were allowed to adhere for 20 min. The culture medium was replaced every 2–3 days. These methods supplied >98% Vimentin-positive (Vimentin+) ESCs.

Immunocyte isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from the blood samples of healthy fertile women (n=43) by Ficoll-Hypaque density gradient centrifugation. According to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), CD14+ monocytes were purified from PBMC by magnetic affinity cell sorting using the human CD14 MicroBeads kit; NK cells were obtained through negative selection by an NK cell isolation kit. The purity of CD45+CD14+ monocytes and CD45+CD3−CD56+ NK cells was >90% as determined by flow cytometry (FCM).

NK cells were cultured in RPMI-1640 medium (Gibco) containing with recombinant human IL-2 protein (10 ng/mL, R&D system) before co-culture. In addition, we obtained the monocyte-derived macrophages as described previously (Shao et al. 2016). The monocytes were stimulated with macrophage colony-stimulating factor (M-CSF, 20 ng/mL, R&D Systems) and granulocyte macrophage colony-stimulating factor (GM-CSF, 5 ng/mL, R&D Systems) in RPMI-1640 medium (Gibco) containing 10% FBS and 2 mM L-glutamine for up to 6 days. The medium that contained M-CSF and GM-CSF was changed every 3 days.
**Cell co-culture unit**

ESCs of ectopic lesion from patients with EMS were cultured in 24-well plates (Corning) at a density of 1 × 10^5 cells/well for 48h. In the contact co-culture unit, the monocyte-derived macrophages were added to the wells directly at the same density as the ESCs. ESCs or macrophages of 1 × 10^5 cells/well were cultured alone as controls. The medium was aliquoted and stored at −80°C for the cytokine assay. To evaluate the effect of ESCs and macrophages on NK cells, after 48h of co-culture of ESCs (1 × 10^5 cells/well) and macrophages (1 × 10^5 cells/well), NK cells (1 × 10^5 cells/well) were directly added to the co-culture system for another 48h. Then, all suspension cells in the co-culture system were collected, and FCM was used to detect the expression of functional molecules in NK cells.

To further investigate the possibly regulatory mechanism of ESCs and macrophages on NK cells, after 48h of co-culture of ESCs and macrophages, NK cells were then placed in the upper compartment of the transwell chamber inserts (0.4 μm aperture, 12 mm diameter; Corning) at the same density as the ESCs for another 48h in the non-contact transwell co-culture unit. After serum starvation for 8h, the non-contact co-culture unit was then incubated with human IL-10R antibody (rhIL-10Rβ, 1 μg/mL; R&D Systems), human IL-1p/IL-1F2 antibody (rhIL-1β, 1 μg/mL; R&D Systems) or human LAP (TGF-β1) antibody (rhTGF-β1, 5 μg/mL; R&D Systems), and vehicle (1% PBS) was used as the control. The total volume of medium in each well was 1 mL. After 48h, cell viability assay and FCM were carried out to analyze the levels of viability, proliferation and apoptosis-related molecules, and functional molecules in these NK cells.

**Enzyme-linked immunosorinet assay (ELISA)**

In order to evaluate the cytokine secretion level of macrophages (1 × 10^5 cells/well) after co-culture with ESCs (1 × 10^5 cells/well) for 48h and the controls, the culture supernatant from co-culture unit or control wells was harvested, centrifuged to remove cellular debris and stored at −80°C until being assayed by ELISA for determination of TGF-β (R&D Systems), IL-1p (R&D Systems) and IL-10 (R&D Systems).

