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Original Article

IL-22 in the endometriotic milieu promotes the proliferation of endometrial stromal cells via stimulating the secretion of CCL2 and IL-8

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Abstract: Interleukin-22 (IL-22) is a member of the IL-10 cytokine family and plays critical roles in inflammation, immune surveillance, and tissue homeostasis. However, whether IL-22 regulates the growth of endometrial stromal cells (ESCs), and participates in the pathogenesis of endometriosis remain unclear. In this study, we found that the expression of IL-22 and it receptors (IL-22R1 and IL-10R2) in eutopic endometrium and ectopic lesion of women with endometriosis was higher than that from healthy control. Recombinant human IL-22 (rhIL-22) stimulated the proliferation of ESCs in a dosage-dependent manner. On the contrary, anti-human IL-22 neutralizing antibody inhibited the proliferation of ESCs *in vitro*. The stimulatory effect of IL-22 on the proliferation of ESCs could be reversed by inhibitor of STAT5, ERK1/2 or AKT signal pathway. However, blocking STAT3, JNK or P38 signal pathway had no these effects. By Enzyme-linked immunosorbent assay (ELISA) and flow cytometry assay, we demonstrated the rhIL-22 not only stimulate the secretion of CCL2 and IL-8, but also significantly up-regulate the expression of IL-8 receptor CXCR1 on ESCs. Meanwhile, STAT5, ERK1/2 and or AKT signal inhibitors could abrogate the increase of CCL2, IL-8 and CXCR1 levels induced by rhIL-22. However, rhIL-22 had not similar influence on CCL2 receptor CCR2. Our current results suggested that the higher level of IL-22 and it receptors in eutopic endometrium may stimulate the expression of CCL2, IL-8/CXCR1, and further promote the growth of ESCs possibly through activating STAT5, MAPK/ERK1/2 and or AKT signal pathways, which may be involved in the occurrence and development of endometriosis.

Keywords: IL-22, endometrial stromal cells, proliferation, CCL2, IL-8, endometriosis

Introduction

Endometriosis is classically defined as the presence of endometrial glands and stroma implants outside the uterine cavity, primarily the pelvic peritoneum, ovaries, and rectovaginal septum. It affects 10-15% of women of reproductive age and is still the most enigmatic gynecological disease [1]. The stigmata of endometriosis include dysmenorrheal, dyspareunia, chronic pelvic pain, irregular uterine bleeding, and/or infertility [2, 3]. However, the pathogenesis of endometriosis still remains controversial despite extensive research. More and more evidences suggest that the primary defect in endometriosis can be located in the eutopic endometrium. Abnormalities inherent to the eutopic endometrium that are not found in the endometrium of women without endometriosis might therefore contribute to ectopic growth outside the uterine cavity [4, 5]. Different characteristics of eutopic endometrium of women with endometriosis, such as aberrant production of cytokine, growth, adhesion and angiogenic factors as well as specific cancer-related molecules, are believed to contribute to the occurrence and maintenance of this disease.

Interleukin-22 (IL-22) is a member of the IL-10 family of cytokines along with IL-10, IL-19, IL-24, IL-26, IL-28 (α and β), and IL-29 [6, 7]. It is pro-

duced by $CD4^+$ Th17 cells, NK cells, $CD11c^+$ myeloid cells, and lymphoid tissue inducer-like cells [8-11]. The IL-22 receptor is composed of the IL-22R1 and IL-10R2 subunits. The IL-22 receptor is found on cells of nonhematopoietic origin in the skin, kidney, liveAr, lung, and gut, allowing for IL-22-mediated regulation of local epithelial, endothelial, and stromal cell responses after infection or exposure to inflammatory stimuli [12, 13]. These functions, including up-regulation of anti-apoptotic pathways and increased proliferation, are protective in acute inflammatory conditions and in tissue injury [14, 15]. Recently, a growing body of evidences indicated the IL-22 in tumor microenvironment stimulates the growth of cancer cells [16, 17].

Endometriosis is also regarded as an autoimmune disease, because of its chronic inflammatory process, as well as malignancies, for its capacity of infiltrative and destructive growth [18, 19]. However, under a large number of pro-inflammatory cytokines in the endometriotic milieu, such as, IL-23 [20], IL-6 [21], IL-1β [22], TNF-α [22], whether there was high level IL-22 still unclear. Therefore, the aim of this study is to explore the expression and the function of IL-22 in the endometriotic milieu and its relevance to endometriosis.

