Immune Netw. 2023 Apr;23(2):e14
https://doi.org/10.4110/in.2023.23.e14
pISSN 1598-2629·eISSN 2092-6685

IMMUNE NETWORK

Original Article



# IL-17A and Th17 Cells Contribute to Endometrial Cell Survival by Inhibiting Apoptosis and NK Cell Mediated Cytotoxicity of Endometrial Cells via ERK1/2 Pathway

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**Received:** Dec 29, 2021
**Revised:** Nov 23, 2022
**Accepted:** Dec 12, 2022
**Published online:** Jan 30, 2023

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## ABSTRACT

Immune status including the immune cells and cytokine profiles has been implicated in the development of endometriosis. In this study, we analyzed Th17 cells and IL-17A in peritoneal fluid (PF) and endometrial tissues of patients with (n=10) and without (n=26) endometriosis. Our study has shown increased Th17 cell population and IL-17A level in PF with endometriosis patients. To determine the roles of IL-17A and Th17 cells in the development of endometriosis, the effect of IL-17A, major cytokine of Th17, on endometrial cells isolated from endometriotic tissues was examined. Recombinant IL-17A promoted survival of endometrial cells accompanied by increased expression of anti-apoptotic genes, including Bcl-2 and MCL1, and the activation of ERK1/2 signaling. In addition, treatment of IL-17A to endometrial cells inhibited NK cell mediated cytotoxicity and induced HLA-G expression on endometrial cells. IL-17A also promoted migration of endometrial cells. Our data suggest that Th17 cells and IL-17A play critical roles in the development of endometriosis by promoting endometrial cell survival and conferring a resistance to NK cell cytotoxicity through the activation of ERK1/2 signaling. Targeting IL-17A has potential as a new strategy for the treatment of endometriosis.

**Keywords:** IL-17A; Th17 cells; Endometriosis; Apoptosis; NK cell cytotoxicity; ERK

## INTRODUCTION

Endometriosis is a gynecologic disease in which tissue that normally grows inside the uterus grows outside the uterus, causing pelvic pain and infertility. Although endometriosis is generally considered a steroid-sensitive disease, defects in the immune system as well as

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### Conflict of Interest

The authors declare no potential conflicts of interest.

### Abbreviations

IL-17R, IL-17 receptor; IRB, Institutional Review Board; p-, phospho-; PF, peritoneal fluid; PMA, phorbol-12-myristate-13-acetate; RORC, retinoic acid-related orphan receptor C; RORγt, second isoform of RAR-related orphan receptor gamma.

### Author Contributions

Conceptualization: Kang YJ, Yoon SR; Data curation: Jung H, Choi I; Formal analysis: Kang YJ, Cho HJ, Park A, Kim MJ; Investigation: Kang YJ, Lee Y; Methodology: Kang YJ, Cho HJ; Resources: Jeung IC, Jung YW; Supervision: Lee HG, Yoon SR; Writing - original draft: Kang YJ, Cho HJ, Lee Y, Park A; Writing - review & editing: Lee HG, Yoon SR.

genetic and epigenetic predisposition are also thought to be involved in the development of endometriosis (1). It has been suggested that the immune environment plays an important role in both the initiation and progression of endometriosis (2,3). In particular, immune cells such as T lymphocytes, B lymphocytes and NK cells play essential roles in determining the fate of implantation and in affecting the proliferation of endometrial and endometriotic cells (4,5). An immunological/inflammatory etiology has been proposed due to the increased concentrations of activated macrophages, T cells, B cells and inflammatory cytokines present in endometriosis (6,7).

The recent identification of the Th17 subset that is characterized by the production of IL-17A and the related cytokine IL-17F has advanced the understanding of the roles of various T cell subsets in endometriosis (8). Th17 cells express the lineage-specific transcription factor, retinoic acid-related orphan receptor C (RORC) and represent a distinct lineage of CD4 T cells. Th17 cells are involved in the pathogenesis of various human diseases, including various autoimmune conditions, inflammatory diseases, allergies and cancer (9,10). Th17 cells can rapidly initiate an inflammatory response by recruiting, activating, and eliciting the migration of neutrophils (9). Importantly, tumor-associated Th17 cells have been suggested to have anti-tumoral activity (11) and pro-tumoral activity (12). Moreover, tumors, tumor-derived fibroblasts, and antigen-presenting cells secrete several key cytokines including TGF-β, IL-23, IL-6, IL-1β and IL-21 that form a cytokine milieu to regulate and expand Th17 cells (13).

IL-17A, the major cytokine produced by Th17 cells, is a pro-inflammatory cytokine that has been implicated in the pathogenesis of various chronic inflammatory diseases. It has been reported that increases in the level of IL-17A in peritoneal fluid (PF) correlated with the severity of endometriosis and infertility associated with this disorder (14) and IL-17A stimulated IL-8 secretion, cyclooxygensase-2 expression, and cell proliferation of endometriotic stromal cells (15). In addition, the presence of IL-17 in endometrial cyst fluid and high levels of IL-17 in aromatase-positive endometriosis samples have been reported (16). A recent study has demonstrated that IL-17A contributes to the pathogenesis of endometriosis by triggering pro-inflammatory cytokines and angiogenic growth factors (17). Despite recent progress, the functional roles of IL-17A on cell survival of endometrial cells and NK cell mediated cytotoxicity have not been elucidated. Thus, we constructed endometrial stroma cells from endometriotic tissue and investigate the effect of IL-17A on endometriotic stromal cells and NK cells. In this study, we first propose that IL-17A promotes cell survival of endometrial cells by conferring anti-apoptotic activity and resistance to NK cytotoxicity, which may contribute to pathogenesis of endometriosis.

## MATERIALS AND METHODS

### Subjects and PF samples

This study included 26 women with endometriosis (endometriosis group) and 10 women without endometriosis (control group). Women of reproductive age (between 20 and 40 years of age) who underwent laparoscopic examination for endometriosis were included. Endometriotic tissues and PF were obtained from patients with ovarian endometrioma who were undergoing laparoscopy. All samples were collected informed consent form each patient. This study was approved by the Institutional Review Board (IRB) of The Catholic University of Korea (IRB ID: DC12TAS10022).