**FCM**

The suspension cells in direct three-cell co-culture system or NK cells in indirect co-culture system as described earlier were collected from all wells and centrifuged immediately at 1500 rpm for 8 min. The expression of CD45, CD3, CD56, CD16, NKG2D, Perforin, Ki-67, IFN-γ, Fas and FasL was analyzed by FCM (all antibodies were from Biolegend, San Diego, CA, USA). Specifically, these NK cells were fixed, permeabilized and stained with BV421-conjugated anti-human perforin antibody, BV510-conjugated anti-human IFN-γ antibody or PE-conjugated anti-human Ki-67 antibody after labeling with fluorescein-isothiocyanate-conjugated anti-human CD45 antibody, phycocerythrin-cyanine 7 (PE-Cy7)-conjugated anti-human CD3 antibody, Brilliant Violet 421 (BV421) anti-human CD56 antibody and allopurinol-cyanin

(1% positive cells were allowed beyond the statistical marker in the appropriate controls.

**Cell viability assay and cell-mediated cytotoxicity assay**

A cell counting kit-8 (CCK-8) assay was used to evaluate the viability of NK cells. After culturing for 24h at 37°C, the medium was replaced with 90 μL fresh RPMI-1640 medium and 10 μL CCK-8 solution and incubated for about 2h. The absorbance at 450 nm was measured using the Thermo Scientific Varioskan Flash spectrophotometer (Thermo Scientific) every 30 min. The mean value of 3 wells was used as the result of each sample. A cell-mediated cytotoxicity assay was used to measure ESC death following incubation with NK cells from upper compartment of the transwell or control group. Set up 96-well assay plates containing ESCs (1 × 10^5 per well) and NK cells (ESC/NK cell was 1/1 or 1/3) and prepare wells for NK cell or ESC spontaneous LDH release control, ESC maximum LDH release control, volume correction control and co-culture medium background control. Incubate the cytotoxicity assay plate for 4h at 37°C, 5% CO₂. After the 4-h incubation, centrifuge the plate at 250 g for 4 min. Transfer 50 μL aliquots from all wells using a multichannel pipettor to a fresh 96-well fat-bottom plate. Add 50 μL of CytoTox 96® Reagent (Promega) to each well and cover the plate with foil and incubate for 30 min at room temperature. After adding 50 μL Stop Solution (Promega) to each well, the absorbance at 490 nm was recorded within 1 h.

**Statistical analysis**

The results were representative of multiple experiments and were presented as mean ± s.e.m. The variables were analyzed by one-way ANOVA using Tukey’s post hoc test in multiple groups (SPSS, version 11.5). The differences were considered as statistically significant at P < 0.05.

**Results**

Co-culture with ESCs and macrophages downregulates the expression of CD16, NKG2D, perforin and IFN-γ in NK cells

To explore the ability of NK cell activation by ESCs, NK cells were purified from peripheral blood and further co-cultured with normal ESCs from healthy control, eutopic ESCs or ectopic ESCs from EMS. As presented in Fig. 1A, compared with normal ESCs and eutopic ESCs, ectopic ESCs led to the lowest ratio of CD16-NK and
Co-culture with ESCs and macrophages downregulates the expression of CD16, NKG2D, perforin and IFN-γ in NK cells. (A) NK cells were purified from peripheral blood and further co-culture with norESCs, eutESCs or ectESCs (n = 6) for 48h, and then the expression of CD16, NKG2D, perforin and IFN-γ of NK cells was analyzed by flow cytometry (FCM). norESCs: ESCs of normal endometrium from healthy control; eutESCs: ESCs of eutopic endometrium from patients with EMS; ectESCs: ESCs of ectopic lesion from patients with EMS. (B) The procedure for constructing direct co-culture unit. (C and D) After co-culture with ESCs from ectopic lesions (n = 9) and monocyte-derived macrophages, the expression of CD16, NKG2D, perforin and IFN-γ of NK cells was analyzed by FCM. The flow clustering graphs (C) and the statistical graphs (D) are shown. ESC: ESCs from ectopic lesions; Mo: monocyte-derived macrophages. Statistical description: Mean ± S.E.M., *P < 0.05, **P < 0.01 or ***P < 0.001. NS: no significant difference.
ESC and Mφ impair NK cell's cytotoxicity in EMS by IL-10 and TGF-β

