An increased level of IL-6 suppresses NK cell activity in peritoneal fluid of patients with endometriosis via regulation of SHP-2 expression

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STUDY QUESTION: Is the decreased natural killer (NK) cell cytolytic activity in the peritoneal fluid (PF) of endometriosis patients due to primary cytokine activity?

SUMMARY ANSWER: An increased level of interleukin-6 (IL-6) in the PF of patients with endometriosis suppresses NK cell cytolytic activity by down-regulating cytolytic granule components, such as granzyme B and perforin, through the modulation of Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) expression.

WHAT IS ALREADY KNOWN: Endometriosis is known to be related to a defect in NK cell cytolytic activity. Additionally, the levels of inflammatory cytokines are elevated in the PF of women with endometriosis.

STUDY DESIGN, SIZE, DURATION: The effects of PF on the differentiation and functional activity of NK cells were investigated in patients with or without endometriosis, and cytokines that reduce NK cell cytolytic activity in endometriosis patients were examined. The study included women who underwent laparoscopic examination for the diagnosis of endometriosis from August 2012 to July 2013 (33 women with, and 15 women without, endometriosis).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Women of reproductive age (20–40 years old) who underwent laparoscopic examination for endometriosis were included. Cytokines present in the PF were identified by enzyme-linked immunosorbent assay. The cytolytic activity of NK cells in the PF was also analyzed using a calcein-acetoxy methyl ester (AM) release assay.

MAIN RESULTS AND THE ROLE OF CHANCE: PF from patients with endometriosis suppressed the differentiation and cytotoxicity of NK cells compared with PF from controls ($P < 0.05$). Increased levels of IL-6 were also found in the PF of patients with endometriosis ($P < 0.01$), and IL-6 levels were negatively correlated with the cytolytic activity of NK cells ($r_s = -0.558$, $P = 0.03$). Furthermore, IL-6 reduced the cytolytic activity of NK cells, concomitantly with the down-regulation of granzyme B and perforin ($P < 0.05$), by modulating SHP-2. Importantly, the addition of anti-IL-6 to the PF of endometriosis patients restored the activity of NK cells ($P < 0.01$), suggesting that IL-6 plays a crucial role in the reduction of NK cell activity in the PF of patients with endometriosis.

LIMITATIONS, REASONS FOR CAUTION: PF contains various inflammatory cytokines in addition to IL-6 and so it is possible that other cytokines may affect the differentiation and activity of NK cells.

WIDER IMPLICATIONS OF THE FINDINGS: Our results imply that the suppression of IL-6 using an anti-IL-6 antibody or soluble IL-6 receptor could rescue the impairment of NK cell activity in patients with endometriosis.

† The authors consider that the first two authors should be regarded as joint First Authors.

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Key words: IL-6 / natural killer cell / cytotoxicity / endometriosis / SHP-2

Introduction

Endometriosis is a chronic gynecological disorder characterized by the presence of endometriotic tissue outside the uterine cavity. Endometriosis affects 1 in 10 women of reproductive age, causing pain and infertility (Momoeda et al., 2002; Osuga et al., 2002). The most commonly accepted theory regarding the pathogenesis of this disease is retrograde menstruation and implantation of ectopic endometrium (Bricou et al., 2008); however, this theory cannot explain why only certain women develop endometriosis. Although the etiopathogenesis of endometriosis appears to be very complex, particularly in terms of environmental factors (Gazvani and Templeton, 2002a,b; Giudice and Kao, 2004), the immune status has been considered to play an important role in the initiation and progression of the disease. 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 (D’Hooghe et al., 2003; Khan et al., 2008). Recently, natural killer (NK) cells have also been suggested to play an essential role in the pathogenesis of the disease by either allowing or inhibiting the survival, implantation and proliferation of endometrial cells (Osuga et al., 2011; Sikora et al., 2011). Decreases in NK cell cytotoxicity have been observed among the peritoneal cells of patients with endometriosis compared with healthy controls (Oosterlynck et al., 1991, 1992). This finding implies that a defect in NK cell cytotoxic function, preventing the cells from eliminating endometrial cells at ectopic sites, may cause endometriosis. However, the exact mechanisms of NK cell impairment are not fully understood.

NK cells are important components of the immune system, and particularly the innate immune system, which play protective roles against tumor development and viral infections through the cells’ cytolytic and immunomodulatory capabilities (Sun and Lanier, 2009). NK cells are known to destroy cells by secreting lytic granules containing granzymes and perforin at the immune synapse (Berke, 1997; Trambas and Griffiths, 2003). NK cell effector functions are regulated by multiple activating and inhibitory NK cell receptors, which are known to recruit various phosphatases due to their immunoreceptor tyrosine-based motifs. Activating receptors such as NKp44, NKp46, NK2D and CD16 contain immunoreceptor tyrosine-based activation motifs, and inhibitory receptors such as KIR2DL, KIR3DL and NKG2A contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Killer cell inhibitory receptors (KIRs) are representative inhibitory receptors that recognize major histocompatibility complex class I molecules on target cells and regulate NK cell cytotoxicity against target cells. Recently, Wu et al. (2000) have reported increased KIR expression on peritoneal NK cells from women with endometriosis, which may contribute to the decreased peritoneal NK cell activity observed in these patients.

It has been reported that NK cells that are preincubated with the peritoneal fluid (PF) from women with severe endometriosis have decreased cytotoxicity (Oosterlynck et al., 1993). This finding suggests the presence of immunosuppressive factors that affect NK cytotoxicity in the PF of endometriosis patients. Because PF is an immunologically dynamic environment that contains not only immune cells, including macrophages and NK cells, but also various soluble factors and cytokines, the mechanism of the decreased NK cell cytotoxicity in the PF of patients with endometriosis could be mediated by cytokines.

