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IL-17A Contributes to the Pathogenesis of Endometriosis by Triggering Proinflammatory Cytokines and Angiogenic Growth Factors

Soo Hyun Ahn,* Andrew K. Edwards,* Sukhbir S. Singh,† Steven L. Young,‡ Bruce A. Lessey,§ and Chandrakant Tayade*

Endometriosis is a chronic, inflammatory disease characterized by the growth of endometrial tissue in aberrant locations outside the uterus. Neoangiogenesis or establishment of new blood supply is one of the fundamental requirements of endometriotic lesion survival in the peritoneal cavity. IL-17A is emerging as a potent angiogenic and proinflammatory cytokine involved in the pathophysiology of several chronic inflammatory diseases such as rheumatoid arthritis and psoriasis. However, sparse information is available in the context of endometriosis. In this study, we demonstrate the potential importance of IL-17A in the pathogenesis and pathophysiology of endometriosis. The data show a differential expression of IL-17A in human ectopic endometriotic lesions and matched eutopic endometrium from women with endometriosis. Importantly, surgical removal of lesions resulted in significantly reduced plasma IL-17A concentrations. Immunohistochemistry revealed localization of IL-17A primarily in the stroma of matched endometrial tissue samples. In vitro stimulation of endometrial epithelial carcinoma cells, Ishikawa cells, and HUVECs with IL-17A revealed significant increase in angiogenic (vascular endothelial growth factor and IL-8), proinflammatory (IL-6 and IL-1β), and chemotactic cytokines (G-CSF, CXCL12, CXCL1, and CX3CL1). Furthermore, IL-17A promoted tubulogenesis of HUVECs plated on Matrigel in a dose-dependent manner. Thus, we provide the first evidence, to our knowledge, that endometriotic lesions produce IL-17A and that the removal of the lesion via laparoscopic surgery leads to the significant reduction in the systemic levels of IL-17A. Taken together, our data show a likely important role of IL-17A in promoting angiogenesis and proinflammatory environment in the peritoneal cavity for the establishment and maintenance of endometriosis lesions. The Journal of Immunology, 2015, 195: 000–000.

Endometriosis, one of the most prevalent causes of hysterectomy, infertility, and chronic pelvic pain (1), is characterized by the growth of endometrial tissue at ectopic sites, including the peritoneal cavity. Among multiple factors involved in the complex pathophysiology of this disease (2), both a proinflammatory peritoneal environment and active neoangiogenesis at the site of lesion development are thought to be integral to endometriotic lesion establishment and persistence. This notion is supported with the effectiveness of anti-inflammatory and antiangiogenic drugs in minimizing pain and lesion size in mice and humans (3–5). Theorized to originate from menstrual material normally shed into the peritoneal cavity through the patent fallopian tube (6), the endometrial fragments, only in some women, initiate inflammatory response associated with tissue damage (7). This leads to the recruitment and activation of neutrophils and macrophages (8), which secrete and increase the concentrations of chemotactic and angiogenic cytokines such as CCL11 (9) and vascular endothelial growth factor (VEGF) (10) in the peritoneal fluid. Indeed, the immune system of women who develop endometriosis is postulated to be abnormal, and the atypical activation of macrophages and subsequent recruitment of neutrophils (8), dendritic cells (11), Th (12), and NK cells are suggested to play a crucial role in the pathogenesis of endometriosis (13, 14).

IL-17A, a cytokine that was once believed to be primarily produced by Th17 cells, is the most studied among the family of IL-17 cytokines. Since its discovery as a proinflammatory mediator (15), IL-17A has been recognized in its critical role in the promotion of disease progression and pathogenesis of autoimmune diseases (16). Furthermore, expression of IL-17A is associated with the pathogenesis of a variety of tumors, where it has been documented to exhibit both tumorigenic and antitumor effects depending on the tumor microenvironment (17). IL-17A mediates its action by binding to a heterodimeric complex of receptor composed of IL-17RA and IL-17RC for downstream cell signaling (18). Early in its discovery, the majority of its production was thought to originate from Th17 cells; however, further investigations have documented the expression of IL-17A from innate immune cells such as γδ T (19–21), NK (22), neutrophils (16, 23) as well as mast cells (24), adding complexity to our understanding of the pleiotropic functions of this cytokine.