IFN-γ⁺ NK cells (P < 0.001) (Fig. 1A), but all ESCs had no influence on the expression of NKG2D and perforin in NK cells (P > 0.05) (Fig. 1A). These data suggest that ectopic ESCs have a stronger inhibitory effect on cytotoxicity of NK cells compared with normal ESCs and eutopic ESCs. To further imitate the immune microenvironment in ectopic lesions, NK cells were directly co-cultured with or without ESCs from EMS and or monocyte-derived macrophages (Fig. 1B). As shown, the ratio of CD16⁺ NK cells/CD16⁻ NK cells, the percentage of NKG2D⁺ NK cells and perforin⁺ NK cells, as well as the proportion of IFN-γ⁺ NK cells were reduced after incubation with ESCs only, macrophages only or both of them compared with control group (non-co-cultured NK cells) (P < 0.01 or P < 0.001) (Fig. 1C and D). However, the levels of other molecules associated with NK cells’ cytotoxic activity (e.g., KIR2DL1, KIR3DL1, NKP30, NKP44 and NKP46) were not changed (data not shown). Particularly, in three-cell co-culture group, these molecules (CD16, NKG2D, perforin and IFN-γ) associated with the cytotoxicity of NK cells were downregulated remarkably compared with control groups (P < 0.001) (Fig. 1C and D). These data suggest that the co-culture of ESCs from ectopic lesion and macrophages may impair cytotoxicity of NK cells by downregulating CD16, NKG2D, perforin and IFN-γ expression.

Co-culture with ESCs and macrophages inhibits the viability and cytotoxicity of NK cells

To further investigate the viability and cytotoxicity activity of these ESC- and macrophage-educated NK cells to ESCs, we collected these non-cultured and co-cultured NK cells for cell viability assay, or further

Figure 2 Co-culture with ESCs and macrophages inhibits the viability and cytotoxicity of NK cells. (A) The procedure for constructing indirect co-culture unit. (B) NK cells after co-culture with ESCs (n = 11) or/and macrophages were collected for cell viability assays. (C) NK cells indirectly co-cultured with ESCs and macrophages were collected and further co-cultured with fresh ESCs (n = 8) at the ratio of 1:1 or 1:3 for cell-medium cytotoxicity assays. Statistical description: Mean ± s.e.m., *P < 0.05, **P < 0.01 or ***P < 0.001.
co-cultured with fresh ESCs for cytotoxicity assays (Fig. 2A). In cell viability assays, the viability of NK cells was downregulated remarkably after co-culture with ESCs and macrophages (P<0.001) (Fig. 2B). In addition, the viability of NK cells after co-culture with ESCs or macrophages was also decreased compared with non-culture control group (P<0.001) (Fig. 2B). However, there was no significant difference among three different co-culture groups (P>0.05) (Fig. 2B). The results of cytotoxicity assays showed that the cytotoxicity of NK cells co-cultured with ESCs and macrophages was significantly decreased (P<0.001) (Fig. 2C) compared with the controls, as well as a decreasing trend (P<0.05 or P<0.01) (Fig. 2C) in contrast with that in the ESC or macrophage culture unit.

**Co-culture of ESCs and macrophages leads to a higher secretion of IL-1β, IL-10 and TGF-β**

To investigate the regulatory mechanism of ESCs and macrophages on NK cells, we cultured monocyte-derived macrophages with ESCs and detected cytokine levels in co-culture unit by ELISA. Studies showed that higher expression of IL-1β derived from ovarian endometrioma (Kao et al. 2011), IL-10 derived from regulatory T (Treg) cells and IL-10/Th17 cells in endometriostic milieu (Li et al. 2014, Chang et al. 2017) and platelet-derived (Guo et al. 2016) or Treg cell-secreted (Li et al. 2014) TGF-β1 contributed to the development of EMS. Therefore, IL-10, IL-1β and TGF-β were considered as candidate cytokines that might be involved in regulating the killing activity of NK cells. As depicted in Fig. 3, we found that as a cell population of cytokine production, macrophage secreted IL-1β, IL-10 and TGF-β. Cytokines derived from ESCs of ectopic lesions were also detected, indicating an intrinsic powerful ability of secretory characteristics of ESCs under pathological conditions. When co-culturing macrophages with ESCs, the levels of these cytokines were significantly elevated compared with controls (P<0.01 or P<0.001) (Fig. 3).