Although many factors, such as KIR receptors, soluble intracellular adhesion molecule-1 (Fukaya et al., 1999) and human leukocyte antigen-I (Vernet-Tomas Mdel et al., 2006), have been reported to be associated with decreased NK cytotoxicity in endometriosis, the primary cytokine in PF that inhibits NK cell activity in endometriosis and this cytokine’s mechanism remain unknown. Therefore, we aimed to identify the primary cytokine that suppresses NK cytotoxicity in the PF of patients with endometriosis and to elucidate this cytokine’s mechanism.

Materials and Methods

Subjects and PF samples

This study included 33 women with endometriosis (endometriosis group) and 15 women without endometriosis (control group). Women of reproductive age (between 20 and 40 years of age), who underwent laparoscopic examination for endometriosis from August 2012 to July 2013 were included. Endometriosis was classified according to the staging system defined by the American Society for Reproductive Medicine as Stages I–IV. Women with endometriosis Stages I–IV were 6, 8, 11 and 8 cases, respectively. The control group consisted of women with benign ovary cysts (serous cyst adenoma accounted for nine cases, mucinous cyst adenoma for three cases and benign mature cystic teratoma for three cases). Patients with other accompanying diseases of the uterus and adnexa, infectious diseases or autoimmune diseases and patients who had previously undergone other treatments for endometriosis such as immunosuppressants or had received chemotherapy for malignant diseases were excluded. The day of laparoscopic surgery was selected between the complete end of menstrual bleeding and before the ovulation day to prevent the influence of menstrual blood regurgitation or ovulation fluid. Patients who had a history of taking other medications including oral contraceptives, progestins or gonadotrophin-releasing hormone analogs within 3 months before surgery were excluded.

All of the patients were provided with all available information on PF sampling, and informed consent to the use of PFs in this study was obtained. This study was approved by the Institutional Review Board of the Catholic University of Korea according to the Bioethics and Safety Act and the Declaration of Helsinki (IRB ID-DC12TAS10022).

Preparation of PF cells

No antibiotics or steroids were administered prior to the laparoscopic operation because these treatments might have an effect on immune cells. Under general anesthesia, a pneumoperitoneum was created with a penetration tube and PF in the cul-de-sac was harvested without any treatment in the dorsal lithotomy position. The clinical stage of endometriosis was determined using the revised American Society for Reproductive Medicine
classification system. PF samples were first subjected to centrifugation at 600× g for 10 min at 4°C. The cell-free supernatant was then collected and stored frozen at −80°C until being assayed. After the lysis of red blood cells with Red Blood Cell Lysis Buffer (Roche, Mannheim, Germany), the cells were resuspended in RPMI medium containing 10% FBS at 1× 10⁸ cells/ml. The cells were stimulated with 100 ng/ml hIL-2 for 12 h for the cytotoxicity assay.

**Differentiation of NK cells from CD34+ cord blood cells**

Human cord blood was obtained from healthy women with full-term pregnancies, with the consent from mothers. Written informed consent was obtained from all volunteers in accordance with the Declaration of Helsinki. CD34+ cells were isolated using hematopoietic progenitor isolation kit (Miltenyi Biotec, Teterow, Germany) according to the manufacturer’s instructions. CD34+ cells were cultured in MyeloCult H5100 (STEMCELL Technologies, Vancouver, BC, Canada) supplemented with 10⁻⁶ M hydrocortisone (STEMCELL Technologies), stem cell factor (SCF, 30 ng/ml), Flt-3 ligand (FL, 50 ng/ml) and hIL-7 (10 ng/ml) for 14 days. Then, the medium was changed into differentiation medium, MyeloCult H5100 containing human IL-15 (30 ng/ml) and 10⁻⁶ M hydrocortisone, and cultured for 14 days. All cytokines used for NK cell differentiation were purchased from PeproTech (Rocky Hill, NJ, USA). Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) inhibitor, NSC87877, was purchased from Calbiochem (San Diego, CA, USA).

**Analysis of peritoneal cells by flow cytometry**

Single-cell suspensions from PF were stained with appropriate antibodies for further analysis. For surface staining, FITC-anti-CD4, PE-anti-CD8, APC-anti-CD3, PE-anti-CD11b, PE-Cy7-anti-CD56, APC-anti-CD11c, PE-Cy7-anti-CD14, APC-anti-CD19, FITC-anti-CD15, FITC-anti-CD16, FITC-anti-CD27, PE-anti-Fas ligand (FASL) and PE-anti-tumor necrosis-related apoptosis-inducing ligand (TRAIL) were used. 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 with hIL-2 (100 ng/ml; PeproTech) for 12 h and stimulated with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (1 μg/ml; Sigma-Aldrich) for 4 h. Then the cells were treated with GolgiPlug (BD Biosciences) for 1 h. The cells were collected, fixed and permeabilized with Fixation/Permeabilization buffer (BD Biosciences) for 40 min. For surface staining, FITC-anti-CD4, PE-anti-CD3 and PE-Cy7-anti-CD56 antibodies were used. For intracellular staining, PE-anti-interferon-γ (IFN-γ), FITC-anti-tumor necrosis factor-α (TNF-α), APC-anti-IL-17, FITC-anti-granzyme B and PE-anti-perforin antibodies were used. The data of samples were acquired by Canto II (BD Biosciences, Sparks, MD, USA) and analyzed using software FlowJo (TreeStar, Inc., Ashland, OR, USA).

**Evaluation of cytotoxicity**

Cytotoxicity was evaluated using a calcein-AM release assay (Somanchi et al., 2011). Briefly, K562 target cells were labeled with calcein (Invitrogen, Carlsbad, CA, USA) for 1 h. Calcein-labeled target cells (1× 10⁶ cells) and serially diluted effector cells were then co-cultured in 96-well round-bottom plates for 4 h. “Maximum release” was simulated by adding 2% Triton X-100 to the target cells, and ‘spontaneous release’ was simulated by adding culture media to the target cells. The calcein released into the supernatant was measured by a multi-mode microplate reader ( Molecular Devices, Chicago, IL, USA). The percent-specific lysis was calculated according to the formula [(test release – spontaneous release)/(maximum release – spontaneous release)] × 100.

