The role of myeloid-derived suppressor cells in endometrial cancer displaying systemic inflammatory response: clinical and preclinical investigations

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Introduction

Endometrial cancer is the second most common gynecological malignancy (after cervical cancer) in Japan and the sixth most common form of cancer in women worldwide.1 The prognostic factors for patients with endometrial cancer have been investigated intensively: histopathological findings including histological subtype, grade of the tumor, lymphovascular space involvement, myometrial or cervical stromal invasion, lymph node metastasis, protein or microRNA expression levels, and genetic alterations have all been reported to be significant prognostic factors.2,3 Moreover, accumulating evidence has suggested that cancer stem cells (CSCs) are involved in the progression of tumorigenic potential and chemoresistance of endometrial cancer, and that the number of CSCs in a tumor can be an indicator of poor prognosis.3,4 However, the majority of these factors cannot be assessed preoperatively, and some of them are available only as research purposes. Thus, identifying prognostic factors that can be used preoperatively to predict treatment outcomes is of great importance.

Recently, systemic inflammatory responses including thrombocytosis, leukocytosis, or neutrophilia have gained attention as prognostic indicators in patients with various solid malignancies. According to previous reports, pretreatment concurrent leukocytosis and thrombocytosis are observed in approximately 10–20%5 and 20–40%6 of patients with gynecological cancer, respectively, and are associated with aggressive clinical behavior or poor prognosis.7

Myeloid-derived suppressor cells (MDSCs), which can be induced by pro-inflammatory cytokines growth factors, are a heterogeneous population of the myeloid lineage that can enhance cancer progression by stimulating cancer cell invasion, metastasis and tumor angiogenesis.8 In studies of uterine cervical cancer, it has been demonstrated that tumor-related leukocytosis (TRL) is caused by tumor-derived granulocyte colony-stimulating factor (G-CSF), and that MDSCs induced by G-CSF are responsible for the rapid growth and radio/
chemoresistant nature of TRL-positive cancers.\textsuperscript{9–11} Thrombopoiesis can also be stimulated by various tumor-secreted cytokines or growth factors. A previous study of ovarian cancer suggested that tumor-secreted IL-6 enhances thrombopoiesis, leading to thrombocytosis and tumor progression.\textsuperscript{12}

In the area of endometrial cancer, recent investigations have shown that pretreatment leukocytosis or thrombocytosis are observed in approximately 5–20\% of patients.\textsuperscript{13,14} It has also been reported that pretreatment leukocytosis or thrombocytosis are independently associated with poor prognosis.\textsuperscript{13–16} Although both leukocytosis and thrombocytosis can be caused by tumor-derived cytokines or growth factors,\textsuperscript{9–12} and concurrent leukocytosis and thrombocytosis has been observed in endometrial cancer patients,\textsuperscript{7} the association between platelet counts, leukocyte counts, and patients’ prognosis has not been fully investigated. Moreover, the underlying mechanisms of leukocytosis or thrombocytosis in patients with endometrial cancer, as well as the role of MDSCs in endometrial cancer progression, remain to be elucidated.

To address these unsolved issues, in the current study, using clinical data obtained from patients with endometrial cancer, we first investigated the prognostic significance of pretreatment leukocytosis, thrombocytosis, and their combination. Then, using clinical samples, endometrial cancer cell lines, and mouse models of endometrial cancer, we performed laboratory investigations focusing on tumor-derived G-CSF, G-CSF-induced MDSCs, and MDSC-mediated CSC induction.

Materials and methods

Patients and clinical samples

Permission to proceed with the data acquisition, tumor/blood samples collection, and analysis was obtained from Osaka University Hospital’s institutional review board. A list of surgically treated patients with endometrial cancer at Osaka University Hospital from April 2000 to March 2016 was generated from our institutional tumor registry, and their clinical data were analyzed retrospectively. Appropriate informed consent was obtained from each patient. Progression-free survival (PFS) was measured as the time from the operation to disease progression. Overall survival (OS) was defined as the time from operation to death or the last observation.

Cell culture

Ishikawa and Hec1B human endometrial cancer cell lines were purchased from the American Type Culture Collection. The cell lines were passaged in our laboratory soon after they were received from the cell bank, before being divided and stored in liquid nitrogen vessels. Each experiment was carried out using thawed cells without further authentication. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (FBS). MDSCs were maintained in Roswell Park Memorial Institute-1640 (Nacalai Tesque, Kyoto, Japan) medium supplemented with 10\% FBS.

Clone selection

The expression vector for the mouse G-CSF gene (pCAMG-CSF), empty vector (pCAZ 2),\textsuperscript{17,18} mouse IL-6 gene (pEF-mIL6),\textsuperscript{19} and empty vector (pEF-BOS-EX)\textsuperscript{20} were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The expression of these genes was driven by the CAG promoter, as reported previously.\textsuperscript{17,18} Transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions. Clonal selection was performed by adding G-418 to the medium at a final concentration of 500 µg/mL. Ishikawa endometrial cancer cells stably transfected with the G-CSF expression vector, IL-6 expression vector, and the empty vector were designated as Ishikawa-G-CSF, Ishikawa-IL-6, and Ishikawa-Control, respectively. Hec1B endometrial cancer cells stably transfected with G-CSF expression vector and the empty vector were designated as Hec1B-G-CSF and Hec1B-Control, respectively.

