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Role of interleukin-32 in the pathogenesis of endometriosis: *in vitro*, human and transgenic mouse data

Mi-Young Lee[1], Sung Hoon Kim[1,*], Young Sang Oh[1], Seung-Ho Heo[2], Kang-Hyun Kim[2], Hee Dong Chae[1], Chung-Hoon Kim[1], and Byung Moon Kang[1]

[1]Department of Obstetrics and Gynecology, University of Ulsan College of Medicine, Asan Medical Center, 388-1, Pungnap-2dong, Songpa-gu, Seoul 138-736, Korea [2]Asan Institute for Life Sciences, University of Ulsan College of Medicine, Asan Medical Center, 388-1, Pungnap-2dong, Songpa-gu, Seoul 138-736, Korea

*Correspondence address. Department of Obstetrics & Gynecology, University of Ulsan College of Medicine, Asan Medical Center, 388-1, Pungnap-2dong, Songpa-gu, Seoul 138-736, Korea. Tel: +82-2-3010-3647; Fax: +82-2-476-7331; E-mail: kimsung@amc.seoul.kr

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STUDY QUESTION: Does interleukin-32 (IL-32) play a role in the pathogenesis of endometriosis?

SUMMARY ANSWER: IL-32 might be involved in the pathogenesis of endometriosis through increased viability, proliferation and invasion of endometrial cells.

WHAT IS KNOWN ALREADY: Endometriosis is characterized as a chronic inflammatory disease and several proinflammatory cytokines are suggested to be involved in its pathogenesis and pathophysiology. IL-32, recognized as a new proinflammatory cytokine and a strong inducer of other proinflammatory cytokines, has been shown to serve as a key modulator in several chronic inflammatory diseases.

STUDY DESIGN, SIZE, DURATION: This study included comparison of IL-32 levels in the peritoneal fluids between women with and without endometriosis, *in-vitro* experiments using Ishikawa cells and endometrial stromal cells (ESCs), and experiments on IL-32 transgenic mice and wild-type mice with induced endometriosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: IL-32 levels in the peritoneal fluids were measured using enzyme-linked immunosorbent assays. Cell viability, expression of proliferating cell nuclear antigen (PCNA), and cellular invasiveness were analyzed following *in-vitro* treatment of Ishikawa cells and ESCs with recombinant IL-32 alpha (α) and gamma (γ). Ectopic endometriotic lesions were compared between IL-32 transgenic mice and wild-type mice after autologous endometrial transplantation with immunohistochemistry for Ki-67 antigen and PCNA.

MAIN RESULTS AND THE ROLE OF CHANCE: The peritoneal fluid concentration of IL-32 was significantly higher in patients with advanced stage endometriosis compared with the controls. *In-vitro* treatment with IL-32 α and γ caused significant increases in cellular viability, PCNA expression, and invasiveness in Ishikawa cells and ESCs. The IL-32 transgenic mice had a significantly larger size of the ectopic endometrial lesions with higher expression of Ki-67 antigen and PCNA compared with wild-type mice.

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: It is still unclear whether IL-32 is a main regulator, or one of several downstream proinflammatory cytokines, causing establishment and/or progression of endometriosis.

WIDER IMPLICATIONS OF THE FINDINGS: Further investigation on IL-32 signaling pathways may contribute to development a more effective treatment of endometriosis.

STUDY FUNDING/COMPETING INTEREST(S): This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant number: HI16C1682). None of the authors has anything to disclose.

Key words: cytokine / endometriosis / interleukin-32 (IL-32) / pathogenesis / inflammatory disease

Introduction

Endometriosis is characterized by the presence and proliferation of functional endometrial tissue outside the uterine cavity, usually on the pelvic peritoneum, ovaries and rectovaginal septum and is often responsible for pelvic pain and infertility (Giudice and Kao, 2004). Although its etiologies and mechanisms still remain unclear, it is widely accepted that endometriosis is an estrogen-dependent chronic inflammatory disease, and several proinflammatory cytokines have been suggested to play a crucial role in inflammatory reactions associated with endometriosis (Fakih *et al.*, 1987; Eisermann *et al.*, 1988; Hill and Anderson, 1989; Harada *et al.*, 1997; Bulun, 2009; Mori *et al.*, 1991; Ho *et al.*, 1997; Suen *et al.*, 2014).

IL-32 has been recognized as a new proinflammatory cytokine and a strong inducer of other proinflammatory cytokines, such as TNF-α and IL-8 (Kim *et al.*, 2005). IL-32 is located on human chromosome 16p13.3, and at least nine splice variants of IL-32 have been identified so far; IL-32 α, β, γ, δ, ε, ζ, η, θ and ι (Kim *et al.*, 2005; Goda *et al.*, 2006; Kang *et al.*, 2014). Although these isoforms may have different biological activities, not all of the functions of each isoform have been well described. The most abundant isoform in human NK cells seems to be α, and the most active isoform is γ, which is the longest splice variant containing all exons (Choi *et al.*, 2009). IL-32 is expressed in most human tissues including brain, bone marrow, immune systems, endocrine tissues, lungs, gastrointestinal tract, liver, pancreas, urological tract, skin, adipose tissues and reproductive organs (The human protein atlas) and the expression is more prominent in immune cells than in non-immune tissues (Hong *et al.*, 2017).

Originally it was shown that IL-32 activates typical cytokine signal pathways of nuclear factor-kappa B and p38 mitogen-activated protein kinase (Kim *et al.*, 2005). However, the detailed downstream signaling pathway of IL-32 remains undefined since the IL-32 receptor has not been identified (Ribeiro-Dias *et al.*, 2017). IL-32 is a mediator of the inflammatory response and has been shown to control immune function, host defense and cell death (Bai *et al.*, 2010; Li *et al.*, 2010; Joosten *et al.*, 2013). IL-32 is also involved in several inflammatory diseases, such as rheumatoid arthritis, chronic obstructive pulmonary disease, chronic rhinosinusitis, ankylosing spondylitis and inflammatory bowel disease (Shioya *et al.*, 2007; Calabrese *et al.*, 2008; Ciccia *et al.*, 2012; Soyka *et al.*, 2012).

Based on the emerging importance of IL-32 in several inflammatory conditions, IL-32 expression may also have a potential role in the pathogenesis of endometriosis, which is characterized by chronic inflammation. However, the role of IL-32 has not been reported in endometriosis so far. Therefore, the present study aimed to evaluate the possible role of IL-32 in the pathogenesis of endometriosis. We compared IL-32 levels in peritoneal fluid (PF) between women with and without endometriosis, evaluated the *in-vitro* effects of IL-32 on viability, proliferation and invasiveness of endometrial cells, and investigated the effect of IL-32 on endometriosis using an IL-32 transgenic (TG) mouse model.