Materials and methods

Tissue collection

All tissue samples were obtained with informed consent in accordance with the requirements of the Research Ethics Committee in Yixing Second People's Hospital and Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College. Samples of the endometriotic ovarian lesion (n = 12) were obtained from women age 28-45 years undergoing laparoscopy for pain or other benign indications. The endometrial tissues were obtained from fertile women (age 25-44 years) with (n = 26) or without (n = 12) endometriosis as control. None of the women had received hormonal medication in the 3 months prior to the surgical procedure. All the samples were confirmed histologically according to established criteria.

Cell isolation and culture

The eutopic endometrial tissue from women with endometriosis were collected under sterile conditions and transported to the laboratory on ice in DMEM (Dulbecco's modified Eagle's medium)/F-12 (Gibco, USA). The ESCs were isolated according to the previous methods [23-25].

Immunohistochemistry

The IL-22, IL-22R1 and IL-10R2 protein levels in the endometriotic lesions (n = 12) and eutopic endometrial tissues from women with (n = 12) or without (n = 12) endometriosis in the proliferative or secretory phase of the cycle were dehydrated in graded ethanol and incubated with hydrogen peroxide in 1% bovine serum albumin in Tris-buffered saline (TBS) to block endogenous peroxidase. The samples were then incubated with mouse anti-human IL-22 monoclonal antibody (25 ug/ml, R&D system, Inc., MN, USA), mouse anti-human IL-22R1 (15 ug/ml, R&D system), mouse anti-human IL-10R2 (25 ug/ml, R&D system) or mouse IgG isotype antibody overnight at 4°C in a humid chamber. After washing three times with TBS, the sections were overlaid with peroxidase-conjugated goat anti-mouse IgG antibody (SP-9002; Golden Bridge International, Inc., Beijing, China) and the reaction was developed with 3,3'-diaminobenzidine (DAB); the sections counterstained with hematoxylin. The experiments were repeated five times.

BrdU cell proliferation assay

The BrdU cell proliferation assay was utilized to evaluate the effects of IL-22 on ESCs proliferation. ESCs (n = 6) were re-suspended in DMEM/F-12 supplemented with 10% FBS, and seeded at a density of 1×10^4 cells/well in 96-well flat-bottom microplates. Thereafter, the cells were starved with DMEM containing 1% FBS for 12 h before treatment, and then were stimulated with recombinant human (rhIL-22) protein (0, 1, 10, or 100 ng/ml, R&D system), anti-IL-22 neutralizing antibody (0, 0.05, 0.5 or 5 ug/ml, R&D system) for 48 h. Vehicle was added to some wells as negative control.

Moreover, in order to analyze whether the effect of IL-22 on proliferation is dependent on downstream signals and molecules, we treated ESCs (n = 6) with or without rhIL-22 (10 ng/ml), WP1066 (STAT3 inhibitor, 10 uM, santa cruz biotechnology, inc., USA), N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (STAT5 inhibitor, 20 uM, santa cruz biotechnology), U0126 (MAPK/ERK1/2 inhibitor, 30 uM,