Animal experiments

All procedures involving animals were approved by the animal care and usage committee of Osaka University, in accordance with the relevant institutional and National Institutes of Health guidelines (Approved No; 26-072-010). To evaluate the role of MDSCs in the endometrial cancer microenvironment, we employed 5–7-week-old Balb/c nude mice that were subcutaneously inoculated with $1 \times 10^7$ of Ishikawa-Control, Ishikawa-G-CSF, Hec1B-Control, or Hec1B-G-CSF cells in 150 µL of phosphate-buffered saline (PBS) into their left flanks. This was based on previous reports showing that tumor-derived G-CSF increases the number of MDSCs in mice and that a significant number of MDSCs can be obtained from these mice for experimental use.\textsuperscript{7}

The first set of experiments investigated the anti-tumor activity of doxorubicin. Ishikawa-Control-derived or Ishikawa-G-CSF-derived tumor-bearing mice were treated intravenously with 5 mg/kg of doxorubicin (every 3 weeks). The second set of experiments was conducted to investigate whether MDSCs contribute to the induction of CSC \textit{in vivo}. Ishikawa-Control-derived or Ishikawa-G-CSF-derived tumor-bearing mice were injected intraperitoneally with anti-Gr-1-neutralizing antibody or isotype control at a dose of 200 µg/mouse every 2 days. The third set of experiments examined the effect of PGE2 inhibition on the induction of CSCs \textit{in vivo}. Hec1B-G-CSF-derived tumor-bearing mice were treated intraperitoneally with 2.5 mg/kg of daily celecoxib for 3 weeks. The fourth set of experiments was conducted to investigate the antitumor effects of combination treatment involving celecoxib and doxorubicin. Hec1B-G-CSF-derived tumor-bearing mice were treated intravenously with doxorubicin (5 mg/kg, every 3 weeks) or with doxorubicin (5 mg/kg, every 3 weeks) plus celecoxib (2.5 mg/kg, daily). Doxorubicin was diluted in double-distilled water just before intravenous infusion. Celecoxib was dissolved in double-distilled water and polyethylene glycol 400 ($v/v = 1.2$) just before intraperitoneal infusion. Mice were randomized into treatment groups when the tumors had reached about...
50 mm³ in size, then treatments were initiated. At the end of the experiment, the mice were sacrificed by carbon dioxide asphyxiation, and their tumors were collected for analysis. Tumor growth was assessed in three dimensions using calipers, and tumor volume was calculated using the formula 

\[ V = \frac{L \times W \times D}{2} \]

where \( V \) indicates volume, \( L \) length, \( W \) width, and \( D \) depth. White blood cells and granulocytes were counted using a VetScan HM2 automatic cell counter (Abaxis, Union City, CA, USA). Furthermore, serum PGE2 concentrations were evaluated using an enzyme-linked immunosorbent assay (ELISA).

Reagents and antibodies

The following labeled monoclonal antibodies were used for the staining experiments: anti-human/mouse antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (Tonbo Biosciences, San Diego, CA, USA); anti-mouse antibodies: allophycocyanin (APC)-conjugated anti-Ly6G (Tonbo Biosciences) and phycoerythrin (PE)-conjugated anti-Ly6C (Tonbo Biosciences). Purified anti-mouse CD32/16 Ab (BioLegend, San Diego, CA) was utilized for blocking FcγRII (CD32) and FcγRIII (CD16) binding of IgG. A neutralizing antibody against Gr-1 (RB6-8C5) was purchased from BioXCell (West Lebanon, NH, USA). PGE2 was obtained from Cayman Chemical (Ann Arbor, MI, USA). PF-04418948 (a PGE2 receptor [EP2 receptor] antagonist) was obtained from Cayman Chemical (Ann Arbor). ONO-AE3-208 (an EP4 receptor antagonist) was kindly provided by Ono Pharmaceutical (Osaka, Japan). Celecoxib, doxorubicin, and recombinant mouse IL-6 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human G-CSF was kindly provided by Kyowa Hakko Kirin (Tokyo, Japan). Recombinant mouse CXCL2 was purchased from Wako (Osaka, Japan). An anti-CD3ε antibody was purchased from Tonbo Biosciences (San Diego, CA, USA). Antibodies against Stat3, phospho-Stat3 (Y705), β-actin, and anti-rabbit and anti-mouse secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) were used for the western blotting analysis. G-418 was purchased from Life Technology (Grand Island, NY, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted from cells using TRIzol (Life Technologies, Grand Island, NY, USA). The resultant total RNA (1 μg) was used to synthesize cDNA using ReverTraAce qPCR RT Master Mix (Toyobo, Osaka, Japan). The cDNA was amplified by qRT-PCR using the included TaqMan Gene Expression Master Mix and the specific TaqMan primer/probe assay designed for the investigated genes: SOX2 (Hs01053049_s1), NANOG (Hs00179605_m1), OCT4 (Hs00353768_g1), and PGE2 was purchased from Cayman Chemical (Ann Arbor). GAPDH and were expressed as the fold change relative to the expression level in untreated cells. Quantification was performed using the ΔΔCt calculation method.

Isolation of MDSCs

MDSCs were isolated from the spleen of G-CSF-derived tumor-bearing Balb/c nude mice using a Myeloid-Derived Suppressor Cell Isolation Kit and an MS column (Miltenyi Biotec, Auburn, CA, USA). The purity of the isolated cell population was determined previously using flow cytometry, and the frequency of CD11b⁺ Gr-1⁺ cells was > 99%.9

Flow cytometry

Single-cell suspensions were prepared from mouse spleens and tumor specimens. Red blood cells were removed using ammonium chloride lysis buffer. Then, the cells were filtered through 40-μm nylon strainers, incubated with antibodies, and analyzed by flow cytometry. Fragment crystallizable receptor (FcR) blocking was not performed during flow cytometry, because our preliminary experiments showed no differences between the experiments with FcR blocking and those without detecting MDSCs (Figure S8). Flow cytometric data were acquired using a FACScant II flow cytometer and analyzed using the FACSDiva software (BD Biosciences, San Jose, CA, USA). Cells that had been incubated with irrelevant isotype-matched antibodies and unstained cells served as controls.
**Aldefluor assay**

An Aldefluor Assay Kit (Stem Cell Technologies, Vancouver, Canada) was used to determine the percentage of tumor cells expressing high levels of aldehyde dehydrogenase (ALDH; ALDH-high cells), in accordance with the manufacturer’s instructions. Briefly, $1 \times 10^6$ cells were incubated with the Aldefluor substrate for 45 minutes at 37°C, with and without the ALDH inhibitor diethylaminobenzaldehyde. After incubation, ALDH-high cells were detected in the FITC channel of the flow cytometer using FACS Diva software.