Materials and Methods

Tissue and sample collection

For endometrial stromal cell (ESC) cultures, endometrial samples were obtained from fertile women ($n = 10$) diagnosed with intramural leiomyoma at the time of hysterectomy. Women with endometrial abnormalities, adenomyosis or pelvic endometriosis, and those who had taken any hormonal medication in the preceding 3 months were excluded. The endometrial samples were placed in Hank's balanced salt solution (HBSS) and transported to the laboratory for ESC isolation and culture.

PF was collected in the follicular phase from women with advanced stage endometriosis ($n = 45$) and from controls ($n = 40$) during laparoscopic surgery. PF was obtained from the posterior cul-de-sac or uterovesical pouch through laparoscopic cannula. We excluded any PF samples with blood-contamination. The cellular components of the PF were removed by centrifugation at 3500 revolutions per minute for 15 min. The PF supernatant was then collected and stored in aliquots at –70 to 80°C until analyzed. All of the patients in the endometriosis group were confirmed as having ovarian endometrioma by histological report. All of the patients in the control group were confirmed as having no endometriotic lesions by laparoscopy, and they had histological diagnoses of uterine leiomyoma ($n = 29$) and benign ovarian cyst ($n = 11$). The clinical characteristics of cases and controls are summarized in the Table I.

Written informed consent was obtained from each patient using consent forms and protocols were approved by the Institutional Review Board for Human Research of Asan Medical Center.

Cell line, cytokines and animals

The Ishikawa cell line (a well-differentiated endometrial adenocarcinoma cell line) was purchased from Sigma-Aldrich. Recombinant IL-32 alpha (α) and gamma (γ) were purchased from R&D Systems. IL-32γ TG mice on a C57BL/6 background were obtained from Professor Kim's laboratory (Konkuk University, Korea) (Choi *et al.*, 2010). C57BL/6 mice were purchased from SLC Inc.

Isolation and culture of human ESC

ESCs were separated and maintained in monolayer culture as described previously (Kim *et al.*, 2009). Following isolation, ESCs were passed by standard methods of trypsinization, plated in culture dishes, and grown in DMEM supplemented with 10% charcoal-stripped calf serum (Flow Laboratories). ESCs after the first passage were assayed immunocytochemically using specific cell-surface markers and we have previously shown that the purity of isolated ESCs was more than 95% (Kim *et al.*, 2009; Lee *et al.*, 2009). We utilized only the cells after the first passage in all experiments using ESCs.

Table I Clinical characteristics of patients and controls.

	Control group ($n = 40$)	Endometriosis group ($n = 45$)	*P*-value
Age[a]	39.4 ± 1.2	36.2 ± 1.3	0.065[b]
No. of deliveries[a]	1.3 ± 0.2	0.7 ± 0.1	<0.001[b]
Body mass index[a] (kg/m^2)	22.5 ± 0.6	21.0 ± 0.5	0.102[b]
Smokers (%)	2 (5%)	4 (8.9%)	0.485[c]
Married women (%)	30 (75%)	25 (55.6%)	0.080[c]
Infertility (%)	0	2 (4.4%)	0.177[c]
AFS classification of endometriosis			
Stage III		34	
Stage IV		11	

[a]Values are mean ± SE.
[b]Derived from Mann–Whitney *U* test.
[c]Derived from chi-square test.
AFS, American Fertility Society.

Experimental setup for IL-32 treatment

In each experiment, different cell batches of Ishikawa cells or separate passages of ESCs from different patients were utilized. After growth of the Ishikawa cells and the ESCs to 70% confluence, they were treated with serum-free, phenol red-free medium (Sigma-Aldrich) for 24 h before treatment with IL-32 α and IL-32 γ. To evaluate the effect of IL-32 on the cellular viability, proliferating cell nuclear antigen (PCNA) expression and cellular invasiveness, cells were treated with vehicle (control), IL-32α (25 or 50 ng/ml) or IL-32γ (25 or 50 ng/ml).

Cell viability assay

Cell survival was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Titer 96 Aqueous Cell Proliferation Assay kit; Promega). Absorbance was assessed in a microplate reader at a wavelength of 490 nm, and the results were expressed as a percentage of the absorbance observed in control (untreated) cells.

Protein extraction and western blot analysis

The cells were resuspended in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protease inhibitor cocktail (complete mini tablet, Roche). The membranes were blocked by incubation for 1 h at room temperature prior to overnight incubation at 4°C with primary antibodies raised against PCNA (Cell Signaling Technology) and β-actin (Cell Signaling Technology). After three washes, the membranes were further incubated with a secondary horseradish peroxidase-conjugated anti-IgG antibody (Invitrogen, Carlsbad, CA, USA) and visualized using a Pierce chemiluminescent substrate (Life Technologies, Carlsbad, CA, USA). Densitometric quantification of the bands was performed using a laser densitometer. Bars represent the mean of three replicates.

Invasion assay

Cell invasion was analyzed using a 96-well transwell plate containing 8-μm pore size inserts (Corning) coated with Cultrex basement membrane extract (Trevigen). After starvation of the cells in serum-free DMEM for 18 h, 50 μl of cell suspension (50,000 cells/well) was added to the top chamber and 100 μl of serum-free DMEM with vehicle, IL-32α (25 or 50 ng/ml) or IL-32γ (25 or 50 ng/ml) were added to the bottom chamber. The chambers were incubated in humidified air with 5% CO_2 at 37°C for 24 h. After 24 h incubation, the top chamber inserts were washed with washing buffer to remove non-invading cells, and the inserts were transferred to an assay chamber plate to analyze the number of invaded cells. The invaded cells were labeled with 5 μg/ml calcein-AM (Trevigen) in cell dissociation solution at 37°C for 1 h. Cell invasion was assessed by measuring the absorbance of samples at 485 nm excitation and 520 nm emission using the same parameters (time and gain) with a Victor X3 multilabel plate reader (PerkinElmer).

Enzyme-linked immunosorbent assay (ELISA) for IL-32, IL-1β and TNF-α

IL-32, IL-1β and TNF-α levels in the PF as well as IL-32 levels in the cell supernatants and cell lysates were quantitated using each ELISA kit (R&D Systems) according to the manufacturer's instructions. Absorption of the streptavidin-horseradish peroxidase color reaction was measured at 450 nm and then the optical density was corrected at 570 nm. The optical density was compared with serial dilutions of recombinant human IL-32, IL-1β and TNF-α as a standard.