### Preparation of PF cells and endometrial cells

PF samples were centrifuged at 600×*g* for 10 min at 4°C. The cell-free supernatant was then collected and stored at −80°C until analysis. After the red blood cells were lysed with Red Blood Cell Lysis Buffer (Roche, Mannheim, Germany), the remaining cells were resuspended in RPMI medium containing 10% FBS at $1\times10^6$ cells/ml and cultured. Human endometrial cells were isolated and cultured as previously described (18,19). Briefly, the tissue was rinsed, minced into small pieces and incubated in DMEM containing type I collagenase (0.25%) and deoxyribonuclease I (15 IU/ml) for 2 h at 37°C. The resultant dispersed endometriotic tissue cells were separated by filtration through a 70-μm nylon cell strainer. The cells were collected by centrifugation, resuspended in DMEM containing 10% FBS, plated onto 100-mm dishes and incubated at 37°C.

### Analysis of PF cells by flow cytometry

Single cell suspensions from PF were stained with appropriate antibodies for further analysis. FITC-CD4, PE-CD8, APC-CD3, PE-CD11b, PE-Cy7-CD56, APC-CD11c, PE-Cy7-CD14, APC-CD19, FITC-CD15, FITC-CD16, FITC-HLA-ABC, APC-HLA-APC, PE-HLA-G and PE-MIC-AB antibodies were used for surface staining. Antibodies for immunostaining were purchased from BD Biosciences (San Jose, CA, USA) and eBioscience (San Diego, CA, USA). To measure cytokine production, cells were incubated for 12 h and then stimulated with phorbol-12-myristate-13-acetate (PMA, 50 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (1 μg/ml; Sigma-Aldrich) for 3 h. The cells were then treated with GolgiPlug (BD Biosciences) for 1 h. The cells were collected, fixed and permeabilized with Fixation/Permeabilization buffer (BD Biosciences) for 40 min. FITC-anti-CD4 antibodies were used for surface staining, and PE-Cy7-anti-CCR6, PE-anti-second isoform of RAR-related orphan receptor gamma (Rorγt) and APC-anti-IL-17 antibodies were used for intracellular staining. The staining data were acquired using a FACS Canto II instrument (BD Biosciences, Sparks, MD, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

### Evaluation of cytotoxicity

Cytotoxicity was evaluated using a calcein-AM release assay (20). Briefly, endometrial target cells were labeled with calcein (Invitrogen, Carlsbad, CA, USA) for 1 h. Calcein-labeled target cells ($1\times10^4$ cells) and serially diluted effector cells (NK cells) were then co-cultured in 96-well round-bottom plates for 4 h. “Maximum release” was induced by adding 2% Triton X-100 to the target cells, and “spontaneous release” was evaluated by adding culture media to the target cells. The calcein released into the supernatant was measured using a multi-mode microplate reader (Molecular Devices, Chicago, IL, USA). The percent specific lysis was calculated according to the following formula: Percent Specific Lysis=[(Test Release−Spontaneous Release)/(Maximum Release−Spontaneous Release)]×100.

### Cytokine measurement by ELISA

The IL-1β, IL-6 and IL-8 cytokines were quantitated in the PF supernatants using a specific ELISA kit purchased from eBioscience. Samples from each patient were tested in duplicate according to the detailed protocol provided by the manufacturer.

### mRNA analysis by quantitative RT-PCR

Total RNA was extracted from cells using Trizol (Invitrogen), and the RNA was reverse transcribed using a cDNA Reverse Transcription Kit (Toyobo, Osaka, Japan). Real-time quantitative PCR was performed using a TaKaRa Analyzer with SYBR Green Master Mix (TaKaRa, Tokyo, Japan) and the following primers: GAPDH,

5′-GCCATCAATGACCCCTTCAT-3′ and 5′-GCTCCTGGAAGATGGTGATG-3′; IL-1β, 5′-GAATCTCCGACCACCACTAC-3′ and 5′-TCGTTATCCCATGTGTCGAA-3′; IL-6, 5′-TACCCCCAGGAGAAGATTCC-3′ and 5′-TTTTCTGCCAGTGCCTCTTT-3′; IL-8, 5′-GTGCAGTTTTGCCAAGGAGT-3′ and 5′-AATTTCTGTGTTGGCGCAGT-3′; IL-17A, 5′-CATGAACTCTGTCCCCATCC-3′ and 5′-CCCACGGACACCAGTATCTT-3′; IL-23, 5′-CTCTGCTCCCTGATAGCCCT-3′ and 5′-TGCGAAGGATTTTGAAGCGG-3′; RORC, 5′-TTTTCCGAGGATGAGATTGC-3′ and 5′-AAGGCCAGCTCCAGATTGTA-3′; MMP9, 5′-CCTGGGCAGATTCCAAACCT-3′ and 5′-GTACACGCGAGTGAAGGTGA-3′; VEGF, 5′-CCCACTGAGGAGTCCAACAT-3′ and 5′-TTTCTTGCGCTTTCGTTTTT-3′; GM-CSF, 5′-GGGAGCATGTGAATGCCATC-3′ and 5′-GGCTCCTGGAGGTCAAACAT-3′; HLA-A, 5′-TGGCAGCTCAGATCACCAAG-3′ and 5′-GGCCCTCCAGGTAGACTCTC -3′; HLA-B, 5′-CCTAGCAGTTGTGGTCATCGG-3′ and 5′-TGTGAGAGACACATCAGAGCC-3′; HLA-C, 5′-TCAAGAGCCTCTGGCATCTC-3′ and 5′-AGATGATCGGGGAGGGAACA-3′; HLA-E, 5′- TCATTCCCAATGGGTGTCGG-3′ and 5′- CTTCTTGAGTCCGGATGGGG-3′; HLA-G, 5′-GTGGCTGAACAAAGGAGAGC-3′ and 5′- CTCAGGGTGGCCTCATAGTC-3′; Twist, 5′-TCTCGGTCTGGAGGATGGAG-3′ and 5′-GTTATCCAGCTCCAGAGTC-3′, SLUG, 5′- GAGCATTTGCAGACAGGTCA-3′ and 5′-CCTCATGTTTGTGCAGGAGA-3′, SNAIL, 5′-CTTCCAGCAGCCCTACGAC-3′ and 5′-CGGTGGGGTTGAGGATCT-3′. Target mRNA expression was normalized to GAPDH expression.