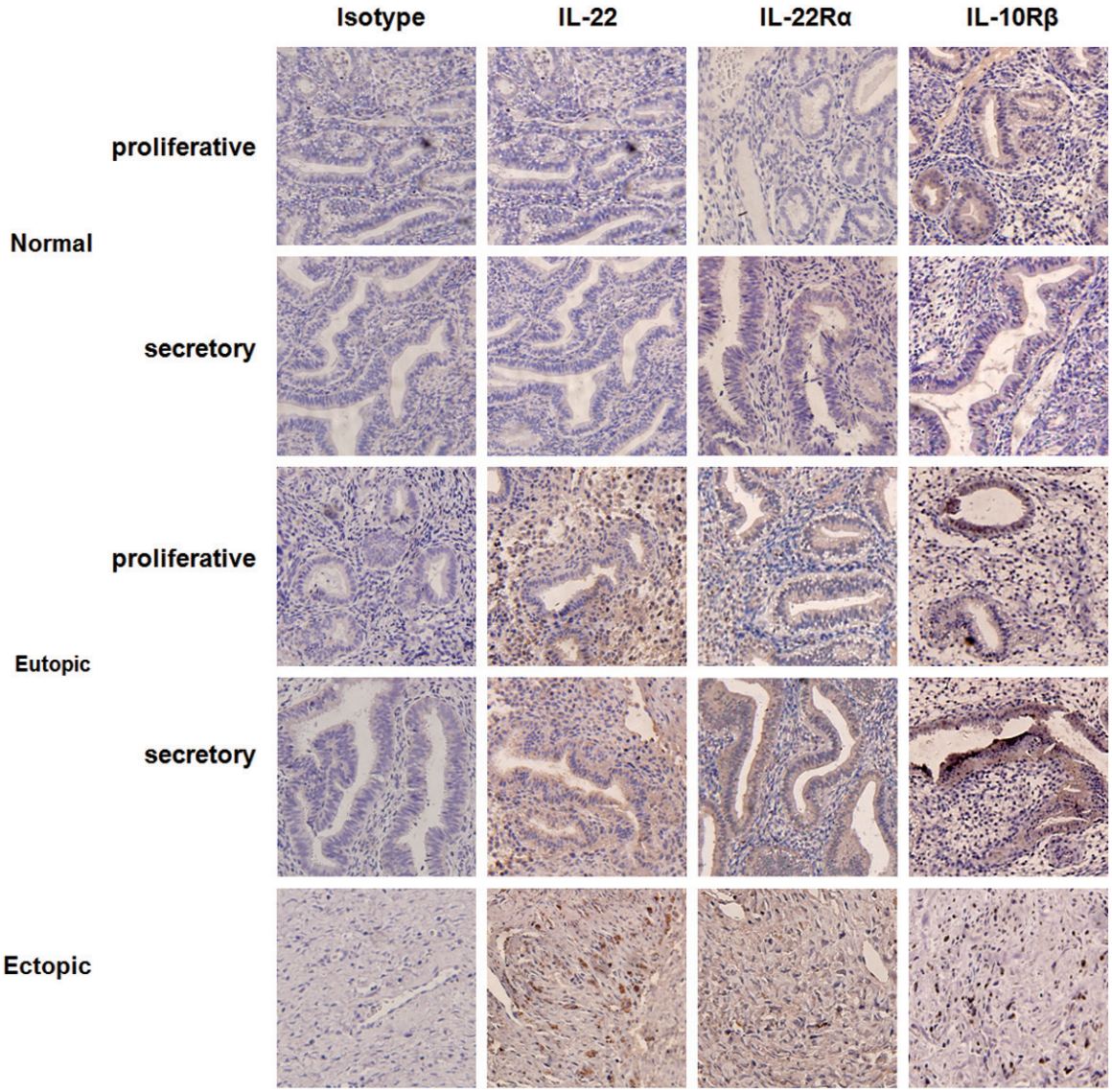

Figure 1. *The expression of IL-22 and its receptors is increased in the eutopic endometrium and ectopic lesion from women with endometriosis.* The expression of IL-22 and it receptors (IL-22R1 and IL-10R2) in the endometrium from women of healthy control (n = 12), eutopic endometrium (n = 12) and lesion (n = 12) from women with endometriosis was analyzed by *immunohistochemistry.* Original magnification: × 200. Here normal: normal endometrium from women of healthy control; eutopic: eutopic endometrium from women with endometriosis; ectopic: ectopic lesion from women with endometriosis.

cell signal technology, USA), SP600125 (JNK inhibitor, 10 uM, cell signal technology), SB203508 (P38 inhibitor, 10 uM, cell signal technology) and or LY294002 (AKT signal pathway, 50 uM, cell signal technology) for 48 h, with vehicle as control.

The ability of ESCs to proliferate was detected with BrdU cell proliferation assay (Millipore, USA) according to the manufacturer's instruction. Each experiment was performed in triplicate, and repeated four times.

ELISA

The ESCs of eutopic endometrium from women with endometriosis were treated with rhIL-22 (10 ng/ml), N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (20 uM), U0126 (30 uM) and or LY294002 (50 uM) for 48 h, with vehicle as control. Then the secretion of CCL2 and IL-8 by the supernatant of ESCs were detected by ELISA (Shanghai ExCell Biology, Inc, Shanghai, China), according to the manufacturer's instruction.