The effect of ESCs and macrophages on killing-related molecules of NK cells is dependent on IL-10 and TGF-β

NK cells were treated with αhIL-10Rβ, αhIL-1β, αhTGF-β or vehicle in the indirect co-culture unit with ESCs and macrophages. Co-culture with ESCs and macrophages significantly downregulated the expression of CD16, NKG2D, perforin and IFN-γ of NK cells. However, as shown, exposure with αhIL-10Rβ and αhTGF-β resulted in the decrease in these molecules in NK cells (P<0.05) (Fig. 4), indicating that they can restrict the effect of ESCs and macrophages on the expression of CD16, NKG2D, perforin and IFN-γ in NK cells. Whereas, no significant difference was found when adding αhIL-1β to the co-culture unit (P>0.05) (Fig. 4), suggesting that IL-1β may be not involved in regulating killing-related molecules of NK cells mediated by ESCs and macrophages.

![Figure 3](image-url) Co-culture of ESCs and macrophages leads to a higher secretion of IL-1β, IL-10 and TGF-β. Cytokine (IL-1β, IL-10 and TGF-β) levels in the co-culture unit of monocyte-derived macrophages with ESCs (n=6) by ELISA. Statistical description: Mean ± s.e.m., **P<0.01 or ***P<0.001.

**Figure 4** The effect of ESCs and macrophages on killing-related molecules of NK cells is dependent on IL-10 and TGF-β. NK cells were treated with αhIL-10Rβ, αhIL-1β, αhTGF-β or vehicle in the indirect co-culture unit with ESCs (n=6), macrophages and NK cells for 48h, and then the expressions of CD16, NKG2D, perforin and IFN-γ in NK cells were evaluated by FCM. Statistical description: Mean ± s.e.m., *P<0.05 or **P<0.01.

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ESC and Mφ impair NK cell’s cytotoxicity in EMS by IL-10 and TGF-β

Co-culture of ESCs and macrophages inhibits the cytotoxicity of NK cells partly by elevation of IL-10 and TGF-β

Finally, to further investigate whether the impact of co-culture of ESCs and macrophages on NK cells’ behavior is dependent on upregulation of IL-10 and TGF-β, cell viability assays and cytotoxicity assays were performed to examine the viability and killing ability of NK cells. As shown, treatment with αhIL-10Rβ, αhIL-1β or αhTGF-β1 did not influence the expression of Ki-67 in NK cells ($P > 0.05$) (Fig. 5A) as well as the viability of NK cells ($P > 0.05$) (Fig. 5B) from the co-culture system. Similarly, the levels of Fas and FasL on NK cells were unaffected by these treatments ($P > 0.05$) (Fig. 5A).

Consistent with the level of killing-related molecules, αhIL-10Rβ and αhTGF-β1 but not αhIL-1β could markedly reverse the decreased cytotoxicity of NK cells from ESC–macrophage co-culture unit ($P < 0.05$ or $P < 0.01$), regardless of the ratio of ESCs to NK cells which was 1:3 or 1:1 (Fig. 5C). These results suggest that ESCs and macrophages inhibit the cytotoxicity of NK cells partly by IL-10 and TGF-β promotions. Neither the viability nor the apoptosis of NK cells was dependent on IL-10 and TGF-β from ESCs and macrophages.

