IL15 promotes growth and invasion of endometrial stromal cells and inhibits killing activity of NK cells in endometriosis

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Abstract

Endometriosis (EMS) is associated with an abnormal immune response to endometrial cells, which can facilitate the implantation and proliferation of ectopic endometrial tissues. It has been reported that human endometrial stromal cells (ESCs) express interleukin (IL)15. The aim of our study was to elucidate whether or not IL15 regulates the cross talk between ESCs and natural killer (NK) cells in the endometriotic milieu and, if so, how this regulation occurs. The ESC behaviors in vitro were verified by Cell Counting Kit-8 (CCK-8), Annexin/PI, and Matrigel invasion assays, respectively. To imitate the local immune microenvironment, the co-culture system between ESCs and NK cells was constructed. The effect of IL15 on NK cells in the co-culture unit was investigated by flow cytometry (FCM). In this study, we found that ectopic endometrium from patients with EMS highly expressed IL15. Rapamycin, an autophagy inducer, decreased the level of IL15 receptors (i.e. IL15Rα and IL2Rβ). IL15 inhibits apoptosis and promotes the invasiveness, viability, and proliferation of ESCs. Meanwhile, a co-culture with ESCs led to a decrease in CD16 on NK cells. In the co-culture system, IL15 treatment downregulated the levels of Granzyme B and IFN-γ in CD16+ NK cells, NKG2D in CD56dimCD16− NK cells, and NKP44 in CD56brightCD16− NK cells. On the one hand, these results indicated that IL15 derived from ESCs directly stimulates the growth and invasion of ESCs. On the other hand, IL15 may help the immune escape of ESCs by suppressing the cytotoxic activity of NK cells in the ectopic milieu, thereby facilitating the progression of EMS.

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Introduction

Endometriosis (EMS) is one of the common estrogen-dependent inflammatory diseases, which affects 10–15% of women of reproductive age (Rizner 2009). The following is the main pathological process of EMS: endometrial cells grow outside the uterine cavity, causing pelvic pain, dyspareunia, and infertility. In recent years, researchers pay more attention to the immunological mechanisms of EMS (Shi et al. 2013). The dysfunction of the immune cells in the microenvironment of the abdominal cavity improves the adhesive and invasive ability and enhances the proliferation and angiogenesis of the endometrial cells. Therefore, it not only helps the immune escape but also promotes the growth of ectopic lesions within the abdominal cavity (Javierre et al. 2011, Veillat et al. 2012, Shi et al. 2013, Li et al. 2014).

Natural killer (NK) cells are closely related with the development of EMS. It has been proven that the number of peripheral blood and abdominal NK cells in EMS patients has no significant difference (Oosterlynck et al. 1994, Matsuoka et al. 2005). However, the activity visibly weakens, and the impaired degree of NK cell activity positively correlates with EMS development. The deficiency of NK cell function will activate endometrial cells failed to be cleared and settled in the abdominal cavity.

IL15 is reported to be produced by a wide variety of cells and tissues, including epithelial cell lines, monocytes, macrophages, and decidual and endometrial tissues (Kitaya et al. 2000, Okada et al. 2000a, Dunn et al. 2002, Ohteki 2002). As IL2 and IL15 share the same heterodimeric transducing receptor made of IL2/15Rβ (CD122) and CD132, IL15 also belongs to the IL2 cytokine family (Fehniger & Caligiuri 2001). IL15 is identified as a pleiotropic cytokine that plays important roles in enhancing the production of Th1-predominant pro-inflammatory...
cytokines (Strengell et al. 2002), promoting the proliferation of T cells and NK cells (Fehniger et al. 2002), and regulating the differentiation, development, and killing activity of NK cell (Barreira et al. 2011, Yang et al. 2015).

It has been reported that the IL15 level in peritoneal fluid (PF) and ectopic endometrium from women with EMS was increased (Arici et al. 2003, Chegini et al. 2003). Differentially, the study by Lin and coworkers indicates that IL15 was decreased in PF in the advanced stage of EMS (Lin et al. 2006). However, it is still unclear whether or not IL15 participates in the regulation of EMS pathogenesis by strengthening the interaction between ESCs and NK cells. Therefore, this study was performed to investigate the expression of IL15 and its receptors in EMS, and to further explore the role of IL15 in the cross-talk between ESCs and NK cells in vitro.