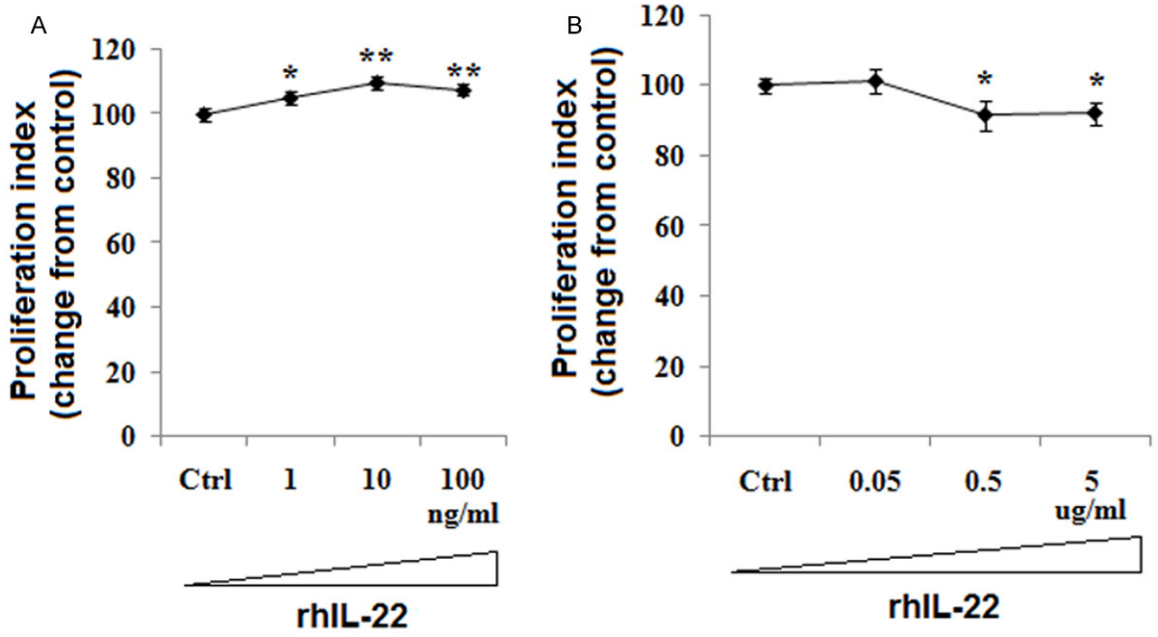

Figure 2. *IL-22 stimulates the proliferation of ESCs.* The primary ESCs of eutopic endometrium from women with endometriosis were incubated with recombinant human IL-22 (rhIL-22) (0, 1, 10 or 100 ng/ml) (A) and mouse anti-human IL-22 neutralizing antibody (0, 0.05, 0.5 or 5 ug/ml) (B) for 48 h, then the BrdU proliferation assay was used to detect the proliferation of ESCs. Results were highly reproducible in three independent experiments. Data are mean ± SD. $^{*}P < 0.05$ or $^{**}P < 0.01$ compared to the vehicle control.

Flow cytometry

The ESCs of eutopic endometrium from women with endometriosis were incubated with rhIL-22 (10 ng/ml), N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (20 uM), U0126 (30 uM) and or LY294002 (50 uM) for 48 h, with vehicle as control. Then digested with 0.25% trypsin only for 30-50 s, and blown off gently and washed with phosphate-buffered saline (PBS). After blocking with 10% FBS, the recovered cells were mixed with mouse anti-human CCR2-Percp5.5 monoclonal antibody and CXCR1-fluorescein isothiocyanate (FITC) monoclonal antibody (Biolegend, USA), meanwhile, the isotypic control was used. After incubation in darkness for 30 min at room temperature, the cells were analyzed immediately by a flow cytometer (FACS Calibur, BD). Statistical analysis was conducted by using isotype matched controls.

Statistics

All values were shown in the mean ± SD. Data were analyzed by using one-way analysis of variance and least significant difference (equal variances assumed), or Tamhane's test (equal variances not assumed) was used post hoc for multiple comparisons with Statistical Package for the Social Sciences software version 11.5. Differences were considered as statistically significant at $P < 0.05$.

Results

The expression of IL-22 and its receptors is increased in the eutopic endometrium and ectopic lesion from women with endometriosis

In order to explain the regulatory role of IL-22 in the biological behavior of ESCs, at first, we compared the expression of IL-22 and its receptors (IL-22R1 and IL-10R2) by immunohistochemistry. As shown in **Figure 1**, the expression of IL-22 in eutopic endometrium and ectopic lesion from women with endometriosis was higher than that of normal endometrium from healthy control, especially in the stromal cells. The positive staining of IL-22 was shown both in the secretory and proliferative periods. However, we had not detected the positive staining of IL-22 in all healthy endometrium samples. Moreover, relative to the stromal cells, glandular epithelium preferentially expressed IL-22R1 and IL-10R2. Compare to healthy control, IL-22R1 and IL-10R2 staining