The proinflammatory nature of IL-17A was recognized in a seminal experiment conducted by Fossiez et al. (15), in which rheumatoid synovial fibroblasts produced IL-6, IL-8, G-CSF, and PGE2 in response to human rIL-17A treatment. This effect was promptly abolished with the addition of anti–IL-17A mAb. Since then, IL-17A

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Abbreviations used in this article: EECC, endometrial epithelial carcinoma cell; EPC, endothelial progenitor cell; VEGF, vascular endothelial growth factor.
has been extensively linked to the pathogenesis of chronic inflammatory diseases, including rheumatoid arthritis and psoriasis (25). In addition, IL-17A promotes vascular remodeling of the airways in the mouse model of asthma via the recruitment of endothelial progenitor cells (EPCs) to the allergen-exposed airways (26). Furthermore, it confers tumor resistance to anti-VEGF therapy via induction and secretion of angiogenic and inflammatory factors such as VEGF and IL-8 from tumor stromal cells (27). Recently, IL-17A was demonstrated to be involved in the promotion of ovarian cancer growth in mice via upregulation of proangiogenic and inflammatory mediators from small peritoneal macrophages (21). Despite much advancement made in understanding the role of IL-17A in other disease pathogenesis, little is known regarding its role in endometriosis. So far, IL-17A has been documented to induce the production of IL-8 and cyclooxygenase-2 from endometriotic stromal cells, in addition to promoting proliferation of the cells (28). Furthermore, IL-17A concentration in the peritoneal fluid of women with endometriosis correlated with endometriosis disease severity and infertility of the patients (29). These reports clearly point toward the potential involvement of IL-17A in the pathogenesis of endometriosis that warrants further investigation.

Endometriosis is a chronic disease characterized by the elevation of proinflammatory cytokines in the peritoneal fluid. Concentrations of cytokines such as TNF-α, IL-6, and IL-8 are increased in the peritoneal fluid and are augmented in production from activated peritoneal macrophages, which primarily contribute to the inflammatory milieu associated with pathogenesis of the disease. Furthermore, secretory factors from immune cells mediate vascularization of endometriotic lesions through both neoangiogenesis stimulated by VEGF (30) and de novo vasculogenesis at the site of implantation through recruitment of bone marrow–derived EPCs (31, 32). Emerging evidences from cancer and autoimmune literature suggest that IL-17A contributes to the neoangiogenesis and perpetuates inflammatory responses (25, 33), but there are few reports investigating the role of IL-17A in the pathogenesis of endometriosis. In this study, we show that endometriotic lesions are capable of producing variable amounts of IL-17A, depending on the disease severity, and that systemic concentrations of IL-17A drop significantly in patients with endometriosis after surgical removal of endometriotic lesions. In addition, we document the effects of IL-17A on the induction of proangiogenic, chemotactic, and growth promoting cytokines from endometrial epithelial carcinoma cells (EECCs), Ishikawa cells, and HUVECs.

Materials and Methods

Ethics approval for human samples

Human eutopic endometrial, ectopic endometriotic tissue samples, and plasma samples from patients with endometriosis and normal eutopic endometrium and plasma samples from women without known pathologies were collected and stored after informed consent using approved protocols by the Institutional Review Committees at Greenville Health System, Greenville, SC; University of North Carolina, Chapel Hill, NC; and Ottawa General Hospital, Ottawa, ON, Canada. Normal endometrial tissue and ectopic endometriotic tissue sections embedded in paraffin were obtained from the Kingston General Hospital, Kingston, ON, Canada, with informed consent from the patients. Ethics approval for this study was provided by the Health Sciences Research Ethics Board, Queen’s University, Kingston, ON, Canada.