Materials and methods

Subjects and sample collection

The 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 (21–46 years), attending the Obstetrics and Gynecology Hospital of Fudan University between January 2015 and April 2016. None of the patients took any medications or received hormonal therapy within 6 months before surgery, and none of the patients had experienced any complications related to pelvic inflammatory disease. EMS (n = 37) was diagnosed by laparoscopic examination and pathological findings. Of these 37 patients with EMS, 10 had early stage (stage I + II) and 27 had advanced stage (stage III + IV). In the vast majority of cases, the primary location of EMS was in the ovaries (n = 29), followed by the Pouch of Douglas (n = 6) and the lateral pelvic wall (n = 2). The endometrial tissues were obtained from patients with leiomyoma by a laparoscopic hysterectomy (age 27–48 years, n = 18) and were used as controls. All of the samples were collected only in the proliferative phase of the cycle.

Among these, 12 cases of control endometrium tissues and 12 cases of ectopic endometrium tissues were collected for immunohistochemistry (IHC) analysis. Moreover, all other control and ectopic endometrium samples were obtained from the patients during surgery under sterile conditions and were transported to the laboratory on ice in DMEM (Dulbecco's modified Eagle's medium)/F-12 (Gibco) for isolating and culturing ESCs and for using for in vitro assays.

Peripheral blood samples from 12 healthy volunteers were taken sterilely in heparinized Hank's buffer solution (Gibco). The samples were immediately transported to a laboratory on ice for bead sorting of the NK cells.

IHC analysis for detecting IL15 expression in endometrium

Paraffin sections (5 μm) of normal (n = 12) and ectopic (n = 12) endometrium tissues were dehydrated in graded ethanol.

After blocking the endogenous peroxidase activity by 3% H2O2 for 15 min, samples were incubated with a goat human IL15 antibody (25 μg/mL; R&D Systems) or a goat IgG isotype in a humid chamber overnight at 4°C. All sections were washed three times with phosphate-buffered saline (PBS) and then overlaid with peroxidase-conjugated anti-goat IgG (Golden Bridge International, Inc, Beijing, China). The color reaction was developed with 3,3-diaminobenzidine (DAB) and then counterstained with hematoxylin. The results were observed using an Olympus BX51+DP70 microscope (Olympus).

Cell culture

We purified and cultured the ESCs from the control and ectopic endometrium tissues as described previously (Mei et al. 2012). The endometriotic tissues from the patients were minced into 2 mm pieces and incubated in DMEM/F12 containing collagenase type IV (0.1%; Sigma) and deoxyribonuclease type I (DNase I; 3000 U; Sigma) with constant agitation for 70 min at 37°C. The resulting dispersion was filtered in turn through 100 and 200 μm nylon strainers. The filtrate was then centrifuged at 800 g for 15 min to further remove the leukocytes and erythrocytes and was washed with PBS. The ESCs were resuspended in DMEM/F-12 containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), plated on culture flasks, and incubated at 37°C in 5% CO2. The culture medium was replaced every 2–3 days. The purity of the Vimentin+ ESCs was >98%.

Enzyme-linked immunosorbent assay (ELISA) for determination of IL15

In order to evaluate the secretion level of IL15, the primary ESCs (2 × 10^5 cells/well) from the control (n = 6) or ectopic (n = 6) endometrium tissue were seeded in 24-well flat-bottom plates and cultured for 48 h, and then the culture supernatant was harvested, centrifuged to remove cellular debris and stored at −80°C until being assayed by ELISA for determination of the IL15 (R&D Systems).

Isolation of human NK cells

The peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood samples by Ficoll-Hypaque density gradient centrifugation. The NK cells were obtained through negative selection by an NK cell isolation kit, according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The cell population purity was detected by FCM using a CD3-FITC (2.5 μL; BD Biosciences, San Diego, CA, USA) and CD56-PE (2.5 μL; BD Biosciences, San Diego, CA, USA) and was found to be >90%.