**Sphere formation assay**

Sphere formation assay was conducted as reported previously. Endometrial cancer cells (Ishikawa or Hec1B) were plated in ultra-low attachment surface 6-well plates with serum-free medium supplemented with basic fibroblast growth factor (10 ng/ml; ReproCELL, Inc., Kanagawa, Japan), epithelial growth factor (20 ng/ml; R&D Systems), and B27 supplement. After 2 weeks, the number of spheres in each well was counted using a phase-contrast microscope.

**T cell proliferation assay**

A T cell proliferation assay was conducted as reported previously. A 24-well plate was coated with 1 μg/well of anti-CD3e antibody. CD8+ T cells were purified from the spleen of a Balb/c mouse using T-cell isolation columns (R&D systems, Minneapolis, MN) in accordance with the manufacturer’s instructions and labeled with carboxyfluorescein succinimidyl ester (CFSE). In order to assess the impact of MDSCs on CD8+ T cell proliferation, CD8+ T cells were co-cultured with or without MDSCs for 48 hours. T-cell proliferation was evaluated by flow cytometry. CFSE was purchased from Tonbo Biosciences (San Diego, CA).

**Chemotaxis assay**

To evaluate the in vitro migration activity of MDSCs, chemotaxis assays were conducted as reported previously. Cancer cells ($5 \times 10^4$/well) were seeded in the top chamber of a 24-well plate. The top chamber contained an 8-mm pore membrane. The indicated concentrations of recombinant mouse CXCL2 were placed in the bottom chamber of the 24-well plate as a chemoattractant. After 3 hours’ incubation, the frequency of migrated MDSCs was quantitated using a CyQUANT® assay.

**Western blotting analysis**

Cells were washed twice with ice cold PBS and lysed in radioimmunoprecipitation assay lysis buffer. The protein concentrations of the cell lysates were determined using Bio-Rad protein assay reagent. Equal amounts of protein were applied to 5–20% polyacrylamide gels, and the electrophoresed proteins were transferred onto nitrocellulose membranes. After the membranes were blocked, they were incubated with various primary antibodies. The immunoblots were visualized with horseradish peroxidase-coupled immunoglobulins using an enhanced chemiluminescence western blotting system (PerkinElmer, CA, USA).

**Immunohistochemistry**

Tumor samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and processed for immunohistochemical staining. The primary antibodies used were anti-human G-CSF polyclonal antibody (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human CD33 antibody (NCL-L-CD33, Leica Biosystems, Wetzlar, Germany), anti-ALDH1A1 antibody (EP 1933Y)-C-terminal (ab52492), anti-IL-6 antibody (ab6672), and anti-human PGE2 antibody (ab2318) (Abcam, Cambridge, UK). The secondary antibody was a Histone Simple Stain Max-PO (MULTI) (Nichirei Bioscience, Tokyo, Japan). Optical image capture was performed using a PROVIS AX80 (Olympus, Tokyo, Japan).

The slides were examined using a bright field microscope. The immunoreactivities of endometrial cancer for G-CSF, IL-6, and PGE2 were classified as weak or strong: “weak” indicates no or focal staining (less than 50% of the cells were stained) and “strong” indicates clearly positive staining (more than 50% of the cells were stained) or intensely positive staining as described in detail elsewhere. The number of tumor-infiltrating CD33+ cells was scored manually at higher magnification (× 40). A mean score of duplicate cores from each individual tissue was calculated. The intratumoral CD33+ cells were quantified and expressed as the numbers of CD33+ cells per 0.6 mm² of tumor section, as reported previously. The concentrations of human G-CSF and mouse G-CSF were measured using a Human G-CSF Quantikine ELISA Kit (Cat No. DC550) and a Mouse G-CSF Quantikine ELISA Kit (Cat No. MC500) obtained from R&D systems (Minneapolis, MN, USA), respectively. The concentrations of human IL-6 and mouse IL-6 were measured using a Human IL-6 ELISA Ready-SET-Go! Kit (Cat No. 501128847) and a Mouse IL-6 ELISA Ready-SET-Go! Kit (Cat No. 5017218) obtained from eBioscience (San Diego, CA, USA), respectively. The concentration of human PGE2 and mouse PGE2 were measured using a Prostaglandin E2 Express ELISA Kit (Cat No. 500141) obtained from Cayman Chemical (Ann Arbor, MI, USA) and a Mouse Prostaglandin E2 (PGE2) ELISA Kit (Cat No. MBS266212) obtained from MyBioSource (San Diego, CA, USA), respectively. Absorbance values were measured using a microplate reader (iMark Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, CA).

**Enzyme-linked immunosorbent assay (ELISA)**

The concentrations of human G-CSF and mouse G-CSF were measured using a Human G-CSF Quantikine ELISA Kit (Cat No. DC550) and a Mouse G-CSF Quantikine ELISA Kit (Cat No. MC500) obtained from R&D systems (Minneapolis, MN, USA), respectively. The concentrations of human IL-6 and mouse IL-6 were measured using a Human IL-6 ELISA Ready-SET-Go! Kit (Cat No. 501128847) and a Mouse IL-6 ELISA Ready-SET-Go! Kit (Cat No. 5017218) obtained from eBioscience (San Diego, CA, USA), respectively. The concentration of human PGE2 and mouse PGE2 were measured using a Prostaglandin E2 Express ELISA Kit (Cat No. 500141) obtained from Cayman Chemical (Ann Arbor, MI, USA) and a Mouse Prostaglandin E2 (PGE2) ELISA Kit (Cat No. MBS266212) obtained from MyBioSource (San Diego, CA, USA), respectively. Absorbance values were measured using a microplate reader (iMark Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, CA).

**Evaluation of arginase activity**

Arginase activity was determined using a QuantiChrom arginase assay kit (BioAssay Systems, CA, USA) in accordance with the manufacturer’s instructions.
Statistical analysis

Continuous data were compared between groups using Student’s t-test or Tukey’s honestly significant difference test. We compared the Kaplan–Meier curves for each sub-group using the log rank test. P-values of < 0.05 were considered statistically significant. All analyses were performed with JMP software version 13.0 (SAS Institute Inc.)