## Western blot analysis

Endometrial cells were stimulated with 100 ng/ml IL-17A or PD0325901 (Sigma-Aldrich) for 10 or 30 min, and the cells were lysed in 1% Triton X-100 lysis buffer. The cell lysates were resolved by 10% or 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Darmstadt, Germany). The membrane was blocked for 1 h at room temperature in 5% nonfat skim milk in Tris-buffered saline containing 0.05% Tween 20 (Sigma-Aldrich) and then incubated overnight at 4°C with monoclonal rabbit anti-phospho (p)-p44/p42 MAPK, Erk1/2, monoclonal rabbit anti-p44/p42 MAPK Erk1/2, anti-GAPDH, anti-p-AKT, anti-AKT, anti-Mcl-1, anti-Bcl-2, anti-p-p38, or anti-p38 antibodies (Cell Signaling, Danvers, MA, USA). After incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Thermo, Rockford, IL, USA), the signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo).

## Cell migration assay

Cell migration was evaluated using transwell culture plate system (BD Biosciences). Endometrial cells ($2\times10^4$) were seeded on the upper compartment of transwell chambers (24-well insert; 8-μm pore size) in 0.1% BSA medium emented with recombinant human IL-17A (100 ng/ml; PeproTech, Rocky Hill, NJ, USA) at 37°C. Medium containing 10% FBS was added to the lower compartments of the chambers. After incubation for 8 h, the non-migratory cells were removed from the upper surface of the membrane by wiping with a cotton bud. The membrane was fixed with methanol for 10 min and stained with 0.1% crystal violet for 5 min. Migrated cells to the lower surfaces were counted at 100× magnification.

## Statistical analysis

The data were analyzed using a 2-tailed unpaired Mann-Whitney *U* test in PRISM (San Diego, CA, USA) and Student's *t*-test. The p-values less than 0.05 were considered statistically significant.

## RESULTS

### Th17 cells are increased in PF from patients with endometriosis

To study the distribution of immune cells in endometriosis, the subpopulations of mononuclear cells in PF from women with or without endometriosis were analyzed and compared. The population of Th17 ($CD4^+IL\text{-}17^+ROR\gamma t^+$) cells was significantly increased in PF from patients with endometriosis (1.15%±0.13% vs. 4.48%±0.56%, p=0.0008; **Table 1**, **Supplementary Figure 3A**). In addition, the proportions of NK cells were lower in PF from patients with endometriosis; this result was consistent with that in our previous report (21). To analyze the Th17 cell populations in PF, CD4-positive T cells were isolated by magnetic activated cell sorter and stimulated with PMA and ionomycin for 3 h in RPMI 1640 containing 10% FBS. As shown in **Fig. 1A**, there was more Th17 cell population ($CCR6^+$, $IL\text{-}17^+$ and $ROR\gamma t^+$ cells) in PF from patients with endometriosis. Furthermore, the mRNA expression of IL-17A, IL-23 and RORC was increased in PF cells from patients with endometriosis compared to control patients (**Fig. 1B**). Immunohistochemistry of IL-17A and CD4 also showed that there was more infiltration of $IL\text{-}17A^+$ cells and CD4 T cells into endometrial tissue from patients with endometriosis compared to control patients (**Supplementary Fig. 1**).

Because Th17 cells are classified as $CD4^+$ T helper cells that primarily secrete the cytokine IL-17 and their developmental program is controlled by IL-6, and RORγ (22), we measured the levels of several cytokines in PF. The levels of inflammatory cytokines, such as IL-1β, IL-6 and IL-8, were increased in PF from patients with endometriosis at both protein and mRNA levels (**Fig. 1C** and **1D**). Together, these results demonstrated that Th17 cell proportion and IL-17A mRNA expression are increased in PF cells from patients with endometriosis.

### IL-17A promotes endometrial cell survival

In order to investigate the effect of IL-17A on endometrial cells, endometrial cells were isolated from endometriotic tissues and IL-17 receptor (IL-17R) expression was examined on endometrial cells. IL-17R expression of endometrial cells was increased in endometrial cells treated with IL-17 compared to untreated control (**Fig. 2A**). Since previous reports have shown that IL-17A promotes tumor growth (23,24), we determined whether IL-17A promotes the growth or inhibits the apoptosis of endometrial cells. Endometrial cells were treated with IL-17A, and proliferation was determined by Ki-67 and 7-AAD staining. The apoptosis of IL-17A-treated cells was also measured by Annexin-V staining (**Supplementary Fig. 3B and C**). There was no significant difference in the number of Ki-67-positive cells (proliferating cells)

**Table 1.** Lineage distribution in the PF of patients with endometriosis and control

| Variables | % of positive cells | Control | Endometriosis |
|---|---|---|---|
| T cells | CD3 | 27.77±5.56 | 22.72±4.17 |
| T cells | CD4 | 50.43±6.95 | 55.7±5.51 |
| T cells | CD8 | 18.72±5.24 | 15.07±2.62 |
| B cells | CD19 | 2.55±1.72 | 1.68±0.38 |
| Macrophage | CD16 | 26.16±5.07 | 34.02±4.88 |
| Macrophage | CD11b | 49.33±7.73 | 67.02±2.14 |
| Monocyte | CD14 | 31.37±6.29 | 40.00±4.27 |
| Neutrophils | CD15 | 0.54±0.07 | 1.69±0.40 |
| NK cells | $CD3^-CD56^+$ | 18.55±3.24 | 13.91±2.57 |
| DCs | $CD14^+CD11c^+$ | 28.80±6.15 | 38.66±4.64 |
| Th17 cells | $CD4^+IL\text{-}17^+ROR\gamma t^+$ | 1.15±0.13 | 4.48±0.56*** |