Treatment with rapamycin

The ESCs (2 × 10^5 cells/well) during the logarithmic phase were gently placed in 24-well flat-bottom plates and were treated with rapamycin (1 μM, R&D Systems) for 48 h. Then, FCM was applied to evaluate the IL15 receptor level on the ESCs.
BrdU cell proliferation and Annexin/PI apoptosis assays

The ESCs were seeded at a density of $5 \times 10^5$ cells/well in 96-well flat-bottom plates (for proliferation assay) or $2 \times 10^5$ cells/well in 24-well flat-bottom plates (for apoptosis assay). Before treatment, the ESCs were starved for 12 h, with DMEM/F12 that included 1% BSA as a culture medium. Then, the cells were stimulated by recombinant human IL15 (rhIL15, 0.1, 1, or $10 \text{ ng/mL}$) or anti-IL15 neutralizing antibody ($\alpha$-IL15, 0.05 or 0.5 $\mu$g/mL) for 48 h. In addition, a vehicle was added to some wells as the negative control. All of those reagents were purchased from R&D Systems. Then, BrdU cell proliferation (Millipore) and annexin V-FITC apoptosis assays (Invitrogen) were used to evaluate the proliferation and apoptosis ability of the ESCs according to each manufacturer’s instructions, respectively. Each experiment was performed in triplicate, and repeated six times.

Matrigel invasion assay

The transwell plates (24-well, pore size 8 $\mu$m) for invasion assay were purchased from Becton Dickinson Discovery Labware. First, the inner bottoms of the upper chambers were coated with a 15$\mu$L/well matrigel solution (BD Biosciences, San Diego, CA, USA). Then, the plates were placed in a humid 37°C incubator for matrigel solidification. Four hours later, a DMEM/F12-conditioned medium (600 $\mu$L/well) with rhIL15 (10 $\text{ ng/mL}$) or $\alpha$-IL15 (0.5 $\mu$g/mL) was added into the lower chambers of the plates. At the same time, the ESCs ($2 \times 10^5$ cells, 200 $\mu$L medium) were seeded directly into the upper chambers. The plates were incubated for 48 h at 37°C in a 5% CO$_2$ incubator, so the cells could migrate through the matrigel membrane. After incubation, the cells in the upper chambers were removed using cotton-tipped swabs, while the other cells that invaded through the matrigel membrane and reached the outer surface of the upper chambers were carefully handled. We fixed the cells with 10% formalin for 10 min and then stained them with hematoxylin for 15 min. After rinsing the upper chambers twice with distilled water, the results were observed under an Olympus BX51+DP70 microscope (Olympus). We counted the number of cells that had passed through the membrane and clung to the bottom side of the upper chambers. Each experiment was performed in triplicate and repeated four times.

Co-culture system of ESCs and NK cells

The ESCs were cultured in 24-well plates (Corning) at a density of $2 \times 10^5$ cells/well. The NK cells were subsequently added to the wells directly at the same density as the ESCs. Meanwhile, the NK cells of $2 \times 10^5$ cells/well in 24-well flat-bottom plates (for apoptosis assay) were observed under an Olympus BX51 microscope. We counted the number of cells that had passed through the membrane twice with distilled water, the results were observed under an Olympus BX51+DP70 microscope (Olympus). We counted the number of cells that had passed through the membrane and clung to the bottom side of the upper chambers. Each experiment was performed in triplicate, and repeated four times.

FCM analysis

The ESCs stimulated with or without rapamycin were washed with PBS, and then mixed with a mouse anti-human IL15$\alpha$-APC-conjugated antibody (BD Biosciences, San Diego, CA, USA) and IL2$\beta$-PE-conjugated antibody (Biolegend, San Diego, CA, USA). Isotypic control antibodies were used. After incubation in the dark for 30 min at room temperature, the cells were analyzed immediately by FCM (FACS Calibur, BD Biosciences, San Diego, CA, USA).

After a co-culture with the ESCs for 5 days, the expression of KIR2DL1, KIR3DL1, NKG2D, NKP46, NKP44, NKP30, Granzyme B, Perforin, and IFN-$\gamma$ (all antibodies were from Biolegend) in the NK cells was analyzed by FCM.