Autotransplantation of endometrial tissues in IL-32 transgenic and wild-type mice

Five-week-old female IL-32γ TG mice and C57BL/6 mice were ovariectomized and had an acclimatization period of two weeks. They were housed in laminar flow filtered hoods and fed with sterilized food and water. After ovariectomy, sterile 60-day release capsules containing 0.05 mg 17 β-estradiol (Innovative Research of America) were inserted subcutaneously at a site just below the scapula. Before invasive procedures, the mice were anesthetized via an intra-peritoneal injection of a mixture of 40 mg/kg tiletamine/zolazepam (Zoletil, Virbaclab) and 10 mg/kg xylazine (Rumpum, Bayer Korea). Endometriotic lesions were experimentally induced by autotransplantation of right uterine horn onto the peritoneal wall. A mid-ventral incision was made and then a right uterine horn was excised. The uterine horn was opened longitudinally and then cut into square pieces measuring 4 × 4 mm^2. Each of endometrial fragments of equal size was sutured onto the peritoneal wall. There was no visible difference in the gross morphology of uterus between TG and wild-type (WT) mice. Thirty-one days after transplantation, the mice were sacrificed and then the lesions were photographed, removed and measured before fixation for immunostaining. The protocol was approved by Institutional Animal Care and Use Committee of Asan Medical Center.

Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded tissue blocks of endometriotic lesions were cut (3 μm thickness) and mounted on glass slides. Immunohistochemistry was carried out using an automated slide preparation system Benchmark XT (Ventana Medical systems, Inc.). Deparaffinization, epitope retrieval, and immunostaining were performed according to the manufacturer's instructions by using cell-conditioning solutions (CC1) and the BMK ultraVIEW diaminobenzidine detection system (Ventana Medical Systems). Lesion sections were stained with PCNA (1:2000) and Ki-67 (1:100) (Abcam). Positive signals were amplified using ultra-VIEW copper, and sections were counterstained with hematoxylin and blueing reagent. The tissue slides were imaged using Vectra intelligent Slide Analysis System (version 2.0.8, PerkinElmer Inc.) and then these images were used to train the in Form Advanced Image Analysis Software (version 2.2, PerkinElmer Inc.) for quantitative image analysis.

Statistical analysis

Categorical variables were compared using the chi-square test or Fisher's exact test. All data were assessed by the Kolmogorov–Smirnov test to evaluate whether they were normally distributed. If the data were normally distributed, continuous variables were compared using Student's *t* test (two groups) or analysis of variance and Fisher's least significant difference post-hoc test for pairwise comparisons (three groups). If the data were not normally distributed, they were compared using the Mann–Whitney *U* test (two groups). Correlations between IL-32 and IL-1β or TNF-α levels were analyzed by Spearman correlation analysis. Statistical computations were conducted using the Statistical Program for the Social Sciences version 14.0 software. $P < 0.05$ was considered statistically significant.

Results

Comparison of IL-32, IL-1β and TNF-α levels between PF from women with and without endometriosis

IL-32, IL-1β and TNF-α levels were compared between PF collected from women with ($n = 45$) and without endometriosis ($n = 40$). The PF concentration of IL-32 was significantly higher in the endometriosis group compared with the control group ($P = 0.03$) (Fig. 1A), whereas there were no significant differences in IL-1β and TNF-α levels between the two groups (Fig. 1B and C). When we analyzed the IL-32 levels with IL-1β or TNF-α to see whether there exists any correlation between them, we could not see any significant correlation.

IL-32 levels in cell supernatant and cell lysate of cultured endometrial cells

To see the endogenous levels of IL-32 expressed by endometrial cells, we measured IL-32 levels in cell supernatant and cell lysate of cultured Ishikawa cells as well as ESCs after 24 h incubation without any treatment (Fig. 2A). In supernatants, IL-32 levels were 17.5 ± 4.2 pg/ml (mean ± S.E) in Ishikawa cells ($n = 6$) and 24.1 ± 6.4 pg/ml in ESCs ($n = 6$). In cell lysates, IL-32 levels were 117.4 ± 5.1 pg/ml in Ishikawa cells ($n = 6$), and 192.7 ± 8.4 pg/ml in ESCs ($n = 6$).

Cell viability following IL-32 treatment

Cell viability was evaluated in Ishikawa cells ($n = 8$) and ESCs ($n = 8$) treated with the vehicle, IL-32 α (25 or 50 ng/ml), or IL-32 γ (25 or 50 ng/ml), respectively. In Ishikawa cells, viability was significantly increased after treatment with IL-32α 25 ng/ml ($P = 0.015$ at 24 h and $P < 0.001$ at 48 h, respectively) as well as IL-32α 50 ng/ml ($P = 0.019$ at 24 h and $P = 0.026$ at 48 h, respectively) compared with cells treated with vehicle (Fig. 2B). Treatment of Ishikawa cells with IL-32 γ 25 ng/ml and 50 ng/ml also caused a significant increase in viability at 24 h compared with cells treated with vehicle ($P < 0.001$ and $P = 0.034$, respectively) (Fig. 2D). In ESCs, treatment with IL-32α 25 ng/ml led to a significant increase in viability at 24 h ($P = 0.008$) compared with the controls (Fig. 2C). IL-32 γ also caused a significant increase in viability with the concentration of 25 ng/ml at 24 h ($P < 0.001$) and with 25 and 50 ng/ml at 48 h ($P < 0.001$ and $P < 0.001$, respectively) compared with the controls (Fig. 2E).

PCNA expression following IL-32 treatment

PCNA expression was analyzed in Ishikawa cells ($n = 7$) and ESCs ($n = 7$) treated with vehicle or IL-32α (25 or 50 ng/ml). PCNA expression was significantly increased at 24 h after treatment with IL-32α 25 and 50 ng/ml in both Ishikawa cells ($P = 0.026$ and $P = 0.026$, respectively) and ESCs ($P = 0.025$ and $P = 0.025$, respectively) compared with the controls (Fig. 3A and B). PCNA expression was also compared in Ishikawa cells ($n = 8$) and ESCs ($n = 6$) among those treated with vehicle, IL-32γ 25 ng/ml or IL-32γ 50 ng/ml. Compared with the controls, PCNA expression was significantly increased at 24 h after treatment with IL-32γ 25 and 50 ng/ml in ESCs ($P = 0.021$ and $P = 0.016$, respectively), whereas there was no significant difference of PCNA expression following IL-32γ treatment in Ishikawa cells (Fig. 3C and D).

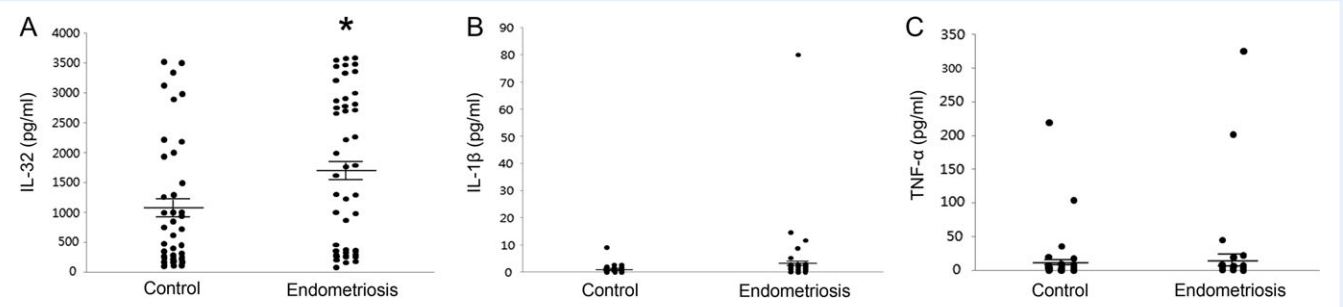

Figure 1 The peritoneal fluid concentrations of interleukin-32 (IL-32), interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) in women with and without endometriosis. *$P < 0.05$ vs control.