Data are presented as the mean ± SEM (control: n=10, endometriosis: n=26). The p-values were calculated using a 2-tailed unpaired Mann-Whitney *U* test.
DC, dendritic cell.
***Significant difference between groups, p<0.001.


https://doi.org/10.4110/in.2023.23.e14

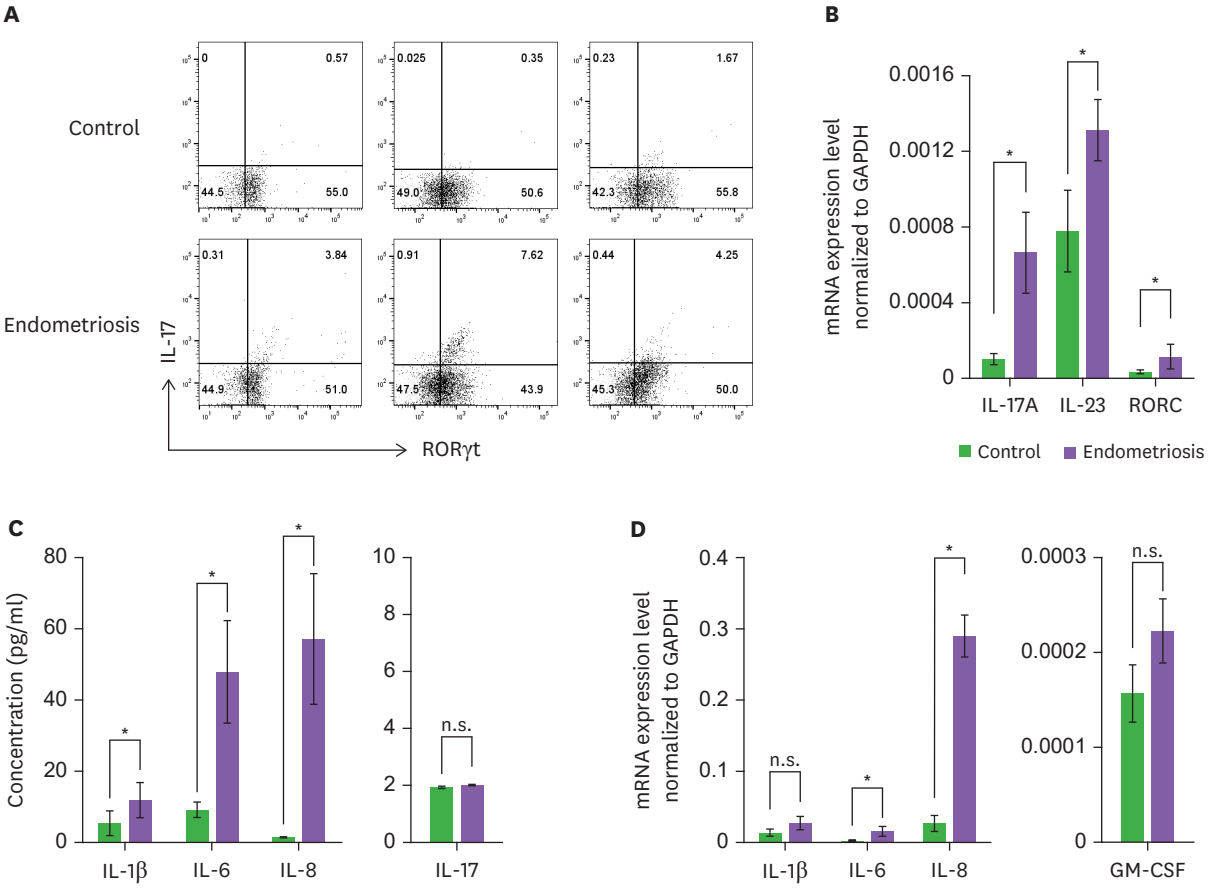


**Figure 1.** Th17 cell population is increased in PF from patients with endometriosis. (A) CD4 T cells in PF were selected by MACS and cultured with PMA and ionomycin. The data were analyzed after staining with anti-CCR6-PE-Cy7, anti-RORγt-PE and anti-IL-17A-APC using FACSCanto. The representative dot plots show Th17 cell populations ($CCR6^+IL\text{-}17A^+ROR\gamma t^+$) in $CD4^+$T cells from patients with (endometriosis=9) or without endometriosis (control=6) (number of $CD4^+$ T cells, control: $1.92X10^6$ cells vs endometriosis: $4.61\times10^6$ cells). (B) The mRNA expression of cytokines and transcription factors in Th17 cells was measured by RQ-PCR (IL-17A: 0.0001±0.00002 vs. 0.0007±0.0002; IL-23: 0.0008±0.0002 vs. 0.0013±0.0001; RORC: 0.00003±0.00001 vs. 0.0001±0.00006; control: n=8, endometriosis: n=8). (C) The levels of the cytokines IL-1β, IL-6, IL-8 and IL-17 in PF samples were determined by ELISA (IL-1β: 5.41 pg/ml±3.48 pg/ml vs. 11.9 pg/ml±4.9 pg/ml; IL-6: 9.19 pg/ml±2.16 pg/ml vs. 47.88 pg/ml±14.38 pg/ml; IL-8: 1.47 pg/ml±0.15 pg/ml vs. 57.13 pg/ml±18.35 pg/ml; IL-17: 1.93 pg/ml±0.04 pg/ml vs. 2.013 pg/ml±0.023 pg/ml; control: n=9, endometriosis: n=23). (D) The mRNA expression of the cytokines IL-1β, IL-6, IL-8 and GM-CSF were measured (IL-1β: 0.014±0.005 vs. 0.027±0.009; IL-6: 0.0022±0.0007 vs. 0.0157±0.006; IL-8: 0.026±0.01 vs. 0.29±0.029; control: n=8, endometriosis: n=8) on endometrial PF cells. The data are shown as the mean ± SEM. The p-values were calculated using a 2-tailed unpaired Mann-Whitney *U* test.
MACS, magnetic activated cell sorter; RQ-PCR, real-time quantitative polymerase chain reaction; n.s., not significant.
*$p<0.05$.