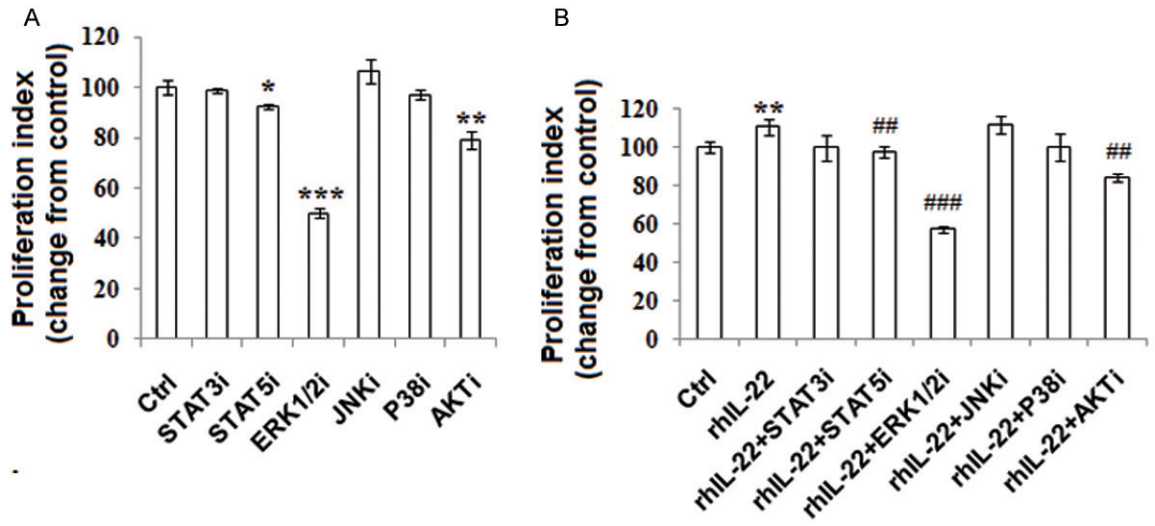

Figure 3. *rhIL-22 promotes the proliferation of ESCs through activating STAT5, ERK1/2 and AKT signal pathways.* A. We treated the primary ESCs of eutopic endometrium from women with endometriosis with STAT3 (WP1066, 10 uM), STAT5 (N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide, 20 uM), ERK1/2 (U0126, 30 uM), JNK (SP600125, 10 uM), P38 (SB203508, 10 uM) or AKT (LY294002, 50 uM) inhibitor for 48 h; B. We treated ESCs with rhIL-22 (10 ng/ml), STAT5 (20 uM), ERK1/2 (30 uM), and or AKT (50 uM) inhibitor for 48 h, and then BrdU proliferation assay was used to detect the proliferation of ESCs. Data are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ compared to the vehicle control. $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ compared to rhIL-22 treatment.

was stronger in eutopic endometrium and ectopic lesion from women with endometriosis. These results indicated that the abnormal levels of IL-22 and its receptor might be involved in the origin and development of endometriosis.

IL-22 stimulates the proliferation of ESCs

To further study the functions of IL-22 on the ESCs, we treated ESCs with rhIL-22 and anti-human IL-22 neutralizing antibody at the different concentration for 48 h, BrdU proliferation assay showed that rhIL-22 promoted the proliferation of ESC, especially at 10 ng/ml ($P < 0.01$) (**Figure 2A**). In contrast, blocking IL-22 with anti-human IL-22 neutralizing antibody from 0.5 to 5 ug/ml inhibited the proliferation of ESCs ($P < 0.05$) (**Figure 2B**). These data suggested that IL-22 in endometriotic milieu may stimulate the growth of ESCs in autocrine and paracrine manners.

rhIL-22 promotes the proliferation of ESCs through activating STAT5, ERK1/2 and AKT signal pathways

The AKT, MAPK and STATs signaling pathways are involved in regulation of cell growth. To further clarify the action of IL-22 on ESCs proliferation, we treated ESCs with WP1066 (STAT3 inhibitor, 10 uM), N′-((4-Oxo-4H-chromen-3-yl) methylene)nicotinohydrazide (STAT5 inhibitor, 20 uM), U0126 (MAPK/ERK1/2 inhibitor, 30 uM), SP600125 (JNK inhibitor, 10 uM), SB203508 (P38 inhibitor, 10 uM) and or LY294002 (AKT signal pathway, 50 uM) for 48 h, and found that blocking STAT5 inhibited the proliferation ($P < 0.05$) (**Figure 3A**), both ERK and AKT inhibitors could significantly decrease the proliferation of ESCs ($P < 0.01$ or $P < 0.001$) (**Figure 3A**). In addition, the increase of ESCs proliferation induced by rhIL-22 could be partly or completely abolished by STAT5, ERK1/2 and AKT inhibitors ($P < 0.01$ or $P < 0.001$) (**Figure 3B**). However, other inhibitors for STAT3, P38 and JNK signaling pathways had not influenced ESCs proliferation and the role of IL-22 in regulating ESCs growth ($P > 0.05$) (**Figure 3A** and **3B**).

rhIL-22 stimulates the secretion of CCL2, and the expression of IL-8 and CXCR1 of ESCs

Our previous work showed that either CCL2 or IL-8 promotes the proliferation of ESCs [24, 25], so we further analyzed the effect of rhIL-22 on the production of CCL2 and IL-8, and the expression of it receptors CCR2 and CXCR1. Results in **Figure 4** showed that rhIL-22 increased the secretion levels of CCL2 and IL-8