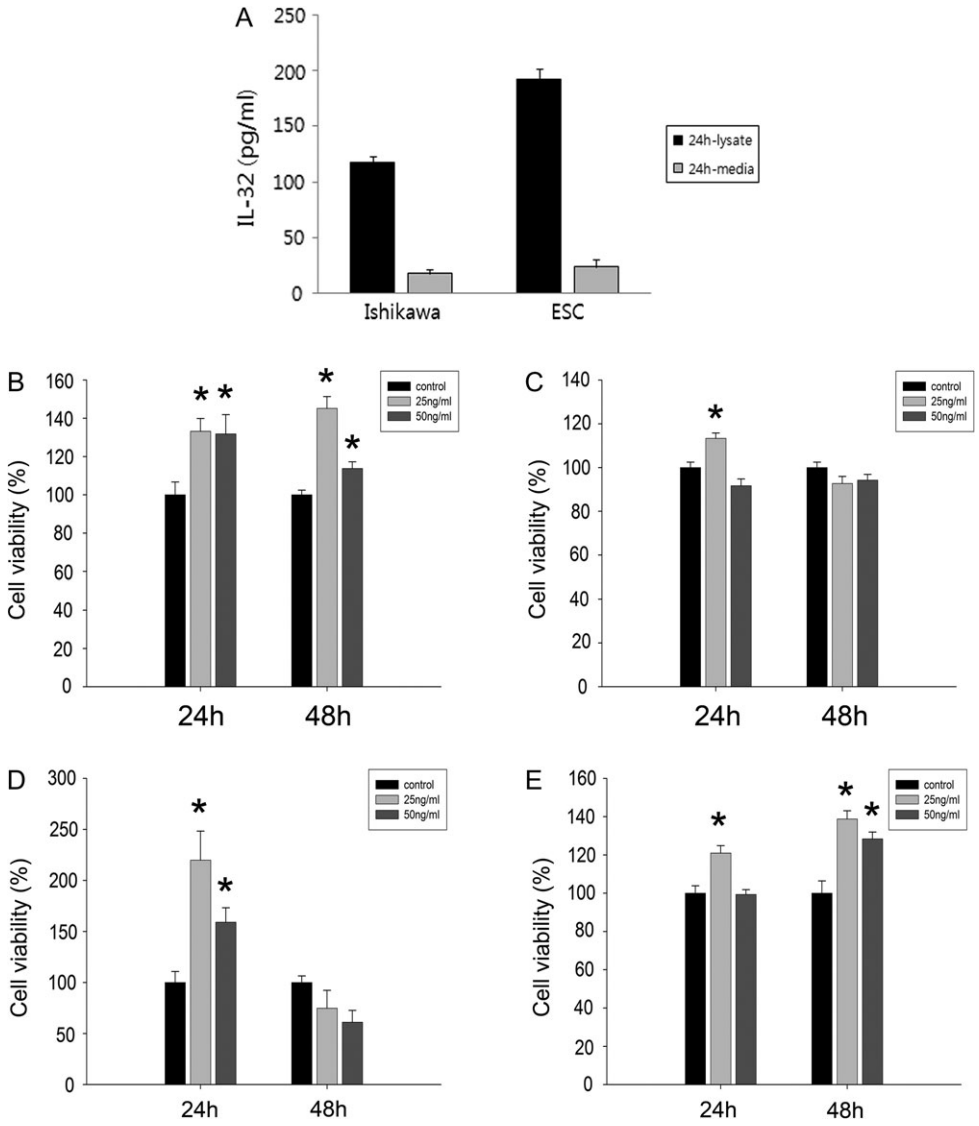

Figure 2 Interleukin-32 (IL-32) levels in cell supernatant and cell lysate of cultured endometrial cells (**A**), Cellular viability of Ishikawa cells (**B**, **D**) and endometrial stromal cells (**C**, **E**) following treatment with IL-32 alpha (B, C) or IL-32 gamma (D, E). Error bars show the mean ± SEM. *$P < 0.05$ vs control. Data are expressed as a percentage, wherein cells treated with vehicle are normalized to 100% (B, C, D, E). Ishikawa: Ishikawa cell line; ESC: endometrial stromal cell; 24 h: 24 h treatment; 48 h: 48 h treatment.

Cell invasiveness following IL-32 treatment

Cell invasiveness was analyzed in Ishikawa cells ($n = 6$) and ESCs ($n = 6$) treated with vehicle or IL-32α (25 or 50 ng/ml). Invasiveness was significantly increased at 24 h after treatment with IL-32α 50 ng/ml in Ishikawa cells ($P < 0.001$) compared with controls (Fig. 4A). Treatment of ESCs with IL-32α 25 and 50 ng/ml also led to a significant increase in invasiveness at 24 h ($P = 0.023$ and $P < 0.001$, respectively) (Fig. 4B). Cell invasiveness was also evaluated in Ishikawa cells ($n = 10$) and ESCs ($n = 10$) treated with vehicle or IL-32γ (25 or 50 ng/ml). In Ishikawa cells, IL-32γ treatment with 25 or 50 ng/ml for 24 h did not cause a significant difference compared with the controls (Fig. 4C). In the ESCs, cell invasiveness was significantly increased at 24 h after treatment with IL-32γ 25 and 50 ng/ml ($P = 0.047$ and $P = 0.018$, respectively) compared with the controls (Fig. 4D).

Comparison of endometrial tissue implants between IL-32γ TG mice and WT mice with immunohistochemical staining

The ectopic endometrial tissues of IL-32γ TG mice ($n = 7$) and WT mice ($n = 6$) have well-preserved gland and stroma without any necrotic areas (Fig. 5A). When we compared the total volume of the ectopic endometrial tissues between IL-32γ TG mice and WT mice, we could see that the volume of the ectopic endometrial tissues was significantly larger in IL-32γ TG mice compared with WT mice ($P = 0.041$) (Fig. 5A). Immunohistochemical staining showed that the expression levels of Ki-67 and PCNA were significantly higher in IL-32γ TG mice than in WT mice ($P = 0.032$, and $P = 0.012$, respectively) (Fig. 5B and C).