Human endometrium, endometriosis, and plasma samples

Human eutopic endometrium, ectopic lesion tissue samples, and plasma samples were obtained from Greenville Health Systems after informed consent from patients between the ages of 21 and 39 years with confirmed endometriosis at the time of laparoscopic surgery. Patients received no hormonal therapy for minimal of 3 mo prior to laparoscopic surgery and for the duration of participation in the study. Patient characteristics including age, BMI, parity, and stage of endometriosis are provided in Table 1. Eutopic endometrium samples from patients were obtained by Pipelle sampling, and ectopic lesions were obtained during laparoscopic surgery by excision. Patients were consented for additional blood draws at the 2-wk postoperative visit and at 3 mo. Upon collection, samples were snap-frozen in liquid nitrogen and stored in −80°C. All plasma samples obtained from the patients and healthy women were separated from peripheral blood and stored in −80°C.

Multiplex cytokine assay

To assess the concentration of IL-17A in human samples, a Bioplex Prohuman cytokine 27-plex assay (M50-OKCAY0; Bio-Rad, Mississauga, Canada) was conducted on eutopic endometrial and ectopic endometriotic tissue plasma and peritoneal fluid samples from women with endometriosis as per kit instructions. Approximately 20 mg endometriotic tissue samples or normal endometrial samples were placed in 1.5 ml microcentrifuge tubes containing protease inhibitor (1 μl/0.01 g tissue; Sigma-Aldrich, St. Louis, MO) with Tissue Extraction Reagent I (100 μl/0.01 g tissue; Invitrogen, Carlsbad, CA). The tissue was homogenized using a rotor-stator homogenizer on ice for 1 min and then centrifuged at 18,000 rpm for 15 min at 4°C. The supernatant was collected and protein concentration measured using Pierce BCA Protein Assay Kit as per kit protocol (Pierce Biotechnology). The samples were normalized and stored at −80°C until later assessment. Plasma samples from healthy subjects were also included as control for the plasma samples from patients with endometriosis samples. Briefly, the 96-well culture plate was coated with assay buffer and magnetic beads, followed by two wash steps using the wash buffer. Samples, blanks, and diluted standards were transferred onto the 96-well plate. The plate was then incubated in the dark at room temperature on shaker for 30 min. After three washes using the wash buffer, detection Ab was added to the plate for 30 min followed by a wash step. The plate was incubated with streptavidin–phycoerythrin for an additional 10 min. Finally, the plate was washed before analysis using the Bioplex 2000 Suspension Array System (Bio-Rad).

IL-17A immunohistochemistry on matched eutopic endometrium and ectopic lesions

Immunohistochemistry was performed on paraffin-embedded sections of matched eutopic endometrium and ectopic lesion samples obtained from patients. The slides with tissue sections cut at 5 μm were deparaffinized in Cytroisolv (Fisher Scientific, Ottawa, ON, Canada) and subsequent rehydration in decreasing gradients of ethanol. Heat-induced Ag retrieval was conducted using sodium citrate buffer (0.01 mol [pH 6]) heated to 95°C in a water bath. Endogenous peroxide activity was blocked using 3% H2O2, followed by incubation with 1% BSA to block nonspecific binding. Sections were incubated with anti-human IL-17A rabbit polyclonal Ab (1:250 in 1% BSA/PBS; ab79056; Abcam, Cambridge, MA) or isotype Ab as a negative control. After overnight incubation in a humidified chamber at 4°C, sections were incubated with biotinylated secondary polyclonal goat anti-rabbit IgG Ab (1:500; DakoCytomation, Glostrup, Denmark), stained with DAB chromogen (DakoCytomation), and then counterstained with Harris hematoxylin (Fisher Scientific, Ottawa, ON, Canada). The sections were dehydrated in increasing gradients of ethanol and Cytroisolv, coverslipped with Permount mounting media (Fisher Scientific), and then viewed under the microscope.

Cell culture

EECCs (CRL-1671; American Type Culture Collection, Manassas, VA), HUVECs (200-05F; Cell Applications, San Diego, CA), and Ishikawa cells (99040201-1VL; Sigma-Aldrich) were incubated in a standard cell incubator at 37°C with 5% CO2. EECCs and Ishikawa cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (Sigma-Aldrich). HUVECs were maintained in All-in-one ready-to-use Endothelial Cell Growth Medium (211-500; Cell Applications). All cell lines were grown in T75 cell culture flasks (Corning, Corning, NY) up to 70–80% confluence prior to experimental use.