Results

Prognostic significance of systemic leukocyte/platelet count in patients with endometrial cancer

A total of 900 patients with endometrial cancer who underwent surgical treatment were included in the current study. The clinicopathological characteristics of these patients are shown in Table S1.

We first investigated the clinical significance of pretreatment leukocyte count and platelet count. For this purpose, we divided the patients in four groups: normal leukocyte/platelet counts (Group 1), leukocytosis alone (Group 2), thrombocytosis alone (Group 3), and concurrent leukocytosis and thrombocytosis (Group 4). Postoperatively, 39.3% (Group 1), 40.0% (Group 2), 45.7% (Group 3), and 68.7% (Group 4) of patients received adjuvant chemotherapy due to pathological risk factors exhibited by the resected tumor, strongly indicating the highly aggressive nature of endometrial cancer in Group 4. Consistent with this, as shown in Figure 1(a), patients in Group 4 showed significantly shorter PFS (p < 0.0001) and OS (p < 0.0001) than those in the other three groups. Moreover, as shown (Figure 1(b)), patients in Group 4 showed significantly higher recurrence and mortality rates than the other three groups (recurrence rates, 9.1% in Group 1, 10.0% in Group 2, 11.4% in Group 3, and 48.6% in Group 4; mortality rates; 7.8% in Group 1, 10.0% in Group 2, 11.43% in Group 3, and 34.3% in Group 4). Among patients who received postoperative adjuvant chemotherapy, 10.1% (Group 1), 12.5% (Group 2), 15.6% (Group 3) and 56.5% (Group 4) of patients developed recurrent disease within 1 year, strongly indicating the highly chemoreistant nature of endometrial cancer in Group 4.

Tumor-derived G-CSF as a cause of leukocytosis and thrombocytosis in endometrial cancer

To investigate the cause of concurrent leukocytosis and thrombocytosis in endometrial cancer, using tumor samples obtained from randomly selected patients, immunohistochemical staining was performed. As shown, the tumors obtained from patients in Group 4 exhibited significantly strong G-CSF expression than those in Group 1 (Figure 1(c(i))). Consistent with this, patients in Group 4 showed significantly higher serum G-CSF concentrations than those in Group 1 (Figure 1(c(ii))). Moreover, tumors from patients in Group 4 exhibited significantly stronger IL-6 expression than those in Group 1 (Figure 1(d(i))). Consistent with this, patients in Group 4 showed significantly higher serum IL-6 concentrations than those in Group 1 (Figure 1(d(ii))). Collectively, these results clearly indicated that tumor-derived G-CSF and IL-6 are responsible, at least in part, for the development of concurrent leukocytosis and thrombocytosis in patients with endometrial cancer.

To investigate the potential effects of tumor-derived G-CSF or IL-6 on granulopoiesis and thrombopoiesis, we established mouse experimental models in which nude mice were inoculated with endometrial cancer cells that had been stably transfected with G-CSF, IL-6, or control vector (Figure 1(e(i)),(f(i)) and Figure S1A). As expected, Ishikawa-G-CSF-derived tumor-bearing mice showed significantly higher leukocyte counts than Ishikawa-Control-derived tumor-bearing mice. Moreover, Ishikawa-G-CSF-derived tumor-bearing mice exhibited significantly higher platelet count and serum IL-6 level than Ishikawa-Control-derived tumor-bearing mice (Figure 1(e)). In contrast, although Ishikawa-IL-6-derived tumor-bearing mice showed significantly higher platelet count than Ishikawa-Control-derived tumor-bearing mice, the number of leukocytes nor serum G-CSF level were increased compared with Control tumor-bearing mice (Figure 1(f)). Collectively, these results strongly indicated the existence of a G-CSF-mediated paracrine pathway in the development of concurrent leukocytosis and thrombocytosis (Figure S1D).

The role of tumor-derived G-CSF in the induction of MDSCs in TRL-positive endometrial cancer

We next investigated the mechanism responsible for the aggressive and chemoresistant nature of endometrial cancer displaying concurrent leukocytosis and thrombocytosis. It has been reported that tumor-derived G-CSF induces MDSCs and stimulates the progression of uterine cervical cancer. Consistent with this, as shown, Ishikawa-G-CSF-derived tumor-bearing mice displayed markedly increased CD11b+ Gr-1+ cells in their bone marrow, blood, spleens, and tumors compared with Ishikawa-Control-derived tumor-bearing mice (Figure 2(a)). The same results were obtained in experiments in which Hecl1B cells were employed (Figure S1A–C). Consistent with the findings obtained in mice, as shown, endometrial cancers obtained from patients in Group 4 showed significantly higher number of tumor-infiltrating CD33+ cells, than those in Group 1 (Figure 2(b)). Importantly, CD11b+ Gr-1+ cells isolated from the spleen of Ishikawa-G-CSF-derived tumor-bearing mice significantly inhibited the CD8+ T cell proliferation (Figure 2(c)). Moreover, endometrial cancers obtained from patients in Group 4 showed significantly lower numbers of tumor-infiltrating CD8+ T cells than those in Group 1 (Figure 2(d)), all of which are consistent with the immunosuppressive nature of MDSCs. Moreover, endometrial cancers obtained from patients in Group 4 showed significantly lower numbers of tumor-infiltrating CD8+ T cells than those in Group 1 (Figure 2(d)), which was consistent with previous studies showing that tumors with increased MDSCs showed lower number of tumor-infiltrating CD8+ T cells. Collectively, the results from Figure 2(c,d) indicate the immunosuppressive nature of MDSCs.

We next investigated the mechanisms by which G-CSF-induced MDSCs migrate into endometrial cancers. As shown, MDSCs obtained from endometrial cancer-bearing mice expressed C-X-C motif chemokine receptor 2 (CXCR2) (Figure 2(e(i))), and endometrial cancer cells (Ishikawa and Hecl1B) expressed CXCL2 (Figure 2(e(ii))). Moreover, treatment endometrial cancer cells with CXCL2
significantly stimulated the migration activity of MDSCs (Figure 2(f)). Collectively, these results indicated that MDSCs induced by tumor-derived G-CSF may migrate into the tumor microenvironment via the CXCL2/CXCR2 axis.