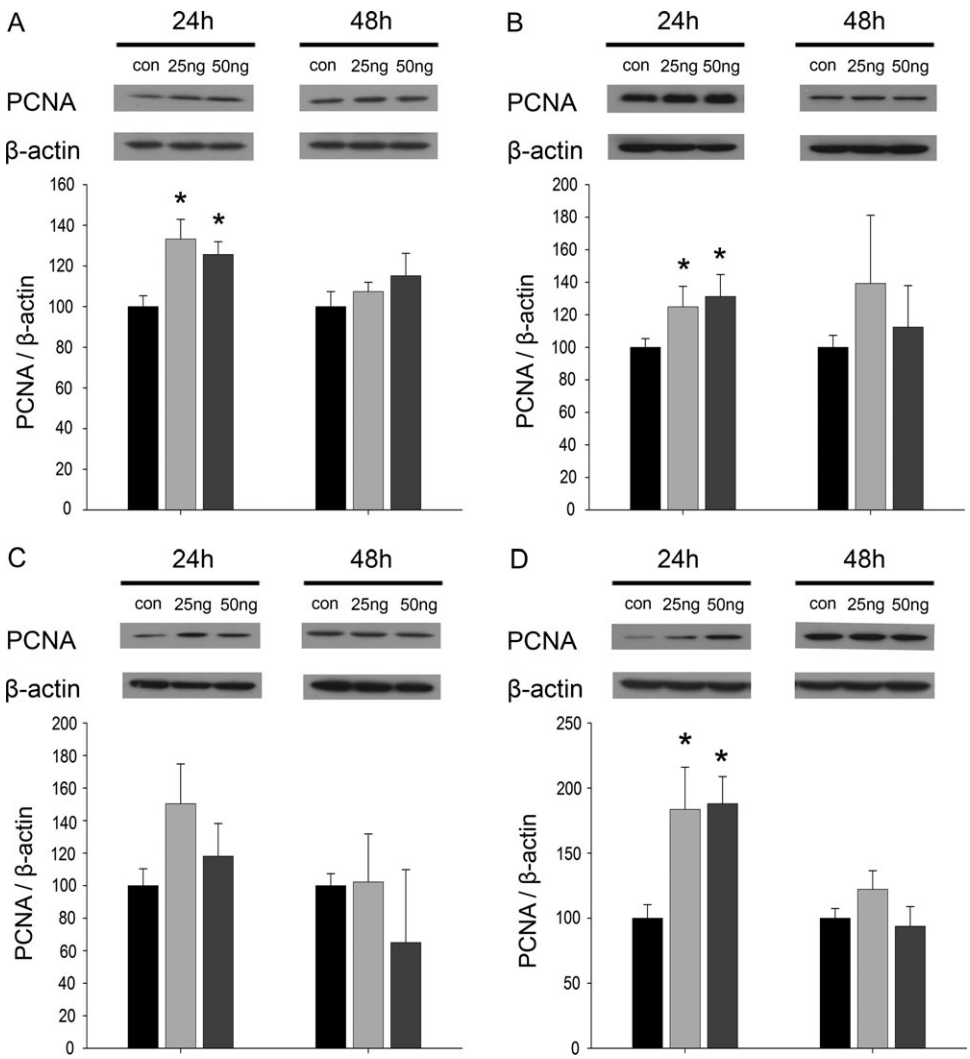

Figure 3 Effects of *in-vitro* interleukin-32 (IL-32) treatment on PCNA expression in endometrial cells. PCNA expression levels were measured by western blot analyses in Ishikawa cells (**A**, **C**) and endometrial stromal cells (B, D) following treatment with IL-32 alpha (**A**, **B**) or IL-32 gamma (**C**, **D**). Error bars show the mean ± SEM. *$P < 0.05$ vs control. Data are expressed as a percentage, wherein cells treated with vehicle are normalized to 100%. con: control; 25 ng: 25 ng/ml; 50 ng: 50 ng/ml; 24 h: 24 h treatment; 48 h: 48 h treatment.

Discussion

To the best of our knowledge, the current study is the first one to demonstrate the possible role of IL-32 in the pathogenesis of endometriosis. We found that IL-32 levels were significantly increased in the PF from women with endometriosis compared with the controls. We also showed that *in-vitro* IL-32 treatment of endometrial cells led to significant increases in cellular viability, proliferation and invasiveness. Moreover, we could see that IL-32γ TG mice had a significantly larger size of the ectopic endometrial lesions with higher Ki-67 and PCNA expression compared with WT mice. These findings strongly suggest that IL-32 may play a critical role in the pathogenesis of endometriosis through increasing viability, proliferation and invasion of endometrial cells.

Given that endometriosis is well characterized as an estrogen-dependent chronic inflammatory disease, we hypothesized that IL-32 might play a role in the pathogenesis and/or pathophysiology of endometriosis. We compared the IL-32 levels in the PF between women with and without endometriosis along with TNF-α and IL-1β levels, and could see that only the IL-32 level was significantly higher in the endometriosis group compared with the controls. While several studies have shown increased TNF-α and/or IL-1β levels in the PF of women with endometriosis (Fakih *et al.*, 1987; Eisermann *et al.*, 1988; Hill and Anderson, 1989; Mori *et al.*, 1991), others found no significant difference in TNF-α and/or IL-1β levels between the PF of women with and without endometriosis (Cheong *et al.*, 2002; Dziunycz *et al.*, 2009; Barcz *et al.*, 2012). Barcz *et al.* (2012) suggested that this discrepancy among various studies might have resulted from unrevealed differences between the subjects such as disease activity, type of the lesions, menstrual phase and genetic diversity. Although the present study showed a more prominent increase of IL-32 than TNF-α and IL-1β in the PF of women with endometriosis, a further study is necessary