between control and IL-17A-treated cells (**Fig. 2B**), whereas the number of Annexin-V-positive and PI negative endometrial cells (apoptotic cells) were decreased after treatment with IL-17A (**Fig. 2C**). Furthermore, the expression levels of anti-apoptotic genes, such as Bcl-2, increased in endometrial cells treated with IL-17A (**Fig. 2D**). These results imply that IL-17A promotes endometrial cell survival by decreasing apoptosis rather than by increasing proliferation.

## IL-17A increases resistance of endometrial cells to NK cell mediated cytotoxicity

Since NK cells have been shown to development of endometriosis, we examined IL-17A effect of NK cell cytotoxicity. NK cell cytotoxicity against IL-17A-treated endometrial cells was lower than NK cell cytotoxicity against untreated endometrial cells (**Fig. 3A**). These suggest that IL-17A confers resistance to NK cell-mediated killing as well as apoptosis on endometrial cells. Furthermore, the expression of HLA-G which is a ligand of NK cell inhibitory receptor,


https://doi.org/10.4110/in.2023.23.e14

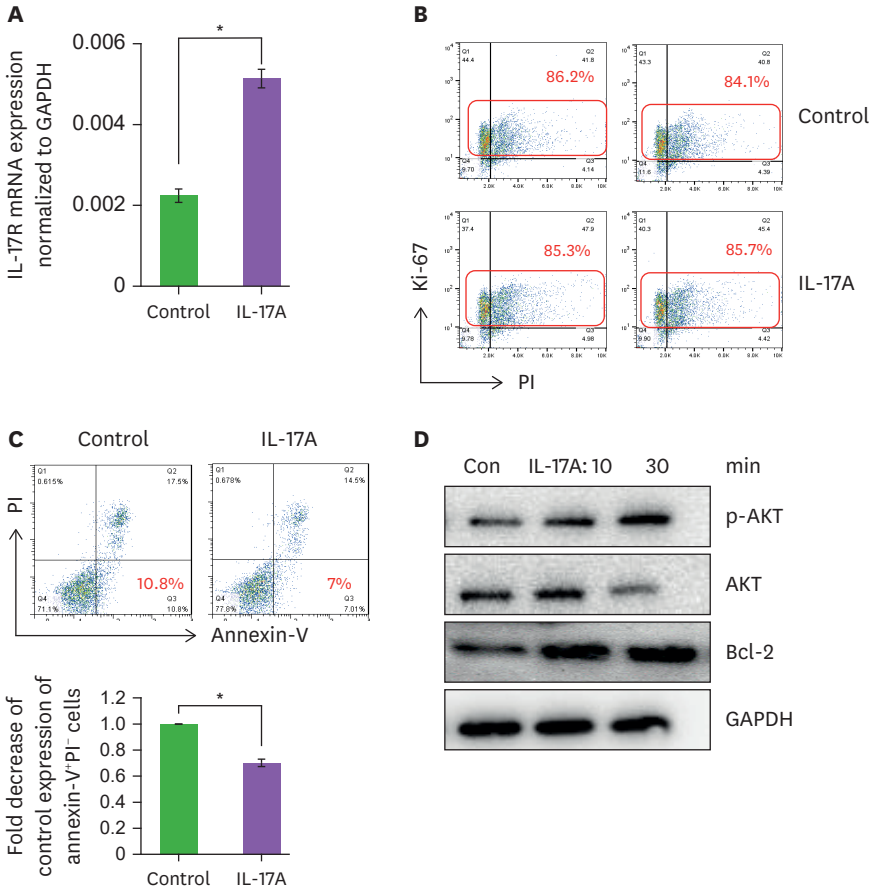


**Figure 2.** IL-17A promotes endometrial cell survival by inhibiting apoptosis of endometrial cells. (A) The mRNA expression of IL-17R on endometrial cells from ectopic endometrial tissue. The data are represented as the mean ± SEM of 3 experiments. (B, C) Endometrial cells were cultured for 48 h in complete medium with or without 100 ng/ml IL-17A. The cells were harvested and then stained with Ki-67, Annexin-V-FITC and PI. The data are represented as the mean ± SEM of 3 experiments. (D) Western blot analysis of p-AKT, total AKT and anti-apoptotic proteins (Mcl-1 and Bcl-2) in endometrial cells treated with or without 100 ng/ml IL-17A for 10 or 30 min. The data are presented as the mean ± SEM of 3 experiments. The p-values were calculated using a 2-tailed unpaired Mann-Whitney *U* test. PI, propidium iodide.
*p<0.05.

KIR2DL4 on IL-17A-treated endometrial cells was increased compared to untreated endometrial cells (0.092±0.009 vs. 0.13±0.006, p=0.006; **Fig. 3B and C**), which may partially contribute to increasing resistance of endometrial cells to NK cell cytotoxicity.