WST-1 proliferation assay

EECCs and HUVECs were harvested with Trypsin-EDTA and seeded onto a 96-well tissue-culture plate (Sarstedt Newton) at 1.25 × 104 cells/well, followed by IL-17A stimulation at different concentrations (1, 5, 25, 50, and 100 ng/ml; R&D Systems, Minneapolis, MN) in triplicates. PBS was used as a control. Post-24-h incubation, WST-1 cell proliferation reagent (Roche Diagnostics, Laval, QC, Canada) was added to each well for an additional 2 h at 37°C. Using a spectrophotometer, absorbance at 450 and 690 nm was measured. OD was calculated by subtracting absorbance at 690 from 450 nm.
Table I. Endometriosis study patient and healthy control subject characteristics

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Endometriosis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients participating in study</td>
<td>n = 24*</td>
<td>n = 10</td>
</tr>
<tr>
<td>Age (y)</td>
<td>26.7 ± 7.6</td>
<td>27.3 ± 5.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.3 ± 6.1</td>
<td>22.9 ± 2.4</td>
</tr>
<tr>
<td>Parity (%)</td>
<td>25*</td>
<td>20*</td>
</tr>
<tr>
<td>Stage of endometriosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages I to II</td>
<td>n = 16</td>
<td></td>
</tr>
<tr>
<td>Stages III to IV</td>
<td>n = 8</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SD unless otherwise indicated.

*Patients on oral contraceptive therapy, progestins, gonadotrophin-releasing hormone agonist/antagonist, aromatase inhibitors, or any other medications used toward the management of endometriosis during the study were excluded.

Six out of twenty-four endometriosis patients were parity 1; all other patients were nulliparous.

Two out of ten control subjects were parity 1; all other control subjects were nulliparous.

BMI, body mass index.

Propidium iodide cell-cycle analysis
EECCs were plated onto a six-well plate (Sarstedt Newton) at 5 × 10³ cells/well and incubated with different concentrations of IL-17A in triplicates (25, 50, and 100 ng/ml; R&D Systems) and PBS control for 24 h at 37°C with 5% CO₂. Posttreatment, cells were harvested from the plate, pelleted, and fixed in 70% ethanol at 4°C overnight. Postfixation and centrifugation to pellet the cells, 1 ml propidium iodide (0.04 mg/ml; BioShop Canada, Burlington, ON, Canada) and RNase A (0.625 mg/ml; Sigma-Aldrich) were added to each condition and incubated for 3 h at 4°C in the dark. A Beckman Coulter Cytomics FC500 (Beckman Coulter, Mississauga, ON, Canada) was used to conduct single-color analysis.

Flow cytometric analysis for IL-17RA
After removal of the growth media from the flask, EECCs and HUVECs were harvested using Trypsin-EDTA, and washed with cell staining buffer (1% BSA and 0.1% sodium azide in PBS). Approximately 1 × 10⁶ cells were incubated with either mouse anti-human IL-17RA Ab conjugated with PE (1:50; FAB177P; R&D Systems) or mouse IgG1 isotype control conjugated with PE (1:100; IC002P; R&D Systems) for 30 min at room temperature. Cells were fixed with ice-cold 2% paraformaldehyde in PBS for 15 min and kept at 4°C prior to conducting flow cytometric analysis (Beckman Coulter Cytomics FC500; Beckman Coulter).

Cell-culture supernatant cytokine analysis from EECCs, Ishikawa cells, and HUVECs treated with IL-17A
EECCs and Ishikawa cells were seeded at 5 × 10⁴ cells/well on a six-well plate in triplicate (Sarstedt Newton) and stimulated with different concentrations of IL-17A (10, 25, 50, and 100 ng/ml; R&D Systems) and PBS as control. HUVECs were seeded at 1 × 10⁵ cells/well on a six-well plate in triplicate (Sarstedt Newton) and incubated with different concentrations of IL-17A (5 and 50 ng/ml; R&D Systems) and PBS as control. The cells were incubated for 24 h in a standard cell-culture incubator at 37°C with 5% CO₂. The conditioned media was collected and analyzed using Human multiplex cytokine analysis (Eve Technologies, Calgary, AL, Canada).