**Cytokine measurement by enzyme-linked immunosorbent assay**

Cytokines, including interleukin-6 (IL-6), TNF-α, tumor growth factor-β (TGF-β) and IFN-γ, were evaluated in the supernatants of PF by specific enzyme-linked immunosorbent assay (ELISA) kits purchased from eBioscience and soluble UL16-binding protein (s-ULBP). IL-1β and IL-8 were evaluated by specific ELISA kits purchased from R&D systems (Minneapolis, MN, USA). Samples from each patient were tested in duplicates.

**mRNA analysis by quantitative RT–PCR**

Total RNA was extracted from cells using Trizol (Invitrogen), and the RNA was reverse transcribed with a cDNA Reverse Transcription Kit (TOYOBO, Osaka, Japan). Real-time quantitative PCR was performed using an iCycler (BioRad, Hercules, CA, USA) with the following primers as follows: GAPDH 5′-GCCATCAATGACCGCTTCTT-3′, 5′-GCT CCTGGAAAGATGTGT-3′; IL-1β 5′-GAGTCTGGGACACCACTC-3′, 5′-TCGTTATCCCAGTGTGCAA-3′; IL-6 5′-TACCCCCAGAGAGAAGA TTC-3′, 5′-TTTTCTGCCAGTGCTCTTTT-3′; IL-8 5′-TGTGCGATCC-3′, 5′-AAATTTCTGGTGTGCGCAGT-3′; STAT3 5′-CC TTTGGAACGAAGGGGTA-3′, 5′-CGGACTGTTGCTGCTTCA-3′; TNF-α 5′-CTCTTCTCTCTCTCATG-3′, 5′-GGTTGCTCACAACAT GGCT-3′; IFN-γ 5′-CTTAAAAGATGACCGAGA-3′, 5′-CGGCAG AGTTCAGCCCATC-3′; granocyte B 5′-AAGTGGCTCTCCTGGG-3′, 5′-AAGATCTGCTGAGCCACT-3′; perforin 5′-GCAGCTGAGAAAGA CCTATTACAGG-3′, 5′-TCTGAGGCGCTTTGAAGTC-3′, 5′-MC5′-TC AAGGAGGCAAACACACAC-3′, 5′-GGCTTTTATTGGTTTCTCA-3′, SOCS 5′-GCCACCTACTGACACCTCT-3′, 5′-AAACAGGCGAG GAGGACTCTCT-3′; SP-1 5′-TCTCAAGAGACGGGGATG-3′, 5′-CGCAC TCTGCTCCTGTTC-3′, 5′-SP-2 5′-CATCAAGGGAGAGATTGA-3′, 5′-GGCTGTAGTACCTCAGCTC-3′. The data of mRNA expressions were normalized to the amount of GAPDH transcript.

**Luciferase assay**

The proximal promoter regions of SHP-1 and SHP-2 were generated by PCR from human cord blood genomic DNA using the primers as follows: SHP-1 (1,800 bp) 5′-AAAAGATGATCGAGAAGAGTGAC-3′, 5′-GCCACTACATGAGTTTCTCTCTT-3′, 5′-AACAGGCGAGGAGGACTCTCT-3′. The PCR products were verified by sequencing, then subcloned into the pGL3 luciferase vector (Promega, Madison, WI, USA) using KpnI and HindIII sites, respectively. The resulting constructs were transfected into HEK 293 cells along with control thymidine kinase-pRL Renilla plasmid (Promega) using lipofectamine (Invitrogen). After 48-hr transfection, cells were lysed and luciferase activity was measured using a luminometer (MicroLumat Plus LB 96V, Berthold Technologies, Bad Wildbad, Germany). The same constructs were transfected into mature NK (mNK) cells using an Amaxa kit. The luciferase expression was measured by quantitative RT–PCR.

**Statistical analysis**

The data were analyzed using a two-tailed unpaired Mann–Whitney U-test in PRISM (San Diego, CA, USA) and a two-tailed paired Student t-test by Microsoft. Relationships between tested variables were assessed on the basis of Spearman’s correlation coefficients (r) and their probability levels (P). A P-value of <0.05 was considered statistically significant.
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Results

Immunophenotypic analysis of PF cells

The subpopulations of mononuclear cells in PF obtained from women with or without endometriosis were analyzed and compared. As shown in Table I, the PF of patients with endometriosis exhibited moderately increased populations of macrophages (CD16, CD11b), monocytes (CD14) and neutrophils (CD15) compared with the PF of controls; other immune cells, such as T cells (CD3, CD4, CD8), B cells (CD19) and dendritic cells (CD14+CD11c+) did not show any differences between the PF of controls and the PF of patients with endometriosis (Table I). However, the proportion of NK cells (CD3+CD56+) was significantly decreased in the PF of patients with endometriosis compared with the PF of controls (Fig. 1A and B, 13.91 ± 2.56 versus 24.07 ± 3.26%, P = 0.0023). NK cells (CD3+CD56+) can be further defined by the presence of CD11b and CD27; CD11b+CD27− NK cells display an immature phenotype and potential for differentiation, CD11b+CD27+ and CD11b−CD27+ NK cells have the best ability to secrete cytokines and CD11b−CD27− NK cells exhibit high cytolytic function (Fu et al., 2011). Our study demonstrated that the proportion of CD11b−CD27− immature NK cells was increased in the PF of endometriosis patients; however, the proportion of CD11b+CD27− mNK cells did not differ between the PF of patients with and without endometriosis, suggesting defective NK cell maturation in endometriosis patients (Fig. 1C). Previous reports have indicated that the functions of NK cells can be evaluated based on the expression of KIRs and killer cell activating receptors (KARs) and that both receptors can transduce positive and negative signals to regulate NK cell expression of KIRs and killer cell activating receptors (KARs) and that both receptors can transduce positive and negative signals to regulate NK cell immaturity and potential for differentiation, CD11b+CD27+ and CD11b−CD27+ NK cells have the best ability to secrete cytokines and CD11b−CD27− NK cells exhibit high cytolytic function (Fu et al., 2011). Our study demonstrated that the proportion of CD11b−CD27− immature NK cells was increased in the PF of endometriosis patients; however, the proportion of CD11b+CD27− mNK cells did not differ between the PF of patients with and without endometriosis, suggesting defective NK cell maturation in endometriosis patients (Fig. 1C). Previous reports have indicated that the functions of NK cells can be evaluated based on the expression of KIRs and killer cell activating receptors (KARs) and that both receptors can transduce positive and negative signals to regulate NK cell cytotoxicity and cytokine release (Cheent and Khakoo, 2009). We therefore analyzed KAR and KIR expression on NK cells. Although the expressions of KIRs were varied with patients, NK cells in the PF of endometriosis patients tended to have reduced expression of KARs including NKp46, NKG2D and increased expression of KIR, especially KIR3DL1, compared with those in the PF cells of controls (Fig. 1D). In some cases, decreased expressions of KIRs such as NKG2A and KIR2DL2 were observed in the PF cells of endometriosis patients compared with those in the PF cells of control (data not shown).