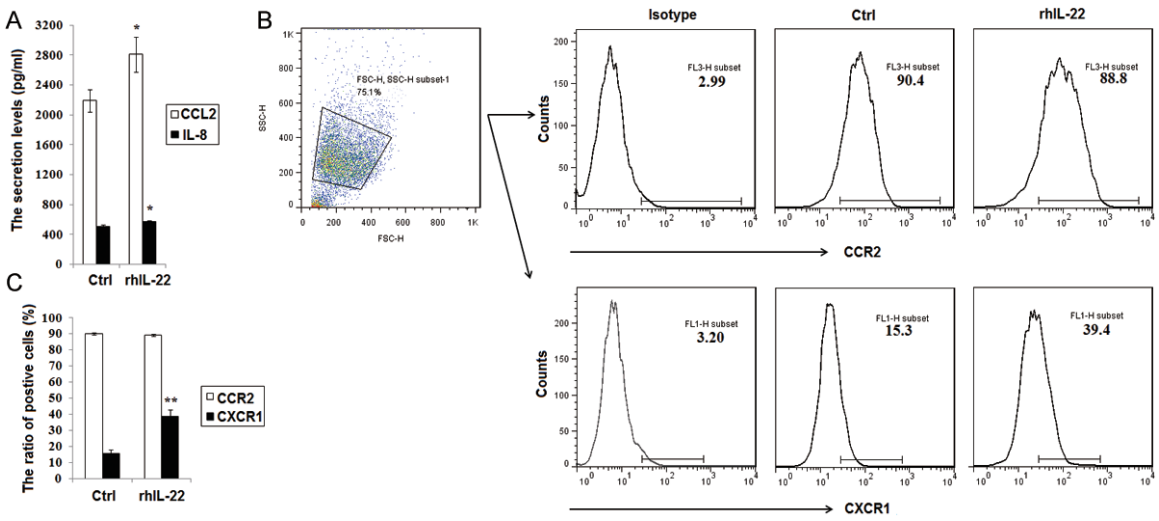

Figure 4. *rhIL-22 stimulates the secretion of CCL2, and the expression of IL-8 and CXCR1 of ESCs.* The primary ESCs of eutopic endometrium from women with endometriosis were incubated with rhIL-22 (10 ng/ml) for 48 h, and then the secretion levels of CCL2 and IL-8 in the supernatant, and the expression of CCR2 (CCL2 receptor) and CXCR1 (IL-8 receptor) were analyzed by ELISA (A) and flow cytometry (B, C), respectively. Data are mean ± SD. $^{*}P < 0.05$ or $^{**}P < 0.01$ compared to the vehicle control.

of ESCs ($P < 0.05$) (**Figure 4A**). Meantime, the results of flow cytometry showed that rhIL-22 obviously up-regulated the expression of IL-8 receptor CXCR1 ($P < 0.01$) (**Figure 4B** and **4C**), but not changed the expression of CCL2 receptor CCR2 ($P > 0.05$) (**Figure 4B** and **4C**). These data suggested that rhIL-22 may stimulate the proliferation of ESCs through stimulating CCL2 secretion and IL-8/CXCR1 signals, and promote the growth and survival of ESCs in the endometriotic milieu.

The effect of rhIL-22 on the CCL2, IL-8 and CXCR1 of ESCs are dependent of different signal pathways

To further investigate the molecular mechanism of rhIL-22 on the expression of CCL2 and IL-8/CXCR1, we incubated ESCs with rhIL-22 and STAT5, ERK1/2 or AKT inhibitor for 48 h, and analyzed the secretion of CCL2 and IL-8 in the supernatant by ELISA, and the expression of CXCR1 on ESCs by flow cytometry. As shown in **Figure 5**, only AKT inhibitor directly decreased the production of CCL2 and IL-8 of ESCs ($P < 0.05$ or $P < 0.01$) (**Figure 5A** and **5B**). Blocking STAT5, ERK1/2 or AKT could inhibit the stimulatory effect of rhIL22 on the production of CCL2 ($P < 0.05$ or $P < 0.001$) (**Figure 5A**), but STAT5 inhibitor had no similar function on IL-8 secretion ($P > 0.05$) (**Figure 5B**). In addition, the increase of CXCR1 expression on ESCs induced by rhIL-22 could be abrogated by either STAT5 or AKT inhibitor ($P < 0.01$) (**Figure 5C** and **5D**).