Endothelial cell tubulogenesis assay
Tubulogenesis assay was conducted as per kit protocol (CBA-200; Cell Applications). HUVECs grown to 70–80% confluence in T75 cell culture flask (Corning) were trypsinized, pelleted, and seeded onto Matrigel-coated ibidi µ-angiogenesis slide (ibidi USA) at a density of 5 × 10³ cells/well with a complete medium containing different concentrations of IL-17A (1, 5, and 50 ng/ml; R&D Systems) in triplicate. VEGF (50 ng/ml; R&D Systems) and PBS were used as a positive and negative control, respectively.

Statistical analysis
All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad). Ordinary one-way ANOVA with Tukey post hoc test was used to analyze the results of WST-1 proliferation assay, supernatant cytokine analysis, and tubulogenesis assay. Unpaired t test was used to analyze the results of human tissue and plasma data and flow cytometric data. Data are represented as mean ± SD, unless otherwise stated in the figure. The p values ≤ 0.05 were considered statistically significant.

Results
IL-17A concentration in matched tissue sample and plasma sample from women with endometriosis is variable compared with healthy controls
In the current study, we evaluated the concentration of IL-17A in tissues and plasma samples from patients with or without endometriosis (Fig. 1A–C). Patients were further stratified into stage I, II, III, or IV of endometriosis as per the guidelines from the American Society for Reproductive Medicine. We further grouped stage I and II patient into early and stage III and IV into advanced disease categories. Although statistically nonsignificant, we observed a trend of increasing IL-17A concentration in the ectopic lesions compared with the matched eutopic endometrium obtained from same patients (Fig. 1A). In the plasma samples, IL-17A concentration was significantly increased in women with endometriosis compared with healthy controls (Fig. 1B). When the matched ectopic lesions and eutopic endometrium were stratified by disease severity, we observed persistent increase in IL-17A in ectopic lesion samples compared with eutopic endometrium across severity (Fig. 1C). Note that the concentration of IL-17A from normal endometrial tissues of disease-free women was <10 pg/ml (n = 4; 3.13 ± 1.92 pg/ml; data not shown), and the concentration of IL-17A in the peritoneal fluid was negligible (n = 24; data not shown).

The plasma concentration of IL-17A diminishes after laparoscopic lesion removal
The systemic concentration of IL-17A in women with endometriosis significantly declined after surgical removal of lesions, with mean IL-17A plasma concentrations of 395.1 ± 93.20 pg/ml presurgery and 228.6 ± 78.33 pg/ml 2 wk after surgery (Fig. 2). Immunolocalization of IL-17A in human eutopic endometrium and ectopic endometriotic lesions
In the extant literature, there are no reports of IL-17A immunolocalization in the eutopic endometrium or peritoneal endometriosis lesions. A single study showed localization of IL-17A–positive cells in the stroma of an ovarian endometrioma lesion; however, expression in peritoneal lesions or matched eutopic endometrium was not examined (28). In this study, we show the localization of IL-17A–positive cells within the stroma and surrounding the vasculature in matched eutopic endometrium and ectopic lesion samples from women with endometriosis (Fig. 3A, 3B). Because of the heterogeneous nature of the ectopic lesions, we were unable to concretely conclude whether IL-17A staining was increased in ectopic lesions as compared with matched eutopic endometrium from same patients.

IL-17RA is expressed in EECCs and HUVECs
IL-17RA, the primary receptor for IL-17A, is reported to be ubiquitously expressed in different cell types in mouse (34) and human (35). Before conducting functional assays with IL-17A, we first wanted to establish whether IL-17RA is present on EECCs and HUVECs to rationalize their responsiveness to IL-17A. Both EECCs and HUVECs were incubated with anti–IL-17RA Ab conjugated with PE at 4°C overnight. Flow cytometric analysis revealed that EECCs (Fig. 4A) and HUVECs (Fig. 4B) indeed express IL-17RA on the cell surface, which suggests their capacity to specifically respond to IL-17A stimulation.