Table I Lineage distribution in the peritoneal fluid of patients with endometriosis and controls.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Control (%)</th>
<th>Endometriosis (%)</th>
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<tbody>
<tr>
<td>T cells</td>
<td>CD3 27.25 (±5.56)</td>
<td>27.25 (±5.56)</td>
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<tr>
<td>T cells</td>
<td>CD4 54.75 (±6.95)</td>
<td>55.7 (±5.51)</td>
</tr>
<tr>
<td>T cells</td>
<td>CD8 18.73 (±5.24)</td>
<td>15.06 (±2.62)</td>
</tr>
<tr>
<td>B cells</td>
<td>CD19 2.79 (±1.72)</td>
<td>1.67 (±0.37)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>CD16 25.3 (±5.06)</td>
<td>34.01 (±4.88)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>CD11b 54.68 (±7.73)</td>
<td>67.02 (±2.14)</td>
</tr>
<tr>
<td>Monocyte</td>
<td>CD14 31.72 (±6.78)</td>
<td>40.00 (±4.27)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>CD15 0.30 (±0.06)**</td>
<td>1.69 (±0.39) **</td>
</tr>
<tr>
<td>NK cells</td>
<td>CD3+CD56+ 24.07 (±3.26)**</td>
<td>34.01 (±4.88)**</td>
</tr>
<tr>
<td>DCs</td>
<td>CD14+CD11c+ 31.65 (±6.14)</td>
<td>38.65 (±4.63)</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM (control: n = 10, endometriosis: n = 23). P-values were calculated using a two-tailed unpaired Mann–Whitney U test. **Significant difference between groups, P < 0.01.

NK cell defects in the PF of patients with endometriosis

Oosterlynck et al. (1992) have previously demonstrated decreased NK cell-mediated cytotoxicity in the PF of patients with endometriosis. In the current study, to confirm the functional activity of NK cells in the PF of patients with endometriosis, we examined NK cell cytotoxicity using a calcein-AM release assay. As shown in Fig. 2A, NK cytolytic activity of PF cells against K562 cells was decreased in endometriosis patients compared with controls (21 ± 4.15 versus 34 ± 6.99%, P = 0.035). However, the decreased population of NK cells in the PF of endometriosis (Fig. 1B) may contribute to the reduction of NK cell activity. To exclude the influence of different NK cell numbers in PF cells, CD3−CD56− NK cells were isolated from PF cells by using MACS and performed cytotoxicity assay. The numbers of samples were not enough to be statistically analyzed because of a difficulty in obtaining required numbers of purified NK cells for the cytotoxicity assay from PF cells. Nevertheless, the results showed that the cytotoxicity of isolated CD3−CD56− NK cells from PF cells with endometriosis tended to be decreased compared with CD3−CD56− NK cells from control PF cells, even though the difference is not significant (control: n = 3, endometriosis: n = 3, Supplementary data Fig. S1). This suggests that reduced NK cell cytolytic activity of PF cells in endometriosis patients is not merely dependent on the lower number of NK cells in PF cells.

Given the reports indicating that NK cells rapidly release cytolytic granules at the immunological synapse, inducing target cell death (Cooper et al., 2001), we studied whether isolated CD3−CD56− NK cells in PF from endometriosis patients are defective for either the cell death pathway or granule release. The expression of FasL in peritoneal NK cells did not differ between patients with and without endometriosis (Fig. 2B). However, TRAIL expression was decreased in the peritoneal NK cells of patients with endometriosis. Lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) lines the membrane of cytolytic granules and is used as a marker of NK cell degranulation (Winchester, 2001). CD107a expression was also decreased in the peritoneal NK cells of patients with endometriosis compared with controls (Fig. 2C, 19 ± 2.42 versus 35.65 ± 9.5%, P = 0.019). In addition, the mRNA expression of cytolytic granule components, such as granzyme B and perforin, tended to be reduced in the PF cells of patients with endometriosis (Fig. 2D). Taken together, these results suggest that NK cell activity is suppressed in the PF of patients with endometriosis, perhaps due to the decreased expression of cytolytic granule components, such as perforin and granzyme B, as well as of TRAIL and CD107a.