Discussion

IL-22 is a member of the IL-10 cytokine family and plays critical roles in inflammation, immune surveillance, and tissue homeostasis at mucosal sites [13, 26, 27]. Moreover, Kobold *et al* reported that IL-22 promotes growth in chemotherapy-resistant lung cancer cells [16]. Endometriosis results from increased cellular proliferation, adhesion and invasion of the retrograde endometrium in response to appropriate stimuli. These differences between the biological phenotype of the eutopic endometrium from women with endometriosis, and that of women without endometriosis may contribute to the survival and ectopic implantation of the regurgitated endometrial cells into the peritoneal cavity and thus to the development of endometriosis. Based on the similarity between cancer and endometriosis in a certain degree, therefore, our current work was undertook to investigate whether IL-22 plays a regulatory role in the growth of ESCs, and participates the origin and development of endometriosis.

The results of immunohistochemistry showed that the positive staining of IL-22 in endometrium from women of healthy control was undetectable. Compare to healthy endometrium, the

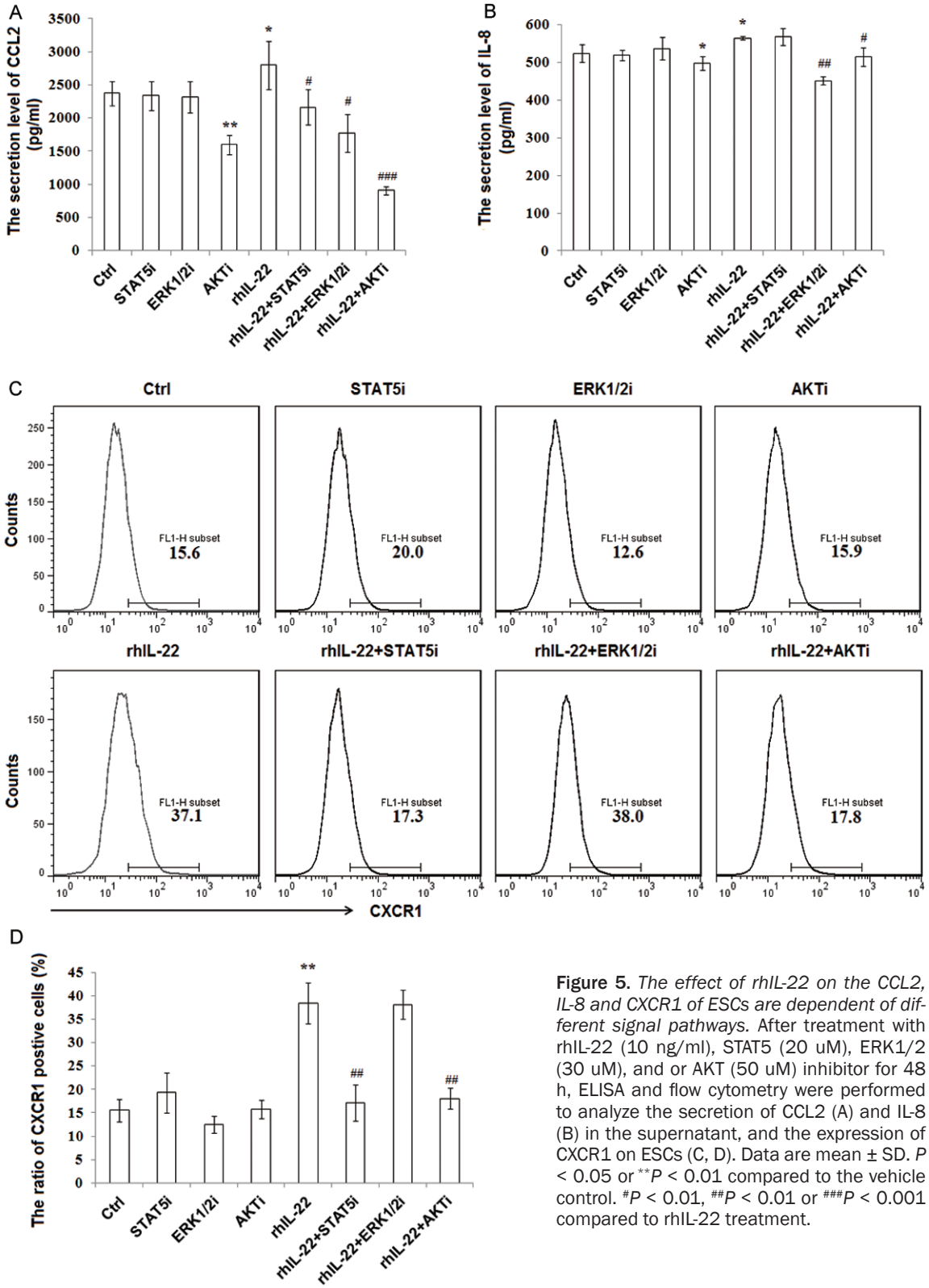

Figure 5. *The effect of rhIL-22 on the CCL2, IL-8 and CXCR1 of ESCs are dependent of different signal pathways.* After treatment with rhIL-22 (10 ng/ml), STAT5 (20 uM), ERK1/2 (30 uM), and or AKT (50 uM) inhibitor for 48 h, ELISA and flow cytometry were performed to analyze the secretion of CCL2 (A) and IL-8 (B) in the supernatant, and the expression of CXCR1 on ESCs (C, D). Data are mean ± SD. $P < 0.05$ or $^{**}P < 0.01$ compared to the vehicle control. $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ compared to rhIL-22 treatment.

expression of IL-22 and it receptors (IL-22R1 and IL-10R2) were prefer expressed at the eutopic endometrium and ectopic lesion from women with endometriosis. However, recent

research had found that the IL-22 level in serum was decreased in women with ovarian endometrioma [28]. This difference of IL-22 expression between peripheral and local sites remains to be further studied. Many researches had established that the highly elevated IL-22 expression was an inflammation driver in either a direct or indirect manner [29, 30]. Endometriosis is associated with increased secretion of pro-inflammatory cytokines, impaired cell-mediated immunity. Thus, we speculated that the increase of IL-22 expression in ectopic lesion from women with endometriosis possibly due to pelvic inflammatory.

The ligand-dependent transcription factor AHR has been described to be essential for IL-22 expression in Th17 cells, γδT cells, and human Th22 cells [7]. The ligand-dependent transcription factor AHR acts as a sensor for environmental toxins, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and for phytochemicals such as indol-3-carbinol, but it also recognizes endogenous ligands, such as the tryptophan photo-metabolite 6-formylindolo (3,2-b)carbazole (FICZ). Evidence has begun to accumulate that TCDD exposure promotes occurrence of endometriosis [31, 32]. Research work on primates has shown that exposure to the TCDD is associated with an increased prevalence and severity of endometriosis [33]. So TCDD might regulate the biological behavior of ESCs by stimulating the production of IL-22.