IL-17A does not induce proliferation in EECCs or HUVECs
To investigate whether IL-17A exhibit mitoticeffect on epithelial and endothelial cells, we incubated EECCs (Fig. 5A) and HUVECs (Fig. 5B) with different concentrations of rIL-17A and PBS control. After 24 h of incubation at 37°C, we did not observe any significant
difference in proliferative capacity compared with the PBS-treated wells on either cell line. Therefore, IL-17A does not directly induce mitosis on the EECCs or HUVECs in vitro. To determine whether rIL-17A would induce apoptosis in EECCs, we performed propidium iodide flow cytometric assay to determine DNA abundance in EECCs treated with IL-17A (25, 50, and 100 ng/ml) and PBS control. We did not observe any differences between IL-17A–treated and PBS control groups (data not shown). This further strengthens the notion that IL-17A may not directly have proliferative or apoptotic effects of epithelial and endothelial cells.

IL-17A induces the production of chemokine and angiogenic cytokines from EECCs

IL-17A is known to induce a variety of cytokines from different tissue types with pleiotropic downstream effects. We wanted to investigate the cytokine profile of EECCs when induced with varying concentration of IL-17A. Stimulation of EECCs with IL-17A (10, 25, 50, and 100 ng/ml) led to the significant increase in the production of angiogenic and chemotactic cytokines, namely VEGF, platelet-derived growth factor-AA, CXCL12, and G-CSF (Fig. 6A–D, respectively). The cytokine profile suggests a potential involvement of IL-17A in mediating neoangiogenesis and recruitment of lymphocytes and bone marrow–derived cells to the site of lesion development. In earlier studies, we (31) and others (32) showed that CXCL12 contributes to the recruitment of EPCs at the endometriotic lesions and aids neoangiogenesis.

IL-17A induces the production of proinflammatory cytokines and chemokines from HUVECs

Similar to EECCs, different concentrations of IL-17A (5 and 50 ng/ml) induced the production of proinflammatory cytokines and chemokines from HUVECs, namely IL-1α, CXCL1, IL-6, and CX3CL1 (Fig. 7A–D, respectively) in a dose-dependent fashion. These cytokines are well known for their potent proinflammatory actions. These data suggest the ability of IL-17A to regulate the expression of proinflammatory, angiogenic cytokines, and chemokines in the peritoneal environment.

IL-17A induces the production of proinflammatory cytokines from Ishikawa cells

In the endometriosis literature, EECCs and Ishikawa cells have been widely used to understand molecular mechanisms involved in the pathogenesis of endometriosis. We wanted to establish how Ishikawa cells would respond to IL-17A stimulation. In this study, we report that stimulation of Ishikawa cells with different concentrations of IL-17A lead to increased expression of proinflammatory and chemotactic cytokines in a dose-dependent manner, namely IL-1β, IL-8, IL-9, and CCL11 (Fig. 8A–D, respectively). It is well described in literature that IL-1β, IL-8, and CCL11 are increased in concentration in the peritoneal fluid of women with endometriosis and are thought to contribute to the pathogenesis of the disease.

IL-17A promotes tubulogenesis from HUVECs in a dose-dependent fashion

The endothelial tube formation assay is a fast, quantifiable method for measuring in vitro angiogenesis and a standard method used to investigate the effects of an angiogenic stimulant or inhibitor on endothelial cells. To investigate whether IL-17A can induce direct tubulogenesis, HUVECs seeded on a coat of Matrigel were treated with different concentrations of IL-17A in complete endothelial cell growth medium for 16 h at 37°C prior to the analysis of tubulogenesis, unlike other methods in which endothelial cells are serum starved.
prior to tubulogenesis assay (26). Our data showed that IL-17A induces tubulogenesis of HUVECs on Matrigel in a dose-dependent manner (Fig. 9A, 9B), suggesting that IL-17A is capable of inducing direct tubulogenesis of endothelial cells in vitro.

Discussion

IL-17A has been implicated in several chronic, inflammatory, and autoimmune disorders; however, its association with the pathogenesis of endometriosis has not previously been well described. We provide the first evidence, to our knowledge, that endometriotic lesions produce IL-17A protein in variable amounts depending on the stage of the disease. Traditionally, IL-17A was thought to be produced only by Th17 cells; however, recent reports suggest that variety of cell types produce IL-17A including stromal cells, fibroblasts, and endothelial cells (34). Immunohistochemical analysis provides further evidence that IL-17A is expressed by the endometriotic lesions, but the complexity and heterogeneous nature of the endometriotic lesions precludes identification of the specific cell

FIGURE 3. IL-17A–positive cells are detected in the matched eutopic (A) and ectopic lesion (B) samples from women with endometriosis. Immunohistochemistry images are representative of five matched tissue samples immunostained with anti-human IL-17A. Original magnification ×200 with digitally magnified inlet. Scale bar, 100 μm.