PF of endometriosis patients showed suppressed NK cell differentiation and cytotoxicity

Women with endometriosis are characterized by increases in inflammation, the volume of PF and the concentrations of white blood cells and macrophages (D’Hooghe et al., 1996). These activated peripheral mononuclear cells are reported to secrete various cytokines (de Boer et al., 1993), which may contribute to a peritoneal microenvironment that favors the implantation of endometrial cells and the establishment of endometriosis (Pizzo et al., 2002). Thus, we examined whether PF affects the differentiation of mNK cells from CD34+ hematopoietic stem cells in culture. The suppression of cytotoxicity of NK cells was
more effective at 50% dilution of PF than at 25% dilution of PF. Therefore, cells were treated with 50% PF during NK cell differentiation (data not shown). The population of NK cells (CD56+NK46+) was analyzed on the 14th day of differentiation using flow cytometry. As shown in Fig. 3A, the population of NK cells was reduced by co-culture with the PF of endometriosis patients compared with the PF of controls (P < 0.05). In addition, the cytotoxicity of mNK cells co-cultured with the PF of endometriosis patients was significantly decreased compared to controls.}

**Figure 1** NK cell populations were decreased in the peritoneal fluid (PF) of endometriosis patients. (A) The representative dot plots show CD3−CD56+ NK cell populations among PF cells from patients with or without endometriosis. (B) The graph shows the percentages of CD3−CD56+ NK cells in the PF of controls and that of patients with endometriosis (control: n = 10, endometriosis: n = 23). (C) Among CD3−CD56+ NK cells, the proportions of NK cells in each developmental stage were analyzed. CD27−CD11b− cells are the immature phenotype of NK cells. CD27+CD11b− and CD27+CD11b+ cells have the ability to secrete cytokines and exert cytolytic functions (control: n = 10, endometriosis: n = 23). The data are shown as the mean ± SEM. P-values were calculated using a two-tailed unpaired Mann–Whitney test in PRISM. *P < 0.05, NS, not significant. (D) Representative histograms show the expression of NK cell activating receptors (NKG2D, NKp44, NKp46) and inhibitory receptors (NKG2A, KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1) (control: n = 8, endometriosis: n = 20).
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Figure 2  NK cell functions were defective in the PF of patients with endometriosis. (A) NK cell cytolytic activity in the PF of patients with endometriosis (n = 16) and of controls (n = 10) was evaluated using a calcein-AM release assay. PF cells were stimulated with 100 ng/ml of hIL-2 for 12 h. Calcein-labeled target K562 cells were co-cultured with effector cells at a 1:5 ratio in 96-well U-bottom plates for 4 h. The calcein released into the supernatant was measured with a multi-fluorescence reader. (B) The representative histograms (control: n = 10, endometriosis: n = 23) show the expression of proteins in the death-signaling pathway, including FASL and TRAIL (dotted line, isotype control). (C) The graph shows the CD107a degranulation of NK cells (CD3^+CD56^+). Lymphocytes in PF were co-cultured with target K562 cells in 96-well U-bottom plates for 4 h. CD107a^+ cells were identified by flow cytometry (control: n = 6, endometriosis: n = 11). (D) The mRNA levels of granzyme B and perforin (control: n = 5, endometriosis: n = 19, NS, not significant) were analyzed by quantitative RT–PCR. The data are shown as the mean ± SEM.
with that of mNK cells co-cultured with the PF of controls (46.43 ± 5.89 versus 82.06 ± 2.61%, \( P = 0.001 \)) on the 14th day of differentiation (Fig. 3B). Granzyme B expression by mNK cells was also decreased by co-culture with the PF of endometriosis patients (Fig. 3C). Together, these data suggest that the PF of endometriosis patients suppresses the differentiation and activation of NK cells.

**IL-6 levels were increased in the PF of endometriosis patients**

The peritoneal microenvironment is considered to be an important immunological factor in the development of immune cells regulated by cytokines. In particular, PF contains soluble factors and cytokines that may interfere with NK cells and influence their development and function. To identify the microenvironmental factors, and particularly cytokines, which contribute to the suppression of NK cell differentiation and activity in the PF of endometriosis patients, various inflammatory cytokines were examined by ELISA. Among these cytokines, IL-6 levels were significantly increased in the PF of endometriosis patients compared with the PF of controls (Fig. 4A, \( P < 0.01 \)). Because the major source of IL-6 appears to be peritoneal exudate cells, and particularly macrophages (Harada et al., 2001), this result is consistent with the increased proportion of macrophages (CD16, CD11b, CD14) in the PF of endometriosis patients, as shown in Table I. Other inflammatory cytokines such as IL-1β and IL-8, were also increased in the PF of endometriosis patients at both the protein and mRNA levels (Fig. 4A and B). Although IFN-γ and TNF-α were not detected by ELISA, the mRNA levels of these cytokines were also increased in the PF cells of endometriosis patients compared with the PF cells of controls (Fig. 4B). To confirm that IL-6 induces mRNA expressions of transcription factors (TFs) that are involved in IL-6 signaling, the gene expressions of STAT3, c-Myc and SOCS-3 were determined by quantitative RT-PCR. The mRNA expression levels of c-Myc \( (P < 0.01) \) and SOCS-3 \( (P < 0.05) \) were increased in the PF cells of endometriosis patients compared with the PF cells of controls (Fig. 4C). Furthermore, statistical analysis showed a negative correlation between the IL-6 concentration in the PF of endometriosis patients and NK cell cytolytic activity (Fig. 4D, \( r_s = -0.558, P = 0.03 \)), indicating that IL-6 might be a suppressive factor that influences NK cell cytolytic activity in the PF of endometriosis patients.
Neutralization of IL-6 restored NK cell cytotoxicity in the PF of endometriosis patients

To determine whether IL-6 is the primary factor that reduces cytolytic activity in the PF of endometriosis patients, 5 μg/ml of anti-IL-6 antibody was added to PF during NK cell differentiation in culture to neutralize the IL-6 in the PF. The PF samples of endometriosis patients, which contained 8 pg/ml IL-6, were selected for effective neutralization. Five microgram per milliliters of anti-IL-6 antibody specifically neutralized IL-6 without significant effects on PGE2 and IL-1β production (data not shown). IL-6 ELISA was performed using diluted culture supernatant to measure amount of IL-6 in the supernatant. Culture supernatant supplemented with 30 ng/ml of IL-6 was measured as 8 ng/ml of IL-6 (data not shown); this is likely due to the decrease in the amount of IL-6 during mNK differentiation culture (data not shown).