Subsequently, we analyze the effect of IL-22 on the proliferation of ESCs, and found that rhIL-22 promoted the proliferation of ESCs, on the contrary, blocking IL-22 by anti-human IL-22 neutralizing antibody down-regulated the proliferation. These results above indicated the abnormal increase level of IL-22 and it receptors might participate in the development of endometriosis through promoting the proliferation and growth of ESCs in the endometriotic milieu.

Previous studies about IL-22 have showed that the downstream signaling is mediated through STAT3 and to a lesser extent through STAT1 and STAT5, as well as the AKT and MAPK pathways [34-36]. However, in our study, we demonstrated that the stimulatory effect of IL-22 on the proliferation of ESCs was mediated mainly through STAT5, ERK1/2 and AKT signal pathways. These results suggested that the downstream signaling of IL-22 in ESCs is not identical with the lymphocyte.

IL-22 promotes the production of inflammatory mediator, such as IL-6 [7]. Moreover, IL-22 is essential for the relase of chemokine such as CXCL1, CXCL5, CXCL9, and CCL2 [37, 38]. A series of research showed that chemokines produced in the endometriotic milieu may contribute to a feed-forward cascade of events, which accentuates the recruitment of leukocytes into the peritoneal cavity of patients with endometriosis [39]. CCL2 (also known as monocyte chemoattractant protein-1) is a specific factor that chemoattracts and activates monocytes and macrophages that is a major ligand of receptor CCR2. Our previous studies also confirmed that both chemokine CCL2 [24] and IL-8 [25] are involved in regulation of ESCs behavior, and associated with endometriosis. Therefore, to further study the detailed functions of IL-22 on ESCs, we treated ESCs with rhIL-22 and found that rhIL-22 increased the secretion of CCL2 and IL-8 of ESCs, and the expression CXCR1. And these effects of IL-22 on the CCL2, IL-8/CXCR1 were dependent of different signal pathways, including STAT5, ERK1/2 and or AKT signaling.

Collectively, the increase of IL-22 in eutopic endometrium owing to local inflammatory and or external environment (TCDD), on the one hand, directly aggravate the inflammatory through stimulating the release of inflammatory mediator, and form this vicious cycle; on the other hand, cause the elevated levels of CCL2 and IL-8/CXCR1 of ESCs, and further promote the proliferation and growth of ESCs through STAT5, ERK1/2 and or AKT signal pathways, and finally lead to the formation of the immune microenvironment, which is conducive to the formation and development of ectopic foci.

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