FIGURE 4. IL-17RA is expressed on EECCs and HUVECs. EECCs (A) and HUVECs (B) were stained with either PE-conjugated mouse anti-human IL-17RA or PE-conjugated mouse IgG isotype control in room temperature. On average, 65.2 ± 3.9% of EECCs stained for IL-17RA, whereas 58.1 ± 14.6% cell surface staining was seen for HUVECs. Representative of three separate experiments. *p < 0.05 compared with isotype.
Strikingly, we demonstrated significant decline in plasma concentration of IL-17A after surgical removal of endometriotic lesions. These findings strongly suggest that IL-17A may be a contributory factor to the inflammatory peritoneal milieu associated with endometriosis. The association between the removal of the lesion and decrease in IL-17A further suggest the role of the ectopic lesion as a potential reservoir of IL-17A or the removal of the lesion simply leads to the diminishment in the proinflammatory environment, where IL-17A is a component that mediates the proinflammatory status. It is likely that, with the removal of the lesion, the tissue-resident Th17 cells and other potential producers of IL-17A are also removed, thereby contributing to the significantly lowered concentration of IL-17A detected in the peripheral blood.

The source of IL-17A can be speculated by analyzing the data available on the peritoneal fluid of women with endometriosis. If the main source of IL-17A is from peritoneal fluid-resident immune cells, the concentration of IL-17A in the peritoneal fluid will be elevated. Zhang et al. (29) documented elevated concentration of IL-17A in the peritoneal fluid in minimal or mild endometriosis as compared with severe disease, ranging between 5 and 6 pg/ml. This study did not find a significant difference in the peritoneal fluid concentration of IL-17A between women with endometriosis and without disease. On the contrary, we could not detect measurable levels of IL-17A in the peritoneal fluid from women with endometriosis (n = 24; data not shown). Because we show that the removal of the lesion led to the significant decrease of IL-17A in the plasma, the effect of removal likely involves changes within the systemic immune system. It is possible that, unlike other proinflammatory cytokines, IL-17A is primarily produced by tissue-resident immune cells and as such may not be detectable in the peritoneal fluid. To clarify these findings, we need to establish whether IL-17A–positive, tissue-resident immune cells in women with endometriosis are indeed the source of IL-17A in this disease.

Endometriosis is a disease mediated by inflammatory and angiogenic peritoneal environment. Using cell lines well established in the endometriosis literature, we document the unique cytokine response to IL-17A in vitro.
signatures induced by IL-17A in EECCs, Ishikawa cells, and HUVECs. In particular, IL-17A induced the production of chemotactic and angiogenic factors including G-CSF, VEGF, CXCL12, and IL-8 from EECCs and Ishikawa cells. In addition, IL-17A induced the production of chemotactic and proinflammatory cytokines such as CXCL1, CX3CL1 and IL-6 from HUVECs. Taken together, our data suggest that IL-17A may be involved in the orchestration of a paracrine network of cytokines between the adjacent cells that leads to the promotion of angiogenesis and inflammation in the peritoneal cavity. Specifically, our data suggest that IL-17A has the potential to enhance vascularization of the lesion through VEGF- and IL-8–mediated pathways. Evidence for de novo vasculogenesis at endometriosis lesions has been previously suggested by a report of the recruitment of EPCs to the lesion site via a CXCL12-mediated pathway (36).