The proportion of NK cells was hardly affected by anti-IL-6 treatment (Fig. 5A), whereas treatment with anti-IL-6 antibody rescued the reduced NK cell cytotoxicity in the PF of endometriosis patients (Fig. 5B, P < 0.01). In addition, granzyme B expression by NK cells was also increased by the addition of anti-IL-6 antibody to PF compared with cells cultured only in the PF of endometriosis patients (Fig. 5C). Together, these results indicate that treatment with anti-IL-6 antibody can restore impaired NK cell cytotoxicity in the PF of endometriosis patients, suggesting that IL-6 plays a crucial role in the defective cytotoxicity of NK cells in the PF of these patients.

IL-6 suppressed NK cell functions

NK cells express IL-6 receptors (Rabinowich et al., 1993) and IL-6 enhances the adhesion and proliferation of NK cells when combined with IL-15 in vivo (Lin, et al., 2008). However, remarkably little is known about the effects of IL-6 on NK cell functions. Therefore, the direct effects of IL-6 on the differentiation and activity of NK cells were investigated. The optimal dose of IL-6 for effectively suppressing NK cytotoxicity and increasing expression of SHP-2 was determined to be 30 ng/ml. Therefore, 30 ng/ml IL-6 was added during the in vitro differentiation of mNK cells and the cells were analyzed by flow cytometry on the 7th, 10th and 14th days of differentiation. IL-6 actually increased the CD56+ NK cell population on the 7th and 10th days of culture, e.g. at the beginning of differentiation, and the population of mNK cells reached a similar level to that of the IL-6-untreated control after 14 days of culture (Fig. 6A). However, the cytolytic activity of IL-6-treated NK cells was significantly decreased compared with that of untreated
controls (Fig. 6B, P < 0.05). The expression of the NK inhibitory receptor KIR2DL3 was increased by IL-6 treatment, whereas TRAIL and granzyme B expression by NK cells were decreased (Fig. 6C). In addition, the mRNA expression levels of the cytolytic granule contents granzyme B and perforin were decreased by IL-6 treatment (Fig. 6D, P < 0.05 and P < 0.01, respectively). IFN-γ production, another functional activity of NK cells, was measured by ELISA or quantitative RT–PCR but was not significantly affected by IL-6. However, the mRNA levels of IFN-γ were decreased in IL-6-treated NK cells (Fig. 6E, P < 0.05). These data indicate that IL-6 inhibits the functional activities of mNK cells.

IL-6 regulated the expression of SHP-2 in NK cells

It has been reported that IL-6 induces the tyrosine phosphorylation of phospholipase Cγ and SHP-2 (Stahl et al., 1994) and that SHP-2 expression negatively regulates NK cell functions, including cytotoxicity and cytolytic granule release (Yusa and Campbell, 2003; Yusa et al., 2004; Purdy and Campbell, 2009). SHP-2 is a widely expressed cytoplasmic protein tyrosine phosphatase that shares high structural and primary sequence homology with Src homology region 2-containing protein tyrosine phosphatase-1 (SHP-1) (Hof et al., 1998). Thus, we investigated whether IL-6 regulates SHP-1 and SHP-2 mRNA expression in mNK cells. As shown in Fig. 7A, the expression levels of SHP-1 and SHP-2 were increased by treatment with IL-6. To examine the role of IL-6 in the regulation of SHP-1 and SHP-2, a promoter assay was performed. Fragments obtained from the 800 bp and 1.6 kb regions of the 5′-untranslated region of the human SHP-1 and SHP-2 genes, respectively, were inserted into the pGL3 luciferase vector to generate promoter–reporter constructs. We then transfected HEK 293 cells with the SHP-1 or pGL3 or SHP-2/pGL3 DNA and examined luciferase activity after treatment with or without IL-6. The SHP-1 promoter was unaffected by IL-6. Addition of IL-6 induced the activation of the proximal SHP-2 promoter, but the difference was not significant. However, in the absence of IL-6, basal luciferase activity was relatively high, which may have been due to constitutive IL-6 production by HEK 293 cells (Fig. 7B). We also performed SHP-2 gene promoter transfection in human NK cells and N9K2 cell line using an Amaza kit. Due to the low transfection efficiency of N9K2 and mNK cells, however, we could not detect luciferase activity...
using luminometer both in NK92 cells and mNK cells. Therefore, we checked the RNA level of luciferase by quantitative RT–PCR, instead (Dai et al., 1996) and performed western blot analysis to examine whether IL-6 up-regulates SHP-2 expression at the protein level. IL-6 induced a significant 1.5-fold increase in luciferase mRNA (SHP-2/pGL3) compared with no treatment (Supplementary data, Fig. S2A, P < 0.05), and SHP-2 protein expression was also up-regulated by IL-6 in mNK cells (Supplementary data Fig. S2B). Next, to confirm that IL-6 suppresses NK cytotoxicity through SHP-2 regulation, an SHP-2 inhibitor, NSC87877 was added during NK cell differentiation in the presence of IL-6. As shown in Fig. 7C and D, the suppression of cytotoxicity induced by IL-6 in mNK cell was restored by the treatment of