Role of G-CSF-induced MDSCs and MDSc-derived PGE2 in the induction of endometrial CSCs in vitro

We next investigated the mechanism responsible for the chemoresistant nature of endometrial cancer displaying...
concurrent leukocytosis and thrombocytosis, with a focus on CSCs. For this purpose, we employed ALDH1 as a CSC marker and confirmed that ALDH-high endometrial cancer cells have CSC-like properties in vitro (Figure S2, S3). We first investigated whether MDSCs enhance ALDH activity of endometrial cancer cells in vitro. When Ishikawa cells were co-cultured with MDSCs extracted from the spleens of Ishikawa-G-CSF-derived tumor-bearing mice, the frequency of ALDH-high Ishikawa cells was significantly increased (Figure 3(a)). Because PGE2, a mediator of inflammation, has recently been reported to enhance the stemness of cervical, colorectal, or bladder cancer cells, we examined whether PGE2 can enhance the stemness of endometrial cancer cells. As shown in Figure 3(b) and Figure S4A, both Ishikawa and Hec1B cells expressed EP2 and EP4 receptors, which are known to be involved in PGE2 signaling. Consistent with this, the numbers of ALDH-high Ishikawa and Hec1B cells were significantly increased in response to treatment with PGE2 (Figure 3(c) and Figure S4B). The effect of PGE2 for inducing ALDH-high cells was greatest at a dose of 50 ng/ml, which was consistent with a previous study of colorectal cancer. Moreover, PGE2-mediated induction of ALDH-high cells was significantly inhibited by treatment with an EP antagonist (Figure 3(d) and Figure S4C). We next assessed whether MDSCs express PGE2 in vitro. As shown in Figure 3(e), MDSCs extracted from the spleens of Ishikawa-G-CSF-derived tumor-bearing mice expressed PGE2, which was in clear contrast to splenocytes (excluding MDSCs) extracted from the same mice. Furthermore, the production of PGE2 from MDSCs was inhibited by treatment with celecoxib, a cox-2 inhibitor (Figure 3(f)). Importantly, the MDSC-mediated induction of ALDH-high cells was significantly inhibited by treatment with celecoxib in vitro (Figure 3(g) and Figure S4D).

**In vivo effects of G-CSF-induced MDSCs and MDSC-derived PGE2 in the induction of endometrial cancer stem cells**

We next investigated whether MDSCs enhanced the stemness of endometrial cancer cells in vivo. As shown in Figure 4(a,b), significantly increased tumor-infiltrating MDSCs, elevated serum PGE2 level, and ALDH-high endometrial cancer cells were observed in Ishikawa-G-CSF-derived tumors more than in Ishikawa-Control-derived tumors. The same results were obtained in experiments in which Hec1B cells were employed (Figure S5A-B). We then examined the association between MDSCs and ALDH-high tumor cells in endometrial cancer specimens obtained at initial surgery. As shown, the endometrial cancer specimens obtained from patients exhibited various degrees of immunoreactivities for ALDH1 and CD33 (Figure 2(c,i) and (ii)). Importantly, as shown in Figure 4(c(iii)), Tumors that exhibited higher numbers of tumor-infiltrating CD33+ cells showed significantly greater immunoreactivity for ALDH1. Moreover, the patients whose tumors exhibited higher numbers of tumor-infiltrating CD33+ cells showed significantly higher serum PGE2 levels (Figure 4(d)). The results from representative cases are shown in Figure S6; patients with endometrial cancer in Group 4 showed significantly increased MDSCs (CD11b+ CD33+ HLA-DR− cells) in peripheral blood than those in Group 1 (Figure S6A). Furthermore, in immunohistochemical analyzes, significantly greater immunoreactivity for G-CSF, CD33, PGE2, and...
ALDH1 were observed in tumors obtained from patients in Group 4th a ni nt o f r o mf a t i e n si nG r o u p1 ( FigureS 6 B ) .

To further investigate the significance of MDSC-mediated CSC-induction in endometrial cancer, we next evaluated the sensitivity of G-CSF-producing endometrial cancer to doxorubicin, a key anti-cancer agent in the treatment of endometrial cancer. As shown, Hec1B-G-CSF-derived tumors containing increased MDSCs were less sensitive to doxorubicin than Hec1B-Control-derived tumors (Figure 4(e)). The same results were obtained from the experiment using Ishikawa-G-CSF-derived tumors (Figure S5C). Consistent with this, patients with endometrial cancer
Figure 3. The mechanism responsible for the enhancement of stemness by MDSCs.

(a) Effect of MDSCs on the induction of CSC in vitro. Ishikawa cells (8 × 10^5/well) were cultured with or without MDSCs (1 × 10^5/well) in the presence of 0.1% FBS for 18 hours in 6-well dishes. The frequencies of ALDH-high Ishikawa cells were assessed using an Aldefluor assay (n = 6). Bars, SD. ***p < .001, using two-sided Student’s t-test. (b) Expression levels of EP receptors in Ishikawa cells. EP1–EP4 receptors and β-actin mRNA levels of Ishikawa cells that had been incubated in the presence of 10% FBS were assessed by RT-PCR. (c) Effect of PGE2 on the induction of CSCs in vitro. Ishikawa cells were treated with the indicated concentrations of PGE2 in the presence of 0.1% FBS for 18 hours. The frequencies of ALDH-high Ishikawa cells were assessed using an Aldefluor assay (n = 6). (d) Significance of EP receptors in the PGE2-mediated CSC induction. Ishikawa cells were treated with 50 ng/ml PGE2 with or without EP2 antagonist (200 nM) and EP4 antagonist (20 nM) in the presence of 0.1% FBS for 18 hours. The frequencies of ALDH-high Ishikawa cells were assessed using an Aldefluor assay (n = 6). (e) Production of PGE2 by MDSCs. MDSCs (1 × 10^6/well) that had been isolated from spleens of Ishikawa-G-CSF-derived tumor bearing mice were cultured in the presence of 2% of FBS for 24 hours. The PGE2 concentrations of the culture media were measured using a Prostaglandin E2 Express ELISA Kit. (n = 3). Splenocytes (1 × 10^6/well, including and excluding MDSCs) were also used as a comparison. Bars, SD. *p < .05, **p < .01, using Tukey’s honestly significant difference tests. (f) Effect of celecoxib on the production of PGE2 by MDSCs. MDSCs (5 × 10^5/well) were treated with celecoxib (20 μM) in the presence of 0.1% FBS for 18 hours in vitro. PGE2 concentrations in the culture media were measured using a Prostaglandin E2 Express ELISA Kit. (n = 3). Bars, SD. ***p < .001, using two-sided Student’s t-test. (g) Effect of PGE2-inhibition on the MDSC-mediated CSC induction. Ishikawa cells (8 × 10^5/well) and MDSCs (1 × 10^5/well) were co-cultured in 6-well dishes and treated either with celecoxib (20 μM), EP2 antagonist (200 nM), or EP4 antagonist (20 nM) in the presence of 0.1% FBS for 18 hours in vitro. Then, the frequencies of ALDH-high Ishikawa cells were assessed using an Aldefluor assay (n = 6). Bars, SD. ***p < .001, using Tukey’s honestly significant difference tests.
who showed strong immunoreactivity for CD33 and ALDH1 showed significantly shorter PFS and OS than those who showed weak immunoreactivity for CD33 and ALDH1 (Figure 4(f,g)).