IL-17A may also play a crucial role in the promotion of inflammation via the recruitment of immune cells by inducing the production of chemokines such as G-CSF, CCL11, CXCL1, and CX3CL1 from the endometriotic lesion. For instance, IL-17A–induced CX3CL1 may play a critical role in the mobilization of proinflammatory monocytes and other immune cells into the lesion. CX3CL1 is both an adhesion molecule and chemotactic cytokine for T cells and monocytes that acts by adhering and immobilizing the cells to the endothelial cell surface (37, 38) and for which production

**FIGURE 7.** IL-17A induces the production of proinflammatory cytokines and chemokines from HUVECs in a dose-dependent manner. HUVECs were plated onto a six-well plate in triplicate at a density of $1 \times 10^5$ cells/plate and incubated with different concentrations of IL-17A (5 and 50 ng/ml) for 24 h in a standard cell-culture incubator at 37˚C with 5% CO₂. Conditioned supernatants were collected and screened for cytokine expression, from which IL-1, IL-6, CX3CL1, and G-CSF (A–E, respectively) showed statistical significance. *$p < 0.05$ compared with PBS.

**FIGURE 8.** IL-17A induces production of chemokine, angiogenic, and proinflammatory cytokines from Ishikawa cells. Ishikawa cells were plated onto a 96-well cell-culture plate in triplicate at a density of $5 \times 10^5$ cells/well and incubated with different concentrations of IL-17A (5 and 50 ng/ml) or PBS control for 24 h at 37˚C with 5% CO₂. The conditioned supernatants of each treatment were collected and screened for cytokine expression, from which IL-1β, IL-8, IL-9, and CCL11 (A–D, respectively) showed statistical significance. *$p < 0.05$ compared with PBS.
by HUVECs can be induced by IFN-γ (39). Thus, IL-17A may not only initiate the process of inflammation in endometriosis, but also sustain it through the indirect mobilization of proinflammatory immune cells by inducing the production CX3CL1 from the endothelial cells of the lesion. Taken together, the data suggest a potential therapeutic effect of IL-17A blockade in endometriosis. Decoding the paracrine network established between different cell types in the lesion will enhance our understanding of the mechanisms employed by IL-17A in establishing the proinflammatory and proangiogenic environment in endometriosis.

Current research suggests inherent molecular differences in eutopic endometrium of women with endometriosis that allows the menstrual exudate to escape immune surveillance and develop into ectopic foci in the pelvic cavity. In addition, the immune system of women with endometriosis behaves curiously in the presence of endometrial fragments. As such, the pathogenesis of endometriosis may be 2-fold: women with endometriosis have endometrial cell dysfunction that stimulate aberrant innate and adaptive immune responses toward the endometrial fragments found in ectopic locations, allowing for the fragments to survive and implant to grow into...
endometriosis. These immune cells are not only producing increased amount of proinflammatory and growth promoting cytokines, but also exhibit diminished cytotoxicity/adaptive responses toward the fragments. Such immune cell activity is reflected in the inflammatory milieu known to be associated with the peritoneal environment of endometriosis. We hypothesize that the immune cells would be the major producer of IL-17A, which trigger other cells in the vicinity that express its receptor to make cytokine that are typically found in endometriosis environment.

In literature, IL-17A is shown to promote direct endothelial cell tubulogenesis in vitro (26, 40). In this study, we also show direct effect of IL-17A in promoting tubulogenesis of HUVECs. Typically, other studies use serum-starved media on Matrigel to study the angiogenic effect of IL-17A. In this study, we opted to use complete growth media supplemented with growth factors and FBS. The rational for using the complete endothelial cell growth media for our experiment is to achieve the similar angiogenic environment of peritoneal fluid that bathes endometriotic lesions in the peritoneal cavity. This allows us to elucidate if the presence of IL-17A, in addition to other growth factors, would elicit an additive effect in driving tubulogenesis. This method also allows our data to be translatable to in vivo situation as endometriotic lesions are exposed to growth promoting cytokines in the peritoneal fluid.

Overall, the limitation of the current study is inherent in endometriosis research in general. To conduct proper interpretation and comparison of data, the tissue samples between patients and controls must be matched in menstrual stage, disease stage, and age of the individual. In addition, the medical history of individuals and therapeutic regimens needs to be taken into account to consider the effect of estrogen and progesterone on the disease state. The analysis is driving tubulogenesis. This method also allows our data to be translatable to in vivo situation as endometriotic lesions are exposed to growth promoting cytokines in the peritoneal fluid.

Disclosures

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