Statistical analysis

The results were representative of multiple experiments and are presented as mean $\pm$ S.E.M. The variables were analyzed by a t-test or one-way ANOVA by Graphpad Prism 5 software. The differences were considered to be statistically significant if $P < 0.05$. 

Figure 1 ESCs in ectopic endometrium from women with EMS highly express IL15. (A) IHC analysis for IL15 expression in normal endometrium ($n=12$) and ectopic endometrium ($n=12$). Original magnification: $\times 400$. Blank arrow: glandular epithelial cells; Red arrow: stromal cells. (B) The average area of the IL15-positive sites from 10 randomly chosen views per sample was counted at a magnification of $\times 400$ using Image-Pro Plus image analysis software. The data are expressed as whiskers: Min to Max. Normal E: normal endometrium; Ectopic E: Ectopic endometrium. (C) The secretion level of IL15 in the supernatants from normal ESCs ($n=6$) and ectopic ESCs ($n=6$) by ELISA. The data are presented as the mean $\pm$ S.E.M. $**P < 0.001$ compared with the normal group (student t-test).
Results

**ESCs in ectopic endometrium from women with EMS highly express IL15**

To determine the expression and localization of the IL15 in ESCs, we performed an IHC analysis on the paraffin-embedded endometrium with or without EMS. An abundant expression of IL15 was observed in the ectopic endometrium. As depicted in Fig. 1, compared with the normal endometrium, the ectopic endometrium from EMS exhibited strong staining for IL15 (Fig. 1A and B, \( P < 0.001 \)). It was located mainly in the stromal cells, not the glandular epithelial cells, which is in contrast to the previous report (Chegini et al. 2003). The cause for the difference may be due to the different phases of the specimens. The results of ELISA also showed that the secretion level of IL15 from the ectopic ESCs was significantly higher than that from the normal ESCs (Fig. 1C, \( P < 0.001 \)). These data suggested that a high level of IL15 from the ectopic endometrium might play a regulatory role in the biological behavior of the ESCs.

**ESC autophagy induced by rapamycin downregulates IL15 receptors levels**

Autophagy is one of the main mechanisms for maintaining cellular homeostasis. Our previous work showed that estrogen repressed the autophagy of the ESCs by upregulating CXCL12/CXCR4 signaling, and further promoted ESC growth (Mei et al. 2015). In order to analyze the expression of IL15 receptors (i.e., IL15RA and IL2RB) on the ESCs and the effect of autophagy on the IL15 receptors in the ESCs, we treated the primary ESCs from the ectopic lesions with rapamycin (an autophagy inducer) and then detected the expression of the IL15 receptors. As shown, the purity of the primary vimentin*ESCs was more than 98% (Fig. 2A). The ratio of the IL15RA*ESCs and IL2RB*ESCs was about 60% and 40%, respectively (Fig. 2B, C, D and E). Rapamycin (1 μM) significantly decreased the level of IL15RA (Fig. 2B and C, \( P < 0.001 \)) as well as IL2RB (Fig. 2D and E, \( P < 0.01 \)), which was expressed by the ESCs when compared with the control group. These results suggest that downregulation of the ESC autophagy in EMS may promote the reactivity of ESCs to the IL15 by increasing the expression of the IL15 receptors.

**IL15 promotes growth and invasion of ESCs in an autocrine manner**

To explore whether or not IL15 impacts the biological behaviors of ESCs, we performed BrdU proliferation, apoptosis, and matrigel invasion assays to evaluate the effect of IL15 on the proliferation, apoptotic ability,
and invasiveness of the ESCs, respectively. Our results showed that blocking IL15 with α-IL15 at 0.5 μg/mL decreased ESCs’ proliferation (Fig. 3A, *P < 0.05). By contrast, the IL15 protein at a dose of 10 ng/mL stimulated ESCs’ proliferation (Fig. 3B, *P < 0.05), but restricted ESCs’ apoptosis (Fig. 3C and D, *P < 0.05). Moreover, rhIL15 significantly enhanced the invasiveness of the ESCs (Fig. 3E and F, *P < 0.01). Overall, these data suggest that IL15 is an important regulator for the growth and invasiveness of the ESCs.