**G-CSF/IL-6/PGE2 network in the tumor microenvironment of TRL-positive endometrial cancer**

We next examined the mechanism by which IL-6 production is stimulated in patients with endometrial cancer who have
increased MDSCs. PGE2 is a known stimulator of IL-6 production; therefore, endometrial cancer cells were treated with the indicated concentration of PGE2. As shown, human IL-6 production from Ishikawa cells was significantly enhanced in response to PGE2 treatment in a dose-dependent manner (Figure 5(a(i))). Next, we co-cultured endometrial cancer cells with MDSCs, which have been shown to produce PGE2. As can be seen, human IL-6 concentrations in the culture medium were significantly increased by co-culturing with MDSCs. Moreover, the MDSC-mediated increase in human IL-6 production from Ishikawa cells was canceled by treatment with celecoxib and EP antagonists (Figure 5(a(ii))). Moreover, when MDSCs were co-cultured with Ishikawa-G-CSF-derived cells, the secretion of mouse IL-6 was significantly increased (Figure 5(b(i))), which was in clear contrast to the situation when MDSCs were co-cultured with Ishikawa-Control-derived cells. In line with this, mouse IL-6 production from MDSCs was significantly enhanced in response to exogenous G-CSF treatment in a dose-dependent manner (Figure 5(b(ii))). Collectively, these results indicated the significance of tumor-derived G-CSF and PGE2 in the secretion of IL-6 from MDSCs.

We further investigated whether G-CSF collaborates with IL-6 in the activation of MDSCs. It has been reported that G-CSF inhibits spontaneous neutrophil apoptosis through induction of the Janus kinase 2 (JAK2)-Signal transducer and activator of transcription 3 (stat3) pathway, and that stat3 signaling induces the expression of arginase-1 in MDSCs, which is a hallmark of MDSC-mediated immunosuppressive activity. As mouse G-CSF receptor and mouse IL-6 receptor were expressed in MDSCs (Figure 5(c)), we investigated the effect of exogenous G-CSF and/or IL-6 on MDSC activation by assessing stat3 phosphorylation in MDSCs and arginase or PGE2 production by MDSCs. As expected, treatment of MDSCs with G-CSF or IL-6 alone stimulated the phosphorylation of stat3. Notably, when MDSCs were treated with G-CSF together with IL-6, the phosphorylation of stat3 was further enhanced (Figure 5(d)). Consistent with these findings, the activities of MDSCs were also enhanced by co-administration of G-CSF and IL-6 (Figure 5(e,f)). Moreover, the ability of MDSCs in the induction of ALDH-high endometrial cancer cells was also enhanced by co-administration of G-CSF and IL-6 (Figure 5(g)). Collectively, these results strongly suggested that G-CSF collaborates with IL-6 in stimulating the immunosuppressive (stat3-arginase axis) and CSC-inducing (MDSC-PGE2-CSC axis) activities of MDSCs (Figure 5(h)).

**In vivo effects of MDSC-inhibition on the stemness and the chemoresistance of endometrial cancer**

Finally, we investigated the effects of MDSC depletion using an anti-Gr-1 antibody in a mouse model of endometrial cancer. As shown, when Ishikawa-G-CSF-derived tumor-bearing mice were treated with an anti-Gr-1 neutralizing antibody, G-CSF-mediated increases in MDSCs and ALDH-high endometrial cancer cells were significantly inhibited. Furthermore, the anti-Gr-1 neutralizing antibody significantly decreased serum PGE2 concentrations in Ishikawa-G-CSF-derived derived tumors to doxorubicin. In this experiment, treatment with the anti-Gr1 neutralizing antibody or celecoxib only did not significantly decrease the number of platelets in peripheral blood mononuclear cells. However, when combined with doxorubicin, celecoxib significantly decreased the number of platelets (Figure S7A, S7B(ii), S7C(ii)). Of note, drug treatments were well tolerated, with no apparent toxicity throughout the studies.

**Discussion**

In the current study, we have shown that concurrent pretreatment leukocytosis and thrombocytosis is an indicator of poor prognosis in patients with endometrial cancer treated with standard treatments, including surgery and adjuvant chemotherapy.

This clinical finding is partially consistent with previous reports. Including ours, increasing numbers of reports have suggested that pretreatment leukocytosis or thrombocytosis are independent prognostic factors in patients with endometrial cancer. However, none of these studies have investigated the association between leukocytosis and thrombocytosis. In the current study, we showed that patients who exhibit only leukocytosis or thrombocytosis have favorable prognosis, with survival rates and response to chemotherapy equivalent to those who exhibit normal leukocyte/platelet counts. Similar results have been reported from a study of ovarian cancer. Thus, we consider that the “high risk” patients that we need to pay special attention are not patients with leukocytosis or thrombocytosis alone but those with concurrent leukocytosis and thrombocytosis. Moreover, our clinical findings strongly indicated the existence of certain factors that can cause both leukocytosis and thrombocytosis, and stimulate the aggressiveness of endometrial cancer.