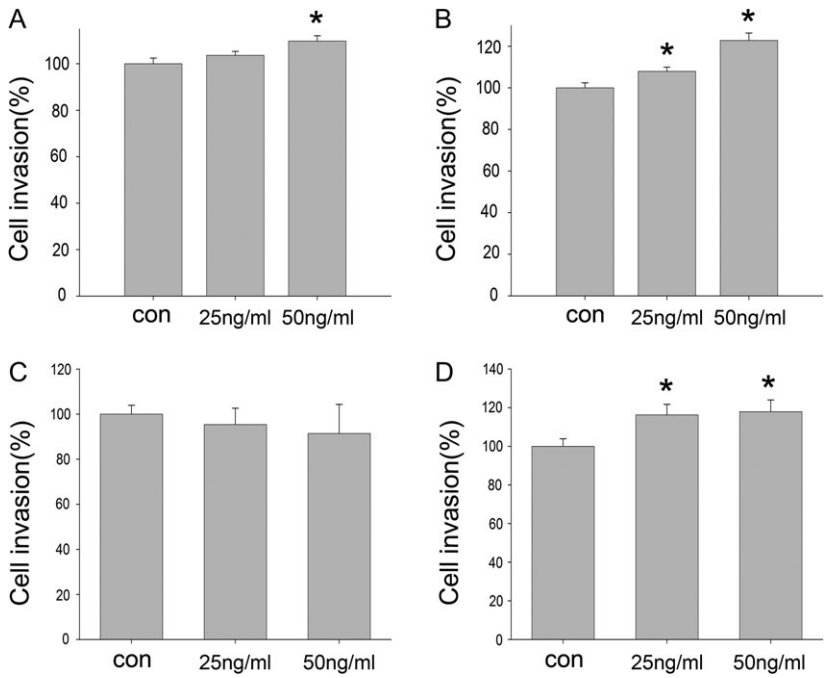

Figure 4 Cellular invasiveness of Ishikawa cells (**A**, **C**) and endometrial stromal cells (**B**, **D**) at 24 h after treatment with interleukin-32 alpha (A, B) or interleukin-32 gamma (C, D). Error bars show the mean ± SEM. *$P < 0.05$ vs control. Data are expressed as a percentage, wherein cells treated with vehicle are normalized to 100%.

to confirm this finding by recruiting much larger number of samples that can make adjustment for several confounding variables possible. It is also noteworthy that the IL-32 levels are extraordinarily higher in PF of controls and endometriosis group (~1.0 ng/ml and 1.5 ng/ml, respectively) than TNF-α or IL-1β in the present study. Other previous studies on IL-32 also have reported very high levels of IL-32 in other body fluids, synovial fluid (~12 ng/ml in ankylosing spondylitis), sputum (~7 ng/ml in healthy controls) and serum (~2.5 ng/ml in of healthy controls) (Bang *et al.*, 2014; Lee *et al.*, 2015), which suggests that IL-32 concentrations might be much higher in various body fluids compared with well-known proinflammatory cytokines such as TNF-α or IL-1β.

We have shown that viability and PCNA expression of endometrial cells were significantly increased after treatment with both IL-32α and IL-32γ. We could also see that cellular invasiveness was obviously increased following both IL-32α and IL-32γ treatment in the ESCs. Taken together with the findings that the IL-32 level is elevated in PF of women with endometriosis, it can be suggested that endometrial cells refluxed to the pelvis through retrograde menstruation can survive and grow at ectopic locations due to the action of IL-32 and they can invade to adjacent tissues, eventually leading to establishment and progression of endometriosis.

The effects of IL-32 α and γ on the viability of both cell types seem to be more obvious by 25 ng/ml treatment compared with 50 ng/ml treatment. Since the concentration of IL-32 in the PF of endometriosis group is ~1.5 ng/ml, the concentrations used in the present study (25 and 50 ng/ml) may seem to be very high levels. Although we do not have any data on the effect of IL-32 in lower concentrations, it is possible that the endometrial cells may have increased cellular viability by IL-32 in a specific range of concentrations, beyond which no obvious differences can be found. This might be due to desensitization of the downstream signaling pathway of IL-32 at a certain concentration of IL-32, although there is no data as yet to support this. We could see ~20% increases in cellular invasiveness of ESCs following IL-32α or γ. Considering that the cellular invasion can be increased to ~50% or 150% level by *in-vitro* IL-8 or IL-1β treatment in other studies (Zhang *et al.*, 2011; Luo *et al.*, 2015), the effect of IL-32 on cellular invasion might be weaker than other proinflammatory cytokines. Alternatively, it is also possible that the all of the IL-32 isoforms together might lead to a much greater increase in invasiveness compared to treatment with each isoform alone, since we treated the ESCs with either the IL-32α subunit or the IL-32γ subunit.

In the present study, we have utilized the Ishikawa cell line as a surrogate of human endometrial glandular cells. We could see that the Ishikawa cell data are consistent with those of ESCs in that both IL-32 α and γ led to significant increases in cellular viability. However, PCNA expression was significantly increased by IL-32γ treatment only in ESCs, and there was no significant difference in PCNA expression following IL-32γ treatment in Ishikawa cells. Moreover, only ESCs have a significantly increased cellular invasiveness following IL-32γ treatment, whereas Ishikawa cells showed no difference of invasiveness with IL-32γ treatment. Although we have no data explaining these discrepancies between the two cell types in the present study, it is possible that there might be some differences in the cellular response to IL-32γ between endometrial glandular cells and ESCs in terms of cellular proliferation and invasiveness. Also, it can be suggested that the Ishikawa cell line, which is a well-differentiated endometrial adenocarcinoma cell line, may have biological characteristics of cancer cells, and might have different response to IL-32γ from normal endometrial glandular cells as well as ESCs.