**ESCs trigger differentiation of CD16+ NK cells**

Nearly 90% of the peripheral blood NK cell phenotype is CD56+CD16−; the other 10% of NK cells are CD56+CD16+ (Cooper et al. 2001). CD16 has been identified as the Fc receptors FcγRIIIa (CD16a) and FcγRIIIb (CD16b). These receptors bind to the Fc portion of IgG antibodies, which then activate the NK cells for antibody-dependent cell-mediated cytotoxicity. In order to investigate whether or not ESCs are involved in regulating the CD16 expression, we isolated the CD3−CD56−NK cells from the PBMCs (Fig. 4A) and co-cultured these cells with the ESCs. As shown, compared with the NK cells only group, the CD16+CD56− NK cells were significantly decreased in the co-culture group (Fig. 4B and C, *P < 0.001). Moreover, we calculated and compared the ratio of the CD16+ NK cells to the CD16− NK cells between the NK cells alone and the co-culture groups. We found similar significant differences (Fig. 4B and C, *P < 0.001). The difference was that the co-culture group with the ESCs led to an obvious decrease in CD16+ NK cells. These results suggest that the ESCs from ectopic lesions may restrict the activity of the NK cells by suppressing CD16+ NK cell differentiation within the abdominal cavity.

**IL15 derived from ESCs downregulates killing activation of NK cells**

Finally, to define the relationship between the IL15 expression of the ESCs and the function of the NK cells, we cultured NK cells from the peripheral blood from healthy women with ESCs, and treated them with or without α-IL15 or rhIL15 for 5 days. Although the ESCs promoted the differentiation of the NK cells to the CD16+ NK cells, treatment with α-IL15 or rhIL15 had no significant influence on the CD16+ CD56− NK cells (Fig. 5A and B, *P > 0.05), suggesting that IL15 is not involved in regulating the CD16 levels by ESCs.

Further analysis showed that rhIL15 decreased Granzyme B (Fig. 5C and D, *P < 0.01) and IFN-γ (Fig. 5C and F, *P < 0.001), but not perforin (Fig. 5C and E, *P > 0.05), in the CD16+ NK cells of the co-culture system. In addition, IL15 stimulation resulted in decreases of NKG2D in CD56dimCD16− NK (Fig. 6A and B, *P < 0.01) and Nkp44 in CD56brightCD16− NK (Fig. 6E and F, *P < 0.01) cells. However, the expression of KIR2DL1 (Fig. 6C, *P > 0.05), KIR3DL1 (Fig. 6D, *P > 0.05), Nkp46 (Fig. 6G, *P > 0.05), and Nkp30 (Fig. 6H, *P > 0.05) on the NK cells did not change when exposed to IL15. Collectively, these data indicate that the abnormally high IL15 expression decreases the killing activity of the NK cells by downregulating Granzyme B, IFN-γ, activating receptor NKG2D and natural cytotoxicity receptor Nkp44 expression.
and may further contribute to the immune escape of the ESCs within the peritoneal cavity, finally promoting the progress of EMS.

**Discussion**

EMS results from increased cellular proliferation, adhesion, and invasion of the retrograde endometrium in response to appropriate stimuli. The etiology of EMS remains an enigma, and the incidence of EMS shows an obvious increase in recent years. While Sampson's theory of retrograde menstruation is widely accepted (Sampson 1925), the immune theory is still recognized as explaining the causes of women with EMS. Accumulating evidence suggests that immune escape plays a key role in developing and spreading endometriotic foci.

In this regard, immunosuppressive peritoneal fluid mononuclear cells (i.e., regulatory T cells, macrophages, and NK cells) and related cytokine patterns may address the response against the foci. The breakdown of peritoneal homeostasis may allow the escaping from immune surveillance of the endometriotic cells, which can implant and proliferate to avoid the apoptotic pathways (Sturlese et al. 2011, Salmeri et al. 2015).