In laboratory investigations, we demonstrated that tumor-derived G-CSF is responsible for the development of leukocytosis. This finding was consistent with a previous study of cervical cancer. Importantly, we found, for the first time, that tumor-derived G-CSF stimulates the production of IL-6 from an endometrial cancer microenvironment, which can be a cause of thrombocytosis. Moreover, we have found that G-CSF-induced MDSCs are involved in the progression of TRL-positive endometrial cancer, by inhibiting CD8+ T cells and enhancing the stemness of endometrial cancer cells through the production of PGE2. We also demonstrated that
Figure 5. The mechanism of tumor-derived factors on MDSC function.

(a) (i) The effect of PGE2 on the production of IL-6 from endometrial cancer cells. Ishikawa cells (8 × 10^5/well) were cultured in 6-well dishes and treated with the indicated concentration of PGE2 in the presence of 0.1% FBS for 18 hours in vitro. The human IL-6 concentration in culture medium according to ELISA assay is shown. Bars, SD. *p < .05, **p < .01, ***p < .001, using Tukey's honestly significant difference tests. (ii) The effect of MDSCs on the production of IL-6 from endometrial cancer cells. Ishikawa cells (8 × 10^5/well) and MDSCs (1 × 10^5/well) were co-cultured in 6-well dishes containing 0.1% FBS in the presence or absence of celecoxib (20 μM), EP2 antagonist (200 nM), or EP4 antagonist (20 nM) for 18 hours. Human IL-6 concentration in culture medium was assessed using an ELISA assay. Bars, SD. *p < .05, using Tukey's honestly significant difference tests. (b) (i) Effect of tumor-derived G-CSF in the production of mouse IL-6 from MDSCs. Ishikawa-G-CSF or Ishikawa-Control cells (8 × 10^5/well) and MDSCs from the spleen of mice bearing G-CSF-expressing endometrial cells (5 × 10^5/well) were co-cultured in 6-well dishes in the presence of 0.1% FBS for 18 hours in vitro. The mouse IL-6 concentration in culture medium was assessed using an ELISA assay. Bars, SD. **p < .01, using Tukey's honestly significant difference tests. (ii) The effect of exogenous G-CSF treatment on the production of mouse IL-6 from MDSCs. MDSCs isolated from the spleens of mice bearing G-CSF-expressing endometrial cells were treated with the indicated concentrations of G-CSF in the presence of 2% FBS for 18 hours in vitro. The mouse IL-6 concentration in the culture medium was assessed using an ELISA assay. Bars, SD. **p < .01, using Tukey's honestly significant difference tests. (c) The expression levels of mouse G-CSF receptor, mouse IL-6 receptor, and β-actin mRNA levels in mouse MDSCs. Ishikawa cells were used as a negative control and mouse bone marrow cells were used as a positive control. (d) The effect of G-CSF and IL-6 on Stat3 activation in MDSCs. MDSCs isolated from the spleens of mice bearing G-CSF-expressing endometrial cells were treated with recombinant human G-CSF (5 ng/mL) or recombinant mouse IL-6 (100 ng/mL) in the presence of 2% FBS. The activation of Stat3 in MDSCs was assessed by western blotting. (i) Phospho-stat3, (ii) total stat3, (iii) β-actin. (e) The effect of G-CSF and IL-6 on mouse PGE2 production from MDSCs. MDSCs were treated with recombinant human G-CSF (5 ng/mL) or recombinant mouse IL-6 (100 ng/mL) in the presence of 2% FBS for 24 hours. The PGE2 concentrations of the culture medium were measured using a Prostaglandin E2 Express ELISA Kit. (n = 3). Bars, SD. **p < .01, ***p < .001, using Tukey's honestly significant difference tests. (f) The effect of G-CSF and IL-6 on the arginase activity. MDSCs were treated with recombinant human G-CSF (5 ng/mL) or recombinant mouse IL-6 (100 ng/mL) in the presence of 2% FBS. Then, the frequencies of ALDH-high Ishikawa cells were assessed using an Aldefluor assay. (n = 6). Bars, SD. *p < .05, **p < .01, ***p < .001, using Tukey's honestly significant difference tests. (ii) Representative dot plots are shown. (h) Proposed paracrine mechanism responsible for the induction of endometrial cancer stem cells by MDSCs.
the inhibition of MDSCs or PGE2 effectively inhibited the induction of CSCs and enhanced the efficacy of doxorubicin in experimental models of endometrial cancer. To the best of our knowledge, this is the first report investigating the role of MDSCs in endometrial cancer progression. Our previous studies demonstrated that polymorphonuclear (PMN) MDSCs (also known as granulocytic MDSC) are the dominant subset in G-CSF-producing cancer-bearing mice. Thus, the results obtained from our investigations suggest that PMN-MDSCs play a significant role in endometrial cancer progression.

The strength of the current study was that the results from our in vitro and in vivo experiments could be verified in patients with endometrial cancer. As shown in analyzes using patient-derived tumors, we confirmed that the frequency of tumor-infiltrating MDSCs was positively correlated with: the tumor expression of G-CSF and PGE2, frequency of CSCs in tumor, and PGE2 concentration in patients with endometrial cancer (Figure S5B). These results indicated that the G-CSF-MDSC-PGE2-CSC axis demonstrated in our in vitro or in vivo experiments is applicable to human endometrial cancer.

The findings obtained in the current study could have important clinical implications. Our investigations indicated that, by performing a simple, low-cost peripheral blood examination, it might be possible to identify patients who are at high risk of developing recurrence after initial treatment including surgery plus adjuvant chemotherapy. For the early detection of recurrent disease in these patients, careful post-treatment follow-up can be recommended. Moreover, the results of this study may provide a scientific rationale for future clinical trials of MDSC-targeting therapies in patients with endometrial cancer exhibiting concurrent leukocytosis and thrombocytosis.