## IL-17A activates the ERK1/2 pathway in endometrial cells

IL-23/IL-17 signaling activated by microbial products promotes activating STAT3 phosphorylation in CRC epithelial cells (25), and IL-17 enhances the claudin-mediated development of the tight junctional barrier in T84 cell monolayers via the ERK/MAPK pathway in the intestine (26). Therefore, we investigated STATs and the ERK/MAPK signaling pathway to elucidate the action mechanism of IL-17A in endometrial cells. As shown in **Fig. 4A**, phosphorylation of ERK1/2 and p38 were increased in endometrial cells after treatment with IL-17A. However, STAT3 phosphorylation was unchanged (data not shown). To examine the role of ERK1/2 MAPK in IL-17A-mediated resistance to cell death, endometrial cells were treated with PD0325901, an ERK1/2 inhibitor. PD0325901 inhibited the IL-17A-induced phosphorylation of ERK1/2 and decreased expression of anti-apoptotic genes such as Bcl-2 (**Fig. 4A**). On the other hand, SB203580 as a p38 inhibitor did not affect expression level of anti-apoptotic genes (data not shown). IL-17A treatment reduced apoptosis level


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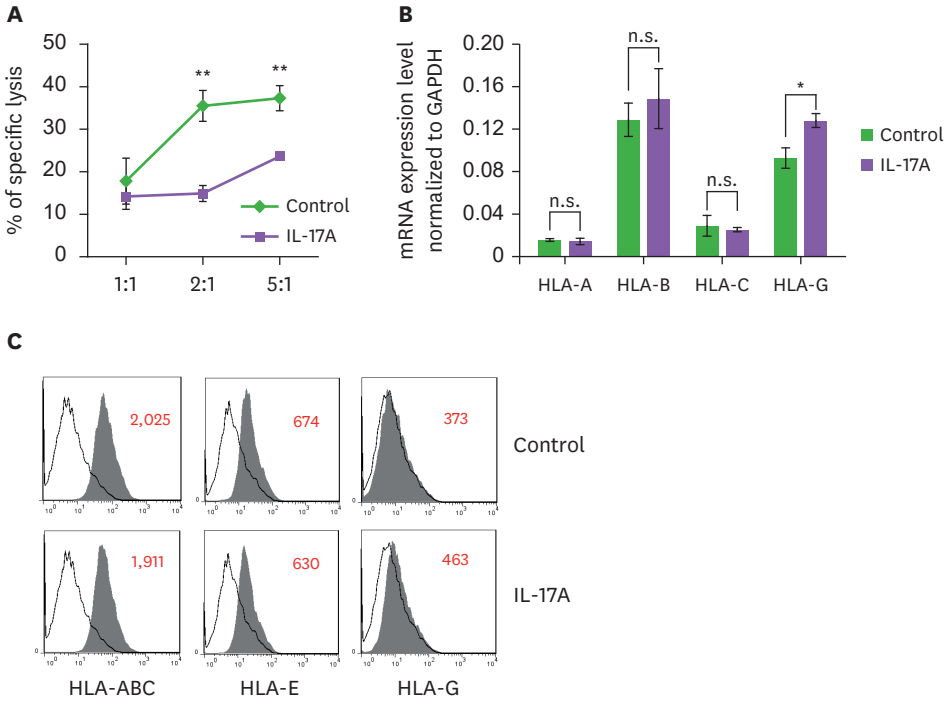


**Figure 3.** IL-17A increases resistance of endometrial cells to NK cell cytotoxicity. (A) NK92 cell cytolytic activity against endometrial cells treated with or without 100 ng/ml IL-17A was evaluated using a calcein-AM release assay. Calcein-labeled endometrial cells were co-cultured with effector cells in 96-well U-bottom plates for 4 h. Calcein released into the supernatant was measured with a multi-fluorescence reader. The data are represented as the mean ± SEM of 3 experiments. (B) The mRNA expression of HLA-G in endometrial cells was measured by RT-PCR. (C) Expression of HLA class on the endometrial cells were determined by FACS. The data are presented as the mean ± SEM of 3 experiments. The p-values were calculated using a 2-tailed unpaired Mann-Whitney *U* test.
n.s., not significant.
*p<0.05; **p<0.01.

of endometrial cells (annexin-V positive cells) and the reduced apoptosis was recovered by PD0325901 treatment (**Fig. 4B**). In addition, PD0325901 treatment with IL-17A decreased the IL-17A-induced resistance of endometrial cells to NK cell cytotoxicity and increased NK cell cytotoxicity, although PD0325901 treatment alone did not affect cytotoxicity (**Fig. 4C**). These results indicate that IL-17A elicits resistance to apoptosis and NK cell-mediated killing by activating the ERK1/2 pathway.

## IL-17A is involved in the migration of endometrial cells *in vitro*

IL-17A has also been reported to stimulate the migration and invasion of several types of cancer cells (24,27). In order to determine whether IL-17A promotes the migration of endometrial cells, cell migration assay was performed using 15 line trans-well culture plate system. As shown in **Fig. 5A**, IL-17A treated endometrial cells were more migrated than untreated endometrial cells showing IL-17A directly enhanced motility of endometrial cells *in vitro*. In addition, mRNA expressions of TWIST, SLUG, and SNAIL, which are known to be regulators of cell motility (28,29) were upregulated in IL-17A treated endometrial cells compared to untreated endometrial cells (**Fig. 5B**). Together, these imply that IL-17A is involved in the migration of endometrial cells *in vitro*.