Discussion

EMS is now considered to be a disease of both endocrine and immune dysregulation. Accumulating...
studies have indicated that immunosuppressive effects of the endometriotic microenvironment facilitate the survival and implantation of endometrial fragments in the refluent menstruation (Kralickova & Vetvicka 2015). NK cell activity in patients with EMS is significantly reduced and this effect can be mediated by factors present in endometrial stroma culture-conditioned medium (Vigano et al. 2001). Our previous work has demonstrated that ESCs can suppress the cytotoxic activity of NK cells via cytokine production in the ectopic milieu (Yu et al. 2016). Indeed, cytokines are the main mediators and communicators of the immune system. Local macrophages in the ectopic tissues are the primary contributors to the elevated pro-inflammatory and chemotactic cytokines found in the PF of women with EMS. Recent studies have identified macrophages in ectopic lesions as responsible for the outcome of the auto-transplantation (Capobianco & Rovere-Querini 2013). Moreover, the crosstalk between macrophages and ESCs exists, which may support the establishment of endometriotic lesions (Wang et al. 2014, Shao et al. 2016).

In this study, we investigated the mutual communication between ESCs from ectopic lesion and macrophages at a cytokine level and their effects on NK cells in the simulative ectopic microenvironment in vitro, finding markedly increased levels of cytokine secretion and downregulated viability and killing activity of NK cells in the co-culture unit of ESCs and macrophages. Among these cytokines under investigation, the levels of IL-1β, IL-10 and TGF-β in the co-culture unit of macrophages and ESCs were significantly elevated, indicating a special immune microenvironment (coexistence of pro-inflammatory and anti-inflammatory factors). In fact, both ESCs and macrophages themselves generate cytokines. TGF-β was obviously highly expressed in ESCs, implying that ESCs may be an important source of TGF-β in ectopic lesions besides immune cells and platelets, which can partly explain why ESCs alone suppressed the viability of NK cells.

**Figure 6** Schematic representation of the effects of IL-10 and TGF-β derived from the crosstalk between ESCs and macrophages on NK cells in EMS. The macrophages and ESCs in endometriotic lesions communicate mutually and produce more IL-10 and TGF-β, which downregulate killing-associated molecules (CD16, NKG2D, perforin and IFN-γ) and cytotoxicity of NK cells locally, and may further trigger the immune escape of ectopic fragments and promote the occurrence and the development of EMS.
EMS is known as a chronic inflammatory disease. However, our recent study suggests that the local microenvironment of women with EMS presents a coexistent state of pro-inflammatory and tolerance factors. The dominant position is pro-inflammatory in the initial stage of EMS, whereas the environment tends toward tolerance during the advanced stage (Li et al. 2014, Chang et al. 2017). The infiltrating monocyte-derived cells exhibit an inflammatory phenotype. However, signals from ESCs may attenuate their colitogenic potential. Local macrophages are a consistent feature of endometriotic lesions. They may undergo activation as a consequence of signals generated within ectopic lesion or possibly of the lack of hormone-regulated anti-inflammatory signals in the ectopic endometrium (Lousse et al. 2008, Novembri et al. 2011). Moreover, ESCs themselves have the ability of cytokine secretion as a self-protective mechanism to facilitate their survival and implantation. In this way, the coexistence of pro-inflammatory and anti-inflammation molecules in the ectopic lesions is rather joint than contradictory. On the one hand, the persistent inflammation may result in a positive feedback loop to accelerate the damage to normal tissues; on the other hand, the anti-inflammatory cytokines may convey immunosuppressive signals to effective immune cells and further promote the immune escape of ectopic lesions.