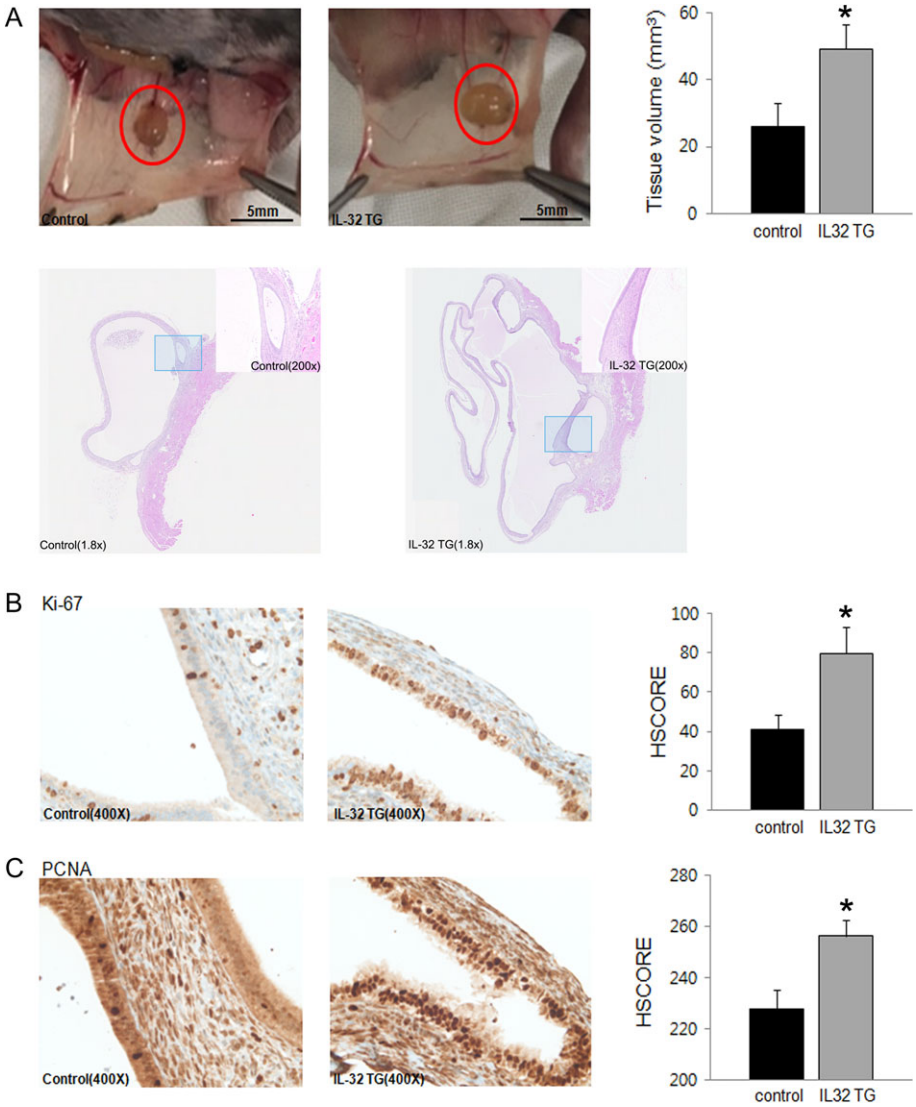

Figure 5 Photographs, graph, micrographs of H&E staining showing ectopic endometrial tissues in wild-type mice and interleukin-32 transgenic mice (**A**), and representative micrographs and HSCOREs of immunostainings for Ki-67 antigen (**B**) and proliferating cell nuclear antigen (**C**). Error bars show the mean ± SEM. *$P < 0.05$ vs control. Control: wild-type mouse; IL-32 TG: interleukin-32γ transgenic mouse; PCNA: proliferating cell nuclear antigen.

Pelch *et al.* (2012) reviewed the mouse autotransplantation model for endometriosis research, and suggested that the timing of necrobiopsy should be determined by particular purpose of research. Specifically, collection of mice for several days following autotransplantaion allows the assessment of the early critical events in the establishment of endometriosis. By 2 weeks, endometriotic lesions are well established and have usually formed cyst-like structures, and they continue to be enlarged in size and volume for two months following autotransplantation. Although there might be some visible differences in the early establishment period between TG and WT mice following several days of surgery, we wanted to compare the endometriotic lesions after longer period (31 days), when the measurement can be much easier due to the larger size of the lesions. Utilizing the mouse autotransplantation model, the present study showed that IL-32 γ TG mice had a significantly larger size of the ectopic endometrial lesions with higher Ki-67 and PCNA expression compared with WT mice. These *in-vivo* findings are quite consistent with the *in-vitro* cell culture data showing increased viability and proliferation following IL-32 treatment.

We utilized the IL-32γ TG mice originally established by Choi *et al.* (2010) in the present study. According to the original study, the IL-32γ TG mice were driven by the chicken β-actin promoter to express human IL-32γ. They were developed without any visible abnormality or runting. It was shown that IL-32γ was expressed strongly in the peripheral white blood cells and the mean serum level was ~3.0 ng/ml in the IL-32 γ TG mice, whereas WT mice had no detectable IL-32γ in white blood cells as well as in the serum. Human IL-32γ was expressed at high levels in the liver, stomach, skeletal muscle, heart, and pancreas, and expressed in lower levels in cerebrum, lymph node, upper

intestine, kidney, testis and thymus. Considering that a high level of human IL-32γ is expressed in the peripheral white blood cells as well as in serum, the ectopic endometrial cells transplanted to peritoneum in IL-32γ TG mice are exposed to higher levels of IL-32γ, which can lead to establishment of larger ectopic lesions compared to WT mice through increased cellular proliferation by IL-32. However, we could not analyze the level of IL-32 expression in the endometrium or peritoneum of IL-32γ TG mice as well as WT mice in the present study. A further study is necessary to investigate the role of IL-32γ in TG mice based on a criss-cross experiment design along with measurement of expression levels of IL-32 in the uterus and the peritoneal cavity of the TG as well as the WT mice.

The strength of the present study is the consistent data based on *in-vitro* and *in-vivo* models as well as analyses on human PF samples, which strongly suggests that IL-32 could play a pivotal role in the pathogenesis of endometriosis. However, further studies are necessary to overcome several limitations of the present study such as the higher concentrations used in the present study (25 and 50 ng/ml) compared with the IL-32 level in the PF and the uncertainity about whether IL-32 is a main regulator or one of several downstream proinflammatory cytokines causing establishment and/or progression of endometriosis. It also has to be determined whether IL-32 has direct effects on the endometrial cells or whether activation of TNF-α and/or IL-1β by IL-32 is the critical process affecting endometrial cells. Furthermore it will be important to investigate whether effective inhibition of IL-32 signaling pathway can contribute to development of a better treatment strategy for endometriosis.

Authors' roles

L.M.Y. drafted the manuscript. S.H.K. designed the study, recruited patients, analyzed the data and revised the manuscript. Y.S.O. performed experimental work. S.H. and K.K. performed all of the experiments on animal models. H.D.C. participated in the study design and edited the manuscript. C.K. participated in the study design and edited the manuscript. B.M.K. participated in the study design and edited the manuscript.

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Conflict of interest

None of the authors has anything to disclose.

References

Bai XY, Kim SH, Azam T, McGibney MT, Huang H, Dinarello CA, Chan ED. IL-32 is a host protective cytokine against *Mycobacterium tuberculosis* in differentiated THP-1 human macrophages. *J Immunol* 2010;**184**:3830–3840.

Bang BR, Kwon HS, Kim SH, Yoon SY, Choi JD, Hong GH, Park S, Kim TB, Moon HB, Cho YS. Interleukin-32γ suppresses allergic airway inflammation in mouse models of asthma. *Am J Respir Cell Mol Biol* 2014;**50**:1021–1030.

Barcz E, Milewski Ł, Dziunycz P, Kamiński P, Płoski R, Malejczyk J. Peritoneal cytokines and adhesion formation in endometriosis: an inverse association withvascular endothelial growth factor concentration. *Fertil Steril* 2012;**97**:1380–1386.

Bulun SE. Endometriosis. *N Engl J Med* 2009;**360**:268–279.

Calabrese F, Baraldo S, Bazzan E, Lunardi F, Rea F, Maestrelli P, Turato G, Lokar-Oliani K, Papi A, Zuin R *et al*. IL-32, a novel proinflammatory cytokine in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008;**178**:894–901.

Cheong YC, Shelton JB, Laird SM, Richmond M, Kudesia G, Li TC, Ledger WLIL-1. IL-6 and TNF-alpha concentrations in the peritoneal fluid of women with pelvic adhesions. *Hum Reprod* 2002;**17**:69–75.

Choi JD, Bae SY, Hong JW, Azam T, Dinarello CA, Her E, Choi WS, Kim BK, Lee CK, Yoon DY *et al*. Identification of the most active interleukin-32 isoform. *Immunology* 2009;**126**:535–542.