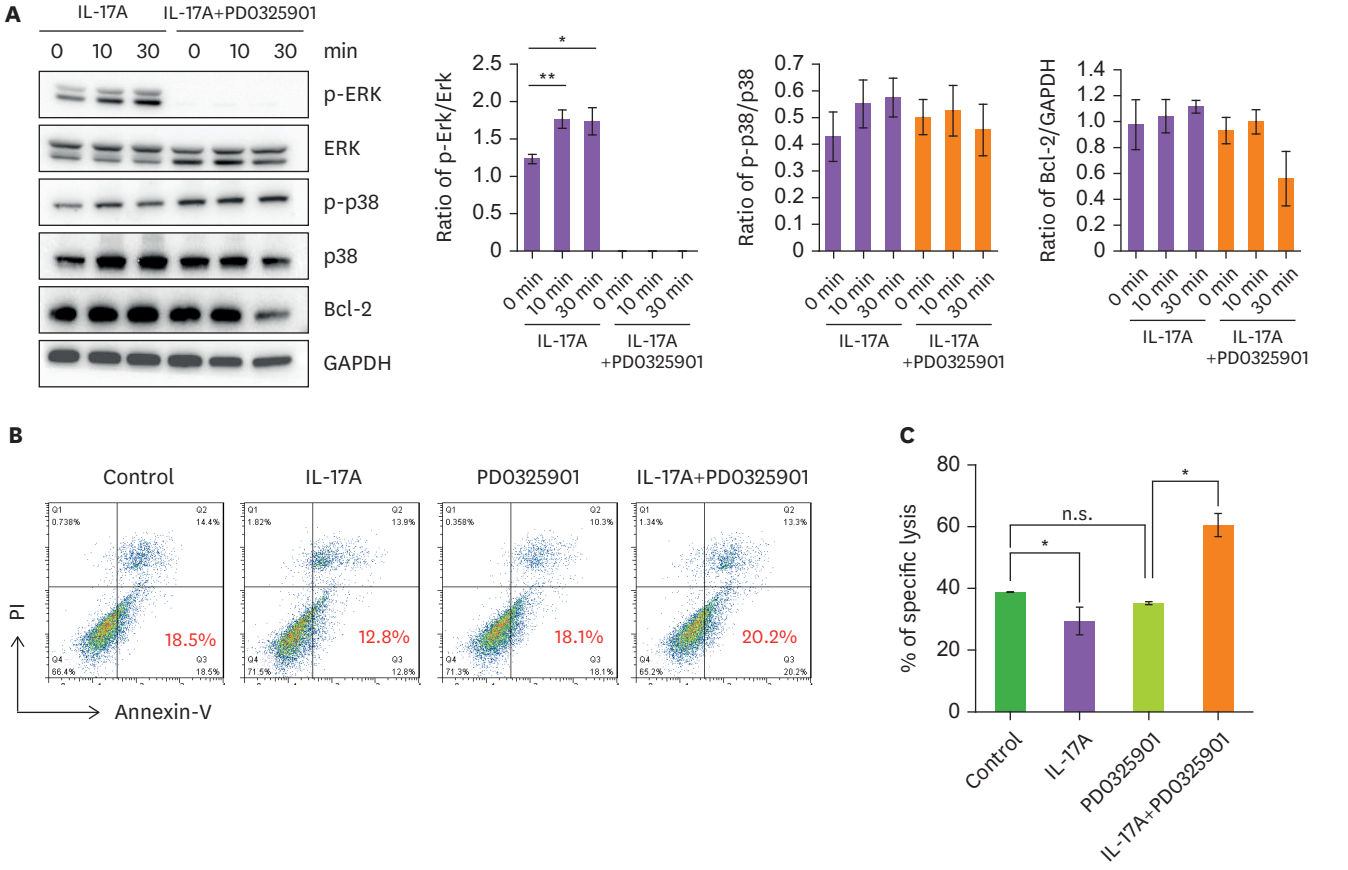


**Figure 4.** IL-17A activates the ERK1/2 pathway in endometrial cells. (A) Endometrial cells were treated with 100 ng/ml IL-17A and an ERK inhibitor (PD0325901). PD0325901 was added at 10 μM 1 h before the IL-17A treatment for 10 or 30 min. Left figure shows representative data of 3 experiments. Right figure shows the ratio of p-Erk/Erk, p-p38/p38, and Bcl-2/GAPDH which were determined using the program CSAnalyzer4. (B) Endometrial cells were cultured for 48 h in complete medium with or without 100 ng/ml IL-17A. PD0325901 was pretreated at 10 μM 1 h before the IL-17A treatment when it is indicated. The cells were harvested and then stained with Annexin-V-FITC and PI for apoptosis analysis. (C) The cytotoxicity was measured using the calcein-AM release assay. The data are representative of 5 experiments and are presented as the mean ± SEM. The p-values were calculated using a 2-tailed unpaired Mann-Whitney *U* test.
n.s., not significant; PI, propidium iodide.
*p<0.05; **p<0.01.

## DISCUSSION

In this study, we first demonstrated that IL-17A promotes the survival of endometrial cells by conferring resistance to apoptosis and NK cytotoxicity on endometrial cells through activating the ERK/MAPK pathway.

It has been generally accepted that cytokines within the endometriotic environment contribute to disease pathogenesis by promoting inflammation surrounding the implantation of endometriotic lesions. For instance, IL-1β, IL-6, IL-8, IFN-γ and TNF-α have been reported to be increased in PF from patients with endometriosis (30). Our previous study reported that IL-6 suppresses NK cell activity by regulating SHP-2 expression in endometriosis patients (21). IL-6 has been reported to promote endometrial cell growth by STAT3 signaling (31). We confirmed that IL-6 stimulates the growth of endometrial cells via STAT3 signaling (**Supplementary Fig. 2**). The number of Ki-67-positive cells (proliferating cells) and the phosphorylation levels of STAT3 were significantly increased in IL-6-treated endometrial cells compared to control cells. Since IL-6 has also been shown to be a critical cytokine that induces the differentiation of Th17 cells (32), the high concentration of IL-6 in PF from patients with endometriosis may elicit Th17

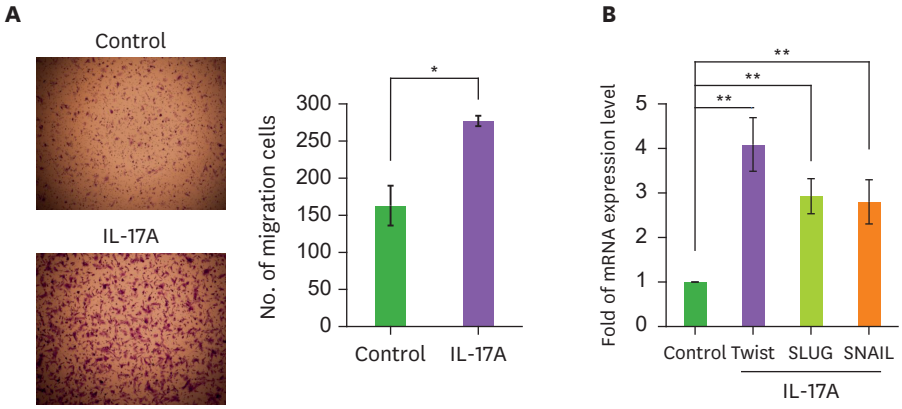


**Figure 5.** IL-17A is involved in the motility of endometrial cells *in vitro*. Cell migration was evaluated by a trans-well migration assay as described in the Materials and Methods. After incubation at 37°C for 8 h, migrated cells were counted at ×100 magnification. (A) The representative pictures are shown and the data are represented as mean ± SEM of 3 independent observations with different cell passages. (B) The mRNA levels of Twist, SLUG and SNAIL were analyzed by RQ-PCR. Data are presented as the mean ± SEM of 3 independent experiments. The p-values were calculated using a 2-tailed unpaired Mann-Whitney *U* test.
RQ-PCR, real-time quantitative polymerase chain reaction.
*p<0.05; **p<0.01.