Herein, we found that cytokine IL15 was abnormally highly expressed in EMS compared with the normal endometrium. The ectopic endometrium from EMS exhibited strong staining for IL15. In normal human endometrial cells, the production of IL15 is regulated by ovarian steroid hormones, suggesting an important role that IL15 plays in human reproductive physiology (Okada et al. 2000). In the current study, the ESCs from ectopic lesions expressed IL15Rα and IL2Rβ, indicating that highly expressed IL15 may influence the biological behaviors of ESCs in an autocrine manner. Then, we designed and conducted a series of experiments. The next finding of the present study is that IL15 can regulate the proliferation, apoptosis, and invasion abilities of ESCs in vitro.

It has been reported that the induction of autophagy exerts a proapoptotic effect on normal human endometrial cells (Choi et al. 2012). Compared with normal endometria, a remarkable activation of the autophagic process was observed in ectopic endometrial lesions (Nasu et al. 2011). It is also evident that transcriptional induction of autophagy-related genes (i.e., LC3B, ATG14, BECN1, and ATG7) and coding for proteins are involved in different steps of the autophagic pathway. A decrease of autophagic activity in ectopic and eutopic endometrial cells leads to less autophagy-dependent degradation of proteins and less programmed cell death. Our previous work also established that autophagy suppression induced by CXCL12 promotes growth of ESCs in vitro (Mei et al. 2015). Mediated via the lysosomal degradation pathway, autophagy is responsible for degrading cellular proteins and is currently the only known process for degrading cellular organelles, recycling them to ensure cell survival (Reggiori & Klionsky 2002). We speculated that the autophagic level of ESCs may be involved in regulating the expression of the IL15 receptors in ESCs. Rapamycin is a specific mTOR inhibitor with an IC50 of ~0.1 nM (Kim & Guan 2015). It specifically binds to immunophilin, FK-binding protein-12 (FKBP12) to form a complex that directly binds to the FRB domain of mTOR, thus inhibiting its activity. The result of our present study showed that rapamycin significantly inhibited the expression of the IL15 receptors as an autophagy revulsant, implicating that a decrease in the ESC autophagic level promotes the expression of IL15 receptors, increases the sensitivity of ESCs to IL15, and improves the stimulatory effect of IL15 on the growth and invasion of ESCs.
As IL2 and IL15 share the same receptor components (i.e. IL2R/IL15Rβ and γc) and use of common JAKs (i.e. JAK1 and JAK3) and STATs (i.e. STAT3 and STAT5) signaling molecules, the two cytokines share several functions (Pelletier et al. 2002, Mishra et al. 2014), including the stimulation of the proliferation of activated CD4−CD8−, CD4+CD8+, CD4+ T cells (Fehniger et al. 2002, Waldmann 2006, Steel et al. 2012, Johnston et al. 1995). They also stimulate the generation, proliferation, and activation of NK cells (Cooper et al. 2002). However, IL15-deficient mice have a marked reduction in the number of peripheral NK, NKT, γδT, and intestinal intraepithelial lymphocytes. According to the important role of the STAT3 signal in EMS (Okamoto et al. 2015), it can be speculated that the STAT3 signal may be involved in the regulatory effect of IL15 on the biological behaviors of ESCs, which needs further research.

NK cells, which comprise ~15% of all circulating lymphocytes, play an important in the innate immune response as effector cells, able to exert a prompt cytolytic activity against malignant or infected cells without prior sensitization. NK cell populations can be distinguished by the cell surface density of the neural cell adhesion molecule CD56. It can be divided into two subsets called CD56bright and CD56dim NK cells. Although the subsets of NK cells have been documented as separate subpopulations, it has been postulated that CD56bright NK cells are the immediate precursors of CD56dim NK cells (Poli et al. 2009). The expression levels of CD56 appear to correlate with NK cell function: CD56dim NK cells mainly exist in the peripheral blood, highly expressing KIR and Fc-γ receptor III (CD16), and are more naturally cytotoxic. By contrast, CD56bright NK cells are potent producers of cytokines, particularly IFN-γ and TNF-α, following the activation by monocytes, but have a low natural cytotoxicity and low or absent levels of the Fc-γ receptor CD16. By contrast, the NK cells in the secondary lymphoid tissues, as well as the