Choi J, Bae S, Hong J, Ryoo S, Jhun H, Hong K, Yoon D, Lee S, Her E, Choi W *et al*. Paradoxical effects of constitutive human IL-32{gamma} in transgenic mice during experimental colitis. *Proc Natl Acad Sci U S A* 2010;**107**:21082–21086.

Ciccia F, Rizzo A, Accardo-Palumbo A, Giardina A, Bombardieri M, Guggino G, Taverna S, Leo GD, Alessandro R, Triolo G. Increased expression of interleukin-32 in the inflamed ileum of ankylosing spondylitis patients. *Rheumatology (Oxford)* 2012;**51**:1966–1972.

Dziunycz P, Milewski Ł, Radomski D, Barcz E, Kamiński P, Roszkowski PI, Malejczyk J. Elevated ghrelin levels in the peritoneal fluid of patients with endometriosis: associations with vascular endothelial growth factor (VEGF) and inflammatory cytokines. *Fertil Steril* 2009;**92**:1844–1849.

Eisermann J, Gast MJ, Pineda J, Odem RR, Collins JL. Tumor necrosis factor in peritoneal fluid of women undergoing laparoscopic surgery. *Fertil Steril* 1988;**50**:573–579.

Fakih H, Baggett B, Holtz G, Tsang KY, Lee JC, Williamson HO. Interleukin-1: a possible role in the infertility associated with endometriosis. *Fertil Steril* 1987;**47**:213–217.

Giudice LC, Kao LC. Endometriosis. *Lancet* 2004;**364**:1789–1799.

Goda C, Kanaji T, Kanaji S, Tanaka G, Arima K, Ohno S, Izuhara K. Involvement of IL-32 in activation-induced cell death in T cells. *Int Immunol* 2006;**18**:233–240.

Harada T, Yoshioka H, Yoshida S, Iwabe T, Onohara Y, Tanikawa M, Terakawa N. Increased interleukin-6 levels in peritoneal fluid of infertile patients with active endometriosis. *Am J Obstet Gynecol* 1997;**176**:593–597.

Hill JA, Anderson DJ. Lymphocyte activity in the presence of peritoneal fluid from fertile women and infertile women with and without endometriosis. *Am J Obstet Gynecol* 1989;**161**:861–864.

Ho HN, Wu MY, Chao KH, Chen CD, Chen SU, Yang YS. Peritoneal interleukin-10 increases with decrease in activated CD4(+) T lymphocytes in women with endometriosis. *Hum Reprod* 1997;**12**:2528–2533.

Hong JT, Son DJ, Lee CK, Yoon DY, Lee DH, Park MH. Interleukin 32, inflammation and cancer. *Pharmacol Ther* 2017;**174**:127–137.

Joosten LAB, Heinhuis B, Netea MG, Dinarello CA. Novel insights into the biology of interleukin-32. *Cell Mol Life Sci* 2013;**70**:3883–3892.

Kang JW, Park YS, Lee DH, Kim MS, Bak Y, Ham SY, Park SH, Kim H, Ahn JH, Hong JT *et al*. Interaction network mapping among IL-32 isoforms. *Biochimie* 2014;**101**:248–251.

Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA. Interleukin-32: a cytokine and inducer of TNFalpha. *Immunity* 2005;**22**:131–142.

Kim SH, Lee HW, Kim YH, Koo YH, Chae HD, Kim CH, Lee PR, Kang BM. Down-regulation of p21-activated kinase 1 by progestin and its increased expression in the eutopic endometrium of women with endometriosis. *Hum Reprod* 2009;**24**:1133–1141.

Lee SR, Kim SH, Lee HW, Kim YH, Chae HD, Kim CH, Kang BM. Increased expression of glutathione by estradiol, tumor necrosis

factor-alpha, and interleukin 1-beta in endometrial stromal cells. *Am J Reprod Immunol* 2009;**62**:352–356.

Lee EJ, Lee EJ, Chung YH, Song DH, Hong S, Lee CK, Yoo B, Kim TH, Park YS, Kim SH *et al*. High level of interleukin-32 gamma in the joint of ankylosing spondylitis is associated with osteoblast differentiation. *Arthritis Res Ther* 2015;**17**:350.

Li W, Sun W, Liu L, Yang F, Li YK, Chen YN, Fang JL, Zhang WJ, Wu JG, Zhu Y. IL-32: a host proinflammatory factor against influenza viral replication is upregulated by aberrant epigenetic modifications during influenza A virus infection. *J Immunol* 2010;**185**:5056–5065.

Luo XZ, Zhou WJ, Tao Y, Wang XQ, Li DJ. TLR4 activation promotes the secretion of IL-8 which enhances the invasion and proliferation of endometrial stromal cells in an autocrine manner via the FAK signal pathway. *Am J Reprod Immunol* 2015;**74**:467–479.

Mori H, Sawairi M, Nakagawa M, Itoh N, Wada K, Tamaya T. Peritoneal fluid interleukin-1 beta and tumor necrosis factor in patients with benign gynecologic disease. *Am J Reprod Immunol* 1991;**26**:62–67.

Pelch KE, Sharpe-Timms KL, Nagel SC. Mouse model of surgically-induced endometriosis by auto-transplantation of uterine tissue. *J Vis Exp* 2012;**6**:e3396.

Ribeiro-Dias F, Saar Gomes R, de Lima Silva LL, Dos Santos JC, Joosten LA. Interleukin 32: a novel player in the control of infectious diseases. *J Leukoc Biol* 2017;**101**:39–52.

Shioya M, Nishida A, Yagi Y, Ogawa A, Tsujikawa T, Kim-Mitsuyama S, Takayanagi A, Shimizu N, Fujiyama Y, Andoh A. Epithelial overexpression of interleukin-32alpha in inflammatory bowel disease. *Clin Exp Immunol* 2007;**149**:480–486.

Soyka MB, Treis A, Eiwegger T, Menz G, Zhang S, Holzmann D, Akdis CA, Meyer N. Regulation and expression of IL-32 in chronic rhinosinusitis. *Allergy* 2012;**67**:790–798.

Suen JL, Chang Y, Chiu PR, Hsieh TH, Hsi E, Chen YC, Chen YF, Tsai EM. Serum level of IL-10 is increased in patients with endometriosis, and IL-10 promotes the growth of lesions in a murine model. *Am J Pathol* 2014;**184**:464–471.

The human protein atlas. https://www.proteinatlas.org/ENSG00000008517-IL32/tissue

Zhang JJ, Xu ZM, Zhang CM, Dai HY, Ji XQ, Wang XF, Li C. Pyrrolidine dithiocarbamate inhibits nuclear factor-κB pathway activation, and regulates adhesion, migration, invasion and apoptosis of endometriotic stromal cells. *Mol Hum Reprod* 2011;**17**:175–181.