IL-10 and TGF-β are known anti-inflammatory cytokines capable of inhibiting the synthesis of inflammatory cytokines. IL-10 has been found in increased levels in the serum and PF of women with EMS compared to normal women. Endometriotic lesion growth can be promoted or decreased by administering or depleting IL-10, respectively, in a mouse model (Ho et al. 1997, Cameron & Kelvin 2003, Suen et al. 2014). TGF-β is critically expressed in endometriotic lesions and has been implicated both in the initiation of menstruation and in the repair of eutopic endometrium (Omwandho et al. 2010). Recently, Han et al. have reported that estrogen receptor β can protect endometriotic cells from immunosurveillance by inhibiting TNF-induced cell death pathways and can promote ectopic lesion growth by enhancing inflammasome-induced IL-1β production (Han et al. 2015). Blocking IL-10 or TGF-β, the decreased levels of killing-associated cytokines and the downregulated cytotoxicity of NK cells were reversed, whereas the same results were not shown by blocking IL-1β in the co-culture unit, indicating that upregulated IL-1β does not involve in the dysfunction of NK cells in EMS. IL-1β may support the implantation and growth of the ectopic foci by regulation of inflammatory process as well as upregulation of COX-2 and VEGF (Huang et al. 2013, Sikora et al. 2016). As shown in Fig. 6, these results reveal that the ESCs and macrophages inhibit the cytokine secretion and cytotoxicity of NK cells partly by upregulating the secretion of IL-10 and TGF-β. Therefore, the increased concentration of IL-10 and TGF-β along with the decreased cytotoxicity of NK cells observed in EMS supports the notion that local cytokine dysregulation allows endometrial fragments to implant in the peritoneal cavity.

IL-10 expression may be modulated via autocrine (Gabrysova et al. 2014). The IL-10 receptor consists of IL-10Rα and IL-10Rβ (Moore et al. 2001). IL-10Rβ is relatively widely expressed, whereas IL-10Rα is restricted to leukocytes and lymphoid organs with particularly high levels in monocytes and macrophages as well as in NK cells, CD4+ and CD8+ T cells, DCs, B cells and mast cells. IL-10 binding to IL-10Rβ on NK cells may activate the IL-10/JAK1/STAT3 cascade, where phosphorylated STAT3 homodimers translocate to the nucleus within seconds to activate the expression of target genes (Hutchins et al. 2013). IL-10 can inhibit the synthesis of inflammatory cytokines IFN-γ, IL-2, IL-3, TNF-α and GM-CSF to counteract excessive inflammatory immune responses (Moore et al. 2001, Cameron & Kelvin 2003).

Here, we show that the local upregulated macrophage-derived IL-10 in the abnormal and complicated ectopic microenvironment can partly impair the cytokine secretory capacity and cytotoxicity of NK cells, which is consistent with the immunosuppressive properties of IL-10 and previous findings (Somigliana et al. 1996).

TGF-β is a major immunosuppressive cytokine that maintains immune homeostasis. TGF-β is critically expressed in human endometrium and is secreted by endometrial cells and macrophages into the uterine fluid, which has been involved in both the initiation of menstruation and in the repair of eutopic endometrium (Jones et al. 2006, Omwandho et al. 2010). It has been demonstrated that platelet-derived TGF-β1 may be responsible for reduced NKG2D expression as well as reduced cytotoxicity of NK cells in women with EMS (Guo et al. 2016). Consistently, our results indicate that abundant TGF-β derived from ESCs and macrophages in women suffering from EMS may be crucial in establishment and maintenance of ectopic lesions via inhibition of cytotoxicity in NK cells. There would be a mutual exchange between ESCs and macrophages and a synergistic effect on TGF-β secretion as well as on immunosuppression of NK cells. Recently, it has been demonstrated that TGF-β inhibits the metabolic activity of NK cells by opposing the induction of mammalian target of rapamycin activity mediated by IL-2 or IL-15 (Viel et al. 2016). TGF-β also inhibits the cytotoxicity of NK cells possibly depending on type I and type II serine-threonine kinase receptors and transcription factors known as SMADs (Flavell et al. 2010). The exact mechanism of TGF-β on cytotoxicity of NK cells in EMS still needs to research.

In conclusion, we demonstrate in this study that IL-10 and TGF-β derived from the co-culture system of ESCs and macrophages may be responsible for reduced killing-associated cytokine secretion as well as...
reduced cytotoxicity of NK cells in women with EMS. This study highlights the critical role of local immune microenvironment in impairing NK cell-mediated anti-endometriotic immunity, which contributes to the development of EMS. More importantly, this study provides evidence that anti-IL-10 or anti-TGF-β treatment may improve NK cell function in treating 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.

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