Figure 5 IL15 downregulates the production of Granzyme B and IFN-γ of NK cells. (A, B, C, D, E and F) The ESCs were co-cultured with the CD3−CD56+ NK cells, and were treated with or without α-IL15 (0.5 μg/mL) or rhIL15 (10 ng/mL) for 5 days. Then, the expression of CD16, Granzyme B, IFN-γ, and perforin in the NK cells was analyzed by FCM. The data are presented as the mean ± s.e.m, NS, no significant difference. **P<0.01 and ***P<0.001 compared with the control group (student t-test or one-way ANOVA).
human endometrium, are phenotypically distinct and are CD56<sup>bright</sup> (King 2000, Freud et al. 2014). These are weakly cytotoxic but important immune regulators. The phenotypes and functions of the NK cells in the peritoneum and endometrium could contribute to the pathology of EMS (Oosterlynck et al. 1992, Ho et al. 1995). In the current study, after co-culture with ESCs, the ratio of CD16<sup>−</sup>CD56<sup>+</sup> NK cells was significantly decreased. These data suggest that ESCs can trigger CD16<sup>+</sup>CD56<sup>+</sup> NK cell differentiation and may participate in the induction and maintenance of phenotypes and functions of the NK cells in the endometriotic milieu.

The functions of NK cells are under the control of signals initiated by the engagement of various combinations of activatory or inhibitory cell surface molecules making up three main mechanisms of NK activation. The missing-self hypothesis proposes that cells that are either transformed or abnormal to the body, such as tumor cells or infected cells, do not express MHC class 1 molecules on their cell surface and are identified as “non-self” by NK cells. Killing mechanisms, including the release of cytotoxic granule components such as perforin, are then activated. Alternatively, ‘induced self’ describes the cells that express high levels of stress-induced activation ligands (i.e. HLA-E ligands), which activate the NK cell killing activity. Moreover, NK cells also respond to effector molecules by releasing cytotoxic mediators (i.e. perforin and Granzyme B) or cytokines (i.e. IFN-γ).

The nature and extent of NK cell activity are, therefore, determined by the target cell, NK cell subtype, and local environment. It has been reported that an increase in the inhibitory killer immunoglobulin-like receptor (KIR) expression on NK cells in women with EMS (Maeda et al. 2002, Zhang et al. 2006, Kitawaki et al. 2007). The relationship between activating receptors of NK cells and EMS is rarely reported. Natural cytotoxicity receptors (NCRs) (i.e. Nkp46, Nkp44, and Nkp30) are known activating receptors of NK cells in female patients with recurrent pregnancy loss (RPL), implantation failure, and pre-eclampsia (Fukui et al. 2009, Fukui et al. 2011). NCRs and NKG2D are main receptors for the activation of NK cells. The percentage of cells expressing KIR2DL1 among NK cells in the peritoneal fluid and peripheral blood was significantly higher in women with EMS than in normal women, suggesting that KIR2DL1 plays a role in the suppression of NK cells with EMS (Maeda et al. 2002). In this study, we found that IL15 led to decreases in Granzyme B and IFN-γ, but it did not lead to a decrease in perforin in CD16<sup>−</sup>NK cells, activating receptor NKG2D in CD56<sup>dim</sup>CD16<sup>−</sup> NK and cytotoxicity receptor Nkp44 in CD56<sup>bright</sup>CD16<sup>−</sup> NK in the co-culture system. However,
stimulation with IL15 had no effect in the expression of KIR2DL1, KIR3DL1, NKp46, and NKp30 on NK cells. Therefore, high levels of IL15 in the ectopic endometrium may suppress cytotoxic activities. However, the exact mechanism for this process is still unclear.

Collectively, as shown in Fig. 7, the present study tells us that the abnormally high levels of IL15 from the ectopic endometrium, on one hand, directly stimulates the proliferation and invasion, and restricts apoptosis in ESCs in an autocrine manner, and, on the other hand, decreases the killing activity of the NK cells by downregulating Granzyme B, IFN-γ, activating receptor NKG2D, and cytotoxicity receptor NKp44 expression in a paracrine manner, and may further contribute to the immune escape of ESCs, finally promoting the ectopic growth and implantation of ESCs within the peritoneal cavity and accelerating the development of EMS. Moreover, the downregulation of the ESC’s autophagy level may amplify the role of IL15 in the dialogue between the ESCs and NK cells in 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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