cell differentiation. In fact, our data showed an increased population of Th17 cells that express IL-17A in PF from patients with endometriosis (**Fig. 1A and B**). Th17 cells that secrete the IL-17A and IL-17F cytokines and express the cell surface IL-23 receptor as well as the RORC lineage-specific transcription factor (RORγt in mice) have been identified as a distinct lineage of CD4 T cells (33). Thus, IL-23 signaling through IL-23R further enhances the activation of STAT3, which coordinates with RORγt to stabilize Th17 cells and their function (34,35). Consistently, our study demonstrated that the mRNA expression levels of IL-17A, IL-23 and RORC were increased in PF cells from patients with endometriosis compared to control patients (**Fig. 1B**), suggesting that Th17 cells participate in the development of endometriosis. Recently, it has been reported that Th17 and IL-17A are correlated with occurrence of endometriosis (14) and IL-17A contributes to the pathogenesis of endometriosis by triggering pro-inflammatory cytokines (17). However, functional role of IL-17A in endometrial cell survival and NK cell mediated cytotoxicity remain to be elucidated. Therefore, we isolated endometrial cells from endometriotic tissues and investigated the effect of IL-17A on apoptosis and migration of endometrial cells. IL-17A decreased apoptosis of endometrial cells, which were accompanied by increasing the expression of anti-apoptotic genes, such as Mcl-1 and Bcl-2 and inducing the phosphorylation of ERK1/2 MAPK. Mcl-1, an anti-apoptotic member of the Bcl-2 family, is phosphorylated at Thr163 by ERK1/2, thus increasing its stability and enhancing its anti-apoptotic activity. However, IL-17A did not affect proliferation of endometrial cells in our study, which is not consistent with previous report from Hirata et al. (15). Interestingly, IL-17A treatment to endometrial cells increased resistance to NK cell mediated killing leading to decrease in NK cell cytotoxicity against the endometrial cells (**Fig. 3A**). Human NK cells are the dominant immune cell type in the endometrium. They express specific markers such as CD9 and CD49a as well as inhibitory receptor, KIR2DL4 which known to interact with HLA-G molecules leading to inhibition of NK cell mediated killing (36). The expression of HLA-G on endometrial cells were increased by treatment of IL-17A (**Fig. 3B and C**), and this may partially contribute to resistance to NK cell mediated killing on endometrial cells. On the other hand, the IL-17A-induced resistance of endometrial cells to apoptosis and NK cell cytotoxicity was abolished by the treatment of ERK/12 inhibitor, PD0325901 (**Fig. 4B and C**). Together, these indicate that IL-17A promotes endometrial cell survival by conferring resistance to apoptosis and NK cell mediated cytotoxicity on endometrial cells through the activation of ERK1/2 pathway.


https://doi.org/10.4110/in.2023.23.e14

Recently, studies have demonstrated that the transcription factors Twist, SLUG and SNAIL increased cell migration (28,29,37). In our data, IL-17A directly promoted migration of endometrial cells in *in vitro* assay system and increased expression of the Twist, SLUG, and SNAIL in endometrial cells (**Fig. 5B**), which might contribute to the development of endometriosis.

In conclusion, the present study suggests that Th17 cells and IL-17A play important roles in the development of endometriosis by promoting endometrial cell survival and migration. These imply that IL-17A might be a candidate target for the treatment of endometriosis.

## ACKNOWLEDGEMENTS

We would like to thank Jung-Hyeon Choi (Medical Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology [KRIBB]) for technical assistance with immunohistochemistry. This work was supported by grants from the grant of National Research Foundation of Korea (NRF) (2019R1A2C1007906) and KRIBB Research Initiative Program from the Korea government.

## SUPPLEMENTARY MATERIALS

### Supplementary Figure 1

IL-17A$^+$ cells and CD4$^+$ T cells infiltrate into endometriosis tissues. Immunohistochemistry was performed on sections of endometrial tissues from 7 patients with endometriosis and 3 control patients. Representative pictures of cyst walls of ovarian endometrioma are shown from (A) anti-IL-17A staining and (B) anti-CD4 staining (magnification ×200). Brown staining cells indicate positive cells for IL-17A (A) and CD4 on paraffin sections (B), respectively. All the sections were counterstained with hematoxylin and examined under DIC imaging system.

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### Supplementary Figure 2

IL-6 promotes proliferation of endometrial cells. (A) Endometrial cells were treated with 100 ng/ml of IL-6 for indicated time and Western blot analysis was performed. IL-6 increased the phosphorylation of STAT3 and AKT in endometrial cells. (B) Endometrial cells were cultured for 48 h in complete medium with 100 ng/ml of IL-6 and proliferation was determined by Ki-67 and 7-AAD staining using FACS. IL-6 increased Ki-67 positive cells in endometrial cells.

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### Supplementary Figure 3

Gating strategy for FACS plots. Total lymphocytes were first gated on a FSC/SSC. (A) And then gated on the CD4$^+$ population. These were then further gated for the subsets of interest, namely, Rorγ(t)$^+$IL-17$^+$ cells. (B) And then gated Annexin-V$^+$ and PI$^+$ cells. (C) And then gated PI$^+$ and Ki-67$^+$ cells.

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