**Figure 6** IL-6 suppressed mNK cell functions. (A) CD56+ cells were analyzed over a time course during mNK cell differentiation on Days 7, 10 and 14 (white diamond: −IL-6, black square: +IL-6). The data are representative of three independent experiments. The data are shown as the mean ± SEM of triplicates. (B) Cytolytic activity was determined by a calcein-AM release assay with K562 target cells (E : T ratio = 1 : 1, 2 : 1). The data are representative of three independent experiments. The data are shown as the mean ± SEM of triplicates. (C) The dot plots show the expression of KIR2DL3, TRAIL and granzyme B in CD56+ cells on Day 20. The figures are representative of three independent experiments. (D) The mRNA levels of the cytolytic granule components, including granzyme B and perforin, were analyzed by quantitative RT–PCR. The data are shown as the mean ± SEM (white bar: −IL-6, n = 3, black bar: +IL-6, n = 3). P-values were calculated using a one-tailed unpaired Mann–Whitney test. (E) IFN-γ production was measured by ELISA, and the mRNA expression of IFN-γ was analyzed by quantitative RT–PCR. The data are shown as the mean ± SEM (−IL-6: n = 3, +IL-6: n = 4). P-values were calculated using a two-tailed unpaired Student’s t-test. *P < 0.05, NS, not significant.
NSC87877. Furthermore, the expressions of granzyme B and perforin were also increased by the treatment of NSC87877 in the presence of IL-6, indicating that IL-6 suppresses NK activities through the regulation of SHP-2. Indeed, increased expression of SHP-2 was found in the PF cells of endometriosis patients compared with the PF cells of controls at the mRNA level, as measured by quantitative RT–PCR (Fig. 8A), and at the protein level, as demonstrated by immunostaining (Fig. 8B). Taken together, these data suggest that IL-6 is involved in the suppression of NK cell functions in the PF of patients with endometriosis through the up-regulation of SHP-2 expression.

Discussion

It is known that endometriosis is primarily caused by inflammation, which is mediated by immunological cytokines, growth factors and adhesion factors (Harada et al., 2001; Kyama et al., 2003) and defective cellular immunity. In particular, the impaired function of NK cells has been reported to contribute to the survival and ectopic implantation of endometrial cells (Sikora et al., 2011). However, the primary cytokine that inhibits NK cell function in the PF microenvironment is unknown, as is the mechanism of action. In this study, we first demonstrated defects in the differentiation and functional activity of NK cells in the PF of patients with endometriosis. In addition, we suggest that IL-6 plays a crucial role in impairing NK cell function via the regulation of SHP-2 expression in endometriosis.

It has been reported that NK cell cytotoxicity is decreased in the PF of women with endometriosis compared with the PF of women without endometriosis and that this phenomenon is mediated by the regulation of KIR expression (Wu et al., 2000). Consistent with the previous report, we observed reduced NK cell cytolytic activity in the PF of

Figure 7 IL-6 regulated Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) expression. (A) Quantitative RT–PCR analyses of the mRNA levels of SHP-1 and SHP-2 in mNK cells treated with IL-6 or left untreated (−IL-6: n = 3, +IL-6: n = 4). The data are shown as the mean ± SEM. (B) Luciferase activity was measured using a luminometer and normalized to Renilla luciferase activity. The graph shows the mean ± SEM of triplicates. HEK 293 cells were transfected with the SHP-1 and SHP-2 luciferase reporter construct (pGL3) and treated with IL-6 (50 ng/ml) or left untreated. (C) Cells were treated with NSC87877 (SHP-2 inhibitor) for 14 days during NK cell differentiation. Cytolytic activity was determined by a calcein-AM release assay. (D) Quantitative RT–PCR analyses of the mRNA levels of granzyme B and perforin in mNK cells treated with NSC87877. The graphs (B), (C) and (D) are representatives of three independent experiments. The data are shown as the mean ± SEM of triplicates. *P < 0.05, NS, not significant.
patients with endometriosis concomitant with increased KIR receptor expression on NK cells. In addition, the NK cell expression levels of granzyme B, perforin, TRAIL and CD107a were decreased in the PF of patients with endometriosis, indicating a functional defect in NK cells in endometriosis patients. Moreover, we observed a decreased number of NK cells (CD3<sup>+</sup>CD56<sup>+</sup>) in the PF of patients with endometriosis compared with controls. This finding is in contrast to a previous report that has shown no difference in the proportion of NK cells in the PF of women with and without endometriosis (Hassa et al., 2009). This difference may be because they determined NK cells by using CD16 that is frequently expressed by macrophages as well. However, Tariverdian et al. (2009) also observed a moderate decrease in NK cell population in the PF of endometriosis patients compared with controls, although it was not significant. We found that the population of NK cells (CD3<sup>+</sup>CD56<sup>+</sup>) was significantly decreased, whereas the proportion of immature NK cells (CD27<sup>-</sup>CD11b<sup>+</sup>) among CD3<sup>+</sup>CD56<sup>+</sup>NK cells was increased in the PF of endometriosis patients compared with the PF of controls. These findings suggest that endometriosis is associated with defects in the differentiation in addition to activation of NK cells. This is the first report that demonstrates a role for NK cell differentiation in endometriosis. Furthermore, during NK cell differentiation in vitro, treatment with the PF of patients with endometriosis suppressed the differentiation and function of NK cells, suggesting the presence of immunosuppressive factors that affect NK cells in the PF. Therefore, we investigated immunological factors in PF, such as soluble factors and cytokines that may inhibit the differentiation and cytotoxicity of NK cells. The levels of the inflammatory cytokines IL-6, IL-8, IL-1β, IFN-γ and TNF-α were increased in the PF of patients with endometriosis, which is consistent with previous reports showing increased levels of inflammatory cytokines in the serum of endometriosis patients (Oral et al., 1996; Harada et al., 2001; Gazvani and Templeton, 2002a,b). Among these levels, the IL-6 level was dramatically increased in the PF of patients with endometriosis compared with the PF of controls. Furthermore, the mRNA expression levels of the IL-6 signaling TFs, c-Myc and SOCS-3 were increased in the PF cells of endometriosis patients. It is known that IL-6 signaling is involved in the regulation of cell growth, differentiation and survival. However, the role of IL-6 in the differentiation and effect of IL-6 on the cytotoxicity of NK cells have remained unknown. Here, we characterized the direct effects of IL-6 on NK cell differentiation from CD34<sup>+</sup> cells and on functional activity of NK cells. Treatment with IL-6 initially enhanced the rate of NK cell differentiation, but the final number of mNK cells was similar to that of the untreated control. However, IL-6 significantly reduced the cytotoxicity of mNK cells, and the concentration of IL-6 in PF was negatively correlated with the cytotoxicity of NK cells, which could explain the defects in NK cell cytotoxicity observed in the PF of patients with endometriosis. However, the finding does not explain the impaired differentiation of NK cells in the PF of endometriosis patients. Furthermore, although treatment with anti-IL-6 antibody restored the NK cell functions, including cytotoxicity and IFN-γ production, that were inhibited by the PF of endometriosis patients, the antibody did not rescue NK cell differentiation, which was also inhibited by incubation with the PF of endometriosis patients. Collectively, these findings suggest that IL-6 plays a critical role in the function of NK cells rather than in NK cell differentiation. PF from patients with endometriosis contains many cytokines, growth factors and soluble factors in addition to IL-6. In certain, but not all, patients with endometriosis, increased levels of s-ULBP, a ligand of the NKG2D receptor and TGF-β were detected (data not shown). TGF-β is a known suppressor of immune cells, including their proliferation and differentiation (Bierie and Moses, 2006), and is known to inhibit the differentiation of human NK cells (Allan et al., 2010). Thus, other factors in PF, such as s-ULBP or TGF-β, could affect the development and activation of NK cells in the PF of endometriosis patients.