The limitations of our study need to be addressed. First, in the present study, we employed ALDH-activity as a marker of CSCs, although CSC markers for solid tumors have not yet been established. Second, although the current study focused on the "MDSC-PGE2-CSC axis", MDSCs are known to produce various mediators, including cytokines, chemokines, and growth factors. Thus, we cannot exclude the possibility that other MDSC-derived factors might also play roles in the induction of CSCs. Third, although the present study attributed the G-CSF-mediated increase in IL-6 to the increased production of IL-6 from cancer cells and MDSCs, we cannot exclude the possibility that other stromal cells are also involved in the production of IL-6 in response to G-CSF. Moreover, although the present study focused on G-CSF induced MDSCs, we cannot exclude the possibility that other stromal cells, such as tumor-associated neutrophils or platelets also play roles in the progression of endometrial cancer in G-CSF-dependent or IL-6-dependent manners, respectively. Moreover, although the present study focused on the CXCL2/CXCR2 axis, other chemokines and their receptors also play roles in the recruitment of MDSCs into the tumor microenvironment. Previous studies have suggested that tumor-derived chemokine C-C motif ligand 2 (CCL2), CXCL5, CXCL12, and their receptors (C-C chemokine receptor [CCR]2, CCR5, and CXCR4) contribute to MDSC recruitment to tumors. Accordingly, the mechanisms by which G-CSF stimulates endometrial cancer progression and the mechanism responsible for MDSC recruitment need to be investigated further. Fourth, we employed an anti-Gr-1-neutralizing antibody to deplete MDSCs in the present study. However, we cannot rule out the possibility that the anti-Gr-1-neutralizing antibody also affects other cells, such as neutrophils. Fifth, although CD33 was employed for the identification of human MDSCs in the current study based on previous studies, CD33-positive cells are not always MDSCs. Thus, clear phenotypic characterization of human MDSCs by immunohistochemistry need to be investigated in the future studies. Lastly, we employed nude mice in the current study, because the inoculation of human endometrial cancer cells into immunocompetent mice did not result in the development of primary/metastatic tumors. We consider that the results from our in vivo experiments should be interpreted with caution, because they might include the artificial interactions between human cancer cells and murine immune cells.

In conclusion, this study is the first to demonstrate that concurrent leukocytosis and thrombocytosis is an
Figure 6. Effect of MDSC depletion on the stemness and the chemoresistance of endometrial cancer.

(a) (i) Effects of an anti-Gr-1 neutralizing antibody on MDSC recruitment and CSC induction in Ishikawa-G-CSF-derived tumors. Balb/c nude mice were inoculated with Ishikawa-G-CSF (n = 12) or Ishikawa-Control cells (n = 6). Six mice bearing Ishikawa-G-CSF-derived tumors were treated with an anti-Gr-1 neutralizing antibody. Three weeks after inoculation, bone marrow, blood, spleen, and subcutaneous tumors were collected. (i) CD11b$^+$ Gr-1$^+$ cell populations in the BM, PBMC, spleen and tumor cells were assessed using flow cytometry. (ii) The frequencies of ALDH-high Ishikawa cells among the tumor cells were assessed using an Aldefluor assay. (iii) Mouse serum PGE2 concentrations were evaluated using an ELISA. Bars, SD. *p < .05, ***p < .001, using Tukey's honestly significant difference tests. (b) Effects of an anti-Gr-1-neutralizing antibody on the doxorubicin sensitivity of endometrial cancer. Balb/c nude mice were inoculated with Hec1B-G-CSF cells. One week after inoculation, the mice were assigned to three treatment groups: Control (water, every 3 weeks combined with isotype control, every 2 days, n = 4), doxorubicin (5 mg/kg doxorubicin, every 3 weeks combined with isotype control, n = 4) or doxorubicin (5 mg/kg, every 3 weeks) combined with an anti-Gr-1-neutralizing antibody (200 μg/mouse, every 2 days, n = 4). (i) The volumes of the tumors were measured for 3 weeks after start of treatment. Bars, SD. *p < .05, using Tukey's honestly significant difference tests. (ii) Three weeks after the start of treatment, subcutaneous tumors were collected. CD11b$^+$ Gr-1$^+$ cell populations in the tumor cells were counted by flow cytometry. (iii) The frequencies of ALDH-high Hec1B cells in the tumor were assessed using an Aldefluor assay. (iv) Mouse serum PGE2 concentrations were evaluated using an ELISA. Bars, SD, ***p < .001, using Tukey's honestly significant difference tests. (c) Effects of celecoxib on the doxorubicin-sensitivity of endometrial cancer. Balb/c nude mice were inoculated with Hec1B-G-CSF cells. One week after inoculation, the mice were assigned to four treatment groups: vehicle (n = 4), doxorubicin (5 mg/kg, every 3 weeks, n = 4), celecoxib (2.5 mg/kg, daily, n = 4), or doxorubicin (5 mg/kg, every 3 weeks) combined with celecoxib (2.5 mg/kg, daily, n = 4). (i) The volumes of tumors were measured for 3 weeks. Bars, SD, *p < .05, ***p < .001, using Tukey's honestly significant difference tests. (ii) Three weeks after the start of treatment, subcutaneous tumors were collected. CD11b$^+$ Gr-1$^+$ cell populations in the tumor were assessed by flow cytometry. (iii) The frequencies of ALDH-high Hec1B cells in the tumor were assessed using an Aldefluor assay. (iv) Mouse serum PGE2 concentrations were evaluated using an ELISA. Bars, SD, ***p < .001, using Tukey's honestly significant difference tests.
independent poor prognostic factor in patients with endometrial cancer. Moreover, the increase in tumor-infiltrating MDSCs induced by tumor-derived G-CSF, MDSC-mediated T cell suppression, and MDSC-mediated CSC induction are responsible for progression and chemoresistance in this type of cancer. We believe that our results provide a scientific rationale for future clinical trials of MDSC-inhibition therapy in patients with endometrial cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed

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