NK cells express receptors, including activating and inhibitory receptors, which recognize cells and enable target cell lysis. In this study, we found that the expression of inhibitory receptors on NK cells in the PF of patients with endometriosis was higher than in controls, which is consistent with a previous report that demonstrated increased KIR

Figure 8 Expression of SHP-2 in the PF cells with endometriosis. (A) The mRNA levels of SHP-1 and SHP-2 were analyzed by quantitative RT–PCR (control: n = 4, endometriosis: n = 6). The data are shown as the mean ± SEM. (B) SHP-2 expression was increased on CD3<sup>+</sup>CD56<sup>+</sup> cells of PF from endometriosis patients compared with control. CD56<sup>+</sup> cells were selected by MACS and collected onto glass slides by cyto-spin. The cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100 and stained with Alexa 546-anti-SHP-2 and FITC-anti-CD3. Left, pictures show the representative pictures from independent experiments (control: n = 2, endometriosis: n = 2, red: SHP-2, green: CD3, blue: DAPI). Right, bar graph shows the intensity of SHP-2 measured using the LSM 5 Image Browser. The data are shown as the mean ± SEM of six different fields. P-values were calculated using a two-tailed unpaired Mann–Whitney test. *P < 0.05, NS, not significant.
expression on peritoneal NK cells isolated from women with endometriosis (Wu et al., 2000). Such inhibitory NK cell receptors contain Ig domains (KIR2DL1, KIR2DL2, KIR3DL1 and KIR3DL2) in their extracellular domains (Maghazachi, 2005) and ITIMs in their cytoplasmic domains (Daeron and Vivier, 1999; Colucci et al., 2002). ITIM phosphorylation results in the recruitment of the phosphatases SHP-1 and SHP-2. After ligand binding, the tyrosine residues in the ITIMs are phosphorylated, facilitating the recruitment of SHP-1 and SHP-2 and suppressing immune responses, including NK cell cytotoxicity (Wang et al., 2002; Tessmer et al., 2007). These phosphatases have also been reported to inhibit the function of activating receptors by dephosphorylating molecules such as Vav-1 (Stebbins et al., 2003). Consistent with this phenomenon, our results showed increased mRNA levels of SHP-1 and SHP-2 in PF cells and increased expression of KIR on NK cells (CD56+ cells) in the PF of patients with endometriosis compared with controls.

It is unclear whether IL-6 directly regulates SHP-1 and SHP-2 expression in NK cells. Recently, it has been reported that IL-6 production is positively regulated by SHP-1 in macrophages (Rego et al., 2011). In our study, IL-6 increased the mRNA expression of SHP-1 and SHP-2 in NK cells. A promoter assay using the luciferase system demonstrated that IL-6 treatment induced activity of the proximal SHP-2 promoter region, indicating the direct regulation of SHP-2 by IL-6. Given that SHP-2 has been reported to negatively regulate NK cell activity (Purdy and Campbell, 2009), IL-6 might inhibit cytolytic activity through the regulation of SHP-2, which is involved in NK cell-mediated killing. A high level of IL-6 has also been shown to decrease the function of NK cells in other clinical situations. In the athymic model, impairment of NK functions induced by IL-6 increased lymphoblastoid cell tumorigenicity (Tanner and Tosato, 1991). Also, in patients with chronic heart failure, the levels of IL-6 produced by unstimulated peripheral blood mononuclear cells in patients were correlated with NK cell cytolytic impairment (Vredevoe et al., 2004).

In conclusion, our data indicate that an increased level of IL-6 in the PF of endometriosis patients decreases the cytolytic activity of NK cells by down-regulating cytolytic granules and the TRAIL death pathway, which are mediated via regulation of SHP-2 expression. These findings suggest that IL-6 plays a crucial role in the pathogenesis of endometriosis and that the suppression of IL-6 or SHP-2 in the PF of endometriosis patients may counteract the impairment of NK cells in these patients.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
Y.-J.K. performed all experiments, analyzed the data and wrote the manuscript. I.C.J. conducted clinical evaluations and collected samples. A.P. assisted with sample preparation and flow cytometry analysis. Y.-J.P., T.-D.K., H.J., H.G.L. and I.C. provided helpful discussion and critical analysis of data. S.R.Y. supervised the overall project, analyzed the data and wrote the manuscript.

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Conflict of interest
None declared.

References


